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## Involvement of cholinergic mechanisms in the behavioral effects of dietary fat consumption

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### Abstract

Clinical reports suggest a positive association between fat consumption and the incidence of hyperactivity, impulsivity and cognitive abnormalities. To investigate possible mechanisms underlying these disturbances under short-term conditions, we examined in Sprague-Dawley rats the influence of 7-day consumption of a high-fat diet (HFD) compared to chow on anxiety, novelty-seeking and exploratory behaviors and also on acetylcholine (ACh) neurotransmission that may mediate these behaviors. The HFD consumption, which elevated circulating fatty acids but produced no change in caloric intake or body weight, stimulated novelty-seeking and exploration in an open field, while reducing anxiety in an elevated plus maze. Using the Ellman assay to measure ACh esterase (AChE) activity that breaks down ACh, the second experiment showed HFD consumption to significantly reduce AChE activity in the frontal cortex, hypothalamus and midbrain. With measurements of [<sup>125</sup>I]-epibatidine or [<sup>125</sup>I]-bungarotoxin binding to nicotinic ACh receptors (nAChRs) containing  $\beta 2$  or  $\alpha 7$  subunits, respectively, the results also showed HFD consumption to increase both  $\beta 2$ -nAChR binding in the medial prefrontal cortex and substantia nigra and  $\alpha 7$ -nAChR binding in the lateral and ventromedial hypothalamus. When treated with an acute dose of the nicotinic antagonist, mecamylamine (0.5 mg/kg, sc), the HFD animals responded with significantly reduced exploratory and novelty-seeking behaviors, whereas the chow-consuming rats exhibited no response. These findings suggest that the exploratory and novelty-seeking behaviors induced by dietary fat may be mediated by enhanced nicotinic cholinergic activity, which is accompanied by increased density of  $\beta 2$ -nAChRs in cortical and midbrain regions associated with impulsivity and locomotor activity and of  $\alpha 7$ -nAChRs in hypothalamic regions associated with arousal and energy balance.

### Keywords

Acetylcholine; nicotinic receptors; rat; novelty-seeking; high-fat diet

### 1. Introduction

The consumption of palatable, fat-rich diets has increased significantly over the past several decades (WHO, 2003). In addition to contributing to the worldwide epidemic of obesity (Harrington and Elliott, 2009), this rise in high-fat diet (HFD) consumption has been linked

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to hyperactivity in children, impulsivity in adolescents, and cognitive deficits in adults (Crichton et al., 2010; Pauli-Pott et al., 2010; Wiles et al., 2009). There are only a few reports in animals examining the behavioral effects of dietary fat intake. Recent studies show chronic consumption of a HFD to cause deficits in memory and learning in adult mice (Greenwood and Winocur, 2005; Valladolid-Acebes et al., 2010; Winocur et al., 2005) and reduce anxiety in adult rats (Maniam and Morris, 2010). Additionally, in very young animals or animals exposed to an acute stressor, HFD consumption stimulates exploration in an open field, a behavior related to novelty-seeking (Boukouvalas et al., 2008; Soulis et al., 2007). Binging on fat is also found to predispose animals to increased cocaine-seeking and -taking behavior (Puhl et al., 2011), suggesting that this diet may generally enhance risk-taking behaviors. Whereas this ability of dietary fat to produce behaviors related to emotion, impulse control and cognition is becoming more generally recognized, the neurobiological mechanisms underlying these effects, especially during the initial pre-obesity stages of unhealthy eating, remain to be determined.

The neurotransmitter, acetylcholine (ACh), which is known to influence behaviors related to novelty-seeking and exploration through its actions in various cortical and subcortical brain areas, is likely to have a role in mediating the changes in emotional behaviors induced by HFD consumption. Pharmacological studies with specific agonists and antagonists suggest that ACh via nicotinic ACh receptors (nAChRs) acts in the medial prefrontal cortex (mPFC) to increase impulsive behaviors (Raybuck and Gould, 2010) and the central nucleus of the amygdala (CeA) to stimulate anxiety (Zarrindast et al., 2008). Additionally, in the substantia nigra (SN) and ventral tegmental area (VTA), the activation of nAChRs stimulates locomotor- and reward-related behaviors (Alburges et al., 2007; Wise, 2009). Within regions of the hypothalamus such as the lateral hypothalamus (LH), ventromedial hypothalamus (VMH) and paraventricular hypothalamic nucleus (PVN), nAChR's have been shown to control arousal, stress-responding and energy balance (Imaki et al., 1995; Pasumarthi and Fadel, 2010; Ribeiro et al., 2007; Takahashi et al., 1995; Yoburn and Glusman, 1984). In the paraventricular nucleus of the thalamus (PVT), cholinergic stimulation regulates states of arousal and cognition (Pasumarthi and Fadel, 2008). These behavioral effects may be receptor- and site-specific, with the high affinity  $\beta 2$  subunit-containing nAChRs ( $\beta 2$ -nAChRs) predominating in extra-hypothalamic regions and the  $\alpha 7$  subunit-containing nAChRs ( $\alpha 7$ -nAChRs) predominating in hypothalamic regions (Marks et al., 1996; Tribollet et al., 2004; Zaninetti et al., 2002). While this evidence is consistent with the idea that nicotinic cholinergic signaling may mediate the behavioral changes induced by dietary fat, the effects that HFD consumption has on this system have yet to be characterized. There is only one animal study directly investigating this possibility, showing long-term (6 months) fat consumption that increased weight gain to reduce the activity in the cortex and hypothalamus of the enzyme, acetylcholinesterase (AChE), which breaks down ACh (Kaizer et al., 2004). Additional *in vitro* studies have also suggested that free fatty acids (FFA), which are elevated both peripherally and centrally by fat intake (Ahren et al., 1997; Buettner et al., 2000), can inhibit AChE activity and enhance the activity of nAChRs (Nishizaki et al., 1997; Nishizaki et al., 1999; Vajreswari et al., 2002).

To investigate the relationship of dietary fat to cholinergic function in the brain, this study in Sprague-Dawley rats, which are similar in their caloric intake and body weight but differ in their serum FFA levels, was designed to: 1) characterize the disturbances in anxiety, novelty-seeking, and exploration produced by short-term HFD consumption; 2) determine whether these HFD-induced behaviors are associated with changes in cholinergic neurotransmission and nicotinic receptor binding in particular brain regions; and 3) test directly whether blocking the nicotinic cholinergic system attenuates the behaviors induced by fat consumption. The overall hypothesis being tested was that fat consumption stimulates

ACh and nAChRs in specific brain regions and that this neurochemical change contributes to the HFD-induced disturbances in anxiety, novelty-seeking and exploratory behaviors.

