

Attenuated fever in rats during late pregnancy is linked to suppressed interleukin-6 production after localized inflammation with turpentine

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An attenuated fever response to pathogens during late pregnancy is a phenomenon that has been described in several mammalian species, and although mechanisms are not completely understood, decreased prostaglandin E₂ (PGE₂) synthesis has been implicated. Upstream of PGE₂, there is evidence to suggest that anti-inflammatory cytokines such as interleukin-1 receptor antagonist (IL-1ra) could play a significant role. In the present study we addressed the role of pro-inflammatory cytokines during late pregnancy, specifically interleukin-6 (IL-6), an important circulating mediator in fever. Turpentine oil (TURP), a very potent pyrogen and activator of IL-6, was injected into the hind-limb muscle of rats at the 18th day of pregnancy (GD 18) or in non-pregnant (NP) age-matched female controls. As expected, TURP injection induced a highly significant fever in the NP animals, which peaked 11 h post-injection and lasted for over 24 h. This was accompanied by a significant rise in circulating IL-6 levels, which correlated with changes in PGE₂ synthesizing enzymes expression in the hypothalamus. In complete contrast, TURP-induced fever was totally absent in GD 18 animals whose body temperature did not deviate from basal values. The lack of response was additionally reflected by the absence of change in IL-6 concentration and by the significant attenuation of PGE₂ synthesizing enzymes expression, which correlated with the suppressed expression of SOCS3, a hypothalamic marker of IL-6 activity. Contrary to the changes in circulating IL-6 levels at GD 18, IL-1ra was induced to levels comparable to those of NP females, suggesting that the influence of this anti-inflammatory cytokine on the fever response to TURP is at best minimal. These data further confirm the importance of IL-6 in fever generation and provide evidence that it may be a key component of the attenuated fever response in late pregnancy.

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Fever is a hallmark response to injury or infection with a well-established adaptive value (Kluger & Rothenburg, 1979; Kluger, 1991; Roth, 2006); its induction, duration, magnitude and relapse are tightly regulated by the balance between the members of a family of endogenous mediators known as cytokines that can act as pro-inflammatory/pyrogenic (interleukin (IL)-1 β ; tumour necrosis factor α (TNF α) and IL-6) or anti-inflammatory/cryogenic intermediaries (the endogenous IL-1 receptor antagonist (IL-1ra) and IL-10 among others) (Kluger, 1991; Leon, 2002; Roth, 2006). In the central nervous system, pyrogenic cytokines induce prostaglandin E₂ (PGE₂) synthesis, the ultimate step in fever induction (Ivanov *et al.* 2002; Ivanov & Romanovsky, 2004; Rummel *et al.* 2006) by the transcriptional induction of cyclooxygenase-2 (COX-2) and microsomal PGE synthase-1 (mPGES-1), the rate-limiting

enzymes of this pathway (Lacroix & Rivest, 1998; Ivanov & Romanovsky, 2004; Matsumura & Kobayashi, 2004). Disruption of this tightly controlled process could result in an abnormal fever response, such as that observed during late pregnancy where an attenuated response to exogenous pathogens has been documented in several mammalian species. In these studies experimental animals including mice, rats, sheep and guinea pigs (Kasting *et al.* 1978; Martin *et al.* 1995), exhibited a reduction in the fever response to infusion of either the viral mimic polyinosinic: polycytidylic acid (poly I:C) (Cooper *et al.* 1988), endotoxin lipopolysaccharide (LPS) (Kasting *et al.* 1978; Zeisberger *et al.* 1981; Martin *et al.* 1995) or endogenous pyrogens such as IL-1 β (Simrose & Fewell, 1995) and PGEs (Stobie-Hayes & Fewell, 1996; Eliason & Fewell, 1997). Several studies have linked this to a reduction in

central PGE₂ synthesis (Imai-Matsumura *et al.* 2002), probably as a result of blunted hypothalamic COX-2 induction (Imai-Matsumura *et al.* 2002; Mouihate *et al.* 2002). Recently, the involvement of central nitric oxide synthase suppressing LPS-induced fever at late pregnancy has been suggested (Begg *et al.* 2007). What remains uncertain is the nature of the peripheral signals responsible for the reduced COX-2 expression and, ultimately, the fever response. We have recently reported (Ashdown *et al.* 2006b) that higher than normal circulating levels of IL-1ra in LPS-treated pregnant rats at the later stages of pregnancy, could be partly responsible for these effects. In support of these findings we further demonstrated a causal link between increased levels of circulating IL-1ra and hypothalamic COX-2 induction. Unlike IL-1ra, however, no consistent differences were reported on the ability of animals at late stages of pregnancy to synthesize pro-inflammatory/pyrogenic cytokines in response to LPS. In these animals the LPS-induced production of IL-1 β and IL-6 were reported to be reduced when compared with virgin females (Fofie *et al.* 2005), but not when compared with earlier stages of pregnancy or in lactating dams, whose febrile response is higher in magnitude than dams in late pregnancy (Mouihate *et al.* 2005).

It has been also suggested that near-term pregnancy suppression of fever may stem from the inability to mount an appropriate/full response to circulating pro-inflammatory cytokines (Chen *et al.* 1999). This, however, has not been supported by the finding that intracellular signalling pathways triggered by LPS/IL-1 β or LPS-induced IL-6 to induce COX-2 remained intact with no differences reported between pregnant dams at early and late stages of pregnancy (Mouihate *et al.* 2005; Harre *et al.* 2006). Particularly surprising about these findings is in regard to IL-6, which we and others have demonstrated to be a critical component of the fever response (Kozak *et al.* 1997, 2006; Cartmell *et al.* 2000; Rummel *et al.* 2006). The circulating levels of IL-6 increase dramatically following a systemic inflammatory challenge and correlate significantly with the fever response. During late pregnancy the levels of IL-6 appear to remain unchanged when compared with GD 15 controls (Harre *et al.* 2006). This observation argues against the importance of this cytokine in the regulation of the fever response. One possibility for this anomaly may lie in the type of stimulus being used. For instance, the majority of studies investigating this aspect (Cooper *et al.* 1988; Fofie & Fewell, 2003; Fofie *et al.* 2005; Mouihate *et al.* 2005; Harre *et al.* 2006), including ours (Ashdown *et al.* 2006b), have used a generalized systemic challenge namely LPS or poly I:C. These immunogens tend to act by triggering a robust immune response, which involves the targeting of multiple organs/systems to induce cytokine release (Givalois *et al.* 1994; Cartmell *et al.* 2001; Turrin *et al.*

2001). Such an overwhelming response may be a factor masking any intricate changes that may occur in the levels of circulating pyrogens such as IL-6. We have previously described a model of localized inflammation, namely the intramuscular (i.m.) injection of turpentine oil (TURP), that induces fever by the activation of febrile effectors in a more defined serial fashion (Luheshi *et al.* 1997; Leon, 2002). The mode of action of this potent pyrogen involves the production of localized necrotic damage (Wusteman *et al.* 1990), which results in the sequential induction of TNF α and IL-1 β at the site of injury (Zheng *et al.* 1995; Luheshi *et al.* 1997). The locally increased cytokines, particularly IL-1 β , induce IL-6 synthesis and release into the circulation (Luheshi *et al.* 1997; Turnbull *et al.* 2003). A particular advantage of this approach is that it facilitates the study of IL-6 without the influence of other circulating cytokines. Given this property, we sought to characterize the particular role of IL-6 in fever generation in late-pregnant rats (gestational day 18 (GD 18)) by measuring the changes in its levels in the circulation and how they correlate with the fever response, and by investigating changes in brain mechanisms regulating the fever response to this important circulating pyrogen.

