DIETARY REGULATION OF SILENT SYNAPSES IN THE DORSOLATERAL STRIATUM

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ABSTRACT

Obesity results in circuit adaptations that closely resemble those induced by drugs of abuse. AMPA-lacking 'silent' synapses are critical in circuit generation during early development, but largely disappear by adulthood. Drugs of abuse increase silent synapses during adulthood and may facilitate the reorganization of brain circuits around drug-related experience, facilitating addiction and relapse Whether obesity causes addiction-related synaptic circuit reorganization via alterations in silent synapse expression has not been examined. Using a dietary-induced obesity paradigm, we show that mice that chronically consumed high-fat diet (HFD) exhibit upregulated silent synapses in both direct and indirect pathway medium spiny neurons in the dorsolateral striatum. Both the onset of silent synapses and their re-silencing after HFD withdrawal occur on an extended time scale of weeks rather than days. Our data suggest that HFD-related silent synapses likely arise from AMPA receptor internalization rather than through *de novo* synaptogenesis of NR2B-containing NMDA receptors. These data demonstrate that chronic consumption of high-fat diet can alter mechanisms of circuit plasticity, likely facilitating neural reorganization analogous to that observed with drugs of abuse.

INTRODUCTION

Obesity has increased in Western society in recent decades with a prevalence of ~42% in adults (Flegal et al., 2016). Most individuals who attempt to diet via caloric reduction tend to fail in the long term, with return to their original weight or rebounding to a higher weight (Dulloo et al., 1997; J. R. Speakman et al., 2011). This failure to maintain diet and weight loss may be caused by the compulsive need to overeat highly palatable food after a period of abstinence, similar to cravings that arise during drug abstinence in chronic drug users (Pickering et al., 2009; Sharma et al., 2013). Dopamine mediates craving and compulsive drug-seeking behavior, and has been suggested to similarly mediate overeating in obesity (Baik, 2013; Salamone, 2003; Seiler et al., 2022; Small et al., 2003; Volkow & Wise, 2005). Like drugs of abuse, palatable food activates the dopaminergic system and acts in a similarly reinforcing manner (Volkow et al., 2017), mediated through actions in reward associated reward areas such as the nucleus accumbens, hypothalamus, and prefrontal cortex (Briggs et al., 2010). The dorsal striatum, though less studied than the nucleus accumbens in appetitive motivation and addiction, has been associated with later, putatively habitual stages of addiction (Everitt & Robbins, 2013; Harada et al., 2021; Volkow et al., 2006) and is believed to be a primary substrate mediating craving during abstinence that later leads to relapse (Contreras-Rodríguez et al., 2017; Volkow et al., 2006).

The neural substrates and mechanisms mediating the persistent craving that drives relapse in drug abstinence and dieting is only partially understood. Work in the last decade has demonstrated that drugs of abuse upregulate silent synapses, activating a substrate for circuit plasticity and remodeling that allows motivational circuits to become reorganized around behaviors associated with drug-taking (Dong, 2016; Graziane et al., 2016a; Y. H. Huang et al., 2015). Silent synapses contain functional NMDA but not AMPA receptors (Hanse et al., 2013). Due to the magnesium block on NMDA at resting potentials, without AMPARs to depolarize the postsynaptic region in response to glutamate, NMDA cannot pass current and thus the synapse remains 'silent' in response to glutamate release. Prevalent in early development, silent synapses act as a substrate for synapse selection and circuit development but are minimally expressed in adulthood (Atwood & Wojtowicz, 1999; X. Huang et al., 2015; J. Isaac, 2003; J. T. R. Isaac et al., 1995). Drugs of abuse, such as cocaine, morphine, and chronic nicotine upregulate silent synapses in medium spiny neurons (MSNs) in a drug- and pathway- specific manner. Cocaine upregulates silent synapses in direct but not indirect pathway MSNs via de novo generation of new, NR2B-containing NMDA only synapses while opioids upregulate silent synapse in the indirect but not direct pathway via the removal of AMPARs from established synapses (Graziane et al., 2016a; Y. H. Huang et al., 2009a). Chronic nicotine increases silent synapses in the indirect pathway via *de novo* generation of NR2B-containing NMDA only synapses (Xia et al., 2017). Thus, different drugs induce alterations in

silent synapses, and presumably circuit plasticity, in different target neuronal populations and by different mechanisms, though the functional significance of these different patterns is poorly understood.

Evidence suggests that the un-silencing of silent synapses following drug withdrawal facilitates incubation of craving during abstinence, suggesting the possibility that these silent synapses effectively encode drug-related associations that are then strengthened when unsilenced (Ma et al., 2014, 2016). During continued drug abstinence, upregulated silent synapses normalize, returning to levels comparable to drug-naive mice; however, re-exposure to drug can induce a rapid return of increased silent synapses, specifically in neurons previously encoding drug-related experience (Koya et al., 2012). Some have theorized that the upregulation of silent synapses induced by drugs of abuse reflect a process by which such drugs reactivate early developmental processes to remodel circuits mediating memory around drug-related experience in a way that makes those memories more robust and resistant to change (Dong & Nestler, 2014).

Here we examined silent synapses in the DLS in response to high fat diet. We select the DLS because of its putative role in mediating craving during abstinence (Contreras-Rodríguez et al., 2017). Moreover, though the nucleus accumbens is commonly studied in both feeding and addiction research, the DLS plays a critical role in regulating feeding behavior (Small et al., 2003; Sotak et al., 2005). Indeed, in dopamine-deficient mice that do not feed themselves and will die unless administered L-DOPA, which facilitates feeding, genetic rescue of dopamine in the DLS but not the NAc can rescue feeding (Sotak et al., 2005). Here we fed mice high fat diet over several weeks and then used ex-vivo patch-clamp electrophysiology to investigate alterations in silent synapses in both direct- and indirect- pathway MSNs. In contrast to cocaine and opioids, which upregulate silent synapses in only one of these pathways (Graziane et al., 2016a; Y. H. Huang et al., 2009a), we find that obesity and HFD upregulate silent synapses in both pathways, though the time course of both emergence of upregulation and normalization on withdrawal of HFD are prolonged compared to drugs of abuse.

