ORIGINAL ARTICLE

Late Pregnancy is a Critical Period for Changes in Phosphorylated Mitogen–Activated Protein Kinase/Extracellular Signal–Regulated Kinase 1/2 in Oxytocin Neurones

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The physiological demands of parturition and lactation lead to the increased pulsatile release of oxytocin (OT) into the circulation from the neurohypophysial axons of OT neurones in the supraoptic (SON) and paraventricular (PVN) nuclei. These states of increased OT release are accompanied by a significant plasticity in magnocellular OT neurones and their synaptic connections, and many of these changes require activation of a central OT receptor. The mitogen-activated protein kinase/extracellular signal-regulated kinase pathway (MAPK/ERK) is assumed to be up-regulated in the PVN during lactation, and many of the effects of OT in peripheral and brain tissue are mediated through a MAPK/ERK pathway. The present study investigated whether this pathway is altered in the SON and PVN during late pregnancy [embryonic day (E)20–21], which is a critical period for OT plasticity induction, and for lactation, when plastic changes are sustained. Based on immunoreactivity for phosphorylated ERK1/2 (pERK1/2), the results suggest an enhanced activation of MAPK/ERK pathway in OT neurones specifically during late pregnancy in both the SON and PVN. Although immunoblots from the SON confirm this pregnancy-associated up-regulation in late pregnancy, they also suggest enhancement into lactation as well. Together, the results suggest an important role for the MAPK/ERK pathway during reproductive changes in the SON and PVN.

Key words: oxytocin, vasopressin, pERK1/2, supraoptic nucleus, paraventricular nucleus

One of the key neurohypophysial hormones, oxytocin (OT), is synthesised and released from the magnocellular cells (MNCs) of the supraoptic nucleus (SON) and paraventricular nucleus (PVN). Although best known to activate peripheral OT receptors (OTRs) mediating the induction of labour through uterine contractions, and the mammary myoepithelial cell contractions necessary for milk ejection during lactation (1), OT also acts as a peptidergic neurotransmitter in the brain (2), which contains an abundance of OTRs (3). In the SON and PVN, OT is released from the dendrites and somata (4,5) to act on OTRs on OT neurones themselves. This autoregulatory activity of OT is assumed to be critical to the induction of the normal OT bursting patterns observed during milk ejection (6,7).

The activity of OT in the brain is likely conditioned by changes in OTRs that occur in late pregnancy after the fall of progesterone (8), a time when local OT secretion itself becomes significantly elevated near parturition (9). Studies also showed that central OTR stimulation during mid and late gestation is essential for the normal OT secretory response during lactation, thus assuring the healthy

development of the offspring (10,11). Central OTR activation during pregnancy is also necessary for plasticity observed in intrinsic (12) and synaptic properties (13) of OT neurones. The fall of progesterone during the last few days of pregnancy may be critical for many OT-related changes in the centrasl nervous system (8,14), as well as in the induction of maternal behaviours (15).

There is increasing evidence that some of the effects of OT in the brain may be mediated through an mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway that also has been implicated in some peripheral actions of OT (16). For example, centrally released OT improves spatial memory in the hippocampus of lactating rats through the phosphorylation of ERK1/2 (17). There are also complex alterations in ERK1/2 signalling in the PVN during lactation, suggesting that this pathway might be involved in the regulation of anxiety-like behaviour in female rats (18). Furthermore, both OT and vasopressin (VP) neurones exhibit an increased phosphorylated ERK1/2 (pERK1/2) in response to osmotic challenge (19) and pERK1/2 is selectively increased by psychological and

systemic stressors in a subset VP neurones that contain interleukin-6 (20). In the present study, we investigated whether pERK1/2 varied during the reproductive cycle in the PVN and SON using immunohistochemistry and western blotting. The results suggest late pregnancy [embryonic day (E)20–21] is a critical period for pERK1/2 elevation in the SON and PVN, specifically within OT neurones.

Materials and methods

Animals

Adult Sprague–Dawley albino female rats (virgins), pregnant rats (E18–19), late-pregnant rats (E20–21) and lactator rats (day 8 lactation) were used (Harlan Labs, Indianapolis, IN, USA) for both western blotting and immunohistochemical analyses. The rats had free access to food and water in the cages housed in a room under a 12 : 12 h light/dark cycle. The Institutional Animal Care and Use Committee approved all protocols used in the study.

