Regulation of *Drosophila* hematopoietic sites by Activin-β from active sensory neurons

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An outstanding question in animal development, tissue homeostasis and disease is how cell populations adapt to sensory inputs. During *Drosophila* larval development, hematopoietic sites are in direct contact with sensory neuron clusters of the peripheral nervous system (PNS), and blood cells (hemocytes) require the PNS for their survival and recruitment to these microenvironments, known as Hematopoietic Pockets. Here we report that Activin-β, a TGF-β family ligand, is expressed by sensory neurons of the PNS and regulates the proliferation and adhesion of hemocytes. These hemocyte responses depend on PNS activity, as shown by agonist treatment and transient silencing of sensory neurons. Activin-β has a key role in this regulation, which is apparent from reporter expression and mutant analyses. This mechanism of local sensory neurons controlling blood cell adaptation invites evolutionary parallels with vertebrate hematopoietic progenitors and the independent myeloid system of tissue macrophages, whose regulation by local microenvironments remain undefined.
In vertebrates, regulation of self-renewing blood cell populations by local organ microenvironments is poorly understood at the cellular and molecular level. In a related Drosophila melanogaster model, blood cells (hemocytes), with similarities to vertebrate tissue macrophages and oligopotent hematopoietic progenitors, form resident clusters in segmentally repeated inductive microenvironments of the larval body wall, also known as Hematopoietic Pockets (HPs). More than 90% of these larval hemocytes of embryonic origin are known as Hematopoietic Pockets (HPs) (Fig. 1a). Based on their functional dependence on sensory neurons of the HPs for their localization and survival, and the elevated proliferation of resident hemocytes compared to those in circulation, we investigated the molecular mechanism of hemocyte induction by the sensory neurons of the peripheral nervous system (PNS).

Here we identify a molecular mechanism by which Drosophila hematopoiesis is controlled by the PNS. Activin-β (Actβ, Act), a Transforming Growth Factor-β (TGF-β) family ligand, is specifically produced by multidendritic neurons and chordotonal organs of the PNS, and acts to regulate the proliferation and long-term adhesion of hemocytes. PNS activation by agonist treatment drives expansion of the blood cell pool, while specific silencing of sensory neurons affects resident hemocyte number and localization. Actβ plays a key role in this regulation, as evidenced by the induction of Actβ expression in response to PNS activity, and a blunted response in Actβ mutants. These findings shed new light on the mechanisms by which local microenvironments regulate blood cell adaptation and may integrate sensory inputs.

**Results**

**Sensory neurons form an interface with the blood cell system.**

First we examined the local anatomy of PNS neurons and resident (sessile) hemocytes. High magnification imaging revealed that PNS neurons form intricate extensions to areas of resident hemocytes (Fig. 1b), suggesting an interface that allows direct neuron-to-hemocyte communication. Using a split GFP approach, GFP Reconstitution Across Synaptic Partners (GRASP) (Supplementary Fig. 1a), we confirmed that hemocytes are anatomically extremely close, and potentially form direct contacts with PNS neurons and glia. To identify specific molecular signals that mediate this communication, we screened components of several key signalling pathways utilizing in vivo RNA interference (RNAi), searching for defects in hemocyte number and/or localization. Based on this, we focused on the role of the TGF-β family-related Dsma2 (Smox) pathway in hemocyte regulation. To identify the responsible ligand, we examined expression of the putative pathway ligands Activin-β (Actβ), Dawdle (daw) and myoglianin (myo) using ligand GAL4 reporter (O’Connor lab) and (GRASP) approach, GFP Reconstitution Across Synaptic Partners (GRASP) (Supplementary Fig. 1a), we confirmed that hemocytes are anatomically extremely close, and potentially form direct contacts with PNS neurons and glia. To identify specific molecular signals that mediate this communication, we screened components of several key signalling pathways utilizing in vivo RNA interference (RNAi) searching for defects in hemocyte number and/or localization. Based on this, we focused on the role of the TGF-β family-related Dsma2 (Smox) pathway in hemocyte regulation. To identify the responsible ligand, we examined expression of the putative pathway ligands Activin-β (Actβ), Dawdle (daw) and myoglianin (myo) using ligand GAL4 reporters (O’Connor lab and ). Actβ was highly expressed by specific subsets of sensory neurons in the HPs, in particular the multidendritic (md) neurons and chordotonal organs (Fig. 1d,e). None of the other ligands showed obvious expression in the sensory neurons. Colabelling confirmed localization of Actβ-producing neurons with resident hemocytes in the HPs (Fig. 1c). In contrast, Actβ was not detectably expressed in other components of the HPs, such as epidermis, muscle and oenocytes. Actβ was also highly expressed in motor neurons and other neurons of the central nervous system (CNS) as described previously, similar to related ligands of the TGF-β family. However, the vast majority of hemocytes in the larva, present as resident hemocytes in the HPs, were physically separated from the CNS, motor and some external sensory neurons.