MATERIALS AND METHODS

Animals

Adult mice (10-11 weeks at start of experiment) of both sexes were used for all experiments. To identify D1-expressing medium spiny neurons (MSNs), mice with a floxed tdTomato (JAX strain: #007914) were crossed with mice expressing cre-recombinase under the D1-promoter (B6.Cg-Tg(Drd1a-cre)262Gsat/Mmcd). Mice used in experiments were hemizygous for both the cre and floxed tdTomato alleles (tdTomato$^{wt/fl}$; D1-cre$^{wt/cre}$). To identify D2-expressing MSNs, we used mice hemizygous for a

green fluorescent protein transgene under control of the Drd2 promoter (JAX strain: #020631). All lines were on a C57Bl/6J background. Mice were group housed in a 12h light/dark cycle facility. All experiments were approved by Queens College, CUNY, Institutional Animal Care and Use Committee in accordance with the National Institutes of Health (NIH) Guidelines for the responsible use of animals in research.

High Fat Diet Feeding

Under dietary induced obesity (DIO), mice received *ad libitum* access to high fat diet (HFD, 60% total calories from fat, Envigo, TD.06414,). HFD was administered for a minimum of 6 weeks prior to electrophysiology. In the time course experiments, mice received HFD for the periods indicated prior to electrophysiology. In the studies of renormalization after discontinuation of HFD, mice received 6 weeks of HFD before being switched to standard chow for HFD discontinuation.

Slice preparation

Mice were anesthetized using intraperitoneal injection of sodium pentobarbital (Euthasol) and decapitated. The brain was removed rapidly from the cranial cavity and placed into ice-cold (4 °C) artificial cerebrospinal fluid (ACSF), in mM: 25 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 1.25 NaH2PO4, 26 NaHCO3, 12.5 glucose, and 1 Na-ascorbate, and maintained at pH 7.4 by oxygenating with 95% O2/5% CO2. Coronal slices (300µm) containing dorsal striatum were obtained using VT1000 S vibratome (Leica Biosystems, Buffalo Grove, IL). Slices were transferred and incubated for at least 60 minutes at a holding temperature of 32-34 °C in ACSF and continuously bubbled with 95% O2/5% CO2. Slices were then maintained at room temperature (~27 °C) for the remainder of experiments.

Electrophysiology

Single hemisphere coronal slices (300 µm) were transferred post incubation into the recording chamber and perfused with oxygenated ACSF at a constant flow rate of 1.5-2mL/min. Temperature of ACSF was maintained at 30±1 °C using automatic temperature control (TC-324B Warner Instrument, Hamden, CT). Whole-cell patch clamp recordings from tdTomato labelled D1-expressing and EGFP-labeled D2-expressing MSNs were performed as previously described (Augustin et al., 2014). Recording pipettes with 4-8 MΩ resistance were filled with an internal solution containing, in mM: 20 CsMeSO3, 15 CsCl, 8 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 10 TEA, 5 QX-314, adjusted to pH 7.3 with CsOH. Picrotoxin (50 µM) was added to ACSF in all experiments to block $GABA_A$ receptor mediated synaptic currents.

Bipolar twisted tungsten matrix microelectrode (FH-Co, Bowdoin, ME) with 500µm tip separation was placed in the striatum, dorsolateral to the recording electrode location. Experiments were performed to establish the percentage of silent synapses in recorded neurons using the minimal stimulation assay, in which failure rates of EPSCs at -70 and +40 mV were compared. In brief, small (<40 pA) EPSCs were evoked at -70 mV (single pulse every 5 seconds), then stimulation intensity was reduced until stimulation response failures and successes could be clearly distinguished and a fail rate of approximately 50% was obtained. EPSCs are recorded at this stimulation intensity (i.e., produces 50% failure rate) across 50 trials at -70 mV before the holding potential is switched +40 mV and another 50 trials are recorded using the same stimulation intensity. Successes and failures were identified visually. The percentage of silent synapses is calculated comparing failures at -70mV to failures at +40mV using the formula: $1-\ln(\text{Failures}_{-70\,\text{mV}}) / \ln(\text{Failures}_{+40\,\text{mV}})$. Spontaneous excitatory post synaptic currents (EPSCs) were recorded using Multiclamp 700B amplifier at a holding potential of -70mV. Miniature EPSCs were recorded (5-min samples, gap-free recording) with picrotoxin (50 µM) and tetrodotoxin (10 µM) applied in bath.

For experiments reported in Fig.3, NMDA subunit specific currents were isolated in $Mg2^{++}$-free ACSF. Test pulses were applied every 20 seconds (plot created using 1 minute average of 3 pulses) at -70mV. Evoked responses were measured under 3 conditions: 1) Five-minute baseline in ACSF containing the AMPAR antagonist NBQX (5 µM) (0-5), 2) the addition of Ro25-6981 to selectively block NR2B (min 6-16), and 3) the addition of NVP-AAM077 (50 nM) to additionally block NR2A (min 17–26). NBQX was present throughout the entire recording.

