The *Drosophila* insulin/IGF receptor controls growth and size by modulating PtdIns$P_3$ levels

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SUMMARY

Understanding the control of size is of fundamental biological and clinical importance. Insulin/IGF signaling during development controls growth and size, possibly by coordinating the activities of the Ras and PI 3-kinase signaling pathways. We show that in *Drosophila* mutating the consensus binding site for the Ras pathway adaptor Drk/Grb2 in Chico/IRS does not interfere with growth whereas mutating the binding sites of the PI 3-kinase adaptor p60 completely abrogates Chico function. Furthermore, we present biochemical and genetic evidence that loss of the homolog of the tumor suppressor gene, Pten, results in increased PtdIns$P_3$ levels and that these increased levels are sufficient to compensate for the complete loss of the Insulin/insulin-like growth factor receptor function. This reduction of Pten activity is also sufficient to vastly increase organism size. These results suggest that PtdIns$P_3$ is a second messenger for growth and that levels of PtdIns$P_3$ during development regulate organismal size.

Key words: Growth, PtdIns$P_3$, Pten, Cancer, Tumor suppressor, Insulin, PI 3 kinase

INTRODUCTION

In *Drosophila*, the homologs of the mammalian insulin receptor (IR)/insulin-like growth factor receptor (IGFR), InR, and insulin receptor substrates (IRS1-4), Chico, control size (via regulation of growth and proliferation), female fertility, and lipid metabolism during development (Fernandez et al., 1995; Chen et al., 1996; Böhni et al., 1999; Brogiolo et al., 2001). Similar to loss of Igfl or Irs1 function in mice, mutations in positive components of the *Drosophila* insulin pathway cause a dramatic reduction in size (Liu et al., 1993; Araki et al., 1994; Tamemoto et al., 1994; Stocker and Hafen 2002). Conversely, overexpression of one of the seven *Drosophila* insulin-like peptides (DILP2) results in flies with increased cell, organ, and body size (Brogiolo et al., 2001). Moreover, the increased total lipid levels and female sterility observed in Irs2-deleted and Neuronal Insulin Receptor Knock-Out (NIRKO) mice are also observed in chico mutant flies (Böhni et al., 1999; Bruning et al., 2000; Burks et al., 2000).

Thus, the *Drosophila* InR signaling pathway, in which these processes are combined into a single prototypical pathway consisting of components encoded by single genes, controls physiological processes reminiscent of both the mammalian IR and IGFR systems, thereby demonstrating evolutionary conservation of function (Oldham et al., 2000a; Garofalo, 2002).

The IR and IGFR act through IRS1-IRS4 proteins, which are multifunctional adaptors that link insulin and IGF signaling to the Ras/MAPK and phosphoinositide 3′-kinase (PI 3-kinase) signaling pathways (Yenush and White, 1997). The pleckstrin homology domain (PH) and phosphotyrosine binding domain (PTB) of the IRS proteins are believed to mediate binding to phosphoinositide phosphates and the juxtamembrane NPXY motif of IR/IGFR, respectively (Yenush et al., 1997). Drk is the *Drosophila* homolog of Grb2, an adaptor protein containing SH2 and SH3 domains. It has been suggested that Grb2 may, via its binding to IRS (Olivier et al., 1993), link insulin/IGF to the Ras/MAPK pathway and thereby control proliferation (Simon et al., 1993; Skolnik et al., 1993; Yenush et al., 1997). The *Drosophila* homolog of the SH2 domain containing p85 PI 3-kinase adaptor subunit, p60, binds Chico/IRS and thereby recruits the p110 catalytic subunit of PI 3-kinase [which converts phosphoinositol(4,5)P$_2$ (PtdIns(4,5)P$_2$) into phosphoinositol(3,4,5)P$_3$ (PtdIns(3,4,5)P$_3$)] to the plasma membrane (Levers et al., 1996; Yenush et al., 1997; Stambolic et al., 1998; Maehama and Dixon, 1999; Weinkove et al., 1999). The p110 PI 3-kinase belongs to the class I PI 3-kinases implicated in the metabolic effects of insulin (Vanhaesebroeck et al., 2001). The classical effectors that mediate the biological outcomes of insulin and IGF downstream of IRS have been divided into two functional branches: the Ras/MAPK proliferation pathway, and the PI 3-kinase metabolic, growth and survival pathway (Yenush et al., 1997; Vanhaesebroeck et al., 2001).

