

Structural and Functional Plasticity within the Nucleus Accumbens and Prefrontal Cortex Associated with Time-Dependent Increases in Food Cue-Seeking Behavior

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Urges to consume food can be driven by stimuli in the environment that are associated with previous food experience. Identifying adaptations within brain reward circuits that facilitate cue-induced food seeking is critical for understanding and preventing the overconsumption of food and subsequent weight gain. Utilizing electrophysiological, biochemical, and Dil labeling, we examined functional and structural changes in the nucleus accumbens (NAc) and prefrontal cortex (PFC) associated with time-dependent increases in food craving ('incubation of craving'). Rats self-administered 60% high fat or chow 45 mg pellets and were then tested for incubation of craving either 1 or 30 days after training. High fat was chosen for comparison to determine whether palatability differentially affected incubation and/or plasticity. Rats showed robust incubation of craving for both food rewards, although responding for cues previously associated with high fat was greater than chow at both 1 and 30 days. In addition, previous experience with high-fat consumption reduced dendritic spine density in the PFC at both time points. In contrast, incubation was associated with an increase in NAc spine density and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA)-mediated transmission at 30 days in both groups. Finally, incubation of craving for chow and high fat was accompanied by an increase in calcium-permeable and calcium-impermeable AMPARs, respectively. Our results suggest that incubation of food craving alters brain reward circuitry and macronutrient composition specifically induces cortical changes in a way that may facilitate maladaptive food-seeking behaviors.

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INTRODUCTION

Environmental stimuli can powerfully motivate behavior. With the massive increase in caloric intake in developed nations (Caballero, 2007), one behavior of special interest is that of feeding. From an evolutionary perspective, opportunities to consume food were often sporadic and it was therefore adaptive for humans to recognize and act upon cues predictive of food availability (Eknoyan, 2006). Unfortunately, overconsumption occurs when the availability of food exceeds the biological need and food-associated cues continue to trigger the instinct to consume.

Indeed, clinical studies have revealed that humans will overconsume food after attending to food-related cues, including visual presentation (Marcelino *et al*, 2001; Fisher and Birch, 2002; Coelho *et al*, 2009) and the smell of food

(Fedoroff *et al*, 1997; Jansen *et al*, 2003; Jansen *et al*, 2008). Cue exposure can even induce overeating when the cue presented is not relevant to the food available (eg, seeing an advertisement for one food item can promote consumption of another; Harris *et al*, 2009). Cue-potentiated feeding is a behavior readily observed in rodents as well. Petrovich *et al* (2007) demonstrated that reintroduction to cues paired with specific food pellets when rats were hungry enhanced consumption of those pellets when the rats were subsequently satiated (Petrovich *et al*, 2007), suggesting that food-paired cues, in addition to signaling the presence of food, may induce a state of 'craving'. Such motivational states can be altered by diet, as evidenced by studies indicating that obese rats exposed to 'junk-food' or palatable high-fat (HF) diets exhibit greater cue-triggered motivation (Brown *et al*, 2015; Robinson *et al*, 2015). These observations indicate that not only are food-predictive cues important for triggering food seeking and consumption in rodents but moreover that such cues are most salient in those that develop obesity. Thus, understanding the mechanisms underlying cue-induced seeking behavior may provide useful insights to the treatment of obesity and other overeating disorders.

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Efforts have been made to identify reward circuit regions that are altered by feeding. For example, consumption of a 'junk-food' diet induces an increase in calcium-permeable α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (CP-AMPA; Oginsky *et al*, 2016) and HF diet-induced obesity elicits an increase in AMPA to N-methyl-D-aspartate (NMDA) ratio (Brown *et al*, 2015) in the nucleus accumbens core (NAc-C). In addition, previous work within our laboratory has shown that non-contingent consumption of a 60% HF diet induces a reduction of thin-type spines in the infralimbic medial prefrontal cortex (IL-mPFC; Dingess *et al*, 2016). Behaviorally, we have shown that there is an increase in lever responding for food-associated cues over time, independent of macronutrient composition (Darling *et al*, 2016). What remains unknown, and this manuscript begins to address, are the neuroadaptations that correspond with this enhanced time-dependent motivation for food cues. This is important to investigate given that unrewarded cue presentations gain salience over time, a phenomenon known as 'incubation of craving', and exposure to such cues is hypothesized to precede relapse behavior (O'Brien *et al*, 1992; Lu *et al*, 2004; Grimm *et al*, 2011), which in the context of feeding behavior, may manifest as a failure to maintain healthy eating habits.

The incubation of craving model has become a useful tool to evaluate cue responsiveness for many drugs of abuse (Grimm *et al*, 2001; Bienkowski *et al*, 2004; Lu *et al*, 2004; Shepard *et al*, 2004; Abdolahi *et al*, 2010) but has been observed for food as well, including sucrose pellets (Grimm *et al*, 2005; Grimm *et al*, 2011), liquid saccharin (Aoyama *et al*, 2014), HF pellets (Darling *et al*, 2016) and even food pellets with a similar macronutrient composition to standard lab chow (Krasnova *et al*, 2014; Darling *et al*, 2016). Briefly, it involves training an animal to press a lever and receive a reward (eg, food pellet) in conjunction with associated cues (eg, light and tone). Following a training period, the animal lever presses for the cues alone, in the absence of the reward. Incubation of craving occurs when the responsiveness towards these cues becomes greater over time. Investigation of this paradigm has led to important discoveries regarding the neural substrates of cue-potentiated reward seeking. Studies using a variety of techniques have implicated the PFC (Koya *et al*, 2009; Ma *et al*, 2014) and NAc (Hollander and Carelli, 2007; Wolf and Ferrario, 2010; Counotte *et al*, 2014) as regions, which undergo plasticity following incubation of craving. The NAc-C in particular has gained attention due to the extensive structural and functional glutamatergic plasticity that occurs following incubation of cocaine craving, including increases in spine density (Christian *et al*, 2016) and AMPA/NMDA ratio (Ortinski *et al*, 2012) as well as an upregulation in CP-AMPA receptors (Conrad *et al*, 2008). In contrast to drug reward, repeated exposure to natural rewards, such as sexual experience (Pitchers *et al*, 2012) or incubation of sucrose craving, induces a decrease in AMPA/NMDA (Counotte *et al*, 2014).

With the exception of sucrose, the neural mechanisms underlying the incubation of food craving have not been investigated. Thus, we utilized behavioral, histological, electrophysiological, and biochemical analyses to examine structural and functional plasticity in primary reward circuit regions, the PFC and NAc, following incubation of craving for HF *vs* chow food rewards. The chow pellets were

manufactured to mimic the macronutrient composition of the standard rodent chow for the purpose of evaluating the effect of incubated food craving more generally. HF was chosen for comparison to assess the influence that palatability might have on the observed neurophysiological adaptations, given that we have previously shown that these pellets are preferred over the chow pellets (Darling *et al*, 2016).