![Figure 1](image1.png)

**Figure 1** | PNS sensory neurons produce Activin-β and are surrounded by hemocytes. (a) Model of a Drosophila larva; hemocytes in red, neurons in green. Boxed area marks a HP. (b) Close-up of a lower part of a HP illustrating intricate neuronal extensions in areas of hemocytes; neurons (Anti-HRP, red), lateral patch area. Middle and lower panel show single channels. (c) Actβ expressing larval PNS neurons marked by reporter Actβ-GAL4; UAS-mCD8GFP (green), Actβ positive neurons co-localize with hemocytes marked by HmlA-DsRed (red). Fillet prep containing all tissue layers. (d) Actβ expression pattern in larval PNS neurons, Actβ-GAL4/+ ; UAS-stinger/+ (green), pan-neuronal anti-elav (red). Fillet prep containing all tissue layers. (e) Model of Actβ expression (green) in most multidendritic neurons (diamonds), chordotonal organs (triangles), and some external sensory neurons (circles). Scale bars, (c), 50 μm; (d), 100 μm.
neurons and axon terminals of motor neurons (Supplementary Fig. 2a,b, and see anatomical description of the HPs in ref. 4).

**Actβ signalling promotes blood cell proliferation and adhesion.**

Next we investigated the effects of Actβ/dSmad2 pathway loss-of-function (lof, gof). Studying the viable null mutant Actβ<sup>Ed80</sup> (ref. 21), or silencing Actβ by in vivo RNAi, driving transgene expression ubiquitously or in sensory md neurons, we found diminished hemocyte numbers in the segmental HPs (Fig. 2a–e). Consistent with this, we observed an increase in the fraction of circulating hemocytes (Fig. 2f), and overall reduced total hemocyte numbers per larva (Fig. 2g), using a method of quantitative hemocyte retrieval from single *Drosophila* larvae<sup>22</sup>. These defects resembled hemocyte phenotypes seen on PNS neuron ablation<sup>4</sup> and suggested defects in hemocyte adhesion, proliferation and/or survival. Silencing of Actβ in motor neurons using the driver OK6-GAL4 (Supplementary Fig. 2d,e,h,i) did not affect the localization of hemocytes in the HPs (Supplementary Fig. 2f,g,j,k), and resulted only in minor reductions of total hemocyte numbers during various time points of larval development, which were non-significant according to Student’s t-test (Supplementary Fig. 2l). Actβ<sup>Ed80</sup> mutants showed partial penetrance (62% mutant phenotype) and were analysed side-by-side with controls as 2nd instar larvae, to avoid compensatory mechanisms that became evident in 3rd instar larvae. In all backgrounds of Actβ lof, PNS neuron clusters were present and appeared normal by cell number and dendritic morphology. This suggested a role for Actβ signalling in hemocytes, rather than indirect effects due to roles of Actβ in the nervous system<sup>11,13,18,19</sup>

To substantiate the role of Actβ/dSmad2 signalling in hemocytes, we systematically determined the effects of hemocyte-autonomous RNAi silencing of the Activin type II receptor *punt* (*put*)<sup>23</sup> and the signal transducer dSmad2 (ref. 11). Similar to the loss of Actβ in the microenvironment, knockdown of Actβ pathway components in hemocytes resulted in diminished numbers of resident hemocytes in the segmental HPs (Fig. 2h–k). Silencing of the Activin type I receptor *baboon* (*babo*)<sup>11</sup> showed similar albeit milder effects on hemocyte localization. Silencing of the pathway in hemocytes by knockdown of *put* or dSmad2 further resulted in increased fractions of circulating hemocytes (Fig. 2l) and reduced total hemocyte numbers per larva (Fig. 2m). With prolonged RNAi silencing of *put* and dSmad2, the reduction in total hemocytes eventually reversed in older larvae (Supplementary Fig. 3), again implying putative compensatory mechanisms that take effect in the prolonged absence of dSmad2 signalling. Silencing of *put* and dSmad2 under the control of HmlΔ-GAL4 had no effect on lymph gland hemocytes in larvae of the developmental window studied (Supplementary Fig. 4a–i), and no concomitant increase in the fraction of crystal cells, neither of the lymph gland nor the embryonic lineage of hemocytes, was observed (for lymph gland see Supplementary Fig. 4c,i). This was examined because in the larva, plasmocytes are known to give rise to small numbers of crystal cells<sup>24,25</sup>. Taken together, our findings suggest that sensory neuron-produced Actβ signals through the dSmad2 pathway in hemocytes, which supports hemocyte numbers and promotes hemocyte localization to the HPs.