Methods

Adult female Sprague–Dawley rats (Charles River, Saint Constant, Quebec, Canada) were used in all experiments; two kinds of animals were employed: randomly cycling non-pregnant (NP) females (250–300 g) and primiparous pregnant females at gestational day 18 (GD 18; 250–340 g). All the individual experimental procedures for this study were approved by the Animal Care Committee of McGill University. Care throughout the duration of the experiment was provided according to the Canadian Council of Animal Care guidelines. Animals were housed individually in a controlled environment at an ambient temperature of $21 \pm 2^\circ\text{C}$; they had free access to food and water and were handled for at least 7 days prior to experimentation. Observations were carried out in a 12 h:12 h light–dark cycle (lights on from 08:00 to 20:00 h).

In all studies, animals received a single intramuscular (i.m.) injection of 100 μl of purified turpentine oil (TURP) (Riedel-deHaën, Sneeze, Germany) or 100 μl of sterile physiological saline (SAL) into the gastrocnemius muscle of the left hind limb. Animals were killed (see details below) 11 h after either treatment.

Measurement of body temperature

Changes in core body temperature were measured using remote radio-biotelemetry (Data Sciences, St Paul, MN, USA) as previously described (Sachot *et al.* 2004). Briefly, anaesthetized animals (i.m.; 50 mg kg⁻¹ ketamine hydrochloride, 5 mg kg⁻¹ xylazine hydrochloride,

0.5 mg kg⁻¹ acepromazine maleate; 1 µl (g body weight)⁻¹) were implanted intraperitoneally (i.p.) with pre-calibrated temperature-sensitive radio transmitters (TA10TA-F40, Data Sciences). The level of anaesthesia was assessed by the withdrawal reflex to a toe pinch. Pregnant females were implanted on GD 7 and allowed to recover until GD 18. Transmitter output frequency (Hz) was monitored, at 10 min intervals, by an antenna mounted in a receiver board, situated beneath the cage of each animal. The output data from each transmitter were transformed into degrees centigrade by Dataquest software (Data Sciences). On the day of the experiment, NP and GD 18 females were separated into SAL or TURP groups ($n = 5-7$ per group). Injections were administered between 08:00 and 09:00 h and the changes in core body temperatures monitored up to 11 h post-treatment. Body temperature data were analysed by calculating the area under the temperature-*versus*-time curve (AUC or fever index) for each animal; mean AUC per group was used for statistical analysis.

Plasma cytokine measurements

Plasma concentrations of IL-6, IL-1 β and IL-1ra were determined from animals killed at the peak of the febrile response, 11 h after TURP injection, in order to measure the maximum humoral responses to the TURP challenge. Blood was collected from animals deeply anaesthetized with a lethal dose of pentobarbital sodium (i.p.; 60 mg (kg body weight)⁻¹) via cardiac puncture ($n = 5-7$ per group) using sterile heparinized syringes and placed into sterile tubes. Samples were then centrifuged (5300 g, 15 min at 4°C), aliquoted and stored at -80°C until assays were performed. Sandwich enzyme-linked immunosorbent assays (ELISA) for IL-6, IL-1 β and IL-1ra (NIBSC, Potters Bar, UK) were performed as previously described (Rees *et al.* 1999), except that plasma samples and secondary antibodies (NIBSC) were diluted in a buffer containing 0.5 M NaCl, 2.5 mM NaH₂PO₄, 7.5 mM Na₂HPO₄, 0.1% Tween 20, 1% ovalbumin and 1 : 100 normal sheep serum (Sigma-Aldrich, St Louis, MO, USA). Intra-assay and inter-assay variations were below 10%. The detection limit for IL-6 and IL-1 β was 40 pg ml⁻¹, and 78 pg ml⁻¹ for IL-1ra. All samples were assayed in duplicate.

Hypothalamus dissection

Killed animals designated for plasma collection were perfused with sterile physiological saline (prepared in DEPC-treated H₂O); brains were dissected, frozen on dry ice and stored at -80°C until used. Hypothalami were microdissected from frozen tissue and divided into right and left hemispheric portions; the left hemisphere was used for RNA extraction and the right for protein extraction.

RNA extraction and RT-PCR in the hypothalamus

In order to assess the changes in transcription of SOCS3, IL-1 β , COX-2 and mPGES-1 genes in the hypothalamus, reverse transcription (RT), followed by PCR were performed as previously reported (Rummel *et al.* 2006). Briefly, total RNA was extracted by disaggregating tissues in 1 ml of TRIzol (Invitrogen, Burlington, ON, Canada). Total RNA was isolated according to the manufacturer's protocol and the air-dried RNA pellet dissolved in 50 µl of DEPC-treated water. One microgram of total RNA was transcribed into cDNA. PCR reactions were performed for COX-2, mPGES-1, SOCS3, IL-1 β and β -actin using 1.8 µl of cDNA and 6 pmol of specific primers (Alpha DNA, Montreal, QC, Canada) for COX-2 (forward: 5'-TGATAGGAGAGACGATCAAGA-3'; reverse: 5'-ATGGTAGAGGGCTTTCAACT-3'), mPGES-1 (forward: 5'-TTTCTGCTCTGCAGCACACT-3''; reverse: 5'-CATGGAGAAACAGGTGAACT-3'), SOCS3 (forward: 5'-CCAGCGCCACTTCTTCAC-3'; reverse: 5'-GTGGAGCATCATACTGGTCC-3'), IL-1 β (forward: 5'-CCCAAGCACCTTCTTTTCTTCATCTT-3', reverse: 5'-CAGGGTGGGTGTGCCGTCTTTC-3') and β -actin (forward: 5'-GCCGTCTCCCCCTCCATCGTG-3'; reverse: 5'-TACGACCAGAGGCATACAGGGACAAC-3') using a Gene Amp PCR system 9700 Thermocycler (Applied Biosystems, Foster City, CA, USA). The following parameters were used: (1) 5 min at 94°C (all primer pairs); (2) 30 s at 94°C, 30 s at 60°C (for SOCS3, IL-1 β and β -actin) or 57°C (for mPGES-1 and COX-2), followed by 45 s at 72°C for 20, 28, 34, 36 or 40 cycles (for β -actin, COX-2, mPGES-1, IL-1 β and SOCS3, respectively); and (3) 72°C for 10 min (for all primer pairs). PCR products were separated by gel electrophoresis (1.5% agarose) and band densities were obtained using GeneTool image analysis software (Syngen, Frederick, MD, USA). Each cDNA data were expressed as a ratio of β -actin optical density and then relative to measurements obtained from the SAL group of NP females ((gene X/ β -actin mRNA)/(mean of gene X/ β -actin from SAL-NP group)) in order to pool data from different gels. Initial PCR experiments using total RNA were performed for each pair of primers to ensure that products did not result from genomic DNA amplification. Additionally, the linear phase of PCR amplification of cDNA was determined by performing RT-PCR on a sample from each treatment group for an increasing number of cycles (20-50 cycles).

