The Role of Hypothalamic NF- κ B Signaling in the Response of the HPT-Axis to Acute Inflammation in Female Mice

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A large proportion of critically ill patients have alterations in the hypothalamus-pituitary-thyroid (HPT) axis, collectively known as the nonthyroidal illness syndrome. Nonthyroidal illness syndrome is characterized by low serum thyroid hormone (TH) concentrations accompanied by a suppressed central component of the HPT axis and persistent low serum TSH. In hypothalamic tanycytes, the expression of type 2 deiodinase (D2) is increased in several animal models of inflammation. Because D2 is a major source of T_3 in the brain, this response is thought to suppress TRH expression in the paraventricular nucleus via increased local bioavailability of T_3 . The inflammatory pathway component RelA (the p65 subunit of nuclear factor- κ B) can bind the *Dio2* promoter and increases D2 expression after lipopolysaccharide (LPS) stimulation in vitro. We aimed to determine whether RelA signaling in tanycytes is essential for the LPS-induced D2 increase in vivo by conditional elimination of RelA in tanycytes of mice (*RelA^{ASTKO}*). *Dio2* and *Trh* mRNA expression were assessed by quantitative in situ hybridization 8 or 24 hours after saline or LPS injection. At the same time points, we measured pituitary *Tsh β* mRNA expression and serum T_3 and T_4 concentrations. In *RelA^{ASTKO}* mice the LPS-induced increase in *Dio2* and decrease in *Trh* mRNA levels in the hypothalamus were reduced compared with the wild-type littermates, whereas the drop in pituitary *Tsh β* expression and in serum TH concentrations persisted. In conclusion, RelA is essential for the LPS-induced hypothalamic D2 increase and TRH decrease. The central changes in the HPT axis are, however, not required for the down-regulation of *Tsh β* expression and serum TH concentrations. (*Endocrinology* 157: 2947–2956, 2016)

The nonthyroidal illness syndrome (NTIS) is frequently observed in critically ill patients admitted to the intensive care unit (1). NTIS is characterized by low serum thyroid hormone (TH) concentrations and a suppressed central component of the hypothalamus-pituitary-thyroid (HPT) axis, both in animal models and in humans (2–4). Although often regarded as an adaptive mechanism during acute inflammation, NTIS may negatively influence dis-

ease outcome in protracted critical illness. Furthermore, local TH metabolism is differentially affected depending on the timing and tissue studied, making it a difficult and complex syndrome to interpret (2).

The exact mechanism involved in the illness-induced suppression of the HPT axis is incompletely understood. Secretion of TRH from the paraventricular nucleus (PVN) is negatively regulated by T_3 , thus forming a negative feed-

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Abbreviations: ARC, arcuate nucleus; D2, type 2 deiodinase; EGFP, enhanced GFP; GFP, green fluorescent protein; GLAST, glutamate aspartate transporter; HPT, hypothalamus-pituitary-thyroid; $\text{I}\kappa\text{B}\alpha$, Inhibitor of Kappa light chain enhancer in B-cells alpha; LPS, lipopolysaccharide; ME, median eminence; NF- κ B, nuclear factor- κ B; NTIS, nonthyroidal illness syndrome; PVN, paraventricular nucleus; qPCR, quantitative PCR; SSC, saline sodium citrate buffer; TAM, tamoxifen; TH, thyroid hormone; TRH-DE, TRH degrading ectoenzyme; WT, wild type; YFP, yellow fluorescent protein.

back loop. The main source of T_3 in the central nervous system is type 2 deiodinase (D2), an enzyme encoded by the *Dio2* gene that converts the prohormone T_4 (secreted by the thyroid gland) from the cerebrospinal fluid and the circulation into the biologically active hormone T_3 (5, 6). D2 is strongly expressed in tanycytes, a specialized glial cell type in the wall of the third ventricle (7, 8). During acute inflammation, the expression and activity of D2 in the hypothalamus is up-regulated (9, 10) independently of the fall in TH concentrations (11). Increased activity of D2 in tanycytes suppresses TRH secretion from the PVN via local increase in T_3 availability in the hypothalamus (9, 12). Mice lacking D2 are unable to adequately suppress TRH expression in response to bacterial endotoxin (lipopolysaccharide [LPS]) (13) and studies in cell specific knock out mice make clear that D2 in tanycytes is essential for proper regulation of the HPT axis (14).