## 2. Results

### 2.1 Experiment 1: Behavioral and metabolic changes induced by a high-fat diet

The fat- and chow-consuming rats were tested for anxiety in an elevated plus maze (EPM) or for novelty-seeking and exploration in an open field activity chamber with a novel object, and also their serum was measured for levels of circulating FFA. The results showed that the rats consuming a HFD for 7 days ( $n=8$ ), compared to the chow rats ( $n=8$ ), ate a similar number of daily calories ( $115\pm 6$  vs  $99\pm 5$  kcal,  $p=0.36$ , *not significant, ns*) and were similar in body weight ( $312\pm 7$  vs  $308\pm 6$  g,  $p=0.58$ , *ns*) but had markedly elevated FFA levels ( $0.31\pm 0.03$  vs  $0.12\pm 0.01$ ,  $p<0.05$ ). Using a RM-ANOVA, we identified a significant interaction between diet and multiple behaviors in the EMP [ $F(2,24)=4.91$ ,  $p<0.05$ ], with the HFD rats compared to the chow rats being less anxious. This was indicated by a 50% increase in percent of time spent in the open arm of the EPM ( $p<0.05$ ) and a significant reduction in percent of time spent in the center square ( $-27\%$ ,  $p<0.05$ ), with no difference in percent of time spent in the closed arm (*ns*) (Fig. 1a). In addition, the measures of open field activities revealed a significant interaction [ $F(3,32)=4.21$ ,  $p<0.05$ ] between diet and behaviors such as the rats' interaction with a novel object (time with object and zone entries) and their rearing behavior (vertical counts). The HFD-consuming rats spent more time interacting with the novel object ( $+38\%$ ,  $p<0.05$ ) and displayed enhanced rearing behavior ( $+40\%$ ,  $p<0.05$ ), a sign of exploration, and they showed no differences in entries made into the quadrant containing the novel object (*ns*) or in ambulatory distance (*ns*) (Fig. 1b), suggesting a lack of effect on general motor-related behaviors. These findings demonstrate that short-term consumption of fat, while significantly elevating FFA levels without affecting caloric intake and body weight, reduces anxiety and promotes certain behaviors related to novelty-seeking and exploration.

### 2.2 Experiment 2: Changes in AChE activity induced by a high-fat diet

This experiment was designed to examine cholinergic neurotransmission by measuring the activity of AChE, an enzyme responsible for the breakdown of ACh, in the frontal cortex, hypothalamus, midbrain region and cerebellum. The animals eating HFD ( $n=8$ ) compared to chow diet ( $n=8$ ) for 7 days showed similar values of caloric intake ( $121\pm 5$  vs  $129\pm 6$  kcal,  $p=0.75$ , *ns*) and body weight ( $347\pm 10$  vs  $335\pm 8$  g,  $p=0.54$ , *ns*). The data demonstrated that the two diets differed in their effects on AChE [ $F(3,32)=4.0$ ,  $p<0.05$ ]. Specifically, the HFD, which significantly elevated FFA ( $0.36\pm 0.02$  vs  $0.16\pm 0.009$  mEq/L,  $p<0.05$ ), markedly reduced the activity of AChE in the four brain areas (Fig. 2). This change was somewhat larger in the frontal cortex ( $-48\%$ ,  $p<0.05$ ) than the hypothalamus ( $-28\%$ ,  $p<0.05$ ) and midbrain area ( $-22\%$ ,  $p<0.05$ ), with no change observed in the cerebellum (*ns*). With reduced AChE activity normally associated with increased ACh content in the synaptic cleft (Hartmann et al., 2007), these results suggest that ACh levels may be enhanced in these brain regions in rats ingesting a fat-rich diet.

### 2.3 Experiment 3: Changes in nAChR binding induced by a high-fat diet

Quantitative autoradiography was used to measure the binding of [ $^{125}$ I]-epibatidine to  $\beta 2$ -nAChRs and of [ $^{125}$ I]-bungarotoxin (BTX) to  $\alpha 7$ -nAChRs in several brain nuclei of fat- and chow-consuming rats ( $n=8$ /group). Once again, these two groups had similar caloric intake ( $118\pm 8$  vs  $112\pm 5$  kcal,  $p=0.68$ , *ns*) and body weights ( $393\pm 12$  vs  $379\pm 10$  g,  $p=0.41$ , *ns*) during their 7-day feeding regime. In both groups, the pattern of specific binding of these two ligands rarely overlapped, with intense [ $^{125}$ I]-BTX binding found in the hypothalamus and amygdala and [ $^{125}$ I]-epibatidine binding found in the thalamus and midbrain, with only

the cortex showing binding of both ligands, as previously described (Pauly et al., 1991; Tribollet et al., 2004). The specific binding of [<sup>125</sup>I]-epibatidine to β2-nAChRs was dense in the mPFC region, SNc, PVT and VTA, yielding binding values within a range of (229–328 fmol/mg brain protein) but very little binding in the hypothalamus and amygdala, and the binding of [<sup>125</sup>I]-BTX to α7-nAChRs was clearly visible in the different hypothalamic areas as well as the mPFC and CeA, although highest in the LH and mPFC (260–331 fmol/mg brain protein), lower in the PVN, VMH and CeA (80–165 fmol/mg brain protein), and absent in the PVT.

When comparing the HFD- to chow-consuming rats (n=8/group), quantitative analysis of [<sup>125</sup>I]-epibatidine binding (fmol/mg brain protein) showed an interaction between brain area and diet [ $F(3,32)=4.23, p<0.05$ ]. Post hoc comparisons revealed a small but significant increase in β2-nAChRs, specifically in the mPFC (+10%,  $p<0.05$ ) and SNc (12%,  $p<0.05$ ) but not the VTA (*ns*) (Table 1). A significant interaction effect between brain area and diet was also detected with [<sup>125</sup>I]-BTX binding [ $F(4,40)=4.08, p<0.05$ ]. In contrast to β2-nAChR density, the binding of [<sup>125</sup>I]-BTX to α7-nAChR sites in the HFD compared to chow rats, while unaltered in the mPFC (*ns*) and CeA (*ns*), was significantly increased in two areas of the hypothalamus, the LH (11%,  $p<0.05$ ) and VMH (25%,  $p<0.05$ ), but not in the PVN (*ns*) (Table 1). This stimulatory effect of HFD consumption on β2-nAChR binding in the mPFC and SNc and α7-nAChR binding in the LH and VMH is clearly depicted in the autoradiographs presented in Fig. 3. Collectively, these binding data indicate that the consumption of a HFD can lead to both a receptor-specific and site-specific stimulation of nAChR binding.

#### 2.4 Experiment 4: Attenuation of HFD-induced behaviors by administration of mecamylamine

With evidence in Experiments 1 and 3 showing high fat consumption to increase novelty-seeking behavior and nicotinic receptor binding in cortical, midbrain and hypothalamic regions, this last experiment was performed to determine whether peripheral administration of a general nicotinic antagonist can block the effects of one-week HFD consumption on behaviors related to novelty-seeking and exploration. A two-way ANOVA was used to compare four specific behaviors (ambulatory distance, vertical counts, time in zone with novel object, and number of entries into novel object quadrant) of animals (n=8/diet/treatment) fed a HFD or chow diet after a single injection of saline or mecamylamine at a low dose of 0.5 mg/kg. Collectively, the results demonstrated that mecamylamine, while not altering these behaviors in chow-consuming rats, was able to attenuate the novelty-seeking and exploratory behaviors induced by the HFD (Fig. 4). While the four treatment groups showed no differences in ambulatory distance [ $F(1,30)=1.23, ns$ ], analysis of their rearing behavior (vertical counts) revealed a significant interaction between diet and drug [ $F(1,30)=5.94, p<0.025$ ], with post-hoc comparisons showing the 35% increase ( $p<0.05$ ) in rearing of the HFD-saline compared to chow-saline rats to be significantly attenuated by mecamylamine (*ns* vs. chow-saline) (Fig. 4). This is in contrast to the chow-fed animals, which showed no changes in rearing behavior (*ns*) after this drug, with only an upward trend ( $p=0.06$ ) detected between the chow-saline and chow-mecamylamine rats in measures of vertical counts. This significant drug × diet interaction, while not seen with the measure of time spent in the zone containing the novel object [ $F(1,30)=1.89, ns$ ], was clearly evident with the measure of entries made into this zone [ $F(1,30)=5.32, p<0.025$ ], which revealed a 32% increase in entries ( $p<0.05$ ) in the HFD-saline group compared to the chow-saline rats. Similar to the results with rearing behavior, mecamylamine had no effect in the chow animals (*ns*) but, in the HFD rats, significantly reduced the number of entries into the quadrant containing the novel object by 40% ( $p<0.05$ ) compared to the HFD-saline group, bringing this measure to similar values as the chow-saline control group (*ns*). These results

provide evidence that acute treatment with this nicotinic cholinergic antagonist can attenuate the novelty-seeking and exploratory behaviors induced by the HFD.

