In vivo oestrogenic modulation of Egr1 and Pitx1 gene expression in female rat pituitary gland

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Abstract

EGR1 and PITX1 are transcription factors required for gonadotroph cell Lhb promoter activation. To determine changes in Egr1 and Pitx1 mRNA levels in central and peripheral pituitary stimulations, an in vivo model based on i.c.v. pulsatile (1 pulse/0.5 h over 2 h) GnRH agonist (1.5 nM buserelin) or antagonist (2 nM antide) microinjections was used. The microinjections were given to ovariectomised and 17β-oestradiol (E2) (3–20 μg), ERA (ESR1) agonist propyl pyrazole triol (PPT) (3–0.5 mg), ERB (ESR2) agonist diarylpropionitrile (DPN) (3–0.5 mg) s.c. pre-treated rats 30 min after last pulse anterior pituitaries were excised. Relative mRNA expression was determined by quantitative RT-PCR (qRT-PCR).

Results revealed a gene-specific response for GnRH and/or oestrogenic stimulations in vivo. Buserelin pulses enhanced Egr1 expression by 66% in ovariectomised rats, whereas the oestradiol-supplemented C i.c.v. NaCl-microinjected group showed a 50% increase in Egr1 mRNA expression. The oestrogenic signal was transmitted via ERA (ESR1) and ERB (ESR2) activation as administration of PPT and DPN resulted in 97 and 62%, respectively, elevation in Egr1 mRNA expression. A synergistic action of GnRH agonist and 17β-oestradiol (E2) stimulation of the Egr1 gene transcription in vivo were found. GnRHR activity did not affect Pitx1 mRNA expression; regardless of NaCl, buserelin or antide i.c.v. pulses, s.c. oestrogenic supplementation (with E2, PPT or DPN) consistently decreased (by −46, −48 and −41% respectively) the Pitx1 mRNA in the anterior pituitary gland. Orchestrated Egr1 and Pitx1 activities depending on specific central and peripheral regulatory inputs could be responsible for physiologically variable Lhb gene promoter activation in vivo.

Key Words

- oestrogenic modulation
- Egr1
- Pitx1
- gene expression
- rat pituitary gland

Introduction

Upon binding to its specific G protein-coupled receptor, gonadoliberin (GnRH) induces a signal that then flows through MAPK cascades, leading to the phosphorylation of a number of downstream targets including several DNA-binding proteins. The culmination of the GnRH transcriptional signal results in regulated expression of four gonadotroph signature genes, Cga, Lhb, Fshb and Gnrhr, located within the tertiary network of
GnRH-responsive genes, because changes in their transcription depend on the proteins encoded by the primary and secondary response genes (Salisbury et al. 2008).

The early growth response (Egr1) gene resides within the GnRH transcriptional network and belongs to the primary response gene family as detectable changes in Egr1 transcription occur as early as possible within 1 h of GnRH stimulation (Yuen et al. 2002). As EGR1 protein accumulates, it regulates the transcription of secondary-like MAPK phosphatase 2 (Zhang et al. 2001) and tertiary-like Lhb response genes (Call & Wolfe 2002). Several cis-acting regulatory sites were identified on the 5′-flanking Egr1 gene sequence: two oestrogen response element (ERE) half-palindromic sites, two AP1 sites located in an encompassing the consensus sequence CC(A/T)6GG (de Jager et al. 2001, Duan et al. 2002) and multiple binding sites for ternary complex factors having the Ets consensus core sequence (Mayer et al. 2008). The importance of EGR1 in the pituitary is emphasised by the consequences of Egr1 gene knockout in mice. These animals, although viable, exhibit reduced body mass, are infertile owing to defects in hormone regulation and have pituitary glands that are considerably smaller than normal. The anterior lobe in particular is limited in size owing to a reduced number of growth hormone-positive cells. Furthermore, the expression of LHB is completely blocked (Topilko et al. 1998). Substantial progress has been made in the identification of the transcription factors that are required for basal, tissue-specific and GnRH-activated expression of the Lhb subunit gene. In the highly conserved proximal 140 bp Lhb promoter region, several specific cis-acting regulatory elements were found to interact with trans-acting early growth response protein 1, the orphan nuclear receptor – steriodogenic factor 1 (SF1) – and a paired-like homeodomain protein PITX1, whereas regulatory elements for SP1 protein and CArG box were identified in the distal domain of the rat sequence (Jorgensen et al. 2004).

