Pituitary adenylate cyclase-activating polypeptide stimulates glucose production via the hepatic sympathetic innervation in rats

Running title: PACAP regulation of glucose production

Chun-Xia Yi¹, Ning Sun¹,², Mariette T Ackermans³, Anneke Alkemade⁴, Ewout Foppen¹,⁴, Jing Shi², Mireille J Serlie⁴, Ruud M Buijs⁵, Eric Fliers⁴ & Andries Kalsbeek¹,⁴

1. Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience, Amsterdam, The Netherlands;
2. Dept Neurobiology, Tongji Medical College of Huazhong University of Science and Technology, Hubei, China.
3. Dept Clinical Chemistry Lab Endocrinology, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands;
4. Dept Endocrinology & Metabolism, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands.
5. Instituto De Investigaciones Biomedicas UNAM, Ciudad Universitaria, Mexico City, Mexico.

Correspondence:
Chun-Xia Yi MD
Email: c.yi@nin.knaw.nl

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Objective - The unraveling of the elaborate brain networks that control glucose metabolism presents one of the current challenges in diabetes research. Within the central nervous system the hypothalamus is regarded as the key brain area to regulate energy homeostasis. The present study aimed to investigate the hypothalamic mechanism involved in the hyperglycemic effects of the neuropeptide Pituitary Adenylyl Cyclase-Activating Polypeptide (PACAP).

Research Design and Methods - Endogenous glucose production (EGP) was determined during intracerebroventricular (i.c.v.) infusions of PACAP-38, VIP or their receptor agonists. The specificity of their receptors was examined by co-infusions of receptor antagonists. The possible neuronal pathway involved was investigated by: 1) local injections in hypothalamic nuclei, 2) retrograde neuronal tracing from the thoracic spinal cord to hypothalamic pre-autonomic neurons together with Fos immunoreactivity, and 3) specific hepatic sympathetic or parasympathetic denervation to block the autonomic neuronal input to liver.

Results - I.c.v infusion of PACAP-38 increased EGP to a similar extent as a VIP/PACAP-2 (VPAC2) receptor agonist. I.c.v. administration of VIP had significantly less influence on EGP. The PACAP-38 induced increase of EGP was significantly suppressed by pre-infusion of a VPAC2 but not a PAC1 receptor antagonist; as well as by hepatic sympathetic but not parasympathetic denervation. In the hypothalamus, Fos immunoreactivity induced by PACAP-38 was co-localized within autonomic neurons in paraventricular nuclei (PVN) projecting to preganglionic sympathetic neurons in the spinal cord. Local infusion of PACAP-38 directly into the PVN induced a significant increase of EGP.

Conclusions - This study demonstrates that PACAP-38 signaling via sympathetic pre-autonomic neurons located in the PVN is an important component in the hypothalamic control of hepatic glucose production.
To maintain glucose homeostasis, a complex glucose sensing and regulatory system has developed within the central nervous system (CNS), involving hypothalamic and hindbrain areas (1;2). Also a recent study in humans evidenced the sensitivity of the hypothalamus to small changes in blood glucose levels (3). Despite evidence from animal data for roles in glucose homeostasis of several hypothalamic nuclei such as the arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and paraventricular nucleus (PVN), the major part of the neurochemical make-up of this hypothalamic network is largely unknown.

Pituitary Adenylyl Cyclase-Activating Polypeptide (PACAP) is a highly conserved peptide from the secretin-glucagon superfamily (4), which exerts a plethora of peripheral and central effects. PACAP also affects food intake and glucose and lipid metabolism (5;6). PACAP-immunoreactive neurons and their receptors (5) are distributed widely, including the CNS and peripheral tissues such as adipose tissue and pancreas. Highest concentrations in the CNS are found in the hypothalamus (7), where PACAP-38 is produced abundantly in VMH neurons and high release rates are reported for the PVN (7-9). PACAP from retinal ganglion cells is released primarily in the hypothalamic suprachiasmatic nuclei (SCN) (10). However, since tissue specific knock-out animals are not available, it is unclear whether the effects of PACAP on glucose metabolism are mediated via central or peripheral pathways.

PACAP is structurally related to vasoactive intestinal peptide (VIP). PACAP and VIP receptors have been classified as 3 different subtypes: PACAP-1 receptors (PAC1R) (11), VIP/PACAP-1 (VPAC1R) and -2 receptors (VPAC2R) (12;13). VIP and PACAP have a comparable affinity for VPAC1 and VPAC2 (14), while PACAP also has a high affinity for the PACAP-specific PAC1 receptor (15). Both PAC1R and VPAC2R are broadly expressed in the brain, including the hypothalamus, while VPAC1R is mainly expressed in the cerebral cortex and the hippocampus (16). The demonstration that central administration of PACAP-38 not only induces Fos immunoreactivity in several brain areas, including the PVN (17), but also increases plasma glucose levels (18), suggests that central PACAP-38 may play an important role in (glucose) metabolism.