To determine whether Actβ/dSmad2 signalling has trophic and/or proliferative roles in hemocytes, we focussed on the effects of Actβ overexpression and *babo* gain of function. Moderate Actβ overexpression in PNS neurons, or ectopic sites in oenocytes and epidermis using the driver *Spalt* (*Sal*)-GAL4, resulted in increased total hemocyte numbers per larva (Fig. 3a–g), while silencing of Actβ in these locations had no significant effect according to Student’s t-test (Supplementary Fig. 5a–c). The increase in total hemocyte numbers on Actβ overexpression was accompanied by increased *in vivo* EdU incorporation, suggesting enhanced hemocyte proliferation (Fig. 3i). Consistently, silencing of *put* and dSmad2 in hemocytes resulted in reduced EdU incorporation of hemocytes *in vivo* (Fig. 3j). To substantiate a direct role of Actβ in hemocyte proliferation, we also examined larval hemocytes *ex vivo* under conditions of Actβ stimulation. Indeed, we found that Actβ promoted EdU incorporation, indicative of increased hemocyte proliferation (Fig. 3k). In contrast, high overactivation of the pathway by hemocyte specific expression of the constitutively activated receptor *babo-CA* resulted in reduced hemocyte numbers per larva (Supplementary Fig. 6a–c) and drove hemocytes into apoptosis (Supplementary Fig. 6d). Neither Actβ overexpression nor dSmad2 pathway silencing in hemocytes increased the rate of hemocyte apoptosis (Supplementary Fig. 6c,d). Based on this, we conclude that the level of Actβ/dSmad2 signalling may determine the nature of the hemocyte response. At moderate activation levels, Actβ/dSmad2 signalling is a positive regulator of haemocyte number that promotes proliferation, while high overactivation of the pathway drives cells into apoptosis. Consequently, we anticipate that the amplitude of Actβ expression is likely to be tightly regulated.

Next we examined the role of Actβ/dSmad2 signalling in hemocyte localization. Ectopic expression of Actβ in areas typically devoid of hemocytes, such as the *Sal* expressing ventral areas of the epidermis and oenocytes (Fig. 3e,f) or imaginal discs, did not result in a uniform adhesion or attraction of hemocytes, i.e., in the alternating gap areas of the epidermis where no sensory neuron clusters are located (Fig. 3e,f). Further, uniform overactivation of the pathway by hemocyte expression of *babo-CA* showed a largely unaffected overall pattern of resident hemocytes, despite the above-mentioned apoptosis of hemocytes (Supplementary Fig. 6a,b). This argued against a function of Actβ in hemocyte chemotraction by gradient formation and led us instead to focus on a potential role of Actβ in the induction of hemocyte adhesion. Ectopic expression of Actβ produced an overall trend of decreasing the fraction of circulating hemocytes, although this effect remained statistically insignificant by t-test (Fig. 3h), and accumulation of hemocytes in ectopic areas seemed minimal (Fig. 3c–i). Seeking a more sensitive assay to quantify hemocyte adhesion, we took advantage of the fact that resident hemocytes of the HPs can be mobilized by mechanical disturbance and spontaneously re-adhere to the body wall within 30–45 min (ref. 4). Using an established protocol for this assay<sup>24</sup>, we examined the adhesive properties of hemocytes under various Actβ/dSmad2 pathway conditions. Indeed, dSmad2 pathway knockdowns in hemocytes, or Actβ silencing in PNS neurons, diminished hemocyte re-adhesion as evidenced by increased fractions of circulating hemocytes (Fig. 4a). However, dSmad2 pathway activation did not promote increased re-adhesion, suggesting an additionally needed, rate-limiting step in hemocyte adhesion. Moreover, we found that hemocyte-autonomous knockdowns of dSmad2 pathway components, but not Actβ silencing in neurons, produced a lack in hemocyte cluster formation and self-adhesion (Fig. 4b–e). Taken together, we conclude that the Actβ/dSmad2 pathway directly or indirectly promotes hemocyte adhesion to the microenvironment (Fig. 4f, Model), a process that we predict requires in addition another, rate-limiting step. DSmad2 signalling may have an additional autonomous roles in hemocyte clustering/self-adhesion, which is revealed only when the pathway is blocked in hemocytes, thereby excluding alternative signalling by other Act family ligands as might be the case in Actβ lof backgrounds.

