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Downregulation of Type 3 Deiodinase in the Hypothalamus during Inflammation

de Vries, Emmely M.; Surovtseva, Olga; Vos, Winnie G.; Kunst, Roni F.; van Beeren, Mieke; Kwakkel, Joan; Chassande, Olivier; Ackermans, Mariette T.; Fliers, Eric; Boelen, Anita

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Thyroid

Regulation of type 3 deiodinase in the hypothalamus during inflammation

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Keyword	Basic Research, Thyroid Hormone Metabolism, Thyroid Hormone Action- Brain, Basic
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Abstract	 BACKGROUND: Inflammation is associated with marked changes in cellular thyroid hormone metabolism in triiodothyronine (T3) target organs. In the hypothalamus, type 2 deiodinase (D2), the main T3 producing enzyme, increases upon inflammation, leading to an increase in local T3 availability which in turn decreases TRH expression in the paraventricular nucleus (PVN). Type 3 deiodinase (D3), the T3 inactivating enzyme, decreases during inflammation, which might also contribute to the increased T3 availability in the hypothalamus. While it is known that D2 is regulated by nuclear factor κB (NF-κB) during inflammation, the underlying mechanisms of D3 regulation are unknown. The aim of the present study therefore was to investigate inflammation induced D3 regulation using in vivo and in vitro models. METHODS: Mice were injected with a sub lethal dose of bacterial endotoxin (lipopolysaccharide, LPS) in order to induce a systemic acute phase response. A human neuroblastoma (SK-N-AS) cell line was used to test the involvement of the thyroid hormone receptor alpha 1 (TRa1) as well as the Activator Protein-1 (AP-1) and NF-κB inflammatory pathways in the inflammation induced decrease of D3. RESULTS: D3 expression in the hypothalamus was decreased 24 hours after LPS injection in mice. This decrease was similar in mice lacking the TRa. Incubation of SK-N-AS cells with LPS robustly decreased both D3

LPS-induced D3 decrease.

mRNA expression and activity. This led to increased intracellular T3 concentrations. The D3 decrease was prevented when NF-kB or AP-1 was inhibited. TRa1 mRNA expression decreased in SK-N-AS cells incubated with LPS but knock down of the TRa in SK-N-AS cells did not prevent the

CONCLUSIONS: We conclude that the inflammation induced D3 decrease

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in the hypothalamus is mediated by the inflammatory pathways NF-κB and AP-1, but not TRa1. Furthermore, the observed decrease modulates intracellular T3 concentrations. Our results suggest a concerted action of inflammatory modulators to regulate both hypothalamic D2 and D3 activity in order to increase the local TH concentrations.
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Regulation of type 3 deiodinase in the hypothalamus during inflammation.

3	Emmely M de Vries ¹ , Olga Surovtseva ¹ , Winnie G Vos ¹ , Roni F Kunst ¹ , Mieke van Beeren ¹ ,
4	Joan Kwakkel ¹ , Olivier Chassande ^{2,} Mariette T Ackermans ¹ , Eric Fliers ³ and Anita Boelen ^{1,3}
5	Affiliations ^{: 1} Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
6	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
7	Netherlands. ² University of Bordeaux, Tissue Bioengineering, U1026, F-33076, Bordeaux,
8	France. ³ Amsterdam UMC, University of Amsterdam, Department of Endocrinology &
9	Metabolism, Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism,
10	the Netherlands.
11	Emmely M de Vries, PhD, Amsterdam UMC, University of Amsterdam, Endocrinology
12	Laboratory, Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism,
13	The Netherlands. E-mail: <u>em.d.vries@cbg-meb.nl</u>
14	<u>Olga Surovtseva</u> , Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
15	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
16	Netherlands. E-mail: <u>o.v.surovtseva@amsterdamumc.nl</u>
17	Winnie G Vos, Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
18	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
19	Netherlands. E-mail: <u>w.g.vos@amc.uva.nl</u>
	1

1 2		
3 4	20	Roni F Kunst, Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
5 6 7	21	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
8 9	22	Netherlands. E-mail: <u>r.f.kunst@amc.uva.nl</u>
10 11	1 2	Micko van Booron, PhD, Amsterdam LIMC, University of Amsterdam, Endecrinology
12 13	25	<u>Mileke van Beeren</u> , PhD, Amsterdam Olvic, Oniversity of Amsterdam, Endocrinology
14 15	24	Laboratory, Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism,
16 17 18	25	The Netherlands. E-mail: <u>hcvanbeeren@upcmail.nl</u>
19 20	26	Joan Kwakkel, PhD, Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
21 22 23	27	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
24 25 26	28	Netherlands. E-mail: g.j.kwakkel@amsterdamumc.nl
27 28 20	29	Olivier Chassande, PhD, University of Bordeaux, Tissue Bioengineering, U1026, F-33076,
30 31	30	Bordeaux, France. E-mail: <u>chassande@bordeaux.inserm.fr</u>
32 33 34	31	Mariette T Ackermans, PhD, Amsterdam UMC, University of Amsterdam, Endocrinology
35 36 37	32	Laboratory, Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism,
38 39 40	33	The Netherlands. E-mail: m.t.ackermans@amsterdamumc.nl
41 42	34	Eric Fliers, PhD MD, Amsterdam UMC, University of Amsterdam Department of
43 44 45	35	Endocrinology & Metabolism, Research institute Amsterdam Gastroenterology,
46 47 48	36	Endocrinology & Metabolism, the Netherlands. E-mail: <u>e.fliers@amsterdamumc.nl</u>
49 50 51	37	Anita Boelen, PhD, Amsterdam UMC, University of Amsterdam, Endocrinology Laboratory,
52 53	38	Research institute Amsterdam Gastroenterology, Endocrinology & Metabolism, The
54 55 56	39	Netherlands. Email: a.boelen@amsterdamumc.nl
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Abstract

