Central relay of bitter taste to the protocerebrum by peptidergic interneurons in the *Drosophila* brain

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Bitter is a taste modality associated with toxic substances evoking aversive behaviour in most animals, and the valence of different taste modalities is conserved between mammals and *Drosophila*. Despite knowledge gathered in the past on the peripheral perception of taste, little is known about the identity of taste interneurons in the brain. Here we show that hugin neuropeptide-containing neurons in the *Drosophila* larval brain are necessary for avoidance behaviour to caffeine, and when activated, result in cessation of feeding and mediates a bitter taste signal within the brain. Hugin neuropeptide-containing neurons project to the neurosecretory region of the protocerebrum and functional imaging demonstrates that these neurons are activated by bitter stimuli and by activation of bitter sensory receptor neurons. We propose that hugin neurons projecting to the protocerebrum act as gustatory interneurons relaying bitter taste information to higher brain centres in *Drosophila* larvae.
Detailed knowledge exists on the anatomical distribution and function of gustatory receptors in mammals and *Drosophila*[^1-3]. In *Drosophila* 60 gustatory receptor genes encode 68 gustatory receptors[^4-9], with the majority of these receptors detecting bitter compounds[^5]. Although gustatory receptors in *Drosophila* share no homology to mammalian taste receptors, the strategy used in both to detect a taste molecule, process its information and the valence of aversive bitter and appetitive sweet stimuli share similarities[^1]. In contrast to the extensive knowledge on the peripheral coding of taste in flies and mammals, much less is known about the central pathways that relay these signals into meaningful behaviour. Although broad regions in different parts of the brain have been shown to respond to various taste cues, there is little information on the molecular identity of specific neurons that convey different taste modalities to the higher brain centres[^10,11]. Recently, secondary neurons that relay sweet taste from subesophageal zone (SEZ) to the antennal mechanosensory motor centre of adult *Drosophila* were characterized[^12]. Analogous secondary neurons for other taste modalities have not yet been identified.

A candidate for conveying bitter taste from the SEZ to higher brain centres are neurons that express the hugin neuropeptide[^13,14] (referred to as hugin neurons), whose arborizations in *Drosophila* larvae overlap with that of bitter gustatory receptor neurons (GRNs) expressing the caffeine receptor GR66a[^15-17]. In adult *Drosophila*, GR66a was shown to represent a bitter receptor for detection of caffeine[^9,16,17] and inactivation of GR66a positive neurons leads to impairment of caffeine aversion[^17]. In *Drosophila*

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**Figure 1 | Hugin neurons are part of bitter gustatory pathway.** (a) Expression analysis of hugin neurons and GR66a positive dendrites in the SEZ using Hug-YFP, UAS-mRFP line crossed to GR66a-Gal4. Scale bars, 10 μm for upper two panels and 50 μm for lowest panel. (b) Close proximity of GR66a positive dendrites and hugin positive dendrites located in the SEZ using GRASP (Hug1.2lexA attp40 driving lexAop-CD4::spGFPI10) crossed to GR66a-Gal4 driving UAS-CD4::spGFPI. Scale bar, 10 μm. a—P = anterior—posterior. (c) Two-choice assay with 200 mM caffeine. Left representative plates are time projections of the last 5 min of the 20 min experiment. Activating hugin neurons with UAS-dTrpA1 (n = 10) leads to impairment of choice behaviour (P < 0.001, compared with the controls OrgR x dTrpA1 (n = 10) and Hugin-Gal4 (n = 11), Mann-Whitney-U-Rank-Sum-Test (MWU-TEST)). Controls did not significantly differ from each other (P = 0.275, MWU-TEST). (d–f) Two-choice assay with 200 mM caffeine. Ablation of hugin neurons by expression of UAS-rpr::hid (n = 10) leads to impairment of bitter substrate avoidance compared with HugG3-Gal4 (n = 10) and UAS-rpr::hid (n = 14) controls (P < 0.001, MWU-Test). Controls did not differ from each other (P = 0.728, MWU-Test). Silencing hugin neurons by expression of UAS-shibireTS (n = 10) shows same impairment on gustatory bitter choice compared with HugG3-Gal4 (n = 10) and UAS-shibireTS (n = 10) controls (P < 0.001, MWU-Test). Controls did not differ from each other (P = 0.168, UAS controls P = 0.082, MWU-Test). All two-choice experiments were performed at 32 °C. Boxplots were generated from PI values of the last 5 min of the 20 min experimental time. Significances are indicated as ***P < 0.001, **P < 0.01 and *P < 0.05. Line plots showing the time course of the two choice experiments are displayed as mean (line) ± s.e.m. (transparent areas). Details of descriptive statistics and statistics against chance levels for experimental lines are shown in Supplementary Table 1.

