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

Running title: Energy states modulate OSN sensitivity

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

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ABSTRACT

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.

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.

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.

102 **MATERIALS AND METHODS**

103 ***Animals***

104 Wild type zebrafish were maintained at 28.5˚C on a 14 h light/10 h dark schedule in 10
105 L and 3 L holding tanks in a recirculating aquarium system (Aquatic Habitats). For
106 studies on zebrafish larvae, the eggs were obtained by setting a cross in 3L breeding
107 tanks (Aquatic Habitats). Eggs were maintained in E3 medium (5 mM NaCl, 0.17 mM
108 KCl, 0.33mM $CaCl_2$, 0.33 mM $MgSO_4$, 0.00001% methylene blue) in an incubator at
109 28.5˚C till 78 hpf. Unless otherwise indicated, adult fish were fed *ad libitum* twice daily
110 with freshly hatched brine shrimp. Larvae are were fed thrice daily with either the
111 cultured paramecia water or synthetic larval food.The Institutional Animal Ethics
112 Committee approved all the procedures employed in this study.

113 ***Chemicals***

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

122 ***Intracerebroventricular (icv) and intranasal delivery***

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

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

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

142 ***Amino acid stimulation of the OE for evaluation of OSN activation***

143 Post 30 mins icv delivery of pharmacological agents, fish were transferred into the
144 habitat tank containing 1 liter aquarium water. A mixture of 8 amino acids (Ala, Cys, His,
145 Lys, Met, Phe, Trp, and Val; 0.8 mM each in distilled water or solvent was introduced
146 into these tanks using a syringe. For dose-response experiments, 0 mM, 0.008 mM,
147 0.08 mM and 0.8 mM concentrations of each of the amino acids was used in the
148 odorant mixture. Following the 8 min exposure, the fish were immediately anesthetized
149 and processed for pERK immunofluorescence.

150 ***Immunofluorescence***

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

161 The frozen sections were rehydrated and permeabilized using 0.5% Triton-X in
162 phosphate buffer saline (PBST). The sections were treated with 5% heat inactivated
163 goat serum (HIGS) or 5% BSA in PBST for 1 hour and then incubated with the primary
164 antibody overnight at 4 ^{o}C. The sections were washed using 0.1% PBST and pre-
165 incubated with 5 % HIGS or 5 % BSA in PBST for 1 hour and was incubated with
166 desired secondary antibody for 3 hours at room temperature (RT). Then the sections
167 were washed and mounted with mounting media (70% glycerol, 0.5% N-propyl gallate in
168 1 M Tris, pH 8.0, DAPI). The following primary antibodies were used in this study -
169 polyclonal anti-NPY antibody (1:2000; Sigma), monoclonal anti-calretinin antibody
170 (1:3000; Swant), monoclonal anti-NPY antibody (1:2000; Sigma), polyclonal anti- $G_{\alpha/olf}$
171 antibody (1:1000; Santa Cruz), anti-Hu antibody (1:100; Abcam), anti-SV2 antibody
172 (1:50; Developmental Studies Hybridoma Bank), monoclonal anti-phosphorylated ERK
173 1/2 (1:500; ab50011; Abcam) and polyclonal anti-TrpC2 (1:50; Abcam). Anti-rabbit
174 Alexa-488 (1:500; Invitrogen) and anti-mouse Alexa-568 (1:500; Invitrogen) were used
175 as secondary antibodies. Sections were observed using either an upright
176 epifluorescence microscope (Axioimager.Z1 with Axiocam HRm camera; Carl Zeiss) or
177 inverted laser scanning confocal microscopes (LSM 710 or LSM 780; Carl Zeiss).

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

184 ***Whole-mount immunofluorescence***

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

191 PBS for 60 min each at RT. The tissue was permeabilized with proteinase K (10 µg/ml
192 in PBS) for 5 min followed by three washes in PBS. Blocking was carried out for 8 hours
193 at 4 ^{o}C in 5% BSA, 5% DMSO in 0.5 % Triton X-100. The tissue was then incubated in
194 primary antibody against neuropeptide Y (1:5000) and synaptic vesicle-2 (1:50) for 48
195 hours at 4 ^{o}C. Following three washes with PBST (15 min each; 4 ^{o}C, the tissue was
196 incubated overnight with secondary antibody Alexa Fluor-488 (1:500) and Alexa Fluor-
197 568 (1:500) at 4 ^{o}C. The tissue was rinsed three times in PBST (2 hours each; RT) and
198 once in PBT (RT) and finally mounted in 70% glycerol, 0.5% N-propyl gallate in 1 M
199 Tris, pH 8.0, DAPI on a coverslip-bottomed petridish. Images were taken using the
200 inverted laser scanning confocal microscope (LSM 780; Carl Zeiss).

201 ***Odorant preference assay in adult zebrafish***

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

222 1200h to 1700h.The swimming paths were analyzed from the recorded videos using an
223 automated video tracking system (Ethovision XT version 8.0; Noldus). Preference
224 indices were calculated using the formula: Preference index (PI) = ((Total time spent in
225 the odorant zone) – (Total time spent in the control zone)) / (Total time). The data were
226 evaluated for the period of 5 mins before and after odorant stimulation.

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

228 The protocol described by (Vitebsky *et al.*, 2005) was employed with modifications.
229 Briefly, 78 hpf zebrafish larvae (n=10-20) were placed in a 35 mm Petri dish containing
230 5 ml of E3 medium. A circle of 1 cm diameter was drawn on white paper and placed
231 under the petri dish such that the circle was at the center of the dish. The larvae were
232 gently swirled to position them within the perimeter of the circle. L-cysteine (10 ul of 3 x
233 10^{-5} M) was gently introduced over the surface of the water, approximately at a point
234 above the center of the circle (ensuring minimal vibration of the water surface). The
235 entire procedure was video recorded and the number of larvae that moved out of the
236 area of the circle within 30 secs of addition of L-cysteine was scored. The larvae that
237 remained within the circle were considered as non-responsive. The larvae once used for
238 the assay were not used in further experiments and the entire experiment was carried
239 out at 27-28.5 ^{o}C.

