Genetic tools to study juvenile hormone action in Drosophila

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The insect juvenile hormone receptor is a basic helix-loop-helix (bHLH), Per-Arnt-Sim (PAS) domain protein, a novel type of hormone receptor. In higher flies like Drosophila, the ancestral receptor germ cell-expressed (gce) gene has duplicated to yield the paralog Methoprene-tolerant (Met). These paralogous receptors share redundant function during development but play unique roles in adults. Some aspects of JH function apparently require one receptor or the other. To provide a foundation for studying JH receptor function, we have recapitulated endogenous JH receptor expression with single cell resolution. Using Bacteria Artificial Chromosome (BAC) recombineering and a transgenic knock-in, we have generated a spatiotemporal expressional atlas of Met and gce throughout development. We demonstrate JH receptor expression in known JH target tissues, in which temporal expression corresponds with periods of hormone sensitivity. Larval expression largely supports the notion of functional redundancy. Furthermore, we provide the neuroanatomical distribution of JH receptors in both the larval and adult central nervous system, which will serve as a platform for future studies regarding JH action on insect behavior.

Juvenile hormone (JH) drives various aspects of insect physiology. A well-documented role for this hormone is to prevent early metamorphosis. In most insects, JH prevents early induction of key metamorphosis-initiating genes. As larvae gain competence to undergo metamorphosis, the JH titer drops, reducing expression of the antimetamorphic, JH response gene Kr-h1, which lifts suppression on pupal or adult developmental programs. This aspect of JH activity has promoted the synthesis of batteries of compounds employed to combat pest insects. These JH analog (JHA) insecticides have been used in various formulations to combat medical and agricultural pests since their exogenous application can disrupt normal development. Methoprene, a JHA that shares structural similarity with endogenous JH, has proven valuable as both a research tool and as a key component of flea and tick preventative products for companion animals.

Unlike beetles and moths, in which JHAs can induce supernumerary larval instars, JH exposure does not override metamorphosis in flies, which instead die as pharate adults that fail to eclose from the puparium. Furthermore, the best candidate for a Drosophila JH receptor, the bHLH PAS protein Met, was demonstrated to be encoded by a nonvital gene. Thus, despite its utility as a powerful genetic model, progress toward understanding JH signaling in Drosophila has been frustratingly slow. However, Konopova and Jindra demonstrated that suppressing the presumed Met ortholog in Tribolium using RNAi induced developmental phenotypes that were typical of a loss of JH signaling; specifically, TcMet-deficient animals experienced precocious metamorphosis. Sequence analysis demonstrated that TcMet is ancestral to another Drosophila bHLH PAS gene called gcc, and that DmMet arose during dipteran evolution. Met27 gcc28 double null larvae suffer developmental arrest during pupariation, which phenocopies the effects of the loss of JH achieved through genetic ablation of the JH-producing gland, the corpus allatum (CA) (allatectomy, CAX19). Unlike CAX larvae, Met27 gcc28 larvae cannot be rescued by supplying exogenous JHA12. However, they can be rescued with a single ectopic copy of either gene, demonstrating redundancy. Recent work has demonstrated that Met and Gce proteins each bind JH with high affinity and that this binding is eliminated when the ligand-binding pocket is mutated. As bona fide paralogous JH receptors (JHR), these proteins represent a novel class of metazoan hormone receptor.

Paralog-specific function for each JH receptor has primarily been described in adults. In addition to its developmental and reproductive roles, JH signaling also influences insect behaviors, including courtship, aggression, and the onset of foraging behavior in honey bees. While studies have demonstrated how JH acts,
during nervous system development\textsuperscript{14, 22}, we understand virtually nothing about how JH influences the neuronal circuits underlying various insect behaviors.

To provide a framework for addressing the tissue- and cell-specific action of JH, we determined the spatio-temporal expression of JH receptors through \textit{Drosophila} development using a series of genetic tools. Specifically, we recapitulated endogenous \textit{Met} and \textit{gce} expression in both larval and adult \textit{Drosophila melanogaster} using both recombinered BAC drivers and a viral T2A peptide-mediated transgenic knock-in to drive the expression of a variety of fluorescent reporter constructs. Our results suggest that \textit{Met} and \textit{gce} share considerable expressional overlap in larval \textit{Drosophila}, supporting the notion of functional redundancy during preadult development\textsuperscript{12}. There were a few key exceptions to this finding, in which one receptor was expressed at substantially higher levels than, or in exclusion to, its paralog. Furthermore, we demonstrate JH receptor expression in known JH target tissues, the temporal dynamics of which are consistent with periods of hormone sensitivity. In both larvae and adults, paralog-specific expression was evident predominantly in the central nervous system (CNS), suggesting that they have distinct contributions to JH-regulated behavior and CNS development. Here, we provide a neuroanatomical atlas of JHR expression, which will ultimately facilitate future studies regarding JH action in CNS development and function.

**Results**

**BAC Validation.** In this study we recapitulated endogenous JHR expression using transgenic \textit{D. melanogaster} carrying either (1) one of several BACs comprised of a gce or \textit{Met} coding sequence, interrupted by a GAL4::p65 or LexA::p65 driver fragment replacing the first exon, and flanked bilaterally by 40 kb of genomic DNA (Fig. 1a,b), or (2) a T2A-GAL4 fusion construct inserted in frame into the native gce genomic locus (Fig. 1a). These constructs were used to drive the expression of a number of fluorescent reporter constructs. We used several experimental approaches to validate the reported expression patterns.

