Central regulation of glucose metabolism
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A novel, double intracarotid cannulation technique to study the effect of central nutrient sensing on glucose metabolism in the rat

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ABSTRACT

Background:
The hypothalamus plays a key role in central nutrient sensing and glucose homeostasis. Due to its position next to the third ventricle, intracerebroventricular (ICV) injections or osmotic minipumps are widely applied techniques in studying effects of hormones and other molecules on the hypothalamus and glucose metabolism.

New methods:
The intracarotid catheter technique in which a catheter is placed in the carotid artery, pointing towards the brain, provides a physiological route to centrally infuse blood-borne molecules in an undisturbed animal. To measure effects of central interventions on peripheral glucose metabolism, endogenous glucose production (EGP) and insulin sensitivity can be measured using a stable isotope technique. To combine both techniques, it is necessary to combine different catheters. We here describe a novel cannulation technique for the carotid artery, enabling stress-free infusions towards the brain and blood sampling from the carotid artery concomitantly, and infuse a stable isotope via the jugular vein.

Results:
We showed accurate EGP measurements when intracarotidically infusing saline towards the brain. The stress-hormone corticosterone, as well as energy expenditure, did not alter upon central infusion.

Comparison existing method(s):
ICV infusions bypass the blood-brain-barrier (BBB) and are thus a less physiological approach when studying central effects of blood-borne factors. Furthermore, ICV injections can elicit a stress response which can interfere with outcomes of glucose metabolism. We described a stress-free, physiological method to study effects of central infusions on peripheral parameters.

Conclusions:
This technique provides new opportunities for studying central effects of, for instance, hormones and nutrients, on glucose metabolism.
INTRODUCTION

Blood-borne molecules like hormones and nutrients can cross the blood brain barrier (BBB) to affect central sites to influence behavior and metabolism. To study these central effects, many use cannulae implanted into different brain ventricles and infuse hormones, nutrients or drugs via intracerebroventricular injections (ICV) or osmotic minipumps. However, with this direct delivery in one of the ventricles, BBB is bypassed and thus infusions do not reflect the physiological route by which blood-borne factors are affecting central sites. The ICV infusions however are used frequently because, for example, certain drugs are unable to cross the BBB [1, 2] and surgery to implant ICV cannulae and osmotic minipumps is relatively quick. Also when studying the central effect of metabolic hormones and substrates such insulin, leptin and glucose, this technique is widely accepted and applied [3-5].

To administer molecules/substrates more physiologically to the brain, it is possible to place an ascending catheter in the carotid artery, pointing towards the brain. Infusions via this route have proven effective in the field of central nutrient sensing [6, 7], and infused molecules, for instance glucose, have been shown to reach hypothalamic structures bilaterally [8].

As mentioned above, hormones and nutrients affect the brain to regulate feeding behavior and metabolism. Specifically, the hypothalamus plays an important role in nutrient sensing and is involved in regulating glucose metabolism. It contains glucose responsive neurons which either increase or decrease their firing rate, upon changes in blood glucose levels [9]. In addition it has been shown that hypothalamic neurons are also responsive to fatty acids [10]. Consequently to the modified activity of these neurons, glucose homeostasis is maintained. This can be established either by altering glucose uptake by peripheral tissue under the control of insulin, or by affecting endogenous glucose production (EGP). To measure the effects of central interventions on glucose homeostasis, EGP can be measured using stable isotopes. In this procedure, [6,6-2H2]-glucose is infused via a jugular vein catheter and blood samples are drawn via the carotid artery, where after isotope enrichment is measured by gas chromatography-mass spectrometry (GCMS) [11] and EGP calculated using Steele equations [12]. In addition, during a hyperinsulinemic euglycemic clamp, an insulin bolus is infused through the jugular vein and the subsequent amount of exogenous unlabelled glucose that needs to be infused via the jugular vein to maintain euglycemia, is a measure for insulin sensitivity [13]. The method with stable isotopes to measure glucose metabolism is extensively validated using these descending catheters in jugular vein and carotid artery that allow continuous infusion and withdrawal of blood at the same time. It is possible to draw blood from other sides than the artery, for instance via tail incision, but to be able to perform these experiments in a freely moving and undisturbed animal, it is necessary to combine the carotid (ascending) catheter towards the brain with the (descending) catheter towards the heart to draw blood samples, and, infuse a stable isotope, insulin and/or glucose via the jugular vein catheter. We here describe how we have combined existing cannulation techniques and developed
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a never before shown ascending and descending catheter at the same location in the carotid artery making it possible to measure insulin sensitivity with stable isotopes at the same time as continuous infusions towards the brain. Moreover, we were able to conduct the experiment using this technique, in air-tight controlled metabolic cages and measure energy expenditure as well, without eliciting a stress response.