The inflammatory pathway component RelA (the p65 subunit of nuclear factor- κ B [NF- κ B]) was reported to increase D2 expression in vitro (15) and to bind to the *Dio2* promoter region (16). We recently showed that RelA is up-regulated in vivo in the mediobasal hypothalamus of mice after LPS administration and that inhibition of RelA in a primary tanycyte culture prevents the LPS induced increase of *Dio2* mRNA expression (17). However, evidence for a causal role for RelA in the regulation of D2 during acute inflammation in vivo is still missing.

We aimed to address this question using temporally controlled tanycyte-specific elimination of RelA. To this end, we crossed *RelA^{fllox}* mice with mice expressing a tamoxifen (TAM)-inducible Cre recombinase (CreERT²) under the control of the glutamate aspartate transporter (GLAST) promoter. Acute inflammation was induced by LPS injection. Illness-induced hypothalamic *Dio2* and *Trh* expression was determined by in situ hybridization. In addition, pituitary *Tsh β* mRNA expression and serum TH were measured using quantitative PCR (qPCR) and a RIA, respectively.

Materials and Methods

Animals

Animals were kept under controlled conditions in a pathogen-free environment and provided with food and water ad libitum. All animal interventions were performed in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local ethics committee. Conditional *RelA^{fllox}* mice were a kind gift of Roland M. Schmid (Technical University Munich, Munich, Germany) (18). To target tanycytes, transgenic mice with a GLAST-controlled CreERT2 expression were used (19). Mice were backcrossed to a C57BL/6 (B6) background for at least 10 generations. A GLAST-

CreERT²-YFP reporter mouse (20) was used to verify TAM-induced Cre expression in tanycytes. A NF- κ B-enhanced green fluorescent protein (EGFP)-reporter mouse (21), expressing EGFP under the control of an artificial NF- κ B-responsive promoter was used to verify LPS induced activation of RelA signaling in tanycytes. Male and female NF- κ B-EGFP-reporter mice received an ip injection of 10 μ g LPS per gram of body weight (LPS, *Escherichia coli* O127:B8; Sigma) diluted in 0.9% NaCl. Control mice received 0.9% NaCl ($n = 2$ –3 mice per group). Mice were perfused transcardially with $1 \times$ PBS followed by 4% paraformaldehyde/PBS. Brains were collected and postfixed in 4% paraformaldehyde for 24 hours at 4°C. After postfixation, the brains were cryoprotected by immersion in 30% sucrose/PBS at 4°C. Thirty-micrometer coronal brain sections were cut on a cryostat (Leica CM 3050s). Serial sections were collected free floating in PBS.

Female mice (7–14 wk of age) with a homozygous Cre/loxP-based deletion of *relA* alleles (*RelA^{fl/fl;tg/+}*, designated as *RelA^{ASTKO}*) and floxed littermate controls (*RelA^{fl/fl;+/+}*, designated as wild type [WT]) were used. Cre recombination was induced by five consecutive ip injections (once every 24 h) of 80 mg tamoxifen per kilogram of body weight, applied at a concentration of 10 mg/mL in an oil/ethanol (9:1) solution, between 30 and 50 days of age (19, 22). Efficient recombination was verified by genotyping showing the expression of a truncated *relA* transcript after tamoxifen treatment. Acute inflammation was induced by an ip injection of 10 μ g LPS per gram of body weight diluted in 0.9% NaCl. Control mice received 0.9% NaCl. Mice ($n = 8$ –10 mice per group) were killed by CO₂ intoxication 8, 24, or 36 hours after LPS administration, respectively. To control for diurnal variation, each time point had its own control group and all experiments were done at the same time of day. Blood was taken by heart puncture, and serum was stored at -20°C . Brains were isolated, snap frozen in dry ice-cooled 2-methyl butane and cut with a cryostat in 20- μ m-thick sections. Pituitaries were isolated, frozen on dry ice, and stored at -80°C for RNA isolation.

