Neuropeptide Y Activity in the Nucleus Accumbens Modulates Feeding Behavior and Neuronal Activity

Supplemental Information

Supplemental Methods and Materials

Surgery for Cannulae in Nucleus Accumbens

Male Wistar rats (Charles River, Germany) weighing 270-300 g were housed in a temperature (21-23ºC) and light-controlled room (lights on 0700–1900). One week after arrival, two cannulae aimed bilaterally at the Acb shell were implanted. Rats were anaesthetized with an i.p. injection of 80 mg/kg ketamine (Eurovet Animal Health, Bladel, The Netherlands), 8 mg/kg xylazine (Bayer Health Care, Mijdrecht, The Netherlands) and 0.1 mg/kg atropine (Pharmachemie B.V., Haarlem, The Netherlands) and fixed in a stereotaxic frame. Permanent 26-gauge stainless steel guide cannulae (Plastics One, Bilaney Consultants GmbH, Düsseldorf, Germany) were implanted at AP:+1.4 mm, ML:+/-2.8 mm, DV:-6.6 mm (coordinates from bregma and using an angle of 10° in the frontal plane). Guide cannulae were secured to the skull using four anchor screws and dental cement and occluded by a 28-gauge stainless steel dummy cannula (Plastics One, Bilaney Consultants GmbH, Düsseldorf, Germany). Immediately after surgery, rats received an analgesic subcutaneously (Carprofen, 0.5 mg/100 g body weight) and were housed individually.

Radioactive *in situ* Hybridization

Sections were defrosted and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 min, washed in PBS, pretreated with 0.25% acetic anhydride in 0.1 M triethanolamine, washed again in PBS and dehydrated in graded ethanol followed by 100% chloroform and 100% ethanol. The sections were hybridized overnight at 72°C with 10^6 cpm ^{33}P-labeled (33P-UTP, Perkin Elmer) antisense ppENK RNA probe in buffer containing 50% deionized formamide, 2× standard saline citrate (SSC), 10% dextrane

sulphate, 1× Denhardt's solution, 5 mM EDTA and 10 mM phosphate buffer, after 5 min heating at 80°C. After hybridization, the sections were washed in 5×SSC (short, 72°C) and 0.2×SSC (2 h, 72°C) and dehydrated in graded ethanol in 0.3 M ammonium acetate. Sections were exposed to X-ray film (Kodak Bio-Max MR, Sigma-aldrich, Zwijndrecht, The Netherlands) for 4 days. The films were developed and expression levels were quantitatively analyzed using an 8800F Canon scanner. All images (600 dpi) were analyzed using ImageJ (Rasband, WS, NIH, Bethesda, MD, USA, http://rsbweb.nih.gov/ij/, 1997–2005). In each section gray values were determined in the region of interest, measured bilaterally and subtracted from background, producing a single value for each brain area on each section.

Fluorescent Immunohistochemistry

Coronal slices of 35 µm were cut in a cryostat and collected and stored until processing in cryoprotectant (30% glycerol, 30% ethylene glycol, 40% 0.1M PBS). Free floating slices were washed in PBS at least 5 times for a total of 60 min and were incubated in blocking buffer (2.5% normal donkey serum, 2.5% normal goat serum, 1% bovine serum albumin, 1% glycine, 1% lysine, 0.4% Triton X-100) for at least 30 min at room temperature (RT). Sections were incubated overnight in rabbit anti-NPY (1:1000, Niepke 091188, Netherlands Institute for Brain Research) primary antibody diluted in blocking buffer. The next day, slices were washed in PBS several times for a total of 60 min. Subsequently, sections were incubated with donkey anti-rabbit Alexa Fluor-488 (Invitrogen) secondary antibody diluted in blocking buffer for 1 h at RT. After 5 washes in PBS for a total of 60 min at RT, sections were mounted on glass slides and embedded in mounting medium containing DAPI (Vectashield, Vector labs, Burlingame, CA, USA). Cholera toxin B (CTB)-alexa-555 conjugate emission was sufficient to visualize under the fluorescent microscope without additional CTB antibodies. Images were first taken by fluorescent microscopy and co-localization across all sections was determined by confocal microscope scanning.

