Behavioural and neurological effects of fructose cessation and re-exposure

by

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Abstract

Fructose is a monosaccharide present in many healthy and unhealthy food items consumed. Fructose consumption has risen over the years, which has been linked to the development of metabolic syndrome, non-alcoholic fatty liver disease, and obesity. Detrimental metabolic impacts of fructose consumption include increased feeding behaviour, body weight gain, and adiposity, among other hallmarks of metabolic distress. The hypothalamus is a brain region mediating feeding behaviour, such as promoting feeding via the NPY/AgRP neurons of the arcuate nucleus (ARC). Recent evidence shows that fructose can act via the hypothalamus to produce a shift towards positive energy balance, and preliminary findings from our lab have shown that fructose consumption produces an obesogenic phenotype and increased excitatory tone onto NPY/AgRP neurons. While this tone is reversible with cessation after one-week of fructose consumption, it has not been determined if increased excitatory tone onto NPY/AgRP neurons is reversible with cessation from chronic fructose consumption. We used a combination of whole-cell patch-clamp recordings and a fructose choice test to assess how cessation of dietary fructose could reverse fructose-mediated increases in excitatory tone at NPY/AgRP neurons or enhance fructose preference upon re-exposure. We have found that fructose abstinence reverted increases in excitatory tone at NPY/AgRP neurons in males, but not females. Additionally, fructose consumption reduced the preference for fructose, which was re-established with a fructose cessation. These findings suggest that maintenance of increased excitatory tone despite fructose cessation may drive feeding behaviour. Additionally, repeated exposures to fructose could enhance preference, which may predispose individuals to consume increased sugars later in life.
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1.0 Introduction

1.1 Sources of dietary fructose

Fructose is a monosaccharide found within food items in our diet. It is naturally occurring in fruits and vegetables (Johnson & Conforti, 2003), but it is also present as an additive in beverages, such as in soda or fruit drinks (Walker, Dumke, & Goran, 2014). A molecule of fructose combined with a molecule of glucose produces sucrose, a disaccharide which is commonly known as “table sugar.” Fructose tastes sweeter than either glucose or sucrose (Tonosaki & Beidler, 1989) and is often added to different food items to improve their taste (Davis, 1973). Fructose is present in its free form in high-fructose corn syrup (HFCS) and in sweetener alternatives as an added sugar, such as honey (Persano Oddo & Piro, 2004) and molasses (Palmonari et al., 2020). While these aforementioned sweetener alternatives are often pitched as “healthy,” they still produce similar metabolic effects as sucrose or HFCS consumption (Raatz, Johnson, & Picklo, 2015). HFCS is used as a sweetener in place of sucrose due to its low production cost and increased sweet taste (Carman, 1982), resulting in its significantly increased use through the years. The source of fructose consumption is relevant, as healthy sources (e.g. fruits) can be beneficial but unhealthy sources (e.g. fruit drinks) can be harmful (Choo et al., 2018), such as reduced risk of diabetes with fruit consumption (Li et al., 2015), and increased risk of diabetes with the consumption of sugar-sweetened beverages (Imamura et al., 2015). This is attributed to healthy sources containing beneficial components, such as vitamins, while unhealthy sources may have increased calories in the absence of nutritional value. Altogether, these findings suggest that regardless of the sources, consumption of fructose can be detrimental.
1.2 Fructose consumption increases development of metabolic diseases

Fructose consumption has increased throughout the years and has been linked to the rise in obesity (Bray, Nielsen, & Popkin, 2004), as well as detrimental metabolic effects such as metabolic syndrome and diabetes (Bray, 2013). HFCS consumption increased by 30% from the 1970s to 2000s, and this rise in consumption preceded the rise in obesity rates in the United States (Bray et al., 2004). This consumption, often in the form of added sugars, is consistently above the World Health Organization (WHO) guidelines for added sugars consumption (Powell, Smith-Taillie, & Popkin, 2016), which is consumption of less than 10% ("WHO calls on countries to reduce sugars intake among adults and children," World Health Organization, 2015). Diseases such as diabetes type II are 20% more prevalent in countries with excessive HFCS consumption, compared to countries with no HFCS consumption (Goran, Ulijaszek, & Ventura, 2013). While many may think that this increased risk is solely due to unhealthy items in the overall diet, evidence shows that food items with added sugars, which are marketed as healthier alternatives, produce similar risk of diabetes (Montonen, Järvinen, Knekt, Heliövaara, & Reunanen, 2007; Palmer et al., 2008), weight gain (Mozaffarian, Hao, Rimm, Willett, & Hu, 2011) and obesity (Malik, Schulze, & Hu, 2006). Overall, it is evident that increased fructose consumption over the years has contributed to the rise in obesity and metabolic diseases.

Excessive fructose consumption is associated with specific hallmarks of metabolic syndrome (Malik et al., 2006; Pang et al., 2021). According to the National Heart, Lung and Blood Institute, metabolic syndrome is a collection of risk factors such as increased adiposity, triglyceride levels and fasting blood glucose, which increases the risk of diabetes and heart disease ("Metabolic Syndrome," NHLBI, 2018). Human study data suggests that four weeks of fructose consumption increased triglyceride levels in healthy individuals (Lê et al., 2006;
Silbernagel et al., 2011), which was further exacerbated in obese individuals (Teff et al., 2009). This increase in triglycerides is not only a hallmark of metabolic syndrome, but also of non-alcoholic fatty liver disease (NAFLD), which has been linked to increased fructose consumption (Jensen et al., 2018; Lim, Mietus-Snyder, Valente, Schwarz, & Lustig, 2010; Ouyang et al., 2008). Fructose consumption increased de novo hepatic lipogenesis (Sobrecases et al., 2010) and suppressed fatty acid oxidation (Topping & Mayes, 1972), resulting in NAFLD. Fructose also increased central adiposity (Lin et al., 2016; Stanhope et al., 2009), which is also seen in animal models (Ramos, Batista, & Albuquerque, 2017). Fructose intake is associated with a hyperinsulinemic state, which is a hallmark of metabolic syndrome (Roberts, Hevener, & Barnard, 2013) and this is seen in both rats (Blakely, Hallfrisch, Reiser, & Prather, 1981) and humans (Aeberli et al., 2013; Ter Horst, Schene, Holman, Romijn, & Serlie, 2016). Feeding studies in rodents confirmed findings of the detrimental impacts of fructose, with similar impairments in triglycerides (Kanarek & Orthen Gambill, 1982; Ramos et al., 2017; Zubiría et al., 2013), body adiposity (Jürgens et al., 2005; Ramos et al., 2017), hyperinsulinemia (Huang, Chiang, Yao, & Chiang, 2004; Zubiría et al., 2013) and glucose intolerance (Huang et al., 2004; Soto et al., 2017). Altogether, these findings suggest that fructose consumption produces hallmarks of metabolic syndrome and is overall detrimental to our metabolic health.

1.3 Fructose acts via the hypothalamus to affect metabolic health and feeding

1.3.1 Relevance of the brain in obesity

The brain is a critical organ for energy balance, given that it receives signals from the periphery, resulting in modulation of feeding behaviour. The blood brain barrier (BBB) is the
most permeable at the median eminence of the hypothalamus (Gross & Weindl, 1987), which is a critical region within the brain involved in maintenance of homeostatic feeding. The hypothalamus effects feeding behaviour based on the energy status of the animals, and the hypothalamic homeostatic neurons will enact quick plastic changes in response to peripheral signals to then meet the metabolic demands at hand. Peripheral signals, such as hormones or nutrients, can be potent signalling molecules of hunger and satiety that will cross the BBB to act upon hypothalamic neurons to modulate feeding behaviour. For example, ghrelin released from the stomach can travel to the brain resulting in increased excitatory input onto orexigenic neurons (Yang, Atasoy, Su, & Sternson, 2011) and feeding behaviour (Tschop, Smiley, & Heiman, 2000), while leptin signalling decreased the activity of the orexigenic neurons (Yang et al., 2011) and decrease feeding behaviour. Additionally, the neurons within the hypothalamus are responsive to specific nutrients, given that among pair-fed mice who consumed the same calories of either chow or a high fat diet (HFD), only HFD-fed mice had increased excitation of their hypothalamic neurons (Wei et al., 2015).

1.3.2 Fructose promotes peripheral signals of hunger, while suppressing signals of satiety

Ghrelin and leptin are heavily influenced by fructose consumption, to result in promotion of feeding behaviour. In lean individuals, consumption of a 30% fructose solution reduced post-prandial suppression of ghrelin (Teff et al., 2004) in comparison to glucose. A similar pattern happened in obese individuals, as obese individuals given a 25% fructose solution have reduced suppression of acyl-ghrelin, while 25% glucose solution suppressed acyl-ghrelin entirely (Van Name et al., 2015). These findings suggest that in humans, fructose consumption impairs the suppression of hunger-promoting ghrelin, which could result in increased fructose intake and
feeding. In contrast, it is difficult to elucidate the impact of fructose on ghrelin function in rodents. HFCS consumption resulted in increased ghrelin levels in rats (Lindqvist, Baelemans, & Erlanson-Albertsson, 2008; Ma et al., 2013). Ghrelin receptor knockout mice did not have any changes in body weight or adiposity (Ma et al., 2013), while ghrelin knockout mice given an 8% HFCS solution had increased adiposity, inflammation, and insulin resistance (Ma et al., 2017). While the impact of fructose may be differential in ghrelin knockout and ghrelin receptor knockout rodents, in combination with human studies, fructose seems to increase the level of ghrelin.