MATERIALS AND METHODS

Animals

For biochemical studies, male Sprague–Dawley rats were purchased from Harlan and for all other studies, bred in house. All animals were housed in a temperature-controlled room with a 12:12-h light–dark cycle. All animals had free access to food and water throughout. In all studies, rats were 60–70 days old at the start of experimentation. All procedures were approved by the UW or UM Institutional Animal Care and Use Committee.

Diets

All rats were maintained on standard lab chow in the home cage throughout experimentation (#5001, Labdiet, St Louis, MO: ~29% protein, ~58% carbohydrate, and ~13% fat by kilocalorie; 3.36 kcal/g). During self-administration, rats either lever pressed for a custom chow pellet manufactured to closely match home-cage chow composition (#F0165, Bio-Serv, Flemington, NJ; ~25.3% protein, 64.5% carbohydrates, and 10.1% fat by kilocalorie; 3.35 kcal/g), or HF pellets (#F07062, Bio-Serv; ~19.4% protein, 60.8% fat, and 19.9% carbohydrates by kilocalorie; 3.72 kcal/g).

Food Self-Administration

Chow or HF self-administration was conducted in standard operant boxes (Med Associates, St Albans, VT; 30.5 cm \times 24.1 cm \times 21.0 cm). Each box contained two retractable levers. A response on the active lever resulted in the delivery of a chow or HF pellet (45 mg) and the presentation of a compound cue (light+tone; 5 s, 2900 Hz), followed by a 25-s timeout period. Responding on the inactive lever had no consequences, but was recorded. Prior to the first session, rats were fasted for 24 h, and placed into the chambers overnight for 14 h from 1800 to 0800 hours during which time they were able to lever press for either HF or chow rewards. After this session, rats were returned to their home cages for the remainder of the day. Subsequently, rats underwent daily food self-administration training (one 6 h session per day for 10 days) during which time active lever presses, inactive lever presses, and total pellets received were recorded. Incubation of craving was measured as previously described (Grimm *et al*, 2011) following 1 or 30 days post training. During this test, no food pellets were available and responding on the previously active lever resulted in the 5-s presentation of the food-paired compound cue followed by a 25-s timeout period.

Quantification of Dendritic Spine Density

Quantification of dendritic spine density followed methods previously described (Ferrario *et al*, 2005; Bloss *et al*, 2011;

Dingess *et al*, 2016). Briefly, immediately following incubation testing (1 day or 30 days), rats were anesthetized with isoflurane and killed via cardiac perfusion (200 ml 0.9% saline followed by 300 ml 1.5% paraformaldehyde (PFA; in 0.1 M phosphate buffer (PBS)). After washing in PBS, brains were coronally sectioned into 200 μ m slices with a Leica VT1200S vibratome (Leica, Buffalo Grove, IL) and collected in PBS. Slices were post-fixed (4% PFA; 20 min), incubated with Vybrant-DiI cell-labeling solution (1 : 200, Invitrogen, Carlsbad, CA; 1 h, RT), rinsed in PBS, and then placed in PBS (4 °C for 48 h) to allow dye diffusion across membranes. Finally, slices were mounted on glass slides with Vectashield (Vector, Burlingame, CA) and imaged using a Zeiss confocal microscope. Cortical pyramidal cells in the PFC were visually identified by their morphology (Yang *et al*, 1996). Dendritic spines were then quantified on third- and fourth-order basal dendrites and second- and third-order apical dendrites in output layer V of the prelimbic medial PFC (PL-mPFC) and IL-mPFC. Layer V of the PFC was chosen for analysis based upon previous findings from our laboratory, indicating a fat-induced decrease in spine density in this region (Dingess *et al*, 2016) and additionally because other rewarding substances have been shown to alter this deep layer specifically (Aghajanian and Marek, 1999; Mair and Kauer, 2007; Goriounova and Mansvelter, 2012; Kasanetz *et al*, 2013). Medium spiny neurons (MSNs) in the NAc were visually identified by their morphology (Preston *et al*, 1980; Wilson and Groves, 1980) and spines were quantified on third- and fourth-order dendrites. In both the PFC and NAc, the terminal 10 μ m of selected dendrites was analyzed. Spines were classified based on parameters previously described (Bloss *et al*, 2011; Dingess *et al*, 2016). Importantly, spines were classified as mushroom type if the ratio of head to neck diameter was >1.1 and the maximum head diameter was $>0.4 \mu$ m. In total, 24–56 dendrites were analyzed per dietary group (eight dendrites per animal, four from each hemisphere) for each brain region. Spine analyses were compared to handled, untrained, age-matched controls, and analyses were conducted blind to the experimental condition. Similar to previous studies (Shen *et al*, 2009; Dingess *et al*, 2016), statistical analyses were performed by averaging spine types from the eight observed dendritic segments within animals per brain region.

Synaptic Electrophysiology

To determine whether there was a change in glutamatergic transmission associated with incubation, whole-cell electrophysiology was conducted on coronal slices through the NAc-C immediately after incubation testing. Only one cell per rat was used for each experiment so that reported *n* values represent the number of animals. Recording conditions and solutions for whole-cell recordings were as previously described (Slaker *et al*, 2015). All experiments utilized cesium chloride (CsCl) internal solution with spermine (0.1 mM). MSNs within the NAc-C were identified by their morphology. For AMPA/NMDA ratios, neurons were held at +40 mV in 100 μ M picrotoxin and 50 μ M D-(–)-2-amino-5-phosphonopentanoic acid (d-APV) was added once a stable baseline was acquired. Peak AMPAR excitatory postsynaptic current (EPSC) amplitudes were measured at 20–25 min in d-APV, and this EPSC was

subtracted from 5 min averages of baseline EPSCs to obtain the peak NMDA receptor EPSC. Current–voltage (*I–V*) curves were generated following a stable responding after the addition of d-APV. All drugs and reagents were obtained from Sigma-Aldrich (St Louis, MO).

BS³ Crosslinking and Western Blotting

Established BS³ crosslinking, SDS-PAGE, and western blotting procedures were used to examine GluA1 and GluA2 surface *vs* intracellular expression, as in Boudreau *et al* (2012), at 1 or 30 days after chow self-administration training (1 or 30 days) without additional testing for incubation of ‘craving.’ Behavioral testing was eliminated to get a ‘snapshot’ of AMPAR expression prior to cue reintroduction as the observed functional changes in rectification (~10%) are at the limit of detection for biochemical analysis. Therefore, this measure was used to assess whether there was an available intracellular pool of CP-AMPA receptors that could be translocated to the synapse if necessary. We focused on GluA1 and GluA2 expression levels as they provide a good indication of changes in CP- and non-CP-AMPA receptor expression due to the fact that CP-AMPA receptors lack the GluA2 subunit (Hollmann *et al*, 1991; Isaac *et al*, 2007). BS³ crosslinked samples were heated in Laemmli sample treatment buffer with 5% b-mercaptoethanol (70 °C, 10 min), loaded (20 μ g protein), and electrophoresed on 4–15% Bis-Tris gradient gels under reducing conditions. Proteins were transferred onto PVDF membranes (Amersham Biosciences, Piscataway, NJ). Membranes were rinsed, blocked (1 h, RT, 5% (w/v) nonfat dry milk in TBS-Tween 20 (TBS-T; 0.05% Tween 20, v/v)), and incubated overnight (4 °C) with primary antibodies (1 : 1000 in TBS) to GluA1 (Thermo Scientific; PA1-37776) or GluA2 (NeuroMab, UC Davis/NIH: 75-002). Membranes were washed in TBS-T, incubated with HRP-conjugated secondary (Invitrogen, Carlsbad, CA; 1 h, RT), washed, and immersed in the chemiluminescence detecting substrate (GE Health-care, Piscataway, NJ). Images were acquired on film and Ponceau S (Sigma-Aldrich) was used to determine total protein. Bands of interest were quantified using Image J (NIH).