Genotyping

Tail biopsies were incubated overnight at 55°C in 300 μ L PCR buffer (50 mM KCl; 10 mM Tris-HCl, pH 8.3; 2.5 mM MgCl₂; 0.1 mg/mL gelatin; 0.45% [vol/vol] Nonidet-P40; 0.45% [vol/vol] Tween 20) containing 40 μ g proteinase K (Merck) while shaking. After incubation, samples were heated for 10 minutes at 95°C to inactivate the proteinase K. Samples were spun down for 5 minutes and the supernatant was used for subsequent PCRs. Blood samples were taken to test for recombination in peripheral immune cells that could potentially affect the LPS response in knockout mice. PCRs to detect floxed (*RelA^{fllox}*) and recombined (*RelA^{del}*) *RelA* alleles as well as the Cre recombinase transgene were performed using the following parameters and primers: 4 minutes at 94°C (45 sec at 94°C, 30 sec at 58°C, 45 sec at 72°C) 35 times, 5 minutes at 72°C. Primers included the following: *RelA^{fllox}* forward, 5'-GAGCGCATGCCTAGCACCAG-3', *RelA^{fllox}* reverse, 5'-GTGCACTGCATGCGTGTCAG-3'; *RelA^{del}* forward, 5'-AGTGCATGTCATGCGTGTCAG-3', *RelA^{del}* reverse, 5'-GTGCACTGCATGCGTGTCAG-3'; Cre forward, 5'-GCCTGCATTACCGGTGATGCAACGA-3', Cre reverse, 5'-GTGGCAGATGGCGCGGCAACAACATT-3', control (*C β*): forward, 5'-CACTGATGTTCTGTG

TGACA-3', reverse, 5'-GAGGATCTGAGAAATGTGACTCC AC-3'. PCR fragments were analyzed on DNA agarose gel.

Confirmation of conditional knockout

To confirm expression of recombined *RelA* transcripts in the target area, the periventricular area containing the tanycyte layer was punched in RNAlater buffer (Life Technologies), and RNA was isolated as described below. Expression of the wild-type and recombined mRNA was confirmed by qPCR with the following primers: *RelA* forward, 5'-tgaccctgtcctctcacat-3' and *RelA* reverse, 5'-gacacacctgttcaggag-3'. The qPCR resulted in the amplification of a 922-bp PCR product for WT *RelA* transcripts, and a 448-bp PCR product for recombined *RelA* transcripts.

Immunohistochemistry

For visualizing yellow fluorescent protein (YFP) in tanycytes of GLAST-CreERT²-YFP reporter mice, perfusion-fixed brains were cut on a vibratome. Sections were blocked as well as permeabilized with 10% normal goat serum in 0.2% Triton X-100 containing PBS. Immunostaining was performed using a rabbit anti-green fluorescent protein (GFP) antibody (1:500; Molecular Probes). Sections were then incubated with fluorescence-labeled secondary antibodies (1:1000, Alexa Fluor 488; Invitrogen) and analyzed using an Olympus AX70 microscope.

For visualizing EGFP in tanycytes of LPS and saline injected NF- κ B-EGFP-reporter mice, sections were washed in PBS followed by permeabilization with 0.2% Triton X-100. After permeabilization, sections were blocked using 3% normal goat serum, followed by overnight incubation with the GFP antibody (1:500, catalog number GFP-1020; Aves) at 4°C. Sections were washed in PBS and incubated with secondary antibody-biotinylated goat antichick IgY (1:500, catalog number ab 6876; Abcam) for 1 hour at room temperature. Subsequently, sections incubated with a complex of avidin and biotin (1.50, Vectastain ABC kit, catalog number PK-6101) and then covered with 3,3'-diaminobenzidine-H₂O₂ solution (one tablet set for 5 mL of deionized water, catalog number D4293; Sigma). After stopping the color reaction with PBS, sections were mounted on coated slides, air dried overnight, and coverslipped using cytooseal XYL mounting medium (catalog number 8312-4; Thermo Scientific). Staining was visualized with the Axio Imager 2 microscope (Carl Zeiss).

Riboprobe synthesis

A cDNA fragment of mouse *Trh* cDNA corresponding to nt 1251–1876 (GenBank accession number NM_009426.2) and a fragment of mouse *Dio2* cDNA corresponding to nt 131–1045 (GenBank accession number AF096875) were generated by PCR and subcloned into the pGEM-T Easy Vector (Promega).

Radiolabeled riboprobes for *Dio2* and *Trh* were generated by performing the transcription reaction in the presence of [³⁵S]uridine 5-triphosphate (Hartmann Analytik) as described before (23).

