Sex differences in the effect of acute fasting on excitatory and inhibitory synapses onto ventral tegmental area dopamine neurons

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Running title: Acute fasting on synaptic transmission onto dopamine neurons.

Key words: ventral tegmental area, fasting, dopamine, inhibitory synaptic transmission, excitatory synaptic transmission, endocannabinoid.

Key Points:

1. Fasting can increases motivation for food and can energize reward-seeking.
2. Ventral tegmental area (VTA) dopamine neurons respond to motivationally relevant information and fasting can influence mesolimbic dopamine concentration.
3. An acute overnight fast differentially alters food approach behaviours and excitatory synaptic transmission onto VTA dopamine neurons of male or female mice.
4. While inhibitory synapses onto VTA dopamine neurons are not altered by fasting in male or female mice, male mice had strengthened excitatory synapses whereas female mice had increased endocannabinoid-mediated short term plasticity at excitatory synapses.
5. These results help us understand how fasting differentially influences excitatory synaptic transmission onto dopamine neurons and may inform different strategies for fasting-induced food seeking by male and female mice.

Abstract:

Dopamine neurons in the ventral tegmental area (VTA) are important for energizing goal directed behaviour towards food and are sensitive to changes in metabolic states. Fasting increases the incentive motivation for food, mobilization of energy stores and have sex-dependent effects. However, it is unknown how acute fasting alters excitatory or inhibitory synaptic transmission onto VTA dopamine neurons. An acute 16h overnight fast induced increased food seeking behaviour that was more predominant in male mice. Fasting increased miniature excitatory postsynaptic current (mEPSC) frequency and amplitude in male, but not female mice. This effect was not due to altered release probability as there was no change in the paired pulse ratio, nor was it due to an altered postsynaptic response as there was no change in the AMPAR/NMDA ratio or response to glutamate uncaging. However, this effect was consistent with an increase in the number of release sites. In addition, depolarization-induced-suppression of excitation (DSE), a measure of short-term endocannabinoid-mediated plasticity, was enhanced in female but not male fasted mice. There were no fasting-induced changes at inhibitory synapses onto dopamine neurons of male or female mice. Taken together, these

results demonstrate that fasting influences excitatory synapses differentially in male and female mice, but preserves inhibitory synapses onto dopamine neurons, indicating that the mesolimbic circuit of male and female mice respond differently to acute energy deprivation.

Introduction

Increased obesity rates have been paralleled with increased dieting amongst the general population (Julia *et al.*, 2014). While achieving modest body weight reduction can improve cardiovascular and glycemic outcomes (Wing *et al.*, 2011), weight loss is rarely maintained. Hypotheses have been put forward to understand why permanent weight loss is seldom achieved. First, obesogenic diets can induce long lasting plasticity in neural circuits that motivate food intake and drive feeding behaviour (La Fleur *et al.*, 2007; Liu *et al.*, 2016). Secondly, food restriction and weight loss can induce metabolic and neural changes that enhance reward-seeking and food intake (Fulton, 2010; Sharma *et al.*, 2013; Matikainen-Ankney *et al.*, 2020). Ventral tegmental area (VTA) dopamine neurons encode motivationally salient information, and dopamine released in the nucleus accumbens enhances motivation for food (Salamone & Correa, 2012). Increased synaptic strength of excitatory inputs in the VTA underlies learning of reward-associated cues (Stuber *et al.*, 2008). Acute exposure to sweetened high fat food strengthens excitatory synapses onto VTA dopamine neurons and drives further food consumption (Liu *et al.*, 2016). However, it is unclear how food restriction and weight loss influence synaptic plasticity in the VTA. Furthermore, a greater understanding of mechanisms underlying food restriction or fasting may inform why weight loss is rarely maintained and perhaps shed light on how eating disorders may develop.

Both chronic food restriction (Reilly, 1999; Carr, 2002) and acute fasting (Jewett *et al.*, 1995) increase motivation for food. Numerous studies have shown that chronically food restricted mice and rats have greater dopamine release in the nucleus accumbens (Heffner *et al.*, 1980; Wilson *et al.*, 1995; Cadoni *et al.*, 2003). In addition, chronically food restricted male mice have greater burst firing of dopamine neurons in the substantia nigra (Branch *et al.*, 2013). This is associated with an increased synaptic strength of dopamine neurons as well as increased sensitivity of dopamine D2 autoreceptors (Branch *et al.*, 2013). Conversely, overnight fasting does not affect burst firing or the AMPAR/NMDAR ratio of substantia nigra dopamine neurons from male mice (Branch *et al.*, 2013). Acute fasting (24-36h) decreases activity of dopamine transporter and decreases dopamine reuptake in the rat striatum (Paterson *et al.*, 1998). In the VTA, a 24 h fast increases somatodendritic dopamine D2 receptor-mediated inhibitory post synaptic current (ISPC) responses (Roseberry, 2015). Thus, while fasting can influence dopamine signaling, there is a lack of understanding how acute fasting can influence excitatory and inhibitory synaptic transmission within the VTA. Furthermore, while there are reported sex differences in the physiological response to fasting in humans (Johnstone *et al.*, 2002) and mice (Jensen *et al.*, 2013; Freire *et al.*, 2020), it is not known how fasting influences synaptic transmission in the VTA differently between male and female rodents. Therefore, we hypothesized that acute overnight fasting influences excitatory and inhibitory synaptic transmission onto VTA dopamine neurons differently in male and female mice.

Methods

All protocols were in accordance with the ethical guidelines established by the Canadian Council for Animal Care and were approved by the University of Calgary Animal Care Committee. Mice 2-4 months old were housed in groups of 2-5 in same sex cages and were maintained on a 12-hour light-dark schedule (lights on at 8 AM MST, zeitgeber time (ZT0)) and were given chow and water *ad libitum*. Mice were fed chow (5062 from Pico-Vac), which is composed of (% of total kCal) 23.189 protein, 21.635 fat (ether extract), and 55.176 carbohydrate. The total energy density of this diet was 4.60 kCal/g. Experiments were performed during the animal's light cycle. Male and female DAT^{cre}Td-Tomato mice, used to identify dopamine neurons, were generated by crossing DAT-Cre (B6.SJL-Slc6a3tm1.1(cre)Bkmn/J mouse line) with Rosa-td Tomato mice (B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J (Ai9)). All mice were bred locally in the Clara Christie Centre for Mouse Genomics. Mice were initially weighed and then isolated 10 hours into their light phase (ZT10), and single housed overnight for a total of 16 hours. During this time, food was removed from the cages of the fasted group and chow was given *ad libitum* to the control group. Water was given *ad libitum* to both groups. Water intake and body weight were recorded for fasted and control groups, and food consumption per body weight was recorded in the control group after the 16h fast (ZT2). In a separate cohort of mice, at ZT1, glucose, ketone, and serum corticosterone were measured from tail vein blood. Electrophysiology or behavioural experiments were performed on mice at ZT2.

Ketone, Glucose and CORT measurements

Glucose measurement was performed using a glucose meter (Accu-check Aviva, Roche) on a drop of tail vein blood. Ketone measurement was performed similarly on a drop of tail vein blood, using a ketone blood monitor with keto testing strips (Freestyle Precision Neo, Abbott Laboratories Inc.). Blood serum was separated after centrifuging blood samples, obtained from the tail using Microvette collection tubes (Microvette CB 300 uL, Clotting Activator/Serum tubes, Sarstedt, Inc.) at 16,100 g, 15 min at 4 °C and serum corticosterone (CORT) was measured with and according to manufacturer's instructions (DetectX Corticosterone Enzyme Immunoassay Kit, Arbor Assays).

Food-seeking behaviour

Mice were pre-exposed to a ~5 g high-fat diet pellet 48 hours prior to the behavioural test to reduce food neophobia. This high-fat food (D12492 from Research Diets) is composed of (% of total calories) 20 protein, 60 fat, and 20 carbohydrate. The total energy density of this diet was 5.21 kcal/g. Following isolation, fasted or control mice were individually placed in a light-dark box apparatus for 10 minutes. The apparatus was an acrylic box (40 cm length x 40 cm width x 12 cm height) divided equally into light (60 lumens) and dark (5 lumens) chambers with an opening (3.5 cm x 3.4 cm) for the mouse to move from zone to zone. A pellet of high-fat food (Research Diets, D12492) was placed at the centre of the light side, and the area around the food (9 cm x 9 cm) was defined as the food zone (Liu *et al.*, 2016). Mice were placed in the light chamber facing the opening into the dark chamber. Movement of the mouse was recorded and analyzed using EthoVision XT software (Noldus) as well as MatLab (code:

https://github.com/borglandlab/Godfrey-and-Borgland-2020.git). The apparatus was cleaned between each subject.

Electrophysiology

All electrophysiological recordings were performed in slice preparations from adult (2-4 months old) DAT^{cre};Td-Tomato mice. In these mice, a red fluorescent marker is expressed exclusively in dopamine neurons, permitting immediate identification of each recorded neuron in the VTA. Mice were deeply anaesthetized with isoflurane and transcardially perfused with an ice-cold NMDG solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 $NaH_2PO_4.2H_2O$, 30 $NaHCO_3$, 20 HEPES, 25 D-Glucose, 5 Sodium Ascorbate, 3 Sodium Pyruvate, 2 Thiourea, 10 $MgSO_4.7H_20$, and 0.5 $CaCl_2.2H_20$ and saturated with 95% O_2/5% CO_2. Mice were then decapitated, and brains were extracted. Horizontal midbrain sections (250 µm) containing the VTA were cut on a vibratome (Leica, Nussloch, Germany). Slices were then incubated in NMDG solution (32°C) and saturated with 95% O_2/5% CO_2 for 10 minutes. Following this, the slices were transferred to aCSF containing (in mM): 126 NaCl, 1.6 KCl, 1.1 NaH_2PO_4, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, 11 glucose (32°C) and saturated with 95% O_2/5% CO_2 and incubated for a minimum of 45 min before being transferred to a recording chamber and superfused (2 mL/min) with aCSF (32-34°C) and saturated with 95% O_2/5% CO_2. Cells were visualized on an upright microscope using "Dodt-type" gradient contrast infrared optics and whole-cell recordings were made using a MultiClamp 700B amplifier. The recorded signal was collected at a sampling rate of 20 KHz. Except for recordings of miniature post-synaptic currents (mPSC), a 2 KHz Bessel filter was applied to the data during collection using the MultiClamp 700B (Molecular Devices, CA). For

mPSC recordings, a 10 KHz Bessel filter was applied to the data during collection and the data was post hoc filtered at 2 KHz after the recordings using Clampfit 10.3 (Molecular Devices, CA). For evoked currents, a bipolar stimulating electrode was placed 100 to 300 µm rostral to the cell being recorded and stimulated at 0.1 Hz. The liquid junction potential was not corrected for.

Recording electrodes (2-5 MΩ) were filled with $CeMeSO_3$ internal solution containing (in mM): 117 $CeMeSO_3$, 2.8 NaCl, 20 HEPES, 0.4 EGTA, 5 TEA, 5 MgATP, 0.5 NaGTP. The liquid junction potential was -10 mV. For miniature excitatory post-synaptic currents (mEPSC), cells were voltage clamped at the reverse potential of inhibitory currents, experimentally determined to be -65 mV, and the current was recorded for 5 minutes. For miniature inhibitory post-synaptic currents (mIPSC), cells were voltage clamped at the reverse potential of excitatory currents, experimentally determined to be +9 mV, and the current was recorded for 5 minutes. mEPSCs and mIPSCs were identified and measured using Mini Analysis 60 (Synaptosoft, Decateur GA) and the following parameters: mEPSC: amplitude > 12 pA, decay time < 3 ms, rise time < 1.75 ms; mIPSC: amplitude > 15 pA, decay time < 10 ms, rise time < 4 ms.