In order to determine the importance of central PACAP signaling in regulating glucose metabolism, we combined intracerebroventricular (i.c.v.) administration of PACAP-38, VIP and/or their receptor specific agonists and antagonists, with measurements of plasma glucose concentration, endogenous glucose production (EGP), metabolic clearance rate (MCR), gluconeogenesis (GNG) as well as glycogenolysis. In order to delineate further the hypothalamic output pathway we combined the i.c.v. administration of PACAP-38 with Fos immunohistochemistry and retrograde tracing from the intermediolateral column (IML) of the thoracic spinal cord, we measured plasma glucose concentrations and EGP after intra-nuclear PVN administration of PACAP-38, and performed i.c.v. infusions of PACAP-38 in rats after a specific hepatic sympathetic or parasympathetic denervation.

RESEARCH DESIGN AND METHODS

Animals. Experiments were conducted with approval of the animal care
committee of the Royal Netherlands Academy of Arts and Sciences. Male Wistar rats weighing 300-350 g (Harlan, Netherlands) were housed four per cage at room temperature (21±1°C) with a 12/12-h light-dark schedule (lights on at 07.00 h). Food and water were available ad libitum, unless stated otherwise.

**Surgery preparation.** Animals underwent surgery under anesthesia with 0.8 ml/kg Hypnorm i.m. (Janssen, High Wycombe, Buckinghamshire, UK), and 0.4 ml/kg Dormicum s.c. (Roche, Almere, Netherlands).

Silicon catheters were inserted into the right jugular vein and left carotid artery for i.v. infusions and blood sampling, respectively. With a standard David Kopf stereotaxic apparatus, i.c.v guiding cannula were placed into the lateral cerebral ventricle, double i.c.v. cannulations were applied for i.c.v. pre-infusion studies (see below). Intra-nuclear infusion guiding cannula were placed into the PVN. All coordinates were adapted from our previous study (Suppl.1 in the online appendix available at [http://diabetes.diabetesjournals.org](http://diabetes.diabetesjournals.org)).

Fixation, anti-clotting and connection of catheters and probes were applied as described before (19), as well as the hepatic sympathetic, parasympathetic and sham denervation (20).

For visualizing the PVN pre-autonomic neurons, 0.05-0.1µl retrograde neuronal tracer cholera toxin subunit B (Conjugated with Alexa Fluar 555, CTB-AF555, Molecular Probed, Eugene, OR) was injected into the thoracic IML unilaterally between T6 and T7 (where the liver-projecting sympathetic motor neurons are found) (21).

Experiments were performed after at least 10 days recovery and with pre-surgery bodyweights regained. Food was restricted overnight at 20g before the experimental day, and experimental blood sampling was started 5 hours after light on.

**Stable isotope tracer dilution, i.c.v infusions and blood sampling schedule.** To compare the effects of the different PACAP-38/VIP receptor (ant)agonists on EGP, the isotope dilution method was applied, using [6.6-2H2]glucose (prime (8.0 µmol/5min) - continuous (16.6 µmol/h) infusion (>99% enriched); Cambridge Isotopes, Andover, USA). To dissect the separate contributions of GNG and glycogenolysis to EGP, in an additional vehicle and PACAP-38 i.c.v. infusion group, the MIDA method was applied using U-13C6-glucose and 2-13C1-glycerol (22;23) (U-13C6-glucose 3.53 mg/ml, 2-13C1-glycerol 22.8 mg/ml, bolus 2910µl/hr for 5 min, and continuous infusion 500µl/hr). Blood samples (0.3ml/sample) were taken at t=-5 min for background tracer enrichment, and at t=90, 95 and 100min for determining basal plasma parameters and isotope enrichment after having reached isotope equilibrium (data are presented by averaging these three time points). After t=100min, single i.c.v. infusions of different drugs (and vehicle, 5 µl/hr), were started immediately and lasted 120 min; direct infusion of PACAP-38 into PVN was performed with a 5 fold lower concentration of PACAP-38 as compared to the i.c.v. infusions and a 2 µl/hr infusion rate. From t=120 - 220min 6 blood samples were taken with 20 min interval for determining plasma parameters. After the last blood sample, liver tissue was collected under deep anaesthesia for quantitative real-time-PCR (RT-PCR) studies, and subsequently animals were perfusion fixed (Suppl.2) for Fos immunoreactivity (Fos-ir) and localizing CTB-AF555 tracer. Single Fos or double
Fos/CTB, and Fos/arginine-vasopressin (AVP) immunohistochemistry was performed. In order to investigate the effect of PACAP-38 on plasma epinephrine concentrations an additional experiment with i.c.v. infusions of PACAP-38 and vehicle was performed. Blood was sampled (2.0ml/sample) only at t=-5 and 90min.

All drugs used for i.c.v. infusions were dissolved in a 5-fold stock solution in purified water containing 30% glycerol, and diluted to working solution by purified water; except for the VPAC2R antagonist which was dissolved in 0.5% acetic acid neutralized by NaHCO$_3$ (this vehicle did not differ from the common vehicle with respect to its effects on plasma glucose concentration (p=0.29), EGP (p=0.30) and MCR (p=0.10)). PACAP-38 for the micro-infusions was dissolved in 0.9% saline.

