Hmgcr in the Corpus Allatum Controls Sexual Dimorphism of Locomotor Activity and Body Size via the Insulin Pathway in Drosophila

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The insulin signaling pathway has been implicated in several physiological and developmental processes. In mammals, it controls expression of 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGR), a key enzyme in cholesterol biosynthesis. In insects, which cannot synthesize cholesterol de novo, the HMGR is implicated in the biosynthesis of juvenile hormone (JH). However, the link between the insulin pathway and JH has not been established. In Drosophila, mutations in the insulin receptor (InR) decrease the rate of JH synthesis. It is also known that both the insulin pathway and JH play a role in the control of sexual dimorphism in locomotor activity. In studies here, to demonstrate that the insulin pathway and HMGR are functionally linked in Drosophila, we first show that hmgcr mutation also disrupts the sexual dimorphism. Similarly to the InR, HMGR is expressed in the corpus allatum (ca), which is the gland where JH biosynthesis occurs. Two p[hmgcr-GAL4] lines were therefore generated where RNAi was targeted specifically against the HMGR or the InR in the ca. We found that RNAi-HMGR blocked HMGR expression, while the RNAi-InR blocked both InR and HMGR expression. Each RNAi caused disruption of sexual dimorphism and produced dwarf flies at specific rearing temperatures. These results provide evidence: (i) that HMGR expression is controlled by the InR and (ii) that InR and HMGR specifically in the ca, are involved in the control of body size and sexual dimorphism of locomotor activity.

INTRODUCTION

The high degree of similarity displayed among species by the insulin signaling pathway highlights its importance in developmental and physiological processes. In mammals, insulin receptor (InR) and insulin like growth factor receptor (IGFR) share the same signaling pathway components and insulin receptor substrates (IRS), which mediate functions, such as growth [1,2], fertility and glucose homeostasis [3]. They also modulate several metabolic pathways, such as cholesterol biosynthesis [3,4] or lipid metabolism [5]. In lower organisms like Drosophila, seven genes coding for insulin like ligands have been described [6]. Four of them are synthesized in neurons of the Pars Intercesibalis (PI) called Insulin Producing Cells (IPCs) [6–9]. In contrast, only one insulin like receptor (InR) has been described [10] with a conserved structure. Functionally, in comparison to mammals, its action is similar, during development, to insulin like growth factor receptor (IGFR) [8,11–18], while in adult stage, to the endocrine function of the InR [19–21]. Indeed, mutations affecting InR or chico, the IRS homologue, or ablation of insulin producing cells (IPCs), cause growth retardation [12,15,16], reduce body and organ size [6,8,16,22], increase sugar level [8,13–15,21], and longevity [15,16,23]. These phenotypes resemble those obtained for IRS-1 [24] or IRS-2 knockout mice [25].

The highly conserved insulin pathway between Drosophila and mammals, and the wide variety of genetic and molecular tools of Drosophila, gives us the opportunity to use this relatively simple model to identify new roles of the insulin pathway. Indeed, recent studies demonstrated that the insulin pathway is an integrative system adapting growth to food availability [7,26,27]. Additionally, Wu and collaborators implicated InR in food intake and noxious food aversion [19,20]. In a previous study, we also reported a new role for insulin pathway in Drosophila, by showing its implication in the control of a sexual dimorphism in locomotor activity [21]. This sexually dimorphic behavior comprises differences in the temporal pattern of locomotor activity, represented by a different number of activity/inactivity periods (or start/stop number) between males and females when flies are freely walking during a determined period (5 hours) [21,28–30].

A disruption in the insulin pathway either in IPCs or at the InR level abolishes sexual dimorphism [21]. In an earlier study, we found that the Juvenile Hormone (JH), one of the main hormones in insects, is involved in the control of start/stop number [21,28–30]. JH is synthesized in the ca [31], while the 3-Hydroxy-3-Methylglutaryl Co Enzyme A Reductase (HMGR) [31] is the key step in JH biosynthesis. Additionally, ectopic application of Fluvastatin, an HMGR inhibitor, abolishes sexual dimorphism [28]. Interestingly, we previously found that InR is expressed in the ca [21], while Tatar and collaborators demonstrated that a mutation affecting the InR leads to a decrease in the JH level [15]. Together, these data suggest that there is a functional link between the insulin pathway and JH synthesis and/or release in the ca. We...
therefore hypothesized that HMGCR could be the link between the insulin pathway and JH in the ca.

In mammals, HMGCR is a transmembrane glycoprotein anchored in the smooth endoplasmic reticulum [32] and is principally found in liver tissues [33]. It catalyzes the production of mevalonate, which represents the rate limiting step of cholesterol biosynthesis in the liver [34]. Tight control on cholesterol production is critical in physiology, since defects reducing cholesterol synthesis lead to the Smith-Lemli-Opitz syndrome [35]. Alternatively, over production causes a predisposition to atherosclerotic vascular diseases [36]. HMGCR has also been implicated in other processes like embryogenesis [37] and cancer [38,39]. The reduction is continuously transcribed and is regulated by a number of factors, including negative feed back from cholesterol [40], or remarkably by insulin that strongly stimulates HMGCR synthesis [40–42]. Regulation involves a family of helix-loop-helix transcription factors, called sterol response element binding protein (SREBP) and particularly SREBP-1c [42,43]. Regulation involves a family of helix-loop-helix transcription factors, called sterol response element binding protein (SREBP) and particularly SREBP-1c [42,43]. Regulation involves a family of helix-loop-helix transcription factors, called sterol response element binding protein (SREBP) and particularly SREBP-1c [42,43].