Surgery for Cannula and Microwire Electrode in Nucleus Accumbens for Neural Recordings in Mice

Anesthesia was initiated with ~4% isofluorane and intraperitoneal injections of ketamine (100 mg/kg) and xylazine (10 mg/kg). A surgical level of anesthesia was maintained over the course of surgery with supplements (30 mg/kg) of ketamine every 45–60 min. The skull was leveled between bregma and lambda and a craniotomy was created over the ventral striatum. A single array of microwire electrodes was placed centered at AP:+1.2, ML:±0.7, DV:−4.5 (coordinates from bregma). A single infusion cannula was then placed at AP:+1.4, ML:±2.1, DV:−4.6 (coordinates from bregma and using an angle of 15° in the frontal plane). Craniotomies were sealed with cyanoacrylate (“Metabond”) and methyl methacrylate (i.e., dental cement; AM Systems, Carlsborg, WA, USA).

Fluorescent *in situ* Hybridization

For the fluorescent *in situ* hybridization analysis, fresh frozen mouse brains were cryosectioned at 14 µm thickness and dried onto slides. The sections were then fixed in ice-cold 4% paraformaldehyde for 20 min, dehydrated in an ethanol series and allowed to air dry. The sections were rehydrated, acetylated for 10 min, dehydrated and air dried again. The hybridization mix (50% formamide, 5×SSC, 5×Denhardt's solution, 250 µg/ml yeast RNA, 0.5 mg/ml salmon testes DNA, and 300 ng/ml RNA probe) was then added to the slides, which were incubated in humidified chambers at 60°C overnight. After washing and blocking with 5% normal rabbit IgG and 1% blocking reagent (Roche), the Y1R probe was first detected by using a 1:200 anti-digoxigenin antibody coupled to HRP (Dako, Carpinteria, CA, USA). The digoxigenin signal was amplified and detected using tyramide signal amplification (TSA)-direct coupled to cyanine-3 (PerkinElmer, Wellesley, MA, USA). Hydrogen peroxide treatment (3%, 15 min) was used to eliminate HRP activity. The fluorescein-labeled probe was detected with 1:500 rabbit anti-fluorescein coupled to HRP (Molecular Probes), followed by amplification with TSA-direct coupled to fluorescein (PerkinElmer). The sections were then dehydrated and mounted in DPX (Fluka, Neu-Ulm, Germany).

Generation of fluorescent in situ *hybridization probes*

Amplification products obtained by PCR were gel-purified and cloned into pCR2-TOPO vector (Invitrogen, Carlsbad, CA) and transformed into One Shot TOP10 competent cells (Invitrogen), using standard techniques. Positive clones were verified by diagnostic restriction enzyme digest and amplicon sequencing. To generate antisense digoxigenin (DIG)-labeled Y1R cRNA probe, plasmid was linearized by digestion with *BAM*HI and subjected to in vitro transcription with T7 RNA polymerase according to the manufacturer's protocol (Roche, Basel, Switzerland). For generation of sense DIG-labeled Y1R cRNA probe, plasmids were linearized by digestion with *Eco*RV and subjected to in vitro transcription with SP6 RNA polymerase. To generate antisense fluorescein-labeled ppENK and pDYN cRNA probes, plasmids were linearized by digestion with *BAM*HI for ppENK and *Eco*RV for pDYN and subjected to in vitro transcription with T7 RNA polymerase (for ppENK) and SP6 (for pDYN) according to the manufacturer's protocol (Molecular Probes, Eugene, OR, USA). Hybridization with sense probes was included as control experiment to confirm the specificity of the *in situ* hybridization protocol. These sense hybridization probes did not show any fluorescent hybridization signal.

Table S1. Primers

	NCBI Reference Sequence	Primers	Product Size	Transcript Nucleotide Number
NPY Y1 receptor (Y1R)	NM_010934	Forward: GAAGCAGGCTAGCCCAGTC Reverse: TCAGGTGGTGACTGCTTTTG	506	1337-1882
preproenkephalin (ppENK)	NM_001002927	Forward: AACAGGATGAGAGCCACTTG Reverse: CTTCATCCGAGGGTAGAGAC	437	568-1041
prodynorphin (pDYN)	NM_018863	Forward: TCTTTTCTCACCCTGACTGC Reverse: CCATAGCGTTTGTACAGGTC	371	510-916

