Hypothalamic regulation of metabolism
Zhang, Z.

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PVN TRH and cold

5 Administration of thyrotropin-releasing hormone (TRH) in the hypothalamic paraventricular nucleus (PVN) of male rats mimics the metabolic cold defense response

Z. Zhang\textsuperscript{1}, F. Machado\textsuperscript{2,5}, L. Zhao\textsuperscript{5}, C.A. Heinen\textsuperscript{1}, E. Foppen\textsuperscript{1}, M.T. Ackermans\textsuperscript{3}, J.N. Zhou\textsuperscript{4}, P.H. Bisschop\textsuperscript{1}, A. Boelen\textsuperscript{1}, E. Fliers\textsuperscript{1}, A. Kalsbeek\textsuperscript{1,5}

\textsuperscript{1} Department of Endocrinology and Metabolism, Academic Medical Centre (AMC), University of Amsterdam, Amsterdam, the Netherlands

\textsuperscript{2} Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

\textsuperscript{3} Department of Clinical Chemistry, Laboratory of Endocrinology, Academic Medical Centre, University of Amsterdam, Amsterdam, the Netherlands

\textsuperscript{4} CAS Key Laboratory of Brain Function and Disease, School of Life Science, University of Science and Technology of China, Hefei, China

\textsuperscript{5} Hypothalamic Integration Mechanisms, Netherlands Institute for Neuroscience (NIN), Amsterdam, the Netherlands
Abstract

During cold exposure, energy sources such as glucose and fatty acids are mobilized to generate heat, at the same time thyrotropin-releasing hormone (TRH) expression in the paraventricular nucleus of hypothalamus (PVN) is increased. TRH effects on glucose metabolism and thermoregulation have been studied in many brain regions but not in the PVN. However, in addition to TRH neurons, the PVN also contains a dense TRH-immunoreactive innervation. We investigated the role of TRH release in the PVN in the control of energy mobilization. Male Wistar rats exposed to a cold environment (4°C) for 2-hours showed increased body temperature, locomotor activity and plasma corticosterone concentrations, but blood glucose concentrations similar to that of room temperature control animals. In line with that, TRH administration in the PVN for 2-hours, at room temperature, also promptly increased body temperature, locomotor activity and plasma corticosterone concentrations. In addition, TRH administration in the PVN markedly increased blood glucose concentrations and endogenous glucose production (EGP) compared to saline controls. Selective hepatic sympathetic or parasympathetic denervation reduced the TRH-induced increase in glucose concentrations and EGP. Gene expression data indicated increased gluconeogenesis in liver and lipolysis in brown adipose tissue (BAT), both after 2-hours of cold exposure and TRH administration in the PVN. We conclude that TRH administration in the rat PVN largely mimics the metabolic and behavioural changes induced by cold exposure indicating a probable link between TRH in the PVN and cold defence.

Key words

Glucose, autonomic nervous system, body temperature, liver, brown adipose tissue
**Abstract**

During cold exposure, energy sources such as glucose and fatty acids are mobilized to generate heat, at the same time thyrotropin-releasing hormone (TRH) expression in the paraventricular nucleus (PVN) of hypothalamus (PVN) is increased. TRH effects on glucose metabolism and thermoregulation have been studied in many brain regions but not in the PVN. However, in addition to TRH neurons, the PVN also contains a dense TRH-immunoreactive innervation. We investigated the role of TRH release in the PVN in the control of energy mobilization. Male Wistar rats exposed to a cold environment (4°C) for 2-hours showed increased body temperature, locomotor activity and plasma corticosterone concentrations, but blood glucose concentrations similar to that of room temperature control animals. In line with that, TRH administration in the PVN for 2-hours, at room temperature, also promptly increased body temperature, locomotor activity and plasma corticosterone concentrations. In addition, TRH administration in the PVN markedly increased blood glucose concentrations and endogenous glucose production (EGP) compared to saline controls. Selective hepatic sympathetic or parasympathetic denervation reduced the TRH-induced increase in glucose concentrations and EGP. Gene expression data indicated increased gluconeogenesis in liver and lipolysis in brown adipose tissue (BAT), both after 2-hours of cold exposure and TRH administration in the PVN. We conclude that TRH administration in the rat PVN largely mimics the metabolic and behavioural changes induced by cold exposure indicating a probable link between TRH in the PVN and cold defence.

**Key words**

Glucose, autonomic nervous system, body temperature, liver, brown adipose tissue

**Introduction**

An efficient cold defence mechanism requires an accurate coordination of energy sources (e.g. glucose and fatty acids) and heat production (e.g. shivering and adaptive thermogenesis). The central TRH system is known to be involved in thermoregulation and glucose metabolism, two important adaptive systems during cold exposure, through both endocrine [1, 2] and neuronal pathways [3, 4]. Indeed, TRH administration, either peripherally or centrally, has strong effects on glucose metabolism [5-9] and body temperature regulation [10-16]. In addition, animals with TRH deficiency exhibit impaired cold tolerance [17-19] and, paradoxically, hyperglycemia [20].

