Regulation of hypothalamic neuronal sensing and food intake by ketone bodies and fatty acids

Running title: Role of ketone bodies and fatty acids in food intake

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ABSTRACT

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 high fat diet (60%; HFD) intake on feeding and serum and VMH FA levels, rats were trained to eat a low fat (13.5%; LFD) or a HFD in 3h/d and were monitored with VMH FA microdialysis. Despite having higher serum levels, HFD rats had lower VMH FA levels but ate less from 3-6 h of refeeding than did LFD rats. However, VMH β-hydroxybutyrate (β-OHB) and VMH/serum β-OHB levels were higher in HFD rats during the first 1h of refeeding suggesting that VMH astrocyte ketone production mediated their reduced intake. In fact, using calcium imaging in dissociated VMH neurons, ketone bodies overrode normal FA sensing, primarily by exciting neurons that were either activated or inhibited by oleic acid. Importantly, bilateral inhibition of VMH ketone production with a 3-hydroxy-3-methylglutaryl-CoA synthase inhibitor reversed the 3-6h 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.
INTRODUCTION

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 (FA), 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 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 low fat diet (LFD) vs. HFD. In addition, although there is a sizeable literature demonstrating that pharmacological and molecular manipulations of FA metabolism in the brain can alter feeding behaviour (8-13), most of it overlooks the fact that the majority of 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). Since neurons can utilize ketone bodies as an alternate energy source (16; 17), we postulated that any effects that ingestion of a 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 both energy and glucose homeostasis (18). Thus, we used microdialysis to monitor VMH brain ketone levels in parallel with serum ketone levels and food intake of HFD vs. LFD and coupled these with in vitro studies of the effects of ketone bodies on the FA sensing in VMH neurons. The results of these studies are presented here and suggest a novel mechanism by which high fat diet 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 Institutional Animal Care and Use Committee of the E. Orange Veterans Affairs Medical Center.

VMH FFA and food intake measurements

Rats (n=8/group; 300-400g) were anesthetized with isoflurane (1.5% at 0.8 l/min) and a unilateral microdialysis probe with a 3mm membrane length and 3000kDa 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 (ARC) and ventromedial hypothalamic (VMN) nuclei (VMH=VMN+ARC; −2.9 mm bregma, ±3.7 mm midline, and −8.5 mm dura). The next day at 0800 (2h before food was presented), microdialysis was begun with probes perfused with artificial cerebrospinal fluid (aCSF, Harvard Apparatus, Holliston, Massachusetts, USA) containing 3% FA free bovine serum albumin (BSA, Sigma Aldrich, St. Louis, Missouri, USA) at 1.0 µl/min. Eluates were collected every 30min and were stored at -80°C until NEFA assay. Probe placement was assessed terminally.

A first set of rats were fasted for 24 h and VMH probes were infused for 1h at each of 5 concentrations (0-400 µmol/L) of OA with monitoring of effluent OA concentrations at 20min. Fasting VMH OA levels were calculated using the zero-net-flux method (19). A second set of rats were trained for 2 wk to eat either 13.5% LFD (Purina #5001) or 60% HFD (Research Diet D12492, New Brunswick, NJ) (n=8/group) in 3 h each day from 1000 to 1300 in BioDAQ© food intake monitoring apparatus. After 1 wk, jugular catheters were implanted and rats were allowed 6–7 d to recover their preoperative body weight on their 3 h/d feeding
schedule. On testing day, rats ate their respective diets for 6 h while having their ongoing food intake monitored and simultaneously undergoing 30 min FA microdialysis and blood sampling.

**VMH β–hydroxybutyrate (β-OHB) and feeding measurements**

LFD rats (n=8/group) had unilateral VMH guide cannulae implanted. After 1-2 wk of recovery, they were fasted for 24 h for zero-net-flux assessment. The next day, at 0800, microdialysis probes (3mm membrane length and 6kDa pore size (CMA 11, Harvard Apparatus, Holliston, MA) were inserted into the guide cannulae and perfused at 1.0 µl/min with β-OHB diluted in aCSF for 40 min at each of 4 concentrations (0-100µmol/L) and effluent β-OHB concentrations were monitored. Next, additional rats were trained for 2 wk to eat either LFD or HFD (n=8/group) over 3 h/d and were implanted with jugular catheters and unilateral VMH guide cannulae. At 0800 on the test day, microdialysis probes were inserted and jugular catheters connected and their respective diets were returned from 1000-1300. At 1300, rats on 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 30min.

