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WAKE-mediated modulation of cVA perception via a hierarchical neuro-endocrine axis in *Drosophila* male-male courtship behaviour

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The nervous and endocrine systems coordinate with each other to closely influence physiological and behavioural responses in animals. Here we show that WAKE (encoded by *wide awake*, also known as *wake*) modulates membrane levels of $GABA_A$ receptor Resistance to Dieldrin (Rdl), in insulin-producing cells of adult male *Drosophila melanogaster*. This results in changes to secretion of insulin-like peptides which is associated with changes in juvenile hormone biosynthesis in the corpus allatum, which in turn leads to a decrease in 20-hydroxyecdysone levels. A reduction in ecdysone signalling changes neural architecture and lowers the perception of the male-specific sex pheromone 11-cis-vaccenyl acetate by odorant receptor 67d olfactory neurons. These finding explain why WAKE-deficient in *Drosophila* elicits significant male-male courtship behaviour.

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Olfaction is one of the primary sensory modality for chemical senses. The brain can detect and decode many external olfactory chemical cues for eliciting suitable behavioural responses. Therefore, many insects rely on various environmental and individually-derived olfactory chemical signals for mating decisions[1–3]. Variability in olfactory perception, associated with many factors such as genetics, age, sex, and nutritional status affects courtship behaviour[1–4]. Similar to all heterotrophic organisms, insects acquire nutrients that are essential for normal growth, development, and physiological maintenance from their food. However, evidence has shown that the nutrients could play a key role in modulating olfactory sensitivity including the adjustment of feeding behaviour according to metabolic demand[5–7].

The nutrient signalling through the insulin/insulin-like growth factor 1 (IGF-1) pathways also serves to regulate physiological decisions associated with reproduction, growth, and ageing[8,9]. Growing evidence shows that the nutrient dependent insulin signalling pathway is crucial for female reproductive maturity and is associated with improved fertility in males[10]. Although insulin plays a major role in directly enhancing reproductive efficiency[11,12], numerous other endocrine hormones affected by insulin signalling also play crucial roles in regulating reproduction. For example, the ecdysteroid hormone 20-hydroxyecdysone (20E, also known as ecdysone), coordinates with the sesquiterpenoid juvenile hormone (JH) and gonadotropic hormones to exert antagonistic effects on the regulation of metamorphosis and fertility[13,14]. JH and ecdysone signalling have also been demonstrated to regulate the courtship behaviour of several insects[15], including *Drosophila*[16,17], and influence male-male courtship behaviour in the case of ecdysone regulation[16,18,19].

The *Drosophila melanogaster* gene, *wide awake* (Gene ID: 42676), an ortholog of human *ankfn1* and mouse *nmf9*, is known to be involved in the regulation of sleep by pigment-dispersing factor (PDF) expressing neurons[20]. GABAergic sleep-promoting neurons suppress the firing of both large and small ventral lateral neurons (l-LNvs and s-LNvs) expressing PDF through the $GABA_A$ receptor resistant to dieldrin (Rdl) to control sleep onset[21,22]. Evidence indicates that WAKE interacts with Rdl to upregulate its levels and promote its localisation to the plasma membrane in l-LNvs, resulting in increased GABA sensitivity and decreased excitability, thereby promoting sleep at dusk[20,23]. In addition to circadian imbalances, mice with *nmf9* mutations exhibit vestibular function deficits and reduced fear conditioning[24]. Apart from pleiotropic physiological functions, there is an indication that WAKE-related neuronal functions are highly conserved across different species. As previous reported that WAKE contributes to up-regulating Rdl levels and promotes the localisation of RDL to the plasma membrane, to effectively maintain the neuron's sensitivity to GABA[20,25]. This study highlights the role of WAKE, as the hierarchical responsive master, through the insulin/insulin-like growth factor signalling (IIS) by modulating Rdl in insulin producing cells (IPCs) in the *Drosophila* brain, which produces three insulin-like peptides (Ilps)–Ilp2, 3 and 5. Further stabilisation of other endocrine hormones, JH and ecdysone, inhibits male-male courtship behaviour. The severe hormone imbalance caused by WAKE deficiency eventually decreases ecdysone signalling in Or67d olfactory sensory neurons (OSNs), in turn affecting their neural architecture and responsiveness to 11-cis-vaccenyl acetate (cVA). Physiological and behavioural responses often depend on endocrine hormone regulation of complex networks that affect neuronal biological processing[26–29]. This cascade may modulate *Drosophila* male-male courtship behaviour in the *wake* mutant. Here we proposed an interesting model whereby WAKE in IPCs, via a long course involving multiple molecular interactions, wherein imbalances in intricate neuro-endocrine networks affect specific nerve cells, which ultimately result male-male courtship behavioural responses.

Results

WAKE in the adult *Drosophila* nervous system modulates male-male courtship behaviour. Increased male-male courtship chaining behaviour was observed in culture vials containing the *UAS-Mob2.eCFP* (BDSC #32099) stock from the Bloomington collection. Inverse PCR analysis was used to identify an insertion mutant in the *wake* (see Supplementary Fig. 1) in this line, which was designated as $wake^{32099}$. To verify that *wake* mutations are associated with male-male courtship behaviour in *Drosophila*, we analysed two other *wake* lines (i.e., $wake^{NP3168}$ and $wake^{GS17103}$) with insertion sites near the $wake^{32099}$ insertion site (Fig. 1a). We observed male-male courtship behaviour in these lines but not in wild-type flies with an identical genetic background as quantified by a Courtship Index (CI) (Fig. 1b; test group no. 1–4; Supplementary Movie 1). Additionally, while $wake^{32099}$ flies exhibited significant male-male chaining behaviour when compared with heterozygous controls, as quantified by a Chaining Index (Fig. 1c; test group no. 1 vs. 2; Supplementary Movie 2), this behaviour was not observed in $wake^{GS17103}$ and $wake^{NP3168}$ males (Fig. 1c; test group no. 3 and 4). However, when these two mutant lines were crossed with $wake^{32099}$ to generate transheterozygous flies, the chaining behaviour was observed (Fig. 1c; test group no. 5 vs. 2 or no. 5 vs. 3; test group no. 6 vs. 2 or no. 6 vs. 4; Supplementary Movie 3).

In the *P*-element inserted $wake^{32099}$, the excision line (namely revertant #13-1; $revrt^{13\text{-}1}$) was identified as a precise revertant line using nucleic acid sequencing (see Supplementary Fig. 2) and further used as a genetic control for the $wake^{32099}$ mutant. Subsequent analysis of this revertant line exhibited that male-male courtship behaviour was significantly reduced (pair test, Fig. 1b, test group no. 2 vs. 5; chaining test, Fig. 1c, test group no. 1 vs. 7).

The *D. melanogaster* WT (2U) strain used for the pairs in this study is a w^{1118} (isoCJ1) Canton-S derivative[30]. We aimed to determine whether *wake*-induced male-male courtship behaviour was associated with a preference for specific genetic backgrounds. Further studies indicated that $wake^{32099}$ flies exhibited male-male courtship behaviour toward the different genetic backgrounds despite white/red eye colouring (see Supplementary Fig. 3). To further verify the association between WAKE and male-male courtship behaviour, we downregulated *wake* using double-stranded RNA interference ($wake^{RNAi}$). Three *UAS-*$wake^{RNAi}$ lines (*UAS-*$wake^{RNAi}$ −1, −2 and −3), based on independent constructs, were obtained from the Vienna *Drosophila* Resource Centre (VDRC), and different small dsRNA fragments were expressed to target various regions of the *wake* transcript (Fig. 2a), making it relatively easy to clarify and rule out the off-target RNAi effects. Following adult eclosion, expression of *wake* dsRNA was induced in most cells by feeding flies RU486 (*actin-GeneSwitch>UAS-*$wake^{RNAi}$) (Fig. 2b1), and differing levels of male-male courtship behaviour were observed in induced males versus corresponding controls (pair test, Fig. 2c, test group no. 1–7, test group no. 3 vs. 1, test group no. 3 vs. 2, test group no. 5 vs. 1, test group no. 5 vs. 4, test group no. 7 vs. 1, test group no. 7 vs. 6; chaining test, Fig. 2d, test group no. 3 vs. 1, test group no. 3 vs. 2).

