Behavioral and neural alterations of the ventral tegmental area by exposure to junk food in rats

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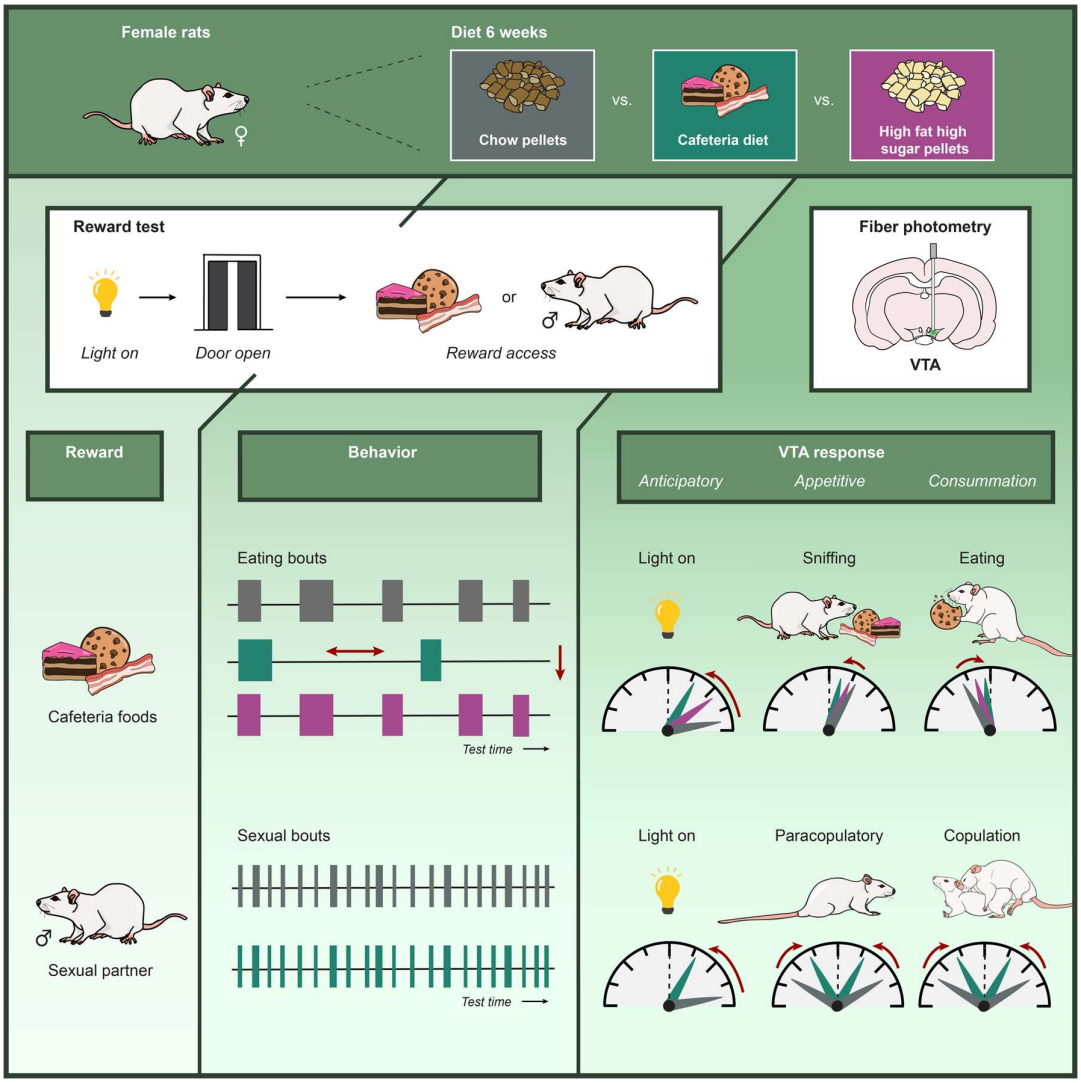

Abstract

The brain reward system is essential for regulating appetitive and consummatory behaviors in response to various incentive stimuli. Junk food, known for its high palatability, is particularly associated with the potential for excessive consumption. While previous studies have shown that excessive junk food intake can impact reward circuitry, the precise mechanisms remain unclear. Additionally, it is unknown whether the functionality of this brain system is similarly altered in response to other natural rewards. In this study, we used fiber photometry combined with a behavioral reward test to investigate how six weeks of excessive cafeteria (CAF) diet consumption affects VTA neural activity and behavioral responses to food and sexual rewards in sexually experienced female rats. For the first time, we demonstrate that prolonged exposure to a CAF diet decreases interest in a food reward, resulting in reduced consumption. These behavioral changes were accompanied by diminished neural activity in the VTA. Similarly, reductions in VTA activity responses were observed in response to a sexual partner, although no significant behavioral differences were detected during sexual interactions. Furthermore, a two-week reversal diet of standard chow was insufficient to restore VTA neural activity in CAF-exposed animals, which continued to show decreased VTA responses to both food rewards and sexual partners. Our findings suggest that prolonged junk food exposure leads to desensitization of the VTA, resulting in reduced responsiveness to natural rewards.

Keywords: cafeteria diet, food, sexual behavior, reward, ventral tegmental area, high fat high sugar diet, rat

1. Introduction

The widespread availability and excessive consumption of processed foods rich in fat and sugar, also called junk food, is a potential cause of the increased prevalence of obesity and overweight around the world[1-3]. The highly palatable characteristics of these foods activate hedonic and motivational mechanisms to promote food-seeking behavior and overeating[1], and it has been shown that modern energy-rich diets are consumed not solely for their nutritional content but also for their hedonically pleasant traits[4; 5]. Besides the risk for obesity, the consumption of these foods is associated with a high risk for other diseases such as cardiovascular disease, type 2 diabetes, infertility, hypertension and some cancers[6-11]. Moreover, multiple studies have found intricate links between the consumption of junk food, and cognitive and neurological changes[12-15]. Impairments in aspects of short-term memory, executive function and reinforcement learning have been found in humans and rodents[16-18].

Excessive junk food consumption can also disrupt neural reward mechanisms[19; 20]. In normal circumstances, evaluation of the palatability and consumption of food are in part regulated by dopamine transmission from the ventral tegmental area (VTA) to the nucleus accumbens (NAc)[21; 22]. VTA dopamine neurons regulate many aspects of reward-related behavior including reinforcement, decision making, attention, and impulsivity[23-26]. These cells are involved in predicting the value of the reward, and in integrating past experiences with the effort required to obtain a reward before approach to that reward is made[27-33]. Studies in humans and rats have shown that acute exposure to palatable foods increases dopamine release in the NAc [34-37] and increases NAc neural activity[38-40], while chronic exposure to food rich in fat and sugar leads to an attenuated NAc response [38; 41; 42] and a lower dopamine turnover in the NAc and VTA at baseline or after a palatable food reward[34; 43-50]. In addition, down-regulation of D2[51-55], but up-regulation of D1 receptors in the NAc were reported after chronic junk food exposure[52-54]. Similarly, it has been found that junk food can alter opioid signaling in the VTA[54; 56-59].

If palatable food can affect the reward system in such a way, it seems plausible that longer-term junk food consumption can also lead to *behavioral* changes. Studies examining the direct effects of junk food in human are scarce, but consumption of yoghurt high in fat and sugar was shown to diminish preference for low-fat food[60]. Furthermore, several studies have found that patients with overweight/obesity show increased sensitivity to immediate rewards[61-63], increased delay-discounting[64], increased eating-related self-control[62; 63], and increased reward cue tracking[65]. Inhibitory responses[66], contingency learning[67], probabilistic learning[67-70], and reward devaluation effects[71; 72], on the other hand, are found to be negatively correlated with overweight and obesity. Although these human studies provide interesting insights in the behavioral

consequences of overweight, the direct link to the actual consumption of junk food cannot be demonstrated. In contrast, many rat studies have investigated the direct effects of junk food consumption on behavior, but with conflicting results. While some studies reported increased levels of motivation to work for food rewards in obesity-prone rats or rats exposed to junk food diets[54; 73-77], others have found no changes or reported reduced interest in cues for reward or attenuated operant responses for palatable food rewards[41; 43; 78-82]. These inconsistencies are most likely caused by variations in diet types, durations, and models used, along with differences in food rewards.

Interestingly, food rewards that are used in behavioral tests are often different from the palatable diet used as initial manipulation. This suggests that the effects of junk food consumption are not limited to the original palatable food they were exposed to. As a matter of fact, it has been shown that long-term junk food exposure could alter the behavioral responses toward other kinds of unnatural drug rewards. For example, reduced levels of ethanol consumption[83-85], amphetamine cue preference[43], conditioned place preference for cocaine[86], or elevated brain stimulation reward thresholds [53] have been found. Similarly, human studies have shown correlations between obesity and less efficient monetary reward responses[69; 87; 88].

Although substantial evidence from studies in both humans and rats indicates that prolonged consumption of junk food can affect neural reward mechanisms, many questions remain unanswered. Human fMRI studies are limited in their ability to conduct high-resolution brain region investigations or to study the consequences of diet manipulations. Rat studies, on the other hand, provide a unique opportunity to explore the neural regulation in greater detail that can be directly linked to behavioral consequences of long-term junk food consumption. Still, many of the rat studies mentioned above explored only a small fraction of the reward-related behavior. In addition, they used operant conditioning designs that are highly dependent on learning and motor skills. How patterns of rewarding behavior change upon junk food consumption when free access to and interaction with a food reward is provided remains unknown. In addition, if different rewarding behaviors make use of the same neural pathways, and prolonged exposure to junk food not only affects food-related behavior, but also the neural substrate behind that behavior, it can be hypothesized that prolonged junk food might also affect other rewarding behaviors. Sexual behavior is one of these rewarding behaviors in which VTA [89-91] and nucleus accumbens [92-94] are involved during different phases and/or aspects. By combining behavioral analysis with real-time VTA neural activity recording, we were able to study the effects of excessive junk food consumption on the different phases involved in rewarding behaviors (anticipatory, appetitive and consummatory behavior), including the VTA response upon a reward-predictive light cue.

Finally, rodents in junk food exposure studies are often fed with pellets rich in fat and/or sugar. The advantage of such diets is the control over the intake of received nutrients and energy. However, an important disadvantage of these pellet diets is that they do not translate to many dietary behaviors that are important to humans in the context of junk food. Humans typically eat unhealthy products due to their taste, texture, novelty, and variety[95]. In addition, the element of choice of what to consume has been shown to play an important role in overeating. For example, studies have shown that the body weight of rats on a high-fat, high-sugar diet with free choice increased more than that of rats on a forced high-fat, high-sugar diet[96], and that the presentation of a wide range of foods induces over-eating[97], meaning that variety in a meal and greater dietary variation also plays a significant role. *Cafeteria diet* (CAF), as used in this study, gives rats *a choice* of the same unhealthy, but highly palatable, products that humans consume (e.g., bacon, muffins, and cookies)[98; 99]. This increases the hedonic properties of the 'wanted' food and the motivation to consume[100; 101].

Our study aimed to investigate whether excessive consumption of junk food, in the form of CAF, could alter neural activity patterns of the VTA in response to food and sexual rewards in rats. We hypothesized that long-term CAF exposure could disrupt VTA function not only in the context of response to food rewards, but also in the context of the processing of other types of reward, such as the pursuit of a sexual partner. Moreover, we expected that CAF diet would have more impact on the functioning of brain reward circuitry more severely than a high-fat, high-sugar (HFHS) pellet diet that is commonly used in obesity research. Finally, we investigated whether reversal to a healthy diet could restore the behavioral and neural changes caused by long-term CAF exposure.

2. Results

To study the impact of long-term CAF diet exposure on the VTA during behavioral responses to food or sexual rewards, we fed three groups of adult, female Wistar rats with either a control diet (CTR, standard chow), a high-fat, high-sugar diet (HFHS, RD Western Diet pellets), or a cafeteria diet (CAF, a selection of palatable, albeit unhealthy, human-like food choices that the animal itself could choose from, see Tables S2-S6 for more detail about the body weights, diets, food intake and nutritional values). After 6-weeks of diet exposure, we tested the rats in a sequence of behavior tests with a food reward (consisting of a selection of the favorite CAF diet items) first, and a sexual reward (a sexually trained male rat) second (Fig 1A/B). Our experimental design facilitated the assessment of distinct phases of reward-related behavior, and included a baseline phase (without any reward), a pre-reward phase (introducing the reward, but without access to the reward), and a reward phase allowing free interaction with the reward (Fig 1C).

