Mex3c mutation reduces adiposity partially through increasing physical activity

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Abstract

MEX3C is an RNA-binding protein with unknown physiological function. We have recently reported that a *Mex3c* mutation in mice causes growth retardation and reduced adiposity, but how adiposity is reduced remains unclear. Herein, we show that homozygous *Mex3c* gene trap mice have increased physical activity. The *Mex3c* mutation consistently conferred full protection from diet-induced obesity, hyperglycemia, insulin resistance, hyperlipidemia, and hepatic steatosis. In *ob/ob* mice with leptin deficiency, the *Mex3c* mutation also increased physical activity and improved glucose and lipid profiles. Expressing cre in the neurons of *Mex3c* gene trap mice, an attempt to partially restoring neuronal *Mex3c* expression, significantly increased white adipose tissue deposition, but had no effects on body length. Our data suggest that one way in which *Mex3c* regulates adiposity is through controlling physical activity, and that neuronal *Mex3c* expression could play an important role in this process.

Key Words

- *Mex3c*
- mice
- physical activity
- adiposity
- high-fat diet

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Introduction

Caenorhabditis elegans MEX3 is an hnRNP K homology (KH) domain-containing RNA-binding protein (Siomi *et al.* 1993), regulating RNA targets such as *pal1* (Draper *et al.* 1996, Hunter & Kenyon 1996, Huang *et al.* 2002), *rme2* (Ciosk *et al.* 2004), and *nos2* (Jadhav *et al.* 2008). It is involved in the cell fate specification process in *C. elegans*, or in totipotency maintenance of the germline in adult worms (Draper *et al.* 1996, Hunter & Kenyon 1996, Musco *et al.* 1996, Ciosk *et al.* 2006). Human and mouse genomes encode four MEX3 homologues: *MEX3A*, *MEX3B*, *MEX3C*, and *MEX3D* (Buchet-Poyau *et al.* 2007). They all have KH RNA-binding domains in the N-terminus (also present in the *C. elegans* homolog) and a zinc finger domain at the C-terminus (absent in MEX3 in *C. elegans*).

MEX3C contributes to genetic susceptibility to hypertension, as shown by linkage analysis and association studies (Guzman *et al.* 2006). It is also a cancer chromosomal instability-repressor gene (Burrell *et al.* 2013). The mechanisms for both observations are unknown. Human *MEX3C* enhances the degradation of *HLA-A2* mRNA through binding to its 3' UTR (Cano *et al.* 2012, Cano & Lehner 2013). These reports suggest that *MEX3C* has multiple functions, most likely through regulation of different mRNA targets in different cell types.

Mouse *Mex3c* is highly similar to its human homolog. We generated *Mex3c* gene trap mice on a C57BL/6 background; homozygous mutant mice had a high postnatal death rate, possibly due to poorly expanded

lungs (Jiao *et al.* 2012a). We bred mice to a mainly FVB/N background; homozygous *Mex3c* mutant mice on this background survived to adulthood and were fertile. However, these mice show growth retardation associated with IGF1 deficiency in developing bones (Jiao *et al.* 2012a), and reduced adiposity due to increased energy expenditure (Jiao *et al.* 2012b). Herein, we further examined the effects of *Mex3c* mutation on physical activity and diet-induced obesity, and explored the roles of neuronal *Mex3c* in this process through expressing *Mex3c* in neurons of *Mex3c* mutant mice, an attempt to partially restore *Mex3c* expression.

Materials and methods

Animals

The *Mex3c* gene trap allele (*Mex3c^{Gt(DD0642)Wtsi}*, written as 'tr' herein, indicating the trapped allele) was described earlier (Jiao *et al.* 2012a). *Lep^{ob/ob}* mice (Zhang *et al.* 1994) and mice with rat Synapsin I promoter-driven *Cre* (*Syn1-Cre*; Zhu *et al.* 2001) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Mex3c^{+/tr};Lep^{+/ob}* mice were intercrossed to obtain *Mex3c/Leptin* double-mutant mice. *Mex3c^{+/tr};Syn1-Cre⁺* mice were intercrossed to obtain *Mex3c^{tr/tr};Syn1-Cre⁺* and *Mex3c^{tr/tr};Syn1-Cre⁻* mice. All mice were backcrossed at least five generations to a mainly FVB/N background. The experiments were conducted in accordance with the National Research Council publication *Guide for Care and Use of Laboratory Animals*, and approved by the Institutional Animal Care and Use Committee of Wake Forest University Health Sciences. A chow diet (Prolab RMH3000, PMI Nutrition International, Henderson, CO, USA) was used for colony maintenance. A high-fat diet (HFD, TD.08811, Harlan), with 14.8, 40.6, and 44.6% calories from protein, carbohydrate, and fat, respectively, was used to induce obesity. Diet administration started when mice were 5–6 weeks old and lasted for 12–16 weeks. Mouse genotyping was performed using lysates of ear biopsies, as previously described (AgoulNIK *et al.* 2002, Jiao *et al.* 2012a).

