FAT/CD36: A major regulator of neuronal fatty acid sensing and energy homeostasis in
rats and mice.

Running title: Role of CD36 in FA sensing

Christelle Le Foll¹, Ambrose Dunn-Meynell², Sergei Musatov³, Christophe Magnan⁴, Barry E
Levin¹,².

¹Dept Neurology and Neurosciences, NJ Medical School, Newark, NJ; ²VA Med. Ctr., East
Orange, NJ; ³Weill Cornell Medical College, New York, NY; ⁴BFA CNRS-University Paris
Diderot, Paris, France.

Corresponding author:

Christelle Le Foll

Neurology Service (127C)

VA Medical Center

385 Tremont Ave

East Orange, NJ 07018-1095

tel: 973 676-1000, x3583

fax: 973 395-7233

email: lefollch@umdnj.edu

Word count (4000 max): 3773

Tables: 6

Figures: 2

Supplemental Tables: 1

Supplemental Figures: 2
ABSTRACT

Hypothalamic “metabolic sensing” neurons sense glucose, fatty acids (FA) and play an integral role in the regulation of glucose, energy homeostasis, the development of obesity and diabetes. Using pharmacologic agents, we previously found that ~50% of these neurons responded to oleic acid, by utilizing the fatty acid (FA) translocator/receptor, FAT/CD36 (CD36). To further elucidate the role of CD36 in neuronal FA sensing, ventromedial hypothalamus (VMH) CD36 was depleted using adeno-associated viral (AAV) vector expressing CD36 shRNA in rats. While their neuronal glucosensing was unaffected by CD36 depletion, their percent of neurons that responded to oleic acid was decreased specifically in glucosensing neurons. A similar effect was seen in total body CD36 knockout mice. Next, weanling rats were injected in the VMH with CD36 AAV shRNA. Despite significant VMH CD36 depletion, there was no effect on food intake, body weight gain or total carcass adiposity on chow or 45% fat diets. However, VMH CD36 depleted rats did have increased plasma leptin and subcutaneous fat deposition and markedly abnormal glucose tolerance. These results demonstrate that CD36 is a critical factor in both VMH neuronal FA sensing and the regulation of energy and glucose homeostasis.
INTRODUCTION

Several lines of evidence support the idea that specialized hypothalamic metabolic sensing neurons can monitor peripheral fuel availability by altering their activity in response to ambient levels of glucose and fatty acids (FA) as a means of regulating energy and glucose homeostasis in the body (1-8). Glucose is the primary energy substrate for neuronal metabolism (9), while astrocytes are the primary source of FA oxidation in the brain (10; 11). However, metabolic sensing neurons possess specialized pathways that allow them to utilize FA as a signalling molecule to regulate their activity (5; 7; 12-14). We previously showed that at least 50% of the FA sensing in ventromedial hypothalamic (VMH= arcuate (ARC) + ventromedial (VMN) nuclei) neurons is attributable to the interaction of long chain FA (LCFA) with FA translocase/CD36 (CD36) acting as a receptor, while only ~20% is attributable to intracellular metabolism of FA (5). In those studies, we utilized both fura-2 calcium imaging and FLIPR membrane potential dye, together with various pharmacological agents to identify some of the mechanisms by which individual VMN neurons responded to the LCFA, oleic acid (OA) (5; 15). In the present studies, we utilized these same techniques, together with molecular manipulations of CD36 expression, to determine the importance of CD36 in the sensitivity of VMN neurons to OA \textit{in vitro} and the overall effect of depleting CD36 in the VMH \textit{on} food intake, body weight gain and adipose gain and glucose tolerance \textit{in vivo}. We confirmed our prior studies showing that CD36 is a critical mechanism for neuronal sensing of LCFA and demonstrate that depletion of VMH CD36 alters the distribution of adiposity and glucose homeostasis in outbred rats.

RESEARCH DESIGN AND METHODS

\textit{Experiment 1: assessment of glucose and OA sensing in dissociated VMH neurons}
Animals

Animals were housed at 23-24°C on a 12:12-h light-dark cycle (lights on at 10:00 h). Male Sprague-Dawley rats and C57/Bl6 mice were purchased from Charles River Laboratories. Total body CD36 knockout mice bred on a C57BL/6J background (CD36KO) (16) were kindly provided by Dr. Maria Febbraio (Lerner Research Inst, Cleveland). All work was in compliance with the animal care and use committee of the E. Orange Veterans Affairs Medical Center.

Adeno-associated viral vectors

Murine CD36 open reading frame was PCR amplified from brain cDNA library with a COOH-terminal FLAG tag and subcloned into an AAV expression plasmid to generate AAV.CD36. A control vector (AAV.mCherry) was designed to express mCherry-FLAG. The expression of both transgenes is regulated by a hybrid cytomegalovirus/chicken β-actin promoter. Virus stocks were prepared by packaging the vector plasmids into AAV serotype 2 particles using a helper-free plasmid transfection system. The vectors were purified using heparin affinity chromatography, dialyzed against PBS supplemented with 2 mmol/l MgCl₂, and diluted to 10¹² genomic particles per milliliter.

Stereotaxic surgery

Cold-anesthetized, postnatal day 5 (P5) rats were stereotaxically injected bilaterally in the border between the VMN and ARC over 10 min with 0.4 µl of saline containing 2 × 10⁸ genomic particles of AAV per side containing either CD36 shRNA + mCherry or a control sequence + mCherry. With level bregma and lambda, coordinates were: 2.6 mm caudal to
bregma, 0.40 mm lateral to midline, 6.6 mm down from dural surface as previously described (20).

**Measurement of OA- and glucose-induced changes in intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) oscillations in dissociated VMN neurons**

Rats were injected in the VMH at P5 with AAV expressing CD36 shRNA. At P21-28, they were perfused, the VMN was bilaterally punched from VMH slices and neurons were dissociated as previously described (5; 17). Evaluation of glucose- and OA-induced alterations in [Ca\(^{2+}\)]\(_i\) oscillations in individual VMN neurons which expressed mCherry (see below) were assessed using fura-2 acetoxy-methyl ester (Molecular Probes, Eugene, OR), as previously described (5; 17). Neurons were classified as glucose excited (GE), glucose inhibited (GI) and non-glucosensing (NG) and as OA-excited (OAE), inhibited (OAI) or non-responsive using previously established criteria for changes in [Ca\(^{2+}\)]\(_i\) area under the curve (AUC) (5; 17). The lipophilic, anionic bis-oxonol dye from the fluorometric imaging plate reader (FLIPR) membrane potential assay kit (Molecular Devices, Sunnyvale, CA) was also used in dissociated VMN neurons to assess OA- and glucose-induced changes in membrane potential as previously described (18). Studies began with neurons held at 2.5 mmol/l glucose unless otherwise specified. Changes in [Ca\(^{2+}\)]\(_i\) fluctuations in response to glucose and OA were assessed over 10 min periods after addition of each substance. All neurons were incubated with 20 nmol/l glutamate terminally to ensure that they were functionally viable.
mRNA expression in dissociated VMH neurons and VMN and ARC micropunches

After determination of glucose- and FA sensing properties, 120 µl of lysis buffer (Ambion, Austin, TX) were added to the dissociated neurons and mRNA was quantified by real-time quantitative PCR (QPCR) as previously described (5; 18; 19). The resultant cDNA was analyzed using TAQman MGB primer/probes sets targeting genes involved in glucose and FA metabolism (Supplemental Table 1). Similar techniques were used to extract and assay mRNA in ARC and VMN micropunches. Data were expressed as the ratio of the gene of interest relative to the housekeeping gene, cyclophilin (5; 18; 19).