Thyroid

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8	BACKGROUND: Inflammation is associated with marked changes in cellular thyroid hormone
9	metabolism in triiodothyronine (T3) target organs. In the hypothalamus, type 2 deiodinase
0	(D2), the main T3 producing enzyme, increases upon inflammation, leading to an increase in
1	local T3 availability which in turn decreases TRH expression in the paraventricular nucleus
2	(PVN). Type 3 deiodinase (D3), the T3 inactivating enzyme, decreases during inflammation,
3	which might also contribute to the increased T3 availability in the hypothalamus. While it is
4	known that D2 is regulated by nuclear factor кВ (NF-кВ) during inflammation, the underlying
5	mechanisms of D3 regulation are unknown. The aim of the present study therefore was to
6	investigate inflammation induced D3 regulation using in vivo and in vitro models.
57	METHODS: Mice were injected with a sub lethal dose of bacterial endotoxin
8	(lipopolysaccharide, LPS) in order to induce a systemic acute phase response. A human
0	neuroblactoma (SK-N-AS) cell line was used to test the involvement of the thyroid hormone
9	neurobiastoma (SK-N-AS) cen inte was used to test the involvement of the thyroid normone
0	receptor alpha 1 (TR α 1) as well as the Activator Protein-1 (AP-1) and NF- κ B inflammatory
51	pathways in the inflammation induced decrease of D3.
52	RESULTS: D3 expression in the hypothalamus was decreased 24 hours after LPS injection in
3	mice. This decrease was similar in mice lacking the TR α . Incubation of SK-N-AS cells with LPS
64	robustly decreased both D3 mRNA expression and activity. This led to increased intracellular
5	T3 concentrations. The D3 decrease was prevented when NF-kB or AP-1 was inhibited. TR α 1
6	mRNA expression decreased in SK-N-AS cells incubated with LPS but knock down of the TR $lpha$
57	in SK-N-AS cells did not prevent the LPS-induced D3 decrease.
8	CONCLUSIONS: We conclude that the inflammation induced D3 decrease in the

69 hypothalamus is mediated by the inflammatory pathways NF-κB and AP-1, but not TR α 1.

Furthermore, the observed decrease modulates intracellular T3 concentrations. Our results

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l 📢	74	Introduction	
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) 7 2	75	Inflammation is associated with profound changes in thyroid hormone metabolism, the	
,) 0	76	ensemble of which is known as the so-called nonthyroidal illness syndrome (NTIS) (1). NTIS is	;
1 2	77	characterized by low serum triiodothyronine (T3) concentrations, in severe illness also by	
3 4 5	78	low serum thyroxine (T4) concentrations while serum thyroid stimulating hormone (TSH)	
6 7	79	does not change or even decreases. The observed alterations result from a variety of	
8 9	80	changes in local thyroid hormone metabolism in T3 target organs and marked alterations in	
20 21 22	81	the hypothalamus-pituitary-thyroid (HPT) axis. Type 2 deiodinase (D2), expressed in	
23	82	tanycytes lining the third ventricle, increases in various NTIS animal models (2-4) thereby	
25 26 27	83	generating T3 locally. Increased availability of T3 subsequently lowers the expression of	
28 29	84	thyrotropin releasing hormone (TRH) in the hypothalamic paraventricular nucleus (PVN)	
50 51 52	85	which has been observed in humans and rodents (5).	
5 4 5	86	Local availability of T3, and thereby T3 target gene expression, is determined by the net	
6 7	87	effect of changes in thyroid hormone transporters, deiodinating enzymes and thyroid	
8 9 0	88	hormone receptor (TR) expression (1). Besides type 2 deiodinase, the thyroid hormone (TH)	
1 2	89	inactivating enzyme type 3 deiodinase (D3) is expressed in the hypothalamus, mainly in	
13 14 15	90	neurons (6). D2 and D3 are often regulated in a reciprocal fashion (7). Indeed, acute and	
6 7	91	chronic inflammation decrease D3 expression in the hypothalamus (8) (9) which may	
8 9 60	92	contribute to increased T3 availability in the hypothalamus.	
51 52 53	93	D2 expression in tanycytes is regulated by nuclear factor кВ (NF-кВ) during inflammation	
54 5	94	(3,10) (11). However, the underlying mechanisms of inflammation-induced D3	
6 7 8 9	95	downregulation are unknown. We hypothesize that inflammatory pathways activated by	
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96	lipopolysaccharide (LPS) such as the NF-kB or Activator Protein-1 (AP-1), are also involved in
97	the regulation of D3 either via a direct effect on D3 transcription or via the TR. TRs are
98	members of the nuclear receptor family, and encoded by two genes, the TR α and TR β gene.
99	Due to alternative splicing and alternative promoter usage, the TR α -gene may give rise to a
100	variety of isoforms; the TR $lpha 1$ isoform is the only bona-fide TR $lpha.$ The TR eta gene encodes the
101	TR β 1 and TR β 2 isoforms, both able to bind T3 (12). In the hypothalamus, TH responsiveness
102	of D3 is mediated by the TR $lpha$ (13). TR expression itself is also affected by inflammation; both
103	hepatic TR $eta1$ and TR $lpha1$ expression is decreased in LPS-injected mice (2) (14) and in a IL-1 eta
104	treated liver cell line (HepG2). The TR eta 1 decrease is mediated by the NF-κB pathway while
105	the TR α 1 decrease requires both NF-kB and AP-1 signalling (15) (16). The LPS-induced
106	hypothalamic D3 decrease is similar in $TR\beta^{0/0}$ and WT mice which also suggest a role for $TR\alpha$
107	in the regulation of hepatic <i>Dio3</i> (17).
108	To investigate the regulation of D3 expression and activity during inflammation we used an
109	in vivo and in vitro approach. Wild type and TR α KO mice were injected with LPS to assess D3
110	expression in the hypothalamus. SK-N-AS cells, a human neuroblastoma cell line expressing
111	D3 (7), were stimulated with LPS in combination with inhibitors of the NF-kB and AP-1
112	pathway. In addition, SK-N-AS cells were transfected with specific Thra siRNA's to see