For experiments that needed pre-infusion and co-infusion of receptor antagonists, a pre-infusion of the receptor antagonist was started immediately after t=100min through the left i.c.v. cannula, 10 min later the PACAP-38 was started via the right i.c.v. cannula.

Analytical methods. Plasma samples were stored at -20°C for analysis. By using radioimmunoassay kits, plasma insulin (t=100, 140, 180, 220min), glucagon (t=90, 120, 160, 200min) [LINCO Research (St. Charles, MO, USA) and corticosterone concentrations (all time points) (ICN Biomedicals, Costa Mesa, CA) were measured. Plasma isotope enrichments were measured using gas chromatography-mass spectrometry (GCMS) and GNG was calculated by mass isotopomer distribution analysis (MIDA) (23-25). Plasma epinephrine and liver noradrenalin were measured by High Performance Liquid Chromatography (HPLC) with fluorescence detection after derivatisation of the catecholamines with diphenylethylenediamine. Glycogen content was measured by spectrophotometry. Liver expression of phosphoenolpyruvate carboxykinase (Pepck) and Glucose-6-Phosphatase (G6Pase) mRNA were examined by RT-PCR (suppl.3) (19). Fos-ir positive cells in the PVN from vehicle, PACAP-38, VIP (5nmol/hr), VPAC1R, VPAC2R agonist i.c.v. infusion and direct injection of PACAP-38 into the PVN were quantified (Suppl.4) (26).

Calculation and statistics. Data from all experiments are presented as means±SEM. EGP was calculated from isotope enrichment using adapted Steele equations (27). Glucose concentration and EGP were analyzed using a repeated-measures analysis of variance (ANOVA), to test for the effects of peptide infusions and time. Plasma epinephrine, corticosterone, glucagon and insulin, as well as liver noradrenalin, glycogen content and mRNA expression were analyzed using one way ANOVA, to compare the average between experimental groups.

RESULTS

I.c.v. PACAP-38 induces hyperglycemia by stimulating endogenous glucose production. To investigate the possible contribution of the hypothalamic PACAP/VIP systems to peripheral glucose metabolism, we administered PACAP-38 and VIP, as well as a specific VPAC1-R agonist ($K^{15},R^{16},L^{27}$VIP/GRF) (28) and VPAC2-R agonist (Hexa-His' VIP (2-27) (29), by i.c.v. infusion into the lateral cerebral ventricle. Upon i.c.v. infusion of PACAP-38 for 120 min (1nmol/hr, n=6), both plasma glucose concentration and EGP were increased in comparison with the basal state at t=100min (~70% and
PACAP regulation of glucose production

ANOVA detected a significant effect of Time (difference between time points is expressed by Time effects \(P_t\)) \((p_t<0.001\) for both parameters). The PACAP-38 induced increase was also significant as compared to the vehicle control group \((n=6)\) (difference between groups is expressed by Group effects \(P_g\)) \((p_g=0.001\) for plasma glucose and EGP, respectively) (Figs.1A&B). I.c.v. infusion of VIP at the same concentration \((1\text{nmol/hr}, n=4)\) did not significantly change plasma glucose concentrations \((p_t=0.15)\), but did cause a significant increase of EGP \((p_g=0.004)\). This increase was slightly higher than vehicle control \((p_g=0.049)\), but significantly lower than PACAP-38 \((p_g=0.04)\). When a 5-fold higher concentration of VIP \((5\text{nmol/hr}, n=5)\) was administered, a clear hyperglycemia was induced \((-50\%, p<0.001)\), which was significantly higher than that in the \(1\text{nmol/hr}\) and vehicle \((p_g<0.001)\) groups, and not significantly different from PACAP-38. EGP also increased significantly \((p<0.001)\) in the \(5\text{nmol/hr}\) group, it was significantly higher than that of the vehicle control group \((p_g=0.02)\), but did not differ from \(1\text{nmol/hr}\) VIP and was still significantly lower than the PACAP-38 group \((p_g=0.03)\). MCR increased significantly in both PACAP38 \((p=0.007)\) and VIP \((1\text{nmol/hr})\) \((p=0.002)\) treated animals and showed a significant decrease from \(t=160\text{min}\) to \(t=220\text{min}\) in the VIP \(5 \text{nmol/hr}\) group \((p=0.004)\) (Fig.1C). The vehicle group showed no effects of Time for the MCR. No Group effect was found, although MCR was higher in PACAP-38 than in vehicle.