Hyperactivation of IGF signaling pathways is associated with a wide variety of tumors (Valentinis and Baserga, 2001). Components in each of these pathways have been implicated in the initiation and progression of a wide variety of human...
malignancies. Because both branches are strongly implicated in contributing to tumorigenesis, it is important to determine the relative contribution of each pathway to the processes of growth and proliferation, and thus tumorigenesis. We used two types of approaches to address the relative contribution of the two major effector branches to the processes of growth, proliferation and organismic size control during development. In the first, we analyzed the effects of amino acid substitutions in the different effector sites of the Chico protein. In the second approach, we analyzed the effects of modulating the activity of the PI 3-kinase pathway by genetically altering the levels of PtdInsP3 within the cells. The results obtained from both experimental strategies provide strong evidence that in Drosophila, the PI 3-kinase branch is necessary and sufficient to control InR-mediated growth and proliferation. Furthermore, the results suggest a pivotal role of cellular PtdInsP3 levels in the control of cell growth.

MATERIALS AND METHODS

Chico effector site mutations

The sites of the mutations in the chico coding region were chosen to substitute important conserved residues that have been determined by structural or biochemical studies carried out in mammalian IRS proteins (Yenush et al., 1997). The mutations were introduced using QuickChange (Stratagene) and transgenic lines were established according to the method of Böhní et al. (Böhní et al., 1999). The mutants were confirmed by PCR analysis and sequencing. The experiments were carried out with two independent insertion lines for each of the constructs tested.

Mutant analysis

Pten2L100 is a viable hypomorphic mutation located outside of the ORF. Pten2L117 is a strong mutation (5 basepair deletion causing a frameshift at amino acid position 165 resulting in the insertion of 35 new codons (AYKGPKRCNPIASICSVFFQTSLFKCSIFESKPK) before the new stop codon located in the catalytic domain) and behaves similar to a null mutation. These mutations fail to complement other previously identified mutations of Pten and the lethality is rescued by a Pten genomic rescue construct, which identifies them as alleles of Pten. Pten2L100 is a weak temperature-sensitive mutation. Heteroallelic mutants are pupal lethal, but when shifted from 25°C to 18°C during wandering 3rd instar stage allow for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs of Ras pathway hyperactivation were observed, such as multiple photoreceptors or extra wing veins. The weighing and metabolic alterations of Ras pathway hyperactivation were observed, such as multiple and over-enlarged legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, shifted from 25°C to 18°C during wandering 3rd instar stage allow for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs of Ras pathway hyperactivation were observed, such as multiple photoreceptors or extra wing veins. The weighing and metabolic alterations of Ras pathway hyperactivation were observed, such as multiple and over-enlarged legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs of Ras pathway hyperactivation were observed, such as multiple photoreceptors or extra wing veins. The weighing and metabolic alterations of Ras pathway hyperactivation were observed, such as multiple and over-enlarged legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs for viable heteroallelic escapers. Slightly rough eyes, concave wings, and over-elongated legs are occasionally observed. However, no signs for viable heteroallelic escapers.

Fertility and starvation assays

All analyses were performed in a y w background. The fertility assay was performed by mating 15 males and females of the genotype y w; chico1/chico2; P[w+ chicoPH1 or chicoPTB2 or chicoDrk2.1 or chico3DR or chicoDMD rescue construct]/+ and after 1 day of egg laying, the number of pupae was determined. The starvation assay was performed according to the methods of Böhní et al. (Böhní et al., 1999), except that Pten hetereoallelic mutant flies were raised at 18°C.

Metabolic labeling

Briefly, 3rd instar non-wandering larvae were phosphate starved in phosphate-free Schneider S2 medium and then labeled with 2 mCi/sample inorganic 32P (50 mCi/ml; NEN Life Science Products). PtdInsP3 and PtdInsP2 levels were determined as described previously (Arcaro and Wymann, 1993).