WAKE expressed in PDF neurons related to regulating sleep behaviour[20]. In addition, the mouse ortholog *Nmf9* is broadly expressed in the inner ear, amygdala, and suprachiasmatic nuclei[24]. Therefore, WAKE may be closely linked to the function of specific nerves. To further examine the roles of WAKE in the

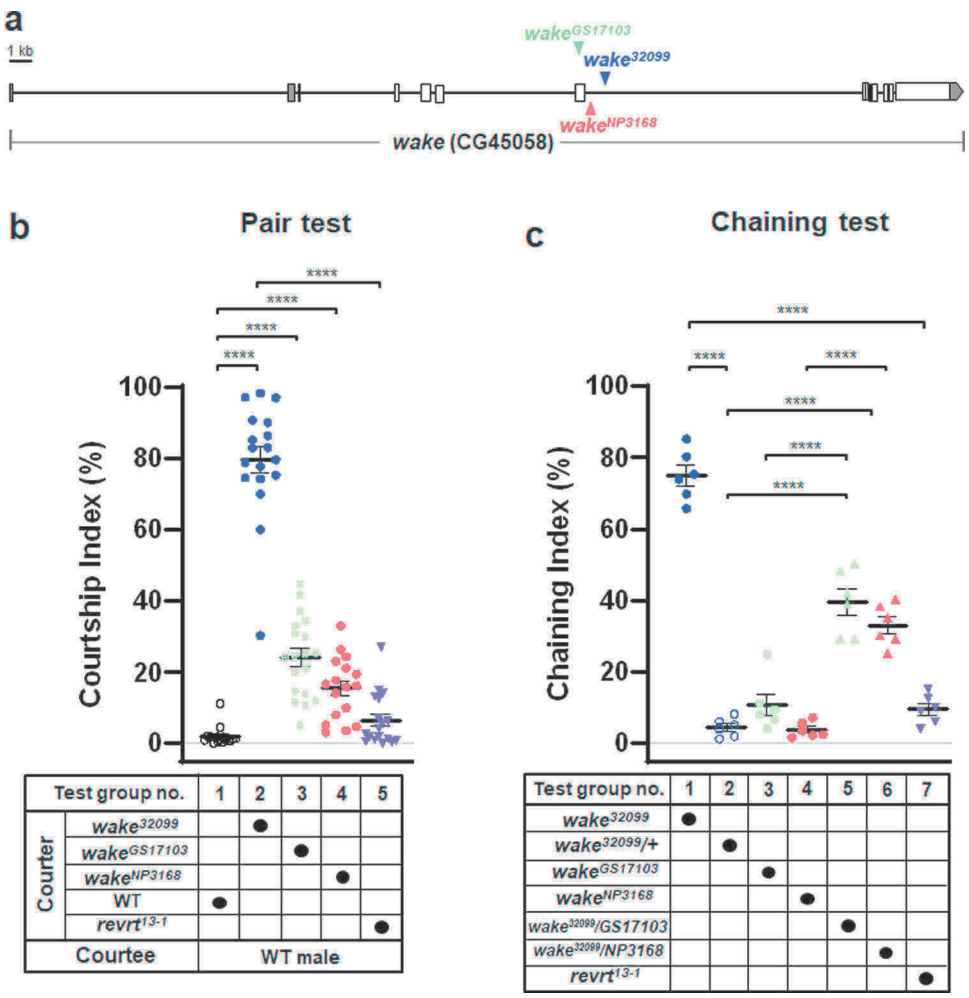


Fig. 1 *wake* influences male-male courtship behaviour. The courtship and chaining indices were defined as the percentages of the 10-min observation period in which the corresponding male-male courtship behaviour was observed. **a** Locations of the insertions in the different *wake* mutants ($wake^{32099}$, $wake^{GS17103}$, and $wake^{NP3168}$) are indicated by differently coloured arrowheads. **b** The *wake* insertion lines have higher male-male courtship behaviour indices than the wild-type (WT) or the $revrt^{13-1}$ precise $wake^{32099}$ transgene excision control lines. $n = 18, 18, 19, 17$ and 18 (from left to right) for each test, $^{****}p < 0.0001$, two-tailed Mann–Whitney U-test. **c** Males homozygous for $wake^{32099}$ also exhibited a higher level of male-male chaining behaviour than both $wake^{32099}$ heterozygous ($wake^{32099}/+$) and $revrt^{13-1}$ males. In addition, males homozygous for $wake^{GS17103}$ or $wake^{NP3168}$ exhibited a very low level of chaining behaviour, which was significantly enhanced in $wake^{32099}/wake^{GS17103}$ and $wake^{32099}/wake^{NP3168}$ transheterozygotes. $n = 6$ for each. $p < 0.0001$, one-way ANOVA. $^{****}p < 0.0001$, *post hoc* Tukey's multiple comparisons test. Scatterplots show error bars (± SEM) for all data points. Source data are provided as a Source Data file.

nervous system, the TARGET system[31] was used for pan-neuronal spatiotemporal knock-down WAKE by increasing the temperature after adult eclosion (*elav-Gal4;tub-Gal80^{ts}>UAS-wake^{RNAi}*) (Fig. 2b2). Both pair tests (Fig. 2c, test group no. 8–15; test group no. 10 vs. 8, test group no. 10 vs. 9, and test group no. 13 vs. 11, test group no. 13 vs. 12, test group no. 15 vs. 11, test group no. 15 vs. 14) and chaining tests (Fig. 2d; test group no. 6 vs. 4, test group no. 6 vs. 5) demonstrated that WAKE in the adult nervous system is involved in the inhibition of male-male courtship behaviour. qPCR characterization of individual *UAS-wake^{RNAi}* lines revealed an effective reduction in relative *wake* RNA levels to Gal4-driver alone (see Supplementary Fig. 4).

WAKE in IPCs modulates male-male courtship behaviour in *Drosophila*. Apart from the $wake^{NP3168}$ mutant described above, we also obtained two Gal4 enhancer trap lines (i.e., $wake^{NP3624}$ and $wake^{NP1350}$) adjacent to the insertion site of $wake^{32099}$ and generated a *wake-Gal4* line (i.e., $wake^{15185}$*-Gal4*) (Fig. 3a) by inserting the *Gal4* gene after the start codon of isoform-*wakeRG* using CRISPR/Cas9 technology (see Supplementary Fig. 5). A cluster of median neurosecretory cells (MNCs) (arrowheads in Fig. 3b–e) were observed in fluorescence images of intact central brains (Fig. 3b–e) and ventral nerve cords (VNC) in adult male flies (see Supplementary Fig. 6a–d) expressing mCD8::GFP driven by these drivers. Additionally, immunolabelling with an anti-Ilp2 antibody was used to verify the expression patterns of these Gal4 drivers and demonstrate that the MNCs included Ilps-expressing IPCs (Fig. 3f–i). Here we use another strategy involving a Gal4- and LexA-based intersectional genetic approach by a combination of the four *wake*-related Gal4 drivers and IPCs-specific *ilp2-LexA* driver (Fig. 3j). Then, *UAS-myr::SNAP* is expressed by GAL4 only in IPCs that are located in the regions targeted by *wake*-related Gal4 and *ilp2-LexA* (Fig. 3k–n). Further, we hypothesized that male-male courtship behaviour might be modulated by WAKE in IPCs. To verify this, we collected an RU486-inducible IPCs-specific driver (*ilp2-GeneSwitch*; Fig. 4a) and validated a cluster of 14 Ilp2-positive IPCs in the pars intercerebralis using anti-Ilp2 staining (Fig. 4b). For further study, these *wake*-related Gal4 and *ilp2-GeneSwitch* drivers were