In insects, cholesterol is not synthesized de novo [44]. However, in the ca, HMGCR catalyzes the synthesis of mevalonate, the precursor of the JH family components [31]. In Drosophila, hnger gene (also named columbus (clb)), has been cloned and is implicated in germ cell guidance during development [45], as well as further in zebrafish [46]. HMGCR also plays a role in the potentiation of hedgehog signaling [47] as well as in neurodegenerative disease [48]. However, little is known about its regulation. Although a homologue of SREBP has been identified in Drosophila [HH1106] and is expressed in the ca [49,50], there is still no clearly established link between HMGCR and this transcription factor.

Here, we show that a mutation affecting the hnger gene in Drosophila, abolishes sexual dimorphism in locomotor activity. We also show that this phenotype can be rescued by over expression of hnger specifically in the ca. For the latter studies, we generated two independent p[hmgcr-GAL4] lines to specifically drive either a RNAi-HMGCR or an RNAi-InR in the ca. In both cases, we show that locomotor behavior mimics the hnger mutant (lack of the sexual dimorphism), highlighting the role of the hnger and the insulin pathway in the ca. Furthermore, we also show that silencing inr gene within the ca results in a reduction of HMGCR, suggesting a transcripional control exerted by insulin signaling pathway on the HMGCR in the ca. Finally, targeting either RNAi-InR or RNAi-HMGCR specifically in the ca, yields dwarf flies, suggesting a very specific action of InR and HMGCR within the ca, in the control of development.

RESULTS

Sexual dimorphism in locomotor activity is abolished in the hmgcr mutant

The number of activity/inactivity phases, equivalent to the start/stop number, has been shown to be different between males and females in normal wild-type (Canton Special (CS)) flies [30]. Application of Fluvastatin, an HMGCR inhibitor [51] abolishes the sexual dimorphism [28]. In an attempt to determine whether HMGCR is directly involved in the control of this dimorphism, we quantified the locomotor activity of flies carrying a homozygous mutation affecting the gene hnger. Since all known hnger mutations were homozygous lethal [45], and heterozygous combinations (P[PZ]hnger+/CS) and (hnger+/+) exhibit a wild-type like phenotype (data not shown), we used an hypomorphic mutation of the hnger gene, a line carrying a P element in the 3’ region of this gene (P[PZ1304684hngerC14]), also known as 11635 line (Bloomington Stock Center number). When reared at 24°C, the 11635 homozygous flies are also lethal. However, when reared at 19°C, 5 and 10% of males and females, respectively, which are developmentally delayed, attempt to survive until adulthood. Quantification of the locomotor activity of these flies indicates that the start/stop number for both sexes is the same (Figure 1a).

However, at this stage of the study, we could not exclude that the P element affects other genes in the 3’ region of hnger. To confirm that the effects observed in the 11635 homozygous mutants are due to a perturbation of the hnger gene, we genetically rescued the phenotype by directing the expression of the the hnger gene in the 11635 homozygous mutant. In these studies, we used the yeast derived UAS/GAL4 system to specifically target the hnger cDNA: p[UAS-hnger] (also known and referred here as: UAS-clb) [45] with different p[GAL4] driver lines in an homozygous 11635 genetic background mutant. The pan-neural p[elav-GAL4] (elav-GAL4) [52] or the head specific fat body p[takeout-GAL4] (to-GAL4) [53] drivers were not able to rescue lethality. However, the p[acti-
GAL4) [act-GAL4] [54] a driver known to be expressed in many tissues and cells (but not considered an ubiquitous driver), completely rescues lethality but, surprisingly, does not rescue the sexual dimorphism (Figure 1b). Finally, the p[daughterless-GAL4] (da-GAL4), an ubiquitous driver [55] was sufficient to rescue both lethality [partly in males (75%) and completely in females (Figure 1b)] and the start/stop number. The rescue of the locomotor activity defect in the P element 11635 mutant flies (hmgcr11635 allele) confirms that this phenotype resulted from mutation to the hmgcr gene by the P element insertion. However, to elucidate the difference in the rescue (lethality and sexual dimorphism) obtained with those two drivers, act-GAL4 versus da-GAL4, we also looked precisely at their respective expression patterns, using the reporter transgene UAS-gfp (green fluorescent protein). These studies showed that GFP was found to be expressed in the ca under the sole control of the da-GAL4 line, but not in the act-GAL4 (data not shown), suggesting a specific role for HMGCR in the ca to control sexual dimorphism. These results suggest firstly, that the HMGCR is involved in the control of sexual dimorphism and secondly, that its action may be required in the ca.

