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Prolactin induces Egr-1 gene expression in cultured hypothalamic cells and in the rat hypothalamus

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Abstract

Prolactin (PRL), the major lactogenic hormone, acts also as neuromodulator and regulator of neuronal and glial plasticity in the brain. There is an increase in synthesis and release of PRL within the hypothalamus during peripartum and in response to stress. To identify mechanisms by which PRL induces neuroplasticity, we studied the ability of PRL to induce the transcription factor Egr-1 in the hypothalamic cell line, 4B, in vitro, and in specific neuronal cell types of the hypothalamus in vivo. PRL induced Egr-1 mRNA expression in 4B cells, an effect which was prevented by the MEK inhibitor, U0126. In vivo, intracerebroventricular PRL (1 µg) increased Egr-1 mRNA levels in the hypothalamic paraventricular (PVN) and supraoptic nuclei (SON) of female rats. The increase in mRNA paralleled elevated Egr-1 protein expression in the PVN and SON. Double staining immunohistochemistry revealed Egr-1 localization in oxytocin neurons of the PVN and SON, but not in vasopressin neurons in these regions. In the PVN, a population of non-oxytocin or -vasopressin cells localized in a region corresponding to corticotropin releasing hormone neurons also showed marked Egr-1 immunoreactivity. The data suggests that PRL modulates plasticity in oxytocinergic neurons, through MAP kinase-dependent induction of Egr-1.

Keywords

signaling; neuroplasticity; stress; hypothalamo-pituitary-adrenal axis; transcription factor; lactation

1. Introduction

The cytokine prolactin (PRL), best known as hormone instrumental in lactation, also acts as neuromodulator in the brain and influences neuronal plasticity of the adult nervous system on multiple levels: PRL promotes neurogenesis [32,43], neuronal survival after stress in the hippocampus [47], and the differentiation of neurospheres into mature neurons [43]. Additionally, PRL reverses myelin damage via increases in oligodendrocyte numbers, myelin basic protein and myelinated axons [14].

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Such neuronal actions of PRL should mainly be attributed to PRL synthesized and released within the brain, e.g. in the hypothalamus [4,11,12,48,49]. Moreover, circulating PRL, produced and secreted by lactotroph cells in the anterior pituitary, can access the brain, surpassing the blood brain barrier via a transport system in the choroid plexus and thus contributing to central PRL effects [33,50]. Different forms of PRL receptors have been demonstrated in several brain regions including the hippocampus and the paraventricular (PVN) and supraoptic (SON) nuclei of the hypothalamus [1,2,10,35]. Classically linked to the Jak/Stat signal transduction pathway via its cytokine family receptor, PRL also activates the ERK1/2 MAP kinase cascade in the hypothalamus [6].

The hypothalamus undergoes a number of neuroplastic changes during pregnancy and lactation to adjust to the altered requirements during this period [16,26]. It has been shown that stimuli leading to long-term changes in gene expression patterns are associated with induction of the immediate early gene early growth response factor (Egr)-1 (also known as zif 268, krox 24, NGFI-A, TIS8), which encodes a transcription factor. Besides Egr-1, the Egr zinc finger family of transcription factors also includes Egr-2 (krox 20), Egr-3 and Egr-4 (NGFI-C, pAT133), proteins sharing a highly conserved DNA binding domain which recognizes a consensus sequence on the DNA. Egr proteins show little homology outside the DNA binding domain, enabling a differentiated response to activation of different signalling pathways [3,18]. Egr-1 is involved in the transcriptional regulation of genes involved in female reproduction [27], wound repair [24] and neurite outgrowth [15], synaptic plasticity in various species [3,22,38], and regulation of the neuronal proteasome [21] and the activity of the hypothalamo-pituitary-adrenal (HPA) axis [52].