Food consumption

Mice were singly caged in cages with lifted wire bottoms for food intake assays. Any food spilled on the bottom of the cages was recovered and subtracted from food intake. Food intake was assayed 1 week after starting the high-fat diet, and normalized to body mass. This normalization is suitable because at the time of assaying, all mice have comparable white adipose tissue (WAT) accumulation.

Monitoring physical activity

Locomotor activity was monitored using the OxyMax CLAMS system (Columbus Instruments, Columbus, OH, USA) when animals were 3 months old. A photobeam-based activity-monitoring system detected and recorded ambulatory (*x* and *y* axes) and rearing (*z* axis) movements. The temperature of the cabinet was 23 °C. All parameters were measured continuously and simultaneously for 72 h after 24 h of adaptation in singly housed mice. Three-day averages for each mouse were used for analysis.

Glucose and insulin tolerance tests

Mice were fasted for 16 h for glucose tolerance tests and 6 h for insulin tolerance tests. Just before glucose or insulin injection, tail vein blood glucose was measured with the ACCU-CHEK active meter and Test Strips (Roche). After mice received an i.p. injection of 10% D-glucose (Sigma, 1 g/kg body weight), or insulin (Novo Nordisk, 0.75 U/kg, pre-diluted to 2.5 U/ml in 0.9% NaCl), blood glucose levels were measured 30, 60, 90, and 120 min post-injection.

Blood biochemical assays

Plasma was collected from mice fasted for 4 h. Commercial kits were used to assay concentrations for blood triglycerides, cholesterol (Cayman Chemical, Ann Arbor, MI, USA), and insulin (Mercodia, Uppsala, Sweden).

Intestinal fat absorption

After 10 weeks of high-fat diet treatment, male mice were fed a test diet similar to TD.08811 (TD.10816; Harlan, Indianapolis, IN, USA), except 5% of fat in TD.08811 was replaced by the nonabsorbable sucrose polybehenate (Jandacek *et al.* 2004). After 5 days on the test diet, fecal samples of individually caged mice were collected for 3 days. The fatty acid content and composition in both the diet and feces were determined by gas chromatography (Section on Lipid Sciences, Wake Forest University Health Sciences), and the fractional absorption of total and individual fatty acids was calculated as described previously (Jandacek *et al.* 2004).

WAT and liver tissue histology

Adipose and liver tissues were fixed in 4% paraformaldehyde at 4 °C overnight for cryosections and paraffin sections. For histological analyses of WAT and liver,

5–8 μm paraffin sections were stained with hematoxylin and eosin. To quantify adipocyte size, 100 adipocytes from each of three mice per group were sampled. Three to four nonsuccessive sections were sampled for each mouse. Adipocyte size was analyzed by ImageJ software on digital images taken under the same magnification.

Quantitative PCR analysis of gene expression and mitochondrial DNA content

Total RNA was extracted from epididymal WAT and brown adipose tissues (BAT) of male mice, treated with Turbo DNase (Ambion, Thermo Fisher Scientific, Waltham, MA, USA) to eliminate DNA, and reverse transcribed as described previously (Jiao *et al.* 2012a). Sybr Green 2 $\times$ PCR mixture (Applied Biosystems) was used for detecting *Ppib*, *Pgc1 α* (*Ppargc1 α*), *Ppar γ* , *Ucp1*, *Ucp2*, *Cd137* (*Tnfrsf9*), and *Tmem26* expression with specific primers described previously (Jiao *et al.* 2012b, Wu *et al.* 2012). *Hprt1* and *Ucp1* Taqman probes (Applied Biosystems) were also used. Five mice from each group were analyzed. Mean $\pm$ S.E.M. were obtained from three to four independent PCR analyses. *Hprt1* and *Ppib* were used as internal controls and obtained similar results.

Quantitative PCR was used to compare the mtDNA content of epididymal WAT and BAT. Total DNA was isolated from tissues with the DNeasy Blood and Tissue Kit (Qiagen). ND5F and ND5R primers amplifying an 87 bp region of the *mt-Nd5* gene were used to amplify the mtDNA. 18sRNA Taqman probe (Applied Biosystems) and *Gapdh* primers, which gave similar results in qPCR, were used for amplifying genomic DNA. Sequences for the primers were described previously (George *et al.* 2012).

