Journal of Neuroendocrinology
From Molecular to Translational Neurobiology

Journal of Neuroendocrinology **24**, 285–291

ORIGINAL ARTICLE

Anatomically-Specific Actions of Oestrogen Receptor in the Developing Female Rat Brain: Effects of Oestradiol and Selective Oestrogen Receptor Modulators on Progestin Receptor Expression

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Journal of Neuroendocrinology

Steroid hormones largely exert their actions by activating nuclear receptors, which, as transcription factors, powerfully influence fundamental processes of neural development. Often, steroid receptor action demonstrates remarkable specificity under different developmental, anatomical or hormonal conditions. Yet, the mechanisms underlying such specificity are poorly understood. The present study examined the anatomically-specific regulation of progestin receptor (PR) expression by oestrogen receptor (ER) activation in the ventromedial nucleus (VMN) of the hypothalamus and the medial preoptic nucleus (MPN) of the neonatal female rat brain, using the selective oestrogen receptor modulators (SERMs), tamoxifen and ICI 182 780 (ICI), in the presence or absence of oestradiol benzoate (EB) treatment. The results demonstrate that PR immunoreactivity (PR-ir) in the neonatal female MPN was significantly increased by EB and this increase was abolished by either tamoxifen or ICI treatment. In contrast, within the VMN of the same animals, EB had no effect on PR-ir and the SERMs only modestly decreased PR-ir. Interestingly, ICI acted as a true antagonist regardless of EB treatment, whereas tamoxifen acted as an ER agonist in the absence of EB in the MPN, but not the VMN, representing one of the first *in vivo* demonstrations of tissue-specific and oestradiol-independent effects of tamoxifen on ER activation. The present results indicate that PR expression is highly dependent on oestradiol and its receptor in the MPN, although it is independent of both oestradiol and ER activation within the neonatal VMN. These findings demonstrate the anatomically-specific actions of oestradiol and its receptor to induce PR in two brain regions controlling different aspects of female reproductive behaviours in adulthood.

Key words: oestradiol, oestrogen receptor, progestin receptor, SERMs, tamoxifen, development, preoptic, hypothalamus, ventromedial nucleus, VMN, VMH.

doi: 10.1111/j.1365-2826.2011.02232.x

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Steroid hormones influence the developing brain through activation of their cognate nuclear receptors which, as transcription factors, regulate gene expression to alter fundamental processes of neural development. Often, steroid hormones act with anatomical specificity, exerting differential effects on gene expression in different brain regions despite similar levels of receptor expression (1, 2). Although the specificity of steroid receptor action is likely critical for normal neural development, the mechanisms by which this specificity is achieved remain poorly understood.

One of the best studied examples of steroid-induced gene transcription is the regulation of progestin receptor (PR) expression by the actions of oestradiol at oestrogen receptor (ER)α (2–5). *In vitro* work has demonstrated that oestradiol can bind directly to oestrogen response element(s) located in the promoter of the PR gene to initiate gene transcription (6–10). The idea that these same processes occur in brain is supported by evidence that the reduction of circulating oestradiol levels by ovariectomy reduced PR mRNA (11–13) and protein (1, 14, 15) in both the ventromedial nucleus of the hypothalamus (VMN) and the medial preoptic nucleus (MPN) of

adult female rats and oestradiol replacement restored PR expression to baseline levels (1).

By stark contrast to the adult female, regulation of PR by oestradiol in the neonatal female brain demonstrates both developmental and anatomical specificity. Exogenous oestradiol benzoate (EB) treatment significantly increased levels of PR immunoreactivity (PR-ir) in both the MPN and VMN of ovariectomised female rats from postnatal day 14 (P14) through P67 (1). However, at P7, although EB treatment increased PR-ir by approximately 50-fold in the MPN, PR levels were unaltered by EB in the VMN of the same animals (1, 2, 16), despite high levels of ERα expression in both regions (2). Recent work from our laboratory suggests that the inhibition of ERα activation by ERβ in the VMN, but not the MPN, may be an underlying mechanism by which this anatomical specificity is achieved (2).

