Sensitivity of Olfactory Sensory Neurons to food cues is tuned to nutritional states by Neuropeptide Y signalling

Running title: Energy states modulate OSN sensitivity

Tarun Kaniganti[1], Ajinkya Deogade[1], Aditi Maduskar, Arghya Mukherjee, Akash Guru, Nishikant Subhedar and Aurnab Ghose[*]

Indian Institute of Science Education and Research (IISER) Pune
Dr Homi Bhabha Road, Pune 411008, INDIA

Keywords: Neuropeptide Y; Olfactory sensitivity; Olfactory Sensory Neurons; nutritional state; neuromodulation

[1] These authors contributed equally to this work.

[*] Correspondence: Dr. Aurnab Ghose, Indian Institute of Science Education and Research (IISER) Pune, Dr Homi Bhaba Road, Pune 411008, INDIA
Email: aurnab@iiserpune.ac.in

ABSTRACT

Background: Modulation of sensory perception by homeostatic feedback from physiological states is central to innate purposive behaviours. Olfaction is an important predictive modality for feeding-related behaviours and its modulation has been associated with hunger-satiety states. However, the mechanisms mapping internal states to chemosensory processing in order to modify behaviour are poorly understood.

Results: In the zebrafish olfactory epithelium, a subset of olfactory sensory neurons (OSNs) and the terminal nerve projections express neuropeptide Y (NPY). We find that NPY signalling in the peripheral olfactory system of zebrafish is correlated with its nutritional state and is both necessary and sufficient for the olfactory perception of food related odorants. NPY activity dynamically modulates the microvillar OSN activation thresholds and acts cooperatively with amino acid signalling resulting in a switch-like increase in OSN sensitivity in starved animals. We suggest that cooperative activation of phospholipase C by convergent signalling from NPY and amino acid receptors is central to this heightened sensitivity.

Conclusions: This study provides ethologically relevant, physiological evidence for NPY signalling in peripheral modulation of OSN sensitivity to food-associated amino acid cues. We demonstrate sensory gating directly at the level of OSNs and identify a novel mechanistic framework for tuning olfactory sensitivity to prevailing energy states.

INTRODUCTION

Modulation of sensory perception by homeostatic feedback is critical for survival. Gating of sensory inputs by internal states, such as energy availability, enables the animal to issue measured responses, like food-seeking and consumptive behaviours. Olfaction is an important sensory modality in food seeking behaviour providing predictive information on the availability, quality and associated reward salience. Nutritional state-dependent modulation of olfactory perception has been reported in a number of species, though the underlying mechanisms are not well understood (Palouzier-Paulignan *et al.*, 2012; Sengupta, 2013). Additionally, dysfunctional olfactory responses are reported in obese animals, including humans, highlighting the importance of this sensory modality in health and disease (Aime *et al.*, 2007; Stafford *et al.*, 2013).

Activity patterns of neuromodulators generate molecular representations of internal states and modify neuronal properties and circuit dynamics to effect homeostatic adjustments. Neuromodulatory inputs may impinge on multiple levels of sensory processing, including the peripheral sensory circuitry. In the olfactory system, ghrelin, orexins, insulin, leptin, GnRH, FMRF-amide, cholecysteokinin and endocannabinoids are known to influence olfactory sensitivity (Eisthen *et al.*, 2000; Park & Eisthen, 2003; Hardy *et al.*, 2005; Baly *et al.*, 2007; Julliard *et al.*, 2007; Lacroix *et al.*, 2008; Savigner *et al.*, 2009; Breunig *et al.*, 2010; Tong *et al.*, 2011; Palouzier-Paulignan *et al.*, 2012). Although gating of sensory information at the level of the peripheral receptor cells is widely accepted for most sensory modalities, less is known about modulation at the level of olfactory sensory neurons (Lucero, 2013). Receptors for leptin and insulin are expressed in the olfactory mucosa, and these peptides alter the firing of olfactory sensory neurons in *in vitro* preparations (Baly *et al.*, 2007; Lacroix *et al.*, 2008; Savigner *et al.*, 2009). GnRH has also been shown to reduce electro-olfactogram (EOG) responses in salamanders (Park & Eisthen, 2003). However, the significance of these observations in behaving vertebrates has not been tested. Further, little is known about the molecular mechanisms that underlie changes in OSN sensitivity.

Neuropeptide Y (NPY) has long been recognized as a central modulator of feeding associated behaviours across invertebrate and vertebrate phyla. In *C. elegans* and

Drosophila, NPY signalling has been implicated in altering chemosensitivity, heightened orexigenic drive, and facilitating presynaptic transmitter release in the OB (de Bono & Bargmann, 1998; Wu *et al.*, 2005; Root *et al.*, 2011; Sengupta, 2013; Ko *et al.*, 2015). In vertebrates, NPY peptide expression in the OE has been reported from teleost fish to mammals (Hansel *et al.*, 2001; Mathieu *et al.*, 2002; Gaikwad *et al.*, 2005). Ectopic application of NPY to OEs isolated from salamanders and rats has been reported to modify the odorant response (Mousley *et al.*, 2006; Negroni *et al.*, 2012).

Using behaving zebrafish, we demonstrate that NPY expression levels in the OE and OB reflect the prevailing energy states, and NPY signalling is necessary and sufficient for gating olfactory responses to food cues. Paracrine signalling by NPY directly modulates the sensitivity of a subset of OSNs to food-associated amino acid cues via a phospholipase C (PLC) dependent mechanism.

MATERIALS AND METHODS

Animals

Wild type zebrafish were maintained at 28.5˚C on a 14 h light/10 h dark schedule in 10 L and 3 L holding tanks in a recirculating aquarium system (Aquatic Habitats). For studies on zebrafish larvae, the eggs were obtained by setting a cross in 3L breeding tanks (Aquatic Habitats). Eggs were maintained in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00001% methylene blue) in an incubator at 28.5˚C till 78 hpf. Unless otherwise indicated, adult fish were fed *ad libitum* twice daily with freshly hatched brine shrimp. Larvae are were fed thrice daily with either the cultured paramecia water or synthetic larval food.The Institutional Animal Ethics Committee approved all the procedures employed in this study.

Chemicals

Synthetic human neuropeptide Y (NPY), small molecule NPY Y1 receptor inhibitor BIBP-3226 (BIBP), D -(+)- glucose monohydrate (glucose), Trp channel blocker 2-aminoethyl diphenylborinate (2-APB), PLC inhibitor U73122 hydrate (U73122), sodium chloride, paraformaldehyde, sucrose, poly L- lysine, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), L- lysine, L- histidine monohydrochloride monohydrate, L-tryptophan, L- cysteine hydrochloride, L- alanine, L- phenylalanine, L- valine and L-methionine were purchased from Sigma Aldrich. Gq specific inhibitor GP2A (371780) was purchased from Millipore.

Intracerebroventricular (icv) and intranasal delivery

Prior to icv delivery, fish were anesthetized following immersion in 2-phenoxyethanol (Sigma-Aldrich, 1:2000). All pharmacological agents were dissolved in 0.9% NaCl or in DMSO depending on solubility. The final concentration of DMSO did not exceed 0.1% for BIBP, 2-APB, GP2A treatments and 0.5% for U73122 treatment. Icv delivery protocol was modified after (Yokogawa *et al.*, 2007). Briefly, 1 µl of solution was delivered directly into the ventricular space using an insulin needle (Becton Dickinson Insulin Syringe U40 - 31G) attached via a catheter to a 10 µl Hamilton microsyringe. After injection, the fish were returned to their tanks and allowed to recover before

proceeding for either behavioural recordings or immunohistochemistry. Unless otherwise indicated, all the behavioural and immunofluorescence studies were undertaken 30 mins following injection. The following doses were used for icv delivery of glucose (8000 ng); BIBP (100 pmol); 2-APB (100 μM); GP2A (20 μM); U73122 (1 μM) and NPY peptide (1 pmol).

Intranasal delivery of BIBP-3226 (66.67pmol) or vehicle was carried out by superfusing both the OE by delivering 5μl of the solution via the nares of anesthetized fish for 30 secs using a Hamilton microsyringe. The mouth and gills were covered with a small piece of tissue paper to avoid the solution from spreading. Following the procedure, fish were returned to the tank after 1 minute, allowed to recover from anesthesia for 2 mins and evaluated in the odorant preference assay.

Amino acid stimulation of the OE for evaluation of OSN activation

Post 30 mins icv delivery of pharmacological agents, fish were transferred into the habitat tank containing 1 liter aquarium water. A mixture of 8 amino acids (Ala, Cys, His, Lys, Met, Phe, Trp, and Val; 0.8 mM each in distilled water or solvent was introduced into these tanks using a syringe. For dose-response experiments, 0 mM, 0.008 mM, 0.08 mM and 0.8 mM concentrations of each of the amino acids was used in the odorant mixture. Following the 8 min exposure, the fish were immediately anesthetized and processed for pERK immunofluorescence.

Immunofluorescence

Adult fish were anesthetized followed by the removal of skull and immersion in 4% paraformaldehyde (for 20 hours at 4 ^{o}C) for all experiments, except those involving pERK staining in the OE. In latter experiments, the fixation time was between 14-16 hours in 3% paraformaldehyde at 4 ^{o}C. The brain and/or the olfactory epithelia were dissected out and transferred to 25% sucrose solution for cryoprotection, embedded in OCT compound (Jung), sectioned in the transverse plane at 10 – 20 μm thickness using a cryostat (CM1950; Leica) and mounted on poly-L-lysine coated slides. Zebrafish larvae (78 hpf) were directly immersed in 4% paraformaldehyde, similarly processed and sectioned in sagittal or transverse planes at 10 μm thickness. The slides containing the sections were stored at -40 ^{o}C until they were processed for immunofluorescence.

147 The frozen sections were rehydrated and permeabilized using 0.5% Triton-X in
148 phosphate buffer saline (PBST). The sections were treated with 5% heat inactivated
149 goat serum (HIGS) or 5% BSA in PBST for 1 hour and then incubated with the primary
150 antibody overnight at 4 ^{o}C. The sections were washed using 0.1% PBST and pre-
151 incubated with 5 % HIGS or 5 % BSA in PBST for 1 hour and was incubated with
152 desired secondary antibody for 3 hours at room temperature (RT). Then the sections
153 were washed and mounted with mounting media (70% glycerol, 0.5% N-propyl gallate in
154 1 M Tris, pH 8.0, DAPI). The following primary antibodies were used in this study -
155 polyclonal anti-NPY antibody (1:2000; Sigma), monoclonal anti-calretinin antibody
156 (1:3000; Swant), monoclonal anti-NPY antibody (1:2000; Sigma), polyclonal anti- $G_{\alpha/olf}$
157 antibody (1:1000; Santa Cruz), anti-Hu antibody (1:100; Abcam), anti-SV2 antibody
158 (1:50; Developmental Studies Hybridoma Bank), monoclonal anti-phosphorylated ERK
159 1/2 (1:500; Abcam) and polyclonal anti-TrpC2 (1:50; Abcam). Anti-rabbit Alexa-488
160 (1:500; Invitrogen) and anti-mouse Alexa-568 (1:500; Invitrogen) were used as
161 secondary antibodies. Sections were observed using either an upright epifluorescence
162 microscope (Axioimager.Z1 with Axiocam HRm camera; Carl Zeiss) or inverted laser
163 scanning confocal microscopes (LSM 710 or LSM 780; Carl Zeiss).