A third set of rats (n=6-8/group) were conditioned to eat the LFD or HFD in 3 h/d 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+0.4% DMSO vehicle or 30 µmol/L hymeglisin, a 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) inhibitor (20), in aCSF+0.4% DMSO at 1.0 µl/min (n=6-8/ group) along with simultaneous microdialysis for ketones was begun. The LFD or HFD was provided from 1000-1300 and the LFD from 1300-1600 with monitoring of food intake, VMH and serum β–OHB levels.
β-OHB and acetoacetate induced changes in intracellular Ca$^{2+}$ ([Ca$^{2+}]_i$) oscillations in dissociated VMN neurons

Postnatal day 21-28 (P21-28) rats were perfused and neurons were dissociated from VMN punches previously described (18; 21; 22). Evaluation of glucose-, oleic acid (OA)- and ketone body-induced alterations in [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-glucosensing (NG), then as OA excited (OAE), inhibited (OAI), non-responsive (OAN) and as β-OHB and acetoacetate (AA)-excited (β-OHBE or AAE), -inhibited (β-OHBI or AAI) or non-responsive using previously established criteria for changes in [Ca$^{2+}]_i$ area under the curve (21; 22). Studies began with neurons held at either 2.5mmol/L or 0.5mmol/L glucose followed by 15nmol/L OA and by 1-100µmol/L β-OHB or 1µmol/L AA. Additionally, a combination of 1µmol/L AA and 20µmol/L β-OHB (their relative ratio in the brain) were also assessed. All neurons were incubated with 20nmol/L glutamate terminally to assess viability.

In vitro effects of FA on VMH astrocyte-induced ketone production

The VMH was dissected from P21-P28 rats and was triturated in Neurobasal-A (Invitrogen, Grand Island, NY) containing 5mmol/L glucose, 0.23mmol/L sodium pyruvate, 100U/ml penicillin/streptomycin, 10µg/ml gentamycin, 10% fetal bovine serum (FBS) at pH 7.4. Dissociated cells were plated in 25 cm$^2$ flasks coated with poly-D-lysine (50µg/ml) until confluence. Astrocytes were then separated from remaining microglia by shaking at 250 rpm for 3 h at 37º C (23). Astrocytes attached to the flask were trypsinized with 0.05 % trypsin-EDTA (Sigma, St Louis, MO), centrifuged and the pellet re-suspended 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), 150µmol/L of OA, palmitic acid (PA) or octanoic acid (OctA) or 30 µmol/L hymeglusin. Hymeglusin was synthesized by H.M. Mizorko.
and is an HMG-CoA synthase inhibitor that is been demonstrated to inhibit ketone production in bacteria (20; 24). Media was then harvested at 0h, 2h and 4h 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**

Using Systat (Chicago, IL) and GraphPad Prism software, La Jolla, CA), one-way and two-way ANOVA and one-way ANOVA for repeated measures with post-hoc Bonferonni corrections were carried out for the *in vitro* and *in vivo* studies. No more than 2 outliers per group were removed if necessary as utilizing Systat software.

**RESULTS**

**Dietary effects on blood and VMH free FA (FFA) levels and food intake**

The efficacy of using microdialysis to measure VMH extracellular FFA levels was established in LFD, 24 h fasted rats using the zero-net-flux method and was found to be 37.12±9.87µmol/L 37.2±9.8µmol/L (Supplementary Fig. 1A; n=5). Thus, VMH FFA levels are ~10-fold less than in the blood. 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 over 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. 1A). In HFD rats, serum FFA remained at relatively high levels over the entire 6 h (Fig. 1A) and were 120% higher than LFD levels during the first 3 h (P=0.001, Fig 1A). Given the marked difference in serum FFA, VMH FFA levels were unexpectedly 37% lower in HFD than LFD rats over much of
the first 3 h of feeding (P=0.045; Fig. 1B, C, 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 at a relatively large set of meals between 4 and 6h (P=0.013, Fig 1D, F). Importantly, even though they were fasted for 18h prior to their 3h intake periods during training, individual rats ate an average of one 15-20 kcal meals and two 5-10 kcal meals during the first 3h period. On test days, rats also ate two 6-10 kcal meals during the second 3h 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**