RESULTS

To analyze the role of the different domains of Chico/IRS under physiological conditions, a panel of effector site mutants was created in a genomic rescue construct for chico that disrupts the PH or the PTB domains or the putative binding sites of Drk/Grb2 and p60 (Böhní et al., 1999) (Fig. 1A). The constructs include the cis-regulatory sequences that permit expression of chico in its normal spatial and temporal pattern. The wild-type chico construct fully restores the defects of chico homozygous null mutants (Böhní et al., 1999). In this manner, the effector site mutants were assayed for the ability to rescue the three different phenotypes associated with complete loss of Chico function: body size reduction, female sterility and lipid alterations. The Drk/Grb2 consensus binding site mutant was able to fully rescue the reduced weight to the same extent as the wild-type rescue construct (Fig. 1B). Therefore, the presence of a functional Drk binding site in Chico and thus the link to the activation of the Ras/MAPK kinase pathway is not required for its wild-type function. In contrast, the PH and PTB domain mutants and the double p60 PI 3-kinase binding site mutant were unable to rescue the reduced body weight (Fig. 1B). The latter result is surprising because InR contains additional functional PI 3-kinase binding sites in its C-terminal

Fig. 1. Chico PH, PTB, Drk, and PI 3-kinase effector mutants. (A) The chico genomic rescue construct and the corresponding PH, PTB, Drk, and PI 3-kinase effector consensus site mutants. Numbers indicate amino acid positions (not drawn to scale). (B) The PH, PTB, Drk, and PI 3-kinase effector consensus site mutants. Numbers indicate amino acid positions (not drawn to scale). (C) The PTB domain is not required for Chico control of female fertility. The number of pupae that developed from eggs laid by an equal number of females of the indicated genotype is shown. (D) All the Chico effectors, except Drk, function in the control of lipid metabolism. All assays were performed three times on at least 10 flies.
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PTEN negatively regulates organismal growth and size (Yenush et al., 1996; Böhni et al., 1999), an extension shared only with the C. elegans InR homolog, Daf-2, and not the mammalian IR or IGFR (Kimura et al., 1997). This suggests that the presence of additional p60 binding sites in the InR C-terminal tail is not sufficient in vivo to mediate wild-type levels of growth and proliferation in the absence of the Chico p60 PI 3-kinase binding sites and that the InR C-terminal tail may contribute only low levels of PI 3-kinase signaling (Fernandez et al., 1995; Chen et al., 1996; Yenush et al., 1996). Although the PTB domain mutant failed to restore normal body weight, it rescued the female sterility associated with the loss of Chico function (Fig. 1C). With the exception of the full rescue of the lipid accumulation observed in Drk/Grb2 mutant, all the other effectors only partially restored the change in lipid accumulation (Fig. 1D).

The inability of the p60 binding site mutant to rescue the size defect indicates that the Chico PI 3-kinase docking sites are necessary for InR/Chico (insulin/IGF) action in size control. However, the issue of whether recruitment of PI 3-kinase to Chico is sufficient to mediate the attainment of wild-type body size is unresolved. It has been reported that overexpression of PI 3-kinase and Akt in Drosophila is sufficient for increased growth but not proliferation (Verdu et al., 1999; Weinkove et al., 1999). Loss of zygotic InR function results in embryonic lethality with some small arrested larvae, but loss of zygotic Chico function results in viable small flies (Fernandez et al., 1995; Chen et al., 1996; Böhni et al., 1999; Brogiolo et al., 2001). Two parsimonious hypotheses could explain this difference. (1) InR activates not only the PI 3-kinase pathway but also another, Chico-independent, signal transduction pathway, or (2) InR signals predominantly through PI 3-kinase, but loss of Chico does not block PI 3-kinase activation completely because of direct interaction of p60 with the InR C-terminal tail. This provides residual PI 3-kinase activation sufficient to rescue viability, but not wild-type size. If the latter hypothesis were true, then increasing PtdInsP3 levels should be sufficient to rescue loss of InR function.