Immunohistochemistry

The rats were deeply anaesthetised with sodium pentobarbital (50 mg/kg) and perfused through the heart with cold 4% paraformaldehyde and 0.2% picric acid in 0.01 M phosphate-buffered saline (PBS). The brains were excised and fixed overnight. Hypothalamic slices (50 μm) were prepared with a vibrating microtome (VT1000 Leica, Bannockburn, IL, USA). The slices were rinsed off fixative with several changes of PBS containing 0.5% Triton X-100 (PBST), then incubated for 24–48 h at 4 °C for double-labelling with rabbit anti-pERK1/2 (dilution 1 : 1000, Catalogue number 4370S, Cell Signaling, Danvers, MA, USA) + anti-OT antibody (PS36 or PS38) raised in mouse against OT-NP (Oxytocin-Neurophysin, dilution 1 : 500, a gift from Dr Harold Gainer, NIH, Bethesda, MD, USA). This experiment was repeated for VP neurones, with sections incubated in mouse anti-pERK1/2 (dilution 1 : 1000, Catalogue number 5726S, Cell Signaling) + anti-VP antibody (dilution 1 : 20 000) raised in rabbit against VP-neurophysin (a gift from Dr Alan Robinson, UCLA, Los Angeles, CA, USA). The sections were rinsed in PBST and incubated in a cocktail of secondary antibodies for 3 h at room temperature. The secondary antibodies used were goat-anti-mouse (Alexa Flour 488 nm; Invitrogen, Carlsbad, CA, USA) and goat-anti-rabbit (Alexa Fluor 568 nm, Invitrogen) conjugated immunoglobulin G at a dilution of 1 : 200 with PBST. Rinsed sections were mounted on a glass slide and cover-slipped using a polyvinyl alcohol (PVA) solution containing 6 g/25 ml glycerol, 2.4 g/25 ml PVA, 0.625 g/25 ml of the anti-fade reagent 1,4 diazabicyclo [2.2.2]octane, brought to 25 ml with 6 ml dH_2O and 12 ml PBS (all reagents, Sigma-Aldrich, St Louis, MO, USA).

Tiled images were acquired from the SON or PVN with a Zeiss 710 confocal microscope (Carl Zeiss, Oberkochen, Germany) using a × 20 objective (0.8 n.a.), with optical section oversampling of approximately 1 μm. The percentages of magnocellular OT and VP neurones that co-localised with pERK1/2 in the PVN and SON were estimated from counts made in these confocal stacks with Zen (Carl Zeiss) or IMAGEJ (NIH, Bethesda, MD, USA). Antibody penetration was approximately 12–15 μm, and each section was tested for penetration depth of each antibody before sampling. Each neurone counted was observed in multiple optical sections to avoid double counts and, similarly, counts were made from slices separated by at least 50 μm to avoid counting the same neurone in two sections. When sampling ranged from the rostral to caudal borders of each nucleus in all groups, we did not estimate the total number of neurones stained for any of the antibodies, focusing only on VP or OT neurones for which the soma was within the focal plane and determining whether they co-localised pERK1/2. Slides were coded by one investigator and counted blind by a second. The number of neurones sampled was:

OT neurones, PVN: virgin (3156), E18–19 (3161), E20–21 (3585), lactators (3377) and SON: virgin (2653), E18–19 (3640), E20–21 (3368), lactators (2866) (n = 6 animals).
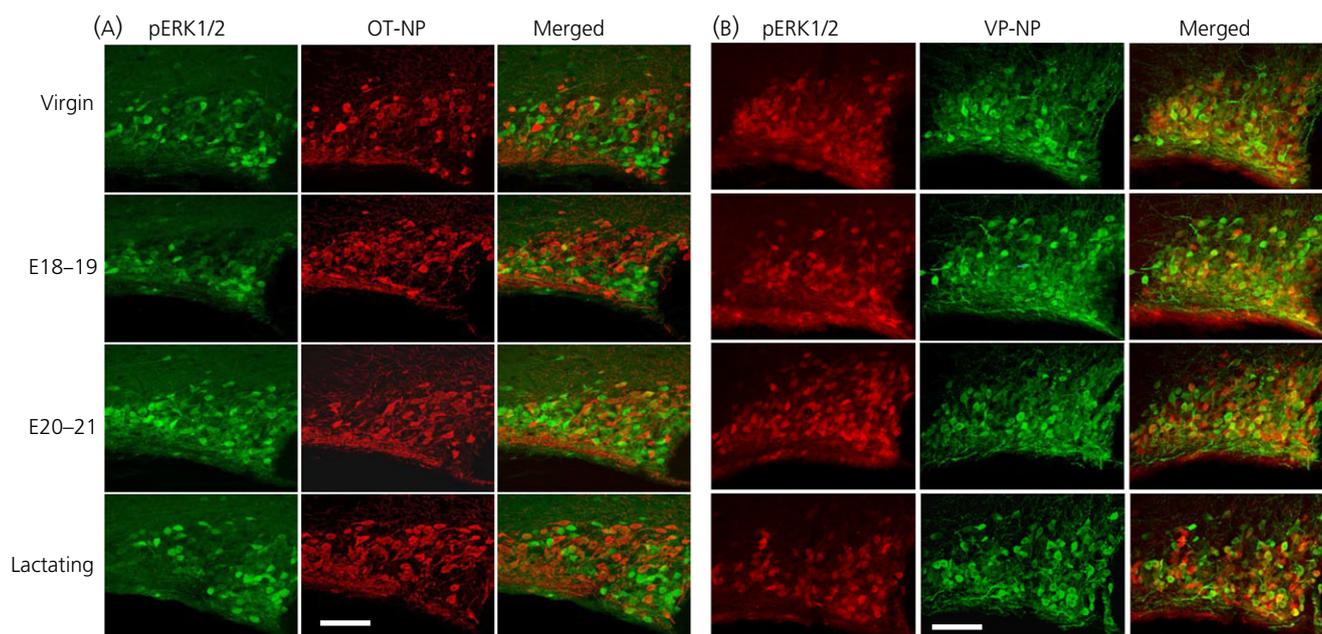

Fig. 1. Immunolocalisation of phosphorylated ERK1/2 (pERK1/2) in oxytocin (OT) (A) and vasopressin (VP) (B) neurones in the supraoptic nucleus (SON) in virgin, embryonic day (E)18–19, E20-21 and lactating rats. Double-immunofluorescence confocal microscopy revealed that OT neurones (A), expressed more pERK1/2 during late pregnancy (E20-21) than at other times. VP neurones co-localised pERK1/2 to the same degree in all groups. Yellow cells/neurones in the merged columns represent double-labelled neurones. Quantitative data are shown in Fig. 2. Scale bar = 100 μm.
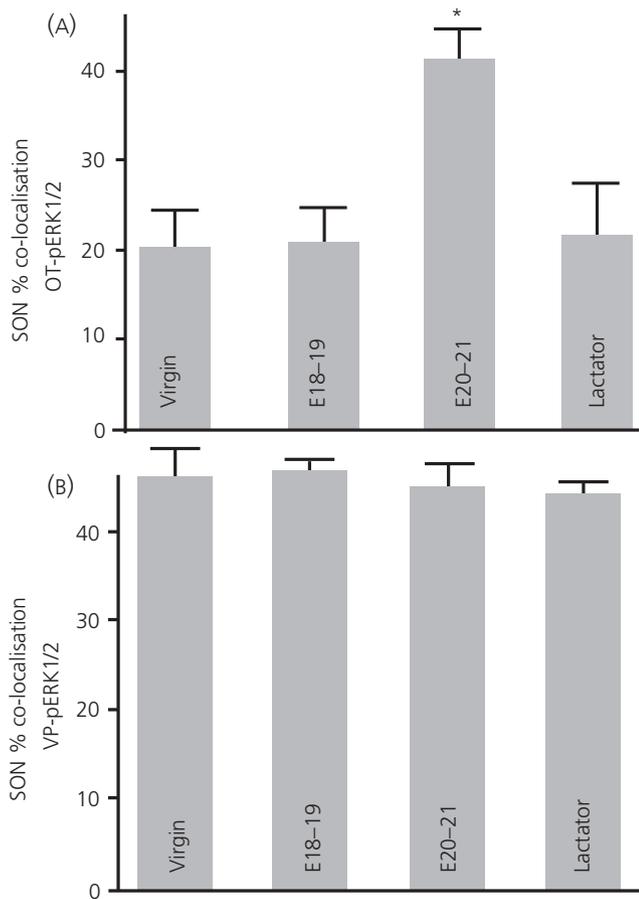
Fig. 2. Co-localisation of phosphorylated ERK1/2 (pERK1/2) with oxytocin (OT) (A) and vasopressin (VP) (B) in the supraoptic nucleus (SON) of virgin, embryonic day (E)18–19, E20–21 and lactating rats. ANOVA indicated significant difference across groups for pERK1/2 co-localisation with OT ($P = 0.006$) but not VP neurones ($P = 0.835$). Co-localisation was highest at E20–21 (*), which was significantly different from virgin ($P = 0.017$), E18–19 ($P = 0.014$) and lactating rats ($P = 0.019$) ($n = 6$ animals per group).