**HmlΔ-GAL4**

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3
Figure 2 | Loss of Activin-β/ΔSmad2 signalling results in hemocyte defects. (a–d) Loss of Actβ in neurons affects hemocytes, lateral view of larva (top panels) with close-up (lower panels). (a) Control Hml1-DsRed/+; (b) Actβ mutant, Hml1-DsRed/+; Actβ ED80/ED80; (c) Actβ RNAi silencing with ubiquitous driver Actin5C-GAL4; (d) Actβ RNAi (UAS-Act RNAi Vienna GD) silencing in PNS neurons, md neuron specific driver 21-7-GAL4. (e) Hemocyte counts in segmental HPs (bracketed areas in a–d top panels), n=4–7 per genotype. (f) Fraction of circulating hemocytes, n=4 to 8 per genotype. (g) Total hemocyte counts per larva, n=4 to 8 per genotype. In e–g experiment numbers relative to matching control cohorts, genotypes Hml1-DsRed/+; Actβ ED80/ED80; Actin5C-GAL4, Hml1-DsRed/+; UAS-Actβ RNAi/+; (matching control: Actin5C-GAL4, Hml1-DsRed/+; 21-7-GAL4, UAS-mCD8GFP, Hml1-DsRed/+; UAS-Actβ RNAi/+; (matching control: 21-7-GAL4, UAS-mCD8GFP, Hml1-DsRed/+). Hemocyte numbers were assessed in larvae 64–75 h AEL (2.2–2.6 mm). (h–j) Hemocyte-specific RNAi silencing of Actβ pathway components, driver Hml1-GAL4, UAS-GFP; He-GAL4. Top panels whole larva lateral view, lower panels closeups. (h) Control; (i) UAS-put RNAi; (j) UAS-ΔSmad2 RNAi. (k) Hemocyte counts in segmental HPs (bracketed areas h–j in top panels), n=4 to 7 per genotype. (l) Fraction of circulating hemocytes, n=7 to 10 per genotype. (m) Total hemocyte counts per larva, n=7 to 11 per genotype; larvae 53–60 h AEL (1.8–2.0 mm). In (k–m) experiment numbers relative to matching control cohorts are shown; genotypes Hml1-GAL4, UAS-GFP/+; He-GAL4/+ (control); Hml1-GAL4, UAS-GFP/+; He-GAL4/UAS-put RNAi or Hml1-GAL4, UAS-GFP/UAS-ΔSmad2 RNAi; He-GAL4/+ (experiment). Scale bars, a–d and h–j 0.5 mm. Error bars represent s.d., and two-tailed t-test values correspond to NS (not significant) P>0.05; *P≤0.05; **P≤0.01; ***P≤0.001; ****P≤0.0001.
**Actβ** links sensory neuron activity with blood cell responses.

Considering the anatomical contacts of PNS neurons with hemocytes, and the role of Actβ as neuron-emanating signal that supports hemocyte proliferation and adhesion, we sought to determine whether PNS neuron activity may regulate blood cell behaviours. Using a diagnostic driver for acetylcholine production, Cha-GAL4, we confirmed previous reports that PNS neurons are cholinergic (Fig. 5a,b). This allowed us to use the pan Acetylcholine Receptor (ACHr) agonist carbachol (carbamoylcholine) for the stimulation of larval sensory neurons. Interestingly, we found that cuticle glia using repo-GAL4 (ref. 29), yet no hemocyte phenotypes were observed (Supplementary Fig. 7a–d), demonstrating specificity of the observed hemocyte responses to neuronal silencing.

In addition to these short-term (15 min–2 h) effects on hemocyte localization to the HPs, we examined the long-term effects of stimulation by carbachol, or neuronal silencing by Kir2.1 or UAS-shi dnr, on hemocytes. Interestingly, we found that carbachol exposure over several days of larval development significantly increased total hemocyte numbers per larva compared to controls according to Student’s t-test (Fig. 5h). Consistently, silencing of PNS neurons through various regimens of heat shock induction of Kir2.1 or the dominant-negative dynamin shi dnr over 20–48 h
had opposite effects, resulting in larvae with reduced total hemocyte numbers (Fig. 5h). To examine whether the carbachol-induced increase in hemocyte number was based on proliferation, we quantified in vivo EdU incorporation of hemocytes. Indeed, carbachol-exposed larvae showed about 1.5-fold increased EdU incorporation over controls, an effect which interestingly was mainly seen in younger 2nd instar larvae (Fig. 5i). Further, carbachol-exposed larvae showed an overall larger increase in the resident hemocyte population of the hematopoietic sites compared to the circulating fraction (Fig. 5j), consistent with our model proposing enhanced proliferation of resident hemocytes in the HPs. Taken together, our data suggest that PNS neuronal activity supports hemocyte localization and expansion by proliferation, and predict a molecular signal from PNS neurons that is inducible by sensory neurons.