Experiment 2: In vivo effects of VMH CD36 depletion

Animals and diet

P21 rats were housed at 23-24 C on a 12:12-h light-dark cycle (lights on at 10:00 h) and injected with AAV expressing CD36 shRNA or control sequence + mCherry bilaterally in the VMH (see below). Rats were fed Purina rat chow (4.5% fat, 3.75kcal/g) for 6 wk followed by 9 wk on 45% fat diet (HFD; 4.75kcal/g, Research Diet D12541). Food and water were available ad libitum. Food intake and body weight were monitored bi-weekly. During the last week on HFD, rats underwent an oral glucose tolerance test. Terminally, rats were decapitated and the brains were harvested for QPCR. Trunk blood was collected for glucose, insulin, leptin, free FA and β-hydroxybutyrate. Retroperitoneal, perirenal, mesenteric and epididymal fat pads were weighed as representative of visceral fat and inguinal pads were taken as representative of subcutaneous fat.
**Stereotaxic surgery**

Chloropent (chloral hydrate+nembutal) anesthetized P21 rats were stereotaxically injected bilaterally with 0.4 µl containing $2 \times 10^8$ genomic particles of AAV expressing CD36 shRNA or control sequence +.mCherry into both the VMN and ARC ($n=8-10$/group). With bregma and lambda level, coordinates were: 2.7 mm caudal to bregma, 0.6 mm lateral to midline, 8.4 mm below dura (VMN) and 0.3 mm lateral to midline and 8.9 mm below dura (ARC).

**Oral glucose tolerance test (OGTT)**

During the last week on HFD, rats were gavaged with 2g/kg glucose and blood was sampled by tail nip at 15, 30, 60, 90 and 120 min for glucose and insulin.

**Assays of Insulin, Leptin, Free Fatty acid, β-hydroxybutyrate and Glucose**

Plasma insulin and leptin levels were analyzed with radioimmunoassay kits (Linco, Carlsbad, CA). Plasma free FA and β-hydroxybutyrate levels were analyzed using a colorimetric assay (Wako, Richmond, VA). Plasma blood glucose was measured using a glucose Analox® instrument.

**Statistics**

Responses of neurons to changes in glucose, oleic acid or drug concentrations were compared using the T-test for nonparametric statistics (GraphPad Prism, La Jolla, CA). One-way and two-way ANOVA and ANOVA for repeated measures with post hoc Bonferonni corrections were used for the in vivo studies. Outliers were removed if necessary (Systat, Chicago, IL).
RESULTS

Experiment 1

Effect of in vivo CD36 VMH depletion on neuronal glucose- and FA sensing.

VMH injection of AAV CD36 shRNA at P5 reduced neuronal CD36 mRNA expression by 45% as compared to controls (Control=0.91±0.09; CD36 AAV=0.50±0.10, P<0.05) at P21-28, but had no effect on body weight gain from P5 to P21 (Figure 1 Supplemental data). This reduction in VMH CD36 did not affect the proportion of GE or GI neurons (Table 1). When considered independently of their glucosensing status, there was no effect of VMH CD36 depletion on the proportion of VMH neurons which was excited or inhibited by 15 nmol/l OA. However, as a function of their glucosensing properties, the proportion of CD36 shRNA GE neurons which was further excited by 15 nmol/l OA was reduced by 49%, while the percent of GE neurons inhibited by OA was decreased by 77% vs. control neurons (Table 1). VMH CD36 depletion had no effect on the percent of GI neurons excited by OA. However, whereas 21% of control GI neurons were inhibited by OA, no CD36 shRNA neurons were inhibited by OA (Table 1).

Since it is possible that OA might alter $[\text{Ca}^{2+}]_i$ oscillations without necessarily altering membrane potential or neuronal activity, neurons were also imaged with FLIPR membrane potential dye for changes in membrane potential induced by glucose and OA (Table 2). Similar to calcium imaging, CD36 depletion did not alter the proportion of GE or GI neurons using FLIPR dye. Also, whereas CD36 depletion reduced the proportion of GE neurons excited by OA using calcium imaging by 49%, these OA-excited GE neurons were deceased by 32% using FLIPR dye (Table 1, 2). With calcium imaging and FLIPR dye, the number of CD36 shRNA GE neurons inhibited by OA was reduced by 77% but there was no effect on the number of OA-
excited GI neurons. On the other hand, whereas CD36 depletion abolished OA inhibition of GI neurons using calcium imaging, there was a 79% decrease in the number of these neurons using FLIPR. Therefore, while there were some differences, the relative proportions of OAE to OAI neurons identified by the two techniques were quite comparable.