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

241 For testing the alarm response in fish under different energy states, the skin extract was
242 prepared as described earlier (Mathuru *et al.*, 2012). Briefly, several shallow cuts were
243 made on the skin of the zebrafish using a sharp blade. The injured fish was immersed in
244 2 ml of 20mM Tris- Cl (pH 8.0) for 30 seconds. This process was repeated for 7-10 fish
245 and the extract was collected into the same volume. The 2 ml of crude extract was then
246 centrifuged at 13.2k rpm and the supernatant was collected. A 25% solution of the
247 alarm pheromone was prepared by diluting in distilled water and 500 µl of the latter was
248 used in the behavioural assay.

249 As stated above in the odorant preference assay, adult zebrafish were singly housed,
250 habituated for 2 days, starved for yet another day, anaesthetized and injected with
251 either saline or glucose (8000ng) via icv route. Following an interval of 30 mins, the fish

252 were subjected to the assay. Control stimulus (500µl of distilled water) was introduced
253 into the tank following the first 5 mins using a plastic dropper. After a period of 10 mins,
254 test stimulus (500 µl of the alarm pheromone extract) was added. The fish were
255 evaluated for their response to the alarm pheromone for 10 minutes. The assay was
256 video recorded (Sony Handycam DCR SR20) and analyzed using an automated video
257 tracking system (Ethovision XT version 8.0; Noldus).

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

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

263 The intensity of NPY immunoreactivity in the olfactory bulb was measured using Image
264 Pro Plus 7.1 software (Media Cybernetics). Briefly, the precise volume measurements
265 of the bulb were made in the 3D constructor using interactive thresholding. The
266 integrated optical density was then measured in the defined volume of the bulb for NPY
267 and SV2 channels separately. For each treatment, the optical density of the NPY
268 immunoreactivity was normalized with respect to the SV2 immunoreactivity and again
269 with a unit volume defined. NPY immunoreactivity in the soma of the terminal nerve
270 neurons was measured using Image J. Relative NPY fluorescence per terminal nerve
271 neuron in each group was plotted with respect to that of the control group. The intensity
272 of NPY immunoreactivity in the olfactory epithelia was measured using Image J. The
273 total fluorescence was calculated by the formula corrected total fluorescence (CTF) =
274 Integrated density – (Area of the selected ROI * Mean fluorescence of the background
275 readings). The CTF was calculated for both NPY immunoreactivity and DAPI labeling
276 and then the NPY fluorescence was normalized to DAPI fluorescence.

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

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

282 For whole-mount RNA *in situ* hybridization (ISH) experiment adult fish were starved for
283 three days and anesthetized followed by the removal of the dorsal cranium and
284 immersion in 4% paraformaldehyde (for 14-16 hours at 4 ^{0}C), olfactory rosettes were
285 dissected out, washed in phosphate buffered saline (PBS), and stored in 100%
286 methanol at -20°C overnight. ISH was carried out as described earlier (Akash *et al.*,
287 2014). Digoxigenin-labeled sense and antisense riboprobes were transcribed *in vitro*
288 using Zebrafish NPY Y1R TOPO pcDNA 3.1 vector template (gift from D. Larhammar)
289 using the following primer pair. Forward: 5'-GACACCAACTCATCCTGCACC-3';
290 Reverse: 5'-TGGGTACAGTTCATCGCCAC-3'. Probes were purified using BioRad
291 Micro Bio-Spin columns as per manufacturer's protocol. Samples were sequentially
292 rehydrated to PTW (phosphate buffered saline, 0.1% Tween-20 pH 7.3) and
293 permeabilized in 2% hydrogen peroxide for 35 minutes (min) at room temperature (RT).
294 Samples were further permeabilized by a 40-min proteinase K treatment and post-fixed
295 for 20 min in 4% paraformaldehyde at RT. Prehybridization, probe hybridization and
296 washes were performed as described previously (Akash *et al.*, 2014) except for the
297 inclusion of 5% dextran sulfate in the hybridization buffer. The OE were embedded in
298 4% low melting point agarose blocks and sectioned (30 µm) using a Vibratome (VT
299 1200; Leica). The sections were blocked in 1% blocking solution (10 min at RT; Roche
300 Applied Sciences) and then incubated overnight at 4 ^{0}C with sheep anti-DIG alkaline
301 phosphatase conjugated antibody (1:2,000; Roche Applied Sciences). Sections were
302 washed thrice with PTW buffer and twice with coloration buffer (100 mM Tris-HCl, pH
303 9.5, 50 mM $MgCl_2$, 100 mM NaCl, 0.1% Tween 20, 2 mM Levamisole). DIG-labeled
304 probes were detected using pre-warmed BM purple solution (Roche). The reaction was
305 continuously monitored at 37^{0} C for 30 min to 2 h or overnight at 4 ^{0}C depending on the
306 strength of the signal. The reaction was stopped by washing three times with PTW
307 buffer. The sections were then mounted in 70% glycerol and were either processed for
308 imaging or stored at 4 ^{0}C. DIC photomicrographs were taken with an upright microscope
309 (Axioimager Z1, Carl Zeiss).

310 ***Statistical analysis***

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

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

322

323 **RESULTS**

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

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

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

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

343 NPY signalling via the NPY Y1 receptor (Y1R) has been implicated in regulating food
344 intake in multiple species (Mercer *et al.*, 2011; Nguyen *et al.*, 2012; Yokobori *et al.*,
345 2012). Further, Y1R mediates olfactory processing in rodents (Negroni *et al.*, 2012).
346 mRNA *in situ* hybridization using antisense probe against Y1R mRNA showed intense
347 signal in subpopulations of OSNs (Fig 1G upper panel) while the sense probe controls
348 did not produce any reaction (Fig 1G lower panel).

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

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

352 NPY promotes hunger and increases food intake across vertebrate phyla, including
353 zebrafish and its expression is elevated in multiple neuronal regions upon fasting (Clark
354 *et al.*, 1987; Marks *et al.*, 1992; Volkoff *et al.*, 2005; Yokobori *et al.*, 2012). Fed (free
355 feeding on an excess of food 30 mins prior to sacrifice; mock icv injection), starved (for
356 3 days; mock icv injection) and adult zebrafish injected with glucose (via icv route) were
357 compared for NPY expression in the olfactory epithelia and olfactory bulb. Glucose icv
358 treatment allowed isolation of central regulation from peripheral homeostatic inputs. The
359 latter treatment also reduced the variability in free feeding fish that may arise from
360 individual proclivities. Total fluorescence intensity of the NPY signal and the number of
361 NPY expressing OSNs in the olfactory mucosa increased upon starvation in comparison
362 to either well fed or glucose injected animals (Figure 2A-C, Figure 2J,$p= 0.0044$ and
363 Figure 2K, $p= 0.0065$). Similarly, both the NPY signal intensity in the OB and the
364 number of NPY-positive glomeruli also increased upon starvation (Figure 2D-F, Figure
365 2L, $p<0.0001$ and Figure 2M, $p= 0.0059$). NPY fluorescence in the olfactory bulb
366 comprises of signals from both the OSN projections onto glomeruli and the terminal
367 nerve. Independent evaluation of the terminal nerve showed an increase in NPY
368 immunofluorescence per TN soma and also in the number of NPY expressing TN cell
369 bodies in starved zebrafish compared either to fed or glucose injected fish (Figure 2G-I,
370 Figure 2N, $p<0.0001$ and Figure 2O, $p<0.0001$).

