Methods On-line

Animals For experiments that did not test illumination-dependence, animals were raised on a 12/12 light/dark cycle on a gray or black background. For experiments testing the effects of illumination, animals were raised as described in the Methods Summary. We quantified skin pigmentation by scoring pixel intensities of lateral views of 200 µm x 600 µm rectangular areas

immediately posterior to the gut of stage 42 larvae, thresholding, and expressing the result as the percentage of black area (Image J; threshold set to 95). The temperature of the water next to the larvae, assessed with a thermistor (Fisher Scientific), was stable across all conditions and lay between 22 and 23° C.

Eye enucleation Experiments were performed to confirm that retinal input controls skin pigmentation. The eye primordium was removed unilaterally or bilaterally at stage 35, leaving the pineal gland intact, and the effect of ambient illumination on pigmentation was tested 24 hr later. Sham surgery consisted of creating a small opening in the skin covering the eye and rubbing it with dull forceps.

Neuropharmacology Bath application of 10 nM sulpiride (Sigma) in 10% MMR saline was used to achieve D_2 dopamine receptor blockade. To test whether activation of D_2Rs in the retina contributes to background adaptation we exposed stage 42 larvae raised in the dark on a black background (DBB) to D_2R agonist 1 μM quinpirole hydrochloride (Sigma) in the dark for 30 min. The effect of quinpirole on pigmentation was compared to eye-enucleated stage 42 larvae also exposed for 30 min to quinpirole in the dark. To test whether D_1Rs can contribute to the larval dark/white adaptation we exposed stage 42 larvae raised for 28 hr in the light on black background (LBB) to either 1 μM D_1R agonist (SKF 38393; Sigma) or 1 μM D_1R antagonist (SCH 23390; Sigma) during 30 min of white (LWB) or dark (DBB) adaptation. Pigmentation displayed by dark- and white-adapted treated larvae was compared to controls. Beads used to deliver drugs to the VSC were washed in 2 mM calcium saline for 2-4 hr at room temperature and loaded for 1 hr in a bi-directional rotator (Barnstead International) with a solution containing 10 μM calcein-AM (Invitrogen), 10 μM BAPTA-AM (Invitrogen), or 10 μM TTX (Sigma). Larvae were anesthetized with tricaine, recovered following bead implantation, and developed for 24 hr before fixation. To quantify diffusion of fluorescent calcein, larvae were first fixed for 40-60 min in a solution of 0.1X PBS containing 40 mg/ml EDAC (Sigma) and then in 4% paraformaldehyde (PFA) for 1.5 hr. Stocks of 10X MMR physiological saline (pH 7.8) were prepared by dissolving NaCl (1 M), KCl (20 mM), $MgSO_4$ (10 mM), Hepes (50 mM), EDTA (1 mM), and $CaCl_2$ (20 mM) in Fisher water and autoclaving for 20 min.

Immunocytochemistry Larvae were fixed in 4% PFA in phosphate-buffered saline (PBS, pH 7.4) for 2 hr at 4°C, soaked in 30% sucrose for 2.5 hr or overnight at 4°C, and embedded in OCT (Fisher Scientific). For dopamine immunocytochemistry, the fixation solution contained 0.1M