In situ hybridization

In situ hybridization was carried out as described before (24). Slides were fixed in 4% paraformaldehyde solution. After washes in PBS, slides were permeabilized in 0.4% Triton-X-100 in PBS. Deacetylation was carried out by incubating the slides in 0.1 M triethanolamine (pH 8) containing 0.25% (vol/vol) acetic

anhydride for 10 minutes. Slides were washed in PBS, dehydrated in increasing ethanol concentrations, and air dried.

Radiolabeled riboprobes were diluted to a final concentration of 1×10^4 cpm/ μ L (*Trh*) or 5×10^4 cpm/ μ L (*Dio2*) in hybridization buffer (50% formamide; 10% dextran sulfate; 0.6 M NaCl; 10 mM Tris/HCl, pH 7.4; 1 $\times$ Denhardt's solution; 100 mg/mL sonicated salmon sperm DNA; 1 mM EDTA-di-Na; and 10 mM dithiothreitol). TRH was diluted with 5% cold probe. After application of the hybridization mixture, slides were coverslipped and incubated overnight at 58°C. The next morning, slides were washed in 2 $\times$ saline citrate buffer (SSC) and coverslips were removed. Subsequently, slides were washed in 1 $\times$ SSC and treated with ribonuclease A (20 μ g/mL) and ribonuclease T1 (1 U/mL) at 37°C for 30 minutes. Successive washes followed at room temperature in 1 $\times$, 0.5 $\times$, and 0.2 $\times$ SSC for 20 minutes each and in 0.2 $\times$ SSC at 60°C for 1 hour. The sections were dehydrated and exposed to Biomax MR film (Kodak). Sections were exposed for 1 day (*Trh*) or 3 days (*Dio2*). Integrated density of the signal in the PVN (for *Trh*) and the arcuate nucleus (ARC)-median eminence (ME) region (for *Dio2*) was determined using ImageJ software (National Institutes of Health, Bethesda, Maryland). A value per brain section was calculated by subtracting the background signal and by dividing the outcome by the area of the calculated signal.

RNA isolation and qPCR

Pituitary mRNA was isolated on the MagnaPure RNA isolation system using the LC mRNA tissue kit (Roche Molecular Biochemicals). cDNA was synthesized with the first-strand cDNA synthesis kit with oligodeoxythymidine primers (Roche Molecular Biochemicals). Quantitative PCR was performed using the Lightcycler 480 and Lightcycler480SybrGreen I master mix (Roche Molecular Biochemicals). Primers used for detection of *Hprt*, *Dio2*, and *Tsh β* are described elsewhere (9). Primers for cyclophilin-B (*Ppib*) were: forward, 5'-GAGACTTCACCAG GGG-3', and reverse, 5'-CTGTCTGTCTTGGTGCTCTCC-3'.

Primers for elongation factor 1- α 1 (*Eef1a1*): forward, 5'-AGTCGCCTTGGACGTTCTT-3', reverse, 5'-ATTGTGA GATCAGGTGGCCG-3'. Quantification was performed using the LinReg software. Samples deviating more than 5% from the mean efficiency value of the assay were excluded. Calculated values were normalized to the geometric mean of three reference genes (*HPRT*, *cyclophilin*, *Ef1a1*) according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. Reference gene expression was not significantly different among treatment groups.

T₃ and T₄ measurements

T₃ and T₄ serum concentrations were measured with an in house RIA (25). All samples of one experiment were measured within the same assay (intraassay variability T₃, 3.6%, and T₄, 6.6%).

In vitro dual-luciferase assay

To test whether inhibition of RelA can have a direct effect on the *Dio2* promoter, we designed an in vitro dual luciferase approach. An H4 human glioma cell line was acquired from American Type Culture Collection and grown in DMEM supplemented with 10% fetal calf serum and 1% penicillin-streptomycin-fungizone (all from Lonza). Cells (5×10^4 per well) were

Figure 1. A, Immunofluorescence image of the tamoxifen-induced Cre mediated YFP expression in the tanyocyte layer lining the hypothalamic third ventricle. B, Analysis of *RelA* PCR products from the circumventricular area of WT and *RelA*^{ASTKO} mice. The 922-bp product represents WT *RelA*, and the 448-bp band represents truncated *RelA*.

