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Oxytocin treatment at birth accelerates an epigenetic shift in the oxytocin receptor gene in the maternal brain

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Abstract

Background To date, nearly one in three births in the United States occurs after a labor induced with synthetic oxytocin. Despite how common this intervention is, little is known about its long-term consequences for maternal health. Existing work has identified a link between labor induction with synthetic oxytocin and increased risk for postpartum depression. For some women, the link between labor induction and postpartum depression risk may be altered functioning of the oxytocin system, including epigenetic modification of the oxytocin receptor gene, *OXTR*. Here we use the prairie vole to understand how pregnancy and birth impact epigenetic control of *Oxtr*, and how a labor induced with synthetic oxytocin may alter this control.

Methods In an unmanipulated birth model, we measured *Oxtr* DNA methylation levels in the brain of virgin females, at term pregnancy, and 90 min postpartum using targeted pyrosequencing at four CpG sites in the *Oxtr* promotor region that are conserved from the human *OXTR*. We used RT-PCR to assess *Oxtr* gene expression levels in the brains of these same subjects. These same methods were next used in a model of labor induction with exogenous oxytocin. In both models, brain regions targeted for analysis included the nucleus accumbens, amygdala, and medial preoptic area. These regions all use oxytocin system activity in regulating aspects of maternal behaviors. 2-way ANOVA, unpaired two-tailed t-tests, and Pearson's and Spearman's correlations were used for analyses.

Results Results identify a regulatory switch in *Oxtr* from term pregnancy to early postpartum that is facilitated in part by oxytocin. *Oxtr* DNA methylation in virgins is negatively associated with *Oxtr* gene expression at all four conserved CpG sites in the nucleus accumbens. At term pregnancy, there is no relationship between these markers. Immediately postpartum, this relationship shifts back to a pre-pregnancy state. Administration of increasing doses of exogenous oxytocin, modeling labor induction, shifts the methylation-expression relationship toward a negative state in the nucleus accumbens, mimicking a postnatal brain but at a prenatal time point.

Conclusions Findings show epigenetic control of *Oxtr* is dynamic across pregnancy and birth and is sensitive to exogenous oxytocin. Results indicate a need to better understand how common birth interventions impact *Oxtr* regulation in the maternal brain.

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Introduction

Exogenous oxytocin (Pitocin) is routinely used to induce or augment labor and/or to combat postpartum hemorrhage in hospitals in the US. In the most recent National Vital Statistics Report [1] 32.1% of hospital births occurred following labor induction. At the same time, 32.2% of births occurred via a cesarean section [2]. One common component of these two birth interventions is an artificial manipulation of the oxytocin system prior to birth, whether through exogenous administration (induction) or eliminating or reducing its functioning, as happens in an elective cesarean. While these interventions are common in obstetric practice and are assumed to reduce morbidity and mortality in both mothers and babies, an understanding of the consequences of these practices on the maternal brain is surprisingly lacking.

In an unmanipulated vaginal birth, oxytocin is released in a pulsatile manner into the blood supply from the posterior pituitary and is also synthesized and released from intrauterine tissues. It acts on oxytocin receptors in the uterine myometrium to stimulate uterine contractions [3–6]. While it is thought that circulating oxytocin levels spike during labor, especially during the second and third stages, measurements have led to inconsistent results [5]. What may be of particular importance for labor onset and delivery is availability of the oxytocin receptor (OXTR). The density of OXTR as well as levels of oxytocin receptor gene (*OXTR*) mRNA increases dramatically in the myometrium across pregnancy and spikes at the onset of labor [7–11]. Little is known regarding regulation of *OXTR* in the brain around the transition to motherhood. The dynamic nature of OXTR in the context of birth suggests its regulation plays a critical role in the birth process and makes it an interesting and useful target to explore the consequences of oxytocin system manipulation in the newly maternal brain as happens with birth interventions.

Our group previously identified a conserved regulatory region in the prairie vole oxytocin receptor gene (*Oxtr*) promoter containing a cluster of CpG sites homologous in humans and voles (CpG sites −934, −924, and −901) [12]. This region, known as the MT2 region, is important for DNA methylation-dependent *OXTR* transcription in humans [13]. Methylation at each of these sites in human populations has been linked to several psychiatric disorders [14–17]. Notably with regard to birth and the maternal brain, this includes a link to risk for developing postpartum depression [18]. Our work in the vole examining these conserved CpG sites has demonstrated a role for DNA methylation in the control of *Oxtr* gene expression in the nucleus accumbens. Methylation at each of these sites is sensitive to varying early life experience and is also correlated between brain and blood tissues, providing a potential translational model for understanding *OXTR* epigenetic regulation in humans [12, 19]. In a series of collaborative studies in our vole model we have also demonstrated that maternally administered oxytocin just prior to giving birth increases *Oxtr* methylation in the fetal brain and leads to adult offspring with a more prosocial behavioral phenotype [20].

To explore the link between birth, birth interventions, and functioning of the oxytocin receptor gene, we used the prairie vole to assess *Oxtr* DNA methylation and gene expression at these conserved CpG sites in the nucleus accumbens, amygdala, and medial preoptic area (MPOA), neural regions involved in maternal and social behavior [21–23]. Data were collected at term pregnancy without laboring (elective cesarean model) and immediately following natural birth and also following exogenous oxytocin administration (labor induction model) in term-pregnant mothers. We also examined these markers of *Oxtr* functioning in whole blood in unmanipulated pregnancy and birth to assess the relationship between central and peripheral markers of *Oxtr* DNA methylation and gene expression. Prairie voles are a unique and useful model for understanding the role of *Oxtr* epigenetic regulation as it relates to birth and the parental brain. In addition to the CpG site conservation with humans that is not present in rat or mouse models, prairie voles also engage in high levels of biparental care of offspring [24–27], more closely modeling human parental care than traditional lab species.

Methods

Subjects

All subjects were laboratory-bred prairie voles (*Microtus ochrogaster*) descended from a wild caught stock originally captured near Champaign, Illinois. Animals were weaned on postnatal day (PND) 20 (birth is PND 0) and housed in same-sex sibling pairs in small polycarbonate cages (27 cm x 16 cm x 16 cm) until paired for breeding. Breeding pairs were housed in large polycarbonate cages (44 cm x 22 cm x 16 cm). Animals were provided food (high-fiber rabbit chow) and water *ab libitum* and Envirodri nesting material. Lights were maintained on a 14:10 light: dark cycle. All procedures in the term pregnant and unmanipulated vaginal birth model were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Indiana University, Bloomington. All procedures in the labor induction model were reviewed and approved by the IACUC at Northeastern University.

Breeding paradigm

To accurately predict birth timing, a timed mating paradigm was used as described in Kenkel et al. [20]. Virgin females (PND 60–90) were weighed and then paired with an experienced breeding male and allowed to cohabitate for 24 h to begin estrus induction. A perforated cage divider was then put in place that allowed for auditory and olfactory communication but prevented direct physical contact. Estrus induction in female prairie voles typically begins after 24–36 h of cohabitation with the male partner. To ensure an estrus state upon reunion, dividers were removed after approximately 72 h and mating was confirmed by an observer. Males remained cohoused with the female throughout gestation. Prairie vole gestational length is approximately 21.5 days, so females were weighed 20.5 days after observed mating (term pregnant and unmanipulated birth model) or 21.5 days after observed mating (labor induction model). Females in the term pregnant and vaginal birth groups were weighed a day before expected birth so as not to unintentionally cause a delay in birth due to any handling stress. At this point, a decision to move forward with birth intervention was made based on characteristics of estimated pregnancy state. This included weight gain since initial pairing, whether mating was observed, and physical conditions such as a rounded abdominal shape and prominence of nipples. Litters were considered term and were included for study if fetal pup weight was greater than 2.0 g/pup (term pregnancy condition) or if the litter was delivered within 24 h of injection (labor induction). Litters in both experiments averaged 4 pups and had an average sex ratio of 2 males:2 females.