Supplemental Results

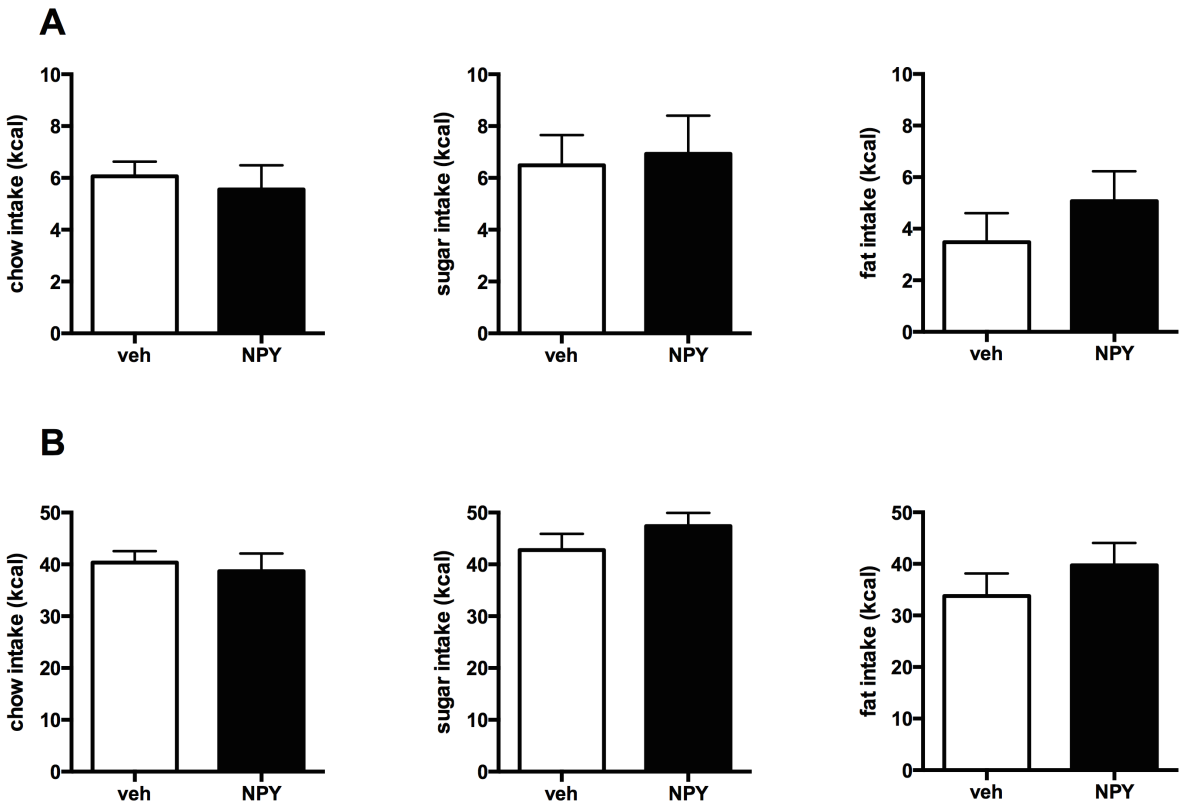

Figure S1. Effect of intra-Acb NPY on 5 h (**A**) and 24 h (**B**) intake of chow, sugar and fat component. The intake of chow, sugar and fat are not increased after 5 h and 24 h after NPY administration in the Acb of rats. Acb, nucleus accumbens; NPY, neuropeptide Y; veh, vehicle.