seeded in a 24-well plate and were transfected with 250 ng PGL3-hd102-luc construct (kindly provided by B. Gereben, Hungarian Academy of Sciences, Budapest, Hungary) and 4 ng P3xflag-CMV-Renilla (kindly provided by M. Vooijs, Maastricht University, Maastricht, The Netherlands) using Jetprime transfection reagent and buffer according to the manufacturer's guidelines (Polyplus Transfection SA). After 24 hours, cells were stimulated with 1 μ g/mL LPS. To inhibit NF- κ B nuclear translocation, cells were treated with JSH-23 (50 μ M, dissolved in 10% dimethylsulfoxide; Calbiochem), or with dimethylsulfoxide alone. After 4 hours, cells were washed with PBS and lysed with passive lysis buffer from the dual-luciferase reporter assay kit (Promega). A dual-luciferase assay was performed according to the manufacturer's instructions, and the samples were analyzed using a Glomax luminometer (Promega).

Statistics

Differences between conditions were evaluated per time point using a two-way ANOVA with two grouping factors (treatment and genotype). To evaluate the response to LPS, data were normalized to saline controls, and a two-way ANOVA with two grouping factors (time and genotype) was performed. Normal distribution of the data was tested using the Shapiro-Wilk test on the residues of the ANOVA. *P* values in the figures represent the effect of LPS treatment (*P*_{treat}) or genotype (*P*_{genotype}). To test pair-wise comparisons, an ANOVA was followed by a Tukey's post hoc test. Symbols in the figures represent the pair-wise *P* values. All tests were performed using GraphPad Prism 6 (GraphPad software Inc).

Results

Characterization of the mouse model

To visualize TAM-induced Cre expression within periventricular tanyocytes, GLAST-CreER^{T2} mice were crossed to a YFP reporter line (20). Treatment of mice with

TAM resulted in a pronounced expression of Cre recombinase in tanyocytes, as indicated by abundant expression of YFP protein in the wall of the third ventricle and median eminence of GLAST-CreER^{T2}-YFP reporter mice (Figure 1A).

To test for efficient recombination of the *RelA* locus in periventricular astrocytes and tanyocytes of *RelA*^{ASTKO} mice, GLAST-CreER^{T2} mice were crossed with *RelA*^{flox} mice and expression of recombined *RelA* transcripts was analyzed by PCR amplification. Truncated *RelA* was expressed in periventricular tissue samples comprising the wall of the third ventricle of *RelA*^{ASTKO} mice but not in tissue samples derived from WT mice (Figure 1B), which

showed solid expression levels for full-length *RelA* transcripts. Moreover, white blood cells of *RelA*^{ASTKO} mice did not show recombination of *RelA* implicating that these peripheral immune cells are not affected by TAM induced *RelA* deletion (data not shown).

LPS-induced acute inflammation leads to NF- κ B activation in tanyocytes

To demonstrate activation of the NF- κ B signaling pathway in periventricular tanyocytes under inflammatory conditions, we used a recently generated NF- κ B-reporter mouse, in which expression of EGFP is under transcriptional control of NF- κ B (21). Analysis of periventricular tissue sections revealed robust expression of EGFP in tanyocytes lining the third ventricle 24 hours after LPS injection (Figure 2, A and B). In contrast, in NF- κ B-reporter mice receiving saline, no NF- κ B activation was observed (Figure 2C). Thus, acute inflammation induced by LPS administration leads to immediate activation of NF- κ B signaling in tanyocytes.

Knockdown of *RelA* in tanyocytes prevents the LPS induced D2 increase in the mediobasal hypothalamus and the TRH decrease in the PVN

LPS administration resulted in a 2-fold increase in *Dio2* mRNA expression in the mediobasal hypothalamus of WT mice compared with saline-treated WT mice but not in the mediobasal hypothalamus of *RelA*^{ASTKO} mice (Figure 3A) compared with saline-treated *RelA*^{ASTKO} mice (basal *Dio2* levels, however, might be slightly but not significantly elevated in *RelA*^{ASTKO} mice compared with WT

Figure 2. Microscopic image of the third ventricle of LPS (A and B) and saline-injected (C) NF- κ B-EGFP-reporter mice are shown. Magnification, $\times 10$ (A) and $\times 40$ (B and C). EGFP expression was visualized with 3,3'-diaminobenzidine staining. Arrows indicate representative activated tanycytes, whereas arrowheads point to EGFP-positive microglial cells.