Recording electrodes (2-5 MΩ) were filled with $CeMeSO_3$ internal solution and the evoked currents were recorded in the presence of picrotoxin (100 µM). The α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) to N-methyl-D-aspartate receptor (NMDAR) ratio was recorded while the cells were voltage clamped at +40 mV, and a single evoked stimulus was delivered. Using Matlab (code: https://github.com/borglandlab/Godfrey-and-Borgland-2020.git) the average of 12 current amplitudes was calculated before and after

the application of the NMDAR receptor antagonist, AP5 (50 µM). NMDAR current amplitude was calculated by subtracting the current amplitude after AP5 (AMPAR only) from the current amplitude without AP5. The AMPAR EPSC amplitude was divided by the NMDAR EPSC amplitude to calculate the AMPAR/NMDAR ratio (Borgland *et al.*, 2006). To elicit glutamatergic current via AMPARs voltage clamped at -70 mV, Rubi-Glutamate (30 µM) was washed on to the slice and glutamate uncaging was triggered by 15 ms pulses of 473 nm light through the 40X microscope objective every minute (0.02 Hz) (Fino *et al.*, 2009). The average of 5 glutamate induced currents was calculated.

To examine short term plasticity, EPSCs recorded at -70 mV were electrically evoked using 40 pulses at 100 Hz. Using Matlab (code: https://github.com/borglandlab/Godfrey-and-Borgland-2020.git) the amplitude of the first 2 stimulations were averaged and the cumulative probability of amplitude was plotted, and a line was fitted to the final 14 pulses. The readily releasable pool (RRP) was calculated as the y-intersect of this best fitting line. The probability of release (p) was calculated by dividing the first peak ($EPSC_0$) by the RRP. The PPR was calculated by dividing the second peak ($EPSC_1$) by $EPSC_0$ (Thanawala & Regehr, 2013, 2016).

Depolarization-induced suppression of excitation.

To evoke depolarization-induced suppression of excitation (DSE), neurons were voltage clamped at -70 mV in the presence of picrotoxin (100 µM), and a paired stimulus was delivered with an interstimulus interval (ISI) of 50 ms. Prior to each 10 s depolarization step to +40 mV, a 1 min baseline was recorded (Melis *et al.*, 2004). The magnitude of DSE was measured as the percentage of the amplitude of the EPSC immediately after depolarization (acquired between 5

and 15 s after the end of the pulse) relative to that of 6 EPSCs (1 min) before depolarization. The depolarization step occurred a minimum of 3 times, and an averaged DSE was calculated from these steps. To confirm DSE was mediated by endocannabinoids, the protocol was repeated in the presence of the CB1R antagonist, AM251 (2 µM). The paired-pulse ratio was calculated by dividing the second pulse by the first pulse during the baseline.

Depolarization-induced suppression of inhibition (DSI)

Recording electrodes (2-5 MΩ) were filled with a KCl internal solution containing (in mM): 144 KCl, 10 HEPES, 2.6 BAPTA tetrapotassium salt, 1 $CaCl_2$, 2.5 Mg_2ATP, 0.25 Na_2GTP, 3 QX 314 – Cl. The liquid junction potential was -4 mV. Neurons were voltage clamped at -70 mV and a paired stimulus (ISI 50 ms) was delivered in the presence of DNQX (10 µM) and AP5 (50 µM). A 5 s depolarization step to +40 mV evoked DSI (Melis *et al.*, 2013). The magnitude of DSI was measured as the percentage of the amplitude of the IPSC immediately after depolarization (acquired between 5 and 15 s after the end of the pulse) relative to that of 6 IPSCs (1 min) before depolarization. The depolarization step occurred a minimum of 3 times, and an averaged DSI was calculated from these steps. To confirm DSI was mediated by endocannabinoids, the protocol was repeated in the presence of the CB1R antagonist, AM251 (2 µM). The paired-pulse ratio was calculated by dividing the second pulse by the first pulse during the baseline.

Inhibitory long-term depression (iLTD) protocol

Recording electrodes (2-5 MΩ) were filled with a K-Gluconate internal solution containing in (mM): 100 K-gluconate, 50 KCl, 10 HEPES, 0.2 EGTA, 0.2 EGTA, 2 $MgCl_2$, 4 MgATP,

0.3 Na_2GTP, 10 Na_2phospocreatine. The liquid junction potential was -11 mV. The paired (ISI 50 ms) evoked currents were recorded at -70 mV in the presence of DNQX (10 µM) and AP5 (50 µM). A 10 min baseline was recorded followed by a 5-min 10 Hz electrical stimulation, a protocol that has been used previously to induce iLTD at VTA synapses after cocaine exposure (Pan *et al.*, 2008). IPSCs were recorded for 25 min following the stimulation protocol. IPSCs normalized to baseline in the 5 min before the 10 Hz stimulation was compared to a 5 min period 20 min after the stimulation.

Data Analysis

All values are expressed as mean ± SD and assessed for normality using a Shapiro-Wilk test. Statistical significance was assessed by using two-tailed unpaired Student's t test for 2 comparisons. A two-way ANOVA followed by a Tukey's multiple comparisons was used for multiple group comparisons. For time course experiments, a repeated measures two-way ANOVA followed by Sidak's multiple comparisons test were used. In all electrophysiology experiments, sample size is expressed as N/n, where N refers to the number of cells recorded from n animals. GraphPad Prism 8.3 was used to perform statistical analysis. Unless otherwise stated, figures were generated using GraphPad Prism 8.3 and Adobe Illustrator CS4 software (Adobe Systems Incorporated).

Results

Changes in body weight and concentrations of blood glucose, blood ketones, and serum CORT following fasting.

To characterize the physiological effects of a 16 h overnight fast on DAT^{cre}Td-Tomato mice, changes in weight, food and water consumption, blood glucose concentration, blood ketone concentration, and serum CORT concentration were measured (Figure 1A). There was a significant difference in chow consumption between male (0.44 ±0.05 KCal/g, n = 8) and female (0.54 ±0.12 kCal/g; n = 8) control mice (t(14) = 2.283, P = 0.0386; Figure 1B). Fasting also decreased water consumption in male (control: 4.3 ±0.5 mL, n = 8; fasted: 3.5 ±1.0 mL , n = 8) and female (control: 5.8 ±0.9 mL, n = 8; fasted: 3.6 ±1.0 mL, n = 8,) mice (sex x fasting interaction: F(1,28) = 5.140, P = 0.0313; fasting effect: F(1,28) = 7.302, P = 0.0116). Furthermore there was a sex difference in water consumption (sex effect: F(1,28) = 23.55, P < 0.0001; Figure 1C). A Tukey's multiple comparison test indicated a significant difference in water consumption between female (P<0.0001) but not male (P = 0.2818) control and fasted mice. Male (control: -0.2 ± 1.2 %, n = 8, fasted = -11.1 ±0.6 %, n = 8) and female (control = +1.1 ± 2.3 %, n = 8; fasted = -10.0 ± 0.7 %, n = 8) mice lost weight during fasting (fasting effect: F(1,28) = 495.2, P < 0.0001, sex effect: F(1,28) = 5.695, P = 0.0240, sex x fasting interaction: F(1,28) = 0.0257, P = 0.8738; Figure 1D).

Following fasting, there was a main effect of fasting on blood glucose concentration in male and female mice (F(1,28) = 101.6, P < 0.0001, male control: 8.6 ±1.2 mmol/L, n = 8; female control: 7.1 ±0.6 mmol/L, n = 8, male fasted: 5.6 ± 0.6 mmol/L, n = 8; female fasted: 4.8 ±0.3 mmol/L, n = 8), and a main effect of sex (F(1,28) = 18.20, P = 0.0002; Figure 1E). There was no sex x fasting interaction (F(1,28) = 1.609, P = 0.2151). There was a main effect of fasting on blood ketone concentrations (F(1,28) = 265.6, P < 0.0001, male control: 0.1 ±0.07 mmol/L, n = 8; female control: 0.15 ±0.05 mmol/L, n = 8, male fasted: 1.2 ±0.2 mmol/L, n = 8; female fasted:

1.8 ±0.4 mmol/L, n = 8). Furthermore, there was a main effect of sex on blood ketones ($F(1,28) = 13.06$, $P = 0.0012$; fasting x sex interaction: $F(1,28) = 12.01$, $P = 0.0017$). A Tukey's multiple comparison test revealed a significant difference between ketone levels between male and female fasted mice ($P = 0.0002$), but not control mice ($P = 0.9996$, Figure 1F). Finally, there was a significant sex x fasting interaction on serum CORT concentrations ($F(1,28) = 10.60$, $P = 0.0030$, main effect of fasting: ($F(1,28) = 53.96$, $P < 0.0001$), main effect of sex ($F(1,28) = 18.46$, $P = 0.0002$). A Tukey's multiple comparison's test reveals a significant difference in CORT levels between control and fasted male ($P = 0.0348$, control: 69.66 ±31.19 nmol/L, n = 8, fasted: 309.8 ±170.1 nmol/L, n = 8) and female mice ($P <0.0001$; control: 130.8 ±141.3 nmol/L, n =8, fasted: 753.3 ±245.9 nmol/L, n = 8; Figure 1G). Furthermore, female fasted mice had greater CORT levels than male fasted mice ($P<0.0001$). Taken together, these results suggest that acute fasting decreased body weight and glucose concentration as well as increased ketones and CORT levels and the metabolic effect was more pronounced in female compared to male mice, regardless of weight loss.

Sex differences in food approach behaviour and food consumption following fasting.

To test if acute fasting altered food seeking differentially in male and female mice, we used a modified light dark box, whereby mice must enter the open light field to seek food (Figure 2A-C). There was no main effect of fasting on distance travelled in the light zone ($F(1,38) = 0.1770$, $P = 0.6763$; Figure 2D). However, there was a main effect of sex on distance travelled in the light zone ($F(1,38) = 9.853$, $P = 0.0033$, sex x fasting interaction: $F(1,38) = 0.0235$, $P = 0.9788$; female control: 2398 ±410 cm, n = 11; female fasted: 2370 ±214 cm, n = 10, male

control: 2082 ±363 cm, n = 11; male fasted: 2021 ±343 cm, n = 10, Figure 2D), suggesting that females have greater locomotor activity than males. Dark zone entries was not influenced by fasting (fasting effect: $F(1,38) = 2.940$, $P = 0.0945$, Figure 2E). However, there was a sex effect on dark zone entries (sex effect: $F(1,38) = 7.063$, $P = 0.0114$, fasting x sex interaction: : $F(1,38) = 1.223$, $P = 0.2757$, female control: 30 ± 10, n = 11; female fasted: 29 ± 7, n = 10, male control: 27 ± 4, n = 11; male fasted: 20 ± 7, n = 10). Although general activity in the light-dark box was not affect by fasting, behaviours within the food-zone were. There was a main effect of fasting on distance travelled in the food zone ($F(1,38) = 11.15$, $P = 0.0019$; Figure 2F). However, there was no sex difference on distance travelled in the food zone (sex effect: $F(1,38) = 1.992$, $P = 0.1663$, sex x fasting interaction: $F(1,38) = 0.0017$, $P = 0.9967$; male fasted: 155 ±59 cm, n = 10; female fasted: 180 ± 65cm, n = 10, male control: 93 ± 53 cm, n = 11; female control: 120 ± 58cm, n = 11; Figure 2F). There were also a greater number of food zone entries following fasting in all mice (fasting effect: $F(1,38) = 13.64$, $P = 0.0007$, male fasted: 37 ± 10, n = 10; female fasted: 43 ± 10, n = 10, male control: 26 ± 10, n = 11; female control: 31 ± 11, n = 11; Figure 2G). However, there was no effect of sex on food zone entries (sex effect: $F(1,38) = 3.15$, $P = 0.08$, sex x fasting interaction: main effect fasting: $F(1,38) = 0.0495$, $P = 0.8250$). The average duration of each food zone entry was greater following fasting (fasting effect: $F(1,38) = 54.60$, $P < 0.0001$, male fasted: 2.3 ± 0.8 s, n = 10; female fasted: 1.5 ± 0.5 s, n = 10, control: 0.8 ± 0.3 s, n = 11; female control: 0.8 ± 0.3 s, n = 11; Figure 2H). There was a sex effect on duration in the food zone (sex effect: $F(1,38) = 6.220$, $P = 0.0171$, fasting x sex interaction: $F(1,38) = 6.561$, $P = 0.0145$). A Tukey's multiple comparison test revealed a significant difference on duration in the food zone between male and female fasted mice ($P = 0.0065$), but not control mice ($P > 0.9999$),

