Peer Review File

Manuscript Title: Gut cytokines modulate olfaction through metabolic reprogramming of glia.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee expertise:

Referee #1: fly neurosensory function, diet, immune function; aging

Referee #2: fly neuron/glia metabolic coupling

Referee #3: fly gut signaling, tissue interaction, metabolism

Referees' comments:

Referee #1:

In this manuscript from Cai et al., the authors provide extensive evidence documenting the effects of intestinal signals on specific populations of glial cells that influence neuronal function. These signals apparently arise from damaging events in the gut, such as pathogen infection and aging, and involve the secretion of Unpaired cytokines that activate JAK/STAT signaling in specific glia to alter lipid metabolism. Extended periods of JAK/STAT in the glia cause loss of olfactory function that is evident in older animals, while short-term activation, such as that caused by acute infection, appears largely reversible once gut damage has subsided. The authors have done a remarkable job tracing cause and effect from the EC cells in the gut to ensheathing glia in the brain, and their expertise in uncovering regulatory mechanisms of JAK/STAT signaling is evident in the overwhelming strength of support for this pathway in modulating glial metabolism. In this sense, the work is a major contribution, and in my opinion, deserves strong support for publication in Nature. The ecological context for this system, and the inference that links this signaling pathway with an adaptive response to infection, however, is less supported and developed. Specific comments in this regard are provided below, and while additional insights in this area would expand the profile and impact of this work, it is also reasonable to consider further investigations of this sort outside the scope of what is already a large and important manuscript.

Minor comments:

Pg 5, line 84: The authors should make clear that an acute oral infection protocol, as opposed to injection or “poking”, was used to create infections. This became clear later on, but it should be mentioned here in the first reference to infection.

Pg 5, Line 89: The authors seek to test whether aversion against Ecc15 also involves changed in olfactory perception. I interpreted this to imply that the authors wanted to determine whether olfaction is involved in the feeding aversion phenotype, which is not tested. This brings up the broader question of whether olfaction has anything in particular to do with the infection-induced changes in feeding preference and/or survival. The authors’ model predicts that it does, but this is never tested, although it may be implied from published work. Nevertheless, it would be insightful to know whether olfactory blind animals (e.g., Orco, Gr63a, Ir25a, Ir8a mutants) exhibit Ecc15- or PE-dependent changes in these two phenotypes as the loss of olfactory discrimination argued in Fig. 1d was never shown to be sufficient for these phenotypes. Or to determine whether manipulation of UPD, JAK/STAT leads to behavioral phenotypes in smell blind animals. Complicating this interpretation is that several of the treatments interpreted as

impacting attraction/aversion to odors appear to alter discrimination rather than eliminate olfactory influence all together, the latter of which should lead to PI=0 and which is often not the case (e.g., Figs 1i, 2f, etc).

Pg 5, line 95: For inference such as that drawn from Fig. 1d, the authors should consider directly testing for a significant statistical interaction term that would indicate that (in this case) time since infection influences the effect of infection on PI. Piecing together pairwise t-tests is generally considered inappropriate in this context. The difficulty associated with the pairwise approach is highlighted by Fig. 1d, right panel, in which the loss of a difference between Mock and Ecc15 treatments 4 days post-infection appears to have as much to do with an increase in PI in the mock-infected as a decrease in the infected. A full two-way ANOVA approach (or its non-parametric analog) would help quantify the importance of such trends.

I was not able to find details on the mock infection protocol, and important details of the infection protocol were also not included. Were mock-infected flies given normal food instead of OD100 in 5% sucrose? Or just 5% sucrose? And how long were they fed this cocktail? Were they fed in capillary tubes? How much did infected and mock-infected flies consume during this period? Would the authors please add these details to the methods.

Do dead bacteria elicit gut-dependent UPD signaling following consumption? If not, it would be important to know whether the bacteria need to be alive to elicit the observed phenotypes (either in the acute infection protocol or in longer term feeding assays). This might in principle, distinguish infection per se, from sensory input.

I believe that the authors' interpretation of the survival data (e.g., Fig. 1k) are that the feeding differences caused by loss of olfaction lead to different “effective doses” among genotypes, which are reflected in differences in survival. If I understand this correctly, the authors might consider establishing it more clearly, ideally by providing a fixed dose via capillary and showing no difference in survival. This would rule out non-specific effects of the genetic manipulations on tolerance. Doses of PE as low as a few cells are often enough to kill flies, so this could be reasonably well-controlled.

Fig. 3e: The authors infer from these data that knockdown of Dome or STAT rescues an age-dependent decline in olfaction sensitivity. While the data are consistent with this interpretation, they are not definitive. Such inference requires that the manipulations do not affect (i.e., increase) PI in young individuals. It is possible that knockdown increases PI in young animals and that PI declines significantly with age (while still ending higher than young animals). This applies to other similar points of inference as well (e.g., Fig. 4c).

Referee #2:

Cai et al. present an interesting analysis of long-distance JAK/STAT signaling between the gut and brain using a Drosophila model. They demonstrate that JAK/STAT signaling can be activated in ensheathing glia of the brain through Unpaired cytokine expressing in the gut upon infection of the enteropathogen, Erwinia Carotovora. Activation of this signaling pathway induces cellular changes, including glial lipid droplet formation, and organismal behavior changes, including altered olfaction sensitivity. The authors go on to identify key glial proteins required for this effect including the apolipoprotein, Glaz, and the monocarboxylate transporter, Outsiders. They demonstrated that reduced expression of these proteins reduced glial lipid droplet formation but, surprisingly, also partially rescued age-related olfaction sensitivity decline. The insights gleaned are of interest to a wide number of readers and novel. However, the authors often make broad claims about neurodegeneration which are unsubstantiated with the data provided. These statements should be removed or altered to reflect what the authors actually assessed. Additionally, the authors need to address the following concerns before the manuscript can be considered for publication in Nature.

Major Points

1. It is unclear why the authors observed such a specific phenotype in ensheathing glia when the cytokines inducing the phenotypes are secreted from the gut, presumably into the hemolymph. There are many different cell types that these cytokines would interact with prior to reaching the antennal lobes. Are the Unpaired receptors only expressed in ensheathing glia? Or is there other data the authors can provide as to why the phenotypes they observed are so specific? It is unclear whether Upd2/3 expression could be derived solely from the gut and not from other tissues (e.g. glia, neurons, fat body, hemocytes). The authors should demonstrate that Upd2/3 RNAi in other tissues (using tissue-specific Gal4 drivers) does not affect the brain phenotypes in order to bolster support of the conclusion that the cytokines are gut-derived, particularly since there is RFP "background" signal seen with NP1-GAL4 in Fig. 2b.

2. In the T-maze assays, the authors should state whether the genotype distributions are different from baseline (zero) in addition to stating whether the groups are different from each other. For example, in Fig. 1d, while there is no difference between mock- and ECC15-treated flies in aversive odor detection 4 days post-infection, it's not clear whether those flies are performing avoidance behaviors or not. Additionally, the authors should assess whether other behaviors that might affect olfactory attraction/avoidance are affected (locomotion, etc.).

3. The authors need to provide full genotypes of the files used in all experiments and ensure that the RNAi and UAS lines used are in the same genetic background as the control, (mCherry RNAi). This is especially important because the mCherry RNAi line used is from the TRiP library and is in a w+ background, while the KK RNAi lines and UAS-lipase-4 used in the study are in w- genetic backgrounds. The white gene encodes an ABC-lipid transporter which may alter the pathways and behaviors analyzed in this study (see ABCG1 (ABC8), the human homolog of the Drosophila white gene, is a regulator of macrophage cholesterol and phospholipid transport. Klucken et al. PNAS, 2000). It is important to note that the mini-w+ reporter expressed with each transgene is a truncated version of the full white gene and thus may not have full function. Please repeat experiments as needed to make sure that control and target RNAi are derived from animals with the same genetic background. The authors should also include a no-RNAi control (e.g. UAS-empty) in all RNAi experiments.

4. In Figs 1e, 2c,d, please provide western blots for STAT:GFP with quantification as this would help to substantiate IF data using an alternative approach. It may be interesting to compare head vs body derived protein to see if STAT:GFP upregulation in the brain is specific to the brain or if this is a general, ubiquitous effect.