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[^1]: Caffeine Pure
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larvae, artificial activation of GR66a positive neurons leads to aversive behaviour\(^{18}\). Thus, hugin neurons were good candidates for acting as a central relay for bitter information from sensory neurons to the protocerebrum, acting as bitter taste interneurons in the *Drosophila* larval brain.

### Results

**Hugin neurons are required for caffeine avoidance response.** We first asked whether the hugin neurons make contacts with caffeine responsive GR66a neurons. Using the GFP reconstitution across synaptic partners (GRASP) approach\(^{19}\), we could indeed observe a GRASP signal in the SEZ, indicating that caffeine receptor neurons and hugin neurons are in close proximity to each other (Fig. 1a,b).

Activating the hugin neurons causes the larvae to stop feeding and move out of a strongly appetitive food source (yeast)\(^{20}\), which could be due to activation of an aversive bitter taste pathway. We therefore tested the behavioural response to caffeine in a two choice assay. When hugin neurons are activated with the temperature sensitive cation channel dTrpA1, the animals showed significantly less avoidance to caffeine (Fig. 1d,e). To exclude potential developmental effects, we also expressed the temperature sensitive mutant form of dynamin (shibire\(^{21}\)) in the hugin neurons, which leads to a block of synaptic release in a

![](image)

**Figure 2 | Hugin neurons are not required for high salt or sweet taste processing.** (a) Two choice assay with 2 M NaCl. Activating hugin neurons with UAS-dTrpA1 (\(n = 11\)) leads to impairment of choice behaviour (\(P < 0.001\) compared with OrgR \(\times\) dTrpA1 (\(n = 10\)) and Hugin-Gal4 (\(n = 10\)) controls, Mann-Whitney-U-Rank-Sum-Test (MWU-Test)). Controls did not differ from each other (\(P = 0.241\), MWU-Test). (b) No significant difference in avoidance behaviour was observed on 2 M NaCl between Hugin > rpr;hid (\(n = 10\)) and UAS-rpr;hid control (\(n = 10\)) (\(P = 0.165\), MWU-Test) or Hugin-Gal4 control (\(n = 10\)) (\(P = 0.838\), MWU-TEST). Controls did not differ from each other (\(P = 0.241\), MWU-Test). (c) Two choice assay with 1 M fructose. Activating hugin neurons with UAS-dTrpA1 (\(n = 11\)) leads to impairment of choice behaviour (\(P < 0.001\) compared with OrgR \(\times\) dTrpA1 control (\(n = 11\)) and Hugin-Gal4 control (\(n = 13\), MWU-Test)). Controls did not differ from each other (\(P = 0.384\), MWU-Test). (d) There was no significant difference on 1 M fructose choice behaviour between Hugin > rpr;hid (\(n = 12\)) and Hugin-Gal4 control (\(n = 10\)) (\(P = 0.668\), MWU-Test). UAS-rpr;hid control larvae (\(n = 10\)) showed significant difference to Hugin > rpr;hid (\(P = 0.013\), MWU-Test). Controls did not differ from each other (\(P = 0.104\), MWU-Test). Sample two choice plates are shown on the left side of each experiment for each genotype for the last 5 min of the experiment. Boxplots were generated from PI values of the last 5 min of the 20 min experiment. Signatures are indicated as **(**\(P < 0.001\), **(**\(P < 0.01\) and **(*\(P < 0.05\). Line plots showing the time course of the two choice experiments are displayed as mean (line) ± s.e.m. (transparent areas). Details of descriptive statistics and statistics against chance levels for experimental lines are shown in Supplementary Table 2.
Distinct hugin neurons modulate taste and feeding behaviour. As the hugin neuronal cluster is composed of different classes with different projection targets\(^{13,14}\), we next wanted to determine which class was responsible for taste processing and feeding regulation. Through promoter deletion analysis, we generated a hugin promoter-Gal4 line which showed target gene expression exclusively in the eight hugin cells (four per hemisphere) that project to the protocerebrum (HugPC-Gal4, Fig. 4a). To see whether this subset of hugin neurons (huginPC neurons) is necessary for proper bitter taste processing, we ablated these neurons (Fig. 4b) and tested larvae in the caffeine two-choice experiment. Ablation of the huginPC neurons resulted in the inability to respond appropriately to caffeine, indicating that hugin neurons process bitter taste information. In sum, when hugin neurons are activated, larvae are no longer able to respond properly to different taste modalities, such as yeast protein\(^{20}\), fructose, high salt and caffeine. This chemosensory response was specific for taste, as olfactory behaviour was not affected (Fig. 3), showing that activation of hugin neurons selectively disrupts gustatory chemosensory processing. However, inhibiting the hugin neurons results in the inability to respond appropriately to caffeine, indicating that hugin neurons process bitter taste information. In sum, when hugin neurons are activated, larvae perceive all tested substrates (water, caffeine, salt and fructose) as bitter tasting. In contrast, when hugin neurons are inactivated, only the bitter substrate choice is affected since proper detection of bitter compounds no longer occurs.