Experiment 1. Term pregnancy and vaginal birth model tissue collection

Upon determination of being term pregnant, females were randomly assigned to either the unmanipulated vaginal birth condition ($n = 17$) or the term pregnancy condition ($n = 15$). Females in the unmanipulated vaginal birth condition were checked every 60 min for the presence of pups, beginning when room lights came on 21.5 days after observed mating. This timing ensured litters were found and maternal tissue samples were collected within 90 min of parturition. When a new litter was found, females were promptly euthanized via cervical dislocation and rapid decapitation under deep isoflurane anesthesia. Whole blood was collected in DNase/RNase-free microcentrifuge tubes and brains were extracted and frozen on dry ice. Tissues were then stored at −80°C until assayed. Females in the term pregnancy condition were euthanized in the same manner prior to the birth of any pups. Immediately following collection of whole blood and brain, a laparotomy was done, the uterine horns were exposed, and fetuses were removed and euthanized. Pup characteristics used as inclusion criteria for determining term pregnancy include pup weight (average pup weight must be >2.0 g), pup appearance (gray body color), and the ability of pups to take breaths with minimal stimulation. Average pup weight across the group was 2.58 g (SD = 0.29 g). Tissue from virgin females ($n = 13$) was collected in the same manner. Average female weights at initial pairing for breeding were: term pregnant: 29.3 g (SD = 3.01 g); vaginal birth: 30.1 g (SD = 3.53 g). Average female weights at tissue collection were: virgin: 28.9 g (SD = 2.51 g); term pregnant: 65.1 g (SD = 5.62 g); vaginal birth: 54.6 (SD = 6.34). A timeline of Experiment 1 tissue collection points and tissues and brain regions used for measures of *Oxtr* DNA methylation and gene expression is presented in Fig. 1.

Experiment 2. Labor induction model, prenatal treatment and tissue collection

Breeding pairs underwent a timed mating procedure as described above. Upon determination of being term pregnant, females ($n = 14$/group) were randomly assigned to one of four treatments: low-dose oxytocin (0.125 mg/kg,); medium-dose oxytocin (0.25 mg/kg); high-dose oxytocin (0.5 mg/kg); or saline. Oxytocin doses were chosen based on previous work in our lab [20, 28]. The medium dose used is equivalent to an approximately 5 IU dose of oxytocin, within the dosing range used for labor induction in obstetric practice [29–31]. On the expected day of birth (21.5 days post-mating) females received a single intraperitoneal injection of drug and were returned to the home cage. Ninety minutes after treatment, females were euthanized via cervical dislocation and rapid decapitation under deep isoflurane anesthesia. Brains were extracted, immediately frozen on dry ice, and stored at −80°C until assayed. Immediately following collection of tissue, a laparotomy was done, the uterine horns were exposed, and fetuses were removed and euthanized. Pup characteristics used as inclusion criteria for determining term pregnancy include pup weight (average pup weight must be >2.0 g), pup appearance (gray body color), and the ability of pups to take breaths with minimal stimulation. Only females with litters meeting criteria for term pregnancy were included for analysis (low-dose oxytocin, $n = 9$; medium-dose oxytocin, $n = 13$; high-dose oxytocin, $n = 9$; saline, $n = 13$). Average pup weights were: saline: 2.35 g (SD = 0.24 g), low dose oxytocin: 2.36 g (SD = 0.32 g), medium dose oxytocin: 2.41 g (SD = 0.34 g), high dose oxytocin: 2.56 g (SD = 0.43 g). Average female weights at initial pairing for breeding were: saline: 34.7 g (SD = 4.02 g); low dose oxytocin: 32.2 g (SD = 3.75 g); medium dose oxytocin: 35.3 g (SD = 4.31 g); high dose oxytocin: 32.6 g (SD = 3.73 g). Average female weights at tissue collection were: saline: 53.3 g (SD = 5.12 g); low dose oxytocin: 53.4 g (SD = 3.72 g); medium dose

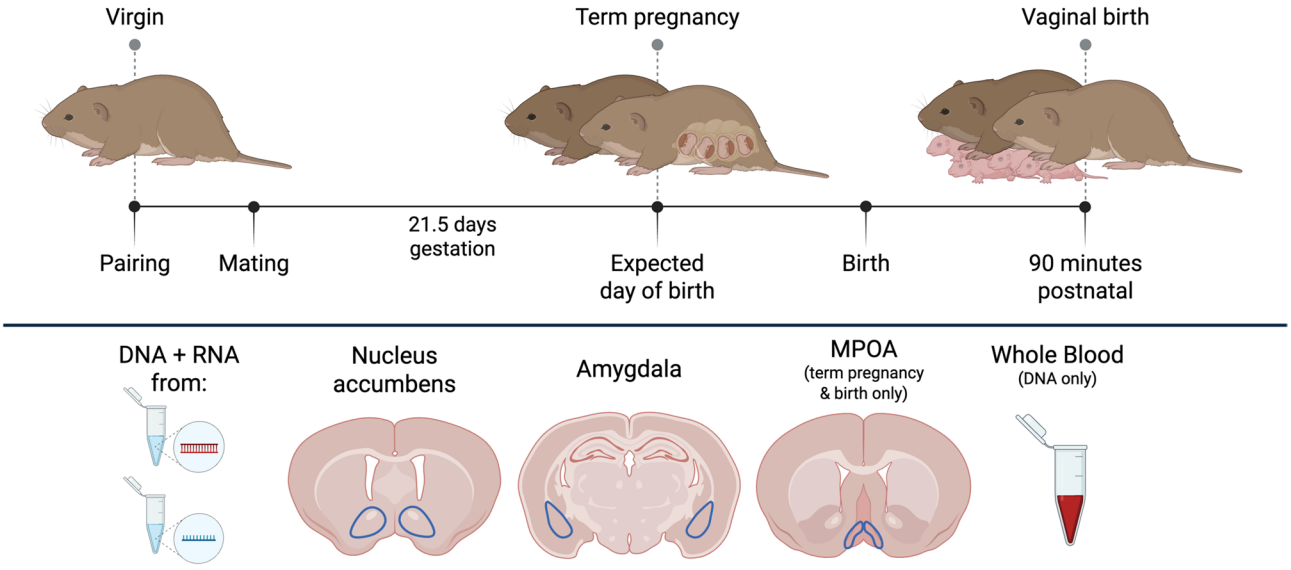

Fig. 1 Experiment 1 timeline and tissue collection. Brain and whole blood tissues were collected from virgin females, females at term pregnancy, or females within 90 min of an unmanipulated vaginal birth. DNA and RNA were extracted for methylation and gene expression analyses, respectively, from the nucleus accumbens, amygdala, and medial preoptic area (MPOA) from the brain. DNA was extracted for methylation analysis from whole blood. Figure created using BioRender