Protein extraction and hypothalamic COX-2 Western blot analysis

The right side of the hypothalamus was disaggregated in lysis buffer (50 mM Tris-HCl, 2 mM EDTA and 1% Nonidet) that included a protease inhibitor cocktail

for general use (Sigma-Aldrich). Protein content was quantified using Bradford's reagent (Sigma-Aldrich) following manufacturer's instructions, aliquoted and frozen at -80°C until used.

On the day of the analytical procedure the protein was mixed (1:1) with Laemmli running buffer (Bio-Rad Laboratories, Mississauga, ON, Canada) mixed with β -mercaptoethanol ($50\ \mu\text{l}$ of β -mercaptoethanol per millilitre of Laemmli buffer; Sigma-Aldrich) and incubated at 95°C for 5 min. This ($50\ \mu\text{g}$) was then loaded into pre-cast acrylamide gels (4–20% Tris-glycine gel, Invitrogen) and electrophoresed for 2 h at 125 V. The protein was then transferred (overnight at 15 V) to nitrocellulose membranes (Hybond ECL, Amersham Biosciences Corp., Piscataway, NJ, USA) using Xcell II Blot Module (Invitrogen), following the manufacturer's instructions for liquid transference.

For immunodetection, membranes were blocked with $1 \times$ Tris-buffer saline (TBS), 10% non-fat dry milk (Bio-Rad Laboratories) and 0.1% Tween 20 for 2 h at room temperature. Membranes were then incubated overnight (at 4°C) with a mixture of antibodies raised against murine COX-2 (1 : 1000; Cayman Chemical, Hornby, ON, Canada) and actin (1 : 10 000, Sigma-Aldrich) diluted in $1 \times$ TBS, 5% milk and 0.1% Tween 20. After, antibody excess was washed ($1 \times$ for 15 min and $4 \times$ for 5 min) with $1 \times$ TBS, 0.1% Tween 20, membranes were incubated with a detection antibody (1 : 2000; donkey anti-rabbit IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, CA,

USA; diluted in $1 \times$ TBS, 1% milk and 0.1% Tween 20) for 1 h at ambient temperature. After a similar series of washing steps, the membranes were incubated with ECL Western blotting detection reagents ($0.125\ \text{ml cm}^{-2}$; Amersham Biosciences Corp.) for 1 min and then exposed to a chemoluminescence sensitive film (Hyperfilm ECL, Invitrogen) for 1–5 min. Films were digitized and optical density was determined using GeneTool image analysis software (Syngen, Frederick, MD, USA). Two bands were detected, one at approximately 42 kDa corresponding to actin and another at $\sim 72\ \text{kDa}$ corresponding to COX-2. Levels of COX-2 expression were expressed as a ratio of the β -actin signal and then, as for PCR data, as relative amounts of the measurements obtained for the SAL-NP females in order to pool data from different membranes.

Data analysis

All data are presented as mean values \pm s.e.m. and were analysed using StatView software (version 4.57, Abacus Concepts Inc., Berkeley, CA, USA). One-way ANOVA was used to analyse the data from the temperature, ELISA, PCR and Western blot studies. In cases where comparisons using ANOVA were significant, Newman-Keuls multiple comparisons test was performed. Correlations between parameters were analysed and expressed as fitness to linear curve (r^2); statistical significance was determined by ANOVA. In all cases, P values less than 0.05 were deemed statistically significant.

Results

TURP-induced fever is abolished in late pregnancy

Basal core-body temperature (T_c) was generally lower in pregnant animals compared with NP females (Fig. 1). This finding is consistent with previous reports that show reduced light-phase basal T_c during late pregnancy (Fewell *et al.* 2002; Fofie & Fewell, 2003). Similar to previous reports (Cooper & Rothwell, 1991; Luheshi *et al.* 1997), i.m. injection of $100\ \mu\text{l}$ of TURP into the left rear-limb of NP females ($n = 7$), induced a significant increase in T_c that started 2.5 h after injection and peaked between 10 and 11 h post-injection ($\sim 39.5^{\circ}\text{C}$), with a maximum increase in temperature of 2.4°C (Fig. 1). Fever persisted during the light phase of the second day but was no longer evident during that day's dark phase (data not shown). In contrast, and somewhat surprising given the potency of the TURP stimulus, rats at a late pregnancy stage (GD 18) exhibited no change in body temperature, which did not deviate from basal over the time course of the study ($n = 6$; Fig. 1). As would be expected given the lack of response in GD 18 animals, the comparison of the fever index showed that NP females injected with TURP had significantly higher fever index

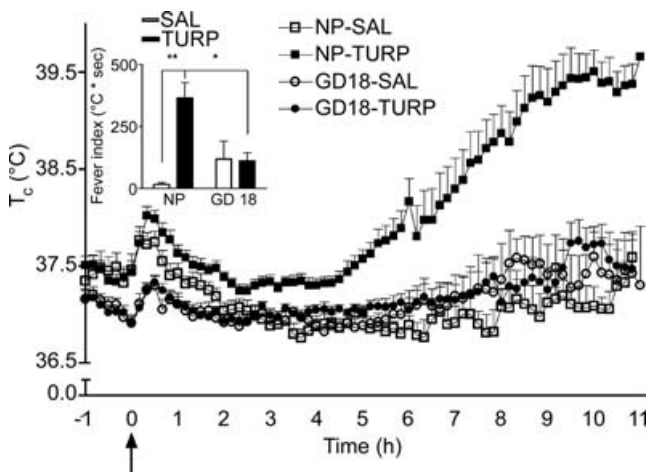


Figure 1. TURP-induced fever is attenuated in late pregnancy

Injection of TURP ($100\ \mu\text{l}$; i.m.) induced a significant rise in core body temperature in NP females (T_c , $^{\circ}\text{C}$). Animals in the 18th day of pregnancy (GD 18) were completely resistant to the febrile effects of TURP. The arrow indicates the time of injection (animals were injected between 08:00 and 09:00 h). Inset, fever indexes (or area under the curve, AUC) were calculated for each group. AUC in the NP females injected with TURP was significantly greater than both SAL-injected NP females and TURP-injected GD 18 dams. NP females *versus* SAL-injected NP females: $**P < 0.01$ and *versus* TURP-injected GD 18 females: $*P < 0.05$.

than NP-SAL ($n = 5$) and GD 18-TURP groups (one-way ANOVA: $F_{3,22} = 8.72$, $P = 0.0008$; Newman-Keuls test *versus* SAL-injected NP females: $P < 0.001$ and *versus* TURP-injected GD 18 females: $P < 0.01$; inset Fig. 1), while that of GD 18-TURP did not differ from the one of GD 18-SAL ($n = 5$; Newman-Keuls test: $P > 0.05$). Interestingly, and despite the absence of the fever response in the pregnant females, TURP-induced oedema of the hind-limb (a hallmark sign of localized inflammation) was present in GD 18 animals, and was similar in severity to that exhibited by the counterpart cycling females. None of these animals showed signs of extreme discomfort that necessitated the premature termination of the study.

