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High Fat Diet Augments Amphetamine Sensitization in Mice: Role of Feeding Pattern, Obesity, and Dopamine Terminal Changes

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Abstract

High fat (HF) diet-induced obesity has been shown to augment behavioral responses to psychostimulants that target the dopamine system. The purpose of this study was to characterize dopamine terminal changes induced by a HF diet that correspond with enhanced locomotor sensitization to amphetamine. C57BL/6J mice had limited (2hr 3d/week) or extended (24h 7d/week) access to a HF diet or standard chow for six weeks. Mice were then repeatedly exposed to amphetamine (AMPH), and their locomotor responses to an amphetamine challenge were measured. Fast scan cyclic voltammetry was used to identify changes in dopamine terminal function after AMPH exposure. Exposure to a HF diet reduced dopamine uptake and increased locomotor responses to acute, high-dose AMPH administration compared to chow fed mice. Microdialysis showed elevated extracellular dopamine in the nucleus accumbens (NAc) coincided with enhanced locomotion after acute AMPH in HF-fed mice. All mice exhibited locomotor sensitization to amphetamine, but both extended and limited access to a HF diet augmented this response. Neither HF-fed group showed the robust amphetamine sensitization-induced increases in dopamine release, reuptake, and amphetamine potency observed in chow fed animals. However, the potency of amphetamine as an uptake inhibitor was significantly elevated after sensitization in mice with extended (but not limited) access to HF. Conversely, after amphetamine sensitization, mice with limited (but not extended) access to HF displayed reduced autoreceptor sensitivity to the D₂/D₃ agonist quinpirole. Additionally, we observed reduced membrane dopamine transporter (DAT) levels after HF, and a shift in DAT localization to the cytosol was detected with limited access to HF. This study showed that different patterns of HF exposure produced distinct dopamine terminal adaptations to repeated AMPH, which differed from chow fed mice, and enhanced sensitization to AMPH. Locomotor sensitization in chow fed mice coincided with elevated DAT function and increased AMPH potency; however, the enhanced behavioral response to AMPH after HF exposure was unique in that it coincided with reduced DAT function and diet pattern-specific adaptations.

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1. Introduction

The increasing prevalence of obesity over the past few decades has prompted efforts to better understand the control of food intake, and has sparked debate as to whether over-consumption of food should be considered an addictive disorder. Evolutionarily, natural rewards such as food motivated humans to seek caloric sources out of necessity; however, now that food availability is at an all-time high, the palatability of food has become a primary motivator of consumption. Sweetened high fat (HF) foods are of particular interest because their consumption has been shown to activate reward circuitry in the brain, leading to increased dopamine release in the ventral striatum (Valdivia et al., 2014) in a manner similar to drugs such as cocaine and amphetamine (Volkow et al., 2009). Intake of highly palatable food in rodents has produced behavioral phenotypes that resemble psychostimulant abuse, namely reward deficiency (Johnson and Kenny, 2010) and increased sensitivity to dopamine receptor agonists (Baladi et al., 2012a for review). Recent studies have identified attenuated dopamine D₂ receptor binding in humans after prolonged consumption of high fat foods which resemble changes observed after chronic drug use (Volkow and Wise, 2005). Additionally, individuals seeking treatment for weight management are commonly prescribed pharmacotherapies that target the dopamine transporter (DAT), highlighting the involvement of the dopamine system in feeding and food seeking behaviors.

Mesolimbic dopamine signaling contributes to the reinforcing efficacy of psychostimulants (Baik, 2013), and elevated dopamine in the nucleus accumbens (NAc) is a neurochemical hallmark of all abused substances, including food (Wang et al., 2009, 2011). Adaptations in the dopamine system following drug abuse are well documented by neuroimaging studies in humans showing reduced D₂ receptor binding (Volkow et al., 1990) and reduced responsiveness to abused drugs (Volkow et al., 1997). Similar changes have been reported in clinical neuroimaging studies with obese patients, suggesting that they exhibit a comparable dopaminergic phenotype to drug addicts (Wang et al., 2009; Volkow and Wise, 2005). Obese individuals display significantly less D₂ binding than normal weight healthy subjects (Wang et al., 2001) and show reduced striatal activation during the consumption of highly palatable food (Stice et al., 2008). Imaging data provide valuable insight into functional changes that occur in the dopamine system with obesity, but the mechanisms that initiate these changes are not well understood.

Several rodent models of diet-induced obesity have been used to characterize dopaminergic changes produced by excessive food intake. Rats fed a HF-high sugar diet for five weeks had reduced dopamine D₁ and D₂ receptor gene expression in the NAc, an effect that persisted up to 18 days after removal of the diet (Alsiö et al., 2010). Similar reductions in D₁ and D₂ gene expression have also been reported in mice after twelve weeks of access to HF (Carlin et al., 2013). Interestingly, increased D₂ receptor binding and decreased DAT protein have been shown after short term (20 day) HF consumption (South and Huang, 2008); however, a marked decrease in ³H-raclopride binding to D₂ receptors and reduced membrane DAT levels were observed in rats given a HF diet for eight weeks (Narayanaswami et al., 2013). Moreover, dopamine reuptake in the NAc was attenuated after six, but not two, weeks of consumption of a HF diet in rats (Cone et al., 2013). Overall these data suggest that the

length of HF exposure contributes to the severity of dopamine system impairment, and that dopaminergic dysfunction persists beyond the removal of an obesogenic diet.

The length of exposure to a HF diet magnifies its impact on the dopaminergic system, but several studies suggest that the temporal pattern of HF diet availability contributes to HF reward salience. For example, rats with restricted access to a HF diet (2hrs/day) show a binge-like escalation in food intake, which did not engender obesity, compared with rats that have continuous access to the same diet (Corwin et al., 1998). Additionally, intermittent access to HF (1hr, 3d/week) increased progressive ratio responding for HF rewards compared with rats that received HF food daily (Wojnicki et al., 2010), indicating that the reinforcing efficacy of HF is partially driven by the pattern of HF diet availability. Intermittent access to HF resembles behavioral outcomes observed with intermittent access drug and alcohol paradigms that show escalated alcohol intake (Rosenwasser et al., 2013) and increased reinforcing efficacy of cocaine (Zimmer et al., 2012). Intermittent paradigms have also been shown to engender sensitization to the effects of cocaine or AMPH at dopamine terminals, and produce cross-sensitization to other drugs (Calipari et al., 2014).