### 3. Discussion

The goal of this study was to determine whether short-term HFD consumption, in rats with normal caloric intake and body weight, can lead to abnormal behavioral patterns that may be linked to changes in cholinergic neurotransmission. The findings demonstrate that rats consuming a diet rich in fat, resulting in increased circulating levels of FFA, exhibit reduced anxiety along with greater novelty-seeking and exploratory behaviors. These behavioral changes in fat-consuming rats are accompanied by an increase in cholinergic activity and nAChR binding in select brain regions and are attenuated with peripheral administration of the nicotinic antagonist, mecamylamine. These findings provide evidence suggesting that short-term consumption of fat can contribute to abnormal patterns of behavior, possibly by stimulating nicotinic cholinergic neurotransmission in specific hypothalamic and extra-hypothalamic brain regions.

#### 3.1 Consumption of a high-fat diet leads to changes in behavioral patterns

In rats consuming a HFD compared to a chow diet with lower fat content, the results of the first experiment revealed a significant reduction in anxiety in an EPM and increase in novelty-seeking and exploratory behavior in an open field activity chamber. This finding is consistent with published studies, showing fat consumption to reduce stress sensitivity in rodent models (Maniam and Morris, 2010; Prasad and Prasad, 1996; Teegarden and Bale, 2007). With the EPM often used to measure exploratory behavior (Moreira et al., 2007; Rodgers and Dalvi, 1997), our finding may also signify enhanced exploration of a novel environment, in this case of the open arm, resulting from reduced anxiety. With more direct measures of exploration and novelty-seeking, our findings demonstrate that rats consuming a HFD are more likely to explore an open field and interact with a novel object. Consistent with this are other reports in prepubescent or acutely-stressed fat-consuming animals, which with measures of sniffing and open field activity provide some evidence for increased exploratory and locomotor behavior (Boukouvalas et al., 2008; Soulis et al., 2007). With our HFD and chow rats exhibiting no baseline differences in their locomotor activity, perhaps because of the two acclimation sessions they had prior to novelty testing in the open field, our results additionally indicate that the changes in anxiety, novelty-seeking and exploratory behaviors induced by the HFD are not due to any non-specific effects of this diet on activity level. This is the first direct evidence, in adult animals of normal body weight and caloric intake, that dietary fat is responsible for the observed reduction of anxiety and increase in novelty-seeking and exploration.

#### 3.2 Increased cholinergic neurotransmission with high-fat diet consumption

With evidence indicating that ACh in extra-hypothalamic and hypothalamic regions functions to influence behaviors related to exploration and novelty-seeking (see Section 1), the next goal was to determine whether cholinergic neurotransmission in these areas may be disturbed by dietary fat. In rats consuming a HFD, measurements of the ACh metabolizing enzyme, AChE, revealed reduced enzymatic activity specifically in the frontal cortex, hypothalamus and midbrain area, reflecting a possible increase in ACh levels in these regions. This result agrees with a previous study, which used a long-term feeding paradigm (6 months on a HFD) that increased weight gain and produced metabolic disturbances and revealed a suppressive effect on AChE activity in the cortex and hypothalamus (Kaizer et al., 2004). It also receives indirect support from another study, showing 8 weeks on a HFD to reverse the Neurochemical effects of neonatal parathion exposure that depletes central ACh stores (Slotkin et al., 2009). The possibility that ACh levels are increased by the HFD



along with this enzymatic change is supported by studies in mutant mice lacking the AChE gene or in mice injected with an AChE antagonist, both of which lead to elevated levels of extracellular ACh (Hartmann et al., 2007). One possible mechanism mediating the effects of fat consumption on cholinergic function may be related to circulating energy molecules, such as FFA. These metabolites are markedly elevated in the HFD-consuming rats and have been shown to reduce the activity *in vitro* of AChE (Tel et al., 2010; Vajreswari et al., 2002). Although not measured in the current study, there is published evidence also suggesting that other energy signaling molecules, such as glucose and ghrelin, can function through cholinergic signaling pathways and cause changes in behaviors such as food reward and memory (Dickson et al., 2010; Ragozzino et al., 1994). Together, this evidence supports the idea that short-term (7 day) fat consumption, which increases metabolic factors such as FFA, is effective in stimulating the cholinergic system in cortical, hypothalamic and midbrain regions and perhaps contributing to the observed changes in behavior.

### 3.3 Increased nAChR binding induced by high-fat diet consumption

Since chronic stimulation of nAChRs with a cholinergic agonist is known to increase nAChR binding in multiple extra-hypothalamic and hypothalamic brain regions (Pauly et al., 1996; Sparks and Pauly, 1999; Trauth et al., 1999), a possible increase in ACh levels resulting from the fat-induced reduction of AChE activity may also be accompanied by changes in receptor binding. Using quantitative autoradiography, we examined the density of [<sup>125</sup>I]-epibatidine ( $\beta$ 2-nAChR) and [<sup>125</sup>I]-BTX ( $\alpha$ 7-nAChR) binding sites in different extra-hypothalamic and hypothalamic brain regions. Our findings with autoradiographic measurements of nAChRs showed the consumption of a HFD to increase the density of  $\beta$ 2-nAChRs specifically in the mPFC and SNc, while having no effect on these receptors in the VTA and PVT, and also to increase the density of  $\alpha$ 7-nAChRs specifically in the LH and VMH, but not the mPFC, CeA or PVN. Similar to these results in rats consuming a HFD, other reports measuring nicotine-induced [<sup>3</sup>H]-ACh or [<sup>3</sup>H]-nicotine binding have demonstrated an increase in receptor density in the hypothalamus, specifically the VMH, and also in the midbrain, specifically the SN region, but not in the thalamus which shows a resistance to such agonist-induced changes in binding (Kellar et al., 1989; Pauly et al., 1991). Although not directly measured in this study, the cellular mechanisms responsible for the receptor upregulation observed in the mPFC, SN, LH and VMH may involve an enhanced rate of synthesis or decrease in degradation of nAChRs. While an early study suggested that the greater susceptibility of specific brain regions to agonist-induced receptor changes may be related to their higher baseline levels of the nAChRs (Pauly et al., 1991), our findings are not consistent with this, showing changes in nAChR binding to occur specifically in regions exhibiting mid-range values of baseline binding. Overall, our results indicate that the  $\beta$ 2-nAChRs specifically in the mPFC and SN and  $\alpha$ 7-nAChRs specifically in the VMH and LH are particularly sensitive to the stimulatory effects of dietary fat.