For leptin, normal weight individuals with 30% fructose solution for two days had reduced leptin (Teff et al., 2004), which was also seen with obese individuals (Teff et al., 2009). When individuals consumed a 25% fructose solution for eight weeks, there was increased fasting leptin levels (Rezvani et al., 2013) but decreased post-prandial levels of circulating leptin. These findings suggest that fructose may promoted feeding via suppression of leptin to reduce signals of satiety. In rodents, short-term consumption of fructose, defined as two weeks, increased serum leptin levels (Lindqvist et al., 2008), while chronic fructose consumption for eight or nine weeks of a 60% solution led to increased leptin levels and leptin resistance (Bursač et al., 2014; Huang et al., 2004). Leptin resistance was also seen with consumption of a 60% high fructose diet (Chotiwat, Sharp, Teff, & Harris, 2007; Shapiro et al., 2008). This indicates that prolonged consumption of fructose in rodents increased leptin levels but that despite this, leptin resistance indicated a lack of responsiveness. While the findings differ between humans and rodents, collectively, they suggest that fructose can promote feeding behaviour by promoting the activity of hunger-inducing ghrelin while suppressing the activity of satiety-promoting leptin.
1.3.3 Fructose can act within the hypothalamus to promote feeding

Fructose can cross the BBB, is metabolized (Oppelt, Zhang, & Tolan, 2017), and has effects within the brain shortly after systemic administration (Oldendorf, 1971). Intracerebroventricular administration of fructose into the brain increased food intake (Cha, Wolfgang, Tokutake, Chohnan, & Lane, 2008; Miller, Martin, Whitney, & Edwards, 2002), as fructose decreased the ATP/AMP ratio, resulting in increased AMPK activity and decreased malonyl-CoA (Cha et al., 2008), which then promoted feeding. Fructose transporters are located within the rodent hypothalamic regions (Kojo, Yamada, & Yamamoto, 2016; Song et al., 2017), such as the orexigenic lateral hypothalamus (LH) and ARC. Fructose also upregulated the presence of its specific transporter, GLUT5, upon fructose consumption (Meng et al., 2016). GLUT5 is located on ependymal cells and neurons within the LH and ARC (Kojo et al., 2016), indicating that neurons within these regions are capable of transporting fructose. While there is a gap in the literature regarding the expression of fructose enzymes within the LH or ARC, there is evidence which shows that other hypothalamic regions, such as the supraoptic nucleus, paraventricular nucleus, and the suprachiasmatic nucleus expressed fructokinase mRNA (Song et al., 2017), indicating the capability of the hypothalamus to utilize fructose. Within humans, GLUT5 expression was seen on the BBB (Vannucci, Seaman, Brucklacher, & Vannucci, 1994) and within the brain (Mantych, James, & Devaskar, 1993; Shepherd, Gibbs, Wesslau, Gould, & Kahn, 1992), but evidence is lacking regarding GLUT5 expression in specific brain regions. The human findings of fructose influencing hypothalamic activity are conflicting. While lean or normal weight individuals had no change in hypothalamic BOLD activity upon fructose infusion (Purnell et al., 2011) or in response to food cues after fructose infusion (Luo, Monterosso, Sarpelleh, & Page, 2015), obese individuals showed mild decrease in hypothalamic activity.
Another study in lean individuals, fructose produced a delayed reduction in hypothalamic activity (van Opstal et al., 2019), while another conflicting study in lean individuals showed fructose increased hypothalamic activity (Page et al., 2013). While the findings are conflicting within human studies, evidence in rodent models still supports the findings that fructose is transported into the brain and can be utilized there.

1.3.4 Fructose increases orexigenic hypothalamic neuropeptide expression

Within the hypothalamus, first-order neurons of the ARC, such as the neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons and the proopiomelanocortin (POMC) neurons are involved in mediating feeding and metabolism in response to fluctuations in nutrients (Wei et al., 2015) and peripheral signals (Yang et al., 2011). NPY/AgRP neurons of the ARC received increased presynaptic glutamatergic inputs (Krashes et al., 2014) in hunger-like states, resulting in increased activation of these neurons (Liu et al., 2012) and feeding behaviour. Additionally, the neurons are susceptible to the impacts of nutrients specifically, and not just the number of calories. In pair-fed mice who consumed the same number of calories of HFD and chow diet, the HFD-fed mice have increased excitability of their NPY/AgRP neurons compared to chow pair-fed controls, indicating the ability of these neurons to respond to nutrients specifically. Altogether, these findings show that NPY/AgRP neurons of the ARC are likely the first candidate to investigate the impact of fructose on feeding.

Studies have shown that fructose can increase gene expression of orexigenic neuropeptides within the hypothalamus. Two weeks of fructose consumption reduced Npy expression (Lindqvist et al., 2008), while prolonged fructose consumption for 8–18 weeks
increased \textit{Npy} expression (Bursač et al., 2014; Nabil, El Demellawy, Mahmoud, & Mahmoud, 2020; Tripathi, Banerjee, Nirala, & Mathur, 2021. Intracerebroventricular NPY administration (Clark, Kalra, Crowley, & Kalra, 1984; Zarjevski, Cusin, Vettor, Rohner-Jeanrenaud, & Jeanrenaud, 1993) or cannula administration (Stanley & Leibowitz, 1985) increased food intake. Additionally, NPY injection increased motivation for food (Flood & Morley, 1991), and increased \textit{Npy} expression is seen in rodent models of obesity (Huang, Han, & Storlien, 2003; Sanacora, Kershaw, Judith, & White, 1990), showing the orexigenic effect of NPY. Taken altogether, it is possible that sustained fructose consumption can increase \textit{Npy} expression to promote feeding behaviour. In terms of anorectic hypothalamic neurons, both short-term (two weeks) (Lindqvist et al., 2008) and long-term (12–18 weeks) of fructose solution consumption decreased \textit{Pomc} expression (Nabil, El Demellawy, Mahmoud, & Mahmoud, 2020; Soto et al., 2017). Together, these findings show that prolonged fructose consumption results increased expression of orexigenic \textit{Npy} and decreased expression of anorectic \textit{Pomc}, which could contribute to fructose-mediated increased feeding.

This thesis focuses on the NPY/AgRP neurons of the ARC, and their involvement in the fructose-mediated increases in feeding behaviour. However, other hypothalamic populations can also be involved in mediating the orexigenic drive produced by fructose. Both ten days (Jacki M. Rorabaugh, Stratford, & Zahniser, 2014) and five weeks of 8–10\% fructose solution consumption increased orexin levels (Franco-Pérez et al., 2018) and orexin neuron activation (Rorabaugh et al., 2014). Given that orexin neurons of the LH project to NPY/AgRP neurons of the ARC (Peyron et al., 1998) to activate them (Yamanaka et al., 2000), increased orexin neuron activity can drive further fructose-mediated feeding. The endocannabinoid system is also involved, given that one week of 23\% fructose solution consumption increased mRNA
expression of endocannabinoid degradation enzymes and cannabinoid receptor 1 (CB₁) (Erlanson-Albertsson & Lindqvist, 2010). The presence of more degradation enzymes could indicate increased levels of endocannabinoids, which can promote food intake (Williams & Kirkham, 1999). Additionally, CB₁ receptor activation is known to promote feeding (Di Marzo & Matias, 2005). These findings suggest that the endocannabinoid system could also be involved in the development of fructose-mediated feeding. Altogether, fructose-mediated feeding can occur via promotion of the activity of multiple hypothalamic orexigenic neuronal populations.

1.3.5 Adaptability of NPY/AgRP neurons

As mentioned previously, the hypothalamus affects feeding behaviour based on peripheral signals to meet the energetic demands of the animal. This plastic capability is very prominent in NPY/AgRP neurons, who will go through physical and functional changes based on peripheral signals. In the case of hunger, NPY/AgRP neurons have increased dendritic spines, which return back down to levels similar to fed controls within 48 h of re-feeding (Liu et al., 2012). Additionally, NPY/AgRP neurons will receive increased excitatory inputs onto their neurons (Liu et al., 2012; Yang et al., 2011), which returned back to normal after a re-feeding period (Liu et al., 2012). These structural and functional changes are dependent on the N-methyl-D-aspartic acid (NMDA) receptor (NMDAR), whose activation mediates post-synaptic plasticity of excitatory synapses (Holtmaat & Svoboda, 2009), as deletion of the NMDAR blocks the fasting–induced increases in excitatory tone (Liu et al., 2012). This is also mediated via the action of peripheral signals of ghrelin and leptin, as ghrelin increased excitatory tone onto NPY/AgRP neurons, while leptin rapidly decreased any increased excitatory tone (Yang et al.,
2011). Altogether, this suggested that NPY neurons are capable of rapid, plastic changes based on the status of hunger or satiety.

It should be noted that cessation from peripheral signals, such as a diet, does not maintain permanent changes. Rather, the peripheral signal itself can produce permanent changes, which can not be reversed or rescued with cessation of the peripheral signal. For example, short-term consumption of a HFD increased activity of NPY/AgRP neurons, which was reduced by leptin application (Wei et al., 2015). However, long HFD consumption sustains the increased activation of NPY/AgRP neurons, but this cannot be reduced by use of leptin (Baver et al., 2014). While this may be a product of leptin-resistance, this still provides evidence that NPY/AgRP neurons are capable of adapting, but that permanent changes can occur within these neurons.

1.3.6 Fructose promotes feeding via reward-related regions

Feeding is not only driven by the nutritive value of the diet, but also by its palatability (Sclafani & Ackroff, 1994), which can then reinforce consumption of a palatable diet, regardless of the status of satiety. Considering this, the impacts of fructose on feeding not only affect brain regions linked to homeostatic feeding, but also brain regions associated with the motivational and emotional processes associated with the hedonic aspect of feeding. These effects are likely on the mesolimbic dopaminergic system comprising primarily of the ventral tegmental area (VTA) and its projection sites, such as the striatum, the prefrontal cortex (PFC) and the orbitofrontal cortex. Fructose produces increased preference for itself or for other foods associated with it via the mesolimbic dopaminergic pathway, indicating that fructose may be involved in increased “liking” for food, which along with its homeostatic impacts, could drive
positive energy balance and feeding. In human studies, intake of a fructose solution in healthy individuals increased appetite and increased activity within the ventral striatum (Luo et al., 2015). Additionally, healthy participants in the aforementioned study shown a palatable food after fructose solution intake have increased appetite in relation to the food images and are more willing to give up long-term monetary rewards for short-term food rewards (Luo et al., 2015). The increased activity and hunger rating could be dependent on the health status of the individuals, as previous studies in normal weight individuals did not show a change in activity within the striatum upon fructose ingestion (Page et al., 2013), while obese individuals had increased striatal activity and hunger ratings (Wurtman & Wurtman, 1979). In animal studies, consumption of an 8% or 12% fructose solution produced fructose binging behaviour (Rorabaugh et al., 2014), as mice with intermittent access to this solution had increased solution consumption within the first hour of access. Of note, the binging of fructose solution was specific to fructose, as a follow-up study showed that mice given intermittent access to glucose or sucrose had lower binging compared to mice given fructose (Rorabaugh, Stratford, & Zahniser, 2015). Sclafani & Ackroff, 1994 showed that grape or cherry flavoured drinks conditioned to fructose were consumed more than a control solution. Altogether, this shows how fructose itself can reinforce fructose intake.