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

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

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

382 significant increase in the preference index (PI) for the baited side (Figure 3C, p=
383 0.0156). However, fed animals displayed no difference in PI between the pre- and post-
384 stimulation periods (Figure 3B).

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

470 Sansone *et al.*, 2014). Unlike classical OSN signal transduction, V2R signalling involves
471 phospholipase C activity and opening of the diacylglycerol (DAG) -gated TrpC2
472 channels resulting in OSN activation (Lucas *et al.*, 2003; Sato *et al.*, 2005; Koide *et al.*,
473 2009; Sansone *et al.*, 2014). To test if amino acid mediated activation of OSNs in
474 zebrafish was limited to the microvillar OSNs, we directly evaluated OSN activation by
475 monitoring ERK1/2 phosphorylation (pERK). We found that OSNs activated by amino
476 acid stimulation, as revealed by ERK activity, exclusively co-localized with TrpC
477 expressing OSNs (Figure 5A-C).

478 We next tested if prevailing energy states directly modulated OSN activation by amino
479 acids. Fed and starved zebrafish were stimulated with amino acid or distilled water and
480 OSN activation was evaluated by scoring for pERK signal. A small increase in the
481 number of activated OSNs was observed in fed fish stimulated with amino acids
482 compared to controls (Figure 5D). However, a significantly larger response was seen
483 upon amino acid stimulation of starved zebrafish suggesting increased OSN sensitivity
484 to odorants in these animals (Figure 5D). Two-way ANOVA revealed a statistically
485 significant interaction between the high NPY signalling (starvation) and V2R activation
486 (amino acid stimulation) denoted as the [NPY x AA] interaction (Figure 5D; $F_{(1,20)}$=112.2,
487 $p< 0.0001$). Post-hoc analysis revealed that while amino acid stimulation had a small
488 effect (increased OSN activation in fed fish where endogenous NPY signalling is
489 reduced compared to the controls), high NPY signalling alone failed to activate OSNs in
490 the absence of odorant (no difference between control treatments of fed and starved
491 fish).

492 To directly test the role of NPY in OSN activation, we used the NPY Y1R antagonist
493 BIBP administered via the icv route to block NPY signalling in starved zebrafish.
494 Compared to distilled water, amino acid stimulation caused a large increase in OSN
495 activation in starved control animals (Figure 5E). However, the OSN activation was
496 significantly attenuated in BIBP pre-treated starved animals compared to those
497 receiving the vehicle, implicating NPY signalling in tuning OSN sensitivity (Figure 5E).
498 As in the previous experiment, comparing fed and starved fish, two-way ANOVA
499 revealed a statistically significant [NPY x AA] interaction (Figure 5E; $F_{(1,20)}$= 28.06, $p<$
500 0.0001). While post-hoc analysis showed an effect for amino acid stimulation (increase

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

505 To test if NPY signalling was sufficient to restore OSN sensitivity to amino acid
506 stimulation, we evaluated OSN activity (pERK-ir) in fish where endogenous NPY
507 signalling is suppressed by administration of glucose via the icv route followed by
508 addition of exogenous NPY peptide. Compared to distilled water, amino acid stimulation
509 resulted in an increase in the number of OSNs activated in glucose-injected fish (Figure
510 5F). However, this change was significantly smaller than the large increase in OSN
511 activation seen upon amino acid stimulation of glucose-injected fish that have also
512 received exogenous NPY (Figure 5F). These data implicate NPY signalling to be
513 sufficient in restoring OSN sensitivity to amino acids. Again two-way ANOVA revealed a
514 statistically significant [NPY x AA] interaction (Figure 5F; $F_{(1,20)}= 31.71$, $p< 0.0001$). As
515 in the last two experiments, amino acid stimulation did have a statistically significant
516 effect (amino acid increased OSN activation compared to distilled water in glucose-
517 injected animals) but this was significantly smaller than the combined effect of high NPY
518 signalling and amino acid stimulation.

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

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

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

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

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

541 Our experiments suggest that NPY signalling facilitates olfactory perception of food-
542 associated amino acids, which is largely limited to microvillar OSNs that employ a V2R-
543 PLC-TrpC2 signalling cascade. Attenuation of OSN activation by blocking TrpC2 activity
544 would be confirmatory but there are no specific TrpC2 inhibitors currently available. We
545 therefore used 2-APB, a general Trp channel blocker (not specific for TrpC2), that has
546 been shown to block OSN responses in the vomeronasal organ in rodents (Lucas *et al.*,
547 2003; Zhang *et al.*, 2010) and amino acid responses in the OE of *Xenopus* (Sansone *et*
548 *al.*, 2014). Pretreatment of zebrafish with 2-APB administered via icv route significantly
549 attenuated OSN activation by amino acids in starved zebrafish (Figure 6A, p<0.0001).

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

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

561 in pERK positive OSNs upon amino acid stimulation even in the presence of exogenous
562 NPY peptide. In contrast, the vehicle treated animals showed the expected increase in
563 OSN activation (Figure 6C, p=0.0014). These results implicate PLC signalling in NPY-
564 mediated lowering of the amino acid sensitivity threshold of OSNs. NPY receptor
565 mediated activation of PLC has been documented previously and is thought to involve
566 $G_{\beta/\gamma}$ signalling (Koch *et al.*, 1994; Selbie *et al.*, 1995; Selbie *et al.*, 1997).