To determine whether Actβ might play a role as this inducible signal, we quantified Actβ expression in sensory neurons of varying states of activity. Indeed, exposure of intact *Drosophila* larvae to carbachol induced a substantial and dose-dependent increase in Actβ expression within 2–3 h, as quantified by the expression of a UAS-luciferase transgene driven by the Actβ-GAL4 reporter (Fig. 6a). For this experiment, we focused on reporter expression in the PNS by carefully removing the CNS to visualize GFP expression yet avoiding lethality by high expression of *Kir2.1*, which in turn drives hemocyte proliferation and adhesion, Scale bars, b, 50 μm. Error bars represent s.d., and two-tailed t-test values correspond to NS (not significant) \( P > 0.05; \) * \( P \leq 0.05; \) ** \( P \leq 0.01; \) *** \( P \leq 0.001; \) **** \( P \leq 0.0001.\)

**Figure 4** | **Actβ promotes hemocyte adhesion.** (a) In vivo re-adhesion of hemocytes after mechanical disturbance, bars illustrate percent of circulating hemocytes; genotypes are Hml-GAL4, UAS-GFP; He-GAL4 × UAS-RNAi transgenes and control; and 21-7-GAL4, UAS-mCD8GFP, Hml-DsRed × UAS-Actβ RNAi and control; \( n = 7 \) to 8. (b–e) Ex vivo released hemocytes show different levels of aggregation; Hml-GAL4, UAS-GFP; He-GAL4 × (b) Control; (c) UAS-put RNAi; (d) UAS-babo RNAi; (e) UAS-dSmad2 RNAi. (f) Model of Actβ production by sensory neurons and induction of hemocyte responses in proliferation and adhesion. Scale bars, b, 50 μm. Error bars represent s.d., and two-tailed t-test values correspond to NS (not significant) \( P > 0.05; \) * \( P \leq 0.05; \) ** \( P \leq 0.01; \) *** \( P \leq 0.001; \) **** \( P \leq 0.0001.\)

**Discussion**

This research identified Actβ as one of the elusive genes that govern hemocyte proliferation in the hematopoietic sites (HPs) of the *Drosophila* larva, as was predicted by previous functional studies. Our work further links Actβ RNA expression to the level of PNS neuronal activity. This model implies that increased expression of Actβ would give rise to higher levels of active Actβ (through tub-GAL80<sup>β</sup>) in limited time windows of 22 h to visualize GFP expression yet avoiding lethality by high expression of *Kir2.1*, we observed induction of GFP in Actβ expressing PNS neurons of controls, while this GFP signal was largely absent in parallel cohorts silenced by coexpressed *Kir2.1* (Fig. 6e–g). In addition to its effects on the PNS, *Kir2.1* coexpression also reduced Actβ-GAL4 driven GFP expression in the CNS (Fig. 6f,g).

Finally, we asked whether Actβ is required for the induction of hemocyte responses on stimulation with carbachol. Comparing Actβ<sup>Ed80</sup> null mutant larvae with Actβ-competent controls, we found that loss of Actβ attenuated the effect of carbachol-induced blood cell expansion (Fig. 6h). This suggested that Actβ plays a major role in the cholinergic regulation of hemocytes, consistent with a model of neuronal activity-induced Actβ expression (Fig. 6k). However, Actβ mutants showed mild albeit by t-test insignificant carbachol-induced blood cell expansion (Fig. 6h) and partial short-term hemocyte recruitment to the HPs on 15 min of carbachol exposure (Fig. 6i,j), suggesting additional inducible signal/s that may contribute to these effects. Taken together, our findings support a model in which PNS neuronal activity promotes Actβ expression, which in turn drives hemocyte expansion and long-term localization to the HPs (Fig. 6k).
protein, although the formal demonstration awaits development of a suitable tool for the detection of Actβ protein. In the future, it will be interesting to study specific sensory stimuli that trigger hemocyte responses. Sensory neurons of the PNS have a prime function in detecting innocuous and noxious sensory stimuli such as mechanical strain, temperature, chemicals and light\textsuperscript{30,31}, many of which signal potentially harmful conditions that may cause tissue damage. Thus, linking the detection of challenging conditions with the adaptive expansion of the blood cell pool may be an efficient system to elevate the levels of macrophages, to remove and repair damaged tissues, enhancing the overall fitness of the animal. Because Drosophila larval hemocytes persist into the adult stage\textsuperscript{6,7}, the mechanism of sensory neuron-induced blood cell responses may allow adaptation of the animal beyond the larval stage.