It has been shown that PRL can stimulate Egr-1 expression in Nb2 lymphoma cells [8]. Also, in HC 11 mouse mammary epithelial cells and Nb2 cells, PRL induced expression of vascular endothelial growth factor via mitogen-activated protein (MAP) kinase and Egr-1 [13]. The increased activity of the central PRL system during lactation occurs at a time of pronounced plastic changes in the hypothalamus, especially in the PVN and SON, changes including glial retraction and remodeling of synaptic contacts of oxytocinergic neurons [16,26]. The above observations prompted us to examine whether PRL induces Egr-1 in the rat hypothalamus, and to identify neuronal targets for PRL-induced Egr-1 in this region. The signaling pathway involved in the PRL effect on Egr-1 transcription was assessed using the hypothalamic neuronal cell line, 4B, which expresses endogenous PRL receptors [6].

2. Results

Effect of PRL on the expression of Egr-1 in the hypothalamus in vivo

The effect of central PRL administration on Egr-1 mRNA in the hypothalamus was examined by *in situ* hybridization. While undetectable levels of Egr-1 mRNA autoradiographic staining were found in hypothalamic sections from vehicle-treated control rats, clear Egr-1 staining was observed in the PVN and SON 4 h after intracerebroventricular (icv) PRL infusion (1 µg/5 µl), (Fig. 1).

Consistent with the levels of Egr-1 mRNA, immunoreactive Egr-1 was undetectable in hypothalamic sections from control rats, as measured by immunohistochemistry. In contrast, icv injection of PRL induced clear Egr-1 immunostaining in both the PVN and the SON, 4 h after injection (Fig. 2).

Neuronal cell types expressing Egr-1 after stimulation with PRL

Double-staining immunohistochemistry using Egr-1 and anti-oxytocin or -vasopressin antibodies showed that induction of Egr-1 following icv PRL injection occurred in a

subpopulation of oxytocinergic neurons in the PVN and the SON (Fig. 3), but not in vasopressinergic neurons (Fig. 4). In the PVN, there was a population of Egr-1 neurons in the dorsomedial region which was not oxytocin-positive. The topographic distribution of these Egr-stained neurons was consistent with corticotropin releasing hormone (CRH) neurons [39](Fig. 3).

Effects of PRL on the expression of Egr-1 in hypothalamic 4B cells

To determine whether PRL can directly induce Egr-1 in neurons, the effects of PRL on Egr-1 mRNA levels were examined in vitro using the hypothalamic neuronal cell line, 4B. Levels of Egr-1 mRNA, measured by quantitative RT-PCR, were readily detectable under basal conditions. Incubation of the cells with PRL (1 µg/ml) for 4h increased levels of Egr-1 mRNA (2-fold increase, $p < 0.05$; Fig 5). The increase in Egr-1 mRNA was prevented by co-incubation with the MEK inhibitor, U0126 (10 µM), added 30 min before PRL. The MEK inhibitor alone significantly reduced basal mRNA levels of Egr-1 (Fig. 5).

3. Discussion

The increases in central PRL that occur during lactation coincide with pronounced neuroplastic changes in the hypothalamus, especially in the oxytocinergic system of the PVN and SON. This study shows that icv administration of PRL induces the expression of Egr-1 in the hypothalamus, specifically in magnocellular oxytocinergic neurons of the SON and PVN. Egr-regulated genes are involved in neuronal plasticity. Thus, it is likely that induction of Egr-1 by PRL contributes to neuronal plasticity in oxytocinergic neurons required during lactation-associated adaptation [26,45]. Also, PRL has been shown to promote plasticity in the brain in different contexts, including olfactory neurogenesis and white matter remyelination [14,32, 43,47]. In contrast to the high expression levels of Egr genes demonstrated in other brain regions such as cortex, amygdaloid nuclei, thalamus, striatum and hippocampus [19,31,41,51], basal levels of Egr-1 mRNA and protein were almost absent in the hypothalamic PVN and SON. This probably reflects the relatively low activity levels of oxytocinergic neurons under basal conditions. The demonstration that PRL induces both ERK 1/2 phosphorylation and Egr-1 expression in oxytocinergic neurons suggests that the increased PRL levels contribute to the adaptive changes in oxytocinergic neurons during lactation [16,26].