Activity of huginPC neurons upon bitter stimulation. To further investigate the connection between bitter taste and hugin neurons, we asked if the huginPC neurons could be activated by caffeine using CaMPARI (Calcium Modulated Photoactivatable Ratiometric Integrator), that allows monitoring of calcium activity in intact animals. Calcium activity and simultaneous presence of ultraviolet-light (405 nm) lead to an irreversible conversion from green to red fluorescence of the neurons of interest\(^{22}\). When we placed intact larvae in solutions containing water and water mixed with caffeine, fructose, high NaCl or yeast (Fig. 6a), huginPC neurons were strongly activated by caffeine (Fig. 6b,c). They also showed a concentration dependent increase in calcium activity with increasing caffeine concentrations (Fig. 6d). Other bitter tastants, such as quinine and denatonium, also activated huginPC neurons (Fig. 6e).

Finally we asked if there is a functional connection, in addition to the anatomical connection (Fig. 1a,b), between the caffeine sensing GR66a neurons and hugin neurons. To this end, we activated the GR66a neurons and then monitored the activity of hugin neurons using the calcium indicator GCaMP6s (Fig. 7a,b). Activation of GR66a by dTrpA1 and simultaneous calcium imaging of huginPC projections resulted in the induction of rhythmically occurring calcium peaks, demonstrating a functional connection between GR66a and huginPC neurons (Fig. 7b).
Discussion

The bitter taste rejection response is important for all animals that encounter toxic or harmful food in their environment. Here we showed that the hugin neurons in the Drosophila larval brain function as a relay between bitter sensory neurons and higher brain centres (Fig. 8). Strikingly, activation of the hugin neurons made the animals significantly more insensitive to substrates with negative valence like bitter (caffeine) and salt (high NaCl), as well as positive valence like sweet (fructose). In other words, when the hugin neurons are active these animals ‘think’ they are tasting a bitter substance. In our previous work, activation of all hugin neurons led to decreased in neural activity of the antennal nerve (AN), and induction of a wandering-like behaviour. We have now pinpointed the neurons responsible specifically to those that project to the protocerebrum. These neurons not only respond to bitter stimuli, but also show a concentration dependent increase in calcium activity in response to caffeine. Dose dependent coding of bitter taste stimuli was previously shown to occur in peripheral bitter sensory neurons, where bitter sensilla exhibit dose dependent responses to various bitter compounds. Larvae in which the huginPC neurons have been ablated still showed some negative valence-like responses to substrates with negative valence like bitter (caffeine) and salt (high NaCl). However, they avoid the empty chamber since the bitter perception has been optogenetically induced in the central nervous system (CNS) and the mice ‘think’ they are tasting a bitter substance.

