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Modulation of Oestrogen Receptor-β mRNA Expression in Rat Paraventricular and Supraoptic Nucleus Neurones Following Adrenal Steroid Manipulation and Hyperosmotic Stimulation

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Abstract

Magnocellular neurosecretory neurones in the hypothalamic paraventricular (PVN) and supraoptic (SON) nuclei express oestrogen receptor β (ERβ) but not ERα. In the PVN, ERβ is strongly expressed in the ventromedial parvocellular neurones projecting to the brainstem. We used quantitative *in situ* hybridization, with ^{35}S-labelled riboprobes, to study heterologous regulation by manipulating adrenal steroid hormones (72 h after adrenalectomy ± corticosterone replacement; repeated stress: halothane inhalation, environmental cold, immobilization, each daily for 3 days) in male rats. Adrenalectomy increased ERβ mRNA expression in the magnocellular PVN and SON, by 2.2 and 2.5-fold, respectively, with no effect in the ventromedial parvocellular PVN neurones. Corticosterone replacement partially prevented the increases in ERβ mRNA expression in magnocellular PVN and SON neurones. Repeated stress over 72 h had no effect on ERβ mRNA expression in the magnocellular PVN or SON, but increased expression 1.4-fold in the ventromedial parvocellular PVN neurones. Although consequences of hydromineral balance derangement after adrenalectomy may stimulate magnocellular neurones, strongly stimulating the neurones by giving intact male rats 2% saline to drink for 72 h decreased ERβ mRNA expression in the magnocellular PVN and SON neurones by approximately 60%, and in the ventromedial parvocellular PVN neurones by 13%. Thus, ERβ mRNA expression is negatively regulated by basal glucocorticoid secretion in magnocellular PVN and SON neurones, and positively regulated by stress in ventromedial parvocellular PVN neurones. However, ERβ mRNA expression in magnocellular neurones is negatively linked to hyperosmotic stimulation of the neurones. The 6.25-fold variation in ERβ mRNA expression in magnocellular neurones from salt-loading to adrenalectomy could alter their sensitivity to oestrogens. Consequently, regulation of oxytocin and vasopressin neurone activity via ERβ is expected to vary according to their functional state and, in particular, on basal glucocorticoid actions.

The magnocellular neurosecretory oxytocin and vasopressin neurones in the paraventricular (PVN) and supraoptic (SON) nuclei project their axons to the neurohypophysis (1). In the rat, both oxytocin and vasopressin neurones are stimulated by increased extracellular fluid osmolarity or sodium concentration, and vasopressin secretion is also stimulated by hypotension or by reduced blood volume (2, 3). Vasopressin stimulates water reabsorption in the kidneys and, in the rat, oxytocin is natriuretic, although its principal roles are in milk ejection and parturition (4). *In vivo* oestrogen has been reported to increase or to decrease vasopressin release, and to promote the production of oxytocin (5–7), perhaps involving oestrogen receptor (ER) regulation of the oxytocin and vasopressin genes (8, 9).

Mechanisms of oestrogen action on these neurones are unclear. The PVN and SON magnocellular neurones accumulate systemically administered radiolabelled-oestrogen, although weakly relative to some brain regions (10), but previous studies have failed to demonstrate expression of ERα in these neurones (11). Furthermore, while the inhibitory action of oestrogen on vasopressin and oxytocin release from hypothalamic-neurohypophysial explants stimulated hyperosmotically is via a membrane action (12), oestrogen evidently acts genomically to inhibit stimulation by NMDA

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(13). Thus, the discovery of ERβ (14), and the finding that the magnocellular neurones prominently express ERβ (15–17) may explain the enigma of how they accumulate oestrogen, and how it acts on them (16). ERβ is expressed in both oxytocin and vasopressin magnocellular neurones in both the PVN and SON (18, 19), but predominantly in vasopressin neurones (19–22). The greatest expression is in the ventromedial and dorsal groups of parvocellular PVN neurones, predominantly oxytocin and nociceptin (orphanin FQ)-containing, that project to the brainstem and spinal cord, and are involved in autonomic regulation (20, 23).

The function of ERβ is not clear. It is a steroid ligand-regulated transcription factor, binding 17β-oestradiol with similar affinity to ERα, but with low affinity for testosterone, progesterone and corticosterone (24); it has a higher affinity for some phyto-oestrogens than ERα (24). Indeed, pharmacological studies indicate that the inhibitory action of oestrogen on vasopressin and oxytocin release *in vitro* is via ERβ (13). It can act on gene transcription as a homodimer, as well as a heterodimer with ERα (25), with which it has considerable homology (15).

Expression of ERβ mRNA in PVN and SON magnocellular neurones varies with their physiological state, decreasing with sustained hyperosmotic stimulation via chronic salt-loading (26) and during lactation (27). It has subsequently been shown that reduced activity of these neurones, in induced hyponatraemia, leads to increased expression of ERβ mRNA, and that these changes in expression are not related to changes in circulating sex steroid levels (21).

In addition to modulating magnocellular neurone activity, salt-loading also inhibits parvocellular corticotrophin-releasing hormone (CRH) PVN neurones and subsequent adrenocorticotropic hormone (ACTH) secretion (28, 29), although it increases morning nadir adrenal corticosteroid secretion (29–31). Moreover, disturbance in hydromineral balance following manipulations of adrenal steroid hormones, such as by adrenalectomy, may regulate the magnocellular neurones by altering activity in their neural inputs (32). Notably, PVN and SON magnocellular neurones do not normally express mineralocorticoid receptor, or progesterone receptor, and glucocorticoid receptor (GR) expression is not consistently reported (33, 34). However, in chronic hyponatraemia, GR is expressed in magnocellular vasopressin neurones, but not in oxytocin neurones, and expression decreases after adrenalectomy (33, 35). It has recently been reported that ERβ mRNA expression in the PVN in female rats is reduced by adrenalectomy (36).

In this study, we examined in male rats the heterologous regulation of ERβ mRNA expression in magnocellular PVN and SON neurones and in the ventromedial caudally projecting parvocellular PVN neurones by imposing changes in endogenous corticosteroid production, ablating secretion by adrenalectomy and increasing it by chronic stress; we compared the effects of these manipulations with those of salt-loading.