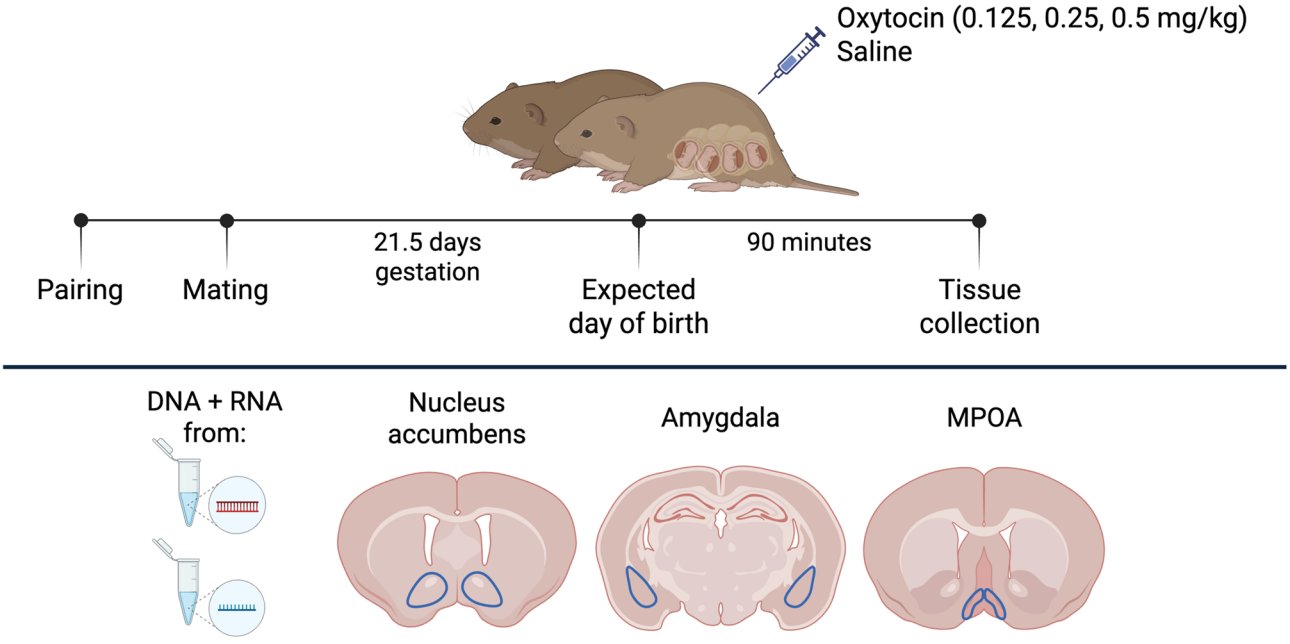

Fig. 2 Experiment 2 timeline and tissue collection. Brain tissue was collected from term pregnant females 90 min after receiving an injection of one of three doses of synthetic oxytocin (0.125 mg/kg, 0.25 mg/kg, or 0.5 mg/kg) or a saline vehicle control. DNA and RNA were extracted for methylation and gene expression analyses, respectively, from the nucleus accumbens, amygdala, and medial preoptic area (MPOA) from the brain. Figure created using BioRender

oxytocin: 54.5 g (SD = 6.06 g); high dose oxytocin: 55.4 g (SD = 4.09 g). A timeline of Experiment 2 tissue collection points and tissues and brain regions used for measures of *Oxtr* DNA methylation and gene expression is presented in Fig. 2.

Brain dissections

Brains were dissected in a −20°C chamber. Initially, a coronal cut was made to remove the olfactory bulbs. A second coronal cut was made 2 mm caudal to the frontal pole to collect punches (1 mm in diameter, 2 mm in depth) of the nucleus accumbens, including both shell and core regions (bilateral) and MPOA regions. An

additional coronal cut was made 5 mm caudal to the frontal pole to collect bilateral punches (1.5 mm in diameter, 2 mm in depth) of the amygdala (punches encompassed central, medial, and basolateral nuclei).

Oxtr DNA methylation analysis

Extraction of DNA was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. DNA was isolated from nucleus accumbens, amygdala, and MPOA regions in all animals and from whole blood in the term pregnant and unmanipulated vaginal birth model. Two hundred nanograms of DNA was subject to bisulfite conversion (Kit MECOV-50, Invitrogen, Carlsbad, CA) following manufacturer instructions. This process allows for the detection of methylated cytosines by sequencing. Twelve nanograms of bisulfite converted DNA was used as a template for PCR using a Pyromark PCR kit (Qiagen, Valencia, CA) and 0.2 μM of primers TSL201F 5'-GGG GATAGGATGGTTAGTTAGTATT-3' and TSL201R 5'-CCAACAACCTCAAAACTCTACT-3'. Each PCR reaction was amplified in triplicate on three identical PCR machines (S1000 Thermal Cycler, Bio-Rad, Hercules, CA). Cycling conditions used to amplify the target *Oxtr* fragment, which included CpG sites −934_1, −934_2, −924, and −901, were: [Step 1: (95ºC/15 min)/1 cycle, Step 2: (94ºC/30s, 58ºC/30s, 72ºC/30 s)/50 cycles, Step 3: (72ºC/10 min)/1 cycle, Step 4: 4ºC hold. Standard controls of 0% and 100% methylated DNA as well as a no DNA control were included for each PCR plate. Pyrosequencing was performed on a Pyromark Q24 using Pyromark Gold Q24 Reagents (Qiagen, Valencia, CA) following manufacturer instructions using two primers: TSL201S 5'-GAGGGAAGGTTTTGGAGTTTTTTAT AT-3' and TSL201S2 5'-AGGGATTGAAAAGTGA-3'. Epigenotypes reported are an average of three replicates for the term pregnant and unmanipulated vaginal birth model and as a single replicate for the labor induction model. For term pregnant and unmanipulated vaginal birth groups, on average, nucleus accumbens samples deviated from the mean by, −934_1: 0.46%; −934_2: 0.42%; −924: 0.56%; −901: 0.52%; amygdala samples deviated from the mean by, −934_1: 0.43%; −934_2: 0.42%; −924: 0.56%; −901: 0.64%; MPOA samples deviated from the mean by, −934_1: 0.58%; −934_2: 0.56%; −924: 0.66%; −901: 0.66%; whole blood deviated from the mean by, −934_1: 0.46%; −934_2: 0.37%; −924: 0.35%; −901: 0.46%.

Oxtr gene expression analysis

Extraction of RNA was done using the Qiagen AllPrep DNA/RNA Mini Kit (Qiagen, Valencia, CA) following manufacturer instructions. RNA was isolated from nucleus accumbens, amygdala, and MPOA. RNA was processed for cDNA synthesis following the protocol provided in the iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real time qPCR was conducted using a 7500 Fast Real-Time PCR System (Applied Biosystems) using *Power* SYBR Green (Applied Biosystems No. 4367659). Cycling conditions used were: for *Oxtr* and *Gapdh* [Step 1: (95ºC/10 min)/1 cycle, Step 2: (95ºC/15s, 63.4ºC/60s)/35 cycles]. All reactions were run in triplicate (replicate standard deviation was < 0.05) and their specificity verified by melting curve analysis and separation on a 2% agarose gel. Primer performance was evaluated using standard serial dilution with 6 points and all primer sets performed within acceptable range for efficiency (*Oxtr* = 99.7%, $R^2 = 0.993$; *Gapdh* = 93.5%, $R^2 = 0.998$). The primer sequences used for *Oxtr* are TSL401_F 5'-GCCTTTCTTCTTCGTGCAGATG-3' and TSL401_R 5'-ATGTAGATCCAGGGGTTGCAG-3', for *Gapdh* TSL403_F 5'-ACTCTTCCACCTTCGATGC TG-3' and TSL403_R 5'-TTCTTACTCCTTGGAGGCC ATG-3'. Relative gene expression is presented using the comparative Ct method, $2^{-\Delta\Delta Ct}$. *Gapdh* was chosen as a reference based on its reliability across brain regions and across developmental time points in the mouse [32] and the prairie vole [20, 33].

Data analysis

Normality of data was assessed by the D'Agostino-Pearson test. *Oxtr* methylation in Experiment 1 was analyzed using a 2-way Group (virgin, term pregnant, vaginal birth) x CpG site (−934_1, −934_2, −924, −901) analysis of variance (ANOVA) with post-hoc testing done using Tukey's Least Significant Difference multiple comparisons tests. A 2-way treatment (saline, low-, medium-, high-dose oxytocin) by CpG site (−934_1, −934_2, −924, −901) ANOVA with Tukey's multiple comparisons post hoc testing was also used in Experiment 2. *Oxtr* gene expression in the MPOA in Experiment 1was assessed using an unpaired two-tailed t test, comparing term pregnancy versus vaginal birth. *Oxtr* expression levels in the nucleus accumbens and amygdala in Experiment 1 comparing virgin, term pregnant, and vaginal birth and in all regions examined (nucleus accumbens, amygdala, MPOA) in Experiment 2 (comparing saline, low-, medium-, and high-dose oxytocin) were assessed using a 1-way ANOVA by Group. Correlation analyses were used to examine the relationships between *Oxtr* DNA methylation and gene expression in both birth models. Pearson's correlation was used when data were normally distributed, while Spearman's rank correlation was used when data were not normally distributed. These same correlation analysis parameters were used to examine the relationship between *Oxtr* DNA methylation in the brain and whole blood in Experiment 1. Statistical analyses were done using GraphPad Prism 9.0. Alpha was set to $p < 0.05$

for all analyses. An observed power analysis is presented in Supplementary Table 4.