These findings suggest that PR expression is 'disengaged' from oestradiol regulation in the neonatal female VMN, although it is highly dependent on oestradiol and ERα in the MPN. In further support of this idea, PR expression is virtually absent in the MPN before the onset of ovarian steroidogenesis, and circulating oestradiol levels are extremely low (17). However, in the VMN of the same animals, levels of PR expression are relatively high despite low levels of oestradiol in circulation (1, 2). Taken together, these findings suggest that PR expression is differentially regulated by oestradiol in the neonatal MPN and VMN and that ERα exerts anatomically-specific actions in the developing female brain.

There are several possible mechanisms by which PR expression may occur in the neonatal VMN in the relative absence of ovarian-derived oestradiol. One possibility is that PR expression is completely independent of ERα activation in the neonatal female VMN and is constitutively expressed in this region during early postnatal life. Alternatively, PR expression may be dependent on ERα activation but independent of serum oestradiol levels. For example, oestradiol has been shown to be synthesised within the brain from pregnenolone within the adult hippocampus (18) and within various regions of the neonatal female brain (19). This locally produced oestradiol could act to induce PR in an anatomically specific fashion within the developing brain. Furthermore, the possibility that the local conversion of testosterone to oestradiol via aromatase activity is possible as well and, indeed, aromatase mRNA is expressed within the developing VMN (20). Along the same lines, ligand-independent activation of ERs induces the expression of PR within the developing brain (21), representing another possible mechanism. Therefore, to distinguish between these possibilities, we examined the levels of PR in the MPN and VMN of neonatal females treated with the ER antagonist ICI 182 780 (ICI) or tamoxifen in the presence or absence of exogenous oestradiol. Although tamoxifen is known to have true selective oestrogen receptor modulator (SERM)-like actions and ICI 182 780 is more commonly known to be a pure antagonist, ICI 182 780 has been shown to have agonist-like effects in specific preparations (22, 23), and therefore the term SERMs will be used for both compounds. It was predicted that the inhibition of ERα activation should reduce PR levels, if indeed, PR expression in the neonatal VMN is dependent on ERα activation. Conversely, PR expression would remain unaltered after SERM treatment if PR expression in the neonatal VMN is independent of ERα activation. The present results indicate that PR expression is highly dependent on oestradiol and its receptor in the MPN, but is independent of both oestradiol and ER activation within the neonatal VMN, suggesting the existence of anatomically-specific mechanisms that regulate the expression of steroid receptors in the developing female brain.

Materials and methods

Animals

Subjects were offspring of timed pregnant female Sprague–Dawley rats (60–80 day of age; Taconic Laboratories, Germantown, NY, London). Animals were housed under a 10 : 14 h light/dark cycle at a constant temperature of 25 ± 2 °C with food and water available *ad lib.* All animal procedures used in the present study were approved by the Institutional Animal Care and Use Committee at the University at Albany. All females were permitted to deliver their pups normally. The day of birth was designated postnatal day 1 (P1).

Treatments

Female neonates received daily injections of tamoxifen (100 μg per animal; Sigma, St Louis, MO, USA), ICI (1.5 mg/kg; Tocris Bioscience, Ellisville, MO, USA), or an equal volume of the vehicle alone (0.01 ml/g body weight; 10% dimethyl sulphoxide in sesame oil, s.c. (Vehicle) on P1, P2 and P3. In addition, female neonates received a single injection of EB (20 μg/kg) or an equal volume of vehicle [sesame oil, s.c. (Oil)] on P2, 48 hr before tissue collection. The dose of tamoxifen was effective at reducing ER activation within neonatal female brain as reported previously (24). In preliminary studies, the dose of ICI was shown to be the most effective at reducing levels of PR within the MPN of males and is consistent with effective doses reported previously (25). Doses of EB consistently induce PR in the MPN of neonatal females in previous studies from our laboratory (1, 2, 26).

Tissue collection

On P4, neonates were anaesthetised by hypothermia by placement on ice and sacrificed by rapid decapitation. Brains were removed from the skull and immersion fixed in 5% acrolein in 0.1 M phosphate buffer (PB; pH 7.6) for 6 h, then cryoprotected in 30% sucrose in 0.1 M PB. Brains were sectioned at 50 μm in the coronal plane using a rotary freezing microtome. Sections were stored in cryoprotectant (30% sucrose, 0.1% polyvinyl-pyrrolidone-40 in 30% ethylene glycol and 0.1 M PB) at −20 °C until immunocytochemical processing.