**hmgcr is expressed in the fat body and the corpus allatum in adult flies**

To determine the expression pattern of hmgcr, we performed immunostaining. In mammals, the enzyme has been found in a wide variety of tissues including brain, testis and liver [40]. Polyclonal antibodies raised against the human HMGCR [33] were used to determine the expression pattern of HMGCR in Drosophila tissues. First, we showed that the reductase is expressed in residual larval fat body (Figure 2a), which corresponds to residual adipocytes inherited from larval stages that disappear in flies within approximately three days after hatching. HMGCR is also detected in some cells at the periphery of the brain (Figure 2b and 2c). The presence of this enzyme in the brain has already been indirectly suggested by Tschape and collaborators [48]. These authors have shown that HMGCR could play a role in neuronal tissues. Indeed, the ectopic expression of the reductase, under the control of the pan-neural driver, elav-GAL4, in flies carrying a mutation leading to neurodegenerescence, is sufficient to rescue the lethal phenotype. In Figure 2c, we can see an immunoreactive cluster of cells located in the pars intercerebralis (P1). Outside the head, the reductase is also found to be expressed in the digestive tract, especially in the cardia (Figure 2d). Finally, the HMGCR is also expressed in the ca (Figure 2c), in accordance with previous results suggesting a role for the reductase in this tiny gland.

**Identification of hmgcr regulatory sequences and genesis of two p[hmgcr-GAL4] lines**

To date, no specific GAL4-driver line has been reported to be specifically expressed in the ca in adult fly. Since we found that hmgcr is expressed in the ca, suggesting a specific role in this structure, we generated two p[hmgcr-GAL4] lines, in which the putative genomic DNA promoter sequence specifically drives the GAL4 gene. The Drosophila hmgcr gene is composed of 8 exons, in which 5 are coding exons. Two mRNA are transcribed according to two different initiation sites (Figure 3a). RNA (3,972 kb) comprises the first, but not the second and third exons. The RNA (3,383 kb) starts only with the second exon. Both mRNA share the other three encoding exons. In order to construct two p[GAL4] lines corresponding respectively to the two putative mRNA promoters, we first selected a sequence of 3.8 kb upstream to the first exon initiation site, and secondly, a sequence of 10.8 kb upstream to the second exon. Each sequence was cloned and fused upstream to the coding sequence of the yeast GAL4 transcription factor gene. In these two sequences, based on the already identified mammalian sequence, specific cis-acting transcription factor recognition sites were identified (Figure 3a), especially E-boxes and SRE (Sterol Responding Elements) that are known to be the targets of SREBP (Sterol Responding Element Binding Protein). The two cloned constructs were then used to generate two independent and specific p[hmgcr-GAL4] transgenic lines called DI-3 and DI-11, corresponding respectively to the RNA and RNA promoters. In an initial step, we determined if the two lines were functional. Using the UAS-gfp as reporter, we determined their expression pattern. Two days old DI-3/UAS-gfp flies were found to express the reporter gene gfp in the residual larval fat body (data not shown). GFP was also detected in some PI cells (data not shown) but not in other brain neurons. Moreover, a strong expression of the reporter gene was found in the ca (Figure 3c). The ca is a very small gland located near the corpus cardiacum (cc) and the cardia, a compartment of the digestive tract (Figure 3b). To confirm that the observed signal corresponds to the ca, a double immunostaining against GFP and the Adipokinetic Hormone (AKH) [56] was performed (Figure 3d). AKH is known to label specifically the cc [56,57], allowing the presence of GFP in the ca. In a second step, double immunostaining against GFP and HMGCR was performed (Figure 3e), allowing colocalization of the two proteins in ca cells, to be shown. Thus, this expression pattern confirms that the 3.8 kb length promoter cloned fragment
drives the GFP in the same cells of the HMGCR are colocalized (right panel: yellow), confirming that the DI-3 (green) or Cy3 (red). When merged, we can see that the GFP and secondary antibody. Those last are labeled respectively with FITC (green) and Cy3 (red). When merged, we can see that the GFP and HMGCR are colocalized in the same cells of the ca (Figure 3f), whereas GFP expression is still found in PI cells (data not shown). It is likely that the residual fat body cells are not detected because of the lack of these cells in 5 day old flies. These results show that DI-3 presents a temporally dynamic expression pattern. This also tends to suggest distinct roles for HMGCR in some tissues of young and older adults. By contrary to DI-3, DI-11 has a very stable temporal expression pattern. The reporter gene UAS-gfp driven by DI-11 shows a strong expression in the ca (Figure 3g). As for DI-3, a double immunostaining reveals that GFP and HMGCR are colocalized in the previously shown endogenous HMGCR expression pattern, as revealed by the immunostaining. The DI-3 follows partially and temporally the expression of the endogenous gene, while the DI-11 seems to follow permanently the expression in the ca. These two lines are therefore valuable tools to specifically target any reporter gene in the ca, so that studies on the putative role of HMGCR in this specific tiny gland can be undertaken.

**Targeted expression of hmgcr cDNA in the corpus allatum rescues the hmgcr mutant phenotype**

We made the hypothesis that the lack of the sexual dimorphism in locomotor activity in the hmgcr11635 mutant could be due specifically to the lack of the hmgcr in the ca. First, driving UAS-clb with DI-3 in the homozygous mutant background (DI-3; UAS-clb, hmgcr11635/hmgcr11635) was not sufficient to rescue lethality at 24°C. As we previously observed that act-GAL4 driving UAS-clb was sufficient to allow (hmgcr11635/hmgcr11635) flies to survive until adulthood, but not to rescue the sexual dimorphism in locomotor activity, we combined these two driver lines (act-GAL4 and DI-3) in the same fly, to drive hmgcr gene expression. Interestingly, the presence of the two drivers, act-GAL4 and DI-3, in the mutant genetic background (hmgcr11635/hmgcr11635) was sufficient to rescue the sexual dimorphism. Indeed, figure 4 shows that 2 days old

![Diagram](image_url)

**Figure 4. Targeted expression of the HMGCR in the corpus allatum rescues the hmgcr mutant phenotype.** p[DIII-GAL4] plus p[act-GAL4] lines are therefore valuable tools to specifically target any reporter gene in the ca to control the sexual dimorphism. Interestingly, this rescue occurs in two days old flies, but not in the same flies of 5 days old.