Statistical analysis

Two-tailed Student's *t*-tests were performed to compare the means of two groups. Tukey's post-tests following

ANOVA were performed for the means of more than two groups. For repeated measurements, two-way ANOVA was performed followed by Bonferroni post-tests. $P < 0.05$ was accepted as statistically significant.

Results

Mex3c mutation significantly increases physical activity

Previously, we observed that heterozygous *Mex3c* gene trap mice (+/*tr*, '*tr*' indicates the trapped allele for simplicity) have increased energy expenditure but normal physical activities. Physical activities of homozygous mutant mice (*tr/tr*) were examined to determine whether *Mex3c* has a dose effect on locomotor activities. In contrast to +/*tr* mice's normal physical activity reported previously (Jiao *et al.* 2012b), *tr/tr* mice showed significantly increased ambulatory activity (Fig. 1A) and rearing activity (Fig. 1B) compared with +/+ mice. Thus, the *Mex3c* mutation was associated with significantly increased physical activity.

tr/tr mice have normal body temperature (Jiao *et al.* 2012b). As they have reduced adiposity, we wondered whether they increase activity to maintain normal temperature. We previously observed that *tr/tr;ob/ob* mice were obese (Jiao *et al.* 2012b). Physical activity of *tr/tr;ob/ob* mice was also significantly increased compared with *ob/ob* mice and +/+ mice (Fig. 1C and D).

Mex3c mutation prevents diet-induced obesity

These findings prompted us to examine the effects of *Mex3c* mutation on diet-induced obesity. Mutant mice were fed a high-fat diet. As *tr/tr* mice were growth retarded (Jiao *et al.* 2012a), they weighed significantly less than age-matched +/+ mice at all time points regardless of diet (Fig. 2A and B). After 5 weeks on the high-fat diet, +/+ males gained significantly more weight than those on

Figure 1

tr/tr mice have increased physical activity compared with normal mice. (A) Ambulatory (Amb.) activity of +/+ and *tr/tr* males. (B) Rearing activity of control and mutant males. (C) Ambulatory activities of *ob/ob* mice and double-mutant males. (D) Rearing activity of *ob/ob* mice and double-

mutant males. Mean $\pm$ S.E.M. are presented. * $P < 0.05$ and ** $P < 0.01$ by two-way ANOVA. (C and D) Data from +/+ mice were also included for reference. Mice ($n \geq 5$ /group) were 3 months old.

Figure 2

Mex3c mutant mice are resistant to diet-induced obesity. (A and B) Growth curves of male (A) and female (B) mice on chow or a high-fat diet (HFD). Six or more animals were tested at each time point. (C and D) Percentage of epididymal and parametrial WAT relative to body weight in male (C) and female (D) mice. Each circle represents one animal. (E) Hematoxylin and eosin (H&E)-stained epididymal WAT sections (representative of five mice observed per group). Scale bar: 50 μ m. (F) WAT adipocyte sizes in

epididymal tissues from control and mutant mice. 300 adipocytes from three mice (100 cells from five different fields per mouse) from each group were sampled and analyzed by ImageJ software. AU, arbitrary unit. Mean $\pm$ S.E.M. are presented except in (E) NS, no significant difference. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ by Bonferroni post-tests (A, HFD-fed vs chow-fed +/+ mice) or Tukey's multiple comparison tests (C, D and F) following ANOVA.

chow diet (Fig. 2A). By contrast, *tr/tr* males did not gain more weight than those on chow diet even after 12 weeks on the high-fat diet. Male mice on an FVB/N background are sensitive to diet-induced obesity, but female mice on this background are not (Hu *et al.* 2004, Chen *et al.* 2005). Consistently, the high-fat diet was not associated with significant weight gain even in *+/+* female mice (Fig. 2B).

After 12 weeks on the high-fat diet, *+/+* males accumulated significantly more epididymal WAT than those on the chow diet (Fig. 2C), while *tr/tr* males did not. The diet treatment increased parametrial WAT mass in some *+/+* females, but overall the difference was not significant due to large intra-group variations. Parametrial WAT was not changed in any *tr/tr* female mice (Fig. 2D). Treatment significantly increased adipocyte size in male *+/+* mice, but not in male *tr/tr* mice (Fig. 2E and F). In female, *+/+* mice fed the high-fat diet, the size of adipocytes increased only in those that became obese (two females examined) but not those remained nonobese (two females examined). Adipocyte size did not increase in the four high-fat diet-fed *tr/tr* female mice examined.