In our previous work, activation of all hugin neurons led to behavioural and physiological phenotypes such as decreased feeding, decrease in neural activity of the antennal nerve (AN), and induction of a wandering-like behaviour. We have now pinpointed the neurons responsible specifically to those that project to the protocerebrum. These neurons not only respond to bitter stimuli, but also show a concentration dependent increase in calcium activity in response to caffeine. Dose dependent coding of bitter taste stimuli was previously shown to occur in peripheral bitter sensory neurons, where bitter sensilla exhibit dose dependent responses to various bitter compounds. Larvae in which the huginPC neurons have been ablated still showed some negative valence-like responses to substrates with negative valence like bitter (caffeine) and salt (high NaCl). However, they avoid the empty chamber since the bitter perception has been optogenetically induced in the central nervous system (CNS) and the mice ‘think’ they are tasting a bitter substance.
avoidance to caffeine. Whether this implies the existence of other interneurons being involved in caffeine taste processing remains to be determined. Interestingly, the huginPC neurons are inhibited when larvae taste other modalities like salt (NaCl), sugar (fructose) or protein (yeast). This may indicate that taste pathways in the brain are segregated, but influence each other, as previously suggested.

Bitter compounds may be able to inhibit the sweet-sensing response to ensure that bitter taste cannot be masked by sweet tasting food. This provides an efficient strategy for the detection of potentially harmful or toxic substances in food26,27. For appetitive tastes like fructose and yeast, bitter interneurons like the huginPC neurons in the CNS may become inhibited to ensure appropriate behaviour to pleasant food. Salt is a bivalent taste modality, that is, low doses of salt drive appetitive tastes like fructose and yeast, bitter interneurons projecting to the protocerebrum represent a hub between bitter pathways in the brain and taste decision to either take up low doses or reject high doses.

Figure 5 | HuginPC neurons modulate feeding and wandering like behaviour. (a) HUGPC-Gal4 line driving UAS-dTrpA1 (n = 75 larvae). Larvae show reduction in food intake compared with OrgR x dTrpA1 (n = 120 larvae) and HUGPC x OrgR (n = 80 larvae) (P < 0.001, MWU-Test). Controls did not differ from each other (P = 0.107, MWU-Test). Activation of huginPC neurons with dTrpA1 induces wandering like behaviour, where larvae leave the appetitive food source yeast. Shown are time projections over 20 min of plates with apple juice agar and a red spot of yeast in the middle. Decrease of food intake was measured as % of red stained gut content compared with the area of the whole larva. (b) Extracellular recordings of the antennal nerve (AN). Activation of huginPC neurons with UAS-dTrpA1 (n = 13 larvae, 27 temperature steps) leads to significant decrease in cycle frequency of the AN motor pattern compared with OrgR x dTrpA1 control (n = 9 larvae, 32 temperature steps) and HUGPC x OrgR control (n = 10 larvae, 26 temperature steps) (P = 0.003, MWU-Test). Controls did not differ from each other. Significances are indicated as ***P < 0.001, **P < 0.01 and *P < 0.05. Details of descriptive statistics are shown in Supplementary Table 4.

Methods
Fly lines. Wild type (OrgR) crossed to UAS-dTrpA1 (Bloomington #26263) served as control in Figs 1, 2, 3 and 5. Hugin-Gal4 (HugS3-Gal4 (ref. 13), Bloomington # 58769), GR66a-Gal4 (second Chr., gift from K. Scott (formerly described as GR66C1)), GR66a-Gal4 (third Chr., Bloomington # 57670 used in Fig. 7b), HuginlexA (Hug2.1lexA attP40 (ref. 32)), HUGPC-Gal4 (see generation of this Gal4 line below), UAS-eNhPr-YFP (Bloomington # 41753, referred here as UAS-YFP in Supplementary Fig. 3, since this homozygous line together with HUGPC-Gal4 was used as fluorescent marker only). UAS-CaMPARi (Bloomington #58761), UAS-rpr;UAS-hid (UAS-rpr (Bloomington# 5823) crossed homozygous to UAS-hid33), UAS-shibire23,4, lexAop-CD4:spGFP11 and UAS-CD4:spGFP1-P10 were gifts from K. Scott19, 13x LexAop-2-IVS-GCamP6s-p10 (Bloomington #44274).