164 Preadsorption with NPY peptide was carried out to demonstrate the specificity of the
165 anti-NPY antibody (Sigma) used in this study. Briefly, anti-NPY antibody was
166 preadsorbed with synthetic human peptide (N5017; Sigma) at 10^{-5} M for 24 hours.
167 Application of the preadsorbed antibody resulted in total loss of the immunoreactivity
168 from the olfactory bulb sections, confirming the specificity of the antibody (data not
169 shown).

170 ***Whole-mount immunofluorescence***

171 Whole-mount immunohistochemistry was carried out using standard protocols. Briefly,
172 adult zebrafish brain (olfactory nerve and OE intact) were fixed in 4% PFA and post-
173 fixed in Dent's fixative (4:1 methanol:DMSO) overnight. The brain was then dehydrated
174 in 100% methanol for 30 min. To promote permeabilization, the tissue was subjected to
175 three freeze (-80 ^{o}C) thaw (RT) cycles in 100% methanol followed by overnight storage
176 at -80 ^{o}C. The tissue was rehydrated serially with 50% methanol, 15% methanol and

177 PBS for 60 min each at RT. The tissue was permeabilized with proteinase K (10 µg/ml
178 in PBS) for 5 min followed by three washes in PBS. Blocking was carried out for 8 hours
179 at 4 ^{o}C in 5% BSA, 5% DMSO in 0.5 % Triton X-100. The tissue was then incubated in
180 primary antibody against neuropeptide Y (1:5000) and synaptic vesicle-2 (1:50) for 48
181 hours at 4 ^{o}C. Following three washes with PBST (15 min each; 4 ^{o}C, the tissue was
182 incubated overnight with secondary antibody Alexa Fluor-488 (1:500) and Alexa Fluor-
183 568 (1:500) at 4 ^{o}C. The tissue was rinsed three times in PBST (2 hours each; RT) and
184 once in PBT (RT) and finally mounted in 70% glycerol, 0.5% N-propyl gallate in 1 M
185 Tris, pH 8.0, DAPI on a coverslip-bottomed petridish. Images were taken using the
186 inverted laser scanning confocal microscope (LSM 780; Carl Zeiss).

187 ***Quantification of NPY fluorescence intensity and cell count analysis***

188 The intensity of NPY immunoreactivity in the olfactory bulb was measured using Image
189 Pro Plus 7.1 software (Media Cybernetics). Briefly, the precise volume measurements
190 of the bulb were made in the 3D constructor using interactive thresholding. The
191 integrated optical density was then measured in the defined volume of the bulb for NPY
192 and SV2 channels separately. For each treatment, the optical density of the NPY
193 immunoreactivity was normalized with respect to the SV2 immunoreactivity and again
194 with a unit volume defined. NPY immunoreactivity in the soma of the terminal nerve
195 neurons was measured using Image J. Relative NPY fluorescence per terminal nerve
196 neuron in each group was plotted with respect to that of the control group. The intensity
197 of NPY immunoreactivity in the olfactory epithelia was measured using Image J. The
198 total fluorescence was calculated by the formula corrected total fluorescence (CTF) =
199 Integrated density – (Area of the selected ROI * Mean fluorescence of the background
200 readings). The CTF was calculated for both NPY immunoreactivity and DAPI labeling
201 and then the NPY fluorescence was normalized to DAPI fluorescence.

202 The number of NPY-ir glomeruli in the olfactory bulb and the number of NPY/pERK-ir
203 OSNs in the olfactory epithelia were manually counted. The number of NPY or pERK
204 expressing OSNs were represented as the average number of cells per lamella across
205 all sections covering both epithelia of each individual fish.

206 ***Odorant preference assay in adult zebrafish***

207 In order to habituate the fish (3-5 months old) to the experimental tank (4 x 20 x17 cm
208 filled with 1400 mL of aquarium water) and the icv injections protocol, a two-day
209 acclimation regime was carried out. On the first day, the fish were anesthetized and
210 injected with saline (0.9 % NaCl) via the icv route. On the second day, a mock injection
211 procedure was followed in which the fish was subjected to the entire exercise as on the
212 first day but no active agent was delivered. During the two-day habituation, the fish were
213 not given any food. The habituated fish were then starved for yet another day and then
214 anaesthetized and injected with either vehicle or BIBP-3226 (100pmol) via the icv route.
215 Similarly, the assay was carried out for the regularly fed fish, starved fish without any
216 injection, and starved fish injected with glucose (8000ng), NPY peptide (1 pmol) or a co-
217 injection of glucose and NPY peptide. Following an interval of 30 mins, the fish were
218 subjected to the odorant preference assay, one at a time, while being video recorded
219 (Sony Handycam DCR SR20) from the side. The olfactory behavioural assay was
220 modified after Koide et al. (2009). Adult zebrafish were assayed for response to a
221 mixture of 8 amino acids (Ala, Cys, His, Lys, Met, Phe, Trp, and Val; 0.1 mM each in
222 distilled water; Sigma). No stimulation was provided for the first 5 mins following which
223 the amino acid mix was introduced in one side of the tank at the rate of 1 ml/min using a
224 syringe pump. Simultaneously, distilled water was delivered at the same rate at the
225 opposite end of the tank. The behaviour of the fish was monitored for a total of 10 mins
226 after the introduction of the odorants. All behavioural assays were performed between
227 1200h to 1700h.The swimming paths were analyzed from the recorded videos using an
228 automated video tracking system (Ethovision XT version 8.0; Noldus). Preference
229 indices were calculated using the formula: Preference index (PI) = ((Total time spent in
230 the odorant zone) – (Total time spent in the control zone)) / (Total time). The data were
231 evaluated for the period of 5 mins before and after odorant stimulation.

232 ***L-cysteine aversion assay in 78 hpf larvae***

233 The protocol described by (Vitebsky *et al.*, 2005) was employed with modifications.
234 Briefly, 78 hpf zebrafish larvae (n=10-20) were placed in a 35 mm Petri dish containing
235 5 ml of E3 medium. A circle of 1 cm diameter was drawn on white paper and placed
236 under the petri dish such that the circle was at the center of the dish. The larvae were
237 gently swirled to position them within the perimeter of the circle. L-cysteine (10 ul of 3 x

238 10^{-5} M) was gently introduced over the surface of the water, approximately at a point
239 above the center of the circle (ensuring minimal vibration of the water surface). The
240 entire procedure was video recorded and the number of larvae that moved out of the
241 area of the circle within 30 secs of addition of L-cysteine was scored. The larvae that
242 remained within the circle were considered as non-responsive. The larvae once used for
243 the assay were not used in further experiments and the entire experiment was carried
244 out at 27-28.5 ^{o}C.

245 ***Response to alarm pheromone (Schreckstoff)***

246 For testing the alarm response in fish under different energy states, the skin extract was
247 prepared as described earlier (Mathuru *et al.*, 2012). Briefly, several shallow cuts were
248 made on the skin of the zebrafish using a sharp blade. The injured fish was immersed in
249 2 ml of 20mM Tris- Cl (pH 8.0) for 30 seconds. This process was repeated for 7-10 fish
250 and the extract was collected into the same volume. The 2 ml of crude extract was then
251 centrifuged at 13.2k rpm and the supernatant was collected. A 25% solution of the
252 alarm pheromone was prepared by diluting in distilled water and 500 µl of the latter was
253 used in the behavioural assay.

254 As stated above in the odorant preference assay, adult zebrafish were singly housed,
255 habituated for 2 days, starved for yet another day, anaesthetized and injected with
256 either saline or glucose (8000ng) via icv route. Following an interval of 30 mins, the fish
257 were subjected to the assay. Control stimulus (500µl of distilled water) was introduced
258 into the tank following the first 5 mins using a plastic dropper. After a period of 10 mins,
259 test stimulus (500 µl of the alarm pheromone extract) was added. The fish were
260 evaluated for their response to the alarm pheromone for 10 minutes. The assay was
261 video recorded (Sony Handycam DCR SR20) and analyzed using an automated video
262 tracking system (Ethovision XT version 8.0; Noldus).

263 Darting was defined as rapid swimming with increased reversal frequency and
264 swimming velocities at least 2.5 times higher than those recorded in the first 5 mins of
265 observation. Freezing was defined as a period of complete immobility, typically at
266 bottom of the tank for at least 5 seconds.

267 ***NPY Y1 Receptor RNA in situ hybridization***

268 For whole-mount RNA *in situ* hybridization (ISH) experiment adult fish were starved for
269 three days and anesthetized followed by the removal of the dorsal cranium and
270 immersion in 4% paraformaldehyde (for 14-16 hours at 4 ^{0}C), olfactory rosettes were
271 dissected out, washed in phosphate buffered saline (PBS), and stored in 100%
272 methanol at -20°C overnight. ISH was carried out as described earlier (Akash *et al.*,
273 2014). Digoxigenin-labeled sense and antisense riboprobes were transcribed *in vitro*
274 using Zebrafish NPY Y1R TOPO pcDNA 3.1 vector template (gift from D. Larhammar)
275 using the following primer pair. Forward: 5'-GACACCAACTCATCCTGCACC-3';
276 Reverse: 5'-TGGGTACAGTTCATCGCCAC-3'. Probes were purified using BioRad
277 Micro Bio-Spin columns as per manufacturer's protocol. Samples were sequentially
278 rehydrated to PTW (phosphate buffered saline, 0.1% Tween-20 pH 7.3) and
279 permeabilized in 2% hydrogen peroxide for 35 minutes (min) at room temperature (RT).
280 Samples were further permeabilized by a 40-min proteinase K treatment and post-fixed
281 for 20 min in 4% paraformaldehyde at RT. Prehybridization, probe hybridization and
282 washes were performed as described previously (Akash *et al.*, 2014) except for the
283 inclusion of 5% dextran sulfate in the hybridization buffer. The OE were embedded in
284 4% low melting point agarose blocks and sectioned (30 µm) using a Vibratome (VT
285 1200; Leica). The sections were blocked in 1% blocking solution (10 min at RT; Roche
286 Applied Sciences) and then incubated overnight at 4 ^{0}C with sheep anti-DIG alkaline
287 phosphatase conjugated antibody (1:2,000; Roche Applied Sciences). Sections were
288 washed thrice with PTW buffer and twice with coloration buffer (100 mM Tris-HCl, pH
289 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 2 mM Levamisole). DIG-labeled
290 probes were detected using pre-warmed BM purple solution (Roche). The reaction was
291 continuously monitored at 37^{0} C for 30 min to 2 h or overnight at 4 ^{0}C depending on the
292 strength of the signal. The reaction was stopped by washing three times with PTW
293 buffer. The sections were then mounted in 70% glycerol and were either processed for
294 imaging or stored at 4 ^{0}C. DIC photomicrographs were taken with an upright microscope
295 (Axioimager Z1, Carl Zeiss).