Materials and methods

Animals and procedures

Adult male Hooded-Lister rats (body weight 180–220 g; Harlan-Olac, Bicester, UK Ltd) were acclimatized to a 12 : 12 h light/dark cycle, ambient temperature 18–20 °C, for at least 2 weeks before experiments, housed in groups of three per cage. Standard rat chow and water were available initially *ad libitum*. We elected to use male rats because, in a preliminary study, we found no difference between males and females in patterns of ERβ mRNA distribution in the PVN and SON (37). Moreover, studying males obviously avoids the variations in oestrogen exposure through the oestrous cycle in females, and testosterone is a precursor for oestrogen in the male brain, including in the SON, which contains abundant aromatase that catalyses testosterone conversion to 17β-oestradiol (38). Furthermore, 5α-androstane-3β,17β-diol, a metabolite of 5α-dihydrotestosterone (formed from testosterone by 5α-reductase action in the hypothalamus) has agonist activity via ERβ (14).

In the first experiment, male rats (n = 5 per group) were treated as follows: (i) sham-adrenalectomy + vehicle injections (corn oil, 0.5 ml/kg) daily; (ii) adrenalectomy (ADX) + vehicle, given 0.9% NaCl to drink; and (iii) ADX + corticosterone (Sigma, Poole, UK) 10 mg/kg s.c. injection daily (at 16.00 h). Surgery was performed under halothane (5%) anaesthesia.

In the second experiment, rats were either: (i) left undisturbed (controls, n = 5) or (ii) exposed to a cycle of stressors over 72 h (exposure to 5% halothane for 1 min; environmental cold (swimming in water at 4 °C for 15 min); restraint in a Perspex cylinder for 60 min: each once daily, in random order at 10.00, 12.00 and 16.00 h (n = 5).

In the third experiment, rats were given only 2% NaCl in water (w/vol) to drink for 72 h (n = 6). The controls (n = 6) continued to receive tap water to drink.

The rats were killed by decapitation between 09.00 and 12.00 h: 72 h after surgery (first experiment) or the start of the stress cycle (second experiment, i.e. 16–19 h after the last stress); or after 2% NaCl ingestion for 72 h; untreated controls were killed at the same time. Trunk blood was collected into tubes containing 5% EDTA (approximately 0.1% final concentration) and plasma was separated by centrifugation and stored at −20 °C for subsequent radioimmunoassay of corticosterone, or measurement of $[Na^+]$. The brains were removed, quickly frozen on dry ice and stored at −80 °C until processed for *in situ* hybridization histochemistry. The thymus gland was removed and weighed to assess integrated corticosterone secretion; thymus weight is inversely related to corticosterone secretion, increasing within 72 h of adrenalectomy unless corticosterone replacement is given (39).

Preparation of oestrogen receptor-β and -α riboprobes

ERβ

A 400-bp *Acc*I-*Eco*RI fragment encoding the 5′-untranslated region of rat ERβ cDNA (the cDNA was generously provided by Dr George G. J. M. Kuiper) (14) was subcloned into pBluescript KS. Sense and antisense riboprobes were generated by *in vitro* transcription, in the presence of ^{35}S-UTP, with T7- and T3-RNA polymerase after plasmid linearization with *Acc*I or *Eco*RI, respectively (40).

ERα

In situ hybridization histochemistry for ERα mRNA was carried out as a control procedure for the ERβ mRNA hybridization, as ERα mRNA is not expressed in the PVN and SON. A 367-bp *Pst*I–*Eco*RI fragment corresponding to part of the ligand-binding domain of the mouse ERα cDNA was subcloned into the pGEM3 plasmid (41); cDNA was kindly provided by Dr K. E. Chapman, Molecular Endocrinology, MMC, Western General Hospital, Edinburgh), and used to transcribe ^{35}S-labelled antisense and sense (control) riboprobes from linearized plasmid, using the T7 or SP6 -RNA polymerase, respectively.

CRH mRNA riboprobe

In situ hybridization for CRH mRNA was used to evaluate the effectiveness of the adrenal steroid manipulations. A cDNA clone (*Bam*HI–*Pvu*II fragment) encoding approximately 518 bp spanning the intron/exon II boundary of rat CRH (42) was subcloned into pBluescript KS. Antisense riboprobe was generated by *in vitro* transcription, in the presence of ^{35}S-UTP, with T3-RNA polymerase after plasmid linearization with *Xba*I (40).

In situ hybridization histochemistry

Coronal cryostat sections (10 μm) were cut through the hypothalamus, thaw-mounted onto poly L-lysine coated microscope slides and stored at −80 °C until use. Every fifth section was separately mounted and stained with

Toluidine Blue-O (Sigma) to locate regions of interest. The hybridization was carried out according to methods previously described (40).

Briefly, mounted sections were fixed in cold 4% paraformaldehyde, rinsed in 1 × phosphate buffered saline (PBS; pH 7.4), immersed in 0.3% acetic anhydride-triethanolamine and dehydrated through a graded series of ethanol solutions. Sections were then hybridized with the ^{35}S-labelled antisense or sense ERβ or ERα riboprobes mixed with 50% formamide in a humidified chamber at 50 °C overnight. After the incubation, slides were washed in 2 × saline sodium citrate (SSC) at room temperature (three times, 5 min each), treated with 30 μg/ml RNase A (bovine pancreas: Roche Diagnostics Ltd, Lewes, UK) at 37 °C for 60 min, then rinsed with 2 × SSC at room temperature for 60 min. Subsequently, slides were washed in 0.1 × SSC at 60 °C for 90 min followed by another wash starting at 60 °C, then allowed to cool to room temperature. Sections were dehydrated through a series of alcohols (50%, 70% and 90%) made by adding 100% ethanol to 0.3 M ammonium acetate. Slides were left to dry at room temperature, then apposed to Kodak Hyperfilm β-max (Amersham Life Science Ltd, Little Chalfont, UK) for 2 weeks. After development of the film, the slides were dipped in NTB2 liquid nuclear emulsion (Anachem, Luton, UK.; diluted 1 : 1 with double distilled water), exposed for 5 weeks, photographically processed, stained with 1% pyronin Y (Sigma) and coverslipped.

From rats in the adrenal steroid manipulation study, some sections through the medial parvocellular division of the PVN, which contains CRH-producing neurones, were hybridized with the ^{35}S-labelled riboprobe for CRH mRNA.