Statistical Analysis

All statistical tests were conducted using Prism 6 (GraphPad Software) using two-way and two-way repeated measures (RMs) ANOVAs as appropriate. When applicable, Sidak’s (behavioral, electrophysiological, and biochemical analyses), or Tukey’s (spine density) multiple comparison tests were used. All data are shown as mean \pm SEM.

RESULTS

Both chow and HF groups demonstrated proficiency of the behavioral task, evidenced by the preferential responding to the active *vs* inactive lever during training (Supplementary Figure S1B and C). Two-way RMs ANOVA revealed a significant day effect and significant diet effect for active lever pressing during testing. Both groups demonstrated an increase in active lever responding (incubation of craving) between 1 and 30 days post training (chow (*n* = 30) 1 day: 22.67 \pm 2.71, 30 days: 64.27 \pm 3.81; HF (*n* = 28) 1 day: 46.89 \pm 4.69, 30 days: 98.00 \pm 8.83, $F_{(1,56)} = 84.12$, $p < 0.001$,

Figure 1b); however, the HF group responded significantly more to the active lever than the chow group at both testing time points ($F_{(1,56)}=25.65$, $p<0.001$) and during training ($F_{(1,779)}=13.51$, $p<0.001$, Table 1). Unsurprisingly, two-way RMs ANOVA also revealed a significant day and diet effect for cue presentations received during testing ($F_{(1,56)}=86.30$, $p<0.001$; $F_{(1,56)}=23.28$, $p<0.001$, respectively, Figure 1d). There were no significant differences in body weight between groups across training or during cue-testing (Figure 1e and Supplementary Figure S1E).

Dendritic spine density and spine type in the PFC and NAc were quantified following incubation of craving testing at 1 and 30 days (Tables 2 and 3). Two-way RMs ANOVA revealed a significant effect of spine type and significant dietary effect on basal and apical dendrites of the IL-PFC at 1 and 30 days. Tukey's multiple comparison's test revealed that rats in the HF group exhibited a significant decrease in thin spine density on basal and apical dendrites of the IL-PFC when compared to naive controls at both 1 day (basal: HF ($n=4$): 6.13 ± 0.22 , naive ($n=3$): 7.50 ± 0.19 , $p<0.001$, Figure 2d; apical: HF: 6.16 ± 0.16 , naive: 7.21 ± 0.11 , $p<0.001$, Figure 2e) and 30 days (basal: HF ($n=6$): 6.39 ± 0.13 , naive ($n=4$): 7.59 ± 0.12 , $p<0.001$, Figure 2d; apical: HF: 6.29 ± 0.17 , naive: 7.44 ± 0.15 , $p<0.001$, Figure 2e). There were no significant differences in spine density observed within the prelimbic PFC (PL-PFC; Supplementary Figure S2).

Tukey's multiple comparison's test revealed that, at 1 day, the chow group exhibited an increase in spine density attributed to an increase in thin spines within the NAc-C compared to naive controls (chow ($n=4$): 9.97 ± 0.08 , naive ($n=3$): 9.38 ± 0.14 , $p<0.01$, Figure 3d). This significant trend was also observed in the NAc-Sh (chow ($n=4$): 9.81 ± 0.08 , naive ($n=3$): 9.29 ± 0.04 , $p<0.01$, Figure 3e). At 30 days, in both the NAc-C and NAc-Sh, the increase in spine density was maintained but was attributed to an increase in mushroom-type spines (NAc-sh: chow ($n=7$): 3.02 ± 0.07 , naive ($n=4$): 2.59 ± 0.11 , $p<0.01$, Figure 3e; NAc-C: chow ($n=7$): 3.07 ± 0.06 , naive ($n=4$): 2.72 ± 0.16 , $p<0.01$, Figure 3d). Curiously, the HF group did not exhibit any structural changes in the NAc-Sh different from the naive controls at either time point (Figure 3e). In the NAc-C, however, although there were no observable differences 1 day after training, the HF group showed an increase in mushroom-type spines 30 days following training (HF ($n=6$): 3.13 ± 0.09 , naive ($n=4$): 2.72 ± 0.16 , $p<0.001$, Figure 3d).

To further investigate the plasticity in the NAc-C, we utilized whole-cell electrophysiology. Two-way RMs ANOVA revealed that there was no significant dietary effect on AMPA/NMDA ratio ($F_{(1,14)}=0.02$, $p=0.88$) but that there was a significant day effect ($F_{(1,14)}=14.55$, $p<0.01$, Figure 4b) such that both groups exhibited an increase in AMPA/NMDA from 1 to 30 days (chow ($n=9$) 1 day: 1.95 ± 0.21 , 30 days: 3.08 ± 0.27 ; HF ($n=7$) 1 day: 1.86 ± 0.29 , 30 days: 3.09 ± 0.37). Interestingly, AMPA EPSCs of MSNs showed a significant increase in rectification index (RI) from 1 to 30 days in chow rats only (1 day ($n=8$): 0.89 ± 0.04 , 30 days ($n=6$): 1.15 ± 0.08 , $p<0.01$, Figure 4c), indicating an increase in CP-AMPA receptor-mediated synaptic transmission. The increase in AMPA/NMDA but the lack of change in RI in HF animals could be interpreted as an increase in