Immunocytochemistry

Immunocytochemistry was performed, as described previously (2, 16, 17) on free-floating sections using a rabbit polyclonal antiserum (Dako Corp. Inc., Glostrup, Denmark) directed against the DNA binding domain of the human PR. This antisera detects both the A and B isoforms of PR and its specificity has been well-documented (27, 28). All incubations were performed at room temperature unless otherwise stated. Sections were rinsed in tris-buffered saline (TBS; pH 7.6; 3 × 5 min each), incubated in 1% sodium borohydride in TBS (10 min), rinsed in TBS (4 × 5 min), incubated in TBS containing 20% normal goat serum (NGS), 1% H_2O_2 and 1% bovine serum albumin for 30 min. PR antiserum was diluted 1 : 1000 in TBS containing 0.3% Triton X-

100, 2% NGS (TTG) for 72 h at 4 °C. Sections were rinsed in TTG (3 × 5 min), incubated in biotinylated goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, CA, USA) at a concentration of 5 μg/ml in TTG for 90 min. Sections were rinsed in TTG (2 × 5 min), in TBS (2 × 5 min) then incubated in avidin-biotin complex reagent (Vectastain Elite Kit; Vector Laboratories) for 60 min. Sections were rinsed in TBS (3 × 5 min), then incubated in TBS containing 0.05% diaminobenzidine, 0.75 mM nickel ammonium sulphate, 0.15% β-D-glucose, 0.04% ammonium chloride, and 0.2% glucose oxidase for approximately 10 min. Sections were rinsed in TBS (3 × 5 min), mounted on gelatin-coated slides and coverslipped with Permount (Fisher Scientific, Pittsburgh, PA, USA).

Image and statistical analysis

A representative, anatomically-matched section through the MPN and ventrolateral VMN of each animal was selected for image analysis by an experimenter who was blind to treatment group. Animals for which an anatomical match could not be found were excluded from the analysis. Microscopic images of the MPN and VMN were captured with a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan) fitted with a SPOT Insight camera (Diagnostic Instruments, Sterline Heights, MI, USA) connected to a Dell Inspiron 8600 laptop. NIH IMAGE software (NIH, Bethesda, MD, USA) was used to analyse captured images. The relative amount of PR-ir in the MPN and VMN was determined as described previously (1, 2, 16, 29) by measuring the area (μm^2) covered by 'thresholded' pixels [i.e. those pixels with a grey level higher than a defined threshold density (specific immunoreactive staining)]. 'Threshold' was determined as a constant function of the background optical density defined as the mean optical density three to five SDs higher than the mean background density (1, 2, 16). The mean background density was measured in a region devoid of PR-ir, immediately lateral to the analysed region containing PR-ir. Statistical analyses were performed using a two-way ANOVA (SERM × EB; $P < 0.05$), followed by pre-planned, pairwise comparisons using Student–Newman–Keuls post-hoc analysis ($P < 0.05$).

Results

MPN

In the MPN, levels of PR-ir were highly dependent on exogenous oestradiol and ER activation (Figs 1 and 2). Two-way ANOVA revealed a significant main effect of EB treatment ($F_{1,62} = 12.888$; $P < 0.001$); a significant main effect of SERM treatment ($F_{2,62} = 23.837$; $P < 0.001$) and a significant interaction between EB and SERM treatment ($F_{2,62} = 31.091$; $P < 0.001$).

In the absence of SERMs, EB treatment increased levels of PR-ir within the MPN compared to oil-treated controls ($P < 0.001$). This EB-induced increase of PR-ir in the MPN was attenuated by both SERMs. Levels of PR-ir were lower in EB-treated females receiving either tamoxifen ($P < 0.001$) or ICI ($P < 0.001$) compared to females receiving EB alone. In the absence of EB treatment, ICI had no effect on levels of PR-ir ($P = 0.583$). By contrast, tamoxifen increased levels of PR-ir in the MPN in the absence of EB compared to oil-treated controls ($P < 0.001$), suggesting that tamoxifen had agonistic properties in the absence of oestradiol, consistent with its classification as a SERM.