Earlier studies have shown that TRH administration in the anterior hypothalamus induces hyperthermia [14, 15, 21] and in the lateral hypothalamus results in hyperglycaemia [7]. These studies indicate an important role for the efferent projections of TRH neurons in thermogenesis and glucose metabolism. TRH receptors are also present in the PVN [22-24], TRH-immunoreactive axon terminals making synaptic contacts with both TRH and non-TRH neurons [22]. The PVN is an important integration centre for the neuro-endocrine and autonomic regulation of energy metabolism [25-27]. It is also one of the prime and first hypothalamic regions to be infected after pseudorabies virus tracing from the liver [28] and BAT [29]. Studies of our own group and others have explicitly implicated the PVN in the central control of glucose metabolism [30-32] and brown adipose tissue (BAT) thermoregulation [33-37] via the sympathetic nervous system (SNS). Intriguingly, in the hypothalamus, early cold exposure increases TRH mRNA and peptide expression exclusively in the PVN [38], pointing to a key role for TRH in the PVN during cold exposure.

In view of the above data, we hypothesized that TRH release in the PVN plays a critical role in the control of two essential responses during cold adaptation, i.e., glucose metabolism and thermoregulation. Therefore, in the present study, we compared the effects of cold exposure and TRH administration locally in the PVN on circulating thyroid hormone, glucose and corticosterone concentrations, body temperature, locomotor activity as well as relevant gene expression in liver and BAT. Our data indicate a central role for TRH in the PVN in regulating glucose metabolism and thermogenesis during a cold challenge.
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Materials and methods

Animals

Male Wistar rats weighing 300-350g (Harlan Nederland, Horst, Netherlands) were housed individually in a 12h-12h light-dark cycle environment (lights on at 07:00), with a room temperature of 23±2°C and 60±5% humidity. Chow and water were provided ad libitum unless stated otherwise. All procedures were approved by the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences.

Surgery

After one week acclimatization in the facility, animals were anesthetized by an intramuscular injection of a mixture of ketamine (80 mg/ml), xylazine (8 mg/ml) and atropine (0.1 mg/ml) (4:2:1, v:v:v) at a dosage of 1 ml/kg body weight. Animals were allowed to recover from the surgery for at least one week and until their body weight had reached pre-surgery levels.

Experimental groups

Experiment-1 - Eighteen animals were used in three experimental conditions. Animals in group 1 (n=6) received nano-thermo loggers (Star-Oddi Ltd., Iceland) both subcutaneously (Tsc) just above the interscapular brown adipose tissue and intraperitoneally attached to the abdominal muscle layer (Tip) for continuous body temperature recording. During the experiment, rats were housed in calorimetric cages within an air-conditioned chamber (TSE Systems GmbH, Bad Homburg, Germany) at either room temperature (23°C) for 3 days with a cold challenge (4°C) for two hours at day 2, or at room temperature for 3 days continuously. Metabolic parameters, i.e., O2 consumption, respiratory exchange rate (RER) and locomotor activity were monitored. Animals were studied in a crossover design in both conditions with a one-week interval (i.e., each animal served as its own control). Experimental groups 2 (n=5) and 3 (n=7) received an intra-atrial silicone cannula implanted through the right jugular vein for blood sampling. During the experiment, rats were exposed to either room temperature (23°C; group 2) or cold temperature (4°C; group 3) for two hours in a modified fridge where blood sampling was possible, without touching the animals, through the jugular vein cannula and a hole in the door.

Experiment-2 - Fifty-eight animals received bilateral microdialysis (MD) probes adjacent to the PVN for retrodialysis using a stereotaxic apparatus (anteroposterior: -1.8 mm, lateral: +2.0 mm, depth: -7.9 mm, angle of arm: 8°). The U-shape tip of MD probe was...
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1mm long, 0.5mm wide and 0.2 mm thick. Probe placements were checked in 20 μm cresyl violet stained brain sections of each animal at the end of the experiment. Animals in Experiment-2A (TRH MD, n=18) also received a jugular vein cannula for stable glucose isotope infusion and a carotid artery cannula for blood sampling. In Experiment-2B (TRH MD, n=20), in addition hepatic sympathectomy (HSX), hepatic parasympathetic (HPX) or sham surgery was performed as reported previously [39]. Briefly, sympathetic or parasympathetic nerve bundles were cut using microsurgical instruments. Any connective tissue attachments between the hepatic artery and portal vein were also dissected, eliminating any possible nerve crossings. Animals with sham denervation surgery, received the same manipulation as described above except for cutting the nerve. During TRH MD experiments, animals were connected to a metal collar hanging by a counter-balanced arm on the day before the actual experiment, i.e., the two-hour TRH administration. Food was restricted to 20 g overnight, which is only slightly less than the average consumption per night (i.e., 24±2 g) for rats with a similar body weight. Two hours before the experiment, rats were connected to tubing for blood sampling, tracer infusion and TRH administration. In Experiment-2A and 2B, three blood samples were taken at -25 min, -20 min and 0 min for basal glucose and corticosterone measurement. At time 0 (between 12:00 and 13:45), TRH (40 mM, Sigma-Aldrich, Germany) or Ringer was perfused via the MD probes into the PVN at a speed of 3 μl/h for two hours. Blood samples were withdrawn every twenty minutes and blood glucose concentrations were determined. To assess endogenous glucose production (EGP), [6, 6-H2] glucose (D2 glucose) was used as a tracer. Blood samples were taken at -125 min for background enrichment, and at -30 min, -25 min and -20 min to determine enrichment during the equilibrium state and every 20 min from time 0 to 120 min to determine enrichment during the experiment. In Experiment-2C (TRH MD, n=20), animals were only provided with subcutaneous nano-thermo loggers and MD probes as described above. TRH or Ringer was perfused via MD probes into the PVN for two hours. Locomotor activity was monitored by pressure-sensitive baseplates below the home cage [40], with a sample rate of once every 6 minutes. No blood samples were taken and the animals were decapitated immediately after the two hours of TRH or Ringer administration.