In Drosophila self-renewing hemocytes, Actβ/dSmad2 signalling has diverse effects on proliferation, apoptosis and adhesion. Our \textit{ex vivo} data indicate that hemocyte proliferation is likely a direct effect, which is consistent with similar roles of babo/dSmad2 in other tissues such as \textit{Drosophila} imaginal discs and brain\textsuperscript{11,21}, and TGF-β family dependent proliferation in vertebrate systems\textsuperscript{32,33}. Echoing our findings of \textit{babo-CA} driven hemocyte apoptosis, TGF-β family mediated direct or indirect effects on apoptosis have been described in invertebrate and vertebrate systems\textsuperscript{13,34}. Overall, TGF-β family signalling is known for its multifaceted biological roles, depending on the cellular contexts and levels of ligand stimulation, which often translate into qualitatively distinct transcriptional and other cellular responses, that are mediated by both Smad and non-Smad signalling mechanisms\textsuperscript{32,33}. While \textit{Drosophila} Actβ and possibly related TGF-β family ligands are known to signal through the induction of ecdysone receptor (EcR) in some but not all \textit{Drosophila} tissues\textsuperscript{15,35}, we found no indication for a link with EcR expression in some but not all tissues\textsuperscript{15,35}, we found no indication for a link with EcR expression in in some but not all tissues\textsuperscript{15,35}. Since hemocyte-autonomous loss of dSmad2 signalling causes a more severe phenotype than Actβ lof, we speculate that other Act family ligands such as \textit{daw} and \textit{myo}, which are expressed in various tissues including surface glia, muscle, fat body, gut, and imaginal discs\textsuperscript{11,17,20,36,37} may partially substitute for Actβ in its absence. Overall, Actβ is likely to be only one player in a more complex regulatory network. Future research will identify other inducible signals from neurons that regulate neuron-blood cell communications. This is predicted from Actβ mutants that only partially block carbachol-induced blood cell responses. Actβ/dSmad2 lof and pathway silencing in hemocytes also reveal an underlying ability of the cells to compensate for the lack of this...
signalling pathway and the associated impairment in proliferation. Time course experiments with various RNAi lines suggest that the amplitude and temporal occurrence of the compensatory response may be proportional to the severity of the block in dSmad2 signalling. Future investigation will address whether the related BMP/Mad pathway might play a part in this, as silencing of Mad in hemocytes appeared to dampen elevated hemocyte numbers seen in dSmad2 null mutants. Similar
observations of dSmad2-lof causing Mad overactivation have been reported in the *Drosophila* wing disc and neuromuscular junction previously\(^{20,38}\).

Larval development may comprise distinct sensitive phases for the regulation of hemocyte responses. This is supported by carbachol-promoting hemocyte proliferation preferentially in the early-mid 2nd instar larva, that is, at a stage when hemocytes are still tightly localized to the HPs\(^{4,22}\). Likewise, the effects of Actβ-lof and pathway silencing in hemocytes are more pronounced in younger larvae, suggesting a possible stronger dependence on the pathway, in addition to the emergence of compensatory mechanisms under lob conditions over time (above). Moreover, it will be interesting to investigate whether Actβ-signalling may not only vary temporally, but also by the ability of cell types to produce active Actβ ligand, thereby influencing signalling outcomes, consistent with the cell type specific processing known for Activins and other ligands of the TGF-β family in both invertebrates and vertebrates\(^{36,39,40}\).

*Drosophila* Actβ has previously been studied for its role in the formation and function of neuromuscular junctions in the *Drosophila* larva, where Actβ expressing motor neurons project axons from the CNS, reaching from the center of the larva to the muscle layers of the body wall\(^{16,17,20}\). However, resident hemocytes are shielded from these areas through the muscle layers of the body wall, which also form the base of the HPs, thereby creating an anatomical space between the muscle layers and epidermis\(^4\) where resident hemocytes and Actβ expressing sensory neurons colocalize (i.e., the Hematopoietic Pocket). The model that sensory neurons signal to adjacent hemocytes in the HPs is further supported by the fact that Actβ silencing in motor neurons did not affect resident hemocyte localization and had, by t-test, no significant effect on hemocyte numbers. However, we cannot completely rule out involvement of alternative or additional scenarios, for example, that experimental manipulations of PNS activity, which also feed back to the CNS, would in turn trigger a signal to motor neurons that may respond by secreting Actβ and/or another factor/s, thereby influencing hemocytes and/or the PNS itself. Likewise, we confirmed the direct effect of Actβ on hemocytes ex vivo, and found no signs of altered sensory neuron morphology under Actβ-lof/silencing, we cannot rule out that in the larva, Actβ may contribute to molecular changes in the PNS that in turn might contribute to the observed hemocyte effects.

Sensory neurons of the HPs project axons to the CNS\(^{11}\), and our work shows that hemocytes are closely adjacent to and/or form direct contacts with sensory neurons, likely along the neuron cell bodies and dendrites, suggesting the communication involves non-canonical mechanisms. In *Drosophila*, as in vertebrates, signal transfer along all neuronal membrane surfaces, including dendritic synapses and dendro-dendritic connections, have been described\(^{12,21}\), which may also form the interface in neuron-blood cell communication. The transcriptional induction of Actβ in response to sensory stimuli recalls previous reports of the transcriptional upregulation of Actβ in the formation of long-term memory in both flies and vertebrates\(^{44,45}\). This suggests parallels between the neuronal regulation within the CNS, and PNS-blood cell circuits, which will be an interesting subject for future study. Based on our findings and another recent report demonstrating that transcriptional regulation of the related BMP Decapentaplegic (Dpp) in the *Drosophila* wing epithelium depends on the K+ channel Irk2 (ref. 46), we propose that cellular electrochemical potential may be a more general theme in the expression of TGF-β family ligands.