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males and females bearing both act-GAL4 and DI-3 have a significantly different start/stop number, compared to control flies, confirming that DI-3 is sufficient to rescue the sexual dimorphism. Most interesting was the observation that the expression of DI-3 disappeared after approximately 4 days. After the first locomotor activity quantification, flies were re-collected and maintained on normal food medium during 3 additional days, after which locomotor activity was again recorded and the start/stop numbers determined. Figure 4 shows that these five days old flies exhibited a mutant phenotype: males and females start/stop number was similar. This result concurs with the observed expression pattern of the gfp transgene under the control of DI-3, suggesting that before 3 days, hunger transgene is expressed in both the ca and the rest of the body (comprising the PI neurons), while after 4 days, the transgene, driven only by act-GAL4 promoter, is not expressed anymore in the ca. This result suggests that HMGCR plays a role in the sexual dimorphism, by acting specifically within the ca. However, due to the reporter gene expression pattern of DI-3 in some other neurons and tissues, we can not exclude if PI and/or larval fat body cells could also be implicated in the control of start/stop number.

Directed expression of RNAi-HMGCR in the corpus allatum abolishes sexual dimorphism and mimics the hmgrc mutant phenotype

To determine more precisely the tissues where HMGCR is required to promote the sexual dimorphism in locomotor activity, we used an interferential RNA (RNAi) against the hmgcr gene. For each experiment, DI-3 line in combination with one of the two p[UAS-RNAi-HMGCR] lines (10367R1 and 10367R3, inserted on the chromosome III and II respectively; a generous gift from R. Ueda, NIG, Japan) were used to avoid non specific P element insertion side effect. We found that flies carrying the UAS-RNAi-HMGCR driven by either the da-GAL4 ubiquitous driver or by the act-GAL4 driver, yields lethality to the second instars larva. Although we can not exclude a non specific toxic effect, this phenotype is comparable to lethality induced by hmgcr mutations. Compared to controls: DI-3/CS and/or UAS-RNAi-HMGCR/CS, no HMGCR was detected in the ca in 2 days old flies when the UAS-RNAi-HMGCR was driven by DI-3 (Figures 5a and 5b). It is worth reiterating, that in 2 days old flies, DI-3 does not drive expression of UAS-gfp in the cardia (see Figure 3f) and we can presume that the expression pattern is the same for UAS-RNAi-HMGCR. Indeed, the cardia (Figure 5g) is sufficiently immuno-reactive independently of the presence of the UAS-RNAi-HMGCR transgene. This result confirms that HMGCR is not detectable in tissues simultaneously expressing the GAL4 driver and the UAS-RNAi-HMGCR and thus, validates the specificity of action of the latter. In accordance with previous results, two days old flies without HMGCR expression in the ca (DI-3/UAS-RNAi-HMGCR) did not show the sexual dimorphism in locomotor activity (Figure 5b). Furthermore, UAS-RNAi-HMGCR/CS control flies expressing HMGCR in the ca, harbor a wild type phenotype, as the sexual dimorphism is present (Figure 5a). As for the former experiment, all the 2 days old recorded flies were kept for further re-evaluation, and then, placed in a new food medium for 3 days before their locomotor activity was once again quantified. As expected, all five days old flies (controls UAS-RNAi-HMGCR/CS or DI-3/UAS-RNAi-HMGCR) present a sexual dimorphism comparable to a wild-type phenotype (Figures 5c and 5d). Indeed, as previously observed with the UAS-gfp, DI-3 does not drive UAS-RNAi-HMGCR in flies older than 4 days. This result together with the data supporting the presence of HMGCR protein in the ca (Figure 5d), suggests that the action of the RNAi is at least partly reversible. However, we can still not exclude a possible action of HMGCR in PI and/or residual larval fat body

Figure 5. Directed expression of p[UAS-RNAi-HMGCR] specifically in the corpus allatum mimics the hmgrc mutation, by disrupting the sexual dimorphism. For all panels (a–g): immunostaining using a primary antibody raised against the human form of the HMGCR, revealed with a secondary antibody labeled with Cy3 (red). a–d) Targeting UAS-RNAi-HMGCR specifically in the ca yields to the lack of the HMGCR protein, and leads to the disruption of the sexual dimorphism. This effect is completely reversible. In 2 days old DI-3/UAS-RNAi-HMGCR flies (b), HMGCR protein is not detectable and the number of start/stop is identical between males and females compared to controls (a) (2 days old UAS-RNAi-HMGCR/CS flies). Interestingly, in 5 days old flies, both DI-3/UAS-RNAi-HMGCR (d) and UAS-RNAi-HMGCR/CS (c) the HMGCR is detectable and the start/stop number is sexually dimorphic. This is in agreement with the temporal expression pattern driven by the DI-3. Additionally, this result strongly suggests the reversibility of the RNAi effect. N.B: results from b) and d) come from the same flies, recorded at 2 and 5 days old respectively. e, f) Expressing the UAS-RNAi-HMGCR in the ca using the DI-11 line (DI-11/UAS-RNAi-HMGCR) also leads to the lack of the HMGCR product (f) and disrupts the sexual dimorphism compared to controls flies (e). g) The HMGCR is expressed both in the cardia and the ca (left panel) in control flies (2 days old UAS-RNAi-HMGCR/CS flies), whereas in 2 days old (DI-3/UAS-RNAi-HMGCR) flies, the HMGCR is detected only in the cardia (right panel). This last staining serves as a positive control, to demonstrate that the DI-3 drives the UAS-RNAi-HMGCR only in the ca. h) Expressing the UAS-RNAi-HMGCR in the ca under the control of DI-11 leads to a strong lethality when flies are reared at 24 °C. Moreover, the only few females that survived are dwarf (left: dwarf female DI-11/UAS-RNAi-HMGCR, middle: female control UAS-RNAi-HMGCR/CS) and right: female CS). Scale bar = 25 μm. doi:10.1371/journal.pone.0000187.g005

