ROUTINE ANIMAL USE PROCEDURES

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Beschreibung von standardisierten Abläufen
im Rahmen von Tierversuchen

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Part I. Descriptions of routine procedures used in our laboratories

1. Use of mammalian species in research on food intake, body weight regulation, and related health disorders.

Non-animal models or models involving infra-mammalian animals are not suitable for modeling the organization of eating by the varied, synergistic physiological controls under investigation in our laboratories. These physiological controls include, for example, orosensory, gastrointestinal, endocrine and metabolic signals that affect food intake. Such signals are transmitted by multiple hormonal and peripheral neural mechanisms and are processed in widespread areas of the brain, from the brainstem to forebrain. The organization and mechanisms of these brain processes are also under investigation in our laboratories. The degree of understanding of these mechanisms is not nearly sufficient to build computer models that can be used to provide useful new information. Rather, physiological analyses of living mammalian species must be performed. We are aware of the legal and ethical restrictions on the use of animals and make every effort to minimize the number of animals we use through efficient experimental design and to maximize the animals' wellbeing during experiments through continuous refinement of our procedural expertise and well informed, humane care.

2. Routine care of experimental animals

Care of animals in adaptation period before experiments or between experiments
According to the Art. 2 TVV, Art. 121 TSchV, the general wellbeing of the animals and the condition of the cages (food / water) is checked daily, and closer assessments whenever the animals are transferred to clean cages. Routine checks include observations of the animals' alertness and activity, the availability of sufficient food and water, as well as clean bedding. This is accomplished by visual observation of all animals (removing of animals from cages is not required) and is recorded in experimental protocols (see attachment). Any kind of impairment is indicated on the cage labels. As confirmed by a special evaluation (approved by the Veterinary Office on 23/05/2011, TVHa-134 for the Institute of Veterinary Physiology, University of Zurich, and currently under evaluation for the Physiology and Behavior Laboratory, ETH Zurich) there is no negative consequence for the well-being of the animals if routine checks are restricted to working days. Therefore routine checks can be omitted on the weekend as long as this approval is valid and no other circumstances necessitate weekend checks (e.g. immediate postsurgical period).

Care of animals being involved in feeding experiments
As part of the experimental procedure, all animals will be weighed individually on the day of experiment. This also allows for the assessment of the general wellbeing of the animal (e.g., alertness, responsiveness, no signs of stress [porphyrin secretion]). After injection of the test substances (see 3.), the animals will be monitored for some immediate signs of discomfort and then put back in their cages. In case food intake is assessed manually, the animals can be briefly checked at the time when feeding cups are weighed. In case food intake is assessed automatically, the animals will be checked immediately after the pre-defined experimental period, but no later than after 24h even if the experiment lasts longer because of the duration of action of the substance to be tested.

Automatic 24h measurements of food intake are usually conducted in non-standard cages that fulfill the required housing conditions for rodents (see table x for cage dimensions).

Special care for animals at higher risk

The well-being of animals that are at higher risk is more carefully and more frequently assessed. This is the case, for example, for animals that are recovering from surgery, have recently received drug injections or other manipulations that might seriously impact their wellbeing, or are maintained on regular schedules of food deprivation. These animals are checked and handled daily to determine responsiveness; possible discomfort or stress (as indicated by guarding particular body areas or other defensive or aggressive behavior, dehydration, porphyrin secretion, wound closure, etc.). They are also weighed daily and fecal output and food and water intakes are checked. Records of abnormalities and respective measures are kept in form of the attached score sheet (see attachment). Procedures (e.g., surgery, injections, etc.) that are part of the experiment are recorded separately, in data books, etc.

3. Single-housing and maintaining animals in wire mesh floor cages

Some measurements or test procedures cannot be conducted when animals are group-housed (e.g., measurements of food and water intake, taste preference tests, aversion tests, measurements in metabolic cages). In these cases, and also during adaptation periods or baseline measurements prior to such experiments, animals have to be single-housed. Records will be kept of start and end of single housing periods. In certain tests it is necessary to single-house rats and mice in cages with wire-mesh floors in order to measure food spillage and to prevent the contamination of the feeders with bedding, or in order to prevent the animals from eating bedding material or feces in experiments involving food deprivation or after certain kinds of surgery. Whenever practical, wire-mesh cages will be fitted with appropriately sized "sleeping tubes" or the equivalent where animals can rest. If rats are equipped with chronic brain canulae, the cages cannot be fitted with sleeping tubes because of the risk of head injury. For mice, empty food cups are sometimes used instead of sleeping tubes. If non-standard cages are used the space requirements for rodents (according to appendix of Art. 10 TschV) will be fulfilled (for cages dimensions see table 1; appendix., unless specified in a specific animal experimentation protocol.

We are aware of the animal-welfare concern regarding housing rats/mice on wire-mesh floors, but believe that our standard feeding procedures decrease our animals' welfare only minimally. There is clear scientific evidence that rats given the choice will spend the majority of their resting time on solid floor cages rather than wire mesh floor cages (e.g., Manser et al., Laboratory Animals 29:353, 1994). However, they do not spend a significantly higher percentage of their *non-resting* time on solid floor. In our case, rats can rest in the "sleeping tubes" or equivalents in their cages. Rats housed continuously on wire-mesh floor cages do not show behavioral abnormalities (including the amount of time spent resting), deficits in food intake or growth, or in a number of physiologial variables related to stress (such as plasma corticosterone and catecholamines; Manser et al., op. cit.; Stauffacher, Proc. 6th FELASA Symposium). The situation in mice is more complicated, with group housing or environmental enrichment sometimes leading to increased behavioral abnormalities, including increased aggression, and stress (Haemisch et al., Physiology and Behavior 56:1041, 1994; Würbel et al., Ethology 102:371, 1996).

All in all, therefore, we will minimize the time that animals are maintained in wire-mesh floor cages and believe that this is justifyable given the scientific importance of the work. Furthermore, before adaptation for the experiment begins, or whenever the experimental conditions (or experiment-free periods of more than three weeks) allow it, animals will have access to a common "play ground" or will be group-housed in enrichment cages. Rats from different groups of animals will not be mixed in these "play grounds". Because our group-housing capacity in enrichment cages is limited, some rats/mice will be housed in standard rat cages (Makrolon).

4. Routine procedures for feeding tests and immunohistological (IHC) studies

The majority of feeding tests and IHC studies follow a more or less fixed design in which the animal may or may not be food deprived (see below), a test substance is administered (drug, hormone, etc., as specified in the particular animal use applications), and then food intake and sometimes other behaviors (e.g., water intake) are measured at intervals over a specified period, either automatically or manually, by weighing the food cups, etc.
All these treatments are compliant with manipulations that can be conducted in rooms where other animals are kept, as defined in Art. 6 TVV. IHC studies are usually terminated by transcardial perfusion (see 17), which is only conducted in rooms where no animals are kept.

4.1 Food deprivation

Food deprivation is used if appropriate to the particular experiment. Deprivation periods will typically last for 6-24 h in rats and 6-12 h in mice (SG 1).

The experimental design may necessitate a pre-test food deprivation for different reasons. For example, short-term food deprivation will help to trigger immediate food intake in all animals at a predetermined time point. This is especially important when testing substances (e.g., peptide hormones) with very short biological half-lives. In these cases, food deprivation of 6 h (mice) to 12 h (rats) is usually sufficient. Short deprivations are also used to ensure that the animal is not in the immediate postprandial state during the test. Food deprivation is also necessary to investigate differences in central nervous system activity between fasted and ad libitum fed animals that might e.g. occur after the administration of hormones acting on the system regulating food intake. These differences may contain important information on the mechanism of action of these hormones. A third example is that food deprivation is necessary to investigate which brain areas are activated by fasting (e.g., 12 h in mice; 24 h in rats) and how this signal is reversed. This is an important method in investigations of the mechanisms of hunger and satiety.

Regular food deprivation schedules (i.e., ad libitum access to food for a certain period per day) are used in some experiments. In this case, the deprivation periods are at most 12 h/d. Animals maintain normal food intake and body weight on such schedules without difficulty. Careful daily attention will be paid to ensure that this is the case.

4.2 Pair-feeding and weight matching procedures

In some experiments, pair-feeding or weight matching procedures are necessary controls. This may e.g. be necessary to distinguish between effects of an anorectic treatment on

metabolic parameters versus the effect of lower body weight *per se* due to the anorexia induced by the treatment. Therefore, in addition to the experimental group receiving the treatment and a control group, a third group that is pair-fed (i.e., receives the same daily amount of food as the experimental group with the lower voluntary food intake) or is weight matched (i.e., receives sufficient food to maintain the same body weight as the experimental animals, but not enough to exceed their weight). Both procedures are standard in the field and are expected controls in many sorts of experiments.

4.3 Routine acute injections

Test substances are given centrally (i.e., into the brain) or peripherally at a specific time of day and subsequent food intake is recorded either by manually weighing the feeding cups or through an automated computer-based system. The animals are adapted to short-term restraint and injections by regular handling. Therefore, injections are nearly stress-free.

Intraperitoneal (IP) or subcutaneous (SC) injection
Peripheral injections can be either IP or SC, with an injection volume of usually 1 ml/kg (rats; i.e., 0.3 ml for a rat of 300g BW) to 10 ml/kg (mice; i.e., 0.3 ml for a mouse of 30g BW), the limits in any case will be 20 ml/kg for rats and 50 ml/kg for mice (GV-SOLAS, 2006). The technique of administration is state-of-the-art and conforms to the methods imparted by the Institute of Laboratory Animals of the University of Zurich. The animals can be used for multiple experiments (e.g., cross-over designs, dose-response studies, etc.). In our experience, at least 20 such experiments per animal can be done without any adverse consequences that are caused by the injection *per se*.

Administration by gavage (IG)
In some experiments, orally active substances will be administered directly into the stomach, again using state-of-the-art techniques. Before the proper experiment, in order to minimize stress during the experiment, the rats will be trained to accept the gavage probe used for IG administration (gavage probe with a 2.0 mm diameter bulb for rats > 200g or flexible plastic gavage probe for mice). Substances are administered in volumes of up to 10 ml/kg rat (e.g., 3 ml for a 300g rat), or a maximum of 20 ml/kg per day.
Subsequent to 12-24 h food deprivation, rats of this body weight will spontaneously ingest more than 5 g of solid food together with 5-10 ml water within the first 30 min when food is returned, or more than 10-25 ml of liquid nutrients. Therefore, the IG infusion of the indicated volumes is not a physiological stress if the animal is adapted to the handling and tube insertion. Some experiments may need repeated IG administration under stress-free conditions, i.e. without any manipulation during the experiment. In these cases, the rats will be provided with a surgically implanted chronic IG cannula (see Section 12).

Intravenous (IV) infusion
Finally, in some experiments substances will be injected IV. This might involve the use of chronic, surgically implanted IV catheters (see below). The injection of 5ml/kg body weight (rats and mice) should not be exceeded for single bolus infusions.