Our findings in the *Drosophila* model pioneer a new concept that has not been shown in any vertebrate system to date— the neuronal induction of self-renewing, tissue-resident blood cells. These cells correspond to the broadly distributed system of self-renewing myeloid cells that are present in most vertebrate organs, which by lineage are completely independent from blood cell formation fueled by hematopoietic stem cells\(^3–6,47,48\). In vertebrates, TGF-β family ligands such as Activin A and TGF-β regulate the activity and immune functions of macrophages, and cellular and humoral immune responses, in multiple ways through autocrine and paracrine signalling\(^{49,50}\). While the autonomic neuronal and glial regulation of hematopoietic stem and progenitor cells in the bone marrow has been recognized\(^{51–55}\), the role of sensory innervation in bone marrow hematopoiesis remains unknown. Even more so, nothing is known about the role of the nervous system in the regulation of the independent, self-renewing myeloid system of tissue macrophages. However, local neurons and sensory innervation of many organs including skin, lung, heart and pancreas\(^{56–59}\) and inducible changes in the self-renewal rates of tissue macrophages\(^2\), suggest that principles of neuronal regulation are likely also at work in vertebrates, providing a link between neuronal sensing and adaptive responses of local blood cell populations.

**Methods**

**Fly strains.** *Drosophila* lines used were *HmlA-DsRed* (2 copies)/CyO (ref. 4), *Actβ-lof/+/unc13GFP* (ref. 21), *tub-GAL80* (ref. 60), *Actin5c-GAL4* (Bloomington), *Actβ-GAL4* (ref. 18), *da-GAL4* (ref. 21), *myo-GAL4* (ref. 13), *Cha-GAL4* (Bloomington), *en-GAL4* (Bloomington), *UAS-Kir2.1* expressing *Drosophila* wild type (ED80/C0–i). Comparison of control and *Actβ-lof/silencing, we cannot rule out that in the larva, Actβ may contribute to molecular changes in the PNS that in turn might contribute to the observed hemocyte effects.

Sensory neurons of the HPs project axons to the CNS\(^{11}\), and our work shows that hemocytes are closely adjacent to and/or form direct contacts with sensory neurons, likely along the neuron cell bodies and dendrites, suggesting the communication involves non-canonical mechanisms. In *Drosophila*, as in vertebrates, signal transfer along all neuronal membrane surfaces, including dendritic synapses and dendro-dendritic connections, have been described\(^{12,21}\), which may also form the interface in neuron-blood cell communication. The transcriptional induction of Actβ in response to sensory stimuli recalls previous reports of the transcriptional upregulation of Actβ in the formation of long-term memory in both flies and vertebrates\(^{44,45}\). This suggests parallels between the neuronal regulation within the CNS, and PNS-blood cell circuits, which will be an interesting subject for future study. Based on our findings and another recent report demonstrating that transcriptional regulation of the related BMP Decapentaplegic (Dpp) in the *Drosophila* wing epithelium depends on the K+ channel Irk2 (ref. 46), we propose that cellular electrochemical potential may be a more general theme in the expression of TGF-β family ligands.

Our findings in the *Drosophila* model pioneer a new concept that has not been shown in any vertebrate system to date— the neuronal induction of self-renewing, tissue-resident blood cells. These cells correspond to the broadly distributed system of self-renewing myeloid cells that are present in most vertebrate organs, which by lineage are completely independent from blood cell formation fueled by hematopoietic stem cells\(^3–6,47,48\). In vertebrates, TGF-β family ligands such as Activin A and TGF-β regulate the activity and immune functions of macrophages, and cellular and humoral immune responses, in multiple ways through autocrine and paracrine signalling\(^{49,50}\). While the autonomic neuronal and glial regulation of hematopoietic stem and progenitor cells in the bone marrow has been recognized\(^{51–55}\), the role of sensory innervation in bone marrow hematopoiesis remains unknown. Even more so, nothing is known about the role of the nervous system in the regulation of the independent, self-renewing myeloid system of tissue macrophages. However, local neurons and sensory innervation of many organs including skin, lung, heart and pancreas\(^{56–59}\) and inducible changes in the self-renewal rates of tissue macrophages\(^2\), suggest that principles of neuronal regulation are likely also at work in vertebrates, providing a link between neuronal sensing and adaptive responses of local blood cell populations.