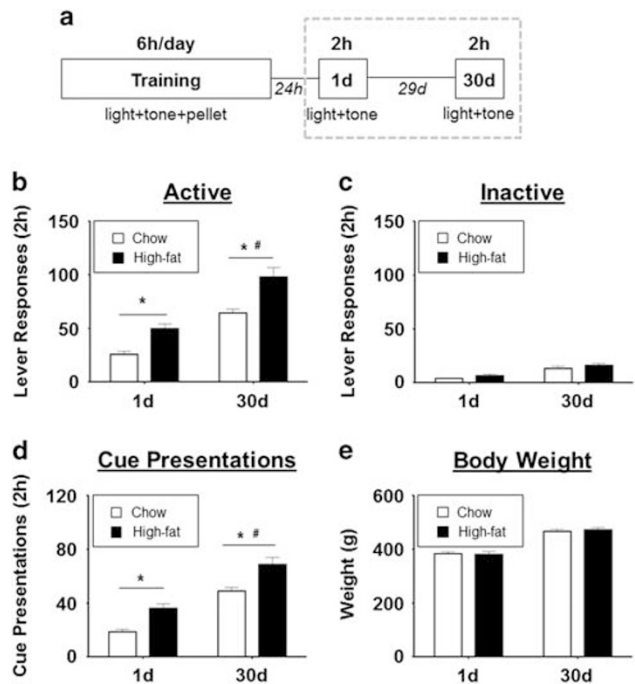


Figure 1 Both groups exhibit an incubation of craving effect but the response for HF pellets is greater than chow pellets at both 1 and 30 days post training. (a) Experimental timeline. Gray dotted line indicates portion of timeline from which data for b–e was measured. (b and c) Responses toward the active (b) and inactive (c) lever by chow ($n=30$, white) and high-fat ($n=28$, black) groups during incubation of craving testing 1 and 30 days post training. (d) Cue presentations received by chow and HF groups during incubation of craving testing 1 and 30 days post training. (e) Body weights during each cue presentation session. Data reflect only animals that underwent testing at both 1 and 30 days. *Indicates significant difference between chow vs HF. # Indicates significant difference between 1 and 30 days in both chow and HF groups. Values represent the mean \pm SEM ($p<0.05$).

Table 1 Active Lever Responses Throughout Experimental Timeline

Day	Chow	HF
1	137.0 \pm 16.8	178.7 \pm 20.2
2	101.0 \pm 11.0	98.4 \pm 11.4
3	90.7 \pm 8.2	109.8 \pm 11.3
4	91.8 \pm 7.6	107.6 \pm 10.4
5	107.2 \pm 9.2	127.1 \pm 9.5
6	116.7 \pm 12.0	132.4 \pm 8.8
7	106.4 \pm 9.4	133.0 \pm 12.4
8	123.4 \pm 13.7	134.8 \pm 12.4
9	103.1 \pm 11.5	109.0 \pm 8.9
10	109.2 \pm 11.1	154.6 \pm 16.5*
1 day	22.7 \pm 2.7	46.9 \pm 4.7*
30 days	64.3 \pm 3.8	98.0 \pm 8.8*

Values represent the mean \pm SEM (* $p<0.05$).

CI-AMPA-mediated transmission or alternatively a change in NMDA receptor strength (Franks and Isaacson, 2005; Tizabi *et al*, 2012; Counotte *et al*, 2014). Indeed, NMDA receptor expression is altered following exposure to both

Table 2 Summary of Dendritic Spine Quantification Following Cue Reintroduction at 1 day

	Basal			Apical		
	Naive	Chow	HF	Naive	Chow	HF
<i>PL-PFC</i>						
Filopodium	0.29 ± 0.04	0.33 ± 0.04	0.22 ± 0.06	0.29 ± 0.04	0.21 ± 0.04	0.22 ± 0.06
Thin	7.42 ± 0.22	7.50 ± 0.07	7.53 ± 0.08	7.29 ± 0.23	7.38 ± 0.07	7.47 ± 0.06
Stubby	1.63 ± 0.19	1.58 ± 0.04	1.66 ± 0.06	1.42 ± 0.17	1.54 ± 0.04	1.50 ± 0.05
Mushroom	2.58 ± 0.11	2.50 ± 0.13	2.56 ± 0.08	2.58 ± 0.11	2.58 ± 0.11	2.47 ± 0.11
Branched	0.25 ± 0.00	0.21 ± 0.08	0.22 ± 0.11	0.25 ± 0.07	0.13 ± 0.00	0.13 ± 0.05
Total	12.17 ± 0.27	12.13 ± 0.07	12.19 ± 0.19	11.83 ± 0.25	11.83 ± 0.18	11.88 ± 0.14
<i>IL-PFC</i>						
Filopodium	0.29 ± 0.08	0.29 ± 0.03	0.31 ± 0.04	0.29 ± 0.04	0.17 ± 0.04	0.34 ± 0.06
Thin	7.50 ± 0.19	7.52 ± 0.10	6.13 ± 0.22	7.21 ± 0.11	7.38 ± 0.07	6.16 ± 0.16
Stubby	1.71 ± 0.11	1.67 ± 0.09	1.72 ± 0.08	1.42 ± 0.15	1.46 ± 0.04	1.34 ± 0.06
Mushroom	2.42 ± 0.18	2.55 ± 0.08	2.38 ± 0.17	2.58 ± 0.11	2.58 ± 0.15	2.47 ± 0.16
Branched	0.17 ± 0.08	0.17 ± 0.07	0.16 ± 0.03	0.13 ± 0.00	0.13 ± 0.00	0.19 ± 0.08
Total	12.08 ± 0.44	12.20 ± 0.11	10.69 ± 0.15	11.63 ± 0.22	11.71 ± 0.15	10.50 ± 0.26
	Core			Shell		
	Naive	Chow	HF	Naive	Chow	HF
<i>Nucleus accumbens</i>						
Filopodium	0.21 ± 0.08	0.31 ± 0.04	0.33 ± 0.11	0.21 ± 0.04	0.34 ± 0.08	0.29 ± 0.04
Thin	9.38 ± 0.14	9.97 ± 0.08	9.50 ± 0.14	9.29 ± 0.04	9.81 ± 0.08	9.38 ± 0.07
Stubby	1.79 ± 0.11	1.66 ± 0.06	1.75 ± 0.07	1.58 ± 0.11	1.53 ± 0.06	1.58 ± 0.11
Mushroom	2.58 ± 0.11	2.75 ± 0.05	2.79 ± 0.15	2.58 ± 0.04	2.72 ± 0.03	2.58 ± 0.08
Branched	0.25 ± 0.00	0.28 ± 0.06	0.29 ± 0.04	0.13 ± 0.07	0.19 ± 0.04	0.33 ± 0.04
Total	14.21 ± 0.27	14.97 ± 0.12	14.67 ± 0.19	13.79 ± 0.15	14.59 ± 0.17	14.17 ± 0.04

Spine quantification in the prelimbic medial prefrontal cortex (PL-mPFC), infralimbic mPFC (IL-mPFC), and nucleus accumbens (NAc), respectively. Bold indicates significant decrease in high-fat (HF)-exposed animals compared to both chow-exposed animals and naive controls. Italics indicate significant increase in chow-exposed animals compared to both HF-exposed animals and naive controls. Values represent the mean ± SEM ($p < 0.05$).

natural rewards and drugs of abuse, which could account for changes in the AMPA/NMDA ratio (Huang *et al*, 2009; Brown *et al*, 2011; Pitchers *et al*, 2012; Counotte *et al*, 2014). Although the expression of NMDA receptors was not conclusively studied here, an evaluation of NMDAR decay revealed no significant time or dietary effects, suggesting a lack of change in subunit composition of this receptor (function). This observation, coupled with the results of our spine analysis (Figure 3), suggests that the observed changes in AMPA/NMDA are likely a result of increases in AMPAR-mediated transmission.