Acute administration into the brain via cannula or freehand

Central (intracranial) application of substances is also usually done via a chronically implanted cannula (see Sections 9, 10 and 19), targeting either into the brain ventricles or into the parenchyma, i.e. specific brain nuclei. The infusion volume is up to 0.5 µl/rat for intraparenchymal administration, and up to 3 µl/rat for intracerebroventricular (ICV) administration. The implanted cannulae are fitted with plastic threads which can be used to screw on the infusion tubing. The tubing is connected to precision microliter syringes and the infusion can either be done manually or via automated precision pumps. Infusion is slow, occurring over few minutes up to several hours, depending on the specific experiment. The animals are trained for these procedures, especially connecting the tubing to the cannula. The animals are only minimally disturbed and can move freely in their cages during infusion. Animals can be used for at least 10 such experiments, with one or more recovery days between tests. Thereafter, granulation tissue sometimes develops at the site of injection so that targeted application can no longer be guaranteed.

Alternatively, without implanted cannula injections may be performed in anesthetized animals in order to administer substances ICV or into the cisterna magna (see below). Sometimes it is necessary to avoid chronic inflammatory processes due to cannula implantation, thus this kind of injections is the alternative of choice.

Chronic or semi-chronic brain infusions (e.g. using osmotic mini-pumps) are described in detail in Section 10 or will be incorporated in particular Applications for Animal Use, as appropriate.

5. Criteria for premature discontinuation of the experiments (criteria for temporary or permanent withdrawal of the animal from the experimental protocol)

General criteria for withdrawal

General criteria (including post-surgical criteria) for withdrawing an animal from experiments are defined in the attached score sheet for animals under special care (see appendix). Affected animals will be taken out of running experiments and given appropriate supportive care. If animals do not improve despite appropriate treatment during the defined time periods the animal will be completely withdrawn from further studies and euthanized (e.g., by an overdose of pentobarbituric acid or using CO_2) if necessary. Records of euthanasia are kept in electronic or hardcopy records. Exceptions or extensions of these criteria have to be defined specifically in the animal experimentation permission if required by the experimental situation.

Special criteria for variations in body weight

Introductory remarks: Considering the inter-individual variation of body weight (which according to growth curves in rats and mice often varies by more than 20% between individuals), it is important to take each individual's body weight as baseline. It also has to be noted that body weight can vary considerably depending on the filling state of the gastrointestinal tract (e.g. body weight can decrease by >20 g due to 12 h food deprivation in rats). Further, it is also important to note under usual laboratory conditions, adult rats and mice are often "normally" considerably overweight, so that such weight loss results merely in a lean animal, not a malnourished one. In this context, it is relevant to mention that ad libitum feeding of laboratory rats often results in an unhealthy degree of obesity that leads to several signs of poor health and shortened life span. As a result, moderate dietary restriction (i.e., a regimen of access to 75% of the amount eaten ad libitum) is considered state of the art in many nutritional and toxicological applications (K Keenan, G Ballam, D Haught, and P Laroque "Nutrition" in G Krinke, editor, The Laboratory Rat; San Diego, CA, Academic Press, 2000). Clearly, such routine restriction cannot be done in experiments in which voluntary

food intake and body weight regulation are the variables under study. But this consideration should be kept in mind in tests where surgical or other manipulations prevent “normal” levels of food intake and body weight.

Variations in body weight may occur as an intended consequence of treatments (experimental parameter) or as an unexpected and undesired event that might reflect an experimental complication associated with an impaired health status. Progressive changes in body weight of the latter category are very rare, at least for all procedures defined in the RAUP. If present such variations typically occur suddenly in single animals due to an experimental problem. If the underlying problem cannot be identified or persists despite intervention those animals are typically excluded from the experiment before the exclusion criterion for body weight defined in the score sheet is fulfilled. If not excluded at an earlier stage animals will be euthanized when body weight changes by more than 20% of the individual body weight at baseline conditions (e.g. before surgical manipulations or other experimental treatments).

For all experiments in which body weight changes are expected or intended based on the experimental procedures or aims, special justifications need to be defined if the body weight change is indicative of any impairment of the animal’s general wellbeing. It is hardly possible to define a single value of body weight change that indicates impaired wellbeing across all experimental situations. If individual body weight is considered, experimentally induced changes can be masked by naturally occurring body growth, which per se varies in different strains and at different ages. The limited usefulness of defining percentage values as experimental endpoints for body weight changes is illustrated by the following example. If growing animals are food-restricted in a way that largely compensates their natural body weight gain under ad libitum feeding conditions, severe malnourishment might occur in the absence of net body weight loss relative the baseline body at the beginning of the experiment. There are numerous other experimental situations, in which percentage values of body weight change alone do not allow a meaningful conclusion about the nutritional status of an experimental animal. Taking the introductory remarks into consideration, changes in body weight within the range of 20-30% relative to the average of control animals usually do not result in any impairment of a healthy animal’s wellbeing, at least under laboratory conditions. However, for the reasons mention above, averaged group means are of limited value for the assessment individual experimental endpoints. Moreover, depending on the design of the study, normal weight control groups are not necessarily part of all experiments. For these reasons the 20-30% range of body weight change might only be used in some cases as a rough guideline but certainly not as a single criterion of general validity.
In some experiments disease-related anorexia/cachexia is studied (e.g. in cancer or infection models). Such experiments are not considered as routine procedures and might lead to stronger decreases in food intake and body weight than the changes described above. The criteria for the discontinuation and the endpoints of those experiments are defined for each specific case in the particular Applications for Animal Use.

6. Anesthesia of rats and mice, pre- and post-operative care

6.1 Pre-operative care and preparation for surgery

Animals will be housed for at least 1 week before surgery to adapt to the laboratory conditions (housing environment, temperature, diet, light/dark schedule, animal handlers). A few hours before surgery, the animals receive broad-spectrum antibiotics for prophylaxis (e.g., trimethoprim-sulfonamide [Borgal 100 µl/rat IP or SC], Baytril [10mg/kg], Cobactan [2mg/kg] or chloramphenicol 20 mg/kg).[1] Animals are pre-treated with atropin (50 µg/kg SC, 15-30 min before anesthesia) [2] and Rimadyl^{R} (carprofen, 5 mg/kg SC) or Finadyne^{R} (flunixin, 3-5 mg/kg SC) 1-2 hours before anesthesia. The operation field will be clipped and disinfected (e.g., Betadine soap followed by Betadine solution). All animals will receive eye ointment to protect their cornea from drying out. Animals will be kept on heating pads to avoid hypothermia.

6.2 Anesthesia protocols

Anesthesia with ketamine/xylazine

Rats: Ketamine (60-100 mg/kg), xylazine (4-10 mg/kg) and acepromazine (1mg/kg) are injected IP in a single syringe (approx. 0.1ml / 100 g). If prolonged anesthesia is necessary (e.g., if operation last >30 min), animals are re-injected after 30 min with about 0.05-0.1 ml ketamine alone.

Mice: Mice receive a mixture of ketamine (about 65 mg/kg [Narketan; dilution 1:5]), xylazine (about 12 mg/kg [Rompun; dilution 1:5]) and acepromazine (1 mg/kg [Prequilan; dilution 1:33]). The three stock solutions are mixed (100µl + 100µl + 100µl) and injected in a single syringe (injection volume for 30g mouse: approx. 300 µl IP depending on the mouse strain).

Anesthesia with pentobarbituric acid

Very deep anesthesia for terminal experiments (e.g., for immunohistochemical studies) can be induced with pentobarbital (50-100 mg/kg IP).

Inhalation anesthesia with isoflurane

Anesthesia will be induced in an induction chamber with 4-5% isoflurane. When surgical tolerance is reached, anesthesia is maintained with 1-3% isoflurane. Analgesia may be supported using buprenorphin (Temgesic; 50-100 µg/kg SC), or alternatively with carprofen (Rimadyl 5 mg/kg SC) when respiratory depression is a factor to avoid (i.e. stereotaxic fixation in mice)

6.3 Monitoring during anesthesia

The depth of anesthesia will be monitored frequently throughout the procedure. This includes test of reflexes (e.g., corneal reflex), reaction to loud noise (during ketamine anesthesia), and regular observation of depth and frequency of respiration and of skin and mucous membrane coloration.

6.4 Post-operative care

[1] For some operations, e.g. ovariectomy or implantation of osmotic minipumps, general treatment of all animals with antibiotics is not considered necessary. In these cases, neither pre- nor post-operative treatment with antibiotics will be done.

[2] Pre-treatment is not used for short lasting operations, e.g., implantation of minipumps.

During immediate recovery from anesthesia, animals will be closely monitored and kept at thermoneutrality by heating pads or an infrared lamp until full recovery. A thermometer will be placed in the recovery area to avoid overheating of the animals. Animals will receive SC or IP fluids (volume: 5%-10% of bodyweight weight, 50% of this volume immediately, remaining volume after 2-3h, fluid volume administered ip at one time should not exceed 3% of body weight).

Post-operatively, the animals will receive antibiotics (e.g., trimethoprim-sulfonamide [Borgal 100 µl/rat IP or SC], Baytril [60mg/kg], Cobactan [2mg/kg] or chloramphenicol 20 mg/kg) and analgesics for 3-4 days (see table below).

	Mouse	Rat	Class
Buprenorphine	0.05 - 0.1 mg/kg SC q 8h	0.05 - 0.1 mg/kg SC q 8h	Opioid
Carprofen	5 mg/kg SC q 24h	5 mg/kg SC q 24h	NSAID
Flunixin	2.5 mg/kg SC q 12h	2.5 mg/kg SC q 12h	NSAID

Animals will be closely monitored by an experienced experimenter and a health score sheet with defined items will used as support and to document this stage (see attached health score sheet). Normally animals will be allowed to recover for at least 1 week. Experiments will then begin only when food intake and body weight have stabilized. Animals undergoing general anesthesia and surgery usually display a transient period of adipsia, anorexia and weight loss after surgery. This usually lasts 2 days but, especially in the case of certain surgeries such as total abdominal vagotomy, can persist for a week or more. If reduced food intake persists beyond 2 days, palatable foods are offered (for example, Ensure (1.5 kcal/ml), ground chow mash made of chow and water, sweetened condensed milk). Hydration will be maintained by administration of saline SC.

Part II. Description of surgical techniques used routinely in our laboratories

Note that each description is followed by consideration of possible side effects and measures for special care during recovery. All severity grades that are defined in the following section represent the expected and non-cumulative severity grades for each single manipulation.

7. Lesion of superficial brain structures, e.g. the area postrema (SG 2)

Area postrema (AP) lesion
Rats will be anesthetized. A dorsal midline incision 3-cm long from just rostral to the occipital crest to approximately the midcervical level will be made and using blunt dissection, the surrounding soft-tissue including muscle layers will gently be retracted. The rat will be transferred into a stereotaxic apparatus and the head placed in 90° ventroflexion. This will allow access to the foramen magnum and the atlanto-occipital joint will be exposed. The dura will be incised and the foramen magnum may have to be enlarged slightly using a bone rongeur in some cases. The AP can then be visualized below the cerebellum on the dorsal surface of the medulla at the caudal extent of the fourth ventricle. The AP can be lesioned by two different methods; both have been proven to be successful in previous experiments. First, lesion can be produced by thermal coagulation of the AP using a small triangular shaped copper wire (0.75 mm) attached to a small soldering iron. The hot copper wire probe will be applied to the AP region for at most 1s. Second, lesion can be produced by using a blunt 21-gauge needle attached to a vacuum line. The AP can easily be sucked off the underlying tissue under visual control. Sham operations are done in the same way except for the actual lesioning procedure. Bleeding only occurs very rarely and can easily be controlled using sterile cotton swabs. Bleeding in the muscle layers or skin will be stopped using a thermocautery. The muscles will be repositioned and affixed then in 2 separate layers with intermittent sutures using 3-0 vicryl. The skin incision will be fastened with 5-0 vicryl and the animal removed from the stereotaxic frame.
AP lesioned animals usually recover from surgery within about 7-10 days. Lesioned animals display reduced body weight gain compared to sham controls.