VP neurones, PVN: virgin (2148), E18–19 (2343), E20–21 (2317), lactators (2165) and SON: virgin (2406), E18–19 (2466), E20–21 (2274), lactators (2466) ($n = 6$ animals).

SON lysate preparation

The rats ($n = 8$ per group) were deeply anaesthetised with sodium pentobarbital (50 mg/kg) and perfused through the heart with cold sucrose solution (in mM: 20 D-glucose, 0.45 ascorbic acid, 2.5 KCl, 1 MgSO$_4$, 1.25 NaH$_2$PO$_4$.H$_2$O, 26 NaHCO$_3$, 210.35 sucrose, and 2 CaCl$_2$). The brains were excised and sectioned coronally at 250 μm on a vibrating microtome (VT1000; Leica, Bannockburn, IL, USA). Small pieces from three or four hypothalamic brain slices were cut containing the SON and the immediate surrounding area, then transferred to sterile vials containing lysis buffer (pH 7.4) (in mM: 20 Hepes, 150 NaCl, 0.01% octylphenoxypolyethoxyethanol) with 0.01% protease inhibitor (#P8340; Sigma-Aldrich,) and 0.01% phosphatase inhibitor 2 (#P5726; Sigma-Aldrich,) cocktails. These SON pieces would include small portions of adjacent regions, in particular the perinuclear zone dorsally, the optic tract and chiasm medially, and the ventral anterior amygdaloid area and medial amygdaloid nucleus laterally (21). These pieces were homogenised manually by mortar and pestle and were passed three times through a 19-gauge needle, and then a 25-gauge needle followed by vortex. The homogenised lysates were then centrifuged at 14 489 g for 15 min and the supernatant was transferred to a fresh sterile vial, followed by protein estimation with BCA protein assay kit (Pierce, Thermo Scientific, Waltham, MA, USA). The entire process of lysate preparation was carried out on ice.

Western blotting

Protein obtained from each SON-lysate (100 μg) was mixed with 5–10 μl of 2 × Laemmli sample buffer and boiled at 95 °C for 5 min and the proteins were separated on 10–12% sodium dodecyl suphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were transferred to nitrocellulose membranes in electrophoresis buffer (25 mM Tris, 200 mM glycine and 20% methanol) for 120 min at 95 V at +4 °C. The membranes were then blocked either with 5% bovine serum albumin or 5% nonfat dry milk in Tris-buffered saline containing (in mM: 1000 Tris, 50 NaCl, pH 8.0 adjusted with HCl and 0.001% Tween 20) (TBST) for 60 min at room-temperature. The primary antibodies to either ERK1/2 or pERK1/2 (Cell Signaling) (dilution 1 : 1000) were added to the blocking buffer and were incubated overnight with the blot at +4 °C. The pERK1/2 antibody was the same rabbit anti-pERK1/2 used for double-labelling OT neurones for immunohistochemistry. The blots were washed three times with TBST buffer and incubated 60 min with goat anti-mouse/rabbit (horse radish peroxidase-conjugated) secondary antibody diluted in the blocking buffer at room temperature. They were washed three times with wash buffer and visualised with enhanced chemiluminescence reagents (Pierce-ECL Western Blotting Substrate; Thermo Scientific) using Classic BX autoradiography film (MIDSCI, Valley Park, MO, USA). Immunoblot densities from film were scanned at 300 dpi, then analysed using IMAGEJ. A lysate from one animal from each group was run in one of the four lanes on a gel (one lane per group, virgin, E18–19, E20–21 and lactators). The experiment was repeated eight times ($n = 8$ for each group). After first processing and imaging for pERK1/2, these gels were stripped by incubating for 30 min at +50 °C in 30 ml of stripping buffer (2% SDS, 62.5 mM Tris HCL, pH 6.7 and 210 μl of β-mercaptoethanol), then re-probed for ERK1/2. For statistical comparisons, pERK1/2 values were normalised against ERK1/2 for each run. To control for variability in exposures across the eight runs, the pERK1/2/ERK1/2 ratios were ranked within each of the eight runs.