PACAP-38 induces hyperglycemia via VPAC2R. I.c.v. infusion of the VPAC1R-agonist \((1\text{nmol/hr}, n=5)\) or the VPAC2R-agonist \((1\text{nmol/hr}, n=6)\) resulted in quite different effects on plasma glucose concentration and EGP (Fig.1D&E). I.c.v. infusion of the VPAC2R agonist caused a significant increase of plasma glucose concentrations \((-40\%, p<0.001)\), which was higher than the effect of vehicle \((p_g<0.001)\) or the VPAC1R agonist \((p_g=0.001)\); it also significantly increased EGP \((-65\%, p<0.001)\), an effect which was again significantly stronger than that of vehicle \((p_g<0.001)\) and VPAC1R agonist \((p_g=0.02)\). Although the effects were shorter lasting and less pronounced than those of PACAP-38, no significant differences were found between the VPAC2R agonist and PACAP-38 with regard to their effects on plasma glucose concentration and EGP. I.c.v. infusion of the VPAC1R agonist also increased plasma glucose concentrations, especially during later stages \((p=0.002)\) and differed significantly from vehicle \((p_g=0.02)\), but this increase was significantly lower than that of PACAP-38 \((p_g=0.01)\), as well as of that of VPAC2R agonist \((p_g=0.001)\). On the other hand, the VPAC1R agonist did not significantly change EGP, its effect did not differ significantly from the vehicle group, and was significantly lower than that of the VPAC2R agonist \((p_g=0.001)\) and PACAP-38 \((p_g=0.004)\) (Figs.1C&D). MCR during VPAC1R agonist decreased \((p=0.001)\) significantly, but did not differ from vehicle and PACAP-38. During VPAC2R agonist infusion, MCR increased \((p<0.001)\) significantly, it was significantly higher than vehicle \((p_g=0.03)\), and not different from PACAP-38. VPAC1R and VPAC2R are also expressed in peripheral tissues, such as pancreas and adipose tissue (30). In order to exclude the possibility that the glucoregulatory effects of PACAP/VIP resulted from leakage of brain infusates into the systemic circulation, we also infused the VPAC1R and VPAC2R agonists (with the same
conditions as used for the i.c.v. administration) directly into the systemic circulation. No significant changes in either plasma glucose concentrations or EGP were detected (Supplemental Figs. 1A&B).

Because no specific PAC1R agonist was available, we tested the possible involvement of the PAC1R in the glucoregulatory effects of PACAP-38 by i.c.v. pre-infusion of the PAC1R specific antagonist PACAP-6-38 (31) (5nmol/hr) together with PACAP-38 (1nmol/hr) (n=4). The co-infusion of PACAP-6-38 with PACAP-38 still significantly increased plasma glucose concentrations ($p_t<0.001$, $p_g=0.70$ vs. PACAP-38), EGP ($p_t<0.001$, $p_g=0.80$ vs. PACAP-38) and MCR ($p_t=0.049$, $p_g=0.80$ vs. PACAP-38) (Figs.1G-I).

To test further the role of the VPAC2R, the VPAC2R specific antagonist myristoyl-K12-VIP(1-26)-KKGGT (32) was pre-infused (5nmol/hr) together with PACAP-38 (1nmol/hr) (n=6). Plasma glucose levels did not rise significantly, but were still significantly higher than the vehicle control ($p_g=0.003$). EGP showed a significant increase over the time ($p_t=0.003$), but was not different from its own vehicle control. Compared with the single i.c.v PACAP-38 infusion group, the co-infusion almost completely blocked the increases of plasma glucose ($p_g=0.03$) and EGP ($p_t<0.001$) evoked by PACAP-38. MCR increased as well ($p_t<0.001$), but significantly less than single PACAP-38 ($p_g=0.02$), and not different from its own vehicle control (Figs.1G-I).

The inhibitory effect of i.c.v. administered PACAP-38 on food intake involves POMC-containing neurons in the ARC, and can be blocked by the co-administration of the MC3-R/MC4-R specific antagonist SHU9119 (18). Here, co-infusion of the MC3-R/MC4-R antagonist SHU9119 (5nmol/hr) with PACAP-38 (1nmol/hr) still increased plasma glucose concentrations ($p_t<0.001$, EGP ($p_t<0.001$) and MCR ($p_t=0.049$), and did not significantly change the hyperglycaemic, EGP and MCR stimulatory effects of single i.c.v. PACAP-38 (Figs.1G-I).

I.c.v. administration of the PAC1R (n=4), VPAC2R (n=5) or SHU9119 (n=6) on their own did not significantly affect plasma glucose concentrations or EGP (Supplemental Figs.1C&1D).

**Contribution of glycogenolysis and GNG to the hyperglycemic action of PACAP-38.** In animals receiving dual-isotope tracer infusions for analyzing glycogenolysis and GNG, i.c.v. administration of PACAP-38 (n=6) significantly increased plasma glucose concentrations and EGP ($p_t<0.001$ and $p_t<0.002$, respectively), i.e., comparable to the previous results with the [6.6-2H2]glucose method (compare Figs.2A&C and Figs.1A& B). In the vehicle group (n=5), EGP slightly decreased during the study period ($p_t=0.034$). In both groups (i.e., vehicle and PACAP-38), the fractional contribution from GNG to EGP slowly increased along the 120 min i.c.v. infusion (from 19% to 28% in vehicle, $p_t=0.002$; and from 18% to 23% in the PACAP group, $p_t=0.008$), without significant differences between the two groups (Fig.2B). The absolute rate of GNG, however, increased significantly more in the PACAP-38 group than in the vehicle group ($p_g=0.027$).