**Blood and plasma analysis**

Blood glucose concentration was determined by a glucose meter (Freestyle™, Abbott, the Netherlands) during the experiment. Plasma [6, 6-H2] glucose enrichment was measured by gas chromatography-mass spectrometry (GCMS) as described previously [41]. EGP was calculated by the method of Steele [42]. Plasma corticosterone concentration was measured with radio-immunoassay kits (MP Biomedicals, Orangeburg, USA).
Plasma triiodothyronine (T3) and thyroxine (T4) concentrations were determined using an in-house radio-immunoassay as reported before (inter-assay variation T3, 6.2% and T4, 7.3%; intra-assay variability T3, 3.6% and T4, 6.6%) [43]. Plasma thyroid-stimulating hormone (TSH) was determined by a Chemiluminescent Immunoassay, using the Immulite 2000 and a rat specific standard (Siemens, Munich, Germany) with an inter-assay variation of ±9% and an intra-assay variation of 3-4%. Within the same experiment, all samples were measured in one run to prevent inter-assay variation.

Table 5.1 Primers used in qPCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>symbol</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
<th>Products length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Gapdh</td>
<td>TGAACGGGAAGCTCAGTG</td>
<td>TCCACCACCCCTGTGTGCTGTA</td>
<td>306</td>
</tr>
<tr>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
<td>Hprt</td>
<td>GCAGTACAGCCCCAAATGG</td>
<td>AACAAGTCTGGGCCTGTATCCAA</td>
<td>84</td>
</tr>
<tr>
<td>Uncoupling protein 1</td>
<td>Ucp1</td>
<td>AATCAGCTTTGGCTCCCTGT</td>
<td>GCTTTGTGCTTGAACAGTACCTGTGATTCTGA</td>
<td>181</td>
</tr>
<tr>
<td>Deiodinase, type 1</td>
<td>Dio1</td>
<td>GAA GTG CAA GTG CTGGA</td>
<td>CTGGCCAGAAGAAGATcccA</td>
<td>59</td>
</tr>
<tr>
<td>Deiodinase, type 2</td>
<td>Dio2</td>
<td>TCCTGGAGCGTTTCTCTTCATTG</td>
<td>GCACCTGTGCTTTTGCTGTTGAT</td>
<td>78</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor alpha</td>
<td>Ppara</td>
<td>TCACACAATGCAATCCGTTT</td>
<td>GGTTGTGGATATGTTTGAACAGTACCTGTGATTCTGA</td>
<td>177</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor gamma</td>
<td>Pparg</td>
<td>CAGGGAAGACAACAGACTAA</td>
<td>GGGGGTGATATTGTGTTG</td>
<td>95</td>
</tr>
<tr>
<td>Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha</td>
<td>Pgc1a</td>
<td>CAATGAATGCAGCGGTCTTA</td>
<td>GTGTGAGGAGGTCTACCTGTAGTACCAGTACCTGTGATTCTGA</td>
<td>195</td>
</tr>
<tr>
<td>Hormone-sensitive lipase 1</td>
<td>Hsl</td>
<td>AAAAGGCCATCGGTGAAGGT</td>
<td>AGGAGGCTCATTCACGCCTGCCTGTGATTCTGA</td>
<td>231</td>
</tr>
<tr>
<td>Lipoprotein lipase 1</td>
<td>Lpl</td>
<td>CACACAGCATGGATTTACACA</td>
<td>ACCTGCAAGACGTGGACAGT</td>
<td>124</td>
</tr>
<tr>
<td>Adrenergic receptor, beta 3</td>
<td>Adrb3</td>
<td>CTTCCACGCTAGCCCTGT</td>
<td>AGCAATTCCCCGATGTCCACACAGCATGGATTTACACA</td>
<td>140</td>
</tr>
<tr>
<td>Glucokinase</td>
<td>Gck</td>
<td>CAAGCTGCACCCGAGCTT</td>
<td>TGATTGGATATTGTGTTGACACAGTACCTGTGATTCTGA</td>
<td>85</td>
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</tbody>
</table>
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<th>Products length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose-6-phosphatase</td>
<td>G6pase</td>
<td>CCCATCTGGTTCCACATTC AA</td>
<td>GGCGCTGTCCAAAAAGA ATC</td>
<td>109</td>
</tr>
<tr>
<td>Phosphoenolpyruvate carboxykinase 1</td>
<td>Pepck</td>
<td>GTGCCCTCTTGCTCTACGA A</td>
<td>GGTGCGCATGATGACC TT</td>
<td>116</td>
</tr>
<tr>
<td>Glucose Transporter Type 4</td>
<td>Glut4</td>
<td>GGGCTGTGAGTGAGTGTGCT TTC</td>
<td>CAGCGAGCAAGGCTAG A</td>
<td>150</td>
</tr>
<tr>
<td>Farnasoid X receptor (Nr1H4)</td>
<td>Fxr</td>
<td>CAAGTGACCTCCACGCACC AA</td>
<td>AAGGAACATGGCCTCGA CT</td>
<td>300</td>
</tr>
</tbody>
</table>