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

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

590

591 **DISCUSSION**

592 We have used zebrafish to study the modulation of olfactory sensitivity to food-related
593 cues by NPY signalling in response to the prevailing nutritional state. As NPY
594 expression in the peripheral olfactory circuitry was tuned to the nutritional state, we
595 investigated the contribution of NPY signalling to olfactory perception in an odorant
596 preference assay using a food cue associated mixture of amino acids. While the starved
597 fish showed a strong preference for the baited side, recently fed fish were agnostic to
598 the amino acid cue suggesting that the positive valence of food-related odorants is
599 tuned to the prevailing nutritional state. Administration of NPY Y1 receptor antagonist to
600 starved animals also reduced the attraction towards amino acids. Since NPY is a strong
601 orexic agent, this behaviour could also be attributed to the loss of feeding drive following
602 NPY inhibition. We tested this possibility using an amino acid based aversion assay and
603 found that NPY activity was necessary for amino acid perception. As central
604 administration of glucose reduced NPY expression in OE, it should mimic the fed state
605 in its response to amino acids. While glucose injected fish showed no preference for
606 amino acids, exogenous administration of NPY was sufficient to restore the response.
607 Collectively, these experiments implicate a central role for NPY as a mediator of
608 nutritional state-dependent tuning of olfactory perception of food-related cues. To
609 investigate if the differences in olfactory response observed in fed and starved states
610 extend to non-food -associated odorants, we evaluated the response to the alarm
611 substance Schreckstoff (a non-amino acid olfactory cue). Irrespective of their nutritional
612 states, the fish responded similarly to Schreckstoff suggesting that the energy state-
613 dependent modulation is specific to food cues. Teleost fish employ a vomeronasal-like
614 amino acid sensing mechanism, largely confined to microvillar OSNs (Sato *et al.*, 2005;
615 Koide *et al.*, 2009), while Schreckstoff is processed by crypt OSNs (Mathuru *et al.*,
616 2012). NPY-based neuromodulation appears to be limited to microvillar OSNs. This
617 claim is substantiated by the observation that almost all the amino acid activated OSNs
618 in starved animals (heightened NPY signalling) co-expressed TrpC2 channels. This cell
619 type -dependent partitioning limits NPY-mediated tuning of olfactory sensitivity to amino
620 acids, the major food-associated odorant cue.

621 Interestingly, we detected little NPY expression in microvillar OSNs. This suggests that
622 NPY, released in a non-synaptic manner from other OSN subtypes and the terminal
623 nerve, acts on NPY receptors on microvillar OSNs and modulates their activity. It
624 remains unclear what triggers the change in NPY expression in response to nutritional
625 states. Although the OB is intrinsically glucosensitive (Fadool *et al.*, 2011; Tucker *et al.*,
626 2013), we do not know which glucoresponsive source influences the NPY expression.

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

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

652 2006; Root *et al.*, 2011). Indeed, externally applied NPY increased the electro-
653 olfactogram (EOG) response to amino acids in OE isolated from starved but not fed
654 salamanders and rats (Mousley *et al.*, 2006; Negroni *et al.*, 2012).

655 However, these studies do not report intrinsic differences in EOG responses between
656 starved and fed animals (Mousley *et al.*, 2006; Negroni *et al.*, 2012). This is possibly
657 because these isolated OE preparations are devoid of endogenous NPY, while an intact
658 behaving animal can access the secreted neuropeptide. The rat study also
659 demonstrated an elevation in NPY receptor expression upon starvation (Negroni *et al.*,
660 2012). Similar increase in sNPF receptor expression has been reported in starved flies
661 (Root *et al.*, 2011). NPY receptor expression may also increase in the OSNs of starved
662 zebrafish. This increase will augment NPY signalling in these animals along the lines
663 indicated in this study. A recent study in *Drosophila* has reported the modulation of OSN
664 responses by NPF (fly orthologue of NPY) signalling, suggesting the conservation of
665 this modality across phyla (Lee *et al.*, 2017).

666 Amino acids are typically perceived by microvillar OSNs expressing the vomeronasal
667 class II receptors (V2Rs) that are coupled to PLC activity. PLC activation, in turn, leads
668 to OSN depolarization via the opening of DAG-gated TrpC2 channels (Lucas *et al.*,
669 2003; Sansone *et al.*, 2014). Zebrafish and *Xenopus,* lack a distinct vomeronasal organ,
670 instead, vomeronasal receptor (VR) expressing OSNs are found in the OE (Sato *et al.*,
671 2005; Koide *et al.*, 2009; Sansone *et al.*, 2014). We show that OSN activation by amino
672 acid stimulation in zebrafish is also dependent PLC signalling and TrpC2 activity.
673 Importantly, NPY-dependent enhancement of OSN sensitivity is also dependent on PLC
674 activity. Canonical stimulation of NPY receptors involves $G_{\alpha i}$ signalling resulting in
675 inhibition of adenylyl cyclase activity (Herzog *et al.*, 1992). However, heterotrimeric G-
676 proteins can potentially generate bifurcating signals, one mediated by $G_{\alpha i}$ and the other
677 by the $G_{\beta\gamma}$ subunits. The latter subunit is known to activate PLC and there is evidence
678 that NPY signalling, in certain contexts, leads to PLC activation (Selbie *et al.*, 1995;
679 Selbie *et al.*, 1997). Thus, NPY and amino acid signalling converge on PLC activation
680 (Figure 6E). This convergence offers a potential mechanism where the additive effect of
681 the two convergent pathways lowers the activation threshold of OSNs (Figure 6E, F).
682 Interestingly, the centrality of PLC activity also offers a potential mechanistic

683 explanation for the co-operative, switch-like increase in microvillar OSN activation
684 observed for co-incident NPY and amino acid signalling. Synergistic activation of PLC
685 isoforms has been extensively reported in studies investigating GPCR signalling cross
686 talk (Zhu & Birnbaumer, 1996; Werry *et al.*, 2003). PLC isoforms, like PLCβ1-3, have
687 two distinct binding sites for $G_{\alpha q}$ and $G_{\beta\gamma}$, and simultaneous occupation of these sites
688 can result in synergistic activation (Zhu & Birnbaumer, 1996; Yoon *et al.*, 1999; Philip *et*
689 *al.*, 2010). In the case of PLCβ3, this cooperative activation has been modeled as a two-
690 state coincidence detector (Philip *et al.*, 2010). NPY signalling, via $G_{\beta\gamma}$ activation, has
691 been demonstrated to potentiate adrenergic and purinergic signalling (Selbie *et al.*,
692 1997). If a similar mechanism were operational in zebrafish OSNs, then it would be
693 expected that the amino acid signalling via V2Rs would involve $G_{\alpha q}$ activity. We tested
694 this possibility and found that GP2A treatment (a specific $G_{\alpha q}$ activity blocker) prevented
695 amino acid mediated OSN activation. This observation offers new insight into olfactory
696 processing of amino acid cues in fish. It is worth noting that while $G_{\alpha i}$ and $G_{\alpha o}$ are
697 predominantly implicated in mammalian VNO transduction, there is also available
698 evidence suggesting the involvement of $G_{\alpha q}$ (Wekesa *et al.*, 2003; Thompson *et al.*,
699 2006).