In summary, EB treatment significantly increased levels of PR-ir compared to oil-treated animals and both tamoxifen and ICI acted as potent ER antagonists, blocking the induction of PR by EB. These findings served as proof of principle that these agents were readily accessing the brain and, in the presence of EB, were acting as potent ER antagonists in our model. These controls are specifically important for ICI 182 780, a compound that has only recently been shown to pass the blood–brain barrier (BBB) (25). Our results support the findings of Alfinito *et al.* (25) in that the systemic administration of ICI blocked the actions of oestradiol within the brain. These results demonstrate that ICI was capable of passing the immature BBB and acting as a potent antagonist to ERα function.

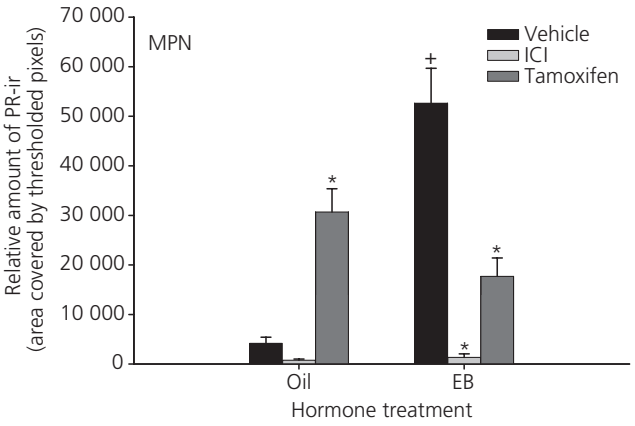

Fig. 1. The relative amount of progestin receptor immunoreactivity (PR-ir) in the medial preoptic nucleus (MPN) of postnatal day 4 (P4) female rats that were treated with either oestradiol benzoate (EB) or the oil vehicle (Oil) and either tamoxifen, ICI 182 780 (ICI) or vehicle alone from the day of birth (P1) to P4. *Significantly different from the vehicle group within the same hormone treatment ($P < 0.05$); $^{+}$significantly different from the Oil/Vehicle group ($P < 0.05$).

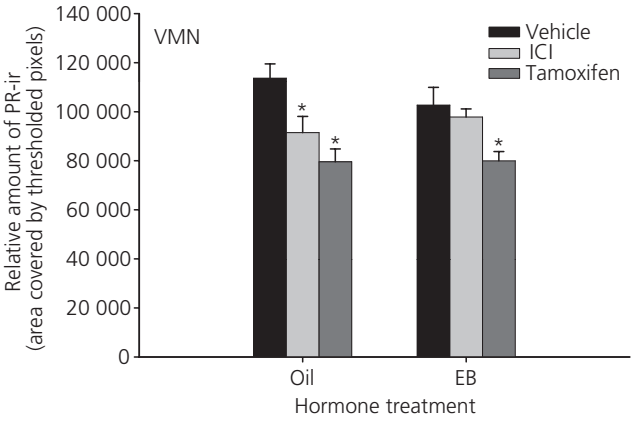

Fig. 2. The relative amount of progestin receptor immunoreactivity (PR-ir) in the ventromedial nucleus (VMN) of postnatal day 4 (P4) female rats that were treated with either oestradiol benzoate (EB) or the oil vehicle (Oil) and either tamoxifen, ICI 182 780 (ICI) or vehicle alone from the day of birth (P1) to P4. *Significantly different from the vehicle group within the same hormone treatment ($P < 0.05$).