Statistical analysis

The data were analysed with JMP PRO, version 12 (SAS Institute Inc., Cary, NC, USA). For the pERK co-localisation studies with VP or OT, we first tested for normality using the Shapiro–Wilk test and homogeneity of variance using Bartlett's test. In all but one case, we could not reject the null hypotheses that the distributions were normal or that the variances were equal (based on $P < 0.05$), and we therefore applied a standard ANOVA followed by the Tukey–Kramer post-hoc test. The exception to this was the co-localisation data for VP with pERK in the PVN, which, although normal, did not satisfy the homogeneity of variance requirement ($P = 0.019$). These data were therefore analysed with Welch's test instead. For the western blot data, based on ordinal rankings, we used the nonparametric Kruskal–Wallis test followed by the Steel–Dwass test for between-group comparisons.

Results

Increased pERK1/2 in OT neurones of the PVN and SON in late pregnancy

We aimed to investigate whether the known plasticity in OT neurones at the end of pregnancy was specifically associated with
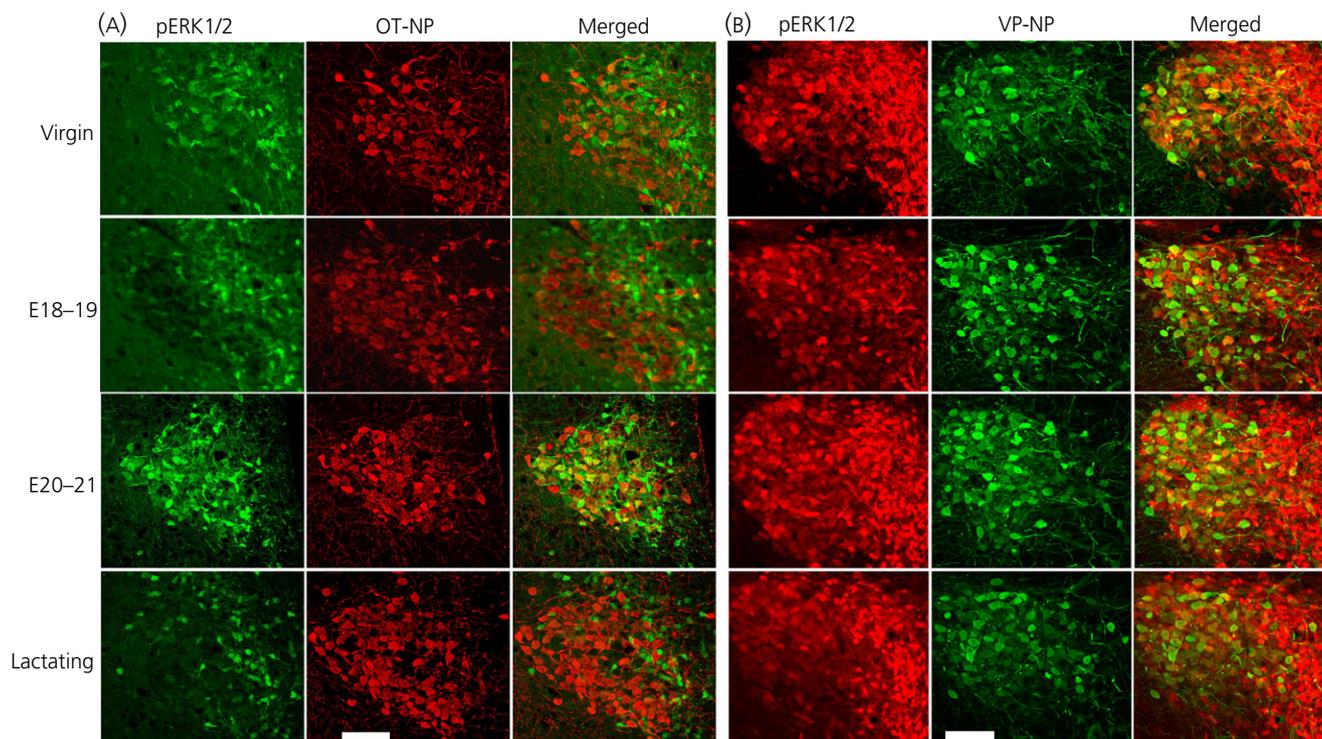
Fig. 3. Immunolocalisation of phosphorylated ERK1/2 (pERK1/2) in oxytocin (OT) (ᴀ) and vasopressin (VP) (ʙ) neurones in the paraventricular nucleus (PVN) in virgin, embryonic day (E)18–19, E20–21 and lactating rats. Double-immunofluorescence confocal microscopy revealed that OT (ᴀ) but not VP (ʙ) increased its co-localisation with pERK1/2 in late pregnancy (E20–21). Yellow cells/neurones in the merged columns represent double-labelled neurones. Quantitative data are shown in Fig. 4. Also note that the level of the PVN is more rostral for OT neurones than VP neurones, and that, medial to VP neurones, large numbers of parvocellular pERK1/2 neurones were visible. Scale bar = 100 μm.