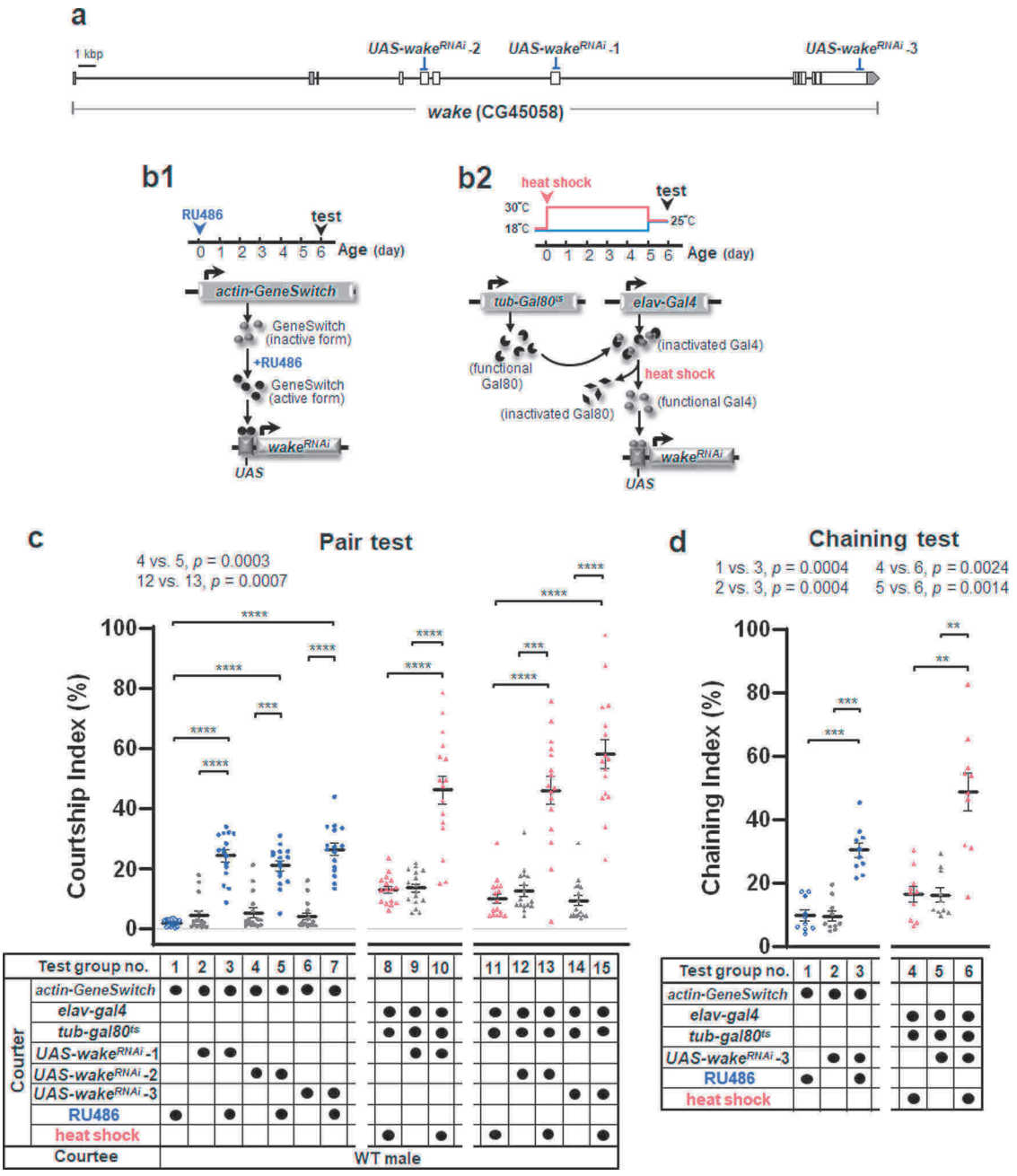


Fig. 2 Downregulation of WAKE in early adulthood prompts male-male courtship behaviour. a The *wake* transcript target locations of *wake* dsRNA (*wake*^{RNAi}) were elicited using the corresponding *UAS-wake* dsRNA stocks (*UAS-wake*^{RNAi}−1, −2, and −3). **b1** Schematic representation of ubiquitous adult-onset activation of *wake*^{RNAi} expression using the *actin-GeneSwitch* and addition of the drug RU486 to food from eclosion to day 5. **b2** Schematic representation of the TARGET system. The pan-neuronal *elav-Gal4* driver was combined with ubiquitously expressed GAL80^{ts} to control the transcriptional activity of GAL4 and drive *wake*^{RNAi} expression in neurons upon heat induction after eclosion for 5 d. The strength of male-male courtship behaviour was tested at the 6-day time point in both regimens. **c** The scatterplots for the courtship indices include ± SEM for all data points, $n = 16$ for test group no. 1–7 and 11–15, $p < 0.0001$, Kruskal–Wallis test. $^{***}p < 0.005$ and $^{****}p < 0.0001$, *post hoc* Dunn's multiple comparisons test. $n = 16$ for test group no. 8–10, $p < 0.0001$, one-way ANOVA. $^{****}p < 0.0001$, *post hoc* Tukey's multiple comparisons test. **d** The chaining indices include ± SEM for all data points, $n = 10$ for each, $p < 0.0001$, Kruskal–Wallis test. $^{**}p < 0.01$, and $^{***}p < 0.005$, *post hoc* Dunn's multiple comparisons test. Source data are provided as a Source Data file.

subsequently used to express *wake* dsRNA for WAKE down-regulation and courtship behaviour assay. WAKE downregulation under the four *wake*-related Gal4 drivers, was associated with courtship behaviour in experimental flies when compared with control flies in both pair tests (Fig. 4c; test group no. 1–9; test group no. 2 vs. 1 or 9; test group no. 4 vs. 3 or 9; test group no. 6 vs. 5 or 9; test group no. 8 vs. 7 or 9) and chaining tests (Fig. 4d; test group no. 15 vs. 13 or 14). Notably, when WAKE down-regulation was restricted to the IPCs of adults after eclosion, male-male courtship behaviour also occurred in RU486-treated flies when compared with the corresponding controls (pair tests, Fig. 4c; test group no. 12 vs. 10; test group no. 12 vs. 11 and chaining tests, Fig. 4d; test group no. 18 vs. 16 or 17). Over-expressing isoform WAKE-RG in IPCs on the WAKE-deficiency