suggesting that fasting influences the food seeking behaviour in female mice differently than male mice. Fasted mice consumed more high-fat food during the test than control mice (fasting effect: $F(1,36) = 108.8$, $P < 0.0001$, male fasted: 0.26 ± 0.07 g, n = 10; female fasted: 0.17 ± 0.09 g, n = 8, male control: 0.009 ± 0.01 g, n = 11; female control: 0.028 ± 0.04 g, n = 11; Figure 2I). Furthermore, there was fasting x sex interaction on food consumption (interaction: $F(1,36) = 8.129$, $P = 0.0072$). A Tukey's multiple comparison test revealed a significant differences in food consumption between control and fasted male ($P < 0.0001$) and female ($P < 0.0001$), as well as a difference fasted male and female mice ($P = 0.0161$), but not in control mice ($P = 0.8762$), suggesting that male mice consume more food during the test. Taken together, fasting increases food approach behaviours in male and female mice. However, male fasted mice spent more time in the food zone and consumed more food than female mice.

Effects of acute fasting on excitatory synaptic transmission.

We next examined whether fasting altered basal synaptic properties at excitatory synapses onto VTA dopamine neurons. To assess the quantal release properties of excitatory inputs to VTA dopamine neurons, miniature excitatory post-synaptic currents (mEPSC) were recorded. There was a main effect of fasting ($F(1,63) = 6.701$, $P = 0.0119$) and sex ($F(1,63) = 5.306$, $P = 0.0246$) on mEPSC amplitude, but no fasting x sex interaction ($F(1,63) = 1.309$, $P = 0.2568$; male control: 15.6 ± 1.2 pA, N/n = 16/5, male fasted: 17.1 ± 2.3 pA, N/n = 18/5; female control: 15.1 ± 1.2 pA, N/n = 16/6, female fasted: 15.7 ± 1.6 pA, N/n = 17/5; Figure 3C). A Tukey's multiple comparison on the main effect of fasting indicated a significant difference in amplitude of male ($P = 0.047$), but not female mice ($P = 0.741$). Furthermore, there was a significant sex x

fasting interaction on mEPSC frequency (interaction: $F(1,63) = 5.150$, $P = 0.0267$) as well as main effects of sex ($F(1,63) = 9.281$, $P = 0.0034$) and fasting ($F(1,63) = 6.085$, $P = 0.014$). Tukey's multiple comparisons tests revealed fasting increased mEPSC frequency in male ($P = 0.0068$), but not female ($P = 0.9999$) mice compared to controls (male control: 0.5654 ±0.4270 Hz, N/n = 16/5; male fasted: 1.5 ± 1.3 Hz, N/n = 18/5; female control: 0.4 ± 0.4 Hz, N/n = 16/6; female fasted: 0.5 ± 0.3 Hz, N/n = 17/5, Figure 3D). These data indicate that fasting differentially affects mEPSC frequency onto VTA dopamine neurons of male and female mice.

An increase in the mEPSC frequency is commonly an indicator of presynaptic changes, which could be in the form of increased release probability (Pr) or increased number or function of the active release sites (n) (Simpson, 1971; Brock *et al.*, 2020). To further examine glutamate release probability, the paired-pulse ratio (PPR) was calculated from evoked EPSCs from dopamine neurons of fasted and control mice. There were no significant group differences in PPR (sex x fasting interaction: $F(1,40) = 0.8292$, $P = 0.3680$, fasting effect: $F(1,40) = 0.04698$, $P = 0.8295$, sex effect: $F(1,40) = 0.1747$, $P = 0.6782$; male control: 0.7 ± 0.2, N/n = 12/3; male fasted: 0.7 ±0.1, N/n = 11/3; female control: 0.7 ±0.1, N/n = 10/4; female fasted: 0.7 ±0.2, N/n = 11/4; Figure 3E).

To test if fasting alters the size of the readily releasable pool and/or release probability, we used a short high frequency train stimulation of 40 pulses at 100 Hz (Thanawala & Regehr, 2013) (Figure 4A). Analysis of these evoked currents can reveal the relative size of the readily releasable pool (RRP(train)) and release probability (P(train)) (Figure 4B). RRP(train) was not changed by fasting (fasting effect: $F(1,31) = 0.1113$, $P = 0.7409$; male control: 1383 ±730, N/n = 9/4; male fasted: 1174 ±481, N/n = 11/3; female control: 1315 ±581, N/n = 8/2; female fasted:

1394 ±422, N/n = 7/2; Figure 4C). P(train) was also not changed following fasting (fasting effect: F(1,31) = 0.001955, P = 0.9650, male control: 0.3803 ±0.2468, N/n = 9/4; male fasted: 0.5 ±0.6, N/n = 11/3; female control: 0.3 ±0.2, N/n = 8/2; female fasted: 0.3 ±0.03, N/n = 7/2; Figure 4D). Furthermore, PPR calculated from these stimulations was not altered by fasting (fasting effect: F(1,31) = 0.08011, P = 0.7790; male control: 0.7791 ±0.3351, N/n = 9/4; male fasted: 0.8±0.3, N/n = 11/3; female control: 0.8 ± 0.2, N/n = 8/2; female fasted: 0.8 ±0.2, N/n = 7/2; Figure 4E). Taken together, these data indicate that fasting does not alter release probability or the size of the ready releasable pool at excitatory synapses onto dopamine neurons.

We next examined whether fasting altered synaptic efficacy of dopamine neurons through postsynaptic mechanisms. We first measured the AMPAR/NMDAR ratio, which reflects a relative change in the contribution of AMPAR, NMDAR or both. Fasting did not change the AMPAR/NMDAR ratio in male or female mice (fasting effect: F(1,32) = 0.01884, P = 0.8917; sex effect: F(1,32) = 1.268, P = 0.2686, male control: 0.6 ±0.3, N/n = 9/4; male fasted: 0.7 ±0.4, N/n = 10/5; female control: 0.8 ± 0.3, N/n = 8/4; female fasted: 0.7 ± 0.2,N/ n = 9/4; Figure 5A,B). To test if fasting altered the number or function of AMPARs that could contribute to increased mEPSC amplitude that now surpasses the threshold of event detection, we used Rubi-glutamate to uncage glutamate at excitatory synapses while recording AMPAR responses at -70 mV (Figure 5C). Responses to optical glutamate uncaging onto VTA dopamine neurons of male mice was not affected by fasting (t(34) = 0.1039, P = 0.9179, male fasted: 22.2 ±10.1 pA, N/n = 18/4, male control: 21.8 ±11.5 pA, N/n = 18/4; Figure 5D,E). Taken together, postsynaptic responses at glutamatergic synapses onto VTA dopamine neurons were not altered by fasting.

Effect of fasting on depolarization-induced suppression of excitation.

Acute fasting could be considered a physiological stressor to mice, as was reflected in increased CORT levels in female mice. In response to glucocorticoids, endogenous cannabinoids (endocannabinoids) induce a rapid suppression of synaptic transmission in the hypothalamus and the basolateral amygdala (Di *et al.*, 2003, 2016). Endocannabinoids act as retrograde transmitters that are synthesized and released on demand and act presynaptically at cannabinoid 1 (CB1) receptors to suppress neurotransmitter release (Wilson & Nicoll, 2002) (Figure 6A). To test if fasting differentially altered endocannabinoid signalling in males and females, we examined depolarization-induced suppression of excitation (DSE), a transient form of plasticity mediated by endocannabinoids. We recorded evoked EPSCs before and after a 10s depolarization. DSE was abolished by the CB1 receptor antagonist, AM251, in both males (drug effect: $F(1,55) = 9.615$, $P = 0.0030$, male control with AM251: 96.8 ±19.3 % baseline, N/n = 5/2; male fasted with AM251: 108.3 ±7.5 % baseline, N/n = 4/2; Figure 6C,D) and females (drug effect: $F(1,58) = 40.27$, $P < 0.0001$, female control with AM251: 94.6 ±11.7 % baseline, N/n = 8/4; female fasted with AM251: 113.2 ± 9.9 % baseline, N/n = 10/4; Figure 6F,G), confirming that DSE is endocannabinoid mediated. There was no effect of fasting on DSE in the VTA of male mice (fasting effect: $F(1,55) = 0.4677$, $P = 0.4968$, male control: 76.6 ±23. 9 % baseline, N/n = 25/10; male fasted: 76.6 ±23. 9 % baseline, N/n = 25/9; Figure 6B,D). However, there was a significant fasting x drug interaction on DSE in female mice (interaction: $F(1,58) = 12.22$, $P = 0.0009$, female control: 79.8 ±22.7 % baseline, N/n = 24/13; female fasted: 61.9 ± 18.1 % baseline, N/n = 20/8; Figure 6E,G). A Tukey's multiple comparisons test indicated a significant difference in DSE between fasted and control female mice in the absence ($P = 0.0119$), but not

in the presence of AM251 (P = 0.1617). When male and female mice were compared together, there was a significant sex x fasting interaction on DSE (interaction: F(1,87) = 4.890, P = 0.0296). A Tukey's multiple comparisons test indicated a significant difference between female fasted and control mice (P = 0.0482), but not male fasted and control mice (P = 0.9683, Figure 6I). Taken together, fasting increases DSE in female, but not male mice.

To determine if the increased DSE in female mice is due to an increase in the CB1 receptor-expression or a change in receptor efficacy, we applied a maximal concentration of CB1 receptor agonist, WIN 55,212 (10 µM) to VTA slices. If there was an increase in receptor expression or receptor efficacy, the maximal response to the agonist should be greater. Although WIN 55,212 inhibited EPSCs in the VTA of female mice (drug effect: F(1,6) = 66.71, P = 0.0002), this effect was not altered by fasting (fasting effect: F(1,6) = 0.1244, P = 0.7364, control: 79.7 ±4.3 % baseline, N/n = 4/4; fasted: 77.9 ± 9.5 % baseline, N/n = 4/3; Figure 7A-C). Consistent with a presynaptic locus, WIN 55,212 increased PPR (drug effect: F(1,6) = 10.90, P = 0.0164; control PPR baseline: 0.75 ±0.2, N/n = 4/4; control PPR WIN 55,212: 0.82 ±0.2, fasted PPR baseline: 0.79 ±0.1, N/n = 4/4, fasted PPR WIN 55,212: 0.87 ±0.05, N/n = 4/4; Figure 7D). Taken together, increase in DSE observed in female mice after fasting was not due to a fasting-induced change in receptor number and/or efficacy.

Effects of fasting at inhibitory synapses in the VTA.