### 3.4 Role of $\beta$ 2-nAChRs in mediating behavioral changes induced by dietary fat

The changes in behavioral patterns induced by consumption of fat may stem from disturbances in cholinergic signaling in specific cortical and midbrain regions. Our findings indicate that animals consuming a HFD, while being less anxious, show increased behaviors related to novelty-seeking and exploration and that these behaviors are associated with enhanced cholinergic neurotransmission within the frontal cortex and midbrain area, along with increased  $\beta$ 2-nAChR binding in the mPFC and SNc. The role of  $\beta$ 2-nAChR sites in the mPFC in mediating enhanced novelty-seeking and exploration is suggested by pharmacological evidence, showing reduced impulse-related behaviors in animals administered a specific  $\alpha$ 4 $\beta$ 2 nAChR antagonist in this brain region (Tsutsui-Kimura et al., 2010a; Tsutsui-Kimura et al., 2010b). Also, the fat-induced increase in the density of  $\beta$ 2-nAChR sites in the SNc, a region where nicotine is known to activate dopaminergic

projections to motor regions (Keath et al., 2007), suggests that dietary fat may be promoting novelty-seeking and exploration through direct activation of the midbrain motor circuits. Collectively, our findings in fat- compared to chow-consuming animals suggest that enhanced cholinergic signaling through  $\beta 2$ -nAChRs in the mPFC and SNc can stimulate impulse- and motor-regulating circuits, thereby driving animals to seek more interaction with novel objects and explore an open field. With novelty seeking and exploratory behavior playing an important role in animal survival strategy (Kelley et al., 2004; Korpela et al., 2011), our findings can additionally suggest that a HFD, which is most desirable for its high energy content, also prepares rats for future novel and possibly threatening conditions

### 3.5 Role of $\alpha 7$ -nAChRs in mediating behavioral changes induced by dietary fat

Our results in HFD- compared to chow-consuming animals, showing a reduction in AChE activity within the hypothalamus and increase in  $\alpha 7$ -nAChRs specifically in the LH and VMH, indicate that fat is stimulating hypothalamic pathways related to energy and arousal that, in turn, can promote enhanced exploratory and novelty-seeking behaviors. In addition to affecting feeding behavior (Miyata et al., 1999; Yang et al., 1999), recent literature has focused on the important role of ACh in the LH in promoting behavioral arousal by interacting with local peptide and neurotransmitter systems (Henny and Jones, 2006; Kane et al., 2000; Pasumarthi and Fadel, 2010). This stimulatory effect is most likely mediated by local nicotinic receptors, since treatment with a nicotinic agonist is shown to stimulate orexin expression, a peptide in the LH which is known to promote arousal (Estabrooke et al., 2001; Kane et al., 2000; Li et al., 2000). Similarly, the VMH, a region rich in energy-sensing neurons (Kang et al., 2004) and originally suggested to function as a satiety center (Takahashi et al., 1995), is now believed to have a role in promoting arousal, based on evidence showing this region to be activated during periods of food anticipation and heightened arousal (Ribeiro et al., 2007; Ribeiro et al., 2009). Unlike the stimulatory effect of dietary fat on  $\alpha 7$ -nAChRs in the LH and VMH, no changes in receptor binding occurred in the CeA or PVN, where ACh is associated with the activation of stress responses (Ohmori et al., 1995; Zarrindast et al., 2008; Zhang and Zheng, 1997). Since our current findings reveal reduced anxiety in animals consuming a HFD, this behavior is likely to be mediated by other neurotransmitter systems, such as  $\gamma$ -aminobutyric acid, which is stimulated by feeding (Rada et al., 2003) and known to reduce anxiety and stress responding specifically in the CeA (Sanders and Shekhar, 1995) and PVN (Imaki et al., 1995) regions. Together with these studies, our evidence suggests that dietary fat, by stimulating cholinergic signaling via  $\alpha 7$ -nAChRs, can activate hypothalamic energy-sensing and arousal circuits in the LH and VMH, rather than anxiety circuits, to promote novelty-seeking and exploratory behaviors.

### 3.6 Nicotinic cholinergic mechanisms mediate the HFD-induced behaviors

The current findings suggest that nicotinic cholinergic mechanisms mediate the novelty-seeking and exploratory behaviors induced by a fat-rich diet. In addition to showing an increase in the density of  $\alpha 7$  and  $\beta 2$  nicotinic receptor sites, our results with pharmacological manipulations demonstrate that the nicotinic receptor antagonist, mecamylamine, can attenuate certain HFD-induced behaviors characteristic of novelty-seeking and exploration and that this occurs at a low dose that has little behavioral effect in animals eating a chow diet, as shown here and in previously published studies (Kolokotroni et al., 2011). These results with mecamylamine are consistent with recent reports showing a similar low dose to attenuate nicotine-induced behaviors related to novelty-seeking, such as impulsive choice (Kirshenbaum et al., 2011; Kolokotroni et al., 2011). Whereas there was no difference observed in Experiment 4 with novel object interaction time, this may be due to the marked changes in the number of entries made into the novel object quadrant, which precluded a significant change in quadrant time. In a secondary analysis of zone entries and

time spent in zone for individual animals, we found these two measures to be inversely related ( $-0.62$ ,  $p < 0.05$ ), further supporting the possibility that the increase in number of entries into the novel object quadrant most likely masked a change in time spent interacting with the object. Although not directly measured in the current study, it is important to note that mecamylamine has previously been shown to have anxiolytic properties even at low doses (Newman et al., 2001; Newman et al., 2002) and thus may be having an additive effect to the dietary fat in reducing anxiety. While mecamylamine as a general nicotinic antagonist makes it difficult to identify the receptor-specificity of these observed behavioral changes, the available evidence suggests that both  $\alpha 7$  and  $\beta 2$  nAChRs are involved in impulse and novelty-related behaviors. This is indicated by results with a specific antagonist of the  $\alpha 4\beta 2$  nAChR, which can block the effects of nicotine on premature responding in various tests of impulsivity (Blondel et al., 2000; Tsutsui-Kimura et al., 2010a), and with  $\alpha 7$  nAChR null mice, which demonstrate impaired performance in similar impulse-related tasks (Hoyle et al., 2006). Together with our evidence showing increased density of both  $\alpha 7$  and  $\beta 2$  receptor subtypes after HFD consumption, an effect similar to that occurring with prolonged nicotine treatment (Flores et al., 1997; Rasmussen and Perry, 2006; Schwartz and Kellar, 1983), it is likely that mecamylamine by blocking these receptor sites can attenuate the behavioral abnormalities induced by a diet rich in fat.

### 3.7 Conclusion

We conclude that the behavioral changes induced by HFD consumption, increased novelty-seeking and exploration, may be related to enhanced nicotinic cholinergic signaling in specific cortical, hypothalamic and midbrain regions associated with such behaviors. Evolutionarily, it is possible that dietary fat consumption, by activating cholinergic circuits, stimulates central survival mechanisms that in turn promote these active-coping behaviors such as exploration of novel environments. With our findings suggesting that a brief, 7-day period of HFD consumption leads to such distinct patterns of behavior along with increased cholinergic neurotransmission, it is possible that such changes can occur with even shorter periods of consumption. They may also be a direct consequence of the elevated FFA acting on central cholinergic circuits, rather than the long-term effects of dietary fat on physiological systems related to body weight.

## 4. Experimental Procedures

### 4.1 Animals

Adult, male Sprague-Dawley rats (approximately 2 months of age), weighing in between 250 – 300 g at the start of all experiments (Charles River Breeding Labs, Kingston, NY), were individually housed (22 °C, with lights off at 1:00 p.m. for 12 hr) in a fully accredited American Association for the Accreditation of Laboratory Animal Care facility, according to institutionally approved protocols as specified in the National Institutes of Health Guide to the Use and Care of Animals and also with the approval of the Rockefeller University Animal Care and Use Committee. All animals were given 1 week to acclimate to lab conditions, during which time they were maintained *ad libitum* on laboratory chow (LabDiet Rodent Chow 5001, St. Louis, MO; 12% fat, 60% carbohydrate, and 28% protein) and water. All protocols fully conformed to the Guiding Principles for Research Involving Animals and Human Beings as set forth by the American Physiological Society. All efforts were made to minimize the use and suffering of animals

### 4.2 Diets

For the experimental period, rats were either maintained *ad libitum* on standard rodent chow (12% fat, 3.3 kcal/g) or a HFD (50% fat, 5.2 kcal/g), which was the same as that described in prior publications (Leibowitz *et al.*, 2004; Dourmashkin *et al.*, 2006). It consisted of fat



from 75% lard (Armour, Omaha, NE) and 25% vegetable oil (Wesson vegetable oil, Omaha, NE), of carbohydrate from 30% dextrin, 30% cornstarch (ICN Pharmaceuticals, Costa Mesa, CA), and 40% sucrose (Domino, Yonkers, NY), and of protein from casein (Bioserv, Frenchtown, NJ) and 0.03% L-cysteine hydrochloride (ICN Pharmaceuticals). The diet was supplemented with minerals (USP XIV Salt Mixture Briggs; ICN Pharmaceuticals) and vitamins (Vitamin Diet Fortification Mixture; ICN Pharmaceuticals). The macronutrient composition was calculated as percentage of total kcal, with the HFD containing 50% fat, 25% carbohydrate, and 25% protein. This semi-solid diet was stored at 4°C until use. On a daily basis, fresh diet was weighed out in metal bowls and placed into the appropriate animal cages.