Verification of lesions: Functional test: 3 % (0.5M NaCl) hypertonic saline test. Rats provided with tap water and 3% saline in premeasured bottles. Spontaneous intake of hypertonic saline will be measured after 24 h. AP lesioned rats consume several times more the quantity of hypertonic saline as do sham rats.
After termination of all experiments, animals will be euthanized and subjected to histological analysis of the AP region. Normally, the AP is successfully lesioned in 60-80% of animals.

8. Specific lesion of other brain areas (SG 2)

Anesthetized rats will be fixed in a stereotaxic apparatus. The skin will be incised to expose the skull, the periosteum will be removed by blunt dissection. The surgery will be exemplified with a lesion produced in the lateral parabrachial nucleus (LPBN). Other areas can be lesioned accordingly with the only difference of using other brain coordinates.

8.1 Electrolytic lesion

The electrolytic lesion will be produced with electrodes which will be placed using specific coordinates according to the brain atlas by Paxinos & Watson (1998). The electrodes will be placed after drilling small holes in the skull just above the appropriate target area. In the case of the LPBN, the holes will be drilled bilaterally. Isolated insect pins (size 000) will be used as electrodes with only their tip being exposed. The electrodes will be lowered through the holes to the coordinates determined before by the help of the atlas. The LPBN will then be destroyed by passing anodal current (1 mA for 15 s). The neutral electrode will be placed in the rectum. After lesioning, the skin will be sutured by absorbable suture material (e.g. Vicryl 3-0). The procedure in the sham-operated control animals will be identical except that no current will applied to the electrodes.

8.2 Excitotoxic lesion

Another lesioning technique involves the local bilateral administration of excitotoxins, such as ibotenic acid into the LPBN. This neurotoxin selectively destroys neuronal cell bodies while sparing nerve fibers passing through the target area. Ibotenic acid (0.2 µl, 10 µg/µl NaCl) or control solution (NaCl) will be infused using the same coordinates as described above (Paxinos & Watson, 1998). Rather than using electrodes, acute injection cannulas (31G) will be lowered into the target area and ibotenic acid will be slowly infused over about 15 min with a precision micro-pump. After infusion, the injector will be left in place for about 10min to allow diffusion of ibotenic acid into the target area.

LPBN-lesioned rats usually recover quickly from surgery, and body weight stabilizes within 14 d after surgery. No obvious side effects of the surgery have been observed on previous occasions. Post-surgical care will therefore pay attention to routinely assessed parameters (see above: development of body weight, food and water intake, reactions at the operation wound, etc.).

8.3 Targeted toxin lesion

This lesioning technique involves the central administration of saporin (SAP), a ribosome-inactivating protein, bound to an antibody against a specific antigen; for instance to target noradrenergic neurons, we use anti-dopamine-beta-hydroxylase (DBH) coupled with saporin; anti-DBH-SAP, or 192-saporin for targeting cholinergic neurons. Alternatively, streptavidin conjugated to saporin (streptavin-SAP) can be coupled to biotinylated specific antibodies, to create customized targeted toxins (e.g. streptavidin-SAP-anti-GLP-1 receptors). Any of these immunotoxins will be applied into specific brain loci (e.g., the area postrema) or ventricles (e.g., 4th ventricle). Any of these toxins will selectively target the cells carrying the cell membrane antigen, while sparing the other neuronal populations. Micro-infusion will be performed under regular stereotaxic surgery set up; for anti-DBH-SAP (e.g. 50 ng in 800 nl, for AP) or control equi-volume solution (IgG-SAP) will be micro-infused using the appropriate stereotaxic coordinates. A beveled glass pipette (inner diameter 40 µm) will be lowered into the target area and anti-DBH-SAP will be micro-infused with a pneumatic micro-injector at a pressure of 40 psi using 5 or 10 msec pulses at 3 min intervals. After infusion, the pipette will be left in place for about 10 min to allow diffusion into the target area.
Lesioned rats usually recover quickly from surgery, and body weight stabilizes within 1 week. No obvious side effects of the surgery have been observed on previous occasions. Post-surgical care will therefore pay attention to routinely assessed

parameters (see above: development of body weight, food and water intake, reactions at the operation wound, etc.).

Verification of lesions: After termination of all experiments, the rats will be euthanized and subjected to histological analysis of the targeted region. For instance for DBH immunoreactivity in the area postrema. Normally, about 75% of animals are successfully lesioned and can be included in the statistical analysis of results.

9. Cannulation of brain ventricles or specific CNS nuclei (SG 2)

9.1 Cannulation of brain ventricles or specific CNS nuclei in rats

For chronic and constant central infusion of drugs a central port is required to provide access to the target area. Anesthetized rats will be fixed in a stereotaxic apparatus. Thermoregulation will be provided through a thermostat regulated heating pad and monitored through a rectal thermometer. Head will be shaved of fur, cleaned with iodine and ophthalmic ointment will be applied to the eyes before incision. After skin incision (1.5 to 2 cm long) and removal of all soft tissue from the surface of the skull, the periosteum will be removed by blunt dissection. Hemostasis will be achieved at all bleeding points and a sterile gauze pad will be used to make the skull completely clean and dry. The coordinates for brain ventricles or specific nuclei will be taken from the brain atlas by Paxinos and Watson, 2006. A dental drill will be used to penetrate the skull above the target area. Care will be taken so that the drill bit does not penetrate through meninges or main blood vessels. Additional holes will be drilled nearby to place stainless steel screws allowing a better fixation of the guide cannula. The guide cannula (stainless steel with a plastic pedestal) will be implanted 1 to 2 mm above the proper target area. The guide cannula will be kept in place by fixing it first with cyanoacrylate glue and once cured also further fixed using dental acrylic cement built up around the cannula and the screws. Suturing the wound is usually not necessary because the dental acrylic will fill up the whole operation area. A round plastic ring can be used to reduce sharp edges of the dental acrylic and to improve wound process. When necessary, one or two skin sutures (e.g., Vicryl 5-0) will be used. An obturator or dummy cannula will keep the guide cannula patent, protruding at least 1 mm on the ventral side and the dorsal side screwed to the guide cannula thread.
Post-surgical care will involve special attention to the operation wound. On rare occasions, bleeding occurs around the dental acrylic. If present, animals will be treated appropriately as follows: 1st remove wound debris, 2nd wound disinfection, 3rd antibiotics. If excessive granulation causing distress to the animals at the operation wound occurs, the animal will be taken out of the experiments.

Verification of guide cannula position: Once the animals have recovered well after surgery, a functional test will be performed to confirm the placement of the guide cannula tip. For example in case of 4th ventricle cannulation, 5 thioglucose (5 TG) will be infused in the brain through the guide cannula and the blood glucose will be measured at 0 and 60 min by tail nick. At least doubling of the blood glucose levels as compared to basal levels will confirm placement of the guide cannula in the fourth intracerebral ventricle.

9.2 Mice central cannulation

Anesthetized mice will be fixed in an ultraprecise stereotaxic apparatus (e.g. digital version with 10 microns resolution). The skin will be incised to expose the skull, and the periosteum will be removed by blunt dissection. The coordinates for brain ventricles or

specific nuclei will be taken from the brain atlas by Paxinos and Watson (2006). Cyanoacrylate glue will be used to strengthen and thicken the skull plates. Under surgical microscope a drill will be used to penetrate the skull above the target area. The hole will be slightly enlarged using the drill bit or a trephine. An additional hole (for an anchoring stainless steel screw) will be drilled nearby. Damage to the meninges will be kept to a minimum. The guide cannula (stainless steel with a plastic thread) will be placed approximately 1.5 – 2.0 mm above the proper target area. The guide cannula will be kept in place by fixing it first with additional cyanoacrylate glue and once cured also using dental acrylic cement. Suturing the wound is usually not necessary because the dental acrylic will fill up the whole operation area. A round plastic ring can be used to reduce sharp edges of the dental acrylic and to improve wound healing. When necessary, one or two skin sutures (e.g., Vicryl 3-0) will be used. An obturator or dummy cannula will keep the guide cannula patent, protruding at least 1 mm in one side and in the other screwed to the guide cannula thread.
Post-surgical care will involve special attention to the operation wound. On rare occasions, bleeding occurs around the dental acrylic. If present, animals will be treated appropriately. If excessive granulation occurs at the operation wound causing distress to the animal, that subject will be taken out of the experiments.

Verification of guide cannula position: Once the animals have recovered well after surgery, a functional test will be performed to confirm the placement of the guide cannula tip. For example the strong orexigenic effect of neuropeptide Y (1 μg / mice/ 1 μl) should trigger consumption of at least 0.5 g food within 2 h post i3vt infusion in the middle of the light phase. Alternatively, an i3vt infusion (1 μg / mice/ 1 μl) of the melanocortin agonist, melanotan (MT)-II should induce a strong anorectic response lasting for at least 12 h.

10. Device Implantation (SG 2)

10.1 Implantation of osmotic mini-pumps for chronic continuous infusions.

Osmotic mini-pumps are used for administration of substrates over extended periods. Depending on the type of mini-pump, an exactly defined dose of substrate (0.5-1μl/h) can be administered over 1-3 weeks. The pumps can be implanted IP or SC or can be carried externally in a kind of "backpack". In this case the osmotic mini-pump is fitted in a micro-centrifuge tube filled with saline (for details see: Ruiz de Elvira, Lab Animals 20:329-334, 1986). The mini-pump is connected to an implanted catheter or cannula. This exteriorized alternative allows for semi-chronic infusions without disturbing the animals, or also for an exchange of the pump without additional surgery. The following section describes the IP implantation: Primed (12 h at 37°C in sterile saline) mini-pumps are implanted during short-term anesthesia with isoflurane (see above). The size of the pump (length 1-3 cm; diameter approx. 0.5 cm for adult rats) fits the body size of the animal. After laparotomy (incision length approx. 1 cm), the sterile pumps are placed in the abdominal cavity. Pumps are not anchored. The abdominal wall will then be sutured in two layers using absorbable suture material (e.g., Vicryl 3-0).

This minor surgery usually has little or no effect on food intake. For that reason, and because the mini-pumps are activated immediately when in contact with body fluids, experiments involving implantation of osmotic mini-pumps normally start directly after surgery. For the same reason, and because the implant and surgical procedure are sterile, antibiotics are usually not administered for this type of surgery.

10.2 Telemetry sensors

The sensors can be implanted IP or SC. For instance, IP implanted sensors may monitor core body temperature and activity. The implantation procedure lasts about 5 min and includes a 2 cm midline laparotomy, placing the sensor in the abdominal cavity and suturing the muscle and the skin. Alternatively, bio-potentials can be recorded to retrieve data as electromyogram or electroencephalogram when electrodes are implanted accordingly.

11. Intraperitoneal and gastrointestinal tract surgery (SG2)

11.1 Gastric sham feeding cannula

This device and its use were introduced in 1974 by Prof. G. P. Smith, Cornell University Medical College, New York, and is used now all around the world; Prof. Smith has described the method in detail (Current Protocols in Neuroscience (on-line), 8.6D.1, 1998).