Given that chow groups showed a withdrawal-dependent increase in CP-AMPA, we next used BS³ crosslinking and western blotting approaches to determine whether AMPAR surface vs intracellular expression is altered following training and withdrawal only. An additional cohort of rats was trained on the chow self-administration task and NAc tissue was collected at 1 ($n=6$) or 30 days ($n=8$) post training without further behavioral testing (no incubation of craving assessment). This allowed us to determine whether

AMPA surface expression is altered prior to testing. Western blotting for GluA1 and GluA2 showed a time-dependent increase in GluA1 (two-way RM ANOVA; main effect of time: $F_{(1,12)} = 11.97$, $p = 0.0047$, Figure 5b), but not GluA2 protein expression (data not shown). Interestingly, GluA1 intracellular expression was increased at 30 days (two-way RM ANOVA; sig. time × location interaction: $F_{(1,12)} = 30.62$, $p = 0.0001$; Sidak's multiple comparison, intracellular 1 vs 30 days $p < 0.05$, Figure 5b). This accumulation of intracellular GluA1 in the absence of any changes in GluA2 is consistent with an accumulation of intracellular CP-AMPA across withdrawal. Taken together, with electrophysiological results above, these biochemical data suggest that CP-AMPA may be recruited to synapses from this intracellular pool during testing.

DISCUSSION

The purpose of this study was to determine the neural adaptations associated with the increase in operant

Table 3 Summary of Dendritic Spine Quantification Following Cue Reintroduction at 30 days

	Basal			Apical		
	Naive	Chow	HF	Naive	Chow	HF
<i>PL-PFC</i>						
Filopodium	0.16 ± 0.03	0.15 ± 0.03	0.15 ± 0.02	0.13 ± 0.05	0.20 ± 0.03	0.13 ± 0.01
Thin	7.44 ± 0.08	7.53 ± 0.12	7.54 ± 0.10	7.37 ± 0.14	7.48 ± 0.17	7.48 ± 0.12
Stubby	1.59 ± 0.06	1.68 ± 0.05	1.68 ± 0.07	1.56 ± 0.04	1.65 ± 0.07	1.62 ± 0.04
Mushroom	2.66 ± 0.06	2.53 ± 0.08	2.49 ± 0.05	2.53 ± 0.11	2.35 ± 0.06	2.50 ± 0.10
Branched	0.25 ± 0.05	0.25 ± 0.04	0.23 ± 0.05	0.13 ± 0.05	0.13 ± 0.04	0.23 ± 0.03
Total	12.09 ± 0.13	12.13 ± 0.45	12.09 ± 0.28	11.66 ± 0.16	11.80 ± 0.19	11.95 ± 0.46
<i>IL-PFC</i>						
Filopodium	0.22 ± 0.06	0.23 ± 0.06	0.28 ± 0.05	0.19 ± 0.08	0.20 ± 0.03	0.24 ± 0.02
Thin	7.59 ± 0.12	7.50 ± 0.19	6.39 ± 0.13	7.44 ± 0.15	7.43 ± 0.06	6.29 ± 0.17
Stubby	1.63 ± 0.11	1.70 ± 0.13	1.61 ± 0.09	1.63 ± 0.05	1.43 ± 0.06	1.51 ± 0.07
Mushroom	2.41 ± 0.16	2.53 ± 0.11	2.41 ± 0.08	2.47 ± 0.09	2.53 ± 0.08	2.53 ± 0.13
Branched	0.19 ± 0.04	0.23 ± 0.05	0.26 ± 0.03	0.22 ± 0.06	0.15 ± 0.05	0.15 ± 0.03
Total	12.03 ± 0.22	12.18 ± 0.08	10.94 ± 0.36	11.94 ± 0.12	11.73 ± 0.08	10.72 ± 0.38
	Core			Shell		
	Naive	Chow	HF	Naive	Chow	HF
<i>Nucleus accumbens</i>						
Filopodium	0.19 ± 0.04	0.30 ± 0.04	0.24 ± 0.06	0.22 ± 0.06	0.17 ± 0.03	0.22 ± 0.50
Thin	9.53 ± 0.21	9.60 ± 0.13	9.63 ± 0.09	9.50 ± 0.25	9.65 ± 0.05	9.53 ± 0.12
Stubby	1.72 ± 0.08	1.76 ± 0.06	1.72 ± 0.06	1.53 ± 0.08	1.39 ± 0.06	1.58 ± 0.10
Mushroom	2.72 ± 0.16	3.07 ± 0.06	3.13 ± 0.09	2.59 ± 0.11	<u>3.02 ± 0.07</u>	2.39 ± 0.15
Branched	0.28 ± 0.06	0.20 ± 0.04	0.33 ± 0.08	0.16 ± 0.03	0.23 ± 0.06	0.18 ± 0.03
Total	14.44 ± 0.13	14.94 ± 0.22	15.05 ± 0.22	14.00 ± 0.17	<u>14.47 ± 0.15</u>	13.90 ± 0.32

Spine quantification in the prelimbic medial prefrontal cortex (PL-mPFC), infralimbic mPFC (IL-mPFC), and nucleus accumbens (NAc), respectively. Bold indicates significant decrease in high-fat (HF)-exposed animals compared to both chow-exposed animals and naive controls. Underlined italics indicate significant increase in chow-exposed animals compared to both HF-exposed animals and naive controls. Bold italics indicate significant increase in both chow- and HF-exposed animals compared to naive controls. Italics indicate chow-exposed animals greater than HF-exposed animals only. Values represent the mean ± SEM ($p < 0.05$).

responding for food cues following periods of abstinence from the primary reinforcer. In addition, we aimed to assess whether palatability influenced these neural adaptations. Our results demonstrated a time-dependent increase in active lever responding for both chow and HF (incubation of ‘craving’, Figure 1b). Although the chow pellet was manufactured to mimic the macronutrient composition of the home-cage diet, the incubation of chow craving was not unexpected given that previous studies have indicated that this macronutrient composition is sufficient to induce this phenomenon (Krasnova *et al*, 2014; Darling *et al*, 2016). Research from our laboratory indicates that incubation of craving for chow pellets may be the result of novelty as incubation fails to develop when rats have prior experience with the pellets (achieved by rearing rats exclusively on chow pellets rather than the standard lab chow; Darling *et al*, 2016).

