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Original Research Article

Sex difference effects of acute starvation on excitatory and inhibitory synapses on dopamine neurons

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ABSTRACT

Background: The aim of this study is to assess the influence of acute fasting on synaptic properties in the ventral tegmental area (VTA) with regards to the sex-dependent differences by use of male and female mice. The study aimed to unravel the intricate interplay between fasting, synaptic plasticity, and behavioural changes to enhance our understanding of the underlying mechanisms.
Methods: This study carried out at a tertiary care centre, employed a 16-hour overnight fasting protocol in female rodents and male rodents to examine the effects on physiological parameters, feeding behaviour, and neuronal attributes in the VTA. Various assays, including measurements of blood glucose, ketones, corticosterone (CORT) levels, locomotor activity, and electrophysiological recordings of synaptic currents, were conducted to assess the physiological and synaptic responses to acute fasting.
Results: The study found that acute fasting induced significant metabolic changes, including body mass decrease by about 10%. Altered food-seeking behaviour was evident, with male mice exhibiting a pronounced increase. Moreover, neuronal attributes in the midbrain or VTA showed gender-dependent responses: males displayed substantial 20% increase in the frequency of mEPSC onto the dopaminergic neurons in the midbrain post fasting, while females exhibited a 13% elevation in CORT levels, accompanied by a transient period of depression at stimulatory synapses onto dopaminergic neurons which was mediated by endocannabinoids. Notably, no significant changes were observed at restraining synapses in rodents of both genders. These findings highlight the nuanced influence of short-term fasting/starvation on the VTA's synaptic plasticity, emphasizing sex-specific responses and providing valuable insights into potential mechanisms influencing gender differences in neuropsychiatric conditions.
Conclusions: The study reveals that acute fasting induces sex-specific synaptic changes in the VTA, shedding light on the intricate relationship between metabolism, food-seeking behaviour, and neural plasticity. These findings emphasize the importance of considering gender-specific responses in studies exploring the neurobiological effects of fasting and their relevance to mental health.

Keywords: Fasting, VTA, Synaptic plasticity, Sex differences, Dopamine neurons

INTRODUCTION

The rise in obesity rates has coincided with a surge in dieting practices among the public at large.[1] While even modest reductions in body weight can lead to improvements in cardiac and glycaemic findings, sustaining weight-loss over the long term remains a challenge.[2] Various hypotheses aim to elucidate why permanent weight loss is infrequently achieved. The prominent hypothesis put forth suggests that diets that promote obesity may induce enduring changes in the nerve circuits governing food consumption and dietary

patterns.[3,4] Another one implies that both diet constraints and weight-loss can trigger neural and metabolic alterations that heighten the drive for rewards and food consumption.[5-7]

Dopamine neurons in the midbrain or VTA are essential for encoding motivationally relevant data, with its release in the ventral striatum amplifying the desire for food.[8] The strengthening of excitatory inputs in the VTA is fundamental to the learning of cues associated with rewards.[9] Notably, exposure to sweetened cholesterol-rich foods reinforces facilitatory synapses onto the dopaminergic neurons of the midbrain, promoting enhanced consumption of food.[4] However, the effect of food constraints as well as weight-loss on the plasticity of the synapses in the VTA remains vague.

Furthermore, delving into the mechanisms underlying these diet patterns may provide insights into why maintaining weight loss is a persistent challenge and may offer understanding into the development of eating disorders. Studies reveal chronic food-restricted rodents have heightened dopamine release in the ventral striatum.[10-12] Moreover, male mice with long-term restriction of food show increased elevated firing in the dopaminergic neuronal cluster in the substantia nigra, associated with amplified excitatory synaptic strength via an elevated AMPAR/NMDAR ratio and heightened sensitivity of dopamine D2 auto receptors.[13] Conversely, overnight fasting has no impact on dopaminergic neurons of the nigral region in male rodents.[13]

A short fast (24 to 36 h) reduces dopamine transporter activity in the rat striatum.[14] A short 24 - hour starvation enhances inhibitory postsynaptic current (IPSC) responses within the VTA elicited by the somatodendritic D2 receptor.[15] However, the precise impact of this protocol on facilitatory and inhibitory transmission of synapses in the VTA remain unclear.