PITX1 exerts an essential role not only throughout pituitary development (Zsé et al. 1996, Kurowani et al. 1999), but also in postnatal life, when it regulates several pituitary-specific genes through direct interaction with other specific transcription factors (Tremblay et al. 1998, 2000). In mice homozygous for the deletion of the Pitx1 gene, the subsequent pituitary development is markedly abnormal. In Pitx1-null animals, mRNA levels for Lhb, Fshb and Tshb are substantially reduced (Zséto et al. 1999). Within gonadotrophs, PITX1 has been shown to stimulate the expression of genes that encode the A-subunit (Tremblay et al. 1998), Lhb (Quirk et al. 2001), FSHB (Zakaria et al. 2002) and GnRH receptor (Jeong et al. 2004). Two cis-acting Pitx1 DNA regulatory regions have been identified on the rat Lhb promoter (Jiang et al. 2005) and this transcription factor protein is postulated to interact with SF1 and EGR1 to form a tripartite complex on the mammalian Lhb subunit gene promoter, which is crucial for GnRH-regulated transcription of this gene (Kaiser et al. 2000).

Oestrogen plays a pivotal role in the regulation of female pituitary development and function. It also dually controls secretion of GnRH resulting in suppressed basal gonadotropin expression as well as enhanced GnRH responsiveness of gonadotroph cells at the time of the LH surge (Christian & Moenter 2010). ERA (ESR1) and ERB (ESR2), two oestradiol receptor subtypes encoded by different genes, are nuclear receptor transcription factors that mediate oestrogen action by targeting the transcription of genes whose products will ultimately alter the physiology of the cell (Sanchez et al. 2002). Both ERA (ESR1) and ERB (ESR2) are expressed in the pituitary gonadotrophs and net oestrogen sensitivity in the pituitary critically depends on the balanced expression of ER subtypes. ERA (ESR1) is thought to be the primary mediator of oestrogen-induced reversal of hypertrophied gonadotrophs after ovariectomy (Sanchez-Criado et al. 2006). In support of these findings, Era (Esr1)-knockout female mice are completely infertile, have elevated levels of LH and do not ovulate, whereas Erb (Esr2)-knockout mice can ovulate, although they are sub-fertile (Hewitt & Korach 2003). Although the knockout mouse model provides an excellent approach to the regulatory aspects of both ER receptors in vivo, there are also important physiological limitations related to potential developmental defects as well as an activation of compensatory mechanisms in the absence of one receptor. By applying ER subtype selective ligands, a specific activation of one ER subtype in the physiological presence of the other subtype and ER variants can be studied (Stauffer et al. 2000, Meyers et al. 2001).

Although the impact of GnRH on Egr1 gene activation is now well established, a potential functional connection between GnRH neuron activity and Pitx1 mRNA expression is poorly recognised. Moreover, despite the indispensable regulatory effect of oestrogens on maintaining LH synthesis in gonadotropic cells, there is lack of data on possible gonadal steroid involvement in the regulation of Egr1 and Pitx1 gene transcription in the anterior pituitary gland. Therefore, in this study, we tested a hypothesis that Egr1 and Pitx1 mRNA expression in vivo not only results from endogenous GnRHR stimulation but

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also remains under the regulatory influence of E2 exerted in an ER-dependent manner.