In Experiment 1, one nucleus accumbens sample from the virgin female group was excluded from analyses involving DNA methylation at CpG site −934_2 due to failed pyrosequencing. One additional sample from the MPOA in the term pregnancy group was excluded from analyses involving DNA methylation at CpG site −934_2 due to failed pyrosequencing. In Experiment 2, two saline-treated nucleus accumbens samples were excluded for high replicate variability for gene expression, one sample was excluded because of failed pyrosequencing at −934_2, and three samples were excluded because of failed pyrosequencing at −901. In the low-dose oxytocin group, one nucleus accumbens sample was excluded for failed phyrosequencing at all CpG sites while another was excluded for high replicate variability at −901. In the medium-dose oxytocin group three samples were excluded at −934_2 for high replicate variability and one was excluded at −901 because of failed pyrosequencing.

Results

Experiment 1. Regulation of *Oxtr* expression by DNA methylation varies by birth mode

To understand the role *Oxtr* epigenetic regulation has on the transition to motherhood, we examined *Oxtr* DNA methylation and gene expression immediately prior to and immediately following vaginal birth in the nucleus accumbens and amygdala. DNA methylation is typically inversely related to levels of expression of the associated gene, where increased levels of methylation act to decrease gene expression. No differences were observed in the absolute levels of DNA methylation just before compared to just after birth in either brain region (Supplementary Fig. 1A, B). In the nucleus accumbens, there was a significant effect of group on *Oxtr* gene expression ($F_{(2,\ 33)} = 4.336$, $p = 0.0213$; Fig. 3A). Expression levels in this region were elevated 90-minutes after vaginal birth compared to virgin females ($p = 0.0231$) and compared to term pregnant females ($p = 0.0461$). This effect was not seen in the amygdala (Fig. 3B).

The MPOA is critical in coordinating displays of maternal behavior [34–37] and therefore we included this region in analyses of *Oxtr* methylation and gene expression at term pregnancy and immediately postpartum. As was seen in the nucleus accumbens and amygdala, there was no difference in levels of methylation just prior to compared to just after giving birth (Supplementary Fig. 1C). Like the amygdala, there were also no differences in *Oxtr* gene expression at these two time points (Fig. 3C). Expression of *Oxtr*, then, is dynamic around unmanipulated birth in a region-specific way. When collapsed across groups, expression levels are significantly different between all three brain regions examined ($F_{(2,\ 102)} = 120.2$,

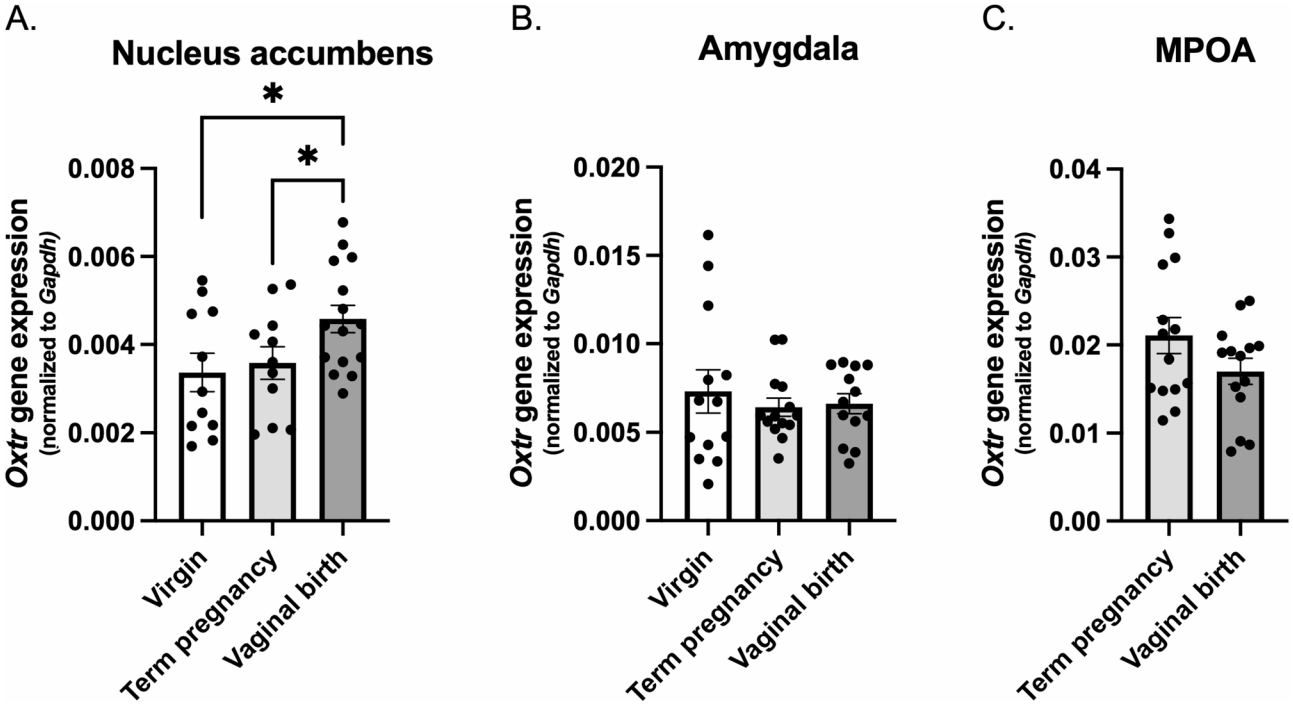

Fig. 3 Expression of *Oxtr* increases following birth in the nucleus accumbens. There is an increase in *Oxtr* expression in the nucleus accumbens within 90 min of giving birth compared to expression levels in virgin females and levels at term pregnancy **A**. This increase after birth is not seen in the amygdala **B** or the MPOA **C**. Note the differing scales for gene expression between brain regions due to differences in expression between regions (see Supplementary Fig. 2). Data presented are mean ± SE with individual data points plotted. * $p < 0.05$

$p < 0.0001$, Supplementary Fig. 2), with the lowest expression seen in the nucleus accumbens and the highest expression in the MPOA. These differing levels may account for differences in these dynamic responses to birth experience.

To examine how the methylation and expression states of each brain region included related to one another at different points in time, the relationship between levels of *Oxtr* DNA methylation between regions were analyzed, as were the relationships between levels of *Oxtr* gene expression between regions. There was a positive correlation between levels of *Oxtr* DNA methylation in all brain regions at all three time points (Supplementary Fig. 6, Supplementary Table 1). No relationship was seen in *Oxtr* expression between different brain regions in virgin females or at term pregnancy (Supplementary Fig. 7A-D, Supplementary Table 2). Within 90 min of giving birth *Oxtr* expression in the MPOA was positively correlated with *Oxtr* expression in the nucleus accumbens ($r(12) = 0.7315$, $p = 0.0045$, Supplementary Fig. 7 F) and in the amygdala ($r(12) = 0.5637$, $p = 0.0448$, Supplementary Fig. 7G). No relationship between *Oxtr* expression in the nucleus accumbens and amygdala was seen at this time point (Supplementary Fig. 7E).

Notably, there was a shift in the relationship between methylation and expression that differed by time point. Adult virgin females displayed the expected negative correlation between *Oxtr* DNA methylation and gene expression in the nucleus accumbens (−934_1: $r(11) = -0.6868$, $p = 0.0118$; −934_2: $r(10) = -0.7343$, $p = 0.0087$; −924: $r(11) = -0.6593$, $p = 0.0169$; −901: $r(11) = -0.6593$, $p = 0.0169$; Fig. 4A), where high levels of DNA methylation presumably act to silence gene expression. Trend lines for each of the 4 CpG sites indicate a similar negative relationship between methylation and expression may be occurring in the amygdala at this time (Fig. 4D), although data did not reach significance.