Consistently, RT-PCR showed a significantly increased mRNA expression of Pepck compared to vehicle, VPAC1R and VPAC2R agonists (Fig.2D); mRNA expression of G6Pase in PACAP-38 showed a trend towards increase in comparison with vehicle (Fig.2E). In the
PACAP-38 group, glycogenolysis significantly increased by 61% ($p<0.001$), whereas in the vehicle group glycogenolysis significantly decreased by 27% ($p=0.005$) (Fig. 2C). In line with the increased glycogenolysis, liver glycogen stores were significantly lower in the PACAP-38 animals (Fig. 2F).

**PACAP-38 induces Fos immunopositive nuclei in sympathetic pre-autonomic PVN neurons.** I.c.v. administration of PACAP-38, VPAC2R agonist or VIP (5nmol/hr), but not the VPAC1R agonist or vehicle, induced Fos-ir in the PVN, periventricular nucleus (Pe) and ARC in the hypothalamus, as well as the nucleus incertus (NI) in the pons (Fig.3&4). In the PVN, the PACAP-38 and VPAC2R-agonist induced Fos-ir nuclei mainly located in its rostro-medial part, with considerable fewer labelled nuclei extending into the lateral magnocellular compartment of the PVN. Fos/AVP double staining showed that few of the VPAC2R agonist-induced Fos-ir neurons contained AVP-ir (Fig.4A&B). Differently, VIP induced strong Fos-ir both in the rostro-medial and lateral magnocellular compartment and co-localization with AVP (Fig.4C&D). Counting Fos-ir showed PACAP-38, VIP and VPAC2R agonist induced similarly significant more Fos-ir in PVN neurons than vehicle and VPAC1R agonist groups (Fig.4E). This specific Fos-ir pattern was also observed after 90 min i.c.v. infusion of PACAP-38, with similar numbers of Fos-ir nuclei in the PVN (Fig.4E).

Among the animals with CTB-AF555 injections in the IML, three showed clear CTB labelling in the CNS, including the corticospinal projection neurons, lateral hypothalamus, PVN, locus coeruleus, parabrachial nucleus, and the nucleus ambiguous. In the PVN, CTB-labelled neurons were concentrated in the dorsomedial and less in the ventrolateral subdivision. Several CTB-labelled neurons co-localized Fos-ir induced by PACAP-38. Among the total of CTB labelled neurons 33±3% per section also contained Fos-ir nuclei. The percentage of CTB labelled neurons among the total of Fos-ir nuclei in PVN per section is ~3%. This co-localization was not observed in other brain areas (Fig.4F).

**Hepatic sympathetic denervation blocks the hyperglycemic action of PACAP-38.** Basal plasma glucose concentrations, EGP and MCR were not influenced by hepatic sympathetic denervation (HSX), parasympathetic denervation (HPX) or sham denervation (shamX) (Figs.5A-C). After HSX, the hyperglycemic and EGP, but not MCR, stimulatory effects of PACAP-38 were significantly reduced. Plasma glucose concentrations of HSX animals no longer showed a significant increase and significantly lower than HPX ($p_{g}=0.02$) and shamX groups ($p_{g}=0.01$). Although EGP still showed a significant increase ($p_{t}<0.001$) in the HSX group, and was higher than that of vehicle-treated non-denervated animals ($p_{g}=0.02$), it was also significantly lower than that of PACAP-38 treated non-denervated ($p_{g}=0.005$), shamX ($p_{g}=0.036$) and HPX ($p_{g}=0.036$) animals. No differences were found between HPX and shamX in plasma glucose concentration and EGP, nor between shamX or HPX and the PACAP-38 treated non-denervated animals in Experiment-1. The PACAP-38 induced increase in MCR was affected in none of the denervated groups. The effectiveness of HSX was validated by a significantly reduced noradrenalin (NA) content in the liver of HSX (26.17±1.32ng/g) as compared to HPX (57.93±5.88ng/g) ($p<0.001$), as well as shamX (58.00±4.30ng/g) ($p<0.001$).
animals. No difference in hepatic NA content was found between HPX and shamX groups. In addition, NA in both HPX and shamX was not different from intact rats without abdominal surgery (58.96±9.24ng/g).

**Local infusion of PACAP-38 into the PVN increases plasma glucose concentration and EGP.** To verify that i.c.v. PACAP-38 induced changes in glucose metabolism largely depend on its direct effects on PVN neurons, we infused PACAP-38 locally into the PVN (n=5). Plasma glucose concentration and EGP (Figs.6A&6B) increased significantly as compared to vehicle control (n=5) (p<0.001 and p=0.01, respectively), as well as compared to animals that had their cannula placed outside the boundaries of the PVN area (n=8) (p=0.001 and p=0.02, respectively). MCR increased significantly as compared to vehicle control (p=0.02, Fig.6C), but did not reach significance as compared to the misplaced group (p=0.07). The number of Fos-ir nuclei in PVN only increased when PACAP-38 was injected into the PVN (Fig.6D-F).