Specificity of hybridization signal

Control procedures with the probes for antisense ERβ and ERα included hybridization of sections with the respective sense probes, or pretreatment with RNase A prior to hybridization with an antisense riboprobe, all conducted under identical conditions to those for antisense probes. There was no detectable labelling in the hypothalamus with the ERβ sense probe. ERα sense labelling was undetectable in all sections we examined. No hybridization signal was detected after RNase pretreatment.

The differential and characteristic distribution of ERβ and ERα mRNA hybridization signals confirmed the specificity of the technique for detection of the ERβ mRNA species. As described previously (17, 37), the distribution of ERβ mRNA in the brain showed some striking differences from that of ERα mRNA, although there was also substantial coincidence of expression within certain structures. The PVN and SON showed no hybridization for ERα mRNA. Cerebellar Purkinje cells and the choroid plexus showed a strong signal for ERβ mRNA, but no labelling for ERα mRNA. ERβ mRNA expression was weak in the arcuate nucleus and absent from the ventromedial nucleus, but there was hybridization signal for ERα mRNA in the arcuate nucleus, particularly dense in the lateral subdivision, and in the ventrolateral part of the ventromedial nucleus. The subfornical organ expressed ERα but not ERβ mRNA. These characteristic signatures for the distributions of ERβ mRNA and ERα mRNA confirm the specificity of the ERβ probe and hybridization technique used here.

The CRH probe has been previously validated and hybridization was detected as expected in the dorso-medial parvocellular PVN (43).

Quantification of autoradiographs

Autoradiographs were evaluated by measuring optical density of film images of regions of interest via a digital camera, or by counting silver-grains over the profiles of individual neurones (× 40 objective), using a computer-aided image analysis system (MCID-4; Imaging Research, Ontario, Canada). For film measurements, optical density was measured for 6–8 images of the PVN and SON per rat. Silver grain counts were made over eight neurones per section, and in four sections per region per rat. Background measurements were made over adjacent tissue with no evident expressing neurones, dorsal to the SON for the PVN and SON, and subtracted. The subdivisions of the PVN were defined after Swanson and Kuypers (44). The slides were coded so that the experimenter was unaware of the treatment of the rats at the time of evaluation. Animal means were calculated for each variable, and these values were used to calculate group means.

Corticosterone radioimmunoassay

Total plasma corticosterone concentrations were measured using a proximity radioimmunoassay (40). The sensitivity of the assay was 1.25 nmol/l and the intra- and interassay coefficients of variation were below 7% and 10%, respectively.

Plasma sodium concentration

Plasma [Na^+] and [K^+] (mmol/l) were measured by flame photometry according to methods and standards supplied by the manufacturer (455 Flame Photometer, Ciba Corning Diagnostics Ltd, Halstead, UK).

Statistical analysis

Student's t-test or one-way analysis of variance (ANOVA or Kruskal–Wallis analysis on ranks), and post-hoc tests (Student–Newman–Keuls or Duncan's tests) were used (SigmaStat, Jandel, San Rafael, CA, USA) as appropriate to compare groups ($P < 0.05$ was considered statistically significant). Results are expressed as the group means ± SEM.

Results

Neurones in the PVN and SON expressed ERβ mRNA, most evident in the PVN (Fig. 1), but were completely devoid of ERα mRNA signal (not shown). As previously reported, expression in the PVN was prominent in the ventromedial parvocellular group of neurones (Fig. 1F) and the dorsal parvocellular part (17), from which neurones project to the brainstem (23, 44), and sparse in the dorsomedial parvocellular zone (Fig. 1F) (18, 19), which contains CRH neurones (Fig. 1E) that project to the median eminence (45). In both the PVN and SON, the expression of ERβ mRNA was less intense in the magnocellular neurones than in the ventromedial parvocellular PVN neurones (Figs 1A,F and 2). ERβ mRNA expression was evident throughout the rostro-caudal extent of the SON, and in both the dorsal (predominantly oxytocin-containing) and ventral (predominantly vasopressin-containing) portions of the nucleus, though weaker in the dorsal part (Fig. 1B).

Adrenalectomy and corticosterone replacement on ERβ mRNA in the PVN and SON

Effectiveness of the treatments was confirmed by the measurements of hypothalamic-pituitary-adrenal (HPA) axis parameters. Corticosterone replacement more than restored plasma corticosterone concentration in adrenalectomized rats (sham-operated controls: 440 ± 67 nmol/l; adrenalectomized: 26.6 ± 16.3 nmol/l; adrenalectomized + corticosterone: 2082 ± 434 nmol/l; Kruskal–Wallis one-way ANOVA, $P < 0.005$; Dunn's Method, $P < 0.05$), and prevented the postadrenalectomy increase in thymus weight (sham-operated controls: 325 ± 23 mg; adrenalectomized: 398 ± 17 mg; adrenalectomized + corticosterone: 298 ± 23 mg; ANOVA, $P < 0.05$; Student–Newman–Keuls, $P < 0.05$). Adrenalectomy increased CRH mRNA expression per neurone in the dorso-medial parvocellular PVN, and expression was decreased by corticosterone replacement (ANOVA, $P < 0.05$) (Fig. 3).

At 72 h after adrenalectomy, ERβ mRNA expression in the SON was significantly increased (Figs 1A,B and 2) (ANOVA, $P < 0.005$); in the sham, adrenalectomy + vehicle and adrenalectomy + corticosterone groups, mean ± SEM optical density was, respectively, 30.9 ± 1.29, 46.6 ± 2.61 and 35.3 ± 1.75 units; adrenalectomy + vehicle versus other groups, $P < 0.05$, Student–Newman–Keuls. This reflected a