To characterise Lhb promoter transcription factor mRNA levels in intact females, pituitary Egr1 and Pitx1 mRNA expressions in proestrus and dioestrus females were first determined. Then, to compare changes in Egr1, Pitx1 and Lhb mRNA levels in an experimental context regarding both central and peripheral pituitary stimulations, an in vivo model based on pulsatile i.c.v. microinjection of GnRH agonist/antagonist given to ovariectomised and oestradiol/ER-specific agonist-supplemented rats was applied. Owing to a direct administration into the third cerebral ventricle, therefore in the vicinity of the hypothalamus and pituitary gland, this approach more closely resembles a physiological context where neuroendocrine GnRH activity is located within a hypothalamic–pituitary unit. In addition, s.c. exogenous E2 injection mimics the physiological pattern of pituitary stimulation by peripheral oestrogens derived from general circulation.

Materials and methods

Chemicals

Propyl pyrazole triol (PPT), an ERA (ESR1) agonist, and diarylpropionitrile (DPN), an ERB (ESR2) agonist, were obtained from Tocris Cookson Ltd (Avonmouth, UK), while E2, buserelin and antide were purchased from Sigma.

Animals and surgical protocol

Female laboratory-strain 4–5-month-old Wistar rats (250–280 g) were kept under controlled light (14 h light:10 h darkness, lights on between 0600 and 2000 h) and temperature (22 °C) with free access to pelleted food and tap water. Thirty non-ovariectomised adult female rats were checked daily for 3 weeks through the examination of vaginal smears between 0900 and 0930 h, animals were primed with E2 (3 × 20 μg/0.2 ml dimethyl sulphoxide (DMSO)), PPT (3 × 0.5 mg/0.2 ml DMSO) and DPN (3 × 0.5 mg/0.2 ml DMSO) as adapted from previous studies (Tena-Sempere et al. 2004) or in controls, with an equivalent volume of DMSO. The following day after receiving the last s.c. injection, the i.c.v. gauges were connected to an automatic pump CMA/100 (CMA Microdialysis AB, Stockholm, Sweden) with 45-cm long silicon tubing (internal diameter, 0.5 mm; outer diameter, 1 mm), allowing the rats to move freely. Ovariectomised (OVX) and OVX/E2 or OVX/selective ER agonist-primed rats received i.c.v. microinjections of 0.9% physiological saline, 1.5 nM GnRH agonist buserelin or 2 nM GnRH antagonist antide. A series of five microinjections were given with a frequency of one pulse per 0.5 h and the flow rate was carefully balanced and set to deliver 10 μl/5 min per pulse.

Sample collection

All rats were killed by decapitation under anaesthesia (ketamine 30 mg/100 g body weight) 30 min after the last i.c.v. microinjection. The anterior pituitary was immediately excised, flash frozen in liquid nitrogen and stored at −80 °C for subsequent RNA extraction. Trunk blood was collected, centrifuged in dry tubes and the serum was stored at −20 °C for RIA.

All animal procedures were approved by the Local Ethics Committee at the Warsaw University of Life Sciences.

RIA

Rat serum LH was measured by RIA using antibodies and LH preparations generously supplied by Dr A F Parlow and NIDDK (Baltimore, MD, USA). Values were expressed in terms of rat LH (RP3) preparations. Intra- and inter-assay variations were 6.5 and 11.5% respectively.