The impact of a HF diet on reward-related behaviors has been examined both independently and in conjunction with abused drugs. Extended access to a highly palatable “cafeteria” diet (e.g. chips, cookies, and other calorie dense convenience foods) increased brain stimulation reward thresholds, suggesting a decrease in reward sensitivity, or anhedonia (Johnson and Kenny, 2010). The study by Johnson and Kenny found that elevated reward thresholds persisted for two weeks after the rats returned to normal chow, and that chow fed rats demonstrated an immediate increase in reward thresholds after D₂ receptor knock down, implicating reductions in D₂ receptor sensitivity in this response. Rats consuming a HF diet also showed greater behavioral sensitivity to repeated methamphetamine administration (McGuire et al., 2011) and increased locomotor activity after cocaine injections compared to controls (Baladi et al., 2012b, 2015; Serafine et al., 2015). Given the resemblance between the consequences of HF diet and drug abuse on the dopamine system, it is possible that consumption of a HF diet may influence the actions of psychostimulants by altering dopamine terminal function. Moreover, the consumption of a HF diet disrupts the anorexigenic hormones insulin and leptin, which have been shown to modulate dopamine neuron function (Kleinridders et al., 2015; Thompson and Borgland, 2013). Therefore, we also sought to identify if changes in anorexigenic hormones corresponded with dopamine system changes. The overall purpose of this study was to characterize dopamine terminal changes after unrestricted or limited access to a HF diet, and to evaluate dopamine terminal plasticity in the NAc of HF-fed mice after exposure to AMPH. We hypothesized that 1) access to a HF diet would enhance behavioral sensitization to AMPH by reducing dopamine reuptake and reducing D₂ autoreceptor function, and 2) the amount of exposure to HF would influence the magnitude of dopamine terminal changes.

2. Methods

2.1. Animals and Diet

C57BL/6 mice originally purchased from Jackson Laboratories (Bar Harbor, ME) were bred in house to produce six-week-old male mice for all experiments. Two groups of mice (n=16

each) were pair housed with free access to water and either standard lab chow (3.46 kcal/g) (5P00 – Prolab RMH 3000; LabDiet, St. Louis, MO) or a high fat (HF) diet (5.24 kcal/g) (DIO - D12492; Research Diets, New Brunswick, NJ) containing 14% and 60% total kcal from fat, respectively. A third group (n=13) had free access to chow, but had limited access (LimA) to the HF diet for 2h/day on M, W, and F. After six weeks of the dietary protocol half of the mice from each group went to slice voltammetry experiments (Chow, HF, and LimA), while the other half underwent locomotor testing for AMPH sensitization before proceeding to voltametric analysis (Chow (AMPH), HF (AMPH), and LimA (AMPH)). All experiments were in compliance with the Wake Forest Animal Care and Use Committee and the National Institutes of Health guide for the care and use of Laboratory animals.

2.2. AMPH Sensitization

Locomotor experiments were initiated during the first hour of the light cycle, and were conducted using Med Associates mouse locomotor chambers (20 cm × 20 cm × 20 cm; Med Associates, St. Albans, VT) with SOF-811 Activity Monitor (v6.05) analysis software. Overall activity was recorded, and the total distance traveled (cm) was used to assess the behavioral response to AMPH. Mice were allowed to habituate to the chambers for 40 minutes, at which point they were given saline (ip), and baseline activity was recorded. At the 80 minute mark, mice were given an ip injection of D-amphetamine sulfate (Sigma, St. Louis, MO) (0.5 mg/kg or 3.0 mg/kg). The total distance traveled was recorded for another 80 minutes and AMPH-induced increases in activity were expressed as percent baseline. Mice first received a low AMPH dose (0.5 mg/kg) followed by five high AMPH dose (3.0 mg/kg) trials administered every other day for ten days. Ten days after the last high-dose trial, mice were challenged with a second low AMPH dose to identify whether behavioral sensitization to AMPH had occurred. Locomotor activity was collected in 5 minute bins and is expressed as percent baseline where locomotor activity associated with each habituation bin was divided by the average habituation activity. Similarly, the locomotor activity associated with the saline injection is normalized to the last 20 mins of the habituation period when locomotor activity associated with the novel environment had stabilized. Increased activity attributed to AMPH administration is expressed as percent change from the average locomotor activity after the saline injection up until the AMPH injection (between minutes 40-80).

2.3. Fast Scan Cyclic Voltammetry

Fast scan cyclic voltammetry was used to identify changes in dopamine signaling within the nucleus accumbens (NAc) core. Voltammetry experiments were conducted after six week access to experimental diets or 24 hrs after completion of the AMPH sensitization procedure. All voltammetry experiments were executed and modeled as previously described (Calipari et al., 2014). Briefly, mice were rendered unconscious using 5% isoflurane, decapitated, with the brain swiftly removed and submerged in ice cold artificial cerebral spinal fluid (aCSF) for slicing on a vibratome (Leica Microsystems Inc.; Buffalo Grove, IL). 300 µm brain slices containing the NAc were used to characterize baseline DA release and reuptake, and to conduct dose response curves for AMPH (0.1-10 µM) and quinpirole (1-100 nM) (Sigma, St. Louis, MO).

2.4. Plasma Hormones

Plasma insulin, leptin, and triglycerides were measured after six-week exposure to each dietary paradigm. Blood was collected into heparinized tubes, centrifuged at $2,500 \times G$ for 10 minutes, and the supernatant was removed and stored at -80°C . Insulin and leptin were measured using enzyme linked immunosorbent assay kits (MyBioSource; San Diego, CA and Enzo Life Sciences; Farmingdale, NY; respectively) following manufacturer's instructions. Plasma triglycerides were detected using a colorimetric assay kit (Cayman Chemicals; Ann Arbor, MI), and all hormone assays were read on a Molecular Devices Spectra Max 384 Plus spectrophotometer (Sunnyvale, CA) using SoftMax Pro software.