113 whether the LPS induced D3 decrease is dependent on TRα signalling. To measure the effect

- 114 of changes in D3 activity on intracellular TH concentrations, we modified our LC-tandem MS
 - 115 method to measure intracellular T3 concentrations in SK-N-AS cells.

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Thyroid

117 Materials and Methods

118 Animal experiments

2 3	117	Materials and Methods
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6 7	118	Animal experiments
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10 11 12 13 14 15 16	119	Male and female 129Sv/Ev (wild type, WT) and TR $\alpha^{0/0}$ mice were used at 6-12 weeks of age.
	120	TR $\alpha^{0/0}$ mice were generated as previously described (18). WT and TR $\alpha^{0/0}$ mice were crossed
	121	and bred separately. All the mice were kept in 12h light/dark cycles in a temperature
17 18	122	controlled room. A week before the experiment the animals were housed in groups
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33	123	according to the experimental setup. Acute inflammation was induced by an intraperitoneal
	124	(i.p.) injection of 200 μg LPS (Lipopolysaccharide <i>, E.coli</i> O127:B8; Sigma, St. Louis, MO, USA)
	125	diluted in 0.5 ml saline. Control mice received 0.5 ml saline. To control for diurnal variations
	126	each time point had its own control and all experiments started at 9 AM. At time points 0, 4,
	127	8 and 24 hours after LPS or saline injection 6 mice per group were anaesthetized under
	128	isoflurane and killed by cervical dislocation as described previously (19). The hypothalamus
34 35	129	was dissected and frozen in liquid nitrogen. The hypothalamic tissue block was used for
36 37 38 39 40 41 42 43	130	dissection of the periventricular area (PE) consisting of the paraventricular nuclei (PVN),
	131	their direct surrounding, and the upper part of the ependymal lining of the third ventricle. All
	132	studies were approved by the local animal welfare committee.
44 45	133	
46 47 48	134	Cell cultures
48 49 50	135	The human neuroblastoma cell line SK-N-AS was cultured in DMEM GlutaMAX [™] (Gibco),
51 52	136	supplemented with 10% Fetal Bovine Serum (Sigma), 1% PSA (penicillin, streptomycin,
53 54 55	137	antimycotic (Thermo fisher)) and 1% NEAA-MEM (Lonza). For the mRNA expression
56 57	138	experiments, 1·10 ⁵ cells per well were grown for 24 hours in a 24-well plate. For the
58 59 60	139	deiodinase activity experiments, 5·10 ⁵ cells per well were grown in a 6-well plate. For UPLC- 8

