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Regulation of Hypothalamic Neuronal Sensing and Food Intake by Ketone Bodies and Fatty Acids

Metabolic sensing neurons in the ventromedial hypothalamus (VMH) alter their activity when ambient levels of metabolic substrates, such as glucose and fatty acids (FA), change. To assess the relationship between a high-fat diet (HFD; 60%) intake on feeding and serum and VMH FA levels, rats were trained to eat a low-fat diet (LFD; 13.5%) or an HFD in 3 h/day and were monitored with VMH FA microdialysis. Despite having higher serum levels, HFD rats had lower VMH FA levels but ate less from 3 to 6 h of refeeding than did LFD rats. However, VMH β-hydroxybutyrate (β-OHB) and VMH-to-serum β-OHB ratio levels were higher in HFD rats during the first 1 h of refeeding, suggesting that VMH astrocyte ketone production mediated their reduced intake. In fact, using calcium imaging in dissociated VMH neurons showed that ketone bodies overrode normal FA sensing, primarily by exciting neurons that were activated or inhibited by oleic acid. Importantly, bilateral inhibition of VMH ketone production with a 3-hydroxy-3-methylglutaryl-CoA synthase inhibitor reversed the 3- to 6-h HFD-induced inhibition of intake but had no effect in LFD-fed rats. These data suggest that a restricted HFD intake regimen inhibits caloric intake as a consequence of FA-induced VMH ketone body production by astrocytes.

Several lines of evidence support the idea that food intake can be altered by ingestion of a high-fat diet (HFD) (1–5). Prolonged intraventricular infusion of the long-chain fatty acid (LCFA), oleic acid (OA), causes a decrease in intake (6). However, the physiological significance of such effects on feeding can be questioned, as can those of direct infusions of fatty acid (FA) into brain areas such as the hypothalamus (7). A major problem is that there is no current information about how brain FA levels change during fasting and feeding. For that reason, we developed a microdialysis technique for assessing changes in ventromedial hypothalamic (VMH) FA levels in parallel with those in serum FA levels and food intake during the intake of a low-fat diet (LFD) versus an HFD.

In addition, although there is a sizeable literature demonstrating that pharmacological and molecular manipulations of FA metabolism in the brain can alter feeding behavior (8–13), most studies overlook the fact that most FA oxidation occurs in astrocytes rather than neurons (14). Importantly, astrocytes are also the only source of ketone body production in the brain, and such production increases when ambient FA levels rise (15). Because neurons can use ketone bodies as an alternate energy source (16,17), we postulated that any effects that ingestion of an HFD might have on food intake might be mediated by astrocyte-produced ketone body actions on nearby FA sensing neurons, which we have shown play a role in the regulation of energy and glucose

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METABOLISM

homeostasis (18). Thus, we used microdialysis to monitor VMH brain ketone levels in parallel with serum ketone levels and food intake of HFD versus LFD and coupled these with in vitro studies of the effects of ketone bodies on FA sensing in VMH neurons. The results of these studies are presented here and suggest a novel mechanism by which an HFD might act via astrocytes to alter neuronal activity and food intake.

RESEARCH DESIGN AND METHODS

Animals

Animals were housed at 23–24°C on a reversed 12:12-h light-dark cycle (lights off at 1000) with ad libitum access to chow (13.5% kcal fat; Purina #5001) and water. Outbred male Sprague-Dawley rats were purchased from Charles River Laboratories. All work was in compliance with the East Orange Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

VMH Free FA and Food Intake Measurements

Rats (n = 8 per group; weight 300–400 g) were anesthetized with isoflurane (1.5% at 0.8 L/min), and a unilateral microdialysis probe with a 3-mm membrane length and 3,000 kDa pore size (MAB 5.15.3PE; Microbiotech/se AB, Stockholm, Sweden) was stereotaxically angled at 20° to the vertical at the junction between the arcuate and VMH nuclei (VMN) (VMH = VMN + arcuate; −2.9 mm bregma, ± 3.7 mm midline, and −8.5 mm dura). The next day at 0800 (2 h before food was presented), microdialysis was begun with probes perfused with artificial cerebrospinal fluid (aCSF; Harvard Apparatus, Holliston, MA) containing 3% FA-free BSA (Sigma-Aldrich, St. Louis, MO) at 1.0 μL/min. Eluates were collected every 30 min and were stored at −80°C until nonesterified FA assay. Probe placement was assessed terminally.

A first set of rats was fasted for 24 h, and VMH probes were infused for 1 h at each of five concentrations (0, 20, 60, 150, 400 μmol/L) of OA, with monitoring of effluent OA concentrations at 20 min. Fasting VMH OA levels were calculated using the zero-net-flux method (19). A second set of rats was trained for 2 weeks to eat a 13.5% LFD (Purina #5001) or a 60% HFD (Research Diet D12492, New Brunswick, NJ) (n = 8 per group) in 3 h each day from 1000 to 1300 in a BioDAQ food intake monitoring apparatus. After 1 week, jugular catheters were implanted, and rats were allowed 6 to 7 days to recover their preoperative body weight on their 3 h/day feeding schedule. On testing day, rats ate their respective diets for 6 h while their ongoing food intake was monitored, and they simultaneously underwent microdialysis and blood sampling at 30 min intervals over 6 h.

VMH β-Hydroxybutyrate and Feeding Measurements

Unilateral VMH guide cannulae were implanted in LFD rats (n = 8 per group). After 1–2 weeks of recovery, they were fasted for 24 h for zero-net-flux assessment. The next day, at 0800, microdialysis probes (3-mm

membrane length and 6-kDa pore size [CMA 11; Harvard Apparatus, Holliston, MA]) were inserted into the guide cannulae and perfused at 1.0 μL/min with β-hydroxybutyrate (β-OHB) diluted in aCSF for 40 min at each of four concentrations (0, 2, 30, 100 μmol/L), and effluent β-OHB concentrations were monitored. Next, additional rats were trained for 2 weeks to eat a LFD or HFD (n = 8 per group) over 3 h/day and were implanted with jugular catheters and unilateral VMH guide cannulae. At 0800 on the test day, microdialysis probes were inserted, jugular catheters were connected, and the rats' respective diets were returned from 1000 to 1300. At 1300, rats being fed the HFD were switched to the LFD, and all rats were allowed to eat for 3 h more, with monitoring of food intake, microdialysis eluates, and blood samples every 30 min over 6 h.