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

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

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899

900

901 **FIGURE LEGENDS**

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

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

914

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

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

931

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

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

951

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

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

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

963

964 **Figure 5. NPY signalling increases the sensitivity of microvillar OSNs to amino**
965 **acids**.

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

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

993

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

996 **(A)** The number of pERK –ir OSNs in starved fish injected with either vehicle or 2-APB
997 (Trp channel blocker) via icv route and exposed to a mixture of amino acids (N=6; error
998 bars represent +/-SEM; ***, p< 0.0001). **(B)** The number of pERK –ir OSNs in starved
999 fish injected with either vehicle or U73122 (selective PLC inhibitor) via icv route and
1000 exposed to a mixture of amino acids (N=6; **, p< 0.001). **(C)** The number of pERK –ir
1001 OSNs in starved fish pre-injected with Glu + NPY followed by administration of vehicle
1002 or U73122 via icv route, exposed to a mixture of amino acids (N=6; **, p< 0.01). **(D)** The
1003 number of pERK –ir OSNs in starved fish injected with either vehicle or GP2A (selective
1004 $G_{\alpha q}$ signalling inhibitor) via icv route and exposed to a mixture of amino acids (N=6; ***,
1005 p< 0.0001). Data are shown as mean +/- SEM and means were compared using
1006 unpaired t-test. **(E)** Schematic showing the convergence of NPY Y1 receptor (NPY Y1R)
1007 signalling and odorant receptor for amino acids (V2R) signalling on phospholipase C
1008 mediated activation of microvillar OSN. **(F)** Increased NPY signalling (starved animals)
1009 results in a sharp increase in the sensitivity of microvillar OSNs to food-associated
1010 amino acid odorant cues. In energy-rich conditions (fed animals), the baseline NPY
1011 signalling is low and the sensitivity of the microvillar OSNs to amino acids is reduced.
1012 AA - amino acid mixture; PLC – phospholipase C; DAG – diacylglycerol; TrpC2 –
1013 transient receptor potential channel C2.

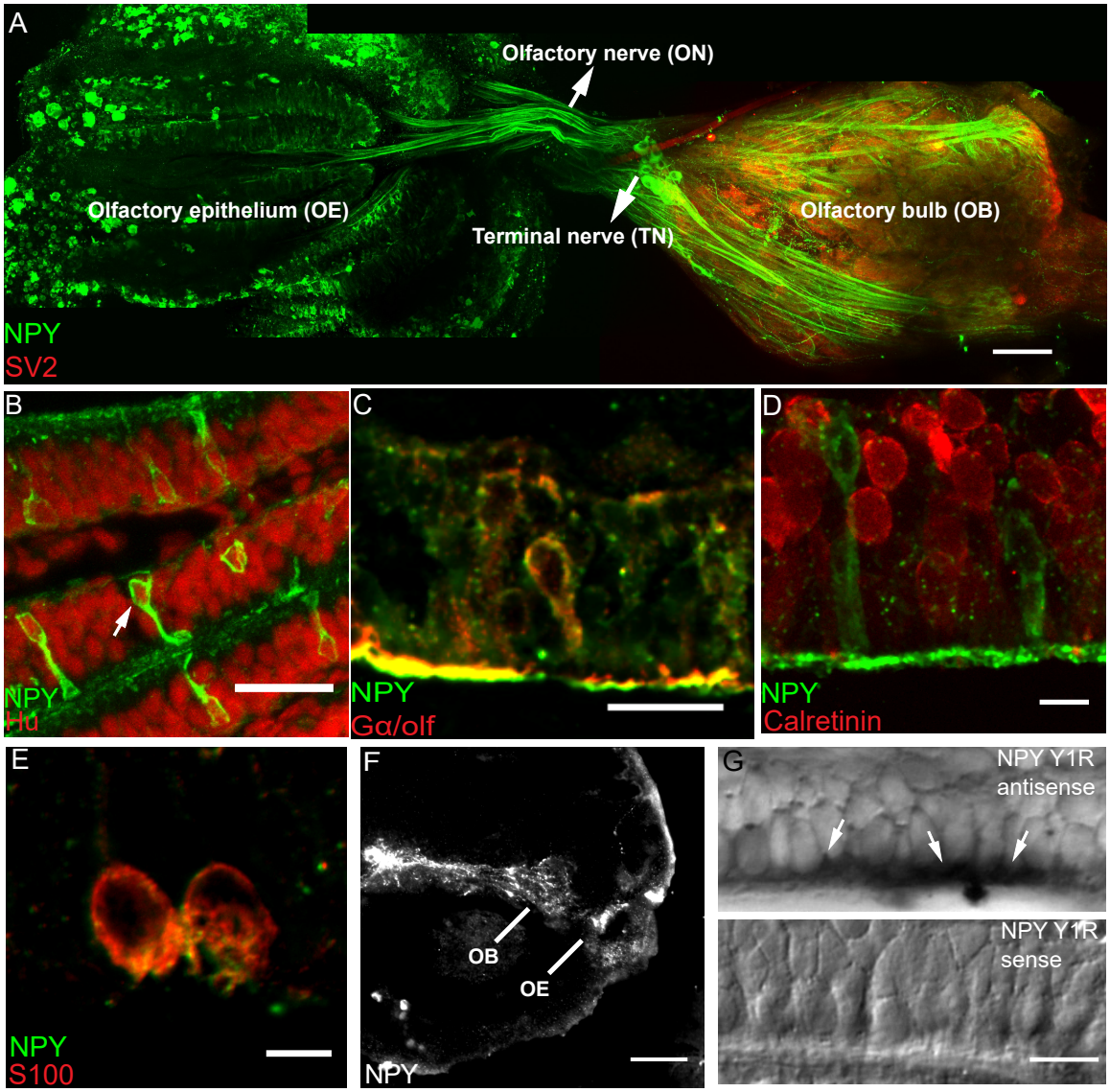
A
Olfactory nerve (ON)
Olfactory epithelium (OE)
Terminal nerve (TN)
Olfactory bulb (OB)
NPY
SV2
B
NPY
Hu
C
NPY
Gα/olf
D
NPY
Calretinin
E
NPY
S100
F
OB
OE
NPY
G
NPY Y1R
antisense
NPY Y1R
sense