5. Generally, the size of all the confocal images in this manuscript should be enlarged for ease of readability and data interpretation. Further, please state and show where in the brain all images were taken. The authors should consider showing whole images of the brain in addition to the zoomed-in images provided. The authors should also consider including additional, representative images from other animals in Extended Data.

6. For RNAi experiments (e.g. Fig. 2e), the authors should quantify knockdown efficiency for all UAS-RNAi lines used. Knockdown should be shown on the protein level by western blot with quantification if possible. For previously published RNAi, please state the knockdown efficiency previously reported. Also, the authors should consider testing multiple RNAi per target to confirm specificity of key results versus an off-target effect from a single RNAi, particularly when using KK RNAi lines.

7. The authors emphasize throughout the text that their findings directly pertain to disrupted neuron function and neurodegeneration. However, neuron activity and death were not directly assayed. Rather, olfactory phenotypes were noted and the authors conclude that alterations are due to neuron loss. However, many tissues are likely disrupted by their manipulations and the cytokine pathways, and these may contribute to olfaction deficits. The authors should consider including investigations more directly demonstrating that there are indeed changes in neuron activity and neuron loss to directly demonstrate relevance to neurodegeneration.

Minor points

8. Information in the introduction should be facts that are necessary for one to understand the investigations to follow. Much of the information in the introduction belongs in the discussion, while connections between olfaction and infections, the fly olfactory system, and glia and inflammation should be the key topics discussed in the introduction. For example, neuron loss, AD, and PD are not directly investigated in the paper, yet there are ~4 sentences on neurodegeneration, AD and PD. All information on how the findings from this study impact understandings on diseases not directly tested should be moved to the discussion. Also, paragraphs 1 and 3 are two seemingly disconnected topics until you get to the last paragraph that mentions a common feature of acute vs chronic inflammation which connects pathogen-mediated anorexia and aging and nicely sets the reader's expectations for the following studies. Thus, a paragraph discussing inflammation and the JAK/STAT pathway in the introduction would be helpful for readers to be better prepared for the results section.

9. Line 65: For clarity "…of the Lipocalin Glaz and the Monocarboxylate Transporter (MCT) Outsiders, resulting…" should be rewritten as "…of the lipocalin, Glaz, and the monocarboxylate transporter (MCT), Outsiders, resulting…".

10. Line 101: Please put a comma after "cytokines".

11. Provide a better description of the Stat::GFP lines in the results and figure legends. For example, "Specifically, these express GFP from 2x or 10x STAT92E promoter."

12. It is a bit confusing to see descriptors like "7d" and "4h" on the figures without thorough explanation. What does "7d" mean? Are 7d animals fed pathogen for 4h then immediately assayed? Or was GFP signal assayed 7d after a 4h infection? In this case, the data would be inconsistent with Fig. 1d showing that the effects of infection are transient.

13. In Fig. 7d,e, the authors have two temperatures listed (25 and 29). Please check that this is correct and clarify why two different temperatures are justified in these experiments.

14. In the discussion, the authors should consider a paragraph describing their data in the context of data describing potential connections between infections and dementia.

15. Line 333: please define the term "EGs".

Referee #3:

This manuscript describes a new inter-organ pathway that becomes transiently activated upon infection to reduce olfactory perception. Cytokines made by the intestine of Ecc15-infected flies remotely activate JAK-STAT signalling in ensheathing glia. This leads to metabolic changes within glia, including changes in lipid metabolism, and a reduction in olfactory sensitivity. This pathway promotes avoidance of bacteria-laced food in response to infection, and its prolonged activation (which occurs in aged flies) also leads to a chronic decline in olfactory sensitivity.

The findings are interesting, timely and provocative. I find most papers in this area unsatisfying as they typically report correlative (and potentially meaningless) changes in the brain in response to gut infection/inflammation/microbiota. This paper stands out from this perspective because it leverages the powerful genetics of Drosophila to: 1) show that gut-to-glia communication is (patho)physiologically relevant both in response to infection and in the context of age-related inflammation; and 2) identify the mechanism involved and establish causation. This extends recent work in this area (Kobler et al. and Charroux et al., both mentioned in the manuscript) by revealing an involvement of the intestine and glia as novel post-infection mediators.

There are currently several major issues that need to be addressed so that the main conclusions of the manuscript are supported by the data.

EFFECTS ON FOOD INTAKE VS FOOD/OLFACTORY PREFERENCE. Infection-induced anorexia and a specific reduction in intake of bacteria-laden food are different behaviours (food intake vs preference) likely induced by different mechanisms. The data in this manuscript describes changes in preference, partly because their initial characterisation led the authors to conclude Ecc15 infection does not reduce food intake. However, the data was not very strong (relatively low n numbers, there is a trend toward reduction, Fig. 1b) and contradicts previous findings (e.g. Kobler et al. but also others). This is important because reduced olfactory sensitivity might result from starvation; how does starvation affect olfactory discrimination and/or avoidance of bacteria-laced food? Does food intake change in aged flies? Also, I note that in this regard that the same cytokine that results in bacteria avoidance in this study has been shown to signal the fed state in a different study (PMID: 23021220). Related to this, is it eating more Ecc15 or eating more generally that makes flies sick?

In this context, the authors need to clarify their definitions of olfactory 'sensitivity' and 'perception', which they use interchangeably and could be interpreted as different things - either a general reduction in response to odours as opposed to distinguishing between different odours. They also need to ensure that the abstract and intro are better aligned with the data; at the moment, the focus on anorexia is confusing and somewhat misleading.

STATEMENTS ABOUT GLIA-NEURON METABOLIC COUPLING. The manuscript contains no data linking glial cells and olfactory neuron activity, yet several sentences and even a cartoon in the manuscript give the impression that this hypothesis is part of their findings. Such coupling could be tested by blocking the lactate shunt into olfactory neurons and/or (better) preventing the infection-induced reduction in olfactory neurons sensitivity by increasing lactate availability in olfactory neurons.

QUALITY OF THE DATA AND STATISTICAL ANALYSES. These need to be improved. The background stainings in control vs experimental images (e.g. Stat-GFP panels) rarely match, which makes comparisons of the "real" stainings unconvincing. Contrary to what the manuscript states, MyoI-Gal4 is strongly expressed in quite a few neurons in the brain (also in ENS). t-tests are often used for data that may not be normally distributed and/or for experiments involving multiple genotypes/conditions. Images are not always representative of quantifications/statements (e.g. ED1c more nuclei are apparent after Ecc15 than in mock, there seems to be a lot of Stat-GFP expression in ensheathing glia prior to infection…).

DOES REDUCED OLFACTORY SENSITIVITY DRIVE AVERSIVE BEHAVIOUR? Whilst it is clear that Ecc15 infection causes a decline in olfactory sensitivity, does this decline underlie Ecc15 aversion? How do flies respond to the smell of Ecc15 before and after infection? In Fig. 1d line 98, is aversion to Ecc15 removed after 4 days? Another way to test this (and a previous point at the same time) is by interfering with the lactate shunt from the neuronal side.

Related to this, the authors need to integrate their data with recently reported olfactory remodelling "downstream" of ORNs as well gustatory remodelling (Kobler et al., 2020; Charroux et al., 2020, respectively). The authors seem to put forward a model in which gustatory cues are the drivers of the aversive behaviour, with the loss of olfactory sensitivity re-enforcing this. Their own data, however, argues against this since the rescue of olfactory sensitivity by dome or STAT knock down rescues Ecc15 aversion. Are the gustatory neurons therefore also affected by these knockdowns?

SPECIFIC COMMENTS:

The RNA-seq comparison of Stat-GFP positive vs negative glia in uninfected flies struck me as an odd choice. These glial cells may not be the same glial cells that turn Stat-GFP positive upon infection. Can the authors clarify this?

Line 139, Fig. 1k: Were these flies given a choice of bacteria-laden or normal food? Or is survival increased even when it isn't through the hypothesized host defence behaviour of avoiding bacteria-laden food? This may suggest that something else about decreasing odour sensitivity affects survival.

Lines 249-250: The wording is a bit confusing – it seems to suggest that the reason for the glial lipid changes during ageing is the neurons rather than the intestinal cytokine upregulation.

Line 199: 'loss' should be replaced with 'decline' or 'reduction'.

Lines 189-191: Germ-free may be clearer than sterile, which I first interpreted as infertile…

Line 244: Please make clear that this is in infected flies.