A third set of rats (n = 6–8 per group) was conditioned to eat the LFD or HFD in 3 h/day and implanted with bilateral VMH guide cannulae and jugular catheters. On test day, bilateral microdialysis probes were inserted at 0800, and an infusion with aCSF and 0.4% DMSO vehicle or 30 μmol/L hymeglusin, a 3-hydroxy-3-methylglutaryl–CoA (HMG-CoA) synthase inhibitor (20), in aCSF plus 0.4% DMSO at 1.0 μL/min (n = 6–8 per group), along with simultaneous microdialysis for ketones was begun. The LFD or HFD was provided from 1000 to 1300 and the LFD from 1300 to 1600, with monitoring of food intake, VMH, and serum β-OHB levels.

β-OHB and Acetoacetate Induced Changes in Intracellular Ca^{2+} Oscillations in Dissociated VMN Neurons

Postnatal day 21–28 (P21–28) rats were perfused, and neurons were dissociated from VMN punches, as previously described (18,21,22). Evaluation of glucose-, OA-, and ketone body-induced alterations in intracellular Ca^{2+} [Ca^{2+}]$_i$ oscillations in individual VMN neurons were assessed using fura-2 AM (Invitrogen, Grand Island, NY), as previously described (18,21,22). Neurons were classified first as glucose excited (GE), glucose inhibited (GI), and non–glucose-sensing (NG), then as OA excited (OAE), OA inhibited (OAI), and OA nonresponsive (OAN), and as β-OHB and acetoacetate (AA) excited, inhibited, or nonresponsive using previously established criteria for changes in [Ca^{2+}]$_i$ area under the curve (21,22). Studies began with neurons held at 2.5 mmol/L or 0.5 mmol/L glucose, followed by 15 nmol/L OA and by 1–100 μmol/L β-OHB or 1 μmol/L AA. Also assessed was a combination of 1 μmol/L AA and 20 μmol/L β-OHB (their relative ratio in the brain). All neurons were incubated with 20 nmol/L glutamate terminally to assess viability.

In Vitro Effects of FA on VMH Astrocyte-Induced Ketone Production

The VMH was dissected from P21–28 rats and triturated in Neurobasal-A (Invitrogen) containing 5 mmol/L

glucose, 0.23 mmol/L sodium pyruvate, 100 units/mL penicillin/streptomycin, 10 μg/mL gentamicin, and 10% FBS at pH 7.4. Dissociated cells were plated in 25-cm^2 flasks coated with poly-D-lysine (50 μg/mL) until confluence. Astrocytes were separated from remaining microglia by shaking at 250 rpm for 3 h at 37°C (23). Astrocytes attached to the flask were trypsinized with 0.05% trypsin-EDTA (Sigma-Aldrich) and centrifuged. The pellet was resuspended in Neurobasal-A and plated in 6-well plates. The day before the experiment, astrocytes were washed with PBS, and Neurobasal-A serum free was added overnight. Astrocytes were then treated with vehicle alone (0.4% DMSO), 150 μmol/L OA, palmitic acid (PA), or octanoic acid (OctA), or 30 μmol/L hymeglusin. Hymeglusin (synthesized by H.M.M.) is an HMG-CoA synthase inhibitor that is been demonstrated to inhibit ketone production in bacteria (20,24). Media were harvested at 0, 2, and 4 h, and total ketone levels were assayed.

Assays of OA, β-OHB, and Total Ketones

OA, total ketone, and β-OHB levels were analyzed using a colorimetric assay (Wako, Richmond, VA).

Statistics

Systat (Chicago, IL) and GraphPad Prism software (GraphPad, Inc., La Jolla, CA) were used to calculate one-way and two-way ANOVA and one-way ANOVA for repeated measures with post hoc Bonferonni corrections for the in vitro and in vivo studies. No more than two outliers per group were removed, if necessary, by using Systat software.

RESULTS

### Dietary Effects on Blood and VMH Free FA Levels and on Food Intake

The efficacy of using microdialysis to measure VMH extracellular free FA (FFA) levels was established in LFD rats fasted 24 h by using the zero-net-flux method and was 37.2 ± 9.8 μmol/L FFA (Supplementary Fig. 1*A*; *n* = 5). Thus, VMH FFA levels are ~10-fold less than blood levels. Next, the effect of dietary intake and composition was assessed in rats eating their daily intake within 3 h of dark onset. The purpose of using this restricted intake paradigm was to assure that there would be sufficient intake within 3 h to elevate VMH FFA to measurable levels. As expected from the 18-h fast, serum FFA levels were elevated, fell rapidly during the first 30 min of meal initiation by 76% in LFD rats, and remained at this lower level over the entire 6 h of monitoring (Fig. 1*A*). In HFD rats, serum FFA remained at relatively high levels over the entire 6 h (Fig. 1*A*) and was 120% higher than LFD levels during the first 3 h (*P* = 0.001; Fig. 1*A*). Given the marked difference in serum FFA, VMH FFA levels were unexpectedly 37% lower in HFD than in LFD rats over much of the first 3 h of feeding (*P* = 0.045; Fig. 1*B*, *C*, and *E*). Despite their lower VMH FFA, HFD rats had

comparable intake to LFD rats during the first 3 h of feeding, but intake was 65% lower during the next 3 h when LFD rats ate a relatively large set of meals between 4 and 6 h (*P* = 0.013; Fig. 1*D* and *F*). Importantly, even though they were fasted for 18 h before their 3-h intake periods during training, individual rats ate an average of one 15–20 kcal meal and two 5–10 kcal meals during the first 3-h period. On test days, rats also ate two 6–10 kcal meals during the second 3-h time period. However, because of the necessity to average data across rats for graphical presentation (e.g., Fig. 1*D* and *F*), these individual meals were obscured. Thus, there was no apparent correlation between serum or VMH FFA levels and caloric intake.