qRT-PCR analysis

Total RNA was extracted using the TRIZOL reagent (Invitrogen) in accordance with the manufacturer's
protocol. The yield of RNA was estimated spectrophotometrically from absorbance at a wavelength of 260 nm, and RNA purity was evaluated according to the A_{260}/A_{280} and A_{260}/A_{230} ratios. The integrity of total RNA was confirmed by the presence of sharp bands in u.v. light (Vilber Lourmat, Marne-la-Vallée, France) corresponding to 18S and 28S rRNAs when separated by electrophoresis on a 1.5% agarose gel. To remove residual genomic DNA contamination, samples of RNA before RT reaction were subjected to DNaseI treatment (Sigma). cDNA was synthesised from 800 ng of the total RNA using anchored random hexamer primers and Moloney murine leukaemia virus reverse transcriptase according to the protocol of DyNAmo cDNA synthesis kit (Finnzymes, Espoo, Finland). Primers were designed to span over intron sequences using the Primer3 software (The Whitehead Institute, Boston, MA, USA) and their specificity was confirmed by a BLAST software-assisted search of a non-redundant nucleotide sequence database (National Library of Medicine, Bethesda, MD, USA). Specific primer sequences were as follows: tLhb: Acc No.: NM_012858; L(115): CCTGGCTGCAGAATAAGTAT, R(247): GTAGTGCA-CACTGGCTGAAG, amplicon size: 133 bp; tEgr1: Acc No.: NM_012551; L(549): AAACACCTACGACACCTG, R(677): AGCGGCCAGTATA GGTGATG, amplicon size: 129 bp; tPitx1: Acc No.: NM_053624; L(424): AACTCAGC-GAGGACCAGGTTGCTC, R(562): CGCCTTTCTTCTTCTGG, amplicon size: 138 bp; tGapdh: Acc No.: NM_017008 XM_216453; L(1675): GAGGACCAGGTTGCTC, R(1835): ATGTAGGCCATGAGGTCCAC, amplicon size: 161 bp. All HPLC-grade oligonucleotides were synthesised by Genosys (Sigma-Genosys, Steinheim, Germany).

PCR amplification was carried out using a SYBR Green 2-step qRT-PCR Kit (Finnzymes) in a final volume of 20 μl. One tube contained 10 μl PCR Master Mix (2×), 4–7 μl RNase-free water, 2 μl primers (1 μl each, working concentration was 0.5 μM) and, depending on gene, 1–4 μl cDNA template. The tubes were run on a Rotor-Gene 6000 (Corbett Research, Mortlake, Australia) and the qRT-PCR conditions were as follows: at 95°C for 15 min (initial denaturation), 37 cycles at 94°C for 15 s (denaturation), 56°C for 20 s (annealing) and 72°C for 30 s (elongation). To determine the specificity of amplification, a final melting curve analysis under continuous fluorescence measurements was performed after each completed cycle. qRT-PCR for each cDNA sample was performed three times in triplicate. Negative controls and NTC controls were included in each reaction. In addition to real-time and melting curve analysis of the reactions, amplified products were separated electrophoretically on 2% agarose gels with ethidium bromide and visualised under u.v. light to confirm proper amplicon size and the absence of non-specific products. All PCR products produced a single specific product. The identity of PCR products was further confirmed by direct sequencing in both directions (Oligo IBB, Warsaw, Poland).

Relative gene expression was calculated using the comparative quantitation option of Rotor Gene 6000 software 1.7. (Qiagen). Relative Egr1, Pitx1 and Lhb gene expressions were determined using Relative Expression Software Tool 2008 according to Pfaffl et al. (2002) and based on a PCR efficiency correction algorithm (Pfaffl 2001). To compensate for the variation in cDNA concentrations and the PCR efficiency between tubes and endogenous control, the glyceraldehyde-3-phosphate dehydrogenase (tGapdh) gene was quantified in each sample and used for normalisation.

Statistical analysis

All results are expressed as a ratio to a calibrator that was chosen to be the OVX/NaCl-microinjected group and presented as the mean values ± S.E.M. Statistical evaluations were carried out using the Mann–Whitney U test. Differences resulting in P < 0.05 were considered statistically significant.

Results

Changes in Egr1, Pitx1 and Lhb mRNA expression and LH serum concentration in proestrus and dioestrus rats

To characterise the physiological changes in Lhb and its specific transcription factor genes’ mRNA expression in the anterior pituitary gland of intact female rats, two opposite stages of the ovarian cycle were compared. In proestrus females, Egr1 mRNA expression was up-regulated by 183% while Lhb and Pitx1 mRNA levels were down-regulated by 26 and 57%, respectively, when compared with the respective mRNA expression found in dioestrus females (Fig. 1). Cycle phase-dependent differences (P < 0.01) in LH serum concentration were found between dioestrus (2.5 ± 0.7 ng/ml) and proestrus females (17.5 ± 3.5 ng/ml).