The cannula, custom made of stainless steel, has a base that is 1.5 mm in diameter, 0.5 mm thick, and 0.5 mm wide, with a 1 mm deep notch along the outer circumference, and a shaft 13 mm long with an ID of 6 mm and an OD of 7 mm that is threaded on both sides. After laparotomy and retraction of the stomach, the flanged end of the tube is inserted through a small stab wound (1 mm long, made with a #11 scalpel) into the limiting ridge of the stomach. The cannula is rotated and the stomach stretched until the flange enters the lumen. A purse string suture of 4-0 Vicryl is placed in the stomach around the shaft of the cannula. An annular piece of Marlex surgical mesh (2 cm OD) is placed over the purse string. This promotes tissue growth between the stomach, abdominal muscle, and skin, which seals the wound. The cannula is exteriorized through a stab wound 2 cm lateral to left of the midline. A threaded washer is screwed down to the skin to maintain the position of the cannula for the first 2 d of healing. A stainless steel screw is placed into the cannula except during tests. Rats tolerate the procedure well and usually eat and drink normally within 2 d of surgery. If the skin around the cannula becomes irritated, it can be treated with Zinc ointment or Betadyne. Between uses, the cannula is closed with a stainless steel screw top and the rat eats and drinks normally. For sham feeding tests, the cannula is opened, the stomach is rinsed with lavages of 5 ml warm saline, and the cannula is attached to a drainage tube that hangs into a collection pan beneath the cage. When the rat ingests liquid food with an open cannula, the food drains into the pan without accumulating in the stomach or entering the intestines in appreciable quantities (“sham feeding”).

11.2 Intragastric (IG) infusion cannula

Chronic IG catheters consist of silicon tubing and a bent V-shaped stainless-steel tubing (20G cannula, protruding end). The proximal end of the silicon tubing (length, 17 cm; ID 0.76 mm; OD, 1.6 mm), is attached to the steel tubing and reinforced with another piece of silicon catheter (length, 1cm; ID, 1.47 mm; OD 1.96 mm. Three drops of silicon glue are applied to the tubing: at 7 mm , 8 mm and 4 cm from the distal end. The reinforced proximal end of the catheter is led through a piece of polypropylene surgical mesh (1.5 x 3 cm, Bard Implants) to improve adhesion to skin and fascia and fixed to it with non-absorbable monofil suture. A 2 cm midline dorsal cutaneous incision is made caudal to the interscapular area, and the protruding V-shaped steel tubing is led subcutaneously to a

puncture wound rostral to the incision between the scapulae, where it is exteriorized and attached to a PE tubing. The distal end of the cannula is then led subcutaneously to a 3 cm midline laparotomy. The stomach is partially retracted through the laparotomy and held in place with cotton swabs dampened with saline.
A 4 mm diameter purse-string suture with non-absorbable 4-0 silk suture is loosely placed on the greater curvature of the gastric corpus (area of thin vasculature, posterior portion of the stomach rumen). The center of the purse-string is punctured with an 18 gauge needle, and the cannula is pushed in until the first drop of silicone glue is inside the stomach. The purse string suture is then tightened and tied, and the stomach wall is anchored between the two drops of silicon glue. An additional silk thread is placed immediately proximal to the second silicon band and connected with the purse-string suture. A 4-0 silk suture is placed proximal to the third silicon glue drop and loosely fixed to the peritoneum on the animal's right side. This avoids any tensile load on the cannula that might cause it to pull out of the stomach. Abdominal muscle layers and skin are closed using absorbable suture (3-0 vicryl and 5-0 vicryl respectively).
After a few weeks the position of the catheter tip can be verified by CT scans. Under isoflurane anesthesia 0.6 mL of diluted contrast agent is infused via gastric catheter and scans are taken immediately afterwards.

11.3 Duodenal infusion (ID) cannula

The duodenum is catheterized via the stomach without puncturing the duodenal wall. The preparation can be adapted to either the gastric infusion cannula (described above) for normal feeding tests or to the gastric sham feeding cannula for sham feeding tests. The latter preparation is described here: The catheter is a 30 cm piece of silastic tubing (ID 0.75 mm; OD 1.66 mm). Gastric sham feeding cannulas are modified by placing two 1.67 mm holes in the inner flange. A 7 cm length of the duodenal catheter is threaded through these holes and anchored by a drop of silastic glue. The intragastric end is then led into the duodenum by external manipulation so that its tip is 3-4 cm distal to the pylorus and fixed in place with a 5-0 suture through the duodenal wall. Normal gastrointestinal motility keeps the distal end of the catheter in the duodenum even if the suture fails. The other end of the tubing is externalized through the gastric cannula during tests. Between tests the outer end is friction fit onto a small nub on the inside of the screw top of the catheter. Dilute green vegetable dye is added to intestinal infusates to confirm patency. Rats tolerate both the surgery and chronic testing very well and usually eat and drink normally within 3 d or surgery.

11.4 Intraperitoneal (IP) infusion cannula

Chronic IP catheters consist of silicon tubing and a bent V-shaped stainless-steel tubing (20G cannula, protruding end). The proximal end of the silicon tubing (length, 20 cm; ID 0.51 mm; OD,0.94 mm), is attached to the steel tubing and reinforced with another piece of silicon catheter (length, 1cm; ID,0.76mm; OD 1.65 mm) The reinforced end of the catheter is led through a piece of polypropylene surgical mesh (1.5 x 3 cm, Bard Implants) mesh (to improve adhesion to skin and fascia) and fixed to it with non-absorbable monofil suture. Twelve small holes are punched with a 26 G cannula within 1.0 cm of the distal end that remains inside the abdominal cavity. The catheter headpiece is implanted as described above for gastric infusion catheters. The distal end of the cannula is then led subcutaneously to a 3 cm midline skin incision. A 16 G steel cannula is inserted through the abdominal musculature and the tip is carefully passed across the abdominal cavity and forced out through the muscle wall 2cm from the midline on the

opposite side. The tip of the catheter is threaded into the cannula and the tubing guided into the abdominal cavity. A small drop of tissue adhesive is applied to the insertion area of the silicon tubing into the abdomen. The skin in the abdomen and in the neck is closed using absorbable suture (5-0 vicryl).

11.5 Intrajejunal (IJ) infusion cannula

Chronic (IJ) catheters are assembled (except that no holes at the end of the tubing are made) and the headsets implanted as described above for IP catheters. The distal end of the cannula is then led subcutaneously to a 3 cm midline laparotomy and led through a puncture hole in the abdominal wall. 1.5cm from the distal end a small piece of soft mesh (5 x 7mm) is attached to the tubing with non-absorbable suture. The jejunum is gently picked up and a small loop is exteriorized on, and covered with, gauze and kept moist with NaCl.
The anti-mesenteric side of the jejunum is punctured with a 22 G cannula and the catheter is inserted. The mesh is then fixed to the jejunal serosa with 4 stitches of non-absorbable suture. The entry point of the catheter is sealed with tissue adhesive. Abdominal muscle layer and skin is closed using absorbable suture (3-0 vicryl and 5-0 vicryl, respectively).
After a few weeks the position of the catheter tip can be verified by CT scans. Under isoflurane anesthesia 0.6 mL of diluted contrast agent is infused via the intrajejunal catheter, and scans are taken immediately afterwards.

12. Implantation of chronic vascular catheters (SG2)

12.1.A Hepatic portal vein (HPV) catheter in rats

Chronic HPV catheters are assembled and the headsets implanted as described above for intrajejunal infusion canulae. The distal end of the catheter is led subcutaneously from the back to a 3 cm midline laparatomy. At present we use two different cannulation sites in our laboratory: A) at the distal end of the mesenteric vein (near the ileocolic vein, for infusion experiments) and B) near the hepatic portal vein (just distal to the splenic vein) for blood sampling experiments.
A) The small intestines are gently retracted to expose the ileocolic vein, covered with gauze and kept moist with sterile saline. A loose ligature is placed on the mesenteric vein and the distal veins (collecting vein, ileocolic vein) are ligated (5-0 silk). The mesenteric vein is pierced with a 21 G syringe needle and the catheter is inserted and advanced until its tip reaches the level of the gastroduodenal vein, about 1 cm from the liver. The catheter is fixed with silk thread and a drop of tissue adhesive is placed over the entry point of the catheter into the vein.
B) The liver is retracted towards the diaphragm and kept out of the way with a piece of wet gauze. The mesenteric vein beginning at the level and distal to the splenic vein is freed from surrounding tissue. A horizontally placed cut and polished 18 G needle placed under the vein may be helpful for reducing the blood flow and also minimizes blood loss. A small incision in the mesenteric vein is made with a 21 G needle, immediately thereafter the catheter is inserted and advanced about 1.5 cm, so that its tip lies 2-3 mm downstream to the gastroduodenal vein. The catheter is fixed with silk thread and a drop of tissue adhesive is placed over the entry point of the catheter into the vein.
After both procedures, the intestines are rinsed with warm sterile saline and repositioned in the abdomen. Skin and muscle are closed with absorbable sutures (3-0 vicryl and 5-0 vicryl respectively). The rats are allowed to recover for at least one week, during which the catheters are flushed daily with sterile saline and filled with either heparinized saline

(100 I.U. / ml) or heparinized 50% glycerol (100-200 I.U./ml). After the first week the catheters are flushed every second day and after 3 weeks twice a week.
Many weeks after implantation, the catheter tip may be overgrown by connective tissue that can form a pouch around the catheter tip. Therefore, catheter patency needs to be verified with an "anesthesia test". Procedure: 0.6-0.8 mL/kg of a diluted xylazine (0.7-0.9 mg/kg) – ketamine (27–35 mg/kg) mix is infused via the hepatic portal vein within 20 sec. Rats which do not loose muscle tone completely within 1 min after completion of the infusion fail the anesthesia test and data from these animals are excluded from analysis.

12.1B Hepatic portal vein (HPV) catheter in mice

Chronic HPV vein catheters in mice consist of polyurethane tubing (Renathane®) and a bent V-shaped stainless-steel tubing (12 mm, 26 G cannula, protruding end). The tip of the polyurethane catheter (length 95 mm; ID 0.3 mm; OD 0.6 mm) is tapered to 0.25 mm OD by pulling in 135°C hot vegetable oil. The catheter tubing is attached to the steel tubing and reinforced with a piece of slightly bigger silicon tubing. The reinforced end with the steel tubing is led through a piece of soft surgical mesh (0.7 x 1 cm, Premilene® or Optilene®) to improve adhesion to skin and fascia and fixed to it with non-absorbable monofil suture. The headset is implanted as described above for intrajejunal infusion cannulas and the distal end of the catheter is led subcutaneously from the back to a 1.5 cm midline laparotomy. The small intestines are exteriorized, placed on and covered with wet gauze and kept moist with NaCl. The liver is retracted towards the diaphragm and kept out of the way with a piece of wet gauze. The mesenteric vein beginning at the level and distal to the splenic vein is freed from surrounding tissue. A horizontally placed cut and polished 25 G needle placed under the vein may be helpful for reducing the blood flow and also minimizes blood loss. A small incision in the mesenteric vein is made; immediately thereafter the catheter is inserted and advanced about 8 mm, so that the tip lies 1-2 mm downstream to the gastroduodenal vein. The catheter is fixed with 8-0 polyamid suture to the mesenteric tissue. The intestines are rinsed with warm sterile saline and repositioned in the abdomen; skin and muscle are closed with absorbable suture (6-0 vicryl).
The mice are allowed to recover from surgery for at least one week, during which the catheters are flushed daily with sterile saline and filled with heparinized 50% glycerol (200IU/ml). Afterwards the catheters are flushed every second day, and after 3 weeks twice a week.