**MATERIAL AND METHODS**

**Animals**
Male Wistar rats (250-275g on arrival, Harlan, Belgium) were housed five per cage in a temperature (±21°C) and light controlled room (lights on: 7 am, lights off: 7 pm). They received an acclimatization period of 7 days. All animals had ad libitum access to laboratory chow (SAFE, Augy, France) and tap water. Animals were housed individually (35 x 25 x 25 cm plastic cages) after surgery. The experimental protocol was approved by the institutional animal care and use committee of the Paris Diderot University (CEEA40), under the agreement # CEB-20-2015.

**Catheters**
Catheters were made of 10 cm pieces of silicone tubing (0.6x1.2 mm, Rubber BV, the Netherlands). Silicone glue was used as an anchor ring at 4.2 cm for the jugular, 1 cm for the carotid towards the brain (hereafter called ascending carotid) and 1.2 cm for the carotid towards the heart (descending carotid). At the end of the catheter two small holes were made with a blunted 20G (gauge) needle to prevent a vacuum during blood sampling and catheters were stored in 70% ethanol and rinsed with saline before surgery. Lengths were based on male rats weighing 275-300g.

**Surgery**
Rats underwent surgery under anesthesia induced with an i.p. injection of 80 mg/kg Ketamin (Eurovet Animal Health, the Netherlands), 8 mg/kg Xylazin (Bayer Health Care, the Netherlands) and 0.1 mg/kg Atropin (Teva Pharmachemie, the Netherlands). To maintain anesthesia during surgery, we first injected 1/3 of the mix that was used to induce anesthesia. Thereafter, only when needed, ketamin was injected to maintain anesthesia. The animals were put on a heat mat during the surgery. Insertion of the right jugular vein and tunneling of all three catheters to the head was performed according to the method described in detail in [14]. Hair on top of the head and in the neck was removed. A cut of 2 cm was made on top of the head using a surgical scalpel. The periosteum was anaesthetized locally using lidocaine and gently pushed aside. Four small holes were drilled using a dental drill in a square leaving enough space in between the two in front and in between the two in the back to fit the cannulas in between. Screws (1.2x3 mm, Fabory, the Netherlands) were inserted. We used four screws to ensure firm attachment of the cement, since a bigger cement is needed to embed the three cannula’s and the metal connector.
A novel, double intra-carotid cannulation technique

The right jugular vein was cannulated according to the method of Steffens (figure 1 and [14]). After externalization of the catheter on the head, a 90° bend and blunted 20G needle was pushed in the end of the catheter and the catheter was checked and flushed; first some blood was withdrawn and saline was pushed back to be sure the catheter was clean. Thereafter it was filled with 0.05 mL PVP solution (0.9 g PVP, polyvinylpyrrolidone K25 (81400), Sigma; 0.3 mL heparin, 5000IE; 0.7 mL amoxicillin, solved overnight or 2h in 37°C) and closed with a cap made of a piece of cauterized flexible tubing (0.6 mm inside diameter (ID), Technilab instruments, the Netherlands).

For the carotid catheters (figure 1B), an opening of about 1 cm was cut about 0.3 cm left of the trachea, and the left carotid artery was dissected and gently separated from the Vagus nerve using two small forceps, resulting in a clean part of the carotid artery of about 1.5 cm. A surgical thread (4-0 Mersilene fiber suture, Ethicon, USA) was put underneath the carotid and cut in half, on one side a loose knot was made while the other closed the carotid in the middle. For both cannulations a new thread was used and the second thread was knotted one on top of the first one to maximize free space and create the possibility to stretch the artery.

To insert the descending catheter, the artery was temporarily closed towards the heart with a Micro serrefine (FST, Germany) in such a way that a small space of about 0.5 cm was left to insert the catheter. The artery was gently stretched/put on tension with a bulldog