**RNA isolation and Real Time PCR (qPCR)**

Total RNA from BAT and liver was isolated using Machery-Nagel RNA isolation kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) and RNA yield was determined using the DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, Delaware USA). cDNA synthesis was performed with equal RNA input using the Transcriptor First Strand cDNA synthesis kit (Roche Molecular Biochemicals, Mannheim, Germany). As a control for genomic DNA contamination, we included a cDNA synthesis reaction without reverse transcriptase. Quantitative PCR was performed using the LightCycler 480 (Roche Molecular Biochemicals) and SensiFAST™ SYBR No-ROX mix (Bioline, GmbH, Germany). Disposables used during PCR were from Greiner Bio-one (Alphen aan den Rijn, Netherlands). The primers used during PCR were from Greiner Bio-one (Alphen aan den Rijn, Netherlands). The primers used for qPCR are listed in Table 1. Quantification was performed using the LinReg software. PCR efficiency was checked individually and samples with a deviation of more than 5% of the mean were excluded from analysis. Values were expressed relative to the geometric mean of the reference gene: Gapdh and Hprt.

**Statistical analysis**

Data are expressed as mean ± standard error of the mean (SEM). All analyses were performed with GraphPad Prism 6.05. Differences between two groups were analyzed using an independent two-tailed Student’s t test. The difference between TRH and control groups during time-series blood sampling experiments was evaluated by two-way ANOVA with repeated measurements (ANOVA RM) followed by Sidak post hoc analysis. Significance was defined at a level of P<0.05.
Results

Cold exposure increases plasma thyroid hormones, TSH and corticosterone concentrations

Plasma T3 and T4 concentrations were significantly increased 30 minutes after cold exposure (Treatment, $F_{(1, 4)} = 27.44$, $P=0.006$; Time*Treatment, $F_{(3, 12)} = 8.264$, $P=0.003$ and Treatment, $F_{(1, 4)} = 9.531$, $P=0.037$; Time*Treatment, $F_{(3, 12)} = 5.412$, $P=0.014$) (Figure 1A and B). The increases of T3 and T4 continued until the end of the experiment. Plasma TSH was also markedly increased during cold exposure with a peak value about 60 minutes after the start of the cold exposure (Treatment, $F_{(1, 4)} = 38.63$, $P=0.003$; Time*Treatment, $F_{(3, 12)} = 16.37$, $P<0.001$) (Figure 1C), as were plasma corticosterone concentrations (Treatment, $F_{(1, 9)} = 10.35$, $P=0.015$; Time*Treatment, $F_{(4, 36)} = 3.084$, $P=0.028$) (Figure 1D).

![Figure 5.1](image-url) Effects of cold exposure on plasma thyroid hormones and corticosterone concentrations. T3 (A), T4 (B), TSH (C) and corticosterone (D) concentrations were determined at 0 min, 30 min, 60 min and 120 min after starting the cold exposure. Data are shown as the absolute increase (delta) compared to the basal samples before treatments. Basal values are shown in Table 2. N= 5-7 per group. Stars indicate significant differences between groups at individual time points. Post-hoc * $P<0.05$, ** $P<0.01$, *** $P<0.001$. 

Chapter 5
Cold exposure increases plasma thyroid hormones, TSH and corticosterone concentrations

Plasma T3 and T4 concentrations were significantly increased 30 minutes after cold exposure (Treatment, $F(1, 4) = 27.44$, $P=0.006$; Time*Treatment, $F(3, 12) = 8.264$, $P=0.003$ and Treatment, $F(1, 4) = 9.531$, $P=0.037$; Time*Treatment, $F(3, 12) = 5.412$, $P=0.014$) (Figure 1A and B). The increases of T3 and T4 continued until the end of the experiment. Plasma TSH was also markedly increased during cold exposure with a peak value about 60 minutes after the start of the cold exposure (Treatment, $F(1, 4) = 38.63$, $P=0.003$; Time*Treatment, $F(3, 12) = 16.37$, $P<0.001$) (Figure 1C), as were plasma corticosterone concentrations (Treatment, $F(1, 9) = 10.35$, $P=0.015$; Time*Treatment, $F(4, 36) = 3.084$, $P=0.028$) (Figure 1D).