VMN

In the VMN, levels of PR-ir were independent of oestradiol and largely independent of ER activation (Fig. 2). Two-way ANOVA revealed a main effect of SERM treatment ($F_{2,57} = 13.554$;

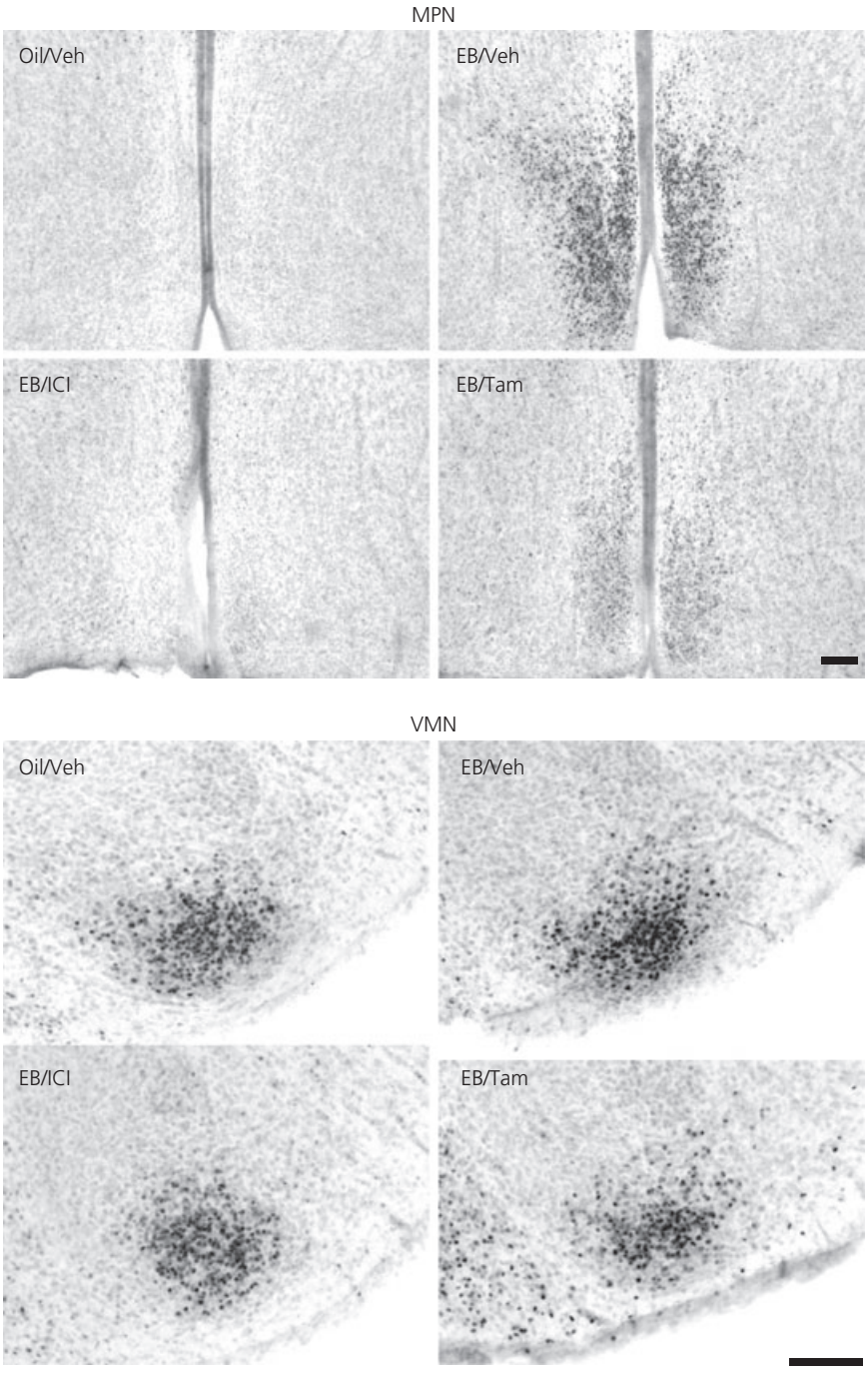

Fig. 3. Progestin receptor immunoreactivity (PR-ir) within the medial preoptic nucleus (MPN) and ventromedial nucleus (VMN) of postnatal day 4 (P4) females treated with oil and vehicle (Oil/Veh), oestradiol benzoate (EB) and vehicle (EB/Veh), EB and ICI 182 780 (EB/ICI) or EB and tamoxifen (EB/Tam). Scale bar = 100 μm.