Term pregnant females no longer showed a negative relationship between *Oxtr* DNA methylation and gene expression in the nucleus accumbens at any of the CpG sites of interest and in fact show trends toward a positive correlation between methylation and expression at sites − 934_1 and − 924 [−934_1: $r(9) = 0.5714$, $p = 0.0663$; −934_2: $r(9) = 0.3712$, n.s.; −924: $r(9) = 0.5630$, $p = 0.0713$; −901: $r(9) = 0.5023$, n.s.; Fig. 4B]. This same lack of relationship was also seen in the amygdala [−934_1: $r(12) = -0.0843$, n.s.; −934_2: $r(12) = -0.1057$, n.s.; −924: $r(12) = -0.0472$, n.s.; −901: $r(12) = -0.0055$, n.s.; Fig. 4E] and in

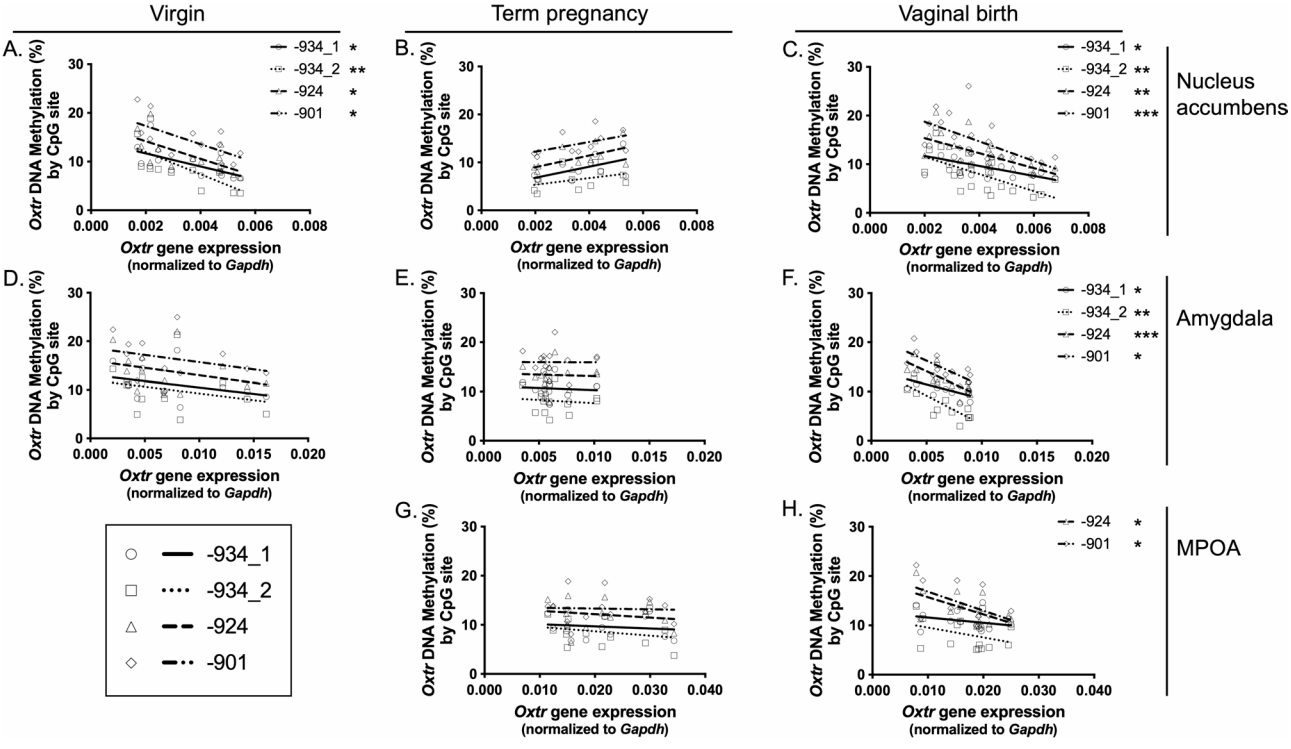

Fig. 4 The relationship between *Oxtr* DNA methylation and gene expression changes across pregnancy and with birth. Virgin females show the expected negative correlation between methylation and expression at all 4 homologous CpG sites in the nucleus accumbens **A**. At term pregnancy this relationship shifts to no significant correlation or even towards a positive correlation in sites − 934_1 and − 924 in this region **B** and then shifts back to a pre-pregnancy state within 90 min of giving birth **C**. This shift from no relationship between methylation and expression at term pregnancy to a negative correlation immediately following vaginal birth is also found in the amygdala **E**, **F** and the MPOA **G**, **H**. Individual data points are plotted. Trend lines are plotted predicting DNA methylation from gene expression for data visualization purposes only. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

the MPOA [−934_1: r(12) = −0.1448, n.s.; −934_2: r(11) = −0.2389, n.s.; −924: r(12) = −0.1858, n.s.; −901: r(12) = −0.0424, n.s.; Fig. 4G]. Data from all regions examined suggests another epigenetic mechanism may be acting to influence gene expression at this stage of pregnancy.

Within 90 min following an unmanipulated vaginal birth, females again displayed a negative correlation between *Oxtr* DNA methylation and gene expression in the nucleus accumbens at each of the CpG sites [−934_1: r(19) = −0.4878, $p = 0.0249$; −934_2: r(19) = −0.6574, $p = 0.0012$; −924: r(19) = −0.6587, $p = 0.0012$; −901: r(19) = −0.6829, $p = 0.0006$; Fig. 4C], similar to what was observed in virgin females. This pattern of *Oxtr* DNA methylation and gene expression was also seen at all CpG sites in the amygdala[−934_1: r(11) = −0.6429, $p = 0.0208$; −934_2: r(11) = −0.7620, $p = 0.0035$; −924: r(11) = −0.8462, $p = 0.0005$; −901: r(11) = −0.6978, $p = 0.0101$; Fig. 4F] and at sites − 924 and − 901 in the MPOA [−924: r(12) = −0.5761, $p = 0.0311$; −901: r(12) = −0.5370, $p = 0.0477$; Fig. 4H]. Unmanipulated vaginal birth, then, rapidly shifts epigenetic control of *Oxtr* back to a pre-pregnancy state in brain regions that control aspects of maternal behavior and social behavior more broadly. These epigenetic markers appear to be very dynamic during pregnancy and in the postpartum period, although at which point in gestation the shift in the relationship between them occurs is not yet known.

Experiment 1. Peripheral levels of *Oxtr* methylation are correlated with central levels of *Oxtr* methylation

We sought to replicate previous findings in whole blood and the nucleus accumbens of a positive association between levels of *Oxtr* DNA methylation and expand them to other relevant neural tissues by exploring associations between peripheral whole blood and the amygdala and MPOA. Mothers here showed a positive relationship in the nucleus accumbens as well as the amygdala and MPOA (Supplementary Fig. 3). Central levels of *Oxtr* DNA methylation are positively correlated with *Oxtr* DNA methylation in the peripheral whole blood at each of the homologous CpG sites in each of these three tissues when collapsed across term pregnant and vaginal birth groups [nucleus accumbens: −934_1: r(30) = 0.8438, $p < 0.0001$; −934_2: r(30) = 0.8753, $p < 0.0001$; −924: r(30) = 0.8350, $p < 0.0001$; −901: r(30) = 0.7720, $p < 0.0001$; amygdala: −934_1: r(30) = 0.8442, $p < 0.0001$; −934_2: r(30) = 0.8510, $p < 0.0001$; −924: r(30) = 0.7848, $p < 0.0001$; −901: r(30) = 0.7308, $p < 0.0001$; MPOA: −934_1: r(30) = 0.7225, $p < 0.0001$; −934_2: r(30) = 0.8574, $p < 0.0001$; −924: r(30) = 0.7692, $p < 0.0001$; −901: r(30) = 0.7697, $p < 0.0001$]. All CpG sites in all brain regions are still significantly positively correlated when analyzed separately by birth group (Supplementary Table 3). This replication and extension of a central/peripheral methylation level relationship points toward the usefulness of peripheral DNA measures in informing central levels in this context and brings about the opportunity for repeated measures sampling in our animal model. Given the CpG site homology present between human and prairie vole *Oxtr*, this also identifies a useful translational methods approach to understanding the newly maternal brain in human mothers.