Figure 1. A: left, two catheters are placed in the carotid artery, one pointing in the direction of the brain (red line), one pointing downwards for blood sampling (blue line). Right, a jugular vein catheter is placed to infuse a glucose isotope (green line); B: representation of both carotid catheters. Catheters are hold in place by thread knots on both sides of the silicone anchor ring (white circles). Knots of both carotid catheters in the middle of the artery are as close together as possible. In addition, thread endings of the knots from both sides of silicone anchor ring can be secured to each other; C: catheters are tunneled behind the ear to the head and attached to the skull with dental cement (according to [14]).
serrefine (FST, Germany) hanging at the end of the surgical thread of the middle knot. This puts tension on the carotid, otherwise the carotid would be pushed away during the insertion of the catheter. A small opening was made in the artery with a bent 23G needle by pushing it in and taking it out with the tip slightly up. A forceps (Dumont #5, FST, Germany) was used to keep the artery open during insertion of the catheter, and as the tip of the catheter was inserted the forceps was removed and the catheter was gently pushed further up till the micro serrefine. The micro serrefine was removed, and by keeping the catheter closed and in the artery with a forceps, the catheter was gently pushed further in the artery till the silicon anchor ring with the other free hand. The micro serrefine is placed back around the carotid and the catheter, to leave enough space to make the knot and keep the catheter secure. On both sides of the anchor ring, the catheter was attached to the artery using surgical thread, the ends of both knots were attached to each other to further prevent the catheter slipping out. Like the jugular, the catheter was exteriorized at the vertex of the head, filled with a bent and blunted 20G needle, checked and flushed and filled with PVP (0.05 mL) and closed with a cap.

For placing the ascending carotid catheter, the rat was turned and further the insertion of the catheter was similar to the carotid towards the heart, except that less tension was put on the artery than when inserting downwards. However, a little bit of tension downwards could facilitate insertion of the catheter. Insertion and externalization occurred the same way as the other catheters. Catheter was filled with 0.03 mL PVP solution and closed with a cap.

Each catheter cap was marked with an unique color. Catheters were attached to the skull with dental cement (Simplex Rapid, Kemdent) (figure 1C). In addition, a small metal connector was placed on top of the cement, posterior to the bent catheters needles for connecting animals to linked connectors during the experiment, in order to avoid pressure on the catheters themselves. Cement was flushed with saline to remove small pieces, to avoid irritation. The wounds in the neck of the rats were stitched with a 5.0 stitching wire (Ethicon, USA). Animals received subcutaneous carprofen (5 mg/kg) and 5 ml saline subcutaneously at the end of surgery. The total procedure from initiation of anesthesia to closing the animal took about 1.5 h.

**Recovery and maintenance of catheters**

Animals were allowed to recover for a minimum of 7 days to ensure that they were back to pre-surgery body weight. The PVP in cannulas was changed 2 times a week (only the jugular vein and ascending carotid). To do so, caps were taken off, PVP and blood was pulled out gently with a piece of 0.6 mm ID tubing on a 2,5 mL syringe with a 20G blunted needle. Then, saline (containing 0.04% heparin for the ascending carotid, for the jugular catheter no heparin was added) was flushed through the tubing. After, tubing was removed and quickly replaced (to avoid air bubbles) by a new syringe and tubing was filled with PVP. The PVP was pushed in gently and catheter was closed with the cap. When the catheter appeared to be blocked, in some cases, some pressure with the syringe with saline was held on the catheter and generally blockage was relieved and new PVP was infused thereafter.
Experiment – Connection to infusion pumps

The night before the experiment, rats were weighed and placed in the metabolic cages (48 x 37.5 x 20 cm, TSE systems, Germany) to get used to their new environment. They were fasted overnight but had free access to tap water. The next morning, around 9 AM, animals were first connected a linked connector using the connector in the cement to avoid pressure on the catheters. This linked connector is attached to a double-bore swivel (Instech, USA) with a counter-balanced beam, located on top of the cage (figure 2B). Because the lines are attached to the metal connector (with tape, Leukoplast, BSN medical GmbH, Germany), out of sight of the rat (on the back of the head) the animal was not disturbed by the lines. There was no pressure on the tubing itself due to the metal connector. In addition, because we used a swivel device with a counterbalanced beam, the connecter plus cannnulae moved with the animal when the animal lifts; in this way it could not catch the cannulae in case it would see them above its head.

Carotid and jugular lines were filled beforehand with saline/heparin 1% (to avoid blood clothing) and cauterized on top of the swivel (or in the case of the blood drawing line, closed with a stainless pin). The ascending carotid was filled with the substance to be infused towards the brain, as a control saline/heparin 0.04% was used. Filling of the lines before the start of the experiment facilitates the connection process and avoids air bubbles in the line during connection.