Gender differences exist in the natural response to starvation or abstinence from food in human beings and mice.[16-18] Yet, the manner in which it uniquely influences transmission of nerve synapses in the midbrain between female and male mice remains unknown. Our hypothesis suggests that acute fasting for overnight exerts distinct effects on excitatory and inhibitory synaptic transmission onto VTA dopamine neurons in female and male mice.

METHODS

Study design

The study was carried out at a tertiary care centre for a period of 1 year, between July 2022 to July 2023, and involved 2-4-month-old mice, group-housed in same-sex cages with unlimited access to water and food. Local bred male and female DATcreTd-Tomato rodents, identifying dopamine neurons, were considered for this study. The study included measurements of body weight, water intake, and glucose, ketone, and serum CORT levels, as well as electrophysiology and behavioural experiments at specific time points within the light cycle.

Inclusion and exclusion criteria

Inclusion criteria for the study involved 2-4-month-old mice, group-housed in same-sex cages, and maintained on a day-night cycle for 12 hours with uncontrolled availability to water and food. Locally bred male and female DATcreTd-Tomato rodents, identifying dopamine neurons, were included.

Exclusion criteria encompassed mice outside the specified age range, those not conforming to the housing conditions, or lacking the genetic markers for dopamine neuron identification.

Glucose, ketone, and CORT data assessments

Glucose levels were assessed using a glucose meter on a small amount of blood extracted from the tail vein. Ketone levels were similarly measured from blood collected from the tail vein using a monitor for observing ketone levels in blood which is equipped with testing strips for keto detection. Serum was obtained by centrifugation of the samples collected in Microvette CB 300 uL tubes with clotting activator at 16,100 gm for 15 minutes at 4 ºC. CORT levels in the serum were quantified using the DetectX CORT enzyme immunoassay kit.

Appetite-driven activity of mice

Rodents were previously exposed to fat-rich food pellet (5-gram) 48 hrs before the behavioural test to minimize aversion to new foods. This diet consists majorly of fat-60%, and equal amounts of carbohydrates, and protein (20 % each), summing to give an energy density equal to 5.21 kcal per gm. Following this exposure, isolated mice, whether fasted/starved or in the control populace, underwent a 10-minute session in a light-dark box equipment made of acrylic, with dimensions in cm being 40×40×12. This box split into 60 lumens of light and 5 lumens of dark chambers, was connected by an opening of dimensions in cm being 3.5×3.4, and the mice were individually introduced to the bright chamber located opposite to the entrance of the dark chamber. Movements were recorded using MatLab and EthoVision XT software. The box was cleansed following every use, and the mice had one hour of availability to a diet rich in fats after the test, and their intake was recorded.

Electrophysiology

Electrophysiological recordings utilized slice preparations from adult DATcre;Td-Tomato mice (2 to 4 months old), featuring a red fluorescent marker exclusive to dopamine neurons in the VTA. After anesthesia and brain extraction, horizontal midbrain sections were

obtained. Slices were incubated and shifted to the monitoring area, in which whole-cell recordings were conducted on identified dopamine neurons using an amplifier. The recorded signals were sampled at 20 KHz, and a Bessel filter was applied. Miniature post-synaptic currents were recorded, and for evoked currents, a bipolar electrode was used. Recording electrodes filled with CeMeSO3 were employed. Short-term plasticity was examined by inducing electrically evoked EPSCs, and Matlab was used for data analysis. The experimental setup was validated, and the apparatus was cleaned between subjects.

Study size

The study included n=16 male and n=16 female DATcreTd-Tomato mice, subjected to a 16-hour overnight fast, examining various physiological parameters.