First, we generated genomic rescue stocks containing the native BAC clones for \textit{Met} or \textit{gce}, which lacked a driver cassette. We crossed these lines to \textit{Met}\textsuperscript{27} or \textit{gce}\textsuperscript{25}/FM7c females and scored the survival of progeny. \textit{Met}\textsuperscript{27} or \textit{gce}\textsuperscript{25} animals die early in pupal development, but can be rescued to adulthood with an ectopic \textit{Met} or \textit{gce} transgene\textsuperscript{12}.  

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\caption{Construction of BAC and T2A-GAL4 reagents and validation of GAL4 and LexA iterations through co-expression. (a) The region of the X chromosome that contains the corresponding sequence contained on the native gce BAC, CH321-09E09. The gce locus is shown in red, while surrounding genes are shown in tan, showing proper syntenic organization according to the FlyBase GBrowse function (www.FlyBase.net). The overall segment corresponding to the sequence contained on the BAC is shown in navy, and under this are representative cartoons for the gce-GAL4::p65 BAC and gce-T2A-GAL4 constructs. (b) The Met-GAL4::p65 BAC was constructed similarly. (c-g”) Co-expression experiments reveal the degree of reporter overlap when two independent reporters with similar architecture were driven by gce-GAL4::p65 and gce-LexA::p65 in the same line. (c) Expression pattern of UAS-IVS-myr-GFP-p10 and (d) LexAop-IVS-myr-tdTomato-p10 in the CNS of wandering third instar larvae. (e) Coexpression in the adult CNS, and (e’) GFP or (e”) tdTomato channel only. Coexpression across the ellipsoid body (f-f”) and the fan-shaped body (g-g”) of the adult central complex. Yellow arrowheads indicate cells that show qualitatively higher expression under the particular driver-reporter combination, (GAL4/UAS or LexA/LexAop) relative to the other. GFP (green or white), \(\alpha\)-dsRed (magenta).}
\end{figure}
Crosses of Met27gce2/FM7c females to w1118 control males resulted in only FM7c/Y males, and their vials showed 25.9% pupal mortality (n = 194), accounting for wvMet27gce2/FM7c/Y males that did not complete metamorphosis. Males carrying the native gce BAC eclosed at levels comparable with balancer sibs, indicating complete rescue (n = 174). By contrast, transformants carrying the native Met BACs rescued only 12% of Met27gce2 double mutants (n = 164) and the rescued males showed mosaic w+ expression in their compound eyes. Therefore, while we were satisfied that the native gce BAC contained sufficient regulatory sequence to direct expression of functional gce, the native Met construct either could not restore JH signaling or contained genes which, when duplicated in the genome via ectopic expression, were incompatible with the JH receptor mutant background.

To determine whether Met-GAL4::p65 could rescue Met phenotypes, we crossed the Met-GAL4::p65 reagent into the Met27 mutant line, but this stock was fragile and could not be maintained. We elected to sequence the genome of the Met-GAL4::p65 reagent to evaluate the integrity of the construct. We discovered that the BAC contained small deletions in a 5′ gene, CG1703 (see Fig. 1b for position of CG1703). However, adjacent sequence corresponding to that contained in either the St-H fragment or the p[EN71] genomic rescue construct was intact. We therefore proceeded under the assumption that the Met expression reported using this BAC might represent the endogenous pattern.

Since we successfully generated multiple gce drivers, we compared the expression patterns reported among these various iterations. We compared gce-LexA::p65 and gce-GAL4::p65 directly in the same line: gce-LexA::p65 (attP40 > 13xLexAop2-IVS-myrdTomato-p10 (attP40); gce-GAL4::p65 (VK00033) > 10xUAS-IVS-myr-GFP-p10 (attP2). Confocal analysis revealed considerable overlap between LexA::p65 and GAL4::p65 reagents, both in adults and larvae (Fig. 1c–g”). Observed differences in spatial partitioning or expression strength of GFP
Figure 3. Confocal scans of JHR expression across the larval central nervous system, including neuronal expression in the ring gland. (a) Met-GAL4::p65 > myr-GFP-p10 expression across the larval CNS (wandering stage). Strong GFP signal was evident in the mushroom bodies (mb), one of which is outlined. The developing medulla (me) and lobula/lobula plate (lo/lp) neuropils are indicated by yellow chevrons. The ring gland (rg) is shown above the larval brain lobes. (b) gce-GAL4::p65 > myr-GFP-p10 expression across the larval CNS shows some restricted GFP signal in the larval mushroom bodies and no GFP in the developing optic lobe neuropils. gce-GAL4::p65 (c,f') and gce-T2A-GAL4 (d,e) exhibited markedly different expression patterns in the larval CNS. Notably, the T2A reagent showed strong mushroom body (mb) and surface glia (sg) (yellow chevrons) expression (e); gce-LexA::p65 showed no mb expression (f), and gce-GAL4::p65 GFP expression in the mushroom bodies was substantially weaker than in the knock-in (f'). Anti-GFP antibody (green or white), αN-cadherin (magenta), Fas2 (magenta: f). Key ring gland neurons are identified as expressing Met/
Gce using a variety of driver and reporter combinations. (g) gce-T2A-GAL4 > GFP-p10 expression in the larval ring gland (wandering stage) shows GFP in the CA-LP1/2 cells that innervate the corpus allatum (white arrowheads), as well as moderate expression in the PTTH cells (yellow arrowheads). (h) gce-LexA::p65 > myr-GFP, (i) gce-GAL4::p65 > mCD8-GFP, and (j) Met-GAL4::p65 > myr-GFP-p10 expression in CA-LP1/2 and PTTH neurons. In each case, CA-LP1/2 neurons are indicated with white arrowheads, while PTTH cells are indicated with yellow arrowheads. Insets in (h–j) serve to resolve PTTH expression. Met > nls-GFP (k) and gce > GFP (l) expression colocalizes with PTTH-HA in PTTH cell bodies; (white arrowheads and insets). (m) Colocalization of R69B06 > myr-GFP-p10 expression and gce-LexA > td Tomato in CA-LP1 and CA-LP2 cells (white arrowheads). All scale bars, 50 µM. GFP (green or white), α-Histone H1 (magenta), dsRed (red).

reporters driven by the GAL4 and LexA BAC constructs were likely due to a combination of position (chromatin) effects, driver strength, and reporter architecture, as previously reported. In contrast, there were substantial differences in BAC- and T2A-reported GFP CNS expression for the gce gene, discussed below.