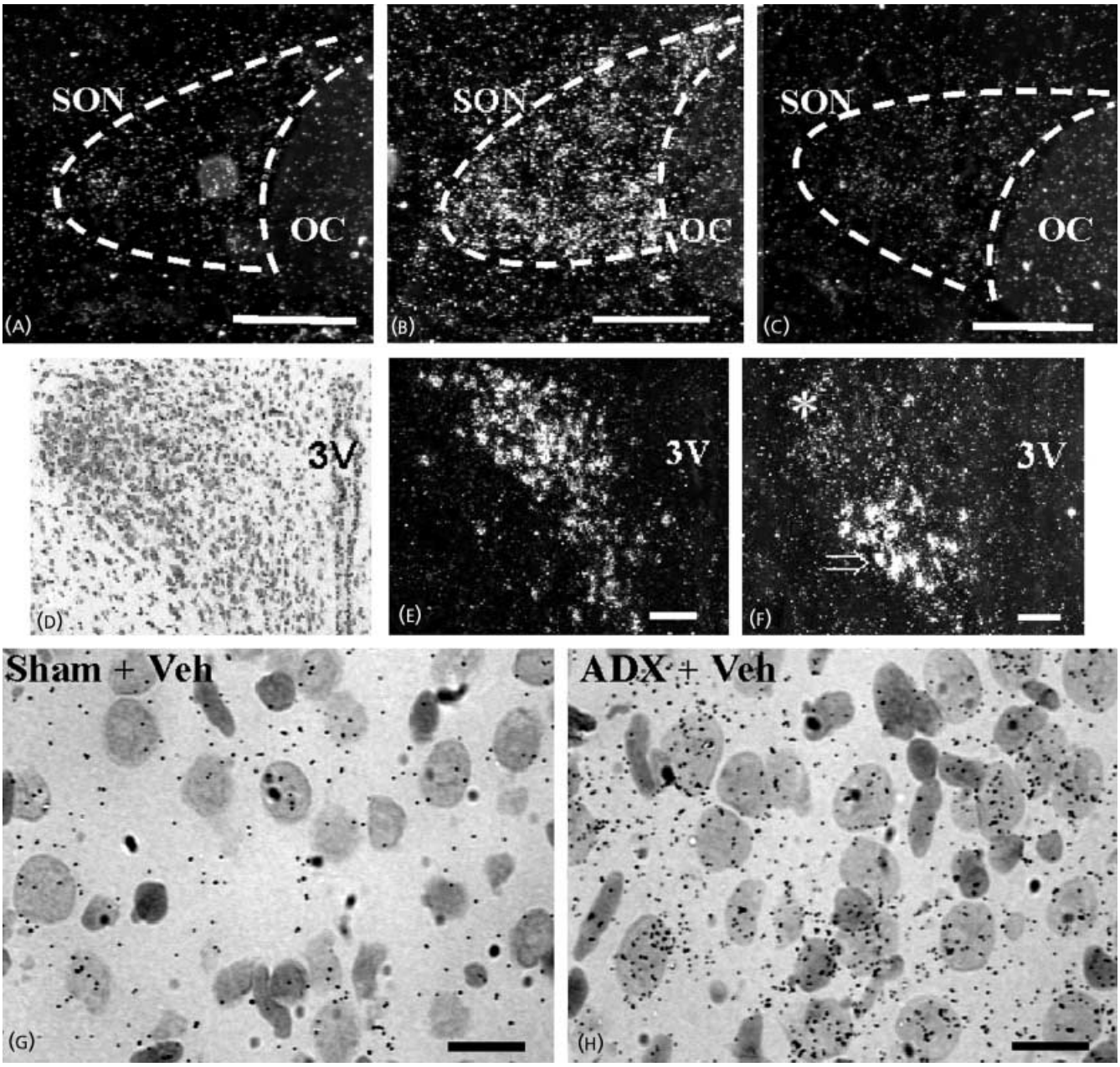

FIG. 1. Oestrogen receptor β (ERβ) mRNA in the supraoptic nucleus and ERβ mRNA and corticotrophin-releasing hormone (CRH) mRNA expression in the paraventricular nucleus: 72 h adrenal steroid manipulations. Photomicrographs (digitized) of coronal sections of hypothalamus; for ease of comparison, only one side is shown per box. (A–C) Supraoptic nucleus (SON; boundary marked by dashed line). Autoradiographs of ERβ mRNA *in situ* hybridization in dark field: (A) sham-operated control; (B) adrenalectomy; (C) adrenalectomy + corticosterone. OC, Optic chiasm. Scale bar = 100 μm. Note increase in ERβ mRNA signal in the SON after adrenalectomy, and decrease with corticosterone replacement. (D–H) Paraventricular nucleus. Sections in (D–F) are from the same brain, 10 μm apart, from a sham-operated control rat: (D) stained with Toluidine blue; (E) autoradiograph of hybridization with ^{35}S-antisense riboprobe for CRH mRNA, showing expression in the dorsomedial parvocellular division, dark-field; (F) autoradiograph of hybridization with ^{35}S-antisense riboprobe for ERβ mRNA; note strong expression in the ventromedial parvocellular subdivision (arrow) and weaker expression in the lateral magnocellular subdivision (*); (G,H) high-power image of lateral magnocellular subdivision bright-field: (G) from sham-operated control, * in (F); (H) same region from an adrenalectomized/vehicle-treated rat. 3V, Third ventricle. Scale bar = 100 μm in (A–F) and 20 μm in (G,H). Note increase in ERβ mRNA signal in the magnocellular subdivision after adrenalectomy.

2.5-fold increase in expression per SON neurone (ANOVA $P = 0.0001$; Student–Newman–Keuls, $P < 0.05$ versus sham) (Fig. 2). Expression per neurone was similarly increased 2.2-fold in the lateral magnocellular division of the PVN (ANOVA $P < 0.005$; Student–Newman–Keuls, $P < 0.05$ versus sham) (Figs 1G,H and 2); expression in the ventromedial parvocellular division of the PVN showed the same trend, but was not increased significantly (Fig. 2). The increases in ERβ mRNA expression following adrenalectomy were partially reversed by corticosterone replacement (significantly for the SON, 36% decrease; ANOVA $P = 0.0001$; Student–Newman–Keuls, $P < 0.05$) (Fig. 2).