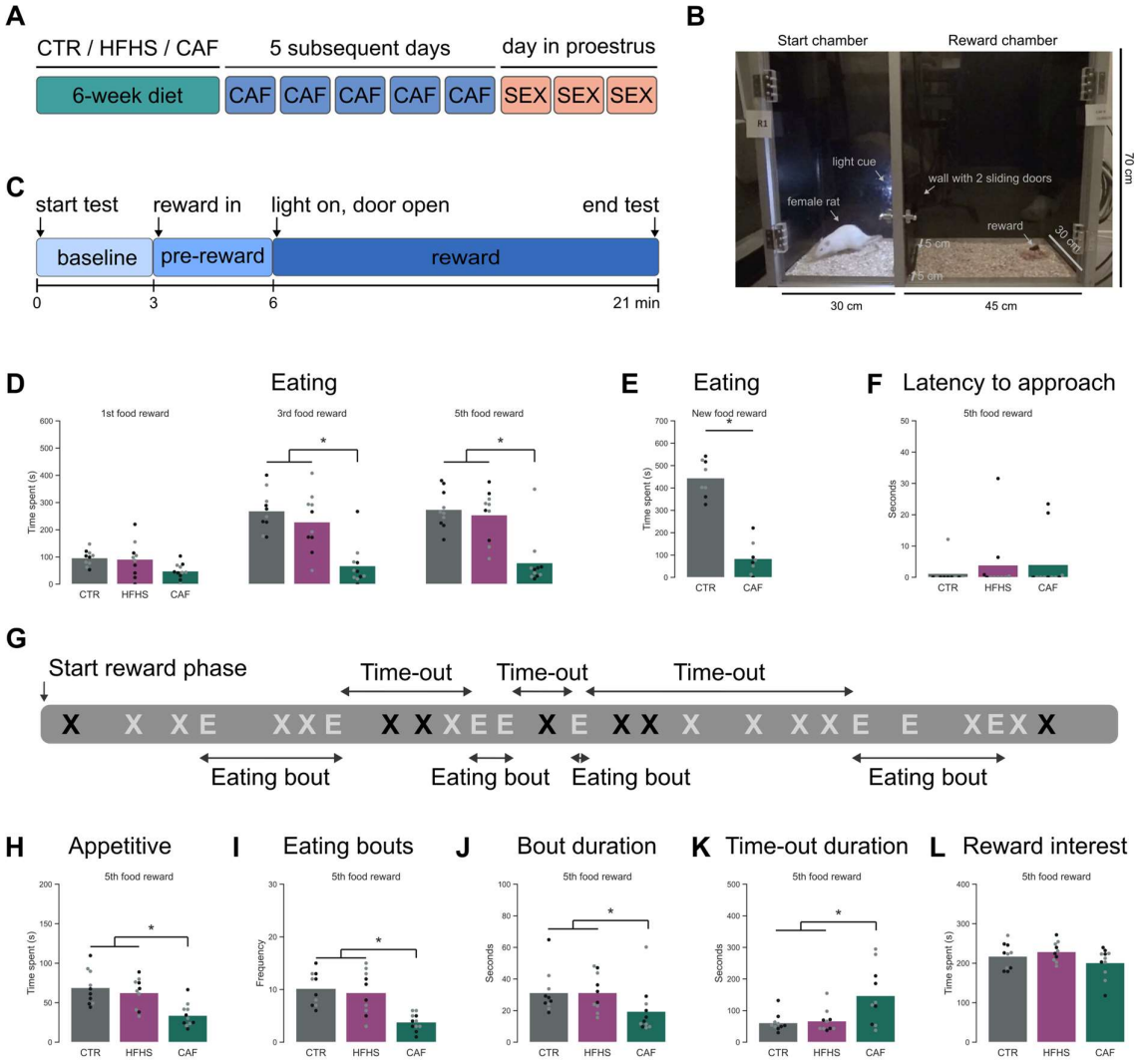

Figure 1. The effects of Cafeteria, High-fat high-sugar, and control diet on behavioral responses during a food reward test. **A)** Schematic representation of the experimental design. The female rats received 6 weeks of either a control (CTR), cafeteria (CAF), or high-fat, high-sugar (HFHS) diet, after which they were exposed to 5 sessions with a food reward (CAF reward) and 3 sessions with a sexual reward (male rat). **B)** Picture of our behavioral reward test box. **C)** Schematic representation of the behavioral reward test. Female rats are introduced into the left chamber with doors closed. After a baseline period of 3 minutes, the reward is placed into the right chamber while the doors remain closed (pre-reward phase). 3 minutes later, the light turns on for 10 seconds before the door opens. The subject then has 15 minutes of free access to the reward and both chambers (reward phase). **D)** Time spent eating during the reward phase of the 1st, 3rd and 5th behavioral reward test with a food reward. **E)** Time spent eating a novel CAF reward during the reward phase. CTR: n=8, CAF: n=8. **F)** Latency to interact with the CAF reward after door opening. **G)** Schematic overview of eating bouts during the reward phase of the behavioral reward test with a food reward. An eating bout is defined as a sequence of eating episodes (E), with or without reward-related behaviors (X in white) in between, that are not interrupted by non-reward related behaviors (X in black). As soon as the attention is withdrawn from the food, an eating bout ends with the last eating episode, and the next eating bout starts with the next eating episode. Time-outs represent the breaks between eating bouts. **H)** Number of appetitive behaviors towards the food reward during the reward phase of the 5th food reward test. Appetitive behaviors include approaching and sniffing the reward. **I)** Number of eating bouts during the reward phase of the 5th food test. An eating bout is defined as a sequence of eating episodes that is not interrupted by non-reward related behaviors. **J)** Mean duration of eating bouts during the reward phase of the 5th food test. **K)** Mean duration of time-outs between eating bouts during the reward phase of the 5th food test. **L)** Time spent on behaviors that indicate interest in the reward in the other chamber during the pre-reward phase of the 5th behavioral reward test with a food reward. Reward interest behaviors include sniffing and rearing the door, head oriented at the door, and sitting close to the door. **All)** Figures show rats on control (CTR, grey bar), high-fat, high-sugar pellet (HFHS, purple bar), and cafeteria (CAF, green bar)

diet. Dot colors representing their body weight category (grey: lighter than average, black: heavier than average). CTR n=10, HFHS n=10, CAF n=11. * p<0.05 compared to by line connected group.

2.1 Long-term CAF diet exposure, in contrast to HFHS, reduces interest in a food reward.

First, we investigated whether long-term CAF diet exposure resulted in altered behavioral responses towards a food reward (Table S1). We found that CAF-exposed rats exhibited a reduced frequency of eating (Fig S1A, Diet: $F_{(2,28)}$=25.121, p<0.001, Test: $F_{(2,56)}$=30.493, p<0.001, Diet*Test: $F_{(4,56)}$=5.734, p<0.001, post-hoc: 3rd test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, 5th test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, CTR: 1st vs 3rd test: p<0.001, 1st vs 5th test: p<0.001, HFHS: 1st vs 3rd test: p<0.001, 1st vs 5th test: p<0.001 (note: only the significant post-hoc results are reported in the result section) and spent notably less time consuming the reward compared to both CTR and HFHS rats (Fig 1D, Diet: $F_{(2,28)}$=29.791, p<0.001, Test: $F_{(2,56)}$=28.911, p<0.001, Diet*Test: $F_{(4,56)}$=4.754, p=0.002) during the reward phase. This disparity was not evident from the initial exposure to the food reward, when the rats were not yet familiar with the test setup, but became apparent in the subsequent tests when HFHS and CTR rats increased their time spent eating while CAF rats remained stable. (3rd test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, 5th test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, CTR: 1st vs 3rd test: p<0.001, 1st vs 5th test: p<0.001, HFHS: 1st vs 3rd test: p<0.001, 1st vs 5th test: p<0.001). Since CAF rats, in contrast to CTR and HFHS rats, were already familiar with the CAF reward after 6 weeks of diet, one could hypothesize that novelty might play a role in the differences between diet groups. However, this idea would be in contradiction with the concept of taste neophobia which would predict the opposite, i.e., longer latencies expected when novel foods are presented[102]. Still, to investigate the potential influence of novelty on reward consumption, we repeated the study with CTR and CAF rats and added one food reward test with a novel CAF reward (with different food products than present in their diet). Again, we found that CAF rats spent less time eating a novel CAF reward compared to CTR rats (Fig 1E, *t*-test: *t*=-8.78, p<0.001). This, along with the observation that HFHS and CTR rats did not reduce their CAF consumption over subsequent reward tests, suggests that the increased interest for the CAF reward of CTR and HFHS rats compared to CAF rats was not caused simply by the novelty of food items.

When eating-related behaviors were explored in more detail, we found that the latency to approach the food rewards was the same for CAF, HFHS and CTR rats in all tests (Fig 1F, 5th test, Diet: $F_{(2,28)}$=0.987, p=0.385, Test: $F_{(2,56)}$=0.733, p=0.485, Diet*Test: $F_{(4,56)}$=0.786, p=0.539). However, once the reward was explored, the CAF rats spent less time sniffing and approaching the food (appetitive behavior, Fig 1H, 5th test, Diet: $F_{(2,28)}$=16.296, p<0.001, Test: $F_{(2,56)}$=6.666, p=0.003, Diet*Test: $F_{(4,56)}$=3.010, p=0.026, post-hoc: 3rd test: CAF vs HFHS: p=0.002, CAF vs CTR: p<0.001, 5th test: CAF vs

HFHS: p=0.003, CAF vs CTR: p<0.001, CTR: 1st vs 3rd test: p<0.001, 1st vs 5th test: p=0.074, HFHS: 1st vs 3rd test: p=0.033). Similarly, a decrease in appetitive behaviors was found in CAF rats across tests, while CTR rats increased their number of appetitive behaviors (Fig S1B, Diet: $F_{(2,28)}$=12.382, p<0.001, Test: $F_{(2,56)}$=4.381, p=0.017, Diet*Test: $F_{(4,56)}$=11.795, p<0.001, post-hoc: 3rd test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, 5th test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, HFHS vs CTR: p=0.051, CAF: 1st vs 3rd test: p=0.024, 1st vs 5th test: p<0.001, CTR: 1st vs 3rd test: p=0.001, 1st vs 5th test: p<0.001, HFHS: 1st vs 3rd test: p<0.001). Overall, this suggests that CAF diet exposure induces a reduction in interest toward the CAF reward.

In contrast to more traditional conditioning tests with limited food exposure, providing the rats with continuous free access to the reward allowed us to perform a more sophisticated behavioral assessment in which we divided the eating episodes into distinct eating bouts to study the temporal patterning of behavior (similar to our previously used sexual bout analysis for analyzing sexual behavior[103-106]). Here, we defined an eating bout as a sequence of eating episodes that are not interrupted by non-reward related behaviors (Fig 1G). As soon as attention was withdrawn from the food reward (e.g., exploring the environment, self-grooming, or turning the head away from reward), an eating bout would end at the last eating behavior. The next eating bout would start with the next eating behavior. Our findings revealed that CAF rats divided their eating episodes into fewer and shorter eating bouts (frequency: Fig 1I and Fig S1C, Diet: $F_{(2,28)}$=17.893, p<0.001, Test: $F_{(2,56)}$=26.634, p<0.001, Diet*Test: $F_{(4,56)}$=5.287, p<0.001, post-hoc: 3rd test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, 5th test: CAF vs HFHS: p<0.001, CAF vs CTR: p<0.001, CTR: 1st vs 3rd test: p<0.001, 1st vs 5th test: p<0.001, HFHS: 1st vs 3rd test: p<0.001; mean duration: Fig 1J and Fig S1D, Diet: $F_{(2,27.981)}$=7.622, p=0.002, Test: $F_{(2,54.709)}$=5.478, p=0.007, Diet*Test: $F_{(4, 54.709)}$=1.321, p=0.274, post-hoc: 3rd test: CAF vs CTR: p=0.023, 5th test: CAF vs HFHS: p=0.029, CAF vs CTR: p=0.029, CTR: 1st vs 3rd test: p=0.016, 1st vs 5th test: p=0.005). Instead, the time-outs between the eating bouts were longer (Fig 1K and Fig S1E, Diet: $F_{(2,27.327)}$=8.978, p=0.001, Test: $F_{(2,53.157)}$=8.354, p<0.001, Diet*Test: $F_{(4, 53.141)}$=0.492, p=0.741, post-hoc: 5th test: CAF vs HFHS: p=0.005, CAF vs CTR: p=0.003, HFHS: 1st vs 3rd test: p=0.026). This indicates that the reduced time spent eating coincides with a decreased number and duration of eating bouts. Interestingly, no such effects were found in rats exposed to HFHS diet, suggesting that long-term CAF diet is a stronger manipulation than HFHS diet.

The observed changes in behaviors during the reward phase were not attributable to differences in baseline activity. All rats spent the same amount of time exploring the chamber (Fig S1F, Diet: $F_{(2,28)}$=1.705, p=0.200, Test: $F_{(2,56)}$=6.395, p=0.003, Diet*Test: $F_{(4, 56)}$=0.879, p=0.483, post-hoc: CAF: 1st vs 5th test: p=0.031, CTR: 1st vs 5th test: p=0.050) and engaging in grooming activities (Fig S1G, Diet: $F_{(2,28)}$=0.264, p=0.770, Test: $F_{(2,56)}$=0.268, p=0.766, Diet*Test: $F_{(4, 56)}$=0.801, p=0.529). Similarly, no

differences in behavior were found during the pre-reward phase in which the reward was presented in the opposite chamber while the door remained closed. CAF rats did not spend more or less time on behaviors indicating interest in the reward compared to HFHS and CTR rats (Fig 1L, Diet: $F_{(2,28)}$=3.195, p=0.056, Test: $F_{(2,56)}$=10.240, p<0.001, Diet*Test: $F_{(4, 56)}$=1.186, p=0.327, post-hoc: CTR: 1st vs 5th test: p=0.008, HFHS: 3rd vs 5th test: p=0.028), such as exploring the door or orienting themselves toward the reward chamber. However, as expected, all rats spent approximately twice as much time engaging in these behaviors during the pre-reward phase compared to the baseline phase when no reward was present behind the doors (Fig 1L compared to Fig S1H, Diet: $F_{(2,28)}$=1.622, p=0.216, Test: $F_{(2,56)}$=2.737, p=0.073, Diet*Test: $F_{(4, 56)}$=0.357, p=0.838).