changes in pERK1/2 in the SON and PVN. Rat hypothalamic sections were labelled with pERK1/2 and OT-NP antibodies. The expression of pERK1/2 in the SON (Fig. 1ᴀ) and PVN (Fig. 3ᴀ) varied across the groups studied. Although OT neurones were sampled throughout both nuclei, most of the OT neurones in PVN were found in the medial magnocellular group (Fig. 3ᴀ). The percentage of OT neurones double-labelled for pERK1/2 was calculated and is shown in Figs 2(ᴀ) (SON) and 4(ᴀ) (PVN). The results from both PVN and SON revealed a two-fold increase in the co-localisation (of OT and pERK1/2) among late-pregnant rats (E20-21) compared to all the other three groups studied.

Immunohistochemistry of ERK1/2 and VP neurones

In many previous studies, changes in the electrophysiological properties of SON neurones during the reproductive cycle were found to be specific to OT neurones (22). To determine whether changes in pERK1/2 were similarly specific to OT and not VP neurones, sections of rat hypothalamus were reacted with a mouse pERK1/2 antibody and rabbit antibodies raised against VP-NP. The mouse pERK1/2 antibody stained the SON and PVN in similar fashion to the rabbit antibody used, and gave identical bands in western blots (not shown). When sampling throughout both nuclei, in the PVN, most of the VP neurones were found in the lateral wing, just caudal to where most of the OT neurones were found (Fig. 3ʙ). Examples of

such double-staining are shown in Figs 1(ʙ) and 3(ʙ). No significant differences were found in co-localisation in either the SON (Fig. 2ʙ) or the PVN (Fig. 4ʙ). However, the two nuclei appeared to behave differently in late pregnancy and lactation, where co-localisation in the PVN trended downward (Fig. 4ʙ). Regardless, no significant changes were found in VP neurones. In the PVN, we also noted large numbers of pERK1/2 neurones in the medial, parvocellular portion of the PVN, immediately adjacent to the lateral wing of the VP-rich portion of the PVN (Fig. 3ʙ). At the level of most of the OT neurones (which is rostral and medial to the largest cluster of VP neurones), these parvocellular pERK1/2 expressing neurones were less obvious (Fig. 3ᴀ).