Preference for fructose seems to involve many projections sites of the VTA, including the nucleus accumbens (NAc), the PFC and the amygdala. Broadly, preference for a fructose solution can be blocked via the systemic use of dopamine receptor D_1 and D_2 antagonists (Baker, Shah, Sclafani, & Bodnar, 2003). Within the NAc, use of D_1 and D_2 antagonists reduces the acquisition of fructose preference, as animals with D_1 and D_2 receptor antagonism consumed significantly less flavoured fructose solution in training sessions, compared to vehicle treated
mice (Bernal et al., 2008). In the same study, D₁ and D₂ receptor antagonism hastened the extinction of preference of a flavour previously conditioned to fructose, as consumption of the fructose-associated flavour was significantly decreased by the third day of testing, in comparison to control mice (Bernal et al., 2008), with a similar effect being seen in the amygdala (Bernal et al., 2009). Additionally, the medial PFC may be involved in this preference, as D₂ antagonism within the medial PFC reduced preference for fructose (Malkusz et al., 2015). Altogether, these findings show that the actions of fructose can be mediated via not only homeostatic, but also hedonic pathways within the brain to promote feeding and increased preference for fructose.

1.4 Rationale

We and others have shown that fructose consumption results in hyperphagia and metabolic syndrome (Huang et al., 2004; Jürgens et al., 2005; Payant, Campbell, Hebert, Maratos-Flier, & Chee, 2019; Ramos et al., 2017; Soto et al., 2017). Fructose consumption increased excitatory tone onto NPY/AgRP neurons (Payant et al., 2019), thus suggesting the susceptibility of synaptic inputs to NPY/AgRP neurons to dietary fructose. Interestingly, the elevated synaptic input to NPY/AgRP neurons seen after short-term fructose feeding can be reversed by terminating fructose feeding. Additionally, one week of fructose consumption enhanced and increased ad libitum fructose consumption upon re-exposure after a one-week cessation period, suggesting a potential sensitization of these neurons. It is not known if NPY/AgRP neurons remain adaptable and capable of plastic changes after chronic fructose consumption. Additionally, it has also not been determined if chronic fructose consumption can prime the NPY/AgRP neurons for future exposure to fructose, resulting in increased fructose consumption and preference. My thesis will determine if abstaining from fructose after chronic
fructose feeding could reverse synaptic changes at NPY/AgRP neurons (Aim 1) or increase the preference for fructose (Aim 2).

1.5 Specific aims

1.5.1 Aim 1: Determine if cessation of chronic fructose intake reversed increased excitatory input to NPY/AgRP neurons

Acute fructose consumption increases excitatory input onto NPY/AgRP neurons, but cessation of fructose feeding can return synaptic input to control levels seen in chow-fed mice. We will determine if increased excitatory tone sustained by chronic fructose feeding for four weeks could be reversed by a one- or four-week chow intervention.

1.5.2 Aim 2: Determine if cessation from fructose intake increased fructose preference

Mice display hyperphagia when re-exposed to a palatable diet (Carlin et al., 2016), and our findings showed that mice re-exposed to fructose ate even more than mice fed fructose continuously. This suggested that subsequent exposure to fructose can further elevate the drive or preference for fructose. We will establish if an initial, sustained fructose feeding period would influence the preference for fructose and assess the duration of abstinence required to enhance fructose preference.
2.0 Methods

All animal procedures described were approved by the Carleton University Animal Care Committee. All animals were single housed in ambient temperature (21–22°C) and 40–60% humidity on a 12:12 light-dark cycle.

2.1 Aim 1: Determine if cessation of fructose consumption can reduce fructose-mediated increases in excitatory inputs at NPY/AgRP neurons

2.1.1 Study design

Male and female C3HeJ mice (JAX 000659) and Npy-hrGfp mice (JAX 006417), expressing human Renilla green fluorescent protein (GFP) under the Npy promoter were used for the studies. All mice began the study at four weeks of age and were assigned to three experimental groups: Chow (CW), Fructose (FrD) or Reversal (Rev). In this case, chow-fed animals were given *ad libitum* access to a chow diet (Teklad 2014, Envigo, Madison, WI) composed of 20% protein, 13% fat and 67% carbohydrate, while fructose-fed mice were given access to a 60% high-fructose diet (TD. 89247, Envigo, Madison, WI) composed of 18.3% protein, 5.2% fat and 60.4% carbohydrate. Weekly body weight and food intake measurements were taken between Zeitgeber time (ZT) 0 and ZT 3 to ensure that chow-fed and fructose-fed mice followed similar body weight and food intake changes as historical data. At the end of the study, animals were sacrificed for tissue collection for electrophysiology (See Section 2.1.2) at five weeks on diet or eight weeks on diet.

For Experiment 1A, we determined if one week of intervention following fructose consumption produced changes in physiology or neuronal activity. Chow-fed or fructose-fed
mice were given *ad libitum* access to chow or 60% fructose diet for five weeks (*Figure 1Ai, ii*), while Reversal mice were given access to fructose for four weeks, followed by one week of chow diet (*Figure 1Aiii*).

For Experiment 1B, given that one week of chow intervention may not produce changes in physiology or neuronal activity, the timeline for chow intervention was extended to four weeks of chow intake. Therefore, chow-fed and fructose-fed mice were given *ad libitum* access to chow or 60% fructose diet for eight weeks (*Figure 1Bi, ii*), while Reversal mice were given access to fructose diet for four weeks, followed by four weeks of diet (*Figure 1Biii*).

![Figure 1. Schematic of experimental design to determine if chow intervention for one (A) or four weeks (B) would revert synaptic changes seen following chronic fructose feeding.](image)

### 2.1.2 Aim 1: Electrophysiology

**Tissue preparation:** Mice were euthanized with an intraperitoneal injection of chloral hydrate (700 mg/kg) dissolved in saline (0.9% NaCl) and were perfused with an ice-cold, carbogenated (95% O₂, 5% CO₂) slice artificial cerebrospinal fluid (ACSF) solution (in mM: 118 NaCl, 3 KCl,
1.3 MgSO₄, 1.4 NaH₂PO₄, 5 MgCl₂, 10 glucose, 26 NaHCO₃ and 0.5 CaCl₂; 300 mOsm/L, pH 7.4). The brain was extracted and sectioned on an iced-packed petri dish prior to the hypothalamus and the cerebellum. The brain was then glued (Loctite 295; Acklands Grainger, Ottawa, Canada) onto an agarose piece on a specimen dish. Coronal brain tissue slices (250 µm) containing the ARC were sliced in a vibratome chamber (Vibratome VT1000s; Leica, Weltzlar, Germany) containing an ice-cold carbogenated slice-ACSF solution. The slices were then transferred to a warm (37°C) bath-ACSF solution (in mM: 124 NaCl, 3 KCl, 1.3 MgSO₄, 1.4 NaH₂PO₄, 10 glucose 26 NaHCO₃ and 2.5 CaCl₂ (300 mOsm/L, pH 7.4) for 5–10 minutes, before they were recovered in carbogenated bath-ACSF solution at room temperature (21–23°C) for one hour prior to use for whole-cell patch-clamp recordings.

**Whole-cell patch-clamp recordings:** Slices containing the ARC were placed and held down on to a recording stage using a platinum harp and were maintained in a warmed (TC-324B; Warner Instruments, Holliston, MA; 32 °C), carbogenated bath-ACSF solution. Borosilicate glass micropipettes (BF-150-86-10; Sutter Instruments, San Francisco, CA) were pulled using a Flaming/Brown micropipette Puller Model P-1000 (Sutter Instruments; San Francisco, CA) and backfilled with a K-gluconate internal pipette solution (in mM: 120 K-gluconate, 10 KCl, 10 HEPES, 1 MgCl₂, 1 EGTA, 4 MgATP, 0.5 NaGTP and 10 phosphocreatine; 285–295 mOsm/L, pH 7.24), which was filtered using a 4 mm syringe filter (Nalgene; Rochester, NY). After location of the ARC using a microscope (Examiner A1; Carl Zeiss, Thornwood, NY) and confirmation of green fluorescence (HXP120: Carl Zeiss, Thornwood, NY; Figure 2), whole-cell patch-clamp recordings of ARC cells commenced. Resting membrane potential (RMP) is calculated with no current injection, but all other voltage-clamp and current-clamp recordings were taken a holding membrane potential of –60mV. Recordings were limited to within two
hours after the recovery period and were obtained using Clampex 10.7 (Molecular Devices, San Jose, CA) using the Multiclamp 700B amplifier (Molecular Devices, San Jose, CA), digitized with a Digidata 1440A (Molecular Devices, San Jose, CA), and were filtered at 1 kHz.

Figure 2. Representative image of coronal slice from Npy-hrGfp mice with a patch pipette approaching fluorescent NPY/AgRP neurons within the ARC. Scale, 200 µM.

Electrophysiological Parameters: A variety of electrophysiological parameters were assessed to determine the impact of fructose cessation on the electrical properties of the NPY/AgRP neurons.

*Spontaneous excitatory post synaptic currents (sEPSCs):* sEPSCs were assessed in cells held at a holding potential of −60mV over the course of 5 minutes, and appeared as downwards
deflections in the recording trace. Cumulative probability plots for the interevent intervals and amplitude were produced by collecting the data from 200 events during a period of the recordings which had a similar frequency to the average sEPSC frequency of each individual cell.

*Current–voltage curve (IV curve):* The current–voltage relationship was determined in voltage clamp, where cells were held at –60 mV and were provided with a series of 250 ms voltage steps. The voltage steps ranged from +20 mV to –50 mV from a holding potential of –60 mV, and were elicited in increments of –10 mV steps.

*Resting membrane potential (RMP):* The resting membrane potential of the cell was assessed in current-clamp recordings without the injection of any holding current (I = 0 mode) over the course of 2 minutes. The RMP was determined by taking an average of the voltage output for the entire trace.