**Cold exposure increases body temperature, energy expenditure and locomotor activity, but does not affect blood glucose**

When exposed to a cold environment, both subcutaneous ($T_{sc}$) and core ($T_{ip}$) body temperature were increased within the first hour ($T_{sc}$: Treatment, $F(1, 5) = 10.97$, $P=0.021$;
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Time*Treatment, $F(8,40) = 1.733$, $P=0.121$; Time$^{\text{ip}}$: Treatment, $F(1,5) = 10.95$, $P=0.021$; Time*Treatment, $F(8,40) = 3.000$, $P=0.010$) (Figure 2A and B). $T_{\text{ip}}$ was increased earlier (i.e., 20 min after the onset of cold exposure) and for a longer period (up to 80 minutes) than $T_{\text{sc}}$, but both normalized within two hours (Figure 2A and B), i.e., when still exposed to cold. Energy expenditure as measured by $O_2$ consumption was significantly increased in the cold treatment group ($Treatment, F(1,5) = 61.92, P<0.001$; Time*Treatment, $F(8,40) = 11.92$, $P<0.0001$) (Figure 2C), as was locomotor activity ($Treatment, F(1,5) = 11.89$, $P=0.018$; Time*Treatment, $F(8,40) = 3.339$, $P=0.005$) (Figure 2E). The RER in the cold exposed group slowly decreased during the first hour of cold exposure and stabilized at this lower value during the second hour of cold exposure ($Treatment, F(1,5) = 4.414$, $P=0.090$; Time*Treatment, $F(8,40) = 3.124$, $P=0.008$) (Figure 2D). Blood glucose concentrations remained stable throughout the two-hour exposure to a cold environment ($Treatment, F(1,10) = 2.026$, $P=0.185$; Time*Treatment, $F(4,40) = 1.099$, $P=0.370$) (Figure 2F).

Retrodialysis of TRH in the PVN increases TSH and corticosterone concentrations, and increases plasma thyroid hormones after two hours

During the two-hour period of TRH MD in the PVN, plasma T3 and T4 concentrations did not change (Figure 3A and C) ($Treatment, F(1,6) = 0.2682$, $P=0.62$; Time*Treatment, $F(3,18) = 0.7414$, $P=0.54$ and $Treatment, F(1,6) = 4.573$, $P=0.08$; Time*Treatment, $F(3,18) = 1.932$, $P=0.16$), although at the final time point ($t=120$ min) plasma T4 was significantly higher in the TRH group as compared to the control group ($P=0.022$). TSH concentrations in plasma were significantly increased from $t=20$ min onwards ($Treatment, F(1,6) = 12.77$, $P=0.013$; Time*Treatment, $F(3,18) = 9.016$, $P<0.001$) (Figure 3E). In Experiment-2C, plasma T3, T4 and TSH concentrations at sacrifice, i.e., two hours after the start of the TRH administration, were significantly increased (Figure 3B, D and F). Plasma corticosterone concentrations also increased after TRH administration, reaching peak levels after 80 min ($Treatment, F(1,9) = 33.11$, $P<0.001$; Time*Treatment, $F(8,72) = 7.025$, $P<0.0001$) (Figure 3G).
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Figure 5.3 Plasma thyroid hormones and corticosterone concentrations during and after TRH administration in the PVN. Plasma T3 (A), T4 (C), TSH (E) and corticosterone (G) concentrations at different time points during TRH administration. Data are shown as the absolute increase (delta) compared to the basal samples before treatments. N=2-6 per group. Basal values are shown in Table 2. Plasma T3 (B), T4 (D) and TSH (F) concentrations at sacrifice after two-hours of TRH administration in the PVN. N=8-10 per group. Stars in A, C and E indicate significant difference between groups at individual time points. Stars in B, D and F indicate t-test significance between treatments. Post-hoc *, P<0.05; **, P<0.01; ***, P<0.001.
Figure 5.4 Effects of TRH administration in the PVN on subcutaneous body temperature and locomotor activity. A: Daily rhythms in body temperature before and during the experiment. Grey areas without pattern indicate the active phase (i.e., 12-h dark period). The two striped bars marked by arrows indicate the times that the animals were connected to respectively the chain and tubing. The dark grey stippled bar indicates the 2-h period with TRH or vehicle administration. B: Zoomed in figure from A showing body temperature changes two hours before and during the 2-h TRH or vehicle administration. C: Locomotor activity two hours before and during the 2-h infusion experiment. Shadows along the line denote the standard error. N=8-10 per group. Two-way ANOVA was performed from t=0 till t=120min. Stars indicate the overall significance of the Treatment effect. ** P<0.01.

Retrodialysis of TRH in the PVN increases body temperature and locomotor activity

Heat production is one of the most critical adaptations in coping with cold. To test whether TRH could mimic the effects of cold on body physiology, we measured body temperature and locomotor activity before and after TRH administration. As expected, T_sc showed a stable day/night rhythm in both the TRH group and the control group with an amplitude of about 1.5°C and higher levels being present in the active phase (night) than in the inactive phase (day) (Figure 4A). On the day of the experiment, we observed a fast increase in T_sc already 15 minutes after starting the TRH administration (Treatment, F (1, 17) = 60.10, P<0.0001; Time*Treatment, F (24, 408) = 19.92, P<0.0001). Moreover, the T_sc was up to 1.6°C higher after TRH administration compared to the pretreatment
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period (Figure 4B). Along with increased body temperature, locomotor activity was also significantly increased in the TRH group compared to the pretreatment period ($F_{(1, 16)} = 18.90, P<0.001$) (Figure 4C). Body temperature and locomotor activity were not affected in the Ringer perfused control group (Figure 4B and C).