An important question is whether PRL acts directly in the PVN and SON neurons or indirectly through activation of afferent neural pathways. Since PRL receptors are present in neurons of the PVN and SON [10,25a], it is likely that PRL exerts these neuronal actions directly within these hypothalamic nuclei. In support, the in vitro studies confirmed that PRL is capable to induce Egr-1 mRNA directly in neurons. Moreover, PRL receptor mRNA, protein and binding sites for ^{125}I -labelled PRL have been localized in neurons of the PVN and SON [1,2,10,35], making direct local PRL actions possible. We have previously shown that PRL activates the ERK1/2 MAP kinase pathway in hypothalamic neurons in vivo and in vitro [6]. The present demonstration that a MEK inhibitor, which blocks the MAP kinase pathway, prevents the stimulatory effect of PRL on Egr-1 expression in the hypothalamic cell line 4B indicates that this effect of PRL is mediated via activation of the ERK1/2 MAP kinase pathway. MAP kinase activation by PRL has also been shown in cell lines of peripheral origin such as Nb2 lymphoma cells [8] and HC11 mouse mammary epithelial cells [13]. Some of the target genes of Egr include growth factors and receptors like transforming growth factor, insulin-like growth factor, platelet-derived growth factor A and B [24,25,44] and the p75 NGF receptor [37], the neuroplasticity-associated gene, arc [28] and other proteins involved in synaptic plasticity like synapsin I [46]. Whether any of these Egr-dependent genes play a role in oxytocinergic neuronal plasticity observed during lactation remains to be elucidated.

In contrast to central PRL infusion in female rats, peripheral PRL injection in male rats was shown to induce *nur77* (NGFI-B) in the arcuate nucleus, but not to stimulate Egr-1 expression in any hypothalamic nuclei from 0.5 to 4h post injection [40]. This suggests that PRL can activate different neural pathways depending of the route of administration. Since the induction of Egr-1 by PRL in the present experiments was not evident at 1h and it required a relatively long period of time (4h), it is possible that peripherally administered PRL would require longer periods of time to be taken up by the circumventricular organs and choroid plexus and act in the brain. Also, gender-dependent differences between the two studies have to be taken into account, as we used female rats in the present study, whereas males were used before [40]. However, this is rather unlikely, since icv PRL also causes robust activation of the MAP kinase cascade within the PVN and SON of male rats (Blume and Neumann, unpublished results).

It is noteworthy that elevated Egr-1 mRNA and immunoreactivity was also found in the dorsomedial region of the PVN, area corresponding to the location of CRH neurons. Since we have previously demonstrated that PRL induces ERK phosphorylation in CRH neurons in the PVN, it is likely that Egr-1-labeled cells in this region are indeed CRH neurons [6]. PRL potentiates the stimulatory effect of the adenylate cyclase stimulator, forskolin, on CRH transcription in primary cultures of hypothalamic cells, in a MAP kinase dependent manner [6]. However, an involvement of Egr in the transcriptional regulation of CRH by PRL is unlikely, since the raise in Egr occurs hours after CRH transcription had returned to basal levels [30,42]. It needs to be shown whether PRL, locally released within the hypothalamic PVN during stress [48] contributes to the neuroplastic changes observed in stressed animals in both the hypophysiotropic neurons of the parvocellular PVN, which control HPA axis activity, and magnocellular neurons of the PVN, which release neurohypophysial peptides into the peripheral circulation [20]. Chronic stress has recently been associated with a high degree of neuronal plasticity within the PVN [20]. Thus, it is tempting to speculate that not only suckling-induced stimulation of intracerebral PRL release contributes to lactation-associated hypothalamic neuroplasticity, but that also stress-induced morphological alterations require the action of intracerebral PRL.