*Neuronal excitability:* To determine neuronal excitability, the cell was held in current-clamp at a membrane potential of –60 mV, and was provided with increasing current steps of +5 pA or +10 pA for 3 seconds, until 70 pA. The rheobase was determined as the minimum amount of current required to elicit an action potential. The number of action potentials was determined as the number of action potentials reaching threshold for each increasing current step.

*Input resistance:* Cells were held in current-clamp at a membrane potential of –60 mV and were provided with 3 s hyperpolarizing steps of 10 pA, until –40 pA. The input resistance was determined based on the voltage output at –10 pA of current, as many NPY/AgRP cells spontaneously depolarize beyond –10 pA of current.
Exclusion criteria: Cells were excluded from all parameters if their action potentials did not reach 0 mV. For sEPSCs analysis, cells were excluded if the frequency of sEPSCs continuously decreased or increased over the course of the 5 minutes. If the leak suddenly changes in a cell (e.g. from $-20$ pA to $-200$ pA), I stopped recording from this cell in voltage-clamp. For current-voltage curve analysis, cells were excluded if their current leak was below $-50$ pA. Additionally, Grubbs’ outlier test was used to remove any outliers from datasets.

2.1.3 Analysis

Aim 1: Repeated measures two-way ANOVA with a post-hoc Bonferroni’s test (Prism 9; GraphPad, La Jolla, CA), was used to determine overall groups difference and differences at weekly time points in body weight and food intake for all experiments.

Recording data obtained from Clampfit 10.7 (Molecular Devices, San Jose, CA) were analyzed using MiniAnalysis (Synaptosoft, Decatur, Georgia) for glutamatergic events, while current-clamp recordings were analyzed using Clampfit 10.7 and Excel 2016 (Microsoft Corporation, Redmond, WA). Sample traces of electrophysiological recordings were produced using OriginPro 2020 (OriginLab, Northampton, MA) and figures were assembled using Adobe Illustrator 2022 (Adobe Inc., San Jose, CA). Repeated-measures one-way ANOVA with a post-hoc Bonferroni’s test was used to compare mean differences for synaptic events and electrical properties of cells. Kolmogorov-Smirnov (K-S) test was used to assess the distribution of interevent intervals and amplitude and from a cumulative probability plot of synaptic events.
2.2 Aim 2: Determine if fructose re-exposure would increase the preference for fructose consumption after a period of cessation.

2.2.1 Aim 2: Physiology methods

All animal procedures were done as described in section 2.1.1. Along with weekly measurements during the study, daily measurements of body weight and food intake were taken during the fructose choice period (see 2.2.2). Fructose choice was used to determine the preference for fructose and was conducted at the end of the fifth week on diet for experiment 2. For experiments 3 and 4, fructose choice was conducted at the beginning of the study at four weeks of age and at the end of the fifth or eighth week on diet, respectively.

2.2.2 Fructose choice

To assess preference for fructose, mice were given *ad libitum* access to a choice of the chow or 60% FrD diet in white ramekins over the course of one week. The ramekins were counterbalanced every day to ensure there is no preference for a side of the cage. Daily measurements of body weight, chow and fructose intake were taken, and the final day of the fructose choice was used to assess fructose preference in the mice. Fructose preference was calculated by dividing the number of fructose calories by the total calories of both chow and fructose diet and converting it into a percentage.
2.2.3 Experiment 2: Fructose preference with one week of fructose cessation

To determine if a cessation from fructose consumption can produce an increase in preference, mice were assigned to three experimental groups: Chow, Fructose or Reversal mice. Mice were assigned to each diet group by matching their body weights so that each group had similar mean body weight at the beginning of the study. Chow-fed and fructose-fed mice had *ad libitum* access to chow or fructose diet, respectively, for five weeks (Figure 3i, ii). The Reversal mice had *ad libitum* access to fructose for four weeks followed by one week of chow intake (Figure 3iii). For all experimental groups, animals were given a fructose choice (see 2.2.1) at the end of the 5-week period.

![Figure 3](image.png)

**Figure 3.** Schematic of experimental design to determine if chow intervention for one-week (iii) can enhance fructose preference compared to chow-fed (i) controls. fructose-fed mice (ii).

2.2.4 Experiment 3: Changes in fructose preference with one week of fructose cessation

It would be relevant to determine how innate fructose preference may have influenced within our study design. Experiment 3 has a similar design as experiment 2 (see 2.2.2), however mice are given a week of fructose choice prior to assignment to the three diet groups: Chow, Fructose or Reversal (Figure 4A). Mice were assigned to their diet groups based on their body weight and innate preference to ensure that all groups had similar average body weights and preference. Chow-fed and fructose-fed mice had *ad libitum* access to chow or fructose diet,
respectively, for five weeks (Figure 4Ai, ii), while the Reversal mice had access to fructose for four weeks followed by one week of chow intake (Figure 4Aiii). Following the ad libitum phase of the study, mice were given a fructose choice test to determine their preference at the end of the study. The innate preference for fructose (Pre) was compared to the preference at the end of the study (Post).

2.2.5 Experiment 4: Changes in fructose preference with four weeks of fructose cessation

One week of fructose cessation is a short intervention, which may not yield a difference in preference. Thus, the time of fructose cessation was extended from one week to four weeks of chow intake. For experiment 4, mice were first given a fructose choice for one week, followed up assignment to three diet groups: Chow, Fructose or Reversal (Figure 4B). Mice were again assigned to their diet groups as described in experiment 3 (see 2.2.3). Chow-fed and fructose-fed mice had ad libitum access to chow or fructose diet for eight weeks (Figure 4Bi, ii). Reversal mice had access to fructose for four weeks, followed by four weeks of chow intake (Figure 4Biii). Following the ad libitum phase of the study, mice were given a fructose choice test to determine their preference at the end of the study. The innate preference for fructose (Pre) was compared to the preference at the end of the study (Post).
2.2.6 Analysis

Aim 2: Repeated measures one-way ANOVA with a post-hoc Bonferroni’s test was used to compare the body weight of animals for diet group assignment for all experiments and was also used to compare the innate preferences for fructose in experiments 3 and 4.

Repeated measures two-way ANOVA with a post-hoc Bonferroni’s test (Prism 9; GraphPad, La Jolla, CA), was used to determine overall groups difference and differences at weekly time points in body weight and food intake for all experiments. Repeated measures two-way ANOVA with a post-hoc Bonferroni’s test was also used to determine differences in fructose preference from the Pre and Post time points of the study in experiments 3 and 4. Repeated measures one-way ANOVA with a post-hoc Bonferroni’s test was also used to compare fructose preference at the end of all experiments. Paired t-test was used to compare the change in preference between Pre and Post time points, while an unpaired t-test was used to compare fructose preference at the end of the diet study between experiments 3 and 4.
3.0 Results

3.1 Aim 1: Determine if cessation of fructose consumption can reduce fructose-mediated increases in excitatory inputs at NPY/AgRP neurons

3.1.1 Short-term cessation from fructose consumption returns excitatory tone in male but not female mice

We determined if fructose-mediated increase in excitatory tone at NPY/AgRP neurons can be reduced to chow-fed levels with fructose cessation. Male and female mice were given *ad libitum* access to chow or fructose diet (Figure 1Ai, ii). Mice with fructose cessation were given access to fructose diet for four weeks, followed by a diet switch to chow diet for one week (Figure 1Aiii).

Fructose consumption does not change passive membrane properties such as the RMP, input resistance, or current-voltage relationship in both sexes (Table 1).

Table 1. Descriptive statistics of the impact of five weeks fructose consumption passive membrane properties of NPY/AgRP neurons

<table>
<thead>
<tr>
<th>Property</th>
<th>Sex (n)</th>
<th>Chow</th>
<th>Fructose</th>
<th>Reversal</th>
<th>F-test, p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential</td>
<td>Male (39)</td>
<td>−44.1 ± 2.29</td>
<td>−45.9 ± 2.58</td>
<td>−45.3 ± 2.84</td>
<td>F2, 36 = 0.11, p = 0.893</td>
</tr>
<tr>
<td></td>
<td>Female (36)</td>
<td>−42.2 ± 2.96</td>
<td>−41.4 ± 3.27</td>
<td>−44.9 ± 4.91</td>
<td>F2, 33 = 0.22, p = 0.806</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>Male (44)</td>
<td>1851 ± 440</td>
<td>2388 ± 348</td>
<td>3460 ± 476</td>
<td>F2, 31 = 2.96, p = 0.067</td>
</tr>
<tr>
<td></td>
<td>Female (33)</td>
<td>1819 ± 276</td>
<td>2064 ± 303</td>
<td>2543 ± 449</td>
<td>F2, 30 = 1.14, p = 0.333</td>
</tr>
<tr>
<td>Current–voltage relationship</td>
<td>Male (33)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>F2, 30 = 2.22a, p = 0.126</td>
</tr>
<tr>
<td></td>
<td>Female (26)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>F2, 24 = 0.79a, p = 0.465</td>
</tr>
</tbody>
</table>

All values are indicated as mean ± SEM.

*Mean values are not applicable. Statistical comparisons reflect the main effect of diet.
Fructose consumption does not change active membrane properties such as the rheobase
\((\text{Male: } F_{2,38} = 0.41, p = 0.664, \text{ Figure 5Ai, iii}; \text{ Female: } F_{2,32} = 0.89, p = 0.421; \text{ Figure 5Aii, iv})\)
or the number of elicited action potentials \((\text{Male: } F_{2,38} = 0.69, p = 0.507, \text{ Figure 5Bi}; \text{ Female: }\nF_{2,23} = 2.93, p = 0.068, \text{ Figure 5Bii})\).