140	MS/MS analysis cells were grown in a 75 cm ² flask until confluent. Cells were stimulated with
141	1 mg/ml LPS (<i>E.coli</i> O26:B6, eBioscience, ThermoFisher) dissolved in PBS. To inhibit NF-кВ, 2
142	mM sulfasalazine (a chemical inhibitor of NF-κB, Sigma-Aldrich) or 50 μ M JSH-23, an inhibitor
143	of nuclear internalization of NF-κB (Calbiochem, Darmstadt, Germany), were dissolved in
144	dimethyl sulfoxide (DMSO; Sigma-Aldrich). To inhibit the AP-1 complex, 50 μ M SP600125
145	(Sigma-Aldrich) was used. As a control, the cells were incubated in 0.1-0.8 % DMSO. Cells
146	were pre-incubated with one of the inhibitors for 30 minutes before LPS stimulation. RNA
147	knockdown of Thra (TR $lpha$) was performed by introducing small interfering RNA (siRNA) via
148	transfection by Lipofectamine (Invitrogen, 20 pmol/well). Thra (ID 138870, ID4526, and
149	ID289416) and control siRNAs (scrambled siRNAs with matching GC content) were pre-
150	designed by Ambion, Life Technologies. Following transfection, cells were plated at 5x10 ⁴
151	cells/ml and rested for 24 hours before stimulation. siRNA introduction resulted in an overall
152	knockdown of appr. 65-75% of TRα1 determined using qPCR.
153	
154	RNA isolation and RT-PCR
155	Hypothalamic mRNA was isolated on the Magna Pure (Roche Molecular Biochemicals,
156	Mannheim, Germany) using the Magna Pure LC mRNA tissue kit according to the
157	manufacturers protocol. RNA from SK-N-AS cells was isolated using High Pure RNA Isolation
158	Kit (Roche Life Science). cDNA synthesis was performed using the AMV First Strand cDNA
159	Synthesis Kit for RT-PCR with oligo d(T) primers (Roche). Real Time PCR was performed using
160	the Lightcycler480 and the Lightcycler 480 Sybr Green I Master kit (Roche) for the
161	hypothalamus and Sensifast SYBR no-rox kit for the cell experiments (Bioline). Primer pairs
162	and PCR conditions for mouse hypoxanthine phosphoribosyl transferase (HPRT) and mouse
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3 4 5 6 7 8 9 10 11 12 13 14 15 16 17	163	D3 and D2 are previously described (2). For the SK-N-AS cell-experiments of human origin we
	164	used: hD3 F: 5'-AACTCCGAGGTGGTTCTGC-3' and R: 5'-TTGCGCGTAGTCGAGGAT-3', hTR α 1 F:
	165	5'- CATCTTTGAACTGGGCAAGT-3'and R: 5'- CTGAGGCTTTAGACTTCCTGATC-3', hHPRT F: 5'-
	166	CCTGCTGGATTACATCAAAGCACTG-3'and R: 5'- TCCAACACTTCGTGGGGTCCT-3', hTBP F: 5'-
	167	CCCGAAACGCCGAATATAATCC-3' and R: 5'- AATCAGTGCCGTGGTTCGTG-3'. hEF1 α 1 F: 5'-
	168	TTTTCGCAACGGGTTTGCC-3'and R: 5'- TTGCCCGAATCTACGTGTCC-3'. Primers were either
17 18 19	169	intron-spanning or genomic DNA contamination was tested using a cDNA synthesis reaction
20 21	170	without the addition of Reverse Transcriptase. Samples were corrected for their mRNA
22 23 24	171	content using HPRT as a reference gene for the animal study (20) or the geometric mean of
24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40	172	HPRT, TBP and EF1 α 1 for the cell culture experiments. Reference gene expression was
	173	statistically evaluated and not affected by treatment.
	174	Deiodinase activity
	175	For D3 activity measurements SK-N-AS cells were washed, scraped in ice cold PBS, spun
	176	down, and the pellet was homogenized in PED50 (0.1 M sodium phosphate, 2 mM EDTA pH
	177	7.2, 50 mM DTT) using a polytron (Kinematica, Luzern, Switzerland). Homogenates were
41 42	178	snap frozen in aliquots and stored in -80°C until further use. Protein concentration was
43 44 45	179	measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard
46 47	180	following the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, The
48 49	181	Netherlands). D3 activity was measured as described before (21) on a Shimadzu HPLC system
51 52	182	in combination with a Waters Symmetry C18 column (4.6x250mm, 5 μ m), coupled to a Perkin
53 54	183	Elmer Radiomatic scintillation analyzer (150 TR Flow).
55 56 57	184	
58 59 60	185	Intracellular T3 concentrations

186	SK-N-AS cells were washed with cold PBS, scraped and homogenized in PBS using a Polytron.
187	Protein concentration was measured as described above. 300 μ L cell homogenates was used
188	for the UPLC-MS/MS determination of the thyroid concentrations as described by
189	Ackermans et al (22) with minor adaptation for cell homogenates instead of tissue. Briefly,
190	the sample pretreatment was adapted using the basic Biotage Evolute Express AX 30 mg
191	Solid Phase Extraction (SPE) as described by Williams <i>et al.</i> (<u>www.biotage.com/literature</u> :
192	Comparison of SPE Approaches for the Extraction of Thyroid Hormones: T3, rT3 and T4 prior
193	to LC-MS/MS analysis, P107 MSACL-EU, Biotage Uppsala Sweden).
194	Data analysis and statistics
195	Animal experiment: differences between LPS and saline or between TR $\alpha^{0/0}$ and WT mice
196	were evaluated by two-way ANOVA with two grouping factors (time and treatment or time
197	and genotype). Normal distribution was tested with the Shapiro-Wilk test on the residues of
198	the ANOVA. If not normally distributed data were ranked. To test pair-wise comparisons
199	ANOVA was followed by students t-test or Mann-Whitney U tests if not normally
200	distributed. Symbols in the figures represent pair wise P-values. P-values ≤0.05 were
201	considered significant. All tests were performed using SPSS (SPSS, Chicago, IL, USA).
202	Cell experiments: Cell experiments were carried out at least three independent times in
203	different passages of SK-N-AS cells. Means of 3-6 technical replicates (relative to assay
204	mean) were calculated per experiment. Unless otherwise specified, data presented in the
205	graphs is the mean +/- SEM of the total number of experiments. One-way or two-way
206	ANOVA was followed by Sidak's multiple comparison to test the difference between control
207	and LPS groups per experimental treatment.