Fly care. Adult flies and larvae were kept on 25 °C under 12 h light/dark conditions. For electrophysiological and food intake experiments 4 h egg collections were made on apple juice agar plates containing a spot of yeast–water paste. After 48 h, larvae were transferred into food vials containing lab standard fly food. For other experiments (two-choice, CaMPARi, GCamP) larvae were raised in vials containing standard fly food with a spot of yeast for 4 days. All larvae used for the experiments were 98 ± 2 h old. Only feeding third instar larvae were used for the experiments.

Generation of transgenic flies. For Hugi-Gal4 line, a 548 bp Hugin fragment 155 bp upstream of the ATG was amplified with primer1 (AAC CCT TGG GTT TAA TTT ATT TAT GTC ATATA) and primer2 (GAG CCT GAT TAG GTC CCT GAT TAA ACT T) and cloned into pCaSpeR-AUG-Gal4-X vector (Addgene plasmid 8,378. The construct was injected into w(1118).

Two-choice gustatory assay. To measure the preference index of larvae towards given appetitive or aversive substrates, 9 mm diameter petri dishes were filled with 20 ml warm water agar (2.125% Agar-Agar, Kobe). After 20 min of air drying half of the agar was cut away and discarded. Compounds (2 M NaCl, 1 M Fructose, 200 mM Caffeine, 10 mM Denatonium) were diluted in warm agar in the given concentrations until the agar fluid was clear, and filled in the other half of the petri dish (10 ml). After 20 min of air drying, the agar was cut away and discarded. For each experiment 30 larvae were taken out of standard fly food and washed with tap water. Larvae were then placed on the water side of the two-choice dishes and videotaped for 20 min. Videos were processed with Fiji (ImageJ) and analysed using a custom written script for Fiji. Analysis of PI values started after the start of the experiment to ensure proper tracking of larvae due larval accumulation at the beginning by placing them on the pure agar side. PIgustatory was calculated as (#larvaeabsorb – #larvewater)/#larvawater.

One-choice olfactory assay. For testing response to an attractive odour (apple vinegar), a one-choice assay was performed. Agar plates were used (as described earlier for two-choice petri dishes) and placed into the incubator at 32 °C for 1.5 h. Larvae were videotaped at 32 °C for 5 min, and then an Eppendorf cup (1.5 ml) with filter paper soaked with apple vinegar was placed on one side of the water agar plate above the larvae such that they were not able to reach the odorant source. Movement of vinegar was analysed using the custom made Fiji macro.
Food intake assay. Apple juice agar plates were prepared with a spot of red yeast paste in the middle of the plate. Plates were then placed in incubators precooled to 18 °C or prewarmed to 32 °C for 2 h. After 30 min starvation, five larvae were transferred on top of the yeast paste of a plate and videotaped for 20 min. After 20 min of videotaping, larvae were transferred into a small cell strainer and washed with 60 °C hot water. Larvae were then transferred to glass slides for photoconversion only when larvae were placed in caffeine solution. Scale bars, 5 μm. (d) Measurement of huginPC neurons expressing UAS-CaMPARI in larvae confronted with different concentrations of caffeine. HuginPC neurons display increasing red/green ratio with increasing caffeine concentration (10 mM: P < 0.001, 20 mM: P = 0.003, 50 mM: P < 0.001, 100 mM: P < 0.001, 200 mM: P < 0.001 compared with water alone, MWU-Test). Dots represent mean, whiskers represent s.e.m. (e) HuginPC neurons expressing UAS-CaMPARI display high calcium activity in 10 mM quinine (P < 0.001, MWU-Test) or 10 mM Denatonium (P < 0.001, MWU-Test). Numbers beneath boxplots indicate number of larvae used for each experiment. Scale bars: 5 μm. (f) Two-choice assay with 10 mM denatonium. Ablating huginPC neurons with UAS-rpr;hid (n = 10) leads to impairment of gustatory choice on denatonium compared with HuginPC-Gal4 control (n = 10, P < 0.001, MWU-Test) or UAS-rpr;hid control (n = 12, P = 0.002, MWU-Test). Controls show significant difference to each other (P = 0.027, MWU-Test). Boxplots were generated from PI values of the last 5 min of the 20 min experiment time for two-choice experiment. Significances are indicated as **p < 0.001, ***p < 0.01 and *p < 0.05. Line plots showing the time course of the two choice experiment and dot plots are displayed as mean (line) ± s.e.m. (transparent areas). Details of descriptive statistics and statistics against chance levels for experimental lines are shown in Supplementary Table 5.