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

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 LFD for 3 h more. In the fasting condition, serum ketone levels were comparably high in both groups (LFD: 319±42µmol/L, HFD: 351±32µmol/L). By 30 min after food exposure, β-OHB levels fell to ~70-75% of their respective baselines (Fig. 2A). However, β-OHB levels then rose progressively from 3-6 h in the HFD rats but remained relatively stable in the LFD rats even though both groups were fed LFD during this period. As with serum ketone levels, fasting VMH ketone levels were comparably high in both groups (LFD: 19.8±3.4µmol/L, HFD: 20.1±4.2µmol/L). For 30 min after food exposure, VMH β-OHB levels remained stable relative in HFD rats, while levels fell in LFD rats (Fig. 2B). This resulted in a 2-fold higher VMH/serum ratio at
30 min and was still 70% higher 60 min after onset of feeding in HFD vs. LFD rats (P<0.05; Fig. 2C). This elevated ratio of VMH to serum ketone levels on a 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 initial HFD intake, the fact 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 over the first 3 h on their respective diets (Fig. 2D, E). However, when switched to chow, the rats previously eating HFD ate 76% less than LFD rats during the first hour, 82% less over the second hour and 60% less over the entire second 3 h period (P<0.05; Fig. 2D, E). This effect in HFD rats was unlikely due to neophobia since a similar pattern was observed when HFD rats were maintained on that diet for 6 h (Fig. 1D, F). Finally, there were no differences in serial plasma glucose levels between the groups over 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 from 3-6 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 long chain (oleic and palmitic) and medium chain (octanoic) FA (Fig. 3). We next demonstrated for the first time that, as in bacteria (20; 24), hymeglusin does indeed completely inhibit ketone production from oleic and palmitic acids, but only partially by octanoic acid 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 either hymeglusin or its vehicle bilaterally into the VMH of rats trained to eat LFD or HFD in 3 h/d. 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 it did not alter their food intake (Fig. 4G, 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. 4E). This inhibition had no effect on intake over the first 3 h but was associated with a doubling of intake from 3-6 h (Fig. 4G, H) to levels comparable to those seen in rats fed LFD for the entire 6h period (Fig. 4G, H). Taken together, these results confirm the supposition that VMH astrocytes produce ketones in animals fed a HFD and 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 3-6 h period of intake, we assessed the effects that ketones (β−OHB and acetoacetate (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 glucosensing capacity, 5- and 2-fold more were excited than inhibited by β−OHB at 2.5mmol/L and 0.5mmol/L glucose, respectively (Table 1). Among those neurons which were glucosensing, 3-fold more GE neurons were further excited than were inhibited by β−OHB at 2.5mmol/L glucose. Similarly, more than half the GI neurons held at 2.5 mmol/L glucose (at which concentration most are inactive) were excited while none were further inhibited by β−OHB (Table 1). At 0.5mmol/L glucose, the overall percentages of GE neurons excited and inhibited by β−OHB was similar to those at 2.5mmol/L glucose. On the other hand, GI neurons, which are primarily activated at 0.5mmol/L glucose, so that only 25% of those neurons were further excited, while 13% were inhibited by β−OHB at that glucose concentration. Similar to its primarily excitatory effect on VMN glucosensing and non-glucosensing neurons, β−OHB had a predominant excitatory vs. inhibitory effect on both OA excited and inhibited VMN FA
sensing neurons held at 2.5mmol/L glucose in the presence of 15nmol/L OA (Table 2). On the other hand, in 0.5mmol/L glucose and 15nmol/L OA, β-OHB excited and inhibited equal percentages of OA excited and inhibited neurons. Thus, as with glucosensing, the effects of β-OHB on VMN FA sensing neurons was primarily excitatory at glucose levels comparable to those seen in the fed state, while at fasting glucose levels, β-OHB had equivalent excitation and inhibition.