As reported previously (Jiao *et al.* 2012b), the absolute amount of food consumed by *tr/tr* mice was reduced compared with normal control mice. However, food intake per gram body mass did not differ between the two groups (Table 1). Control mice absorbed $97 \pm 0.7\%$ of total lipids from high-fat diet ($n=4$) and mutant mice absorbed $94.6 \pm 1.0\%$ of total lipids from that diet ($n=4$). Considering the sensitivity and variation of the assay and the fact that over 94% of total food lipids were absorbed by both groups, it is unlikely that the lean phenotype of *tr/tr* mice was caused by the differences in lipid absorption.

Mex3c mutation improves blood glucose and lipid maintenance on high-fat diet

After 12 weeks on the high-fat diet, male *+/+* mice had significantly higher blood glucose concentrations than *+/+* mice on chow diet, as well as male *tr/tr* mice on either

diet (Fig. 3A). The diet treatment did not change blood glucose concentrations in *tr/tr* males. Although *+/+* male mice on the high-fat diet had hyperinsulinemia, *tr/tr* males on that diet had insulin levels similar to chow-fed *tr/tr* males (Fig. 3B). The high-fat diet caused glucose intolerance in *+/+* males but not in *tr/tr* males (Fig. 3C and D), as well as insulin resistance in *+/+* males, but not in *tr/tr* males (Fig. 3E and F). *+/+* females had higher blood glucose concentrations than *tr/tr* females on either diet (Fig. 3G). The high-fat diet failed to induce hyperglycemia (Fig. 3G) or hyperinsulinemia (Fig. 3H) in most *+/+* females.

Plasma triglyceride concentrations in *+/+* males fed both diets were similar, and both were significantly higher than that in *tr/tr* mice (Fig. 4A). Although the diet treatment increased total plasma cholesterol concentrations in *+/+* and *tr/tr* males (Fig. 4B), the increase was larger in *+/+* males (84 mg/dl) than in *tr/tr* males (59.6 mg/dl). In addition, plasma cholesterol concentrations of *tr/tr* males on the high-fat diet were not significantly different from those of chow-fed *+/+* males, but they were significantly lower than those of *+/+* males on the high-fat diet (Fig. 4B). Both *tr/tr* and *+/+* females on the high-fat diet had lower plasma triglyceride concentrations than their counterparts on the chow diet (Fig. 4C). The high-fat diet significantly increased total plasma cholesterol concentrations in *+/+* but not *tr/tr* females (Fig. 4D).

Normal mitochondrial and beige cell content in adipose tissues of Mex3c mutant mice

Mitochondrial content in WAT or BAT did not differ between *+/+* and *tr/tr* mice (Fig. 4E). The high-fat diet similarly reduced mitochondrial DNA content in WAT of *+/+* and *tr/tr* mice, consistent with previous observations on normal mice (Sutherland *et al.* 2008). The high-fat diet also significantly increased mitochondrial DNA content in BAT of normal mice, but not in *tr/tr* mice (Fig. 4E).

Table 1 Food intake of control and mutant mice. Each group had six or more mice. Mean $\pm$ S.E.M. are presented

Sex	Diet	Food intake (g/mouse per day)		Daily food intake/body weight	
		<i>+/+</i>	<i>tr/tr</i>	<i>+/+</i>	<i>tr/tr</i>
Male	Chow	4.6 ± 0.25	$3.0 \pm 0.17^{**}$	0.18 ± 0.01	0.17 ± 0.01
	HFD	4.0 ± 0.74	$2.5 \pm 0.11^{**}$	0.14 ± 0.03	0.12 ± 0.01
Female	Chow	3.9 ± 0.25	$2.9 \pm 0.14^{**}$	0.18 ± 0.01	0.17 ± 0.01
	HFD	2.9 ± 0.16	$2.1 \pm 0.14^*$	0.14 ± 0.01	0.15 ± 0.01

* $P < 0.05$ and ** $P < 0.01$, *+/+* vs *tr/tr*. HFD, high-fat diet.

Figure 3

Mex3c mutation prevents diet-induced glucose intolerance and insulin resistance. (A and B) Hyperglycemia (A) and hyperinsulinemia (B) occurred in +/+ but not *tr/tr* males on the high-fat diet (HFD) vs chow diet. (C and D) Glucose intolerance occurred in +/+ but not *tr/tr* males on the high-fat diet (HFD) vs chow diet. As baseline blood glucose levels differed among groups (C), data are also presented as relative values normalized to time 0 (D). (E and F) Insulin resistance occurred in control but not mutant males on the high-fat diet (HFD). Data are also presented as relative values normalized to time 0 in F. (G and H) Glucose (G) and insulin (H) concentrations of female mice on

different diets. For C, D, E and F, animal numbers (*n*) in each group are listed.