**Larval expression.** Met-GAL4::p65 and gce-GAL4::p65 or gce-T2A-GAL4 each exhibited widespread tissue expression during larval growth including in epidermis, fat body, muscles, salivary glands (Fig. 2) and the central nervous system (CNS; Fig. 3). As in most other larval tissues, JHR expression was widespread in the larval CNS (Fig. 3a–d). One significant distinguishing characteristic was that Met > myr-GFP-p10 showed strong expression in the mushroom bodies (mb) (Fig. 3a,e), while gce > myr-GFP-p10 showed moderate mb expression (Fig. 3b,f), and gce-LexA > myr-TdTomato-p10 showed none (Fig. 3f). In contrast to the BAC construction, the gce knock-in, gce-T2A-GAL4, showed obvious expression in the mushroom body (Fig. 3e). Met > myr-GFP-p10 showed broad expression in the developing optic lobe, including the nascent lobula and medulla neuropils in WL3 stage larvae (Fig. 3a,e), whereas gce neuronal expression using all three gce constructs and different reporters was essentially absent from the developing optic lobes (Fig. 3b–e,f and f’), but was evident in surface glia (Fig. 3d,e,f and f’). Both receptors were present in key peptidergic neurons whose axons project into the ring gland, including the prothoracicotrophic hormone (PTTH) neurons that stimulate ecdysteroid production by the prothoracic glands (PG) (Fig. 3g,h,k and l) and the CA-LP1 and –2 neurons whose axons terminate in the corpus allatum (see Fig. 3g–j and m).

In wild type flies, JH I or JH III application during the sensitive period severely disrupts adult abdominal differentiation by suppressing the proliferation and spreading of the abdominal histoblasts that normally give rise to the adult abdominal epidermis. Met mutants are refractory to this effect of JH treatment. We detected both JHRs in wandering stage larva (WL3) histoblasts, with expression persisting through pupariation, and then tapering off by 9h APF (Fig. 4).

**Imaginal discs.** Met-GAL4::p65 was expressed in wing, eye, leg, and genital discs (Fig. 5a–c and data not shown). In contrast, there was no gce-GAL4::p65 or gce-T2A-GAL4 expression in the wing, leg, eye, or genital imaginal discs of larvae (Fig. 5d–f) with any driver/reporter combination. To validate these results, we performed qPCR analyses with Met- and gce-specific primers, using cDNA libraries prepared from either pooled leg discs and wing discs, or from larval CNSs which included associated leg discs. We employed two tissue-specific control genes to verify the integrity of the disc-specific sample: Ntum was chosen for its enrichment in wing discs (ModEncode: 10) while bruchipilot (brp), which marks presynaptic active zones in the CNS (21), was used as a CNS-specific control gene. Consistent with our confocal data, we detected no gce transcript in wing/leg disc samples, while Met showed low expression in these tissues (Fig. 5g).

In contrast to the effects of exogenous JH on the differentiation of the adult abdomen derived from the histoblasts, structures derived from imaginal discs are not affected by JH treatment. We hypothesized that an absence (gce) or modest level (Met) of JHR expression in the discs might explain their JH insensitivity. To test this, we attempted to induce JH sensitivity, and presumably a reinduction of pupal cuticle, in wing discs, by supplying ectopic gce, Met, or both transgenes, using the c765-GAL4 driver, which expresses strongly in wing discs, and rearing these larvae on food containing 1 ppm pyriproxyfen [a potent JHA for Drosophila] (15). However, none of these manipulations shifted the response of the wing discs to JHA (Fig. 5h and data not shown), suggesting that simply supplying additional JHR to imaginal discs is not sufficient to confer JH sensitivity to these tissues.

**Ring gland.** The ring gland of higher dipteran larvae contains the endocrine cells of the JH-producing corpus allatum (CA) and the paired, lateral prothoracic glands (PG), which synthesize and secrete ecdysone (32). As shown in Fig. 6a, we observed strong Met and gce expression in both the CA and PG during larval life; gce-T2A-GAL4 > nls-GFP additionally showed strong expression in the corpus cardiaca (Fig. 6b, bottom row).

We sought to address the significance of JHR expression specifically in the PG, since this organ is considered a size-assessing tissue that regulates the time of metamorphosis. We used RNAi to suppress Met expression specifically in the PG of gce mutants, effectively eliminating both receptors from the PG. Met RNAi was functionally validated since its expression driven by Act5C-GAL4 in gce mutants produces the double mutant phenotype; gce > Met RNAi larvae (either 1x Met RNAi in gce (28) or 2x Met RNAi in gce (28); Act5C > 45854 or 2x Met RNAi in gce (28); Act5C > 45854, 45852) showed nearly 100% pupal lethality (Fig. 6c, n = 195, 1x Met RNAi; n = 183, 2x Met RNAi), while balancer sibs showed approximately 100% survival to adulthood (n = 214, 1x Met RNAi cross; n = 142, 2x Met RNAi). Met suppression in the PG using gce RNAi phm > 45852 was inconsequential for survival (n = 152/152).