Study setting

To induce depolarization-induced suppression of excitation (DSE), 100 µM of picrotoxin was used to keep the neurons-70 mV potential, and paired stimuli were applied for every 50 ms interstimulus interval (ISI). The stimulated EPSCs were noted for every one minute before every 10 sec depolarization steps to reach + 40 mV potential, and the mean of the cumulative six EPSCs was used to establish a baseline. DSE magnitude was calculated as the EPSC amplitude % right after depolarization relative to the pre-depolarization baseline. The depolarization step was repeated at least thrice, and an average DSE noted. Further to validate endocannabinoid mediation, the protocol was repeated with 2 µM of AM251, a CB1 receptor antagonist. The paired-pulse ratio (PPR) was determined by division of the amplitude of the 2nd pulse by that of the 1st pulse during the baseline.

For validation, 10 µM of WIN 55,212, a CB1 receptor agonist, was applied to VTA slices succeeding a 10-minute baseline. The paired stimulus was applied after 50 ms ISI. The thirty EPSCs from the 5 minutes before agonist application were taken together to establish a baseline. EPSCs were noted for 20 minutes post-delivery, and the mean of the final 30 ones from the last 5 min was contrasted to the baseline. The PPR was calculated during both the 5-minute baseline and final 5 min of recording.

Neurons were voltage-clamped at -70 mV using electrodes of 2 to 5 MΩ containing a potassium chloride internal solution. Paired stimuli (50 ms ISI) were delivered with 10 µM of DNQX and 50 µM of AP5. Evoked IPSCs were recorded preceding each 5 sec polarization reversal to +40 mV.[19] DSI magnitude, confirming endocannabinoid mediation, was quantified as the % of IPSC amplitude immediately after polarization reversal relative to the pre-depolarization baseline, with the CB1 receptor antagonist AM251 (2 µM) used for validation. The PPR during the baseline was determined accordingly.

Statistical analysis

Various tests, including a two-tailed unpaired t test, ANOVA, and two-way ANOVA with Sidak's multiple comparisons were used for obtaining the statistical significance. Electrophysiology experiment cohort size is denoted by N/n, and significance in figures is represented by asterisks (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

RESULT

Effects of fasting on body weight and levels of blood ketones, serum CORT, and blood glucose

For investigating the influence of the 16-hour fast (overnight) on DATcreTd-Tomato mice, various parameters were recorded. Initial body weight differences were noted, with male fasted rodents of 30.7±1.2 gm and female fasted mice of 23.0±0.9 gm weight. Despite similar caloric intake during isolation, fasting reduced water intake in male rodents (control: 5.3±0.4 mL, fasted: 4.5±1.2 mL) and female rodents (control: 4.8±0.5 mL, fasted: 2.6±1.2 mL). Blood glucose concentrations also changed, with male control mice having levels of 8.6±1.2 mmol/L and male fasted mice having levels of 5.6±0.6 mmol/L. Additionally, serum CORT concentrations significantly increased in both male rodents (control: 64.56±30.13 nmol per litre, fasted: 304.5±161.3 nmol per litre) and female rodents (control: 131.4±150.1 nmol per litre, fasted: 751.2±236.3 nmol per litre) after fasting. These findings indicate that acute fasting led to notable changes in physiological parameters, with sex-specific variations.

Gender disparities in feeding behaviour and food intake after fasting

This investigation demonstrated the impact of transient fasting on food-foraging behaviour in rodents of both genders. For this, we employed an altered light-dark box paradigm where rodents were put to go into the open light field for food. Initial weights differed between sexes, with male rodents weighing more than females (gender effect: F (1,38)=319.4, p<0.0001; male control: 31±1 gram, n=22; male fasted: 31±0.3-gram, n=20; female control: 23±4 gm, n=22; female fasted: 22±4 gm, n=20). While fasting did not significantly affect the area traversed by the rodents in the light chamber, there was a gender difference, indicating greater locomotor activity in females (F (1,38)=9.853, p=0.0033; female control: 2394±413 cm, n=22; female fasted: 2371±204 cm, n=20; male control: 2132±293 cm, n=22; male fasted: 2131±352 cm, n=20).