FA Diet Content Effects on Blood and VMH β-OHB Levels and Food Intake

Because VMH FFA levels did not appear to correlate with changes in food intake, we postulated that systemic or local ketone production by astrocytes after intake of the HFD might be an important regulator of intake. First, zero-net-flux microdialysis established fasting VMH ketone levels of 19.5 ± 3.4 μmol/L with concomitant serum levels of ~320 μmol/L (Supplementary Fig. 1*B*).

Next, serum and VMH β-OHB levels and LFD or HFD food intake were assessed over 3 h, and the HFD rats were then switched to the LFD for 3 h more. In the fasting condition, serum ketone levels were comparably high in both groups (LFD: 319 ± 42 μmol/L, HFD: 351 ± 32 μmol/L). By 30 min after food exposure, β-OHB serum levels fell to ~70–75% of their respective baselines (Fig. 2*A*). However, β-OHB serum levels then rose progressively from 3 to 6 h in the HFD rats but remained relatively stable in the LFD rats even though both groups were fed the LFD during this period. As with serum ketone levels, fasting VMH ketone levels were comparably high in both groups (LFD: 19.8 ± 3.4 μmol/L, HFD: 20.1 ± 4.2 μmol/L). For 30 min after food exposure, VMH β-OHB levels remained relatively stable in HFD rats but fell in LFD rats (Fig. 2*B*). This resulted in a twofold higher VMH-to-serum ratio at 30 min and was still 70% higher 60 min after onset of feeding in HFD versus LFD rats (*P* < 0.05; Fig. 2*C*). This