Since an earlier study showed that Met suppression in the PG did not alter timing from the L2/L3 transition to pupariation, we tested the hypothesis that gce expression may provide functional redundancy. We staged larvae in 6-hour egg collections and scored time to pupariation as the proportion of puparia to wandering larvae
expression in the EB (Fig. 8b) was comparatively weaker than global expression across the FB, with strongest expression shown in layers 1 and 5 (Fig. 8c). which are also GFP-positive. W: wandering; P0: white puparium stage; P(n): hours after pupariation. Scale bars, 50 µM.

Met noduli (NO) displayed prominent dorso-ventral segregation of JHRs. In contrast, gce-GAL4::p65 and similar reporter expression for the myr-GFP-p10 gce receptors showed expression in the follicle cells of stage 8–10 oocytes, but phm-GAL4.

Adult expression. Recent studies have revealed that reproductive behaviors are influenced by JH signaling, some of which require either Met or Gce. The driver lines indicated that both JHRs were widely expressed in adults, with wide expressional overlap in many tissues. Here, we focus on expression in reproductive tissues and the adult CNS. Met mutants suffer substantial reproductive deficiency, while gce mutants show only minor reductions in reproductive capacity. Figure 7 provides an overview of Met and gce expression in male and female reproductive tissues as reported by the nuclear reporter UAS-nls-GFP. In males, both receptors show widespread expression; the ejaculatory duct and ejaculatory bulb, accessory glands and vas deferens all showed moderate to high level of Met and gce expression (Fig. 7a,b,g and k). In females, both receptors showed strong expression in the spermathecae (Fig. 7f and m), regardless of mating status, as well as in the seminal receptacle (Fig. 7c,m), uterus (Fig. 7c,m), and ovaries (Fig. 7d,i), but not in early stage oocytes (vO in Fig. 7d,i, and data not shown). However, both receptors showed expression in the follicle cells of stage 8–10 oocytes, but gce UAS-nls-GFP expression in these cells was moderate compared to that of Met (Fig. 7j,l). We boosted expression with either cytoplasmic GFP or myr-GFP-p10 to unequivocally identify gce in stage 8–10 oocytes (vO; Fig. 7d,i). Reproductive tissues showed similar reporter expression for the gce-GAL4::p65 and gce-T2A-GAL4 drivers (Fig. 7a–j).

To evaluate Met/gce expression in the brain, we employed a series of reporters specifically tailored for neuroanatomy. Overall, both receptors expressed broadly across the adult CNS, with each showing a unique and reproducible pattern. Indeed, the most striking examples of divergent JHR expression were observed across the various cell types of the adult CNS. For example, we observed distinct partitioning across compartments in the central complex and optic lobes (OL). An overview of both JHRs as reported with myr-GFP-p10 is shown in Fig. 8(a,af,k and k').

In the central complex, Met and gce expressed with variable intensity across the ring neurons of the ellipsoid body (EB), and either shared or occupied discrete layers of the fan-shaped body (FB). Across the layers of the FB, gce > myr-GFP-p10 showed the most intense expression in layers 4 and 6, and with comparatively weak expression in layer 5 (Fig. 8h), and also expressed in the asymmetrical body (AB), a unilateral structure associated with long term memory formation and retrieval, gce-T2A-GAL4 > myr-GFP exhibited a similar expression pattern, with weaker GFP signal evident in layers 2, 3, and 5 relative to other layers (Fig. 8m). Met > myr-GFP-p10 showed global expression across the FB, with strongest expression shown in layers 1 and 5 (Fig. 8c). Met > myr-GFP-p10 expression in the EB (Fig. 8b) was comparatively weaker than gce > myr-GFP-p10 and gce-T2A-GAL4 > myr-GFP (Fig. 8g,l), the latter of which was exceptionally strong relative to expression across the entire brain. The paired noduli (NO) displayed prominent dorso-ventral segregation of JHRs. Met > myr-GFP-p10 showed highly enriched expression in the ventral NO3, with moderate expression in NO1 and NO2 (Fig. 8d'). In contrast,
gce-GAL4::p65 > myr-GFP-p10 signal across the NO was moderate to strong in NO2 and NO1 (Fig. 8i), while gce-T2A-GAL4 showed minimal expression in NO2, with moderate expression in both NO1 and NO3 (Fig. 8n).

Given the staggering diversity of cell types in the OL, we focused on a handful of distinctive features that characterized the expression pattern of each receptor. Met > myr-GFP-p10 and gce > myr-GFP-p10 either coexpressed or expressed individually in the various cell types that constitute the layers of two of the major OL neuropils, the lobula and the medulla37, thus establishing a paralog-specific banding pattern in each neuropil (Fig. 8e,j). Some features of optic lobe expression were consistent between the BAC and knock-in gce reagents. None of the gce constructs drove reporter expression in the T4 or T5 layers of the medulla or lobula, respectively (Fig. 8j,o). There were also notable differences in the gce drivers. For instance, while BAC reagents showed moderate lobula plate expression (Fig. 8j), the T2A-GAL4 reagent did not drive myr-GFP expression in the lobula plate (Fig. 8o).