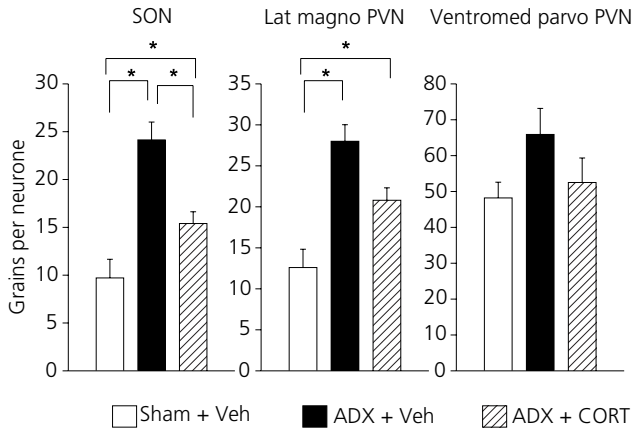

FIG. 2. Effects of bilateral adrenalectomy, with or without corticosterone replacement on oestrogen receptor β (ERβ) mRNA expression in the supraoptic (SON) and paraventricular (PVN) nuclei. Values are group mean ± SEM number of silver grains per neurone. In the PVN, measurements were made in the lateral magnocellular (lat magno) and ventromedial parvocellular (ventromed parvo) subdivisions (Fig. 1). Male rats were sham-operated and given daily vehicle (Sham + Veh; n = 5), or adrenalectomized and given vehicle (ADX + Veh; n = 5) or corticosterone (10 mg/kg s.c.; ADX + CORT; n = 5) injections and killed 72 h later. Cryostat brain sections were hybridized with a ^{35}S-antisense riboprobe (Fig. 1). *P < 0.05 versus group indicated (one-way ANOVA, Student–Newman–Keuls).

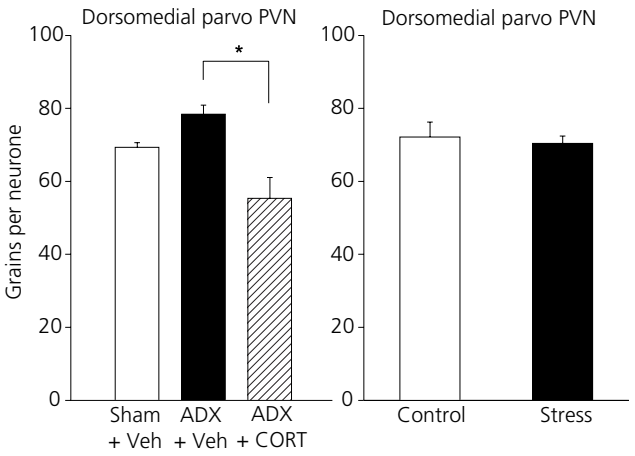

FIG. 3. Effects of bilateral adrenalectomy with or without corticosterone replacement, or of repeated stress, on corticotrophin-releasing hormone (CRH) mRNA expression in the dorsomedial parvocellular paraventricular nucleus. Values are group mean ± SEM number of silver grains per neurone. Left: Male rats were sham-operated and given daily vehicle (Sham + Veh; n = 5), or adrenalectomized and given vehicle (ADX + Veh; n = 5) or corticosterone (10 mg/kg s.c.; ADX + CORT; n = 5) injections, and killed 72 h later (same rats as in Fig. 2). Right: Male rats (same rats as in Fig. 4) were undisturbed (Control, n = 5), or exposed thrice to halothane, cold and restraint over 72 h (Stress; n = 5).Cryostat brain sections were hybridized with a ^{35}S-antisense riboprobe, and autoradiographs quantified (Figs 1E and 4B); *P < 0.05 versus group indicated (Kruskal–Wallis ANOVA, Duncan's tests).

Effects of repeated stress on ERβ mRNA in the PVN and SON

To extend the above studies, we examined the effects of repeated stress for 72 h. Twenty hours after the last stressor plasma corticosterone concentration was increased 3.8-fold (controls: 143.7 ± 27.5 nmol/l; stress: 452.2 ± 118.1 nmol/l; Student's t-test, P < 0.05; this is similar to the 4.7-fold increase over control basal in adrenalectomized rats given corticosterone above), and thymus weight decreased by 35% [controls: 364 ± 26 mg; stress: 289 ± 19 mg; Student's t-test, one-tailed (t = 2.19, 9 d_f.), P < 0.05; for comparison, thymus weight in adrenalectomized rats given corticosterone decreased by 9.2% versus sham controls, and by 25% versus adrenalectomized untreated rats, above]. CRH mRNA expression in the dorsomedial parvocellular PVN was not evidently increased at this time (Fig. 3).

Repeated stress over 72 h significantly increased ERβ mRNA expression in only the ventromedial parvocellular division of the PVN (by 41.4%; Student's t-test, P = 0.02) (Figs 4C,D and 5). Stress had no significant effect, in either the SON (measured as film optical density; control: 40.7 ± 4.0, stress: 33.6 ± 2.49, mean ± SEM units or grains per neurone) (Fig. 5) or in the magnocellular division of the PVN (Fig. 5).

Effects of salt loading for 3 days

Incompletely corrected sodium loss in adrenalectomized rats might reduce stimulation of magnocellular oxytocin and vasopressin neurones through hyponatraemia, but might stimulate magnocellular neurones through reduced blood volume and pressure (3, 46). Accordingly, in the same study, we compared the effects of salt-loading to stimulate the neurones by hyperosmolarity and intracellular dehydration. Salt-loading increased plasma [Na^+] by 6.6% [13 mmol/l; Student's t-test, one tailed, P < 0.05, (t = 1.93, 11°F)], and decreased plasma [K^+] by 16% (0.84 mmol/l; Student's t-test P < 0.05).

By contrast to the effects of adrenalectomy, salt-loading for 72 h significantly decreased ERβ mRNA expression in the SON and lateral magnocellular PVN (Figs 6 and 7). In the SON and magnocellular PVN, ERβ mRNA expression was reduced, relative to the control values, to 59.2 and 57.5% (Student's t-test, P < 0.0005 and P < 0.0001) (Fig. 7A), and expression per neurone was reduced to 39% and 38%, respectively (Student's t-test, P = 0.003, P < 0.0001) (Fig. 7B). There was a significant decrease with salt-loading in the ventromedial parvocellular PVN in expression per neurone, but only to 87% of control (Student's t-test, P = 0.02) (Fig. 7B), with no change in density over this region as a whole (Fig. 7A).

Discussion

This study confirms that the PVN and SON express ERβ but not ERα mRNA (17, 47, 48), while corresponding expression of ERβ protein has been demonstrated by others (16, 18, 21). ERβ expression correlates with radio-labelled oestrogen binding in the PVN and SON (16). Others have previously noted a lack of ERα mRNA and protein expression in the magnocellular PVN and SON (17, 48–50).