Figure 1

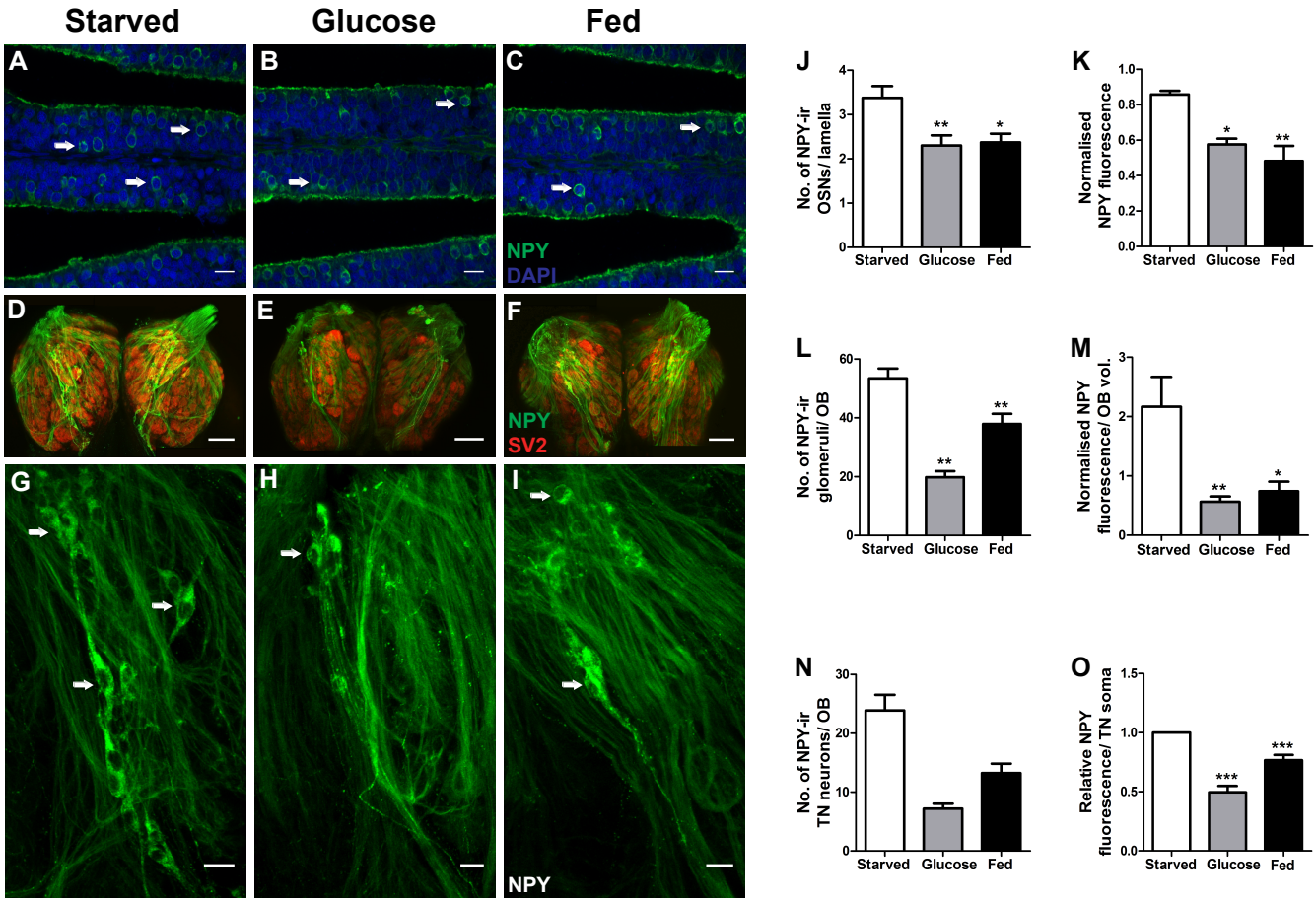
Starved
Glucose
Fed
A
B
C
NPY
DAPI
D
E
F
NPY
SV2
G
H
I
NPY
J
No. of NPY-ir OSNs/ lamella
K
Normalised NPY fluorescence
L
No. of NPY-ir glomeruli/ OB
M
Normalised NPY fluorescence/ OB vol.
N
No. of NPY-ir TN neurons/ OB
O
Relative NPY fluorescence/ TN soma
Starved
Glucose
Fed