Somewhat differing responses of VMN neurons to AA at 1µmol/L (26) were seen at 2.5 and 0.5mmol/L glucose (Supplementary Table 1 and 2). For all VMN neurons, AA exerted an excitatory effect in high and low glucose. When assessed with regard to their glucosensing properties, most GE and all GI were excited by AA. In the presence of 15nmol/L OA, VMN OA excited neurons were further exited while OA inhibited neurons were further inhibited. Thus, as with β-OHB, the predominant effect of AA was excitation of VMN OA excited neurons. On the other hand, while β-OHB predominantly excited OA inhibited neurons, AA largely inhibited them. These results demonstrate that, while their actions differed somewhat, both ketone bodies largely overrode the effects of both 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 vs. inhibitory effect in OA inhibited VMN FA sensing neurons held at 2.5mmol/L glucose in the presence of 15nmol/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. While it is generally inferred that these effects are due to changes in neuronal FA metabolism, the fact is that 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 utilized 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 the levels of FFA and ketones appear in the hypothalamus or other brain areas during fasting or intake of a LFD vs. 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 following an 18h fast and then during the first 6h of intake of LFD vs. 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 a LFD had lower levels of serum FFA but, surprisingly, they had higher levels of VMH FFA than did HFD rats over a 6h period of intake following their 18h fast. Despite these higher levels, caloric intake was similar over the first 3h, but rats on a LFD underwent a second major set of feeding bouts during the second 3h that was not seen in the HFD rats when each groups was continued on their original diet or when both were continued on LFD. This suggested that FA, per se, were not responsible for differences in intake during this second 3h 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 1h of HFD intake which was independent of serum ketone levels. This suggested that there was local production of ketones and that the reduced food intake during the second 3h 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 3h 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 a HFD.
To explore the potential mechanism of this ketone effect, we next assessed the effect of ketones glucose and FA sensing in VMN neurons. We found that ketones overrode the actions of both glucose and OA, with a predominantly excitatory over inhibitory effect on both glucose and FA sensing. However, this generalization is dependent to some degree on whether those neurons were excited or inhibited by glucose or FA, on the ambient glucose concentration and 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 it is this overriding of these normal metabolic sensing pathways that explains the ketone-induced reduction in feeding that follows ingestion of a HFD. Thus, while we have shown that VMH FA sensing neurons utilize FAT/CD36 and glucosensing 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 intracellular metabolism of glucose and FA (18; 34-36).

The studies reported here are the first to utilize 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 which is not dependent upon a calculation of probe efficiency (19). These results demonstrate that 24h 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 vs. HFD, despite the fact that serum FFA levels were higher in the HFD rats. This paradoxical finding could be due either to increased transport and/or reduced uptake and metabolism of FA into the VMH of rats ingesting a LFD after fasting. As opposed to FFA levels, absolute levels of VMH ketones remained at fasting levels for the first 30min after the onset of feeding in HFD rats despite a rapid drop in serum ketones producing a spike in
VMH/serum ratio over the first 60 min of feeding which was not seen in the LFD rats. This strongly suggests that there was local production of ketones 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 hydroxyl-β-methylglutaryl-CoA, a precursor of ketone bodies (24), inhibited ketone production in both cultured astrocytes and locally in the VMH of rats ingesting a HFD.

Although it appears that VMH astrocytes do produce ketones from dietary FA during the first 60 min of ingesting a HFD, the suppressive effects on feeding do not appear until 3-6 h of refeeding after an 18 h fast, regardless of the diet they ingested during this period. We showed here that ketones can readily override normal glucose and fatty acid sensing in VMN neurons and that inhibition of VMH ketone production reversed the reduced feeding of HFD rats during the 3-6 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, while our data demonstrate that a HFD suppresses refeeding after an 18-24 h fast, clearly chronic ingestion of HFD 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 3-4 discrete meals over the first 3 h period and 2 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 following LFD or HFD intake, a large load of HFD can act to moderate later intake by increasing local production of ketones by astrocytes which 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 utilized 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 other chain length 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. Finally, caloric intake by rats undergoing microdialysis for FA measures were appreciably lower in the first 3h 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 3h epoch in both sets of studies.