Circulating levels of cytokines: dampened IL-6 induction is associated with the absence of fever response

At the peak of the fever response, 11 h after TURP injection, NP females showed a 5.4-fold increase in the concentration of IL-6, from 27.4 ± 9.2 pg ml⁻¹ in the SAL group ($n = 5$) up to 146 ± 29 pg ml⁻¹ in the TURP-injected animals ($n = 7$; one-way ANOVA: $F_{3,19} = 6.42$, $P < 0.01$;

Newman-Keuls test *versus* SAL-injected NP females: $P = 0.0012$; Fig. 2A). IL-6 levels in the SAL-injected GD 18 group ($n = 5$; 38.6 ± 2.2 pg ml⁻¹) did not differ from those of the SAL-injected NP females (Newman-Keuls test: $P = 0.74$). Late-pregnant rats did not show a significant induction of IL-6 after TURP injection ($n = 6$; 60.5 ± 21.9 pg ml⁻¹; Newman-Keuls test *versus* SAL-injected GD 18 dams: $P = 0.5$), and compared with TURP-injected NP-females, these levels were significantly lower (Newman-Keuls test: $P = 0.0094$; Fig. 2A).

NP females showed a significant correlation between plasma IL-6 concentration and T_c ($r^2 = 0.74$, $P = 0.0003$; top panel Fig. 2B) which is consistent with the proposed role of this cytokine in driving the fever response after localized infection or inflammation (Cartmell *et al.* 2000; Rummel *et al.* 2006). In contrast, no such correlation was observed in the GD 18 pregnant dams (bottom panel Fig. 2B) in concordance with their suppressed febrile response.

Circulating IL-1 β and IL-1ra levels were also assayed. IL-1 β levels were found to be below detection limits of the assay in all animals tested (NP and GD 18 females; data not shown). TURP-injected groups had a similar rise in IL-1ra concentrations (one-way ANOVA:

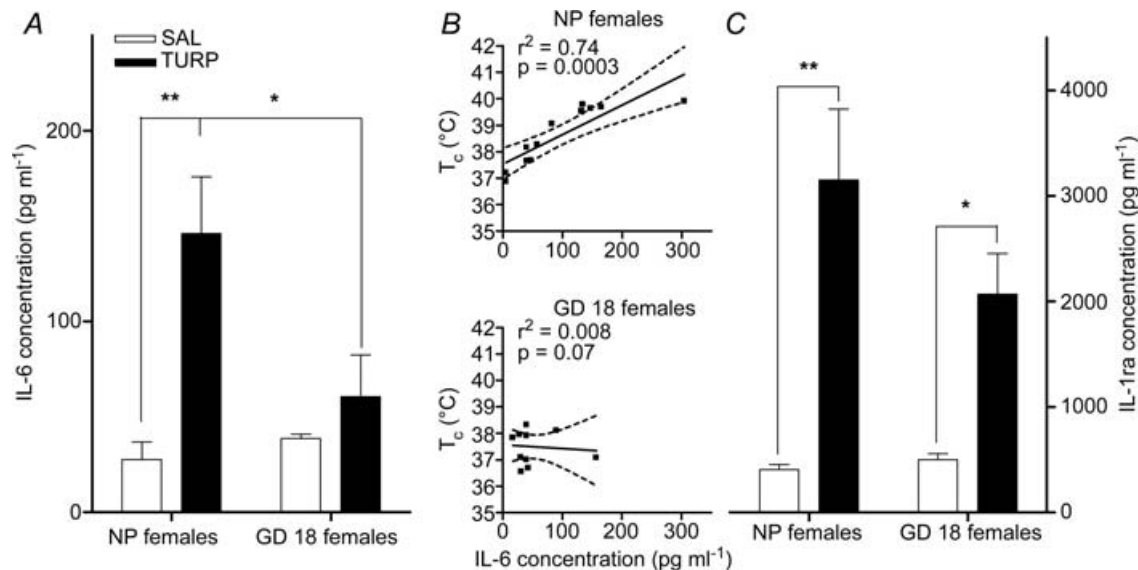


Figure 2. Circulating IL-6 and IL-1ra levels after TURP injection are differentially modulated in late pregnant dams

A, 11 h after the injection of TURP (100 μ l; i.m.), IL-6 concentration was significantly increased in NP females; this increase was not evident in late-pregnant dams (GD 18), whose levels did not differ from those found in the corresponding SAL-injected controls. TURP-injected NP females *versus* SAL-injected NP females: $**P < 0.01$ and *versus* TURP-injected GD 18 females: $*P < 0.05$. B, top panel, correlation analysis between plasma IL-6 levels (x axis) and temperature 11 h after injection (y axis) from NP females revealed a significant relationship between these parameters ($n = 12$ including SAL- and TURP-injected animals; $r^2 = 0.74$, $P = 0.0003$). Bottom panel, the same analysis showed that in GD 18 dams this correlation was not evident ($n = 11$; $r^2 = 0.08$, $P = 0.07$). C, IL-1ra concentration was significantly increased in both NP and GD 18 females after TURP injection; there was no significant difference in the induced levels of the anti-inflammatory cytokine between these two groups. TURP-injected *versus* SAL-injected NP females: $**P < 0.01$; TURP-injected *versus* SAL-injected GD 18 females: $*P < 0.05$.

$F_{3,17} = 9.86$, $P = 0.0005$; Newman–Keuls test: $P > 0.05$), from 406 ± 49 pg ml⁻¹ and 500 ± 55 pg ml⁻¹ under the SAL treatment up to 3151 ± 668 pg ml⁻¹ and 2067 ± 386 pg ml⁻¹ 11 h after TURP injection in NP and GD 18 animals, respectively (Newman–Keuls test SAL- versus TURP-injected NP females: $P < 0.01$; and SAL- versus TURP-injected GD 18 dams: $P < 0.05$; Fig. 2C).

Changes in circulating IL-6 levels correlate with hypothalamic SOCS3 mRNA expression

To further confirm that circulating IL-6 was acting on the hypothalamus, SOCS3 mRNA was measured in the same animals used for cytokine determination. SOCS3, which is induced by the IL-6-activated intracellular signalling pathway (Lebel *et al.* 2000), is part of a negative feedback

mechanism that limits the pro-inflammatory actions of this cytokine (Crocker *et al.* 2003).

In NP females, 11 h after TURP injection, SOCS3 mRNA was induced ~ 1.8 -fold compared with SAL-injected females (one-way ANOVA: $F_{3,19} = 8.79$, $P = 0.0007$; Newman–Keuls test versus SAL-injected NP group: $P < 0.001$; Fig. 3A); these changes correlated significantly with circulating IL-6 levels and T_c ($r^2 = 0.53$, $P = 0.0074$ for IL-6; $r^2 = 0.55$, $P = 0.0059$ for T_c ; top panel of Fig. 3B and C). In contrast, pregnant females did not show any significant increase in SOCS3 mRNA (Newman–Keuls test versus SAL-injected GD 18 dams: $P > 0.05$; Fig. 3A) nor a correlation between this mRNA and T_c or IL-6 levels (bottom panels Fig. 3B and C). Similar to plasma IL-6, SOCS3 mRNA levels in TURP-injected GD 18 dams were significantly lower compared with those of TURP-injected NP females (Newman–Keuls test: $P = 0.019$; Fig. 3A).