Altogether, these findings suggests that long-term exposure to a CAF diet alters both appetitive and consummatory behavioral responses to a food reward, while leaving anticipatory behaviors and reward-unrelated behaviors unaffected. Interestingly, long-term exposure to a HFHS diet did not produce the same effects and resulted in comparable levels of behavioral interest in the food reward as observed in CTR rats.

2.2 Long-term CAF diet exposure reduces VTA activity in response to food rewards

To investigate the impact of long-term CAF diet exposure on the neural activity patterns of the VTA during these reward responses, all rats underwent stereotaxic surgery before the experiment to induce GCaMP6s expression in VTA neurons (Fig 2A). Following a one-week recovery period, the rats were placed on their respective diets for six weeks, after which behavioral testing commenced.

First, we explored the effects of long-term CAF diet exposure on the VTA in response to cues that were paired with access to the rewards. During the first food reward test, when the rats were not yet familiar with the test setup, an increase in VTA neuronal activity was found in all rats in response to a light cue signaling the imminent opening of the doors after a 10-second delay (Fig 2B). In contrast, the actual opening of the doors (allowing access to the CAF food reward) did not elicit an activity change in the VTA. However, heatmap analysis of these trials per rat showed that the increase in light cue associated VTA activity was only observed in a selection of rats, and differed across dietary groups (Fig 2B). As the rats became more familiar with the behavioral reward test and associated the light cue with gaining access to the food reward (on the 5th test day with a food reward), a surge in neural activity in the VTA was observed in CTR rats (Fig 2C), whereas only a subset of HFHS and CAF rats displayed a similar response. Further analysis of the area under the curve (AUC) revealed that CAF rats, but not HFHS rats, displayed an attenuated response compared to CTR rats (Fig 2D, Light: Diet: $F_{(2,34)}$=4.591, p=0.017, Epoch: $F_{(1,34)}$=38.576, p<0.001, Diet*Epoch: $F_{(2, 34)}$=2.976, p=0.064, post-hoc: Pre-light vs Light: CTR: p<0.001, CAF: p=0.026, HFHS: p=0.002, Light:

CAF vs CTR: p=0.003, Door: Diet: $F_{(2,34)}$=3.364, p=0.046, Epoch: $F_{(1,34)}$=1.071, p=0.308, Diet*Epoch: $F_{(2,34)}$=0.023, p=0.977).

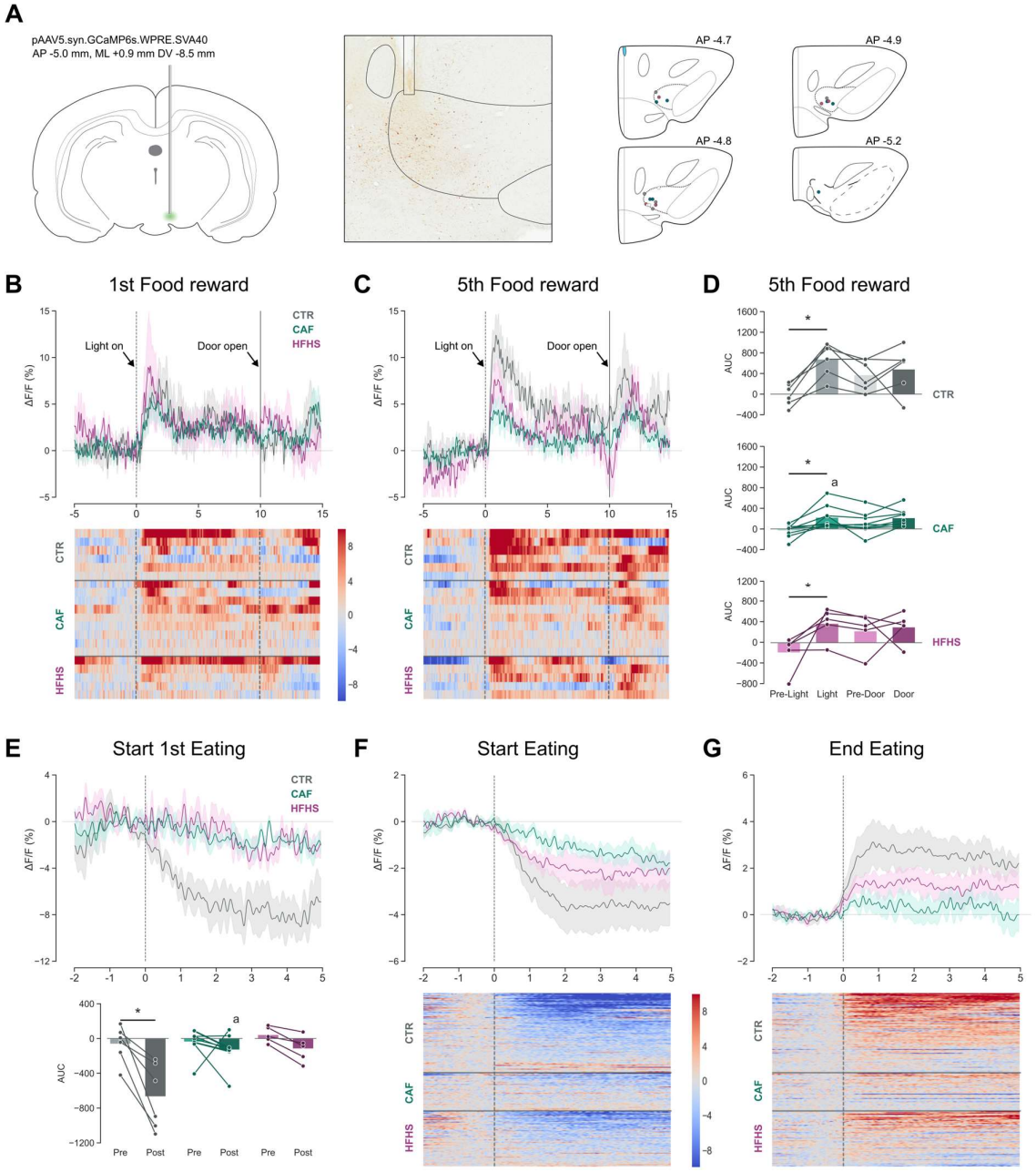

Figure 2. The effects of Cafeteria, High fat high sugar, and control diet on the ventral tegmental area (VTA) during a food reward test. A**)** Schematic representation of surgical details and histologically determined fiber locations and AAV vector expression in the VTA. **B)** Upper panel: group data from CTR, CAF and HFHS rats of the 1st food reward test showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Shaded areas represent SEM. Lower panel: heat maps for the 1st food reward test of all CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Trials are sorted by largest responses. **C)** Upper panel: group data from CTR, CAF and HFHS rats of the 5th food reward test showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Shaded areas represent SEM. Lower panel: heat maps for the 5th food reward test of all CTR, CAF and HFHS

rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Trials are sorted by largest responses. **D)** AUC for the 5th food reward test of CTR, CAF and HFHS rats before (pre-Light) and after (Light) the light cue, and before (Pre-door) and after (Door) the door opening. **E)** Upper panel: Group data from CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the start of eating episodes of the 1st eating in the 5th food reward test). Shaded areas represent SEM. Lower panel: AUC of CTR, CAF and HFHS rats before (pre) and after (post) the start of the very first eating episodes in the 5th reward test with a food reward. **F)** Upper panel: group data from CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the start of eating episodes of all food reward tests). Shaded areas represent SEM. Lower panel: heat maps for each eating episode of all CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the start of eating episodes of all food reward tests). Trials are sorted by largest responses. **G)** Upper panel: group data from CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the end of eating episodes of all food reward tests). Shaded areas represent SEM. Lower panel: heat maps for each eating episode of all CTR, CAF and HFHS rats showing mean GCaMP signals (ΔF/F in percentages, calculated from fluorescent changes aligned to the end of eating episodes of all food reward tests). Trials are sorted by largest responses. **All)** Figures show rats on control (CTR, grey color bars and lines), cafeteria (CAF, green color bars and lines), and high-fat, high-sugar pellet (HFHS, purple color bars and lines) diet. CTR n=6, CAF n=9, HFHS n=5. * $p<0.05$ compared to by line connected group. [a] $p<0.05$ compared to same bar of CTR.

Since the rats had unrestricted access to the food reward, we were able to examine neural responses in the VTA during the specific behaviors. Analysis revealed that CTR rats show a significant reduction in VTA activity (relative to baseline) upon the initial consumption of the reward (Fig 2E, AUC: Diet: $F_{(2,32)}=3.399$, $p=0.046$, Epoch: $F_{(1,32)}=7.074$, $p=0.012$, Diet*Epoch: $F_{(2, 32)}=2.762$, $p=0.078$, post-hoc: Pre vs Post: CTR: $p=0.002$, Post: CAF vs CTR: $p=0.005$). In contrast, long-term exposure to HFHS and CAF was not associated with any change in activity upon this 1st eating episode. Furthermore, when assessing VTA responses to all eating episodes across all tests, rats exposed to the CAF diet displayed a pronounced attenuation of ΔF/F event-related transients in the VTA compared to CTR rats, while HFHS rats had an intermediate response (Fig 2F and S2A, AUC: Diet: $F_{(2,32)}=2.956$, $p=0.066$, Epoch: $F_{(1,32)}= 31.102$, $p<0.001$, Diet*Epoch: $F_{(2, 32)}=2.778$, $p=0.076$, post-hoc: Pre vs Post: CTR: $p<0.001$, HFHS: $p=0.010$, CAF: $p=0.045$, Post: CAF vs CTR: $p=0.005$).

Moreover, the decrease in VTA activity appears to be sustained for the duration of eating episodes. When aligning the fiber photometry signal to the end of each episode of eating, a return to baseline was evident in CTR rats (Fig 2G and S2B, shown as an increase due to ΔF/F normalization to baseline at t = 0, AUC: Diet: $F_{(2,32)}=0.723$, $p=0.493$, Epoch: $F_{(1,32)}= 0.685$, $p=0.414$, Diet*Epoch: $F_{(2, 32)}=0.535$, $p=0.591$). The alterations in VTA neural activity upon eating persisted throughout the entirety of the behavioral reward test. CTR rats consistently displayed activity decreases in the VTA upon eating during the initial, middle, and final five-minute intervals of the test (Fig S2C), whereas CAF rats demonstrated an attenuated response in all intervals. This effect was also reflected in the AUC analysis, with CAF rats showing a significant attenuation compared to CTR rats in all test intervals (Fig S2D, AUC: Diet: $F_{(2,100)}=7.104$, $p=0.001$, Epoch: $F_{(1,100)}= 85.933$, $p<0.001$, Part: $F_{(2,100)}= 0.644$, $p=0.528$, Diet*Epoch*Part: $F_{(12, 100)}=1.429$, $p=0.165$, post-hoc: pre vs post: CTR: part 1: $p<0.001$, part

2: p<0.001, part 3: p<0.001, HFHS: part 1: p=0.014, part 2: p=0.006, part 3: p=0.021, CAF: part 1: p=0.008, part 2: p=0.041, Post: part 1: CAF vs CTR: p=0.011, HFHS vs CTR: p=0.040, part 2: CAF vs CTR: p=0.004, part 3: CAF vs CTR: p=0.022).

We also analyzed changes in neural activity during appetitive behaviors such as sniffing. While CTR- and HFHS-rats demonstrated an elevation in ΔF/F at the onset of a sniffing episode, in comparison, the CAF-exposed rats showed attenuated activity (Fig S2E), resulting in a lack of effect on the AUC post versus pre sniffing (Fig S2F, AUC: Diet: $F_{(2,34)}$=0.717, p=0.496, Epoch: $F_{(1,34)}$= 13.456, p<0.001, Diet*Epoch: $F_{(2, 34)}$=0.706, p=0.500, post-hoc: Pre vs Post: CTR: p=0.012, HFHS: p=0.042).

Our findings suggest that prolonged exposure to HFHS and CAF diets significantly impairs the VTA's response to food rewards and predictive cues associated with food. Typically, an elevation in VTA activity reflects the perceived value of a cue associated with a reward. However, this response is compromised in rats consuming HFHS or CAF diets, suggesting that the value of food rewards might be diminished in these groups. Moreover, long-term CAF diet intake diminishes VTA responsiveness to both the anticipation and consumption of food rewards more profoundly compared to exposure to a HFHS diet. This underscores that the *choice of palatable food*, in combination with fat and sugar content (see Table S3-S5), is a major contributor to disrupting the neural mechanisms governing rewarding behaviors.

2.3 Long-term CAF diet also affects VTA neural responses to other natural rewards

Next, we investigated whether long-term CAF diet consumption would influence both neural and behavioral responses to a natural reward other than food. To address this, we conducted three additional behavioral reward tests, this time with a sexual partner (a sexually experienced male rat) as reward. One limitation of using naturally cycling females in the study is the potential occurrence of pseudopregnancies following copulation sessions. In the event of pseudopregnancy, female rats are unable to copulate again for approximately two weeks. To mitigate this issue, we conducted all three sexual reward tests on a single day. Regrettably, not all females reached the proestrus phase and these females were excluded from the analysis. Notably, only six out of ten females of the HFHS group reached proestrus, and two of these females did not engage in copulation with the stimulus male. Due to these insufficient copulation occurrences in the HFHS group, in addition to the observation that HFHS did not differ greatly from CTR in food reward responses, we proceeded to analyze only the CTR and CAF rats. In the CAF group, ten females reached the proestrus phase, but three of them did not exhibit any paracopulatory behaviors during the first sexual reward test. It is possible that CAF diet consumption delays the onset of the receptive phase in naturally cycling females. To be able to make proper inter-rat comparisons, we analyzed the photometry and

behavioral data from the most successful sexual reward test. The *best sexual reward test* was defined as the test (either the first, second, or third) in which the female displayed the highest number of paracopulatory behaviors.