296 ***Statistical analysis***

297 All the raw measurements were imported to GraphPad Prism 5 (GraphPad Software
298 Inc.) for graphical representation and statistical analysis. In the odorant preference
299 assay, the preference index for each group is represented as boxes and whiskers; the
300 box ranges from the 25th percentile to the 75th percentile and the whiskers represent the
301 minimum and maximum values of the group. The horizontal line in the box denotes the
302 median. The medians of the preference indices, before and after the odorant
303 stimulation, were tested for significant deviation from 0 using Wilcoxon signed-rank test.

304 The effect of energy status on amino acid sensitivity in the OSNs and the amino acid
305 dose response on animals with different energy states was compared using Two-way
306 ANOVA followed by Bonferroni's post-hoc test. The confidence limit for statistical
307 significance in all the analyses was $p < 0.05$.

308

309 **RESULTS**

310 ***NPY is enriched in the olfactory epithelium and olfactory bulb in zebrafish***

311 Whole mount immunofluorescence analysis of the intact olfactory epithelium (OE) and
312 olfactory bulb (OB) of adult zebrafish with antibodies against NPY and the pre-synaptic
313 marker SV2 (synaptic vesicle transmembrane proteoglycan 2) revealed two sources of
314 NPY in the peripheral olfactory circuitry. First, a subset of the olfactory sensory neurons
315 (OSNs) with NPY fiber projections over the olfactory nerve to the bulb, finally
316 terminating as glomeruli in the OB (Figures 1A, B). The second source of NPY was the
317 terminal nerve (TN). TN soma and their axonal projections innervating the OE as well as
318 the extrabulbar telencephalic regions, showed extensive NPY expression (Figure 1A).

319 Double immunostaining with NPY and neuronal marker Hu (Figure 1B) (Iqbal & Byrd-
320 Jacobs, 2010) confirmed the neuronal identity of the NPY expressing cells. Co-
321 localization with OSN-subtype markers revealed that NPY expression was largely
322 limited to the crypt (co-localization with S100) and G_{olf} positive ciliated OSNs. However,
323 it was excluded from calretinin-expressing microvillar and ciliated OSN populations
324 (Figures 1C-E).

325 Expression of NPY in the olfactory anlage of larval zebrafish was tested using
326 immunofluorescence in 78 hpf zebrafish. Consistent with the observations in adult fish,
327 sagittal sections of 78 hpf zebrafish larvae showed NPY expression in both the OE and
328 OB (Figure 1F).

329 NPY signalling via the NPY Y1 receptor (Y1R) has been implicated in regulating food
330 intake in multiple species (Mercer *et al.*, 2011; Nguyen *et al.*, 2012; Yokobori *et al.*,
331 2012). Further, Y1R mediates olfactory processing in rodents (Negroni *et al.*, 2012).
332 mRNA *in situ* hybridization using antisense probe against Y1R mRNA showed intense
333 signal in subpopulations of OSNs (Figure 1G upper panel) while the sense probe
334 controls did not produce any reaction (Figure 1G lower panel).

335 These data demonstrate that NPY and the NPY Y1 receptor is expressed in the
336 peripheral olfactory circuitry of zebrafish.

337 ***Energy status-dependent expression of NPY in the olfactory system***

338 NPY promotes hunger and increases food intake across vertebrate phyla, including
339 zebrafish and its expression is elevated in multiple neuronal regions upon fasting (Clark
340 *et al.*, 1987; Marks *et al.*, 1992; Volkoff *et al.*, 2005; Yokobori *et al.*, 2012). Fed (free
341 feeding on an excess of food 30 mins prior to sacrifice; mock icv injection), starved (for
342 3 days; mock icv injection) and adult zebrafish injected with glucose (via icv route) were
343 compared for NPY expression in the olfactory epithelia and olfactory bulb. Glucose icv
344 treatment allowed isolation of central regulation from peripheral homeostatic inputs. The
345 latter treatment also reduced the variability in free feeding fish that may arise from
346 individual proclivities. Total fluorescence intensity of the NPY signal and the number of
347 NPY expressing OSNs in the olfactory mucosa increased upon starvation in comparison
348 to either well fed or glucose injected animals (Figure 2A-C, Figure 2J,$p= 0.0044$ and
349 Figure 2K, $p= 0.0065$). Similarly, both the NPY signal intensity in the OB and the
350 number of NPY-positive glomeruli also increased upon starvation (Figure 2D-F, Figure
351 2L, $p<0.0001$ and Figure 2M, $p= 0.0059$). NPY fluorescence in the olfactory bulb
352 comprises of signals from both the OSN projections onto glomeruli and the terminal
353 nerve. Independent evaluation of the terminal nerve showed an increase in NPY
354 immunofluorescence per TN soma and also in the number of NPY expressing TN cell
355 bodies in starved zebrafish compared either to fed or glucose injected fish (Figure 2G-I,
356 Figure 2N, $p<0.0001$ and Figure 2O, $p<0.0001$).

357 These results indicate that NPY expression levels in the olfactory epithelia and bulb
358 reflect the prevailing energy status in the brain.

359 ***Nutritional state-dependent olfactory perception of amino acids is mediated by***
360 ***NPY Y1 receptor signalling***

361 Energy state-dependent NPY expression and availability of NPY-receptors in the
362 peripheral olfactory circuitry of zebrafish could be indicative of a role for NPY signalling
363 in nutritional state dependent olfactory processing. We evaluated olfactory responses of
364 fed and starved adult zebrafish in an odorant preference assay using a mixture of amino
365 acids, a major food-associated attractive odorant cue in fish (Figure 3A). The percent
366 time spent in the odorant half by starved animals was more in the post-odorant period
367 compared to the pre-odorant stimulation period and was reflected in the statistically

368 significant increase in the preference index (PI) for the baited side (Figure 3C, p=
369 0.0156). However, fed animals displayed no difference in PI between the pre- and post-
370 stimulation periods (Figure 3B).

371 To test the role of NPY signalling in olfaction, we evaluated odorant preference
372 behaviour of starved fish (high NPY expression in OE and OB) while abrogating NPY
373 signalling through the NPY Y1 receptor (Y1R; the major receptor associated with NPY-
374 dependent appetitive behaviours (Yokobori *et al.*, 2012)). The PIs for animals receiving
375 Y1R antagonist BIBP-3226 (BIBP) via the icv route showed no difference between pre-
376 and post- amino acid stimulation periods (Figure 3E). However, fish receiving the
377 control treatment (saline via icv route) displayed an increased preference for the baited
378 side following odorant stimulation (Figure 3D, p= 0.0313). Analysis of locomotor
379 behaviour revealed no differences between BIBP and control animals (data not shown).

380 As NPY expression in the OE and OB was reduced in fed and glucose administered
381 animals compared to starved animals (Figure 2), we tested whether NPY-signalling was
382 sufficient to restore olfactory sensitivity to amino acids in energy-rich conditions. Starved
383 fish receiving glucose via the icv route (shown previously to reduce endogenous NPY
384 expression) showed no difference in PI before and after amino acid baiting (Figure 3F).
385 However, administration of exogenous NPY to glucose-injected animals was sufficient
386 to restore odorant preference (Figure 3G, p= 0.0313).

387 To test whether NPY signalling modulates nutritional state -dependent olfactory
388 sensitivity specifically for food-associated cues, we evaluated the response to the alarm
389 cue, Schreckstoff. The skin of conspecifics produce Schreckstoff, which is sensed by
390 the crypt subtype of OSNs and robustly triggers a sequence of behaviours that include
391 vigorous darting movements followed by freezing (Mathuru *et al.*, 2012). We compared
392 the response of Schreckstoff between fed and starved adult zebrafish. Schreckstoff
393 application initiated strong behavioural responses, but no differences were detectable
394 between the two nutritional states. The duration of both darting movements (Figure 3I;
395 ns) or immobility (Figure 3J; ns) were comparable between starved and fed animals. As
396 vigorous darting preceded the freezing behaviour, we evaluated the latency to display

397 the darting behaviour as a measure of sensitivity to the alarm cue. However, the latency
398 for darting was also similar between fed and starved zebrafish (Figure 3H; ns).

399 These experiments suggest that endogenous NPY signalling via the NPY Y1 receptor is
400 subject to regulation by the brain energy status and is sufficient to specifically modulate
401 the olfactory sensitivity to food-associated amino acid cues.

402 ***NPY signalling modulates olfactory responses to amino acids in larval zebrafish***

403 Reduction in NPY signalling has been associated with the attenuation of the feeding
404 drive. Thus, the use of Y1R antagonist and food-associated attractive odorant cues
405 (amino acid mixture) in the odorant preference assay may potentially confound an
406 olfactory response with a generalized loss of the feeding drive. To exclude this
407 possibility, we employed an amino acid –dependent aversive assay. 78 hpf zebrafish
408 were stimulated with L-Cysteine, an aversive olfactory signal at high concentrations
409 (Vitebsky *et al.*, 2005), and the escape response monitored (Figure 3K). While a large
410 proportion of larvae responded to L-cysteine (Figure 3I; $p<0.05$), pre-treatment with
411 BIBP abolished the aversive response, which was indistinguishable from the control
412 groups (Figure 3L; ns). This assay, using an amino acid-based aversive cue,
413 demonstrates that NPY-mediated behavioural responses are not an artifact of reduced
414 feeding drive. Rather, NPY signalling modulates amino acid sensing.

415 The L-cysteine aversion assay in larvae also addressed a second potential confound.
416 While the adult odorant preference assay is largely specific to the olfactory system
417 (Koide *et al.*, 2009), it has also been reported that the fish gustatory system can sense
418 amino acids (Oike *et al.*, 2007). However, as the gustatory system is not functional at
419 the developmental time of 78 hpf (Lindsay & Vogt, 2004), the L-cysteine aversion assay
420 at this developmental stage indicates that the olfactory system is the sole sensory
421 modality involved. Collectively, the larval L-cysteine aversion assay implicates NPY
422 signalling in the olfactory processing of amino acid cues.

423 ***Peripheral modulation of olfactory behaviour by NPY***

424 The icv mode of delivery of NPY signalling antagonists in the previous experiments
425 does not allow discrimination between central and peripheral contributions. In order to

426 isolate the role of peripheral modulation, we developed a nasal delivery protocol
427 selectively targeting the peripheral olfactory circuitry. Olfactory epithelia of starved, adult
428 zebrafish were bilaterally bathed with BIBP for 30 secs, introduced via the naris and
429 tested in the odorant preference assay. While the BIBP treated fish were agnostic to the
430 amino acid cues, the control animals were attracted to the amino acid baited side
431 (Figures 4A,B).

432 Phosphorylated ERK1/2 (pERK) is a sensitive marker of neuronal activation (Hussain *et*
433 *al.*, 2013; Randlett *et al.*, 2015). The dorsolateral region of the telencephalon (Dl)
434 produce spontaneous neural activity detectable by pERK immunoreactivity. We used
435 this observation to compare the influence of BIBP on the zebrafish telencephalon
436 between the icv and nasal delivery routes. Compared to control treatment, BIBP
437 administered to starved fish via the icv route consistently resulted in a decrease in
438 pERK-positive cells in the dorsolateral region of the telencephalon (Dl) (Figure 4C,D,G,
439 p= 0.0280), suggesting a role for NPY signalling in promoting activity in this region.
440 However, nasal lavage of BIBP, under the same conditions that abrogate odor
441 preference in the behavioural test, failed to decrease ERK activation in the Dl neurons
442 (Figure 4E,F,H, ns). These experiments suggest that the conditions optimized for the
443 nasal delivery of BIBP led to selective inactivation of NPY signalling that did not extend
444 to telencephalic regions.