Spine morphology/DiI labeling

Mice were anesthetized by intraperitoneal injection of Euthasol and perfused transcardially with PBS and 1.5% paraformaldehyde (PFA) in PBS. Brains were removed and postfixed in 1.5% PFA for one hour. Coronal slices of 150 um were made using a vibratome and collected in PBS solution. Fluorescent DiI (1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate ('DiI'); DiIC18(3)) was introduced into the tissue by ballistic delivery using the PDS-1000/He System, using 1,100 psi rupture discs (Bio-Rad, California, #1652329). Once the dye was introduced into the tissue the tissue was stored in the dark at 4°C for 24 hours to allow for the dye to diffuse through the neuronal membranes. DiI was prepared as follows:

1.5 mg of DiI was dissolved in 50 µl of methylene chloride. The solution was then poured on 12.5 mg of tungsten particles and allowed to dry. The dye was then dissolved in 3 ml of deionized water and

sonicated for 20 minutes. Afterwards one drop of the solution was added to the center of a microcarrier film and allowed to dry and then stored in the dark at 4°C.

Immunohistochemistry and confocal imaging

Immunohistochemistry (IHC) was performed after DiI labeling to relabel D2-expressing MSNs. To label D2-expressing cells, one of either two IHCs were performed: (1) chicken anti-GFP to enhance the endogenous expression of GFP in D2 neurons or (2) rabbit anti-D2R to label D2 neurons. Both IHCs used an Alexa Fluor 488 secondary antibody as well: (1) goat anti-chicken Alexa Fluor 488 or (2) donkey anti-rabbit Alexa Fluor 488. Tissue was permeabilized in .01% Triton-X with PBS for 15 minutes, blocked in .01 Triton-X and a 10% normal serum with PBS for 30 minutes, then incubated in the primary antibody at 1:1000 in blocking solution for 2 hours. Tissue was then washed 3 times for 10 minutes each in PBS. The secondary antibody was applied at 1:1000 in blocking solution followed by 4 10-minute washes in PBS. A confocal microscope (Olympus Fv10i) was used to image neurons. Imaging was done with Alexa Fluor 488 (Excitation 495 nm; Emission 519 nm) and DiI (Excitation 549 nm; Emission 565) filters using 60x oil immersion lens.

Dendritic Spine Analysis

Secondary and tertiary spine segments between 60-90 um were sampled as primary dendritic branches contain sparse amounts of spines. A maximum sampling of 3 branches were taken from each labeled cell. Spines were classified as follows: (1) stubby spines: less than 1 µM, no neck protrusion from dendrite (2) mushroom spines: between 1-2 µM, with neck and head (3) thin spines: length between 2-3 µM with neck and head (4) filopodia: neck greater than 3 µM, no head. Analysis was completed using Neurolucida. One-way ANOVAs were used to compare differences in spine density and type.

RESULTS

Silent synapses are upregulated in the dorsolateral striatum under chronic high-fat diet.

Mice were placed on a high-fat diet for a minimum of 6 weeks before electrophysiology testing. In a preliminary study, we demonstrate significant weight gain at 6 weeks (Fig 1A, $F_{(1,26)}$ =34.37, p<.01), consistent with prior reports in mice that show that 5-6 weeks of high-fat diet induces significant weight gain and alters metabolic regulation (Krishna et al., 2016). Silent synapses were assessed using the minimal stimulation assay targeting the dorsolateral striatum. To identify direct and indirect pathway medium spiny neurons (MSNs), we used mice expressing red fluorescent protein in D1-expressing MSNs (tdTomato x D1-Cre) or a green fluorescent protein under the Drd2 promotor (D2-EGFP).