**Methods**

**Fly strains.** *Drosophila* lines used were *HmlA-DsRed* (2 copies)/CyO (ref. 4), *Actβ-lof/+/unc13GFP* (ref. 21), *tub-GAL80* (ref. 60), *Actin5c-GAL4* (Bloomington), *Actβ-GAL4* (ref. 18), *da-GAL4* (ref. 21), *myo-GAL4* (ref. 13), *Cha-GAL4* (Bloomington), *en-GAL4* (Bloomington), *UAS-Kir2.1* (GMR85E05-GAL4) insertion in

**Figure 6 | Neuronal activity promotes Actβ expression which drives hemocyte expansion.** (a–d) Carbachol induces dose-dependent transcriptional induction of an Actβ reporter. (a) Quantification of luciferase expression in larval fillets of Actβ-GAL4, UAS-Cd4-tdGFP/UAS-luciferase animals, stimulated by increasing doses of carbachol as intact larvae (2.5 mg ml\(^{-1}\), 10 mg ml\(^{-1}\)). Pools of 2–3 larval fillets per sample in biological replicates; carbachol-stimulated conditions were normalized to unstimulated condition (total n = 4–6 per condition); statistics show average of four such normalized independent experiments. (b) Carbachol-induced increase of GFP expression in fillets of Actβ-GAL4, UAS-Cd4-tdGFP larvae, signal quantification over three independent sets of images, normalized to unstimulated condition and averaged (n = 3). (c) Sample set of images as in b. (c) Control, (d) 4 h stimulation with carbachol as intact larva, 2.5 mg ml\(^{-1}\). (e) Transient PNS silencing with *UAS-Kir2.1* reduces GFP expression; induction for 22 h at 27 °C. (f) Fraction of larvae showing visible GFP expression in PNS neurons, genotype tubGAL80\(^{i+h}\); *Actβ-GAL4*, UAS-Cd4-tdGFP/+; control and tubGAL80\^{i+h}; *Actβ-GAL4*, UAS-Cd4-tdGFP/UAS-Kir2.1 (experiment); n = 20 to 22, average of 2 independent biological replicates. (f) Sample images of control and *Kir2.1* expressing larva. Note that *Kir2.1* coexpression reduces GFP expression in PNS and CNS on left side (anterior). (h) Comparison of hemocyte expansion in *Actβ-lof* mutant larvae and controls, genotypes *HmlA-DsRed*/+; *Actβ-lof/+/Drospos* and control *HmlA-DsRed*/+. Total hemocyte counts per larva of carbachol-treated cohorts (0.2 mg ml\(^{-1}\)) and control food cohorts; n = 6–8 per genotype and condition. (i) Comparison of control and *Actβ-lof* mutant ratio of hemocytes to HPs after short-term carbachol exposure (10 mg ml\(^{-1}\), 15 min), genotypes as in h, dorsal view. (k) Model. Actβ in sensory neuron-induced blood cell adaptation. PNS neuronal activity triggered by carbachol elevates Actβ expression in neurons, resulting in enhanced hemocyte proliferation and localization to HP. Neuronal silencing has opposite effects. Scale bars, c, d, 100 μm; g, 0.5 mm; i, 0.2 mm. Error bars represent s.d., and two-tailed t-test values correspond to NS (not significant) P > 0.05; *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001; ****P ≤ 0.0001.
UAS-Act

UAS-Stinger

Hml
(ref. 61),
He-GAL4
(ref. 28). UAS-RNAi lines for
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protocols, staining overnight with gentle agitation4,5. Lymph gland dissections and
were removed, and the fillet was fixed for 20 min in 4% PFA. Fillets were washed in
inspection of intact live larvae, excluding the compact clusters of hemocytes in the
blood cells were visualized by fluorescence microscopy and counted by external
immunocytochemistry4,5. For larval fillet preps, larvae were pinned down on
allowed to leak out, avoiding to apply pressure. To prevent dislodging of resident
hemocytes or contamination with lymph gland hemocytes, larvae were monitored
through a fluorescence stereomicroscope. Resident hemocytes were released by
opening the remainder of the larva and scraping the body wall with a needle under microscopy
and guidance, avoiding the lymph gland. For the release of total hemocyte numbers, both procedures were combined. Hemocytes were allowed to attach to
glass slides for 15–30 min, followed by standard fixation (4% PFA) and
immunocytochemistry4,5. For larval fillet preps, larvae were pinned down on
Sylgard plates and ventrally filleted in a drop of PBS. Gut, fat body and trachea were removed
d Geometry for neuronal silencing experiments
from the corresponding author on reasonable request.
study are available within the article and its Supplementary Information files or
Data availability. The authors declare that all data supporting the findings of this
study are available within the article and its Supplementary Information files or
from the corresponding author on reasonable request.

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Author contributions

K.B. and K.M. designed experiments. K.M., B.A., D.R., S.P., L.H., K.K., I.B., S.W., K.S.G., C.W. and K.B. performed experiments. M.B.O. provided Activin-ß pathway tools and expertise. K.B. conceived the study. K.B., K.M., B.A., D.R., S.P., L.H., K.K., I.B., S.W. and K.S.G. evaluated the data and K.B. wrote the manuscript with input from all authors.

Additional information

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