2.5 Western Blotting

Tissue from the NAc was excised following six-week exposure to the respective diets. DAT protein levels were quantified using Western blot hybridization following methods described previously (Jorgason et al., 2016). The tissue was homogenized in an ice cold sucrose fractionation buffer (250 mM Sucrose, 20 mM HEPES, 10mM KCl, 1.5mM MgCl_2 , 1mM EDTA, 1mM EGTA, 1 mM DTT, with Halt™ protease inhibitors(ThermoScientific, Rockford, IL)). The tissue was then centrifuged at $720 \times G$ for 5 minutes to pellet nuclear content and cellular debris. An aliquot was saved from the supernatant to represent total tissue content. The supernatant was transferred and centrifuged at $10,000 \times G$ for 10 minutes, transferring the resulting supernatant to new tubes for centrifugation at $100,000 \times G$ for 60 minutes. The supernatant collected was saved to represent the cytosolic compartment, and the resulting pellet was re-suspended in fractionation buffer and centrifuged again at $100,000 \times G$ for 60 minutes. The final pellet was suspended in RIPA (150 mM NaCl, 1.0% Triton-X-100, 0.5% Sodium Deoxycholate, 0.1% Sodium Dodecyl Sulfate (SDS), 50 mM Trizma Base, pH 8.0) lysis buffer and saved as the membrane fraction. Protein concentrations were determined using a BCA protein assay kit (ThermoScientific, Rockford, IL). Fifteen g of protein was loaded into 4–12% NuPAGE Bis-Tris gels (NP0321BOX; ThermoFisher Scientific, Grand Island, NY) along with a MagicMark™ XP molecular weight ladder (LC5602; ThermoFisher Scientific, Grand Island, NY). Proteins from the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane and probed using the following primary antibody dilutions: DAT (1:4000) (2231; EMD Millipore, Billerica, MA) and β -actin (1:4000) (ab8229; Abcam, Cambridge, MA). A horse radish peroxidase-conjugated secondary antibody was used (1:5000) for DAT and β -actin (ThermoFisher Scientific, Grand Island, NY), in combination with Pierce ECL chemiluminescence (ThermoFisher Scientific, Grand Island, NY). Blots were exposed using a Bio-Rad Chemidoc imaging system, and the band signal intensity was assessed using QuantityOne software (Bio-Rad, Hercules, CA).

2.6 Microdialysis

Microdialysis guide cannulae (BAS, Bioanalytical Systems Inc, West Lafayette, IN) were stereotactically implanted above the NAc at coordinates anteroposterior +1.1 mm, lateral +0.80 mm, ventral -3.0 mm relative to bregma, midline, and dura in mice from each dietary treatment. Mice recovered for 24 h before the start of experimentation. Concentric microdialysis probes (1 mm membrane length; BAS) were inserted ~ 8 h before the

beginning of sample collection and continuously perfused at 1.0 $\mu\text{L}/\text{min}$ with artificial cerebrospinal fluid (aCSF) (pH 7.4) containing: NaCl (148 mM), KCl (2.7 mM), CaCl_2 (1.2 mM), MgCl_2 (0.85 mM). Samples were collected every 20 minutes for 4 h and stored at -80°C until HPLC analysis. Dialysate was analyzed for dopamine using an HPLC ESA Coulochem III detector running Chromeleon analysis software (ThermoFisher Scientific, Grand Island, NY).

2.7. Statistics

Graph Pad Prism v.5 (La Jolla, CA) was used for all statistical analyses. Two-way repeated measures analysis of variance (ANOVA) was used to identify significant differences within and between treatment groups for body weight, food intake, AMPH sensitization, microdialysis, and voltammetry data using Bonferroni's or Tukey's post-hoc analysis to identify significant variations between groups at individual data points, when applicable. One-way ANOVA tests were used to analyze area under the curve data for total distance traveled, plasma assays, western blot densitometry, and behavioral sensitization between chow, HF, and LimA groups.

3. Results

3.1. Food intake patterns and body weight

Mice with unlimited access to a HF diet consumed significantly more calories per day than mice fed chow or mice with LimA to HF ($p < 0.0001$) (Figure 1A). Moreover, the LimA group consumed significantly fewer kcals than the chow group ($p < 0.001$), but displayed binge like intake when given access to the HF diet consuming 31% and 60% of their daily kcals within 30 minutes and 2hrs, respectively (Figure 1B). The HF group gained significantly more weight compared to the chow fed and LimA groups (Figure 1C). Main effects of time ($F_{18, 756} = 244.17$, $p < 0.0001$) and diet ($F_{2, 756} = 24.92$, $p < 0.0001$), and a time $\times$ diet interaction was observed ($F_{36, 756} = 30.85$, $p < 0.0001$). The total change in body weight was significantly higher in HF-fed mice compared to chow fed and LimA mice ($p < 0.0001$) (Figure 1D). Overall, six-week exposure to HF engenders diet-induced obesity consistent with previous reports using this HF diet protocol (Chuang et al., 2012), and LimA to HF results in binge like intake that is not accompanied by obesity.

3.2. Plasma insulin, leptin, and triglyceride levels

Because a HF diet modifies metabolic hormones that have central effects on food intake and dopamine neurotransmission, we measured plasma insulin, leptin, and triglycerides to identify relationships between these hormones and any dopamine terminal or behavioral changes associated with AMPH exposure. Plasma insulin, leptin, and triglyceride levels were significantly elevated in mice with extended access to HF compared to LimA or chow-fed mice ($p < 0.001$ for insulin and leptin; $p < 0.01$ for triglycerides) (Figure 2). Circulating insulin levels in chow, extended HF, and LimA mice were 131.7 ± 10.8 , 264.4 ± 18.9 , and 114.2 ± 7.2 pmol/L, respectively (Figure 2A). Leptin levels were 5.6 ± 0.3 , 31.0 ± 1.4 , and 4.3 ± 1.9 ng/mL for chow, HF, and LimA access mice, respectively (Figure 2B). Finally, triglyceride content in the plasma was 72.2 ± 4.8 , 120.2 ± 11.9 , and 66.9 ± 5.9 mg/dL for chow, HF, and LimA mice, respectively (Figure 2C). Plasma hormone levels in the extended

access to HF group were consistent with previous reports, suggesting leptin and insulin resistance in these mice (Lin et al., 2000).