Expression of the JHRs in the medulla was complex with some layers showing expression of both receptors, other showing expression of neither, and still others with enhanced expression of Met or gce. Both Met > myr-GFP-p10 and gce > myr-GFP-p10 as well as gce-T2A-GAL4 expressed in axon bundles of cells crossing the inner chiasm (Fig. 8e,j and o). In the lobula, Met > myrGFP showed highest expression in layer 1 (Fig. 8e). By contrast, gce > myr-GFP-p10 and gce-T2A-GAL4 are lacking in layer 1 but are at high levels in the other layers of the lobula (Fig. 8j,o). Also, Met > myr-GFP-p10 showed moderate expression in the medial-most layer 10 of the medulla occupied by arborizations of T4 cells, where gce was absent (Fig. 8j,o).

Discussion
In this study we used a set of genetic tools to probe developmental expression of the duplicate Drosophila JH receptors Met and gce, by recapitulating their expression with fluorescent reporters. We have systematically surveyed larval and adult tissues, with emphasis on neuronal expression. Coexpression experiments in which the GAL4 and LexA gce reagents drove simultaneous GFP and tdTomato constructs demonstrated extensive expression overlap between these reagents, but also illustrated some differences (Fig. 1e–g'). The T2A-GAL4 knock-in and BAC reagents showed substantially divergent expression patterns in key CNS structures in both larvae and adults. The intense staining was outlined by the white dotted line in (d) and are adherens cells that adhere to the underside of the eye disc. (g) Quantitative PCR analysis of Met and gce expression in wing/leg discs vs. expression in CNS+ attached leg disc samples. brp serves as a CNS-specific control gene. Notum is enriched in wing discs relative to the CNS. Unpaired t-test *p < 0.05, ***p < 0.005; n = 135 for each gene: 3 technical replicates, each consisting of 3 biological replicates using material extracted from 15 animals. Error bars: SEM. (h) Normal development of the adult wing in animals fed 1 ppm dietary pyriproxyfen. Left: TM3 balancer sib control fly. Right: C765 > gce fly expressing ectopic gce in the wing disc. Arrowheads indicate the anterior margin of the adult wing. Scale bars, 50 µM.

Figure 5. JHR expression in larval imaginal discs. (a) Met-GAL4::p65 > mCD8-GFP expression in the eye disc, showing Met expression in the developing photoreceptors (yellow arrowheads), which show αHRP staining (a'). mCD8-GFP also shows Met expression globally in the genital disc (b) and wing disc (c) of wandering L3 animals. GFP expression was not detected in the eye (d) (αHRP staining is indicated by white arrowheads), genital (e) or wing discs (f) of wandering L3 animals, using either the gce-GAL4::p65 or gce-T2A-GAL4 drivers. The intensely-staining GFP-positive cells outlined by the white dotted line in (d) are epithelial cells that adhere to the underside of the eye disc. (g) Quantitative PCR analysis of Met and gce expression in wing/leg discs vs. expression in CNS+ attached leg disc samples. brp serves as a CNS-specific control gene. Notum is enriched in wing discs relative to the CNS. Unpaired t-test *p < 0.05, ***p < 0.005; n = 135 for each gene: 3 technical replicates, each consisting of 3 biological replicates using material extracted from 15 animals. Error bars: SEM. (h) Normal development of the adult wing in animals fed 1 ppm dietary pyriproxyfen. Left: TM3 balancer sib control fly. Right: C765 > gce fly expressing ectopic gce in the wing disc. Arrowheads indicate the anterior margin of the adult wing. Scale bars, 50 µM.
adults, likely a consequence of position effects and construct architecture, though there were shared features. Peripheral tissue expression was essentially the same between the T2A and BAC iterations of the gce construct, indicating that at least in non-CNS tissues, BAC expression is a reliable proxy for endogenous gce expression. We obtained only a single Met-GAL4::p65 BAC reagent; several efforts to produce a Met knock-in reagent were fruitless. Given the differences between BAC and knock-in data for gce, we propose that the Met BAC reagent faithfully recapitulates peripheral tissue expression but likely only approximates Met expression in the CNS, since we were unable to generate the “gold standard” genomic knock-in reagent for Met. Overall, our confocal data are consistent with characterized aspects of tissue-specific JH action and therefore represent a working foundation for future studies regarding JH action at the cellular level in Drosophila. An overview of key expression features in the larva is given in Fig. 9a–a’; JHR redundancy during larval life is supported by their near global coexpression. A key exception to this finding was that gce but not Met was conspicuously absent from the imaginal discs (Fig. 5a,b and c). Following JHA challenge, disc-derived structures develop normally in Drosophila6,27, 28. In contrast, exogenous JHA reprograms the abdominal histoblasts by maintaining the expression of the pupal specifier Broad, promoting a second pupal, rather than adult, epidermis38. We demonstrate here that Met and gce coexpress in histoblasts during the window of JH sensitivity28, following which their expression declines, with gce levels declining followed by Met, beginning at 9 h APF (Fig. 4). Under the rationale that JH sensitivity requires both receptors, we created lines in which Met, gce, or both were ectopically expressed specifically in the wing disc. However, following dietary JH treatment, wing development in both experimental and balancer flies was normal (Fig. 5h), indicating that ectopic JHR expression was not sufficient to confer JH sensitivity to this tissue. To address the possibility that another component of the JH receptor machinery was absent in wing discs, we labeled the discs with antibodies against the JH-dependent Met binding partner Taiman (Tai)39, 40. Tai was observed in discs in late wandering larvae, before the onset of pupariation (Fig. S1). Therefore,
the canonical JH signaling pathway is present. Perhaps unidentified inhibitory factors block JH action in wing discs or downstream components are missing.