To test whether increasing PtdInsP3 levels in an InR or PI 3-kinase p110 mutant background is sufficient to restore growth, we eliminated the function of a negative regulator of the insulin pathway. The 3′-phosphoinositol-specific lipid phosphatase, PTEN acts as a negative regulator of the PI 3-
kinase pathway by converting PtdIns$_3$ generated by PI 3-kinase into PtdIns$_2$ (Stambolic et al., 1998; Maehama et al., 1999). We utilized a null (Pten$^{2L117}$) and a hypomorphic (Pten$^{2L100}$) allele of Pten which were identified in a screen for genes involved in growth control (Oldham et al., 2000b). As shown by HPLC analysis of the phospholipids in extracts of Pten mutant larvae, the loss of PTEN function results in a 2-fold increase in PtdIns$_3$ levels (Fig. 2A). This is consistent with the increase in PtdIns$_3$ seen in Pten-deleted murine fibroblasts (Stambolic et al., 1998). One prominent biological effect of these increased PtdIns$_3$ levels in Drosophila is a substantial increase in size in both larvae and pupae (Fig. 2B).

To test whether loss of PTEN function, and consequently increased PtdIns$_3$ levels, is sufficient to restore growth or viability in InR null mutants, we first generated InR and Pten double mutants by creating mosaic animals using the eyeless-Flipase (eyFlp) tissue-specific recombination system (Newsome et al., 2000). In such animals, the head consists of homozygous mutant tissue, whereas the rest of the body is heterozygous for the same mutation. While loss of PTEN function (Pten$^{2L117}$) in the head results in a fly with a disproportionately larger head (with more and larger cells), loss of InR function (InR$^{327}$) results in flies with smaller heads (pinhead) compared to the wild type (Fig. 2C,D,E, Fig. 3A). Heads doubly mutant for Pten$^{2L117}$ and InR$^{327}$, however, are almost the size of heads singly mutant for Pten$^{2L117}$ (Fig. 2F, Fig. 3A). Secondly, two different lethal heteroallelic InR combinations (InR$^{304}$/InR$^{327}$ or InR$^{304}$/InR$^{25}$) (Fernandez et al., 1995; Chen et al., 1996), which arrest at the 2nd larval instar stage, develop to the pupal stage (15-17% of 33% expected) and even to pharate adults in the presence of reduced PTEN levels (Pten$^{2L117}$/Pten$^{2L100}$) (Fig. 2G,H). These results demonstrate that complete loss of PTEN function can largely substitute for InR-mediated growth and proliferation in the absence of InR function and that the Ras/MAPK pathway plays little or no role in the InR mediated control of cell growth. This notion is further supported by the observation that complete loss of InR function in the compound eye does not result in a loss of photoreceptors, a hallmark of loss of Ras pathway function (Brogiolo et al., 2001).

As increasing PtdIns$_3$ levels can rescue loss of InR function, these results suggest that the level of PtdIns$_3$ may be critical in determining the amount of growth. We explored this possibility by examining genetic interactions between Pten and PI 3-kinase p60 and p110. The lethality associated with the complete loss of PI 3-kinase p110 function, cannot be rescued by Pten$^{2L117}$/Pten$^{2L100}$ (data not shown). It is possible that without any PI 3-kinase p110 function, PTEN function becomes obsolete. In order to test this possibility, double mosaic clones were generated with the strong loss of function Pten$^{2L117}$ allele and a null mutation for PI 3-kinase p110 or its p60 adaptor (Weinkove et al., 1999). As shown in Fig. 3A,C,D, loss of PTEN function (Pten$^{2L117}$) is unable to rescue the pinhead phenotype caused by loss of PI 3-kinase p110 function. Clones that are doubly mutant for PI 3-kinase p60 and Pten$^{2L117}$, however, are of wild-type size (Fig. 3A,B,E,F). In the absence of PI 3-kinase p60 function, PI 3-kinase p110 might have residual activity as suggested by the weaker phenotype of the PI 3-kinase p60 null mutant (Weinkove et al., 1999). Indeed, flies doubly mutant for PI 3-kinase p60 and Pten$^{2L117}$/Pten$^{2L100}$ flies are viable (data not shown). These data provide strong genetic support for the close relationship between PTEN and PI 3-kinase and indicate that the
intracellular levels of PtdInsP_3 define the amount of cellular growth.