Egr1 mRNA expression

In the applied experimental model, bidirectional modulation of endogenous GnRH activity by its agonist or antagonist microinjections in vivo resulted in significant
and opposite effects exerted on anterior pituitary Egr1 mRNA expression in ovariectomised rats. When 30 min pulses of buserelin were administered into the third cerebral ventricle, the Egr1 mRNA level increased by 66%, whereas antide microinjections induced its significant reduction (−23%) as early as possible within 2.5 h from the first respective pulse, in comparison with the control, the OVX+i.c.v. NaCl-treated group (Fig. 2).

To establish whether peripheral stimulus resulting from oestrogen background could also affect Egr1 gene transcriptional activity, the effect of s.c. E2 treatment was also examined in OVX rats receiving i.c.v. saline pulses. As shown in Fig. 2, a 50% increase (vs OVX+i.c.v. NaCl-treated group) in the Egr1 mRNA level was found. Then, as two ER subtypes are present in the anterior pituitary, their potential involvement in the regulation of Egr1 mRNA expression was also examined. The results revealed that agonist-specific stimulation of both ERA (ESR1) and ERB (ESR2) receptors potently up-regulated the Egr1 mRNA level which, in comparison with the OVX+i.c.v. NaCl-microinjected group, increased by 97 and 62% as found after s.c. PPT or DPN treatment respectively.

To evaluate the effects of combined GnRH and ER receptor stimulation in vivo, changes in Egr1 mRNA expression resulting from pulsatile i.c.v. buserelin/antide microinjections given to E2-, PPT- or DPN-supplemented rats were also determined. Combined central and peripheral receptor activation intensified Egr1 mRNA expression in the anterior pituitary gland in vivo. In E2/buserelin-microinjected rats, a 20% increase (vs OVX+i.c.v. buserelin-treated group) and a 34% increase (vs E2+i.c.v. NaCl-treated group) were observed. Furthermore, PPT+buserelin treatment enhanced the Egr1 mRNA level by 67% when compared with 98% in the PPT+i.c.v. NaCl or OVX+i.c.v. buserelin-microinjected groups. Similarly, when buserelin was applied to DPN-supplemented rats,
Egr1 gene expression increased by 58% (vs DPN i.c.v. NaCl-treated rats) and by 54% in comparison with the OVX i.c.v. buserelin-treated group (Fig. 2). A significant elevation (by 49, 116 and 72% respectively) of Egr1 mRNA levels was also found in E2-, PPT- and DPN-supplemented groups receiving i.c.v. antide pulses when compared with the level of expression detected in OVX i.c.v. antide-receiving rats (Fig. 2).

Pitx1 mRNA expression

Under the same experimental conditions, Pitx1 mRNA expression in the anterior pituitary gland of female rats in vivo was also determined. As shown in Fig. 3, neither buserelin nor antide pulses given for 2 h every 30 min directly to the third ventricle had any effect on the expression of this gene mRNA in the ovariectomised rats. By contrast, in i.c.v. NaCl-treated rats supplemented with E2, a significant decrease (−46%) in Pitx1 gene expression was found when compared with the level detected in OVX + i.c.v. NaCl-treated animals. Taking the same group as the control, comparable levels of Pitx1 mRNA expression reduction occurred both after s.c. PPT (−48%) and DPN (−41%) treatment. As presented in Fig. 3, consistently inhibitory effects induced by oestradiol, as well as by both ER subtypes, were exerted at a similar level. Moreover, Pitx1 mRNA expression found in PPT- or DPN-supplemented animals receiving i.c.v. buserelin or antide pulses also confirmed that the regulatory inputs affecting Pitx1 gene transcriptional activity were only due to the negative oestrogen impact exerted by E2 via both ERA (ESR1)/ERB (ESR2) subtype activation. Indeed, in all oestrogen-treated groups receiving buserelin or antide microinjections, Pitx1 mRNA expression in each group revealed the same level as determined in respective E2/PPT/DPN + i.c.v. NaCl-treated animals (Fig. 3).