**Figure 5. No impact of fructose consumption on NPY/AgRP neuron excitability.** Ai, ii, Sample traces of action potentials elicited from \(V_h = -60\) mV by 5 pA current step in male (Ai) and female (Aii) NPY/AgRP neurons. Aiii, iv, Comparison of the average rheobase in male (Aiii) and female (Aiv) NPY/AgRP neurons. Bi, Bii, Comparison of the number of elicited action potentials with increasing +10 pA current steps in male (Bi) and female (Bii) mice. The number of cells is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences are determined by one-way ANOVA (Aiii, iv) or two-way ANOVA (Bi, ii).
While there were no differences in NPY/AgRP neurons properties, there was a difference in excitatory tone at NPY/AgRP neurons across the diet groups. Fructose consumption produced a trend upwards in the average sEPSC frequency in male mice (Figure 6Ai, Bi), while it increased the sEPSC frequency in female mice (Figure 6Aii, Bii). Fructose consumption produced a leftward shift in the distribution of interevent (IE) intervals in both male (p < 0.001; Figure 6Ci) and female mice (p < 0.001; Figure 6Cii), with these findings being confirmed with the Kolmogorov-Smirnov test. Fructose consumption also increases the amplitude onto NPY/AgRP neurons, as there is a rightward shift in the distribution of sEPSC amplitudes in both males (p < 0.001; Figure 6Di) and females (p < 0.001; Figure 6Dii).

Interestingly, we found that the excitatory inputs onto NPY/AgRP are different in males and females returned to chow diet for one week. Male mice with one week of fructose cessation had a decrease in their sEPSC frequency, a rightward shift in the distribution of the IE intervals and a leftward shift in the distribution of amplitudes, returning back to levels comparable to chow-fed mice. Female mice with one week of fructose cessation maintained an elevated level of sEPSC frequency, similar to fructose-fed female mice. Additionally, despite cessation from fructose consumption, there was a leftward shift in the distribution of IE intervals and a rightward shift in the distribution of sEPSC amplitudes.

Altogether, these findings show that five weeks of fructose consumption does not change passive or active membrane properties. Additionally, we found that one week of fructose cessation is sufficient to revert the excitatory tone at NPY/AgRP neurons in male mice but is not sufficient in female mice.
Figure 6. One week fructose cessation reverts excitatory tone in males. Ai, ii, Sample traces of excitatory inputs at NPY/AgRP neurons in male (Ai) and female (Aii) neurons. Bi, Bii, Comparison of the average sEPSC frequency with five weeks of chow or fructose consumption, and with one week of fructose cessation in male (Bi) and female (Bii) neurons. Average sEPSCs were calculated over the course of 5 minutes. Ci, Cii Comparison of the distribution and mean (inset) sEPSC interevent intervals in male (Ci) and female (Cii) mice. Cumulative distribution of interevent intervals were binned every 100 ms, and mean interevent intervals were determined from a sample of 200 sEPSC events selected from each cell. Di, Dii Comparison of the distribution and mean (inset) sEPSC amplitude in male (Di) and female (Dii) neurons. Cumulative distribution of interevent intervals were binned every 5 pA, and mean amplitudes were determined from a sample of 200 sEPSC events selected from each cell. The number of cells per group is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences determined by one-way ANOVA (B, C, D) and Kolmogorov–Smirnov test (C, D) are represented as: *, p < 0.05, **, p < 0.01, ****, p < 0.0001.

3.1.2 Longer fructose consumption increases male NPY/AgRP neuron excitability.

Longer fructose cessation does not reduce excitatory tone at female NPY/AgRP neurons

Five weeks of fructose consumption increased excitatory tone at NPY/AgRP neurons in all mice. However, only males saw a reversal in the excitatory tone with the cessation of fructose feeding. To determine if the excitatory tone could be recovered with an extended period of fructose cessation, mice were given ad libitum access to chow or fructose diet (Figure 1Bi, ii) for eight weeks. Mice with fructose cessation were given access to fructose diet for four weeks, following by a diet switch to chow diet for four weeks (Figure 1Bii).

We see some differences in passive membrane properties but only in male mice. While both sexes do not differ in RMP or input resistance, we see a difference in the current-voltage relationships. Male mice with four weeks of fructose cessation have reduced current flow. Female mice do not have a difference in their current-voltage relationship among all diet groups (Table 2).
Table 2. Descriptive statistics of the impact of eight weeks fructose consumption passive membrane properties of NPY/AgRP neurons

<table>
<thead>
<tr>
<th>Property</th>
<th>Sex (n)</th>
<th>Chow</th>
<th>Fructose</th>
<th>Reversal</th>
<th>F-test, p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting membrane potential (mV)</td>
<td>Male (28)</td>
<td>−40.8 ± 2.31</td>
<td>−41.8 ± 1.80</td>
<td>−45.7 ± 2.25</td>
<td>F₂, 25 = 1.52, p = 0.238</td>
</tr>
<tr>
<td></td>
<td>Female (39)</td>
<td>−40.6 ± 3.70</td>
<td>−46.4 ± 2.15</td>
<td>−48.3 ± 2.95</td>
<td>F₂, 45 = 1.77, p = 0.183</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>Male (26)</td>
<td>1334 ± 232</td>
<td>2028 ± 321</td>
<td>3027 ± 776</td>
<td>F₂, 23 = 2.39, p = 0.114</td>
</tr>
<tr>
<td></td>
<td>Female (47)</td>
<td>2307 ± 352</td>
<td>2297 ± 297</td>
<td>3164 ± 351</td>
<td>F₂, 44 = 2.14, p = 0.129</td>
</tr>
<tr>
<td>Current–voltage relationship</td>
<td>Male (26)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>F₂, 24 = 5.06, p = 0.015*</td>
</tr>
<tr>
<td></td>
<td>Female (41)</td>
<td>N/A</td>
<td>N/A</td>
<td>N/</td>
<td>F₂, 38 = 1.63, p = 0.209</td>
</tr>
</tbody>
</table>

All values are indicated as mean ± SEM. Statistical differences determined by two-way ANOVA are represented as: *, p < 0.05.

*aMean values are not applicable. Statistical comparisons reflect the main effect of diet.

Fructose consumption reduced the rheobase (F₂, 25 = 4.98, p = 0.015; Figure 7Ai, ii) in male mice but not female mice (F₂, 46 = 1.35, p = 0.271; Figure 7Aii, iv). Additionally, fructose consumption increased the number of elicited action potentials in male mice (F₂, 26 = 5.88, p = 0.008; Figure 7Bi) but had no impact in female mice (F₂, 46 = 1.65, p = 0.204; Figure 7Bii).

Despite fructose cessation for four weeks, the increased excitability was maintained in male mice.
Figure 7. Increased male NPY/AgRP excitability with chronic fructose consumption. Ai, ii Sample traces of elicited action potentials elicited from $V_h = -60$ mV by 5 pA current step in male (Ai) and female (Aii) NPY/AgRP neurons. Aiii, iv, Comparison of the average rheobase in male (Aiii) and female (Aiv) NPY/AgRP neurons. Bi, Bii, Comparison of the number of elicited action potentials with increasing +10 pA current steps in male (Bi) and female (Bii) mice. The number of cells is indicated in parentheses. All values are represented as mean ± SEM Statistical differences between groups for one-way (Aiii, iv) or two-way ANOVA (Bi, ii) are represented as: *, $p < 0.05$, **, $p < 0.01$, Bonferroni’s post-hoc differences between chow and fructose are represented as: #, $p < 0.05$, ##, $p < 0.01$, ###, $p < 0.001$. Bonferroni’s post-hoc differences between chow and Reversal mice are represented as: $\$, $p < 0.05$, $$, $p < 0.01$, $$$, $p < 0.001$.

Eight weeks of fructose consumption increased the average sEPSC frequency at both male ($F_{2, 21} = 6.80, p = 0.005$; Figure 8Ai, Bi) and female ($F_{2, 34} = 5.26, p = 0.010$; Figure 8Aii, Bii) NPY/AgRP neurons. We also saw a leftward shift in the distribution of IE intervals in both male
(p < 0.001; Figure 8Ci) and female mice (p < 0.001; Figure 8Cii). Interestingly, while male mice had a leftward shift in the distribution of sEPSC amplitudes (Figure 8Di), female mice had a rightward shift (Figure 8Dii). Four weeks of fructose cessation reduced the excitatory tone at NPY/AgRP neurons in male mice, produced a rightward shift compared to fructose-fed males, and a leftward shift in the distribution of sEPSC amplitude. Meanwhile, four weeks of fructose cessation did not reduce the average sEPSC frequency, maintained a leftward shift in the distribution of IE intervals and a leftward shift in the distribution of sEPSC amplitudes.

Altogether, our findings show that extended fructose consumption increased the excitability in males, but not female NPY/AgRP neurons. Four weeks of fructose cessation reverted the excitatory input at NPY/AgRP neurons to chow-fed levels in male mice, but female mice still maintained an elevated level of excitatory tone at NPY/AgRP neurons. This indicates that despite a longer period of abstinence from fructose consumption, the excitatory inputs of female mice remain persistent.
Figure 8. No reduction in excitatory tone in females after four-week fructose cessation. Ai, ii, Sample traces of excitatory inputs at NPY/AgRP neurons in male (Ai) and female (Aii) neurons. Bi, Bii, Comparison of the average sEPSC frequency with eight weeks of chow or fructose consumption, and with four week of fructose cessation in male (Bi) and (Bii) neurons. Average sEPSCs were calculated over the course of 5 minutes. Ci, Cii Comparison of the distribution and mean (inset) of sEPSC interevent intervals in male (Ci) and female (Cii) mice. Cumulative distribution of interevent intervals were binned every 100 ms, and mean interevent intervals were determined from a sample of 200 sEPSC events selected from each cell. Di, Dii, Comparison of the distribution and mean (inset) sEPSC amplitude in male (Di) and female (Dii) neurons. Cumulative distribution of interevent intervals were binned every 5 pA, and mean amplitudes were determined from a sample of 200 sEPSC events selected from each cell. The number of cells per group is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences determined by one-way ANOVA (B, C, D) and Kolmogorov-Smirnov test (C, D) are represented as: *, p < 0.05, **, p < 0.01, ***, p < 0.001, ****, p < 0.0001.

3.2 Aim 2: Determine if fructose re-exposure would increase the preference for fructose consumption after a period of cessation.