Figure 2

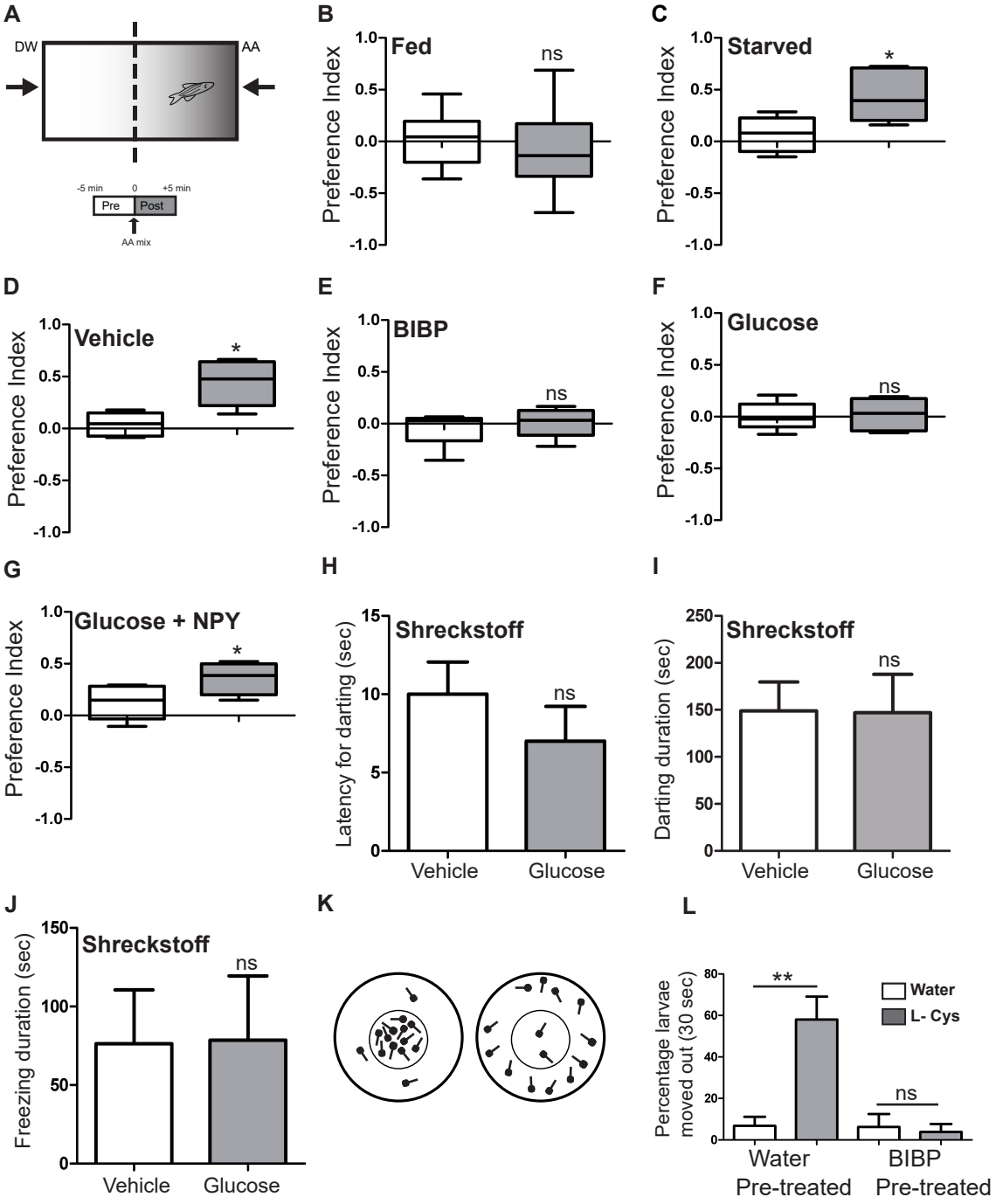
A
DW
AA
-5 min
0
+5 min
Pre
Post
AA mix
B
Fed
ns
Preference Index
C
Starved
*
Preference Index
D
Vehicle
*
Preference Index
E
BIBP
ns
Preference Index
F
Glucose
ns
Preference Index
G
Glucose + NPY
*
Preference Index
H
Shreckstoff
ns
Latency for darting (sec)
Vehicle
Glucose
I
Shreckstoff
ns
Darting duration (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

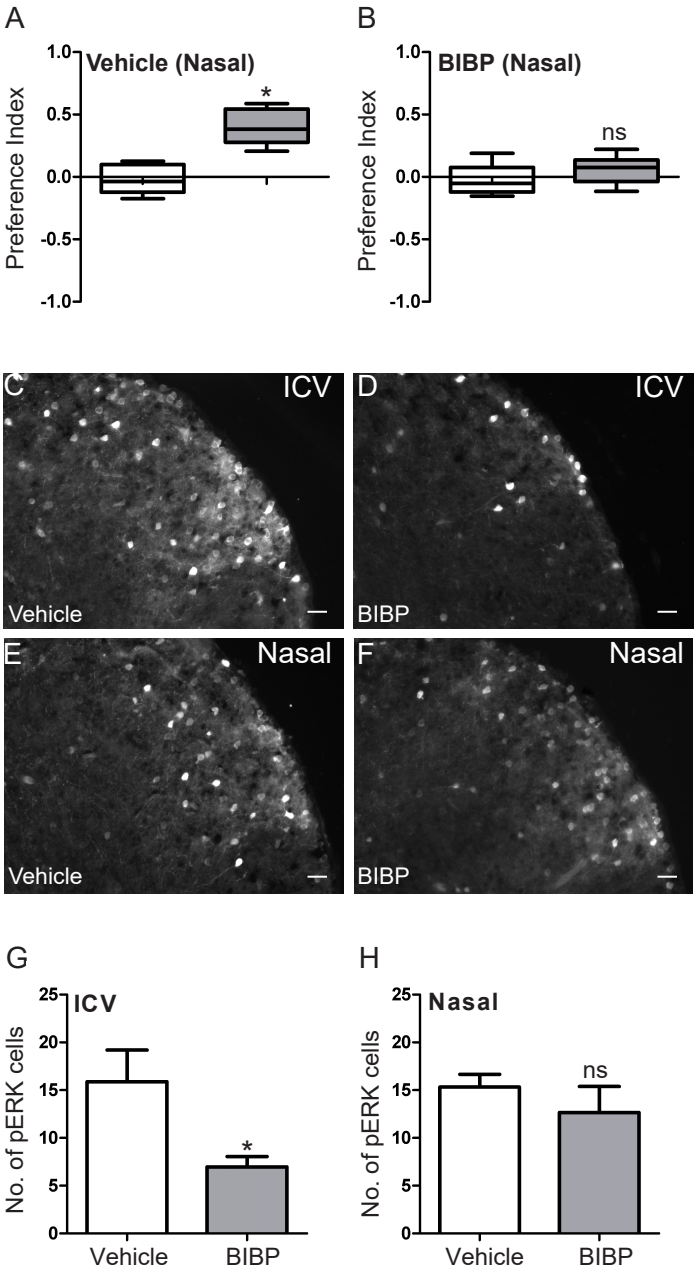
A
Vehicle (Nasal)
Preference Index
1.0
0.5
0.0
-0.5
-1.0
*
B
BIBP (Nasal)
Preference Index
ns
C
ICV
Vehicle
D
ICV
BIBP
E
Nasal
Vehicle
F
Nasal
BIBP
G
ICV
No. of pERK cells
25
20
15
10
5
0
*
Vehicle
BIBP
H
Nasal
No. of pERK cells
ns
Vehicle
BIBP