$P < 0.001$), although no main effect of EB treatment ($F_{1,57} = 0.0952$), and no significant interaction between EB treatment and SERM treatment ($F_{2,52} = 1.127$). EB treatment had no effect on levels of PR-ir compared to oil treatment in animals that did not receive SERM treatment. In the absence of EB treatment, both tamoxifen and ICI modestly reduced levels of PR-ir ($P < 0.05$). However, in EB-treated animals, there was no effect of ICI treatment compared to vehicle controls, although there was a small but significant effect of tamoxifen treatment decreasing PR-ir levels ($P < 0.05$).

Discussion

The present findings suggest that the expression of a transcription factor, the steroid receptor PR, is regulated in an anatomically-specific manner by oestradiol and ER activation within the developing

brain. More specifically, PR expression is highly dependent upon oestradiol within the MPN, but not within the VMN of the developing female rat. This is in stark contrast to the adult female rat in which PR is highly regulated by oestradiol within both brain areas (1), suggesting a developmentally-specific regulation of PR expression as well. The present results are consistent with previous work from our laboratory (1), demonstrating that: (i) in the absence of circulating oestradiol, PR-ir is at relatively high levels in the neonatal VMN, and (ii) exogenous oestradiol administration dramatically increased PR in the MPN but had no effect on PR in the VMN. Interestingly, the results from the present study reveal that SERMs (i.e. tamoxifen and ICI) attenuated the ability of oestradiol to increase levels of PR-ir in the MPN but had little effect on the actions of oestradiol in the VMN. Taken together, these results demonstrate that PR expression in the MPN is highly dependent on oestradiol and ER activation but that, in the VMN of the same animals, PR expression is 'disengaged' from oestradiol and ER, thereby elucidating a striking anatomical specificity in steroid receptor action in the developing brain.

Dependence of PR-ir on oestradiol is anatomically-specific: actions of the SERM, ICI 182 780

ICI is widely accepted as a potent ER antagonist with a similar binding affinity for both ERα and ERβ (30) and has recently been shown to cross the BBB (25). To increase the ability of ICI to enter the brain, animals were treated with ICI on P1–P4, before the full maturation of the BBB (31). Oestradiol induces PR in the MPN (Fig. 3) and (1, 2), whereas systemic administration of ICI completely blocked this ability of EB to induce PR-ir in the MPN, demonstrating that ICI readily and effectively entered the brain. Despite the ability of ICI to act as a potent ER antagonist in the MPN, ICI did not attenuate levels of PR-ir in the VMN in the presence of oestradiol. Interestingly, ICI elicited a small decrease in PR-ir in control animals (Fig. 1), indicating that ICI decreases PR expression in the absence of ER activation. These findings suggest that, in stark contrast to the adult female VMN, PR expression in the neonatal VMN may be independent of oestradiol and ER activation.

Agonist/antagonist properties of tamoxifen are anatomically-specific and hormonally-dependent

Tamoxifen is a true SERM in that it can exert both agonist and antagonist effects on ER activation depending on tissue type, the presence or absence of oestradiol (32) or the availability of coactivators (33, 34). One possible mechanism by which this happens is the differential expression of specific coregulatory proteins such as SRC-1 and SMRT. For example, Klinge *et al.* (33) demonstrated that the expression of SRC-1 and SMRT, *in vitro*, mediated the agonist-like effects of tamoxifen in CHO-k1 cell lines. In further support, tamoxifen-bound ERα recruited SMRT at the PR promoter in chromatin immnoprecipitation assays using MCF-7 cells (35). Because SRC-1 and SMRT are expressed in brain (36), and SRC-1 has been shown to mediate levels of PR-ir within the adult hypothalamus (37), these mechanisms may mediate the agonist-like behaviour of tamoxifen in the brain. In the present study, tamoxifen acted as an ER antagonist in the MPN in the presence of oestradiol treatment but acted as a relatively robust ER agonist in the MPN in the absence of exogenous oestradiol. In comparison, tamoxifen treatment decreased levels of PR-ir in the VMN suggesting that this drug acted as a weak antagonist both in the presence and absence of oestradiol