### 4.3 Test procedures

Animals were first adapted for one week to standard housing conditions and then split into two groups matched for body weight. One group was trained to consume a HFD, first by receiving a small 15-kcal meal of this diet each day for 3 days with chow also available and then by being placed on the HFD *ad libitum* for an additional 7 days. The other group was maintained on the chow diet for the entire experimental period. Caloric intake and body weight was recorded daily, and to avoid any non-specific effects of calories or weight on the behavioral and neurochemical measures, rats in both groups were specifically chosen to have similar caloric intake and equal body weights. This was done first by rank ordering the rats by caloric intake, which positively correlated with body weight ( $r=+0.68 - +0.75$ ) and then eliminating the top tertile or bottom tertile for the HFD and chow groups, respectively. Thus, with this subgrouping, the number of subjects in each experiment, generally starting at 12 per group, was reduced to approximately 8 per group.

In Experiment 1, the effects of HFD versus chow consumption on anxiety and novelty-seeking behaviors were examined. Shortly after dark onset on day 7, rats consuming a HFD or chow ( $n=8/\text{group}$ ) were placed on EPM for 5 min, and then, at the same time on day 8, they were each tested for novelty-seeking and exploration in an open field activity chamber for 20 min. After all behavioral testing was completed; tail vein blood was collected and analyzed for serum levels of circulating FFA (see below).

In Experiment 2, rats ( $n=8/\text{group}$ ) were allowed to consume either a HFD or chow as described above and then, shortly after dark onset on day 7 of diet consumption, were sacrificed by rapid decapitation. Brain regions of interest were dissected and analyzed for AChE activity, as described below and tail vein blood was collected for measures of circulating FFA (see below)

To examine the effects of dietary fat on nAChR binding in specific brain nuclei, Experiment 3, in an additional set of animals ( $n=8/\text{group}$ ) exposed to chow or a HFD for 7 days, used quantitative autoradiography to measure the binding of [ $^{125}\text{I}$ ]-epibatidine to  $\beta 2$ -nAChRs and [ $^{125}\text{I}$ ]-bungarotoxin (BTX) to  $\alpha 7$ -nAChRs. On day 7 of diet exposure shortly after dark onset, animals were sacrificed using rapid decapitation, and their brains were removed, dipped into cold isopentane ( $-25^\circ\text{C}$ ) for 30 s, and then kept at  $-80^\circ\text{C}$  until further use. Additionally, tail vein blood was collected on day 7 for measurements of circulating FFA.

Experiment 4 examined the ability of a nicotinic receptor antagonist to reduce the novelty-seeking behavior induced by a HFD in Experiment 1. Mecamylamine hydrochloride (Sigma Aldrich, St. Louis, MO) was diluted in saline and injected subcutaneously at a dose of 0.5 mg/kg in a 0.5 ml volume. This low dose of mecamylamine was specifically chosen based on literature suggesting that it can block impulsive behaviors produced by low dose nicotine while having limited behavioral effects on its own (Kolokotroni et al., 2011). Animals were initially separated into chow and HFD groups and allowed to consume their respective diets

for a total of 7 days. After this, they were matched for caloric intake and body weight, yielding 16 animals/diet group. On day 8, animals from the chow and HFD groups were injected (s.c.) with either saline vehicle or mecamlamine in a between-subject design and, 20 minutes later, were tested for 20 minutes in the open field for locomotor, exploratory and novelty-seeking behavior, as described below.

#### 4.4 Elevated plus maze

The EPM apparatus consisted of two open arms (10 × 50 cm), which lay perpendicular to two closed arms (10 × 50 cm) with 30 cm high wooden walls, and a common central platform as previously described (Avena et al., 2008; Walf and Frye, 2007). The whole apparatus was placed 55 cm above the floor. The test was carried out by an experimenter blind to the manipulation. Each rat was placed at the central platform of the apparatus and allowed to explore the maze for 5 min under dim red light. A video camera recorded movements of the rats, which were later analyzed for the percent of time spent in the open or closed arms as well as the central platform out of the total 300 seconds of the test. The criterion used to determine a full arm entry was both forepaws into an arm. Anxiety was determined by more time spent in the closed arms of the EPM, while the time spent in the open arms represented a reduction in anxiety behavior. The apparatus was thoroughly cleaned with soap and water and dried between each run.

#### 4.5 Open Field Measures

The open field activity chamber consisted of a computerized Plexiglas box (43.2 × 43.2 cm) with a white floor, black walls, and infrared photocells (MED Associates, Georgia, VT, USA). Novelty-seeking testing occurred in a test room separate from the vivarium under dim red light. All animals were acclimated, for 20 min each day, to the testing chambers on 2 consecutive days (day 6 and day 7 of diet consumption). They were then tested for novelty-seeking (20 min) in these same chambers, with a novel square object placed in the far right quadrant of the chamber and measurements taken of entries made into the quadrant containing the novel object and time spent in the quadrant with the object. During this same 20 minute session, locomotor behavior (ambulatory distance), a measure of motor function, and rearing behavior (vertical counts), a measure of enhanced exploration (Prut and Belzung, 2003), were also recorded.

#### 4.6 Fatty acid determinations

In Experiments 1–3, serum from tail vein blood was assayed for non-esterified fatty acid levels, with an E-Max Microplate Reader using a colorimetric assay kit from WAKO Pure Chemical Industries, Ltd (Osaka, Japan).

#### 4.7 Acetylcholinesterase activity

Animals were sacrificed using rapid decapitation, and their brains were quickly dissected on ice to remove the entire frontal cortex, hypothalamus, midbrain area and cerebellum for determinations of AChE activity. Tissue from the brain regions of interest was then individually homogenized at 4°C in filtered 50 mM TrisHCl buffer, pH 7.4 (1:10 w/v) and centrifuged at 1,000 × g for 10 min to remove nuclei and debris. The protein content of each sample's supernatant was determined using the Bradford Assay (Bradford, 1976). AChE activity was then measured using acetylthiocholine iodide and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), according to the methods of Ellman and colleagues (Ellman et al., 1961). Briefly, 0.32 mM DTNB (color reagent) and 1.0 mM acetylthiocholine iodide (substrate) were added to the homogenates, and the absorbance at 412 nm was measured using an E-max microplate reader. Enzyme activity of AChE is expressed as  $\mu\text{mol}/\text{min}/\text{mg}$  protein at 25°C.