Lhb mRNA expression

As illustrated in Fig. 4, buserelin microinjections given to ovariectomised rats resulted in a 36% increase in Lhb mRNA expression, whereas s.c. E2 treatment lowered expression by 44% when compared with OVX/i.c.v. NaCl-treated controls. The inhibitory effects of oestrogen were then partially reversed by buserelin application as Lhb mRNA expression increased by 38% when compared with oestradiol-primed and NaCl-microinjected rats. The down-regulatory effect of E2 on Lhb transcriptional activity was strictly dependent on ERA (ESR1) activation. Indeed, only after PPT/i.c.v. NaCl supplementation was Lhb mRNA expression reduced by 58% in comparison with the OVX/NaCl microinjected group. Moreover, the ERA (ESR1) agonist maintained Lhb gene expression at a comparable level to that detected in rats supplemented with E2 (Fig. 4).

LH serum concentration

As shown in Fig. 5, GnRH receptor stimulation by i.c.v. buserelin pulses significantly enhanced serum LH concentration (29.50 ± 5.52 ng/ml), whereas it was decreased by receptor antagonist antide (2.72 ± 0.3 ng/ml), when
compared with ovariectomised and i.c.v. NaCl-treated rats (6.37 ± 0.77 ng/ml). Taking the same group as the control, an expected negative feedback of E$_2$ on LH release was observed (2.08 ± 0.23 ng/ml) and this effect was exerted via ERA (ESR1) activation (1.50 ± 0.27 ng/ml).

**Discussion**

In intact rats, cycle-induced changes in pituitary Egr1 and Pitx1 mRNA expression were found. However, both examined genes responded differentially to proestrus-specific synchronic GnRH and oestradiol gonadotroph stimulation: Egr1 mRNA was up-regulated, whereas the Pitx1 mRNA level decreased in comparison with respective dioestrus values (Fig. 1). The results not only confirmed gonadotroph transcriptional network sensitivity for endogenous GnRHR activation but also validated the usefulness of an in vivo model to study transcription factor mRNA expression in a physiological context.

Therefore, to focus on both the central (GnRH network stimulation) and the peripheral (gonadal steroids background) impacts on Lhb promoter transcription factor (Egr1 and Pitx1) mRNA expression in the anterior pituitary gland in vivo, an experimental model which preserves functional hypothalamic–pituitary–peripheral connections was then applied.

