Supplemental Experimental Procedures

Stereotaxic Guide Cannula Implantation and Intracerebroventricular Injection

Guinea pigs underwent stereotaxic surgery 4-14 days prior to experimentation, during which we inserted a 22-gauge guide cannula ~1mm above the third cerebroventricle. This was performed while the animal was under ketamine (33mg/kg; s.c.)/ xylazine (6mg/kg; s.c.) anesthesia that was supplemented by isoflurane (1.5-2%) over the course of the procedure. Animals were secured in a stereotaxic frame by fitting the incisors over a tooth bar and inserting blunt ear bars into the ear canals. The surgery was performed using aseptic techniques. First, the scalp was opened by making a 2-2.5 cm incision down the midline of the skull beginning at the front of the orbits towards the occipital lobe with a scalpel blade. A single hole was drilled and the dura layer cut so that a guide cannula could be slowly lowered at an angle 4° from the vertical plane (thereby avoiding the puncture of the mid-sagittal sinus) to its desired location using the following coordinates (in mm, modified from Luparello et. al., 1964 and Tindal J.S., 1965; measured from bregma and the top of the cerebral cortex) - M/L: -0.7, A/P: -2.1, D/V: -9.8, tooth bar: -5.5. Three 3.2 mm bone anchor screws were inserted into pre-drilled holes and then dental cement/acrylic was used to affix the stainless steel screws and cannula to the skull. Finally, a stylette was inserted into the guide cannula to prevent cerebrospinal fluid from entering the shaft of the cannula. Animals were allotted a minimum of four days of recovery prior to experimentation. At the time of intracerebroventricular (ICV) injection the stylette was removed and a 28-guage injection needle inserted so that it protruded one mm beyond the distal end of the guide cannula. Either purified guinea pig insulin (4 mU) or its filtered, 0.9% saline vehicle (2 µl) was slowly administered into the third cerebroventricle over the duration of one minute, after which the stylette was reinserted.

Tissue Collection and Fixation

Brains were quickly removed, rinsed in ice-cold Sorenson's buffer and placed in a brain matrix (Ted Pella, Inc.; Redding, CA, USA); from which 3 mm (guinea pigs) or 2 mm (mice) blocks, containing the preoptic area (POA), rostral mediobasal hypothalamus (RBH) and caudal mediobasal hypothalamus (CBH), were cut in the coronal plane. These blocks were prepared for histology and immunohistochemistry by fixing them in 4% paraformaldehyde for six hrs, followed by submersion in 20% sucrose buffer for 48 hr (changed at 24 hr intervals) for cryoprotection prior to snap freezing in isopentane at -55°C. To verify ventricular cannula

placement, coronal sections (20 μm) were cut on a cryostat and thaw-mounted onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) and stored at −20°C.

Only animals with verified third cerebroventricle cannula placement were included in the data analysis. *In vivo* data were analyzed using a repeated measures, two-way ANOVA followed by the Least Significant Difference (LSD) test. If we found a significant interaction among the variables, then we would subsequently run a one-way ANOVA followed by the LSD test to determine significance at the individual time points. Differences were considered statistically significant if $p < 0.05$.

Eight gonadally intact male POMC-EGFP mice received a third cerebroventricle injection (250 µl/min) of either insulin (4 mU; n=4) or saline vehicle (1.5 µl; n=4) under isoflurane anesthesia. Mice were anesthetized using 3% isoflurane + oxygen for 3 minutes and received 1.5 % isoflurane + oxygen for the duration of the surgical procedure. After securing in the stereotaxic apparatus, a single hole was drilled into the skull at designated coordinates from bregma (M/L: 0 mm; A/P: -0.80 mm). A 1.7 cm 25 gauge guide cannula was positioned at M/L: 0 mm and A/P: -0.80 mm lowered to D/V: -4.25 mm and then a 1.8 cm injection needle equipped with sylastic tubing connected to a 5 µl Hamilton syringe preloaded with the drug treatment was inserted into the guide cannula (final injection depth of -5.25 mm). The injection needle was left in place for 10 minutes following the injection and then slowly withdrawn from the brain. The tissue was collected as described above.

Image Analysis of c-fos Staining

Hypothalamic sections containing the arcuate nucleus were evaluated for c-fos positive staining and photographed under fluorescent illumination using a Zeiss LSM 510 Meta confocal microscope equipped with a 20x (NA 0.75) APO objective with Zen 2008 software. The number of c-fos positive neurons in two sections, separated by 100 µm, from each guinea pig (n=3-4 guinea pigs/group) within 250 µm of cannula placement was determined by using Adobe Photophop and Image J (W.S. Rasband, Image J, U.S. National Institutes of Health, Bethesda, MD; http://rsb.info.nih.gov/ij/, 1997–2008). The percentage of POMC neurons that coexpressed c-fos was determined in four sequential sections separated by 40 µm within 250 µm of injection site in POMC-EGFP male mice (4 mice/group). Three dimensional reconstruction of co-localization was obtained from serial images taken at 2 µm increments through each tissue section. Fluorescent images were illustrated in Adobe Photoshop and Macromedia FreeHand

(Macromedia, San Francisco, CA).

Serum Measurements of Glucose, Cholesterol and Triglyceride

Serum concentrations of glucose, cholesterol and triglyceride in guinea pigs were determined by the Endocrine Technology and Support (ETS) Core at the Oregon National Primate Research Center (http://www.ohsu.edu/xd/research/centers-institutes/onprc/research-services/research-support/endocrine-technology.cfm), using ELISA kits from Cayman Chemicals (Ann Arbor, MI; Cat.# 10009582, Lot # 0445166 for glucose; Cat # 10007640, Lot # 0444819 for cholesterol; Cat # 10010303, Lot # 0445707 for triglyceride) after validation with guinea pig serum for parallelism with the standard curves. The lower limit of detection and range for the glucose, cholesterol and triglyceride assays were 2.5 – 25 mg/dl, 2.0 – 20 µM, and 3.1 – 200 mg/dl, respectively. All samples were analyzed in one assay kit, with high or low values re-analyzed with the same kit at dilutions of 1:5 or 1:25 for glucose, 1:100, 1:200 or 1:400 for cholesterol, and 1:1 or 1:2 for triglyceride. The intraassay variation, calculated by inclusion of internal control serum pool (n=4) was 0.9%, 7.1% and 2.1% for glucose, cholesterol and triglyceride assays, respectively; the interassay variation for the initial and re-analyzed samples was 10.3% (n=2), 17.8% (n=3) and 6.1% (n=3), respectively.

Preparation of Kiss1-Cre-GFP Hypothalamic Slices

Coronal hypothalamic slices through the arcuate nucleus from male and female Kiss1-Cre-GFP mice were prepared as previously described (Gottsch et al., 2011).

Supplemental References

Luparello,T.J., Stein, M., and Park, C.D. (1964). A stereotaxic atlas of the hypothalamus of the guinea pig. J. Comp. Neurol. *122*, 201-217.

Tindal,J.S. (1965). The forebrain of the guinea pig in stereotaxic coordinates. J. Comp. Neurol. *124*, 259-266.

Gottsch, M.L., Popa, S.M., Lawhorn, J.K., Qiu, J., Tonsfeldt,K.J., Bosch, M.A., Kelly, M.J., Rønnekleiv, O.K., Sanz, E., McKnight, G.S., Clifton, D.K., Palmiter, R.D., and Steiner, R.A. (2011). Molecular properties of Kiss1 neurons in the arcuate nucleus of the mouse. Endocrinology *152*, 4298-309.