mice). The response was less pronounced after 24 hours (Figure 3A). The relative increase of *Dio2* in response to LPS was absent in mice lacking RelA. *Trh* expression in the PVN decreased 8 hours after LPS administration in the WT mice but not in the *RelA*^{ASTKO} mice compared with saline-treated mice, and no differences were observed after 24 hours (Figure 3B). Basal *Trh* expression was signifi-

cantly lower in the *RelA*^{ASTKO} mice compared with WT mice. In summary, the LPS-induced *Trh* decrease was absent in *RelA*^{ASTKO} mice.

The effect of RelA knockdown on basal *Dio2* and *Trh* expression is not a direct effect on the *Dio2* promoter

Because we observed a trend toward increased basal *Dio2* expression and a decreased basal *Trh* expression in the *RelA*^{ASTKO} mice compared with WT mice, we wondered whether the inhibition of RelA function has a direct effect on the *Dio2* promoter. We therefore tested the effect of the RelA inhibitor JSH-23 (retains RelA in the cytoplasm, thereby inhibiting the activity) on promoter activity of a human *Dio2* promoter construct in human H4 glioma cells using a dual-luciferase assay. LPS slightly increased *Dio2* promoter activity after 4 hours, whereas JSH-23 decreased basal promoter activity (Figure 4). This was, however, marginal compared with the 300-fold increase we observed after forskolin stimulation (data not shown).

Knockdown of RelA in tanycytes does not affect *Tsh β* and *Dio2* mRNA expression in the pituitary

Pituitary *Tsh β* mRNA expression decreased 24 hours after LPS administration to a similar extent in both WT and *RelA*^{ASTKO} mice (Figure 5, A–C). Pituitary *Dio2* mRNA expression was increased 8 hours after LPS injection in the WT but not in the *RelA*^{ASTKO} mice, and it significantly decreased 24 hours after LPS injection in WT mice (Figure 5, D–F).

Knockdown of RelA in tanycytes does not affect the LPS-induced decrease in serum *T₃* and *T₄*

LPS injection had pronounced effects on serum TH concentrations. Serum *T₄* decreased to a similar extent 8, 24, and 36 hours after LPS injection in the WT and *RelA*^{ASTKO} mice (Figure 6, A–D). Serum *T₃* decreased 24 and 36 hours after the LPS administration, and the observed decrease was similar in both genotypes (Figure 6, E–H).

Discussion

In the present study, we show the importance of the classical NF- κ B pathway, and specifically RelA, in the inflammation-induced *Dio2* expression in tanycytes. To this end, we used a tamoxifen-inducible conditional knockout mouse that lacks RelA specifically in astrocytes and tanycytes. In mice carrying the inducible GLAST-CreER^{T2} transgene, Cre-recombinase activity showed a high ta-

Figure 3. A, Expression of *Dio2* mRNA in the ARC-ME area (dotted lines) 8 and 24 hours after LPS injection. B, Expression of *Trh* in the PVN 8 and 24 hours after LPS injection. Shown are representative autoradiography pictures of coronal brain sections at 8 hours after injection and the quantification of the signal measured as the integrated density. White bars represent saline-injected mice, and black bars represent LPS-injected mice (10 μ g/g body weight). In the lower graphs, relative responses to LPS injection of WT (dotted bars) and *Rela*^{ASTKO} (checked bars) mice is presented. Dotted line represents the saline-treated groups. Mean values $\pm$ SEM are depicted. *P* values indicate the effect of LPS injection (*P*_{treat}) and genotype (*P*_{genotype}) as evaluated by a two-way ANOVA. Differences between groups (*n* = 5–7 mice per group) were analyzed post hoc with Tukey's test. *, *P* $\leq$.05; **, *P* $\leq$.01; b, *P* $\leq$.01. KO, knockout.

moxifen responsiveness, particularly in the wall of the third ventricle. In a GLAST-CreER^{T2} YFP reporter line, GLAST-driven Cre expression has been recently described to be primarily present in the α -subset of tanycytes (26), lining the lower part of the ventricular zone adjacent to hypothalamic nuclei. Although we also detected some Cre-recombinase expression in the β -subset lining the ME and infundibulum, we observed the most pronounced expression in α -tanycytes. Crossing the GLAST-CreER^{T2}

mice with *Rela*^{fllox} mice led to specific expression of truncated *Rela* in the ventricular walls, in which the tanycytes are located.