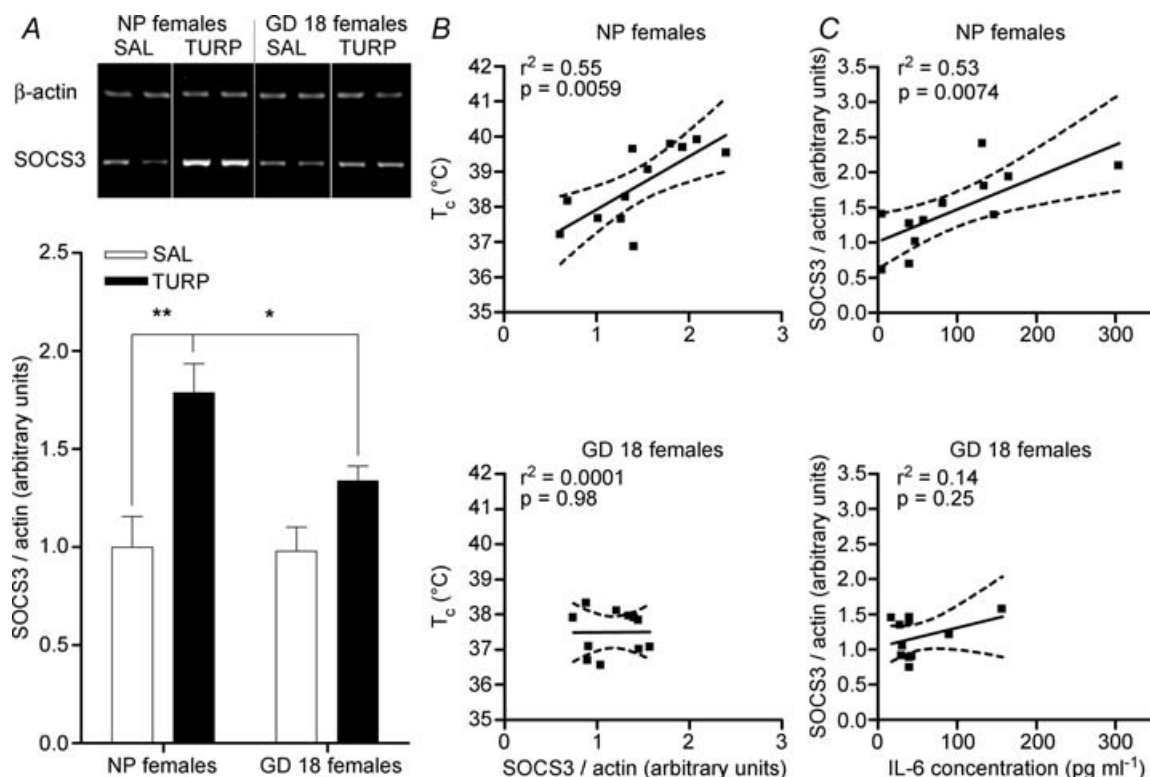


Figure 3. Hypothalamic SOCS3 mRNA, a marker of IL-6 activity, is not induced after TURP challenge during late pregnancy

A, in NP females, SOCS3 mRNA expression in the hypothalamus was significantly induced 11 h after TURP injection (100 μ l; i.m.); in contrast, this increase was blunted in GD 18 dams. TURP-injected NP females versus SAL-injected NP females: ** $P < 0.01$ and versus TURP-injected GD 18 females: * $P < 0.05$. Each band in the gel represents one animal; two animals per group are shown. B, top panel, correlation analysis between hypothalamic SOCS3 mRNA levels (x axis) and temperature 11 h after injection (y axis) from NP females revealed a significant relationship between these parameters ($n = 12$ including SAL- and TURP-injected animals; $r^2 = 0.55$, $P = 0.0059$). Bottom panel, the same analysis showed that in GD 18 dams this correlation was not evident ($n = 11$; $r^2 = 0.0001$, $P = 0.89$). C, top panel, in NP females, correlation analysis as in B for plasma IL-6 levels (x axis) and hypothalamic SOCS3 mRNA levels (y axis) revealed a significant link between these parameters ($n = 12$ including SAL- and TURP-injected animals; $r^2 = 0.53$, $P = 0.0074$). Bottom panel, in contrast, in GD 18 dams this correlation (IL-6 versus hypothalamic SOCS3 mRNA) was not evident ($n = 11$; $r^2 = 0.14$, $P = 0.25$).

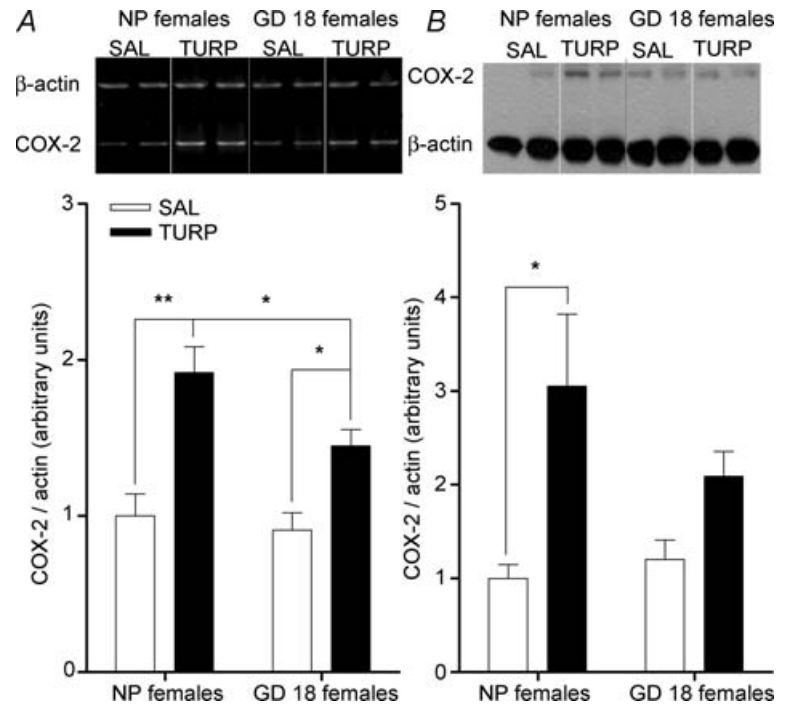


Figure 4. COX-2 expression, at the level of mRNA and protein, is differentially regulated in NP and GD 18 females after TURP injection

A, TURP injection (100 μ l; i.m.) induced a significant increase of COX-2 mRNA expression in the hypothalamus; this induction was reduced, but still present, in GD 18 dams. TURP-injected NP females *versus* SAL-injected NP females: $**P < 0.01$; TURP-injected GD 18 females *versus* SAL-injected GD 18 females and *versus* TURP-injected NP females: $*P < 0.05$. Each band in the gel represents one animal; two animals per group are being shown. B, COX-2 protein levels (measured by Western blot) in the hypothalamus of NP females were increased 11 h after TURP injection (100 μ l; i.m.); this increase was not present in GD 18 dams. TURP-injected NP females *versus* SAL-injected NP females: $*P < 0.05$. Each band in the gel represents one animal; two animals per group are shown.