Fasting had no effect on entry into the dark zone, but there was a gender effect (F (1,38)=7.063, p=0.0114), and

the former influenced food zone behaviours. Fasting increased the area traversed in this region (F (1,38)=11.15, p=0.0019), the number of food zone entries (F (1,38)=13.64, p=0.0007), and the mean of each visit to the food area (F (1,38)=54.60, p<0.0001). Although male rodents stayed longer food zone and had more intake than females in this evaluation, fasting increased food approach behaviours in both sexes. One hour after the test, starved mice utilized more kcal per body weight (gram) of fat-rich diet than control group (F (1,38)=23.90, p<0.0001), with females consuming more than males (gender effect: F (1,38)=24.79, p<0.0001). Female rodents of this test also consumed more than male counterparts post test (p=0.0018), in line with their baseline feeding pattern.

Impact of short-term fasting on excitatory synaptic transmission

Changes due to starvation in excitatory synapses onto VTA dopaminergic neurons were observed in this study. Males exhibited increased miniature excitatory post-synaptic current (mEPSC) amplitude, while females did not display this effect. Fasting also elevated mEPSC frequency in males, suggesting a presynaptic influence. However, no alterations were detected in the PPR, indicating unchanged release probability. Examination of the readily releasable pool and release probability through high-frequency train stimulation showed no significant changes due to fasting. Moreover, post synaptically, fasting did not impact AMPAR/NMDAR ratio or responses to glutamate uncaging. This comprehensive analysis suggests distinct effects of fasting on excitatory synapses, emphasizing both presynaptic and postsynaptic aspects (Table 1).

Table 1: Impact of short-term fasting on excitatory synaptic transmission.

Parameter	Male	Female
mEPSC amplitude	Increased	No effect
mEPSC frequency	Increased	No effect
Paired-pulse ratio (PPR)	Unchanged	Unchanged
AMPAR/NMDAR ratio	Unchanged	Unchanged

Influence of fasting on the suppression of excitation induced by depolarization

Acute fasting serves as a physiological stressor in mice, evident in elevated CORT levels in females. Endocannabinoids, prompted by glucocorticoids, swiftly inhibit synaptic transmission, acting presynaptically at cannabinoid 1 (CB1) receptors. To explore if fasting affects the signalling by endocannabinoids differently based on gender, we investigated the DSE facilitated by these signalling compounds. DSE was nullified by the antagonist AM251, indicating its endocannabinoid mediation. Fasting didn't impact DSE in male mice (male control: 79.46±22.9% baseline; male fasted: 78.56±22.9% baseline), but significantly increased it in females (female control: 78.8±21.7% baseline; female fasted: 60.9±17.1% baseline), demonstrating a notable sex x fasting interaction. Further examination using a CB1 agonist, WIN 55,212, showed no such change in CB1 receptor's efficiency or expression (control PPR WIN 55,212: 0.92±0.1; fasted PPR WIN 55,212: 0.97±0.07). Thus, fasting heightened DSE in female mice without altering CB1 receptors, indicating a nuanced impact of fasting on synaptic plasticity.

Influence of fasting on inhibitory synaptic activity in VTA

The investigation into the impact of starvation on GABAergic nerve synapses onto the dopaminergic neurons of the midbrain in rodents of both genders revealed consistent mIPSC amplitude across control and fasted groups (male control: 22.9±0.9 pA, female control: 22.6±0.7 pA, male fasted: 23.2±1.3 pA, female fasted: 23.3±1.5 pA). Fasting exhibited no significant effect on mIPSC frequency (male control: 1.5±1.2 Hz, female control: 1.2±0.8 Hz, male fasted: 1.6±1.3 Hz, female fasted: 1.3±1.3 Hz). The PPR analysis showed no fasting effect (male control: 0.9±0.3, female control: 1.1±0.2, male fasted: 0.9±0.2, female fasted: 0.9±0.2), but a significant sex effect on PPR suggested potential differences in probability of release at diminishing synapses between both genders of rodents (Table 2). In summary, fasting did not induce alterations in inhibitory synaptic properties in both male and female mice, with notable sex-related distinctions in release probability.