First, we looked at the VTA response evoked by the light cue that preceded the opening of the door. Interestingly, as in the test with food reward, the light cue elicited a more pronounced increase in neural activity in CTR rats compared to CAF rats (Fig 3A). While the AUC increased significantly after the light cue compared to before the cue in CTR rats , no such effect was found in CAF rats (Fig 3B, Light: Diet: $F_{(2,24)}$=2.085, p=0.162, Epoch: $F_{(1,24)}$=14.409, p<0.001, Diet*Epoch: $F_{(2, 24)}$=2.597, p=0.120, post-hoc: Pre-light vs Light: CTR: p=0.002, Light: CAF vs CTR: p=0.041, Door: Diet: $F_{(2,24)}$=3.307, p=0.081, Epoch: $F_{(1,24)}$=2.454, p=0.130, Diet*Epoch: $F_{(2, 24)}$=0.074, p=0.788)).

In addition, we explored activity patterns of the VTA during sexual behaviors. Female rats show paracopulatory behaviors that can be distinguished in hops and darts [107-109]. When the start of a dart (as the moment the female started running ending in the typical dart position) was aligned to the GCaMP signaling, we found an increase in VTA activity upon the start of dart in CTR rats. CAF rats, on the other hand, showed an attenuated VTA response upon darting (Fig 3C/D, AUC: Diet: $F_{(1,22)}$=2.310, p=0.143, Epoch: $F_{(1,22)}$=15.669, p<0.001, Diet*Epoch: $F_{(1,22)}$=2.107, p=0.161, post-hoc: Pre vs Post: CTR: p=0.001, CAF vs CTR: Post: p=0.047). With regards to the short hops, no such effects were found in the VTA (Fig 3E, AUC: Diet: $F_{(1,20)}$=3.201, p=0.089, Epoch: $F_{(1,20)}$=0.246, p=0.625, Diet*Epoch: $F_{(1,20)}$=0.392, p=0.539).

Behaviorally, however, no differences were observed during the sexual reward test. CAF rats exhibited a comparable number of darts (Fig 3G, *t*=0.282, p=0.781) and hops (Fig 3H, *t*=1.088, p=0.291) as CTR rats in the reward test. Moreover, when the micropatterns of sexual bouts were assessed (Fig 3F), no differences were found in the number of sexual bouts (Fig 3I, *t*=0.733, p=0.473), the mean duration of sexual bouts (Fig 3J, *t*=0.576, p=0.572), or the mean duration of the time-outs (Fig 3K, *t*=0.392, p=0.700). Consequently, CAF females received a similar number of copulations from male partners as CTR females (Fig 3KL, *t*=-0.371, p=0.715), even when the number of mounts, intromissions, and ejaculations were considered separately (data not shown).

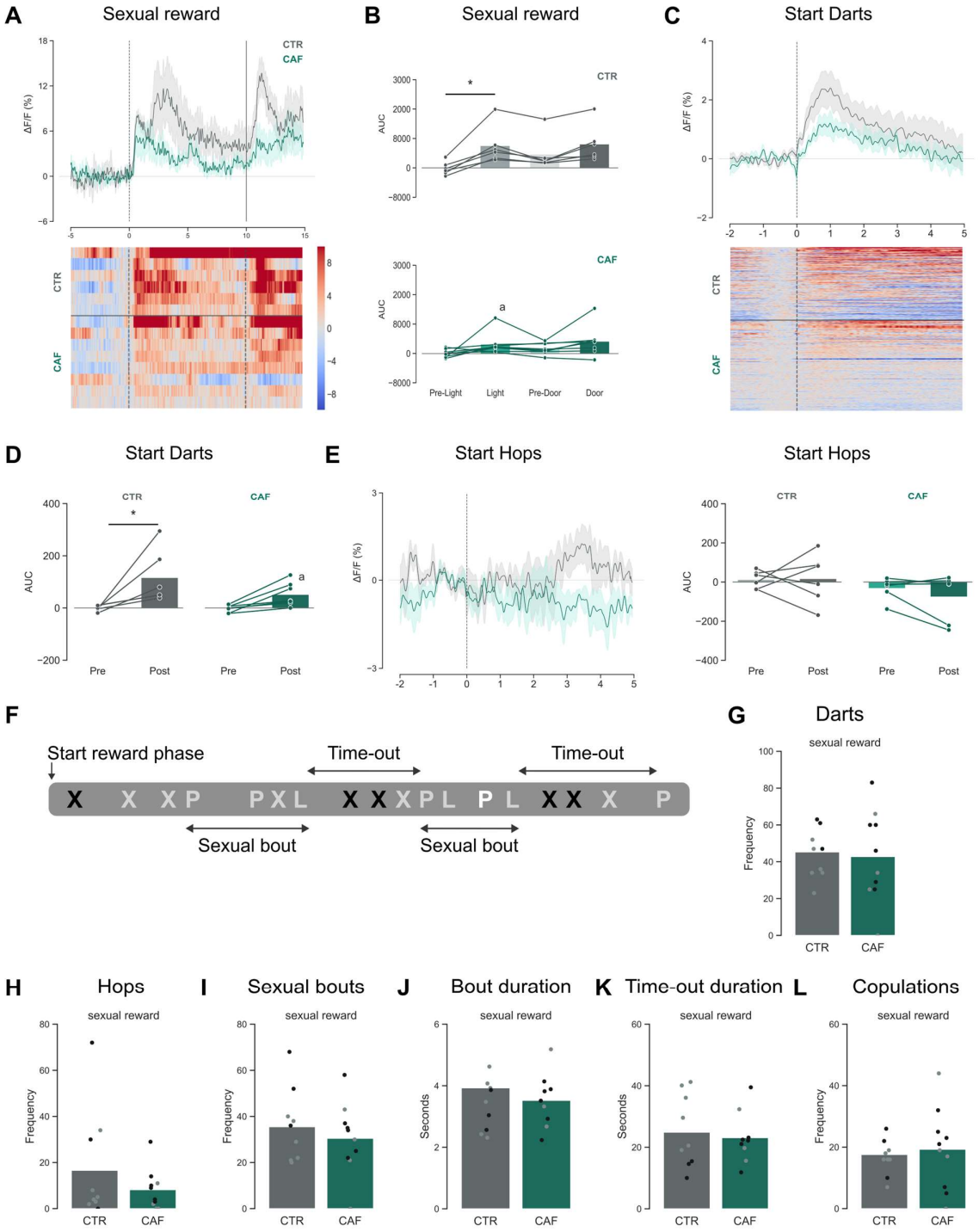

Figure 3. The effects of Cafeteria diet on the ventral tegmental area and behavioral responses during a sexual reward test. **A)** Upper panel: Group data from CTR and CAF rats of the best sexual reward test showing ΔF/F percentages calculated from fluorescent changes aligned to the light cue at t=0. At t=10, the door opened allowing access to the reward. Solid lines indicate mean. Shaded area represents SEM. The best test was defined by the reward test (1st, 2nd or 3rd) in which the female had most paracopulatory behaviors. Lower panel: heat maps for best sexual reward test of CTR and CAF rats, showing ΔF/F percentages calculated from fluorescent changes aligned to the light cue at t=0. At t=10, the door opened allowing access to the reward. Trials are sorted by largest responses. **B)** AUC for the best sexual reward test of CTR (upper) and CAF (lower) rats before (pre-Light) and after (Light) the light cue, and before (Pre-door) and after (Door) the door opening. **C)** Upper panel: Group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages) calculated

from fluorescent changes aligned to the start of darting behaviors in the best sexual reward test. Shaded areas represent SEM. Lower panel: Heat maps for each dart episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of darting behaviors of best sexual reward tests). Trials are sorted by largest responses. **D)** AUC of CTR and CAF rats before (pre) and after (post) the start of darting behaviors of best sexual reward tests. **E)** Left panel: Group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages) calculated from fluorescent changes aligned to the start of hopping behaviors in the best sexual reward test. Shaded areas represent SEM. Right panel: AUC of CTR and CAF rats before (pre) and after (post) the start of hopping behaviors of best sexual reward tests. **F)** Schematic overview of sexual bouts. A sexual bout is defined as a sequence of paracopulatory behaviors (P) and lordosis responses (L), with or without reward-related behaviors (X in white) in between, that is uninterrupted by non-reward related behaviors (X in black). As soon as the attention withdraws from the male rat, a sexual bout ends with the last paracopulatory or lordosis behavior, and the next bout starts with the next paracopulatory or lordosis behavior. **G)** Total number of darts during the reward phase of the best sexual reward test. **H)** Total number of hops during the reward phase of the best sexual reward test**. I)** The total number of sexual bouts during the reward phase of the best sexual reward test. **J)** Mean duration of a sexual bout during the reward phase of the best sexual reward test. **K)** Mean duration of time-outs during the reward phase of the best sexual reward test. **L)** Total number of received copulations (mounts, intromissions and ejaculations) during the reward phase of the best sexual reward test. **ALL)** Figures show rats on control (CTR, grey color bars and lines) and cafeteria (CAF, green color bars and lines) diet. CTR n=6, CAF n=8 for fiber photometry data and CTR n= 10, CAF n=9 for behavioral data. The dots in the behavioral data have colors representing their body weight category (grey: lighter than average, black: heavier than average) * $p<0.05$ compared to by line connected group. [a] $p<0.05$ compared to same bar of CTR.

While paracopulatory behavior (darts and hops) is often defined as appetitive or proceptive behavior, lordosis is considered a consummatory or receptive behavior. Our data revealed that long-term exposure to CAF diet did not alter the receptive responses toward a sexual reward, resulting in similar levels of lordosis quotient (Fig 4A, t=-0.425, p=0.676) and lordosis score (Fig 4B, t=0.778, p=0.447) in CTR and CAF rats. Similarly, when we explored the role of the VTA during these behaviors we found no change in VTA activity upon the display of a lordosis behavior (Fig 4C). However, lordosis behavior is usually reflexive and triggered by a touch from the male. It is displayed throughout a copulation behavior, with considerable variability in both duration and quality (also quantified as a lordosis score[110]). Because these factors could be relevant for analysis of activation patterns of the VTA, we classified the lordosis behaviors based on intensity and type of stimulation when linking these behavioral epochs with the fiber photometry data. It is important to mention that the fiber photometry signals are normalized for baseline at t = 0. As a consequence, signals that appear as an activity increase, may be a rebound from a period of decreased activity. In any case, we found that changes in neural activity in the VTA corresponded best with the end of lordosis responses and that only high intensity lordoses result in increases in VTA activity (Fig 4D), although no significant effects were detected in the AUC due to substantial response variability (Fig 4D, AUC: Diet: $F_{(1,12)}$=0.001, p=0.971, Epoch: $F_{(1,12)}$=0.128, p=0.727, Diet*Epoch: $F_{(1,12)}$=0.085, p=0.776). Low intensity lordosis responses, on the other hand, fail to elicit changes in VTA activity (Fig 4E, AUC: Diet: $F_{(1,18)}$=0.289, p=0.598, Epoch: $F_{(1,18)}$=0.022, p=0.884, Diet*Epoch: $F_{(1,18)}$=0.618, p=0.442). Overall, this suggests that the VTA does not encode the onset of a lordosis behavior, but rather responds to the received stimulation.