445 In summary, the data from nasal delivery of the NPY Y1 receptor antagonist implicate a
446 significant contribution of NPY-signalling in peripheral modulation of olfactory responses
447 to amino acid cues. The nasal delivery experiments implicate regions upstream of the
448 telencephalon, namely the OE and/or the OB as the site/s of action of NPY modulation.
449 This observation led us to directly evaluate the response of OSNs, as described in the
450 next section.

451 ***Peripheral modulation of amino acid sensitivity of OSNs by NPY signalling***

452 The indication that NPY may act in the periphery to modulate olfaction prompted us to
453 investigate the response of the OSNs to amino acids under different conditions. The
454 microvillar subtype of OSNs employ vomeronasal type 2 receptors (V2Rs) to mediate
455 amino acid sensing in teleost fish and amphibia, like *Xenopus* (Koide *et al.*, 2009;

456 Sansone *et al.*, 2014). Unlike classical OSN signal transduction, V2R signalling involves
457 phospholipase C activity and opening of the diacylglycerol (DAG) -gated TrpC2
458 channels resulting in OSN activation (Lucas *et al.*, 2003; Sato *et al.*, 2005; Koide *et al.*,
459 2009; Sansone *et al.*, 2014). To test if amino acid mediated activation of OSNs in
460 zebrafish was limited to the microvillar OSNs, we directly evaluated OSN activation by
461 monitoring ERK1/2 phosphorylation (pERK). We found that OSNs activated by amino
462 acid stimulation, as revealed by ERK activity, exclusively co-localized with TrpC
463 expressing OSNs (Figure 5A-C).

464 We next tested if prevailing energy states directly modulated OSN activation by amino
465 acids. Fed and starved zebrafish were stimulated with amino acid or distilled water and
466 OSN activation was evaluated by scoring for pERK signal. A small increase in the
467 number of activated OSNs was observed in fed fish stimulated with amino acids
468 compared to controls (Figure 5D). However, a significantly larger response was seen
469 upon amino acid stimulation of starved zebrafish suggesting increased OSN sensitivity
470 to odorants in these animals (Figure 5D). Two-way ANOVA revealed a statistically
471 significant interaction between the high NPY signalling (starvation) and V2R activation
472 (amino acid stimulation) denoted as the [NPY x AA] interaction (Figure 5D; $F_{(1,20)}$=112.2,
473 $p< 0.0001$). Post-hoc analysis revealed that while amino acid stimulation had a small
474 effect (increased OSN activation in fed fish where endogenous NPY signalling is
475 reduced compared to the controls), high NPY signalling alone failed to activate OSNs in
476 the absence of odorant (no difference between control treatments of fed and starved
477 fish).

478 To directly test the role of NPY in OSN activation, we used the NPY Y1R antagonist
479 BIBP administered via the icv route to block NPY signalling in starved zebrafish.
480 Compared to distilled water, amino acid stimulation caused a large increase in OSN
481 activation in starved control animals (Figure 5E). However, the OSN activation was
482 significantly attenuated in BIBP pre-treated starved animals compared to those
483 receiving the vehicle, implicating NPY signalling in tuning OSN sensitivity (Figure 5E).
484 As in the previous experiment, comparing fed and starved fish, two-way ANOVA
485 revealed a statistically significant [NPY x AA] interaction (Figure 5E; $F_{(1,20)}$= 28.06, $p<$
486 0.0001). While post-hoc analysis showed an effect for amino acid stimulation (increase

487 in OSN activation by amino acid compared to distilled water stimulation in BIBP treated
488 fish), increase in NPY signalling alone was not enough to change OSN activation above
489 baseline (no difference between distilled water stimulated BIBP and vehicle
490 administered fish).

491 To test if NPY signalling was sufficient to restore OSN sensitivity to amino acid
492 stimulation, we evaluated OSN activity (pERK-ir) in fish where endogenous NPY
493 signalling is suppressed by administration of glucose via the icv route followed by
494 addition of exogenous NPY peptide. Compared to distilled water, amino acid stimulation
495 resulted in an increase in the number of OSNs activated in glucose-injected fish (Figure
496 5F). However, this change was significantly smaller than the large increase in OSN
497 activation seen upon amino acid stimulation of glucose-injected fish that have also
498 received exogenous NPY (Figure 5F). These data implicate NPY signalling to be
499 sufficient in restoring OSN sensitivity to amino acids. Again two-way ANOVA revealed a
500 statistically significant [NPY x AA] interaction (Figure 5F; $F_{(1,20)}= 31.71$, $p< 0.0001$). As
501 in the last two experiments, amino acid stimulation did have a statistically significant
502 effect (amino acid increased OSN activation compared to distilled water in glucose-
503 injected animals) but this was significantly smaller than the combined effect of high NPY
504 signalling and amino acid stimulation.

505 In all the conditions tested, two-way ANOVA revealed a consistent statistically
506 significant [NPY x AA] interaction. This strongly suggests a cooperative effect between
507 NPY signalling and amino acid triggered signal transduction (via the V2Rs), which is not
508 merely additive. This conclusion is further strengthened by the observation that
509 artificially increased NPY signalling by injecting starved fish (high endogenous NPY
510 expression) with additional NPY peptide fails to show any OSN activation in the
511 absence of amino acid stimulation (data not shown).

512 These experiments suggest that increase in NPY in the peripheral olfactory system of
513 energy-deficient animals may lower the threshold for OSN activation by food-related
514 odorants (amino acids), while the converse holds for the energy-rich state. To test the
515 prediction that nutritional states alter OSN sensitivity to amino acid cues, we compared
516 the extent of OSN activation in glucose administered (via icv) and starved fish (saline

517 via icv) in response to varying concentrations of the amino acid cues. Two-way ANOVA
518 revealed a statistically significant difference between the two dose-response curves
519 (Figure 5G; $F_{(1,16)}$= 273.27, p< 0.0001) with the starved fish showing a sharp increase in
520 sensitivity to amino acid cues compared to glucose treated animals. This result supports
521 the conclusion that heightened NPY signalling in starved animals increases the
522 sensitivity of OSNs to amino acid cues.

523 Taken together these experiments underscore the dynamic modulation of the OSN
524 activation threshold in different energy states and implicate NPY signalling acting
525 cooperatively with amino acid signal transduction in tuning OSN sensitivity to food cues.

526 ***NPY and amino acid signalling converges on PLC to activate OSNs***

527 Our experiments suggest that NPY signalling facilitates olfactory perception of food-
528 associated amino acids, which is largely limited to microvillar OSNs that employ a V2R-
529 PLC-TrpC2 signalling cascade. Attenuation of OSN activation by blocking TrpC2 activity
530 would be confirmatory but there are no specific TrpC2 inhibitors currently available. We
531 therefore used 2-APB, a general Trp channel blocker (not specific for TrpC2), that has
532 been shown to block OSN responses in the vomeronasal organ in rodents (Lucas *et al.*,
533 2003; Zhang *et al.*, 2010) and amino acid responses in the OE of *Xenopus* (Sansone *et*
534 *al.*, 2014). Pretreatment of zebrafish with 2-APB administered via icv route significantly
535 attenuated OSN activation by amino acids in starved zebrafish (Figure 6A, p<0.0001).

536 As phospholipase C is central to V2R signal transduction, inhibition of PLC should
537 attenuate OSN activation by amino acids. Indeed, pretreatment with a generic PLC
538 inhibitor, U73122 (introduced via the icv route), resulted in a strong reduction in the
539 number OSNs showing amino acid induced pERK expression compared to animal
540 pretreated with the vehicle control (Figure 6B, p= 0.0058). This suggests a role for PLC
541 signalling in processing amino acid cues in the olfactory epithelia.

542 It has been shown earlier that exogenous NPY is sufficient to restore both the
543 behavioural response to amino acids, and activation of OSNs by amino acids under
544 conditions where endogenous NPY is reduced by glucose administration (Figures 3G
545 and 5F). We tested if the restoration of OSN activation by exogenous NPY was
546 dependent on PLC activity. Pretreatment with the PLC inhibitor prevented the increase

547 in pERK positive OSNs upon amino acid stimulation even in the presence of exogenous
548 NPY peptide. In contrast, the vehicle treated animals showed the expected increase in
549 OSN activation (Figure 6C, p=0.0014). These results implicate PLC signalling in NPY-
550 mediated lowering of the amino acid sensitivity threshold of OSNs. NPY receptor
551 mediated activation of PLC has been documented previously and is thought to involve
552 $G_{\beta/\gamma}$ signalling (Koch *et al.*, 1994; Selbie *et al.*, 1995; Selbie *et al.*, 1997).

553 We have shown earlier that NPY receptor and amino acid stimulated V2R signalling
554 have a cooperative effect of OSN activation (Figure 5D-F) and involve PLC activity
555 (Figure 6B, C above). Interestingly, there is a significant body of literature implicating
556 synergistic activation of PLCβ isoforms in GPCR signalling crosstalk (Zhu &
557 Birnbaumer, 1996; Selbie *et al.*, 1997). Cooperative activation of PLCβ necessitates
558 simultaneous occupancy of the $G_{\beta/\gamma}$ and $G_{\alpha q}$ binding sites on PLCβ. NPY receptor
559 signalling via $G_{\beta/\gamma}$ has been implicated in potentiation of adrenergic and purinergic
560 signalling responses via synergistic activation of PLC (Philip *et al.*, 2010). Assuming a
561 similar cooperative mechanism is operational in zebrafish OSNs where our data show
562 convergent signalling by NPY and amino acids onto PLC, V2R activation is expected to
563 provide the $G_{\alpha q}$ component. We used a $G_{\alpha q}$ specific inhibitory peptide, GP2A, to test the
564 involvement of $G_{\alpha q}$ in amino acid odorant transduction. Starved animals (high
565 endogenous NPY expression) were pretreated with GP2A or vehicle control and
566 stimulated with amino acid mixture. While amino acid stimulation caused a large
567 increase in OSN activation in control fish, GP2A treatment significantly attenuated
568 amino acid activation of OSNs (Figure 6D, p<0.0001). The residual number of activated
569 OSNs was comparable to that seen on amino acid stimulation of BIBP- or glucose-
570 injected animals.

571 These data implicated PLC signalling as a point of convergence of NPY Y1R and amino
572 acid-sensing V2R signalling. Further, the possibility of cooperative activation of PLC by
573 the two signalling cascades as the biochemical basis of cooperativity observed at the
574 levels of OSN activation is strengthened by the identification of a critical role of $G_{\alpha q}$
575 signalling in amino acid signal transduction in zebrafish OSNs.