3.2.1 One week of fructose cessation did not lead to increased fructose preference

We wanted to determine if one week of fructose cessation can produce enhanced fructose preference compared to mice given chow or fructose throughout the entire study. We determined fructose preference in animals fed chow or fructose for five weeks (Figure 3Ai, ii) as well as animals fed fructose for four weeks followed by one week of chow (Figure 3Aiii). There was no difference in body weight change over the 5-week period among male diet groups ($F_{2, 21} = 0.12, p = 0.884$; Figure 9Ai) or female ($F_{2, 9} = 2.62, p = 0.127$; Figure 9Aii) diet groups. Fructose-fed male mice cumulatively ate more calories than fructose-naïve mice ($F_{2, 16} = 3.94, p = 0.042$; Figure 9Bi), but no main effect of diet was seen in female mice ($F_{2, 7} = 0.44, p = 0.648$; Figure 9Bii). Interestingly, there was no difference in fructose preference among male ($F_{2, 21} = 2.60, p = 0.098$; Figure 9Ci) or female ($F_{2, 9} = 1.96, p = 0.196$; Figure 9Cii) diet groups.
Figure 9. No body weight gain or hyperphagia with 5-week fructose consumption. Ai, ii, Comparison of body weight gain in all diet groups in male (Ai) and female (Aii) mice. Each dot represents an average body weight gain in grams at weekly time points. Bi, ii, Comparison of cumulative food intake in all diet groups in male (Bi) and female (Bii) mice. Each dot represents an average
cumulative food intake in kilocalories at weekly time points. **Ci, ii**, Bar graph comparison of fructose preference in percentage in male (**Ci**) and female (**Cii**) mice. Each dot represents the fructose preference at the end of the fructose choice week and is calculated as percent fructose intake out of total food intake. Beige bar in graphs **A** and **B** indicates when Reversal mice were switched from fructose to chow diet. The number of animals is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences determined by two-way ANOVA (**A, B**) or one-way ANOVA (**C**).

### 3.2.2 One week of fructose cessation normalized fructose preference in males, but not females

In the previous experiment, male and female displayed a wide range of responses to fructose, such having low or high fructose-preference (**Figure 9Ci, ii**). To address the inherent differences in fructose preference, we determined if fructose consumption would increase inherent preference for fructose by comparing preference before and after the *ad libitum* diet period. Initial fructose preference was determined, followed by mice being given access to chow or fructose for five weeks (**Figure 4Ai, ii**) or fructose for four weeks followed by one week of chow (**Figure 4Aiii**).

There was a main effect of diet on body weight gain in male mice (*F*₂, 21 = 3.59, *p* = 0.046; **Figure 10Ai**), but there was no difference between the diet groups with post-hoc testing. Female mice did not have a difference in body weight gain (*F*₂, 21 = 0.70, *p* = 0.508; **Figure 10Aii**). There was also no difference in cumulative caloric intake among diet groups in male (*F*₂, 21 = 2.33, *p* = 0.122; **Figure 10Bi**) or female mice (*F*₂, 21 = 3.06, *p* = 0.067; **Figure 10Bii**).

We again observed a wide range of fructose preference in all male and female mice (0–65%) but saw differing responses in male and females re-exposed to fructose after a cessation period. Chow-fed male mice had no difference in their fructose preference (*t*(7) = 0.65, *p* = 0.538; **Figure 11Ai**), but all chow-fed females increased their fructose preference upon re-
Figure 10. No body weight gain or hyperphagia with five weeks of fructose intake. Ai, ii. Comparison of body weight gain in grams in male (Ai) and female (Aii) mice across all diet groups. Each dot represents an average body weight gain at each respective weekly time point. Bi, ii. Comparison of cumulative food intake in kilocalories in male (Bi) and female (Bii) in all diet groups. Each dot represents an average, cumulative food intake at each weekly point. Beige bar in graphs indicates when Reversal mice were switched from fructose to chow diet for one week. The number of animals is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences determined by two-way ANOVA for all graphs.

exposure after five weeks ($t(7) = 4.69, p = 0.002$; Figure 11Aii). This increase in preference was not due to age, given that age-matched 11-week-old fructose-naïve females tended to have a lower fructose preference ($21.01 ± 4.98\%$, $n = 7$; $t(13) = 2.15, p = 0.051$; Figure 11Aiii). All fructose-fed males had a decrease in their fructose preference after five weeks of fructose
consumption ($t(7) = 3.82, p = 0.007; \textbf{Figure 11Bi}$), while most female had a non-significant decrease in preference ($t(7) = 1.42, p = 0.200; \textbf{Figure 11Bii}$). Fructose-fed mice returned to chow diet for one week did not display a difference in preference in both male ($t(7) = 1.73, p = 0.127; \textbf{Figure 11Ci}$) and female ($t(7) = 0.42, p = 0.688; \textbf{Figure 11Cii}$) mice.

We found differing responses between male and female mice returned to chow diet for one week following four weeks of fructose intake. While male mice displayed similar preference to chow-fed mice ($F_{2, 21} = 7.12, p = 0.004; \textbf{Figure 11Di}$), female mice displayed significantly lower preference compared to chow-fed counterparts ($F_{2, 21} = 14.96, p < 0.001; \textbf{Figure 11Dii}$). Taken altogether, these findings suggest that prior exposure to fructose may increase fructose preference after long periods of abstinence, as seen with chow-fed females. Additionally, one week of fructose cessation may be sufficient to normalize preference in males, but not in female mice.
Figure 11. Sex difference in fructose preference with 1-week fructose cessation. Ai, ii, Comparison of fructose preference prior to and at the end of the study in chow-fed males (Ai) and females (Aii). Aiii, Comparison of fructose preference between chow-fed females and age-matched 11-week-old females. Bi, Bii, Comparison of fructose preference prior to and at the end of the study in fructose-fed males (Bi) and females (Bii). Ci, Cii, Comparison of fructose preference prior to and at the end of the study in male (Ci)
and female (Cii) mice with one-week fructose cessation. Di, Dii, Comparison of fructose preference at the end of the 5-week diet study in male (Di) and female (Dii) mice. Across all graphs, each dot represents the fructose preference at the end of the fructose choice week and is calculated as percent fructose intake out of total food intake. Pre indicates initial fructose preference, while Post indicates preference at the end of the diet study. The number of animals is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences determined by pairwise comparisons (Ai-ii, B, C), unpaired t-test (Aiii) and one-way ANOVA (D) are represented as: *, p < 0.05, **, p < 0.01, ***, p < 0.001.

3.2.3 Four weeks of fructose cessation increased fructose preference

Our findings from the previous experiment showed that while male mice had their fructose preference normalize to chow-fed controls after one week of fructose cessation, female mice had significantly lower preference compared to chow-fed controls.

We prolonged the duration of fructose cessation from one week to four weeks to determine if fructose preference can be further enhanced in male mice and normalized in female mice. After initial fructose preference was determined, mice were given access to chow or fructose for eight weeks (Figure 4Bi, ii) or fructose for four weeks followed by four weeks of chow (Figure 4Biii).

There was no difference in body weight gain in male mice (F2,28 = 1.60, p = 0.220; Figure 12Ai), but fructose-fed females gained more body weight (F2,20 = 3.61, p = 0.046; Figure 12Aii). Fructose-fed male (F2,28 = 11.71, p < 0.001; Figure 12Bi) and female (F2,20 = 5.79, p = 0.010; Figure 12Bii) mice had increased cumulative food intake.

We again saw a wide range of fructose preference in male and female mice (0–90 %). Both chow-fed males (t(9) = 4.50, p = 0.002; Figure 13Ai) and females (t(7) = 6.64, p < 0.001; Figure 13Aii) increased their fructose preference upon re-exposure after eight weeks of chow intake. Most fructose-fed males (t(9) = 1.62, p = 0.141; Figure 13Bi) and females (t(7) = 1.81, p
Figure 12. Hyperphagia with eight weeks of fructose consumption. Ai, ii, Comparison of body weight gain in grams in male (Ai) and female (Aii) mice across all diet groups. Each dot represents an average body weight gain at each respective weekly time point. Bi, ii, Comparison of cumulative food intake in kilocalories in male (Bi) and female (Bii) in all diet groups. Each dot represents an average, cumulative food intake at each weekly point. Beige bar in graphs indicates when Reversal mice were switched from fructose to chow diet for four weeks. The number of animals is indicated in parentheses. All values are represented as mean ± SEM. Statistical differences between diet groups determined by two-way ANOVA are represented as: *, p < 0.05. Bonferroni’s post-hoc comparisons between chow and fructose-fed mice are represented as: #, p < 0.05, ##, p < 0.01, ###, p < 0.001. Post-hoc differences for chow and Reversal mice are represented as: $, p < 0.05, $$, p < 0.01, $$$, p < 0.001, $$$$ p < 0.0001.

= 0.114; Figure 13Bii) surprisingly had a trend upwards in increased preference, but this was not significantly higher than initial preference. Male (t(10) = 7.09, p < 0.001; Figure 13Ci) and
female \((t(6) = 4.72, \ p = 0.003; \textbf{Figure 13Cii})\) fructose-fed mice returned to chow for four weeks all had an increase in their fructose preference. Four weeks of fructose cessation is sufficient to normalize the preference for fructose in both sexes, as male \((F_{2,28} = 8.75, \ p = 0.001; \textbf{Figure 13Di})\) and female mice \((F_{2,20} = 4.93, \ p = 0.018; \textbf{Figure 13Dii})\) following four weeks of fructose cessation have similar preference to chow-fed female controls.

These findings suggest the prior exposure to fructose can increase preference for fructose after long periods of abstinence in both males and females. Four weeks of fructose cessation is sufficient time to increase innate fructose preference in both sexes but does not further enhance preference to be higher than chow-fed controls.
Figure 13. Four-week fructose cessation increases fructose preference. Ai, ii, Comparison of fructose preference prior to and at the end of the study in chow-fed males (Ai) and females (Aii). Bi, Bii, Comparison of fructose preference prior to and at the end of the study in fructose-fed males (Bi) and females (Bii). Ci, Cii, Comparison of fructose preference prior to and at the end of the study in male (Ci) and female (Cii) mice with four-week fructose cessation. Di, Dii, Comparison of fructose preference at the end of the 8-week diet study in male (Di) and female (Dii) mice. Across all graphs, each dot represents the fructose preference at the end of the
fructose choice week and is calculated as percent fructose intake out of total food intake. Pre indicates initial fructose preference, while Post indicates preference at the end of the diet study. The number of animals is indicated in the parentheses. All values are represented as mean ± SEM. Statistical differences determined by pairwise comparisons (A, B, C) and one-way ANOVA (D) are represented as: *, p < 0.05, **, p < 0.01, ***, p < 0.001, ****, p < 0.0001.