Enhanced pERK1/2 activation in the late pregnant and lactating rats

Because it is a more homogenous population of magnocellular neurones compared to the complex PVN (i.e. note the large numbers of parvocellular pERK1/2 neurones in the PVN in Fig. 3ʙ), protein lysates were analysed from the SON of virgin, pregnant (E18–19), late pregnant (E20–21) and lactating (day 8) rats. A significant increase in the activation of pERK1/2 was observed in the SON-protein lysates of late pregnant (E20–21) rats compared to those at E18–19 (Fig. 5). However, in contrast to the immunohistochemical results, pERK1/2 levels remained significantly higher in lactators
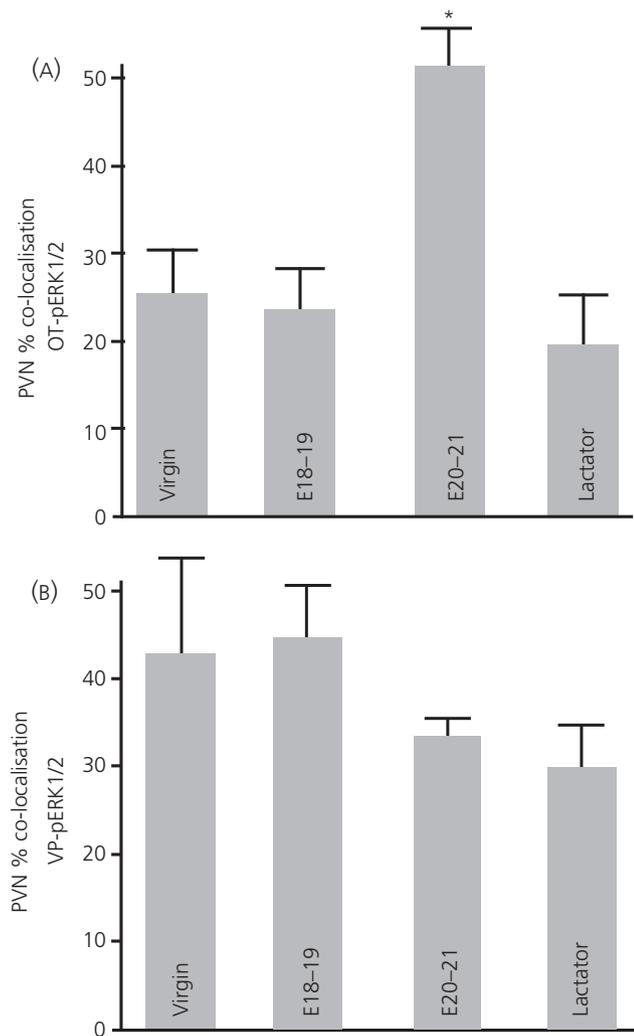
Fig. 4. Co-localisation of phosphorylated ERK1/2 (pERK1/2) in oxytocin (OT) (A) and vasopressin (VP) (B) neurones in the paraventricular nucleus (PVN) in virgin, embryonic day (E)18–19, E20–21 and lactating rats. ANOVA indicated significant difference across groups for pERK1/2 co-localisation with OT (P = 0.001) but not VP neurones (Welch's test, P = 0.216). Co-localisation of OT-pERK1/2 was highest at E20–21(*), which was significantly different from virgin (P = 0.008), E18–19 (P = 0.006) and lactating (P = 0.002) rats. n = 6 animals per group.

Fig. 5. Phosphorylated ERK1/2 (pERK1/2) activation in supraoptic nucleus (SON) of embryonic day (E)20–21 and lactating rats. Histograms of the ranking of the ratio of pERK1/2 over ERK1/2 intensity for each of eight runs (n = 8 animals), with each run including lysates from one of the four groups. Inset: 100 μg of protein sample from hypothalamic SON lysates were probed on western blots (inset) with pERK1/2-antibody (upper panel, inset) and the lower panel shows the blots used for normalisation with ERK1/2 antibody. A Kruskal–Wallis test indicated a difference among the groups (P = 0.0002). Comparisons between all pairs with Steel-Dwass indicated: *Lactator versus Virgin (P = 0.0387). **Lactator versus E18–19 (P = 0.0274). +E20–21 versus E18–19 (P = 0.0062). ++E20–21 versus Virgin (P = 0.0025).

compared to E18–19 rats. These results suggested that pERK1/2 in regions immediately adjacent to the SON may be differentially regulated compared to OT neurones. To further investigate this issue, we examined histological sections for additional pERK1/2 neurones near the SON that might contribute to this difference. The largest groups of pERK1/2 neurones near the SON that might have been partially sampled with western blots were dorsally in the perinuclear zone, immediately adjacent to the anterior amygdala rostrally and the medial nucleus of the amygdala and the bed nucleus of the olfactory tract more caudally. We examined these regions in all animals, and could see no obvious, consistent difference in the number of cells and/or the intensity of labelling that might account for this particular difference.
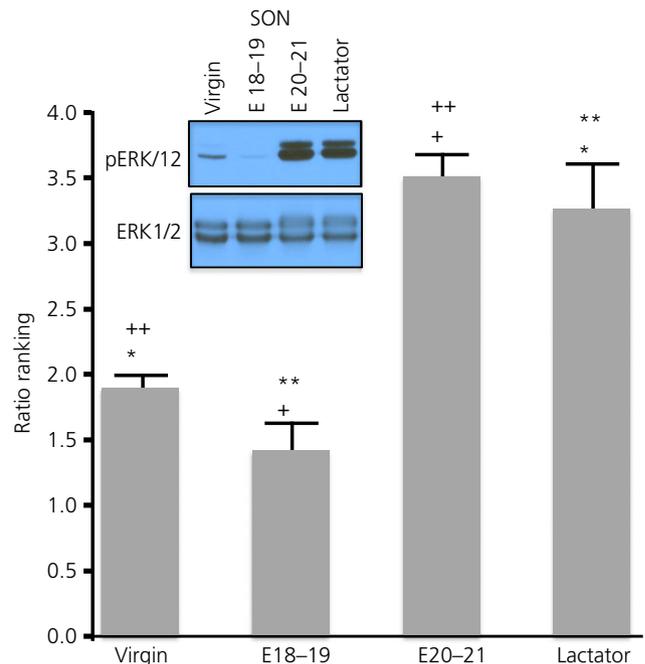
Discussion