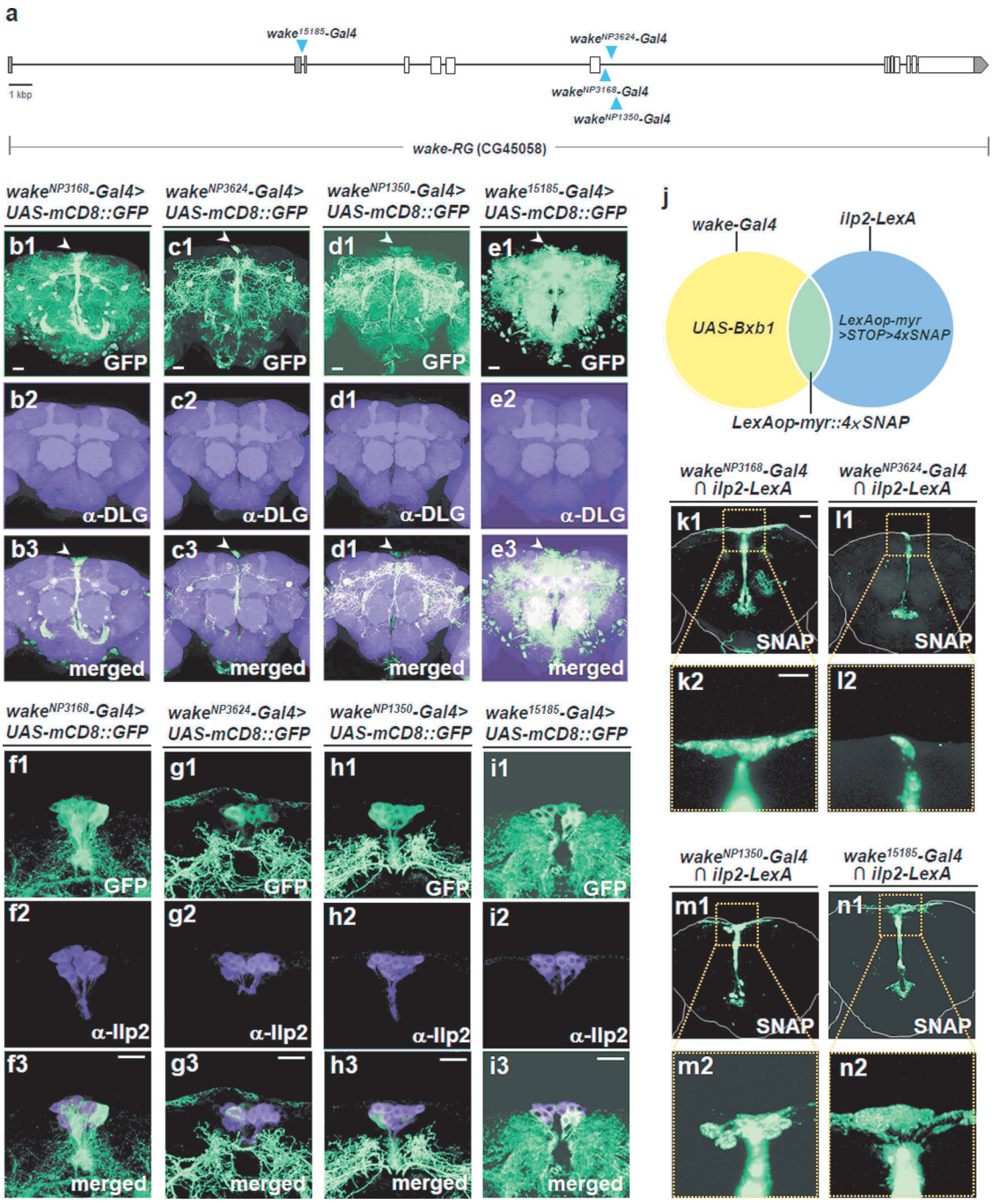


Fig. 3 Identification of *wake-Gal4* drivers expressed in insulin-producing cells (IPCs) of the adult male brain. a *Gal4* transgene insertion locations on the *wake* gene in the different enhancer trap NP lines (*wake*^{NP3168}, *wake*^{NP1350}, and *wake*^{NP3624}) and the *wake*^{15185}*-Gal4* line generated using the CRISPR/Cas9 system are indicated using blue arrowheads. **b**–**e** Patterns of **b** *wake*^{NP3168}, **c** *wake*^{NP3624}, **d** *wake*^{NP1350}, and **e** *wake*^{15185}*-Gal4* expression, revealed using *UAS-mCD8::GFP*, in the adult male brain, represented in green in **b1**–**e1**, which show the cell bodies of median neurosecretory cells (MNCs; indicated by the arrowheads). The neuropil was immunostained using an anti-DLG antibody (magenta in **b2**–**e2**); merged in **b3**–**e3** ($n = 6$ for each). **f**–**i** The MNCs cell bodies exhibiting with four independent drivers (green in **f1**–**i1**) are IPCs, as evidenced by anti-insulin-like peptide 2 (anti-Ilp2) immunolabelling (magenta in **f2**–**i2**); merged in **f3**–**i3** ($n = 6$ for each). **j** Diagrammatic representation of the gene targeting strategy for restricting myr-4xSNAP expression to IPCs specifically using a Gal4 driven Bxb1 recombinase and a specific IPC-expression LexA intersectional method. The blue and yellow circles represent the patterns of *ilp2-LexA* and *wake-Gal4* expression, respectively. The resulting overlapping cell populations that express myr-4 × SNAPs are limited to the regions where Gal4 induced *UAS-Bxb1* expression, as the transcriptional stop cassette is removed (*LexAop-myr > STOP > 4 × SNAP → LexAop-myr::4 × SNAP*) to allow LexA-induced expression, which represents the SNAP proteins that can be labelled specifically with molecular probes. **k**–**e** Representative images showing expression in IPCs with four independent drivers; green indicates the chemically labelled 4 × SNAPs expression pattern resulting from the intersectional *ilp2-LexA* and independent *wake-Gal4* drivers: **k** *wake*^{NP3168}, **l** *wake*^{NP3624}, **m** *wake*^{NP1350}, and **n** *wake-Gal4*^{15185}. Cell bodies of IPCs are indicated by dotted squares (**k1**–**n1**, respectively) (magnified in **k2**–**n2**; $n = 8$ for each). Scale bars, 20 μm.

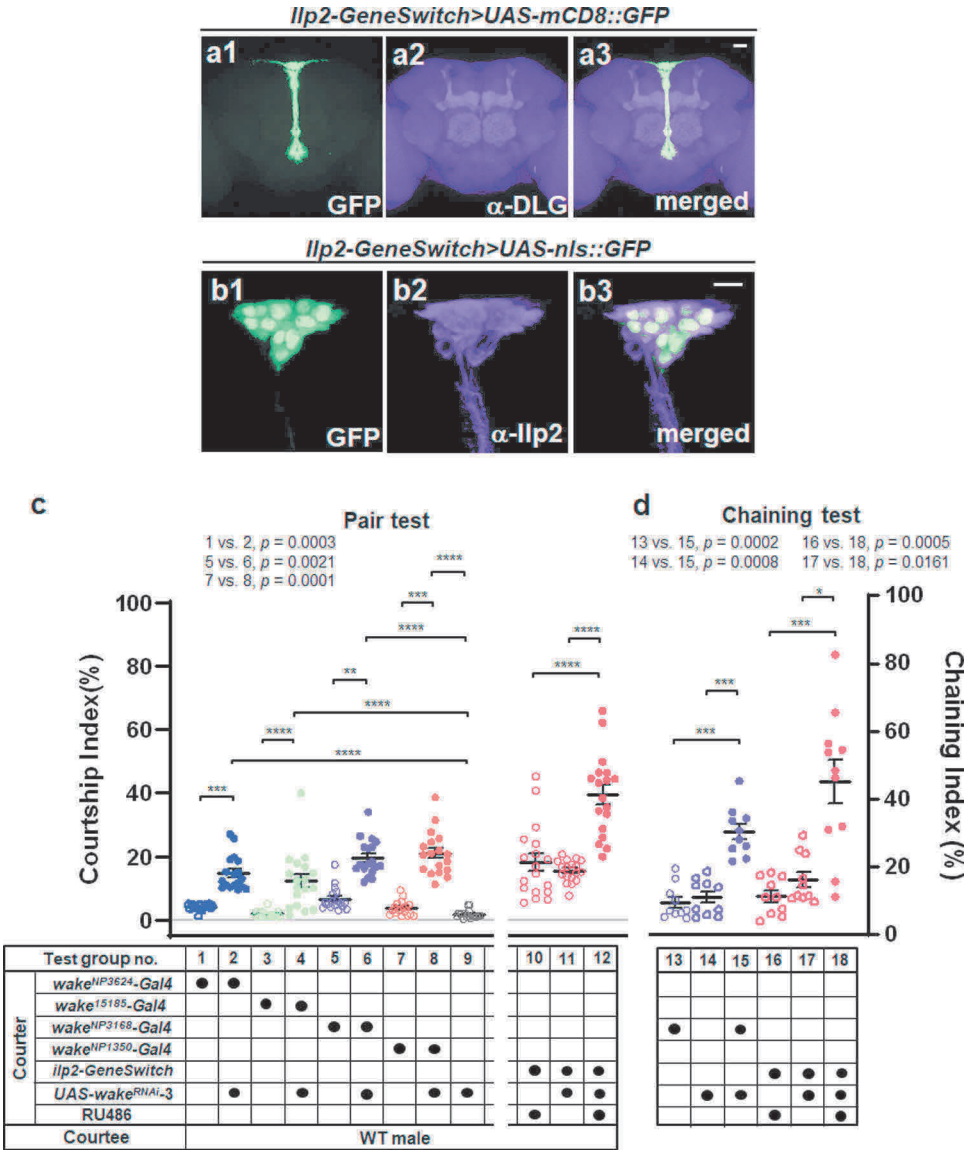


Fig. 4 Downregulation of WAKE in insulin-producing cells in early adulthood modulates male-male courtship behaviour. a Representative images showing the pattern of expression for an RU486-inducible IPC-specific *Ilp2-GeneSwitch* driver; expression patterns in the adult brain (10 days) revealed using *UAS-mCD8::GFP* are shown as green ($n = 6$ for each). The neuropil was immunostained using an anti-DLG antibody (magenta). **b** The cell bodies of IPCs can be observed due to *UAS-nls::GFP* for the nuclei (green) and were confirmed via anti-Ilp2 antibody staining (magenta) ($n = 6$ for each). Scale bars, 20 µm. **c**–**d** Comparison of genetically manipulated ($wake^{NP3624}$>$UAS\text{-}wake^{RNAi}$, $wake\text{-}Gal4^{15185}$>$UAS\text{-}wake^{RNAi}$, $wake^{NP3168}$>$UAS\text{-}wake^{RNAi}$, $wake^{NP1350}$>$UAS\text{-}wake^{RNAi}$) and RU486-treated ($ilp2\text{-}GeneSwitch$>$UAS\text{-}wake^{RNAi}$) 5-day-old males with the corresponding controls. Scatter plots include ± SEM for all data points for **c** the Courtship Index ($n = 18$ for each) and **d** Chaining Index ($n = 10$, 10, 10, 10, 10 and 11; from left to right for test group no. 13–18), $p < 0.0001$, Kruskal–Wallis test. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.005$, and $^{****}p < 0.0001$, *post hoc* Dunn's multiple comparisons test. Source data are provided as a Source Data file.