^aSignificant difference between chow-fed *tr/tr* mice vs HFD-fed *tr/tr* mice;

^bSignificant differences between chow-fed *tr/tr* mice vs chow-fed +/+ mice;

^cSignificant differences between HFD-fed *tr/tr* mice vs HFD-fed +/+ mice ($P < 0.05$ by Bonferroni post-tests following two-way ANOVA). For A, B, G, and H, each circle represents one mouse and mean $\pm$ S.E.M. are indicated.

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ by Tukey's multiple comparison test following one-way ANOVA. Blood glucose and insulin were measured after 16 and 6 h-fasting respectively.

Figure 4

Biochemical and histological analyses of tissues from mutants on high-fat diet. (A) Blood triglyceride concentrations of control and mutant males. (B) Total cholesterol concentrations of control and mutant males. (C) Blood triglyceride concentrations of female mice. (D) Blood cholesterol concentrations of female mice. (E) Mitochondrial DNA content by quantitative PCR. 28S rDNA was used as the genomic DNA control. (F) Beige cell composition in epididymal WAT by quantitative RT-PCR. *Cd137* and *Tmem26* were beige cell markers; *Ppib* was the internal control. (G) *Pgc1*, *Ppar-γ*, *Ucp1*, and *Ucp2* expression by quantitative RT-PCR. (H) Liver tissue from a +/+ male mouse on high-fat diet showing macrovesicular steatosis (hematoxylin and eosin stained). (I) Liver tissue from a tr/tr male mouse

showing microvesicular steatosis. (J) Liver tissue from a tr/tr male mouse without steatosis. (K) Liver tissue from a +/+ female mouse showing macrovesicular steatosis. (L) Liver tissue from a tr/tr female mouse with the worst steatosis. In A, B, C, and D, each circle represents one mouse; mean $\pm$ S.E.M. are indicated. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ by Tukey's multiple comparison test following one-way ANOVA. ns, no significant difference. In E, F, and G, mean $\pm$ S.E.M. of 3–5 independent PCR assays are presented. Equal amounts of DNA (E) or RNA (F and G) from each mouse ($n = 5$ /group) were used for quantitative PCR. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.0001$ for +/+ mice vs tr/tr mice on the same diet analyzed by two-way ANOVA.

Beige cells are thermogenic cells in WAT (Petrovic *et al.* 2010), and *Cd137* and *Temem26* are markers for these cells (Wu *et al.* 2012). The expression of these two genes was not increased in *tr/tr* mice (Fig. 4F), excluding the possibility that WAT contained more beige cells.

In mice on the high-fat diet, *tr/tr* mice had significantly higher expression of *Pgc1α* and *Pparγ* in both types of adipose tissues when compared with *+/+* mice (Fig. 4G). The high-fat diet did not change *Ucp1* expression in the BAT of *+/+* mice, but significantly increased its expression in *tr/tr* mice. Epididymal WAT of control and mutant mice had lower but detectable *Ucp1* expression compared with BAT (assayed both with specific *Ucp1* primers and *Ucp1* Taqman probe), but it was reduced in *tr/tr* epididymal WAT regardless of diet. *Ucp2* expression in both tissues was significantly downregulated in *tr/tr* mice on the high-fat diet. Increased expression of *Pgc1α* and *Pparγ* in the adipose tissues of *tr/tr* mice on a high-fat diet is consistent with their resistance to diet-induced obesity and associated metabolic syndromes. The expression profiles of *Ucp1* and *Ucp2* in *tr/tr* suggest that they do not play a major role in the leanness of the *tr/tr* mice.

Mex3c mutation prevents diet-induced steatosis

Hepatic steatosis was not seen in chow-fed *+/+* and *tr/tr* mice. However, macrovesicular steatosis was observed in over 50% of hepatic lobules of the 4 *+/+* males we examined on the high-fat diet (Fig. 4H), but in none of the 4 *tr/tr* males on that diet. Of the 4 *tr/tr* males on high-fat diet, only one showed microvesicular steatosis (Fig. 4I), and the rest had normal livers (Fig. 4J). Six of seven female *+/+* mice on the high-fat diet showed steatosis, five of which had macrovesicular steatosis (Fig. 4K). On the contrary, four of the seven female *tr/tr* mice on the high-fat diet had only milder hepatic damage (Fig. 4L) and the rest three mice had no steatosis at all.