12.2 Inferior vena cava (VC) catheter in rats

This is another technique with which we have had long experience (for example, N. Geary et al., Am. J. Physiol. 264:R116-R122, 1993). The animal is prepared as for hepatic portal vein cannulation, except all the intestines are retracted to the animal's left side to expose the vena cava. The fascia covering the vena cava and aorta is removed with blunt dissection. The ventral surface of the vein is grasped with a forceps and lifted a few mm; the caudal aspect of the resulting tent-shape is pierced with a 21 G syringe needle, and the tip of the cannula inserted. The cannula is led rostrally 3-4 cm to a point near the junction of the hepatic vein. The cannula is anchored to the psoas muscle posterior to its entry site with non-absorbable 3-0 sutures. The intestines are repositioned in the abdomen, skin and muscle are closed with absorbable sutures (3-0 Vicryl). The rats are allowed to recover for one week, during which time catheters are flushed with 0.3 ml 0.9% sterile saline and filled with 150 µl heparinized saline (100 I.U. / ml) daily, afterwards every second day.

12.3 Jugular vein (JV) catheter

The method is based on the classical method of A. Steffens, "A method for frequent sampling of blood and continuous infusion of fluids in rats without disturbing animal". Physiol. Behav. 4:833-836, 1969, as slightly modified here (B. Ferrari, M. Arnold, R.D. Carr, and W. Langhans, "Subdiaphragmatic vagal deafferentation affects body weight gain and glucose metabolism in male Zucker obese (fa/fa) rats", Am. J. Physiol. 289:R1027-R1034, 2005).

12.3A Jugular vein (JV) catheter in rats

Catheters are assembled from silicone tubing (0.94 mm OD, 0.51 mm ID), a 20 G Vacutainer cannula, and a polypropylene surgical mesh. The protruding end of the catheter is placed as above. The catheter is then led subcutaneously from the neck to the right clavicle. The jugular vein is exposed and the catheter inserted and advanced to the right atrium. Skin and muscle are closed with 5-0 Vicryl absorbable sutures. Caps are then put on the headpiece to close the catheter. The jugular catheter is filled with a 60% polyvinylpyrrolidone solution in saline with 500 I.U. /ml heparin. During the first week after surgery catheters will be flushed every day, during the second week every second day and thereafter every third day.

12.3B Jugular vein (JV) catheter in mice

Chronic jugular vein catheters for mice consist of a polyurethane tubing (Renathane®, 0.3 mm ID x 0.6 mm OD, length 42 mm) and a bent stainless-steel tubing (12 mm, 26 G cannula, slightly bent to a 120° angle, protruding end). The catheter tubing is attached to the steel tubing and reinforced with of silicon tubings. The reinforced end with the steel tubing is led through a piece of soft surgical mesh (0.7 x 1 cm, Premilene® or Optilene®) to improve adhesion to skin and fascia and fixed to it with non-absorbable monofil suture (6-0 Prolene®).

The protruding end of the catheter is placed as described above for jugular vein catheters in rats. The catheter is then led subcutaneously from the neck to the right clavicle. The catheter is filled with sterile NaCl and, 12 mm distant from the tip, a suture is placed around the catheter. The jugular vein is exposed and two ligatures (the caudal ligature is a loose loop) with 5/0 silk are placed around the vein. An incision is made on the ventral surface of the vein, and the catheter is inserted and advanced until the tip reaches the right atrium (about 12mm). The catheter is fixed in place with 5/0 silk around the catheter and connected with the caudal suture thread. Both incisions are sutured with 6/0 Vicryl. The catheter is filled with heparinized 50% glycerol solution (100 - 200 I.U. heparin/mL). The first few days after surgery catheters are flushed every day, later on every 3-4 days.

12.4 Mesenteric artery catheter in rats

The catheter consists of a 23 cm polyurethane catheter (Microrenathane, Braintree Scientific, Braintree, MA, Art. MRE-025; 0.3 x 0.64mm, tapered to ~0.15mm OD by pulling in 125°C vegetable oil). The proximal end of the catheter is fitted with an infusion port made from 26 G (0.45 x 25 mm) surgical stainless-steel tubing (Sterican; B. Braun, Melsungen, Germany) that is cut to 2.0 cm and bent into U-shape; both ends are polished. The connection between the catheter and the infusion port is shielded with a 1.5 cm piece of silicon tubing (Gore W.L., Newark, DE, USA; ID 0.508 mm, OD 0.914 mm). The infusion port is then led from below through a 1.5 x 2 cm square of polypropylene surgical mesh, which is subsequently cut into an oval shape (Marlex; Bard Implants, Billerica, MA, USA) to improve adhesion to the skin and fascia. The headset is implanted as described for IP catheters. The distal end of

the catheter is then led subcutaneously from the neck to a 3cm midline rostral-caudal abdominal skin incision and midline laparatomy.
The catheter is led through a puncture hole in the abdominal wall. The small and large intestines are exteriorized, placed on and covered with wet gauze that is kept moist with warm saline. The liver is retracted towards the diaphragm and kept out of the way with a piece of wet gauze. The catheter is filled with sterile NaCl. The superior mesenteric artery is identified and freed from connective tissue. Special care is taken not to damage the adjacent major intestinal lymph duct. A loop of 5/0 silk thread is placed around the artery upstream of the catheter insertion site and slightly lifted in order to reduce blood flow. After a little puncture hole is made, the tip of the polyurethane catheter is inserted 3-4 mm into the mesenteric artery and fixed in place with 5/0 silk. This catheter is non-occlusive, i.e., it occupies less than 30% of the artery lumen. The intestines are rinsed with warm Ringer-Lactate® solution (B. Braun, Germany) and replaced into the abdominal cavity. Abdominal skin and muscle are closed with absorbable sutures (Muscle: 3-0, Skin: 5-0 Vicryl, Ethicon, Norderstedt, Germany).
During the first few days after the mesenteric artery cannulation, the catheters are flushed daily with 100 µL of 0.9% sterile saline (infused over 20–30 sec), and every 2nd day thereafter. After flushing, the catheters are filled with 50 µL of heparinized 50% glycerol (200 IU/mL).

12.5 Intestinal lymph duct catheter

The headsets are assembled as described above for mesentery artery catheters.
In addition, a small tubing made of BD Insyte TM I.V. Catheters (0.9 x 25mm REF 381223, or 0.7 x 19mm REF 381212, Becton Dickinson S.A. Madrid, Spain) Vialon TM tubings, is cut into a 5-7 mm long section and a little side hole (~0.15 mm) is made prior to implantation. Four g pellets of a high fat diet are given 1-2 hours prior to lymph cannulation surgery in order to stimulate lymph flow. This allows for easy identification of the mesenteric lymph duct based on the whitish-opaque lymph fluid inside. The headset is implanted and the catheter is led into the abdominal cavity as described for mesenteric artery catheters. The small and large intestines are exteriorized, placed on and covered with wet gauze that kept moist with warm saline. The liver is retracted towards the diaphragm and kept out of the way with a piece of wet gauze. The superior mesenteric lymph duct runs parallel to the mesenteric artery and extends laterally from the right kidney to the small intestine, at which point it terminates in an intestinal lymph node. The inferior mesenteric lymph duct, when present, is located directly caudal to the artery. This duct will not be cannulated, but severed with a ligature (10-0 Polyamid suture, Ethilon®, Ethicon, Norderstedt, Germany) in order to increase lymph flow in the superior mesenteric duct. The superior mesenteric lymph duct is freed from the layers of surrounding connective tissue. A loose loop of 10-0 Polyamid thread is placed around the previously prepared VialonTM tubing. A small incision with a 22 G steel cannula (0.7 mm OD) is made into the anterior wall of the mesenteric lymph duct and the VialonTM tubing is inserted. This is necessary to stabilize the extremely thin wall of the lymph. The catheter is filled with heparinized saline (100 IU/mL), and the thin diameter tip is then inserted via the little side hole 2-3 mm into the VialonTM tubing and fastened with the polyamid thread. The size of the insertion hole of the anterior lymph duct wall is reduced with a few stitches with polyamid thread, and a small drop of tissue adhesive (Histoacryl ®, B. Braun, Germany) is applied to seal and secure the implantation side.

The intestines are rinsed with warm Ringer-Lactate® solution (B. Braun, Germany) and replaced into the abdominal cavity. Abdominal skin and muscle are closed with absorbable sutures (Muscle: 3-0, Skin: 5-0 Vicryl, Ethicon, Norderstedt, Germany).

During the first 5 d after the lymph duct cannulation the catheters are flushed daily with 30 µL of 0.9% sterile saline (infused over 20 – 30 sec), and every 2nd day thereafter. After flushing, the catheters are filled with 20 µL of heparinized 50% glycerol (100 IU/mL).

13. Streptozotozin (STZ) treatment for induction of diabetes mellitus (SG 2)

Animals are rendered diabetic by a single IP administration of streptozotocin (STZ; 50-100 mg/kg IP) dissolved in citrate buffer (pH 4.0). Despite low pH, the injection is not painful and can be performed as any IP injection in non-restrained animals. Blood glucose levels will be measured 48-72 h after injection to assess the diabetic state. Blood (0.1-0.2 ml) will be obtained by standard procedure from the tail vein or (if larger quantities of blood are required for measuring additional parameters) retroorbital plexus in short-term isoflurane-anesthetized animals (see below).

Animals will be closely monitored until full recovery from anesthesia which usually occurs within few minutes. Using this method, recipients will be reliably diabetic 2-3 days after STZ treatment. Only animals with a blood glucose level above 20 mmol/l will be used for further experiments. STZ treated animals will be closely monitored for their wellbeing throughout the study. Due to their diabetic state, animals will loose body weight or show reduced body weight gain. Further, they will show profound polyuria and polydipsia. Therefore, in addition to the routine procedures and special attention to the development of body weight and availability of water, the monitoring also involves a regular skin fold test to check the animal for possible dehydration. If this test is positive, the animal will be treated immediatedly with parenteral fluid administration. If dehydration does not resolve within 2 to 3 days or if dehydration is recurrent after successful initial treatment, the animal will be eliminated from further experiments.

14. Ovariectomy (SG 2)

14.1 Via the flank for rats or mice

After a dorsal midline skin incision, access to the abdominal cavity is obtained behind the last rib through a lateral incision (approx. 7 mm in rats and 3 mm in mice) in the abdominal muscles. Using blunt forceps, the ovary and tip of the uterus are exposed. The uterus is ligated with 3-0 Vicryl suture and the ovary is removed using a scissor incision. After checking for possible bleeding, the uterine horn is returned into the abdominal cavity. The muscle is sutured with 3-0 Vicryl. The same procedure will then be performed on the contralateral side. The whole operation lasts approximately 10 - 15 min. The skin incision will be closed by surgical wound clamps.