Endogenously produced GnRH is present in the cerebrospinal fluid (Skinner et al. 1998) and tanycytes have been shown to participate in the release of GnRH to the portal blood system (Rodriguez et al. 2005). Moreover, the passage of microinjected GnRH from the third cerebral ventricle to the hypophyseal portal system, associated
with a rise in LH release, has been documented in the rat and was suggested to occur via tanycytic transport (Ben-Jonathan et al. 1974, Barrera et al. 1991). It is now well established that exogenous GnRH induces ultra-short loop inhibition of GnRH neurons in vivo and ex vivo (DePaolo et al. 1987, Zanisi et al. 1987, Padmanabhan et al. 1995) and GnRH can directly modify the activity of GnRH neurons by either depolarising (Todman et al. 2005) or hyperpolarising (Xu et al. 2008) the membrane potential. The addition of GnRH agonist (buserelin) to explanted superfused hypothalami resulted in a decrease in GnRH release via GABAergic and taurinergic neurons (Feleder et al. 2008). Having established our model with respect to the transcriptional response of Egr1 and Lhb genes to exogenous GnRH agonist/antagonist stimulation, we then examined whether E2 and its specific receptors affect Egr1 mRNA expression in the anterior pituitary gland. As presented, regardless of the i.c.v. microinjections (saline, buserelin or antide), the Egr1 mRNA level was significantly increased in oestradiol-supplemented rats and both ER subtypes were involved in inducing oestrogen-specific effects. A profoundly increased Egr1 gene transcriptional response found in a selective ER ligand against E2-treated rats might have resulted from an apparently higher affinity receptor binding for ERA (ESR1) (400-fold) and the ERB (ESR2) (70-fold) ligands (Schreinhofer et al. 2002, Pelletier et al. 2003). In addition, in the GnRH-stimulated LBT2 cell line, oestradiol was shown to amplify Lhb mRNA expression owing to a threefold enhancement of Egr1 mRNA expression, but remained ineffective in promoting Egr1 mRNA synthesis in non-GnRH-activated cells (Kowase et al. 2007). So far, connections between oestrogen and Egr1 gene transcription have been observed in several in vivo and in vitro studies and oestrogen is thought to act as a mediator of the rapid non-genomic effect exerted on Egr1 gene expression. Indeed, a transient increase in Egr1 mRNA level was reported in the uterus of ovariectomised rats as early as possible within 2 h after oestradiol supplementation (Suva et al. 1991), whereas in primary cardiomyocytes, Egr1 mRNA expression reached a maximum at 15 min after oestradiol stimulation. Moreover, co-transfected ERA (ESR1) and ERB (ESR2) rapidly enhanced Egr1 promoter activity in these cells and this effect required SREs but not ERE/AP1 site activation (de Jager et al. 2001). The significance of SRE sites in oestrogen-induced early response gene transcriptional activity was also reported in ER-positive human breast carcinoma MCF-7 cells where both Egr1 (Chen et al. 2004) and c-fos (Fos) promoter (Duan et al. 2001) required SRE-driven activation of intracellular ERK1/2 signalling and subsequent ELK1 phosphorylation. It has been suggested that rapid SRE-mediated Egr1 gene induction by both ERA (ESR1) and ERB (ESR2) represents a mechanism by which oestrogen might exert an array of effects through the modulation of Egr1 target gene expression (de Jager et al. 2001). In this paper, however, we also report that
prolonged 3-day oestradiol exposure led to an increased Egr1 mRNA expression in the anterior pituitary gland. Studies on LBT2 cells revealed that for several genes, including Egr1, the amplitude of the biosynthetic programme induced by GnRH is strictly determined by GnRH concentration (Youen et al. 2002). If this is the case, the relationship between Egr1 transcription rate and physiologically variable oestradiol concentration with subsequent varying ER receptor activation cannot be excluded. As oestradiol exerts a bimodal effect on GnRH – gonadotropin axis activity (Radovick et al. 2012) – the stimulation of Egr1 gene transcription might represent, at least in part, a molecular mechanism involved in an oestradiol-positive feedback effect exerted on LH synthesis in gonadotroph cells.

As a positive control for oestrogenic stimulation, Lhb subunit gene expression was also determined. Contrary to Egr1 gene expression, the Lhb mRNA level was lowered by E2 supplementation. By using ER-selective ligands, we confirmed that the negative effect of oestradiol on Lhb subunit gene transcription was attributed to ERA (ESR1) activation as only PPT was able to mimic the effect of oestrogen supplementation in OVX rats (Fig. 4). This result is consistent with previous reports showing that oestrogen-induced LHB regulation is heavily dependent upon the actions of ERA (ESR1), because AERKO or ABERKO female mice exhibited elevated Lhb gene expression, but BERKO female mice did not (Couse et al. 2003). A strictly ERA (ESR1)-dependent inhibitory effect of oestradiol was observed in ovariecctomised female rats exhibiting gonadotroph-derived aromatase expression (Galmiche et al. 2006). A study on pituitary-specific Era-knockout mice (ERA (ESR1) flox/flox AGSUCRE) revealed that gonadotroph ERA (ESR1) is essential for maintaining oestrous cyclicity (Gieske et al. 2008).