Fever pathways in the hypothalamus: evidence of blunted prostaglandin synthesis and reduced induction of IL-1 β

Expression of COX-2 was determined by RT-PCR; 11 h after TURP injection, hypothalamic COX-2 mRNA was significantly induced (1.9-fold) in NP females compared with SAL-injected ones (one-way ANOVA: $F_{3,19} = 11.39$, $P = 0.0002$; Newman–Keuls test: $P < 0.001$; Fig. 4A). In GD 18 females TURP injection also caused a significant induction of COX-2 mRNA (1.4-fold, Newman–Keuls test *versus* SAL-injected GD 18 dams: $P < 0.05$), although this was significantly lower compared with the induction in NP females (Newman–Keuls test: $P < 0.05$; Fig. 4A).

In order to confirm the RT-PCR data, COX-2 expression was assayed by Western blot. NP females had 3-fold more COX-2 levels after TURP injection (one-way ANOVA: $F_{3,18} = 4.6$, $P = 0.015$; Newman–Keuls test *versus* SAL-injected NP group: $P < 0.01$; Fig. 4B). In contrast, pregnant females injected with TURP did not show such an increase compared with their own control group (Newman–Keuls test: $P > 0.05$; Fig. 4B). Although induced levels of COX-2 were greater in NP females than in GD 18 dams (3.0 ± 0.77 arbitrary units (a.b.) *versus* 2.1 ± 0.29 a.b., respectively) this difference was not statistically significant (Newman–Keuls test: $P = 0.15$).

Similar to the changes in COX-2, mPGES-1 mRNA levels were significantly up-regulated in NP (2.15-fold; one-way ANOVA: $F_{3,19} = 11.8$, $P = 0.0001$; Newman–Keuls test *versus* SAL-NP females: $P < 0.0001$) and GD 18 females (1.7-fold; Newman–Keuls test *versus* SAL-GD 18 dams: $P < 0.05$; Fig. 5). mPGES-1

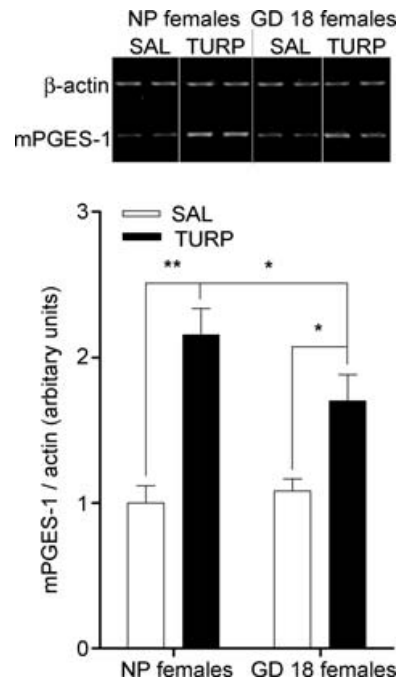


Figure 5. Attenuated mPGES-1 mRNA expression in the hypothalamus of GD 18 females

RT-PCR analysis of mPGES-1 mRNA levels in the hypothalamus revealed a significant induction in NP females after TURP injection (100 μ l; i.m.); in comparison, levels of this mRNA were increased to a lesser extent in GD 18 dams. TURP-injected NP females *versus* SAL-injected NP females: $**P < 0.01$; TURP-injected GD 18 females *versus* SAL-injected GD 18 females and *versus* TURP-injected NP females: $*P < 0.05$. Each band in the gel represents one animal; two animals per group are being shown.

Table 1. Correlation of hypothalamic COX-2 and mPGES-1 expression levels with T_c , plasma IL-6, hypothalamic IL-1 β and SOCS3 mRNAs

	Core temperature (T_c)		Plasma IL-6		Hypothalamic IL-1 β mRNA		Hypothalamic SOCS3 mRNA	
	NP	GD 18	NP	GD 18	NP	GD 18	NP	GD 18
COX-2 mRNA	$r^2 = 0.68^{**}$	$r^2 = 0.01$	$r^2 = 0.47^{**}$	$r^2 = 0.09$	$r^2 = 0.79^{***}$	$r^2 = 0.09$	$r^2 = 0.70^{**}$	$r^2 = 0.72^{**}$
COX-2 protein levels	$r^2 = 0.39^*$	$r^2 = 0.02$	$r^2 = 0.33^*$	$r^2 = 0.12$	$r^2 = 0.26$	$r^2 = 0.26$	$r^2 = 0.70^{**}$	$r^2 = 0.33$
mPGES-1 mRNA	$r^2 = 0.69^{**}$	$r^2 = 0.005$	$r^2 = 0.5^{**}$	$r^2 = 0.05$	$r^2 = 0.84^{***}$	$r^2 = 0.07$	$r^2 = 0.64^{**}$	$r^2 = 0.41^*$

r^2 denotes goodness of fit to a linear curve and significance was evaluated by ANOVA. $n = 12$ for NP group and 11 for GD 18 group.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.0001$.

mRNA levels differed significantly between the two TURP-injected groups (Newman–Keuls test: $P < 0.05$; Fig. 5).

In NP females, expression levels of COX-2 (measured as mRNA or as protein) and mPGES-1 correlated significantly with circulating IL-6 levels (Table 1). SOCS3 mRNA levels correlated with the expression levels of both enzymes (Table 1). In late-pregnant rats no correlation was found between IL-6 and COX-2 or mPGES-1 (Table 1). However, similar to cycling females, SOCS3 mRNA levels correlated with those of COX-2 and mPGES-1 (Table 1).

Although IL-1 β was not detected in the circulation, it has been proposed that centrally it plays a significant role in TURP-induced fever (Luheshi *et al.* 1997). Therefore we decided to measure the expression of its mRNA levels within the hypothalamus. In TURP-injected NP females IL-1 β mRNA was significantly induced (2.4-fold, one-way ANOVA: $F_{3,19} = 13.26$, $P = 0.0001$; Newman–Keuls test *versus* SAL-injected NP group: $P < 0.0001$; Fig. 6A) and correlated with T_c ($r^2 = 0.77$, $P = 0.0002$; top panel Fig. 6B), IL-6 concentration in plasma ($r^2 = 0.45$, $P = 0.017$; top panel Fig. 6C) and SOCS3 mRNA ($r^2 = 0.84$, $P < 0.0001$; figure not shown). Similarly, GD 18 females showed a significant induction of hypothalamic IL-1 β (1.7-fold, Newman–Keuls test *versus* SAL-injected GD 18 females: $P < 0.05$), although these values were significantly lower than those in TURP-injected NP females (Newman–Keuls test: $P < 0.05$; Fig. 6A) and did not correlate with T_c ($r^2 = 0.16$, $P = 0.22$; bottom panel Fig. 6B) or with SOCS3 mRNA ($r^2 = 0.18$, $P = 0.19$; figure not shown). However, hypothalamic IL-1 β mRNA showed a low but significant correlation with circulating IL-6 levels ($r^2 = 0.6$, $P = 0.046$; bottom panel Fig. 6C). T_c and IL-1 β mRNA only correlated with expression levels of COX-2 and mPGE-1 in NP females, and not in GD 18 dams (Table 1).