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cells. To rule out this possibility, we used DI-11 line to drive UAS-RNAi-HMGCR in the ca. When reared at 24°C, DI-11/UAS-RNAi-HMGCR larvae are smaller than control (half size) (Figure 5h) and a strong lethality is observed in third instars larvae, as very few adults survive: less than 1% and females only. Fortunately, when reared at 19°C, 10% and 1% of expected adult females and males, respectively, survive and have normal weight and size. As we can expect for these flies, a lack of sexual dimorphism is observed in correlation with the absence of HMGCR expression in the ca (Figure 5f) compared to the relevant controls (Figure 5e). These results are in accordance with those obtained using the DI-3 line. To exclude possible contributions from other tissues in the sexual dimorphism, and more particularly the involvement of the HMGCR expressed in PI and/or residual larval fat body cells, we used two other GAL4 driver lines, p[GAL4]C316 (C316) and elav-GAL4 to drive UAS-RNAi-HMGCR. C316 induces UAS-gfp expression in the larval fat body, in the cardia and in DPM neurons (data not shown) [58], while elav-GAL4 is a pan-neural GAL4-driver [52]. Flies from either elav-GAL4/UAS-RNAi-HMGCR or C316/UAS-RNAi-HMGCR display a wild type start/stop number compared to controls (data not shown). Altogether, these results strongly suggest that HMGCR expression in the ca is necessary for flies to present the sexually dimorphic locomotor activity.

Directed expression of RNAi-InR in the corpus allatum abolishes the sexual dimorphism and mimics the hmgcr mutation

In a previous study [21], we reported that the insulin signaling pathway is implicated in the start/stop number control, and in parallel, that the insulin receptor (InR) is expressed in the ca. Using the same strategy, we used the DI-3 line to drive a RNAi against the InR gene [p[UAS-RNAi-InR]] in the ca. As before, the DI-3 line and two UAS-RNAi-InR lines (18402R1 and 18402R2, inserted on the chromosome II and III respectively, also a generous gift from R. Ueda, NIG, Japan) have been used to avoid non specific P element insertion side effect. Again here, flies carrying a UAS-RNAi-InR driven either by act-GAL4 or by da-GAL4 lines are lethal in the second instars larva. However, using the DI-3 driver line, we obtained flies from 2 days old (DI-3/UAS-RNAi-InR), which do not have InR expression in ca (Figure 6b), while the start/stop number is not different between males and females (no sexual dimorphism) compared to controls (Figure 6a). Both sexual dimorphism and InR expression are completely restored in 5 day old DI-3/UAS-RNAi-InR flies, which are comparable to control flies: DI-3/CS and UAS-RNAi-InR/CS (Figure 6c) (as previously, the same flies are recorded at 2 and 5 days old). Similarly to the UAS-RNAi-HMGCR/CS flies, UAS-RNAi-InR/CS flies are normal, suggesting a very putative restricted non specific misexpression of RNAi. Another similarity to the RNAi-HMGCR, is that the RNAi-InR effects are at least partly reversible. We previously showed that using the DI-11 in combination with the UAS-RNAi-HMGCR leads to lethality at 24°C. Also here, using UAS-RNAi-InR driven by the DI-11 yields lethality at 24°C. However, when reared at 19°C, we obtained few adult females (less than 1%) that are dwarf and die after about 5 days (Figure 6d). To confirm the specific role of HMGCR within the ca versus the one expressed in the PI or in the residual larval fat body cells, we used again the two GAL4 driver lines, C316 and elav-GAL4 to drive the UAS-RNAi-InR. In both cases, neither InR expression and/or the sexual dimorphism were affected (data not shown). In conclusion, InR expression in the ca seems to be necessary for establishing the sexual dimorphism.