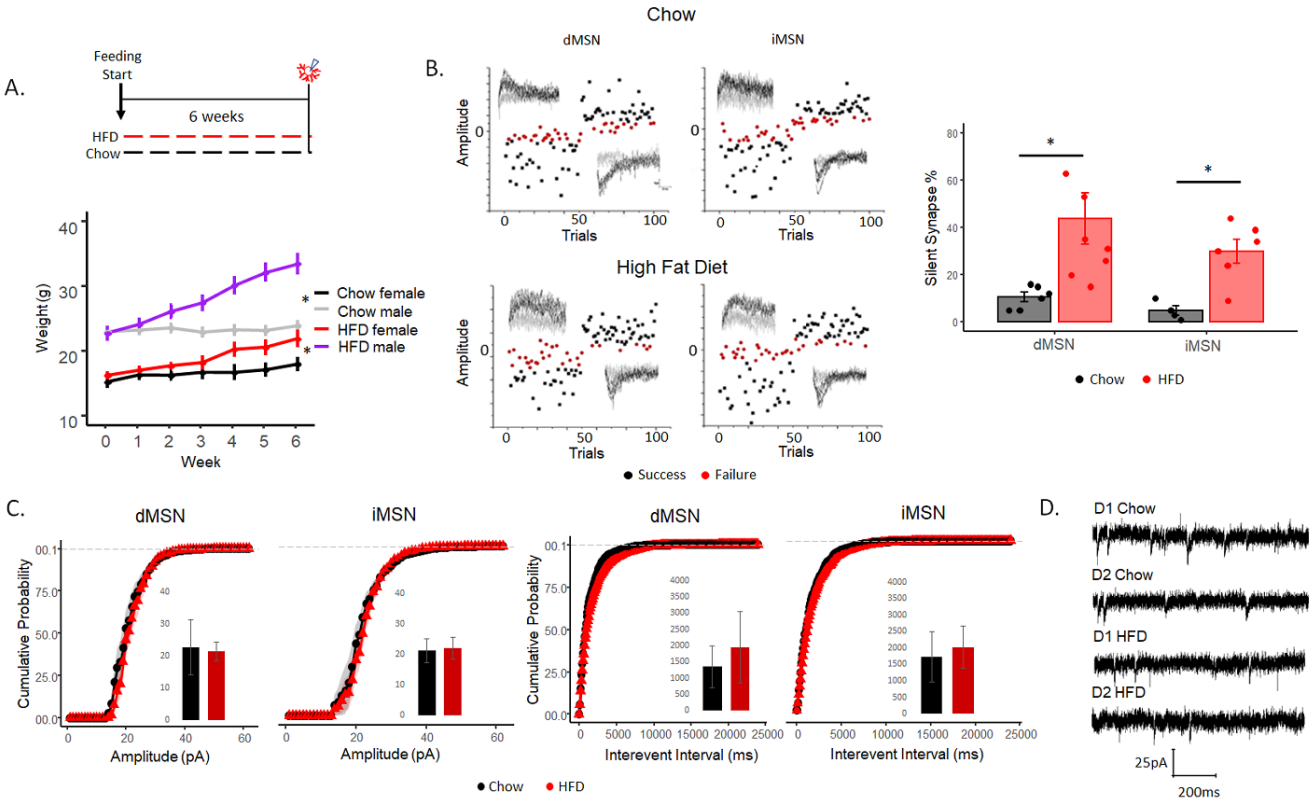

Fig 1. Silent synapses are upregulated in the dorsolateral striatum under chronic high-fat diet. A) Feeding paradigm for chow and HFD mice (top) and weight changes over time (bottom). B) Minimal stimulation assay (left) and the percentage of silent synapses (right) of dMSNs and iMSNs in chow and HFD-fed mice. C) Cumulative probability of amplitude (pA) (left) and interevent interval (ms) (right) in dMSN and iMSNs. D) Example traces of gap-free mEPSCs with 50 µM picrotoxin and 10 µM TTX. Data are presented as mean±SEM. *p< 0.05.

Silent synapses were elevated were increased in mice on high-fat diet compared to controls (Fig. 1B, $F_{(1,22)}$=13.84, p<.01) in both direct- (Fig. 1B, $F_{(1,12)}$ = 6.89, p<.05) and indirect- (Fig. 1B, $F_{(1,8)}$ = 15.02, p<.05) pathway MSNs with no sex differences observed (data not shown, $F_{(1,20)}$ = .21, p=.64). We measured miniature EPSCs in gap-free recordings to assess pre- and postsynaptic changes in synaptic transmission of AMPA-containing (i.e., not silent) synapses at -70mV. High-fat diet did not alter the frequency of miniature EPSCs in the direct (Fig 1D-E, $F_{(1,16)}$=.873, p=.363) or indirect ($F_{(1,13)}$=.443, p=.52) pathways, suggesting no changes in the presynaptic probability of release. Amplitude was not altered in either the direct (Fig 1D-E, $F_{(1,16)}$= .26, p=.61) or indirect ($F_{(1,13)}$= .06, p=.8,) pathway, suggesting no alteration in postsynaptic glutamate response among established AMPA-containing synapses. These data suggest that HFD increases NMDA-only silent synapses without altering presynaptic glutamate release or the strength of transmission in existing non-silent synapses.

Upregulation of silent synapses under high fat diet is delayed compared to drugs of abuse.

Cocaine and morphine induce upregulated silent synapses in MSNs within 5-7 days of once daily injections and, upon drug discontinuation, this upregulation normalizes to baseline (5-15% silent synapses) within 7 days of the last injection (Graziane et al., 2016). We assessed the onset of silent synapse upregulation under high-fat diet by assessing silent synapses after 1, 2, and 4 weeks of HFD. There was no significant difference between high-fat diet fed mice and controls across weeks 1-4 of HFD exposure in either pathway (Fig. 2A, 1 week: $F_{(1,8)}$=.073, p=.79, 2 weeks: $F_{(1,8)}$=2.69, p=.14, 4 weeks: $F_{(1,8)}$=.038, p=.85). These data suggest the underlying mechanism mediating the increase in silent synapses operates on a slower timescale, reflecting more long-term adaptations.

We assessed silent synapses at 1-, 2-, and 4-weeks following withdrawal from high-fat diet, observing a significant effect of time from withdrawal (dMSN: $F_{(4,23)}$=3.39, p<.05; iMSN: $F_{(4,17)}$=4.44, p<.05, Fig.2B). In post hoc comparisons examining the significance of differences between timepoints, neither the 1- or 2-week timepoint were significantly different from silent synapses in mice on HFD in either the direct (1 week: $F_{(1,10)}$=.82, p=.38; 2 week: $F_{(1,12)}$=1.25, p=.28) or indirect (1 week: $F_{(1,9)}$=.004, p=.94; 2 week: $F_{(1,8)}$=1.10, p=.32) pathway. By 4-weeks post withdrawal, silent synapses of mice withdrawn from high fat diet were not significantly different from chow-fed controls in both the direct or indirect pathway medium spiny neurons (Fig.2B, direct: $F_{(1,9)}$=.239, p=.63; indirect: $F_{(1,5)}$=.001, p=.98, respectively), indicating normalization to baseline similar to mice never exposed to HFD occurs approximately four weeks after discontinuation of HFD.