576

577 **DISCUSSION**

578 We have used zebrafish to study the modulation of olfactory sensitivity to food-related
579 cues by NPY signalling in response to the prevailing nutritional state. As NPY
580 expression in the peripheral olfactory circuitry was tuned to the nutritional state, we
581 investigated the contribution of NPY signalling to olfactory perception in an odorant
582 preference assay using a food cue associated mixture of amino acids. While the starved
583 fish showed a strong preference for the baited side, recently fed fish were agnostic to
584 the amino acid cue suggesting that the positive valence of food-related odorants is
585 tuned to the prevailing nutritional state. Administration of NPY Y1 receptor antagonist to
586 starved animals also reduced the attraction towards amino acids. Since NPY is a strong
587 orexic agent, this behaviour could also be attributed to the loss of feeding drive following
588 NPY inhibition. We tested this possibility using an amino acid based aversion assay and
589 found that NPY activity was necessary for amino acid perception. As central
590 administration of glucose reduced NPY expression in OE, it should mimic the fed state
591 in its response to amino acids. While glucose injected fish showed no preference for
592 amino acids, exogenous administration of NPY was sufficient to restore the response.
593 Collectively, these experiments implicate a central role for NPY as a mediator of
594 nutritional state-dependent tuning of olfactory perception of food-related cues. To
595 investigate if the differences in olfactory response observed in fed and starved states
596 extend to non-food -associated odorants, we evaluated the response to the alarm
597 substance Schreckstoff (a non-amino acid olfactory cue). Irrespective of their nutritional
598 states, the fish responded similarly to Schreckstoff suggesting that the energy state-
599 dependent modulation is specific to food cues. Teleost fish employ a vomeronasal-like
600 amino acid sensing mechanism, largely confined to microvillar OSNs (Sato *et al.*, 2005;
601 Koide *et al.*, 2009), while Schreckstoff is processed by crypt OSNs (Mathuru *et al.*,
602 2012). NPY-based neuromodulation appears to be limited to microvillar OSNs. This
603 claim is substantiated by the observation that almost all the amino acid activated OSNs
604 in starved animals (heightened NPY signalling) co-expressed TrpC2 channels. This cell
605 type -dependent partitioning limits NPY-mediated tuning of olfactory sensitivity to amino
606 acids, the major food-associated odorant cue.

607 Interestingly, we detected little NPY expression in microvillar OSNs. This suggests that
608 NPY, released in a non-synaptic manner from other OSN subtypes and the terminal
609 nerve, acts on NPY receptors on microvillar OSNs and modulates their activity. It
610 remains unclear what triggers the change in NPY expression in response to nutritional
611 states. Although the OB is intrinsically glucosensitive (Fadool *et al.*, 2011; Tucker *et al.*,
612 2013), we do not know which glucoresponsive source influences the NPY expression.

613 Modulators like orexin A, insulin and endocannabinoids influence olfactory sensitivity by
614 acting at the level of the OB (Fadool *et al.*, 2000; Apelbaum *et al.*, 2005; Blakemore *et*
615 *al.*, 2006; Soria-Gomez *et al.*, 2014). The *Drosophila* NPY orthologue, sNPF, has been
616 reported to mediate pre-synaptic facilitation at the first order synapse (Root *et al.*, 2011).
617 However, our evaluation of OSN activation revealed an additional mechanism involving
618 direct modulation of OSN sensitivity to odorants. Amino acid stimulation evoked a
619 strong activation of OSNs in starved animals, but this response was blunted in fed fish.
620 Inhibition of NPY signalling by Y1R antagonist or suppression of endogenous NPY
621 expression by glucose administration resulted in attenuation of OSN activation by amino
622 acids. Conversely, application of exogenous NPY peptide to glucose-injected animals
623 was sufficient to restore the OSN activity. Thus energy-deficient animals are more
624 responsive to odorants. Indeed, OSN activation by amino acids showed a sharp
625 increase in starved animals compared to fed fish. In sum, we show that increased NPY
626 activity lowers the threshold for amino acid detection. Strikingly, this study also revealed
627 a robust co-operative effect between NPY and amino acid signalling that resulted in a
628 switch-like increase in OSN sensitivity to amino acids.

629 Elevated sNPF signalling in OSNs of starved flies, via increased expression of the sNPF
630 receptor, has been shown to mediate presynaptic facilitation by increasing glutamate
631 release at the OSN – Projection neuron (PN) synapse (Root *et al.*, 2011). Similar
632 observations are reported from *in vitro* studies on olfactory bulb interneurons
633 (Blakemore *et al.*, 2006). While not excluding a mechanism involving pre-synaptic
634 facilitation of neurotransmitter release, our study uncovers an additional mechanism of
635 dynamic adjustment of OSN activation thresholds by NPY signalling. NPY signalling in
636 the peripheral olfactory circuitry is found across invertebrate and vertebrate olfactory
637 lineages (Hansel *et al.*, 2001; Mathieu *et al.*, 2002; Gaikwad *et al.*, 2005; Mousley *et al.*,

638 2006; Root *et al.*, 2011). Indeed, externally applied NPY increased the electro-
639 olfactogram (EOG) response to amino acids in OE isolated from starved but not fed
640 salamanders and rats (Mousley *et al.*, 2006; Negroni *et al.*, 2012).

641 However, these studies do not report intrinsic differences in EOG responses between
642 starved and fed animals (Mousley *et al.*, 2006; Negroni *et al.*, 2012). This is possibly
643 because these isolated OE preparations are devoid of endogenous NPY, while an intact
644 behaving animal can access the secreted neuropeptide. The rat study also
645 demonstrated an elevation in NPY receptor expression upon starvation (Negroni *et al.*,
646 2012). Similar increase in sNPF receptor expression has been reported in starved flies
647 (Root *et al.*, 2011). NPY receptor expression may also increase in the OSNs of starved
648 zebrafish. This increase will augment NPY signalling in these animals along the lines
649 indicated in this study. A recent study in *Drosophila* has reported the modulation of OSN
650 responses by NPF (fly orthologue of NPY) signalling, suggesting the conservation of
651 this modality across phyla (Lee *et al.*, 2017).

652 Amino acids are typically perceived by microvillar OSNs expressing the vomeronasal
653 class II receptors (V2Rs) that are coupled to PLC activity. PLC activation, in turn, leads
654 to OSN depolarization via the opening of DAG-gated TrpC2 channels (Lucas *et al.*,
655 2003; Sansone *et al.*, 2014). Zebrafish and *Xenopus,* lack a distinct vomeronasal organ,
656 instead, vomeronasal receptor (VR) expressing OSNs are found in the OE (Sato *et al.*,
657 2005; Koide *et al.*, 2009; Sansone *et al.*, 2014). We show that OSN activation by amino
658 acid stimulation in zebrafish is also dependent PLC signalling and TrpC2 activity.
659 Importantly, NPY-dependent enhancement of OSN sensitivity is also dependent on PLC
660 activity. Canonical stimulation of NPY receptors involves $G_{\alpha i}$ signalling resulting in
661 inhibition of adenylyl cyclase activity (Herzog *et al.*, 1992). However, heterotrimeric G-
662 proteins can potentially generate bifurcating signals, one mediated by $G_{\alpha i}$ and the other
663 by the $G_{\beta\gamma}$ subunits. The latter subunit is known to activate PLC and there is evidence
664 that NPY signalling, in certain contexts, leads to PLC activation (Selbie *et al.*, 1995;
665 Selbie *et al.*, 1997). Thus, NPY and amino acid signalling converge on PLC activation
666 (Figure 6E). This convergence offers a potential mechanism where the additive effect of
667 the two convergent pathways lowers the activation threshold of OSNs (Figure 6E, F).
668 Interestingly, the centrality of PLC activity also offers a potential mechanistic

669 explanation for the co-operative, switch-like increase in microvillar OSN activation
670 observed for co-incident NPY and amino acid signalling. Synergistic activation of PLC
671 isoforms has been extensively reported in studies investigating GPCR signalling cross
672 talk (Zhu & Birnbaumer, 1996; Werry *et al.*, 2003). PLC isoforms, like PLCβ1-3, have
673 two distinct binding sites for $G_{\alpha q}$ and $G_{\beta\gamma}$, and simultaneous occupation of these sites
674 can result in synergistic activation (Zhu & Birnbaumer, 1996; Yoon *et al.*, 1999; Philip *et*
675 *al.*, 2010). In the case of PLCβ3, this cooperative activation has been modeled as a two-
676 state coincidence detector (Philip *et al.*, 2010). NPY signalling, via $G_{\beta\gamma}$ activation, has
677 been demonstrated to potentiate adrenergic and purinergic signalling (Selbie *et al.*,
678 1997). If a similar mechanism were operational in zebrafish OSNs, then it would be
679 expected that the amino acid signalling via V2Rs would involve $G_{\alpha q}$ activity. We tested
680 this possibility and found that GP2A treatment (a specific $G_{\alpha q}$ activity blocker) prevented
681 amino acid mediated OSN activation. This observation offers new insight into olfactory
682 processing of amino acid cues in fish. It is worth noting that while $G_{\alpha i}$ and $G_{\alpha o}$ are
683 predominantly implicated in mammalian VNO transduction, there is also available
684 evidence suggesting the involvement of $G_{\alpha q}$ (Wekesa *et al.*, 2003; Thompson *et al.*,
685 2006).

686 These experiments offer a mechanistic framework for NPY-mediated lowering of
687 activation threshold in amino acid sensing OSNs, where the level of NPY signalling
688 tunes the baseline PLC activity in the neurons (Figure 6F). Low NPY signalling (in
689 recently fed or glucose treated fish) results in low basal levels of PLC and the odorant
690 triggered PLC activation has to be substantial to activate the neuron. Elevated NPY
691 signalling in starved animals, along with coincident signalling from amino acid
692 stimulated V2Rs, converges onto PLC. This convergence augments PLC activity,
693 possibly cooperatively, resulting in switch-like lowering of the threshold necessary to
694 stimulate TrpC2 channels in order to activate the OSN.

695 Our study offers ethologically relevant evidence that in vertebrates NPY-signalling is
696 responsive to internal energy states and tunes olfactory sensitivity to food-related
697 odorants. We uncover a novel mechanism involving NPY signalling dependent
698 peripheral modulation of OSN activation thresholds via PLC activity as a central
699 component of tuning olfactory responses to nutritional states.

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885

886

887 **ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

888 All protocols used in this study were approved by Institutional Animal Ethics Committee.

889

890 **CONSENT FOR PUBLICATION**

891 Not applicable.

892

893 **AVAILABILITY OF DATA AND MATERIAL**

894 All data generated or analysed during this study are included in this published article.
895 The raw data are available from the corresponding author on reasonable request.

896

897 **COMPETING INTERESTS**

898 The authors declare that they have no competing interests.

899

900 **FUNDING**

901 Science and Engineering Research Board (SERB), Govt. of India grant [SB/SO/AS-
902 038/2013] to AG and intramural funding from IISER Pune, supported this work.

903

904 **AUTHORS' CONTRIBUTIONS**

905 Conceptualization, N.S. and A.G.; Investigation, T.K., A.D., A.M., Ak.G., Ar.M; Writing,
906 N.S. and A.G.; Funding Acquisition, A.G. All authors gave final approval for publication
907 and agree to be held accountable for the work performed therein.

908

909 **ACKNOWLEDGMENTS**

910 The authors acknowledge the IISER Pune Microscopy Facility and the National Facility
911 for Gene Function in Health and Disease (NFGFHD) at IISER Pune for access to
912 equipment and infrastructure.