background significantly was associated with reduced male-male courtship behaviour (see Supplementary Fig. 7; test group no. 2 vs. 1); qPCR characterization of the *UAS-wake-RG* line revealed an effective increase in relative levels of *wake* RNA (see Supplementary Fig. 4). A previous study demonstrated that WAKE in PDF-expressing neurons affects sleep behaviour in *Drosophila*[20]; therefore, to further verify whether WAKE in PDF-expressing neurons also regulates male-male courtship behaviour, we specifically downregulated WAKE in PDF neurons, although this was not associated with increased male-male courtship behaviour (see Supplementary Fig. 7; test group no. 4 vs. 3). These results indicated that WAKE in IPCs at the adult stage is indeed involved in the inhibition of male-male courtship behaviour.

Moreover, in a competition test with one male test subject and two wild-type targets of different sexes, all male test subjects including $wake^{32099}$ males and males with WAKE downregulation in IPCs exhibited significantly courtship behaviour towards the female target than the male target, with no change in their preference for females (see Supplementary Fig. 8a).

Given that motor activity and courtship behaviour are closely related, we next examined motor activity associated with WAKE expression in flies. Motor activity was assessed using a climbing test and a spontaneous locomotor test, which revealed that such motor abilities persist in $wake^{32099}$ males and males with WAKE downregulation in IPCs (see Supplementary Fig. 8b).

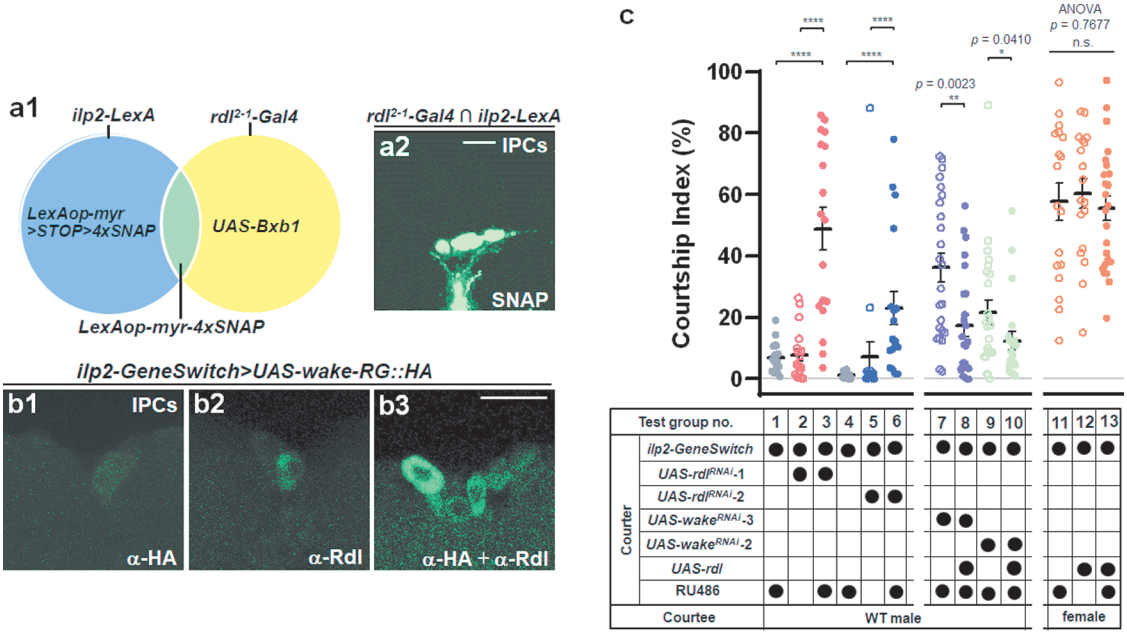
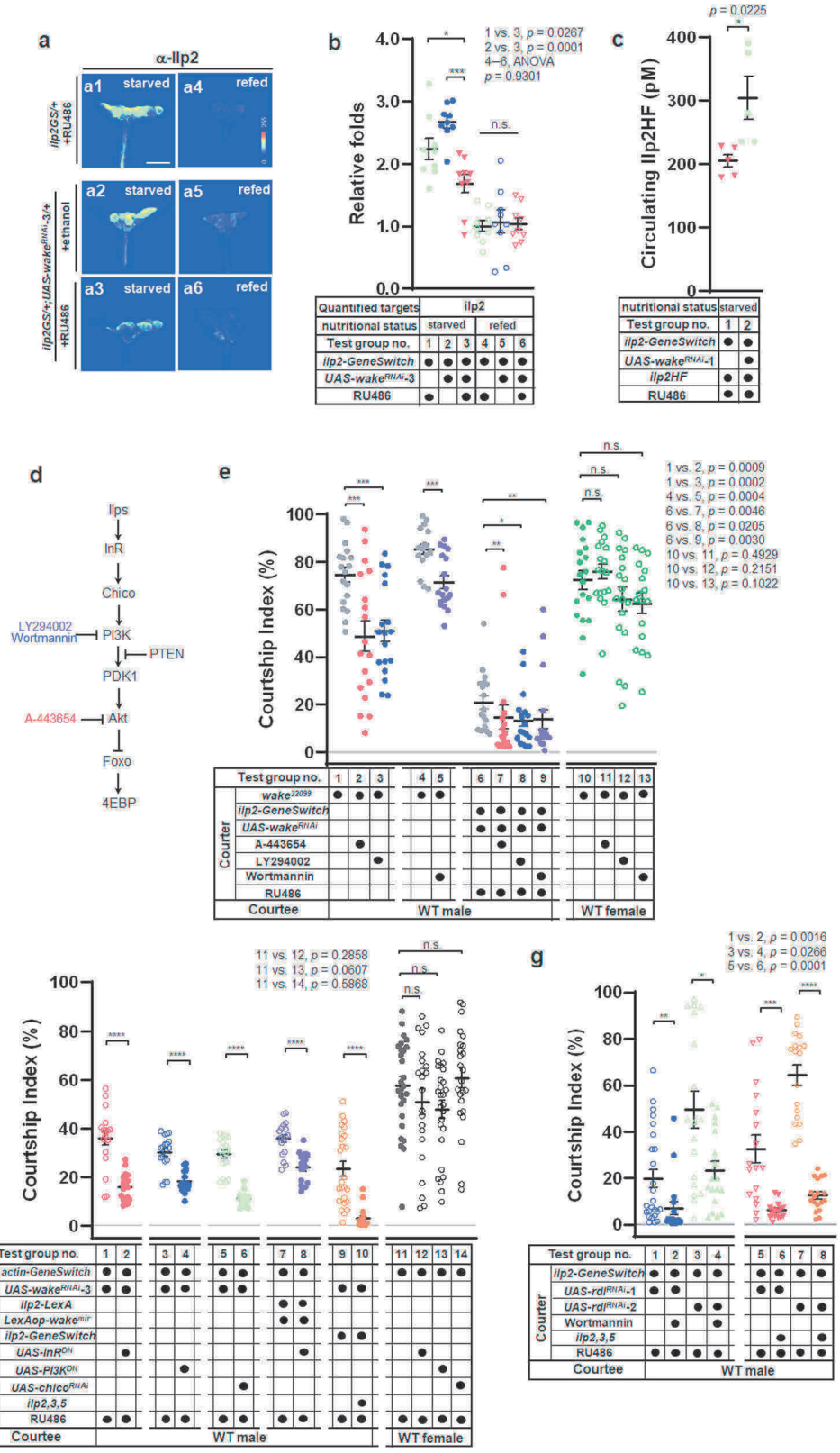