Removing JH signaling in Drosophila larvae, through genetic ablation of the CA [allatectomy (CAX); 13, 14] or by suppressing Met/gce function15, cannot prevent the onset of metamorphosis, but does alter developmental timing in the third instar. During this instar, the PG establishes the timing of pupariation by assessing larval size to determine whether the critical weight checkpoint, which corresponds to the time after which starvation cannot prevent metamorphosis, has been attained33. By suppressing Met expression specifically in the PG, Mirth et al.34 demonstrated that this treatment is insufficient to alter time to pupariation relative to phm/+ control larvae. In this study we found Met and Gce were coexpressed in the PG during larval development (Fig. 6b), suggesting the possibility that Mirth et al.34 failed to override functional redundancy between JHRs in the PG. To address this, we performed the same manipulation in gce mutants, eliminating PG expression of both JHRs. There was no difference in pupariation time between the Met RNAi larvae and the Tm6b balancer sibs although both were delayed slightly in comparison to the parental stock, indicating that the phm-GAL4 construct itself can influence

Figure 7. Confocal scans of reproductive tissues dissected from gce-GAL4::p65 and Met-GAL4::p65 lines driving pStinger (nls:eGFP) expression. Left: gce-GAL4::p65 > nls:eGFP male reproductive tract expression: (a) male reproductive tract: ejaculatory duct (ED), accessory gland (AG), vas deferens (VD) and in the somatic cells of the testes (TS); (b) ejaculatory bulb (EB), gce-GAL4::p65 > nls:eGFP female reproductive tract expression: (c) seminal receptacle (SR), the gross morphology of which is revealed with Phalloidin-568. (d) Previtellogenic oocytes (pqO; yellow arrowheads) showed no expression, while stage 8–10 oocytes (vO) undergoing the vitellogenic checkpoint, showed expression both in the follicle cells surrounding the primary oocyte and in the nurse cells; (e) uterus (common oviduct); (f) spermatheca (ST). Center: overall gce-T2A-GAL4::p65 > nls:eGFP male (g) and female (h) reproductive tract expression, in which GFP expression is strong in paraovaria (PO) and spermathecae (ST). (i) GFP is absent from previtellogenic oocytes, and obvious in checkpoint-stage egg chambers; (j) close-up of the migrating border cells (BC) in a checkpoint-stage egg chamber. Right: Met-GAL4::p65 > nls:eGFP expression was similar to gce-GAL4::p65 > nls:eGFP in the male reproductive tract (k) and EB (k'); a vitellogenic oocyte shows strong GFP expression in the follicle cells surrounding the primary oocyte, in nurse cells, and border cells (BC). (m) The seminal receptacle (SR), uterus (UT) and spermathecae (ST) all showed intense GFP expression. GFP (green or white), Phalloidin-568 (magenta). All scale bars, 50 µM.
Figure 8. Confocal scans of JHR expression across the adult brain, with emphasis on central complex and optic lobe expression. Myr-GFP was used to visualize JHR expression in the nervous system. Left: Two z-slices showing the overall pattern of Met expression in the adult brain (a,a'). The mushroom bodies, which show strong Met expression, are indicated with yellow arrowheads in (a); pharyngeal nerves are indicated with white arrowheads in (a'); inset: minimal expression in a group of peptidergic cells (*). (b) Met-GAL4:p65 > myr-GFP-p10 expression across the compartments of the central complex, including the ellipsoid body (EB), fan-shaped body (FB), and bulb (BU). (c) Met expression across the layers of the FB and nodulus (NO). Arrowheads indicate layers 2/3 (white) and 5 (yellow) of the FB. (d) mCD8-GFP expression and myr-GFP-p10 expression both show that Met is highly expressed in NO3 relative to other subcompartments. (e) Met-GAL4:p65 > myr-GFP-p10 expression across the optic lobe neuropils: lobula (lo), lobula plate (lp), medulla (me), showing strongest expression in the T5 arborizations (white arrowhead) that outline the periphery of the lobula. Yellow asterisk: anterior optical tract. Center: Two z-slices showing the overall pattern of gce expression in the adult brain. The mushroom bodies (yellow arrowheads) show only weak myr-GFP expression with the gce-GAL4:p65 BAC reagent (f,f'); high level myr-GFP expression evident in the ellipsoid body and bulb (f') with one or two yellow asterisks, respectively; (g) inner (white arrowhead) and outer (yellow arrowhead) rings of the EB show intense gce expression; (h) gce-GAL4:p65 > myr-GFP-p10 expression across FB layers is strongest in layers 4 and 6 (white arrowheads) and comparatively weak in layer 5 (yellow arrowhead). Expression was also noted in the asymmetrical body (AB), outlined with a white dotted line. (i) NO3 (white arrowhead) shows minimal GFP expression, while NO1 and NO2 show moderate expression. (j) gce expression across the optic lobe neuropils, labeled as in e, gce was absent in the arbors of the T5 cells (white arrowhead). Right: Two z-slices showing the overall pattern of gce-T2A-GAL4 > myr-GFP expression in the adult brain. Strong GFP expression is seen in the mushroom bodies (k) (yellow arrowheads) and in a subset of peptidergic cells (inset). Central complex expression is shown in (l–n); nodulus expression shows moderate to high GFP signal in NO1 and NO3 using gce-T2A-GAL4. (o) Optic lobe expression shows no GFP in the T5 cells (white arrowhead) or lobula plate (lp); medulla (me) expression is strong in some layers and weak in others. Scale bars, 50 µM. GFP (green or white), αN-cadherin (magenta).
When we attempted to create a vvMet\(^{TM}\)-phm/TM6b line, the stock was fragile and showed extensive preadult mortality, indicating that Met flies are sensitive to PG manipulation, and that expression of GAL4 alone in the PG has developmental consequences.