14.2 Via the ventral midline for rats

This simple approach is especially useful when other visceral surgeries are performed (i.e., when a gastric cannula is installed; e.g., N. Geary et al., Physiol. Behav. 57:155-158, 1995). A 4 cm midline laparotomy is made ending caudally 1 cm rostral to the urethral orifice. The intestines are reflected with a cotton swab and the horns of the uterus visualized and tracked to the ovaries. Each horn is ligated with 3-0 Vicryl about 0.5 cm

from the tip. A clamp is placed on the fatty tissue between the kidneys. The tip of the uterus is cut with scissors distal to the ligation, the clamped fat and veins are cut with a cautery, and the ovary is removed. The clamp is slowly removed, checking carefully for bleeding. The intestines are repositioned in the abdomen, skin and muscle are closed with absorbable sutures (3-0 Vicryl).

15. Acute blood sampling techniques in rats and mice (SG 1)

15.1. Blood sampling from the retrobulbar plexus

This procedure is performed under short term anesthesia using isoflurane. A maximum of 1-1.5 ml of blood will be sampled in rats (0.1-0.2 ml in mice) alternating between the left and right eye. Repeated sampling will be performed with a minimum interval of 2 weeks in between bleedings. Blood is sampled using hematocrit capillaries. These are placed from caudo-laterally on the eyeball in a flat angle. The capillaries are then pushed under the third eyelid and rotated to a vertical angle. Under a slight rotating movement, the capillaries are pushed into the retrobulbar plexus until blood appears in the capillary.

After blood sampling, the capillaries are removed and any bleeding is stopped by applying a sterile cotton swab on the eye for several minutes. Although bleeding usually stops immediately, the animals are carefully checked for 1-2 h after the sampling.

15.2 Blood sampling from the tail vein

Blood samples can be taken by means of a small incision (e.g., by use of a 18-20G cannula) made about 2-3 cm from the end of the rats' tails. The rat is loosely wrapped in a towel; rats are usually very calm in this small and dark surrounding. Gently stroking the tail from the base to the end of the tail helps to reveal the veins. The end of the tail is fixed between two fingers onto the table and a small incision is made into a lateral tail vein or into the dorsal tail vein. After gently stroking from the base of the tail to the end of the tail, with almost no pressure applied, blood drops form at the site of incision. When one stops stroking the tail, bleeding stops and the rat can be placed back into its home cage. Several blood samples can be collected in one day from the same incision. Stroking over the incision with a tissue re-opens it. If longer time intervals occur, new incisions can be made, 1-3 mm away from the last incision, towards the base of the tail. With the above described method, up to 300 µl of blood can easily be collected within 90 s. If greater volumes of blood need to be taken, the tail can be warmed in 40°C water for 1 min for vasodilatation, and up to 1 ml can be collected within 3 min.

The advantages of this method are: (i) anesthesia and surgery or restraint of the animal are not necessary; (ii) the procedure can be considered stress-free as indicated by the low, basal levels of the stress hormone corticosterone, even with frequent sequential blood sampling over 3 h; and (iii) it can be used for longitudinal studies allowing intra-individual comparisons over months and even years. Blood samples collected via an intravenous (jugular vein) catheter and, at the same time, by our tail incision method resulted in comparable amounts of corticosterone (Arnold and Langhans, Physiol. Behav. 99:592-598 2010). The method is modified from that described by M. Fluttert, S. Dalm and M.S. Oitzl, “A refined method for sequential blood sampling by tail incision in rats” Laboratory Animals 34: 372-378, 2000.

15.3 Blood sampling from the vena saphena in mice or rats

Twelve to six hours prior to blood sampling, animals are placed in a restraint tube and the legs are shaved with a clipper around the region of the vena saphena. They are then returned to their cages until the test. For blood sampling animals are again placed in the restraint tube, one person holds the leg while another punctures the vena saphena with a 20G needle and samples the blood into capillaries. The entire procedure requires less than 2 min. Up to 3 samples/leg (i.e., 6 samples/d) can be taken, with a per sample volume of up to 400 µl total per day for mice and up 2 ml total per day for rats. Animals are allowed to recover at least 2 weeks between maximum volume sampling or between sampling from the same leg.

16. Lesions of the vagus nerve (SG 2)

16.1 Selective abdominal vagotomy

To expose the abdominal vagus nerve along the esophagus, a 3 cm midline laparotomy is made, the stomach is retracted caudally using a 3-0 stay sutures placed through the corpus, mesenteric connections between the ventral surface of the stomach and the liver are cut, and the right and central lobes of the liver are reflected rostrally, held in place with gauze pads dampened with warm saline if required. The right (ventral) and left (dorsal) esophageal trunks are identified just below the diaphragmatic hiatus with the aid of an operating microscope (10-25X).

For selective ***hepatic vagotomy***, two 3-0 nonabsorbable sutures are placed 2-5 mm apart around the hepatic branch, which connects the right esophageal trunk and the liver, and the segment between the sutures is cut. For selective ***celiac vagotomy***, sutures are placed 2-10 mm apart around the celiac branch or branches that connect the left esophageal trunk and the celiac ganglion, and the segment between the sutures is cut. For selective ***gastric vagotomy***, sutures are placed 5-10 mm apart around the right esophageal trunk below the bifurcation of the hepatic branch and around the left esophageal trunk below the bifurcation of the coeliac branch, and both segments are cut. The accessory celiac branch, which sometimes bifurcates from the right esophageal trunk, is also sutured and cut.

16.2. Selective vagal de-afferentiation or de-efferentiation

The procedure involves transecting the left afferent vagal rootlet at the brain stem level, and cutting the ipsilateral dorsal trunk of the vagus below the diaphragm (because the vagus decussates in the thorax, the abdominal trunk ipsilateral to the operated rootlet corresponds to the contralateral rootlet). The left afferent vagal rootlet transection cuts all of the vagal afferents arising from the common hepatic, accessory celiac, and ventral gastric branches as they enter the brain stem, while leaving all of the vagal efferent fibers in these branches intact.

The dorsal vagal rootlet transection will be performed as follows. Following anesthesia, the rat is shaved from the chin caudally to the thorax and placed supine in an atraumatic head holder. A midline incision is made from the anterior to the mandible caudally almost to the manubrium, and the skin is pulled laterally with retractors. The left sternohyoid and omohyoid muscles are also retracted to expose the trachea and the external carotid artery. The area of interest lies between the hyoid bone rostrally, the trachea medially, the external carotid laterally, and the superior laryngeal nerve caudally. The muscles under this area are dissected or retracted to expose the occipital bone. The occipital bone medial

to the posterior lacerated foramen is thinned with a dental drill, and then expanded with forceps with care being taken not to damage the underlying dura. An incision is then made in a relatively avascular area over the ventral surface of the medulla, and the resulting cerebral spinal fluid is absorbed. The dura then is retracted exposing the afferent and efferent vagal rootlets below. The efferent rootlets are displaced to gain access to the afferent rootlets, and the afferent nerves are cut with 5-0 forceps. Once the nerve section is completed, the cavity is filled with sterile Gelfoam to reduce CSF drainage and the wound is closed in a single layer. While this procedure alters gut function, rats eat a normal amount of ordinary rat chow following recovery of surgery and grow at the same rate as control rats, so the rats' ability to digest food does not appear to be seriously impaired. To facilitate recovery the following dietary regimen has is applied: 3-4 d prior to surgery the rats are adapted to liquid diet, kept on liquid diet 2 d following surgery, offered wet mash in addition for the following 2 d and chow thereafter. Liquid diet and wet mash is discontinued one week after surgery.

Special postoperative care of vagotomized rats

Abdominal vagotomies, with the exception of hepatic and coeliac vagotomies, often lead to a serious postoperative syndrome related to impaired gastrointestinal function (Kraly, Jerome and Smith, Appetite 7:1-17, 1986). This usually presents as hypophagia, hypodipsia, and weight loss, although sometimes rats continue to eat despite gastrointestinal ileus so that there is little or no weight loss and malnutrition is masked. The syndrome may develop even weeks after surgery, apparently because of a build up in the stomach of solid dry food and hair ingested while grooming. Untreated rat often succumb. On the other hand, if the rats are offered a palatable liquid diet (such as sweetened condensed milk) beginning before surgery, this postoperative syndrome can be avoided and the rats eat, drink and gain weight normally (see, for example, Geary and Smith, Physiol. Behav. 31:391-394, 1983; Le Sauter, Goldberg and Geary, Physiol. Behav. 44:527-534, 1988). The following procedure is highly effective (5 % or fewer rats display any post-vagotomy syndrome):Adaptation of rats to housing conditions for at least 2 weeks; rats are fed a liquid diet (e.g., Ensure®, 1kcal/mL).
Food is removed 4 h before surgery to prevent rats from eating just before surgery.
Access to the dorsal vagus is much easier in animals with an empty stomach.
After total abdominal vagotomy, the animals are kept permanently on the liquid diet.

Histological verification of completeness of vagotomy

Abdominal vagotomies can be verified by post-mortem examination of the surgical site, using the sutures placed during surgery as a guide to the lesion sites. Total abdominal vagotomies and selective de-afferentations or de-efferentations are verified histologically. A combination of two different histological verification methods is used: retrograde transport of the vagal efferent fibers of fluorogold is used to assess the completeness of the subdiaphragmatic dorsal vagal trunk transection and anterograde transport of wheat germ agglutinin-horseradish peroxidase (WGA-HRP) tracer is used to assess the completeness left dorsal vagal rootlet transection at the brain stem level. This requires an additional survival surgery. Rats are injected intraperitoneally with 2mg/mL Fluorogold tracer after treatment with buprenorphin (7.5 µg/100g). One day thereafter, animals are briefly anesthetized with ketamine/xylazin or isoflurane. The rat is placed in supine position and a ventral midline incision is made in its neck. The vagus and nodose ganglion are exposed by blunt dissection, and WGA-HRP (2µL of 2% in distilled H_2O) is

pressure injected (PicoSpritzerII) through a glass micropipette (ID 50 µm) into the ganglion. The wound is closed with a single suture and Carprofen analgesia is given perioperatively and the following day. Two days after the WGA-HRP injection, the animal is deeply anesthetized with pentobarbital sodium and transcardially perfused with 100 ml of 0.9% saline followed by 350 ml of 4% paraformaldehyde in 0.1M sodium phosphate buffer. After perfusion, the brainstem is exposed under a dissecting microscope at x 40, and the integrity of the vagal afferent and efferent rootlets on each side of the brainstem are assessed by visual inspection. The brain, nodose ganglions and esophagus are then removed and processed for verification of complete nerve transections.

17. Terminal experiments

17.1 Transcardial perfusion for immunohistochemical experiments (SG 1)

For immunohistochemical detection of proteins expressed in the brain (e.g., c-Fos which is a transcription factor and a marker of neuronal activation), deeply anesthetized animals are transcardially perfused to recover the fixed brain tissue. Animals are anesthetized with pentobarbituric acid (see above). When deep anesthesia is achieved (e.g., no reflexes can be triggered), the thorax of the animals is opened and the animal will be transcardially perfused. Once perfusion is complete, the brain is excised and further processed for staining.

17.2 Collection of brain for in vitro recording or post-mortem analysis (SG 0)

The crucial step is the rapid collection of the brain for further processing. The animals are euthanized by decapitation using a guillotine. The animals will be used to brief fixation by appropriate handling so that the animals will experience only minimal stress or alternatively placed on a disposable plastic restrainer (e.g. Decapicone). Anesthesia prior to decapitation is not possible because the neuronal function would be impaired.

For post-mortem analysis tissue should be frozen as soon as possible, therefore after decapitation brain will be removed from the skull, placed on a small plastic container (e.g. mini-petri dish), and secured inside of a plastic bag, and immediately afterwards snap frozen on liquid nitrogen.