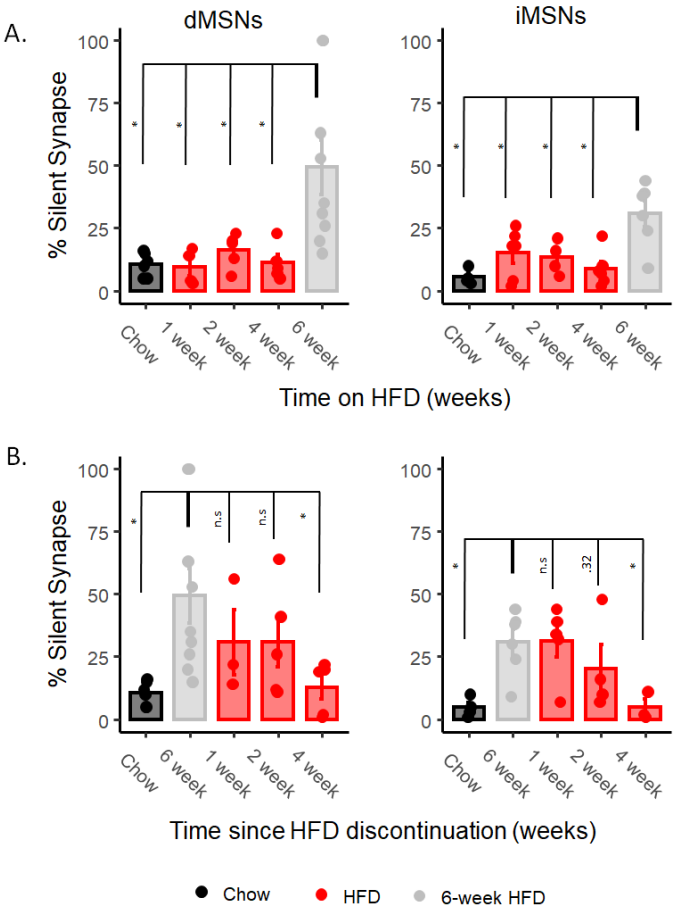

Fig 2. Upregulation of silent synapses under high fat diet is delayed compared to drugs of abuse. A) The onset of silent synapses after starting high fat diet feeding at 1 week, 2 weeks, and 4 weeks using minimal stimulation assay compared to 6 week data from Fig 1(gray bars). B) the percentage of silent synapses after withdrawal from high fat diet at 1 week, 2 weeks, and 4 weeks as measured by minimal stimulation compared statistically to 6 weeks of HFD feeding. Data are presented as mean ±SEM. *p< 0.05.

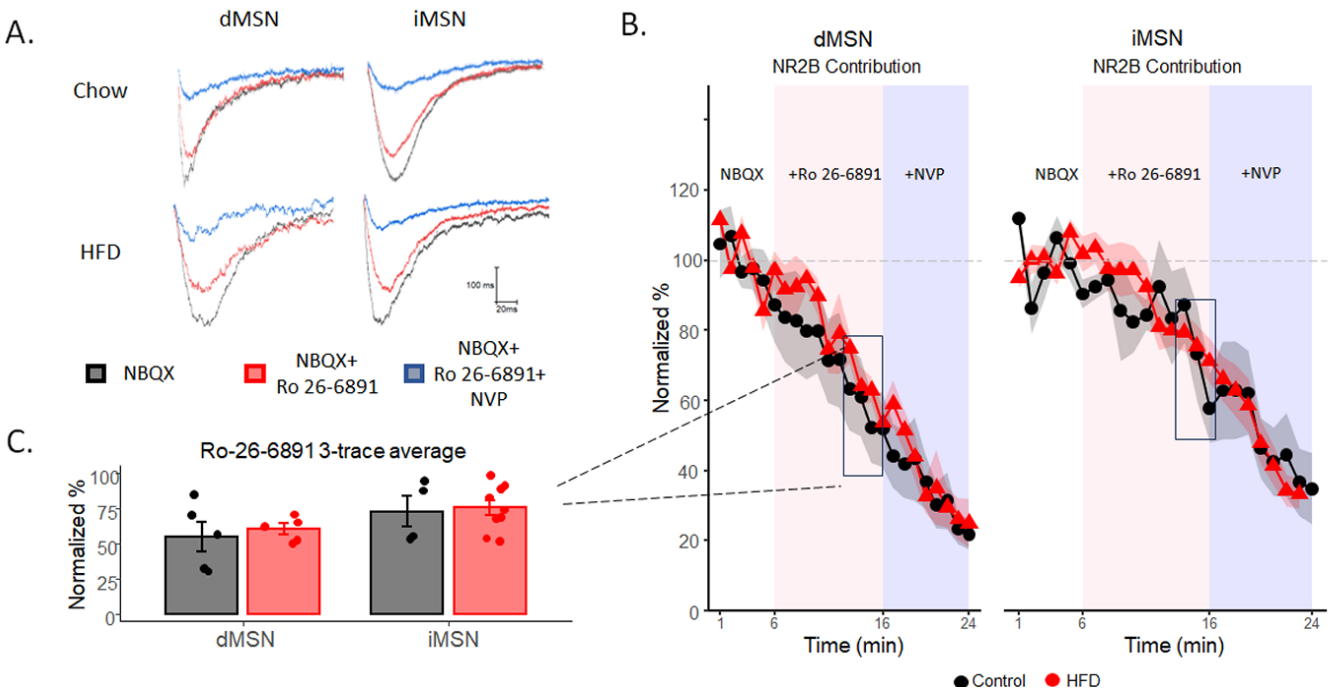

Fig 3. No increase in NR2B subunit prevalence is associated with upregulation of silent synapses. A) Example traces of stimulated EPSCs with the application of NBQX (black), NBQX and NR2B-subunit antagonist Ro 26-6891 (red), and NBQX, Ro 26-6891, and NR2A-subunit antagonist NVP (blue). B) Normalized percent change of NR2B contribution over time. C) The normalized percent at the end of Ro 26-6891 application (the average of 3 minutes) before the application of NVP. Data are presented as mean±SEM. *p< 0.05.

No increase in NR2B subunit prevalence is associated with upregulation of silent synapses.

Increased silent synapses induced by drugs of abuse can arise from two different mechanisms. Cocaine induces increased silent synapses via putative *do novo* generation of new NR2B-subunit containing synapses, as suggested by increased prevalence in NR2B compared to control animals. In contrast, morphine does not induce increased NR2B prevalence but increases silent synapses in the indirect pathway via internalization of AMPA receptors from existing, established synapses (Graziane et al., 2016).