Table 2: Influence of fasting on inhibitory synaptic activity.

Parameter	Male control	Male fasted	Female control	Female fasted
mEPSC amplitude (pA)	22.9±0.9	23.3±1.3	22.6±0.7	23.3±1.5
mEPSC frequency (Hz)	1.5±1.2	1.6±1.3	1.2±0.8	1.3±1.3
PPR	0.9±0.3	0.9±0.2	1.1±0.2	0.9±0.2

Impact of fasting on inhibition's depolarization-induced suppression in the VTA

Fasting influenced the DSI in male midbrain dopaminergic neurons (fasting effect: F (1,42)=3.325, p=0.039, male control: 71.8% baseline, N/n=32/12; male fasted: 59.7% baseline, N/n=30/8). However, no significant effect occurred in female counterparts (fasting effect: F (1,37)=4.221, p=0.09, female control: 59.4% baseline, N/n=26/8; female fasted: 69.9% baseline, N/n=28/8). AM251 inhibition of DSI was evident in male rodents (drug effect: F (1,42)=11.27, p=0.0013, male control with AM251: 82.4% baseline, N/n=14/4; male

fasted with AM251: 78.01% baseline, N/n=16/6) and females rodents (drug effect: F (1,37)=28.67, p<0.0001, female control with AM251: 87.5% baseline, N/n=12/4; female fasted with AM251: 91.6 % baseline, N/n=16/6). While a gender x starvation interaction on DSI was significant (interaction: F (1,54)=9.215, p=0.0037), there were no significant group differences (sex effect: F (1,54)=0.1435, p=0.6896). In summary, starving had no robust effect in the DSI in rodents of both genders.

DISCUSSION

A 16-hour fast (overnight) induced physiological and behavioral changes, impacting females more than males. Female mice showed lower glucose, larger ketones, and increased cortisone levels, with elevated locomotor activity but reduced intake of food during the test compared to males. Although, this led to a rise in food-foraging in both genders, synaptic transmission in the midbrain remained stable, there were subtle gender differences at facilitatory synapses. Male rodents exhibited increased mEPSC amplitude and frequency, while females showed heightened plasticity at facilitatory synapses onto dopaminergic neurons mediated by endocannabinoids. Understanding the synaptic effects of acute fasting is crucial for procedures involving reward learning.

Literature regarding the natural effects of transient fasting, attributed to differences in fasting duration, food restriction levels (total vs. partial), and circadian timing show considerable difference.[17] Our fasting protocol aligns with models in previous studies, resulting in uniform physiological effects linked with transient fasting.[17] This protocol allowed water access during the mouse's dark phase and led to a 10% body weight reduction, attributed to both decreased body weight by 60% and emptying of the gastrointestinal tract by 40%.[20] The observed decline in blood glucose, likely from reduced gastrointestinal glucose absorption during fasting, and increased ketones align with prior findings.[17] Furthermore, our results replicate earlier work, demonstrating increased cortisone levels in starved female rodents unlike male rodents, supporting the reliability of our 16-hour overnight fasting model.[21]

Acute fasting led to a predominant increase in food approach behaviors in male mice, a response associated with elevated mEPSC frequency and the formation of facilitatory synapses onto dopaminergic neurons of VTA.[4] Female rodents exhibited heightened overall locomotor activity, increased entries into the dark zone, and elevated fasting-induced CORT levels, comparable to stressor-induced CORT induction.[22] Despite male mice showing a higher likelihood of engaging with food in the light-dark box test, female counterparts showed more intake than males in an hour following this assessment. This increased consumption in females may be influenced by higher CORT levels, known to drive food intake.[23] Consequently, fasting might pose a stronger physiological stress-induced in females, potentially contributing to decreased food intake throughout the assessment and increased homeostatic food intake afterward.