elevated ratio of VMH to serum ketone levels on an HFD suggests that there was local production of ketones by astrocytes for at least the first hour of HFD intake. Although entry of ketones from the blood into the VMH and/or decreased neuronal ketone utilization might account for the raised ketone levels seen during the initial HFD intake, that the ketone rise was completely blocked by local inhibition of ketone production with hymeglusin demonstrates that the rise was due to local astrocyte ketone production. Nevertheless, food intake was similar between the groups during the first 3 h on their respective diets (Fig. 2*D* and *E*). However, when switched to chow, the rats previously eating HFD ate 76% less than LFD rats during the first
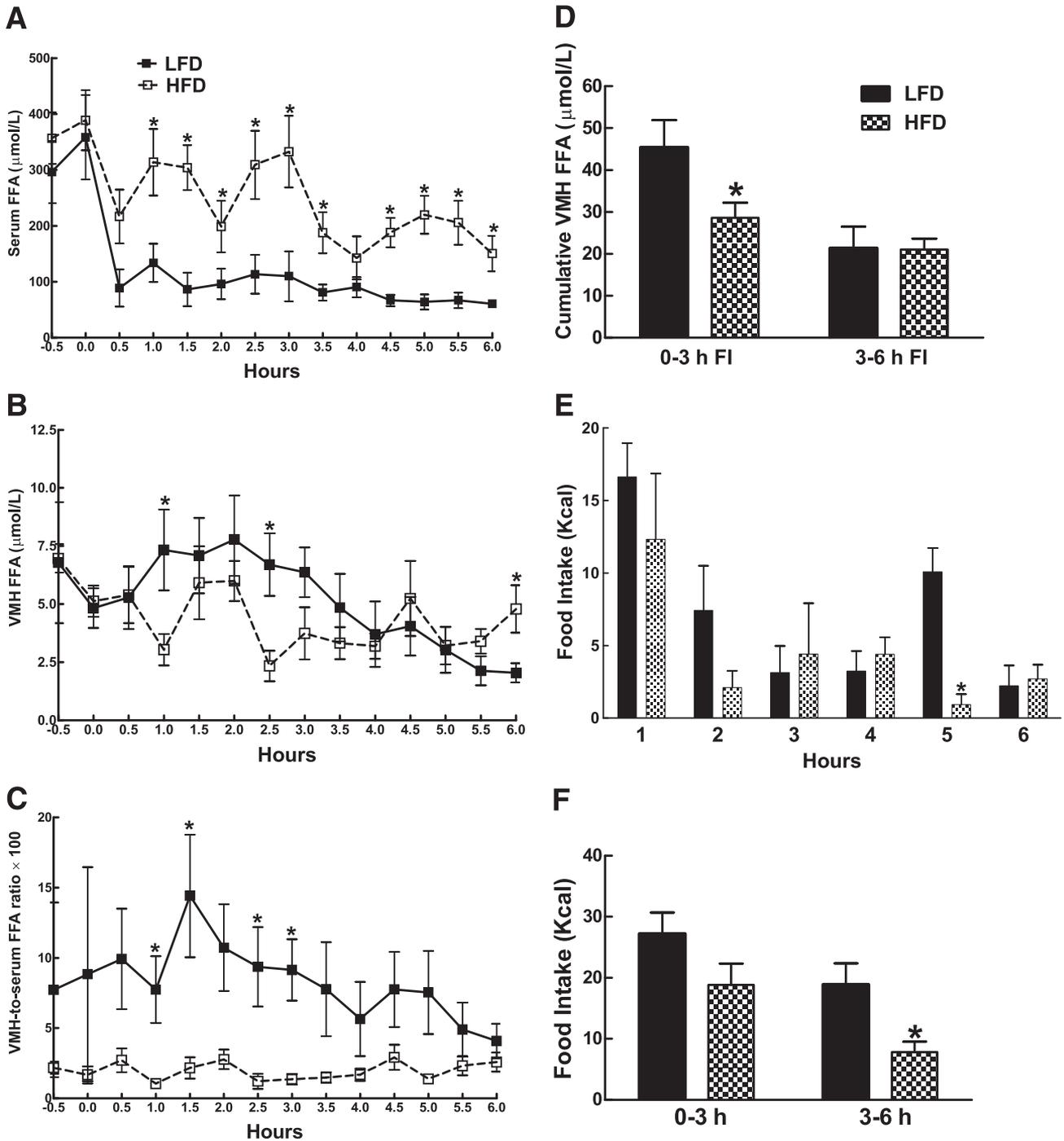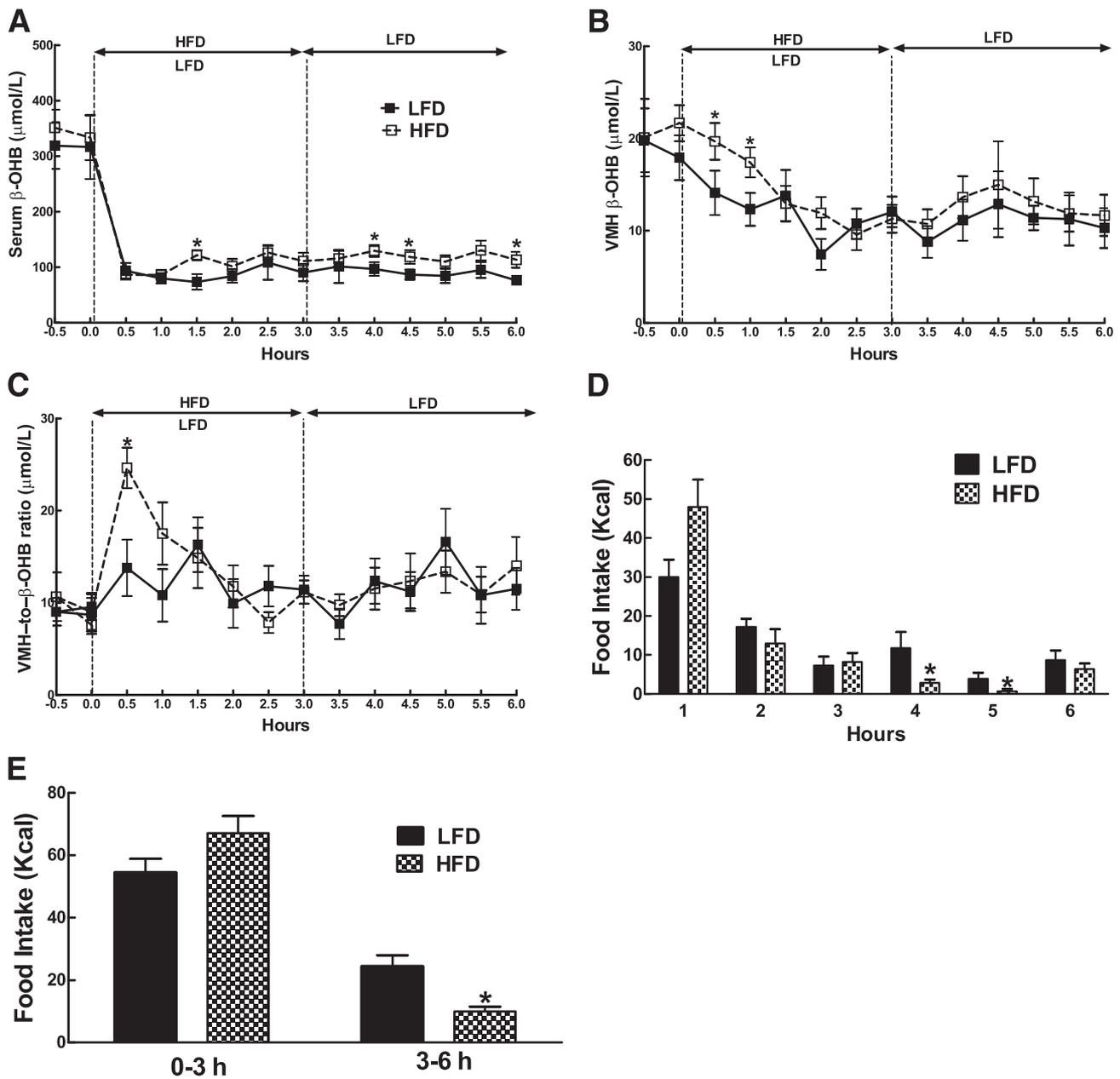
Figure 1—Rats were trained to eat all of an LFD (13.5% fat; $n = 8$) or an HFD (60% fat; $n = 7$) within 3 h of presentation at 0 h on the graph. On the day of testing, FFA levels were measured every 30 min in serum and VMH microdialysates during 6 h of access to their respective diets. A: Serum FFA levels. B: VMH FFA levels. C: VMH-to-serum FFA ratios × 100. D: Cumulative VMH FFA levels. E: Hourly food intake. F: Cumulative food intake during 6 h after presentation of food to rats previously trained to eat all of their food within 3 h of presentation. Data are mean ± SEM ($n = 7-8$). *$P < 0.05$ by one-way ANOVA.