**Plasma glucoregulatory hormones.** Plasma epinephrine concentrations increased significantly after i.c.v. infusion of PACAP-38 (Fig.7). The average concentration of plasma glucagon increased significantly after PACAP-38, VIP (5nmol/hr) as well as VPAC2R, but not the lower concentration of VIP (1nmol/hr; not shown), VPAC1R agonist or vehicle infusion (Fig.8A). Furthermore, no significant difference was found between the glucagon responses of the VIP (5nmol/hr), PACAP-38 and VPAC2R-agonist treated groups. Mean plasma insulin concentrations were mildly elevated upon i.c.v. administration of PACAP-38 and the VPAC2R agonist, but neither of them reached statistical significance (Fig.8B). In HSX, HPX and sham animals, PACAP-38 infusion increased plasma glucagon concentrations to a similar level as did PACAP-38, VIP or VPAC2R-agonist in non-denervated animals. After HSX, mean plasma insulin levels were not increased during the administration of PACAP-38. In HPX and shamX animals only the increase in the shamX group reached significance. Plasma corticosterone concentrations showed a significant increase in all groups, with all drug-induced responses significantly higher than those of the vehicle controls. No significant differences were found between PACAP-38, VIP and their receptor-specific agonists, or between HSX, HPX and shamX groups (Fig.8C).

**DISCUSSION**
The present results clearly show that hypothalamic PACAP signalling is tightly involved in the control of glucose metabolism. I.c.v. administration of PACAP-38 causes a pronounced increase in EGP and lasting hyperglycemia. Follow-up experiments using intra-nuclear infusions, co-infusions of specific VIP/PACAP receptor (ant)agonists, Fos immunohistochemistry, retrograde tracing and specific denervations of the autonomic liver innervation, suggest a role for the hypothalamic PACAP system in the control of glucose metabolism via a specific central pathway involving VPAC2R and pre-autonomic neurons in the PVN.

Although PACAP and VIP share several similar functions, including stimulating the release of prolactin and other pituitary hormones (4), they are basically distinct peptides, with different origins and distributions in both brain and periphery. Hypothalamic VIP-ir fibers, including those in the PVN, almost solely derive from the SCN (33). On the other hand,
PACAP regulation of glucose production

PACAP-ir fibers are widely distributed within the hypothalamus, especially in its medial part (34). Moreover, the hypothalamic PACAP innervation is derived from different origins, including intra-hypothalamic sources such as the VMH, and extra-hypothalamic sources such as the bed nucleus of the stria terminalis (BNST) (9;35), brainstem (9;36) and retina (10). Despite the equal affinity of PACAP and VIP for the VPAC2R as shown by in vitro receptor binding studies (13), we found quite different central effect of PACAP-38 and VIP on glucose metabolism. Similar differences were observed in other studies. In the adrenal, PACAP is approximately 100-fold potent more than VIP in evoking secretion of catecholamine (37), and in the SCN, PACAP is 1000-fold more potent than VIP at altering the phasing of the circadian rhythm (10) despite both tissues expressing VPAC2R (38;39).

Specific denervation of the sympathetic input to the liver obliterated the EGP increase produced by i.c.v. administration of PACAP-38. This indicates that PACAP-38 stimulation of hepatic glucose production partly depends on an intact hepatic sympathetic autonomic innervation. On the other hand, the only partial suppression of EGP in HSX indicates that PACAP-38 also employs other pathways to stimulate liver glucose production, such as increased corticosterone, epinephrine and glucagon release by adrenal and pancreas. PACAP activation of the thoracic IML projecting pre-autonomic neurons in the PVN strongly suggest that the EGP stimulating action involves activation of liver projecting pre-autonomic neurons by PACAP-38 (40). This idea is supported by the presence of VPAC2R-immunoreactivity in the dorsal and ventral PVN (41), the stimulatory effects of focal PVN infusions of PACAP-38 on plasma glucose concentrations and EGP, as well as the effects of HSX.

PACAP-38 also has stimulatory effects on glucose uptake, as the MCR induced by PACAP-38 was higher than in vehicle, and this probably indicates that also PVN pre-autonomic neurons projecting to glucose utilizing tissues such as muscle and adipose tissue are stimulated. MCR was not influenced by any of the denervation procedures, thus when EGP was suppressed by HSX, also the plasma glucose concentration was lowered in the PACAP-38+HSX group (Fig.5A). The VPAC2R-agonist induced the highest MCR response and blocking VPAC2R, but not PAC1R, suppressed the PACAP-38-induced increase of MCR, suggesting that in the CNS PACAP-38 stimulates glucose disposal mainly via VPAC2R. Systemically, however, PAC1R seems to play a major role by regulating glucose disposal via direct actions in pancreas and by changing insulin activity as based on data from PAC1R-KO animals (42). Thus different CNS and peripheral PACAP/VIP receptors may play roles in keeping glucose homeostasis.