Icv PRL specifically induced the hypothalamic expression of Egr-1mRNA and Egr-1 protein in vivo, specifically within oxytocinergic neurons. This suggests a unique role for Egr-1 regulating oxytocinergic and probably CRH neurons in the hypothalamic PVN and SON in the adult female rat. In contrast to the abundant Egr-1 expression in oxytocinergic neurons, there was no expression of Egr-1 mRNA or Egr-1 immunoreactivity in vasopressin neurons of the PVN or SON. This observation contrasts with the prominent PRL-induced activation of ERK1/2 in vasopressin neurons, especially in the SON, and indicates that activation of the ERK1/2 MAP kinase pathway by PRL can initiate distinct pathways in a neuron type specific manner. It has been shown that PRL induces vasopressin release into the circulation [34], an effect which is likely to contribute to the mechanisms for antidiuresis required for fluid conservation during lactation. The present studies demonstrate that, in contrast to the oxytocinergic neuron, MAP kinase-dependent regulation of vasopressinergic neurons does not involve the induction of Egr-1.

Overall, our data demonstrate that PRL induces the expression of the transcription factor Egr-1 in the hypothalamus, specifically in oxytocinergic neurons of the PVN and SON and possibly in CRH neurons of the dorsomedial PVN. The ability of PRL to activate the ERK1/2 MAP kinase pathway and Egr-1 expression in the hypothalamic PVN and SON supports a role for PRL in mediating neuroplasticity of the neuroendocrine system during lactation and/or stress.

4. Experimental Procedure

Virgin female Wistar rats (230–280 g body weight) were obtained from Charles River (Sulzfeld, Germany) and kept under standard laboratory conditions with respect to food, humidity and light periodicity. All animal experiments were approved by the Bavarian local government and in accordance with the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health, Bethesda, MD, USA. Ovine PRL (oPRL) was obtained from the National Hormone and Peptide Program, NIDDK, NIH (Bethesda, MD, U.S.A.). Antisera were obtained from Cell Signaling Technologies (Egr-1, New England Biolabs, Frankfurt, Germany), Chemicon (oxytocin monoclonal antibody, Temecula, CA, U.S.A.) and antibodies online GmbH (vasopressin monoclonal antibody, Aachen, Germany). The anti-Egr-1 antiserum for the Egr-1/OT co-localization was a generous gift from Dr Rodrigo Bravo (Bristol-Myers-Squibb, Princeton, NJ, USA). Secondary antibodies were purchased from Vectastain (goat-anti-rabbit, horse-anti-mouse; Linaris, Wertheim, Germany) or from Molecular Probes Inc. (goat-anti-mouse-IgG, Alexa Fluor 488; goat-anti-rabbit IgG, Alexa 546; Goettingen, Germany). The MEK inhibitor U0126 was obtained from New England Biolabs.

Intracerebroventricular infusions

For icv infusion, chronic guide cannulas (21 gauge) were stereotaxically implanted 2 mm above the lateral brain ventricle under isoflurane anesthesia at least 5 days prior to treatment and closed by a stylet as previously described [36]. Rats were housed individually and handled daily to avoid the effects of experimental manipulation on signaling on the day of the experiment. All icv injections were performed between 8 and 11 AM in conscious, freely moving rats, using a 25-G infusion system lowered into the guide cannula and extending into the lateral ventricle as described in detail before. The injection volume was 5 μ l (1 μ l PRL, followed by 4 μ l isotonic saline, or 5 μ l isotonic saline in group 1).