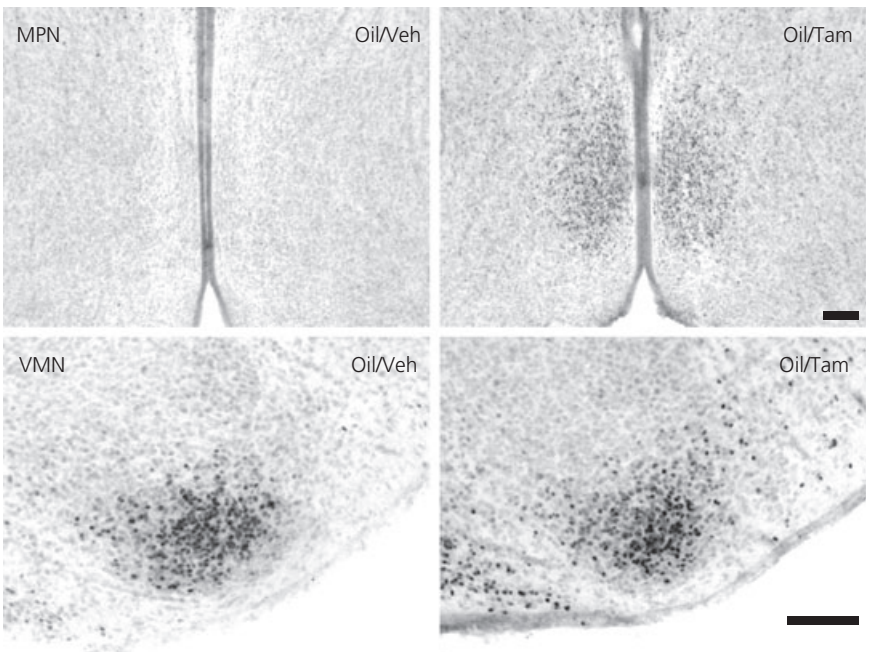

Fig. 4. Progestin receptor immunoreactivity (PR-ir) within the medial preoptic nucleus (MPN) and ventromedial nucleus (VMN) of postnatal day 4 (P4) females treated with oil and vehicle (Oil/Veh) or oil and tamoxifen (Oil/Tam). Scale bar = 100 μm.

(Fig. 4). To our knowledge, these findings represent one of the first *in vivo* studies to demonstrate tissue (i.e. brain region) specific effects of tamoxifen independent of oestradiol levels, which has considerable implications for the use of this drug in the clinical setting.

Conclusions

The actions of oestradiol and ER on the developing brain have been well studied, particularly with regard to the masculinisation of the male rodent brain (38–41). In the developing male MPN, relatively high levels of oestradiol aromatized from testosterone of testicular origin, result in high levels of PR in MPN, whereas PR is virtually absent in the female MPN prior to ovarian oestradiol synthesis (17). The present findings suggest that, unlike the neonatal female MPN, PR expression is high in the VMN and this expression is independent of circulating oestradiol and ER activation. An anatomically-specific regulation of steroid receptor-induced gene expression suggests a mechanism for the differential development of two brain regions that control different reproductive behaviours in adulthood; the VMN is important for female sexual behaviour (42–46) and the MPN for maternal behaviour (16, 47, 48).

Although the expression of PR protein does not imply that PR is active within the developing VMN, the present findings implicate an important role for PR in the development of the female VMN. Consistent with this idea, PR expression is not increased by oestradiol in the VMN, perhaps as a result of the potential for ERβ to inhibit ERα activation (2) and PR expression in the VMN occurs at high levels independent of oestradiol and ER activation, suggesting that PR expression is constitutively expressed or regulated by an unknown factor within the neonatal female VMN. The disengagement of PR expression from oestradiol and ER is transient during early perinatal life because PR expression becomes increasingly dependent on oestradiol after P14 (1). This generates the intriguing idea that progesterone and PR may play an active and important role in the feminisation of the VMN, a process that has typically been viewed as being independent of steroid hormone action.

Acknowledgements

The authors wish to thank Kate Shea and Jessica Wess for their assistance on this project. This study was supported by NSF IOB0447492 and a March of Dimes National Research Grant to C.K.W., as well as NIH NRSA MH08344601A2 to K.L.G. and NIH DK61935 to M.J.T.

Received 5 April 2011,
revised 9 September 2011,
accepted 1 October 2011

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