In conclusion, ingestion of a HFD after an 18h fast leads reduced intake with a delay of 3-6 h. Our results suggest that while ketones produced in the periphery during fasting, when glucose supply is limited, might be utilized within to fuel overall neuronal energy needs, intake of an extremely high fat diet causes an early increase in local astrocyte production of ketones which 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 glucosensing (37), astrocytes play a similar critical role in modulating neuronal FA sensing which is highly dependent upon dietary macronutrient content.

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C.L.F. performed the research, designed the experiments and wrote the manuscript. A.D.M. performed the microdialysis surgeries. H.M.M. provided the hymeglusin. B.E.L. helped designed the experiments and write the manuscript. C.L.F. and B.E.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Table 1: Effect of β-hydroxybutyrate on VMN GE, GI and NG neurons

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<th>2.5 mmol/L glucose</th>
<th>0.5 mmol/L glucose</th>
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<td></td>
<td>% of total</td>
<td>β-OHB Excited</td>
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<td>GE</td>
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<td>12±4</td>
<td>50±9</td>
<td>17±5</td>
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<td>GI</td>
<td>14±3</td>
<td>63±12</td>
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<tr>
<td>NG</td>
<td>74±4</td>
<td>45±7</td>
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<td>Total</td>
<td>100 (170)</td>
<td>48±8 (82)</td>
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At 2.5 and 0.5 mmol/L glucose, freshly dissociated VMN neurons were classified as glucose excited (GE), inhibited (GI) or non-responsive (NG) by alterations in [Ca^{2+}]_{i} oscillations as glucose was changed from 2.5 to 0.5 to 2.5 mmol/L or 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 μmol/L to 100 μmol/L β-hydroxybutyrate (β –OHB) and classified as β-OHB excited or inhibited. Data are in percent of total neurons tested in each category. Total = total percent of each category of neurons for each β-OHB category, irrespective of their glucosensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. Data with differing superscripts in a given category differ from each other by P<0.05 by one way ANOVA followed by post-hoc T-test.
Table 2: Effect of β-hydroxybutyrate on VMN oleic acid (OA) excited (OAE), inhibited (OAI) and non-responsive (OAN) neurons

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<td></td>
<td>% of total</td>
<td>β-OHB Excited</td>
<td>β-OHB Inhibited</td>
<td>% of total</td>
<td>β-OHB Excited</td>
</tr>
<tr>
<td>OAE</td>
<td>43±10</td>
<td>37±7\textsuperscript{a}</td>
<td>14±5\textsuperscript{b}</td>
<td>41±4</td>
<td>21±10\textsuperscript{a}</td>
</tr>
<tr>
<td>OAI</td>
<td>14±7</td>
<td>39±9\textsuperscript{a}</td>
<td>15±6\textsuperscript{b}</td>
<td>10±2</td>
<td>7±5\textsuperscript{b}</td>
</tr>
<tr>
<td>OAN</td>
<td>43±6</td>
<td>47±9\textsuperscript{a}</td>
<td>8±4\textsuperscript{b}</td>
<td>49±4</td>
<td>24±7\textsuperscript{a}</td>
</tr>
<tr>
<td>Total</td>
<td>100 (199)</td>
<td>41±8 (76)\textsuperscript{a}</td>
<td>10±2 (22)\textsuperscript{b}</td>
<td>100 (159)</td>
<td>26±4 (43)\textsuperscript{a}</td>
</tr>
</tbody>
</table>

At 2.5 and 0.5 mmol/L glucose, VMN neurons were classified as OA excited (OAE) or in inhibited (OAI) or non-responsive (OAN) by alterations in [Ca\textsuperscript{2+}]_i oscillations produced by exposure to 15 nmol/L OA. They were then exposed to 1 µmol/L to 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 percent of total neurons tested in each category. Total = total percent 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. Data with differing superscripts in a given category differ from each other by P<0.05 by one way ANOVA followed by post-hoc T-test.