Fig. 1h: Please show GFP as a single panel and quantify; it is the most important co-expression of those shown in the figure.

Author Rebuttals to Initial Comments:

Response to reviewers - 2020-10-18688

We would like to thank the reviewers for the positive reception of our work and for the constructive critiques. We appreciate that the reviewers found the biological questions addressed of considerable potential significance, the study exciting, and our model compelling, and the work to be a major contribution that deserves strong support for publication in *Nature*.

While generally very supportive, the reviewers also raised a few important concerns. The main questions raised by the reviewers related to (i) the role of odor perception in the observed avoidance behavior of flies towards *Ecc15*, (ii) the molecular mechanism underlying the specific response of ensheathing glia to intestinal inflammation, (iii) the role of other tissues (likehemocytes or fatbody) in STAT activation in glia upon infection, (iv) the role of the glia/neuron lipid and lactate shuttles in the regulation of olfaction sensitivity. We have now addressed these questions with additional experiments and by editing the manuscript (we have also had to significantly shorten the manuscript in keeping with Nature's editorial requirements). We believe that our new results resolve the reviewers' concerns, and that they significantly strengthen our manuscript. We summarize these new data here and respond to the reviewers' comments point-by-point below:

(i) Reviewers 1 and 3 raised the important question of whether reduced olfaction sensitivity is required for flies to develop avoidance behavior towards enteropathogens. We have now performed CAFE assays on olfactory deficient flies ($Gr63a^1$ and $Orco^1$) both without and with *Ecc15* infection, and found that uninfected olfactory deficient flies specifically reduced *Ecc15* food intake, phenocopying the infection-induced avoidance behavior of wild-type flies. Infection did not further reduce *Ecc15* food intake of olfactory receptor mutants, supporting the interpretation that downregulation of odor perception promotes the rejection of *Ecc15* containing food in wildtype flies (or reduces their attraction to such food). This is consistent with recent studies showing that flies lacking functional Gr63a or Orco odorant receptors fail to be attracted by *Ecc15* (Kobler, J. M. *et al*. 2020 *Curr Biol*; Charroux, B. *et al*. 2020. *iScience*).

(ii) Reviewer 2 asked why ensheathing glia could specifically respond to gut-derived Upd ligands by upregulating STAT activity, but not other subtypes of glia. We have now performed single-cell RNAseq analysis on all glia and on ensheathing glia purified from the central brain of young and old flies, and found that the mRNA expression level of the *dome* receptor in ensheathing glia is significantly higher than in non-ensheathing glia. Moreover, *socs36E*, a known target gene of JAK/STAT signaling, is found to be

upregulated only in ensheathing glia during aging, but not in other subtypes of glia. These results were further confirmed by additional analysis performed on the whole brain scRNAseq dataset published by the Aerts lab (Kristofer Davie, *et al*. 2018. *Cell*).

(iii) Reviewer 2 asked whether Upd ligands derived from other tissues also contribute to glial STAT activation. In fact, our data suggest that gut-derived Upd ligands are not just sufficient but also required for STAT activation in ensheathing glia and for the regulation of olfaction sensitivity upon infection and during aging. We have now asked whether STAT activity in ensheathing glia at the antennal lobe would also be affected when Upd ligands were overexpressed or knocked down in fatbody or hemocytes. These experiments failed to reveal a response in ensheathing glia to ligands derived from these tissues.

We have now further confirmed our original observations (which were obtained in part using NP1::Gal4 and in part using Mex1::Gal4) by repeating all experiments using Mex1::Gal4 exclusively. As discussed in our original submission, this driver shows noleaky expression in the brain in contrast to NP1::Gal4. Our results further support cross-talk between the gut and antennal lobe glia.

(iv) Reviewer 2 and 3 requested more evidence supporting the role of glia-neuronal metabolic coupling in the observed phenotypes. We agree that this is an important question, and have performed additional olfactory T maze assays, CAFE assays and *PE* survival assays accordingly on young or old flies in which lactate dehydrogenase (*ldh*), lactate shuttle (*out*), or the neuronal lipid binding protein Nlaz were knocked down specifically in projection neurons. We observed that reducing lipid transport or lactate intake in projection neurons alleviates the downregulation of olfactory discrimination upon infection or during aging, consistent with the model we proposed.

In addition to these new experiments, we have also carried out additional control experiments recommended by the reviewers, including adding more control lines for VDRC KK lines and overexpression lines, repeating experiments with additional RNAi lines for important target genes (*dome*, *stat, upd2*, *upd3*, *Glaz* and *out*), validation of the RNAi lines used in the paper by qPCR, performing flow cytometry analysis to quantify STAT::GFP expression in ensheathing glia upon infection to validate the immunostaining results, testing whether only live entheropathogens leadto changes of olfaction sensitivity and to reduced Ecc15 food intake, investigating how starvation influences olfactory discrimination and food choice, improving statistical analysis and the quality of images throughout the manuscript, and adding full fly genotypes for each figure. Experiments

addressing other minor concerns have also been included, as discussed in the point-by-point response below.

Altogether, we believe these new data result in a substantial improvement of the manuscript, addressing the most significant concerns of the reviewers, and further supporting our proposed model.

Specific responses (Bold)

Referees' comments:

Referee #1:

In this manuscript from Cai et al., the authors provide extensive evidence documenting the effectsof intestinal signals on specific populations of glial cells that influence neuronal function. These signals apparently arise from damaging events in the gut, such as pathogen infection and aging, and involve the secretion of Unpaired cytokines that activate JAK/STAT signaling in specific glia to alter lipid metabolism. Extended periods of JAK/STAT in the glia cause loss of olfactory functionthat is evident in older animals, while short-term activation, such as that caused by acute infection, appears largely reversible once gut damage has subsided. The authors have done a remarkable job tracing cause and effect from the EC cells in the gut to ensheathing glia in the brain, and their expertise in uncovering regulatory mechanisms of JAK/STAT signaling is evident in the overwhelming strength of support for this pathway in modulating glial metabolism. In this sense, the work is a major contribution, and in my opinion, deserves strong support for publication in Nature. The ecological context for this system, and the inference that links this signaling pathway with an adaptive response to infection, however, is less supported and developed. Specific comments in this regard are provided below, and while additional insights in this area would expand the profile and impact of this work, it is also reasonable to consider further investigationsof this sort outside the scope of what is already a large and important manuscript.

We would like to thank the reviewer for the comprehensive and positive review of our manuscript. While a deep further exploration of the ecological context of our findings may indeed be beyond the scope of this manuscript, we have performed a series of additional studies to address the specific comments listed below:

Minor comments:

1. Pg 5, line 84: The authors should make clear that an acute oral infection protocol, as opposedto injection or “poking”, was used to create infections. This became clear later on, but it should be mentioned here in the first reference to infection.

We thank the reviewer for pointing this out. We have clarified this, emphasizing “oral infection” throughout.

2. Pg 5, Line 89: The authors seek to test whether aversion against Ecc15 also involves changedin olfactory perception. I interpreted this to imply that the authors wanted to determine whether olfaction is involved in the feeding aversion phenotype, which is not tested. This brings up the broader question of whether olfaction has anything in particular to do with the infection-induced changes in feeding preference and/or survival. The authors’ model predicts that it does, but this is never tested, although it may be implied from published work. Nevertheless, it would be insightful to know whether olfactory blind animals (e.g., Orco, Gr63a, Ir25a, Ir8a mutants) exhibit Ecc15- or PE-dependent changes in these two phenotypes as the loss of olfactory discrimination argued in Fig. 1d was never shown to be sufficient for these phenotypes. Or to determine whether manipulation of UPD, JAK/STAT leads to behavioral phenotypes in smell blind animals. Complicating this interpretation is that several of the treatments interpreted as impacting attraction/aversion to odors appear to alter discrimination rather than eliminate olfactory influence all together, the latter of which should lead to PI=0 and which is often not the case (e.g., Figs 1i, 2f, etc).