913 **FIGURE LEGENDS**

914 **Figure 1. NPY and NPY Y1 receptor expression in the zebrafish olfactory system**

915 **(A)** Whole mount immunofluorescence of adult zebrafish OE and OB. NPY(green) is
916 expressed in the OE, OB, ON (arrow) and TN neurons (arrow) of adult zebrafish. NPY
917 immunoreactive (NPY-ir) axon terminals synapse on glomeruli marked with SV2 (red).
918 **(B)** Transverse section across olfactory epithelium showing the colocalisation (arrow) of
919 NPY-ir OSNs (green) with Hu (red). **(C,D,E)** Transverse sections across olfactory
920 epithelium showing colocalisation of NPY (green) with $G_{\alpha/olf}$ (red, **C**) and S100 (red, **E**)
921 but not with Calretinin (red, **D**). **(F)** Saggital section showing NPY immunoreactivity in
922 the OE and OB of 78 hpf zebrafish larvae. **(G)** Representative RNA *in situ* hybridisation
923 using NPY Y1 receptor mRNA antisense (upper panel) and sense (lower panel) probes
924 showing expression in adult zebrafish OSNs (arrows). [Scale bars: **A**, 200 µm; **B**,**D**,**G** 10
925 µm; **F**, 50 µm; **C**,**E**, 5 µm]

926

927 **Figure 2. NPY expression in the olfactory system is sensitive to nutritional states**

928 **(A,B,C)** Representative transverse sections showing NPY immunoreactivity (green) and
929 DAPI (blue) in the OSNs (arrows) of the olfactory organ for different feeding states
930 (Starved, Glucose and Fed). **(D,E,F)** Whole mount immunofluorescence of olfactory
931 bulb showing NPY (green) and SV-2 (red). **(G,H,I)** NPY-ir in the terminal nerve neurons
932 (arrows) in the olfactory bulb for different feeding states. **(J)** Number of NPY-ir OSNs
933 per lamella across all 20µm sections for both the OEs under different feeding states
934 (N=3). **(K)** Normalised NPY fluorescence in the whole olfactory organ for different
935 feeding states (N=3). **(L)** The number of NPY-ir glomeruli in the whole olfactory bulb for
936 different feeding states (N=6). **(M)** Normalised NPY fluorescence in the whole olfactory
937 bulb for different feeding states (N=5). **(N)** Number of NPY-ir terminal nerve (TN)
938 neurons per each olfactory bulb (N=6) and **(O)** The relative intensity of NPY-ir TN
939 neurons in the whole olfactory bulb for different feeding states (N=6). Data are shown as
940 mean +/- SEM (*, $p<0.5$, **, $p< 0.01$, ***, $p < 0.001$; One – way ANOVA followed by
941 Bonferroni post hoc test analysis for significance in comparison with the starved group).
942 [Scale bars: **A-C**,10 µm: **D-I**, 20 µm].

943

944 **Figure 3. NPY signalling is necessary and sufficient for olfactory responses to**
945 **food-associated amino acid cues**

946 **(A)** Schematic of the odorant preference assay in adult fish using a mixture of amino
947 acids as an attractive odorant cue (see methods for explanation) **(B,C)** Fraction of time
948 spent in the odorant zone as indicated by the preference index in a fed and starved fish
949 (N=6), respectively. The preference index before and after addition of amino acids is
950 tested for significant deviation from 0 using Wilcoxon signed- rank test (*, p< 0.05; ns,
951 no significant difference from 0). **(D-G)** Preference index of starved fish injected via icv
952 with either Vehicle, BIBP, Glucose or co-injected glucose along with NPY peptide (N=6;
953 *, p<0.05 in both D and G; ns, no significant difference from 0). **(H-J)** Starved fish
954 injected with either vehicle (N=10) or glucose (N=7) showed no significant difference
955 (ns) in their response to the alarm cue, Schreckstoff. Data are shown as mean +/- SEM;
956 unpaired t-test was used to test for statistical significance. **(K)** Schematic of the aversion
957 assay in the 78 hpf zebrafish larvae using L- Cysteine as an aversive cue (see methods
958 for explanation). **(L)** 78 hpf larvae pre-treated with water show aversion to L- Cysteine
959 (N=6; **, p< 0.001) while larvae pre-treated with BIBP show no significant (ns)
960 difference between the L-Cysteine treated and control fish. Data are shown as mean +/-
961 SEM; One-way ANOVA followed by Bonferroni's post-hoc test was used to test for
962 statistical significance.

963

964 **Figure 4. NPY signalling modulates olfactory sensitivity at the periphery**

965 **(A,B)** Preference index of starved fish in the odorant preference assay treated either
966 with vehicle or BIBP via the nasal route (N=6). The preference index before and after
967 addition of amino acids was tested for significant deviation from 0 using Wilcoxon
968 signed- rank test (*, p< 0.05; ns, no significant difference from 0). **(C-F)** Representative
969 micrographs of a Dl region from transverse sections across the telencephalon showing
970 pERK immunoreactivity in starved fish pretreated with vehicle/BIBP via icv (**C,D**) or
971 nasal route **(E,F)**. **(G,H)** The number of pERK-ir cells in Dl region in starved fish injected
972 with either vehicle or BIBP via icv route (N=6; G) or via nasal route (N=6; H). Data are

973 shown as mean +/- SEM; unpaired t-test was used to test for statistical significance. *,
974 p< 0.05; ns, no statistically significant difference. [Scale bars: **C-F**, 25 μm].

975

976 **Figure 5. NPY signalling increases the sensitivity of microvillar OSNs to amino**
977 **acids**.

978 **(A-C)** Representative transverse sections showing the immunoreactivity of TrpC2
979 (green), pERK (red) and their colocalisation (yellow) in the OSNs. **(D)** Effect of
980 starvation on amino acid sensitivity in adult zebrafish. Adult zebrafish were either
981 regularly fed (light gray) or starved (dark gray) for 3 days and were exposed to either a
982 mixture of amino acids (AA) or distilled water (DW). The values are means +/- SEM, n=
983 6. Post –hoc analysis of significant odorant × energy status interactions in Two-way
984 ANOVA : $^{*}p< 0.05$, Fed (AA) versus Fed (DW); $^{\#\#\#}p< 0.001$, Starved (AA) versus
985 Starved (DW); $^{\$\$\$}p< 0.001$, Starved (AA) versus Fed (AA). **(E)** Effect of blocking NPY
986 Y1R signalling on amino acid sensitivity in adult zebrafish. Adult zebrafish were starved
987 for 3 days, injected with either BIBP (light gray) or Vehicle (dark gray) and were
988 exposed to either a mixture of amino acids (AA) or distilled water (DW). The values are
989 means +/-SEM, n= 6. Post –hoc analysis of significant odorant × NPY signalling
990 interactions in Two-way ANOVA : $^{***}p< 0.001$, BIBP (AA) versus BIBP (DW); $^{\#\#\#}p<$
991 0.001, Vehicle (AA) versus Vehicle (DW); $^{\$\$\$}p< 0.001$, Vehicle (AA) versus BIBP (AA).
992 **(F)** Effect of energy status on amino acid sensitivity in adult zebrafish. Adult zebrafish
993 were starved for 3 days, injected with either Glucose (Glu; light gray) or co-injected NPY
994 peptide with glucose (Glu + NPY; dark gray) and were exposed to either a mixture of
995 amino acids (AA) or distilled water (DW). The values are means +/-SEM, n= 6. Post –
996 hoc analysis of significant odorant × energy status interactions in Two-way ANOVA :
997 $^{***}p< 0.001$, Glu (AA) versus Glu (DW); $^{\#\#\#}p< 0.001$, Glu + NPY (AA) versus Glu + NPY
998 (DW); $^{\$\$\$}p< 0.001$, Gluc + NPY (AA) versus Glu (AA). In D-F, light grey shading
999 indicates conditions of reduced NPY signalling while dark grey shading indicates
1000 elevated NPY signalling. **(G)** Normalized response of OSNs to different concentrations
1001 of amino acids (log scale) evaluated by counting the number of pERK-ir OSNs in
1002 starved fish injected with glucose (red) and vehicle (blue) (N=3; error bars represent +/-

1003 SEM; individual p-values obtained from 2-way ANOVA followed by Bonferroni's post-
1004 hoc test; **, p<0.01; ***, p<0.001). [Scale bars: **A-C**, 10 μm].

1005

1006 **Figure 6. Amino acid and NPY signalling converge on PLC activity to modulate**
1007 **OSN sensitivity**

1008 **(A)** The number of pERK –ir OSNs in starved fish injected with either vehicle or 2-APB
1009 (Trp channel blocker) via icv route and exposed to a mixture of amino acids (N=6; error
1010 bars represent +/-SEM; ***, p< 0.0001). **(B)** The number of pERK –ir OSNs in starved
1011 fish injected with either vehicle or U73122 (selective PLC inhibitor) via icv route and
1012 exposed to a mixture of amino acids (N=6; **, p< 0.001). **(C)** The number of pERK –ir
1013 OSNs in starved fish pre-injected with Glu + NPY followed by administration of vehicle
1014 or U73122 via icv route, exposed to a mixture of amino acids (N=6; **, p< 0.01). **(D)** The
1015 number of pERK –ir OSNs in starved fish injected with either vehicle or GP2A (selective
1016 $G_{\alpha q}$ signalling inhibitor) via icv route and exposed to a mixture of amino acids (N=6; ***,
1017 p< 0.0001). Data are shown as mean +/- SEM and means were compared using
1018 unpaired t-test. **(E)** Schematic showing the convergence of NPY Y1 receptor (NPY Y1R)
1019 signalling and odorant receptor for amino acids (V2R) signalling on phospholipase C
1020 mediated activation of microvillar OSN. **(F)** Increased NPY signalling (starved animals)
1021 results in a sharp increase in the sensitivity of microvillar OSNs to food-associated
1022 amino acid odorant cues. In energy-rich conditions (fed animals), the baseline NPY
1023 signalling is low and the sensitivity of the microvillar OSNs to amino acids is reduced.
1024 AA - amino acid mixture; PLC – phospholipase C; DAG – diacylglycerol; TrpC2 –
1025 transient receptor potential channel C2.