3.3. HF diet increases behavioral sensitivity to AMPH

To examine whether HF diet consumption alters dopaminergic pathways similarly to drugs of abuse, mice underwent an AMPH sensitization protocol. Total locomotor activity was significantly less during habituation in HF and LimA compared to chow fed mice ($p < 0.0001$) (Figure 3); however, the pattern of activity during habituation was similar in all groups indicating less overall activity, but similar mobility (Figure 3). Total distance traveled after an acute AMPH challenge (3mg/kg) showed similar absolute magnitudes of locomotor output in each group, however this is difficult to interpret considering the significant difference in basal locomotion. To control for the baseline ambulatory difference, and to better interpret the magnitude of locomotor response to AMPH, changes in activity associated with AMPH were normalized to baseline activity.

No significant difference in AMPH-induced activity was observed after the initial low-dose AMPH injection (Figure 4A). Both HF-fed groups showed augmented AMPH-induced locomotor activity after the first, second, and fourth high-dose AMPH injections (Figures 4B, 4C, and 4E) with main effects of diet ($F_{2,561} = 3.99, 4.11, \text{ and } 4.14, p = 0.0381, 0.0363, \text{ and } 0.0354, \text{ respectively}$) and time ($F_{33,561} = 57.07, 63.55, \text{ and } 53.02, p < 0.0001, \text{ each}$), and a diet $\times$ time interaction at each dose ($F_{66,561} = 2.62, 2.94, \text{ and } 3.02, p < 0.0001, \text{ each}$). The percent change from baseline activity declined with each high-dose AMPH trial in all groups, but the decline in locomotor activity was substantially more pronounced in groups with access to HF. The HF and LimA groups did, however, display a significant increase in AMPH induced activity after the low-dose AMPH challenge (Figure 4G) compared to chow with main effects of time ($F_{33,561} = 8.68, p < 0.0001$), diet ($F_{2,561} = 4.70, p < 0.0238$), and a time $\times$ diet interaction ($F_{66,561} = 1.95, p < 0.0001$). The main effect of time suggests that the significant difference between the HF groups and chow were impacted more by the duration of increased activity as opposed to the peak activity increase. When compared within subjects, all groups showed behavioral sensitization to AMPH (Figure 5A-C), but the magnitude of sensitization was greater in the HF ($p < 0.05$) and LimA ($p < 0.01$) groups, identified using one-way ANOVA between the percent change in the area under the curves following the AMPH injection (Figure 5D).

3.4. HF diet does not alter dopamine release, but reduces dopamine uptake

Slice voltammetry was used to characterize dopamine terminal function in the NAc after 6-week exposure to the respective diets. HF diet intake did not alter evoked DA release after six-week exposure to either extended or limited access, compared to chow fed mice (Figure 6A). The maximal rate of dopamine reuptake ($V_{\max}$), however, was significantly reduced in each HF access group compared to chow ($p < 0.01$) (Figure 6B), indicating a deficit in synaptic dopamine clearance.

3.5. HF diet reduces dopamine terminal plasticity

Marked reductions in dopamine terminal plasticity were observed in the NAc of mice consuming a HF diet after AMPH sensitization. Overall, main effects of diet ($F_{2,77} = 10.14$,

$p < 0.0001$), drug ($F_{1,77} = 10.18$, $p < 0.0001$), and a diet $\times$ drug interaction ($F_{2,77} = 6.77$, $p = 0.002$) were observed on dopamine release (Figure 6A). No difference in release was observed between AMPH-naïve groups, but Tukey's post hoc analysis revealed significantly increased dopamine release in chow fed mice after AMPH compared with naïve chow fed controls ($p < 0.001$). Additionally, AMPH sensitized mice fed chow had significantly greater release compared with AMPH-sensitized mice from the HF and LimA groups ($p < 0.001$ each). With respect to the maximal rate of dopamine reuptake (Vmax), main effects of diet ($F_{2,77} = 58.49$, $p < 0.0001$), drug ($F_{1,77} = 26.69$, $p < 0.0001$), and a diet $\times$ drug interaction ($F_{2,77} = 14.46$, $p < 0.0001$) were observed (Figure 6B). Tukey's post hoc analysis indicated: 1) significantly lower Vmax in AMPH-naïve HF and LimA groups compared to chow fed controls ($p < 0.01$ each), 2) increased Vmax after AMPH exposure in chow fed mice ($p < 0.001$), and 3) extended and LimA to HF blocked the AMPH-induced increase observed in chow fed mice exposed to AMPH ($p < 0.001$ each). Interestingly, the HF and LimA mice that displayed enhanced behavioral sensitization to AMPH were resistant to AMPH-induced changes in dopamine terminal function. Representative line plots exported from Demon Voltammetry and Analysis Software (Yorgason et al., 2011, Figures 6C-E) exhibit the difference in dopamine release and reuptake that occurred due to AMPH, where the ascending portion of the curve represents dopamine release and the top third of the descending portion depicts Vmax. Together these data suggest that HF intake dampens the AMPH-induced plasticity that occurs in chow fed mice. This lack of plasticity in HF-fed mice may contribute to enhanced behavioral sensitivity to AMPH.

3.6. HF reduces AMPH potency and alters D₂ autoreceptor function in NAc slices

After collecting baseline parameters we conducted dose response curves for AMPH (dopamine uptake inhibitor and releaser) and quinpirole (D₂/D₃ receptor agonist) to determine whether differences in terminal function or autoreceptor activity in HF or LimA mice reconciled the differences observed in locomotor sensitization to AMPH. Within groups, no significant differences were observed in AMPH's ability to reduce DA release in naïve and AMPH-exposed mice from each dietary group (Figures 7A-C). Between groups, AMPH dose dependently reduced dopamine release in all AMPH-naïve mice ($F_{4,60} = 102.46$, $p < 0.0001$), indicative of AMPH-induced dopamine depletion of vesicles in the terminals (Figure 7D). Bonferroni's post hoc analysis further identified a significant difference in dopamine release with 1 μ M AMPH between the chow and HF groups ($p < 0.05$) which indicates that AMPH was less effective at depleting dopamine in both HF-fed groups at that dose. The effect of AMPH on dopamine release did not differ in slices from mice that underwent the sensitization protocol (Figure 7E). However, the potency of AMPH as an uptake inhibitor (apparent K_m) was significantly enhanced within and between dietary and AMPH-exposed groups (Figure 8). Within dietary groups, chow fed mice showed increased AMPH potency after AMPH exposure with main effects of drug ($F_{1,44} = 6.12$, $p = 0.03$) and dose ($F_{4,44} = 64.97$, $p < 0.0001$), and a drug $\times$ dose interaction ($F_{4,44} = 5.74$, $p = 0.0008$) (Figure 8A). A similar main effect of AMPH dose ($F_{4,44} = 58.73$, $p < 0.0001$), and a drug $\times$ dose interaction was observed between AMPH-naïve and AMPH-exposed HF mice (Figure 8B). Slices from LimA mice showed no difference in AMPH potency as an uptake inhibitor (Figure 8C). Between groups in AMPH-naïve mice, a main effect of dose was identified ($F_{4,56} = 61.28$, $p < 0.0001$), and Bonferroni's post test revealed a significant