**Figure 2.** A: detailed graphical representation of swivel device and connected lines on top of the air-tight metabolic cage. Grey rectangle, swivel device for infusion towards brain and stable isotope; purple line, infusion via ascending carotid; blue line, infusion of glucose isotope. Both are connected to 0.38 mm ID lines coming from syringes on the infusion pump; red line, descending carotid catheter for drawing blood samples. Tubing was tunneled through the condom (dotted line) by a blunted 20G needle or connection point on swivel; B: representation of rat in air-tight metabolic cage with swivel device on top of the cage.
The animal was held inside the cage during the whole time of connection, the top of the cage lifted and hung on a supporting hook, the connector and lines hanging in the cage through the middle plastic socket of the top of the cage. The plastic socket was made air-tight using an ordinary condom and blood drawing line was tunnelled through the condom using a small piece of 20G needle to connect the line in the cage to another line outside the condom (figure 2A and B). To connect the lines from the swivel to the catheters placed on the head of the rat, the cap of the catheter was taken off, and the PVP was pulled out (using a piece of 0.6 mm ID tubing on a syringe with a 20G blunted needle), after which catheters were flushed with saline/heparin 1% (except for the ascending carotid for which we used 0.04% heparin). For the descending carotid catheter, PVP is pushed up due to the blood flow, or gently pulled up with a 2.5 mL syringe and then the catheter is flushed with saline and connected to the line as quickly as possible. The lines were held closed until connection to the infusion pump. After connection, the carotid to the heart was flushed immediately, with saline/heparin 1% solution and checked regularly (every 45 minutes), by taking the stainless pin off, pulling the blood out and flushing it. The top of the cage was closed immediately after connection. During the connection of the rat the cage was open for about 10 minutes, which could be observed in the energy expenditure graphs, but thereafter the system was air-tight for the duration of the experiment.

**Experiment – infusion of stable isotope and blood sampling**

After connecting the animal, the lines, coming through the condom around the swivel were connected to syringes (with a blunted 25G needle) in the infusion pump (KD scientific, USA), by a 0.38 mm ID stiff line (Portex, Smiths medical, Kent, UK). The syringe size is dependent on how much one wants to infuse but in this case we used a 1 mL syringe for infusion towards the brain and 5 mL for the stable isotope. Pieces of 23G needle were used as connectors for 0.38 mm ID line to line coming from the swivel (0.4 mm ID, Technilab instruments, figure 2A).

Before the start of the isotope infusion, around 10 AM, a background (BG) blood sample was taken from the carotid to correct for background isotopic enrichment. To study glucose kinetics, [6,6-²H₂]-glucose was used as a tracer and infused continuously (500 μL/h) during the whole time of the experiment via the jugular vein. A bolus of 3000 μL/h in 5 min was given before the start of the continuous infusion. To be able to follow the tracer up to the catheter, blood from the jugular was taken up, up to the swivel and the pump line was connected on top leaving a tiny air bubble between the isotope and the blood. Timing of the bolus was started when the air bubble disappeared in the catheter. After the bolus and 90 minutes of continuous isotope infusion to reach a steady state, 3 blood samples were taken with a time interval of 5 minutes to measure basal EGP. Thereafter the pump for infusion of saline (with 0.04% heparin) towards the brain was started (5 μL/min) for 2 hours. Blood samples were taken at t = 65, 70, 75, 80, 90 and 120 via the carotid towards the heart. Infusion towards the brain was terminated at t =120 and at t = 125, 150 and 180 another three blood samples were drawn. All the blood
samples were taken as follows; stainless pin of carotid line was taken out, saline was taken up by a 2.5 mL syringe with a blunted 20G needle, was then replaced by a clean 1 mL syringe with blunted 20G needle to take the sample (200 μL). Line was then flushed with saline (1% heparin) and closed. During the whole time of sampling the line was closed well with forceps to avoid bloodspilling.

**Analytical methods**

Blood glucose was measured with a glucose meter (Abbott, the Netherlands). After blood collection and measuring glucose, samples were immediately chilled on ice in tubes containing 5 μL heparin (10%, diluted in saline) and centrifuged for 15 min at 3000 rpm. Plasma was then stored at -20 °C until further analysis. Plasma concentrations of corticosterone were measured in duplicate using radioimmunoassay kits (Biochemicals, California, USA). The amount of sample-, standards-, label-, antibody and precipitating reagent as described in the manufactures protocol, were divided by four. Radioimmunoreactivity was measured with a gamma counter (Wizard 2 PerkinElmer, Waltham, Massachusetts, USA). The intra assay variation-coefficient of the immunoassays was <20%. Plasma [6,6-²H₂] glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS), EGP was calculated using Steele equations [12].

**Statistics**

Statistical analysis was performed using a repeated-measure analysis of variance (rmANOVA) (IBM SPSS statistics 24.0, Chicago, USA) to test for effects of time during the infusion of saline in the metabolic cages. A difference was considered significant when $p<0.05$.