#### 4.8 Quantitative autoradiography

Brains were sectioned (16  $\mu\text{m}$ ) at  $-20^{\circ}\text{C}$  on a cryostat microtome, thaw mounted on gelatin-coated microscope slides, and desiccated overnight at  $4^{\circ}\text{C}$  using an air vacuum. Multiple sets of adjacent sections were collected for measurements of  $\beta 2$ - and  $\alpha 7$ -nAChRs on sections that had consistent anatomical representation. Brain sections were collected from Bregma 3.2 to 2.7 mm for the mPFC; Bregma  $-1.8$  to  $-1.88$  mm for the PVN and the PVT, Bregma  $-2.8$  to  $-3.14$  mm for the LH and CeA; and from Bregma  $-5.6$  to  $-5.8$  mm for the VTA and SN pars compacta (Paxinos and Watson, 1997). All sections, clearly depicting the region of interest, were chosen at the same level for each animal within each group. For each animal, 3 adjacent brain sections were used for the binding assay and averaged for statistical analysis.

The binding of [ $^{125}\text{I}$ ]-epibatidine to  $\beta 2$ -nAChRs was measured using a slightly modified method from Tizabi and colleagues (Tizabi and Perry, 2000). Briefly, brain sections were pre-incubated at room temperature for 15 min in a 50 mM TrisHCl buffer containing (pH 7.4) 120 mM NaCl, 5 mM KCl, 2.5 mM  $\text{CaCl}_2$ , 1 mM  $\text{MgCl}_2$  and were then incubated for 60 min at room temperature in the same buffer solution containing 0.165 nM [ $^{125}\text{I}$ ]-epibatidine (2200 Ci/mmol, Perkin-Elmer, Boston, MA.). Nonspecific binding was determined in adjacent sections with the addition of 300 mM nicotine hydrogen tartrate to the incubation buffer. All sections were then rinsed twice (5 min each) in ice-cold buffer, dipped briefly into ice-cold distilled water, and air-dried at room temperature.

The binding of [ $^{125}\text{I}$ ]-BTX to  $\alpha 7$ -nAChR sites was measured as previously described (Sparks and Pauly, 1999) with slight modification. Briefly, slides containing the brain regions of interest were pre-incubated at room temperature for 15 min in a 50 mM TrisHCl (pH 7.4) buffer containing 0.1% BSA and were then transferred to the incubation solution which contained the same buffer solution in addition to 2 nM [ $^{125}\text{I}$ ]-BTX (120 Ci/mmol, Perkin-Elmer, Boston, MA.). In order to determine non-specific binding, adjacent sections were incubated in this same incubation buffer, but with 300  $\mu\text{M}$  nicotine hydrogen tartrate (Sigma Aldrich, St. Louis, MO) added. Sections were allowed to incubate in these two solutions for 2 h at room temperature and then quickly dipped in ice-cold Tris buffer, rinsed 3 times (10 min each) in ice-cold buffer, dipped in distilled water, and finally air-dried at room temperature.

For both binding assays, the air-dried sections were exposed to Kodak BioMax MR (Sigma Aldrich, St. Louis, MO) film along with [ $^{125}\text{I}$ ] standards (American Radiolabeled Chemicals, St. Louis, MO) for 4 h ( $\beta 2$ -nAChR) or 5 days ( $\alpha 7$ -nAChR). After appropriate exposure times, the films were developed using an automatic developer and images scanned and saved for further analysis. Quantitative densitometric analysis of binding was performed using NIH software, Image J v1.43 for Windows. In order to determine specific binding, non-specific binding, which represented less than 5% of the total binding, was subtracted from total binding in adjacent sections. The values of binding were determined using a standard curve constructed from [ $^{125}\text{I}$ ] standards with a known amount of activity and expressed as (fmol/mg brain protein).

#### 4.9 Statistical analysis

The values in the figures are expressed as mean  $\pm$  SEM. Statistical analyses to compare the effects of dietary fat to chow on caloric intake, body weight and serum fatty acid levels were performed using an unpaired Student's *t*-test, with significant differences accepted at  $p < 0.05$ . Data in the first three experiments, involving behavior, receptor binding or AChE activity, were first analyzed using a repeated measure analysis of variance (RM-ANOVA). In Experiment 4, the effects of mecamylamine on diet-induced behavioral changes were

analyzed using a two-way ANOVA, with diet (two levels) and drug (two levels) as independent variables. Where significant main effects were obtained, follow-up pairwise comparisons were made between groups of interest using either two-tailed t-tests (RM-ANOVA) or a post-hoc Holm-Sidak test (two-way ANOVA), where  $p < 0.05$  was considered significant. The probability values given in the text or legends to the figures and tables reflect the results of these tests.

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## Glossary

<b>ACh</b>	Acetylcholine
<b>AChE</b>	acetylcholinesterase
<b>ANOVA</b>	analysis of variance
<b>BTX</b>	bungarotoxin
<b>CeA</b>	central nucleus of the amygdala
<b>DA</b>	dopamine
<b>EPM</b>	elevated plus maze
<b>HFD</b>	High-fat diet
<b>LH</b>	lateral hypothalamus
<b>mPFC</b>	medial prefrontal cortex
<b>nAChRs</b>	nicotinic acetylcholine receptors
<b>PVN</b>	paraventricular hypothalamic nucleus
<b>PVT</b>	paraventricular thalamic nucleus
<b>SNc</b>	substantia nigra pars compacta
<b>VMH</b>	ventromedial hypothalamus
<b>VTA</b>	ventral tegmental area

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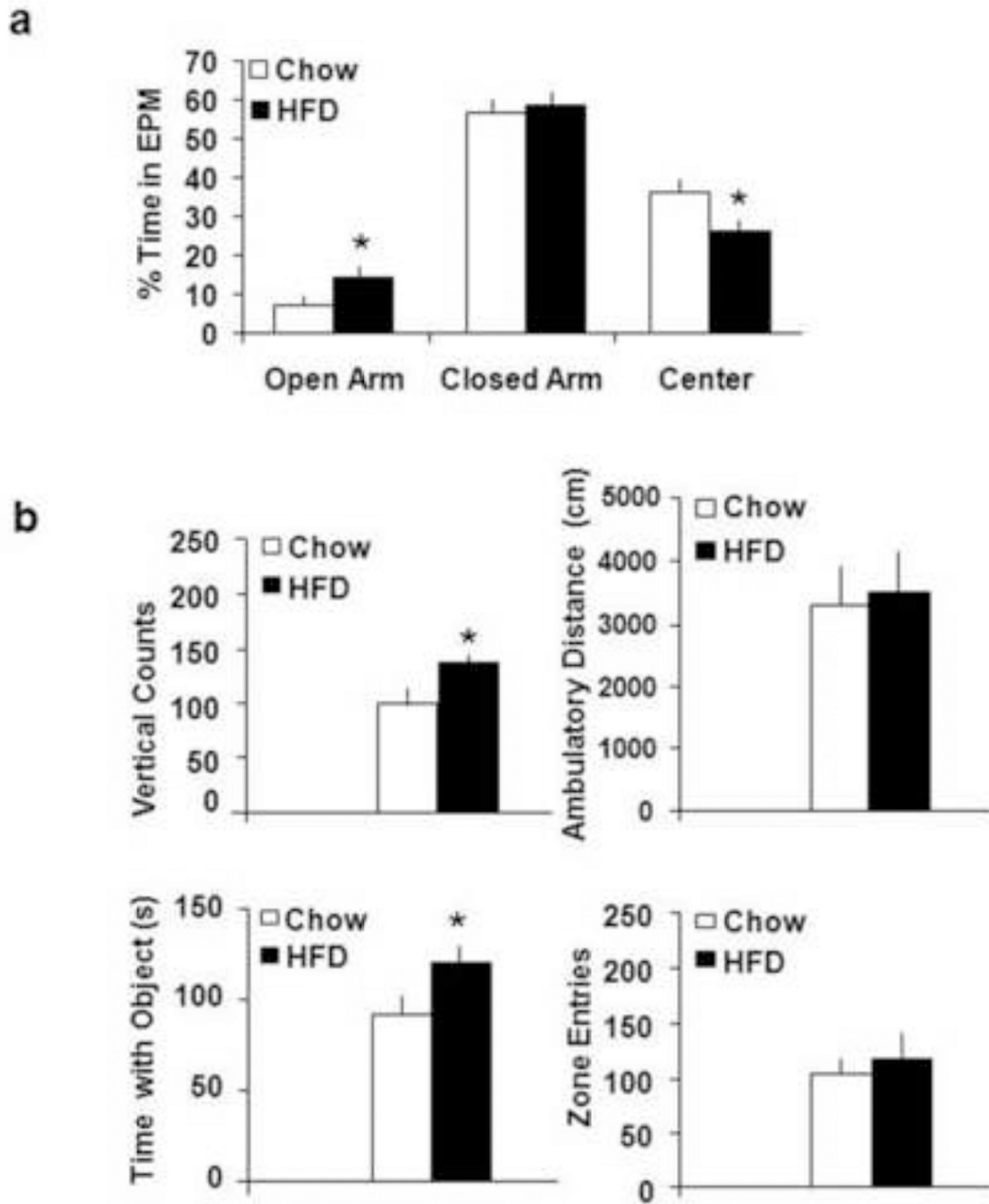
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### Research Highlights