![Figure 5.5](image)

Figure 5.5 Effects of TRH administration in the PVN and hepatic sympathectomy (HSX) or parasympathectomy (HPX) on blood glucose concentrations and EGP. A and C: Blood glucose concentrations. B and D: Endogenous glucose production (EGP). Data are shown as the absolute increase (delta) compared to the basal samples before treatments. Basal values are shown in Table 2. N=5-10 per group. * indicates significant difference between Sham TRH MD and HSX TRH MD groups at individual time points. # indicates significant difference between Sham TRH MD and HPX TRH MD groups at individual time points. Post hoc *, # P<0.05, ** P<0.01, ***, ### P<0.001.

**Retrodialysis of TRH in the PVN increases blood glucose concentrations and endogenous glucose production through the autonomic nervous system**

Blood glucose concentrations increased more than one mmol/L within 40 minutes after the start of the TRH infusion compared to the control group ($Treatment, F_{(1, 9)} = 27.95, P<0.0001$; $Time*Treatment, F_{(8, 72)} = 8.050, P<0.0001$) (Figure 5A). EGP in the TRH group showed a gradual increase after the start of the TRH perfusion, which only reached significance 100 min after the start of the perfusion ($Treatment, F_{(1, 9)} = 6.531, P=0.007$; $Time*Treatment, F_{(8, 72)} = 4.461, P<0.001$) (Figure 5B). To investigate whether the TRH effects on glucose metabolism were mediated via the autonomic nervous system, we repeated the previous experiment in hepatic sympathetic or parasympathetic denervat-
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ed rats. TRH administration significantly increased blood glucose concentrations in sham-operated animals compared to base line \( (P<0.0001) \). HSX greatly reduced this effect (55.2% decrease compared to sham), but did not completely abolish the TRH-induced increase of glucose \( (Treatment, F \ (1, 17) = 12.10, P=0.003; Time*Treatment, F \ (8, 136) = 6.330, P<0.0001) \) (Figure 5C). HPX also attenuated the TRH-induced hyperglycemia (47.6% decrease compared to sham) \( (Treatment, F \ (1, 12) = 5.485, P=0.037; Time*Treatment, F \ (8, 96) = 2.669, P=0.011) \) (Figure 5C). In addition, both HSX and HPX significantly decreased the TRH induced increase in EGP (36.5% and 45.1% decrease compared to sham, respectively) \( (Treatment, F \ (1, 15) = 8.174, P=0.012; Time*Treatment, F \ (8, 120) = 5.315, P<0.0001 \) for HSX vs. Sham and \( Treatment, F \ (1, 12) = 12.16, P=0.005; Time*Treatment, F \ (8, 96) = 7.518, P<0.0001 \) for HPX vs. Sham) (Figure 5D).

Both cold exposure and retrodialysis of TRH in the PVN increase gluconeogenesis and lipolysis gene expression in liver and BAT, respectively

In order to understand the mechanism behind the glucose metabolism and thermogenesis during cold exposure and TRH administration, we analyzed the expression profile of a number of genes that are involved in glucose metabolism and heat production, in liver and BAT, respectively. In the liver (Figure 6A and C), \( Pgc1a \) mRNA expression was not changed by cold exposure, but tended to increase \( (t \ (10.1)=-2.049, P=0.067) \) after TRH administration. Expression of \( Pgc1b \) was significantly increased in both the cold and TRH treatment group. \( Pparg \) gene expression was decreased in response to TRH treatment, but not cold exposure. Gene expression of \( Pepck \), a key enzyme in controlling gluconeogenesis, was greatly increased in both the cold and TRH group. Glucose-6-phosphatase (\( G6pase \)), the enzyme that hydrolyzes glucose-6-phosphate into free glucose, showed a trend towards increased expression after both cold exposure \( (t \ (12)=-2.079, P=0.060) \) and TRH administration \( (t \ (17)=-1.826, P=0.086) \). Farnasoid X receptor (\( Fxr \)) expression was markedly decreased by TRH, but not cold treatment. Expression of the T3 responsive gene type 1 deiodinase (\( Dio1 \)) did not change after either cold exposure or TRH administration.

In BAT (see Figures 6B and D), gene expression of the transcriptional factor \( Pgc1a \) was upregulated both by cold and TRH treatment compared to their controls. Expression of \( Pparg \) was increased by TRH administration, but unchanged during cold exposure. As to glucose metabolism, glucose transporter 4 (\( Glut4 \)) showed a trend towards an increase \( (t \ (9.9)=-2.090, P=0.06) \) and glucokinase (\( Gck \)) mRNA was significantly increased after TRH treatment, but both were unchanged after cold exposure. For lipid metabolism in BAT, lipoprotein lipase 1 (\( Lpl \)) and hormone-sensitive lipase (\( Hsl \)), two enzymes
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involved in triglyceride uptake and hydrolysis, were significantly increased in the TRH group. Cold exposure induced a significant increase in Lpl but not Hsl expression. Type 2 deiodinase (Dio2), an essential enzyme converting T4 into T3, was markedly increased (5-fold) by both cold and TRH treatment. Gene expression of uncoupling protein (Ucp1) did not change by either cold or TRH treatment. Expression of the beta-adrenergic receptor Adrb3 did not differ significantly in either the cold or TRH group compared to their controls. Heat shock protein (Hsp90) was significantly increased by both cold and TRH treatment.