**Effect of total body CD36 knockout (CD36KO) on mouse VMH neuronal glucose and FA sensing.**

VMH neurons were dissociated from P21-28 CD36KO and C57/BL6 wild type control mice. In control mice, 18% of VMH neurons were GE and 11% were GI, while only 11% of CD36KO neurons were GE and 5% were GI (*P<0.001; Data not shown). Thus, CD36KO mice had 39% fewer GE and 55% fewer GI neurons than did control mice. Using OA concentrations from 10 nmol/l to 1000 nmol/l at 2.5 and 0.5 mmol/l glucose, neurons from both genotypes demonstrated similar, but somewhat flat concentration-dependent responses for excitation and inhibition (Figure 1). When considered independently of their glucosensing properties and over the entire range of OA concentrations, there were no significant differences in the percentages of neurons excited or inhibited by OA (Table 3). Also, while there were equivalent numbers of OA inhibited VMH neurons in the Sprague-Dawley rat (Table 1) and the C57/BL6 mouse (Table 3), the mouse had ~50% more neurons that were excited by OA at 2.5 mmol/l glucose, regardless of whether VMH CD36 expression had been decreased or not. As in the rat with VMH knockdown of CD36 (Table 1), CD36KO mice had significant alterations in OA responsiveness when considered with respect to their glucosensing properties (Table 3). In CD36KO mouse GE neurons, there were 53% fewer OA inhibited GE neurons held at 2.5 mmol/l glucose vs. WT mice. As opposed to the outbred rat, the control mouse had no GI neurons that were inhibited by
OA in 2.5 mmol/l glucose, while 13% were inhibited by OA in CD36KO mice (Table 3). At 0.5 mmol/l glucose, CD36KO mouse GE and GI neurons were 30% and 37% more excited by OA than in WT mice (P<0.05; Table 3). As opposed to control mice where OA inhibited 12% and 26% of the GE and GI neurons respectively, no inhibition was induced by OA in CD36KO mice. Overall, in C57/Bl6 mouse, more neurons were responsive to OA when held at 2.5 mmol/l than 0.5 mmol/l glucose whereas in CD36KO mouse no inhibition induced by OA is observed in 0.5 mmol/l glucose as opposite to 2.5 mmol/l glucose. Finally, total body knockout of CD36 was associated with a 26% decrease in FA transport protein 2 (FATP2) and a 50% decrease in glucokinase mRNA expression (Table 4). Overall, these data suggest that germ cell deletion of CD36 has a major effect on both VMN neuronal glucose and CD36-mediated FA sensing which represents compensation for loss of the major FA sensing regulatory pathway.

**Experiment 2**

*In vivo effects of VMH CD36 depletion in rats*

Bilateral VMH (ARC and VMN) injections of AAV expressing CD36 shRNA into 3 wk old rats produced no significant changes in food intake or body weight gain over 6wk of chow intake (Table 5, Figure 2A, B). However, they did have 122%, 12% and 66% higher plasma leptin, glucose and insulin levels, respectively (Table 5), suggesting that they were both fatter and significantly less glucose tolerant than control rats. VMH CD36 depletion also did not significantly alter food intake or weight gain over 9 wk on HFD, although feed efficiency (body weight gain/ caloric intake) was 16% higher and leptin levels were 54% higher than controls (Table 5). While total adiposity (total weight of 5 fat pads as a percent of carcass weight) did not differ, inguinal fat pads, representative of subcutaneous depots, were 60% heavier in the VMH
CD36 shRNA rats suggesting a possible redistribution of fat tissue from visceral to subcutaneous depots in these rats. The most striking effect of reducing VMH CD36 expression was a 232% increase in plasma insulin levels in association with a 98% increase in glucose and 244% increase in insulin AUC during an oral glucose tolerance test after 9wk on HFD (Table 5, Figure 2C, D).

Terminally, VMN and ARC micropunches were assessed for alterations in gene expression due to CD36 depletion (Table 6). In the VMN there was a non-significant 40% decrease in CD36 expression which was associated with increases of 48% in FATP2, 10% in AMP-activated protein kinase (AMPK)α1 and 70% in AMPKα2 mRNA expression with 15% decreases in carnitine palmitoyltransferase 1c (CPT1c) and acyl-CoA synthase 1 expression (Table 6). In the ARC, where CD36 expression was decreased by 71%, agouti-related peptide (AgRP), proopiomelanocortin (POMC), acyl-CoA synthase 5 and 6 and AMPKα2 was reduced by 60%, 58%, 21%, 19% and 32%, respectively, while glucokinase, Kir6.2, FATP2 expression was increased by 23%, 56%, 127% as compared to controls, respectively (Table 6). Other ARC and/or VMN genes involved in FA and glucose metabolism (FA synthase, GLUT4, FATP1,4,6, CPT1a, acyl-CoA synthase 3,4, malonyl-CoA decarboxylase) were measured but did not differ between groups (data not shown).

**DISCUSSION**

The current studies were undertaken to examine the importance of FAT/CD36 in neuronal FA and glucose sensing in freshly dissociated VMH neurons from rats and mice and the effects of reducing VMH CD36-mediated FA sensing on long term energy and glucose homeostasis. Using pharmacological methods, we previously demonstrated that CD36 accounted for at least 50% of
the excitatory and inhibitory effects of OA in VMN neurons, whereas inhibiting any of the steps of neuronal formation of LCFA acyl-CoA, oxidation, formation of reactive oxygen species or activation of the K\textsubscript{ATP} channel accounted for no more than 20% of these effects of OA (5). Here, using \textit{in vivo} AAV shRNA to deplete VMH CD36 in rats and mice with total body depletion of CD36, we show a similar effect of interfering with CD36 signaling, although the results varied somewhat depending upon the method and species utilized. Importantly, in both rats and CD36KO mice, the reduction in FA sensing was completely dependent upon the underlying glucosensing status of the neurons assessed. In rats injected at P5 with AAV CD36 shRNA resulting in a 45% depletion of VMN CD36 mRNA at P21-28, there was no effect on the percentage of glucosensing neurons but there was a 49% reduction in OA excitation and 77% reduction in OA inhibition in GE neurons and a complete loss of OA inhibition in GI neurons held at 2.5 mmol/l glucose. In the CD36KO mouse, there were 40-55% fewer VMH glucosensing neurons in association with a 50% decrease in glucokinase, a critical regulator of glucosensing (1; 21; 22). The KO mouse also had 53% fewer OA inhibited, but no differences in OA excited GE neurons. However, as opposed to the rat, the KO mouse actually had more GI neurons that were inhibited by OA at 2.5 mmol/l glucose and, at 0.5 mmol/l glucose, the CD36KO mouse had 30% and 37% more GE and GI neurons that were excited by OA than did control mice. These differences between the rat and mouse data are likely due to both species differences and the germ cell deletion of CD36 where both early plastic changes (as witnessed by the loss of glucosensing neurons) and altered energy and glucose homeostasis can occur (23; 24). Despite these differences, our cumulative present and previous data (5) strongly suggest that CD36 plays a critical role in the responsiveness of VMH neurons to the LCFA, OA.
This conclusion differs from a previous study demonstrating an important role for the $K_{ATP}$ channel in mediating the effects of LCFA in ARC POMC neurons under high (2.5 to 5 mmol/l) glucose concentrations (25). However, our data clearly demonstrate that, at 2.5 mmol/l glucose, which is comparable to hypothalamic levels seen during the post-ingestive state (26; 27), ~4 fold more GE neurons were excited than inhibited in VMH CD36 depleted than control rats. Since glucokinase and the $K_{ATP}$ channel are the dominant mediators of activation of VMN GE neurons (1; 21; 22), these data and our previous studies (5) suggest that, in those GE neurons that were further excited by OA, this effect is mediated by an alternate pathway that is not dependent upon this mechanism or other pathways involving FA oxidation. In fact, data in FA sensing taste buds suggest that their LCFA-induced activation is dependent upon CD36 acting as a receptor to activate store-operated calcium channels (28). Similarly, our present and previous (5) data support the contention that CD36-mediated FA sensing is similar to that seen in taste buds and is largely independent of intracellular metabolism of LCFA. However, our current studies demonstrate that CD36-mediated FA sensing occurs specifically in VMH neurons that are also responsive to glucose. This supports the idea that such neurons are metabolic sensors which integrate signals from metabolic substrates, as well as hormones and neural inputs from the periphery to regulate their activity (9; 29-31). As opposed to GE neurons, GI neurons are largely inhibited at 2.5 mmol/l glucose and appear to utilize AMPK, nitric oxide and a non-specific cation channel to mediate this effect (32; 33). The fact that 50% of rat GI neurons held at 2.5 mmol/l glucose (where they are largely, but not totally inactive (1; 22)) were excited and 21% were inhibited by OA also suggests that such FA sensing occurs by mechanisms that are largely independent of those used by GI neurons to sense glucose. One possibility is that the increase in neuronal FATP2 gene expression in rats with VMH CD36 depletion might be
associated with increased LCFA transport into the cell and a switch to a dependence on LCFA metabolism in the face of decreased availability of CD36-mediated FA sensing.