3.2.4 Increased fructose preference with longer fructose abstinence

We observed that mice with eight weeks of diet consumption had higher fructose preference compared to mice with five weeks of diet consumption. Thus, we compared the fructose preference at the end of the 5-week study to the preference at the end of the 8-week study.

We found that chow-fed male (t(16) = 1.36, p = 0.193; Figure 14A) and female (t(14) = 1.11, p = 0.286; Figure 14B) with eight weeks between fructose exposures did not have significantly higher preference, compared to 5-week mice. Surprisingly, all fructose-fed mice (Male: t(16) = 2.57, p = 0.021; Female: t(14) = 2.11, p = 0.054) in the 8-week study had higher fructose preference compared to mice consuming fructose for five weeks. Finally, both male (t(17) = 4.20, p < 0.001) and female (t(13) = 3.95, p = 0.002) mice with four weeks of fructose cessation had significantly higher preference compared to mice with only one week of fructose cessation. Altogether, these findings show that longer period of fructose abstinence enhanced fructose preference upon re-exposure.
Figure 14. Comparison of fructose preference with 5-week and 8-week fructose consumption. A, B, Bar graph comparing end of study fructose preference in male (A) and female (B) mice in the 5-week (dark blue, dark pink) and 8-week (light blue, light pink) diet study. Mice in Reversal received four weeks of fructose followed by one week or four weeks of chow in the 5-week and 8-week study, respectively. The white dots represent fructose preference of individual mice at the end of the diet study in both experiments. All values are represented as mean ± SEM. Statistical differences determined by unpaired t-test are represented as: *, p < 0.05, **, p < 0.01, ***, p < 0.001.

4.0 Discussion

Fructose consumption increased the excitatory tone at NPY/AgRP neurons of the ARC. Cessation from fructose consumption reverted increased excitatory tone at NPY/AgRP neurons down to levels comparable to chow-fed mice in males, but not in females. Continuous fructose consumption decreased the preference for fructose, while cessation from this consumption returned this preference back to levels comparable to chow-fed controls. Taken together, these findings suggest that fructose cessation may recover fructose-mediated neurological changes in electrical or synaptic properties and fructose preference.
4.1 Fructose consumption increased excitatory tone and excitability of NPY/AgRP neurons of the ARC

Fructose consumption increased the excitatory tone onto NPY/AgRP neurons in both male and female mice, confirming the previous findings from our lab. Increased excitatory tone at NPY/AgRP neurons resembles the synaptic state seen with fasting and hunger, which may represent a maladaptation of fructose consumption (Kong et al., 2016; Liu et al., 2012; Yang et al., 2011). Mice with an overnight fast received increased excitatory inputs onto their NPY neurons (Kong et al., 2016; Yang et al., 2011), which returned down to levels comparable to fed mice after 48 h (Liu et al., 2012; Yang et al., 2011). These changes are NMDAR dependent, as deletion of the NMDAR blocked the increases in excitatory tone at NPY/AgRP neurons (Liu et al., 2012). Hunger-mediated increases in excitatory tone are also driven by AMPK, given that signals of hunger such as ghrelin will increase excitatory input via AMPK activity (Yang et al., 2011), while signals of satiety such as leptin will decrease any increases in excitatory tone at NPY/AgRP neurons (Pinto et al., 2004; Yang et al., 2011). AMPK will function presynaptically on a neuron projecting to NPY/AgRP neurons, as intracellular AMPK antagonism within patched NPY/AgRP neurons did not reduce hunger-mediated increases in excitatory inputs (Yang et al., 2011). However, bath application of an AMPK antagonist reduced increased excitatory tone onto NPY/AgRP neurons of hungry mice, indicating a presynaptic action. This increased AMPK activity can drive the release of presynaptic $\text{Ca}^{2+}$ stores to increase excitatory synaptic output onto NPY/AgRP neurons (Yang et al., 2011) to drive feeding. Given that
fructose increases AMPK activity within the hypothalamus to promote feeding (Cha et al., 2008) increased AMPK activity would also result in increased excitatory inputs onto NPY/AgRP neurons after fructose consumption.

Chronic fructose consumption increased the excitability of NPY/AgRP neurons in male but not female mice. It is not surprising that fructose consumption can lead to increased excitability, given that fructose increases excitatory tone onto NPY/AgRP neurons. Increased excitatory tone led to depolarization (Purves et al., 2001), and optogenetic findings confirmed that increased excitatory input onto the NPY/AgRP neurons also produced increased firing (Kong et al., 2016; Krashes et al., 2014). In states of hunger (Liu et al., 2012) or stimulation of presynaptic AMPK (Kong et al., 2016), there is increased excitatory tone onto NPY/AgRP neurons, along with a depolarized RMP and increased action potential firing rate, showing that an increase in excitatory tone drives excitability. Therefore, fructose can drive the excitability of NPY/AgRP neurons in male mice by increasing excitatory inputs onto these neurons.

Our studies have not investigated the source of the increased excitatory tone at NPY/AgRP neurons. One potential region of interest is the paraventricular nucleus of the hypothalamus (PVH). The PVH has dense glutamatergic projections to the NPY/AgRP neurons of the ARC, with activation of these fibers promoting feeding behaviour (Krashes et al., 2014). There is evidence of fructokinase, a fructose degradation enzyme, within the PVH (Song et al., 2017), indicating the potential of fructose impacting ARC projecting PVH glutamatergic neurons.
4.2 Fructose cessation reverted the excitatory tone of NPY/AgRP neurons of male mice

When fructose-fed males were returned to a chow diet, their sEPSC frequency was comparable to chow-fed controls. Thus, cessation from fructose consumption in male NPY/AgRP neurons reverted the excitatory tone that was initially enhanced with fructose feeding. However, regardless of the duration of fructose abstinence, female mice maintained a high excitatory tone onto NPY/AgRP neurons, similar to fructose-fed females.

Reversal of the excitatory tone in males is supported by literature, given that the findings of increased excitatory tone due to fasting and reversal of this tone with re-feeding are all done in male mice (Kong et al., 2016; Liu et al., 2012; Yang et al., 2011). There is a gap in the literature about the synaptic adaptability of NPY/AgRP neurons in females, making it difficult to ascertain why the excitatory inputs onto female NPY/AgRP neurons are more persistent. However, there is much evidence showing that changes within the female system can remain persistent within the brain. For example, in cases of metabolic challenges, such as pregnancy, epigenetic changes such as increased histone deacetylation and reduced number of neurons with histone acetylation remained permanent within the ARC (Teixeira, Ramos-Lobo, Furigo, & Donato, 2019). Restricted feeding in females maintained an upregulated number of melanocortin and NPY receptors, even after a period of re-feeding (Méquinion et al., 2017), while stressors permanently increased spine density (McGrath & Briand, 2022) and serum cortisol levels (dos Santos Guilherme et al., 2022), even after the stressor has stopped. In female mice with overnutrition, such as mice raised in small litters, the sEPSC frequency in ARC leptin expressing neurons was permanently increased (Zampieri et al., 2020) compared to control mice. Altogether, these findings do suggest that despite fructose abstinence, permanent changes can be maintained at the level of the NPY/AgRP neurons in female mice.
Despite fructose cessation for four weeks, male NPY/AgRP neurons maintained a high level of excitability, seen as a reduced rheobase and increased number of elicited action potentials. Maintenance of excitability in male NPY/AgRP neurons is consistent with the literature, given that male mice who have consumed palatable diets for long periods of time (Baver et al., 2014; Korgan, Wei, Martin, Kaczorowski, & O’Connell, 2021; Wei et al., 2015) maintained a high level of excitability at their NPY/AgRP neurons, and their activity did not reduce despite signals of satiety. It is entirely possible that within male NPY/AgRP neurons, chronic fructose consumption produces permanent changes within the neuron which maintained a level of excitability, despite a decrease in excitatory tone with fructose cessation. We did not observe a change in excitability in the female Reversal mice despite maintenance of increased excitatory tone. It is possible that despite the persistence in increased excitatory tone within our experiments, female NPY/AgRP neurons may not be responsive to the level of excitatory tone produced within our studies and may require even more excitatory tone to result in increased excitability.

4.3 Fructose exposure enhanced preference for fructose

Exposure to fructose can enhance preference for fructose upon re-exposure, as chow-fed mice increased their fructose preference at the end of their diet studies. Following fructose exposure, mice with fructose abstinence also showed an increase in preference, similar to chow-fed controls and significantly higher than fructose-fed mice. Overall, these findings suggest that while continuous fructose consumption would result in reduced fructose preference, abstinence between fructose exposures can enhance the preference for fructose.
Sugars within our diet have the capability of being reinforcing, and can cause cravings, similar to drugs of abuse. For example, rats given access to saccharin or cocaine consumed more saccharin and increased lever pressing in the face of increased cost (i.e. increased lever presses required for saccharin access) (Lenoir, Serre, Cantin, & Ahmed, 2007). In human studies, individuals meeting the criteria for food addiction had increased food cravings (Davis et al., 2011), which was further enhanced following a taste of a palatable food (Davis, Levitan, Kaplan, Kennedy, & Carter, 2014). Additionally, sugars such as saccharin (Mark, Blander, & Hoebel, 1991) or sucrose (Hajnal, Smith, & Norgren, 2004) have the capability of increasing dopaminergic concentration within the NAc, similar to drugs of abuse (Pontieri, Tanda, Orzi, & Di Chiara, 1996). Taken together, increased preference for fructose after a period of cessation is consistent with the literature, as sugars have the potential to sensitize the mesolimbic dopaminergic system within the brain and influence preference through this pathway. Sclafani & Ackroff, 1994 showed that flavours such as grape or cherry conditioned to fructose are consumed more than control solutions. Additionally, fructose preference involves regions within the mesolimbic dopaminergic pathway, that includes the NAc and the PFC. Systemic dopamine receptor D1 or D2 antagonism can block the preference for fructose-conditioned flavoured solution (Baker et al., 2003), while antagonism within the NAc can block the acquisition of this preference (Baker et al., 2003; Bernal et al., 2008). Additionally, when given access to a fructose solution, mice will drink significantly more fructose (Rorabaugh et al., 2015; Rorabaugh et al., 2014) compared to both glucose and sucrose (Rorabaugh et al., 2015).