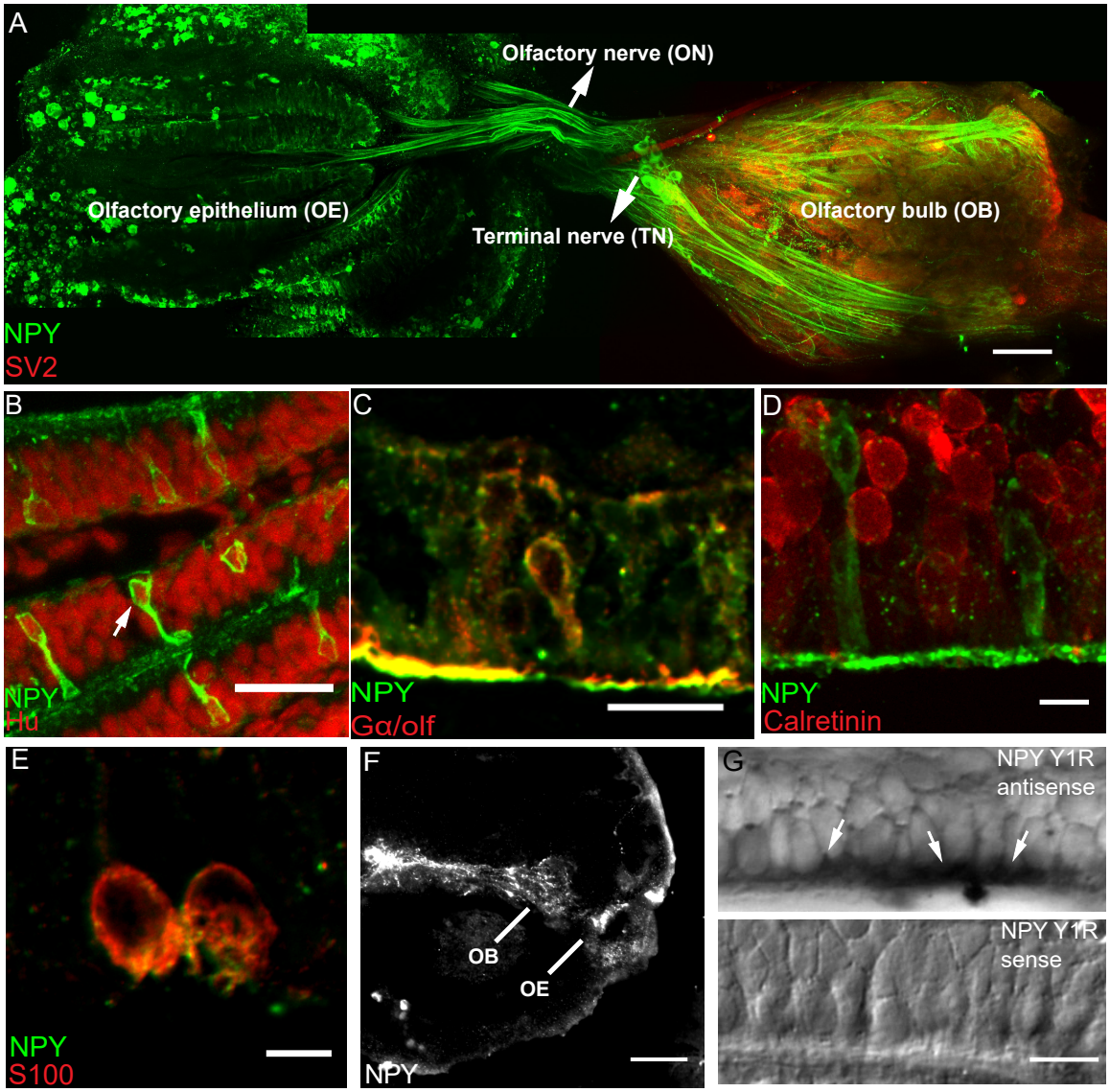
A
Olfactory nerve (ON)
Olfactory epithelium (OE)
Terminal nerve (TN)
Olfactory bulb (OB)
NPY
SV2
B
NPY
Hu
C
NPY
Gα/olf
D
NPY
Calretinin
E
NPY
S100
F
OB
OE
NPY
G
NPY Y1R
antisense
NPY Y1R
sense

Figure 1

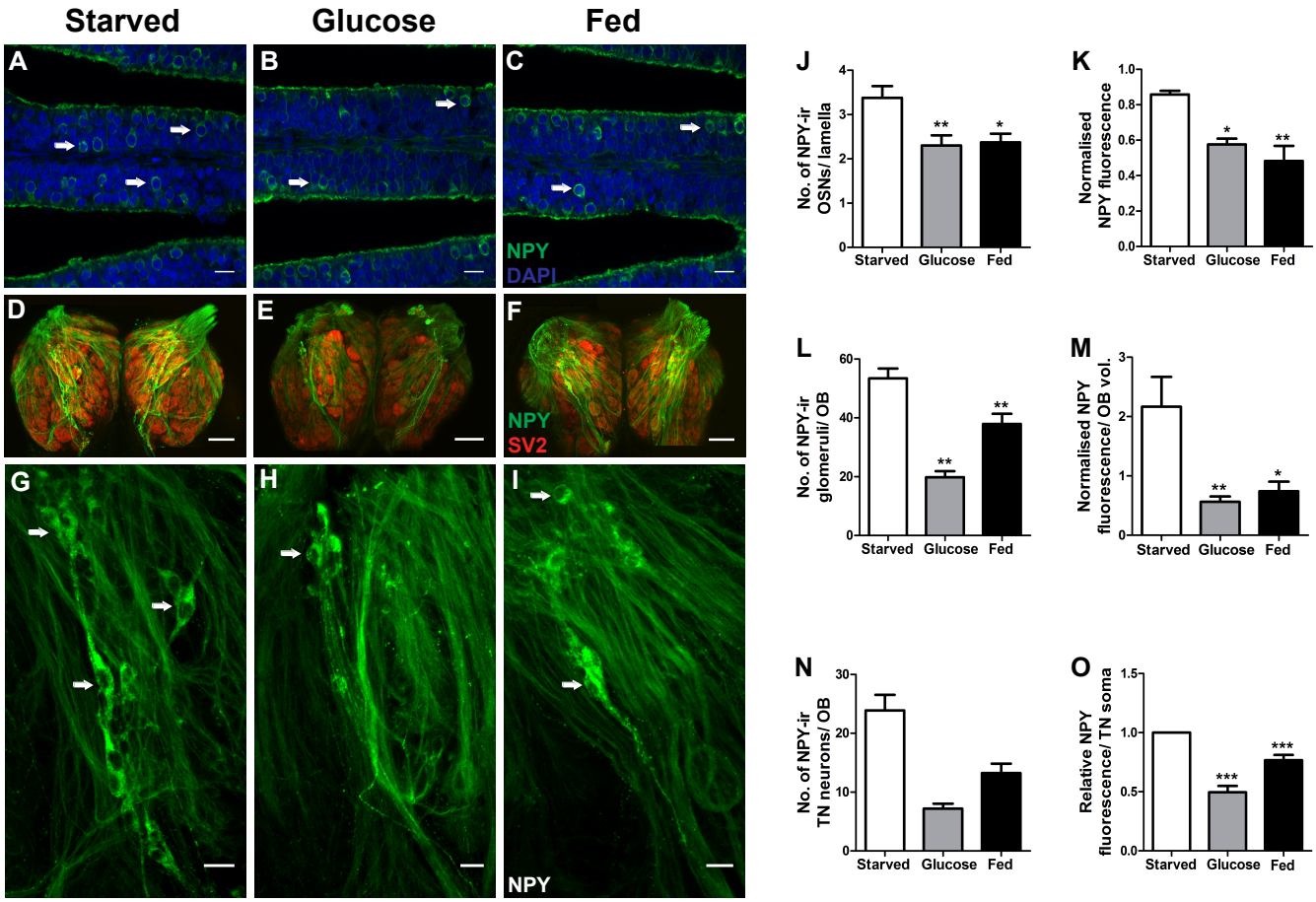
Starved
Glucose
Fed
A
B
C
NPY
DAPI
D
E
F
NPY
SV2
G
H
I
NPY
J
No. of NPY-ir OSNs/ lamella
K
Normalised NPY fluorescence
L
No. of NPY-ir glomeruli/ OB
M
Normalised NPY fluorescence/ OB vol.
N
No. of NPY-ir TN neurons/ OB
O
Relative NPY fluorescence/ TN soma
Starved
Glucose
Fed

Figure 2

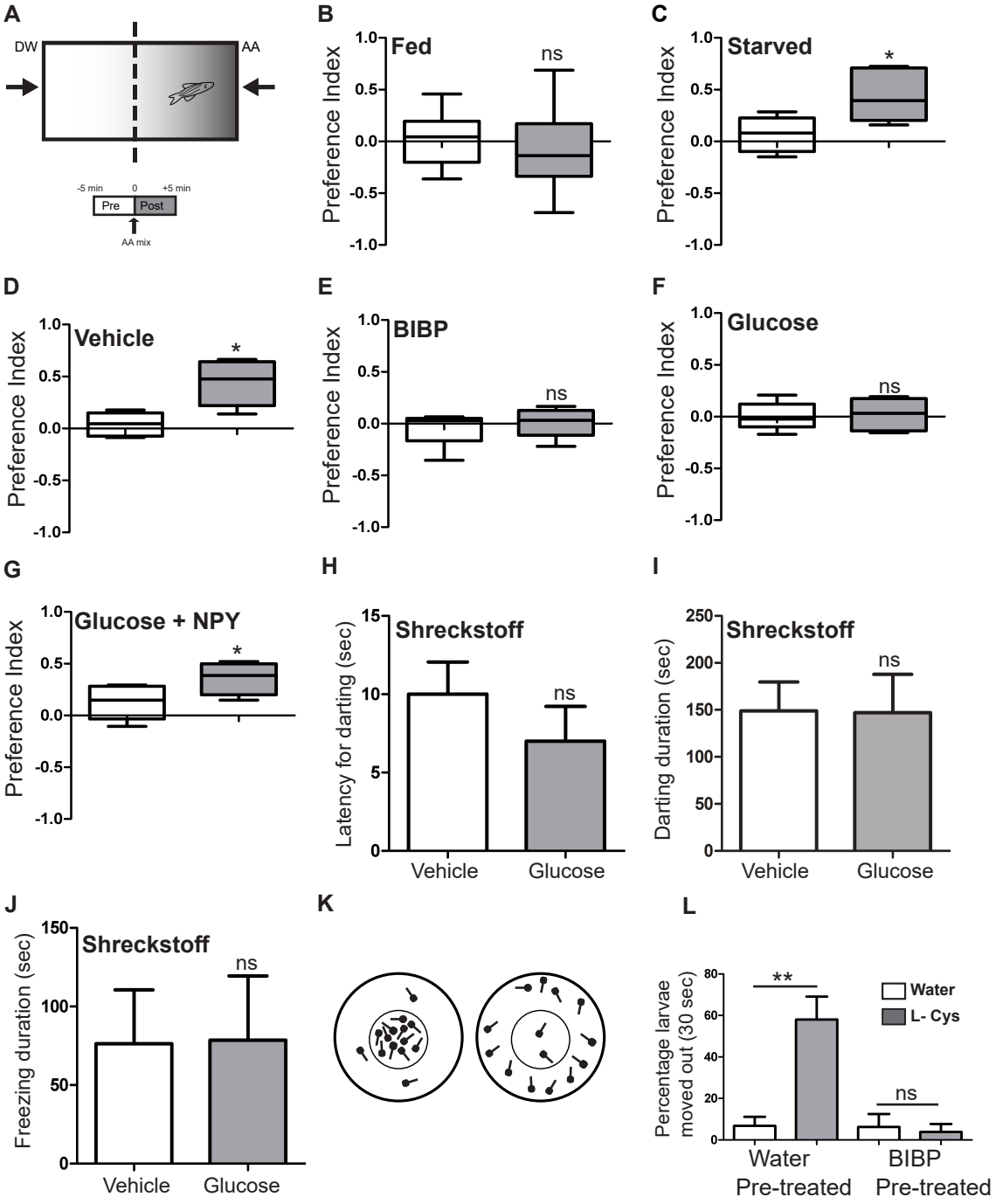
A
DW
AA
-5 min
0
+5 min
Pre
Post
AA mix
B
Fed
ns
Preference Index
C
Starved
*
Preference Index
D
Vehicle
*
Preference Index
E
BIBP
ns
Preference Index
F
Glucose
ns
Preference Index
G
Glucose + NPY
*
Preference Index
H
Shreckstoff
ns
Latency for darting (sec)
Vehicle
Glucose
I
Shreckstoff
ns
Darting duration (sec)
Vehicle
Glucose
J
Shreckstoff
ns
Freezing duration (sec)
Vehicle
Glucose
K
L
**
Water
L- Cys
ns
Percentage larvae moved out (30 sec)
Water Pre-treated
BIBP Pre-treated