Having established a crucial role for CD36 in mediating neuronal FA sensing in the VMH in vitro, we next assessed the importance of VMH CD36-mediated neuronal FA sensing on long term energy and glucose homeostasis. Depletion of VMH CD36 expression using AAV shRNA at 3 wk of age had no significant effects on either food intake or body weight gain when rats were fed low fat chow from weaning for 6 wk, or after an additional 9 wk on a 45% fat diet. However, although they had no increase in the percent of total carcass adiposity (using 5 pads weights as surrogates), VMH CD36 depleted rats did have a 20% increase in feed efficiency, a 125% and 65% increase in plasma leptin levels after 6 wk of chow and 9 wk of HFD and a 60% increase in inguinal fat pad weights relative to carcass weights terminally. These differences from control rats suggests that VMH CD36 depleted rats had a redistribution of fat from visceral to subcutaneous (inguinal) depots, the predominant source of circulating leptin (34; 35) . Assuming this is so and that sparing of visceral depots by shunting fat to subcutaneous depots should improve insulin sensitivity, it was surprising to find that CD36 shRNA rats had markedly elevated plasma insulin levels and impaired glucose tolerance both after chow and HFD. Since visceral adiposity is usually associated with such impairments in insulin sensitivity (36), these results suggest that VMH CD36-mediated neuronal FA sensing is responsible for both the distribution of carcass adiposity and peripheral insulin sensitivity. These differences could not be attributed to changes in ARC neuropeptide expression since there were comparable decreases in AgRP and POMC expression observed in CD36 shRNA rats. The parallel decrease in the expression of both these neuropeptides suggests that this might be due to reduction in their FA sensing ability rather than elevated leptin levels which should decrease AgRP but increase
POMC expression. Although we made no measures of energy expenditure or motor activity, it seems unlikely that either of these could account for differences in fat deposition, although decreased activity might contribute to reduced insulin sensitivity. In fact, despite the fact that CD36 was depleted only in the VMH in rats, total body CD36KO mice also have a similar increase in plasma insulin levels and an impaired whole-body glucose tolerance, as well as impaired FA oxidation, when fed a HFD for several months (23; 24).

One important caveat is that assessment of OA-induced changes in \([\text{Ca}^{2+}]_i\), using calcium imaging provides neither a direct measure of changes in membrane potential nor neuronal activity, per se. In gustatory cells, occupation of the CD36 receptor leads to activation of store-operated calcium channels via phosphorylation of Src-protein-tyrosine-kinases and subsequent release of serotonin and norepinephrine. CD36-mediated release of these neurotransmitters suggests a parallel between CD36 mediated increases in intracellular calcium and neuronal activity produced by exposure to LCFA (28). We previously showed that ~50% of the OA-induced alterations in \([\text{Ca}^{2+}]_i\) oscillations were paralleled by alterations in membrane potential (5). Here we confirm these findings in CD36 shRNA rats. Taken together with studies in FA sensing taste buds (28), our data support a role for LCFA acting on CD36 as a receptor to alter neuronal activity in VMH FA sensing neurons. However, only direct measurements of neuronal activity will settle this issue.

In conclusion, using a variety of molecular manipulations to deplete the FA receptor CD36 from the VMH or whole animal, we have demonstrated its prominence as a mediator of VMH neuronal LCFA sensing specifically in glucosensing neurons \textit{in vitro} and as a regulator of adipose deposition and glucose tolerance, \textit{in vivo}. Thus, whereas VMH glucosensing appears to be most important as a detector and regulator of feeding and neurohumoral responses during
states of low glucose availability (3; 27), CD36-mediated VMH neuronal FA sensing appears to play an important role in the physiological regulation of both energy and glucose homeostasis.
ACKNOWLEDGMENTS

We thank Sunny Lee, VA Med. Center, NJ, Antoinette Moralishvili, VA Med. Center, NJ, and Charlie Salter, VA Med. Center, NJ, for their technical assistance.

This work was supported by the Research Service of the Department of Veterans Affairs (B. E. Levin, A. Dunn-Meynell), the National Institute of Diabetes and Digestive and Kidney Diseases (DK-53181 B. E. Levin. Dr. Maria Febbraio (Lerner Research Inst, Cleveland) kindly supplied the CD36KO mice.

No potential conflicts of interest relevant to this article were reported.

CLF, NJ Medical School performed the research; CLF and BEL, NJ Medical School, designed the experiments and wrote the manuscript. ADM, VA Med. Center, NJ, did all the virus surgeries, SM, Weill Cornell Medical College, NY, provided the CD36 AAV shRNA, CM, BFA CNRS-University Paris Diderot, contributed to the design of the experiments. CLF and BEL 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.
REFERENCES

1. Kang L, Dunn-Meynell AA, Routh VH, Gaspers LD, Nagata Y, Nishimura T, Eiki J, Zhang BB, Levin BE: Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing. Diabetes 2006;55:412-420

2. Lam TK, Pocai A, Gutierrez-Juarez R, Obici S, Bryan J, Aguilar-Bryan L, Schwartz GJ, Rossetti L: Hypothalamic sensing of circulating fatty acids is required for glucose homeostasis. Nat Med 2005;11:320-327

3. Levin BE, Becker TC, Eiki J, Zhang BB, Dunn-Meynell AA: Ventromedial hypothalamic glucokinase is an important mediator of the counterregulatory response to insulin-induced hypoglycemia. Diabetes 2008;57:1371-1379