Expression of Egr-1 mRNA in the hypothalamus

To assess whether PRL also induces the expression of Egr-1 *in vivo*, rats were treated with icv oPRL (1 μ g, n=3) or vehicle (Ringer's solution, 5 μ l; n=3) for 4 h before removal of brains. Serial coronal 12- μ m brain slices were cryosectioned, thaw mounted onto slides and stored at -80°C . In situ hybridization was carried out using 45-oligomer probes directed against Egr-1 (Egr-1: CCGTTGCTCA GCAGCATCAT CTCCTCCAGT TTGGGGTAGT TGTCC). Oligomers were labeled at the 3' end using [^{35}S]deoxy-ATP and terminal DNA transferase as described before [9]. Briefly, slices were thawed, fixed in 4% paraformaldehyde (PFA) for 5 min, washed three times with PBS and acetylated for 10 min (0.25 % acetic anhydride in 0.1M triethanolamine/0.9 % NaCl, pH 8.0). Sections were dehydrated and de-lipidated by sequential transfers through ethanol and chloroform and air dried before hybridization. Sections were incubated in hybridization buffer for 2 h at 42°C and then hybridized at 42°C overnight with the oligomer probes. Slices were then washed three times with $1 \times \text{SSC}$ at 55°C and one time with $1 \times \text{SSC}$ at room temperature, followed by two washes with 50 % ammonium acetate/ethanol and 85 % ammonium acetate/ethanol. Dry sections were exposed to BioMax MR film (Kodak Eastman, Rochester, NY, U.S.A.) for two weeks. Slices at several levels of both the PVN and SON were examined. The sections were chosen according to specific reference markers including the shape and size of the optic chiasm, hippocampus, subfornical organ and anterior comisura.

Expression of Egr-1 protein in the hypothalamus

For Egr-1 or double Egr-1/oxytocin immunohistochemistry, 4 h after the injection of 1 μ g oPRL or vehicle, rats were deeply anesthetized with pentobarbital (0.1 ml per 100 g body weight, intraperitoneally) and perfused intracardially with 4 % PFA. Brains were removed,

post fixed overnight in 4 % PFA and immersed in 30 % sucrose for cryoprotection. Coronal cryostat-cut 20- μ m slices were incubated with the polyclonal Egr-1 antibody (1:4000), followed by a biotinylated secondary antibody (goat-anti-rabbit, Vectastain ABC kit; dilution according to the manufacturer's instructions) and detection with the conventional avidin-biotin complex peroxidase reaction (Vectastain ABC kit) with diaminobenzidine as chromogen as described before [5]. This was followed by incubation with the specific monoclonal antibody against oxytocin (1:1000), visualized with a biotinylated secondary antibody (horse-anti-mouse, Vectastain) and SG substrate (Vectastain) as chromogen.

For Egr-1/vasopressin double immunofluorescence, brains were removed 4 h after the injection of 1 μ g oPRL or vehicle and immersed in 4 % PFA for 24 h. Immunohistochemistry was performed on coronal paraffin slices (4 μ m). Incubation with the primary antibodies (rabbit polyclonal Egr-1, 1:100, in combination either mouse monoclonal anti-vasopressin 1:500 for co-localization of Egr-1 with vasopressin) was followed by signal enhancement with secondary, fluorescence-coupled antibodies (1:1000; goat-anti-rabbit IgG Alexa Fluor 546, goat-anti-mouse IgG Alexa Fluor 488).

PRL-induced, MAP kinase mediated regulation of genes in 4B cells

The hypothalamic cell line 4B [23] was cultured in DMEM supplemented with 10% fetal calf serum, 10% horse serum and 100 IU penicillin/100 μ g streptomycin/ml. For analysis of Egr-1 mRNA regulation, 4B cells cultured in 6-well plates were incubated for 30 min in medium with 0.1% BSA before treatment with vehicle, 1 μ g/ml of PRL, 10 μ M of the MEK inhibitor U0126 (Cell Signaling Technology) or a combination of U0126 (10 μ M) and PRL (1 μ g/ml) for 4h. Total RNA was isolated from 4B cells using the Trizol reagent (Life Technologies, Gaithersburg, MD, U.S.A.). RNA was cleaned using RNeasy kit reagents (Qiagen, Valencia, CA, USA) and subjected to a RNase-free DNase digestion step according to the manufacturer's protocol. RNA concentration and quality of total RNA was determined spectrophotometrically. cDNA was generated from 1–2 μ g RNA per sample using SuperScript III First Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, U.S.A.), followed by RNase H treatment for 20 min at 37 °C.

The expression levels of mRNA for Egr-1 were measured by real-time PCR using TaqMan Gene Expression assays (Egr-1 probe ID 524988) on ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). For each sample and gene, three replicates were run in a 96-well format plate. The probes contain a 6-carboxy-fluorescein phosphoramidite (FAM dye) label at the 5' end of the gene and a minor groove binder and nonfluorescent quencher at the 3' end and are designed to hybridize across exon junctions. 18s rRNA (VIC dye) was used as endogenous control. Real-time PCR reactions were carried out using 1–2 μ l of cDNA for each reaction in a 25 μ l volume following the manufacturer's protocol.