Experiment 2. Prenatal treatment with oxytocin shifts epigenetic regulation of *Oxtr* toward a postnatal state

The shift of *Oxtr* to a postpartum regulatory state, where a negative association between *Oxtr* DNA methylation and gene expression is seen and mimics a pre-pregnancy state, may well be dependent on oxytocin signaling, whether endogenous as a result of unmanipulated labor and vaginal birth, or exogenous as a result of labor induction. Support for this oxytocin dependence comes from our model of labor induction. 90-minutes following an injection of exogenous oxytocin to term pregnant females, there was a main effect of treatment on levels of *Oxtr* DNA methylation in the nucleus accumbens between mothers that received one of three doses of oxytocin (low dose: 0.125 mg/kg; medium dose: 0.25 mg/kg; high dose: 0.5 mg/kg) compared to a saline vehicle injection ($p = 0.0042$; Supplementary Fig. 4A). At this same time point, there was a significant effect of oxytocin treatment on *Oxtr* gene expression in the nucleus accumbens ($F_{(3, 30)} = 3.995$, $p = 0.0166$). Post hoc tests showed a significant increase in expression levels 90-minutes after receiving a medium dose of oxytocin compared to a high dose ($p = 0.0208$; Fig. 5A) and an increase in expression levels after a medium oxytocin dose compared to a low dose at a trend level ($p = 0.0724$).

As in the nucleus accumbens, there was a main effect of treatment on levels of *Oxtr* DNA methylation in both the amygdala ($p = 0.0102$) and MPOA ($p = 0.0009$) (Supplementary Fig. 4B, C). There was a significant effect of treatment on *Oxtr* expression levels in the amygdala ($F_{(3, 33)} = 4.830$, $p = 0.0068$). Post hoc tests revealed a significant increase in expression in saline-treated compared to low dose oxytocin-treated mothers ($p = 0.0047$; Fig. 5B) and also showed an increase in expression after a treatment with the medium dose of oxytocin compared to the low dose at a trend level ($p = 0.0538$). The MPOA also showed a significant effect of treatment on *Oxtr* expression levels ($F_{(3, 33)} = 4.180$, $p = 0.0130$). Post hoc tests showed a significant increase in expression after a medium oxytocin dose compared to a lower dose ($p = 0.0215$; Fig. 5C) and also showed an increase in expression after a medium dose compared to a higher dose at a trend level ($p = 0.0804$). In mothers treated with oxytocin, this pattern of an elevation in *Oxtr* gene

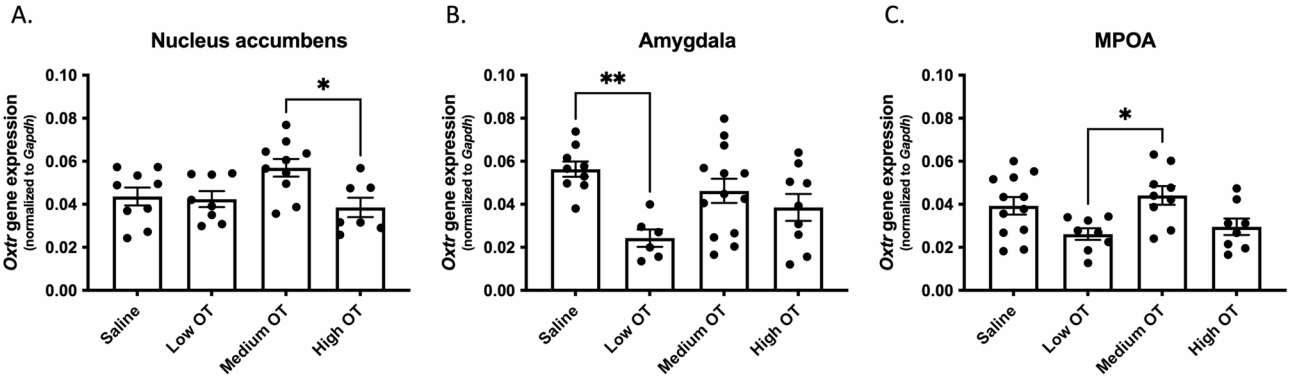

Fig. 5 Labor induction with oxytocin increases expression of *Oxtr*. Females treated with a medium dose of oxytocin on the expected day of birth prior to labor onset have increased *Oxtr* expression in the nucleus accumbens compared to those treated with a high dose of oxytocin **A**. Expression in the amygdala is decreased with a low dose oxytocin treatment compared to saline treatment **B**. A medium dose of oxytocin increases *Oxtr* expression compared to a low oxytocin dose in the MPOA **C**. Data presented are mean ± SE with individual data points plotted. * $p < 0.05$, ** $p < 0.01$

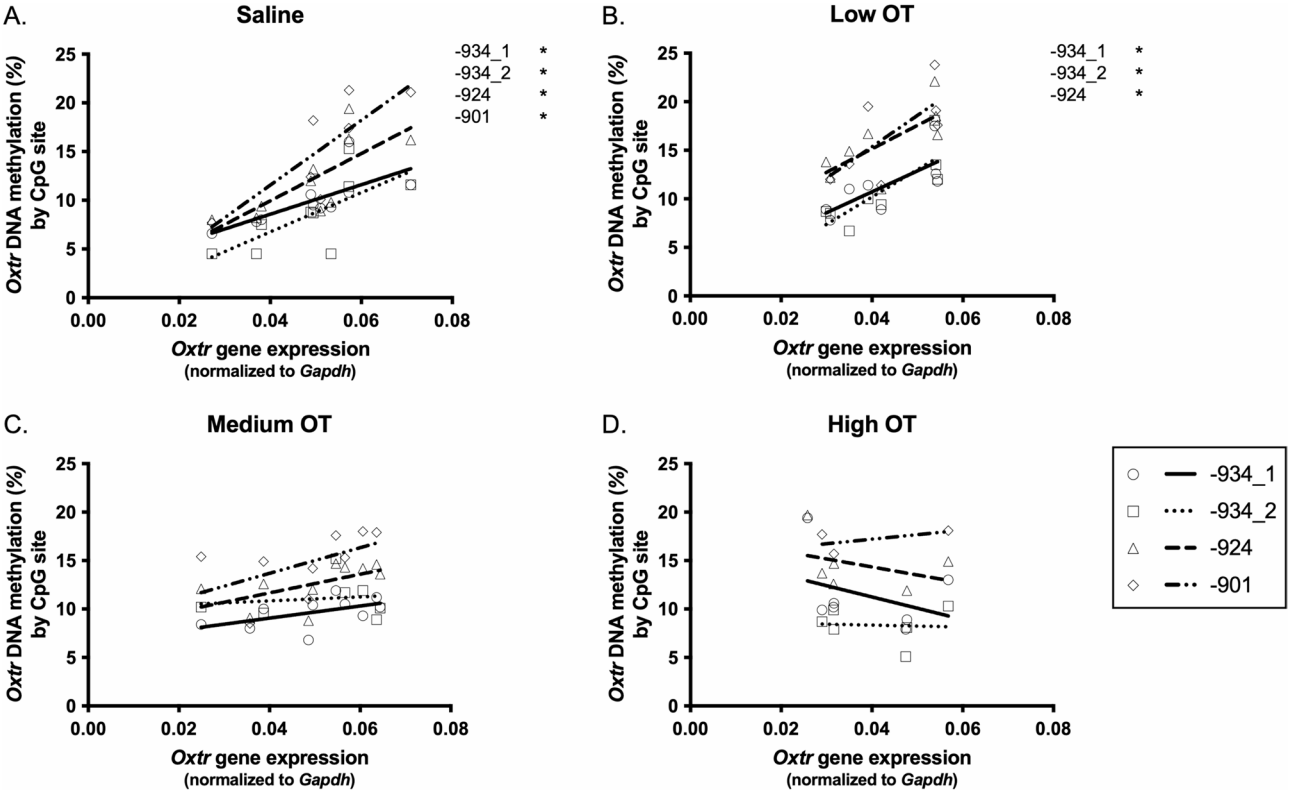

Fig. 6 Labor induction with oxytocin shifts the relationship between *Oxtr* DNA methylation and gene expression in the nucleus accumbens toward a postpartum state. Females treated with saline vehicle just prior to giving birth show a positive correlation between DNA methylation and gene expression at all 4 homologous CpG sites in the nucleus accumbens **A** and at 3 of the 4 sites with a low-dose oxytocin treatment **B**. As oxytocin exposure increases with the medium dose, the relationship between methylation and expression shifts to no significant correlation **C**. A high dose oxytocin treatment results in no correlation, with trend lines beginning to resemble an untreated postpartum state **D**. Individual data points are plotted. Trend lines are plotted predicting DNA methylation from gene expression for data visualization purposes only. * $p < 0.05$

expression after a medium dose of oxytocin compared to lower or higher doses is present across neural regions.