While it is important to point out that the mean P.I. values for flies with reduced olfaction sensitivity were close to 0 in our experiments (while exhibiting a reasonable experimental spread), we do agree that the relationship between olfactory perception and the aversion phenotype needed more work. Previous studies reported that the odorant receptors Orco and Gr63a are required for flies to be attracted to *Ecc15* (Kobler, J. M. *et al*. 2020 *Curr Biol*; Charroux, B. *et al.* 2020. *iScience*). To assess whether these receptors also influence food preference in our experimental paradigm, we performed CAFE assays with these odorant receptor mutants with or without *Ecc15* infection. We found that non-infected odorant receptor mutant flies took in significantly less *Ecc15* food under homeostatic conditions with no changes in normal food intake, consistent with the loss of attraction to *Ecc15* reported earlier (Extended Data Fig.1c). This also phenocopied the infection-induced avoidance behavior seen in wild-type flies, and, accordingly, infection failed to further induce stronger avoidance behaviors in these odorant receptor mutants (Extended Data Fig.1c). Together, these findings support the idea that a loss of odor perception indeed

contributes to the observed avoidance behavior. We discuss a model in which suppressing olfaction is important to prevent continued attraction to the enteropathogen containing food after pathogenicity has been established and aversion is induced by gustatory and immune receptors (as reported by Kobler and Charroux).

As requested by the reviewer, we have also assessed survival of odorant receptor mutant flies when exposed to PE, and found that Gr63a^{1} flies have increased resistance towards *PE* infection compared to wild-type flies, while Orco^{1} flies succumbed faster (Extended Data Fig.3h). As these are germline mutants, it is difficult to attribute these differences in mortality a role for these receptors in olfaction exclusively, as these receptors could be acting in other tissues.

3. Pg 5, line 95: For inference such as that drawn from Fig. 1d, the authors should consider directly testing for a significant statistical interaction term that would indicate that (in this case) time since infection influences the effect of infection on PI. Piecing together pairwise t-tests is generally considered inappropriate in this context. The difficulty associated with the pairwise approach is highlighted by Fig. 1d, right panel, in which the loss of a difference between Mock and Ecc15 treatments 4 days post-infection appears to have as much to do with an increase in PI in the mock-infected as a decrease in the infected. A full two-way ANOVA approach (or its non-parametric analog) would help quantify the importance of such trends.

We thank the reviewer for pointing this out. We have now performed a Kruskal-Wallis test on the data for Fig.1d and other T maze assays instead, as nonparametric tests were also suggested by other reviewers. We have now also repeated and modified that experiment by performing additional T maze assays on independent batches of T maze-naive flies at 1 or 5 days post *Ecc15* infection. The age of these cohorts was controlled to be the same at the timepoint of the T maze test. This allowed us to exclude any potential influence of olfactory memory or of age differences of these flies. The new data are now reported.

4. I was not able to find details on the mock infection protocol, and important details of the infection protocol were also not included. Were mock-infected flies given normal food instead of OD100 in 5% sucrose? Or just 5% sucrose? And how long were they fed this cocktail? Were they fed in capillary tubes? How much did infected and mock-infected flies consume during this period? Would the authors please add these details to the methods.

We have now added a more detailed *Ecc15* infection protocol in the methods. To infect flies, 500ul 5% sucrose solution (mock condition) or 500ul sucrose solution mixed with

***Ecc15* (*Ecc15* was resuspended in sucrose solution, OD100) was added onto Whatman filter paper in an empty vial. Mock flies were fed with 500µl 5% sucrose solution only, whileinfected flies were fed with 500µl *Ecc15* resuspended in 5% sucrose solution. Flies were infected with *Ecc15* for 4 hours or 24 hours, as specified in corresponding figures. Flies were allowed to feed *ad libitum* on these food sources, making calculating exact food consumption impossible. Previous studies had shown that Upd cytokines are induced in enterocytes of infected animals using the same protocol (Jiang, *et al. Cell*. 2009; Buchon,*et al. Cell Host Microbe*. 2009).**

5. Do dead bacteria elicit gut-dependent UPD signaling following consumption? If not, it would be important to know whether the bacteria need to be alive to elicit the observed phenotypes (either in the acute infection protocol or in longer term feeding assays). This might in principle, distinguish infection per se, from sensory input.

We have performed the olfactory T maze assay and CAFE assay on flies infected with heat-killed *Ecc15*, and found no differences in both assays compared to mock flies (Extended Data Fig.1b, 1d). This is consistent with the observation that Upd ligands are produced by damaged enterocytes upon infection by pathogenic bacteria; i.e. dead bacteria are not expected to elicit gut-dependent Upd signaling.

6. I believe that the authors' interpretation of the survival data (e.g., Fig. 1k) are that the feeding differences caused by loss of olfaction lead to different “effective doses” among genotypes, which are reflected in differences in survival. If I understand this correctly, the authors might consider establishing it more clearly, ideally by providing a fixed dose via capillary and showing no difference in survival. This would rule out non-specific effects of the genetic manipulations on tolerance. Doses of PE as low as a few cells are often enough to kill flies, so this could be reasonably well-controlled.

We understand this concern raised by the reviewer. However, we believe that providing a fixed dose of *PE* via capillaries to flies would not guarantee that flies would consume a fixed dose of *PE* food (i.e. they would still have the ability to consume less). As such, it would be impossible to control the volume of food every fly takes in over the period of theexperiment, and the question of non-specific genetic effects on tolerance would remain unresolved. We have acknowledged this in the discussion of these experiments.

7. Fig. 3e: The authors infer from these data that knockdown of Dome or STAT rescues an age-dependent decline in olfaction sensitivity. While the data are consistent with this interpretation,

they are not definitive. Such inference requires that the manipulations do not affect (i.e., increase)PI in young individuals. It is possible that knockdown increases PI in young animals and that PI declines significantly with age (while still ending higher than young animals). This applies to other similar points of inference as well (e.g., Fig. 4c).

To test whether knocking down these target genes would increase olfaction sensitivity in young flies, we have performed the T maze assay with young flies in which *upd2* or *upd3* were knocked down in enterocytes under mock treated conditions, and we found no differences compared to wild-type controls (Extended Data Fig.4d). These results further support our conclusion that reducing Upd/JAK/STAT signaling rescues the age-related decline of olfaction sensitivity.

Referee #2:

Cai et al. present an interesting analysis of long-distance JAK/STAT signaling between the gut and brain using a Drosophila model. They demonstrate that JAK/STAT signaling can be activatedin ensheathing glia of the brain through Unpaired cytokine expressing in the gut upon infection ofthe enteropathogen, Erwinia Carotovora. Activation of this signaling pathway induces cellular changes, including glial lipid droplet formation, and organismal behavior changes, including altered olfaction sensitivity. The authors go on to identify key glial proteins required for this effectincluding the apolipoprotein, Glaz, and the monocarboxylate transporter, Outsiders. They demonstrated that reduced expression of these proteins reduced glial lipid droplet formation but,surprisingly, also partially rescued age-related olfaction sensitivity decline. The insights gleaned are of interest to a wide number of readers and novel. However, the authors often make broad claims about neurodegeneration which are unsubstantiated with the data provided. Thesestatements should be removed or altered to reflect what the authors actually assessed. Additionally, the authors need to address the following concerns before the manuscript can be considered for publication in Nature.

We thank the reviewer for the positive feedback on our study. We have now made changesto the manuscript that remove overly broad statements about neurodegeneration. We have further addressed the other concerns as described below:

Major Points

1. It is unclear why the authors observed such a specific phenotype in ensheathing glia when the cytokines inducing the phenotypes are secreted from the gut, presumably into the hemolymph. There are many different cell types that these cytokines would interact with prior to reaching the antennal lobes. Are the Unpaired receptors only expressed in ensheathing glia? Or is there other data the authors can provide as to why the phenotypes they observed are so specific?

This is indeed an interesting question that remained unresolved in our original submission. We have now sought to gain insight into the specific activation of JAK/STAT signaling in ensheathing glia using single-cell RNAseq. We purified either all glia (labeled using repo::Gal4) or ensheathing glia specifically (labeled using GMR56F03::Gal4) from young and old flies followed by Smart-seq2 analysis. We found that the expression of *dome* is significantly higher in ensheathing glia than in other glia (Extended Data Fig.7c), an observation that may explain the selective sensitivity of ensheathing glia to Upd ligands.

Accordingly, we found specific upregulation of *socs36E*, a known target gene of JAK/STAT signaling, in ensheathing glia, but not in other glial subtypes, in old flies (Extended Data Fig.7d). This was supported by analysis of the whole brain scRNAseq dataset generated by the Aerts lab (Kristofer Davie, *et al*. 2018. *Cell*), where a similar upregulation of *socs36E*in ensheathing glia was found specifically in old animals (Extended Data Fig.7f). These data support our initial observation that was based on a genetic reporter for JAK/STAT activity.