We showed before that stimulation of primary tanycyte cultures with LPS leads to rapid phosphorylation, nuclear translocation, and thereby activation of *Rela* (17). In the present study using a NF- κ B reporter mouse, we show in vivo that LPS-mediated acute inflammation induces target gene expression by the transactivation of NF- κ B specifi-

Figure 4. Luciferase to renilla ratios in H4 glioma cells. H4 cells were transfected with the hDio2-luc construct and a p3xflag-renilla construct to correct for transfection efficiency. Cells were stimulated with LPS (1 μ g/mL) and the RelA nuclear translocation inhibitor JSH-23 (50 μ M) for 4 hours, followed by a dual-luciferase assay. *, $P \leq .05$.

cally in tanycytes. Additionally, histological/morphological data suggest activation of NF- κ B in microglia in the hypothalamic area.

Role of RelA in *Dio2* regulation

Acute inflammation induced by LPS administration is known to markedly increase *Dio2* mRNA expression in

the ARC-ME region of the hypothalamus of mice and rats (9, 10). In mice lacking RelA in tanycytes, the response of *Dio2* expression to LPS was blunted. RelA has been considered a likely candidate for the regulation of D2 during inflammation because it is able to activate the *Dio2* promoter in vitro (10, 16). In addition, we recently showed that RelA regulates the LPS-induced *Dio2* mRNA increase in a primary tanycyte cell culture (17). The present study reveals that this is also the case during the acute-phase response in vivo. The NF- κ B protein family consists of several subunits of which RelA acts as a transcriptional activator that forms hetero- and homodimers. In the cytoplasm, NF- κ B is bound to Inhibitor of Kappa light chain enhancer in B-cells alpha ($I\kappa$ B α), an inhibitory molecule. Upon activation of the signaling cascade, $I\kappa$ B α is phosphorylated by $I\kappa$ B kinase, and NF- κ B is released. NF- κ B can then translocate to the nucleus and activate transcription of target genes (27). The expression of $I\kappa$ B α is also positively regulated by NF- κ B and has been used as a marker for NF- κ B transcriptional activity. A previous study showed that *ikba* mRNA increased 12 hours after LPS administration secondary to the rise in *Dio2* expression (28), suggesting that NF- κ B is not primarily involved

Figure 5. Expression of *Tshβ* mRNA in the pituitary 8 (A) and 24 hours (B) after LPS or saline injection. Expression of *Dio2* mRNA in the pituitary 8 (D) and 24 hours (E) after LPS or saline injection. White bars represent saline-injected mice, and black bars represent LPS-injected mice (10 μ g/g body weight). In the right panels, relative responses to LPS injection of WT (dotted bars) and *RelA*^{ASTKO} (checked bars) mice are presented. Dotted line represents the saline-treated groups. Mean values $\pm$ SEM are depicted. P values indicate the effect of LPS injection (P_{treat}) and genotype (P_{genotype}) as evaluated by a two-way ANOVA. Differences between groups ($n = 5$ –10 mice per group) were analyzed post hoc with a Tukey's test. *, $P \leq .05$; **, $P \leq .01$; a, $P \leq .05$.

Figure 6. Serum T₄ and T₃ concentrations 8 (A and E), 24 (B and F), and 36 hours (C and G) after LPS or saline injection. White bars represent saline-injected mice, and black bars represent LPS-injected mice (10 μg/g body weight). In the right panels (D and H), relative responses to LPS injection of WT (dotted bars) and *RelA*^{ASTKO} (checked bars) mice are presented. Dotted line represents the saline-treated groups. Mean values ± SEM are depicted. *P* values indicate the effect of LPS injection (*P*_{treat}) and genotype (*P*_{genotype}) as evaluated by a two-way ANOVA. Differences between groups (*n* = 7–11 mice per group) were analyzed post hoc with a Tukey's test. *, *P* ≤ .05; **, *P* ≤ .01; a, *P* ≤ .05.

in the initiation of the *Dio2* increase. Our studies, however, show an active role of NF-κB in the LPS-induced increase in *Dio2* mRNA expression. Because we observed a slight increase in basal *Dio2* mRNA expression in the *RelA*^{ASTKO} mice, we tested whether inhibition of the transcriptional activity of RelA by retaining it in the cytoplasm could lead to enhanced *Dio2* promoter activity in an in vitro experiment. However, we showed no such effect.