Following labor induction with exogenous oxytocin, there is a shift in the relationship between *Oxtr* DNA methylation and gene expression that follows the pattern seen at term pregnancy versus vaginal birth. Injection of a saline vehicle leads to a positive relationship between *Oxtr* DNA methylation and gene expression in the nucleus accumbens 90-minutes post-treatment at all CpG sites of interest [−934_1: $r(10) = 0.7188$, $p = 0.0192$; −934_2: $r(9) = 0.7021$, $p = 0.0350$; −924: $r(10) = 0.7519$, $p = 0.0121$; −901: $r(7) = 0.8125$, $p = 0.0263$; Fig. 6A], similar

to what is observed in term pregnant females (Fig. 4B). Presumably, females in both the saline-treated and term pregnant groups have not yet been exposed to high levels of endogenous oxytocin like would occur during natural labor. Mothers exposed to a lower dose of oxytocin look similar to saline-treated mothers 90 min after exposure [−934_1: $r(8) = 0.7448$, $p = 0.0340$; −934_2: $r(8) = 0.8155$, $p = 0.0136$; −924: $r(8) = 0.7082$, $p = 0.0493$; −901: $r(7) = 0.7011$, n.s.; Fig. 6B], while mothers treated with a higher dose of oxytocin no longer show a significant relationship between *Oxtr* DNA methylation and gene expression at any of the CpG sites examined [−934_1: $r(6) = -0.3580$, n.s.; −934_2: $r(6) = -0.0604$, n.s.; −924: $r(6) = -0.3680$, n.s.; −901: $r(6) = -0.5599$, n.s.; Fig. 6D]. Treatment with an intermediate dose of oxytocin results in no significant relationship between *Oxtr* DNA methylation and gene expression at any of the CpG sites [−934_1: $r(10) = 0.5269$, n.s.; −934_2: $r(7) = 0.1435$, n.s.; −924: $r(10) = 0.5815$, n.s.; −901: $r(9) = 0.5233$, n.s.; Fig. 6C] and regression lines for these medium-dose oxytocin mothers suggest a regulatory state that falls between lower and higher dose oxytocin exposure.

This dose-dependent response appears to be at least in part neural region-dependent. Unlike in the nucleus accumbens, there is no shift in the relationship between *Oxtr* DNA methylation and gene expression 90 min after any oxytocin dose in either the amygdala (Supplementary Fig. 5A-D) or MPOA (Supplementary Fig. 5E-G). This lack of a relationship resembles what was seen at term and suggests that, at least in these two regions, oxytocin signaling at the doses used here likely is not contributing to the shift in epigenetic regulation of *Oxtr* from a late gestational state to a postnatal state.

Discussion

Here we present evidence for a rapid change in the epigenetic state of *Oxtr* from term pregnancy to early postpartum in the maternal brain and provide evidence supporting the hypothesis that oxytocin signaling during labor and delivery contributes to this change and prepares the female for motherhood. We observe a shift in epigenetic regulation of *Oxtr* from a virgin female to term pregnancy, a time point that models an elective cesarean delivery without laboring in human women. The birth process rapidly shifted *Oxtr* regulation back to a pre-pregnancy state, suggesting some component of birth facilitates a change in *Oxtr* regulation. Data from our labor induction model indicate the component of birth responsible for this change may well be oxytocin signaling during labor. As increasing doses of oxytocin are administered to term pregnant females, *Oxtr* epigenetic control resembled that of a postpartum mother, pointing toward the increase in oxytocin activity during labor acting not only to facilitate birth but also to create an epigenetic state of *Oxtr* regulation in the maternal brain that is conducive to caring for her new infant. We also replicate previous findings from our lab [12] showing a strong positive correlation between measures of *Oxtr* DNA methylation in whole blood and the nucleus accumbens and extend this finding to positive correlations between measures of DNA methylation in whole blood and in the amygdala and MPOA. This central/peripheral relationship suggests the usefulness of more easily accessible peripheral tissues as a proxy for understanding the epigenetic state of the brain. This provides exciting possibilities both for repeated measures techniques in our animal model of birth and birth interventions and for developing a translational model in humans where blood samples can potentially report on brain state of pregnant women and new mothers. Together, these data suggest labor induction with exogenous oxytocin, a common birth interventions, may have the unintended consequence of facilitating birth at a point that is non-optimal with regard to the epigenetic state of the maternal brain.

One of the primary endocrine drivers of birth is oxytocin, which is synthesized and released in a pulsatile manner from the posterior pituitary and acts on the uterine myometrium to contract uterine muscle [3, 4]. Our data suggest this oxytocin surge during labor and delivery also targets the brain of the new mother and is responsible for the shift in epigenetic regulation of *Oxtr* seen with pregnancy and birth. It appears this shift can be induced at term by administering exogenous oxytocin. As the dose of oxytocin increased from saline vehicle through the highest dose, the relationship between *Oxtr* DNA methylation and gene expression shifted to a more negative relationship, as was seen postnatally in a natural birth. It appears that the exogenous oxytocin used here to model induction of labor in very late gestation mimics the endogenous oxytocin signaling associated with natural birth – as exogenous oxytocin exposure increased to more closely match endogenous levels thought to be present with natural labor, *Oxtr* epigenetic regulation in the maternal brain more closely matched what was observed after natural labor. Oxytocin exposure prenatally, as occurs during labor induction, may be resetting the epigenetic state of *Oxtr* in the maternal brain to a postnatal state prior to the actual birth.

The neural regions explored here are implicated in control of various aspects of maternal behavior [21–23, 38]. This same oxytocin surge that appears to be shifting epigenetic control of *Oxtr* may also facilitate the transition away from the avoidant or infanticidal behavior typically observed in nulliparous female rodents and toward the intense maternal behavior observed following birth of a litter. In the prairie vole, virgin females may be spontaneously allomaternal toward infants [39], although there

is variation in this behavior associated with factors such as age [40, 41] and OXTR density in the nucleus accumbens [42–45]. During pregnancy, nulliparous females often become infanticidal, even if they were allomaternal prior to pregnancy [46]. Immediately upon birth, females are highly maternal, both toward their own offspring to varying degrees [24, 25] and to unrelated infants [47]. This shift in behavior toward infants from allomaternal as virgins to infanticidal as a nulliparous pregnant female to highly maternal immediately following birth mirrors what is seen in the nucleus accumbens with *Oxtr* epigenetic regulation. We observe the expected negative relationship between *Oxtr* DNA methylation and gene expression in virgin females that shifts toward no relationship or even a positive relationship at term and then almost immediately after birth reverts back to a pre-pregnancy state. The shift in epigenetic markers controlling *Oxtr* expression in late gestation may impact OXTR availability in the female brain and alter her pup-directed behavior. The oxytocin surge at birth may then act to reset this system, allowing for the expression of maternal behavior after giving birth.