difference between the HF and chow groups at the highest dose of AMPH (Figure 8D). A nearly significant effect of diet ($F_{2, 56} = 3.65$, $p < 0.053$) was observed on AMPH potency in AMPH-naïve mice (Figure 8D). Main effects of diet ($F_{2, 68} = 10.83$, $p = 0.0009$) and AMPH dose ($F_{4, 68} = 70.25$, $p < 0.0001$) were identified in AMPH-exposed mice, along with a diet $\times$ dose interaction ($F_{8, 68} = 5.24$, $p < 0.0001$) (Figure 8E), and Bonferroni's post hoc analysis revealed significant differences between the chow and HF groups at the highest dose ($p < 0.001$) and the chow and LimA groups as the highest two doses ($p < 0.01$ and 0.001 , respectively) (Figure 8E). These data suggest that dietary HF intake (either extended (HF) or limited access (LimA)) reduced AMPH potency as an uptake inhibitor compared to chow fed mice.

No within group changes in quinpirole-induced dopamine release were observed from naïve or AMPH-exposed chow or HF mice (Figures 9A,B); however, LimA mice exposed to AMPH displayed decreased sensitivity to quinpirole compared to LimA mice that did not receive AMPH, where main effects of drug ($F_{1, 48} = 6.65$, $p = 0.0241$), dose ($F_{4, 48} = 185.31$, $p < 0.0001$), and a drug $\times$ dose interaction ($F_{4, 48} = 3.19$, $p = 0.0209$) were observed (Figure 9C). Between groups, quinpirole reduced dopamine release in AMPH-naïve mice with a main effects of diet ($F_{2, 48} = 4.83$, $p = 0.0289$), dose ($F_{4, 48} = 126.59$, $p < 0.0001$), and a diet $\times$ dose interaction ($F_{8, 48} = 5.12$, $p < 0.0001$) (Figure 9D). Bonferroni's post hoc analysis revealed a significant decrease in dopamine release after quinpirole in the LimA group compared to chow at 3, 10, and 30 nM ($p < 0.05$, 0.001 and 0.001 , respectively). Subinsensitive D_2 autoreceptors in the LimA group but not the HF group suggests that changes in HF availability alters presynaptic D_2 function in a feeding schedule dependent manner, and that D_2 autoreceptor subsensitivity likely contributes to increased behavioral sensitivity to AMPH in the LimA group.

3.7. HF increases in dopamine in response to AMPH in freely moving mice

In order to evaluate whether tonic dopamine levels contribute to the augmented behavioral response to AMPH, and to measure *in vivo* dopamine elevations in response to AMPH, we performed *in vivo* microdialysis in freely moving mice. Basal dopamine levels were similar between chow-fed and HF-fed mice (1.77 ± 0.13 and 1.80 ± 0.14 nM dopamine, respectively), but a one-way ANOVA identified significantly reduced basal dopamine (1.01 ± 0.28 nM) in LimA mice compared to all other groups ($p < 0.05$) (Figure 10A). Administration of AMPH (3mg/kg AMPH i.p.) significantly elevated extracellular dopamine levels from baseline, with main effects of time ($F_{11, 132} = 19.58$, $p < 0.0001$), diet ($F_{2, 132} = 9.36$, $p = 0.004$), and a time $\times$ diet interaction ($F_{22, 132} = 3.54$, $p < 0.0001$) (Figure 10B). Bonferroni's post hoc analysis revealed a significant increase in extracellular dopamine from chow-fed controls between 40-180 minutes following the AMPH injection in mice with extended access to HF ($p < 0.05$ to <0.001), and at 20 and 60 minutes following the AMPH injection ($p < 0.05$) in mice with limited access to HF. These data resemble the locomotor responses to AMPH with the respective diets.

3.8. Membrane and cytosolic DAT protein levels

In order to address whether changes in DAT protein content contributes to enhanced sensitization in the HF and LimA groups, we identified DAT protein compartmentalization

by western blotting for DAT in the membrane, cytosol, and tissue lysate fractions of the NAc. Because the fully glycosylated DAT has enhanced function compared with a less post-translationally modified DAT (Li et al., 2004), we quantified both mature and immature DAT protein bands to identify isoform specific relationships to observed neurochemical outcomes. We found no changes in total membrane DAT between groups; however, extended access to HF reduced the mature 78 kDa DAT compared to chow-fed mice ($p = 0.037$) (Figure 11A). A significant increase in total cytosolic DAT ($p = 0.43$) was found in the LimA group compared to chow and extended HF, largely consisting the mature DAT isoform ($p = 0.011$) (Figure 11B). Total DAT content of NAc tissue lysate was elevated in both HF groups, but was significantly greater with extended access compared to chow-fed controls ($p = 0.036$) (Figure 11C). Interestingly, the immature DAT isoform was the predominant DAT species with extended HF ($p = 0.011$), suggesting a lack of post translational modifications that support mature DAT function. These data show that HF-feeding increases total DAT protein in the NAc, but does not preserve mature DAT levels at the plasma membrane.

4. Discussion

Overall, this study reports that access to a HF diet alters dopamine signaling and produces dopamine terminals that are remarkably different than chow fed mice following AMPH exposure. We showed that a HF diet increased the acute behavioral response to AMPH, which corresponded with increased extracellular dopamine levels. Notably, mice with extended or limited access to HF developed greater behavioral sensitization to AMPH than chow-fed mice, although different mechanisms were likely involved. While both HF groups were resistant to AMPH-induced terminal adaptations that engendered prominent elevations in dopamine release and re-uptake in chow fed mice, extended access to HF was also associated with changes in AMPH potency, but limited access to HF reduced D₂ autoreceptor function. Mice with limited exposure to the HF diet displayed slightly more dopamine release and reuptake following AMPH exposure than extended access, indicating that the amount of fat intake had a graded effect on terminal function. However, increased AMPH potency in mice with extended access to HF was not accompanied by an increase in V_{max} for dopamine uptake, which typically corresponds with enhanced AMPH potency and increased behavioral output (Calipari et al., 2013), demonstrating a unique characteristic of dopamine terminals in HF fed mice. HF-fed mice had greater total DAT protein levels at the plasma membrane, but also had significantly less of the mature DAT isoform, which paralleled reduced V_{max}, and suggests that altered post-translational DAT modifications contribute to functional changes observed after HF. Together these data show that the quantity and pattern of HF intake differentially alter dopamine terminal function, but both lead to an augmented behavioral response to AMPH.