Figure 3

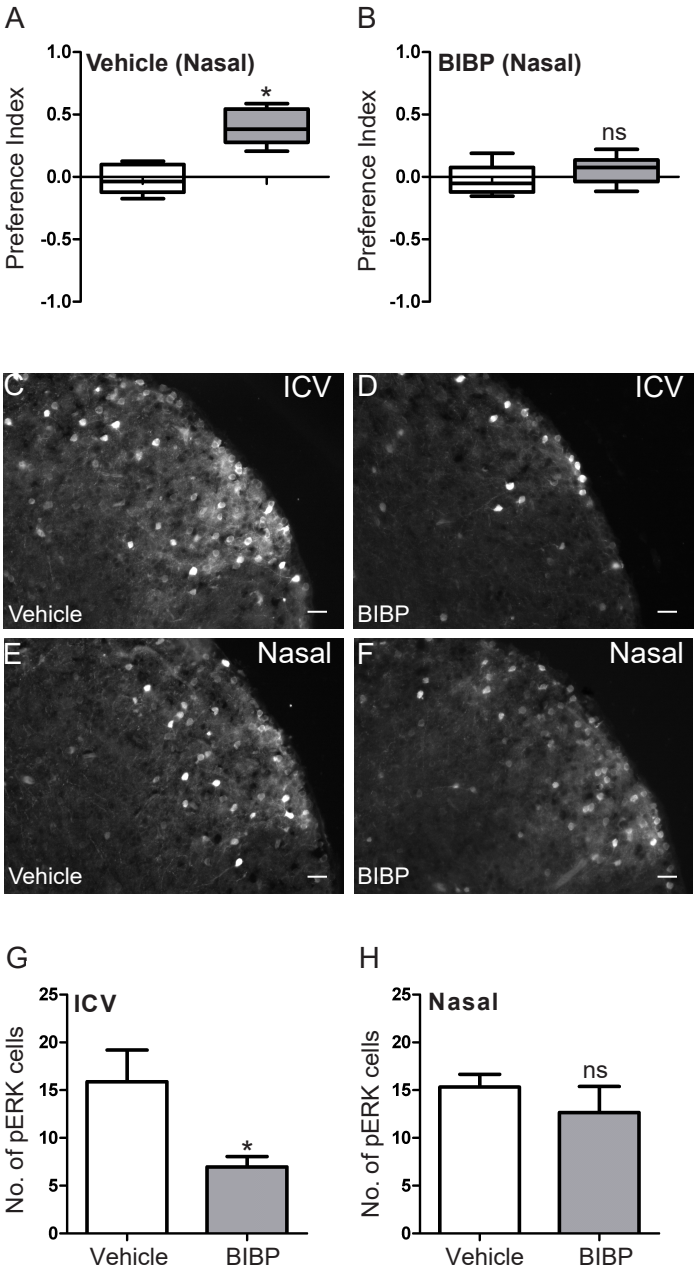
A
Vehicle (Nasal)
Preference Index
1.0
0.5
0.0
-0.5
-1.0
*
B
BIBP (Nasal)
Preference Index
ns
C
ICV
Vehicle
D
ICV
BIBP
E
Nasal
Vehicle
F
Nasal
BIBP
G
ICV
No. of pERK cells
25
20
15
10
5
0
*
Vehicle
BIBP
H
Nasal
No. of pERK cells
ns
Vehicle
BIBP

Figure 4

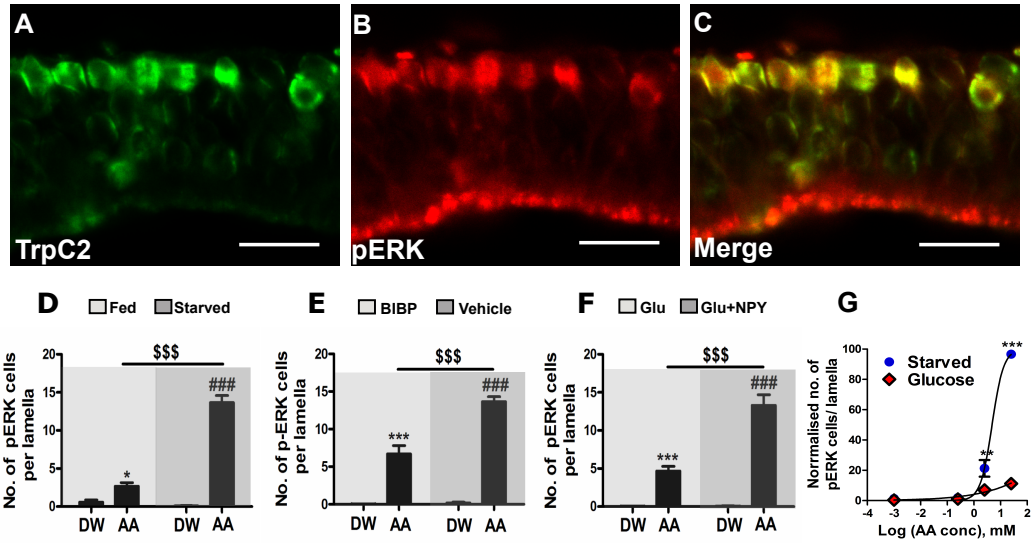
A
TrpC2
B
pERK
C
Merge
D
Fed
Starved
No. of pERK cells per lamella
$$$
###
*
DW
AA
DW
AA
E
BIBP
Vehicle
No. of p-ERK cells per lamella
$$$
###
***
DW
AA
DW
AA
F
Glu
Glu+NPY
No. of pERK cells per lamella
$$$
###
***
DW
AA
DW
AA
G
Starved
Glucose
Normalised no. of pERK cells/ lamella
***
**
Log (AA conc), mM

Figure 5

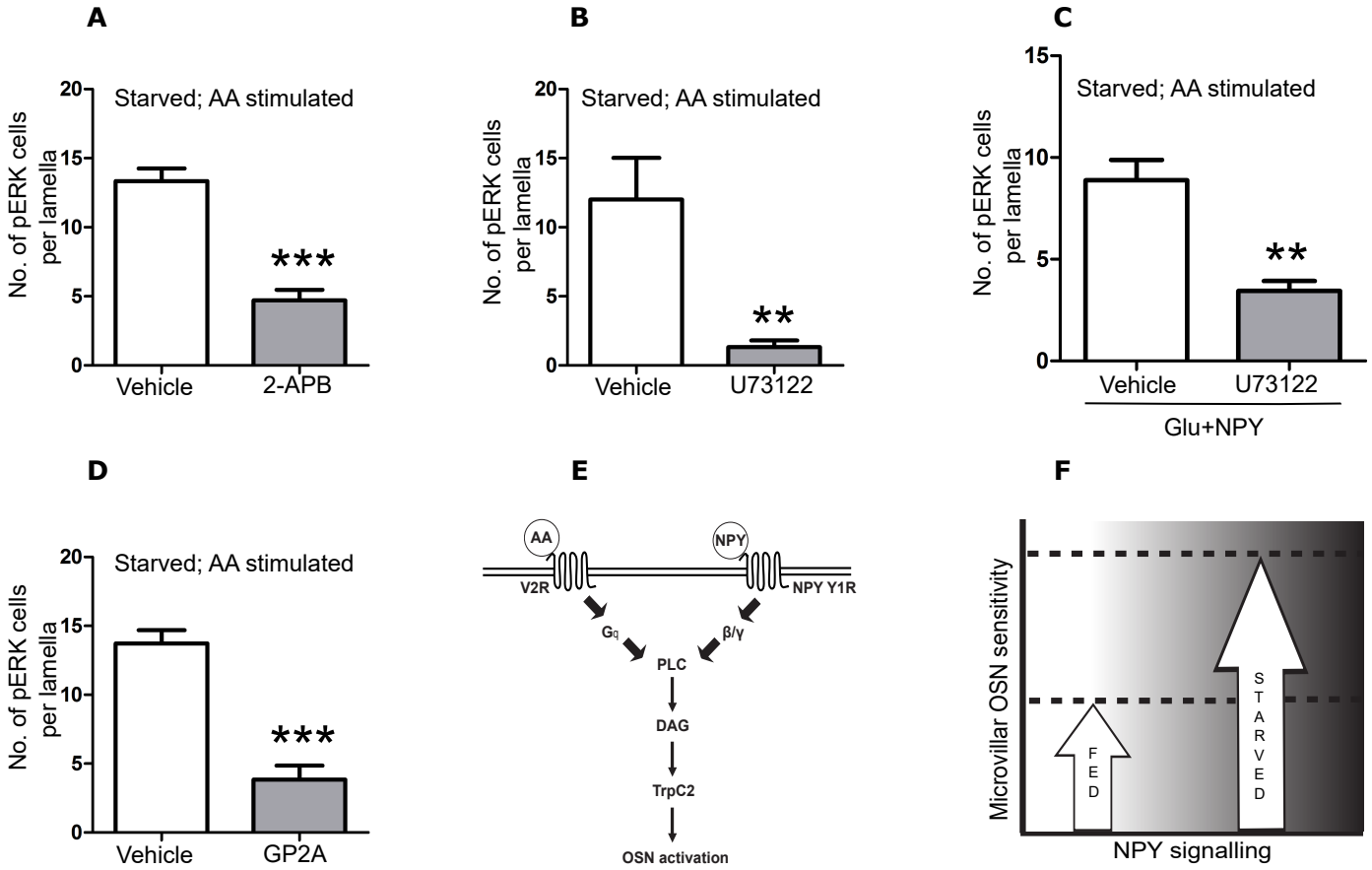
A
Starved; AA stimulated
No. of pERK cells per lamella
20
15
10
5
0
***
Vehicle
2-APB
B
Starved; AA stimulated
No. of pERK cells per lamella
20
15
10
5
0
**
Vehicle
U73122
C
Starved; AA stimulated
No. of pERK cells per lamella
15
10
5
0
**
Vehicle
U73122
Glu+NPY
D
Starved; AA stimulated
No. of pERK cells per lamella
20
15
10
5
0
***
Vehicle
GP2A
E
AA
NPY
V2R
NPY Y1R
Gq
β/γ
PLC
DAG
TrpC2
OSN activation
F
Microvillar OSN sensitivity
FED
STARVED
NPY signalling

Figure 6