Fig. 5 Rdl expressed in insulin-producing cells is involved in WAKE deficiency-dependent male-male courtship behaviour. a1 The blue and yellow circles represent the patterns of *ilp2-LexA* and *rdl*$^{2\text{-}1}$*-Gal4* expression, respectively. The overlapping cells that express 4 × SNAPs are limited to the regions where *rdl*$^{2\text{-}1}$*-Gal4* drives *UAS-Bxb1* expression, as the transcriptional stop cassette is removed to allow LexA-induced expression, which represents the SNAP proteins that can be labelled specifically with molecular probes. **a2** Green indicates a pattern of expression for chemical labelling with 4 × SNAPs, zoomed in on the cell bodies of IPCs, resulting from the intersection of *ilp2-LexA* and the *rdl*$^{2\text{-}1}$*-Gal4* driver ($n = 6$ for each). **b** Self-interaction of WAKE-RG::HA and Rdl was visualized in IPCs using an in situ proximity ligation assay (PLA). Comparison of representative images showing staining with anti-HA (**b1**) or anti-Rdl (**b2**) antibody as negative controls, respectively ($n = 6$ for each). Strong PLA signals for anti-HA and anti-Rdl (green in **b3**). Scale bars, 20 µm. **c** There was a significant difference between the Courtship index of untreated controls and *ilp2-GeneSwitch* tester males exhibiting *rdl* dsRNA expression due to treatment with RU486. $n = 18$ for test group no. 1–6, $p < 0.0001$, Kruskal–Wallis test. $^{****}p < 0.0001$, *post hoc* Dunn's multiple comparisons test. Downregulation of WAKE in IPCs and simultaneous overexpression of Rdl significantly suppressed male–male courtship behavioural activity. $n = 24, 24, 25, 22$ (from left to right) for test group no. 7–10. $^{*}p < 0.05$, and $^{**}p < 0.01$, two-tailed Mann–Whitney U-test. The expression of Rdl alone in IPCs did not affect on male-female courtship behaviour. $n = 18, 18, 25$ (from left to right) for test group no. 11–13. $p > 0.05$ (n.s.), one-way ANOVA using F-test, $F(2, 58) = 0.2656$, $p = 0.7677$. Scatterplots include ± SEM for all data points. Source data are provided as a Source Data file.

WAKE in IPCs affects Rdl to control male-male courtship behaviour in *Drosophila*. As WAKE in PDF neurons affects sleep-related behaviour by regulating Rdl, a $GABA_A$-receptor[20], we next investigated whether WAKE-deficiency in IPCs also affects Rdl. A previous study reported that Rdl is not substantially expressed in IPCs[32]. Here, we performed immunolabelling with an anti-Ilp2 antibody to re-examine whether the *rdl*$^{2\text{-}1}$*-Gal4* expression pattern includes IPCs. Preliminary results showed that in the extensive expression pattern of *rdl*$^{2\text{-}1}$*-Gal4* (see Supplementary Fig. 9a), MNCs with nuclear labelling signals (*rdl*$^{2\text{-}1}$*-Gal4>UAS-nls::GFP*) did not overlap perfectly with Ilp2-positive cells; however, several cells with a relatively weak GFP signal overlapped with Ilp2-positive cells (see Supplementary Fig. 9b). Similarly, using an intersectional recombination strategy, our findings demonstrated that *rdl*$^{2\text{-}1}$*-Gal4* expression overlapped with *ilp2-LexA* expression in IPCs (Fig. 5a). Further, we performed an *in situ* proximity ligation assay (PLA)[33], which provided a sensitive approach to validating WAKE-Rdl interactions in IPCs. We expressed the prey of WAKE-RG tagged with HA in IPCs (*ilp2-GeneSwitch>UAS-wake-RG*$_{HA}$) using available HA and Rdl antibodies and succeeded in detecting PLA signals (Fig. 5b). The specificity of the anti-Rdl antibody was evaluated using immunolabelling based on an ectopic expression study (see Supplementary Fig. 10). This evidence suggests that specifically expressed HA-tagged WAKE physically interacts with endogenous Rdl in IPCs. Whether WAKE is also involved in the stabilisation and localisation of Rdl requires confirmation in further studies. This implies that there is a significant drop in total intensity of Rdl::GFP fluorescence in IPCs with simultaneous down-regulation of WAKE (see Supplementary Fig. 11; quantification results in Supplementary Fig. 11c; test group no. 1 vs. 2). Similarly, we also found that WAKE deficiency significantly affects Rdl::GFP trafficking to the cell surface of IPCs when compared with the corresponding control (see Supplementary Fig. 11c; test group no. 3–6; test group no. 3 vs. 4).

Next, two *UAS-rdl*^{RNAi} lines (*UAS-rdl*^{RNAi}−1 and −2) obtained from the VDRC were used for spatiotemporal control of *rdl* dsRNA expression in IPCs (*ilp2-GeneSwitch>UAS-rdl*^{RNAi}), which was achieved via 5 d of RU486 treatment after eclosion. Male-male courtship behaviour was clearly observed in the RU486-treated flies when compared with corresponding controls (Fig. 5c; test group no. 1–6; test group no. 3 vs. 1; test group no. 3 vs. 2; test group no. 6 vs. 4; test group no. 6 vs. 5). Furthermore, the strength of male-male courtship behaviour was significantly suppressed upon synchronous overexpression of Rdl in IPCs (Fig. 5c; test group no. 7–10; test group no. 8 vs. 7; test group no. 10 vs. 9). However, the CIs of male-female courtship behaviour did not increase when Rdl was overexpressed in IPCs (Fig. 5c; test group no. 11–13; test group no. 13 vs. 11; test group no. 13 vs. 12). These results indicate that WAKE in IPCs specifically inhibits male-male courtship behaviour, which may be related to the modulation of Rdl.

WAKE in IPCs modulates Ilp homoeostasis. Thus far, our results have suggested that WAKE inhibits male-male courtship



behaviour by upregulating Rdl in IPCs. We also aimed to determine whether Ilp release is affected when Rdl levels in IPCs are insufficient to receive inhibitory GABA signals. In the fasting state, insulin secretion can be synchronised and maintained at low levels from individuals. Thus, we aimed to reduce individual variation and effectively quantify the insulin-related reactions during fasting in the subsequent assays. Immunolabelling of brain tissue using an anti-Ilp2 antibody under starvation conditions revealed that the Ilp2 signal in IPCs after WAKE downregulation was significantly lower than that in the corresponding controls (Fig. 6a1–3; quantification results in Fig. 6b; test group no. 1–3; test group no. 3 vs. 1 and test group no. 3 vs. 2). However, if the flies were then allowed to feed for 30 min, secretion of Ilp2 remained normal (Fig. 6a4–6; quantification results in Fig. 6b; test group no. 4–6).