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2 3	209	Results
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9 10	211	Hypothalamic D2 and D3 mRNA expression changes after LPS administration in mice
11 12		
13 14	212	LPS injection increased D2 mRNA and decreased D3 mRNA expression in the periventricular
15 16	213	area (PE) after 24 hours compared to saline injection in mice (Fig. 1).
17 18		
19 20	214	
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22 23	215	D3 mRNA expression and activity upon inflammation in SK-N-AS cells
24 25	24.6	In order to study the machine involved in the LDC induced decreases of hypothelessis D2
26	216	In order to study the mechanism involved in the LPS-induced decrease of hypothalamic D3
27 28 29	217	expression, we used the human neuroblastoma cell line SK-N-AS as a model. Incubation of
30 31	218	SK-N-AS cells with LPS decreased D3 mRNA expression and D3 activity after 24 hours
32 33	219	compared to control treated cells (Fig. 2).
34 35 36	220	Subsequently, we investigated whether decreased D3 activity leads to changes in
37 38	221	intracellular T3 concentrations. To this end, we measured T3 concentrations in SK-N-AS cell
39 40	222	homogenates with a UPLC-MS/MS method. LPS stimulation indeed increased intracellular T3
41 42	223	concentrations markedly (Fig. 3)
43 44	223	concentrations markedly (rig. 5).
45 46	224	Thyroid hormones are transported into the target cell by specific transporters, among the
47	225	west increase the world because the second
49	225	most important thyroid normone transporters are MCT8 and MCT10 which expression is
50 51 52	226	measured in order to exclude the possibility that increased intracellular TH concentrations
53 54	227	were due to altered TH transporter expression; MCT8 mRNA expression increased after LPS
55 56	228	while MCT10 expression decreased, suggesting that this differential altered TH transporter
57 58 50	229	expression does not explain the observed changes in intracellular TH levels (data not shown).
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3 4 5 6	230	In addition, it is unlikely that the increased T3 concentrations are derived from the culture	
	231	medium since T3 concentrations were below the detection limit of our in-house RIA.	
7 8 9 10	232		
11 12 13	233	The involvement of inflammatory pathways.	
14 15 16	234	Pre-incubation with Sulfasalazine and JSH-23, inhibitors of the NF-kB signaling pathway,	
17 18 19	235	decreased basal D3 mRNA and D3 activity and prevented further the LPS-induced decrease.	
20 21	236	Pre-incubation with SP600125, an inhibitor of the AP-1 pathway, also prevented the LPS-	
22 23 24 25 26 27 28	237	induced D3 decrease but without affecting basal levels (Fig. 4).	
	238		
28 29 30 31	239	The role of TR α in the LPS induced D3 mRNA decrease.	
32 33	240	In order to study the role of TR α 1 on the LPS-induced D3 decrease after 24h, SK-N-AS cells	
34 35 36	241	were stimulated with LPS for 6 hours and TR $lpha 1$ mRNA expression was measured. LPS	
36 37 38 39 40 41	242	stimulation decreased TR α 1 mRNA independently of NF- κ B or AP-1 signaling; inhibition of	
	243	the NF- κ B or AP-1 pathway did not prevent the LPS-induced decrease of TR $lpha$ 1 mRNA	
42 43	244	expression (Fig. 5). In order to study the role of TR $lpha$ signaling in the LPS-induced D3	
44 45 46	245	decrease, SK-N-AS cells were transfected with specific siRNA's before stimulating the cells	
40 47 48	246	with LPS. Transfection resulted in a TR $lpha$ knock down (KD) of appr. 65-75% throughout the	
49 50	247	experiments; TR α 1 mRNA expression did not differ between LPS and control TR α siRNA	
51 52 53	248	transfected cells (data not shown). LPS led to a decrease in D3 mRNA expression in control	
54 55	249	cells; inhibition of the TR α gene did not prevent the LPS induced D3 decrease indicating that	
56 57 58	250	the TRα1 did not play a causative role (Fig. 6).	
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1 2 3 4 5 6	251 252	The effect of LPS administration was also studied in mice lacking the TR α gene; LPS injection decreased D3 mRNA expression in the periventricular area (PE) after 24 hours compared to
7 8	253	saline injection in TR $\alpha^{0/0}$ mice. The observed decrease was similar in TR $\alpha^{0/0}$ and WT mice,
9 10 11	254	confirming that TR α 1 does not play a role in decreasing D3 mRNA expression during
12 13	255	inflammation (Fig. 7).
14 15 17 18 19 21 22 23 24 25 27 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 22 23 24 25 26 27 28 29 31 23 34 56 37 38 39 0 41 24 44 45 46 7 89 51 25 55 55 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 55 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 56 57 89 60 12 23 24 55 55 55 56 57 89 60 12 23 24 55 55 56 57 55 56 57 56 57 56 57 56 57 56 57 57 56 57 57 57 57 57 57 57 57 57 57 57 57 57	256	