We assessed the prevalence of NR2B-subunit containing NMDA receptors in HFD mice using Ro25-6981, an NMDA antagonist selective for NR2B-containing NMDA receptors. We isolated NMDA current by applying NBQX and picrotoxin to block AMPARs and GABA activity in a Mg++-free bath ACSF and applied test pulses every 20s over 5 minutes to establish a baseline of NMDA EPSC response. We then applied Ro25-6981 to selectively block NR2B-containing NMDA currents and recorded for 10 minutes. If there is an increased prevalence of NR2B-containing NMDA receptors, as associated with de novo generation of new silent synapses, then Ro-25-6981 will incur a greater decrease in currents in HFD animals. We observe no difference in the effect of Ro-25-6981 in NMDA currents in either pathway (Fig 3B-C, calculated from the averages of the final 3 traces (1 min) before application of NVP; direct: $F_{1,8}$=.21,

p=.65; indirect: $F_{1,11}$=.06, *p*=.81, respectively), suggesting no increase in NR2B-containing NMDA, arguing against increased silent synapses through *de novo* generation of new (silent) synapses as a primary mechanism of HFD induced increases in silent synapses. Finally, we applied NVP-AAM077, an NR2A blocker, to block all NMDA currents and again observed no significant difference between HFD mice and control mice in either pathway (Fig 3B, direct: $F_{1,8}$=.12, *p*=.74; indirect: $F_{1,11}$=.14, *p*=.72, respectively). This lack of increased NR2B in HFD mice suggests that the upregulation of silent synapses HFD is likely due to the internalization of AMPA receptors in existing synapses, converting established synapses to silent synapses.

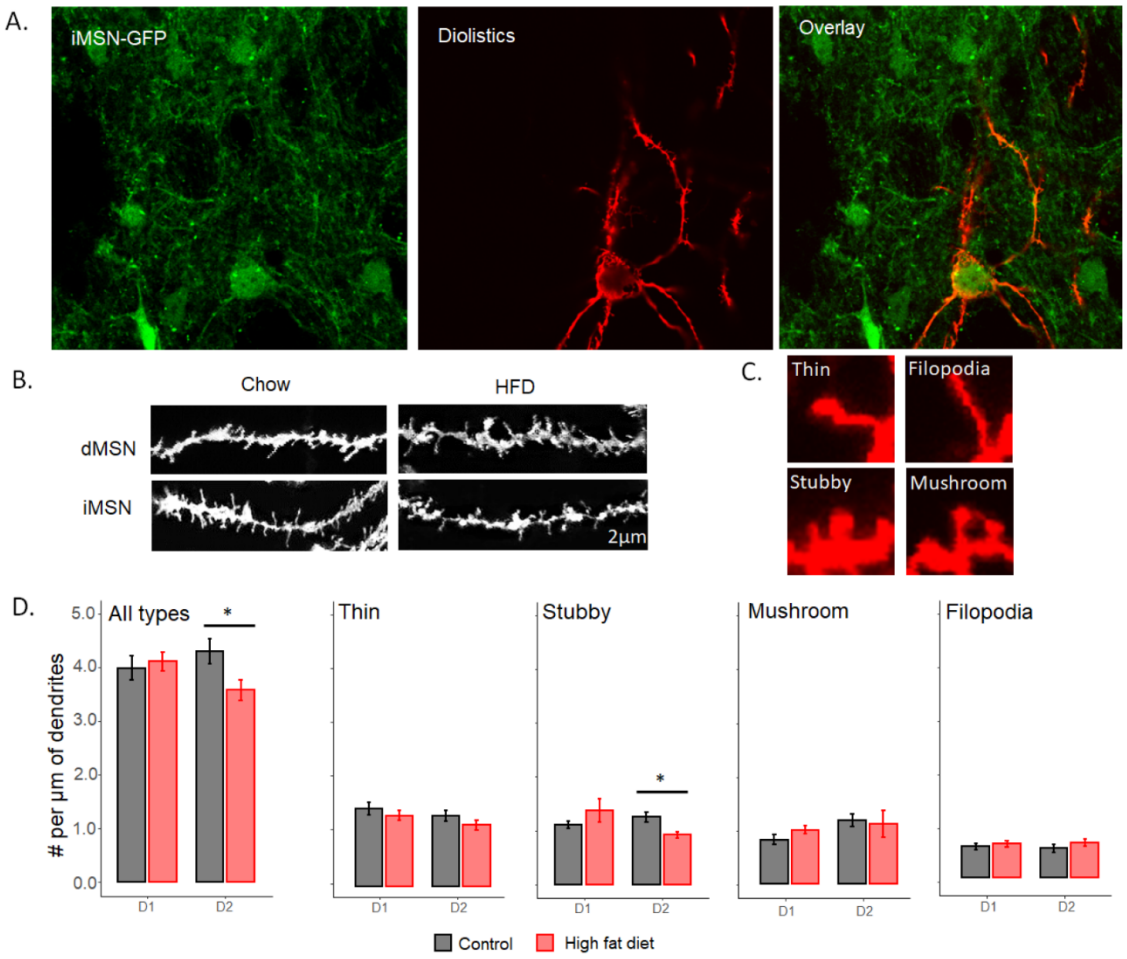

Fig 4. HFD reduces stubby dendritic spines in the indirect pathway. A) Representative images of iMSN-GFP neurons (left), DiI only (middle), and the overlap of iMSN-GFP and DiI labelling (right). B) Representative images of dendritic branch in dMSNs and iMSNs. C) Representative examples of different spine subtypes. D) Number of spines per µm of sampled dendritic branch including all spine subtypes (left) and broken down by individual spine subtype (right 4 graphs). Data are presented as mean±SEM. *p< 0.05.

HFD reduces stubby dendritic spines in the indirect pathway.

The plasticity of dendritic spines plays a role in the shaping of neural circuitry during development and can be further altered during adulthood in an experience-dependent manner (Zito et al., 2010). Both exposure to HFD and withdrawal from drugs of abuse can alter spine density and morphology across brain region in a time- and region-dependent manner (Graziane et al., 2016a; Saiyasit et al., 2020).