We next examined the effect of fasting on GABAergic synapses onto VTA dopamine neurons of male and female mice. To assess the quantal release properties of inhibitory

synapses, we recorded miniature inhibitory post-synaptic currents (mIPSC) in control and fasted mice. There was no effect of fasting on mIPSC amplitude (fasting effect: $F(1,62) = 1.137$, $P = 0.2905$) nor was there an effect of sex on mIPSC amplitude (sex effect: $F(1,62) = 0.0615$, $P = 0.805$, male control: 21.9 ±1.9 pA, N/n = 17/5; female control: 21.6 ±1.7 pA, N/n = 17/6; male fasted: 22.2 ±1.8 pA, N/n = 17/5; female fasted: 22.3 ±1.9 pA, N/n = 15/5; Figure 8A,B). Furthermore, there was no effect of fasting on mIPSC frequency (fasting effect: $F(1,62) = 0.8943$, $P = 0.3480$). There was a potential trending effect of sex on mIPSC frequency (sex effect: $F(1,62) = 3.463$, $P = 0.0675$, male control: 1.6 ±1.3 Hz, N/n = 17/5; female control: 1.1 ±0.5 Hz, N/n = 17/6; male fasted: 1.9 ±1.6 Hz, N/n = 17/5; female fasted: 1.4 ±1.1 Hz, N/n = 16/5; Figure 8A,C).

Because we observed a trend in sex difference on mIPSC frequency, we further examined the PPR of evoked IPSCs, a measure associated with release probability. There was no fasting effect on PPR (fasting effect: $F(1,54) = 0.08796$, $P = 0.7679$; Figure 8D,E). However, there was a significant effect of sex on PPR at inhibitory synapses (sex effect: $F(1,54) = 13.98$, $P = 0.0004$; female control: 0.8 ±0.1, N/n = 13/4; female fasted: 0.8 ±0.09, N/n = 14/4, male control: 0.7 ±0.1, N/n = 16/6; male fasted: : 0.7 ±0.1, N/n = 15/5, Figure 8D,E). Taken together, fasting does not alter release probability at inhibitory synapses in male or female mice. However, female mice may have lower release probability at inhibitory synapses compared to male mice.

Effect of fasting on depolarization-induced suppression of inhibition.

Depolarization-induced suppression of inhibition (DSI) was used to assess endocannabinoid-mediated short-term presynaptic depression at inhibitory synapses. There was a main effect of fasting on DSI of VTA dopamine neurons of male (fasting effect: $F(1,42) = 4.314$, $P = 0.044$, male control: 73.8 ±12.8% baseline, N/n = 16/6; male fasted: 61.7 ±18.0 % baseline, N/n = 15/4; Figure 9A,C), but not female mice (fasting effect: $F(1,37) = 3.218$, $P = 0.08$, female control: 59.8 ±13.6 % baseline, N/n = 13/4; female fasted: 72.5 ±17.0 % baseline, N/n = 14/4; Figure 9D,F). DSI was inhibited by AM251 in both males (drug effect: $F(1,42) = 10.97$, $P = 0.0019$, male control with AM251: 84.5 ±8.1 % baseline, N/n = 7/2; male fasted with AM251: 79.02 ±5.143 % baseline, N/n = 8/3; Figure 9B,C) and females (drug effect: $F(1,37) = 29.69$, $P < 0.0001$, female control with AM251: 88.6 ±7.5 % baseline, N/n = 6/2; female fasted with AM251: 91.6 ±5.7 % baseline, N/n = 8/3; Figure 9E,F). When sexes were compared directly, there was significant sex x fasting interaction on DSI (interaction: $F(1,54) = 9.245$, $P = 0.0036$; Figure 9H). However, there were no significant group differences (sex effect: $F(1,54) = 0.1525$, $P = 0.6977$). Taken together, fasting did not induce robust effects on DSI in male or female mice.

Effects of fasting on inhibitory long-term depression.

We next examined if fasting altered long-term depression (iLTD) at inhibitory synapses onto VTA dopamine neurons of male and female mice. We used a protocol (10 Hz simulation for 5 min) previously established to induce an endocannabinoid-mediated iLTD in the VTA of mice exposed to cocaine, but not naïve mice (Pan *et al.*, 2008). Consistent with previous work in cocaine-naïve mice (Pan et al., 2008), this protocol induced a reversible suppression in male and female control mice (Figure 10A,B,D,E). However, 20 min after the stimulation, there was

an increase in IPSC amplitude in male (stimulation effect: $F(1,8) = 11.41$, $P = 0.0097$), but not female control mice (stimulation effect: $F(1,12) = 0.0708$, $P = 0.9343$, Figure 10B,C,E,F). Fasting did not alter the iLTD in males (fasting effect: $F(1,8) = 11.41$, $P = 0.0097$, male control: 120 ± 21 % baseline, N/n = 5/5; male fasted: 115 ± 8 % baseline, N/n = 5/5), or females (fasting effect: $F(1,12) = 1.716$, $P = 0.2148$, female control: 104 ± 13 % baseline, N/n = 7/5; female fasted: 95 ± 13 % baseline, N/n = 7/6; Figure 10B,C,E,G). When sexes were analyzed together, there was a main effect of sex on iLTD (sex effect: $F(1,20) = 8.641$, $P = 0.0081$), but no effect of fasting (fasting effect: $F(1,20) = 1.301$, $P = 0.2672$, Figure 10G). Baseline PPR was not influenced by either fasting ($F(1,20) = 0.3988$, $P = 0.5349$) or sex ($F(1,20) = 0.3379$, $P = 0.5676$, male control: 0.7 ±0.06, N/n = 5/5; male fasted: 0.7±0.07, N/n = 5/5; female control: 0.7±0.06, N/n = 7/5; female fasted: 0.7 ±0.08, N/n = 7/6; Figure 10H). Furthermore, the change in PPR from baseline to post-stimulation was not influenced by either fasting ($F(1,20) = 0.02744$, $P = 0.8701$) or sex ($F(1,20) = 0.1561$, $P = 0.6970$, male control: 2 ± 9%, N/n = 5/5; male fasted: 3 ± 4%, N/n = 5/5; female control: 2 ±7%, N/n = 7/5; female fasted: 0.5 ± 8%, N/n = 7/6; Figure 10I). Taken together, using a protocol that evokes iLTD in mice exposed to cocaine, fasting did not alter evoked IPSCs in in male or female mice.

Discussion

We observed that an acute overnight 16 h fast induced strong metabolic and behavioural changes that affected females differently from males. Females had lower blood glucose, higher ketones and higher CORT levels than male mice. Furthermore, female mice had

increased locomotor activity in general, but not in the food zone and consumed less food during the test than male mice. While fasting increased food-seeking in both male and female mice, synaptic transmission at inhibitory synapses in the VTA was preserved with only subtle sex differences observed at excitatory synapses. Dopamine neurons from male mice had an increase in mEPSC amplitude and frequency after fasting, whereas female mice had increased endocannabinoid-mediated short-term plasticity at excitatory synapses onto dopamine neurons. Given that many procedures involve acute fasting (i.e. measurement of fasted glucose, initiation of operant conditioning), it is important to understand how this may influence synaptic processes underlying reward learning.

There is a significant variability in the literature associated with physiological effects of acute fasting. This is, in part, due to variability the length of the fast, the level of food restriction (i.e. total vs. partial), and the timing within the circadian rhythm (Jensen *et al.*, 2013). The fasting protocol used here is consistent with fasting models used in other studies and yielded consistent physiological effects associated with an acute fast (Jensen *et al.*, 2013). This procedure allowed for access to water and fasting over the mouse's active (dark) phase. Consistent with similar fasting protocols (Jensen *et al.*, 2013), we observed a 10% reduction in body weight. Weight loss associated with this duration of fast is due both to a reduction in body mass (60%) and gastric emptying (40%) (Prior *et al.*, 2012). In addition, decreased blood glucose, likely due to decreased glucose absorbed from the gastrointestinal tract during the fast, as well as increased ketones is consistent with previous work (Jensen *et al.*, 2013). Moreover, our results replicate previous work using an overnight fast demonstrating increased

CORT in fasted females compared to males (Champy *et al.*, 2004). Thus, a 16 h overnight fast used in our model induces reliable physiological effects consistent with other studies.

Acute fasting increased food approach and food consumption predominantly in male mice. Notably, increased food approach behaviours are associated with increased mEPSC frequency and excitatory synapse formation onto VTA dopamine neurons (Liu *et al.*, 2016). Female mice had increased overall locomotor activity and dark zone entries compared to male mice in addition to higher fasting-induced CORT levels on par with CORT induction from other types of stressors (Bowers *et al.*, 2008). Therefore, it is feasible that fasting is a stronger physiological stressor in female than male mice and this stress may contribute to reduced food consumption during the task compared to male mice. Intriguingly, in a human fasting study, women reduced food intake post-fasting whereas men increased food intake (Zandian *et al.*, 2011), demonstrating the translatability of our findings.

We observed a fasting-induced increase in mEPSC frequency and amplitude in male, but not female mice. Increased mEPSC frequency is typically associated with increased glutamate release (Simpson, 1971; Holz & Fisher, 1999; Brock *et al.*, 2020). However, we did not observe changes in release probability or the size of the readily releasable pool. Furthermore, it is unlikely that the fasting-induced increase in mEPSC frequency is due to an increase in amplitude of AMPAR response that now passes the threshold for mEPSC detection as we did not observe a change in AMPAR/NMDAR ratio or responses to Rubi-glutamate. Thus, fasting does not induce a postsynaptic change in number or function of AMPARs or a post-synaptic unsilencing of glutamatergic synapses in the VTA. An alternative explanation for increased mEPSC frequency could be due to an increase in the number of release sites, which could be

measured with immuno-electron microscopy of excitatory synapses. Notably, an increase in ketones during fasting is associated with increased brain derived neurotrophic factor (BDNF) in the cortex and hippocampus (Marosi *et al.*, 2016; Sleiman *et al.*, 2016; Mattson *et al.*, 2018), which can induce formation of new synaptic release sites in the VTA (Pu *et al.*, 2006). Exposure to sweetened high fat food increased excitatory synapse number onto VTA dopamine neurons, measured with electrophysiology and immuno-electron microscopy. This effect was associated with increased food-seeking behaviour in a light-dark box test (Liu *et al.*, 2016). Decreasing excitatory synaptic strength in the VTA reverses the palatable-food increase in food approach behaviours (Liu *et al.*, 2016). Thus, increased excitatory synapse number onto VTA dopamine neurons of male mice may underlie enhanced food seeking, regardless of whether this is driven by energy density or by food-restriction.

Fasting increased endocannabinoid-mediated DSE at excitatory synapses onto dopamine neurons of female, but not male mice. Others have demonstrated that a 24-h fast induced a loss of endocannabinoid-mediated iLTD at synapses in the dorsomedial hypothalamus, an effect mediated by glucocorticoids (Crosby *et al.*, 2011). Furthermore, increased CORT is associated with increased endocannabinoids in the amygdala or hypothalamus (Di *et al.*, 2003, 2016). A fasting-induced facilitation of DSE was not associated with a change in basal PPR or ready releasable pool in female mice indicating that fasting does not induce a lasting endocannabinoid tone. Furthermore, we did not observe altered CB1 receptor efficacy with fasting of female mice. Endocannabinoid synthesis and release is initiated on demand by an influx in calcium and is influenced by expression of enzymes including d-acyl glycerol lipase (DAGL) and n-arachidonylphosphatidylethanolamine phospholipase D (NAPE-PLD). Future

experiments could test if fasting alters expression or function of DAGL or NAPE-PLD differentially in male or female mice. Taken together, fasting-induced changes in excitatory inputs of dopamine neurons of female mice may be expressed under more depolarized conditions allowing for calcium influx and endocannabinoid synthesis.