Double-labelling studies show that ERβ mRNA and protein expression is greater in magnocellular PVN vasopressin neurones than oxytocin neurones, and in the SON may be expressed in vasopressin (and nociceptin) neurones but in relatively few or no magnocellular oxytocin neurones (18–22, 36). The expression of ERβ mRNA in a minority of

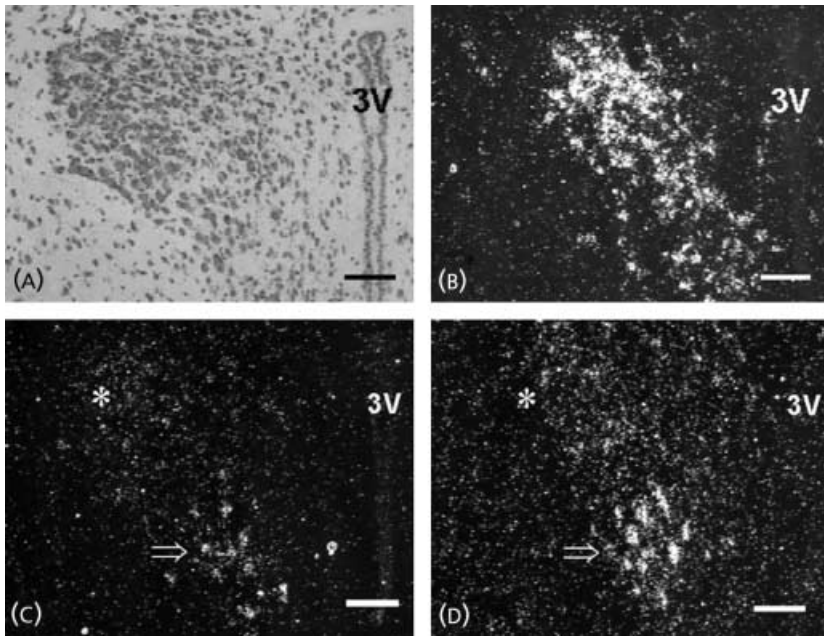

FIG. 4. Oestrogen receptor β (ERβ) and corticotrophin-releasing hormone (CRH) mRNA expression in the paraventricular nucleus and stress. Photomicrographs (digitized) of coronal sections of hypothalamus; for ease of comparison, only one side is shown per box: (A) stained with Toluidine Blue; (B) autoradiograph of hybridization with ^{35}S-antisense riboprobe for CRH mRNA expression, showing signal in the dorsomedial parvocellular subdivision; (C,D) autoradiographs of hybridization with ^{35}S-antisense riboprobe for ERβ mRNA. Sections in (A–C) are from the same brain, 10 μm apart, from an undisturbed rat (Control); (D) from a rat exposed thrice to halothane, cold and restraint over 72 h (Stress). 3V, Third ventricle; asterisk: lateral magnocellular subdivision; arrow: ventromedial parvocellular subdivision. Note increased ERβ mRNA signal after stress in the ventromedial subdivision. Scale bar = 100 μm.

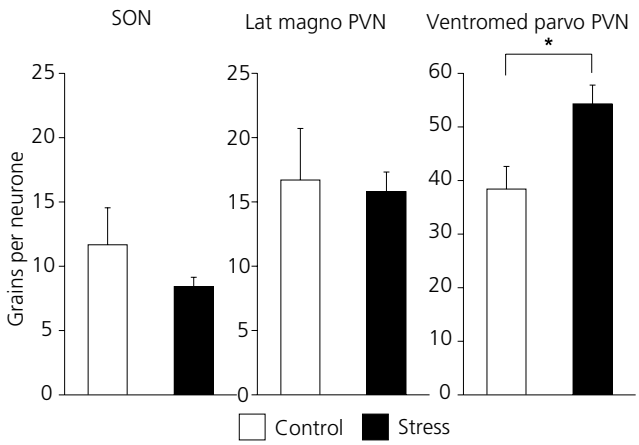

FIG. 5. Effects of repeated stress on oestrogen receptor β (ERβ) mRNA expression in the supraoptic (SON) and paraventricular (PVN) nuclei. Male rats (same rats as in Fig. 4) were undisturbed (Control; n = 5), or exposed thrice to halothane, cold and restraint over 72 h (Stress; n = 5). Cryostat brain sections were hybridized with a ^{35}S-antisense riboprobe, and autoradiographs quantified; values are group means ± SEM of number of silver grains per neurone. In the PVN, measurements were made in the lateral magnocellular (lat magno) and ventromedial (ventromed) parvocellular subdivisions. *P = 0.02 versus group indicated (Student's t-test).

nociceptin-containing neurones in the PVN and SON provides a potential route for oestrogen regulation of oxytocin neurones via local release of nociceptin, which inhibits both oxytocin and vasopressin neurones (51).

The most prominent expression of ERβ mRNA and protein is in the ventromedial and the dorsal groups of parvocellular neurones in the PVN (18, 19, 21), as also seen for ERβ mRNA in this study; these parvocellular neurones project to the brainstem (44). Approximately half of these neurones contain immunoreactive oxytocin and ERβ, detected as mRNA or peptide (17, 21), and a similar proportion contain androgen receptor, which is not detectable in the magnocellular neurones (52). Many of the ventromedial ER β-expressing neurones also contain nociceptin mRNA (20).

In the dorsomedial parvocellular PVN, where CRH neurones projecting to the median eminence are gathered, we found that neurones expressing ERβ mRNA were rare, as previously reported, and CRH neurones coexpressing ERβ mRNA or protein are scarce (17, 18, 20, 53). This indicates that modulatory actions of sex steroids on HPA stress responses (36, 54) are not through ERβ in the CRH neurones.