Discussion

In the present study we demonstrated that a TURP challenge, which would normally induce a highly significant and prolonged fever response in non-pregnant rats, was almost totally ineffective during late pregnancy

(Fig. 1). The absence of this response appeared to be the result of an attenuated IL-6 release into the circulation (Fig. 2A), reflected in a diminished hypothalamic SOCS3 mRNA induction (Fig. 3A) and a reduction in COX-2 and mPGES-1 synthesis in this brain structure (Figs 4 and 5). A functional link between fever, peripheral IL-6 and hypothalamic expression of SOCS3, COX-2 and mPGES-1 was confirmed in the cycling females treated with TURP by the induction of these markers and more crucially by the significant correlation between them and the fever response (Figs 2B, 3B and C and Table 1). Importantly, this relationship was absent in the GD 18 females, where these molecules were scantily induced by TURP. The levels of the anti-inflammatory cytokine IL-1ra were comparable in both NP and GD 18 females injected with TURP (Fig. 2C). This contrasts with our earlier observations made in pregnant females injected with LPS (Ashdown *et al.* 2006b) where it was evident that rather than IL-6, the main factor regulating the attenuated fever response to LPS was in fact a higher than normal production of IL-1ra in the pregnant females. To understand this discrepancy the differences between systemic challenges like i.p. LPS injection and localized activation of the immune system, as occurs with TURP, must be considered. LPS could target directly several tissues that produce IL-1ra (Turrin *et al.* 2001), such as white adipose tissue (WAT), the amount of which increases during pregnancy (Dayer *et al.* 2006), most probably resulting in the over-production of this cytokine during late pregnancy. In contrast, TURP effects are confined to the local site of inflammation thus diminishing the possibility of stimulating fat deposits which are more accessible to a circulating stimulus.

Our current observations demonstrating drastically reduced levels of IL-6 (and consequently hypothalamic SOCS3 mRNA) and COX-2 after TURP injection during late pregnancy, provide a possible mechanism underlying the reported reduction in PGE₂ levels in the brain, the ultimate step in fever generation (Fewell *et al.* 2002; Imai-Matsumura *et al.* 2002; Mouihate *et al.* 2002). These findings are similar to those reported previously in IL-6 'knock out' mice, which were shown to be resistant to TURP-induced fever (Kozak *et al.* 1997), and exhibit significantly attenuated COX-2 induction (Turnbull *et al.*

2003). These results, coupled with ones made in the present study, strongly support our recent observations demonstrating a direct link between circulating IL-6 and brain COX-2 expression (Rummel *et al.* 2006). In those experiments we clearly demonstrated that IL-6 injection induces COX-2 expression in endothelial cells lining the microvasculature of the brain (Rummel *et al.* 2006) and that neutralization of LPS-induced circulating IL-6 almost totally abolishes the expression of this enzyme in the same structures.

In addition to the changes in the levels of COX-2 in the current study, we have observed a significant reduction in the levels of mPGES-1 expression after TURP in late-pregnant animals. This enzyme is involved in the last step of PGE₂ synthesis, downstream of COX-2, and is fundamental in TURP-induced fever (Saha *et al.* 2005). In the central nervous system, regulation of mPGES-1 expression has been shown to be linked with that of COX-2 after LPS challenge (Yamagata *et al.* 2001; Ivanov

et al. 2002; Ivanov & Romanovsky, 2004). Our findings support the hypothesis of overlapping mechanisms of transcriptional regulation for both enzymes (Yamagata *et al.* 2001; Ivanov *et al.* 2002; Ivanov & Romanovsky, 2004), which is likely controlled by IL-6 in the case of TURP-induced inflammation.

Traditionally, a central role in the regulation of COX-2 and mPGES-1 expression has been ascribed to IL-1 β and other molecules classically involved in NF- κ B activation (Laflamme *et al.* 1999; Kojima *et al.* 2004; Nadjar *et al.* 2005; Sooranna *et al.* 2006). The observations made in the current study do not negate this hypothesis since we have demonstrated that hypothalamic IL-1 β is induced in both groups of TURP-treated animals but are reduced significantly in pregnant females 11 h after TURP injection (Fig. 6). However, at earlier time points, 4 and 6 h after TURP injection, when fever and increased COX-2 and mPGES-1 expression were already evident (data not shown), hypothalamic IL-1 β mRNA was not induced,

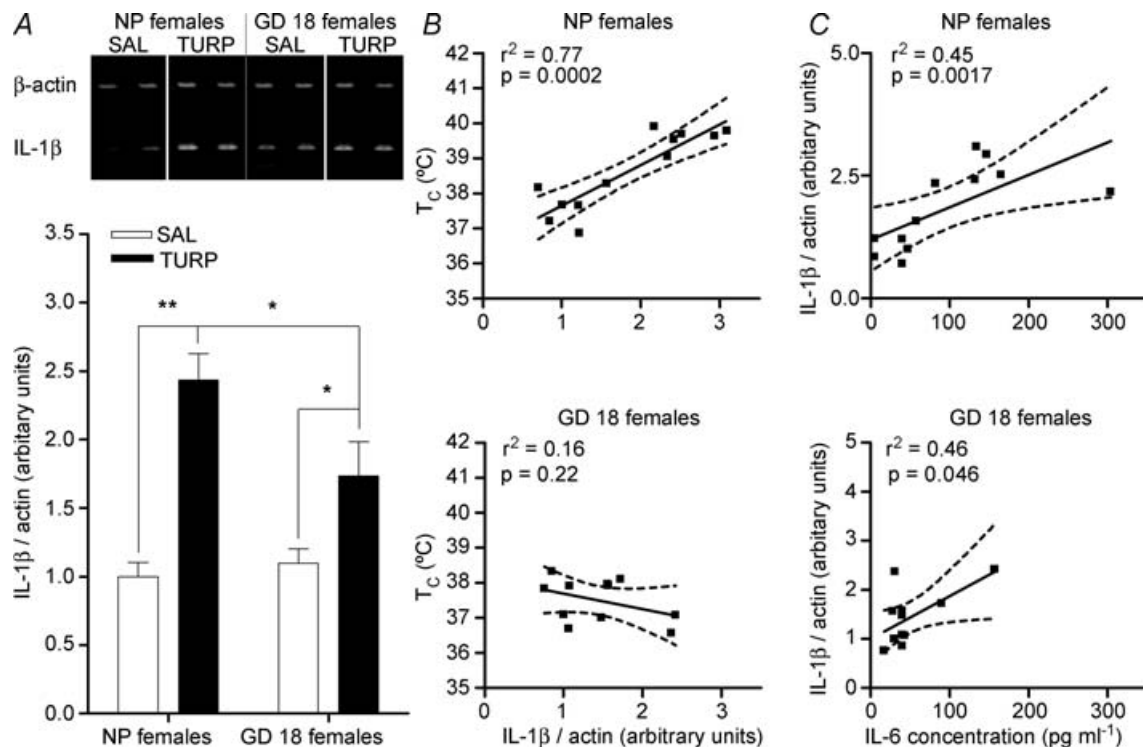


Figure 6. Hypothalamic IL-1 β mRNA induction after TURP is attenuated GD 18-females