We examined dendritic spines in the dorsolateral striatum, comparing these in mice fed a HFD with chow-fed controls using diolistic imaging. DiO (red-dye) was incorporated into tissue of transgenic mice expressing green fluorescence under the Drd2 promotor (D2-GFP) to identify direct vs indirect pathway MSNs. Mice fed high-fat diet for a minimum of six weeks showed a significant reduction in overall spine density (Fig. 7D left, $F_{1,128}$=4.17, p<.043) in iMSN ($F_{1,58}$=5.81, p<.05), but not dMSNs ($F_{1,70}$=.18, p=.66). This density decrease in the indirect pathway is attributable specifically to the loss of stubby spines (Fig. 7D, $F_{1,58}$=10.19, p<.05), with no significant change in spine density in either thin-long spines, mushroom spines, or filopodia in either pathway. Thin-long and filopodia are highly labile and dynamic, whereas stubby and mushroom spines are more stable and mature. Changes in stubby spines can persist over longer periods of time, such as weeks compared to thin-long and filopodia. This decrease in stubby spines in iMSNs is consistent with the loss of mature, previously established synapses (Holtmaat et al., 2005).

DISCUSSION

Our study examined whether chronic high fat diet induces changes in silent synapse prevalence as observed with drugs of abuse. We find that high fat diet increases silent synapses in both direct and indirect pathway medium spiny neurons, in contrast to pathway selective effects of cocaine (D1R-expressing dMSNs) and morphine (D2R-expressing iMSNs). That silent synapses are increased in both pathways may indicate pervasive circuit remodeling that contributes to long-term compulsive behavior associated with energy dense, highly palatable food in obesity. In drugs of abuse, the modification of gene expression contributes to circuit remodeling (Bali & Kenny, 2019) and downregulation of the D2 receptor function is believed to be critical for drug mediated plasticity (Madhavan et al., 2013). Previous studies show that long-term consumption of HFD leads to alterations in both pathways, including decreased D1 receptor gene expression in the NAcc (Alsiö et al., 2010) and downregulation of D2 receptors (Johnson & Kenny, 2010), which may contribute to the circuit remodeling in obesity similarly to that observed in drugs of abuse.

Our examination of presynaptic and postsynaptic transmission in dMSNs and iMSNs after six weeks of HFD feeding showed no alteration in amplitude or interevent interval, consistent with prior studies that similarly lack of difference in mEPSCs between chow and HFD-fed mice. However, further examination found a slower decay constant for EPSCs in the DLS of HFD-fed mice (Fritz et al., 2018), suggesting a dysregulation in the glutamate transporter (GLT-1), as observed in substance abuse disorders (Roberts-Wolfe & Kalivas, 2015). Previous research has found that the AMPAR/NMDAR current ratio is reduced in the DLS during long-term high-fat diet feeding, though whether this is due to possible alterations in AMPA receptor composition or a reduction in NMDA receptor function is not known (Fritz et al., 2018). Our experiment showed that the application of NR2B antagonist Ro-26-6891 did not elicit a significant reduction in NR2B-subunit specific NMDAR contribution, suggesting that the mechanism by which high-fat diet upregulates silent synapses is not the de novo genesis of NR2B-subunit containing NMDA receptors but likely via the internalization of AMPA receptors.

Silent synapse upregulation by HFD takes longer to emerge compared to upregulation observed in response to drugs of abuse. Cocaine and morphine upregulate silent synapses between 5-7 days with daily use (Graziane et al., 2016b) and chronic nicotine exposure increases silent synapses in 21 days (Xia et al., 2017), possibly sooner. In the DLS, silent synapses were increased at 6 weeks of high-fat consumption. This delayed time course may suggest that the changes in silent synapses may be mediated by slowly emerging metabolic changes and adaptations to HFD, including incipient metabolic dysregulation such as developing insulin resistance. Consistent with this possibility, four weeks of high-fat diet feeding can cause the development of hyperglycemia, which is associated with increased fasting insulin levels (Wang & Liao, 2012). Insulin resistance in the brain can impair control over energy metabolism and can further increase the accumulation of fat (Arnold et al., 2014; Kahn & Flier, 2000), but whether increased adiposity is needed for central insulin resistance is still a topic of debate. In early research using cell culture to test whether insulin can affect developmental regulation of silent synapses, incubation of thalamocortical cocultures with insulin during cultivation led to increases in AMPAR EPSC success rate, suggesting that insulin may modulate the maturation of silent synapses to functional synapses (Plitzko et al., 2001). Where some studies find that consumption of HFD can induce central insulin resistance without weight gain (Clegg et al., 2011), others find that increased adiposity is necessary for central insulin resistance (Kahn & Flier, 2000; Kullmann et al., 2012, 2020). In mice, gradual weight gain can be seen around two weeks of high fat diet feeding, most noticeable after 4 weeks (Lutz & Woods, 2012; J. Speakman et al., 2007), and with full manifestation of obesity correlated with downstream physiological effects at 16 weeks with an increase of 20-30% of baseline body weight (Inui, 2003). Insulin resistant mice that had undergone 6 weeks of HFD feedings also experienced impaired

dopamine function, specifically a reduction in reuptake. This effect on dopamine is most likely due to downstream insulin signaling alterations associated with resistance, as blocking that signaling in chow fed mice also leads to reduced reuptake, whereas restoring insulin signaling in HFD fed mice improved reuptake deficits (Fordahl & Jones, 2017).