Diabetes
Table 3: Effect of β-hydroxybutyrate and Aceto-Acetate (AA) on VMN oleic acid (OA) excited (OAE), inhibited (OAI) and non-responsive (OAN) neurons

<table>
<thead>
<tr>
<th></th>
<th>2.5 mmol/L glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>OAE</td>
<td>27±4</td>
</tr>
<tr>
<td>OAI</td>
<td>20±2</td>
</tr>
<tr>
<td>OAN</td>
<td>53±3</td>
</tr>
<tr>
<td>Total</td>
<td>100 (463)</td>
</tr>
</tbody>
</table>

At 2.5 mmol/L glucose, VMN neurons were classified as OA excited (OAE) or in inhibited (OAI) or non-responsive (OAN) by alterations in [Ca\(^{2+}\)] 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 then classified as β-OHB-AA excited or inhibited. Data are in percent of total neurons tested in each category. Total = 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. Data with differing superscripts in a given category differ from each other by P<0.05 by one way ANOVA followed by post-hoc T-test.
FIGURE LEGENDS

**Figure 1:** Rats were trained to eat all of a LFD (13.5% fat; n=8) or HFD (60% fat; n=7) within 3 h of presentation at 0 h on graph. On the day of testing free fatty acid (FFA) levels were measured every 30 min in serum and VMH microdialysates during 6 h access to their respective diets. **A:** Serum FFA levels; **B:** VMH FFA levels; **C:** VMH/serum FFA ratios x 100; **D:** Cumulative VMH FFA levels **E:** Hourly food intake; **F:** Cumulative food intake over 6 h after presentation of food to rats previously trained to eat all of their food within 3 h of presentation. *P<0.05 by one way ANOVA.

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

**Figure 3:** Ketone production in primary cultured VMH astrocytes from P21 rats. **A:** effects of vehicle (0.4%DMSO) and vehicle+30µmol/l hymeglusin (H) **B, C, D:** vehicle+30µmol/l hymeglusin in the presence or not of 150µmol/l OA, 150µmol/l palmitic acid (PA) and 150µmol/l octanoic acid (OctA) on ketone production over 4 h. *P<0.05 by one way ANOVA.

**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 graph. On the day of testing, 0.4% DMSO (Control; n=6-8) or 30µM Hymeglusin (n=6-8) were reverse dialyzed for 2 h before food was introduced. β−hydroxybutyrate (β−OHB) levels were measured every 30 min from serum and VMH microdialysates for 2 h before food was introduced, the 3 h access to HFD
(60% fat) followed by 3h on LFD (13.5% fat). **A:** LFD Serum $\beta$-OHB levels; **B:** HFD Serum $\beta$-OHB levels; **C:** LFD VMH $\beta$–OHB levels; **D:** HFD VMH $\beta$–OHB levels; **E:** LFD VMH/serum $\beta$-OHB ratios x100; **F:** HFD VMH/serum $\beta$-OHB ratios x100; **G:** Hourly food intake for 6h; **H:** Food intake in kcal during the 3 h feeding period followed by 3 h on LFD. *P<0.05 one way ANOVA A-F, *P<0.05 two way ANOVA followed by bonferroni test G-H.
Supplementary Table 1: Effect of acetoacetate on VMN GE, GI and NG neurons

<table>
<thead>
<tr>
<th></th>
<th>2.5 mmol/L glucose</th>
<th>0.5 mmol/L glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total</td>
<td>AA Excited</td>
</tr>
<tr>
<td>GE</td>
<td>11±1</td>
<td>29±6(^a)</td>
</tr>
<tr>
<td>GI</td>
<td>9±2</td>
<td>72±11(^a)</td>
</tr>
<tr>
<td>NG</td>
<td>80±3</td>
<td>22±4(^a)</td>
</tr>
<tr>
<td>Total</td>
<td>100 (330)</td>
<td>27±3 (97(^a))</td>
</tr>
</tbody>
</table>