For in vitro recordings: This method is used to investigate the influence of hormones, neuropeptides and other substrates on neuronal activity under in vitro conditions. Subsequent to brain collection, brain slices are incubated in a temperature-controlled perfusion chamber and maintained viable for several hours by superfusion of artificial cerebrospinal fluid. Spontaneous neuronal activity is then recorded with an extracellular platinum-iridium electrode.

18. In vivo electrophysiological recordings (SG 1)

18.1.A In vivo recording from the cervical vagus

The in vivo technique of measuring the afferent electrical activity is performed under general anesthesia, and the rats are euthanized by an overdose of ketamine immediately after completion of the recordings. Rats are anesthetized with a IP injection of pentobarbital sodium, (50mg/kg), are orotracheally intubated and artificially ventilated (60 breaths à 10mL/kg per min, 95% O_2, 5% CO_2). Body temperature is monitored and maintained at 36-37°C with a warm water heating pad. After the specific nerve preparations described below), small bundles of nerve fibers are peeled off and the distal

cut ends are placed on tungsten metal wire electrodes. After a fiber with a typical response pattern is identified (e.g. gastric load-sensitive or CCK responsive hepatic trunk fibers), testing begins. Test substances (e.g. peptides or metabolites) are administered (intravenously, via mesenteric artery, near celiac artery infusion or hepatic portal vein infusion) using catheters placed during the same anesthesia. Vagal afferent discharges are identified, amplified, and recorded using standard techniques.
To prepare the left vagal trunk in the neck for recording gastric vagal mechano-receptive units, a polyethylene tube is inserted into an incision in the cervical esophagus and advanced distally such that the tip of the tube terminates in the gastric corpus, <1 cm distal to the lower esophageal sphincter. This cannula permits infusion withdrawal of liquid gastric loads. A laparotomy is performed and the duodenum is ligated just distal to the pylorus. The left cervical vagal trunk is detached from the carotid artery and a silicon catheter is inserted in the artery until its tip would lie near the junction of the celiac artery. Teflon tape is placed under the vagal trunk to maintain electrical insulation against emerging fluid. The cavity created in the neck is then filled with warm mineral oil to avoid drying up of the nerve while recording.

18.1.B In vivo recording from the hepatic branch of the vagus
To prepare the common hepatic nerve branch for recording of hepatic vagal units, the portal vein is catheterized using a silicon tubing (OD 0.94mm) and maintained patent by flushing frequently (every 30min) with saline. After lifting the xiphoid process, the ligaments between the liver and the diaphragm or the stomach are transected. The left liver lobe is reflected toward the right side of the esophagus. The stomach is pulled caudally and slightly to the left. The main subdiaphragmatic branches of the abdominal vagus nerve are exposed and the abdominal cavity is filled with mineral oil. The common hepatic nerve branch is freed from adjacent connective tissue under a) using fine forceps. Teflon tape is placed under the nerve bundles to maintain electrical insulation.

18.1.C In vivo recording from the celiac branch of the vagus
After the preparation of the animal as described above, an incision is made in the neck, the esophagus is exposed, an intragastric cannula (Polyethylene; ID 1.4 mm, OD 2.0 mm) is inserted and a 2 mL bolus of 10% glucose is given into the stomach, followed by a continuous infusion of 1 mL of 10% glucose/h throughout the entire experiment. After ventral midline laparotomy, the vena cava inferior is cannulated with 3 silicone catheters, two (ID 0.305mm, OD 0.635mm) catheters for continuous IV administration of methohexital (40 mg/kg/h) and pancuronium bromide (0.4 mg/mL/h), and one (ID 0.635mm, OD 0.94mm) catheter for frequent blood sampling (immediately before start of recording and every 10 min afterwards) for glucose measurement; this latter catheter is kept patent by infusion of saline (0.5 mL/h). The superior mesenteric artery is freed from connective tissue and special care is taken not to damage the adjacent major intestinal lymph duct. A polyurethane catheter (Microrenathane, Braintree Scientific, Braintree, MA, Art. MRE-025; 0.3 x 0,64mm, tip diameter ~0.2mm) is inserted 3-4 mm into the mesenteric artery and fixed in place with 5/0 silk (catheter is non-occlusive, i.e. it fills less than 30% of the artery lumen) and kept patent with a constant flow of saline (5 µL/min). The perfusion area of the cannula is verified at the end of the experiment by infusion of blue food color (100 µL/30 sec). Rats receive pneumothorax to ease artificial ventilation and to reduce nerve movement. Heart rate and blood oxygenation are monitored throughout the experiments by a noninvasive pulse oximeter (Nonin Medical, Inc.), and the level of anesthesia is periodically tested by ensuring that no cardiovascular responses could be

evoked by noxious pinch of the hindpaw. The dorsal celiac branch of the vagus is freed from connective tissue, a piece of Teflon tape is placed under it for electrical insulation and the whole recording site is filled with 37°C mineral oil. A small bundle of nerve fibers is peeled off and cut free from the main branch, and the cut end is placed on a tungsten hook electrode. Some ligaments of connective tissue are placed on the reference electrode.
Fibers with obvious spontaneous activity are screened for sensitivity to a 2.5 µg bolus of serotonin injected into the mesenteric artery (in about 30 µL saline delivered within a few seconds) comparing activity during a 60 sec pre-stimulus baseline and a 10 sec, 30 sec and 1 min post-stimulus period. Serotonin sensitive fibers respond within 2-4 sec with a brief but intense burst of activity. If a nerve bundle fails to respond to serotonin, that bundle is discarded, and a different nerve bundle is tested ("no desensitization of successive doses of serotonin agonists is observed when a minimum interval of 5 min is employed", Hillsley JP 1998, tested in mesenteric afferent bundles). Once serotonin-sensitive afferents are identified, recording begins. Neural activity is filtered with a band with of 300-1000Hz, digitally sampled at 20kHz for computerized spike discrimination and frequency analysis (data interface model 401; Cambridge Electronic Design, Cambridge; MA). Baseline unit activity is recorded for 5-10 min prior to serotonin (see above) administration and followed by 6-10 min recording period. Thereafter, a test compound is infused via the mesenteric artery with an infusion rate of 100 µL/min. Vagal afferent activity is continuously recorded and once again tested for serotonin responsiveness afterwards.

19. Acute central injections in mice or rats (SG 2)

19.1 Cisterna Magna injections
The animal is anesthetized; its ears are placed into a stereotaxic instrument (Stoelting), and the neck is flexed ventrally so that the junction between the skull and the first vertebrae can be palpated. A 28G needle is filled with saline (about 0.25 µl) or artifical CSF, separated from the test drug by a small air bubble, attached via tubing to a microsyringe (Hamilton), placed under slight back-pressure, and lowered until the air bubble moves quickly backward and cerebrospinal fluid can be aspirated, indicating entry into the cisterna magna (about 5 mm or 1-2 mm ventral to the skin in rats and mice, respectively). The drug is then injected (1 µl/min, maximum volume: 10 µl in rats and 2 µl in mice) and the needle withdrawn. The wound is inspected for bleeding or CSF leakage, and the animal monitored until recovery from anesthesia.

19.2 Intracerebroventricular freehand injections in mice
The freehand intracerebroventricular injection technique was introduced long time ago (Haley & McCormick, Br. J. Pharmacol. 12:12–15, 1957), and has been refined more recently (Laursen & Belknap, J. Pharmacol. Methods 16:355-357, 1986). Furthermore, this technique has been used recently to monitor the effect of specific substances on food intake behavior (e.g., Hohmann et al. Am. J. Physiol. 278:R50-R59, 2000; Chartrel et al. PNAS 100:15247-52, 2003), with the advantage of avoiding chronic inflammatory processes related to the metal cannulation that might bias results. Here we describe the general procedure applicable either for mice or rats. The animal needs to be anesthetized with isoflurane and fixed in a stereotaxic frame. A small midline incision is necessary to locate Bregma and drill an initial hole in the skull above the lateral ventricle (mice coordinates: Posterior 0.5 mm, Lateral: 1.0 mm / rat coordinates: Posterior 1.0 mm, Lateral 1.5 mm). Standard pre- and post-operative care is applied (see above). All

injections are made through the same hole that is felt through the skin. On injection days, the animal is anesthetized with isoflurane and slight pressure is applied to the ears (with the fingers to level and stabilize the head). During injection a 27G long needle fitted with a plastic sheath (leaving 0.3 cm needle exposed for mice and 0.5 cm for rats) is attached to the Luer-Lock hub of a Hamilton microsyringe. The injections are given into the lateral ventricle with the needle inserted perpendicularly to the head. After a slow continuous injection, the needle remains in place for several seconds, allowing the solution to disperse and preventing backflow up the needle track. Animals are returned to their homecage and food intake behavior is monitored after recovery from anesthesia (usually less than 5 min).

20 Genomic modifications via viral vectors (SG 2)

20.1 Peripheral viral vectors

The day before virus administration, animals are fasted overnight, with water ad libitum. Animals are equipped with catheters to administer the virus site-specifically. The next morning, 1.5 h prior to virus administration 500 µl PBS are administered into the catheter (e.g., jejunum) to establish a pH around 7.4. The adenovirus is diluted in phosphate buffer and conjugated to monomethoxypoly(ethylene) glycol (PEG) to enhance virus stability, and administered at dilutions of maximal ~5 x 1010 bfu. Animals are water and food deprived for 2 h post-administration, and fed chow ad libitum thereafter. Mice are sacrificed through decapitation at a time point determined by the experiment, and tissue samples (i.e. intestine, liver, etc.) are collected. Verification of viral infection occurs ex vivo of relevant tissue sections using immunohistochemistry

20.2 Viral vectors brain micro-infusion

Antibiotics are given to rats 1 day before surgery. On the day of surgery, animals are anaesthetized with ketamine/xylazine as described above. The rat is secured in ear bars of a stereotaxic frame and positioned according to the target area of viral administration. Stereotaxic injection of Adenovirus is done with a 40 µm beveled tip; 10 msec pulses at 40 PSI, with a 2-3 min interval between pulses. The amount of virus administered is determined based on the experimental conditions (aim, target tissue, virus characteristics, etc.). Upon completion of all injections, muscle or skin layers are sutured. Post-operative care is provided as previously described.

21. Energy expenditure assessment by indirect calorimetry in rats and mice (SG 1)

Measurements of food and water intake and O2 consumption / CO2 production is performed non-invasively using an automatic feeding monitoring system coupled to an open-circuit indirect calorimetry system (TSE Phenomaster System). In addition, via infrared light-beam frames detailed measurements of spontaneous home cage activity can be obtained. Mice are single housed in regular type III cages; food and water are available ad libitum and intake can be constantly monitored. Each cage is connected to the fresh air supply as well as the sample switch unit for drawing air samples from each cage. Cages (n=12) are enclosed in a ventilated cabinet (TSE Systems) to precisely control ambient temperature and light intensity. This feature allows to set up and maintain specific experimental conditions such as:

thermoneurtrality (30°C), regular rodent housing (22°C), hypothermia (4°C) or hyperthermia (36°C). A built in alarm system warns of deviations from individual critical parameters (O2 / CO2 / air flow). Analyses of all these metabolic parameters allows for a precise and reliable estimation of energy intake and expenditure of freely moving mice in a completely stress-free and familiar environment.