It is unclear whether Upd2/3 expression could be derived solely from the gut and not from other tissues (e.g. glia, neurons, fat body, hemocytes). The authors should demonstrate that Upd2/3 RNAi in other tissues (using tissue-specific Gal4 drivers) does not affect the brain phenotypes in order to bolster support of the conclusion that the cytokines are gut-derived, particularly since there is RFP "background" signal seen with NP1-GAL4 in Fig. 2b.

While it is important to note that our data and interpretation did not specifically exclude the possibility that other tissues may also signal to ensheathing glia under specific conditions, we have now performed the requested experiments: we overexpressed and knocked down Upd ligands in hemocytes (using hml::Gal4) or fatbody (using cg::Gal4), asthese tissues are common sources of Upds, and, interestingly, did not observe changes in glial STAT activity at the antennal lobe (Extended Data Fig.4f-4h). This further supportsthe specificity of the gut-glia cross-talk after enteropathogen infection.

As we have indeed observed mild leaky expression of Np1::Gal4 in the brain (noted in our original submission), we have now removed all the data related to Np1::Gal4 and repeated experiments with Mex1::Gal4, for which no background expression in the brain was observed (Fig.2b, 2c and Extended Data Fig.4a-4c). The data obtained using the Mex1::Gal4 driver confirm that gut-derived Upd ligands are not just sufficient but also required for STAT activation in ensheathing glia and for the regulation of olfactory discrimination.

2. In the T-maze assays, the authors should state whether the genotype distributions are different from baseline (zero) in addition to stating whether the groups are different from each other. For example, in Fig. 1d, while there is no difference between mock- and ECC15-treated flies in aversive odor detection 4 days post-infection, it's not clear whether those flies are performing avoidance behaviors or not.

We have now repeated the experiments reported in Fig.1d by performing T maze assays on five independent batches of flies at 1 or 5 days post *Ecc15* infection. The age of these

flies was controlled so that flies were the same age in the 1d and 5d post infection cohorts and naïve flies were used for each cohort, so to rule out an influence of olfactory memory. The new results have less statistical noise and more robustly support the notion that olfaction sensitivity of infected flies recovers 5 days post infection (both aversive and attractive odors resulted in clear differences from baseline). Accordingly, the preference index of flies at 5 days after infection is not statistically different from the Mock treated flies at 1 day (now specifically stated in the figure).

Also, we now performed T maze assays on young flies that express *mCherry*^{RNAi}, *upd2*^{RNAi} and *upd3*^{RNAi} in enterocytes under non-infected conditions. There were no differences in olfactory discrimination in these flies, suggesting that these genotypes do not divert in baseline sensitivity when not infected (Extended Data Fig.4d).

Additionally, the authors should assess whether other behaviors that might affect olfactory attraction/avoidance are affected (locomotion, etc.).

When calculating the preference index in the olfactory T maze assay, we only included flies that moved to either arm of the T maze device. As such, flies that did not move were not taken into account.

We have also directly tested locomotion ofinfected flies that express *mCherry*^{RNAi}, *dome*^{RNAi} and *stat*^{RNAi} in ensheathing glia specifically. We didnot observe differences in locomotion between these flies. We have not included these data in themanuscript due to space considerations, but are showing them here (Fig. R1).

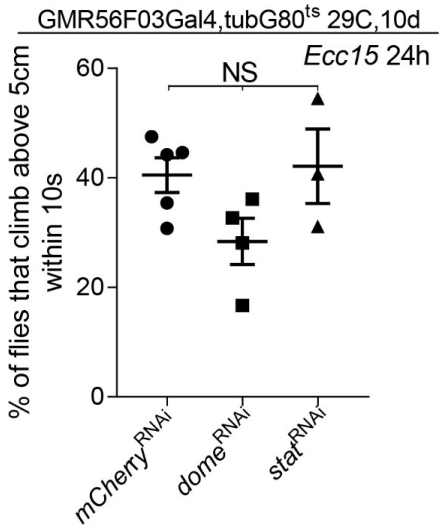

Fig. R1: Percentage of flies that climb above 5cm within 10 seconds. n**=92 flies from 5 replicates for *mCherry*^{RNAi}, 62 flies from 4 replicates for *dome*^{RNAi}, 35 flies from 3 replicates for *stat*^{RNAi}. NS=not significant. Kruskal-Wallis test.**

3. The authors need to provide full genotypes of the files used in all experiments and ensure that the RNAi and UAS lines used are in the same genetic background as the control, (mCherry RNAi).

This is especially important because the mCherry RNAi line used is from the TRiP library and is in a w+ background, while the KK RNAi lines and UAS-lipase-4 used in the study are in w- genetic backgrounds. The white gene encodes an ABC-lipid transporter which may alter the pathways and behaviors analyzed in this study (see ABCG1 (ABC8), the human homolog of the Drosophilawhite gene, is a regulator of macrophage cholesterol and phospholipid transport. Klucken et al. PNAS, 2000). It is important to note that the mini-w+ reporter expressed with each transgene is atruncated version of the full white gene and thus may not have full function. Please repeat experiments as needed to make sure that control and target RNAi are derived from animals withthe same genetic background.

We have now provided full genotypes of the flies used in all experiments (Supplemental Table 1).

Also, we have added two control lines: UAS::lacZ (BL1776) as a control for the overexpression lines with w- genetic backgrounds, like UAS::Hop^{tuml}, UAS::Lip4, UAS::Upd2 and UAS::Upd3; and UAS::*LacZ*^{RNAi} (a gift from Masayuki Miura at University of Tokyo, w- genetic background), as a control for VDRC KK lines. We performed olfactory T maze assays on flies expressing *mCherry*^{RNAi}, UAS::lacZ and *lacZ*^{RNAi} in ensheathing glia (using GMR56F03Gal4) or enterocytes (using Mex1::Gal4) correspondingly, and did not observe significant differences among these control lines (Extended Data Fig.4d and Fig.10a). Moreover, we performed CAFE assays on flies expressing these control constructs in ensheathing glia specifically, and did not observe any differences in food intake (Extended Data Fig.3e).

4. In Figs 1e, 2c,d, please provide western blots for STAT:GFP with quantification as this would help to substantiate IF data using an alternative approach. It may be interesting to compare headvs body derived protein to see if STAT:GFP upregulation in the brain is specific to the brain or if this is a general, ubiquitous effect.

We have performed western blots to measure STAT activity in the brain under mock and *Ecc15*-infected conditions using 10xSTAT::GFP and 2xSTAT::GFP reporters. These experiments did not reveal any significant change in GFP protein levels in whole heads of 10x STAT::GFP flies, while GFP was undetectable in samples from heads of 2xSTAT::GFP reporter flies. Since our imaging data indicated that infection-induced STAT activation is specific to a subset of glia, we were not surprised that assessment of protein levels in samples from whole heads (with background expression of GFP in neurons) did not reveal

a clear change. Western Blots from sorted ensheathing glia, on the other hand, is impossible. To overcome this limitation, we have now performed flow cytometry analysis and found an increase of GFP intensity among ensheathing glia (labelled by nls.mCherry in the presence of GMR56F03::Gal4) 4 hours post *Ecc15* infection (Extended Data Fig.2f). And, in addition, our new scRNAseq analysis reveals specific upregulation of JAK/STAT signaling only in ensheathing glia during aging, but not in other subtypes of glia (Extended Data Fig.7d and 7f), further supporting the immunostaining results.

5. Generally, the size of all the confocal images in this manuscript should be enlarged for ease of readability and data interpretation. Further, please state and show where in the brain all images were taken. The authors should consider showing whole images of the brain in addition to the zoomed-in images provided. The authors should also consider including additional, representative images from other animals in Extended Data.

We have adjusted the images accordingly. All zoomed-in images were from the antennal lobes of the central brain, labelled "AL" in the figures. We now show images of whole brains for more conditions, as much as the figure size limitations allow.

6. For RNAi experiments (e.g. Fig. 2e), the authors should quantify knockdown efficiency for all UAS-RNAi lines used. Knockdown should be shown on the protein level by western blot with quantification if possible. For previously published RNAi, please state the knockdown efficiency previously reported. Also, the authors should consider testing multiple RNAi per target to confirm specificity of key results versus an off-target effect from a single RNAi, particularly when using KK RNAi lines.