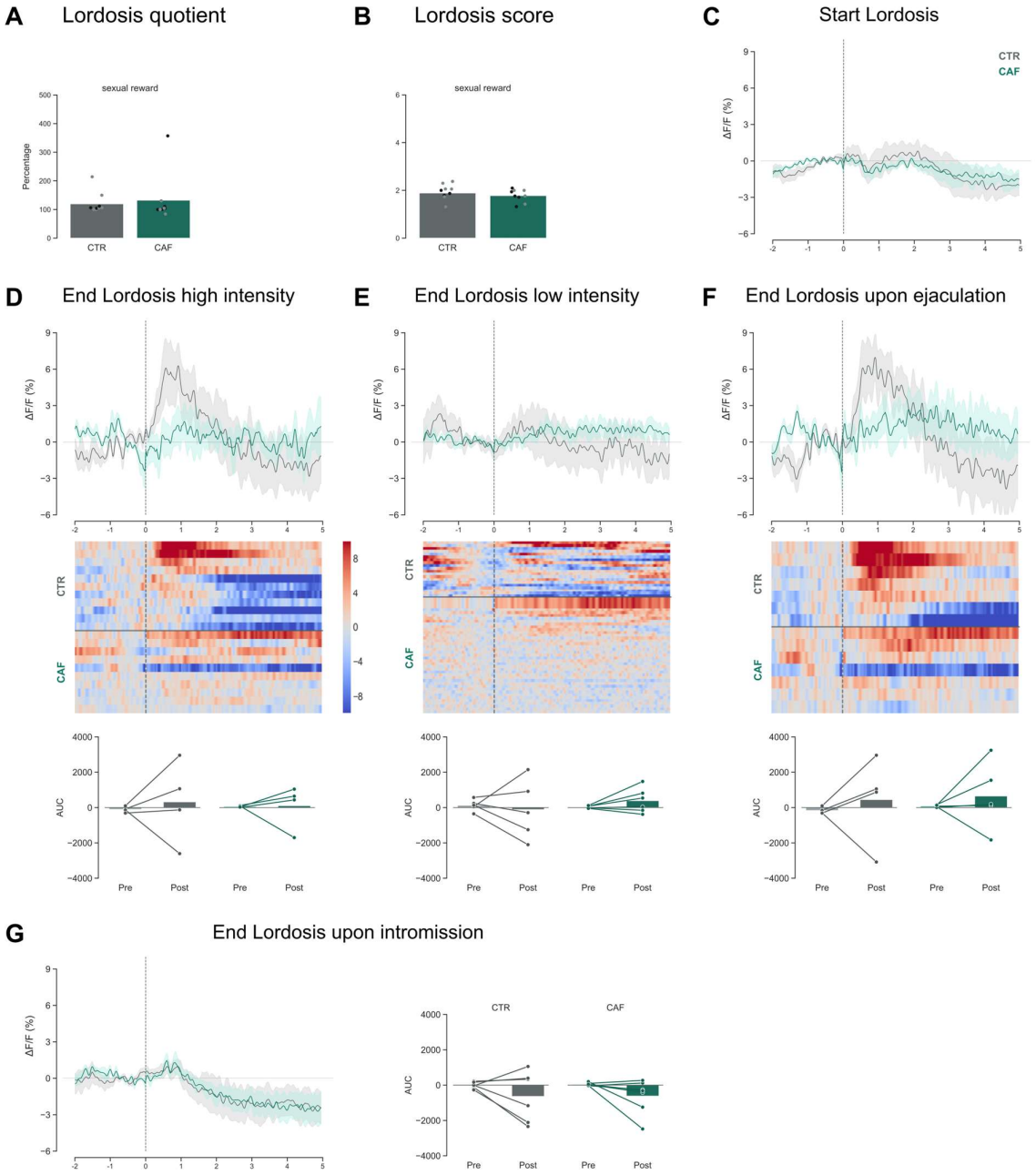

Figure 4. The effects of Cafeteria diet on the ventral tegmental area and lordosis responses during a sexual reward test. **A)** Lordosis quotient during the reward phase of the best sexual reward test. The lordosis quotient was calculated by the sum of lordosis responses (score 1-3) divided by the number of received copulations (mounts, intromissions and ejaculations), multiplied by 100%. **B)** Lordosis score during the reward phase of the best sexual reward test. The lordosis score was calculated by multiplying the quality of the score (0-3 with 0 for no lordosis and 3 for the most intense lordosis) by the number of times it was performed, divided by the total number of lordosis responses. **C)** Group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of lordosis behaviors in the best sexual reward test). Shaded areas represent SEM. **D)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of high-intensity lordosis behavior in the best sexual reward test). Shaded areas represent SEM. Middle panel: heat maps for each lordosis behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the high-intensity lordosis behavior of best sexual reward tests). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the end of high-intensity lordosis behaviors. **E)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent

changes aligned to the end of low-intensity lordosis behavior in the best sexual reward test). Shaded areas represent SEM. Middle panel: heat maps for each lordosis behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the low-intensity lordosis behavior of best sexual reward tests). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the end of low-intensity lordosis behavior of best sexual reward tests. **F)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of lordosis behaviors upon ejaculation in the best sexual reward test). Shaded areas represent SEM. Middle panel: heat maps for each lordosis behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the lordosis behaviors upon ejaculations of best sexual reward tests). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the end of lordosis behaviors upon ejaculation of best sexual reward tests. **G)** Left panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of lordosis behaviors upon intromission in the best sexual reward test). Shaded areas represent SEM. Right panel: AUC of CTR and CAF rats before (pre) and after (post) the end of lordosis behaviors upon intromission of best sexual reward tests. **All)** Figures show rats on control (CTR, grey color bars and lines) and cafeteria (CAF, green color bars and lines) diet. CTR n=6, CAF n=8. * $p<0.05$ compared to by line connected group. [a] $p<0.05$ compared to same bar of CTR.

When we analyzed the photometry data based on the type of copulation the female received, we found an increase in VTA activity after receiving an ejaculation in CTR females (Fig 4F). However, this increase failed to materialize as a significant change in the AUC metric, due to substantial variability of the responses, even when the AUC analysis was limited to the first two seconds post-lordosis (Fig 4F, AUC: Diet: $F_{(1,14)}=0.096$, $p=0.761$, Epoch: $F_{(1,14)}=0.654$, $p=0.432$, Diet*Epoch: $F_{(1,14)}=0.001$, $p=0.972$). Interestingly, CAF rats did not show this activity increase, once again demonstrating an attenuated VTA response. Additionally, no changes in activity levels were observed following a lordosis response to an intromission (Fig 4G). Instead, both CTR and CAF rats exhibited a decrease in neural activity, although this was not reflected in the AUC (Diet: $F_{(1,22)}=0.011$, $p=0.916$, Epoch: $F_{(1,22)}=3.729$, $p=0.066$, Diet*Epoch: $F_{(1,22)}=0.002$, $p=0.963$).

In summary, our findings indicate a role for the VTA in responses to both food and sexual rewards. While long-term exposure to junk food had a profound behavioral effect on the response to food rewards, normal levels of behavior were found upon a sexual reward. Still, rats exposed to CAF diet showed an attenuated VTA activity in response to both a food and sexual reward.

2.4 Two weeks of standard chow diet is not enough to reverse the CAF-induced changes in response to food rewards.

To assess the reversibility of neural and behavioral alterations induced by long-term exposure to CAF diet, all rats were, after their last behavioral test sequence, transitioned to a standard chow diet for a period of two weeks. This was followed by three iterations of the behavioral reward test with a food stimulus (CAF) and one instance with a sexual stimulus (Fig 5A). We observed that for CAF-exposed rats the two-week period of dietary reversal was insufficient to restore the attenuated VTA

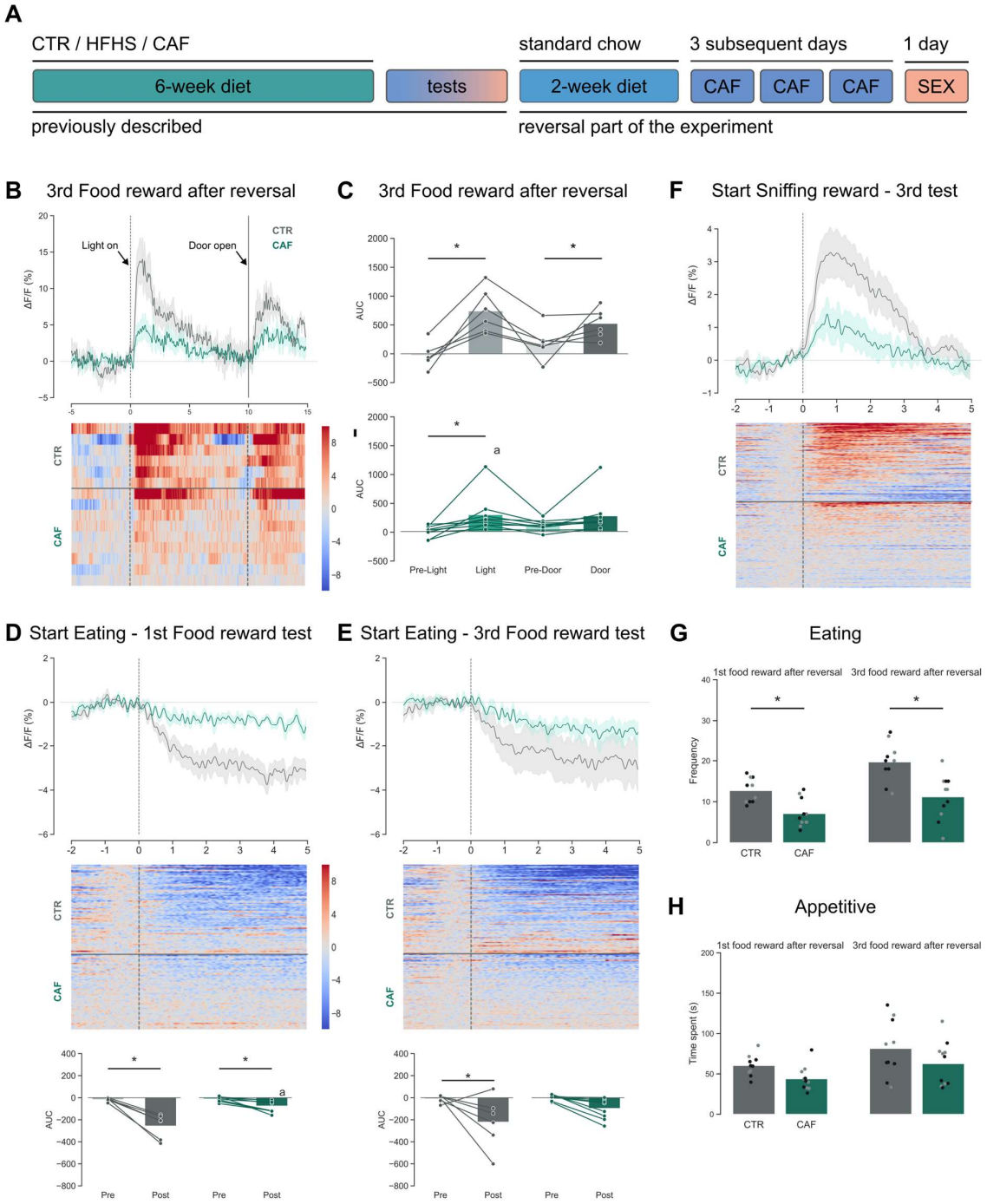

Figure 5. The effects of reversal diet after Cafeteria diet exposure on the ventral tegmental area and behavioral responses during a food reward test **A)** Schematic representation of the experimental design. After the female rats received 6 weeks of either a control (CTR), or cafeteria (CAF) diet, they were tested five times with a food reward (CAF reward) and 3 times with a sexual reward (male rat). All rats were placed on a reversal diet of standard chow for 2 weeks. Next, they were tested three more time with a food reward and once with a sexual reward in the behavioral reward test. **B)** Upper panel: group data from CTR and CAF rats of the 3rd food reward test post-reversal diet showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Shaded areas represent SEM. Lower panel: heat maps for the 3rd food reward test post-reversal diet of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Trials are sorted by largest responses. **C)** AUC from CTR (upper) and CAF (lower) for the 3rd food reward test post-reversal diet before (pre-Light) and after (Light) the

light cue, and before (Pre-door) and after (Door) the door opening. **D)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of eating in the 1st food reward test post-reversal diet). Shaded areas represent SEM. Middle panel: heat maps for each eating behavior episode of all CTR and CAF rats, showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of eating in the 1st food reward test post-reversal diet). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the start of eating in the 1st food reward test post-reversal diet. **E)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of eating in the 3rd food reward test post-reversal diet). Shaded areas represent SEM. Middle panel: heat maps for each eating behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of eating in the 3rd food reward test post-reversal diet). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the start of eating in the 1st food reward test post-reversal diet. **F)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of sniffing the reward in the 3rd food reward test post-reversal diet). Shaded areas represent SEM. Lower panel: heat maps for each sniffing episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of sniffing in the 3rd food reward test post-reversal diet). Trials are sorted by largest responses. **G)** Total number of eating episodes of the food reward during the reward phase of the 3rd food reward test post-reversal diet. **H)** Time spent on appetitive behaviors towards the food reward during the reward phase of the 3rd food reward test post-reversal diet. Appetitive behaviors include approaching and sniffing the reward. **All)** Figures show rats on control (CTR, grey color bars and lines) and cafeteria (CAF, green color bars and lines) diet. CTR n=6, CAF n=9 for fiber photometry data and CTR n= 10, CAF n=11 for behavioral data. The dots in the behavioral data have colors representing their body weight category (grey: lighter than average, black: heavier than average) * $p<0.05$ compared to by line connected group. [a] $p<0.05$ compared to same bar of CTR.

responses elicited by the light cue associated with a food reward (Fig 5B/C, AUC: Light: Diet: $F_{(2,26)}=4.273$, $p=0.049$, Epoch: $F_{(1,26)}=27.659$, $p<0.001$, Diet*Epoch: $F_{(2, 26)}=5.794$, $p=0.023$, post-hoc: Pre-light vs Light: CTR: $p<0.001$, CAF: $p=0.033$, Light: CAF vs CTR: $p=0.004$, Door: Diet: $F_{(2,26)}=3.223$, $p=0.084$, Epoch: $F_{(1,26)}=7.192$, $p=0.013$, Diet*Epoch: $F_{(2, 26)}=0.839$, $p=0.368$, post-hoc: Pre-light vs Light: CTR: $p=0.028$). A similar lack of recovery was evident in the VTA neural responses during the behavioral interactions with the food reward. Specifically, CAF-exposed rats continued to exhibit attenuated neural activity responses during eating both in the 1st (Fig 5D) and 3rd food reward test (Fig 5E) post-reversal diet. The attenuated response persisted in CAF rats during the initial food reward test after dietary reversal, as demonstrated by a significant difference in AUC (Fig 5D, AUC: Diet: $F_{(1,26)}=17.511$, $p<0.001$, Epoch: $F_{(1,26)}=46.771$, $p<0.001$, Diet*Epoch: $F_{(1,26)}=15.952$, $p<0.001$, post-hoc: Pre vs Post: CTR: $p<0.001$, CAF: $p=0.033$, Post: CAF vs CTR: $p<0.001$), but this effect diminished by the third food reward test (Fig 5E, AUC: Diet: $F_{(1,26)}=2.484$, $p=0.127$, Epoch: $F_{(1,26)}=12.877$, $p=0.001$, Diet*Epoch: $F_{(1,26)}=1.678$, $p=0.207$, post-hoc: Pre vs Post: CTR: $p=0.004$), even though it remained visible in the heatmaps. These results suggest a persistent effect of long-term CAF diet on the VTA, which a two-week reversal diet cannot readily undo.