TRH in the PVN

LPS administration decreased *Trh* expression in the PVN in WT mice, which is in agreement with previous studies of our group (29). In the *RelA*^{ASTKO} mice, no decrease in *Trh* expression was seen after LPS stimulation. However, also, basal *Trh* expression was significantly reduced in the *RelA*^{ASTKO} mice. Because we observed simultaneous changes in *Dio2* and *Trh* mRNA expression after 8 hours and did not include earlier time points, we cannot

confirm or exclude a causal role of *Dio2* (via increased local T₃ availability) in the LPS-induced *Trh* decrease. It should be kept in mind that additional factors, eg, IL-1β and corticosterone, may be involved as well because they are capable of mediating the illness-induced TRH decrease, and play a role during the acute phase of the inflammatory response (30, 31).

Pituitary

Although *RelA*^{ASTKO} mice had lower basal *Trh* expression and did not display a drop in *Trh* expression after LPS administration, neither basal nor LPS-induced pituitary *Tshβ* mRNA differed significantly between *RelA*^{ASTKO} mice and WT mice. This remarkable observation might be explained by the fact that *Trh* mRNA levels need not to be indicative for the concentration of the TRH peptide that reaches the pituitary. TRH degrading ectoenzyme (TRH-DE), also known as pyroglutamyl peptidase II, is impor-

Table 1. Antibody Table

Peptide/Protein Target	Antigen Sequence (if Known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised (Monoclonal or Polyclonal)	Dilution Used
GFP		GFP tag antibody	Molecular Probes, A-6455	Rabbit, polyclonal	1:500
GFP		GFP antibody	Aves, GFP-1020	Chicken, polyclonal	1:500
Chicken IgY		Biotinylated goat antichicken IgY	Abcam, ab 6876	Goat, polyclonal	1:500
Rabbit IgG		Goat antirabbit IgG (H + L)	Invitrogen, A-11008	Goat, polyclonal	1:1000
		secondary antibody, Alexa Fluor 488 conjugate			

tant for the regulation of TRH concentrations in the extracellular fluid. TRH-DE is present in tanycytes in which it can enhance the breakdown of TRH in the ME through connections of tanycyte end-feet with neuronal projections (32). In hypothyroid rats, T_3 and T_4 administration leads to TSH suppression, even before the down-regulation of TRH secretion from the PVN via rapid effects on TRH-DE expression in tanycytes. In the case of T_4 , this requires D2 activity (33). Whether basal levels of TRH-DE are lower in *RelA^{ASTKO}* mice or TRH-DE is affected by acute inflammation remains to be investigated. Interestingly, acute inflammation has been shown before to have simultaneous effects on hypothalamic and pituitary gene expression (9) which is in accordance with the present findings.

Serum T_3 and T_4

LPS decreased serum TH concentrations in both WT and *RelA^{ASTKO}* mice. The relatively rapid decrease of T_4 cannot be explained by the observed down-regulation of the central part of the HPT axis. LPS induces a variety of proinflammatory cytokines in multiple organs, including the thyroid gland (9). Cytokines have been shown to inhibit iodide uptake, iodothyronine release, and thyroid peroxidase gene expression in cultured human thyrocytes (34, 35) and might therefore be responsible for the simultaneous decrease in serum T_4 observed in the present study. By contrast, the drop in T_3 after 24 hours could be due either to decreased stimulation of the thyroid gland by TSH because 50% of serum T_3 is derived from the thyroid gland in rodents or to increased peripheral clearance of TH by the liver and kidney. To determine whether the effect on serum TH could be delayed, we measured serum TH concentrations in mice 36 hours after LPS or saline injection. However, at this time point, we still did not observe differences between the WT and *RelA^{ASTKO}* mice.

In conclusion, we show that RelA is essential in the LPS-induced up-regulation of *Dio2* in tanycytes and that central changes in the HPT axis during acute inflammation are not the main determinant of decreased serum TH concentrations observed during acute illness.

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