Mex3c mutation improves glucose and lipid profiles of *ob/ob* mice

The effects of *Mex3c* mutation on metabolism were further examined in *ob/ob* mice. *tr/tr;ob/ob* mice had a similar percentage of WAT to lean tissue and similar histologic features of WAT as those of *ob/ob* mice (data not shown). However, blood glucose levels of *tr/tr;ob/ob* mice, although higher than those of normal mice, were significantly lower than those of *ob/ob* mice (Table 2). Insulin levels of *ob/ob* and *tr/tr;ob/ob* mice were both higher than those of normal control mice; in *tr/tr;ob/ob* mice, they fell between levels of normal control mice and *ob/ob* mice. Blood triglycerides and total cholesterol concentrations were both lower in *tr/tr;ob/ob* mice than in *ob/ob* mice. We confirmed our previous findings of no or milder steatosis in *tr/tr;ob/ob* mice than in *ob/ob* mice (Jiao *et al.* 2012b; data not shown).

Synapsin 1 promoter-controlled cre expression significantly increases adiposity of *tr/tr* mice

The gene trap vector in *tr/tr* mice contains two loxP sites, flanking the splicing acceptor (SA) sequences that disrupt normal *Mex3c* splicing (Fig. 5A). In *tr/tr* mice, three types of transcripts were observed from this locus by RT-PCR and sequencing (Fig. 5A and B). These were the hybrid transcripts between *Mex3c* exon 2 and gal-neo (transcript 1); normal *Mex3c* mRNA transcripts (transcript 2); and transcripts containing a vector-derived exon between *Mex3c* exon 2 and *Mex3c* exon 3 (transcript 3). The vector-derived exon in transcript 3 contains multiple stop codons disrupting the translation of MEX3C^{652AA} and MEX3C^{464AA}, but a product of MEX3C^{372AA} (predicted from multiple human and mouse cDNA records in GenBank; Li X *et al.* 2014, unpublished observations) could be expressed. Thus, removing the SA sequence from the integrated trapping vector by cre-mediated

Table 2 Blood biochemical profiles and food intakes of *ob/ob* mice and *tr/tr;ob/ob* mice.^a Mice were around 3 months old and were fasted for 4 h before blood collection. *+/+* and *ob/ob* data were all from males. *tr/tr;ob/ob* mice included four males and two females, but values for males and females were similar and thus are combined. Tukey's post-tests were performed following one-way ANOVA

Measurement	Levels for each group			P values		
	<i>+/+</i>	<i>ob/ob</i>	<i>tr/tr;ob/ob</i>	<i>+/+ ~ ob/ob</i>	<i>ob/ob ~ tr/tr;ob/ob</i>	<i>+/+ ~ tr/tr;ob/ob</i>
Glucose (mg/dl)	164.8 ± 7.1	551.1 ± 6.4	213.5 ± 5.5	<0.0001	<0.0001	<0.0001
Insulin (ng/ml)	1.0 ± 0.2	12.0 ± 4.0	6.6 ± 2.9	<0.01	NS	<0.05
Triglycerides (mg/dl)	132.0 ± 13	519.1 ± 76.8	138.0 ± 27.5	<0.0001	<0.0001	NS
Total cholesterol (mg/dl)	116.2 ± 6.6	193.0 ± 31.1	151.4 ± 15.2	<0.05	NS	NS

^aSample sizes per group ≥ 6.

Figure 5

Strategy for cre-mediated SA sequence removal in *Mex3c* gene trap mice. (A) Elements of the gene trap vector and three types of transcripts from the trapped *Mex3c* allele. E2 and E3 indicate exon 2 and exon 3 of *Mex3c* gene. In an alternative splicing form (NCBI NM_001039214.4), exon 1 and exon 2 are spliced into one exon (not shown). P1, P2, P3, and P4 indicate primers used for PCR. CE: cryptic exon from the trapping vector. (B) Detection of the three types of transcripts in the hypothalamus and testis tissues of *tr/tr* mice. All PCR products were verified by DNA sequencing. (C) Cre-mediated SA removal eliminates transcript 1.

recombination should eliminate transcript 1 and thus increase the expression of transcripts 2 and 3 (Fig. 5C).