Interestingly, even though two weeks of healthy diet was not sufficient to restore VTA function, a partial behavioral recovery was observed. Analysis revealed that CAF rats still ate less frequently (Fig 5G, Diet: $F_{(2,19)}=18.799$, $p<0.001$, Test: $F_{(1,19)}=35.494$, $p<0.001$, Diet*Test: $F_{(1,19)}=2.442$, $p=0.135$, post-hoc: CAF vs CTR: 1st test: $p=0.006$, 3rd test: $p<0.001$, 1st vs 3rd test: CTR: $p<0.001$, CAF: $p=0.005$) and

have fewer eating bouts (Fig S5A, Diet: $F_{(2,19)}$=7.586, p=0.013, Test: $F_{(1,19)}$=6.038, p=0.024, Diet*Test: $F_{(1,19)}$=0.014, p=0.908, post-hoc: CAF vs CTR: 1st test: p=0.023, 3rd test: p=0.018) than the CTR in both the 1st and 3rd food reward test following the dietary reversal period. However, regarding time spent eating (Fig S5B, Diet: $F_{(2,19)}$=6.640, p=0.018, Test: $F_{(1,19)}$=26.290 p<0.001, Diet*Test: $F_{(1,19)}$=0.303, p=0.588, post-hoc: CAF vs CTR: 1st test: p=0.021, 1st vs 3rd test: CTR: p=0.005, CAF: p<0.001), or mean duration of time-outs (Fig S5C, Diet: $F_{(2,17.9)}$=6.009, p=0.025, Test: $F_{(1,17.309)}$=6.282 p=0.022, Diet*Test: $F_{(1,17.309)}$=0.578, p=0.457, post-hoc: CAF vs CTR: 1st test: p=0.017, 1st vs 3rd test: CAF: p=0.033), CAF rats displayed diminished levels compared to CTR rats only in the 1st food reward test, with no significant differences observed in the third test. Furthermore, consistent with pre-reversal observations, all rats spent an equal amount of time within an eating bout in all tests (the mean duration of eating bouts (Fig S5D, Diet: $F_{(2,19)}$=0.201, p=0.659, Test: $F_{(1,19)}$=19.547 p<0.001, Diet*Test: $F_{(1,19)}$=0.832, p=0.373, post-hoc: 1st vs 3rd test: CTR: p=0.025, CAF: p=0.001). Likewise, although behavioral indications suggested a recovery in appetitive behavior (approaching and sniffing the food reward) among CAF rats in the 1st and 3rd food reward test post-reversal (Fig 5H, Diet: $F_{(2,19)}$=4.016, p=0.060, Test: $F_{(1,19)}$=11.974, p=0.003, Diet*Test: $F_{(1,19)}$=0.008, p=0.931, post-hoc: 1st vs 3rd test: CTR: p=0.024, CAF: p=0.024), neural responses within the VTA remained attenuated compared to CTR rats at both the 1st (Fig 5E, AUC: Diet: $F_{(1,26)}$=17.511, p<0.001, Epoch: $F_{(1,26)}$=46.771, p<0.001, Diet*Epoch: $F_{(1,26)}$=15.952, p<0.001, post-hoc: Pre vs Post: CTR: p<0.001, CAF: p=0.033, Post: CAF vs CTR: p<0.001) and 3rd food reward test (Fig 5F, AUC: Diet: $F_{(1,26)}$=2.484, p=0.127, Epoch: $F_{(1,26)}$=12.877, p=0.001, Diet*Epoch: $F_{(1,26)}$=1.678, p=0.207, post-hoc: Pre vs Post: CTR: p=0.004).

Altogether, our findings suggest that a two-week period of healthy dietary intervention is insufficient to fully restore the immediate behavioral or neural responses to a CAF food reward. However, it does partially improve behavioral effects after repeated exposures, *despite* persistent reduced responsiveness in the VTA.

2.5 Two weeks of standard chow diet inadequate in restoring the CAF-induced changes to sexual rewards.

Lastly, following a two-week exposure to a healthy diet, the rats underwent another sexual reward test. Once more, CAF-exposed rats exhibited no recovery in VTA responsiveness to the light-cue, maintaining an attenuated response compared to control rats (Fig 6A/B, AUC: Light-cue: Diet: $F_{(1,22)}$=1.863, p=0.186, Epoch: $F_{(1,22)}$=11.247, p=0.003, Diet*Epoch: $F_{(1, 22)}$=1.787, p=0.195, post-hoc: Pre-light vs Light: CTR: p=0.010, Door: Diet: $F_{(1,22)}$=0.013, p=0.911, Epoch: $F_{(1,22)}$=3.480, p=0.075, Diet*Epoch: $F_{(1,22)}$=0.007, p=0.933).

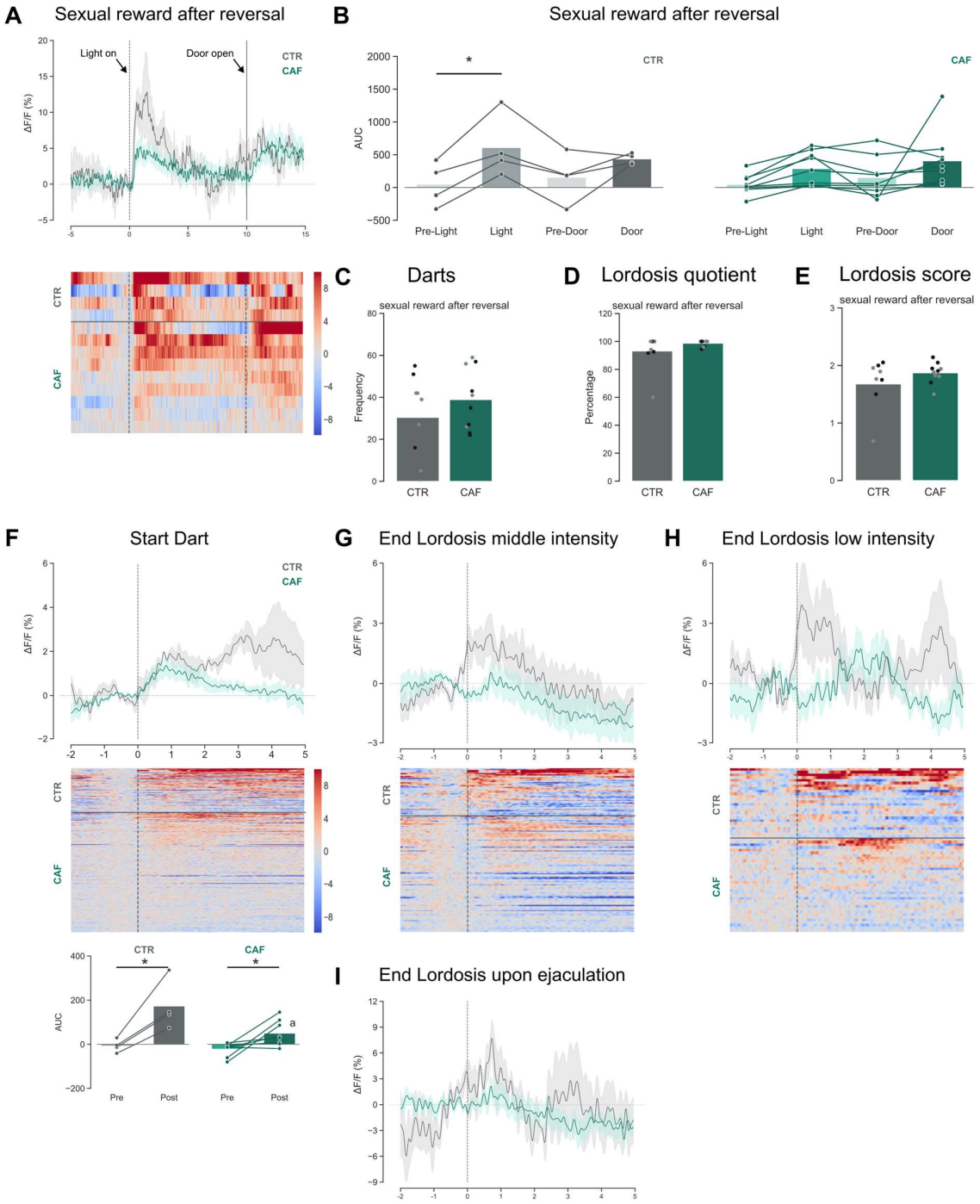

Figure 6. The effects of reversal diet after Cafeteria diet exposure on the ventral tegmental area and behavioral responses during a sexual reward test. **A)** Upper panel: group data from CTR and CAF rats of the sexual reward test post-reversal diet showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Solid lines indicate mean. Shaded area represents SEM. Lower panel: Heat maps for the sexual reward test post-reversal diet of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the light cue at t=0). At t=10s, the door opened allowing access to the reward. Trials are sorted by largest responses. **B)** AUC from CTR (left) and CAF (right) for the sexual reward test post-reversal diet, before (pre-Light) and after (Light) the light cue, and before (Pre-door) and after (Door) the door opening. **C)** Total number of darts during the reward phase of the sexual reward test after reversal diet. **D)** Lordosis quotient during the reward phase of during the sexual reward test after reversal diet. The lordosis quotient was calculated by the sum of

lordosis responses (score 1-3) divided by the number of received copulations (mounts, intromissions and ejaculations) multiplied by 100%. **E)** Lordosis score during the reward phase of the sexual reward test after reversal diet. The lordosis score was calculated by multiplying the quality of the score (0-3 with 0 for no lordosis and 3 for the most intense lordosis) by the number of times it was performed, divided by the total number of lordosis responses. **F)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the start of darting behaviors in the sexual reward test after reversal diet). Shaded areas represent SEM. Middle panel: heat maps for each darting episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the darting behaviors in the sexual reward test after reversal diet). Trials are sorted by largest responses. Lower panel: AUC of CTR and CAF rats before (pre) and after (post) the start of darting behaviors in the sexual reward test after reversal diet. **G)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of middle-intensity lordosis behavior in the sexual reward test after reversal diet). Shaded areas represent SEM. Lower panel: heat maps for each lordosis behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of middle-intensity lordosis behavior in the sexual reward test after reversal diet). Trials are sorted by largest responses. **H)** Upper panel: group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of low-intensity lordosis behavior in the sexual reward test after reversal diet). Shaded areas represent SEM. Lower panel: heat maps for each lordosis behavior episode of all CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of low-intensity lordosis behavior in the sexual reward test after reversal diet). Trials are sorted by largest responses. **I)** Group data from CTR and CAF rats showing mean GCaMP signals (ΔF/F in percentages calculated from fluorescent changes aligned to the end of lordosis behavior upon ejaculation in the sexual reward test after reversal diet). Shaded area represents SEM. **All)** Figures show rats on control (CTR, grey color bars and lines) and cafeteria (CAF, green color bars and lines) diet. CTR n=4, CAF n=9 for fiber photometry data and CTR n= 10, CAF n=9 for behavioral data. The dots in the behavioral data have colors representing their body weight category (grey: lighter than average, black: heavier than average) * $p<0.05$ compared to by line connected group. [a] $p<0.05$ compared to same bar of CTR.

Furthermore, the two weeks of healthy diet did not restore the VTA responsiveness in relation to sexual behavior. While there was still no difference in sexual behavior between CTR and CAF rats (Fig 6C-E, Darts: t=-1.131, p=0.273, Hops: t=-0.161, p=0.874 (data not shown), Lordosis quotient: t=-1.232, p=0.236, Lordosis score: t=-1.197, p=0.249), CAF rats still showed an attenuated VTA response upon darting behavior compared to CTR rats after reversal of diets (Fig 6F, AUC: Darting: Diet: $F_{(1,20)}$=7.066, p=0.015, Epoch: $F_{(1,20)}$=23.791, p<0.001, Diet*Epoch: $F_{(1,20)}$=4.414, p=0.049, post-hoc: Pre vs Post: CTR: p<0.001, CAF: p=0.026, CAF vs CTR: Post: p=0.003, Hopping (data not shown): Diet: $F_{(1,18)}$=0.215, p=0.649, Epoch: $F_{(1,18)}$=6.672, p=0.019, Diet*Epoch: $F_{(1,18)}$=1.274, p=0.274, post-hoc: Pre vs Post: CTR: p=0.032,).

Moreover, the data revealed that CAF rats continued to exhibit a reduced neural response in the VTA during lordosis episodes. While there was an insufficient trial number of the highest lordosis intensity in CTR rats for a robust comparison, the second-highest intensity (Fig 6G) and now also the low intensity of lordosis (Fig 6H) still evoked an increase in activity in CTR rats immediately following the end of a lordosis. However, these increases were too subtle to elicit significant effects in the AUC (data not shown). Likewise, the dietary reversal failed to restore the attenuated responses of CAF rats regarding received ejaculations (Fig 6I).