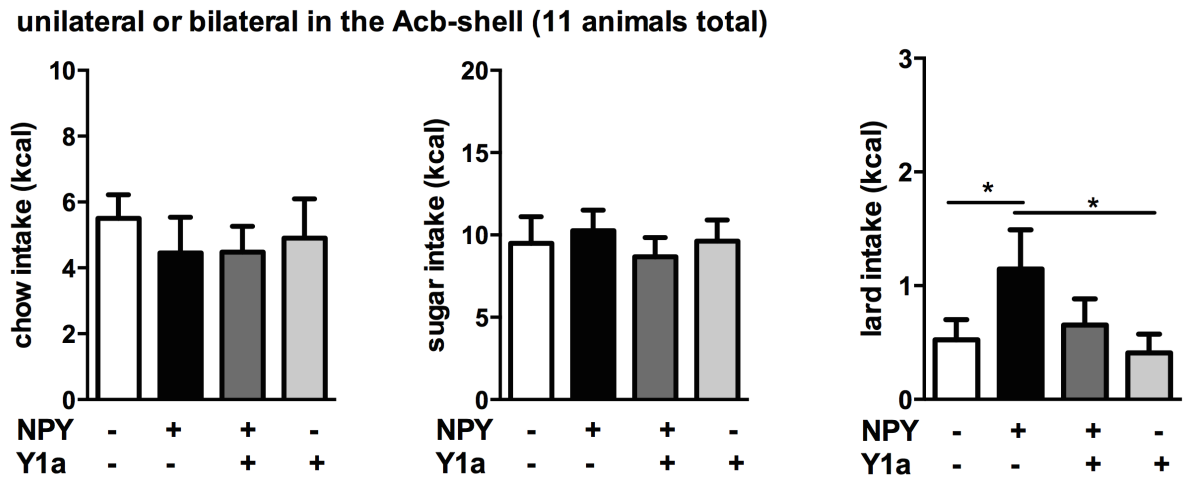

Figure S2. Effect of pretreatment with NPY Y1 receptor antagonist (Y1a) on NPY-induced fat intake. Rats with unilateral or bilateral cannula placement in the Acb shell showed increased fat intake, consistent with Figure 1A. Pretreatment with Y1a did not significantly attenuate the NPY-induced fat intake (Figure 1B; 2-way RM ANOVA: effect of NPY $F_{(1,10)}$ = 4.6; p = 0.06; effect of pretreatment $F_{(1,10)}$ = 5.3; p = 0.04), but no interaction effect of NPY* pretreatment ($F_{(1,10)}$ = 1.0; p = 0.34); post hoc: *p < 0.05. Interestingly, only when the Y1 antagonist was infused bilaterally in the medial shell, NPY's effects on fat intake were completely abolished, suggesting an important role of the medial shell in the Y1 mediated effects and that the Y1R in the Acb-shell is involved in the effect of NPY on fat intake. Acb, nucleus accumbens; NPY, neuropeptide Y.

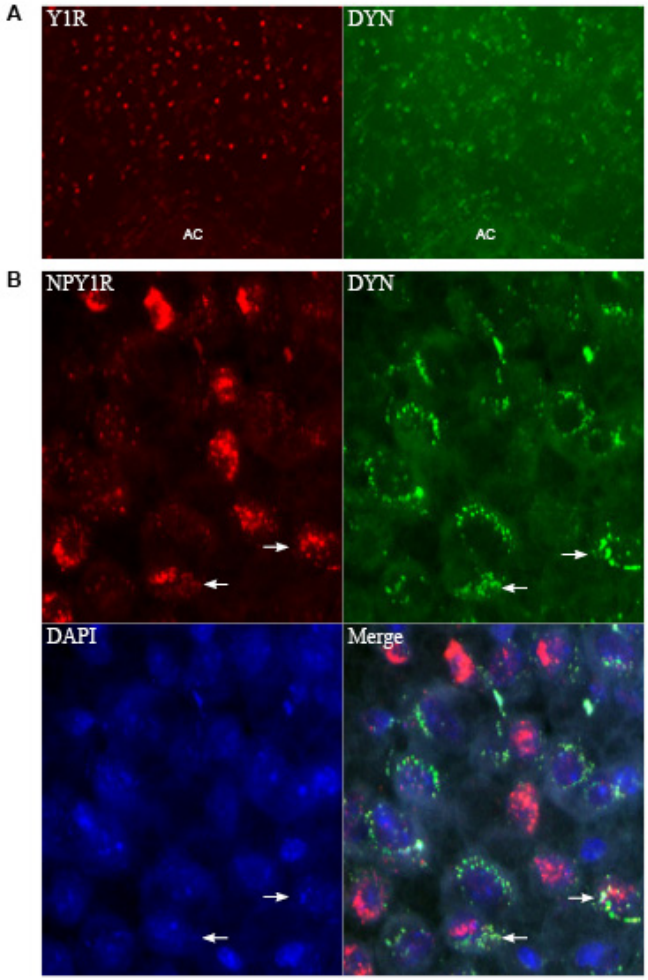

Figure S3. (**A**) Fluorescent microscopy shows the expression pattern of dynorphin (DYN) and NPY Y1 receptor (Y1R) mRNA in the nucleus accumbens (Acb). (**B**) Confocal microscopy shows the colocalization of NPY Y1 receptor (Y1R) on a subpopulation of dynorphin (DYN) neurons in the nucleus accumbens (Acb). Horizontal arrows indicate colocalization. AC, anterior commissure.