Fig. 6 WAKE modulates male-male courtship behaviour through IIS. a Relative Ilp2 immunofluorescence signals in IPCs with WAKE downregulation after 24 h of starvation (**a1**-**a3**) ($n = 9$ for each), and after feeding for 30 min (**a4**-**6**) ($n = 9$ for each); quantification in **b**; $n = 9$ for test group no. 1–3, $p = 0.0002$, one-way ANOVA using F-test, *post hoc* Tukey's multiple comparisons test, $^{*}p < 0.05$, and $^{***}p < 0.005$, $n = 9$ for test group no. 4–6, $p > 0.05$ (n.s.), one-way ANOVA using F-test, $F(2, 24) = 0.07273$, $p = 0.9301$. **c** The concentration of Ilp2HF in haemolymph with WAKE-deficient in IPCs. $n = 5$ for each, $^{*}p < 0.05$, two-tailed unpaired *t*-test. **d** Insulin signalling pathways indicating the targets of inhibitors used in further experiments. **e** In *wake*^{32099} flies or those with WAKE-deficient in IPCs, simultaneous treatment with different inhibitors. $n = 18, 18, 18, 17, 18$ (from left to right) for test group no. 1–5, $n = 19, 18, 18, 17$ for test group no. 10–13. $p > 0.05$ (n.s.), $^{***}p < 0.005$, two-tailed unpaired *t*-test. $n = 18, 18, 19, 18$ for test group no. 6–9. $^{*}p < 0.05$ and $^{**}p < 0.01$, two-tailed Mann–Whitney U-test. **f** In WAKE-deficient background simultaneous expression of InR^{DN}, PI3K^{DN}, or *chico* dsRNA, respectively. $n = 18$ for test group no. 1, 2, 7, 8, $^{****}p < 0.0001$, two-tailed unpaired *t*-test. $n = 18$ for test group no. 3–6, $n = 25, 17$ for test group no. 9, 10, $^{****}p < 0.0001$, two-tailed Mann–Whitney U-test. $n = 27, 22, 27, 24$ for test group no. 11–14, $p > 0.05$ (n.s.), two-tailed unpaired *t*-test. **g** Downregulation of Rdl in IPCs, simultaneous treatment with Wortmannin or those with heterozygous *ilp2, 3, 5* mutants. $n = 25, 18, 18, 18, 18$, and 18 for test group no. 1–4, 7 and 8. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{****}p < 0.0001$, two-tailed Mann–Whitney U-test. $n = 18, 16$ for test group 5 and 6, $^{****}p < 0.0001$, two-tailed unpaired *t*-test. Scatterplots include ± SEM. Source data are provided as a Source Data file.

In *Drosophila*, ligand-activated insulin receptor (InR) phosphorylates a *chico* encoded insulin receptor substrate (IRS), to induce the phosphorylation cascade of phosphoinositide-3-kinase (PI3K), phosphoinositide-dependent-kinase-1, and AKT (protein kinase B) (Fig. 6d). We further analysed fluorescence of tGPH, a PH-GFP fusion protein used as an indicator of PI3K activity, to evaluate insulin signalling[34]. Membrane localisation of tGPH was observed in the fat body, and the *wake*^{32099} background was associated with significant recruitment of the tGPH reporter to the cell membrane, suggesting that PI3K signalling is activated by IIS even in starved flies (see Supplementary Fig. 12a1, 2; quantification results in Supplementary Fig. 12b; test group no. 1 vs. 2). Similarly, these flies were then allowed to feed for 30 min, and a significant increase in tGPH fluorescence at the membrane was still observed in wild-type flies (see Supplementary Fig. 12a3, 4; quantification results in Supplementary Fig. 12b; test group no. 3 and 4). Moreover, enzyme-linked immunosorbent assay (ELISAs) for endogenous dual HA- and flag-tagged Ilp2 (Ilp2HF[35]) also showed that circulating Ilp2HF levels in the haemolymph were significantly higher in IPCs with WAKE downregulation than in the corresponding controls after 24 h of fasting (Fig. 6c; test group no. 1 vs. 2). In *Drosophila*, increased IIS results in a decrease in lifespan and reduced resistance to starvation stress[36,37]. Here, our analysis of *wake*^{32099} flies and those with WAKE downregulation in IPCs or Rdl expression also indicated a substantial reduction in lifespan and starvation tolerance in males (see Supplementary Fig. 13 and Supplementary Table 1).

WAKE modulates IIS to evoke male-male courtship behaviour. We then verified whether enhanced IIS after WAKE deficiency in IPCs is what prompts male-male courtship behaviour in *Drosophila*. First, significant male-male courtship behaviour was observed in males overexpressing Ilp2 in IPCs for 5 d after eclosion (see Supplementary Fig. 12c; test group no. 3 vs. 1; test group no. 3 vs. 2). Next, to examine whether augmented secretion of Ilp2 in WAKE-deficient males leads to male-male courtship behaviour, we inhibited IIS in the canonical PI3K-PKB/AKT pathway using suitable enzyme inhibitors (Fig. 6d); 20 μM A-443654 to inhibit Akt activity[38] and 300 nM LY294002 or 5 mM wortmannin to inhibit PI3K activity[39]. In *wake*^{32099} males (Fig. 6e; test group no. 1–5; test group no. 2 vs. 1; test group no. 3 vs. 1; and test group no. 5 vs. 4) and males with WAKE downregulation in IPCs (Fig. 6e; test group no. 6–9; test group no. 7 vs. 6; test group no. 8 vs. 6; and test group no. 9 vs. 6), the strength of male-male courtship behaviour after 5 days of treatment following eclosion was significantly decreased when compared with that in untreated controls. Although *wake*^{32099} flies were also treated with different IIS inhibitors, this did not substantially influence the CIs of male-female courtship behaviour (Fig. 6e; test group no. 10–13; test group no. 11 vs. 10; test group no. 12 vs. 10; test group no. 13 vs. 10), suggesting that drug treatment did not result in a general inhibition of courtship behavioural activity. Thus, pharmacological inhibition of IIS effectively reduced male-male courtship behaviour even in flies with WAKE deficiency.

Moreover, *actin-GeneSwitch* was used to induce WAKE downregulation at the adult stage (*actin-GeneSwitch>UAS-wake*^{RNAi}) while simultaneously inducing the overexpression of dominant-negative insulin receptor (InR^{DN}), dominant-negative PI3K (PI3K^{DN}), or *chico* dsRNA (*chico*^{RNAi}) for IIS inhibition. Male-male courtship behaviour was also significantly reduced in flies subjected to these genetic regimens when compared with the corresponding controls (Fig. 6f; test group no. 1–6; test group no. 2 vs. 1; test group no. 4 vs. 3; and test group no. 6 vs. 5). Similarly, a reduction in male-male courtship behaviour was observed when overexpression of InR^{DN} was induced by *actin-GeneSwitch* at the adult stage when WAKE was downregulated in IPCs only (*ilp2-LexA>LexAop-wake*^{mir}) (Fig. 6f; test group no. 8 vs. 7). In the *ilp2,3,5* heterozygous background, WAKE downregulation in IPCs at the adult stage (*ilp2-GeneSwitch>UAS-wake*^{RNAi}) also significantly reduced male-male courtship behaviour when compared with that in the corresponding controls (Fig. 6f; test group no. 10 vs. 9). Dominant InR^{DN}, PI3K^{DN}, or *chico*^{RNAi} expression induced in adults by *actin-GeneSwitch* did not significantly alter the CIs of male-female courtship behavioural activity (Fig. 6f; test group no. 11–14; test group no. 12 vs. 11; test group no. 13 vs. 11; test group no. 14 vs. 11). These findings again indicate that decreases in male-male courtship behaviour due to genetic inhibition of IIS are not reflective of general courtship suppression. To further demonstrate that the male-male courtship behaviour observed after Rdl downregulation in IPCs (*ilp2-GeneSwitch>UAS-rdl*^{RNAi}) is caused by the increased secretion of Ilps, flies were treated with wortmannin, which also significantly reduced male-male courtship behaviour (Fig. 6g; test group no. 1–4; test group no. 2 vs. 1; test group no. 4 vs. 3). In the heterozygous *ilp2,3,5* mutant background, Rdl downregulation in IPCs also significantly reduced male-male courtship behaviour when compared with the corresponding controls (Fig. 6g; test group no. 5–8; test group no. 6 vs. 5; test group no. 8 vs. 7).