4. Pocai A, Lam TK, Obici S, Gutierrez-Juarez R, Muse ED, Arduini A, Rossetti L: Restoration of hypothalamic lipid sensing normalizes energy and glucose homeostasis in overfed rats. J Clin Invest 2006;116:1081-1091

5. Le Foll C, Irani BG, Magnan C, Dunn-Meynell AA, Levin BE: Characteristics and mechanisms of hypothalamic neuronal fatty acid sensing. Am J Physiol Regul Integr Comp Physiol 2009;297:R655-664

6. Clement L, Cruciani-Guglielmacci C, Magnan C, Vincent M, Douared L, Orosco M, Assimacopoulos-Jeannet F, Penicaud L, Ktorza A: Intracerebroventricular infusion of a triglyceride emulsion leads to both altered insulin secretion and hepatic glucose production in rats. Pflugers Arch 2002;445:375-380

7. Migrenne S, Cruciani-Guglielmacci C, Kang L, Wang R, Rouch C, Lefevre AL, Ktorza A, Routh VH, Levin BE, Magnan C: Fatty Acid signaling in the hypothalamus and the neural control of insulin secretion. Diabetes 2006;55 Suppl 2:S139-144
8. Obici S, Feng Z, Morgan K, Stein D, Karkanias G, Rossetti L: Central administration of oleic acid inhibits glucose production and food intake. Diabetes 2002;51:271-275

9. Sokoloff L, Reivich M, Kennedy C, DesRosiers MH, Patlak CS, Pettigrew O, Sakurada O, Shinothara M: The $[^{14}\text{C}]$deoxyglucose method for the measurement of local cerebral glucose utilization: theory, procedure, and normal values in the conscious and anesthetized albino rat. J Neurochem 1977;23:897-916

10. Escartin C, Pierre K, Colin A, Brouillet E, Delzescaux T, Guillermier M, Dhenain M, Deglon N, Hantraye P, Pellerin L, Bonvento G: Activation of astrocytes by CNTF induces metabolic plasticity and increases resistance to metabolic insults. J Neurosci 2007;27:7094-7104

11. Edmond J, Robbins RA, Bergstrom JD, Cole RA, de Vellis J: Capacity for substrate utilization in oxidative metabolism by neurons, astrocytes, and oligodendrocytes from developing brain in primary culture. J Neurosci Res 1987;18:551-561

12. Levin BE: Metabolic sensors: viewing gluicosensing neurons from a broader perspective. Physiol Behav 2002;76:397-401

13. Oomura Y, Nakamura T, Sugimori M, Yamada Y: Effect of free fatty acid on the rat lateral hypothalamic neurons. Physiol Behav 1975;14:483-486

14. Wang R, Liu X, Hentges ST, Dunn-Meynell AA, Levin BE, Wang W, Routh VH: The regulation of glucose-excited neurons in the hypothalamic arcuate nucleus by glucose and feeding-relevant peptides. Diabetes 2004;53:1959-1965

15. Le Foll C, Irani BG, Magnan C, Dunn-Meynell AA, Levin BE: Effects of maternal genotype and diet on offspring glucose and fatty acid sensing ventromedial hypothalamic nucleus neurons. Am J Physiol Regul Integr Comp Physiol 2009;297:R1351-R1357
16. Laugerette F, Passilly-Degrace P, Patris B, Niot I, Febbraio M, Montmayeur JP, Besnard P: CD36 involvement in orosensory detection of dietary lipids, spontaneous fat preference, and digestive secretions. J Clin Invest 2005;115:3177-3184

17. Kang L, Routh VH, Kuzhikandathil EV, Gaspers L, Levin BE: Physiological and molecular characteristics of rat hypothalamic ventromedial nucleus glucosensing neurons. Diabetes 2004;53:549-559

18. Kang L, Dunn-Meynell AA, Routh VH, Gaspers LD, Nagata Y, Nishimura T, Eikis J, Zhang BB, Levin BE: Glucokinase is a critical regulator of ventromedial hypothalamic neuronal glucosensing. Diabetes 2006;55:412-420

19. Levin BE, Magnan C, Migrenne S, Chua Jr SC, Dunn-Meynell AA: The F-DIO obesity-prone rat is insulin resistant prior to obesity onset. Am J Physiol 2005;289:R704-R711

20. Levin BE, Becker TC, Eiki J, Zhang BB, Dunn-Meynell AA: Ventromedial hypothalamic glucokinase is an important mediator of the counterregulatory response to insulin-induced hypoglycemia. Diabetes 2008;57:1371-1379

21. Dunn-Meynell AA, Routh VH, Kang L, Gaspers L, Levin BE: Glucokinase is the likely mediator of glucosensing in both glucose excited and glucose inhibited central neurons. Diabetes 2002;51:2056-2065

22. Kang L, Dunn-Meynell AA, Routh VH, Liu X, Levin BE: Knockdown of GK mRNA with GK RNA interference (RNAi) blocks ventromedial hypothalamic (VMH) neuronal glucosensing. Diabetes 2004;53:A43

23. Koonen DP, Sung MM, Kao CK, Dolinsky VW, Koves TR, Ilkayeva O, Jacobs RL, Vance DE, Light PE, Muoio DM, Febbraio M, Dyck JR: Alterations in skeletal muscle fatty acid
handling predisposes middle-aged mice to diet-induced insulin resistance. Diabetes 2010;59:1366-1375

24. Bonen A, Han XX, Habets DD, Febbraio M, Glatz JF, Luiken JJ: A null mutation in skeletal muscle FAT/CD36 reveals its essential role in insulin- and AICAR-stimulated fatty acid metabolism. Am J Physiol Endocrinol Metab 2007;292:E1740-1749

25. Jo YH, Su Y, Gutierrez-Juarez R, Chua S, Jr.: Oleic acid directly regulates POMC neuron excitability in the hypothalamus. J Neurophysiol 2009;101:2305-2316

26. Silver IA, Erecinska M: Extracellular glucose concentration in mammalian brain: continuous monitoring of changes during increased neuronal activity and upon limitation in oxygen supply in normo-, hypo-, and hyperglycemic animals. The Journal of neuroscience : the official journal of the Society for Neuroscience 1994;14:5068-5076

27. Dunn-Meynell AA, Sanders NM, Compton D, Becker TC, Eiki J, Zhang BB, Levin BE: Relationship among brain and blood glucose levels and spontaneous and glucoprivic feeding. J Neurosci 2009;29:7015-7022

28. El-Yassimi A, Hichami A, Besnard P, Khan NA: Linoleic acid induces calcium signaling, Src kinase phosphorylation, and neurotransmitter release in mouse CD36-positive gustatory cells. J Biol Chem 2008;283:12949-12959