Glucose is produced in the liver by glycogenolysis and GNG. It is largely unknown which specific signals, besides circulating glucoregulatory hormones, control the balance between glycogenolysis and GNG. The present study shows that PACAP-38 increased glycogenolysis in line with early studies on the stimulatory effects of electrically stimulated sympathetic nerves on liver glycogenolysis (43). At present it is unclear which group(s) of PACAP-containing neurons is responsible for the brain control of glucose metabolism. PACAP knock-out animals have been shown to have an impaired
PACAP regulation of glucose production

counterregulatory response to hypoglycemia (44). Recovery from hypoglycemia is believed to be mainly processed by the VMH and the sympathoadrenal pathway (45). As PACAP mRNA is highly expressed in the VMH (34), it is possible that activation of PACAP neurons in the VMH, and subsequently the PACAP-containing projections to the PVN, are involved in the hypothalamic mechanism necessary to stimulate hepatic glucose production upon hypoglycemic challenges. PACAP is also involved in acute and chronic stress responses via the PVN-CRH neurons, the HPA-axis and the sympathetic nervous system (46-49). The PACAP system could thus be an important gateway to control hepatic glucose production during stress (46-49). Finally, as PACAP expression in the VMH is also under the influence of estrogen (50) and leptin (51), the currently revealed glucoregulatory effects of PACAP might also be part of a brain circuit that connects the reproductive and adiposity systems with energy metabolism.

In summary, we present a neuronal pathway by which PACAP-38 activates hypothalamic pre-autonomic neurons that control sympathetic nerves innervating the liver, resulting in a hyperglycemia almost entirely due to an increase in hepatic glycogenolysis. Future experiments should reveal the specific physiological stimuli and PACAP neurons responsible for activation of these glucoregulatory neurons.

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FIGURE LEGENDS

**Figure 1** The effects of central administration of PACAP-38, VIP and/or their receptor agonists and antagonists on plasma glucose concentration, endogenous glucose production (EGP) and metabolic clearance rate (MCR). **A-C**, I.c.v. infusion of PACAP-38 increases both plasma glucose concentrations and EGP. Similar responses could not be obtained with equimolar or 5 times higher concentrations of VIP. MCR during PACAP-38 and VIP 1nmol/hr increased and VIP 5nmol/hr decreased along the i.c.v. infusion. **D-F**, I.c.v. infusion of the VPAC2R agonist resulted in significantly higher plasma glucose levels, EGP and MCR responses than the VPAC1R agonist. The almost similar responses induced by the VPAC2R agonist and PACAP-38 indicate that the plasma glucose, EGP and MCR changes induced by PACAP-38 are predominantly mediated via the VPAC2R. **G-I**, Co-infusion of PACAP-38 with the PAC1R or VPAC2R antagonist shows that antagonizing VPAC2R, but not PAC1R, blocks a large part of the plasma glucose (**G**), EGP (**H**) and MCR (**I**) responses evoked by PACAP-38, thus confirming that PACAP-38 mainly acts on central VPAC2Rs to regulate plasma glucose concentrations, EGP and MCR. Co-infusion of PACAP-38 with the MC3R/MC4R antagonist Shu9119 still induces changes of plasma glucose, EGP and MCR. Thus the melanocortin signaling pathway is not likely to be involved in the central PACAP-38 effects on glucose production. Antagonist infusions were started at time point “a” through the left i.c.v. cannula and the PACAP-38 infusion was started 10 min later at time point “b” through the right i.c.v. cannula. The starting and end time points for the i.c.v. infusion of the different drugs in all groups are illustrated in **B** and **H** respectively, i.e., immediately after t=100 min or t=110 min. The gray symbols and lines in figures **D-I** are a repeat of the PACAP-38 and vehicle data in figures **A-C**. Data are presented as mean±SEM.

**Figure 2** Fractional contributions of gluconeogenesis and glycogenolysis to the hyperglycemic action of i.c.v. PACAP-38. **A**, The changes in plasma glucose concentrations in animals with the dual-isotope tracer experiment are very much comparable to those of the single-isotope tracer experiments. **B**, The fractional gluconeogenesis (GNG) of the PACAP-38 group increases slightly, but not significantly, slower than that in the vehicle group. **C**, The absolute flux of GNG and glycogenolysis contribute very differently to the PACAP-38-induced changes in total endogenous glucose production (EGP). **D** and **E**, Relative expression of hepatic phosphoenolpyruvate carboxykinase (Pepck) and Glucose-6-Phosphatase (G6Pase) mRNA after drug infusions. PACAP-38 profoundly increased Pepck mRNA expressions, while vehicle, VPAC1R and VPAC2R did not. **F**, Liver glycogen content at the end of the experiment is significantly lower in PACAP-38 as compared to vehicle treated animals, indicating more pronounced glycogen depletion in the PACAP-38 treated animals. Data are presented as mean±SEM. # P<0.05 vs. Vehicle, * P<0.05 vs. PACAP-38.