The development of behavioral sensitization to AMPH is classically linked with decreased D₂ autoreceptor sensitivity, which impairs feedback inhibition of ventral tegmental area (VTA) neuron firing and attenuates DAT recruitment thereby reducing dopamine reuptake (Steketee and Kalivas, 2011 for review). Reduced D₂ autoreceptor inhibition of spontaneous neuron firing in the ventral tegmental area has been reported in mice after six weeks on a HF diet (Koyama et al., 2014). Interestingly, we did not observe reduced D₂ autoreceptor function at dopamine terminals in the NAc after extended access to HF, and actually

observed increased sensitivity to quinpirole activation of autoreceptors in mice with limited access to HF. The limited access, but not extended access, group also demonstrated escalated HF intake over the six-week period, which has been reported previously in rats (Corwin et al., 1998), and resembles binge-like intake of alcohol (Griffin et al., 2009; Rosenwasser et al., 2013) and cocaine (Oleson and Roberts, 2009) in rodents using intermittent access paradigms. To our knowledge D₂ autoreceptors have not been examined following intermittent HF access, but increased autoreceptor sensitivity has been reported during early ethanol withdrawal after chronic intermittent exposure (Budygin et al., 2003; Karkhanis et al., 2015) and i.p. ethanol administration (Perra et al., 2011). Increased D₂ sensitivity in our limited access mice was observed 24hrs following removal of the HF diet, so it is possible that enhanced D₂ sensitivity may be related to acute withdrawal from HF. AMPH exposure, however, reduced autoreceptor function in the limited access group, in line with the classical development of behavioral sensitization, and other reports showing that repeated psychostimulant exposure is associated with reduced D₂ autoreceptor function (Calipari and Jones, 2014; Mateo et al., 2005). The reduced ability of quinpirole to regulate presynaptic dopamine release suggests that decreased D₂ function after repeated AMPH likely drove the enhanced behavioral sensitization we observed in mice with limited access to HF. Because extended access to HF did not engender altered D₂ function in the same manner as limited access, but similarly enhanced the behavioral response to AMPH, alternate mechanism(s) must contribute to behavioral sensitization after extended HF exposure.

In addition to autoreceptor regulation, the behavioral response to AMPH is strongly influenced by the potency of AMPH as a DAT uptake inhibitor (Calipari et al., 2015). Increased AMPH potency in sensitized chow-fed mice is likely related to elevations in the maximal rate of DA uptake, or V_{max}, as AMPH potency is positively correlated with DAT density (Salahpour et al., 2008; Calipari et al., 2013; Siciliano et al., 2014a). Because we did not observe attenuated D₂ function in the chow group, the significant increase in V_{max} and corresponding AMPH potency, most likely account for the development of behavioral sensitization. Based on previous work showing that AMPH potency corresponds with an increased behavioral response to AMPH (Calipari et al., 2013, 2015), we would have expected a more pronounced increase in AMPH potency in the HF-fed mice compared to chow fed mice. However, this was not the case, as AMPH was less potent in mice with extended access to HF compared to chow fed mice. Interestingly, after the development of behavioral sensitization, a significant increase in AMPH potency was observed in the HF-fed mice (although still less than chow) despite no increase in V_{max}. This suggests that other mechanisms must account for the enhanced behavioral sensitization that we observed. One possibility is reduced basal dopamine reuptake, which is consistent with reports of reduced DAT function following a HF diet (Narayanaswami et al., 2013; Cone et al., 2013). We observed reduced membrane levels of the mature DAT isoform after HF-feeding, particularly in mice with extended access to HF. Because the mature DAT isoform has enhanced transport activity (Li et al., 2004), it is possible that changes in AMPH potency after HF could be related to functional DAT activity at the membrane. Moreover, prolonged consumption of a HF diet has been shown to produce insulin resistance (Arnold et al., 2014; Chuang et al., 2012), and evidence suggests that insulin signaling contributes to the regulation of dopamine uptake by enhancing DAT membrane recruitment and function

(Garcia et al., 2005; Owens et al., 2005; Speed et al., 2011), and increasing dopamine release and reuptake at dopamine terminals (Stouffer et al., 2015). A link between insulin signaling and AMPH effects on DAT function has been demonstrated by Galli and colleagues, who showed that repeated AMPH administration augmented AMPH-induced dopamine efflux in rats following ablation of insulin producing pancreatic β -cells using streptozotocin (Owens et al., 2012). In light of those findings, it is possible that attenuated insulin signaling after HF may reduce DAT function, and only after repeated administration of high-dose AMPH does AMPH potency increase. Neuronal resistance to insulin and leptin after extended access to HF may contribute to altered dopamine neurotransmission (Kleinridders et al., 2015; Thompson and Borgland, 2013). However, mice with limited access to HF had similar plasma leptin and insulin levels as chow fed mice, but showed behavioral sensitization to amphetamine like mice with extended access to HF. This discrepancy indicates that changes in these hormones are not required for augmented sensitization to AMPH, but it is possible that resistance to these hormones exacerbate dopaminergic dysfunction once metabolic impairment emerges. For example, extended access to HF also significantly increased plasma triglyceride levels. Infusion of triglycerides acutely into the NAc has been shown to reduce the locomotor effects of AMPH and disrupt palatable food preference in metabolically normal mice (Cansell et al., 2014); however, once metabolic adaptations like insulin and leptin resistance occur with extended access to HF, we observed that chronically elevated triglycerides do not impact the behavioral response to AMPH. In addition, the impact of a HF diet probably has metabolic and membrane/structural ramifications, both of which can change DAT function. It is puzzling that increased potency was observed in the absence of increased DAT function, but highlights that dysregulation at dopamine terminals after HF centers around the DAT.