We understand the concern raised by the reviewer and have tested the knockdown efficiency for all the RNAi lines used in this paper by qPCR (as western blots would require validated antibodies for each of the targeted proteins) or immunostaining (for *dome*^{RNAi} *and* *stat*^{RNAi} lines, using 10xSTAT::GFP reporter) (Extended Data Fig.3b, 4e, 8f and 8g).

In addition, we have now included additional RNAi lines targeting the same genes,including *dome, stat, upd2, upd3, Glaz and outsiders,* and repeated the olfactory T maze, CAFE food choice and *PE* survival assays on young animals (Fig.4b, 4c, Extended Data Fig.4d, 10a, 10c).

The Dome and statRNAi lines we used have also been used widely in previous studies:

***dome*^{RNAi} (BL34618) in Li *et al*. 2020. *J Cell Bio*; Zhang *et al*., 2019, *Dev Cell*; Gervais *et al*., 2019, *Dev Cell*; Hudry *et al*., 2019, *Cell*; Chen *et al*., 2018, *eLife* ; Recasens-Alvarez *et al*., 2017, *Nat. Commun.*; Liu *et al*., 2015, *J. Cell Biol* ; Ren *et al*., 2015, *PLoS Genet*; Zhang *et al*., 2013, *Dev. Biol*;**

***dome*^{RNAi} (v106071) in Recasens-Alvarez *et al*., 2017, *Nat. Commun*.; Yamamoto *et al*., 2017, *Nature*; Wang *et al*., 2015, *PLoS Genet* ;**

***stat*^{RNAi} (v106980) in Musselman *et al*., 2018, *Genetics* ; Petruccelli *et al*., 2018, *Neuron* ; Recasens-Alvarez *et al*., 2017, *Nat. Commun*.; Péan *et al*., 2017, *Nat. Commun*.; Zhao and Karpac, 2017, *Curr. Biol*.; Terriente-Félix *et al*., 2017, *Dis. Model Mech*.; Tamori *et al*., 2016, *PLoS Biol*; Clemente-Ruiz *et al*., 2016, *Dev. Cell*; Schertel *et al*., 2015, *Genome Res*. Woodcock *et al*., 2015, *Immunity*; Wang *et al*., 2015, *PLoS Genet*; Radyuk *et al*., 2013, *FASEB J.*; Issigonis and Matunis, 2012, *Dev. Biol*. ;**

stat*^{RNAi} (BL35600) in Meng *et al*., 2020, *Stem Cell Reports*; Deng *et al*., 2020, *Nat. Commun*.; Zhai *et al*., 2017, *PLoS Genet*.; Monahan and Starz-Gaiano, 2016, *BMC Dev. Biol*.; Zhai *et al*., 2015, *Nat. Commun.

The qPCR studies have led us to remove data involving tret1-2 and Bsg RNAi lines, as we were unable to unequivocally demonstrate efficient knockdown of these genes. Since expression of the RNAis against these two genes also did not result in any phenotypes different from wild-type controls, we could not conclude whether these genes are requiredor not for changes in olfaction. The removal of these experiments did not change any of our conclusions.

7. The authors emphasize throughout the text that their findings directly pertain to disrupted neuron function and neurodegeneration. However, neuron activity and death were not directly assayed. Rather, olfactory phenotypes were noted and the authors conclude that alterations aredue to neuron loss. However, many tissues are likely disrupted by their manipulations and the cytokine pathways, and these may contribute to olfaction deficits. The authors should consider including investigations more directly demonstrating that there are indeed changes in neuron activity and neuron loss to directly demonstrate relevance to neurodegeneration.

We have removed the broad statements referencing neurodegeneration.

Regarding olfactory degeneration, a previous study has carefully studied the morphological and functional changes of olfactory receptor neurons (ORNs) and

projection neurons (PNs) during aging (Ashiq Hussain, *et al*. 2018. *eLife*). They observed functional decay of PNs, but not ORNs, during aging, which leads to olfactory degeneration. However, what happens to glia during aging and whether glia play a role in olfactory degeneration is not well understood. Our findings contribute new data to that question. -

In this revised version, we have now tested our model for the effects of changes in the lactate shuttle in infection- or age-related projection neuron decline by T maze, CAFE food choice and *PE* survival assays on flies in which lactate dehydrogenase (*ldh*), lactate shuttle (*out*), or the neuronal lipid binding protein *Nlaz* were knocked down specifically in projection neurons. We observed that reducing lipid transport or lactate intake in projection neurons alleviates the inhibition of olfaction sensitivity upon infection or during aging, consistent with the model we proposed (Fig. 4d-4g, Extended Data Fig.10f-10i).

Minor points

8. Information in the introduction should be facts that are necessary for one to understand the investigations to follow. Much of the information in the introduction belongs in the discussion, while connections between olfaction and infections, the fly olfactory system, and glia andinflammation should be the key topics discussed in the introduction. For example, neuron loss, AD, and PD are not directly investigated in the paper, yet there are ~4 sentences on neurodegeneration, AD and PD. All information on how the findings from this study impact understandings on diseases not directly tested should be moved to the discussion. Also, paragraphs 1 and 3 are two seemingly disconnected topics until you get to the last paragraph thatmentions a common feature of acute vs chronic inflammation which connects pathogen-mediatedanorexia and aging and nicely sets the reader's expectations for the following studies. Thus, a paragraph discussing inflammation and the JAK/STAT pathway in th introduction would be helpfulfor readers to be better prepared for the results section.

We thank the reviewer for the extensive suggestions and have now edited our manuscript accordingly. Due to editorial space limitations, the introduction and discussion had to be shortened substantially.

9. Line 65: For clarity "…of the Lipocalin Glaz and the Monocarboxylate Transporter (MCT) Outsiders, resulting…" should be rewritten as "…of the lipocalin, Glaz, and the monocarboxylate transporter (MCT), Outsiders, resulting…".

We have changed the text.

10. Line 101: Please put a comma after “cytokines”.

We have changed the text.

11. Provide a better description of the Stat::GFP lines in the results and figure legends. For example, “Specifically, these express GFP from 2x or 10x STAT92E promoter.”

We have changed the text.

12. It is a bit confusing to see descriptors like “7d” and “4h” on the figures without thorough explanation. What does “7d” mean? Are 7d animals fed pathogen for 4h then immediately assayed? Or was GFP signal assayed 7d after a 4h infection? In this case, the data would be inconsistent with Fig. 1d showing that the effects of infection are transient.

We have clarified this in our manuscript as much as possible. 7d means 7 days, while 4h means 4 hours. To overexpress or knock down gene expression in young flies, we shifted flies from 18C to 29C for 7 days temporarily, followed by infecting these flies with *Ecc15* for 4 hours or 24 hours before dissection or performing other tests, as specified in the figure legends and methods.

13. In Fig. 7d,e, the authors have two temperatures listed (25 and 29). Please check that this is correct and clarify why two different temperatures are justified in these experiments.

We have aged these flies by keeping them at 25C for 14 days and shifted them to 29C for another 14 days to express transgenes (we are using Gal80^{ts} throughout to control expression, 29ºC is the restrictive temperature). Details about aging flies for T maze assay have been described in the methods.

14. In the discussion, the authors should consider a paragraph describing their data in the context of data describing potential connections between infections and dementia.

We thank the reviewer for pointing this out. Unfortunately, the editorial space limitations prevent us from expanding the discussion further. In fact, we have had to significantly reduce our discussion to meet limitations.

15. Line 333: please define the term “EGs”.

We have changed the text.

Referee #3:

This manuscript describes a new inter-organ pathway that becomes transiently activated upon infection to reduce olfactory perception. Cytokines made by the intestine of Ecc15-infected flies remotely activate JAK-STAT signalling in ensheathing glia. This leads to metabolic changes within glia, including changes in lipid metabolism, and a reduction in olfactory sensitivity. This pathway promotes avoidance of bacteria-laced food in response to infection, and its prolonged activation (which occurs in aged flies) also leads to a chronic decline in olfactory sensitivity.