Because loss of Chico function results in increased lipid levels as well as a dramatic decrease in body size (Böhní et al., 1999), we determined whether PTEN might also have a function in metabolic and body size control like Chico. As seen in Fig. 4A,B, partial loss of PTEN function results in flies that are considerably bigger than controls. They weigh approximately 50 percent more than their heterozygous siblings without showing any apparent effect on cell differentiation. The increase in size is due to an increase in both cell size and number as determined by a morphometric analysis of the wing and eye (data not shown). When the levels of lipids and glycogen were measured, a decrease per mass in lipid and glycogen was observed compared to Pten mutant flies rescued by a genomic Pten transgene (Fig. 4C). One biological outcome of this difference is a twofold increase in the rate of mortality under water-only starvation conditions compared to a 2-fold decrease in chico mutant flies (Fig. 4D). Thus lipid and glycogen levels strongly correlate with the survival time under starvation conditions. Since Chico and PTEN activity regulates growth during development and the accumulation of energy stores in the adult, the effects of InR-mediated growth and lipid/glycogen metabolism must diverge downstream of Pten.

DISCUSSION

In this paper, we have used two approaches to investigate, in Drosophila, the relative contributions of the two main effector pathways activated in response of IR/IGF receptor stimulation, the Ras/MAPK and the PI 3-kinase pathway. First, by mutating the consensus binding sites for the two pathway adaptors, Grb2/Drk and p85/p60, in Chico/IRS we showed that the growth promoting function of Chico is dependent on the p60/p85 binding sites but not on the Drk binding site. These results suggest that recruiting PI 3-kinase but not Drk to Chico is essential for cell and organismal growth. Second, we demonstrate that the growth deficit associated with the loss of InR function is fully compensated by loss of PTEN function. This suggests that the levels of PtdInsP_3 in the cell control the amount of cellular growth. Finally, we show that partial loss of PTEN function increases body size and decreases lipid and glycogen stores in the adult suggesting that the levels of PtdInsP_3 also control metabolism in the adult.

Viable allelic combinations of insulin receptor pathway components result in at least three characteristic phenotypes: small body size, female sterility and increased lipid content (Chen et al., 1996; Böhní et al., 1999). The results from the chico effector mutants permit the separation of the three different Chico phenotypes.

(1) Size and fertility. Is there a causal link between the small body size and female sterility? The PTB domain mutant rescues the sterility, but not the size defect, thus separating the growth and the sterility phenotypes. It remains to be resolved whether different levels of PI 3-kinase activation are needed to restore growth and fertility or whether control of fertility involves at least in part the association of Chico with a different receptor which does not require the PTB domain. For the growth regulatory function of Chico, a functional PTB and PH domain are essential. This indicates that in vivo, in the absence of overexpression, these two domains serve non-redundant functions presumably in the localization to the membrane and binding to the insulin/IGF receptor.

(2) Size and lipids. Does the small size cause the increased lipid levels? Chico mutant flies lacking functional p60/PI 3-kinase binding sites, PTB or PH domains are all small, yet the increase in lipid levels is less pronounced than in chico null mutant flies. Also, the Irs2-deleted mice and insulin pathway

Fig. 4. Pten regulates size, metabolism and survival in an opposite manner to positive components of the insulin pathway like Chico. (A) A Pten^{2L117/Pten^{2L100}} mutant fly (right) compared to the y w control (left). (B) Pten^{2L117/Pten^{2L100}} mutant flies weigh more than the y w control (data not shown) or to Pten mutants rescued with the Pten genomic rescue construct (n=10). (C) Pten^{2L117/Pten^{2L100}} mutant flies have decreased glycogen and lipids compared to the y w control (data not shown) or to Pten mutants rescued with the Pten genomic rescue construct (n=10). The glycogen data are transformed by one log10 power. (D) Pten^{2L117/Pten^{2L100}} mutant flies are hypersensitive to water-only starvation conditions compared to a starvation resistant chico^+/chico^+ mutant. All assays were performed at least 2 times independently.
mutants in *C. elegans* are not small, yet display increased lipids (Kimura et al., 1997; Burks et al., 2000). Therefore, there seems to be no direct correlation between developmental growth and energy stores in the adult.