3. Discussion

Our study reveals that rats on a long-term CAF diet, which consists of a *free choice* of highly palatable and unhealthy products, exhibited reduced appetitive and consummatory behavior towards the CAF reward compared to rats on a control diet. These behavioral changes were accompanied by diminished neural activity in the VTA in response to a food-predictive cue and when the rats interacted with the food reward directly. Similarly, reductions in VTA activity were observed in relation to a sexual reward. However, no significant behavioral differences were observed between the control and CAF rats in terms of appetitive (darting and hopping) or consummatory (lordosis) behaviors during sexual interactions. Furthermore, the study confirmed our hypothesis that CAF diet alters the functioning of the reward circuitry more severely than a high-fat, high-sugar diet. This finding coincided with the marked difference that CAF and HFHS diets had on food reward-related behavior: while long-term exposure to CAF limited interactions with the food reward, exposure to HFHS did not. Finally, we found that a reversal diet intervention of two weeks with healthy food was not sufficient to restore the behavioral and neural alterations seen after CAF diet consumption. Overall, these results suggest that prolonged exposure to junk food leads to diminished activity of VTA neurons upon both food and sexual rewards, which corresponds with behavioral changes in response to food rewards but not sexual rewards.

Our data supports our hypothesis that CAF diet more severely affects the VTA than HFHS diet does. This effect is most likely due to the element of *choice* of unhealthy products with different taste, texture, novelty and variety in CAF diet in contrast to HFHS pellets[98; 99]. Consistent with other studies, we observed that the free choice consumption and variation of food items led to greater body weight gain (See Table S2) [96] and increased overeating [97] (See Table S4). Notably, each cage showed different consumption patterns, with preferences for different foods (shown in Table S5). This overeating may contribute to more pronounced alterations in the brain, as indicated by stronger attenuation of VTA responses in CAF-fed rats compared to those on the HFHS diet. Since HFHS rats did not show differences in behavior toward a food reward, one could argue that the alterations in the brain (which were less pronounced than with CAF exposure) were simply not substantial enough to trigger behavioral responses. Overall, CAF diet seems to be a more robust model to study excessive junk food consumption than HFHS pellets, even though implementing it is more laborious and provides less control over the nutritional value of the consumed food.

Furthermore, our study was the first to show that long-term CAF diet can change event-related neural responses in the VTA toward a food and sexual reward, as well as reward-predictive cues. So far, most studies have shown effects of junk food on the hippocampus or nucleus accumbens, but only a few studies have reported potential changes in the VTA[111-114]. Interestingly, all these

studies report reduced levels of receptors [111; 113; 115] or tyrosine hydroxylase[114], supporting our findings of reduced VTA functioning after junk food exposure. In addition, it was recently found that the ways dopamine neurons in the VTA encode food and opposite-sex social motivation show overlap[116]. This study supports our hypothesis that desensitization of the VTA by excessive consumption of CAF diet can disrupt the neural responses toward other natural rewards as well, as shown in our study.

Our findings are in support of the reward hyporesponsivity theory claiming that long-term exposure to foods rich in fat and sugar can cause a blunted behavioral reward response[53; 80; 117]. While some studies have shown an increase in motivation to work for a food reward[54; 73-76], or no effect at all[79], more studies found that rats on a junk food diet show reduced interest in cues for a food reward and show attenuated operant responses[43; 77; 78; 80; 118-122], or longer latencies to collect a reward[77; 82]. In contrast to most of these studies, our experimental design permitted free access to, and interaction with the reward and is therefore considered a 'low effort' paradigm. In this regard, our findings are in line with the other studies in which little or no effort was required to obtain the rewards[56; 73; 80; 81; 123].

Since our behavioral reward test included a light cue indicating free access to the reward, our set-up enabled us to study the effects of excessive junk food consumption on the VTA both during classical conditioning and during reward approach and consumption. Initially, in the first test in all groups, the VTA responded to the light cue in a way previously described for novel and neutral cues[124]. By the fifth and final test however, VTA activity in the control group was higher in reaction to the cue light, relative to the first test, suggesting that the cue light had become a conditioned stimulus. In contrast, light-evoked activity in the VTA for both the CAF and HFHS groups did not differ from the first food reward test. Furthermore, all groups had elevated activity levels following opening of the door, which provided access to the reward, during the fifth food reward test, but not the first. We interpreted this response as a part of the cue-reward sequence that was not yet fully predicted by the cue light, and this might suggest that the VTA is mainly affected in its role to encode the predictive and incentive properties of a reward rather than in its role to signal novel stimuli that could lead to potential rewards[124-126]. After experiencing decreased pleasure and desire for the reward, the adjusted reward prediction after Pavlovian conditioning should typically lead to diminished VTA responses to the light-cue and reward[31; 127; 128]. We hypothesize that this is what happens in the rats exposed to CAF and HFHS diets.

The lack of behavioral changes in responses to a sexual reward, on the other hand, was surprising. The observation that VTA activity was attenuated despite the absence of explicit changes in

behavioral responses to a sexual reward, does support the notion that while long-term CAF exposure may exert effects on some of the same anatomical substrates of different natural rewarding behaviors, redundancy by means of other pathways might contribute to the robustness of sexual behavior. We have previously shown how different natural rewards can recruit similar brain networks to regulate the display of motivated behaviors[105]. Still, the actual display of either feeding or sexual behavior is also regulated by two different and specialized circuitries. While the lateral hypothalamus plays a key role in feeding behavior[129; 130], the ventromedial nucleus of the hypothalamus is the main neural center for female sexual behavior[109; 131-134]. With dense projections between the VTA and the feeding and sexual behavior brain networks[135], it is possible that CAF diet exposure also affects other regions than the VTA related to feeding behavior, while having less effect on the sexual behavior related regions. Alternatively, it can be argued that changes in sexual motivation might be found using more subtle behavioral tests, in which consummatory behaviors are not displayed, such as the sexual incentive motivation test[107; 136], or a test in which the rats have to work to gain access to the sexual reward. It is also plausible that prolonged testing durations can unveil behavioral disparities between CTR and CAF rats. For example, if the motivation to continue copulation is affected, it would likely become more apparent with longer test durations. Another explanation for the lack of effects on sexual behavior can be the fact that our female rats were sexually experienced at the start of testing. Beloate et al. have shown that inhibition of dopaminergic neurons in the VTA does not change the initiation and performance of sexual behavior in male rats, but that it does prevent sexual experience to affect neuronal plasticity and cross-sensitization of amphetamine-induced conditioned place preference[137]. Following this line of thought, it might be possible that the diminished CAF-induced VTA responses might lead to altered sexual behavior in sexually naïve female rats as well.

Furthermore, we would like to stress that this is the first study to investigate how the VTA is activated during female sexual behavior. In our study, we found that an increase in neural activity in the VTA was linked to the start of darting, but not hopping events. The increase in VTA activity was aligned to the start of the running of a darting response, and not the moment the rats placed their buds on the ground for the male to mount. Regarding lordosis behavior, on the other hand, VTA activity was aligned to the end of a lordosis behavior. Lordosis behavior is a response toward a copulatory behavior by the male and when this behavior has ended, an increase in VTA activity was seen exclusively after an ejaculation. This suggests that the VTA has a stimulation-specific response and that it might encode the rewarding elements perceived from such a stimulation.

Finally, we found that a 2-weeks reversal to a healthy diet did not fully restore the behavioral and neural responses caused by long-term CAF diet. This finding is in line with another study that

reported that two weeks of diet withdrawal did not restore the behavioral changes caused by CAF diet exposure[53]. Interestingly though, other studies have reported binge-like eating behavior after withdrawal from junk food diets[112; 114]. Others found a restoration of sucrose preference after 4 weeks of chow feeding[112]. More research is needed to determine whether the behavioral changes will return to normal after longer exposure to healthy chow diet. The findings of unchanged, blunted VTA responses, on the other hand, seem to be rather consistent with other studies that evaluated the persistence of neural changes caused by junk food exposure. It was found that D1 receptor expression and TH levels remained lower after abstinence from CAF diet[59; 114]. Interestingly though, a sex difference was present in these findings. While female rats maintained reduced D1 receptor expression levels, males seem to return to baseline after 72 hours[59].

One limitation of our study was the use of a non-specific GCaMP promotor (hSyn) for the fiber photometry recordings. At the time the study was conducted, no successful cell-specific promotors were available for rats. While transgenic mice are often used to target specific neuronal populations, the behavioral profile in rats offers advantages in this type of study. In addition, rats are a better translational model in studies of obesity and effects of dietary manipulations[138]. Although cell specificity was not reached in this study, previous studies have shown that the VTA consists of both dopaminergic, glutamatergic and GABAergic cells, and many neurons co-release different neurotransmitters[25; 139-142]. It is therefore impossible to determine which population is of most importance for our findings of an attenuated VTA response after prolonged junk food consumption. However, since most neurons are dopaminergic [142] and our responses to cues and rewards are consistent with responses seen when people have measured dopamine neuron firing [127] or dopamine release with voltammetry[36], it is likely that dopaminergic neurons play an important role in our findings. Future studies should reveal the role of the different neurons in the VTA in more detail.

In conclusion, the results indicate that long-term junk food exposure severely affects the reward system by altering behavioral responses to a CAF food reward and desensitizing the VTA to a food and sexual reward. Exposure to a CAF diet in which rats are exposed to a choice of foods rich in fat and sugar has more severe consequences than a HFHS diet. Finally, two weeks of healthy diet is not sufficient to restore the behavioral and neural changes. Our findings support the notion that excessive junk food consumption can have undesirable long-lasting consequences.

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Author contributions

JFL: Experimental design, data gathering, behavioral annotation, data curation, analysis, writing – review and editing, funding acquisition

JCO: Data gathering, behavioral annotation, analysis, writing – review and editing

PTH: Methodology, writing – review and editing

JEM: Methodology, writing - review and editing

RH: Experimental design, methodology, data gathering, data curation, analysis, supervision, writing – original draft, funding acquisition

EMSS: Experimental design, methodology, data gathering , data curation, Programming/software, analysis, supervision, writing – original draft, funding acquisition

Declaration of interests

The authors declare no competing interests.

4. Methods

4.1 Rats

A total of 36 Wistar female rats (200-250 g upon arrival) and 10 male rats (250 g upon arrival) were purchased from Janvier labs (France). All rats were pair-housed in Macrolon IV ® cages on a reversed 12 h light/dark cycle (lights on between 23:00 and 11:00) in a room with controlled temperature (21 ± 1 °C) and humidity (55 ± 10 %), with ad libitum access to standard rodent food pellets (low phytoestrogen maintenance diet, #V1554, Ssniff, Germany) and tap water throughout the experiment (unless otherwise indicated). All animal care and experimental procedures employed in this study were conducted in agreement with the European Union council directive 2010/63/EU and in accordance with the Norwegian Food Safety Authority.

4.2 Experimental design

After sexual training, the females underwent stereotaxic brain surgery in two batches of 18 rats with two weeks in between. Once the females recovered from surgery, they were randomly divided into three experimental groups of control (CTR), high-fat, high-sugar (HFHS), or cafeteria (CAF) diet for six weeks.

After six weeks of diet exposure, the females were tested in the behavioral reward box (Fig S1A), during which they were tethered to the fiber photometry system (RZ10x from Tucker-Davis Technologies, USA). First, five tests (once per day on five consecutive days) with a food reward were conducted, followed by three tests with a sexual reward (on the same day of their proestrus to prevent losing the test subject for data analysis due to pseudopregnancy; 2-hour break between tests). The food reward consisted of small portions of the favorite products selected from the CAF diet (Table S2). A male rat was used as sexual reward.

To rule out the potential effects of pseudopregnancies the female rats remained on their experimental diet for another two weeks after the behavioral reward tests. Subsequently, all female rats were switched to a diet of standard chow for another two weeks (reversal diet), followed by a final round of behavioral reward testing. They were tested three times (on consecutive days) with the food (CAF) reward, and one time with the sexual reward (on a proestrus day).

After completion of the experiment, all rats were perfused and their brains were collected to assess viral infusion location and expression and verification of fiber location.

4.3 Surgeries

4.3.1 Brain surgery. After sexual training, the females underwent brain surgery for AAV vector infusion and fiber implantation. The subjects were anesthetized with isoflurane, placed in a stereotaxic apparatus (Stoelting Europe, Ireland), and received 0.05 mg/kg buprenorphine and 2 mg/kg meloxicam subcutaneously. The scalp was shaved, disinfected with povidone-iodine and locally anaesthetized with bupivacaine (0.15 ml, s.c.). Core body temperature, oxygen saturation and heart rate were monitored throughout the surgery. A hole was drilled above the VTA of the right hemisphere (coordinates: AP -5.0 mm, ML +0.9 mm, DV -8.5 mm relative to bregma[143]) and a 2 µl Hamilton neuros syringe with a 30G blunt needle mounted in a Hamilton injector (Stoelting) on the stereotaxic apparatus was slowly inserted. N=11 of each group was infused with 750 nl, $1x10^{13}$ vg/mL pAAV5.syn.GCaMP6s.WPRE.SVA40 (a gift from Douglas Kim & GENIE Project (Addgene plasmid # 100843 ; http://n2t.net/addgene:100843; RRID:Addgene_100843-AAV5) and N=1 of each group with 750 nl, $1x10^{13}$ vg/mL pAAV5.hSyn.eGFP.WPRE.bGH (a gift from James M. Wilson (Addgene plasmid # 105539 ; http://n2t.net/addgene:105539 ; RRID:Addgene_105539-AAV5)) as

control, both at a rate of 125 nl/min. Following infusion, the needle was left in place for 10 minutes before withdrawal. 4 anchor screws (1 x 3 mm, Agnthos, Sweden) were placed in the skull, after which the fiber-optic cannula (diameter 400 µm, NA 0.66, Doric Lenses, USA) was inserted in the right hemisphere at AP -5.0 mm, ML +0.9 mm, DV -8.4 mm relative to bregma. It was fixed to the skull with super bond (C&B Super bond, Technomedics, Norway) followed by regular dental acrylic (Meliodent, Kulzer GmbH, Germany). Post-operative analgesic treatment consisted of 0.05 mg/kg buprenorphine and 2 mg/kg meloxicam after 24 and 48 hours. Immediately after surgery the rats were pair-housed and recovered for minimum one week before the diet manipulation was started.