The DAT facilitates AMPH-induced reverse transport of dopamine into the synapse as well as competitively inhibiting dopamine reuptake via the DAT (Sulzer et al., 2005; Siciliano et al., 2014b). Therefore, understanding how a HF diet influences DAT activity may shed light on the increased sensitization to AMPH in HF-fed mice. Increased dopamine clearance after AMPH in chow fed mice was presumably caused by adaptations to the dopamine terminals in response to repeated AMPH exposure that increased either membrane trafficking or the efficiency of the DAT to remove dopamine overflow. Changes in V_{max} have been shown to correspond with DAT density (Siciliano et al., 2014a) and provide a neurochemical assessment of overall DAT function. We observed reduced V_{max} in HF-fed mice, which corresponded with reduced membrane levels of the mature DAT isoform and corroborated previous reports indicating decreased membrane DAT in rats consuming a HF diet (Speed et al., 2011, Hryhorczuk et al., 2015). The study by Speed and colleagues also showed that, in rats, four-week access to a HF diet reduced the locomotor response to an acute AMPH injection through attenuated insulin signaling. Our results are not in conflict with this previous study, because we also observed slightly reduced locomotor activity in raw distance-traveled units after acute AMPH in mice consuming HF, compared to chow; however, when our data were normalized to account for significantly lower basal locomotion, which was not observed in the papers by Speed or Hryhorczuk and colleagues, the percent change in activity was significantly greater in HF mice compared to controls. Aside from the potential difference in locomotor response between mice in our study, and

rats in the studies by Speed and Hryhorczuk, a possible explanation for the augmented locomotion we observed could be lower basal dopamine levels in HF-fed mice contributing to lower baseline locomotor activity, but potentially exacerbating the locomotor response to even a modest increase in synaptic dopamine through increased sensitivity of post-synaptic dopamine receptors, or increased D₁ receptor density, which has been shown with increased saturated fat intake (Hryhorczuk et al., 2015). Our microdialysis data suggest that reduced baseline dopamine levels may contribute to enhanced locomotor activity in mice with limited, but not extended, access to HF, perhaps through supersensitivity of post-synaptic dopamine receptors. Additionally, increased cytosolic DATs observed after limited access to HF are potentially available for recruitment to the membrane following acute AMPH, further enhancing dopamine efflux and locomotion (Calipari et al., 2013). Reduced baseline dopamine does not explain the augmented behavioral response to acute AMPH after extended access to HF, but a possible explanation for the difference in baseline dopamine between HF and Lim A mice could be that extended access mice were continuously exposed to the palatable diet while LimA mice were 24 h removed from HF-feeding and potentially in a “withdrawal” state. In addition, it is possible that insulin resistance may contribute to the difference between HF and LimA baseline dopamine levels. Insulin has been shown to dampen dopaminergic neuron firing in the VTA (Mebel et al., 2012; Labouebe et al., 2013) and insulin resistance following extended access to HF may disinhibit this response, increasing dopaminergic output in HF-fed mice. Coupled with AMPH-induced increases in dopamine neuron firing (Shi et al., 2000) and reduced dopamine reuptake, insulin resistance may help explain the locomotor response to acute AMPH in mice with extended access to HF, despite no reductions in basal dopamine tone or increase in AMPH potency.

With respect to the enhanced behavioral sensitization observed following repeated AMPH in mice with extended or limited access to HF, our neurochemical measures of presynaptic dopamine neuron function do not provide a definitive mechanism for the sensitized locomotor response. The increase in AMPH potency partially accounts for the development of sensitization in mice with extended HF intake, but not the augmented locomotor sensitization compared to chow-fed mice. Similarly, reduced autoreceptor activity with limited HF could contribute to sensitization, but because autoreceptor activity was the same in all groups after AMPH, this does not completely explain the augmented sensitization in LimA mice. Overall the lack of plasticity of dopamine signaling was the most remarkable difference between HF and chow-fed mice, suggesting that the ability of chow-fed mice to adapt to a pharmacological challenge, like AMPH, may reduce the development of sensitization. It is likely that changes in other neurotransmitter systems contribute to the increased AMPH sensitization seen in mice with extended or limited HF intake. Indeed, an increase in phosphorylation of the GluR1 subunit of the AMPA receptor has been shown in the NAc of HF-fed rats (Hryhorczuk et al., 2015), suggesting that changes in glutamate signaling may contribute to increased sensitization after extended or limited HF intake. This would be in line with the well-accepted view that increased glutamatergic signaling in the limbic system facilitates locomotor sensitization to psychostimulant administration (for review see Vanderschuren and Kalivas, 2000). Because we found reduced dopamine terminal adaptations in HF-fed mice following AMPH, but observed increased extracellular dopamine in response to AMPH, it is likely that dopamine neuron firing contributed to

enhanced locomotor sensitization to AMPH. AMPH increases dopamine neuron firing (Shi et al., 2000), and has been shown to initiate dopamine transients (Daberkow et al., 2013). Increased synaptic density of glutamate neurons in the VTA reported after HF feeding (Liu et al., 2016) could augment dopamine neuron excitability and exacerbate this response. Together, increased glutamatergic signaling combined with insulin resistance that blocks insulin's ability to dampen VTA dopamine neuron firing after extended HF may contribute to the augmented locomotor response we observed.

In conclusion, our data show that prolonged consumption of a HF diet attenuates dopamine terminal function, altering the effect of pharmacological challenges. Importantly, dopamine terminal changes after a HF diet appear to be obesity independent, as similar neurochemical changes were observed in non-obese mice with limited access to HF. Because HF intake impacts the magnitude of effect AMPH exerts on the dopamine system, prolonged exposure may increase the likelihood of developing abuse disorders. Further studies are warranted to explore pre- and post-synaptic mechanisms that contribute to disrupted dopamine terminal function, and to examine the contribution of anorexigenic hormones, like insulin, in this process. Restoring dopamine terminal function after chronic HF intake may enhance the fidelity of dopaminergic transmission to improve the salience of natural food rewards, reduce compulsive eating behaviors, and block enhanced behavioral responses to psychostimulants like AMPH.

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Highlights

- A high fat (HF) diet enhances behavioral sensitization to amphetamine in mice.
- Dopamine terminals adapt to AMPH differently in HF fed mice compare to chow.
- HF feeding pattern, not obesity, governs dopamine terminal changes.