The results of the current study corroborate our previous work that rats display an amount of incubated craving for novel food pellets, suggesting that the incubation of craving

model may not always reflect ‘craving’ or indicate the level of ‘addiction’ and may rather be inherent to the operant task itself. Thus, we use behavioral responsiveness beyond what is observed for chow pellets as an indicator of reward value. Indeed, although the magnitude of incubation was not different between groups relative to their own baseline, the behavioral response to HF cues was significantly greater than the response to chow cues at both time points (Figure 1b), indicating that the palatability of food enhances the behavioral response to its associated cues. It should be noted that while the chow and HF pellets were distinct in their macronutrient composition, they were similar in their overall caloric density, and thus the greater responsiveness to HF cues is likely due to fat as a macronutrient rather than differences in caloric density. Interestingly, this is different than what we observed in our previous study, which demonstrated that both chow and HF groups trained for 2 h exhibit equivalent lever responding at both 1 and 30 days (Darling *et al*, 2016). This supports work within the drug abuse field that extended access paradigms can escalate

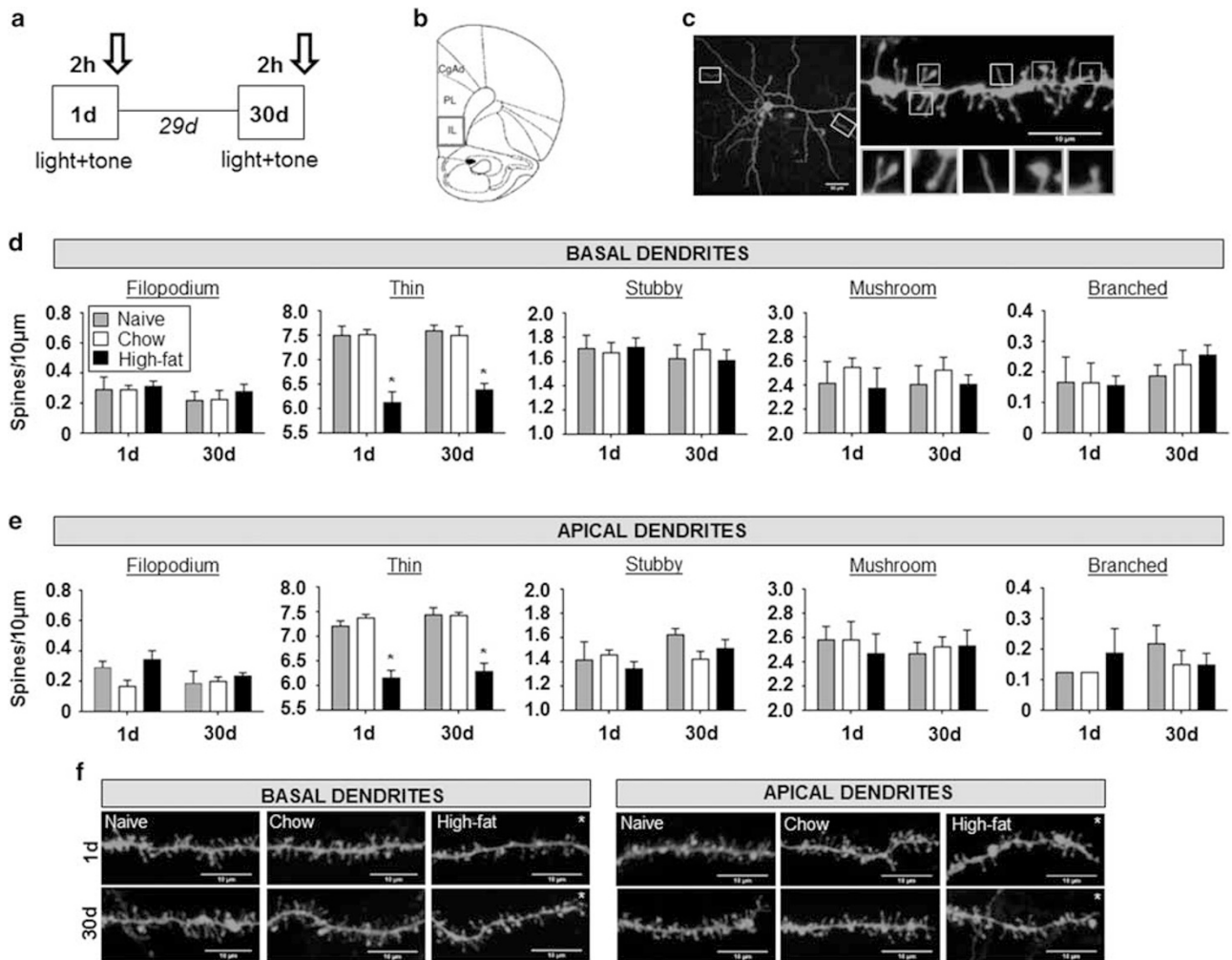


Figure 2 High-fat-exposed animals exhibit a reduction of thin spines in the infralimbic prefrontal cortex (IL-PFC) at both 1 and 30 days. (a) Experimental timeline. Arrows indicate times at which spine analysis was conducted. (b) Schematic of region analyzed. (c) Representative image of a Dil-stained pyramidal cell in the medial PFC (mPFC). White rectangles indicate example regions where spine analysis on basal and apical dendrites was conducted (left). Example basal dendrite of the mPFC acquired from a naive animal (right). Green, orange, yellow, blue, and purple squares highlight branched-, thin-, filopodium-, mushroom-, and stubby-type spines, respectively. (d and e) Quantification of spine types on basal (d) and apical (e) dendrites in the IL-PFC following cue reintroduction at 1 day (naive (gray), $n = 24/3$, where the first and second numbers indicate number of dendrites and animals, respectively; chow (white), $n = 32/8$; high fat (black), $n = 32/8$) and 30 days (naive, $n = 32/4$; chow, $n = 40/5$; high fat, $n = 48/6$) post training. (f) Example dendrites from basal (left) and apical (right) dendrites from each dietary condition at 1 and 30 days. Values represent the mean \pm SEM ($*p < 0.05$). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

reward seeking and more accurately recapitulate compulsive use (Wee *et al*, 2007).

Dendritic spine analyses of the NAC revealed that chow animals exhibited an overall increase in spine density, attributed to an increase in thin-type spines at 1 day and mushroom-type spines at 30 days (Figure 3d and e), compared to naive controls. Mushroom spines are considered to be mature synapses that arise in parallel with long-term potentiation (Toni *et al*, 1999; Yuste and Bonhoeffer, 2001; Matsuzaki *et al*, 2004) and they contain relatively more AMPARs and AMPA-mediated current than other spine types (Matsuzaki *et al*, 2001; Bourne and Harris, 2007), making them a likely candidate location for alterations in AMPAR structure and function to occur. Although direct correlations between spine structure and function were not addressed in this study, and changes in spine density do not always parallel changes in AMPA/NMDA (Shen *et al*, 2011),

it is hypothesized that newly formed synapses on thin spines matured from 1 to 30 days post training, correlating with the shift in spine type as well as the observed alterations in AMPA/NMDA ratio (Figure 4). Because of the observation that similar alterations in spine density have been reported following withdrawal from reward in the absence of cue reintroduction (Robinson and Kolb, 1999; Ferrario *et al*, 2005), we cannot exclude that the observed changes in spine density may be time-dependent rather than cue-dependent. In addition, we cannot rule out that the accumbens associated plasticity, particularly the AMPA/NMDA results, are not the result of the operant procedure itself, independent of reward incentive, as both the chow and HF group showed similar changes. We should point out though that macronutrient profile did have an influence on spine density within the NAc-C and if all the changes could be accounted simply due to operant conditioning we might expect to see