To determine the role of neuronal *Mex3c* expression in energy metabolism, *Mex3c^{tr/tr};Syn1-Cre⁺* and *Mex3c^{tr/tr};Syn1-Cre⁻* mice were generated, where *Syn1-cre* indicates the cre transgene controlled by rat *Synapsin I* promoter (Zhu *et al.* 2001), a cre allele used for knocking out genes in neurons in energy balance studies (Cohen *et al.* 2001, Mori *et al.* 2004). Expression of cre in neurons is expected to remove the SA sequence in neurons of *tr/tr* mice, and thus increase the neuronal expression of transcripts 2 and 3. As antibodies detecting endogenous MEX3C are unavailable, we used qTR-PCR to detect *Mex3c* mRNA expression changes in *Mex3c^{tr/tr};Syn1-Cre⁺* mice. No detectable differences were observed in the expression of transcript 2 (normal *Mex3c* mRNA) between the hypothalamic

tissues of *Mex3c^{tr/tr};Syn1-Cre⁺* and *Mex3c^{tr/tr};Syn1-Cre⁻* mice. This is not surprising, because i) transcript 2 was already expressed at considerable levels in *Mex3c^{tr/tr};Syn1-Cre⁻* mice, making the detection of subtle increase difficult; and ii) *Mex3c* has wide expression in non-neuronal cells in the brain. For example, *Mex3c* is highly expressed in the ependymal cells of the third ventricle (see Fig. 5 in Jiao *et al.* (2012b)), and in smooth muscle cells of the blood vessels (Lu B 2014, unpublished observations), but both do not express cre; iii) cre-mediated recombination is not 100% efficient in neurons expressing both *Mex3c* and cre, a phenomenon previously observed in *LepR^{loxP};Syn1-Cre* mice (Cohen *et al.* 2001); and iv) some transcripts terminate after the SV40 PolyA site, even though the SA sequence is removed by cre-mediated recombination.

Mex3c^{tr/tr};Syn1-Cre⁺ and *Mex3c^{tr/tr};Syn1-Cre⁻* mice both had short stature (Fig. 6A) and were indistinguishable in growth curves (Fig. 6B). However, at the age of 5 months, *Mex3c^{tr/tr};Syn1-Cre⁺* mice had clearly bigger adipose depots than *Mex3c^{tr/tr};Syn1-Cre⁻* mice (Fig. 6C, D, and E), although their adipose depots were still significantly smaller than those of +/+ mice. The low WAT composition in *Mex3c^{tr/tr};Syn1-Cre⁺* and *Mex3c^{tr/tr};Syn1-Cre⁻* mice explains why we could not detect body weight differences between the two groups. Histological examination of epididymal and parametrial WAT tissues revealed that *Mex3c^{tr/tr};Syn1-Cre⁺* mice had larger and more developed adipocytes than *Mex3c^{tr/tr};Syn1-Cre⁻* mice (Fig. 6F). WAT with less developed adipocytes were observed in three of four *Mex3c^{tr/tr};Syn1-Cre⁻* mice examined, but not in 5 *Mex3c^{tr/tr};Syn1-Cre⁺* mice examined (Fig. 6F, right panel). With respect to physical activity, *Mex3c^{tr/tr};Syn1-Cre⁺* mice were less active than *Mex3c^{tr/tr};Syn1-Cre⁻* mice during the light cycle, although they were as hyperactive as *Mex3c^{tr/tr};Syn1-Cre⁻* mice during the dark cycle (Fig. 6G and H).

Discussion

This work builds on our previous findings regarding the role of *Mex3c* in energy balance. In addition to previous observations of increased energy expenditure (Jiao *et al.* 2012b), the *Mex3c* mutation significantly increased locomotor activity. Apparently increased physical activity is the primary result of *Mex3c* mutation, but is not a secondary result of reduced adiposity. Thus, increased physical activity explains in part the decreased adiposity of *tr/tr* mice. Previously, we observed that +/*tr* mice had normal physical activity but increased energy expenditure, suggesting that both basal energy expenditure and

Figure 6

Neuronal expression of cre in tr/tr mice increased adiposity. (A) Cre expression did not affect body length of tr/tr mice. $+/+$ mice ($n=10$) were all females, $Mex3c^{tr/tr};Syn1-Cre^+$ mice ($n=5$) and $Mex3c^{tr/tr};Syn1-Cre^-$ mice ($n=5$) included both females and males, which showed little difference by sex. (B) Body weights of $Mex3c^{tr/tr};Syn1-Cre^+$ and $Mex3c^{tr/tr};Syn1-Cre^-$ mice. $+/+$ mice include Cre^+ and Cre^- mice. (C) Representative of epididymal and perirenal WAT of male mice. (D) Parametrial and perirenal WAT tissues from two females of each group shown. (E) Weight of epididymal/parametrial WAT of normal control and mutant mice.