ERβ mRNA up- and down-regulation

This is the first report regarding changes in ERβ mRNA expression in magnocellular neurosecretory neurones and ventromedial PVN neurones by manipulation of adrenal corticosteroid hormone secretion. The recent study by Isgor *et al.* (36) reported measurements of ERβ mRNA expression over the whole PVN, and the authors found a decrease in expression after adrenalectomy in female rats, but only at proestrus. By contrast, in the present study in male rats, there was a striking increase in ERβ mRNA expression in both the SON and lateral magnocellular division of the PVN after adrenalectomy, both within individual neurones, and across the whole population. The present study showed no change in

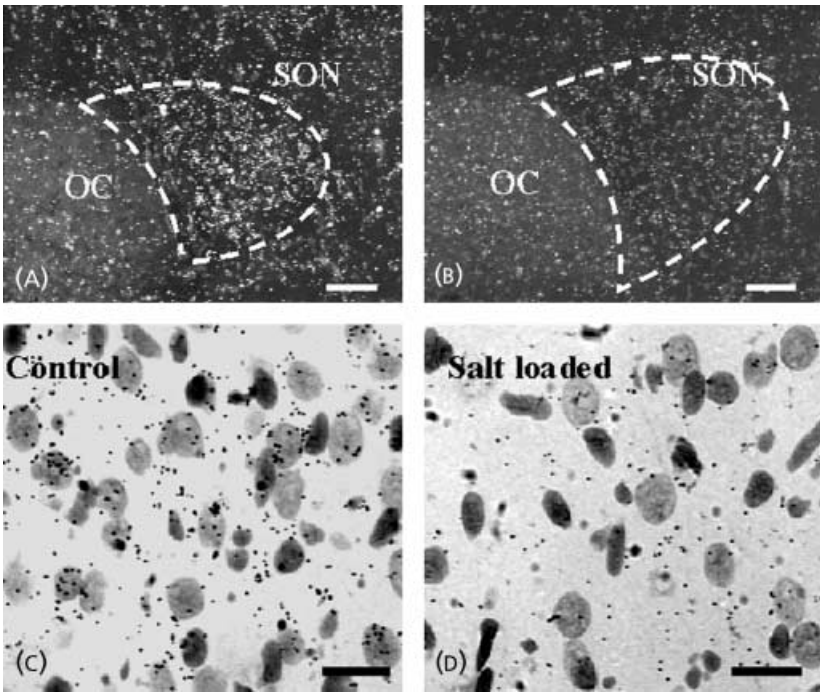

FIG. 6. Oestrogen receptor β (ERβ) mRNA expression in the supraoptic nucleus and salt-loading. Photomicrographs (digitized) of autoradiographs of coronal sections of hypothalamus, hybridized with ^{35}S-antisense riboprobe for ERβ mRNA. Left: control; right: 2% saline to drink for 72 h. (A,B) dark-field; (C,D) high-power, bright-field. OC, Optic chiasm; SON, supraoptic nucleus (boundary marked by dashed line). Note reduced signal after saline drinking. Scale bar = 100 μm in (A,B) and 20 μm in (C,D).

expression in the ventromedial pPVN neurones after adrenalectomy. In measurements of ERβ mRNA expression made over the whole PVN profile, the contribution from the strongly expressing ventromedial PVN neurones is likely to mask changes in the more weakly expressing magnocellular neurones.

By contrast, hypernatraemia induced by salt-loading decreased ERβ mRNA expression in magnocellular PVN and SON neurones. Between adrenalectomy and salt-loading, there was a 6.25-fold difference in ERβ mRNA expression. Other studies have shown that ERβ is predominantly expressed in vasopressin rather than oxytocin neurones, especially in the SON (19–22); hence, changes in ERβ mRNA expression in the magnocellular PVN and SON probably mainly reflect activity of vasopressin neurones.

Hyponatraemia inhibits the secretory activity of vasopressin and oxytocin neurones (2, 55), whereas hypovolaemia and hypotension stimulate vasopressin secretion (1, 3), and also activate oxytocin neurones (46). The net outcome of adrenocortical insufficiency following adrenalectomy is stimulation of vasopressin mRNA synthesis in the magnocellular neurones (56), at least in the PVN (57), and of vasopressin secretion (58), leading to water retention and hyponatraemia. Thus, the context in which ERβ mRNA expression in these neurones was increased after adrenalectomy includes stimulation by hypovolaemia and hypotension, and inhibition by hyponatraemia. These inputs use different pathways and different neurotransmitters (1). Drinking 2% saline instead of water initially causes intracellular dehydration, and leads to sustained activation of magnocellular oxytocin and vasopressin neurones (57). However, under these conditions, parvocellular CRH PVN neurones and ACTH secretion are inhibited (though basal corticosterone secretion is initially stimulated), and this inhibition involves neural input from the lamina terminalis as well as corticosterone feedback (28, 29, 31, 59–61). During such hyperosmotic stimulation, there are increases in expression of a range of genes in the magnocellular neurones, including those for vasopressin and oxytocin (29, 61), for several transcription factors, such as *c-fos*, and Nur 77 (21, 62). However, for ERβ mRNA, the change in expression reported here is unusual because it is a decrease.

Our results indicate that ERβ mRNA expression in magnocellular neurones is regulated in a manner that is inversely related to osmotic drive, rather than by direct action of corticosterone. First, the changes in corticosterone levels were not consistently related to ERβ mRNA expression: adrenalectomy increased expression, but this was only partially reversed by the ample corticosterone replacement; moreover, increasing corticosterone secretion by repeated stress had no effect on ERβ mRNA expression. Any change in ERβ mRNA expression in magnocellular oxytocin neurones consequent on their activation by stress is likely to have been obscured by the greater ERβ expression in vasopressin neurones, which are not excited by the stressors used (63–65). Second, the increased ERβ mRNA expression in magnocellular neurones after adrenalectomy may be a response to hyponatraemia, which is known to increase ERβ expression (21). Third, hypernatraemia inhibits the HPA axis (29) in that it reduces daily glucocorticoid secretion in the same direction as adrenalectomy, but alters ERβ mRNA expression in the magnocellular neurones in the opposite direction to adrenalectomy (Figs 2 and 7) (21).