Figure 1. Food Intake and Body Weight

Mice with 24 hr access to a HF diet ($n=16$) consumed significantly more kcals per pair housed cage over six weeks than mice with access to chow ($n=16$) and mice with limited access (LimA) to HF ($n=13$) (A). Binge like intake of the HF diet was observed in LimA mice on days with 2hr access to HF, ~40% of their daily kcals were consumed within the first 30mins of the two hour HF feeding window (B). Increased food intake in the HF group corresponded with progressive weight gain (C) and a significant change in body weight compared to chow and LimA (D). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 2. Plasma insulin, leptin, and triglyceride levels

Circulating insulin (A), leptin (B), and triglycerides (C) were measured to identify possible relationships between metabolic indexes and behavioral or neurochemical changes associated with AMPH exposure. (**p < 0.01, ***p < 0.001)

Figure 3. Locomotor Habituation

After six weeks of dietary exposure, mice were placed in locomotor chambers, allowed to habituate for 40 min, then administered saline (ip) to identify any injection-stress-induced increases in activity. The patterns of activity during habituation and the saline injection were similar between chow, HF, and LimA mice (A); however, total activity was significantly less in the HF and LimA mice compared to chow ($***p < 0.001$) (B), indicating less overall activity but similar mobility after HF exposure.

Figure 4. AMPH-Induced Locomotor Activity

Mice with access to a standard lab chow ($n=8$), a HF diet ($n=8$), or LimA to a HF diet ($n=7$) for six weeks were tested for behavioral sensitivity to low-dose AMPH (0.5 mg/kg) after repeated high-dose AMPH (3.0 mg/kg) administration. Mice were given an initial low-dose AMPH injection (A) followed by five high-dose AMPH injections every other day for 10 days (B-F). Mice were then given a low-dose AMPH challenge after a ten day abstinence period (G). A significant increase in locomotor activity was observed after AMPH administration in the HF and LimA groups compared to chow during the first (B), second (C), and fourth (E) dose of 3.0 mg/kg AMPH with percent baseline activity regressing to chow levels by the last high-dose injection (F). The final 0.5 mg/kg AMPH challenge revealed a significant increase AMPH-induced locomotor activity in HF and LimA mice compared to chow. Note the Y-axis for (A) and (G) differ from (B-F) to visually discern activity differences between groups. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Blue* denote differences between chow and LimA, Red* denote differences between chow and HF)

Figure 5. Behavioral Sensitization to AMPH

Behavioral sensitization to AMPH was measured by comparing the difference in locomotor activity between the pre- and post- 0.5 mg/kg AMPH injections. Activity patterns from pre and post low-dose AMPH injections for chow (A), HF (B), and LimA (C) groups are overlaid. The post AMPH challenge significantly increased locomotor activity in each group compared to the initial administration of 0.5 mg/kg AMPH; however, the HF and LimA groups displayed greater behavioral sensitization to AMPH (D) compared to Chow, calculated by the percent change in area under the curve following the AMPH injection. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 6. Dopamine Release and Reuptake

Baseline dopamine (DA) function was measured in the NAc using slice voltammetry in mice after six week dietary exposure: chow (n=8), HF (n=8), and LimA (n=6) diets, or 24 hrs following completion of the AMPH sensitization protocol, which was preceded by the six week dietary regimen: Chow AMPH (n=8), HF AMPH (n=8), and LimA AMPH (n=7). (A) Baseline electrically evoked DA release. (B) Baseline maximal rate of dopamine reuptake (Vmax). Representative line traces are shown depicting the difference in baseline DA release and reuptake in slices from AMPH naïve and AMPH exposed chow (C), HF (D), and LimA (E) groups. Peak height represents evoked DA release (μM) and the top third of the curve's downward slope signifies the Vmax plotted over time. (** $p < 0.01$, *** $p < 0.001$)

Figure 7. Effect of AMPH Exposure on Vesicular DA Depletion

Dose response curves for AMPH were conducted in brain slices containing the NAc to evaluate the effect of previous AMPH exposure on AMPH-induced DA release. Prior exposure to AMPH did not alter the ability of AMPH to release DA within each dietary group (A-C). In AMPH-naïve mice, AMPH-induced reductions in DA release were greater in the chow versus the HF groups (D); however, no significant differences in the ability of AMPH to release DA was detected between dietary groups in AMPH exposed mice (E). (* $p < 0.05$)

Figure 8. Effect of AMPH Exposure on Uptake Inhibition

Dose response curves for AMPH were conducted in brain slices containing the NAc to evaluate the potency of AMPH as an uptake inhibitor. Within dietary groups, previous exposure to AMPH increased AMPH potency in brain slices of chow (A) and HF (B) mice, but not LimA mice (C). Between groups, AMPH was more potent as an uptake inhibitor in the chow versus HF group (D). Moreover, slices from AMPH-exposed mice show a significant increase in AMPH potency as an uptake inhibitor in the chow group compared to the HF and LimA groups (E). (** $p < 0.01$, *** $p < 0.001$)

Figure 9. Effect of AMPH Exposure on DA Autoreceptors

Dose response curves for the D_2/D_3 agonist quinpirole were used to identify changes in DA autoreceptor function in NAc slices. Within dietary groups, AMPH exposure did not alter quinpirole sensitivity in the chow (A) or HF (B) groups, but reduced autoreceptor sensitivity in LimA mice (C). Between groups, LimA showed an increased sensitivity to quinpirole compared to chow in AMPH-naïve mice (D), but no differences in quinpirole sensitivity were detected between groups after exposure to AMPH (E). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 10. HF increases *in vivo* dopamine in response to AMPH

Microdialysis detection of DA in the NAc. (A) Average basal dopamine levels collected in 20 minute bins for 1 h prior to AMPH. (B) Extracellular dopamine response to AMPH (3.0 mg/kg i.p.) in chow-fed mice and mice with extended (HF) or limited access (LimA) to a HF diet (n=5 for chow and LimA; n=8 for HF). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Figure 11. Effect of HF on the cellular localization of DAT

Western blot analysis for DAT protein levels in membrane (A), cytosolic (B), and tissue lysate (C) from the NAc. Total DAT levels are calculated by adding the density of the 68 and 78 kDa DAT bands. All data is normalized to β -actin and expressed relative to chow DAT levels. (* $p < 0.05$)