Figure 5.6 Gene expression changes in liver (A and C) and BAT (B and D) after cold exposure (A and B) and TRH administration in the PVN (C and D). Gene expressions are relative to the geometric mean of two reference genes (Gapdh and Hprt) and normalized to the corresponding controls. N=6-10 per group. *, P<0.05; **, P<0.01; ***, P<0.001.

Table 5.2 Basal sample values for figures 1, 3 and 5.

<table>
<thead>
<tr>
<th>Group</th>
<th>T3 (nmol/l)</th>
<th>T4 (nmol/l)</th>
<th>TSH (mU/l)</th>
<th>Cortiosterone (ng/ml)</th>
<th>Glucose (mmol/l)</th>
<th>EGP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1</td>
<td>Control</td>
<td>0.90±0.10</td>
<td>65.80±7.85</td>
<td>1.03±0.12</td>
<td>11.75±5.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cold</td>
<td>0.99±0.06</td>
<td>74.4±1.60</td>
<td>2.41±0.22</td>
<td>6.43±0.37</td>
<td></td>
</tr>
</tbody>
</table>
metabolic and behavioral changes in line with a key role in the cold-defensive response. That the increased release of TRH in the PVN activates a neural mechanism that induces increased liver gluconeogenesis, as suggested by the enhanced gene expression of production, with the liver as the major glucose-producing organ [45, 46]. Despite within the PVN TRH and/or non-TRH neurons are mediating these effects. We conclude which TRH neurons are responsible for this increased release in the PVN and whether by the increased hypothalamic expression of TRH. Future experiments should indicate show that the PVN is one of the target areas needed to effectuate the changes induced in the hypothalamus during cold exposure is well known [1, 38, 44]. Our current results mediated by the autonomic nervous system (ANS). The potent activation of TRH neurons TRH administration. The TRH-induced increase in glucose production was partly gluconeogenesis and thermogenesis, respectively, again after both cold exposure and cold exposure, central TRH administration also resulted in a pronounced increase of plasma corticosterone concentrations, a well-known stimulator of hepatic glucose production [54], HSX/HPX reduced the TRH-induced EGP increases significantly blocked the TRH-induced increase in plasma glucose. This HSX/HPX induced reduction of the TRH-induced hyperglycemia is probably due to a blockage of glucose production in liver, as both HSX and HPX also attenuated the TRH-induced increase in EGP. The neural mechanism of this effect is further stressed by the fact that although not changed, EGP was indeed at the same time increased EGP [45, 50, 51]. However, in contrast to cold increased fatty acid and glucose uptake and the increased expression of genes facilitating increased fatty acid and glucose uptake and the increased expression of genes facilitating energy utilization and metabolic activity in BAT, very much similar to the changes reported in TRH deficient mice [20]. The autonomic control of liver glucose metabolism significantly. Both cold exposure and TRH administration locally in the PVN rapidly, i.e., within 30 minutes, increased body temperature, locomotor activity, glucose production, plasma TSH and corticosterone concentrations. Molecular changes in liver and BAT were in line with these physiological data by showing increased expression of genes involved in gluconeogenesis and thermogenesis, respectively, again after both cold exposure and TRH administration. The TRH-induced increase in glucose production was partly mediated by the autonomic nervous system (ANS). The potent activation of TRH neurons in the hypothalamus during cold exposure is well known [1, 38, 44]. Our current results show that the PVN is one of the target areas needed to effectuate the changes induced by the increased hypothalamic expression of TRH. Future experiments should indicate which TRH neurons are responsible for this increased release in the PVN and whether within the PVN TRH and/or non-TRH neurons are mediating these effects. We conclude that the increased release of TRH in the PVN activates a neural mechanism that induces metabolic and behavioral changes in line with a key role in the cold-defensive response.

Plasma glucose homeostasis is maintained by balancing glucose uptake and glucose production, with the liver as the major glucose-producing organ [45, 46]. Despite increased liver gluconeogenesis, as suggested by the enhanced gene expression of Pepck, G6pase and Pgc1b, blood glucose concentrations did not change during the two-hour
cold exposure. Likely the increased glucose produced was utilized by heat-producing organs such as BAT and muscle, as several studies have shown that cold exposure induces increased glucose uptake and carbohydrate oxidation in muscle and BAT [47-49]. Interestingly, TRH administration locally in the PVN also upregulated gene expression in the gluconeogenic pathway in liver, e.g. *Pepck*, *G6pase* and *Pgc1* family (Figure 6C) and indeed at the same time increased EGP [45, 50, 51]. However, in contrast to cold exposure, central TRH administration also resulted in a pronounced increase of plasma glucose concentrations, probably because these animals were not exposed to cold. The increased glucose production is consistent with the previously reported central hyperglycemic effect of TRH [5, 52], but difficult to reconcile with hyperglycemia reported in TRH deficient mice [20]. The autonomic control of liver glucose metabolism by the PVN has been well recognized [32, 53]. Consistently, both HSX and HPX significantly blocked the TRH-induced increase in plasma glucose. This HSX/HPX induced reduction of the TRH-induced hyperglycemia is probably due to a blockage of glucose production in liver, as both HSX and HPX also attenuated the TRH-induced increase in EGP. The neural mechanism of this effect is further stressed by the fact that although MD TRH increased plasma corticosterone concentrations, a well-known stimulator of hepatic glucose production [54], HSX/HPX reduced the TRH-induced EGP increases despite an intact corticosterone response (data not shown). Notably, HSX and HPX each only blocked approximately half of the TRH-induced EGP and plasma glucose increase; indicating that most likely a total denervation is needed for a complete blocking effect.