Fasting did not induce changes at inhibitory synapses onto VTA dopamine neurons of male or female mice. The probability of release at inhibitory synapses onto VTA neurons was lower in female compared to male mice, regardless of fasting, suggesting that there may be basal differences in release properties. To further explore the effects of fasting at inhibitory synapses we used a 10 Hz stimulation, which induces iLTD of dopamine neurons of cocaine-treated mice, but not vehicle-treated mice (Pan *et al.*, 2008). Consistent with this study, we did not observe an iLTD of dopamine neurons in male or female control mice. Furthermore, fasting was not a sufficient stimulus to facilitate an iLTD. Interestingly, the 10 Hz protocol produced a potentiation of IPSCs only in VTA dopamine neurons of male mice. Future studies could assess if this is due to a sex difference in release probability at inhibitory synapses onto dopamine neurons.

In summary, acute fasting affects metabolic parameters, food-seeking, and synaptic properties in the VTA in a sex-dependent manner. While a 16 h overnight fast increased food-seeking behaviour overall supporting the efficacy of the fasting procedure, this increase was significantly greater in male mice. Furthermore, male mice had increased mEPSC frequency onto VTA dopamine neurons, an effect likely due to increased number of release sites. Female mice on the other hand showed no changes in basal synaptic properties, but rather a facilitation in endocannabinoid-mediated short-term depression at excitatory synapses onto

dopamine neurons. Inhibitory synapses onto dopamine neurons of male or female mice were not changed by fasting. Thus, a 16h fast leads to sexually dimorphic synaptic changes that parallel food-seeking behaviour. These results highlight the sensitivity and importance of VTA dopamine neurons in responding to changes in motivational states. Furthermore, fasting-induced plasticity in the VTA may underlie enhanced food-seeking during food availability and thus, may be a reason why weight loss is rarely maintained. Importantly, major depressive disorder and anxiety are twice as frequently diagnosed in women compared to men, while anorexia nervosa is 13 times more frequent in women (McCarthy *et al.*, 2012). Therefore, understanding sex differences in state dependent changes in synaptic function of VTA dopamine neurons may highlight mechanisms underlying why there is gender and sex bias in these neuropsychiatric illnesses.

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Author Contributions:

NG performed electrophysiological and behavioural experiments. NG and SLB wrote first drafts of the manuscript. NG and SLB revised the manuscript.

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The authors declare no competing financial or other conflicts of interest.

Data Availability

Data is available upon request to the corresponding author.

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Figure Legends

Figure 1. Body weight and metabolic measures after acute fasting in male and female mice.

A) Schematic showing time course of fast and when blood draw and weights were recorded.
B) Chow consumed per bodyweight (kCal/g) by control male and female mice during the 16h overnight period of isolation. Female control mice consume proportionally more chow during period of isolation than male control mice (*P<0.05).
C) Water consumed by male and female mice during the 16h overnight period. There is a sex x fasting interaction on water consumption and main effects of sex (****P<0.0001) and fasting (*P<0.05). A Tukey's multiple comparisons test indicates a difference in female fasting and control mice (***P<0.001).
D) Body weight change between before and after the 16h overnight period. There is a main effect of fasting (****P<0.0001) and a main effect of sex (*P<0.05).
E) Blood glucose concentration (mmol/L) measured after the 16h overnight period. There was a main effect of fasting (****P<0.0001) and a main effect of sex (***P<0.001) on blood glucose concentration.
F) Blood ketone concentration (mmol/L) measured after the 16h overnight period. There was as sex x fasting interaction with main effects of fasting (****P<0.0001) and sex (**P<0.01). A Tukey's multiple comparisons test indicates differences in ketones in fasted and control male (****P<0.0001) and female (****P<0.0001) mice.
G) Serum CORT concentration (mmol/L) measured after the 16h overnight period. The was a fasting x sex interaction and main effects of fasting (****P<0.0001) and sex (***P<0.001). A Tukey's multiple comparisons test indicates differences in serum CORT of fasted and control male (*P<0.05) and female (****P<0.0001) mice.

Symbols represent individual mice, and the bars represent the mean ±SD.

Figure 2. Sex differences in food approach behaviour and food consumption following fasting.

A) Schematic showing time course of fast and when behavioural performance was recorded.
B) Schematic of the light-dark box apparatus with the food zone.
C) Plot illustrating behavior in the light zone (shaded bars), dark zone (filled bars) or food zone (open bars) during the ten minute light-dark box test of individual representative mice.
D) Distance travelled in the light zone during the 10 min test after fasting. There was a main effect of sex on distance travelled (**P<0.01).
E) Dark zone entries during the 10 min test after fasting. There was a main effect of sex on dark zone entries (*P<0.05).
F) Distance travelled in the food zone during the 10 min test after fasting. There was a main effect of fasting on distance travelled in the food zone (**P<0.01).

G) Food zone entries during the 10 min test after fasting. There was a main effect of fasting on food zone entries (***P<0.001).
H) Time in the food zone during the 10 min test after fasting. There was a fasting x sex interaction and main effects of fasting (****P<0.0001) and sex (*P<0.05) on the time in the food zone. A Tukey's multiple comparison test indicated significant differences between fasted and control male (****P<0.0001) and female (**P<0.01) mice in time in food zone.
I) High fat diet consumed during the 10 min test after fasting. There was a sex x fasting interaction and a main effects of fasting (****P<0.0001) and sex (*P<0.05). A Tukey's multiple comparison test indicated significant differences between fasted and control male (****P<0.0001) and female (****P<0.0001) mice in food consumed during the test.

Symbols represent individual mice, and the bars represent the mean ± SD.

Figure 3. Sex differences in quantal release properties at excitatory synapses onto VTA dopamine neurons of male and female mice following fasting.

A) Schematic showing time course of fast and when brain slices were extracted for electrophysiology experiments.
B) Representative examples of mEPSC recordings from male and female fasted and control mice.
C) mEPSC amplitude onto dopamine neurons of male and female fasted and control mice. There was a main effect of fasting (*P<0.05) and a main effect of sex (*P<0.05) on mEPSC amplitude. A Tukey's multiple comparison test indicated a significant difference between mEPSC amplitude of fasted and control male mice (**P<0.01).
D) mEPSC frequency onto dopamine neurons of male and female fasted and control mice. There was sex x fasting interaction and main effects of fasting (*P<0.05) and sex (**P<0.01) on mEPSC frequency. A Tukey's multiple comparison test indicated significant differences between mEPSC frequency of fasted and control male mice (**P<0.01).
E) Paired-pulse ratio of evoked EPSCs was not different between fasted or control male and female mice.
F) Representative example recordings of the paired-pulse evoked EPSC currents.

Symbols represent individual cells, and the bars represent the mean ± SD.

Figure 4. Effect of fasting on ready releasable pool and release probability from male and female mice.

A) Representative example trace of 100 Hz train stimulation used for the readily releasable pool analysis.

B) Example of the plotted cumulative amplitude and best fit line to determine the size of the readily releasable pool (RRP(train)) and the probability of release (P(train)).
C) RRP determined from the y intercept of the fitted line of the cumulative amplitude. There was no effect of fasting or sex on the RRP.
D) Release probability (P(train)) determined from the amplitude of the first evoked EPSC divided by RRP(train).There was no fasting or sex effect on P(train).
E) There was no effect of sex or fasting on PPR (train).

Symbols represent individual cells, and the bars represent the mean ± SD.

Figure 5. The effect of fasting on post-synaptic properties of excitatory synapses of male and female mice.

A) Averaged representative traces of AMPAR and NMDAR EPSCs.
B) The AMPAR/NMDAR ratio of fasted and control male and female mice. There was no effect of fasting or sex on the AMPAR/NMDA ratio.
C) Schematic illustrating the optically-induced (470 nm) uncaging of glutamate using RuBi-Glutamate.
D) Averaged representative traces of glutamate currents recorded at -70 mV.
E) EPSC amplitude in response to photo-uncaging of Rubi-glutamate in control or fasted male mice. There was no difference of EPSCs onto dopamine neurons from control or fasted male mice.

Symbols represent individual cells, and the bars represent the mean ±SD.

Figure 6. Sex differences in DSE of dopamine neurons from fasted and control male and female mice.

A) Schematic illustrating the endocannabinoid mediated depolarization induced suppression of excitation (DSE).
B) Time course of evoked EPSCs before and after the DSE protocol in dopamine neurons of male control and fasted mice.
C) Time course of evoked EPSCs in the presence of the CB1 receptor antagonist AM251 (2 µM) before and after the DSE protocol in dopamine neurons of male control and fasted mice.
D) Averaged responses during DSE of dopamine neurons from male control or fasted mice in the presence or absence of AM251. There is a main effect of AM251 on EPSC amplitude (**P<0.01).
E) Time course of evoked EPSCs before and after the DSE protocol in dopamine neurons of female control and fasted mice.
F) Time course of evoked EPSCs in the presence of the CB1 receptor antagonist AM251 (2 µM) before and after the DSE protocol in dopamine neurons of female control and fasted mice.

G) Averaged responses during DSE of dopamine neurons from female control or fasted mice in the presence or absence of AM251. There is a fasting x drug interaction and a main effect of AM251 on EPSC amplitude (****P<0.0001). A Tukey's multiple comparison test indicates a difference in DSE between control and fasted female mice (*P<0.05).
H) Averaged representative traces of EPSCs during baseline and DSE of dopamine neurons from male and female fasted and control mice.
I) Averaged responses during DSE of dopamine neurons from female control or fasted mice. There is a sex x fasting interaction. A Tukey's multiple comparison test indicates a difference in DSE between control and fasted female mice (*P<0.05).

Symbols represent individual animals, and the bars represent the mean ± SD.

Figure 7. The CB1 receptor agonist, WIN 55,212, supresses EPSCs but responses are not altered by fasting.

A) Averaged example traces of EPSCs of dopamine neurons from female during baseline and after the application of WIN 55,212.
B) Time course of evoked EPSCs before, during and after WIN 55,212 (10 µM) application to dopamine neurons of female control and fasted mice.
C) Averaged responses of EPSCs before and after WIN 55,212 application to dopamine neurons from female control or fasted mice. There is a main effect of WIN 55,212 on EPSC amplitude (***P<0.001).
D) PPR before and after WIN 55,212 application to dopamine neurons of control and fasted female mice. There is a main effect of WIN 55,212 on PPR (*P<0.05).

Symbols represent individual animals, and the bars represent the mean ± SD.

Figure 8. Quantal release properties at inhibitory synapses onto dopamine neurons of male or female mice do not change following fasting.

A) Representative examples of mIPSC recordings from male and female fasted and control mice.
B) mIPSC amplitude onto dopamine neurons of male and female fasted and control mice. There was no effect of fasting or sex on mIPSC amplitude.
C) mIPSC frequency onto dopamine neurons of male and female fasted and control mice. There was no effect of fasting or sex on mIPSC frequency.
D) Paired-pulse ratio of evoked IPSCs was not different between fasted or control male and female mice. However, there was a main effect of sex (***P<0.001) on PPR of evoked IPSCs.
E) Representative example recordings of the paired-pulse evoked IPSC currents.

Symbols represent individual animals, and the bars represent the mean ± SD.

Figure 9. Fasting does not alter DSI at inhibitory synapses onto dopamine neurons of male or female mice.