Reciprocal biosynthetic regulation between JH and ecdysone pathways has been demonstrated in several species, though the mechanisms segregate along taxonomic lines. In the hemimetabolous Diploptera punctata, expression of the ecdysone receptor proteins EcR and RXR (USP) coincides with an elevation in JH titer in 5-day old adult females; suppressing these genes lowers ecdysteroid titer and elevates JH biosynthesis in 6-day old females\(^42\). In holometabolous insects, 20E has an evolutionarily-conserved allatotropic role. JH biosynthesis is irreversibly stimulated in cultured Aedes aegypti CA following 20-hydroxyecdysone (20E) addition at a concentration of 10\(^{-6}\) M\(^43\). Fourth instar Bombyx mori CC-CA complexes likewise increased JH biosynthesis when cultured with 20E in a narrow concentration range, and this JH synthesis profile was concomitant with the elevation and subsequent decline in the transcript abundance of two JH biosynthetic enzymes\(^44\). In both A. aegypti\(^45\) and B. mori, JH biosynthesis is also induced by 20E in a narrow concentration range, and this JH synthesis profile is concomitant with the elevation and subsequent decline in the transcript abundance of two JH biosynthetic enzymes\(^46\).
and *B. mori*, 20E overrides an inhibitory factor from the brain that prevents JH synthesis at a developmentally inappropriate time.

In *Drosophila* such a factor(s) may be a product of the CA-LP1/2 neurons that innervate the CA, and which according to the present study, show JHR expression in WL3 larvae. Further, JH receptor expression in CA/GP cells, as well as in CA-LP and PTTH neurons suggests a potential role in coordinating hormone biosynthesis. For instance, by responding to changes in hormone titer, JHRs may participate in a feedback mechanism through which one or both receptors monitor the rate of biosynthesis or release of JH, ec dysone, or both. Experiments in other insects have shown that JH can inhibit PTTH release to indirectly suppress ecdysone production or, act directly on the PG to suppress ec dysoid steroid synthesis. Met and Gce both express in the PTTH cells (Fig. 3k,l) and PG (Fig. 6b) during the third instar. The coexpression of JH and ec dysone receptor machinery in the endocrine cells and ec dysone responsive genes supports these tissues as critical sites for the convergence of signaling pathways, and the sensitivity of *vwMet*; *phm/TM6b* flies to PG manipulation suggests that Met may be the key player in integrating these signals.

In adult flies, JH-Met regulates reproductive physiology in both sexes. *Met* mutants produce and ovipos it few vitellogenic oocytes than wild type flies, while flies lacking *gce* exhibit considerably milder reproductive consequences. We observed widespread *Met* and *gce* coexpression in both male and female reproductive tracts (Figs 7 and 9b).

In *Drosophila* oogenesis, JH is necessary for entry into the vitellogenic phase. The vitellogenic checkpoint occurs in stage 9 oocytes where JH is required for both yolk protein synthesis by the follicle cells and yolk protein uptake from the hemolymph. It also suppresses 20E-induced apoptosis of the oocyte at stages 6–9 when the females are starved. This apoptosis is mediated by the ec dysone-induced E75A. Both *Met* and *gce* are expressed in the follicle cells surrounding the primary oocyte in stage 8–10 egg chambers, which corresponds with the vitellogenic checkpoint (Fig. 7m and n). Met and gce in stage 8 and 9 follicle cells may mediate the action of JH, 20E, or both to oppose apoptosis of egg chambers. The comparatively severe oogenesis phenotype exhibited by *Met* mutants argues for a scenario under which Met-JH suppresses ec dysone-driven apoptosis, possibly by downregulating the apoptosis inducer E75A, which is a JH and 20E responsive gene, and a demonstrated transcriptional target of JH-Gce in cell culture. If *gce* regulates E75A expression in check-point stage egg chambers, *gce* overexpression, rather than loss of function, might be expected to promote apoptosis in egg chambers.

Virtually nothing is known about *Met* and *gce* function in the brain, but a handful of studies have addressed the more general issue of JH action on nervous system development. Widespread and dramatic neurological damage results from a lethal dose of the JH analog methoprene in OR-C wildtype flies, including fusion of the subesophageal ganglion with the thoracicoabdominal ganglion, an incomplete fusion of the optic lobe neuropils and damage results from a lethal dose of the JH analog methoprene in OR-C wildtype flies, including fusion of the subesophageal ganglion with the thoracicoabdominal ganglion, an incomplete fusion of the optic lobe neuropils and damage.