Consumption of fructose can produce sensitization to other drugs like cocaine (Rorabaugh et al., 2015) or oxycodone (Minhas, Limebeer, Strom, Parker, & Leri, 2021). For example, consumption of fructose can enhance the preference for a chamber conditioned to
cocaine or oxycodone (Rorabaugh et al., 2015; Minhas, Limebeer, Strom, Parker, & Leri, 2021). This sensitization is likely due to fructose, as glucose binging blocks cocaine related conditioned place preference, while fructose and sucrose produces the sensitization. These findings are consistent with findings of other palatable diets, such as a HFD, to sensitize the reward system to other rewarding stimuli (Collins et al., 2015; Naneix et al., 2017), given that rodents sensitized to a HFD will display increased locomotor activity and VTA firing after injections of amphetamine. Thus, fructose can sensitize the reward pathway to result in increased fructose preference upon re-exposure.

Male fructose-fed mice returned to chow diet for one or four weeks had a similar fructose preference as chow-fed controls, and both groups had significantly higher preference than fructose-fed mice. Female fructose-fed mice returned to chow for one week had lower fructose preference than chow-fed controls, similar to fructose-fed females. However, with four weeks of fructose abstinence, the fructose preference was significantly increased, similar to chow-fed controls but higher than fructose-fed females. Few studies have shown the impact of palatable diet consumption and diet reversal on the brain. The studies done on HFD consumption with a short diet reversal (1–2 weeks) showed that the diet reversal did not influence changes made by high diet consumption, such as reduced dopamine receptor expression (Alsiö et al., 2010; Johnson & Kenny, 2010) or decreased dopamine transporter density (South & Huang, 2008). Long diet reversal from high fat diet (4 weeks) normalized disruptions such as in hippocampal (Sobesky et al., 2014) or hypothalamic inflammation (Berkseth et al., 2014) and diminished dopaminergic tone (Carlin, Hill-Smith, Lucki, & Reyes, 2013). Chronic HFD consumption reduced palatable diet preferences, however four weeks of HFD removal normalized this preference (Carlin et al., 2016) and further produced binge behaviour in females preference
(Carlin et al., 2016). Rats returned to chow for ten days after three weeks of junk-food consumption had similar levels of palatable diet consumption as chow-fed controls, and both groups had significantly higher consumption compared to junk-food fed rats (Fetterly & Carrario, 2022). Junk-food deprived rats also frequented the food cup containing the palatable significantly more than junk-food fed or chow-fed mice, indicating upon re-exposure to the palatable diet, rats were more likely to approach and consume the diet (Fetterly & Carrario, 2022). Altogether, these findings support that fructose exposure can cause sensitization, resulting in increased consumption of upon fructose re-exposure after a period of abstinence. This length of the period of abstinence may differ between the sexes, but overall, longer period of abstinence produced increased consumption of fructose upon re-exposure.

4.4 Continuous fructose exposure decreased the preference for fructose

Fructose-fed mice had a lower preference for fructose compared to other diet groups, but only eight weeks of fructose consumption reduced the preference for fructose in both males and females. Chronic fructose consumption decreased dopaminergic output within the striatum (Meyers, Mourra, & Beeler, 2017), which could reduce the preference for fructose. Beyond fructose, continuous exposure to sugars (Frazier, Mason, Zhuang, & Beeler, 2008; Vendruscolo, Gueye, Darnaudéry, Ahmed, & Cador, 2010) or palatable diets (Carlin et al., 2016) reduced the consumption of sugars or palatable diets when given access after ad libitum access. This is different to chow-fed controls mice, who overate when given access to a palatable diet (Carlin et al., 2016). Fetterly & Carrario, 2022 showed that rats with three weeks of junk-food access had significantly less consumption of a palatable diet when given, while chow-fed mice had significantly increased consumption of the palatable diet. In terms of the reward pathway, 

*libitum* access to sugars in adolescent mice reduces the motivation to acquire sucrose in adulthood (Frazier et al., 2008), while in humans, increased palatable diet intake has been associated with reduced striatal activity (Stice, Yokum, Blum, & Bohon, 2010). Taken together, it is likely that continuous exposure to fructose can reduce the preference for fructose, by potentially desensitizing the rewarding aspects of fructose consumption, which could be rescued with removal of fructose.

### 4.5 Comparison of different sugar preferences

Most studies of sugar preferences are done with solutions of sugars, given that most sugar consumption comes in the form of sugar-sweetened drinks. In these studies, it is obvious that sugars are preferred over water (Andres-Hernando et al., 2020) but the type of sugar preferred can vary per strain of mouse (Sclafani, Vural, & Ackroff, 2017). While rats might prefer fructose over other sugars initially (Ackroff & Sclafani, 1991), most rodents would rather consume glucose or sucrose, or conditioned flavours associated with these sugars, compared to fructose (Ascencio Gutierrez, Simental Ramos, Khayoyan, & Schier, 2022; Sclafani & Mann, 1987; Stevanovic et al., 2020; Tordoff, Ulrich, & Sandler, 1990). Stevanovic et al., 2020 showed that while sucrose, fructose and glucose were all preferred to water, the preference for fructose was lower compared to the other two sugars. The study assessed dietary fructose and found that mice will consume equal portions of fructose diet to chow diet while consuming significantly more glucose or sucrose diet compared to chow (Stevanovic et al., 2020). Overall, the literature shows that fructose is the least preferred sugar (Sclafani & Mann, 1987) compared to other sugars available, which is thought to be due to differential post-ingestive impacts of fructose. This could explain why mice within our studies rarely had preference for fructose over chow. However,
despite fructose being the least preferred sugar among other sugars, fructose has reinforcing impacts, such as increased fructose consumption compared to other sugars (Rorabaugh et al., 2015; Rorabaugh et al., 2014) and increased place preference for cocaine (Rorabaugh et al., 2015) or oxycodone (Minhas, Limebeer, Strom, Parker, & Leri, 2021) with fructose consumption. Given these findings, it is possible that despite fructose being the least preferred sugar among sugars, it will still have a reinforcing impact.

4.6 Considerations for preference findings

One variable to consider for our studies is the concentration of fructose consumed. Fructose is a stronger taste sensory stimulus compared to glucose or sucrose (Tonosaki & Beidler, 1989), and while increasing sugar concentrations increased sugar preference in liquid (Ren et al., 2020; Sclafani & Mann, 1987; Stevanovic et al., 2020; Wagner, 1965), the maximum concentration for sugar solution was rarely above 30%. In studies where fructose solution is used, increasing fructose to glucose ratios reinforced the intake of fructose solution (Levy et al., 2015), however this was only seen with two weeks of self-administered fructose consumption, not with continuous and chronic fructose consumption. Thus, it is entirely possible that with a 60% fructose concentration in diet, the high concentration could deter most mice to prefer fructose from chow. Future studies should evaluate if a lower concentration of fructose, such as a 10% or 15% diet, can result in all mice preferring fructose over chow diet, given that the reduced concentration of sugar could reduce any aversion which is seen with a continuous consumption of a 60% diet. It is also possible that dietary fructose preference will not align with solution-based fructose preference, as proven in Stevanovic et al., 2020, where despite higher preference for fructose solution compared to water, 60% fructose diet intake is similar to chow intake.
Age can impact preference for sugars; however, older rodents will have a lower preference for sugars compared to younger rodents (Bertino & Wehmer, 1981; Inui-Yamamoto et al., 2017). Additionally, younger rodents tend to prefer higher concentrations of sugars, with this peak concentration decreasing as rodents age (Bertino & Wehmer, 1981). This indicates that the increased fructose preference in chow-fed mice is likely due to the previous exposure to fructose, given that with increasing age, sugar preference would decrease.

4.7 Potential links between electrophysiological and behavioural findings

It should be noted that our electrophysiology findings are limited to only after fructose cessation, thus making it difficult to link the electrophysiological and behavioural findings together. We have not determined what would occur to the synaptic events or the neuronal excitability of NPY/AgRP neurons if fructose was given again after a cessation period. It is entirely possible that following fructose cessation, ad libitum fructose re-introduction could re-instate the increased excitatory tone in male mice, or further enhance it in female mice. When we reintroduced fructose to mice following an abstinence period, all mice preferred fructose more than fructose-fed controls. This indicates that re-exposure after an abstinence period has the potential to reinstate any impacts of fructose.

There is a potential neural correlate which we have not explored that could link the activity at the NPY/AgRP neurons with fructose preference. The mesolimbic dopaminergic pathway receives projections from NPY/AgRP neurons to the VTA (Alhadeff et al., 2019; Dietrich et al., 2012) and given that fructose can impact the reward circuitry to reinforce fructose consumption (Ackroff & Sclafani, 1991; Minhas et al., 2021; Rorabaugh et al., 2015; Rorabaugh
et al., 2014), it is possible that fructose actions at both the ARC and reward circuitry could explain the electrophysiological findings. Altogether, this suggests a potential neural correlate between the NPY/AgRP neurons of the ARC and the fructose preference we have discovered.

5.0 Conclusion

We have shown that fructose consumption is associated increased excitatory inputs onto NPY/AgRP neurons of the ARC, which is reversible with fructose abstinence in males, but not females. Despite fructose abstinence, sustained excitatory inputs can maintain increased excitability at NPY/AgRP neurons, which could drive feeding behaviour. Additionally, while chronic fructose consumption can reduce the preference for fructose, cessation can alleviate this impact, resulting in an increase fructose preference and consumption. This cessation could have a sensitizing impact, resulting in increased reinforcement and consumption of fructose feeding. These findings may have a significant implication for the early childhood exposure to sugars like fructose, which may predispose an individual to exaggerated sugar intake later in life. Furthermore, our findings suggest that repeated or constant exposure to fructose could enhanced preference for this specific sugar. Our findings suggest that these obesogenic outcomes have neurological origins, where homeostatic and/or hedonic neural circuits may become compromised with sustained fructose exposure.
6.0 References


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