4.3.2 Vasectomy. To prevent pregnancies in experimental female rats while maintaining their reproductive integrity, the stimulus male rats were subjected to vasectomy by ligation of a small portion of the vas deferens of both testes, as previously described[144]. On each side, a small incision was made in the scrotal skin covering the testes and the tissue layer covering the testicle was opened. The testicle, epididymis and vas deferens were exposed and the vas deferens was thermally cauterized. The organs were returned inside the incision and the wound was closed with stitches. Immediately after surgery the male rats were pair-housed and were allowed to recover for two weeks before undergoing sexual training.

4.4 Diet manipulation

After at least one week of surgery recovery, experimental rats were randomly assigned one of the three experimental diets which they received for a period of six weeks. Standard Chow (CTR): standard laboratory chow. High-Fat, High-Sugar (HFHS): laboratory chow with high content of fat and sugar that simulates the nutritional values of junk food, but in the shape of regular pellets (Western Diet #D12079B, Research Diets Inc., USA). Cafeteria diet (CAF): composed of standard chow and 16 different food items obtained in a supermarket, including biscuits, cookies, processed meat and salty snacks (Table S2 and S3), based on a proposed protocol for cafeteria diet (Lalanza & Snoeren, 2021). The food items were divided into 4-daily-rotated plates balanced by type of food, texture, taste, and nutritional composition. All three experimental diets were provided *ad libitum* to the rats The rats had access to tap water ad libitum.

4.5 Behavioral testing

4.5.1 Sexual training. The natural estrous cycle of the females was monitored using an impedance meter (Muromachi MK-12, Japan). When in proestrus, the females were sexually trained in a copulation box (60 x 40 x 40 cm, with a Plexiglas front) in a room with dimmed lighting conditions. A receptive female was first habituated to the copulation box for five minutes, followed by the introduction of a male, until ejaculation was achieved or for a maximum of 30 minutes. During

training, all females were exposed to one ejaculation, except for one rat that received 20 intromissions without ejaculation. Stimulus males achieved at least three ejaculations in different sessions with different females.

4.5.2 Behavioral reward test. All rats were habituated to the behavioral reward test box for 10 minutes on the day before testing began. On a behavior test day, the food was removed from the cage at 9 am, while the animals continued to have access to water. Between 12:00 and 19:00, the behavioral testing was conducted, after which fresh food was placed back in the cage. The diet groups were randomly divided across the day and no effects of order were detected.

The behavioral reward test was conducted in a box (75x30x70 cm, Fig S1A) in which two chambers (30x30x70 cm and 45x30x70 cm) were separated by a wall containing 2 sliding doors. The side of the dividing wall facing the left chamber (starting chamber, not containing the reward) had a light between the two sliding doors. Small apertures in these doors enabled the subject rat to see, hear and smell the reward in the right chamber. The floors were covered with fresh wood chip bedding. The front wall of the box facing the camera was made of transparent methacrylate.

First, a subject rat was connected to the fiber photometry system with a fiber-optic cable and placed in a small cage for approximately 2 minutes to allow the GCaMP and isosbestic signals to stabilize. The reward test started when the female subject was placed in the left chamber, with the doors in the dividing wall closed (Baseline phase, Fig 1B). After 3 minutes, a reward (either food or a male rat) was placed in the right chamber, while the doors remained closed for another 3 minutes (Pre-reward phase). Subsequently, the light turned on for 10 seconds, after which the doors opened. The light turned off again, and the rat was allowed to interact freely with the reward for a total of 15 minutes (Reward phase). The doors opened for 5 cm allowing the females to cross easily between compartments. As this was more difficult for the males, they spent more time crossing . This way, the females were able to pace their sexual activity a little bit more than with completely open doors. After the test, the female was returned to the home cage and the reward box was cleaned with ethanol. Fresh bedding was provided for the next rat.

All behavioral reward tests were recorded on video with a digital video camera (Sony HDR-CX240E handycam) for behavioral assessment.

4.6 Fiber photometry

To assess the activity of VTA neurons during the behavioral reward tests, the bulk fluorescence signal generated by GCaMP6s expressing cells was recorded using fiber photometry[145; 146]. Signal processing and acquisition hardware (RZ10x; Tucker-Davis Technologies, USA) was used to control a

465 nm Lux RZ10x integrated LED for fluorophore excitation, and a 405 nm Lux RZ10x integrated LED for isosbestic excitation. The excitation lights were modulated at 210 Hz (465 nm) and 330 Hz (405 nm), filtered and combined by a fluorescence mini cube (Doric Lenses, USA), and delivered through a 400 µm core, 0.57 NA, low-autofluorescence fiber optic patch cable (Doric, MFP_400/430/1100-0.57_5m_FCM-MF2.5_LAF) to an implanted fiber (Doric, MFC_400/430-0.66_9mm_MF2.5_FLT). LED power was set at a level which resulted in a ~15 and ~30 mV amplitude output signal for 405 and 465 nm demodulated channels, respectively. All signals were acquired using Synapse Essentials software (Tucker-Davis Technologies). Fluorescence was sampled at 1017 Hz and demodulated by the processor. The start of the behavioral reward test and the moment the light was turned on were time stamped by registering TTL signals generated by the USB-I/O box 12 system (Noldus, The Netherlands). With the help of The Observer XT 12.5 (most behavioral data) and XT 16 (Novel food reward data, Noldus), the behavioral events were time stamped and aligned with the fiber photometry data.

Data were exported to Python from Synapse using a script provided by Tucker-Davis Technology. Custom Python scripts were used to independently normalize the demodulated 465 nm and 405 nm signals. To calculate ΔF/F, a least-squares linear fit was applied to the 405 nm signal to align it to the 465 nm signal, producing a fitted 405 nm signal that was used to normalize the 465 nm as follows: ΔF/F = (465 nm signal − fitted 405 nm signal)/fitted 405 nm signal[146]. Fractions of the signal recording in which noisy signals were recorded (e.g., when the cable detached or hit the wall or door) were removed from the recordings when an interquartile range of more than 2 was found. We then aligned the ΔF/F to baseline zero by subtracting the average of a ΔF/F signal collected at the start of each of the events. The area under the curve (AUC) was calculated from this corrected signal. To compare the AUC before and after the event, the AUC was corrected per second.

4.7 Behavioral assessment

From the videos, which were recorded at a frequency of 25 fps, the frequency and duration of the multiple behaviors (Table S1) were annotated, and the start of the behaviors was time-locked on the exact video frames using The Observer XT12.5 and Observer XT16 (Noldus).

Then, some additional parameters were calculated, such as the lordosis score ((number of lordosis with score 1 times 1 + number of lordosis with score 2 times 2 + number of lordosis with score 3 times 3) / number of received copulations)[110], lordosis quotient (number of lordosis responses (1-3) / number of received copulations * 100%) , and the mean contact-return latency after a received mount, intromission or ejaculation (latency to 1st paracopulatory behavior after receiving a copulation) were calculated per rat.

Moreover, a more detailed behavioral assessment paradigm was used to explore the micropatterns in reward-related behaviors. For this, the behavior was divided into eating bouts (Fig 2D, food reward tests) and sexual bouts (Fig 3F, sexual reward test). An eating bout consisted of all eating episodes that were not interrupted by any behavior that was not focused on the food reward. As soon as a behavior undirected at the food reward was displayed, the last eating episode was considered the ending of the eating bout. The break until the next eating episode is called a time-out. A similar approach was used for the sexual bout, but now both a lordosis and paracopulatory behavior could start and/or end a sexual bout. The number of bouts, the mean duration of bouts, and the mean time-out duration were determined.

To mitigate the effects of potential pseudopregnancies, all three sexual reward tests were conducted on a single day and with the same male for each female rat. Regrettably, not all females reached the proestrus phase and these were consequently excluded from the analysis. Notably, only six out of ten females on the HFHS diet reached proestrus, and two of these females never engaged in copulation with the male. Furthermore, although ten of our CAF-exposed females reached the proestrus phase, three of them did not exhibit any paracopulatory behaviors during the initial sexual reward test. This observation suggests a potential slight delay in the onset of the receptive phase compared to CTR females as the first reward test was conducted right after the lights went off. To address these challenges in conducting a reliable behavioral reward test with a sexual reward, we analyzed the neural and behavioral data from the most successful sexual reward test. The *best sexual reward test* was defined as the test (either the first, second, or third) in which the female displayed the highest number of paracopulatory behaviors. In practice this meant that two 1st, four 2nd and four 3rd tests were included in the analysis for the CTR rats; one 1st, two 2nd and three 3rd tests for the HFHS rats; and two 1st, four 2nd, and three 3rd tests for the CAF rats. The sex reward test after reversal was conducted 2.5 hours after the lights went off to avoid the delay in the onset of the receptive phase.

4.8 Brain processing, immunostaining and imaging

At the end of the experiments, rats were injected intraperitoneally with a lethal dose of pentobarbital (100 mg/kg), and transcardially perfused with 0.01 M phosphate buffered saline (PBS; pH 7.4) followed by 4% formaldehyde in 0.01 M PBS. Brains were removed and post-fixed in 4% formaldehyde for 48 hours. Then, the brains were transferred to 20% sucrose in 0.01 M PBS for one day and placed in 30% sucrose in 0.01 M PBS. When sunken, the brains were snap frozen in isopentane cooled by dry ice and stored at -80 °C until sectioning on a sliding microtome (SM2010R, Leica Biosystems, Germany) into 40 µm coronal sections. These sections were stored in

cryoprotectant solution (30% sucrose w/v, 30% ethylene glycol v/v in 0.01 M PBS, pH 7.4) until further use.

To assess the expression of GCaMP in the VTA, immunohistochemistry was performed on selected sections. These were washed in 0.1M Tris-buffered-saline (TBS), blocked for 30 minutes in 0.5% BSA in TBS, and incubated with polyclonal chicken anti-EYFP (1:100 000), Abcam, cat. ab13970) antibody solution containing 0.1% Triton-X and 0.1% BSA in TBS on an orbital shaker for 24 hours at room temperature + 24 hours at 4 °C. The sections were then washed four times in TBS and subsequently incubated in biotinylated goat anti-chicken (1:400, Abcam, cat. ab6876) antibody solution containing 0.1 % BSA in TBS for 30 min, followed by incubation with avidin-biotin-peroxidase complex (VECTASTAIN ABC-HRP kit, Vector laboratories, cat. PK-6100, dilution: 1 drop A + 1 drop B in 10 mL TBS) solution for 30 min, and 3,3'-diaminobenzidine solution (DAB substrate kit (HRP), Vector laboratories, cat. SK-4100, dilution: 1 drop R1 (buffer solution) + 2 drops R2 (3,3'-diaminobenzidine solution) + 1 drop R3 (hydrogen peroxide solution) in 5 mL water) for 5-10 min, with TBS washes between all steps. The slides were left until dry, after which the sections were dehydrated with 50-99.8% ethanol baths, cleared with xylene, and coverslipped using Entellan mounting medium (Sigma, St. Louis, USA).

Finally, the location of the fiber and GCaMP expression were visually verified on the sections with a microscope (Primo Star, Carl Zeiss, Germany). Only the rats that had both GCaMP expression and a fiber within the VTA (between AP -4.7 mm and AP -5.2 mm) were included in the fiber photometry data analysis.

4.9 Statistical analysis

All rats were included in the behavioral analysis, except for three rats that had lost their fiber caps at an early stage of the experiment. For the fiber photometry analysis, however, seven rats were excluded from analysis as they did not express GCaMP or had a wrong fiber placement, resulting in 6 CTR-, 7 HFHS- and 9- CAF included rats. In addition, a small number of sessions (2 x first sexual reward tests; 3 x second sexual reward tests; 2 x third sexual reward tests; and 1 x sexual reward test after the reversal diet, coming from different rats) had to be excluded from the analysis due to insufficient quality of the fiber photometry recording.

Both the behavior and fiber photometry data were analyzed with customized Python 3.9 scripts (available upon request). Statistical analysis of the behavioral data and area under the curve were conducted using SPSS software (version 29, IBM, Armonk, USA) with a level for statistically significant difference set at $p < 0.05$. For the behavioral analysis in which CTR, CAF, and HFHS were

compared, a linear mixed model was used to investigate the diet, test and diet*test interaction effects. In the case of a significant effect, a Bonferroni-corrected post hoc test was conducted. When CTR rats were compared to CAF rats only, an independent sample t-test was used. The linear mixed model analyzing the AUC data comprised the factors Diet (CTR, HFHS, and CAF or CTR and CAF) and Epoch (pre versus post). The pre vs post effects on AUC for the light and door cue were analyzed separately. In case the test was divided into 3 parts, the factor Parts (part 1, part 2, and part 3) was also included in the analysis. Again, in the case of a significant effect, a Bonferroni-corrected post hoc test was conducted.

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