**Figure 3** I.c.v. infusion of PACAP-38, VIP or their receptor agonists result in different Fos-ir patterns in different brain areas. **A**, PACAP-38 (1nmol/hr) induced Fos-ir in paraventricular nucleus (PVN) and periventricular area (Pe). A similar Fos-ir pattern can be seen after VIP (5nmol/hr) (**B**, and Fig.4C and D) and VPAC2R (1nmol/hr) (**Fig.4A and 4B**), but not VIP (1nmol/hr) (not shown), VPAC1R (1nmol/hr) (**C**) or vehicle (**D**). **E & F**,
I.c.v. administration of PACAP-38 (E), VIP (5nmol/hr) and VPAC2R agonist, also induced Fos-ir in the nucleus incertus (NI) located in the pons. No such Fos-ir was visible in the NI with vehicle (F) or VPAC1R agonist infusion (not shown). OT: optic tract, III: third cerebral ventricle, IV: fourth cerebral ventricle. Scale bar: A-D, 400 µm; E and F, 200 µm.

**Figure 4** Fos-ir neurons induced by i.c.v. administration of PACAP-38 and the VPAC2R agonist mainly locate in the medial paraventricular nucleus (PVN). VIP induced Fos-ir also extends to the lateral PVN. A, Few of the VPAC2R agonist induced Fos-ir neurons contain arginine vasopressin (AVP) (high magnification in B). C, VIP induced strong Fos-ir both in the rostro-medial and lateral magnocellular compartment of the PVN with considerable co-localization with AVP (arrow in D, with high magnification). E, The number of Fos immunoreactive (Fos-ir) positive nuclei in the PVN was very much comparable between the PACAP-38, VIP 5nmol/hr and VPAC2R agonist groups. Moreover, animals perfused 90 min instead of 120 min after the start of the i.c.v. PACAP-38 infusion had identical numbers of Fos-ir nuclei in the PVN. F, Retrogradely labelled pre-autonomic neurons in the PVN (red, as pointed by arrow head) after an injection of Cholera toxin subunit B (CTB) into the thoracic intermediolateral column (IML). Some of the retrogradely labelled neurons are also activated by the i.c.v. administration of PACAP-38 as indicated by the co-localized Fos-ir (green, pointed by arrow). III: third cerebral ventricle. Scale bar: A and C 100 µm; B, D and F, 50 µm.

**Figure 5** Selective denervation of the sympathetic innervation (HSX), but not the parasympathetic innervation (HPX) to the liver or sham denervation (ShamX), largely blocks the hyperglycemic and endogenous glucose production (EGP) stimulatory effects of PACAP-38. Metabolic clearance rate (MCR), however, was not influenced by any type of denervation. The starting and end time point for the i.c.v. infusion of PACAP-38 or vehicle in all groups is illustrated in B.

**Figure 6** Local infusion of PACAP-38 into the paraventricular nucleus (PVN) increases plasma glucose concentration (A), endogenous glucose production (EGP) (B) and metabolic clearance rate (MCR) (C). The starting and end time point for the i.c.v. infusion of PACAP or vehicle in all groups is illustrated in B. Misplacement of infusion cannula outside the boundaries of the PVN area produced a significantly smaller response. The effects of local administration of PACAP-38 or vehicle in the PVN on Fos-ir in the PVN are indicated in (D) and (E), respectively. Local infusion of PACAP-38 produced a Fos-ir response in the PVN that was very much similar to the one produced by the i.c.v. administration of PACAP-38 (compare Fig.6D and 3A). Cannula positions are indicated by asterisks in (D) and (E). F, Counting of Fos-ir nuclei in PVN from the three injection groups. #, p<0.001 vs. PACAP-38-PVN group. Scale bar: 100µm.

**Figure 7** Plasma epinephrine concentrations increased significantly after 90 min i.c.v. infusion of PACAP-38. Data are presented as mean±SEM, # P<0.05 vs. Basal state, *P<0.05 vs. Vehicle control.

**Figure 8** Plasma insulin, glucagon and corticosterone responses to the i.c.v infusion of PACAP-38, VIP and their receptor agonists, in liver intact and denervated animals. A, I.c.v. infusion of PACAP-38, VIP and VPAC2R agonist significantly increased plasma glucagon concentrations as compared to the basal state as well as compared to the vehicle control. Hepatic sympathetic, parasympathetic or sham denervation has no influence on the stimulatory effect of PACAP-38 on plasma glucagon. B, In the same animals only the plasma insulin increase in the sham-denervated animals reached
statistical significance. C, All groups showed increased plasma corticosterone concentrations (as compared to basal), but no difference was found between the different drug infusion groups, although all differed significantly from the vehicle infusion. Data are presented as mean±SEM, # P<0.05 vs. Basal state, *P<0.05 vs. Vehicle control.

Figure 1
Figure 2

A

B

C

D

E

F

PACAP regulation of glucose production

Figure 2
Figure 5

A

B

C

Figure 6

A

B

C

D

E

F

PACAP regulation of glucose production
Figure 8

A

B

C

PACAP regulation of glucose production