Figure 4

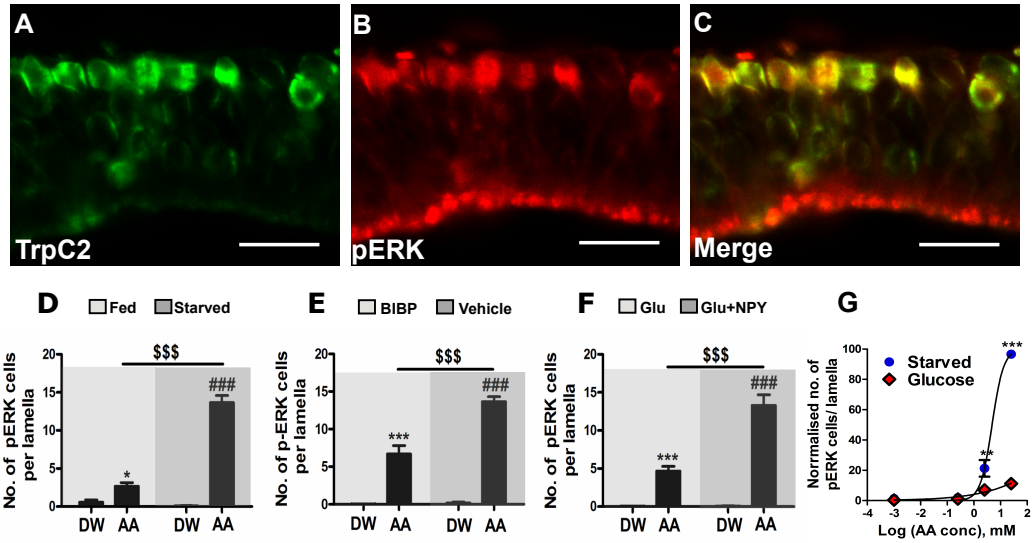
A
TrpC2
B
pERK
C
Merge
D
Fed
Starved
No. of pERK cells per lamella
$$$
###
*
DW
AA
DW
AA
E
BIBP
Vehicle
No. of p-ERK cells per lamella
$$$
###
***
DW
AA
DW
AA
F
Glu
Glu+NPY
No. of pERK cells per lamella
$$$
###
***
DW
AA
DW
AA
G
Starved
Glucose
Normalised no. of pERK cells/ lamella
***
**
Log (AA conc),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

Figure 5

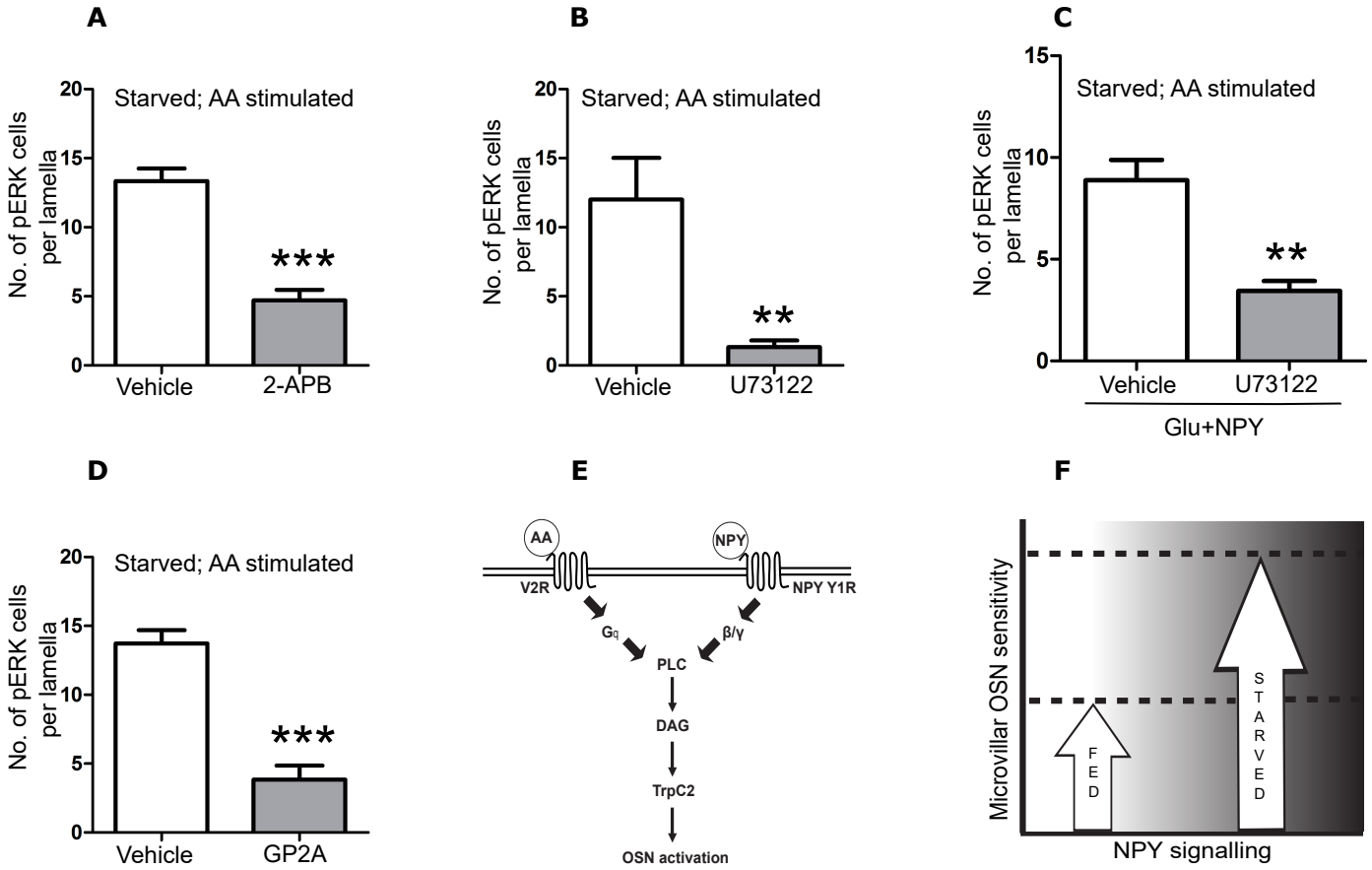
A
Starved; AA stimulated
No. of pERK cells per lamella
20
15
10
5
0
***
Vehicle
2-APB
B
Starved; AA stimulated
No. of pERK cells per lamella
20
15
10
5
0
**
Vehicle
U73122
C
Starved; AA stimulated
No. of pERK cells per lamella
15
10
5
0
**
Vehicle
U73122
Glu+NPY
D
Starved; AA stimulated
No. of pERK cells 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