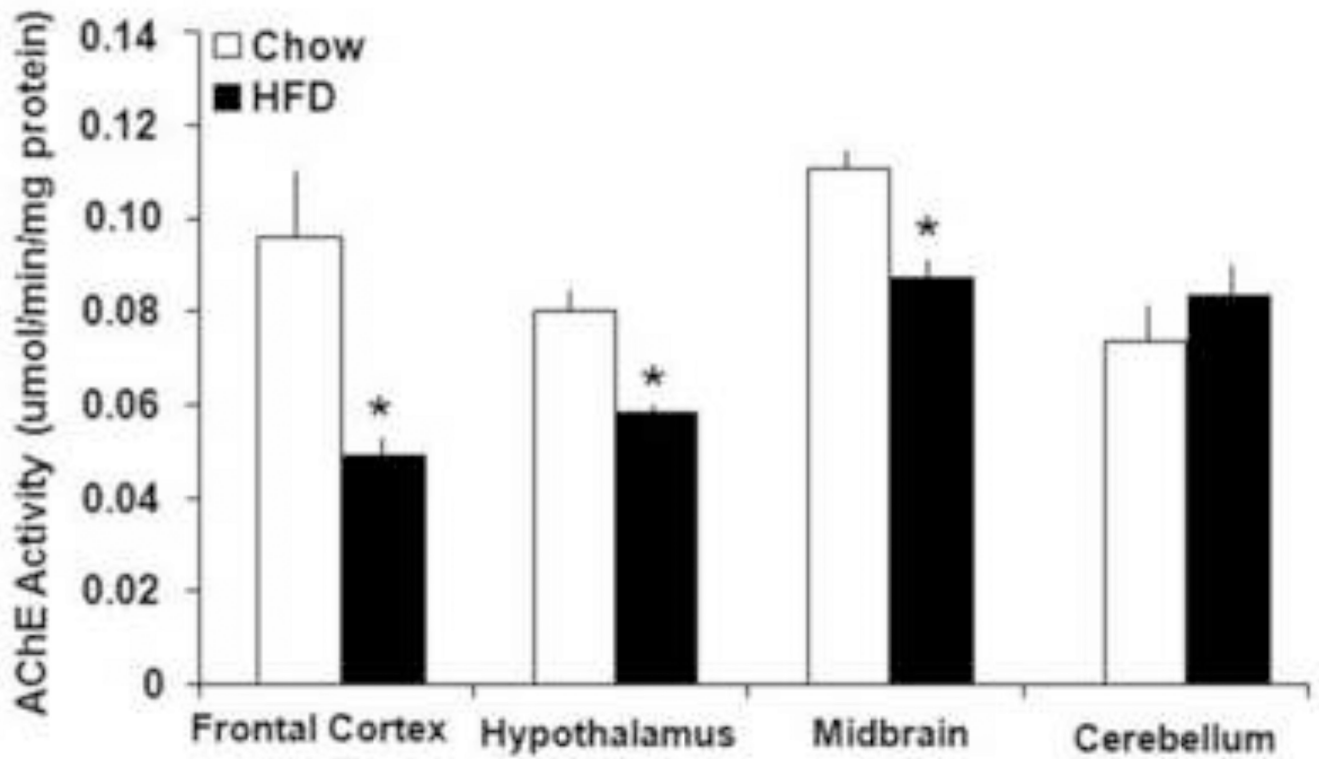
- Consumption of a high-fat diet reduces anxiety and enhances novelty-seeking behavior in rats.
- Dietary fat intake stimulates central cholinergic activity and nicotinic receptor binding.
- Pharmacological blockade of nicotinic receptors attenuates fat-induced behavioral changes.
- Nicotinic cholinergic mechanisms are involved in behavioral effects of dietary fat consumption.



**Figure 1. Consequences of dietary fat consumption on behavioral patterns**

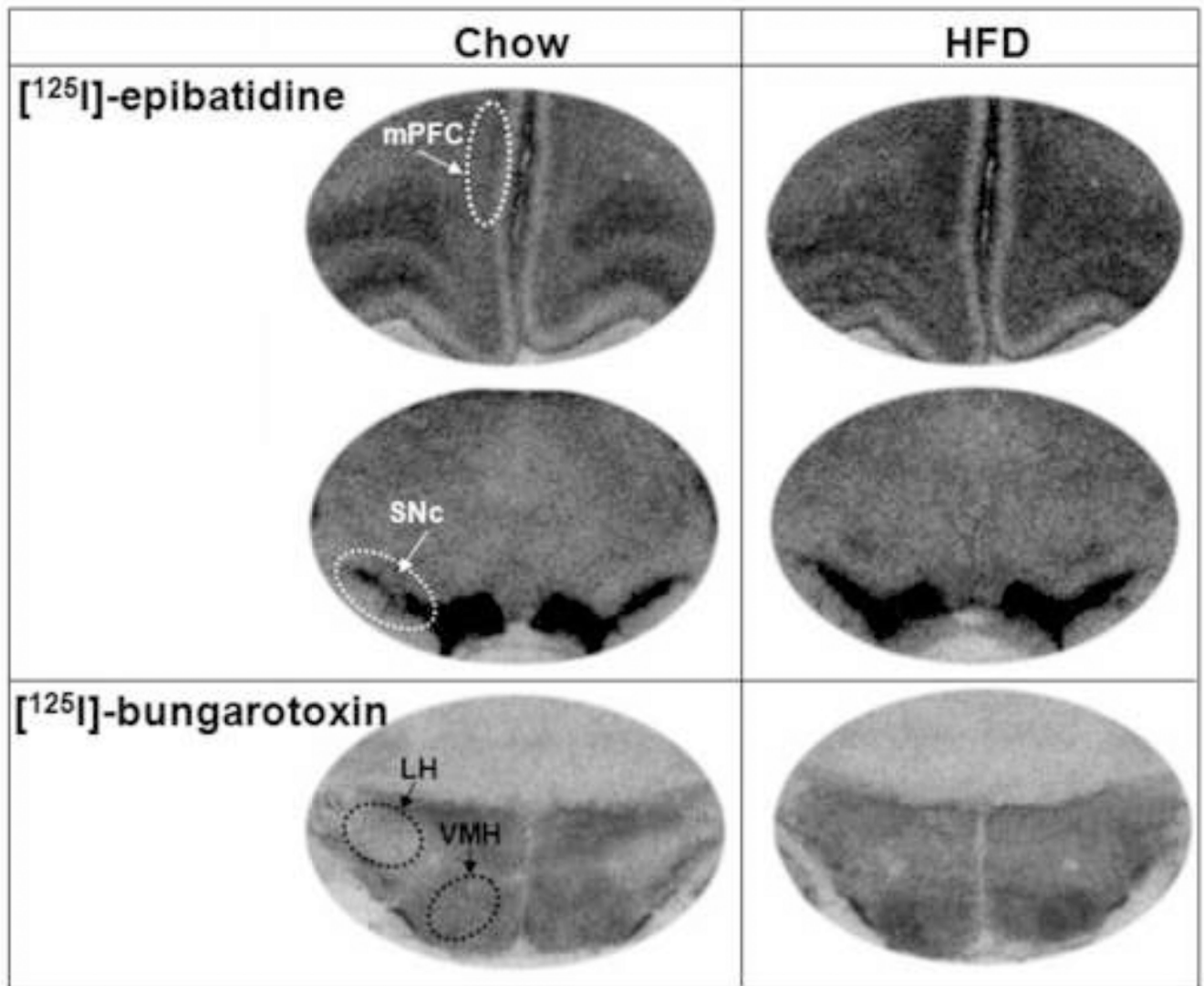
a) Effects of HFD consumption on anxiety, as measured by percent time spent in the open arm, closed arm, or center of an EPM. The data showed a significant increase in time spent within the open arm (\*  $p < 0.05$ ) and a decrease in the time spent in the center of the maze (\*  $p < 0.05$ ) in animals consuming HFD compared to chow diet. b) Effects of HFD consumption on novelty-seeking and exploratory behavior. The data showed that fat compared to chow consumption increased both the time spent interacting with this object (\*  $p < 0.05$ ) and rearing behavior in the open field (\*  $p < 0.05$ ), while having no effect on ambulatory distance or zone entries. Abbreviations: HFD - high-fat diet