WAKE deficiency in IPCs promotes JH biosynthesis via IIS in the corpus allatum, resulting in male-male courtship behaviour in *Drosophila*. Based on the evidence described above, we assumed that WAKE in IPCs modulates the activity of Rdl to maintain Ilps homoeostasis; thus, our next focus was to explore how Ilps evoke male–male courtship behaviour. In *Drosophila*, IIS is triggered by eight different Ilps that have varying spatio-temporal expression patterns and functions, and its pleiotropic effects regulate growth, development, metabolism, ageing, and stress responses[40]. However, only two Ilp receptors (InR and

Lgr3) have been identified[41,42]. The ovarian development of female insects is associated with IIS in a programmed response by which they initiate their reproductive function after nutrient acquisition[43]—Ilps modulate the corpus allatum (CA) to synthesize JH via IIS, which in turn initiates ovarian development[44]. If this mechanism also modulates JH expression in male flies at the adult stage, WAKE-deficient males may also exhibit increased JH production from the CA via enhanced insulin signals. Indeed, a previous study reported that JH is required for courtship behavioural activity in adult *Drosophila* males[17], and mutation of *methoprene-tolerant* (*met*), a JH receptor gene, also results in defects in mating behaviour and reproduction[45].

The role of JH signalling in male-male courtship behaviour has rarely been discussed in *Drosophila*[46]. We observed increased activity of IIS in the CA through the expression of constitutively active insulin receptor (InR^{CA}) by CA-expressing drivers (*Aug21-Gal4* or *Jhamt-Gal4*). Both drivers significantly prompted male-male courtship behaviour when compared with the corresponding controls (Fig. 7a; test group no. 1–6; test group no. 3 vs. 1; test group no. 3 vs. 2; and test group no. 6 vs. 4; test group no. 6 vs. 5). Conversely, WAKE downregulation in IPCs (*ilp2-LexA > LexAop-wake^{mir}*) and simultaneous overexpression of InR^{DN} or *chico* dsRNA to block IIS in the CA led to significant inhibition of male-male courtship behaviour (Fig. 7a; test group no. 7–16; test group no. 9 vs. 7; test group no. 9 vs. 8; test group no. 11 vs. 7; test group no. 11 vs. 10; and test group no. 14 vs. 12; test group no. 14 vs. 13; test group no. 16 vs. 12; test group no. 16 vs. 15). These results demonstrate that WAKE deficiency in IPCs is likely to cause male-male courtship behaviour in *Drosophila* by facilitating IIS expression in the CA.

Because the CA promotes JH biosynthesis via IIS, we further aimed to determine whether enhanced JH signalling evokes male-male courtship behaviour. We first treated *wake^{32099}* flies with precocene I, an anti-juvenoid that inhibits JH synthesis[47], for 5 days immediately after eclosion. The strength of male-male courtship behaviour was significantly suppressed in the treated flies when compared with that in the corresponding controls (Fig. 7b; test group no. 2 vs. 1). Alternatively, WAKE downregulation in IPCs (*ilp2-LexA > LexAop-wake^{mir}*) and simultaneous overexpression of two dsRNAs of JH biosynthetic enzymes —3-hydroxy-3-methylglutaryl CoA reductase (*hmgcr*)[48] or juvenile hormone acid *o*-methyltransferase (*jhamt*)[49] in the CA were used to inhibit JH production, leading to a significant reduction in male–male courtship behaviour (Fig. 7b; test group no. 3–12; test group no. 5 vs. 3; test group no. 5 vs. 4; test group no. 7 vs. 3; test group no. 7 vs. 6; and test group no. 10 vs. 8; test group no. 10 vs. 9; test group no. 12 vs. 8; test group no. 12 vs. 11). In *wake^{32099}* males treated with precocene I, the expression of *jhamt^{RNAi}* or *hmgcr^{RNAi}* in the CA did not lead to significant changes in the CIs of male-female courtship behaviour (Fig. 7b; test group no. 14 vs. 13; test group no. 16 vs. 15; test group no. 17 vs. 15), again indicating that the observed in male-male courtship behaviour was not reflective of general courtship behaviour suppression. Interestingly, a direct comparison of relative JH action using qPCR was associated with slight increases in the expression of the early response *kr-h1* in flies with WAKE downregulation in IPCs (*ilp2-GeneSwitch>UAS-wake^{RNAi}*) on the 5th day after induction (Fig. 7c). Thus, the male-male courtship behaviour in *Drosophila* prompted by WAKE deficiency in IPCs is likely due to the enhancement of JH biosynthesis by the CA via IIS.

WAKE deficiency in IPCs modulates JH signalling to reduce ecdysone signalling. During insect growth and development, JH and ecdysone signalling exert antagonistic effects on many biological processes, including moulting and reproduction. Especially in the developmental period from the larval stage to adulthood, precise regulation of JH and ecdysone levels is necessary for normal metamorphosis[50]. However, although extremely low ecdysone levels are maintained during the adult stage[51], the ecdysone receptor (EcR) is still widely expressed in various tissues, including the brain and other peripheral tissues[52]. Consistent with a previous report[53], our immunolabelling experiments indicated that EcR type A (EcRA)-positive cells were also detected throughout the adult brain (see Supplementary Fig. 14a–c). Therefore, ecdysteroids may retain their physiological functions in adult *Drosophila*, and they are involved in several physiological responses, including those related to oogenesis (i.e., germline development)[54,55], the circadian clock[55,56], stress resistance and longevity[57]. Moreover, male-male courtship behaviour has been observed in *Drosophila* upon the inhibition of ecdysteroid biosynthesis or mutation of the EcR in male flies[16,18,19]. Additional research has demonstrated that courtship behaviour is regulated by EcR in *fruitless* (*fru*) *P1*-expressing neurons[16].

The present findings indicate that activation of IIS in the CA may lead to male-male courtship behaviour in WAKE-deficient flies promoting JH signalling (Fig. 7). Since JH and ecdysone exhibit antagonistic actions in the regulation of many physiological processes, we then examined whether JH biosynthesis promoted by IIS in the CA results in male-male courtship behaviour due to the inhibition of ecdysone signalling. Relative quantification of the expression of the early response genes *Br-C* and *E75* by qPCR was used to verify the strength of ecdysone signalling[13]. Ecdysone signalling was significantly reduced under conditions of IIS activation in the CA (*Aug21-Gal4>UAS-InR^{CA}*), increased JH synthesis (*Aug21-Gal4>UAS-Jhamt*[58]) (Fig. 8a1; test group no. 1–3 and 6–8), and WAKE downregulation in IPCs (*ilp2-LexA>LexAop-wake^{mir}*) (Fig. 8a1; test group no. 4, 5 and 9, 10). Moreover, quantitative analysis using liquid chromatography with tandem mass spectrometry (LC-MS-MS) clearly showed that 20E levels were much lower than the detection limit in terms of per individual or per milligram of dry weight in *wake^{32099}* flies. In the controls (*revrt$^{13\text{-}1}$*), however, 20E levels were approximately 22 ± 3pg/fly or 84 ± 6 pg/mg (Fig. 8a2; test group no. 11 vs. 12). These results demonstrate that WAKE deficiency induces the CA to increase JH levels via IIS, which may reduce 20E synthesis via JH signalling and thereby reduce ecdysone signalling.

WAKE deficiency reduces ecdysone signalling in Or67d OSNs and affects their responsiveness to cVA. In adult male *Drosophila*, *fruP1*-expressing neurons participate in the inhibition of male-male courtship behaviour via ecdysone signalling[16]. This phenomenon was also clearly observed in the current study upon expression of *EcR* dsRNA in *fruP1*-expressing neurons (*fruP1-Gal4>UAS-EcR^{RNAi}*), which was used to downregulate ecdysone signalling (Fig. 8b; test group no. 1–3; test group no. 3 vs. 1; test group no. 3 vs. 2). To verify that the reduction in ecdysone signalling causes male-male courtship behaviour after WAKE deficiency, we treated *wake^{32099}* males with 20E or chromafenozide[59] (a non-steroidal ecdysteroid agonist) just after eclosion for 5 d, which significantly reduced the strength of male-male courtship behaviour (Fig. 8b; test group no. 5 vs. 4; test group no. 7 vs. 6). Alternatively, in the genetic background of WAKE downregulation in IPCs (*ilp2-LexA>LexAop-wake^{mir}*), EcR-A was overexpressed in *fruP1*-expressing neurons to enhance ecdysone signalling, which also suppressed male-male courtship behaviour when compared with that in the corresponding controls (Fig. 8b; test group no. 8–10; test group no. 10 vs. 8; test group no. 10 vs. 9).

Previous evidence has shown that *fruP1-Gal4* is expressed in subsets of OSNs that innervate different glomeruli, including the