In the present study, we found an up-regulation of pERK1/2 expression in OT neurones during late pregnancy, 24–48 h prior to expected delivery, a time associated with hormonal changes that are considered critical to the induction of maternal behaviour and OT neuronal plasticity. Bridges (15) first showed that, although both progesterone and oestrogen gradually increase during pregnancy, approximately 2 days before parturition, progesterone levels dramatically decrease. In steroid replacement experiments in ovariectomised rats, the withdrawal of progesterone in the presence of oestrogen produces an increase in maternal behaviour (15), an up-regulation of OTR binding (8) and increased OT mRNA (23). Similarly, in intact animals, this late gestational period and lactation are also associated with an up-regulation of OT mRNA, OTR mRNA, OTR binding, and significant morphological and synaptic plasticity in magnocellular neurones (1). These events are also accompanied by increased somatodendritic OT release (9,24). Thus, the fall of progesterone likely triggers dramatic changes in OT neurones, and our data show that the induction of pERK1/2 specifically within OT neurones in the PVN and SON is temporally associated with the onset of changes in OT neurone morphology and electrophysiology.

Changes in pERK1/2 within OT neurones are of particular interest because the local release of OT itself is implicated in the development of reproductive plasticity, probably by acting directly on OT neurones (12,14,22,25,26). In addition to the canonical role that MAPK pathways play in cell growth and differentiation (27), induction of pERK1/2 occurs in mature neurones and is associated with synaptic plasticity, both acutely as an intracellular messenger and by targeting nuclear transcription factors (28). Although OTR activation is classically G-protein coupled and most often associated with increased intracellular calcium, it also leads to pERK1/2 signalling (16,29,30). Central OTR activation and its induction of pERK1/2 have been associated with range of functions, including spatial memory in the hippocampus of lactating rats (17) and anxiolysis mediated through the PVN (18,30,31). It was recently demonstrated that pERK1/2 is induced in both OT and VP neurones in response to hyperosmotic challenge (19). Indeed, pERK1/2 induction appears necessary for the acute membrane potential changes that MNCs demonstrate to direct osmotic challenge *in vitro,* and pERK1/2 levels in the SON are decreased below normal in response to hypotonic challenge, suggesting a constitutive role for pERK1/2 (19). Furthermore, both psychological and systemic stressors also activate cytoplasmic pERK1/2 in an interleukin-6-containing subset of magnocellular VP neurones, as well as in parvocellular PVN neurones (20), suggesting that MAPK signalling pathways serve a variety of roles in the SON and PVN.

We also examined pERK1/2 by western blotting and, although again finding a significant increase in the SON at late pregnancy, levels did not return with lactation. However, these two methods measure different endpoints. It is possible that pERK1/2 may change its amount within individual neurones, without contributing to a change in double-labelling. This caveat would include VP neurones, where co-localisation with pERK1/2 did not change in these groups. In addition, there are regions adjacent to the SON (e.g. the perinuclear zone and the most medial aspects of the amygdala) that have some pERK1/2 neurones and which might contribute to the elevation during lactation, although these populations were unlikely to be consistently sampled to a large degree.

Morphological plasticity is induced by central OT administration (14,25), as is the specialised bursting pattern that OT neurones exhibit during parturition and lactation (6,32). The specialised bursting observed during lactation is also suppressed by OTR antagonism (6). Although probably not exclusively, the relevant central OTRs likely include those found on OT neurones themselves in the SON and PVN (33,34) and these are likely activated by somatodendritically released OT (35–37). Further evidence of the role of OT in this regard is its ability to induce synaptic plasticity (38) and bursting *in vitro* (39). In our own laboratory, we found that plasticity in the calcium-dependent afterhyperpolarisations of OT neurones is dependent on central OTR activation during pregnancy (12) and also that OT can induce this change *in vitro* in slices from pregnant rats, specifically in OT neurones (22). Although the present study does not directly address the role of pERK1/2 in OT plasticity, its elevation specifically within OT neurones during this critical period of late pregnancy suggests that pERK1/2 induction could be important for the dynamic changes that OT neurones will soon undergo in preparation for enhanced secretion during parturition, and lactation.

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