The findings are interesting, timely and provocative. I find most papers in this area unsatisfying as they typically report correlative (and potentially meaningless) changes in the brain in responseto gut infection/inflammation/microbiota. This paper stands out from this perspective because it leverages the powerful genetics of Drosophila to: 1) show that gut-to-glia communication is (patho)physiologically relevant both in response to infection and in the context of age-related inflammation; and 2) identify the mechanism involved and establish causation. This extends recent work in this area (Kobler et al. and Charroux et al., both mentioned in the manuscript) by revealing an involvement of the intestine and glia as novel post-infection mediators.

We would like to thank the reviewer for the positive comments and the thorough review of the manuscript.

There are currently several major issues that need to be addressed so that the main conclusionsof the manuscript are supported by the data.

1. EFFECTS ON FOOD INTAKE VS FOOD/OLFACTORY PREFERENCE. Infection-induced anorexia and a specific reduction in intake of bacteria-laden food are different behaviours (food intake vs preference) likely induced by different mechanisms. The data in this manuscript describes changes in preference, partly because their initial characterisation led the authors to conclude Ecc15 infection does not reduce food intake. However, the data was not very strong (relatively low n numbers, there is a trend toward reduction, Fig. 1b) and contradicts previous findings (e.g. Kobler et al. but also others). This is important because reduced olfactory sensitivity might result from starvation.

We agree that the avoidance behavior towards enteropathogens we describe is different from outright anorexia, and we have changed our introduction and discussion accordingly.

With respect to statistical power in Fig.1b (now in Extended Data Fig.1a), we would like to point out that we used 21 flies per condition (n=3 flies per replicate, 7 replicates for each

condition per experiment) to measure food intake. The overall experiment was then repeated three times. We have now better explained the experimental design in the figure legend. The reduction in *Ecc15* containing food intake was significant for infected compared to non-infected flies, while overall food intake was almost identical between the two groups (as food intake of normal food increased), suggesting a selective aversion against *Ecc15* containing food when flies were infected.

We do not think that these data are contradictory to previous findings. The two studies mentioned by the reviewer characterized how wildtype flies react to *Ecc15* food under homeostatic conditions, and they found that the odor of *Ecc15* is attractive to naïve flies, followed by avoidance mediated by gustatory neurons or by the activation of PGRP signaling in the mushroom body. Our data are consistent with this observation, as when given a choice in the CAFE assay, naïve flies eat more *Ecc15* containing food than normal food initially (Fig.1b). Only later (16-40h) or when testing infected flies, the preference of Ecc15 food is reduced and flies prefer normal food over *Ecc15* containing food. This is consistent with the data reported by Kobler et al and Charroux et al.

how does starvation affect olfactory discrimination and/or avoidance of bacteria-laced food?

We have now tested whether starvation would influence olfactory discrimination. We starved wild-type flies for 24 hours on water before testing olfactory discrimination and food intake. We did not observe any differences in olfactory discrimination (Extended DataFig.1d). Starved flies increased both *Ecc15* food intake and normal food intake (Extended Data Fig.1b).

Does food intake change in aged flies?

Yes, a previous study has shown that food intake of wild-type flies decreases with age (Li,*et al*. 2016. *Cell Host Microbe*). This study showed that the age-related development of gastric metaplasia in the intestinal epithelium contributes to changes in food intake duringaging.

Also, I note that in this regard that the same cytokine that results in bacteria avoidance in this study has been shown to signal the fed state in a different study (PMID: 23021220).

We have now discussed this paper in the discussion. It should be noted that this study found that the effect of Upd2 is mediated by GABAergic neurons in the brain, while our data indicate that the effects on olfactory discrimination are caused by JAK/STAT

activation in ensheathing glia. Since starvation does not influence olfactory discrimination (Extended Data Fig.1d), and since direct perturbation of JAK/STAT signaling in ensheathing glia affect both olfactory discrimination and *Ecc15* aversion (Fig.1h) we do not believe that the phenotypes observed here are related to the role of Upd2 in satiation.

Related to this, is it eating more Ecc15 or eating more generally that makes flies sick?

This is an interesting question. We measured the intake of both *Ecc15* containing and normal food, and found that flies lacking *dome* or *stat* in ensheathing glia consumed more *Ecc15*$^{+}$ food (Fig.1i), but without significant changes in normal food intake (Extended Data Fig.3c, 3d). Accordingly, total food intake of these flies was also increased (Extended Data Fig.3c, 3d). Similarly, elevating JAK/STAT activity in ensheathing glia reduced intake of *Ecc15* containing food selectively, reducing overall food intake (Fig.1i, Extended Data Fig.3c, 3d). In our mortality assays, flies are exposed to food containing enteropathogens, and they can feed on that single food source *ad libitum*, making it difficult to clearly quantify food intake. The correlation of selective increases in ingestion of bacteria containing food with increased mortality, however, led us to interpret that it is the selective reduction in bacteria ingestion that results in protection when olfaction is suppressed. It should be noted however that this correlation is lost in whole-body Orco1 mutant flies, which eat less bacteria containing food, yet still succumb faster to infection (Extended Data Fig.3h). It is possible that this discrepancy is due to an unrelated (non-olfactory) roleof Orco in other tissues.

In this context, the authors need to clarify their definitions of olfactory ‘sensitivity’ and ‘perception’, which they use interchangeably and could be interpreted as different things - either a general reduction in response to odours as opposed to distinguishing between different odours. They also need to ensure that the abstract and intro are better aligned with the data; at the moment, the focus on anorexia is confusing and somewhat misleading.

We thank the reviewer for pointing this out. To be more precise in our discussion of the results, we have now changed olfactory perception to olfactory discrimination in our text, and replaced anorexia with avoidance behavior towards pathogens.

2. STATEMENTS ABOUT GLIA-NEURON METABOLIC COUPLING. The manuscript contains no data linking glial cells and olfactory neuron activity, yet several sentences and even a cartoon in the manuscript give the impression that this hypothesis is part of their findings. Such coupling could be tested by blocking the lactate shunt into olfactory neurons and/or (better) preventing the

infection-induced reduction in olfactory neurons sensitivity by increasing lactate availability in olfactory neurons.

We would like to thank the reviewer for this suggestion. We have now performed T maze assays, CAFE food choice assays and *PE* survival assays on flies of which lactate dehydrogenase (*ldh*), lactate shuttle (*out*), and the neuronal lipid binding protein *Nlaz* were knocked down specifically in projection neurons (Fig.4d, Extended Data Fig.10f-10h). We observed that blocking lipid transport or lactate intake in projection neurons alleviates the inhibition of olfactory discrimination upon infection or during aging, consistent with the model we proposed.

3. QUALITY OF THE DATA AND STATISTICAL ANALYSES. These need to be improved. The background stainings in control vs experimental images (e.g. Stat-GFP panels) rarely match, which makes comparisons of the "real" stainings unconvincing. Contrary to what the manuscript states, MyoI-Gal4 is strongly expressed in quite a few neurons in the brain (also in ENS). t-tests are often used for data that may not be normally distributed and/or for experiments involving multiple genotypes/conditions. Images are not always representative of quantifications/statements (e.g. ED1c more nuclei are apparent after Ecc15 than in mock, there seems to be a lot of Stat-GFP expression in ensheathing glia prior to infection…).

We have replaced representative images with images where background signals are comparable, replaced all the data using Np1::Gal4 in the manuscript with new dataobtained from flies using Mex1::Gal4, which does not show leaky expression in the centralbrain. And we have now used the nonparametric Mann-Whitney test for comparisons between two groups and the nonparametric Kruskal-Wallis test for multiple comparisons.The results and interpretations of our experiments remain the same.

4.DOES REDUCED OLFACTORY SENSITIVITY DRIVE AVERSIVE BEHAVIOUR? Whilst it is clear that Ecc15 infection causes a decline in olfactory sensitivity, does this decline underlie Ecc15 aversion?

Our data suggested that the modulation of olfactory function is always coupled with specific changes in *Ecc15* food intake, suggesting that olfactory perception may underly *Ecc15*-mediated avoidance behavior. To directly test whether reduced olfactory sensitivitydrives avoidance behavior, we tested odorant receptor mutant flies, including $Gr63a^{1}$ and$Orco^{1}$. As shown in previous studies that odorant receptors Orco and Gr63a are required for flies to be attracted by *Ecc15* (Kobler, J. M. *et al*. 2020 *Curr Biol*; Charroux, B. *et al.* 2020.