Animals in a cold environment reduce their heat loss and enhance their metabolic rate in order to maintain thermal homeostasis [55, 56]. In line with this, we observed an accelerated metabolic rate as reflected by the increased O_2_ consumption during cold exposure and a resultant transient increase in body temperature. Cold exposure also led to increased locomotor activity, which may contribute to an increase in body temperature. These effects might be mediated by TRH as suggested by our current results and those of previous studies [57-60] [10] [14]. In fact, TRH administration in the PVN increased body temperature in a rather prompt and sustained way. At the molecular level, TRH administration in the PVN stimulated the expression of genes facilitating energy utilization and metabolic activity in BAT, very much similar to the changes induced by cold exposure. Elevated expression of *Lpl* and *Glut4* mRNA in BAT suggests increased fatty acid and glucose uptake and the increased expression of *Hsl*, an important enzyme for triglyceride hydrolysis, will result in an increased availability of free fatty acids for energy production. Taken together, the transcriptional changes show that both cold exposure and TRH administration in the PVN stimulate fuel recruitment
and mobilization (e.g., lipids and glucose) in BAT, an essential preparation for BAT thermogenesis [47, 61, 62]. Other changes in BAT in response to cold are mitochondrial biogenesis and adipocyte proliferation, which at the molecular level are revealed by an increased expression of transcription factors such as \( Pgc1a \) and \( Pparg \) [63-65]. TRH administration in the PVN indeed also significantly increased \( Pgc1a \) and \( Pparg \) mRNA, suggesting a boost of BAT activation. Additionally, the remarkable increase of \( Dio2 \) mRNA by both cold and TRH treatment, indicating an increase of local T3 production, provides another important step towards increased BAT thermogenesis [66, 67]. Surprisingly, expression of \( Ucp1 \), the key protein in BAT that uncouples substrate oxidation from ATP synthesis to heat production, did not change under either cold or TRH treatment. Of note, an increased BAT adrenergic responsiveness could serve as a pre-UCP1 step enhancing BAT thermogenesis [68]. It is possible that cold exposure or TRH administration increased adrenergic responsiveness, so that BAT heat production was elevated without changing UCP1. An earlier study by Lin showed that hyperthermia after an intrahypothalamic TRH injection in rats was due to both increased heat production and cutaneous vasoconstriction [69]. It is likely that a decreased heat loss also contributed to the increased temperature observed in our results.

TRH administration in the PVN increased plasma TSH concentrations shortly after the start of the perfusion. TSH has been suggested to have a thermogenic effect and can increase \( Ucp1 \) expression in rat brown adipocytes [70, 71]. At present, we cannot exclude that in addition to the neural effects of TRH, plasma TSH has a direct effect on glucose metabolism and thermoregulation, although \( Ucp1 \) expression in BAT was not upregulated. One may argue that the increased concentrations of plasma thyroid hormones caused the effects observed during TRH administration. However, we believe that the effects of thyroid hormone in the TRH MD experiment, if any, were subtle. First, the changes in TH levels occurred only at the end of the 2-hour TRH perfusion, i.e., long after the onset of the effects on glucose, corticosterone, locomotion and body temperature. Secondly, TRH induced hyperthermia cannot be mimicked by thyroid hormone administration alone as shown by previous studies [59]. Thirdly, expression of \( Dio1 \), a T3 sensitive gene in the liver, was not changed.

In addition to its hypophysiotrophic function, a separate population of TRH neurons in the PVN sends extensive projections to the brainstem and spinal cord to control autonomic responses, as well as to other brain areas important for glucose metabolism and thermoregulation [3, 72]. Several early studies demonstrated that the preoptic area of the anterior hypothalamus (POA), which is particularly known for its involvement in
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thermoregulation, played a key role in the TRH effects on thermoregulation [12, 16, 73]. However, although a number of other studies showed that also other hypothalamic areas might be involved in the thermoregulatory effects of TRH [14, 15], the role of the PVN has never been investigated. On the other hand, the PVN has been implicated in both stimulatory [33, 36, 37] and inhibitory effects on thermoregulation [34, 35, 74, 75] by different neurotransmitters. Moreover, TRH receptors and TRH terminals are found on PVN neurons [22, 23], indicating an anatomical basis for TRH signaling within the PVN neural circuit. Interestingly, a recent study by Song et al showed that a group of preoptic Vglut2+ neurons inhibited thermogenesis by exciting CRH but not TRH neurons in the PVN [76]. Therefore, together the study of Song et al. [76] and our current results suggest a differential control of thermoregulation by distinct neuronal populations in the PVN[76], i.e., CRH neurons inhibiting and TRH neurons stimulating thermogenesis. However, whether the TRH released in the PVN is derived from TRH neurons within the PVN or extra PVN areas cannot be deduced from the current experiments. Similarly, it is not clear whether the ultimate effects are mediated through TRH-containing or non-TRH PVN neurons [23].

In conclusion, our study demonstrates that TRH administration in the PVN induces metabolic and behavioral changes mimicking those during cold exposure, which places the PVN in the center of the cold defense mechanism.

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References


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