258	Discussion	١

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3	258	Discussion
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6 7	259	To investigate the regulation of D3 expression and activity during inflammation we used an
8 9 10	260	in vivo and in vitro approach. Wild type and TR α KO mice were injected with LPS to assess the
11 12	261	D3 expression in the hypothalamus. SK-N-AS cells, a human neuroblastoma cell line
13 14 15	262	expressing D3 (7) were stimulated with LPS in combination with inhibitors of the NF- κ B and
16 17	263	AP-1 pathway. In addition, SK-N-AS cells were transfected with specific siRNA's to see
18 19 20	264	whether the LPS induced D3 decrease is dependent on TR α signaling. To measure the effect
21 22	265	of changes in D3 activity on intracellular TH concentrations, we set up a LC-tandem MS
23 24 25	266	method to measure intracellular T3 concentrations in SK-N-AS cells.
26 27 28	267	Inflammation results in profound changes in transcriptional and translational activity of
29 30	268	genes involved in thyroid hormone transport and local thyroid hormone metabolism ranging
31 32 33	269	from inhibition to activation. These peripheral changes vary per tissue as well as per type
34 35	270	and severity of illness ultimately resulting in specific changes in local thyroid hormone
36 37 38	271	concentrations (23). The effects of inflammation are often studied in rodents by
39 40	272	administering a sub lethal dose of bacterial endotoxin or lipopolysaccharide (LPS). LPS is a
41 42 43	273	component of gram negative bacteria and it elicits a strong inflammatory response that lasts
44 45	274	approximately 24 hours. LPS binds to the toll like receptor 4 (TLR4), a pattern recognition
46 47 48	275	receptor essential for the sensing of pathogens, and activation of this receptor leads to
49 50 51	276	activation of a variety of intracellular inflammatory pathways (24).
52 53	277	In the hypothalamus, type 2 deiodinase (D2) increases upon inflammation, leading to an
54 55 56	278	increase in local T3 availability which in turn decreases TRH expression in the paraventricular
57 58 59	279	nucleus (PVN), one of the hallmarks of NTIS (1). The illness-induced hypothalamic T3
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3 4	280	increase might also be effective in other illness related phenomena like diminished food
5 6 7	281	intake. It has been shown recently that selective knockdown of the TR eta isoform in the
8 9	282	ventromedial hypothalamus (VMH) leads to severe obesity due to hyperphagia, suggesting
10 11 12	283	that activation of the TR β diminishes food intake as observed during illness (25).
13 14 15	284	We and others have shown that <i>in vitro</i> and <i>in vivo</i> D2 expression in tanycytes is regulated
16 17	285	by NF-кB during inflammation (3,10,11). The present study shows that LPS increased
18 19 20	286	hypothalamic D2 and decreased hypothalamic D3 expression in mice as observed before
21 22	287	(17), and lowered D3 expression and activity in SK-N-AS cells. The D3 decrease was
23 24 25	288	prevented when the cells were incubated with a specific NF-kB and AP-1 inhibitor. Our
26 27	289	results suggest a concerted action of inflammatory modulators to regulate both
28 29 20	290	hypothalamic D2 and D3 activity at the same time in order to increase hypothalamic TH
30 31 32	291	levels. A similar phenomenon is seen in muscle precursor cells; D2 expression is markedly
33 34	292	increased during myoblast differentiation whereas D3 is decreased. The group of Dentice
35 36 37	293	showed that an enzyme involved in chromatin modification (histone H3 demethylating
38 39	294	enzyme, LSD-1 or kdm1a) is essential for transcriptional induction of D2 and repression of D3
40 41 42	295	via interaction with FoxO3 (26). LSD-1 relieves repressive marks on the <i>Dio2</i> promoter and
43 44	296	activates specific marks on the <i>Dio3</i> promoter thereby regulating gene transcription. It is
45 46 47	297	unknown at the moment whether LSD-1 is regulated by NF-κB. However, a study of De
47 48 49	298	Santa et al showed that Jmjd3, an efficient H3K27me3 demethylase and also involved in
50 51	299	chromatin modification, is induced in macrophages depending on direct binding of NF-κB to
52 53 54	300	a cluster of three kB sites in the Jmjd3 gene promoter (27). It is therefore tempting to
55 56	301	speculate that NF-κB is also able to regulate LSD-1. This assumption in combination with the
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302 fact that LSD-1 is highly expressed in murine neurons and astrocytes (28) suggests a link 303 between inflammation, LSD-1 and reciprocal regulation of D2 and D3.

304 One of the few mechanisms published on NF-κB mediated suppression of genes is RNA sequence-dependent, posttranscriptional downregulation. If multiple copies of the mRNA 305 306 cis-regulatory motif 5'-ACUACAG-3' are present in the promoter of a NF-kB sensitive gene 307 (29) that ACTACAG motif may form a binding site for a factor that regulates the stability of 308 the mRNA. Sitcheran et al. suggests that the ACTACAG motif may form part of a stem-loop structure likely via the expression of a factor that regulates RNA stability. We scanned the 309 310 D3 promoter locus for that motif and found only one that is located about 1.5 Kb upstream of the transcription start site of the "regular" 2.2 kb transcript. It seems therefore unlikely 311 312 that this mechanism is markedly involved in the NF-κB dependent downregulation of D3. 313 The LPS-induced decrease in D3 in SK-N-AS cells is also prevented by inhibition of the AP-1 pathway. The AP-1 transcription factor complex, which consists of dimers of the Jun and Fos 314 315 protein families, is activated by Jun N-terminal kinase 1 (JNK), via phosphorylation which in 316 turn is activated by stimulation of the TLR4 by LPS and proinflammatory cytokines (30). 317 Simultaneous inhibition of NF- κ B and the AP-1 complex is necessary to prevent the IL-1 β 318 induced D1 decrease in a human liver cell line (HEPG2 cells) (16). This is in contrast with the 319 observation that inhibition of AP-1 solely is sufficient to block the LPS-induced decrease of 320 D3 in SK-N-AS cells. The mechanism involved in AP-1 mediated repression is unknown. Mittelstadt and Patel showed that AP-1 mediates transcriptional repression of a

metalloproteinase (MMP-9) gene by recruitment of histone deacetylase to the promoter in

323 response to IFN β (31) pointing to a role of chromatin modification.