These factors may also play a role in the normalization of silent synapses following cessation of high fat diet feeding. In both cocaine and morphine, normalization of silent synapses occurs ~7 days after the cession of drug injections (Graziane et al., 2016b; Y. H. Huang et al., 2009b). In our data, we see that silent synapses were still upregulated at 14 days after the switch to chow diet, implying that the slow metabolic changes that putatively effected the initial upregulation of silent synapses may also play a part in their normalization. Weight loss is shown to improve insulin sensitivity and possibly reverse insulin resistance (Begg et al., 2013; Clamp et al., 2017; Oakes et al., 1997). Though insulin seems to induce the maturation of silent synapses in early development in culture (Plitzko et al., 2001), whether insulin resistance affects the upregulation and normalization of silent synapses during adulthood is unknown.

Silent synapses have been associated with changes in spine density and morphology. In studies on drugs of abuse, cocaine causes an increase in spine density (Graziane et al., 2016b) whereas morphine causes a decrease in spine density (Robinson et al., 2002) in the NAc. These observations are consistent with the *de novo* addition of new synapses in the direct pathway or the removal of AMPARs from established synapses—silencing them, suggested to be a precursor of synapse elimination—in the indirect pathway. Alterations in spine density have been studied less in the context of HFD consumption. Previous studies in the NAc show that high fat diet consumption did not alter spine density after 3 weeks, but did reduce spine density in the prefrontal cortex (Dingess et al., 2017). In the hippocampus, HFD causes a decrease in dendritic spine density (Hao et al., 2016). Though spine alterations have been previously studied in areas such as the NAc, prefrontal cortex, and hippocampus, the DLS has not been fully explored. In our studies examining spine density in the DLS, we find that 6 weeks of high fat diet consumption led to a decrease in spine density in indirect pathway MSNs, primarily due to the loss of stubby spines.

Spine type plays a significant role in addiction related plasticity and glutamatergic responses, specifically changes in spine type can lead to associated changes in strength of synaptic response (Runge et al., 2020). Thin- and filopodia type spines are considered more labile and have smaller responses compared to the more stable stubby- and mushroom-type spines. Stubby spines are less dynamic than filopodia and thin-type spines and alterations in stubby spines typically have a longer time course, weeks compared to minutes (Holtmaat et al., 2005). Mushroom type spines, which are considered the most

stable have stability in the time course of months (Matsuzaki et al., 2004). Drugs of abuse, which elicit silent synapses in a matter of days, primarily find relatively fast alterations in thin-type and filopodia spines (Graziane et al., 2016b), where we find no significant changes in these spine types under HFD. In contrast, we find reduced stubby spines, consistent with the extended timescale of weeks vs. days in our study and also consistent with the alterations in and loss of established synapses. Given the slower timescale of mushroom type spines, 6 weeks of HFD exposure may be insufficient to alter these. Whether we would observe loss of mushroom spines of more extended exposure to HFD, or for that matter whether exposure to drugs of abuse over longer periods than a few days could affect more stable stubby and mushroom spines, remains an open question. Our finding that this loss of stubby spines only occurs in the indirect pathway is of interest. In animal models of Parkinson's disease (PD), striatal dopamine loss is associated with decreased glutamatergic afferents and loss of spine density specifically in the indirect pathway (Gagnon et al., 2017), though other research suggests that direct pathway spinal pruning may be time dependent with loss of spines occurring 2 months post lesion in the direct pathway, but not at 1 month (Graves & Surmeier, 2019).

Our findings suggest that chronic consumption of high fat diet can, similarly to drugs of abuse, induce an upregulation of silent synapses that might provide a mechanism by which energy-dense, highly palatable may reorganize neural circuits to generate compulsive, addiction-like behavior around eating, though this might arise primarily through the loss of established synapses. Future work might examine the relationship between metabolic changes and altered regulation of silent synapses and the role these alterations may play in mediating a potential incubation of food craving caused by dieting.

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FIGURE LEGENDS

Fig 1. Silent synapses are upregulated in the dorsolateral striatum under chronic high-fat diet. A) Feeding paradigm for chow and HFD mice (top) and weight changes over time (bottom). B) Minimal stimulation assay (left) and the percentage of silent synapses (right) of dMSNs and iMSNs in chow and HFD-fed mice. C) Cumulative probability of amplitude (pA) (left) and interevent interval (ms) (right) in dMSN and iMSNs. D) Example traces of gap-free mEPSCs with 50 µM picrotoxin and 10 µM TTX. Data are presented as mean±SEM. *$p < 0.05$.

Fig 2. Upregulation of silent synapses under high fat diet is delayed compared to drugs of abuse. A) The onset of silent synapses after starting high fat diet feeding at 1 week, 2 weeks, and 4 weeks using minimal stimulation assay compared to 6 week data from Fig 1(gray bars). B) the percentage of silent synapses after withdrawal from high fat diet at 1 week, 2 weeks, and 4 weeks as measured by minimal stimulation compared statistically to 6 weeks of HFD feeding. Data are presented as mean ±SEM. *$p< 0.05$.

Fig 3. No increase in NR2B subunit prevalence is associated with upregulation of silent synapses. A) Example traces of stimulated EPSCs with the application of NBQX (black), NBQX and NR2B-subunit antagonist Ro 26-6891 (red), and NBQX, Ro 26-6891, and NR2A-subunit antagonist NVP (blue). B) Normalized percent change of NR2B contribution over time. C) The normalized percent at the end of Ro 26-6891 application (the average of 3 minutes) before the application of NVP. Data are presented as mean±SEM. *$p< 0.05$.

Fig 4. HFD reduces stubby dendritic spines in the indirect pathway. A) Representative images of iMSN-GFP neurons (left), DiI only (middle), and the overlap of iMSN-GFP and DiI labelling (right). B) Representative images of dendritic branch in dMSNs and iMSNs. C) Representative examples of different spine subtypes. D) Number of spines per µm of sampled dendritic branch including all spine subtypes (left) and broken down by individual spine subtype (right 4 graphs). Data are presented as mean±SEM. *$p< 0.05$.