Figure 6. Directed expression of p[UAS-RNAi-InR] specifically in the corpus allatum, mimics the hmgcr mutation. For all panels (a–c): immunostaining using a primary antibody raised against the human form of the InR, revealed by a secondary antibody labeled with FITC (green). a–c) Targeting the UAS-RNAi-InR specifically in the ca blocks the InR expression and disrupts the sexual dimorphism. This effect is completely reversible. In 2 days old DI-3/UAS-RNAi-InR flies (b), InR is not detectable and the number of start/stop is identical between males and females compared to controls flies (a) (2 days old UAS-RNAi-InR/CS). Interestingly, in 5 days old DI-3/UAS-RNAi-InR flies (c) the InR is detectable and the start/stop number is sexually dimorphic. This result corroborates the temporal expression pattern driven by the DI-3. Additionally, this strongly suggests, like for the RNAi-HMGCR, the reversibility of the RNAi-InR effect. N.B.: again here, results from b) and d) come from the same flies, recorded at 2 and 5 days old, respectively. d) Expressing the UAS-RNAi-InR in the ca under the control of DI-11 leads to a strong lethality when flies are reared at 24°C and 19°C. However, at 19°C, few females survive, but they are dwarf (left: dwarf female DI-11/UAS-RNAi-InR, right: female control (UAS-RNAi-InR/CS). Scale bar = 25 µm.
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Insulin signaling pathway controls the sexual dimorphism via hmgcr

We have shown that InR and hmgcr are both expressed in the ca. When a RNAi-HMGCR or a RNAi-InR is driven specifically in the ca by DI-3 or DI-11 drivers, this leads to a disruption of the sexual dimorphism in locomotor activity. To demonstrate that the InR and HMGCR are expressed in the same ca’s cells, we performed a double immunostaining against HMGCR and InR. Figure 7a shows that those two proteins are colocalized. Using the DI-3 and/or the DI-11 as drivers, we first showed that expressing the RNAi-HMGCR in the ca does not affect InR expression (Figure 7a). Conversely, expression of an RNAi-InR completely suppresses hmgcr expression. Similarly to previous reports in mammalian cells [42], these results suggest a putative transcriptional control exerted by InR on hmgcr. Furthermore, since DI-3 drives expression of UAS-RNAi only up to 3 days, then expression of both InR and/or HMGCR, which correlates to the sexual dimorphism, is restored at 5 days. This confirms that the RNAi effects are reversible (Figure 7a). However, no direct evidence that the disruption of the sexual dimorphism could be attributed to the lack of HMGCR rather than to the InR. Indeed, both HMGCR and InR could be needed for the sexual dimorphism, but in independent pathways. In order to determine more precisely a functional link between these two proteins in relation to their respective roles in the control of sexual dimorphism, we expressed both hmgcr (using UAS-clb) and UAS-RNAi-InR under the control of the DI-3 driver. We found that InR was still undetectable whereas HMGCR was well present in the ca in 3 days old flies (Figure 7a) and the sexual dimorphism of these flies was determined to be wild type (Figure 7b). Taken together, these results strongly suggest, as in mammals [42], a transcriptional control of HMGCR by the insulin signaling pathway in the ca. In addition, InR seems to control the sexual dimorphism through the HMGCR enzyme.

DISCUSSION

A specific physiological role for the HMGCR

We have shown, in Drosophila, by immuno-histological staining, that HMGCR is expressed in a variety of tissues, including the digestive tract, the brain and the ca. Moreover, this expression pattern appears to be temporally dynamic. Here, the genesis of the RNAi-HMGCR in the DI-3 and/or the DI-11 as drivers, this leads to a disruption of the sexual dimorphism in locomotor activity. To determine more precisely a functional link between these two proteins in relation to their respective roles in the control of sexual dimorphism, we expressed both hmgcr (using UAS-clb) and UAS-RNAi-InR under the control of the DI-3 driver. We found that InR was still undetectable whereas HMGCR was well present in the ca in 3 days old flies (Figure 7a) and the sexual dimorphism of these flies was determined to be wild type (Figure 7b). Taken together, these results strongly suggest, as in mammals [42], a transcriptional control of HMGCR by the insulin signaling pathway in the ca. In addition, InR seems to control the sexual dimorphism through the HMGCR enzyme.

Indeed, DI-3 drives RNAi-HMGCR in the ca in flies younger than 3 days old, and in this case, HMGCR was not detectable in ca. In parallel, the start/stop number was found to be the same for males and females. However, as expected, 5 days old DI-3/RNAi-HMGCR flies behave strictly as controls, since HMGCR is, de novo, expressed in the ca. Altogether, these results confirm that HMGCR expression in the ca is necessary for a sexually dimorphic start/stop number. In addition, RNAi effects are reversible: the same DI-3/RNAi-HMGCR flies were recorded at 2 and 5 days old. This reversibility indicates that the specific effects observed in adults after RNAi induction on locomotion are physiological rather than developmental consequences resulting from the lack of HMGCR.

Insulin pathway controls the sexual dimorphism in the corpus allatum

Previous studies have shown that mutations in Insulin Receptor (InR) decrease JH level [15,59], a hormone synthesized in the ca and implying the HMGCR enzyme. Moreover, we have shown, using a genetic approach, that InR is implicated in the control of the sexual dimorphism in locomotor activity [21]. Here, driving an RNAi-InR specifically in the ca abolishes the sexual dimorphism.
Similarly to the HMGCR, these results suggest that InR is required in this tissue to promote its effect on the sexual dimorphism. Furthermore, the reversibility of the effect observed with DI-3/UAS-RNAi-InR in 5 days old flies compared to 2 days old flies, tends to exclude an IGF-like effect of InR pathway on ca cells during development that could alter the functional capacity of ca. This is the first time that a "issue specific action" of insulin, not directly related to development or carbohydrate metabolism, is reported in Drosophila.