Electrophysiology. Third instar larvae were dissected in 35 mm petri dishes coated with 5 ml two-component silicone (Elastosil RT). Larvae were pinned down dorsal side up at the anterior and posterior end using 77 μm thick sharp etched tungsten needles. The larva was cut open longitudinally along the dorsal midline and the cuticle was pined aside with 40 μm tungsten needles. Interior organs like fat body, intestine or salivary glands were removed except for the cephalopharyngeal skeleton and CNS with attached nerves of interest. Eye and leg imaginal discs were also removed. A transversal cut of the cuticle was performed beneath the CNS to reveal the AN. Nerves not needed for the respective recording were cut. A piece of thinned Parafilm was placed beneath the nerve of interest. This nerve was isolated from the surrounding solution with two adjacent jelly pools. Motor output of the AN was measured using custom made silver wire electrodes connected to a preamplifier (Model MA103, Ansgar Büschges group electronics lab). The preamplifier was connected to a four-channel amplifier/signal conditioner (Model MA 102, Ansgar Büschges group electronics lab). All recorded signals were amplified (amplification factor: 5,000) and filtered (bandpass: 0.1–3 kHz). Recordings were sampled at 20 kHz. Data were acquired with Micro3 1,401 A/D board (Cambridge Electronic Design) and Spike2 software (Cambridge Electronic Design).

Calcium imaging with CaMPARI. High-power LED of 405 nm (Thorlabs, M405L2-UV (405 nm) Mounted LED, 1,000 mA, 410 mW) was driven with a LED...
**Figure 7 | HuginPC neurons are functionally connected to GR66a neurons.** (a) Example of huginPC neuronal arborizations in the protocerebrum expressing GCaMP6s in inactive and active state. Scale bars, 20 μm. (b) Activation of GR66a neurons by dTrpA1 in larvae expressing Hugin-lexA-lexAop-GCaMP6s. Sample traces of calcium currents show rhythmic activity of huginPC projections when GR66a neurons are activated at 30 °C. Quantification of calcium spikes per min showed no significant difference between control (ctrl, n = 13) and experimental genotypes (exp, n = 14) at 20 °C (P = 0.241, Mann-Whitney-U-Rank-Sum-Test (MWU-Test)); control at 20 and 30 °C showed no significant difference between control (ctrl, expressing GCamP6s in inactive and active state. Scale bars, 20 μm without being exposed to ultraviolet light does not convert green to red fluorescence.

**Figure 8 | HuginPC neurons act as gustatory interneurons for bitter taste.** Bitter taste, detected in peripheral taste organs, activates huginPC neurons. Activity of huginPC neurons projecting to higher brain centres leads to a decrease in food intake and in the cycle frequency of feeding related motor patterns in the antennal nerve (AN). Activation of huginPC neurons also leads to aversion to different gustatory substrates, including yeast, thereby triggering aversive behaviour. Thus, huginPC neurons act as relay of bitter taste to the protocerebrum in the Drosophila larval brain.

Data availability. The authors declare that the data supporting the findings of this study are available within the article (and its Supplementary Information files), or available from the authors upon request.

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Author contributions

S.H. and M.J.P. designed experiments and wrote the manuscript. S.H. carried out the experiments and analysed the data. M.P. performed the GRASP experiment.

Additional information

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