A) Time course of evoked IPSCs before and after the DSI protocol in dopamine neurons of male control and fasted mice.
B) Time course of evoked IPSCs in the presence of the CB1 receptor antagonist AM251 (2 µM) before and after the DSI protocol in dopamine neurons of male control and fasted mice.
C) Averaged responses during DSI of dopamine neurons from male control or fasted mice in the presence or absence of AM251. There are main effects of AM251 (**P<0.01) and fasting (*P<0.01) on IPSC amplitude.
D) Time course of evoked IPSCs before and after the DSI protocol in dopamine neurons of female control and fasted mice.
E) Time course of evoked IPSCs in the presence of the CB1 receptor antagonist AM251 (2 µM) before and after the DSI protocol in dopamine neurons of female control and fasted mice.
F) Averaged responses during DSI of dopamine neurons from female control or fasted mice in the presence or absence of AM251. There is a main effect of AM251 on IPSC amplitude (****P<0.0001).
G) Averaged representative traces of IPSCs during baseline and DSI of dopamine neurons from male and female fasted and control mice.
H) Averaged responses during DSI of dopamine neurons from female control or fasted mice. There is no effect of sex or fasting on DSI.

Symbols represent individual animals, and the bars represent the mean ± SD.

Figure 10. Fasting does not alter inhibitory synaptic transmission after a 10 Hz stimulation of dopamine neurons of male or female mice.

A) Averaged representative traces of IPSCs from baseline and following the 10 Hz stimulation of dopamine neurons of control or fasted male mice.
B) Time course of evoked IPSCs before and after a 10 Hz stimulation in dopamine neurons of male control and fasted mice.
C) Averaged responses during before and after a 10 Hz, 5 min stimulation in control or fasted male mice. There is a main effect stimulation (**P<0.01) on IPSC amplitude.
D) Averaged representative traces of IPSCs from baseline and following the 10 Hz stimulation of dopamine neurons of control or fasted female mice.
E) Time course of evoked IPSCs before and after a 10 Hz stimulation in dopamine neurons of male control and fasted mice.
F) Averaged responses during before and after a 10 Hz, 5 min stimulation in control or fasted female mice.

G) Averaged responses after a 10 Hz, 5 min stimulation of dopamine neurons from female or male control or fasted mice. There is a main effect of sex (**P<0.01) on IPSC amplitude post-stimulation.
H) Averaged PPR post-stimulation of dopamine neurons from male or female control or fasted mice.
I) The change in PPR between pre- and post-stimulation is not different in male or female control or fasted mice.

Symbols represent individual animals, and the bars represent the mean ± SD.

Figure 1

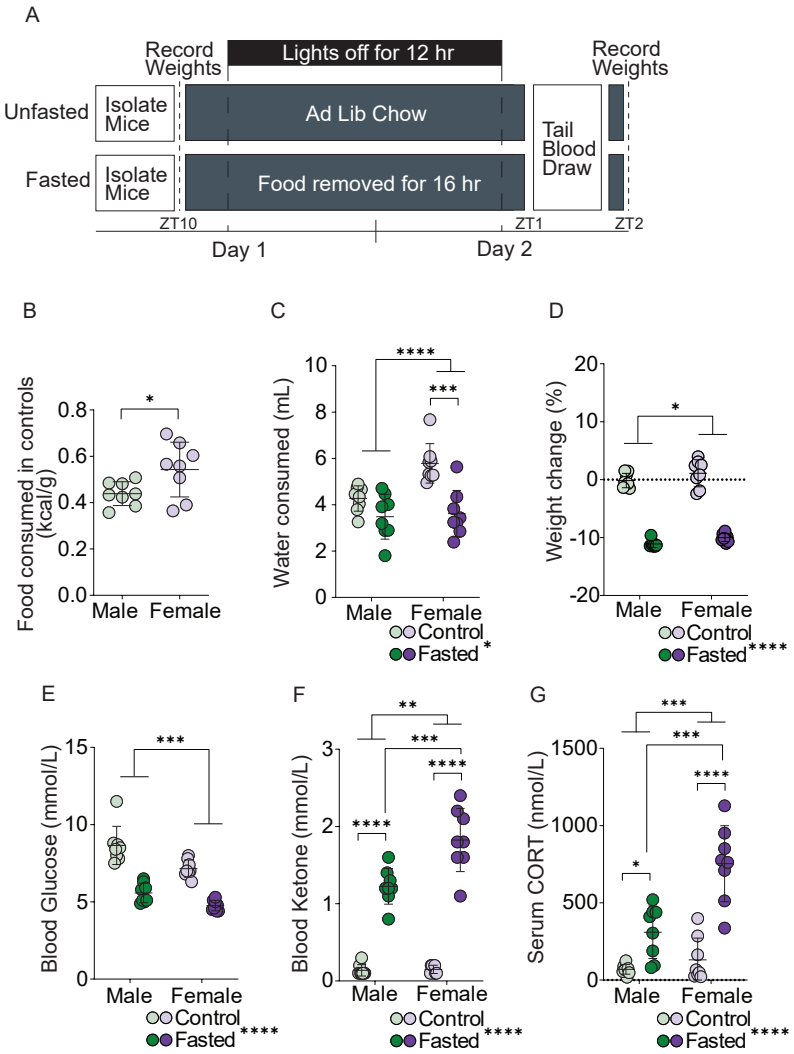

A
Record Weights
Lights off for 12 hr
Record Weights
Unfasted
Isolate Mice
Ad Lib Chow
Tail Blood Draw
Fasted
Isolate Mice
Food removed for 16 hr
ZT10
ZT1
ZT2
Day 1
Day 2
B
Food consumed in controls (kcal/g)
Male
Female
C
Water consumed (mL)
Male
Female
Control
Fasted
D
Weight change (%)
Male
Female
Control
Fasted
E
Blood Glucose (mmol/L)
Male
Female
Control
Fasted
F
Blood Ketone (mmol/L)
Male
Female
Control
Fasted
G
Serum CORT (nmol/L)
Male
Female
Control
Fasted

Figure 2

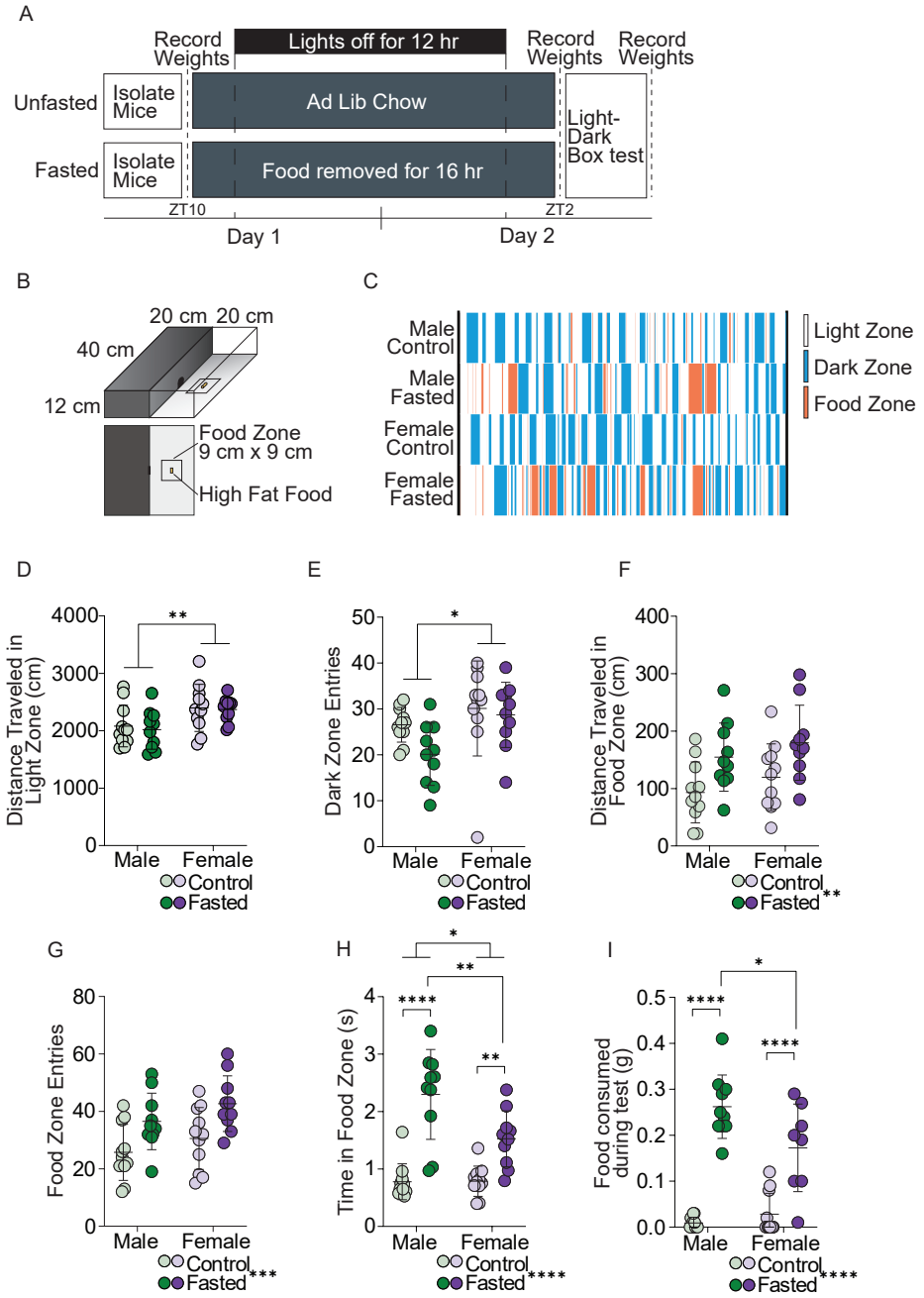

A
Record Weights
Lights off for 12 hr
Record Weights
Record Weights
Unfasted
Isolate Mice
Ad Lib Chow
Light-Dark Box test
Fasted
Isolate Mice
Food removed for 16 hr
ZT10
ZT2
Day 1
Day 2
B
20 cm
20 cm
40 cm
12 cm
Food Zone
9 cm x 9 cm
High Fat Food
C
Male Control
Male Fasted
Female Control
Female Fasted
Light Zone
Dark Zone
Food Zone
D
Distance Traveled in Light Zone (cm)
Male
Female
Control
Fasted
E
Dark Zone Entries
F
Distance Traveled in Food Zone (cm)
G
Food Zone Entries
H
Time in Food Zone (s)
I
Food consumed during test (g)

Figure 3

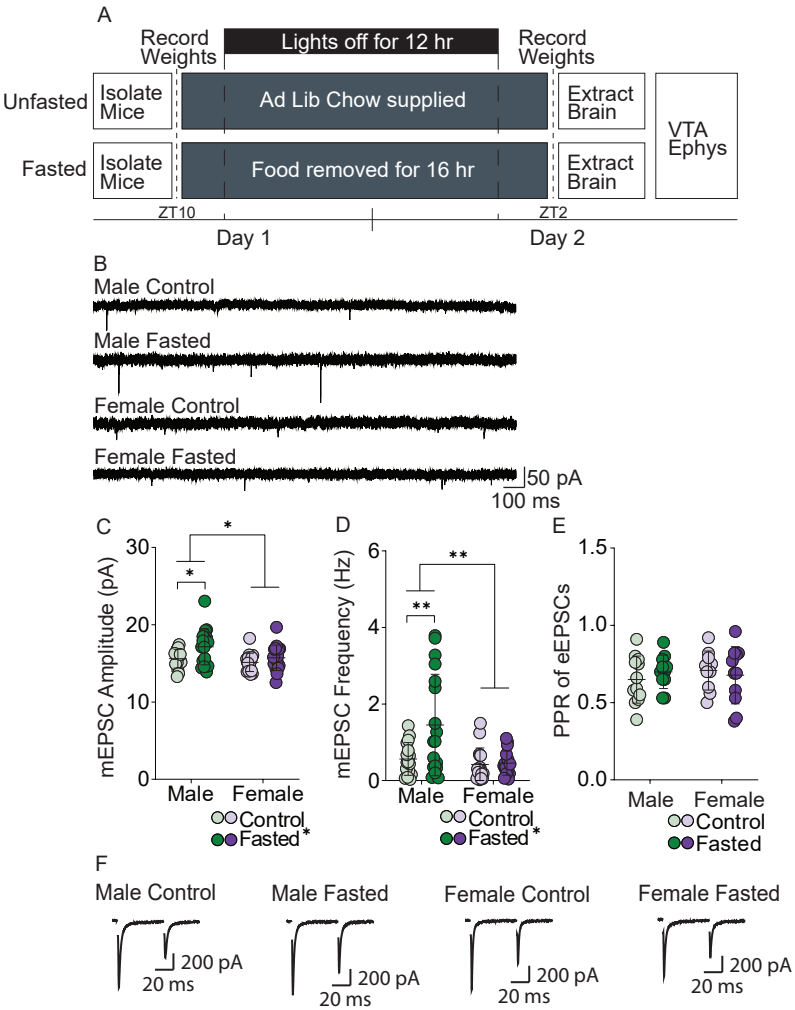

A
Record Weights
Lights off for 12 hr
Record Weights
Unfasted
Isolate Mice
Ad Lib Chow supplied
Extract Brain
VTA Ephys
Fasted
Isolate Mice
Food removed for 16 hr
Extract Brain
ZT10
ZT2
Day 1
Day 2
B
Male Control
Male Fasted
Female Control
Female Fasted
50 pA
100 ms
C
mEPSC Amplitude (pA)
Male
Female
Control
Fasted
D
mEPSC Frequency (Hz)
Male
Female
Control
Fasted
E
PPR of eEPSCs
Male
Female
Control
Fasted
F
Male Control
Male Fasted
Female Control
Female Fasted
200 pA
20 ms