For $Mex3c^{tr/tr};Syn1-Cre^-$ mice, $n=1$ male and 5 females; for $Mex3c^{tr/tr};Syn1-Cre^+$ mice, $n=1$ male and 4 females; for control mice, $n=14$ (all females). *** $P<0.001$ by t-test between Cre^+ and Cre^- mutants. (F) Hematoxylin and eosin-stained epididymal WAT sections. Scale bars: 50 μ m. (G) Ambulatory activity of cre-positive and negative mutant females. (H) Rearing activity of cre-positive and negative mutant females. (G and H) Data were collected from 4 $Mex3c^{tr/tr};Syn1-Cre^+$, 4 $Mex3c^{tr/tr};Syn1-Cre^-$, and 9 $+/+$ female mice. Due to limited numbers of mutant mice available, statistical testing was not done.

physical activity accounted for decreased adiposity of *tr/tr* mice. *+tr* mice had normal physical activity but both *tr/tr* mice and *tr/tr;ob/ob* mice had increased physical activity, suggesting a dose effect of *Mex3c* in the regulation of physical activity. Food intake of *tr/tr* mice is reduced in absolute amount but not after normalized to body mass. Due to the large difference of body size between control and mutant mice, it is still unclear whether food intake is affected in *tr/tr* mice.

The CNS most likely plays an important role in the perturbed energy balance of *Mex3c* mutant mice. This idea is supported by the observations of essentially normal adipose tissue gene expression profiles, and normal absorption of food lipids in *tr/tr* mice. More importantly, neuronal expression of cre significantly increased WAT accumulation in *tr/tr* mice (although cre-positive mutant mice still have less fat accumulation compared with normal control mice).

Many factors (e.g., early termination at the PolyA site, and introduction of additional transcript forms) may explain our failure to observe increased in *Mex3c* expression in *Mex3c^{tr/tr};Syn1-Cre⁺* mice compared with *Mex3c^{tr/tr};Syn1-Cre⁻* mice. *Mex3c* could only be partially re-activated even in cre⁺ neurons of mutant mice, which explained why *Mex3c^{tr/tr};Syn1-Cre⁺* mice still had significantly reduced adiposity compared with normal control mice. The greater adipose accumulation in cre-positive mutants vs cre-negative mutants is most likely the result of cre-mediated SA sequence removal, which increased *Mex3c* expression in a subpopulation of *Mex3c*-expressing neurons. Cre-positive mutants also had reduced physical activity compared with cre-negative mutants during the light cycle, which might be one reason for their greater adiposity. Due to challenges associated with generating larger numbers of Cre⁺ and Cre⁻ mutant mice of the same sex and similar age, we only compared 4 Cre⁺ and 4 Cre⁻ mutant mice. More mutant mice need to be compared to confirm the data.

While *Syn1-Cre* mice also express cre in the testis (Cohen *et al.* 2001), this does not explain their increased adiposity. *Syn1-Cre* mice have been used to knock out *Socs3* and *LeptinR* in neurons to study energy balance (Cohen *et al.* 2001, Mori *et al.* 2004), and a recombination-independent effect of cre on adiposity has not been observed. In addition, many cre transgenic strains are maintained on an FVB/N background (the background of mice used in this study), and cre is not reported to cause increased adiposity in this background. Cre expression increased adiposity, but did not affect body length of *tr/tr* mice, confirming that different systems are affected by the mutation to cause these phenotypes. The CNS is involved

in the regulation of physical activity and basal energy expenditure (Kong *et al.* 2012, Xi *et al.* 2012). The role of neuronal *Mex3c* in basal energy expenditure remains unclear. A direct comparison of energy expenditure between control and mutant mice is problematic as they have significantly different body sizes and adiposity. We collected energy expenditure data for four cre⁺ and four cre⁻ mutant mice, but saw no differences between the two groups. Values varied widely, even among individuals of the same genotype. Thus, these data were inconclusive and not reported. We do not know whether neuronal MEX3C deficiency fully underlies the reduced adiposity. Answering these questions awaits the availability of a *Mex3c*-conditional knockout mouse model.

Our data explain why the *Mex3c* mutation fully protected mice in this study from diet-induced obesity and associated syndromes, and significantly improved glucose and lipid metabolism in *ob/ob* mice. These observations also suggest that genes regulated by *Mex3c* could be therapeutic targets for obesity and diabetes. *Agrp* and POMC neurons have no or low *Mex3c* expression (Jiao *et al.* 2012b). Determining the identity of the neurons expressing *Mex3c* and the neural circuits affected in mutant mice will shed new light on how energy expenditure and voluntary activities are regulated. Identifying mRNA targets of the RNA-binding protein MEX3C that are involved in energy metabolism will not only improve our understanding of MEX3C function at the molecular level, but could help to develop new drugs to control and treat obesity in the future.

In summary, we found that one mechanism through which the *Mex3c* mutation decreases adiposity is increased locomotor activity. Neuronal *Mex3c* may play an important role in regulating energy balance.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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