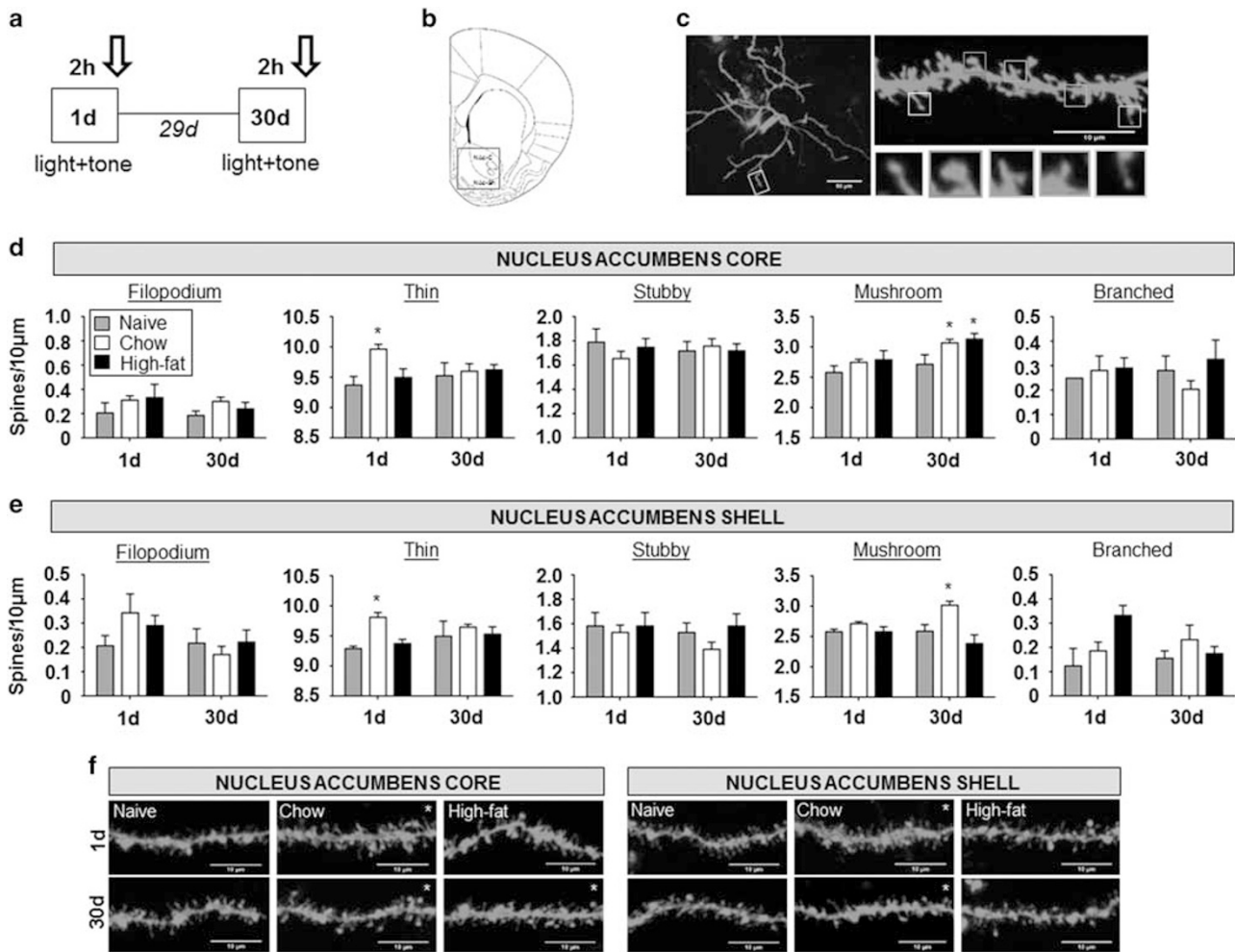


Figure 3 Incubation of chow craving is associated with an increase in thin and mushroom spines in the nucleus accumbens (NAc) at 1 and 30 days, respectively. (a) Experimental timeline. Arrows indicate times at which spine analysis was conducted. (b) Schematic of region analyzed. (c) Representative image of a Dil-stained medium spiny neuron in the NAc. White rectangle indicates example region where spine analysis on dendrites was conducted (left). Example dendrite of the NAc acquired from a naive animal (right). Yellow, blue, green, purple, and orange squares highlight filopodium-, mushroom-, branched-, stubby-, and thin-type spines, respectively. (d and e) Quantification of spine types from dendrites of the NAc core (d) and NAc shell (e) following cue reintroduction at 1 day (naive (gray), $n = 24/3$, where the first and second numbers indicate number of dendrites and animals, respectively; chow (white), $n = 32/8$; high fat (black), $n = 24/3$) and 30 days (naive, $n = 32/4$; chow, $n = 56/7$; high fat, $n = 48/6$) post training. (f) Example dendrites of the NAc core (left) and NAc shell (right) from each dietary condition at 1 and 30 days. Values represent the mean \pm SEM (* $p < 0.05$). A full color version of this figure is available at the *Neuropsychopharmacology* journal online.

similar spine alterations in both groups immediately following training (1 day). Curiously, despite demonstrating an increase in mushroom spines at 30 days, the HF group failed to exhibit an increase in thin spines at 1 day in the NAc-C (Figure 3). Moreover, the dendritic spine changes we observed in the PFC recapitulated changes we previously saw in animals that were non-contingently exposed to 60% HF in their home cages (Dingess *et al*, 2016), suggesting that the spine alterations in the cortex are independent of learning and rather the result of macronutrient exposure.

In the NAc-Sh, the HF group did not exhibit any changes in spine density compared to naive controls. Although further investigation would be required to decipher the cause of this result, it is possible that the loss of thin-type spines in the IL-PFC, a region that projects to the NAc-Sh, may be a culprit. The staining technique employed in this study however does not allow for the identification of specific output targets and thus cannot be used to answer this

question. It is worth noting that based upon our previous research (Dingess *et al*, 2016), we suspected that fat exposure might alter spine density in the PFC, but it is intriguing that a relatively brief exposure to HF (6 h daily for 10 days) is sufficient to induce this plasticity independent of obesity, and that it is so long-lasting.