InR mutations or IPCs ablation lead to a diabetes like phenotype: trehalosemia is increased in both larvae and adults [8,13–15,21]. Interestingly, neither hunger mutant nor RNAi-HMGCR or RNAi-InR expressed in the ca increase haemolymph sugar level (data not shown). This reveals a new and very specific functional role for the insulin signaling pathway in the ca. Moreover, when driven by DI-3, RNAi-InR blocks hunger expression and this action is completely reversed in flies older than five days. Although we can not exclude an IGF like action of insulin in ca during development, inr mutations did not affect ca size [59] and the reversibility of RNAi effects in adult flies strongly suggest that the implication of the InR and HMGCR in such physiological processes, is dynamically active.

**Insulin pathway controls HMGCR expression in the corpus allatum**

A number of clues suggests an interaction between the insulin pathway and JH [15,21]. In mammalian hepatocytes, HMGCR has been shown to be partially under control of insulin [40]. In Drosophila, the HMGCR is the key step in JH biosynthesis, which is known to occur in the ca. We report here that specifically silencing the inr gene in ca, under the control of DI-3 or DI-11, leads to decreased HMGCR, which abolishes sexual dimorphism. Moreover, this phenotype can be rescued by directed expression of the hunger gene within the ca. Similarly to results found in mammalian hepatocytes, this strongly suggests that the insulin signaling pathway might control the expression of the hunger gene. In hepatocytes, insulin is reported to act on hunger transcription through a helix-loop-helix transcription factor called SREBP-1c (Sterol Response Element Binding Protein) [40]. Although three SREBP isoforms have been identified, it appears that only the SREBP-1c form responds to insulin. The two others are more sensitive to cholesterol, which inhibits hunger gene expression through a negative feedback loop. In Drosophila, only one SREBP homolog has been identified: inr-106 (dSREBP) [49,50]. In a recent study, dSREBP was found to be expressed in ca cells [49]. We used DI-3 and DI-11 lines to drive a dominant negative form of this gene: p[UAS-dsrebpDN] (UAS-dsrebpDN) in the ca and observe a lack of sexual dimorphism (Figure S1). Furthermore, when using DI-3 to drive the UAS-dsrebpDN cDNA gene, the sexual dimorphism was retrieved in flies older than 5 days. These results resemble those obtained after the disturbance of InR or HMGCR expression in the ca. SREBP may therefore be a molecular link between InR and HMGCR in ca (Figure 8).

**Body size depends of the hmgcr**

In Drosophila, the insulin signaling pathway regulates growth in accordance with the nutritional availability during development [7,16,26,27]. Disruption of various components of this pathway leads to delayed and small (dwarf) flies [6,8,12,16]. However, very little is known about the precise specificity of the insulin pathway’s action requirement. In fact, in contrast to mammals which display a wide variety of growth factors [60], the predominant role of insulin pathway in development points to an action occurring in all cells of the organism [16,17,26]. However, in some cases, some organs seem to be more or less affected compared to others [61], while a recent study showed dwarf and delayed larva in flies lacking SREBP in some specific tissues [49]. In certain temperature conditions, we observed delayed and small (dwarf) flies by disrupting InR and/or HMGCR expression or using dominant negative forms of SREBP specifically targeted in the ca. Taken together, these results strongly suggest a crucial role of these components (InR-HMGCR-SREBP), specifically in the ca for controlling body size. Although these components are linked to the insulin pathway, our results suggest a new mechanism, probably involving neuroendocrine control by JH, which would be independent of the IGF-like role of insulin occurring in all cells, to determine the body size.

**The sexual dimorphism in locomotor activity**

We showed that HMGCR and InR (as well as the first results obtained with SREBP), specifically within the ca, are implicated in the control of the sexual dimorphism. Moreover, we have not detected any sexual difference neither in InR, nor in HMGCR expression pattern or in the efficiency of inr and/or hunger RNAi silencing, suggesting that the sexual dimorphism in locomotor activity does not rely in the expression pattern of these genes. Previous studies have shown the implication of some PI neurons in the control of the sexual dimorphism, as those neurons have been feminized by the directed expression, in males, of the female version of the sexual determination factor transformer (tra), leading to name those cells “feminizing cells” (FCs) [26,29]. Moreover, we have shown that these cells act on the sexual dimorphism through a neuroendocrine factor, by regulating the synthesis of JH release, probably by acting on the ca’s cells. Here, in this study, we observed that the directed expression of tra in the IPCs does

![Figure 8. Model of the insulin pathway regulating the expression of the HMGCR in the corpus allatum. We hypothesize that the two different populations of cells, the Insulin Producing Cells (IPCs) and the feminising cells (FCs), located in the pars intercerebralis control, by two distinct ways, the JH synthesis/release by the corpus allatum. The informative way (in yellow), which could be feminised, might control the level or the timing of the JH release, while the permissive way (in green) might control or allow the presence of the molecular machinery, as the transcription of the HMGCR, to promote the JH synthesis. In magnification: in the corpus allatum, insulin binds to its receptor (InR), which activates SREBP. In turn, SREBP regulates the transcription of hmgcr gene.](doi:10.1371/journal.pone.0000187.g008)
not influence the sexual dimorphism (data not shown). Thus, it is likely that two independent mechanisms could control the sexual dimorphism. The first, composed by the FCs, might act on the cells of the ca and consequently on the JH. This could be qualified as the “informative” way. The second way composed by the IPCs, might act on the cells of the ca that express the InR and HMGCR components (Figure 8). This could be qualified as the “permissive” way that allow the HMGCR activity and consequently, the synthesis of the JH. In brief, the informative way may control the level of synthesis and/or the timing of JH release, while the permissive way could allow the synthesis of JH. This could suggest that the informative, but not the permissive way is tra-sensitive. Additionally, this could also suggest that the disruption of the permissive way abolishes the sexual dimorphism. The precise interaction between these two putative ways still remains to be thoroughly characterized.