Figure 4

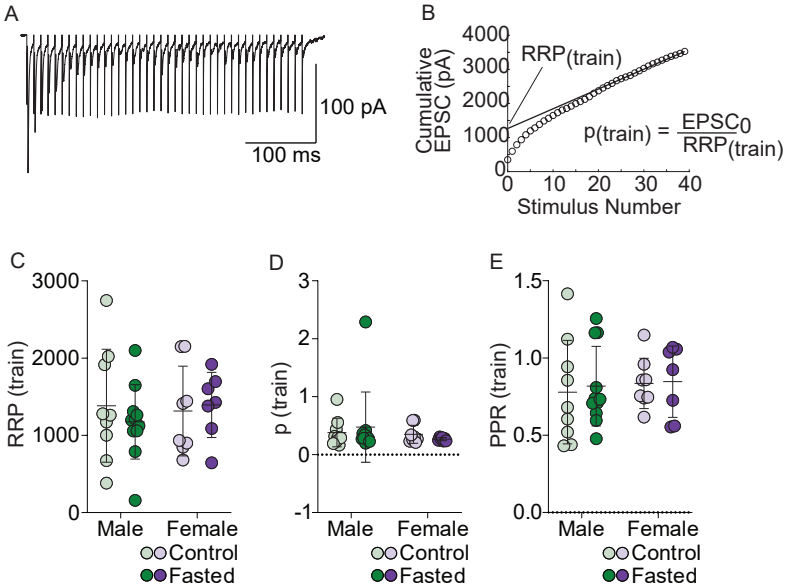

A
100 pA
100 ms
B
Cumulative EPSC (pA)
4000
3000
2000
1000
0
RRP(train)
p(train) = EPSC0 / RRP(train)
0 10 20 30 40
Stimulus Number
C
RRP (train)
3000
2000
1000
0
Male Female
Control
Fasted
D
p (train)
3
2
1
0
-1
Male Female
Control
Fasted
E
PPR (train)
1.5
1.0
0.5
0.0
Male Female
Control
Fasted

Figure 5

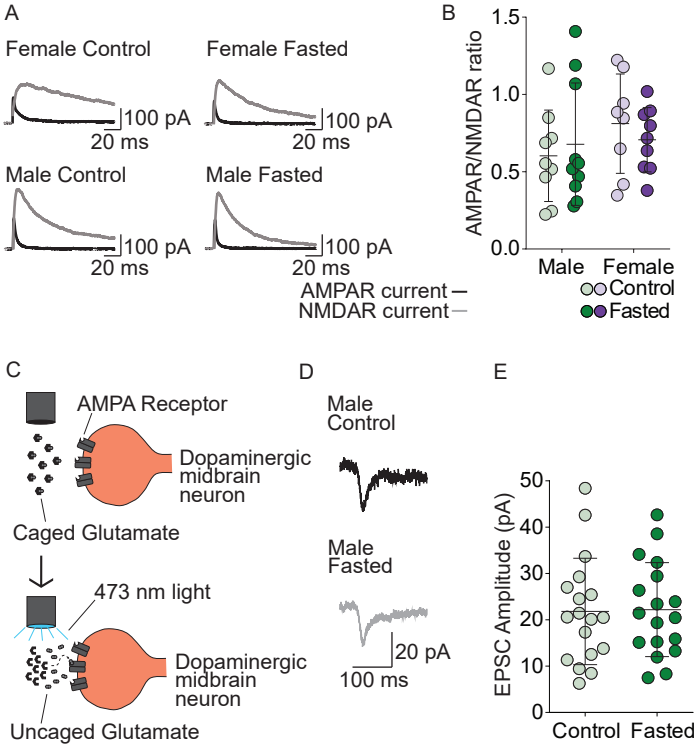

A
Female Control
Female Fasted
100 pA
20 ms
100 pA
20 ms
Male Control
Male Fasted
100 pA
20 ms
100 pA
20 ms
AMPAR current
NMDAR current
B
AMPAR/NMDAR ratio
1.5
1.0
0.5
0.0
Male
Female
Control
Fasted
C
AMPA Receptor
Dopaminergic midbrain neuron
Caged Glutamate
473 nm light
Dopaminergic midbrain neuron
Uncaged Glutamate
D
Male Control
Male Fasted
20 pA
100 ms
E
EPSC Amplitude (pA)
50
40
30
20
10
0
Control
Fasted

Figure 6

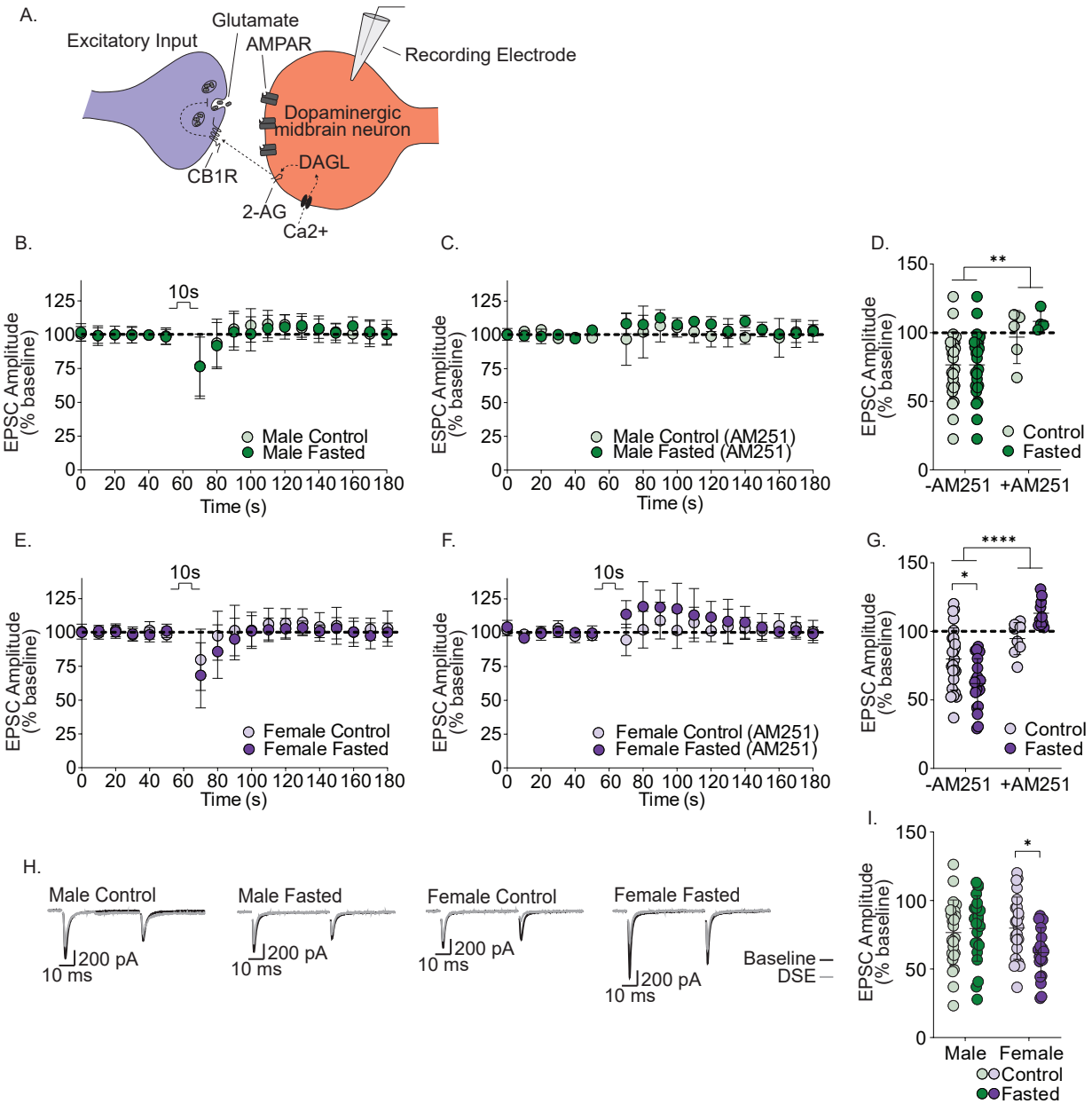

A.
Excitatory Input
Glutamate
AMPAR
Recording Electrode
Dopaminergic midbrain neuron
DAGL
CB1R
2-AG
Ca2+
B.
10s
EPSC Amplitude (% baseline)
Time (s)
Male Control
Male Fasted
C.
ESPC Amplitude (% baseline)
Male Control (AM251)
Male Fasted (AM251)
D.
**
EPSC Amplitude (% baseline)
Control
Fasted
-AM251
+AM251
E.
10s
Female Control
Female Fasted
F.
10s
Female Control (AM251)
Female Fasted (AM251)
G.
****
*
Control
Fasted
-AM251
+AM251
H.
Male Control
Male Fasted
Female Control
Female Fasted
200 pA
10 ms
Baseline
DSE
I.
*
Male
Female
Control
Fasted

Figure 7

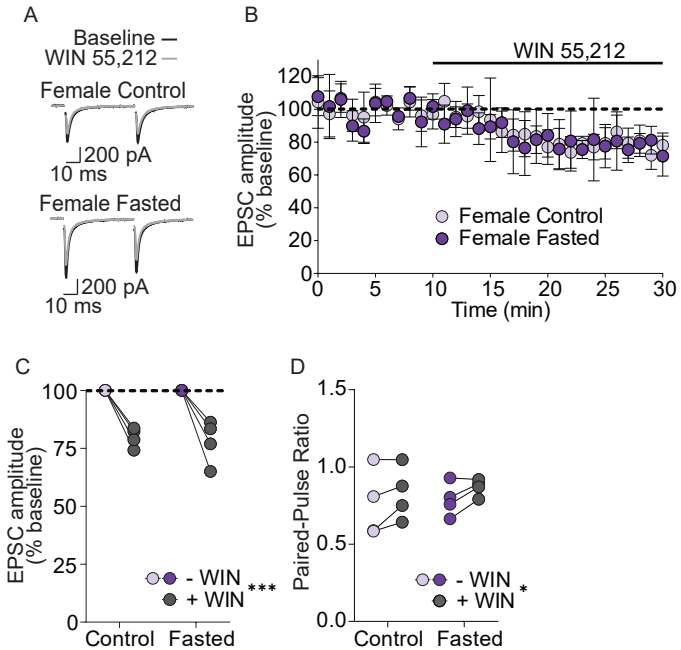

A
Baseline
WIN 55,212
Female Control
200 pA
10 ms
Female Fasted
200 pA
10 ms
B
WIN 55,212
EPSC amplitude (% baseline)
Female Control
Female Fasted
Time (min)
C
EPSC amplitude (% baseline)
- WIN
+ WIN
***
Control
Fasted
D
Paired-Pulse Ratio
- WIN
+ WIN
*
Control
Fasted