**RESULTS AND DISCUSSION**

We present a novel technique in which we combine three already described cannulation techniques [7, 15] but with clear technical advancements, since we combined a never before shown ascending and descending catheter at the same location in the carotid artery, making it possible to combine infusions towards the brain and the assessment of EGP and insulin sensitivity. The two catheters in the carotid artery were successfully placed in 37 of 41 rats and together with the insertion of a jugular vein catheter, the total time of surgery from initiation of anesthesia to the end of the surgery took about 1.5 hours. We noticed that it was important to insert the carotid catheters as quick as possible, since the carotid artery will constrict over time.

All animals lost body weight in the first 2 days after surgery (max 15% of body weight) but recovered and gained body weight quickly thereafter, and 7 days post-surgery all animals had regained their pre-operative weight (figure 3A). Animals showed no signs of discomfort after surgery.

Part of the animals (n = 13) were put in the metabolic cages to measure energy
During the experiment, we found no difficulties in connecting the animals in the metabolic cages. All the animals reached the steady state for glucose enrichment after 90 minutes, and infusion of saline did not elicit changes in EGP as measured by rmANOVA (figure 3B, p=0.43) or blood glucose (rmANOVA, p = 0.58, data not shown) in this period. Furthermore, plasma corticosterone concentrations did not significantly change while animals were in the metabolic cage nor during the infusion of saline towards the brain (figure 3C, rmANOVA, p=0.24). This indicates, together with a lack of changes in energy expenditure during infusion (figure 3D; rmANOVA, p=0.12) and no signs of discomfort, that the animals were not disturbed during this period. In addition, energy expenditure was comparable (between 5 and 7 kcal/kg/h during lights on) to earlier findings using the same rat strain, in the same metabolic system [16].

We are aware of the disrupted blood flow after insertion of the ascending carotid catheter, which is expected to recover within several days. This is evidenced by [8], who show that, when infusing glucose via the intracarotid artery, there is an increase in neuronal activation bilaterally in the hypothalamus as measured by c-fos immunocytochemical stainings.
In addition they report a bilateral distribution of India ink to midline hypothalamic structures following unilateral carotid infusions. Thus, infusions via this method can bilaterally reach the brain.

Concomitantly infusing the stable isotope and taking blood samples from the jugular vein with a double-bore catheter [17], could have been used as an alternative method, excluding the need of the descending carotid catheter. However, this method is not suitable for the continuous infusion of the stable isotope since part of the isotope will be taken out during sampling and will interrupt the continuous flow. Furthermore, after every blood sample, saline is infused through the lines to flush the dead space of the line, which again would also interfere with a continuous infusion.

We measured plasma corticosterone concentrations in the samples taken according to the schedule to measure blood glucose and EGP [18], which was the main outcome of this novel method. This gave us the possibility to determine whether during the sampling period the HPA-axis was activated as a sign of disturbing the animals during the infusion. We did not observe clear differences during infusion as compared to the baseline measure. We did not, however, measure corticosterone during the first hour, and could have missed initial changes in plasma corticosterone concentrations as corticosterone increases and recovers quickly after a stressor [19]. Of note, animals were given at least one hour to recover from handling – the stressor, before drawing the first basal blood samples and start the continuous infusions. Another method to draw blood samples, which could give a more accurate of corticosterone levels, is automated serial sampling in which up to 8 animals at a time can be sampled at a fixed time interval throughout the experiment [17]. This technique could well be used with a jugular vein cannula, however in our view, automated and serial sampling via de carotid artery is technically not possible due to the high pressure of the artery and in addition the variation in flowing rate of the artery between rats. Furthermore, serial sampling with a large amount of samples as described in [19], is only possible when assays require a relatively small amount of plasma, which is impossible when one wants to measure blood glucose, EGP and corticosterone.

Several technical challenges may occur during procedures. Blockage or leakage of the ascending carotid catheter is observed in about 15% of the animals. These animals were directly taken out of the experiment. In some cases, it was not possible to draw blood from the descending carotid (could be due to a small block somewhere in the catheter). But, in case infusion via this catheter is still possible, animals may receive the stable isotope via the carotid artery and blood can be withdrawn via the jugular vein. When this switch was used, we did not observe differences in glucose parameters.

In summary, we describe a novel double cannulation technique, which enables to measure glucose turnover in basal and/or insulin stimulated conditions while infusing substances towards the brain via systemic circulation, without disturbing the animal. This physiological route is preferable above ICV injections which bypass the BBB and can only be given in small amounts, cause tissue damage and can be a stressful procedure, which can interfere with outcomes on glucose metabolism.
REFERENCES