In addition to structural plasticity, we examined functional adaptations in the NAc-C. This region was chosen for analysis because increases in mature mushroom spines were found at 30 days in both chow and HF groups and also because AMPAR transmission in the NAc-C is tightly linked to incubation of reward craving (Conrad *et al*, 2008; Wolf and Ferrario, 2010; Counotte *et al*, 2014; Christian *et al*, 2016). The AMPA/NMDA ratio increased from 1 to 30 days in both the chow and HF groups, consistent with the addition of mature synapses (Figure 4b) and with a role for glutamatergic transmission in the incubation of craving. It should be noted that, although the AMPA/NMDA ratio has

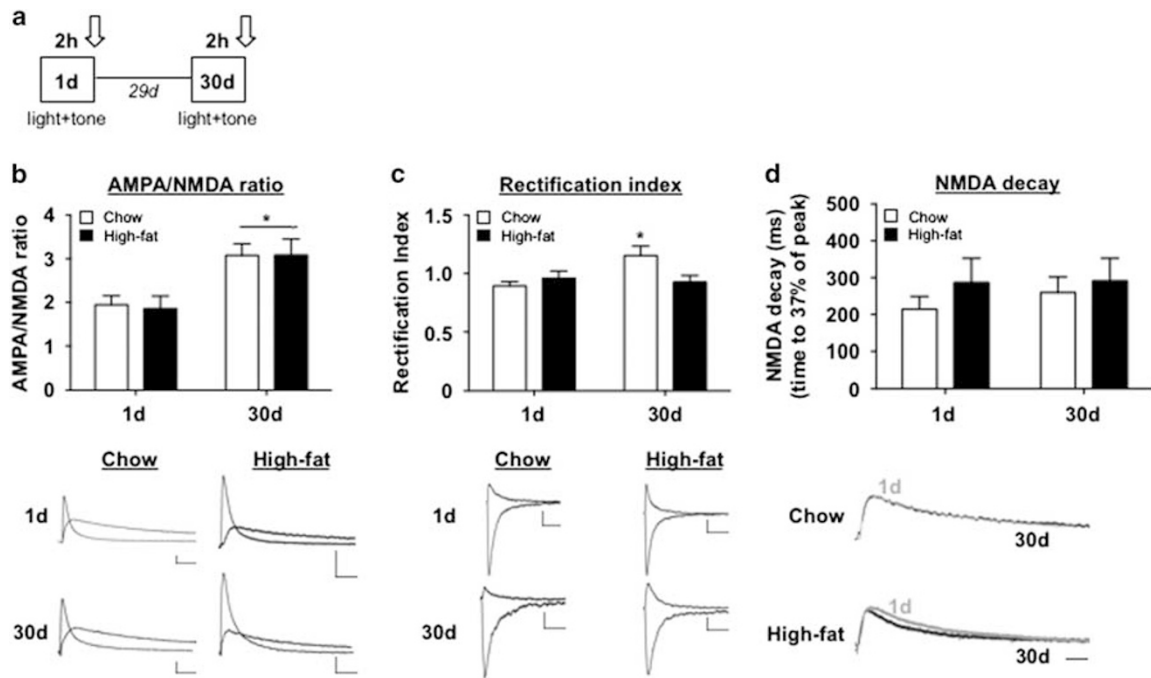


Figure 4 In the NAc-C, incubation of chow and HF craving is associated with an increase in AMPA/NMDA ratio and an increase in CP-AMPA and CI-AMPA, respectively. (a) Experimental timeline. Arrows indicate times at which analysis was conducted. (b) Average α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) to *N*-methyl-D-aspartate (NMDA) ratio in the nucleus accumbens core (NAc-C) following cue reintroduction 1 or 30 days post training in chow ($n=9$, white) and high fat ($n=7$, black) groups. Representative AMPA and NMDA traces (below). Vertical scale bar indicates 50 pA; horizontal scale bar indicates 50 ms. (c) Average rectification index of AMPA receptor EPSCs for chow ($n=8$) and high fat ($n=6$). Representative traces of AMPA EPSCs (below). (d) NMDA decay. Example traces (below). Values represent the mean \pm SEM ($*p < 0.05$).

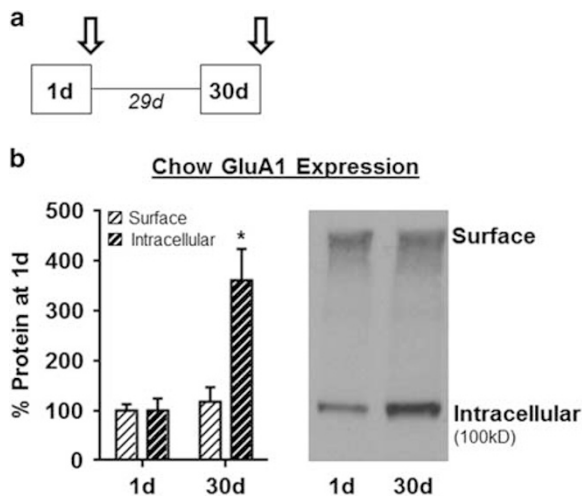


Figure 5 Reintroduction to chow-paired cues elicits an increase in intracellular GluA1 expression at 30 days post training. (a) Experimental timeline. Arrows indicate times at which analysis was conducted. (b) GluA1 surface (white dashed) and intracellular (black dashed) expression in the NAc (combined core and shell) following 1 ($n=6$) or 30 days ($n=8$) removal from reward and cues (left). Representative western blot (right). Values represent the mean \pm SEM ($*p < 0.05$).

been demonstrated to increase over the developmental period (Hsia *et al*, 1998), it stabilizes at the onset of adulthood and, thus, the observed increase in AMPA/NMDA is unlikely to be age-dependent and rather be an effect of the incubation of craving for food cues. As all the rats utilized for

electrophysiological analysis underwent self-administration training, we cannot rule out that the AMPA/NMDA ratio is elevated at 1 day beyond what might be expected for naive rats. However, the electrophysiological studies were employed specifically to further investigate whether the spine-type shift in the NAc from thin to mushroom (Figure 3) was correlated to functional changes in AMPAR transmission. Naive animals were excluded from this experiment due to their lack of significant change in spine type.

To further investigate the type of AMPA-mediated current involved in the AMPA/NMDA ratio shift, *I-V* plot analysis was conducted. Interestingly, our results revealed an increase in inwardly rectifying AMPA receptor currents and therefore RI (both hallmarks of CP-AMPA) from 1 to 30 days in the chow group but not the HF group (Figure 4c). The lack of an increase in CP-AMPA-mediated transmission in the HF group was curious given that others have demonstrated that exposure to palatable food induces an increase in CP-AMPA (Tukey *et al*, 2013; Oginsky *et al*, 2016) in this region. A key distinction however is that in our study, analysis was conducted following reintroduction to food cues, rather than mere exposure to the food. In fact, our rats had not been exposed to 60% HF for 30 days.

A separate group of rats was trained to self-administer chow pellets and NAc tissue was collected on 1 or 30 days without additional testing (ie, no cue exposure). Western blotting for GluA1 and GluA2 showed a time-dependent increase in intracellular but not surface expression of GluA1 and no change to GluA2 protein expression (Figure 5b), a result that suggests an increase in the availability of intracellular CP-AMPA. It is therefore hypothesized that

the increase in CP-AMPA-mediated transmission following the expression of incubation of chow craving is the result of rapid synaptic insertion of CP-AMPA receptors from this intracellular pool in response to testing. This possibility will be addressed in future studies.

In conclusion, for the first time, our results demonstrate reward circuit neuroadaptations that were associated with time-dependent increases in lever responding for chow and HF food rewards (incubation). Although food-associated cues and/or food novelty may drive neuronal adaptations in the NAc that facilitate general food-seeking behavior, we suspect that fat-induced plasticity in the PFC accounts for the robust response to HF cues and ultimately promotes maladaptive feeding. Future studies will examine specific inputs that may be driving this behavior and utilize this knowledge to develop novel therapeutic interventions.

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