The binding of trans-acting PITX1 protein to cis-sites on the Lhb promoter raised the question of possible regulatory inputs exerted on Pitx1 gene transcriptional activity in the anterior pituitary gland in vivo. In this study, we proposed that Pitx1 mRNA expression is also a molecular target sensitive for both central and oestrogenic stimulation. Although buserelin/antide i.c.v. pulses did not affect Pitx1 mRNA expression, E2, PPT and DPN supplementation significantly decreased its mRNA level in the anterior pituitary gland (Fig. 3). Data concerning the GnRH impact on Pitx1 gene promoter activity are limited and a dichotomy exists in research about the necessity for the Pitx1 element in conferring GnRH response to the Lhb promoter in vitro vs in vivo. An unchanging expression of the endogenous Pitx1 gene after forskolin, cyclic ADP-ribose and GnRH stimulation was found in sT3-1 cells (Tremblay & Drouin 1999). A functional Pitx1 binding site was shown not to be a direct target of the GnRH signalling pathway also in transiently transfected LβT2 cells (Quirk et al. 2001). However, in the transgenic mice harbouring mutated Pitx1 element within the Lhb promoter, this gene transcriptional activity was minimal and was not rescued by physiologically elevated concentrations of GnRH, thus suggesting Lhb gene transcriptional dependence on a PITX1-binding site (Quirk et al. 2001). In our study, however, pituitary Pitx1 mRNA expression was not dependent on GnRH system activity. If unaffected, Pitx1 mRNA expression might be a consequence of inadequate gene stimulation, whose expression was checked as early as possible within 2.5 h after the first i.c.v. pulse or rather may reflect Pitx1 gene refactoriness for GnRH activation in vivo that requires further investigation. The consistently decreased Pitx1 mRNA level found in rats supplemented with E2 or with specific ER agonists, as well as in proestrus females, indicates an inhibitory oestrogenic impact exerted on Pitx1 transcriptional activity. This is the first report on oestrogenic-dependent regulation of Pitx1 mRNA expression in the anterior pituitary gland in vivo. By contrast, in positive breast cancer cell lines in human ERA (ESR1), a robust up-regulatory effect of oestradiol on Pitx1 mRNA expression has been recently reported, and Pitx1 was shown to interact with ERA (ESR1) to suppress ERA (ESR1) transcriptional activity on a subset of ERA (ESR1) target genes (Stender et al. 2011). Such a discrepancy between results derived from in vitro and in vivo models was also found in research focused on steroidogenic factor 1 gene sensitivity to GnRH stimulation. Although studies on LBT2 gonadotroph cells consistently underscored SF1 refactoriness to GnRH regulation (Szeto et al. 1996, Tremblay & Drouin 1999), evident GnRH pulse frequency-dependent stimulation of steroidogenic factor 1 gene transcription in the pituitary gland has been reported in female (Haisenleder et al. 1996) and recently in male (Burger et al. 2011) rats. Further research is required to determine whether the extent of the opposite effects of E2 exerted on Pitx1 mRNA expression in vivo and in vitro results from differences existing between physiological (anterior pituitary gland) and pathological (cell lines) context.

In conclusion, the presented data support the usefulness of an applied experimental model to focus on modulatory inputs exerted on pituitary transcription factor gene activity in vivo. The results revealed a gene-specific impact of E2 exerted on Egr1 and Pitx1 mRNA expression.
expression in the anterior pituitary gland. Regardless of the i.c.v. microinjection content (saline, buserelin or antide), an oestradiol-induced activation of both ERA (ESR1) and ERß (ESR2) subtypes concurrently increased Egr1 and decreased Pitx1 mRNA levels in female rats. Moreover, a synergistic action of GnRH agonist and E2 stimulation on Egr1 gene transcription in vivo was also observed. Taken together, an orchestrated activity of transcription factor genes receiving specific central and peripheral regulatory inputs could be ultimately responsible for physiologically variable Lhb gene promoter activation in vivo.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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