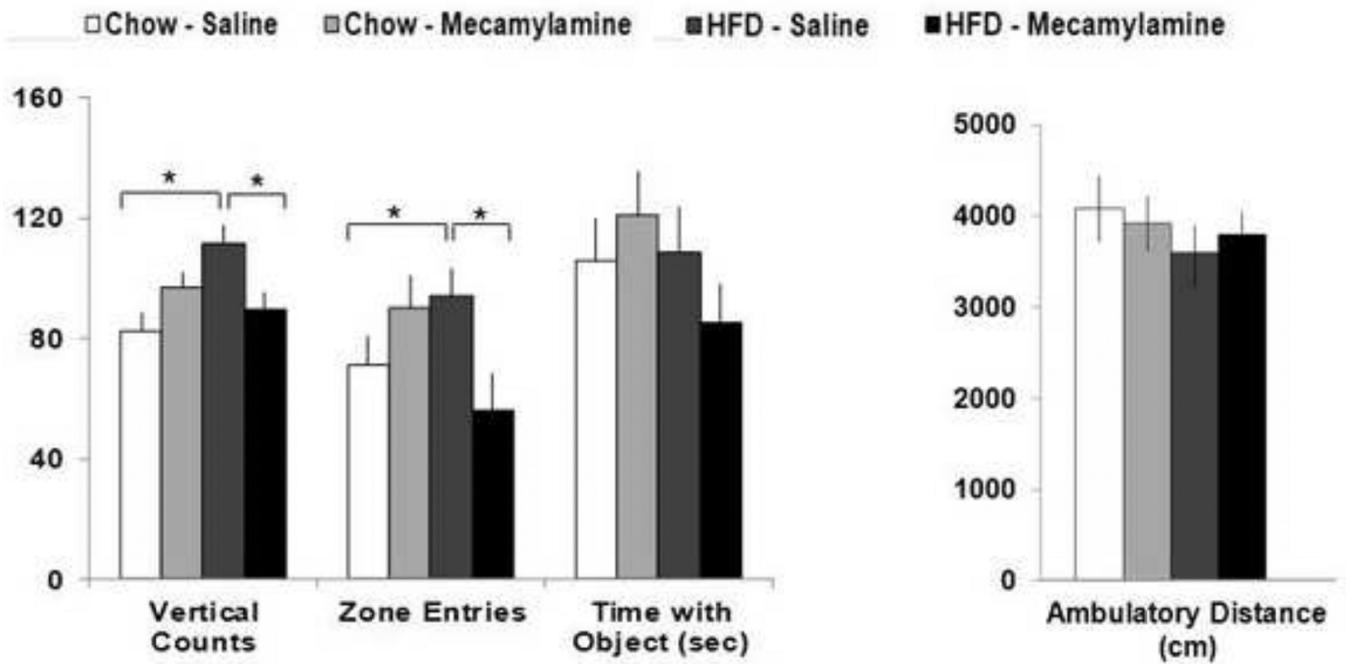


**Figure 2. Changes in AChE induced by HFD consumption**

Reduced AChE activities in homogenized frontal cortex, hypothalamus and midbrain region, but not cerebellum of rats exposed a HFD or chow diet. \* Represents significant differences between HFD and chow diet animals ( $p < 0.05$ ). Abbreviations: HFD - high-fat diet; AChE - acetylcholinesterase



**Figure 3. Autoradiographs illustrating the effects of HFD consumption on nAChR binding** Images depicting a stimulatory effect of dietary fat on  $[^{125}\text{I}]$ -epibatidine binding in the mPFC and SNc regions (top 2 panels) and  $[^{125}\text{I}]$ -bungarotoxin binding in the LH and VMH regions (bottom panel). Abbreviations: HFD - high-fat diet; mPFC - medial prefrontal cortex; LH - lateral hypothalamus; VMH - ventromedial hypothalamus; and SNc - substantia nigra pars compacta



**Figure 4. Effects of mecamlamine on HFD-induced novelty-seeking and exploration**  
 Data showing an attenuation of HFD-induced increases in vertical counts and zone entries in response to an acute mecamlamine injection. \* Represents significant differences between designated groups ( $p < 0.05$ ). Abbreviations: HFD - high-fat diet

**Table 1**

Effects of HFD consumption on the binding of [<sup>125</sup>I]-epibatidine and [<sup>125</sup>I]-bungarotoxin in several brain regions

	[ <sup>125</sup> I]-epibatidine		[ <sup>125</sup> I]-bungarotoxin	
	Chow	HFD	Chow	HFD
<b>mPFC</b>	238±6.6	261±3.9*	323±7.3	319±8.4
<b>CeA</b>	-----	-----	162±2.4	161±3.9
<b>PVT</b>	294±13.3	315±6.2	-----	-----
<b>PVN</b>	-----	-----	123±10.8	111±8.7
<b>LH</b>	-----	-----	265±16.2	296±5.8*
<b>VMH</b>	-----	-----	94±8.5	118±4.9*
<b>VTA</b>	298±10.3	311±8.9	-----	-----
<b>SNe</b>	255±10.9	285±8.1*	-----	-----

All values are expressed as fmol/mg brain protein, with an asterisk (\*) representing a significant effect of diet on nAChR binding ( $p<0.05$ ) and dashes representing brain regions where no ligand binding was observed.

Abbreviations: mPFC - medial prefrontal cortex; CeA – central nucleus of the amygdala; PVT - thalamic paraventricular nucleus; PVN - hypothalamic paraventricular nucleus; LH - lateral hypothalamus; VMH - ventromedial hypothalamus; VTA - ventral tegmental area; and SNe - substantia nigra pars compacta.