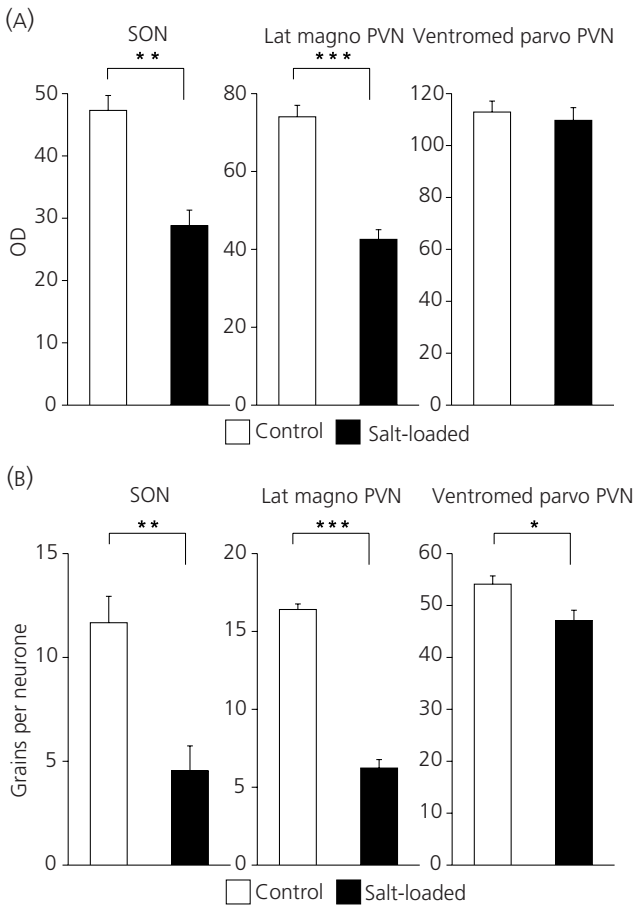

FIG. 7. Effects of salt-loading on oestrogen receptor β (ERβ) mRNA expression in the supraoptic and paraventricular nuclei. Male rats were given either water (Control; n = 6) or 2% saline (Salt-loaded; n = 6) to drink and killed 72 h later (same rats as in Fig. 6). Cryostat brain sections were hybridized with a ^{35}S-antisense riboprobe, and autoradiographs quantified; values are group means ± SEM of (A) film optical density (OD units), and (B) number of silver grains per neurone. Abbreviations as in Fig. 5. (A) **P = 0.0005, ***P < 0.0001; (B) *P = 0.02, **P = 0.003, ***P < 0.0001, versus groups indicated (Student's t-test).

The mechanisms by which ERβ expression in magnocellular SON and PVN neurones may be regulated by hyper- and hyponatraemia are not clear. There are no accompanying changes in circulating sex steroid levels that would account for this (21). The simplest explanation of our results is that osmoresponsive input, including direct effects on the magnocellular neurones (66, 67), is the predominant regulator of ERβ mRNA expression in these neurones (68).

The likely consequence of decreased ERβ expression in magnocellular PVN and SON neurones with hyperosmotic stimulation would appear to be that the regulation of the neurones by sex steroids that bind to ERβ (14) is decreased. Studies performed *in vitro* suggest that these actions include inhibition of vasopressin and oxytocin secretion stimulated osmotically or with NMDA (12, 13) and studies performed *in vivo* suggest that oestrogen inhibits oxytocin mRNA expression in the PVN, but not the SON (16). However, other *in vivo* findings indicate stimulatory actions of ERβ. In gonadectomized animals, hyperosmotic stimulation fails to stimulate oxytocin and vasopressin mRNA expression and peptide release by the magnocellular neurones, unless oestrogen and testosterone (or 5α-DHT) replacement is given (8); decreased vasopressin mRNA expression in hyponatraemia is not affected by gonadectomy (69). Furthermore, oestrogens rapidly increase release of oxytocin and vasopressin from the dendrites of SON neurones (70) and, *in vivo* stress-induced oxytocin secretion is enhanced in females treated chronically with oestrogen (63). Studies in mice show that ERβ expressed alone mediates stimulatory actions of oestrogen on oxytocin mRNA expression in the PVN, but inhibitory actions on vasopressin gene expression (71).

The upstream regulatory elements of the oxytocin and vasopressin genes include a composite hormone response element, with oestrogen-response element-like sequences (72), through which ERβ has been shown *in vitro* to stimulate the oxytocin gene promoter (73). Cellular mechanisms for *in vivo* ERβ-mediated actions on vasopressin and oxytocin neurones remain to be revealed, but actions on, for example, the oxytocin gene are likely to be indirect and to involve cofactors or intermediate transcription factors that interact with ERβ (74). It is also possible that ERβ functions as a ligand-independent transcription factor, being activated similar to some other steroid receptors by phosphorylation in the absence of steroid ligand (75).

Ventromedial parvocellular PVN neurones

These neurones project to the brainstem and spinal cord, including directly to sympathetic preganglionic neurones, and are involved in autonomic regulation by the PVN, defending against plasma volume expansion (76), and modulating stress responses (77). Many of these neurones contain oxytocin, and others nociceptin, and both coexpress ERβ mRNA (20, 21). Oxytocin evokes hypertensive responses via the dorsal vagal complex (78), and the parvocellular ventromedial PVN neurones increase expression of oxytocin after oestrogen priming and progesterone treatment (79).

Ventromedial parvocellular PVN neurones responded differently from magnocellular neurones to the challenges used in the present study. They were the only neurones studied that showed increased ERβ mRNA expression after repeated stress for 72 h, but they showed no significant change after adrenalectomy. It appears likely that these neurones are activated during repeated stress to coordinate the autonomic responses (77, 80). Whether the decrease in ERβ mRNA expression in PVN extracts in female rats given lipopolysaccharide 6 h previously occurs in these neurones is not known (81), although they are expected to be activated by this stimulus (77). It is interesting that there was no change in PVN ERβ mRNA level in a strain that shows no HPA axis response to the endotoxin (81). In the present study, ventromedial parvocellular PVN neurones showed a modest decrease in ERβ mRNA expression after salt-loading, a condition in which autonomic drive to the cardiovascular system and input from baroreceptors will change. Thus, it appears that the expression of ERβ mRNA may be related to their activity for these neurones also. It is possible that ventromedial PVN neurones expressing ERβ and nociceptin mediate oestrogen potentiation of HPA axis stress responses

because an oestrogen receptor antagonist delivered into the PVN reduces the corticosterone response to restraint stress (36), and nociceptin given centrally stimulates ACTH secretion (82). If this is the case, then the up-regulation of ERβ mRNA expression in the ventromedial PVN neurones during chronic stress, as shown here, may amplify oestrogen potentiation of stress responses. This contrasts with the up-regulation of ERβ mRNA expression in the magnocellular neurones after removal of adrenal steroids.

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