cacodylate, 10g/L sodium metabisulfite, and 3% glutaraldehyde (all from Sigma). Ten micron cryostat sections were made through the entire brain and regions of interest were identified anatomically afterwards. For whole-mount experiments, larvae were fixed and incubated in PBS-0.5% Triton for 3 days. The CNS was then dissected and carried through the immunostaining protocol below. Slides and whole-mounts were incubated in a blocking solution of 2% goat serum, 1% fish gelatin for 0.5-2 hr at 20°C, followed by overnight incubation with primary antibodies to TH (Imgenex), NPY (Immunostar Inc), DAT (Advanced Targeting Systems), VMAT2 (Santa Cruz Biotechnology Inc), DA (Novus Biologicals), BrdU (Calbiochem), POMC (Novus), Pax6 (Covance), and Lim1,2 (DSHB) at 4°C; specimens were incubated for 2 hr with fluorescently tagged secondary antibodies at 20°C. Immunoreactivity was examined on a Zeiss Axioskop with a 20x air objective using a Xenon arc lamp, attenuated by neutral density filters and the appropriate excitation and emission filters for Alexa 350, 488, 555, 594, and 647, Cy3, DAPI and DRAQ5 fluorophores. Images were acquired and analyzed with Image J, Axiovision, and Corel Photopaint. The TH antibody was used as a marker for DA neurons of the VSC, given demonstration of TH/dopamine double labeling[1] and TH mRNA/TH colocalization. Differences in labeling were considered significant at $P<0.05$ (Student's *t*-test). The small size of the VSC, with a transverse diameter of ~250 µm and a rostrocaudal extent of 30-50 µm, facilitated counting the neurons.

Ion channel overexpression hKir2.1 and $rNa_v2a\alpha\beta$ DNA constructs were generous gifts of Eduardo Marban and William Catterall and were subcloned, transcribed and injected as previously described[3]. Control and activity-manipulated larvae were raised in a 12/12 hr light (331 LUX)/dark cycle on a gray background.

Calcium imaging The CNS was dissected from larvae at stages 35-42 using jewelers' forceps, tungsten needles and glass probes, and pinned with fine tungsten wires in a small chamber. Midbrain dopaminergic neurons were exposed by sectioning through the hypothalamus with micro-scalpels made of sharp razor blades. Fluorescence of the calcium indicator Fluo-4AM (Invitrogen) was used to study elevations of $[Ca]_i$ in neurons. Brains were incubated for 30 min to 1 hr in culture medium containing 2-5 µM dye and 0.01% Pluronic F-127 and washed in saline prior to imaging. For study of calcium spikes, images were acquired at 0.2 Hz for 1 hr periods with a BioRad MRC 1024 or Leica SP5 laser confocal system with a 20x water

immersion objective. Image stacks were imported into Image J (W. Rasband, NIH) for analysis. Using the line-drawing tools in the program, a region of interest (ROI) was traced around the site of the transient and a measurement of the average pixel intensity within the ROI was acquired. Data were exported to Excel (Microsoft) for analysis. To retain Fluo-4AM fluorescence of the imaged cells, preparations were fixed for 30 min at room temperature with 40 mg/ml EDAC (Sigma) and 1 hr 4% PFA prior to immunocytochemistry processing.

BrdU and TUNEL staining To understand whether TH/NPY annular neurons represent newly born cells or pre-existing neurons, cells proliferating during white adaptation were identified by 5-bromo-2'-deoxyuridine (BrdU; Sigma) labeling. Stage 41 larvae were exposed to BrdU by immersion in 4 mg/ml BrdU/10% MMR for 6 hr. Specimens (St 42) were fixed and sucrose cryoprotected as described above. 10 μm cryostat sections were treated for 20 minutes in 2 N hydrochloride acid (HCl; Fisher Scientific) for antigen retrieval, then washed and incubated with mouse anti-BrdU antibody (Calbiochem) overnight. To determine whether white adaptation influences cell death in the hypothalamus, *in situ* Cell Death Detection KIT fluorescein TUNEL staining (Roche) of 2 hr-white-adapted larvae was compared to staining of black-adapted larvae. Positive TUNEL controls were obtained by 10 min incubation with DNase I (3U/ml in 50 mM Tris-HCl, Ambion) at 25 °C to induce DNA breaks prior to labeling procedures.

Reference for Methods On-line

1. Gonzalez, A. & Smeets, W. Comparative analysis of dopamine and tyrosine hydroxylase immunoreactivities in the brain of 2 amphibians, the anuran *Rana ridibunda* and the urodele *Pleurodeles waltlii*. *Journal of Comparative Neurology* **303**, 457-477 (1991).