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1 2 3	224	D2 is a T2 responsive gape, as T2 treatment induces D2 mPNA expression which is mediated
4	324	DS is a 15 responsive gene, as 15 treatment induces DS miking expression which is mediated
5 6 7	325	by TR $lpha$ (13). It should be noted that TR $lpha$ mRNA expression by itself is affected by
7 8 9 10 11 12 13 14 15 16	326	inflammation; liver TR α 1 mRNA expression decreased rapidly upon LPS administration,
	327	followed by decreased nuclear TR protein expression (14). We have shown that the IL-1 eta -
	328	induced decrease of TR α 1 is a direct effect of decreased promoter activity, probably via
	329	phosphorylation-dependent repression of the TR $lpha$ promoter (16). Hypothalamic TR mRNA
17 18 19	330	expression, however, does not change dramatically after LPS administration (2) but changes
20 21	331	in specific TR isoform expression in PVN neurons and tanycytes have not been carefully
22 23 24	332	studied yet. The present study shows that TR $lpha$ 1 mRNA rapidly decreases after LPS
25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41	333	administration in SK-N-AS cells. Inhibition of the NF-ĸB signaling or the AP-1 pathway did not
	334	prevent the LPS-induced decrease of TR $lpha 1$ mRNA expression in those cells suggesting
	335	tissue/cellular specific regulation of TR expression during inflammation. This is in agreement
	336	with a study of Castro <i>et al.</i> , they showed decreased TR β mRNA expression in heart and liver
	337	of pigs after LPS while NF-κB was not activated (32). The observed decrease in TR $lpha$ 1 mRNA
	338	could be due to the activation of other inflammatory pathways, epigenetic changes, or
	339	increased mRNA degradation. In addition, inhibition of the TR α gene did not prevent the
42 43	340	LPS-induced D3 decrease indicating that the expression of the TR $\alpha 1$ did not play a causative
44 45 46 47 48 49 50 51	341	role. This is confirmed by the observation in mice lacking the TR $lpha$ gene; LPS injection
	342	decreased D3 mRNA expression in the periventricular area in TR $\alpha^{0/0}$ mice to the same extent
	343	as in WT mice.
52 53 54	344	In conclusion, the present study shows that the inflammation-induced D3 decrease in the
55 56	345	hypothalamus is mediated by the inflammatory pathways NF-κB and AP-1. The mechanism
57 58 59	346	involved is currently unknown, but modification of the chromatin of the D3 promoter by
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3 4 5	347	specific enzymes is an exciting possibility. The simultaneous increase in hypothalamic D2 and
5 6 7	348	decrease in hypothalamic D3 is expected to result in local increased TH concentrations
8 9	349	subsequently lowering TRH expression in the PVN as observed before (5). We indeed show
10 11	350	for the first time that the LPS-induced D3 decrease in SK-N-AS cells results in elevated
12 13 14 15	351	cellular T3 levels in the same cells.
16 17	352	
18 19 20	353	Acknowledgements
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33 34	359	
35 36 37	360	Corresponding author:
38 39	361	Anita Boelen, PhD
40 41 42	362	Amsterdam University Medical Centers, location AMC, University of Amsterdam
43 44	363	Laboratory of Endocrinology K2-286/ Dept Endocrinology and Metabolism
45 46	364	Meibergdreef 9, 1105 AZ Amsterdam
47 48 49	365	The Netherlands
50 51	366	Phone: +31 20 5666701
52 53 54 55 56 57 58 59 60	367	Email: a.boelen@amsterdamumc.nl
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1 Legends:

Figure 1: Figure 1: Relative D2 and 3 mRNA expression in the periventricular area of mice 0,
4, 8 and 24 hours after LPS (black circles) or saline (clear circles) injection. Mean values ±
SEM (n = 6) are shown. Symbols indicate differences at separate time points (* P < 0.05, ** P < 0.01).

Figure 2: Relative expression of D3 mRNA and D3 activity in SK-N-AS cells after LPS
stimulation. White symbols represent controls and black symbols LPS. Expression and
activity levels of each sample are calculated as relative value of the assay mean, three
independent experiments have been performed and represented are the means ± SEM of
these experiments. Symbols represent differences between control and treatment groups
tested with Student's t-test.

Figure 3: Intracellular T3 concentrations (measured as fmol/mg protein) in SK-N-AS cells
stimulated with control (white dots) or LPS (black dots) for 24h. The experiment consisted of
6 replicates and was analysed by students t-test (*** P ≤0.001).