Figure 8

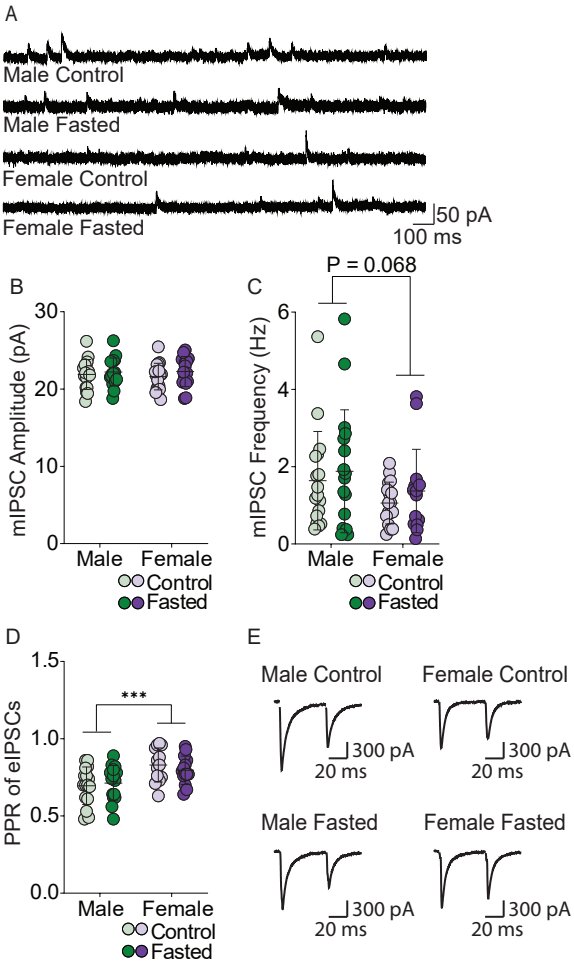

A
Male Control
Male Fasted
Female Control
Female Fasted
50 pA
100 ms
B
mIPSC Amplitude (pA)
30
20
10
0
Male
Female
Control
Fasted
C
P = 0.068
mIPSC Frequency (Hz)
6
4
2
0
Male
Female
Control
Fasted
D
PPR of eIPSCs
1.5
1.0
0.5
0.0
***
Male
Female
Control
Fasted
E
Male Control
Female Control
Male Fasted
Female Fasted
300 pA
20 ms

Figure 9

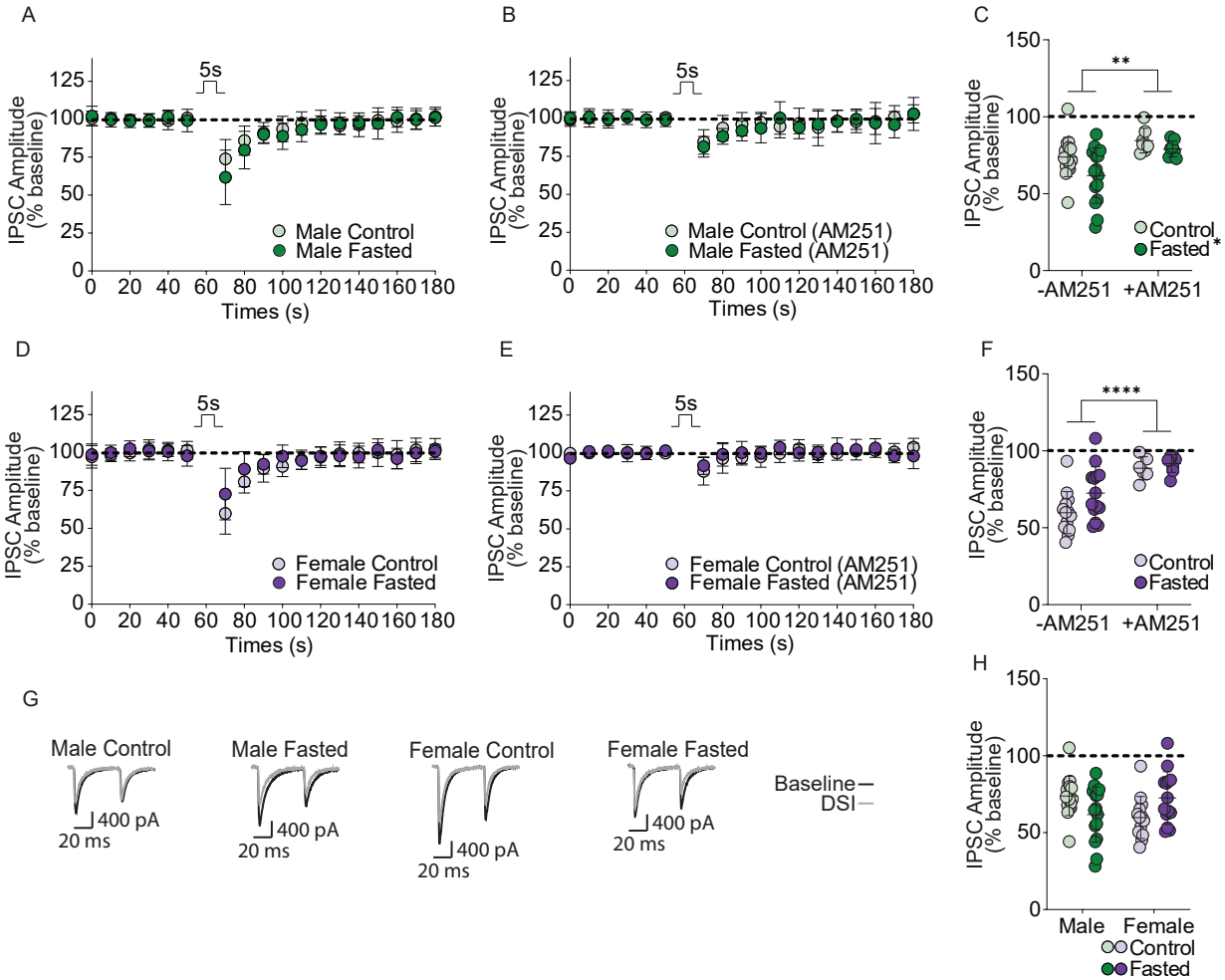
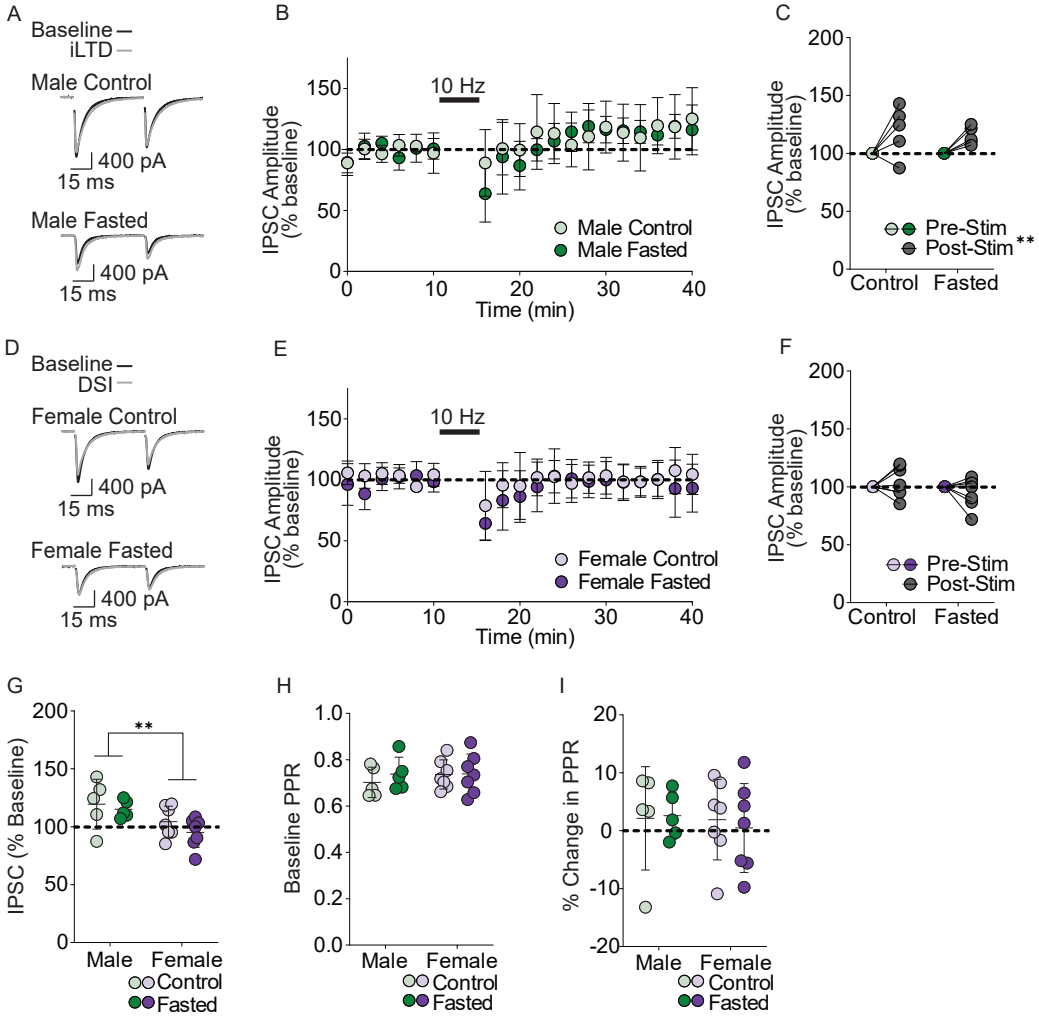

A
5s
IPSC Amplitude (% baseline)
Times (s)
Male Control
Male Fasted
B
Male Control (AM251)
Male Fasted (AM251)
C
**
Control
Fasted*
-AM251
+AM251
D
Female Control
Female Fasted
E
Female Control (AM251)
Female Fasted (AM251)
F
****
G
Male Control
Male Fasted
Female Control
Female Fasted
400 pA
20 ms
Baseline
DSI
H
Male
Female
Control
Fasted

Figure 10


A
Baseline
iLTD
Male Control
400 pA
15 ms
Male Fasted
400 pA
15 ms
B
10 Hz
IPSC Amplitude (% baseline)
Male Control
Male Fasted
Time (min)
C
IPSC Amplitude (% baseline)
Pre-Stim
Post-Stim
**
Control
Fasted
D
Baseline
DSI
Female Control
400 pA
15 ms
Female Fasted
400 pA
15 ms
E
10 Hz
IPSC Amplitude (% baseline)
Female Control
Female Fasted
Time (min)
F
IPSC Amplitude (% baseline)
Pre-Stim
Post-Stim
Control
Fasted
G
IPSC (% Baseline)
**
Male
Female
Control
Fasted
H
Baseline PPR
Male
Female
Control
Fasted
I
% Change in PPR
Male
Female
Control
Fasted