hour, 82% less during the second hour, and 60% less during the entire second 3-h period ($P < 0.05$; Fig. 2D and E). This effect in HFD rats was unlikely due to neophobia because a similar pattern was observed when HFD rats were maintained on that diet for 6 h (Fig. 1D

and F). Finally, there were no differences in serial plasma glucose levels between the groups during the entire 6-h period (data not shown).

The next step was to test the hypothesis that the early increase in VMH ketones and the reduced intake of chow

Figure 2—Rats were trained to eat all of an LFD (13.5% fat; $n = 8$) or HFD (60% fat; $n = 7$) within 3 h of presentation at 0 h on the graph. On the day of testing, β-OHB levels were measured every 30 min in serum and VMH microdialysates during 3 h of access to their respective diets, followed by 3 h on an LFD. Serum (A) and VMH (B) β-OHB levels in μmol/L. C: VMH-to-serum β-OHB ratios × 100. D: Hourly food intake. E: Cumulative food intake during the 6-h period. Data are mean ± SEM ($n = 7–8$). *$P < 0.05$ by one-way ANOVA for A–C, *$P < 0.05$ by t test for D.

from 3 to 6 h in HFD rats was due to local astrocyte production of ketones. To first assess the efficacy of hymeglusin as an inhibitor of astrocyte ketone production, we needed to verify results from prior studies showing that cultured astrocytes produce ketones from FA (15,25). Indeed, cultured astrocytes synthesized ketones from both LCFA (OA and PA) and medium-chain (OctA) FA (Fig. 3). We next demonstrated for the first time that, as in bacteria (20,24), hymeglusin does indeed completely inhibit ketone production from OA and PA,

but only partially by OctA in cultured astrocytes ($P < 0.05$; Fig. 3). Having verified the efficacy of hymeglusin's ability to inhibit ketone production in astrocytes, we next reverse-dialyzed hymeglusin or its vehicle bilaterally into the VMH of rats trained to eat the LFD or HFD in 3 h/day. On testing day, they were given 3 h of LFD or HFD, followed by 3 h of LFD. As expected, hymeglusin decreased VMH ketones relative to serum ketone in LFD rats over the second hour after LFD was presented ($P < 0.05$; Fig. 4E) but did not alter their food intake (Fig. 4G
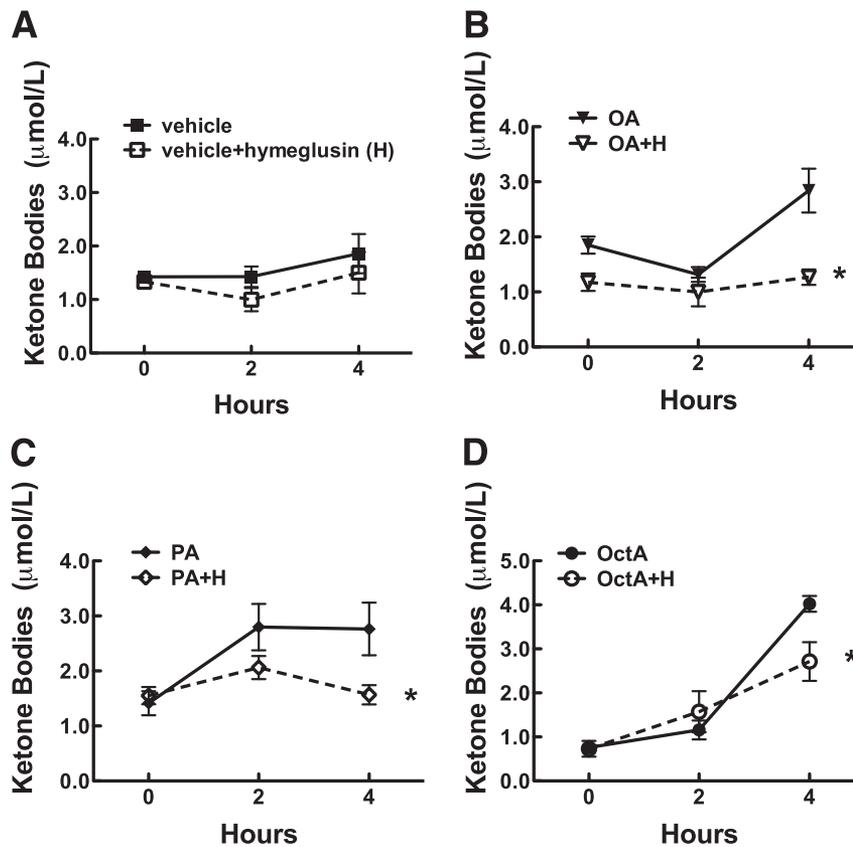
Figure 3—Ketone production in primary cultured VMH astrocytes from P21 rats. *A*: Effects of vehicle (0.4% DMSO) and vehicle plus 30 μmol/L hymeglusin (H). Vehicle plus 30 μmol/L hymeglusin in the presence or not of 150 μmol/L OA (*B*), 150 μmol/L PA (*C*), and 150 μmol/L OctA (*D*) on ketone production during a 4-h period. Data are mean ± SEM (*n* = 6). *$P < 0.05$ by one-way ANOVA.

and *H*). However, in HFD rats, hymeglusin completely inhibited the rise in VMH ketones relative to serum ketone levels over the first 2 h after HFD presentation ($P < 0.05$; Fig. 4*E*). This inhibition had no effect on intake over the first 3 h but was associated with a doubling of intake from 3 to 6 h (Fig. 4*G* and *H*) to levels comparable to those seen in rats fed the LFD for the entire 6-h period (Fig. 4*G* and *H*). Taken together, these results confirm the supposition that VMH astrocytes produce ketones in animals fed an HFD and that local VMH ketone production is, indeed, responsible for the decrease in food intake seen during a second 3-h period of refeeding after an 18-h fast.

Effect of Ketone Bodies on VMN Neuronal Glucose- and FA-Sensing

Finally, to test the hypothesis that the effects of HFD-induced VMH ketone production could provide a mechanism for the reduction in feeding seen during the 3- to 6-h period of intake, we assessed the effects that ketones (β-OHB and AA) have on the ability of dissociated VMN neurons to respond to glucose and FA in vitro. Of all the VMN neurons assessed, regardless of their glucose-sensing capacity, five- and twofold more were excited