The absence of RNA detection when the RT step was omitted indicated the lack of genomic DNA contamination in the RNA samples. Relative quantification of Egr-1 mRNA levels was performed using the comparative threshold cycle method for Egr-1 mRNA and 18rRNA, as described in the users manual [29].

Statistics

For statistical analysis, a one-way ANOVA, followed by Student-Newman-Keuls test was carried out. $P < 0.05$ was considered statistically significant. Data are presented as means $\pm$ SEM.

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Figure 1. Induction of Egr-1 mRNA by PRL in the rat hypothalamus

To visualize Egr-1 mRNA in vivo, conscious female Wistar rats were treated with either vehicle (Veh) or PRL (1 μ g, icv; n=3 animals per group). After 4 h, brains were frozen at -40°C , and 12 μm cryosections processed for in situ hybridization using ^{35}S labeled oligoprobes. SON, supraoptic nucleus, PVN, paraventricular nucleus, OC, optic chiasm, 3V, 3rd ventricle.

Figure 2. PRL induces Egr-1 protein in the rat hypothalamus

For the immunohistochemical localization of Egr-1, conscious female rats were treated with Veh or PRL (1 μ g, icv) for 4h (n=3 animals per group). Brain slices (40 μ m) were incubated with primary antibody (Egr-1, 1:4000), followed by Vectastain ABC with diaminobenzidine (DAB) as chromogen. OC, optic chiasm, 3V, 3rd ventricle.

Figure 3. PRL-induced Egr-1 protein in the rat hypothalamus: co-localization with oxytocin

For the immunohistochemical co-localization of Egr-1 and oxytocin, conscious rats were treated with Veh or PRL (1 μ g, icv) for 4h (n=3 animals per group). Brain slices (40 μ m) were incubated with primary antibody (Egr-1, 1:4000), followed by Vectastain ABC with diaminobenzidine (DAB) as chromogen. Oxytocin was detected using a primary antiserum (anti-oxytocin monoclonal antibody, 1:1000) with SG substrate as chromogen (blue cytoplasm). OC: optic chiasm, 3V, 3rd ventricle. Yellow arrows: oxytocin cell; white arrows: Egr-1 in the nucleus; red arrows: co-localization of oxytocin and Egr-1.

Figure 4. PRL-induced Egr-1 protein in the rat hypothalamus: lack of co-localization with vasopressin

For co-localization of Egr-1 with vasopressin, rats were treated with PRL (1 μ g, icv) or Veh for 4h (n=3 animals per group). PRL-induced Egr-1 was visualized using an Egr-1 polyclonal antibody (1:100) followed by goat-anti-rabbit IgG Alexa Fluor 546 (red nuclei); vasopressin was detected using an anti-vasopressin monoclonal antibody, 1:500 with donkey-anti-mouse IgG Alexa Fluor 488 (green cytoplasm). Yellow arrows: vasopressin cell; white arrows: Egr-1 in the nucleus. OC: optic chiasm, 3V, 3rd ventricle.

Figure 5. ERK1/2 mediates the induction of Egr-1 mRNA by PRL in 4B cells

To measure Egr-1 mRNA levels, hypothalamic 4B cells were changed into serum-free medium containing 0.1% BSA for 30 min prior to pre-incubation with Veh or the MEK inhibitor U0126 (10 μ M) for 30 min, followed by an additional 4 h incubation with Veh or PRL (1 μ g/ml). RNA was extracted, reverse transcribed and the cDNA analyzed using real time PCR with TaqMan Gene Expression assays on ABI PRISM 7900HT Sequence Detection System. (n=4 experiments per data point), * $p < 0.05$ vs Veh/Veh, ** $p < 0.01$, *** $p < 0.001$ vs. Veh/Veh, Veh/PRL.