Materials and Methods

Bacterial Artificial Chromosome (BAC) generation for making driver lines. BAC recombinering was performed in SW102 cells using a kanamycin/streptomycin positive/negative-selection cassette modified from pSK + RpsL-kana<sup>+</sup>. Landing-site cassettes were created by ligating ~500-base-pair GAL4 or LexA<sup>+</sup> and 3′ homology arms amplified from pBGU<sup>+</sup> and pBPnL<sup>+</sup> using primers that added 30 bases of 5′ and 3′ Met and gce homology to the GAL4/LexA-flanked selectable markers. These cassettes were recombined into the *Met* or *gce* BACs (CH321-28005 and CH321-09609<sup>+</sup>) (obtained from Children's Hospital Oakland Research Institute, Oakland, CA), and recombinants were selected on kanamycin medium. Putative clones were analyzed by colony PCR to ensure that proper recombination had occurred. Full-length GAL4, GAL4-p65, or NLS::LexA::p65 transgene cassettes were recombined into these modified BACs making use of the 500-bp homology arms added earlier. Potential recombinants were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. 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Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions. Correct BACs were identified by faster growth on streptomycin medium. Clones were analyzed by colony PCR, and putative recombinant BACs were sequenced at the recombined regions.
Fly lines. Reporter lines used in this study include the membrane-bound GFP reporters 10xUAS-IVS-mCD8-GFP (pJFRC2; attP2), 10xUAS-IVS-myr-GFP (pJFRC12; attP2), 10xUAS-IVS-myrm-GFP-p10 (pJFRC28; attP2), 10xUAS-IVS-myr-GFP-p10 (pJFRC29; attP2), 13xLexAop2-IVS-myr-GFP-GFP (pJFRC19; attP2), LexAop2-IVS-myr-tDrona-tGFP (attP40), and the nuclear GFP reporter UAS-nls-eGFP (pStinger).65 Other Drosophila lines used in this study include: wgc251, plbm-GAL413. Met RNAi lines (BDSC #45852 (w1118, P(GD4384)v45852) and #45854 (w1118, P(GD4384)v45854), Act5C-GAL4 (BDSC# 4414, P[w+m = Act5C-GAL4]25FO/CyO, y+), w; UAS-Met (attP40), and w; UAS-gce (attP2). The PTTH-HA line was a gift from Dr. Michael O’Connor.

Quantitative PCR. Quantitative real-time PCR was performed on a LightCycler 480 (Roche Molecular Diagnostics, Basel, Switzerland) using SYBR Green detection. Data were analyzed using the ∆∆CT method.

Gce-T2A-GAL4 construction. To knock-in the T2A-Gal4 sequence immediately preceding the gce stop codon66,67, S’ and 3’ homology arms approximately 3 kb in length were amplified with the following primers: gce_55_AgeI: TACGACCGGTCCACATTATTCTGACATTTTAGTCTGAG; gce_53StuI: AAGGCCGTCCGTCGTGTCCTCCTCGT; gce_35Pmel: TACGGTTAAACTAGATGATGGAGACGCC; gce_33MluI: TACGACGGTACACCTTGCAACAATGACAC BAC from clone BACR48D17, and cloned into pT7-7.68 T2A-Gal4 was generated by cloning Gal4 from pBPGAL4.2Uw-26 into pTL1 (StuI/SacI) using a forward primer containing the T2A coding sequence. Donor DNA was integrated at attP40 to create the corresponding transgenic fly, which was then mated with +/Y, hs-hid; 5X-ri6TS-Rac112; hs-FLP, hs-I-Scel/TM3, Sb66. The progeny were heat shocked (37 °C for 1 hour) twice, once during the second instar (L2) and once during the mid-third instar (L3) to promote donor release. Females, Donor/5X-ri6TS-Rac112; hs-FLP, hs-I-Scel+/+, were crossed to nSyb-LexA-p65 for lethality selection. Surviving candidates were subjected to chromosomal mapping and genomic PCR confirmation. An identical approach was taken to construct a Met-T2A-GAL4 reagent, but we were unsuccessful in generating transforest lines.

JH treatment. Five µg pyriproxyfen (Sumitomo Chemical Company, Osaka, Japan) (a JHA) in 50 µl 95% ethanol was mixed into 5 ml molten standard molasses/cornmeal/yeast diet after cooling to 60 °C to make a 1 ppm pyriproxyfen diet. Control food contained 50 µl of 95% ethanol only. Flies were monitored for pharate adult mortality, the typical wild type response to high doses of JHAs.

Genomic rescue. Native BACs (CH321–28005 and CH321–09E0961, called “Native Gce” or “Native Met”) lacking a driver cassette were used to transform the same injection line used for driver line construction. Transformants were homozygosed in a w1118; Met and Gce are partially redundant in transducing juvenile hormone action.

Immunocytochemistry. Animals were dissected in phosphate-buffered saline (PBS; pH 7.4, from 10x biology grade PBS, Corning 46–013-CM) and fixed in 4% formaldehyde in PBS for at least 2 hours. Following three washes with PBS +1% TritonX (1% PBST), tissues were blocked in normal donkey serum at a final concentration of 1:50 for at least 2 hours. Primary antibodies were supplied in the following concentrations: chicken anti-GFP monoclonal at 1:1000; mouse anti-Histone H1 at 1:1000; rat anti-N-cadherin (DNEX-8) at 1:20; mouse anti-Fas2 of 1:50 for at least 2 hours. Primary antibodies were supplied in the following concentrations: chicken anti-GFP monoclonal at 1:1000; mouse anti-Histone H1 at 1:1000; rat anti-N-cadherin (DNEX-8) at 1:20; mouse anti-Fas2 of 1:50 for at least 2 hours. After overnight incubation at 4 °C for at least one night, tissues were washed three times in 1% PBST and secondary antibodies in 1% PBST were added at the following concentrations: goat anti-chicken AlexaFluor 488 at 1:1000, donkey anti-mouse Cy5 at 1:1000, donkey-anti rabbit AlexaFluor 594 at 1:1000, and cleared through an ethanol/xylene series, mounted in DPX mounting medium, and visualized by confocal microscopy on a Zeiss 510 LSM.60

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**Author Contributions**

A.B. and L.M.R. wrote the paper. A.B., L.M.R. and J.T. analyzed the data. A.B., M.T., H.M.C., R.W., S.P., J.E., D.M. and R.W. performed the experiments.

**Additional Information**

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**Competing Interests:** The authors declare that they have no competing interests.

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