than inhibited by β-OHB at 2.5 mmol/L and 0.5 mmol/L glucose, respectively (Table 1). Among those neurons that were glucose-sensing, threefold more GE neurons were further excited than were inhibited by β-OHB at 2.5 mmol/L glucose. Similarly, more than half the GI neurons held at 2.5 mmol/L glucose (at which concentration most are inactive) were excited, whereas none were further inhibited by β-OHB (Table 1). At 0.5 mmol/L glucose, the overall percentages of GE neurons excited and inhibited by β-OHB was similar to those at 2.5 mmol/L glucose. However, GI neurons, which are primarily activated at 0.5 mmol/L glucose, so that only 25% of those neurons were further excited, whereas 13% were inhibited by β-OHB at that glucose concentration. Similar to its primarily excitatory effect on VMN glucose-sensing and non–glucose-sensing neurons, β-OHB had a predominant excitatory versus inhibitory effect on both OAE and OAI VMN FA-sensing neurons held at 2.5 mmol/L glucose in the presence of 15 nmol/L OA (Table 2). However, in 0.5 mmol/L glucose and 15 nmol/L OA, β-OHB excited and inhibited equal percentages of OAE and OAI neurons. Thus, as with glucose-sensing, the effects of β-OHB on VMN FA-sensing neurons was primarily excitatory at
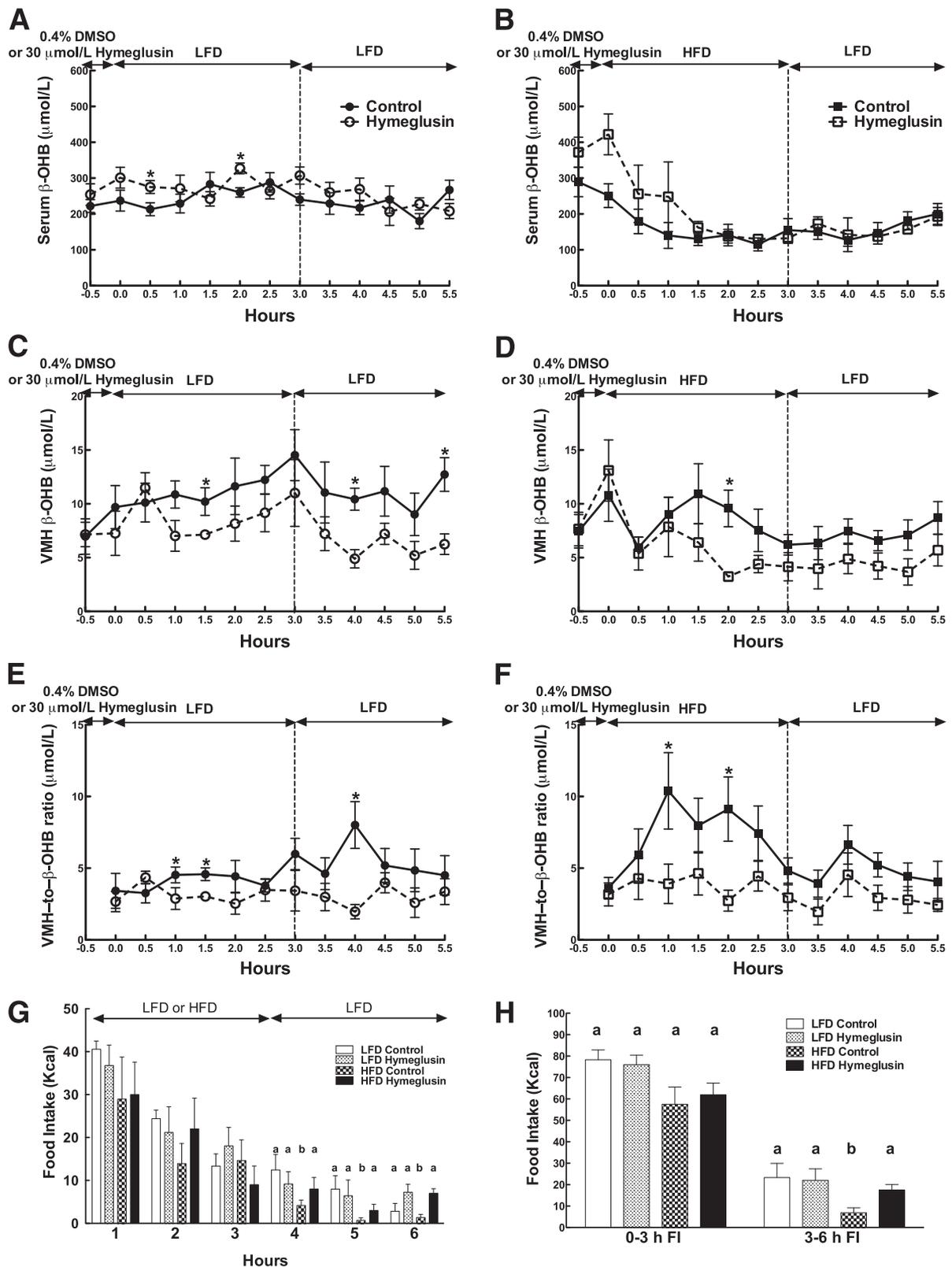
Figure 4—Rats were trained to eat all of their food (LFD, 13.5% fat [n = 12]; HFD, 60% fat [n = 16]) within 3 h of presentation at 0 h on the graph. On the day of testing, 0.4% DMSO (control; n = 6–8) or 30 μmol/L hymeglusin (n = 6–8) were reverse dialyzed for 2 h before food was introduced. Levels of β-OHB were measured every 30 min from serum and VMH microdialysates for 2 h before food was introduced, after the 3-h access to the HFD, followed by 3 h on the LFD. *A*: LFD serum β-OHB levels. *B*: HFD serum β-OHB levels. *C*: LFD VMH β-OHB levels. *D*: HFD VMH β-OHB levels. *E*: LFD VMH-to-serum β-OHB ratios $\times$ 100. *F*: HFD VMH-to-serum β-OHB ratios $\times$ 100. *G*: Hourly food intake for 6 h. *H*: Food intake in kcal during the 3-h feeding period, followed by 3 h on the LFD. Data are mean $\pm$ SEM (n = 6–8). *P < 0.05 one-way ANOVA (*A–F*); [a,b]differ from each other at the P < 0.05 level after two-way ANOVA, followed by Bonferroni test (*G* and *H*).

Table 1—Effect of β-OHB on VMN GE, GI, and NG neurons

	2.5 mmol/L glucose			0.5 mmol/L glucose		
	% of total	β-OHB excited	β-OHB inhibited	% of total	β-OHB excited	β-OHB inhibited
GE	12 ± 4	50 ± 9[a]	17 ± 5[b]	10 ± 4	32 ± 8[a]	18 ± 11[a]
GI	14 ± 3	63 ± 12[a]	0 ± 2[b]	8 ± 2	25 ± 9[c]	13 ± 6[c]
NG	74 ± 4	45 ± 7[a]	8 ± 3[b]	82 ± 3	23 ± 4[c]	14 ± 6[c]
Total	100 (170)	48 ± 8 (82)[a]	9 ± 3 (15)[b]	100 (235)	27 ± 5 (67)[c]	14 ± 4 (35)[c]

Data are mean ± SEM. At 2.5 and 0.5 mmol/L glucose, freshly dissociated VMN neurons were classified as GE, GI, or NG by alterations in $[Ca^{2+}]_i$ oscillations as glucose was changed from 2.5 to 0.5 to 2.5 mmol/L or from 0.5 to 2.5 to 0.5 mmol/L, respectively. They were then held at 2.5 or 0.5 mmol/L and exposed to 1–100 μmol/L β-OHB and classified as β-OHB excited or inhibited. Data are in percentage of total neurons tested in each category. Total indicates total percentage of each category of neurons for each β-OHB category, irrespective of their glucose-sensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. [a,b,c]Differs from each other in each glucose-sensing category by $P < 0.05$ by one-way ANOVA, followed by post hoc t test.

glucose levels comparable to those seen in the fed state, whereas at fasting glucose levels, β-OHB had equivalent excitation and inhibition.