29. Edmond J: Energy metabolism in developing brain cells. Can J Physiol Pharmacol 1992;70 Suppl:S118-129

30. Levin BE, Routh VH, Kang L, Sanders NM, Dunn-Meynell AA: Neuronal glucosensing: what do we know after 50 years? Diabetes 2004;53:2521-2528
31. Pellerin L, Pellegrin G, Martin JL, Magistretti PJ: Expression of monocarboxylate transporter mRNAs in mouse brain: support for a distinct role of lactate as an energy substrate for the neonatal vs. adult brain. ProcNatlAcadSci 1998;95:3990-3995

32. Murphy BA, Fakira KA, Song Z, Beuve A, Routh VH: AMP-activated protein kinase and nitric oxide regulate the glucose sensitivity of ventromedial hypothalamic glucose-inhibited neurons. Am J Physiol Cell Physiol 2009;297:C750-C758

33. Fioramonti X, Marsollier N, Song Z, Fakira KA, Patel RM, Brown S, Duparc T, Pica-Mendez A, Sanders NM, Knauf C, Valet P, McCrimmon RJ, Beuve A, Magnan C, Routh VH: Ventromedial hypothalamic nitric oxide production is necessary for hypoglycemia detection and counterregulation. Diabetes 2010;59:519-528

34. Barichello T, Milioli G, Generoso JS, Cipriano AL, Costa CS, Moreira AP, Vilela MC, Comim CM, Teixeira AL, Quevedo J: Imipramine reverses depressive-like parameters in pneumococcal meningitis survivor rats. J Neural Transm 2012;119:653-660

35. Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S: Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 1997;387:903-908

36. Gastaldelli A, Cusi K, Pettiti M, Hardies J, Miyazaki Y, Berria R, Buzzigoli E, Sironi AM, Cersosimo E, Ferrannini E, Defronzo RA: Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. Gastroenterology 2007;133:496-506
FIGURE LEGENDS

**Figure 1.** VMN neurons from C57/Bl6 and CD36KO mice were held at 2.5 mmol/l (A and B) or 0.5 mmol/l (C and D) glucose and exposed to three concentration of oleic acid 10, 100 and 1000 nmol/l. They were then classified as A and C) oleic acid excited (OAE), B and D) inhibited (OAI) or non-responsive (OAN, not shown). Data are mean ± SEM percent of total neurons tested in each category. There were no significant intergroup differences.

**Figure 2.** A) Food intake and B) body weight gain in 3wk old rats injected in the VMH with either control AAV or AAV expressing CD36 shRNA. C) Oral glucose tolerance (2g/kg) test was performed in control (n=7) and CD36 AAV shRNA rats (n=6) after 6 wk on chow diet and 9 wk on HFD; glucose concentration in mg/dl over 120 min D) Insulin concentration in ng/ml over 120 min. *P ≤ 0.05.
Neurons were classified by glucosensing categories by alterations in \( [\text{Ca}^{2+}]_i \) oscillations as glucose was changed from 2.5 to 0.5 to 2.5 mmol/l. They were then held at 2.5 mmol/l glucose and exposed to 15 nmol/l oleic acid (OA) concentrations and classified as OA excited (OAE) or inhibited (OAI). Data are mean ± SEM percent of total neurons tested in each category. GE=glucose excited, GI=glucose inhibited, NG=non-glucosensing. Total=total percent of each category of neurons at each glucose concentration, irrespective of their glucosensing properties with the number of neurons tested in each group divided by the total number tested in parentheses. \( N=6 \text{ rats/group.} \) *P<0.05 t-test vs. control.
Table 2. *Effect of oleic acid on VMH neurons from 3 wk old male Sprague-Dawley rats injected at P5 with CD36 AAV shRNA in the VMH using FLIPR membrane potential.*

| Glucosensing Category | 2.5 mmol/l glucose |
|-----------------------|---------------------|
|                       | Control N=132        | CD36 AAV shRNA N=135 |
|                       | % of total OAE OAI  | % of total OAE OAI  |
| GE                    | 13±4 47±4 22±4      | 12±3 32±5* 5±5*     |
| GI                    | 11±3 50±5 14±5      | 8±2 49±20 3±1*       |
| NG                    | 62±21 32±22 15±10   | 74±17 40±12 10±9     |
| Total                 | 100 (132) 45 (61) 11 (15) | 100 (135) 44 (60) 12 (16) |

Neurons were classified by glucosensing categories by alterations in membrane potential as glucose was changed from 0.5 to 2.5 mmol/l. They were then held at 2.5 mmol/l glucose and exposed to 15 nmol/l OA and classified as OA excited (OAE) or inhibited (OAI). Data are mean ± SEM percent of total neurons tested in each category. GE=glucose excited, GI=glucose inhibited, NG=non-glucosensing. Total=total percent of each category of neurons at each glucose concentration, irrespective of their glucosensing properties with the number of neurons tested in each group divided by the total number tested in parentheses. N=3 rats/group. *P<0.05 vs. control.
Table 3. Effect of oleic acid on VMH GE, GI and NG neurons in C57/Bl6 wild type and C57/Bl6 CD36KO mice.

|           | 2.5 mmol/l glucose |          | 0.5 mmol/l glucose |          |
|-----------|--------------------|----------|--------------------|----------|
|           | C57/Bl6 (n=177)    | CD36KO (n=239) | C57/Bl6 (n=306)    | CD36KO (n=127) |
| %         |                    |          |                    |          |
| OAE       | 96±4               | 72±15    | 64±4               | 83±7†    |
| OAI       | 15±3               | 7±1*     | 12±3               | 0†       |
| GE        |                    |          |                    |          |
| OAE       | 70±10*             | 13±4*    | 67±7               | 92±8†    |
| OAI       | 61±10*             | 26±6     | 26±4               | 11±3     |
| GI        |                    |          |                    |          |
| OAE       | 75±6               | 57±8     | 47±8               | 78 (99)  |
| OAI       | 5±4                | 9±3      | 21±4               | 6 (8)    |
| Total     | 77 (59)            | 72 (172)| 50 (153)           |          |
|           | 10 (8)             | 8 (18)  | 20 (62)            |          |

VMH neurons from CD36KO and wild type C57/Bl6 mice were classified by glucosensing categories by alterations in \([\text{Ca}^{2+}]_i\) 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 glucose. They were then classified as OAE or OAI at either 2.5mM or 0.5mM by alterations in \([\text{Ca}^{2+}]_i\) oscillations with OA concentrations from 10 nmol/l to 1000 nmol/l. Data are mean ± SEM percent of total neurons tested in each category. Total=total percent of each category of neurons for each OA category, irrespective of their glucosensing properties, with the number of neurons tested in each group divided by the total number tested in parentheses. *P<0.05 C57/Bl6 vs. CD36KO at 2.5 mmol/l. †P<0.05 C57/Bl6 vs. CD36KO at 0.5 mmol/l.
Table 4. mRNA expression in VMN freshly dissociated neurons from CD36KO and C57/Bl6 mice.