3. Lipids and sterility. Like *chico* flies, mice mutant for IRS2 or lacking insulin receptor function in the brain (NIRKO) display increased lipids and are female sterile. Are the increased lipid levels a sign of metabolic dysfunction that leads to the female sterility? The *chico* PI 3-kinase, PTB and PH effector mutants have similar lipid increases, yet the PTB mutant is fertile while the PI 3-kinase mutant is not. Therefore, there appears to be no direct correlation between lipid accumulation and sterility in *Drosophila*.

The rescue of lethal, null InR mutant combinations to near viability by reducing PTEN activity strengthens the argument that a PtdInsP3-dependent signaling pathway is the primary effector for InR-derived growth and proliferation. In support of this observation, PI 3-kinase and Akt have been isolated as retroviral oncogenes, suggesting that activation of PI 3-kinase and Akt is sufficient to mediate growth, proliferation, and oncogenesis in vertebrate systems (Bellacosa et al., 1991; Chang et al., 1997). In *Drosophila* and mammals, overexpression of PI 3-kinase causes increased growth; but this is not sufficient for proliferation as is removal of *Pten* (Klippel et al., 1998; Goberdhan et al., 1999; Huang et al., 1999; Weinkove et al., 1999; Gao et al., 2000). From this premise, it has been proposed that PI 3-kinase and PTEN regulate similar yet distinct pathways (Gao et al., 2000). Alternatively, it is possible that they do function uniquely in the same pathway and that the difference may be due to altered location and function because of overexpression, or to differential feedback of PI 3-kinase versus PTEN. For example, as PI 3-kinase has been shown to act as a serine/threonine protein kinase on IRS, this may have a negative feedback effect on the insulin pathway that might not be evident in *Pten* loss-of-function mutations (Pirola et al., 2001). Nevertheless, PI 3-kinase is absolutely critical in controlling size because using an allelic series of PI 3-kinase mutants in combination with the ey-Flp system resulted in a range of different head sizes (data not shown). Furthermore, expressing an activated and dominant-negative form of PI 3-kinase in *Drosophila* imaginal discs or the heart of the mouse also leads to a corresponding increase or decrease in cell and organ size (Weinkove et al., 1999; Shioi et al., 2000). Thus, the PI 3-kinase/PTEN cycle can be considered a dedicated growth rheostat, and the InR pathway is an evolutionary conserved module for regulating the range of growth and size.

Loss of PTEN function results in a metabolically similar phenotype to loss of murine *Ptp1b* (*Ptpn1*), an IR-specific tyrosine phosphatase, in that hyperactivation of the IR pathway causes resistance to high-fat-diet-induced obesity because of increased basal metabolism (Elchebly et al., 1999; Klaman et al., 2000). These metabolic lipid effects have likely been conserved during evolution because the increased lipid levels in *chico* mutants are reminiscent of the enhanced lipid content in *Irs2* deleted and NIRKO mice (Bruning et al., 2000; Burks et al., 2000).

Collectively, these data firmly establish *Drosophila* as a valid model organism for the study of metabolic diseases like diabetes and obesity as well as for the study of growth disorders like cancer. *Pten* mutant flies are larger in size due to increased cell size and number, but have a corresponding decrease in energy stores, a situation completely opposite to mutations in positive components of the insulin signaling pathway like *InR, chico, PI 3-kinase*, and *Akt*. These large viable *Pten* mutants show that a reduction of PTEN function is sufficient for increased organism size. This fact suggests that the four-fold size difference between viable *InR* and *Pten* mutants can simply be controlled by the amount of PtdInsP3 and this phenomenon may possibly be extended to vertebrate size regulation. Thus, in *Drosophila*, the InR/PI 3-kinase/PTEN pathway combines both metabolism and growth control into one pathway that later diverged into two separate, yet interacting systems in mammals.

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