Somewhat differing responses of VMN neurons to AA at 1 μmol/L (26) were seen at 2.5 and 0.5 mmol/L glucose (Supplementary Tables 1 and 2). For all VMN neurons, AA exerted an excitatory effect in high and low glucose. When assessed with regard to their glucose-sensing properties, most GEs and all GIs were excited by AA. In the presence of 15 nmol/L OA, VMN OAE neurons were further exited, whereas OAI neurons were further inhibited. Thus, as with β-OHB, the predominant effect of AA was excitation of VMN OAE neurons. However, although β-OHB predominantly excited OAI neurons, AA largely inhibited them. These results demonstrate that, although their actions differed somewhat, both ketone bodies largely overrode the effects of glucose and OA on all VMN neurons and specifically on glucose- and FA-sensing neurons. Finally, a combination of AA and β-OHB had a predominantly excitatory versus inhibitory effect in OAI VMN FA-sensing neurons held at 2.5

mmol/L glucose in the presence of 15 nmol/L OA (Table 3; Supplementary Fig. 2).

DISCUSSION

Several lines of evidence suggest that FA can act on the brain, and particularly the hypothalamus, to decrease food intake over relatively short periods of time (6,27,28). Similarly, altering FA metabolism in the periventricular brain areas in general (29), or in the hypothalamus specifically (11,30), alters intake. Although these effects are generally inferred to be due to changes in neuronal FA metabolism, astrocytes are the major source of FA oxidation and metabolism in the brain (14,17,31,32). Furthermore, as others have shown (15,25) and we show here, astrocytes produce ketone bodies from FA and these can then be exported to be used by neurons to alter their activity. In addition, no studies have shown that neurons are able to produce ketone bodies. A major problem with many previous studies is that there is no way to know what levels of FFA and ketones appear in the hypothalamus or other

Table 2—Effect of β-OHB on VMN OAE, OAI, and OAN neurons

	2.5 mmol/L glucose			0.5 mmol/L glucose		
	% of total	β-OHB excited	β-OHB inhibited	% of total	β-OHB excited	β-OHB inhibited
OAE	43 ± 10	37 ± 7[a]	14 ± 5[b]	41 ± 4	21 ± 10[a]	16 ± 5[b]
OAI	14 ± 7	39 ± 9[a]	15 ± 6[b]	10 ± 2	7 ± 5[b]	7 ± 7[b]
OAN	43 ± 6	47 ± 9[a]	8 ± 4[b]	49 ± 4	24 ± 7[a]	12 ± 4[b]
Total	100 (199)	41 ± 8 (76)[a]	10 ± 2 (22)[b]	100 (159)	26 ± 4 (43)[a]	13 ± 3 (21)[b]

Data are mean ± SEM. At 2.5 and 0.5 mmol/L glucose, VMN neurons were classified as OAE, OAI, or OAN by alterations in $[Ca^{2+}]_i$ oscillations produced by exposure to 15 nmol/L OA. They were then exposed to 1–100 μmol/L β-OHB in the presence of 15 nmol/L OA and were then classified as β-OHB excited or inhibited. Data are in percentage of total neurons tested in each category. Total indicates total percentage of each category of neurons for each β-OHB category, irrespective of their OA-sensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. [a,b]Differs from each other by $P < 0.05$ by one-way ANOVA followed by post hoc t test.

Table 3—Effect of β-OHB and AA on VMN OAE, OAI, and OAN neurons

| | | 2.5 mmol/L glucose | |
| | | β-OHB–AA | |
	% of total	Excited	Inhibited
OAE	27 ± 4	16 ± 4[a]	27 ± 3[a]
OAI	20 ± 2	54 ± 10[a]	13 ± 3[b]
OAN	53 ± 3	37 ± 6[a]	8 ± 2[b]
Total	100 (463)	33 ± 5 (151)[a]	13 ± 1 (60)[b]

Data are mean ± SEM. At 2.5 mmol/L glucose, VMN neurons were classified as OAE, OAI, or OAN by alterations in $[Ca^{2+}]_i$ oscillations produced by exposure to 15 nmol/L OA. They were then exposed to 20 μmol/L β-OHB and 1 μmol/L AA in the presence of 15 nmol/L OA and were classified as β-OHB–AA excited or β-OHB–AA inhibited. Data are in percentage of total neurons tested in each category. Total indicates total percent of each category of neurons for each β-OHB–AA category, irrespective of their OA-sensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. [a,b]Differs from each other by $P < 0.05$ by one-way ANOVA, followed by post hoc t test.

brain areas during fasting or intake of an LFD versus HFD or how these levels might affect the intake of these diets.

To address these issues, we first used microdialysis to measure ongoing levels of VMH FFA and ketone bodies after an 18-h fast and then during the first 6 h of intake of LFD versus HFD. We used this paradigm to maximize the chances of being able to detect changes in FFA and ketone levels by forcing the rats to eat all of their calories in this restricted period. We found that rats eating the LFD had lower levels of serum FFA but, surprisingly, had higher levels of VMH FFA than did HFD rats over a 6-h period of intake after their 18-h fast. Despite these higher levels, caloric intake was similar during the first 3 h, but LFD rats underwent a second major set of feeding bouts during the second 3 h that was not seen in the HFD rats when each group was continued on its original diet or when both were continued on the LFD. This suggested that FA, per se, were not responsible for differences in intake during this second 3-h period. For that reason, we postulated that astrocyte-derived ketone production was responsible for these differences in food intake. In fact, there was a spike of increased VMH ketone levels during the first 1 h of HFD intake that was independent of serum ketone levels. This suggested that local production of ketones had occurred and that the reduced food intake during the second 3-h epoch of feeding was related to this spike. To test this hypothesis, we inhibited local VMH ketone production in HFD rats with a resultant doubling of their intake to levels comparable to LFD rats during the second 3-h epoch. These results strongly suggest that locally produced ketone

bodies by VMH astrocytes exposed to dietary FA are responsible for a delayed decrease in intake in rats fed the HFD.