A, similar to other central markers of inflammation, IL-1 β mRNA expression increased in the hypothalamus of NP females, 11 h after TURP injection (100 μ l; i.m.). In TURP-injected GD 18 dams the levels of this mRNA were lower, but still significantly induced. TURP-injected NP females *versus* SAL-injected NP females: ** $P < 0.01$; TURP-injected GD 18 females *versus* SAL-injected GD 18 females and *versus* TURP-injected NP females: * $P < 0.05$. Each band in the gel represents one animal; two animals per group are being shown. B, top panel, hypothalamic IL-1 β mRNA levels (x axis) correlated significantly with temperature 11 h after TURP injection (y axis) in NP females ($n = 12$ including SAL- and TURP-injected animals; $r^2 = 0.77$, $P = 0.0002$). Bottom panel, the same analysis showed that no such correlation existed in GD 18 dams ($n = 11$; $r^2 = 0.16$, $P = 0.22$). C, top panel, as in B, in NP females hypothalamic IL-1 β mRNA levels (y axis) showed a low, but significant correlation with plasma IL-6 levels (x axis) ($n = 12$ including SAL- and TURP-injected animals; $r^2 = 0.45$, $P = 0.017$). Bottom panel, in GD 18 dams this correlation was still present ($n = 11$; $r^2 = 0.46$, $P = 0.046$). The expression of microsomal PGE synthase-1 is attenuated in GD 18 dams after TURP injection.

while circulating IL-6 and hypothalamic SOCS3 mRNA levels were already significantly up-regulated (data not shown). At the later time point tested (11 h), however, there was a high degree of correlation between COX-2 and mPGES-1 expression levels and those of IL-1 β mRNA in the hypothalamus (Table 1) similar to the one found for IL-6 and SOCS3 mRNA (Table 1). Given the established role of IL-1 β in COX-2 and mPGES-1 regulation, it is tempting to suggest that its central induction may contribute to regulation of PGE₂-synthesizing enzymes expression in a sustained febrile response as occurs after TURP injection. The anomaly of this hypothesis is that there are no data suggesting a direct link between peripheral IL-6 and brain IL-1 β , and our preliminary observations (data not shown) do not support the existence of such an association.

The observation that pregnant animals responded to the TURP challenge with a significant induction of both hypothalamic COX-2 and mPGES-1 mRNAs (Figs 4A and 5), although this was not reflected in changes in core temperature (Fig. 1), is paradoxical. There are two possible explanations for this finding: (i) The existence of an additional regulatory step that uncouples transcription from translation of COX-2, by which COX-2 protein levels are not increased even with augmented transcription of the COX-2 gene; accordingly, our results showed that hypothalamic COX-2 protein levels were not significantly elevated in late-pregnant dams (Fig. 5B); (ii) The sensitivity to the pyrogenic effect of central PGE₂ could be attenuated in near-term pregnant rats. This, in fact, has been previously demonstrated by infusing PGE₂ directly into the lateral ventricles of near-term pregnant rats, which resulted in an attenuated pyrogenic effect (Eliason & Fewell, 1998; Chen *et al.* 1999; Eliason & Fewell, 1999). The mechanisms underlying the attenuated febrile response to PGE₂ are not completely clear, although they may involve alteration in PGE receptor expression (Mouihate *et al.* 2002), changes in brain prostaglandin clearance and/or catabolism (Ivanov & Romanovsky, 2003), or increased expression of the antipyretic arginine-vasopressin (Eliason & Fewell, 1998, 1999; Chen *et al.* 1999).

In previous reports, the inability to find altered molecular fever pathways in the central nervous system after LPS during late pregnancy, upstream of PGE₂ synthesizing enzymes (Mouihate *et al.* 2005; Harre *et al.* 2006), could stem from the nature of the given stimulus. As mentioned earlier, LPS is capable of stimulating several organs to produce fever mediators that act in a highly redundant fashion, as is further evidenced by the lack of effect of targeted mutations ('knock out') of cytokines on LPS-febrile response (Kopf *et al.* 1994; Zheng *et al.* 1995; Kozak *et al.* 1997; Leon, 2002). Several of these mediators could activate similar intracellular signalling pathways, responsible for COX-2 induction,

which could have masked our ability to detect alterations in such pathways. In contrast, in TURP-induced fever, the respective situation is such that elimination of any of the identified components in the cytokine pathway (TNF α , IL-1 β and IL-6) abolishes this response (Zheng *et al.* 1995; Kozak *et al.* 1997; Luheshi *et al.* 1997; Leon, 2002) and this is readily reflected in the activation state of downstream mediators, as we report in the present study. Additionally, the use of different controls groups (NP females in the current study and GD 15 and lactating females in Mouihate *et al.* 2005 and Harre *et al.* 2006) could explain some of these inconsistencies.

The mechanisms underlying the blunted IL-6 production during pregnancy were not directly addressed in our study; however, several interesting possibilities arise. TURP-induced fever depends largely on IL-6 induction and release into the circulation, as a direct result of localized release of other pyrogenic cytokines such as TNF α and IL-1 β (Luheshi *et al.* 1997; Turnbull *et al.* 2003). Our previous demonstration that IL-1ra induction is enhanced after LPS injection in late-pregnant rats (Ashdown *et al.* 2006b), does not extend to all inflammation models such as the one used in the current study; however, it is still feasible that IL-1ra induction in the local site of inflammation (where subcutaneous adipose tissue may be activated) may be enhanced, thus indirectly preventing IL-6 synthesis by inhibiting the action of IL-1 β at the level of the necrotic tissue. Other plausible candidates for the reduced IL-6 production during near-term pregnancy are progesterone and oestrogen, that act as generalized modulators of both innate and adaptive immunity (Beagley & Gockel, 2003; Doria *et al.* 2006). Oestrogen levels rise towards the end of pregnancy (from GD 17 onwards), while progesterone levels, that are maintained at a very high level throughout most of the duration of pregnancy, diminish around GD 19 (Mann & Bridges, 2001). Oestrogen has been shown to decrease the inflammatory effects of carrageenan (Cuzzocrea *et al.* 2001), IL-1 β (Ospina *et al.* 2004) and LPS (Vegeto *et al.* 2001; Mouihate & Pittman, 2003). Its anti-inflammatory effect seems to stem from its ability to arrest NF- κ B-mediated transcription (Ghisletti *et al.* 2005) of genomic targets that include IL-6 (Galien & Garcia, 1997) and COX-2 genes (Mouihate & Pittman, 2003; Ospina *et al.* 2004). This could account for the abolished effects of TURP in dams at GD 18 described in our study. Mouihate & Pittman (2003) showed that oestrogen and progesterone replacement reduced circulating IL-6 induction by IL-1 β , but not after LPS injection, although fever and COX-2 expression were modulated in the opposite direction. However, the effect of different doses of oestrogen, in the presence of low levels of progesterone, has not been determined.

The physiological significance of reduced fever or immune activation during pregnancy is generally regarded

as a protective mechanism against the detrimental effects of an abnormal rise in core body temperature or cytokines on fetal development. This is based on a growing body of evidence suggesting that immune activation during mid- or late-pregnancy is a risk factor strongly associated with increased incidence of psychiatric disorders such as cerebral palsy, autism and schizophrenia (Boksa, 2004; Fortier *et al.* 2004; Meyer *et al.* 2005, 2006; Ashdown *et al.* 2006a). Accordingly, prenatal exposure to IL-6 was shown to induce abnormalities in hippocampal structure, deficits in spatial working memory, hypertension and alterations in stress response in the adult offspring (Samuelsson *et al.* 2004; Samuelsson *et al.* 2006). Based on these observations and the results from the current study, it would appear that this cytokine, which has been shown to cross the blood-placental barrier (Dahlgren *et al.* 2006), plays a significant role in the reported neurodevelopmental defects associated with maternal infection, stress or injury during gestation and could provide a promising pharmacological target to help meliorate these effects.

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