| Name                                      | C57/Bl6      | CD36KO       |
|-------------------------------------------|--------------|--------------|
| Fatty acid translocator/CD36              | 0.011±0.004* | ND           |
| Fatty acid transport protein (FATP) 1     | 1.51±0.60    | 1.09±0.52    |
| FATP2                                     | 1.10 ±0.17   | 0.81±0.17*   |
| FATP4                                     | 0.016±0.002  | 0.015±0.001  |
| Glucokinase                               | 1.00±0.06    | 0.502±0.62*  |
| NPY                                       | 0.405±0.09   | 0.421±0.06   |
| POMC                                      | 0.751±0.19   | 0.525±0.09   |
| AgRP                                      | 0.500±0.109  | 0.398±0.08   |
| Lepr-b                                    | 0.151±0.02   | 0.157±0.02   |

Freshly dissociated VMH neurons were harvested for quantitative real-time PCR. Data are mean ± SEM of triplicate determinations expressed relative to the amount of the mRNA expression of the housekeeping gene, cyclophilin. ND=non-detectable. N=5/group: *P<0.05.
Table 5: Morphometric and biochemical data for 19 wk old male Sprague-Dawley injected at 3 wk old with control AAV shRNA or CD36 AAV shRNA in the VMH. Rats were maintained on chow diet for 6 wk followed by 9 wk on 45% fat (HF) diet.

|                              | Control (n=7) | CD36 AAV shRNA (n=7) |
|------------------------------|---------------|----------------------|
| **6wk Chow diet**            |               |                      |
| Initial body weight (g)      | 67.9±1.0      | 67.8±1.4             |
| Body weight (g)              | 368±11        | 374±21               |
| Body weight gain (g)         | 300±10        | 306±21               |
| Food intake (kcal)           | 3880±178      | 3719±165             |
| Feed Efficiency, BWG(g)/FI(kcal)*1000 | 78±2     | 78±4                 |
| Leptin                       | 8.43±1.14     | 18.7±3.7*            |
| Glucose (mg/dl)              | 144±2         | 161±4*               |
| Insulin (ng/ml)              | 1.54±0.21     | 2.55±0.52*           |
| **9wk high fat diet**        |               |                      |
| Body weight gain (g)         | 308±23        | 347±37               |
| Food intake                  | 9980±462      | 9846±651             |
| Feed Efficiency, BWG(g)/FI(kcal)*1000 | 31±1     | 36±1*                |
| **Total 6wk chow, 9wk high fat diet** |           |                      |
| Final body weight (g)        | 675±33        | 720±57               |
| Total Body weight gain (g)   | 607±32        | 653±57               |
| Total food intake (kcal)      | 13860±613     | 13769±777            |
| Feed Efficiency total, BWG(g)/FI(kcal)*1000 | 44±1     | 48±1*                |
| Leptin (ng/ml)               | 31.8±0.9      | 52.6±3.6*            |
| Retroperitoneal (g)          | 24.5±2.9      | 33.9±3.9             |
|                  | Control       | CD36 AAV shRNA |
|------------------|---------------|----------------|
| Mesenteric (g)   | 21.1±2.8      | 24.4±2.1       |
| Inguinal (g)     | 30.9±4.2      | 52.0±5.0*      |
| Inguinal (%BW)   | 4.5±0.4       | 7.2±0.3*       |
| Epididymal (g)   | 24.3±3.1      | 24.1±2.6       |
| Perirenal (g)    | 6.9±1.3       | 8.4±1.2        |
| Total fat pads (g)| 111±15       | 135±14         |
| TFP/BW %         | 16.1±1.4      | 18.0±1         |
| Glucose (mg/dl)  | 158±4.4       | 154±9.6        |
| Insulin (ng/ml)  | 3.57±0.48     | 11.86±2.42*    |
| Glucose AUC (mg/dl/120min) | 9949±1715   | 19790±3089*    |
| Insulin AUC (mg/dl/120min) | 304±94      | 1047±245*      |
| FFA (µmol/l)     | 904±158       | 894±163        |
| βOHB (µmol/l)    | 357±99        | 372±84         |
| Liver (g)        | 18.3±0.9      | 20.3±2         |

Data are mean ± SEM. *P<0.05 or less comparing control to CD36 AAV shRNA values.
Table 6. mRNA expression in VMN and ARC micropunches harvested from male SD rats injected at 3 wk old with control AAV shRNA or CD36 AAV shRNA in the VMH. Rats were maintained on chow for 6 wk followed by 9 wk on 45% fat diet.

|                | VMN Control | VMN CD36 AAV shRNA | ARC Control | ARC CD36 AAV shRNA |
|----------------|-------------|--------------------|-------------|--------------------|
| CD36           | 2.55±0.30   | 1.51±0.02          | 3.29±0.74   | 0.96±0.14*         |
| NPY            | 0.70±0.07   | 0.71±0.07          | 1.14±0.20   | 0.84±0.11          |
| AgRP           |             |                    | 1.52±0.21   | 0.60±0.12*         |
| Glucokinase    | 1.01±0.03   | 0.97±0.03          | 0.87±0.04   | 1.07±0.03*         |
| Kir6.2         | 1.05±0.08   | 1.18±0.29          | 0.79±0.07   | 1.23±0.12*         |
| FATP2          | 0.87±0.02   | 1.28±0.08*         | 0.59±0.05   | 1.35±0.14*         |
| CPT1c          | 1.11±0.05   | 0.95±0.04*         | 1.02±0.03   | 1.05±0.01          |
| acyl-CoA synthetase 1 | 1.10±0.03 | 0.92±0.04*         | 1.13±0.09   | 0.90±0.05*         |
| acyl-CoA synthetase 5 | 0.83±0.05 | 0.96±0.05          | 0.84±0.02   | 1.03±0.07*         |
| acyl-CoA synthetase 6 | 1.11±0.06 | 1.07±0.09          | 1.15±0.04   | 0.93±0.03*         |
| AMPKα1         | 0.94±0.03   | 1.03±0.03*         | 0.99±0.03   | 0.96±0.03          |
| AMPKα2         | 0.69±0.07   | 1.18±0.06*         | 1.09±0.04   | 0.74±0.04*         |

Data are mean ± SEM of duplicate determinations expressed relative to the amount of the mRNA expression of the housekeeping gene, cyclophilin. N=7/group: *P<0.05 One way ANOVA.
**SUPPLEMENTAL DATA**