***iScience*), we performed CAFE assays on these odorant receptor mutant flies with or without *Ecc15* infection. We found that odorant receptor mutant flies took in significantly less *Ecc15* food under homeostatic conditions with no changes in normal food intake (Extended Data Fig.1c), phenocopying the infection-induced avoidance behavior of wild-type flies. Moreover, infection failed to further induce avoidance behaviors on these odorant receptor mutants (Extended Data Fig.1c), suggesting that the downregulation of odor perception contributes to the avoidance behavior. We interpret these results as indicating that the switch from attraction to avoidance of *Ecc15* containing food requires both gustatory and immune receptors (described by Charroux and Kobler) to trigger aversion, as well as suppression of olfactory discrimination to prevent attraction. We havediscussed this now in the discussion.**

How do flies respond to the smell of Ecc15 before and after infection? In Fig. 1d line 98, is aversionto Ecc15 removed after 4 days? Another way to test this (and a previous point at the same time)is by interfering with the lactate shunt from the neuronal side.

Previous studies have shown that the smell of *Ecc15* is attractive to naïve flies in 4-field olfactory choice assay (Kobler, J. M. *et al*. 2020 *Curr Biol*; Charroux et al 2020, *iScience*). However, this smell is no longer attractive for flies after *Ecc15* infection (Kobler, J. M. *et al*. 2020 *Curr Biol*).

We have now improved the data in Fig.1d by performing T maze assays on another five independent batches of flies 5 days post *Ecc15* infection. The age of these flies was controlled to be the same as flies 1 day post *Ecc15* infection, and the influence from olfactory memory was ruled out. Our new data further support that olfactory discriminationis restored 5 days after infection.

We have tried to test attraction to *Ecc15* odor in our T maze device, but found that supplying *Ecc15* solution as odor in our T maze device is not a strong enough trigger for files make a choice, and results were thus inconclusive. A more sensitive 4-field olfactoryarena choice device may be required to test olfaction sensitivity to odors emanating from*Ecc15* solution.

To test the role of the lactate shuttle from the neuronal side, we have now knocked down lactate dehydrogenase (*ldh*), lactate shuttle (*out*), and the neuronal lipid binding protein (*Nlaz*) specifically in projection neurons, and found that blocking lipid transport or lactate intake in projection neurons alleviates the inhibition of olfactory discrimination upon

infection or during aging (Fig.4d, Extended Data Fig.10f-10h), consistent with the model we proposed (Fig.4g).

Related to this, the authors need to integrate their data with recently reported olfactory remodelling "downstream" of ORNs as well gustatory remodelling (Kobler et al., 2020; Charroux et al., 2020, respectively). The authors seem to put forward a model in which gustatory cues arethe drivers of the aversive behaviour, with the loss of olfactory sensitivity re-enforcing this. Their own data, however, argues against this since the rescue of olfactory sensitivity by dome or STATknock down rescues Ecc15 aversion. Are the gustatory neurons therefore also affected by theseknockdowns?

This is an interesting question. We have now better discussed our findings in the contextof the Kobler and Charroux studies. We believe that, consistent with these studies, flies are attracted to *Ecc15* initially and that this attraction is mediated by olfactory receptors. The decline in olfactory discrimination once the flies are infected, in turn, reinforces the aversion mediated by gustatory neurons and other mechanisms (as it reduces attraction). Accordingly, when olfactory discrimination is restored in the *dome* and *stat* knockdown animals, flies again eat more *Ecc15* containing food even while they are already infected. This is consistent with the attraction of flies to *Ecc15* and with our new data with olfactory receptor mutants, as well as with a model in which the balance between attractionmediated by olfactory perception and aversion mediated by gustatory receptors and immune signaling ultimately determines the animal's behavior. We have tried to explain this model better in our discussion.

The role of ensheathing glia in the gustatory circuit is not clear, yet it is possible that ensheathing glia could also affect gustatory neuron functions. We have discussed this possibility in the discussion. Whether JAK/STAT signaling plays a role in the activation of gustatory neurons would be interesting for future investigation, yet we believe that it is beyond the scope of this paper.

SPECIFIC COMMENTS:

The RNA-seq comparison of Stat-GFP positive vs negative glia in uninfected flies struck me as an odd choice. These glial cells may not be the same glial cells that turn Stat-GFP positive upon infection. Can the authors clarify this?

We have now included the Bulk RNA sequencing data from STAT::GFP+ glia and STAT::GFP- glia from both mock flies and infected flies. Based on PCA analysis, the transcriptomes of STAT::GFP+ glia from infected and uninfected animals were more similar to each other than to STAT::GFP- glia of either condition, indicating that JAK/STATinduction has a stronger influence on glial transcriptomes than other infection-related changes (Extended Data Fig.7h). We had thus only analyzed the transcriptomic differencesbetween STAT::GFP+ glia and STAT::GFP- glia during homeostasis. Of note, the genesetsenriched in STAT::GFP+ glia during homeostasis were also enriched in STAT::GFP+ glia after infection.

Line 139, Fig. 1k: Were these flies given a choice of bacteria-laden or normal food? Or is survival increased even when it isn't through the hypothesized host defence behaviour of avoiding bacteria-laden food? This may suggest that something else about decreasing odour sensitivity affects survival.

During the survival assay, flies are typically fed with the enteropathogen *PE* which is resuspended in 5% sucrose solution and added to filter paper in the vial. While flies have no choice of the food source (and hence all animals ultimately succumb to the infection), our interpretation is that the delayed mortality of the Hop^{tumL} expressing animals or the accelerated mortality of the $dome^{RNAi}$ expressing animals is a consequence of theirdifferent aversion to enterpathogen containing food (as measured in the CAFE assay).

Lines 249-250: The wording is a bit confusing – it seems to suggest that the reason for the glial lipid changes during ageing is the neurons rather than the intestinal cytokine upregulation.

We have clarified this passage. Based on previous studies, we believe that projection neurons are the source of lipid production, while JAK/STAT activation in ensheathing glia increases lactate transport to neurons and lipid transfer from neurons back to glia, thus contributing to glial lipid accumulation. Our new results have also confirmed this notion, as knockdown of the lactate shuttle, Outsiders, or the neuronal lipid binding protein, Nlaz,in projection neurons alleviated the decline of olfaction sensitivity upon infection or during aging, consistent with our results in glia.

Line 199: 'loss' should be replaced with 'decline' or 'reduction'.

We have changed the text.

Lines 189-191: Germ-free may be clearer than sterile, which I first interpreted as infertile…

We have changed the text.

Line 244: Please make clear that this is in infected flies.

We have changed the text.

Fig. 1h: Please show GFP as a single panel and quantify; it is the most important co-expression of those shown in the figure.

We have now shown GFP as a separate panel (now moved to Extended Data Fig.2e). The quantification is shown in Extended Data Fig.2c. We have now also included flow cytometry quantification of the GFP signal (Extended Data Fig.2f).

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have adequately addressed my concerns. I like this manuscript and consider it an important contribution.

Referee #2 (Remarks to the Author):

The authors adequately and appropriately addressed all of the concerns addressed in my review and the manuscript is now acceptable for publication.

Referee #3 (Remarks to the Author):

The authors have addressed my concerns very comprehensively and the manuscript is now much improved and ready to be published in its current form. Their findings are exciting and supported by the data provided, and the wording of the manuscript is now more nuanced and better aligned with the data.

I would only like to ask the authors to carefully go through the legends and displays as there are a few typos/panel inconsistencies (not sure what the colours in Fig2a mean, Fig 2c legend is missing, sometimes unclear which data comparisons are/are not shown - e.g. asterisks displayed for upd3RNAi but not for upd2RNAi in 2c).

Author Rebuttals to First Revision:

Response to reviewers - 2020-10-18688A

We would like to thank the reviewers for the positive reception of our work and strong support for publication in *Nature*.

POINT-BY-POINT RESPONSES TO REVIEWERS' COMMENTS:

Referee #1 (Remarks to the Author):

The authors have adequately addressed my concerns. I like this manuscript and consider it an important contribution.

Referee #2 (Remarks to the Author):

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Following this request as well as editorial policy, we have reviewed and corrected the legends for each figure and extended data figure, and ensured that the exact p values were stated when available, that the type of data comparisons performed are explained, statistical tests are clearly described, and that typos/panel inconsistencies are corrected.