Figure 4: Relative expression of D3 mRNA (A) and D3 activity (B) in SK-N-AS cells stimulated
 with saline (white symbols) or LPS (black symbols) for 24 hours after pre-incubation with
 DMSO control (Control), JSH-23 (JSH), Sulfasalazine (Sulf), SP600125 (SP) or a combination of
 JSH-23 and SP600125 (JSH + SP). Expression and activity levels of each sample are calculated
 as relative value of the assay mean, at least three independent experiments have been
 performed and represented are the means of 12 (control) or 3 (all other inhibitor groups)
 experiments +/- SEM. P-values represent the outcome of two-way ANOVA with two grouping

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factors (LPS and inhibitors). Symbols represent differences between saline and LPS per
 treatment group as tested with Sidak's multiple comparison test (*** P ≤0.001).

Figure 5: Relative expression of TRα1 mRNA in SK-N-AS cells stimulated with saline (white
symbols) or LPS (black symbols) for 6 hours after pre-incubation with DMSO control
(Control), JSH-23 (JSH), Sulfasalazine (Sulf), SP600125 (SP) or a combination of JSH-23 and
SP600125 (JSH + SP). Expression of each sample is calculated as relative value of the assay
mean, at least three independent experiments have been performed and represented are
the means of 12 (control) or 3 (all other inhibitor groups) experiments +/- SEM. P-values
represent the outcome of two-way ANOVA with two grouping factors (LPS and inhibitors).
Symbols represent differences between saline and LPS per treatment group as tested with
Sidak's multiple comparison test (*P ≤0.05, ** P ≤0.01 *** P ≤0.001).

Figure 6: Relative expression of D3 mRNA in SK-N-AS cells stimulated with saline (open
symbols) or LPS (black symbols) for 24 hours after knock down of the TRα gene using
siRNA's. Expression of each sample is calculated as relative value of the assay mean, at least
three independent experiments have been performed and represented are the means of 3
(control/LPS) and 8 (TRα knock down) experiments ± SEM. P-values represent the outcome
of one-way ANOVA and symbols represent differences groups as tested with Sidak's multiple
comparison test (** P ≤0.01 *** P ≤0.001).

Figure 7: Relative expression of D3 mRNA in the periventricular area of TRα0/0 mice 0, 4, 8
and 24 hours after LPS (black circles) or saline (clear circles) injection (A). Relative D3
expression (normalised to the mean value of each time point of the control group which was
set at 100% and given as a dotted line) in LPS treated WT (■) compared to TRα0/0 (□) mice

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3 4	(B). Mean values \pm SEM (n = 6) are shown. P values indicate differences between treatment	
5 6	50 or genotype by ANOVA. Symbols indicate differences at separate time points (* P < 0.05. **	
7 8	51 P < 0.01).	
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Figure 2: Relative expression of D3 mRNA and D3 activity in SK-N-AS cells after LPS stimulation. White symbols represent controls and black symbols LPS. Expression and activity levels of each sample are calculated as relative value of the assay mean, three independent experiments have been performed and represented are the means ± SEM of these experiments. Symbols represent differences between control and treatment groups tested with Student's t-test.

146x64mm (600 x 600 DPI)





Figure 3: Intracellular T3 concentrations (measured as fmol/mg protein) in SK-N-AS cells stimulated with control (white dots) or LPS (black dots) for 24h. The experiment consisted of 6 replicates and was analysed by students t-test (*** $P \le 0.001$).

78x68mm (600 x 600 DPI)

в

Control

Figure 4: Relative expression of D3 mRNA (A) and D3 activity (B) in SK-N-AS cells stimulated with saline (white symbols) or LPS (black symbols) for 24 hours after pre-incubation with DMSO control (Control), JSH-

23 (JSH), Sulfasalazine (Sulf), SP600125 (SP) or a combination of JSH-23 and SP600125 (JSH + SP).

Expression and activity levels of each sample are calculated as relative value of the assay mean, at least

three independent experiments have been performed and represented are the means of 12 (control) or 3

(all other inhibitor groups) experiments +/- SEM. P-values represent the outcome of two-way ANOVA with

two grouping factors (LPS and inhibitors). Symbols represent differences between saline and LPS per

treatment group as tested with Sidak's multiple comparison test (*** $P \le 0.001$).

338x190mm (96 x 96 DPI)

Α



Thyroid



Figure 5: Relative expression of TRo1 mRNA in SK-N-AS cells stimulated with saline (white symbols) or LPS (black symbols) for 6 hours after pre-incubation with DMSO control (Control), JSH-23 (JSH), Sulfasalazine (Sulf), SP600125 (SP) or a combination of JSH-23 and SP600125 (JSH + SP). Expression of each sample is calculated as relative value of the assay mean, at least three independent experiments have been performed and represented are the means of 12 (control) or 3 (all other inhibitor groups) experiments +/- SEM. P-values represent the outcome of two-way ANOVA with two grouping factors (LPS and inhibitors). Symbols represent differences between saline and LPS per treatment group as tested with Sidak's multiple comparison test (*P ≤ 0.05 , ** P ≤ 0.01 *** P ≤ 0.001).

338x190mm (96 x 96 DPI)

2.5

2.0

1.5

1.0

0.5

0.0

relative D3 mRNA

pLPS≤0.05

KD LPS/KD





LPS

Ċ

338x190mm (96 x 96 DPI)



**

_{0.00}∫p<0.05

 time (h)

wт



NS 0 ∣

> > time (h)

188x93mm (600 x 600 DPI)