In parallel, other studies have demonstrated that disrupting the insulin pathway increases longevity [13,15,62], while in some cases, it also disrupts the sexual dimorphism [21]. It will be relevant to determine if the inactivation of the expression of either the ins and hinger genes, specifically in the ca, using the RNAi technology [DI-11/RNAi-InR and DI-11/RNAi-HMGCR] could also increase longevity. In summary, we investigated the role of the HMGCR in adult flies in these studies and find a functionally conserved link between InR and HMGCR. This could lead to the establishment of a new model to study the molecular and physiological roles of the reductase in a relatively simple organism.

**MATERIALS AND METHODS**

**Flies stock**

All *Drosophila melanogaster* lines were maintained at 24°C on standard food medium. Wild-type Canton Special (CS) flies were used. p[GAL4]C316 was kindly provided by S. Waddell [58] and p[UAS-clb] by R. Lehmann [43]. *sreb*<sup>hyv</sup>, hmgcr<sup>1033</sup>, p[EN1];*w*<sup>1118</sup>h+, p[UAS-gfp], p[elav-GAL4], p[daughterless-GAL] and p[actin-GAL4] lines were obtained from Belgacem and Martin [21]. Seven-micrometer sections were blocked for one hour in normal horse serum (PBT0.05 and 1% Triton X-100). A primary anti-HMGCR antibody raised from the human form (1/20 in PBT0.05) was added, and incubated overnight at 4°C. After three washes in PBT0.05, a secondary antibody Cy3-labeled anti-rabbit (1/500 in PBT0.05; Amersham) was applied for one hour, at room temperature. After three washes in PBT0.05 and one in PBS, slices were mounted in DABKO and observed by fluorescence microscopy.

**Immunohistochemical techniques**

Adult *Drosophila* heads were fixed (Carnoy) for mass histology (paraffin section) or in 4% paraformaldehyde (PFA) (for cryostat section) as described in Belgacem and Martin [2006] [21]. Seven-micrometer sections were blocked for one hour in normal horse serum (PBT0.05: PBS+0.05% Triton X-100). A primary anti-HMGCR antibody raised from the human form (1/20 in PBT0.05) (rabbit polyclonal antibody) (a courtesy of P. Edwards [33]), was added, and incubated overnight at 4°C. After three washes in PBT0.05, a secondary antibody Cy3-labeled anti-rabbit (1/500 in PBT0.05; Amersham) was applied for one hour, at room temperature. After three washes in PBT0.05 and one in PBS, slices were mounted in DABKO and observed by fluorescence microscopy.

**Cardia and corpus cardiacum/corpus allatum**

Dissected in 4% PFA at 4°C, incubated 30 min and washed in PBS (4°C) and twice in PBT0.05. For double immunostaining (HMGCR and InR), a primary anti-HMGCR antibody (1/20 in PBT0.05) (rabbit polyclonal antibody) and a primary anti-InR antibody (1/20 in PBT0.05) (a mouse monoclonal antibody against the human InR z-subunit; Chemicon) were added, and incubated overnight at 4°C. The first antibodies were recognized by Cy3-labeled anti-rabbit secondary antibody (1/500 in PBT0.05; Amersham) and FITC-conjugated anti-mouse (1/500 in PBT0.05 Sigma) secondary antibodies for anti-GFP or anti-InR. After three washes in PBS, the first antibodies were recognized by Cy3-labeled anti-rabbit secondary antibody (1/500 in PBT0.05; Amersham) for anti-HMGCR, and FITC-conjugated anti-mouse (1/500 in PBT0.05 Sigma) secondary antibodies for anti-GFP or anti-InR. After three washes in PBT0.05 and one in PBS, slices were mounted in DABKO and observed by fluorescence microscopy.

For the double immunostaining (AKH and GFP), *cardia and corpus cardiacum/corpus allatum*, have been prepared as described above, except for antibodies. Primary antibodies, anti-HMGCR
(1/20 in PBT0.05) and anti-AKH labeled with rhodamine (1/800 in PBT0.05) [56] were added and, after washes (in PBT0.05), anti-
HMGR (Rabbit) were recognized by FITC-conjugated anti-
mouse (1/300 in PBT0.05) secondary antibody.

SUPPORTING INFORMATION

Figure S1 Targeted expression of a dominant negative form of SREBP in the corpus allatum abolishes the sexual dimorphism. Expressing p[UAS-srebpDN] in ac under the control of DI-11 abolishes the sexual dimorphism (b) comparing to appropriated controls p[UAS-srebpDN]/CS (a).

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Author Contributions

Conceived and designed the experiments: JM YB. Performed the experiments: YM YB. Analyzed the data: YM YB. Contributed reagents/materials/analysis tools: JM. Wrote the paper: YM YB.
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