**Supplemental Table 1.** mRNA sequences of genes involved in glucose and fatty acid metabolism and glucose sensing in rats and mouse.

| Gene          | GenBank Accession no. | Forward          | Reverse             | Probe         |
|---------------|-----------------------|------------------|---------------------|---------------|
| Cyclophilin   | NM_017101.1           | AATGGCActGGTG    | GCCAGGACCTGT        | TCTACGGGAGAGA|
|               |                       | GCAAAGTC         | ATGCCTTCAG          | AATT          |
| GK            | NM_012565             | GGAGGCCACCAAG    | CCGGCTCATCACC       | CAGCTGGAACCTCT|
|               |                       | AAGGAAAA         | TTCTTCAG            | GCC           |
| FAS           | NM_017332.1           | GGCTCGCGCGAGTCT | TGCAGCCATCCTGT      | CCGGCATTCAGA |
|               |                       | ATGC,            | GTCG                | ATAG          |
| CPT1a         | NM_031559.2           | AAAGATCAGTCGG    | CGCCGCTCAAT         | ACTGGCCGCAATG|
|               |                       | ACCCTAGACA       | GTTCCTTC            | CAA           |
| CPT1c         | NM_001034925.2        | TGAGAGCCATGGA    | AGGGCCCTGGTG         | CACGGCTTCCCCG|
|               |                       | AAAACAAGGA       | TGGTC               | AACA          |
| FATP1         | NM_053580.2           | TTTCTGCGTATCCTG | CCGCACGGCGGAT       | ACCTCTTTGGCCT|
|               |                       | CTGCAAGA         | CAGA                | CTCTG         |
| FATP2         | NM_031736.1           | GCGGCCTCATGAT    | CTGGCTGGCTGA        | CCCCAACCAAA   |
|               |                       | TGGG               | AAATTTGCT           | TGC           |
| FATP4         | NM_001100706.1        | GAGGACGAGATTG    | CCGGCATCCAAT        | AACAGACGGGTC |
|               |                       | CGGAAGGA         | ATAGAAGAGT          | AAAGC         |
| FATP 6        | NM_001106145.1        | AGTGAACCCCAAAG   | GAGGCTGGGA          | TCCAAGCAATCT  |
|               |                       | CCATGGT          | GGATTTCCT           | TCG           |
| CD36          | NM_031561.2           | TGGAGACCTACTCA   | GCCCGTTCACC         | TTCAAGGACAA   |
|               |                       | TTAGAAGAGACA     | CAGTTTT             | ATCTCC        |
| NPY           | NM_012614.1           | TCGTGTGTATTGGGC  | GCGAGTATGAT         | ACAATCCGGGCG |
|               |                       | ATTCTG            | CTGGCCATGT          | AGGA          |
| AgRP          | NM_033650.1           | TCTCCCGCTGCTG    | CGCAGCAAAGGTAG      | AGGACTCTGTGCA|
|               |                       | TGTA              | CCGTGTGTC           | GCC            |
| POMC          | NM_139326.2           | GCCGTGCCGAGGA    | GCCCTCCCCTGGA       | TGGCCGTCCCGGA|
|               |                       | AGAG              | CTTG                | GC            |
| Protein Name                  | Accession Number | 5' end sequence | 3' end sequence |
|------------------------------|------------------|-----------------|-----------------|
| Glut4                        | NM_012751.1      | CTATGCTGGCAAC   | GAACCGTCCGAG    |
|                              |                  | AATGTCTTG       | AATGATGATCT     |
|                              |                  | AATGC           | ATGGGCCTAGCC    |
|                              |                  | AATGTCTTG       | CTGTACCACCCA    |
|                              |                  | AATGC           | CACCGTT         |
| Kir6.2                       | NM_031358.3      | AGGTGGACATCCC   | TCGATGACGTGGT   |
|                              |                  | CATGGA          | AGATGATGAGT     |
|                              |                  | AATGAGTATCT     | CTGTACCACCCA    |
|                              |                  | AATGC           | AACC            |
| acyl-CoA synthetase 1        | NM_012820.1      | GTACCCCTTCAACC  | GGGTGCGATACC    |
|                              |                  | AACACACT        | AGAAGGTT        |
|                              |                  | AATGAGTATCT     | AAGCCCCGAAGGC   |
| acyl- CoA synthetase 3       | NM_057107.1      | GAGTCAAAACAAG   | GTTGCAGACCT     |
|                              |                  | AGAAACCAAACCA   | GTATGCAGAGT     |
|                              |                  | AATGAGTATCT     | CCTGTCAAGTCAA   |
|                              |                  | AATGAGTATCT     | ACCAAAACCACCA   |
| acyl- CoA synthetase 4       | NM_053623.1      | CCCAGGAGATTGA   | TGTCTGAAGTGG    |
|                              |                  | CCTGTCTTAAA     | GCTTAGCTTTT     |
|                              |                  | AATGAGTATCT     | ACCATTCAGTCAAA  |
|                              |                  | AATGAGTATCT     | ACCAAAACCACCA   |
| acyl-CoA synthetase 5        | NM_053607.1      | CAGCCAGTCTTACC  | TCTCCGTGCTCT    |
|                              |                  | TCTCATTGAC      | CCTTCCTTCTC     |
|                              |                  | AATGAGTATCT     | CGATTCCTTC      |
|                                  |                  | AATGAGTATCT     | ACCATTCAGTCAAA  |
| acyl-CoA synthetase 6        | NM_130739.1      | CTATCGGACTTGGG  | CCAGTGCACCAT    |
| malonyl- CoA decarboxylase   | NM_053477.1      | CCAGTGCACCAT    | TCTGTCAAGTCAAA  |
| AMPKα1                       | NM_019142.1      | GCCCTCAAGCTGT   | CACTCGGACAGC    |
| AMPKα2                       | NM_023991.1      | GGAAGTACTCAAAC  | ATGTCTTTT       |
| Lepr-b                       | NM_012596.1      | AAGATACTCAAAC   | CTCCGTTCATTTC   |
|                              |                  | GCCAGAAGATTC    | CCACGTCCAGGCT   |
|                              |                  | AATGATGATGAGT   | TCAG            |
Supplemental Figure 1. Body weight gain of control and CD36 AAV shRNA VMH injected pups from P5 (injection day) to P21 (study day). Data are expressed as mean ±SEM; N=10/group.
Supplemental Figure 2. Ventromedial (VMN) and Arcuate (ARC) nucleus mCherry-FLAG fluorescent image at magnification 10X representing the AAV shRNA injection sites in control and CD36 AAV shRNA injected in P21 rats after 6 wk on chow and 9 wk on 45% fat diet. A and B are control AAV shRNA injected rats; C and D are CD36 AAV shRNA injected rats.