In male mice, we observed a rise in mEPSC amplitude and frequency post fasting. Typically linked to increased glutamate release, elevated mEPSC frequency was not accompanied by changes in the probability of release or the size of the readily releasable pool. Moreover, this surge in mEPSC frequency did not seem to result from an amplified AMPAR response amplitude, as indicated by the unchanged ratio of AMPAR to NMDAR and responses to Rubi-glutamate. Consequently, starving did not stimulate post-synaptic alterations in the function or number of AMPA receptors or a postsynaptic stimulation of glutamate-mediated synapses in the midbrain. This enhanced mEPSC frequency could also involve an augmentation in the density of the synaptic release regions, a factor that can be explored through immuno-electron microscopy of facilitatory synapses. The surge in ketone levels throughout starving, linked to heightened brain-derived neurotrophic factor (BDNF), might result in the formation of release sites. Previous research has associated rise in facilitatory synapse numbers onto dopaminergic neurons of VTA with enhanced food-seeking behavior, supporting the notion that this synaptic adaptation in male rodents may be due to heightened food foraging during fasting, irrespective of whether stimulated by food restriction or energy density.

The investigation delved into the repercussions of transient fasting on transmission of synapses within the VTA of both groups of mice. The fasting regimen triggered notable metabolic and behavioural alterations, with females displaying a more pronounced response. Specifically, females exhibited diminished blood glucose levels, elevated ketones, and heightened cortisol (CORT) levels compared to their male counterparts. In addition to increased locomotor activity in females, both genders demonstrated augmented food-seeking behaviour during the fasting period.

Upon closer scrutiny of synaptic transmission, distinctive effects emerged between males and females. Males displayed heightened excitatory synaptic strength following fasting, evidenced by increased mEPSC amplitude and frequency onto VTA dopamine neurons. In contrast, females showcased an augmentation in temporary plasticity at facilitatory synapses onto dopaminergic neurons mediated by endocannabinoid.

However, inhibitory synapses in the VTA remained unaffected by fasting in both genders. Notably, the study's findings underscore sex-specific responses to acute fasting, shedding light on potential variations in synaptic regulatory mechanisms.

Limitations

Limitations of this study include the focus on acute fasting, requiring further investigation into chronic fasting effects. Additionally, the study primarily explores

synaptic changes in the VTA, necessitating broader investigations to comprehend the full spectrum of fasting's impact on neural circuits and behaviour.

CONCLUSION

The study on the sex differences on the impact of acute fasting on inhibitory and excitatory synapses onto VTA dopaminergic neurons reveals that acute fasting induces sex-specific changes in metabolic parameters, food-seeking behaviours, and VTA synaptic properties. Males exhibit increased mEPSC frequency, indicating more release sites, while females show enhanced endocannabinoid-mediated depression at excitatory synapses. Inhibitory synapses remain unaffected. These gender-specific synaptic alterations correlate with variations in food-seeking, emphasizing the crucial role of VTA dopamine neurons. Fasting-induced synaptic plasticity may contribute to heightened food-seeking during food availability, potentially explaining challenges in sustaining weight loss. Understanding these sex-specific synaptic changes provides insights into gender biases in neuropsychiatric illnesses, such as major depressive disorder, anxiety, and anorexia nervosa.

Recommendations

This study recommends further exploration into the enduring impacts of fasting on synaptic plasticity and behaviour, emphasizing the need for longitudinal investigations. Moreover, it suggests examining the therapeutic potential of modulating VTA dopamine neuron function in the context of metabolic and mental health disorders.

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