22. Adiposity assessment by computer tomography (SG 1)

A La Theta LCT-100 (Aloka) is used. The X-ray source tube voltage is set at 50 kV with a constant 1 mA current. Aloka software estimates the volumes of AT, bone, air, and the remainder using differences in X-ray density, and it distinguishes intra-abdominal and subcutaneous adipose tissue. Animals are scanned either under isoflurane anesthesia or just after killing with CO2. Pilot experiments indicated that computed AT weights are similar in anesthetized animals and animals scanned within 30 min of killing. Anesthesia is induced in a small acrylic box using a flow of 500 (rats) or 400 (mice) ml/min O2 with 5% isoflurane and maintained in the scanner via a nose cone providing 200 ml/min 2.5% (rats) or 100 ml/min 1% (mice) isoflurane. Eyes are protected with ointment. Animals are placed supine position in the appropriate holders with inner diameters of 120 (rats) and 48 mm (mice). First, a sagittal image of the entire animal is made to ensure proper placement in the holder and to set the scan area, either whole-body or the abdominal region. Abdominal scans are performed between vertebrae L1 and L6, L1 and L5, or L4 and L5 inclusive (i.e., from the anterior end of the former to the posterior end of the latter vertebra). To avoid artifactually including subcutaneous leg fat in the abdominal area, animals' hind limbs are extended so that the angle between the femur and the pelvis and spine is ~90°. Rats' hind limbs maintain their position after this manipulation; whereas mice hind limbs do not and are extended and fixed to the holder with tape. Rat tails are curled back on the animals so that they fit in the machine; mouse tails are left extended. Accuracy, reliability and sensitivity of this procedure have been experimentally tested in our laboratory (Hillebrand et al. Obesity 18:848-853, 2010).

23. Evaluation of glucose homeostasis

23.1 Hyperinsulinemic glucose clamp in mice and rats (SG 2)

Mice are equipped with jugular vein catheters during anesthesia (see jugular vein catheter). After a recovery of a minimum of 5 days, mice are deprived of food for 6-12 h and the catheters are connected to an infusion pump immediately after water deprivation. Animals are maintained in their home cages and are infused stress-free. Also, animals are adapted to the infusion room and the noise of the pumps prior to the experiment. For the first 30-40 min, baseline blood glucose levels are established with tail vein punctures (see above). The glucose infusion rate is adjusted to reach a constant blood glucose concentration. Then a bolus of 14C labeled glucose is administered through the catheter, which is then connected again to the pump. After 60 min, mice are killed with an overdose of sodium pentobarbital (150-200 mg/kg) given through the catheter, and organs and blood are taken to measure the uptake of 14C labeled glucose.

23.2 Glucose tolerance test in mice (SG 1)

The oral glucose tolerance test (OGTT) measures the clearance of a standardized glucose load from the body. Since the glucose bolus enters the body via the natural

route—it is absorbed from the intestinal tract—this test also takes into account intestinal aspects of glucose absorption. Animals undergo fasting for a maximum of ~14 to 16 hr (water should be always available), then a glucose solution (10 µl/g BW of 20%) is administered by oral gavage or alternatively the animals voluntarily drink glucose solution, if they are well trained and pre-exposed to it. Blood is withdrawn by tail vein incision (see above) at different time points (before glucose and 15, 30, 60, 90, 120, 150 and 180 min after glucose ingestion/administration and glucose is measured. At the end of the experiment, plenty of food is provided and it is ensured that no animal is bleeding excessively. If necessary, the test can be repeated after at least 3 intervening days because the loss of blood is usually minimal. Instead of orally, the glucose solution may also be injected intraperitoneally (IP).

23.3 Intraperitoneal insulin sensitivity test in mice (SG 1)

The intraperitoneal insulin sensitivity test (IPIST) measures glucose levels subsequent to a standardized insulin load. It gives an estimate of the insulin sensitivity of the animals. Animals are fasted for 14 to 16 hr (water should be always available), a bolus of insulin is administered intraperitoneally (IP, 1U/kg BW). Blood is withdrawn by tail vein incision (see above) at different time points (before insulin and 15, 30, 60, 90, 120, and 150 min after insulin administration), and glucose is measured. At the end of the experiment plenty of food is provided and it is ensured that no animal is bleeding excessively. If necessary, the test can be repeated after at least 3 intervening days because the loss of blood is normally minimal.

24. Roux-en-Y gastric bypass operation (RYGB) in rats (SG 2)

Rats are fasted overnight and are then anesthetized with isoflurane. RYGB is performed using a modified omega loop technique as shown in the graph. The oesophago-gastric junction is anastomosed to a loop of jejunum 7 cm distal to the ligament of Treitz in an end-to-side fashion. A 7 mm side-to-side small bowel anastomosis is performed between the biliopancreatic and the alimentary limbs to create a common channel of 25 cm. Anastomoses are performed using prolene 6/0 and the gastric remnant is closed with prolene 4/0. The sham procedure comprises a laparotomy, a 7 mm gastrotomy on the anterior wall of the stomach and resuturing of the gastrotomy with 4/0 prolene. At the end of all the operations, 5 mL of normal saline is instilled IP before closure to compensate for fluid loss. The animals are housed individually and receive ad libitum standard chow and water. Body weight and food intake are measured daily, and twenty-four hour stool collections are performed.

Body weight loss. Criteria will be used as specified in these RAUPs. However, we expect body weight in the RYGB animals to drop rapidly, which may go beyond the limits specified for all other experiments in the RAUP. In the case of this particular study design, this massive drop in body weight is necessary to achieve the desired results. It also corresponds to the situation in human patients after RYGB surgery. We ensure daily surveillance of the animals throughout the experimental period in order to detect deterioration of the general well-being of animals as soon as possible. If the loss of body weight in an animal exceeds the expected range by more than 5-10%, we will eliminate this animal from the study.

Use of pair-fed controls and use of body weight-matched controls. Rats subjected to RYGB will markedly loose body weight compared to the sham-operated controls. We expect a decrease in body weight of about 30% over 3-4 weeks. We are aware of the fact that this decrease in body weight is massive, but this is consistent with the weight loss after RYGB seen in humans of between 15 and 35%. Special care is therefore taken to carefully observe the animals for any abnormality that may occur during this period. By experience, the general behavior and well-being of rats is expected to be basically undisturbed after RYGB. The decrease in body weight is at least in part due to a marked reduction in appetite and eating. To compensate for this factor, which itself influences energy expenditure, some rats are pair fed to the RYGB group, i.e., sham-operated controls will only receive the amount of food that is consumed by the RYGB animals. Consequently, the pair-fed controls will also loose body weight. In previous studies, it has been observed that body weight loss in pair-fed animals is markedly less than in rats after RYGB. We therefore need an additional control group of sham-operated body weight-matched animals. These animals need to be severely food-restricted to achieve similarly low body weight as in RYGB animals. Again, we are aware of the fact that this is a stressful situation for the animals, but this is consistent with the severe calorie restrictive diets that many obese patients are placed on. We firmly believe that this control group is necessary, as without this group the validity of the other experiments could be questioned.

25. Fat Transplants (SG 2)

Adipose tissue transplantation is performed according to the method of Gavrilova et al. J Clin Invest;105(3):271-8. (2000). Only littermate donors will be used. Mice are anesthetized with pentobarbital (0.01 mL/g body weight of 5 mg/mL in 5% ethanol/PBS, IP). Donor fat pads (subcutaneous or mesenteric) from euthanized mice are placed into sterile PBS, cut into 100-150 mg pieces and immediately implanted into the recipient either subcutaneously through a small incision in the shaved skin of the flank or directly into the mesenteric fat capsule of the recipients, with 1 piece per incision. Incisions are closed using 4-0 silk sutures. About 1g of subcutaneous fat or about 0.5 g of mesenteric fat will be transplanted. After surgery, the mice are housed individually for a week, and then at 2–3 mice per cage. We expect that placing the transplant in contact with the recipients own fat will increase the effect of the transplant, as has been reported in a hamster model (Lacy & Bartness, Am J Physiol Reg.;289(2):R380-R388 2005). Transplanted fat will be visually inspected at sacrifice to ensure vascularization and absence of necrosis. Transplants will then be removed and weighed.

Appendix

Tabe 1: Non-standard cage dimensions for animals in experiments

Cage Type	(width x depth x height)	animals
Institute of Veterinary Physiology (UZH)		
1. Wire mesh floor cages rats	47cm x 33cm x 20cm	1 rat; any body weight
2. Wire mesh floor cages rats	47cm x 25cm x 18cm	1 rat; < 400g body weigh
3. Wire mesh floor cages rats	23cm x 39cm x 20cm	1 rat; < 400g body weigh
4. Wire mesh floor cages mice	25cm x 28cm x 18cm	1 mouse
5. Metabolic cages rats *	42cm x 42cm x 30cm	1 rat; any body weight
6. Metabolic cages mice * (Tecniplast cage type II)	21cm x 27cm x 14cm	1 mouse
7. BioDAQ rats (Tecniplast cage type IV S)	48cm x 38cm x 21cm	1 rat, any bodyweight
8. BioDAQ mice (Tecniplast cage type II L)	37cm x 21cm x 14cm	1 mouse
Physiology and Behavior Laboratory (ETH)		
9. Wire mesh floor cages rats	55cm x 33cm x 36cm	1 rat; any body weight
10. Wire mesh floor cages rats	24cm x 40cm x 21cm	1 rat; < 400g body weigh
11. Wire mesh floor cages mice	25cm x 28cm x 18cm	1 mouse
12. Metabolic cages rats*	42cm x 42cm x 30cm	1 rat; any body weight
13. Metabolic cages mice* (Tecniplast cage type III)	21cm x 27cm x 14cm	1 mouse

* cages are used for indirect calorimetry and the measurement of body temperature (telemetric), physical activity (telemetric) and ingestive behavior

Score sheet for animals under special care

Score sheet for animals under special care

Permission:

Animal or cage ID:

Date	0	1	2	Body weight	Researcher	Measures
	☐	☐	☐			
	☐	☐	☐			
	☐	☐	☐			
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Score	Symptoms		Measures
0			No measures required
		No abnormalities	
1	Appearance	Insufficient grooming, feces stains, ocular or nasal discharge	Close monitoring of health status *
	Dehydration	Decreased skin turgor for less than 24h	Re-hydration **
	Behavior	Defensive behavior, vocalization, reduced activity	Close monitoring of health status *
	Infection	Signs of mild infection	Local disinfection or antibiotic treatment
	Wounds / devices	Wound opening, missing staples or suture, improper fitting of devices	Surgical or technical correction
2	Body weight	Weight loss of more than 20% within relative to individual control weight	Euthanasia
	Dehydration	Decreased skin turgor for more than 24h	
	Behavior	Self mutilation, no reaction to environmental stimuli	
	Infection	Signs of severe or treatment resistant infection	
	Locomotion	Inability to move	
	Wounds / devices	Excessive bleeding, ulcer, irreversible malfunction or improper fitting of devices	

* Responsible project leader will be informed. Symptoms will be accepted for a maximum of 7 days if no substantial improvement is achieved

** Sc or ip infusion of pre-warmed saline or lactated Ringer solution (volume: 5%-10% of bodyweight weight, 50% of this volume immediately, remaining volume after 2-3h, fluid volume administered ip at one time should not exceed 3% of body weight)

Exceptions from these criteria may be specifically defined in the animal experimentation permission.