To explore the potential mechanism of this ketone effect, we next assessed the effect of ketone-, glucose-, and FA-sensing in VMN neurons. We found that ketones overrode the actions of glucose and OA, with a predominantly excitatory overinhibitory effect on glucose- and FA-sensing. However, this generalization depends to some degree on whether those neurons were excited or inhibited by glucose or FA, on the ambient glucose concentration, and on the type of ketone used. Regardless of these details, the important point is that ketone bodies can override normal glucose- and FA-sensing in VMN neurons. We postulate that this overriding of these normal metabolic-sensing pathways explains the ketone-induced reduction in feeding that follows ingestion of an HFD. Thus, although we have shown that VMH FA-sensing neurons utilize FA translocase/CD36 and glucose-sensing neurons utilize glucokinase as major regulators of their ability to respond to LCFA (18,21,33) and glucose (22,34), respectively, our current studies clearly demonstrate that ketones can override these mechanisms, probably by providing an alternate source of ATP and/or reactive oxygen species that would modulate the ATP-sensitive channel or other channels that are capable of responding to the intracellular metabolism of glucose and FA (18,34–36).

The studies reported here are the first to use microdialysis to assess serial brain FFA and ketone levels during ongoing ingestion of different diets. We first demonstrated the validity of using this method using zero-net-flux, a technique that does not depend on a calculation of probe efficiency (19). These results demonstrate that 24-h fasting levels of VMH FFA and ketones are in the low micromolar range. Curiously, when rats were given food after an 18-h fast, VMH FFA levels rose to higher levels in rats ingesting the LFD versus the HFD, even though serum FFA levels were higher in the HFD rats. This paradoxical finding could be due to increased transport and/or reduced uptake and metabolism of FA into the VMH of rats ingesting an LFD after fasting. As opposed to FFA levels, absolute levels of VMH ketones remained at fasting levels for the first 30 min after the onset of feeding in HFD rats, despite a rapid drop in serum ketones producing a spike in the VMH-to-serum ratio during the first 60 min of feeding, which was not seen in the LFD rats. This strongly suggests that local production of ketones occurred within the VMH resulting from the HFD intake. In strong support of the supposition that such local production occurred in astrocytes, we showed that inhibition of HMG-CoA synthase, the penultimate step in the ketogenesis pathway that transforms acetoacetyl-CoA into HMG-CoA, a precursor of ketone bodies (24), inhibited ketone production in cultured astrocytes and locally in the VMH of rats ingesting the HFD.

Although VMH astrocytes appear to produce ketones from dietary FA during the first 60 min of ingesting an HFD, the suppressive effects on feeding do not appear until 3–6 h of refeeding after an 18-h fast, regardless of the diet the rats ingested during this period. We showed here that ketones can readily override normal glucose- and FA-sensing in VMN neurons and that inhibition of VMH ketone production reversed the reduced feeding of HFD rats during the 3- to 6-h period. This lag between ketone production and suppression of feeding suggests that VMH FA-sensing neurons are not involved in the direct and immediate regulation of ongoing feeding. One possibility is that altering the ability of VMH neurons to normally sense FA in the presence of a large influx of ketones leads to a series of events that alters the production and subsequent release of gut satiety hormones during feeding bouts that follow the initial large intake of food subsequent to the 18-h fast. Alternatively, a large pulse of ketones might alter gene expression within VMH neurons such that they change the mechanisms by which they sense FA upon subsequent dietary intake.

Finally, although our data demonstrate that an HFD suppresses refeeding after an 18- to 24-h fast, chronic ingestion of an HFD clearly causes increased intake and obesity in most rodents. One possibility is that the artificial imposition of a forced 3-h feeding period does not mimic natural feeding rhythms and may impose stresses on the animals. However, even in the face of such a restricted regimen, our rats still ate three to four discrete meals during the first 3-h period and two smaller meals during the second 3-h feeding period. Thus, our results demonstrate that HFD refeeding after a substantial fast is significantly reduced and that this reduction can be reversed by inhibiting focal ketone production by VMH astrocytes. Physiologically, this means that, while fasting serum ketone levels drop precipitously after LFD or HFD intake, a large HFD load can act to moderate later intake by increasing local production of ketones by astrocytes that surround and provide substrates to VMH FA-sensing neurons. This provides for diet-dependent local control of neuronal activity.

There are some caveats to the methods used here. First, although LCFA are the major source of FA oxidation and ketone production in astrocytes (15,17,25), the assay used here from microdialysates measured all FA, regardless of chain length, so that we cannot be certain of the exact proportion of various LCFA or the chain lengths of other FA arriving in the VMH. Second, we used calcium imaging as a surrogate for glucose-, FA-, and ketone-induced changes in neuronal activity. In fact, this method correlates well with changes in membrane potential induced by FA (18,21), but we cannot state with certainty that this necessarily correlates with changes in neuronal activity. Nevertheless, we have shown that interfering with VMH neuronal responses by altering CD36-mediated FA sensing has major effects on long-term energy and glucose homeostasis (18).

Finally, caloric intake by rats undergoing microdialysis for FA measures were appreciably lower in the first 3 h than were those dialyzed for ketone measures. This lower intake might have been a result of some tissue damage due to the much larger microdialysis probe required to measure FA. The important point is that their feeding patterns did not differ from those in the ketone studies (data not shown), and we found the same delayed decrease in intake over the second 3-h epoch in both sets of studies.

In conclusion, ingestion of an HFD after an 18-h fast leads to reduced intake with a delay of 3–6 h. Our results suggest that although ketones produced in the periphery during fasting, when glucose supply is limited, might be utilized to fuel overall neuronal energy needs, intake of an extremely HFD causes an early increase in local astrocyte production of ketones that subverts normal nutrient-sensing specifically in FA-sensing neurons. The mechanism by which this produces delayed inhibition of feeding remains unclear, but it is clear that inhibition of this early peak in ketone production selectively in the VMH effectively reverses the delayed inhibition of feeding. Thus, as for the effect of astrocyte-produced lactate from glucose on neuronal glucose-sensing (37), astrocytes play a similar critical role in modulating neuronal FA-sensing that is highly dependent on dietary macronutrient content.

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