At 2.5 and 0.5 mmol/L glucose, neurons were classified by glucosensing categories by alterations in \([Ca^{2+}]_i\) oscillations as glucose was changed from 2.5 to 0.5 to 2.5 mmol/L or 0.5 to 2.5 to 0.5 mmol/L respectively. They were then held at 2.5 or 0.5 mmol/L glucose and exposed to 1µmol/l acetoacetate (AA) and classified as AA excited or inhibited or non-responsive. Data are in percent of total neurons tested in each category. Total = total percent of each category of neurons for each AA category, irrespective of their glucosensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. Data with differing superscripts in a given category differ from each other by P<0.05 by one way ANOVA followed by post-hoc T-test.
Supplementary Table 2: Effect of acetoacetate on VMN oleic acid (OA) excited (OAE), inhibited (OAI) and non-sensing (OAN) neurons

2.5 mmol/L glucose | 0.5 mmol/L glucose
---|---
| % of total | AA Excited | AA Inhibited | % of total | AA Excited | AA Inhibited |
---|---|---|---|---|---|---|
OAE | 24±7 | 64±15\textsuperscript{a} | 16±10\textsuperscript{b} | 37±6 | 74±14\textsuperscript{a} | 4±5\textsuperscript{b} |
OAI | 13±6 | 5±5\textsuperscript{a} | 45±13\textsuperscript{b} | 10±4 | 16±11\textsuperscript{b} | 44±13\textsuperscript{b} |
OAN | 62±12 | 30±8\textsuperscript{a} | 27±5\textsuperscript{a} | 53±12 | 39±13\textsuperscript{a} | 10±6\textsuperscript{a} |
Total | 100 (252) | 34±8 (85)\textsuperscript{a} | 27±5 (71)\textsuperscript{a} | 100 (347) | 50±13 (150)\textsuperscript{a} | 11±5 (45)\textsuperscript{b} |

At 2.5 and 0.5 mmol/L glucose, VMN neurons were classified as OA excited (OAE) or in inhibited (OAI) or non-responsive (OAN) by alterations in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations produced by exposure to 15 nmol/L OA. They were then exposed to 1 µmol/L acetoacetate (AA) in the presence of 15 nmol/L OA and were then classified as AA excited or inhibited. Data are in percent of total neurons tested in each category. Total = total percent of each category of neurons for each 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. Data with differing superscripts in a given category differ from each other by P<0.05 by one way ANOVA followed by post-hoc T-test.
Supplementary Figure 1: Representative Zero-Net Flux studies for VMH FFA (A) β-OHB levels (B) in two different individual rats 24h fasted rats. Y-axis represents the difference between the FFA concentrations infused into and coming out of the microdialysis probe (Δ μM). X-axis represents the FFA perfusate concentration (amount infused into the probe). Zero-net flux (ZNF) represents the point at which the calculated regression line crosses zero on the Y-axis.
Supplementary Figure 2: Representative changes in $[\text{Ca}^{2+}]_i$ oscillations following exposure to oleic acid and ketone bodies (β-OHB and AA) in freshly dissociated VMN neurons from 3-4 wk old male Sprague-Dawley rats. The neurons were first characterized by their glucosensing category in response to altering glucose concentrations from 2.5 (2.5G) to 0.5 (0.5G) to 2.5 mM (2.5G) followed by 15 nmol/L oleic acid (OA), 20 µmol/L β-OHB and 1 µmol/L AA. Neurons
were tested terminally with 20 nM glutamate (Glut) to ascertain viability. **A:** Glucose-excited/ OAI neuron and ketone bodies excited showing increased \([\text{Ca}^{2+}]_i\) oscillations; **B:** Glucose-inhibited/ OAE neuron/ Ketone bodies inhibited showing decreased \([\text{Ca}^{2+}]_i\) oscillations. Areas under the curve (AUC) for \([\text{Ca}^{2+}]_i\) oscillations in response to changes in conditions are given over each 10 min segment of tracing.