Many of the findings here appear to be specific to the nucleus accumbens, including changing *Oxtr* expression in unmanipulated birth and the methylation/expression relationship shift in response to labor induction. It may be that the nucleus accumbens is especially important for fine tuning social behavior in this species. As mentioned above, OXTR in the nucleus accumbens is critical for displays of alloparenting behavior in virgin females. Evidence in rat and mouse models of maternal behavior also indicate a critical role for OXTR in this region. Blocking OXTR function in the nucleus accumbens shell in new mothers increases pup retrieval latency in rats [48] and increases pup abandonment and infanticide in mice [49]. In the present study, *Oxtr* expression levels in the nucleus accumbens are lower prior to giving birth compared to levels in the amygdala and MPOA at the same time point, and the nucleus accumbens is the only region examined that showed an increase in *Oxtr* expression immediately following birth. This lower prenatal expression may give the nucleus accumbens more opportunity for dynamic change in the early postpartum, potentially leading to an upregulation of OXTR in this region to facilitate the onset of maternal behavior. We also observed different oxytocin dose responses in gene expression across brain regions. This suggests that, at term pregnancy, there may be an optimal level of endogenous oxytocin signaling to upregulate *Oxtr* expression and presumably OXTR protein in these regions to help facilitate the onset of maternal behavior, but that this optimal level may vary by region.

As discussed above, methylation of cytosines at CpG sites prevents binding of transcription factors, which then results in decreased transcription of the gene and lower measurable expression of that gene. This expected inverse relationship between *Oxtr* DNA methylation and gene expression was seen in both virgin females and new mothers, but not at term pregnancy. We offer two potential hypotheses to understand what is happening in the female brain as she prepares for birth. One possibility is that at term pregnancy there is a rapid switch in CpG sites of interest here from a methylated state to a hydroxymethylated state, where methylated CpG sites undergo oxidation by ten-eleven translocation (TET) enzymes to become hydroxymethylated CpG sites. This change in epigenetic marker reverses the impact on gene expression from inhibitory to faciliatory, thereby increasing gene expression as levels of hydroxymethylation increase. Given the strong correlation between methylation levels in brain and blood, this shift may be happening in multiple tissue types. The pyrosequencing method used here does not differentiate between methylation and hydroxymethylation. It is possible that what the assay is reading as methylation at term pregnancy is actually hydroxymethylation, which would explain the neutral-to-positive relationship between methylation and gene expression seen at term pregnancy. We are currently exploring this possibility using alternate techniques.

A second possibility is that there are alternate *Oxtr* gene transcripts that are active during late gestation that our primers do not currently capture or differentiate from the primary transcript. These alternate transcripts may then have differential effects on gene transcription and measured expression in the tissues of interest. Eight *Oxtr* transcripts have now been identified in a line of *Tet1* mutant mice [50], although whether these alternate transcripts are translated into functional OXTR is not known. Similar transcripts are present in the prairie vole genome [19, 33]. We believe that this is a likely explanation for the results seen in females at term and that expression of alternate transcripts may vary with brain region. When looking at the relationship of *Oxtr* expression between our brain regions of interest at term pregnancy, the different regions show either no correlation or a negative correlation with one another. Immediately following birth all regions show a positive correlation in *Oxtr* expression with one another. This shift in relationship between regions from just before to just after giving birth is not seen in *Oxtr* DNA methylation, where a positive correlation is maintained regardless of birth status or brain region. We hypothesize that *Oxtr* is being upregulated in the female brain at birth to prepare her for her new role as a mother, but that this change is not occurring in the same manner and along the same time course for each region of interest. In addition, we believe that there may be differential expression of alternate *Oxtr* transcripts during late gestation that result in differential

transcription of *Oxtr* in different regions. Given the different aspects of maternal behavior that are regulated by the nucleus accumbens, amygdala, and MPOA, it is likely the *Oxtr* system plays related yet still unique roles in shaping each region in preparation for motherhood. Having differential expression of the different transcripts across neural regions may be important in fine-tuning the female brain in preparation for a shift in behavior from infanticidal or indifferent to intensely maternal and provides an avenue for future exploration of connections between factors that influence transcript expression and risk for adverse maternal mental health outcomes.

Finally, our data focus solely on oxytocin pathways. However, there is abundant evidence suggesting that oxytocin can bind to vasopressin receptors with effects that differ from those when OXTR is stimulated (reviewed [51, 52]). Vasopressin also has been implicated in birth, uterine contractions and especially premature labor [5]. Under some conditions vasopressin also may influence parental behavior [53, 54]. The findings presented here suggest further need to explore the potentially disruptive consequences of exposure to high levels of exogenous oxytocin, as well as the role of vasopressin and other hormones associated with the birth process and the onset of maternal care.

There is a growing body of work linking common birth interventions with development of postpartum depression (PPD), a potentially severe maternal mental health condition that impacts up to 19% of mothers [55, 56]. Labor induction with synthetic oxytocin has been associated with an increase in PPD and anxiety [57–60]. Similarly, cesarean delivery has been linked to increases in the rates of postpartum anxiety, depression, and stress [61–64]. The factors contributing to the use of these birth interventions in women are of course incredibly varied. We propose that the common thread between these birth interventions and PPD risk for some women may well be altered functioning of the oxytocin system. Unlike the pulsatile release of oxytocin experienced during natural, unmanipulated labor, synthetic oxytocin used to induce or augment labor is typically given continuously. This prolonged exposure can cause the downregulation of *OXTR* expression [65] and eventually desensitize myometrium OXTR, decreasing the ability of the oxytocin peptide to facilitate uterine contractions at the strength and duration needed for birth [66, 67]. This same desensitization of OXTR may occur in the brain of some women. In women with the GG genotype of *OXTR* SNP rs53576, increasing levels of *OXTR* DNA methylation at CpG site −934 (homologous to the prairie vole sites −934_1 and −934_2) increases their risk for developing PPD [18]. Increased *OXTR* methylation at site −934 is also associated with an increased need for oxytocin administration during labor and increased risk for postpartum hemorrage in women who receive oxytocin during labor [68]. Risk for postpartum hemorrage itself is increased in rs53576 A carriers [69], pointing toward an interaction between genotype, reproductive physiology, and maternal health outcomes.

As mentioned above, DNA methylation is typically inversely related to transcript of the associated gene. This gene transcription controls translation of the gene into a protein. DNA methylation levels, then, would also be inversely related to protein production. We have previously demonstrated that early life experiences that increase *Oxtr* DNA methylation and decreased *Oxtr* gene expression in the nucleus accumbens of prairie voles also decreased density of OXTR in this region [12]. While there is not currently a way to measure this, it is very possible these women with increased *OXTR* methylation have an associated decrease in central OXTR availability. Altered availability of OXTR would impact the ability of circulating oxytocin to bind to OXTR and activate signaling pathways. This may ultimately decrease oxytocin signaling and disrupt processes reliant on oxytocin system activity. The doses of oxytocin used here are all subclinical doses in the prairie vole, showing no signs of potentiating labor. It is possible that as dosing increases beyond even our highest dose and combines with the endogenous oxytocin released during labor, it moves toward a supraclinical level of oxytocin, subsequently causing the downregulation of *Oxtr* gene expression and the desensitization of OXTR in the brain in a manner similar to what occurs in the myometrium during an extended induction or augmentation with synthetic oxytocin.

Summary of findings

By utilizing models of both unmanipulated birth and of labor induction with oxytocin, we present evidence for three key findings that demonstrate a switch in the regulatory state of *Oxtr* at term pregnancy that is likely sensitive to exposure to oxytocin in a dose-dependent manner. We first show that the negative relationship between *Oxtr* DNA methylation and *Oxtr* gene expression observed in virgin females becomes a non-significant neutral relationship at term pregnancy. Unmanipulated vaginal birth immediately resets this *Oxtr* methylation/expression relationship to a negative state. We next show that giving exogenous oxytocin to mothers at term pregnancy, a model of labor induction, shifts the non-significant *Oxtr* methylation/expression relationship at term pregnancy toward the negative relationship that was previously seen postpartum, even without these mothers giving birth. We finally demonstrate a strong positive relationship between brain and blood levels of methylation, suggesting that peripheral blood is a useful proxy for understanding the *Oxtr* methylation state of the brain.

Conclusions

Natural birth requires pulsatile spikes in oxytocin to activate oxytocin receptors in the uterine myometrium, facilitating uterine contractions for birth. This process naturally exposes the mother to very high levels of endogenous oxytocin at parturition. Administration of exogenous oxytocin just prior to birth, as would occur during an induced labor, acts similarly on the myometrium and also exposes the mother to very high levels of oxytocin. Our data show shifts in *Oxtr* epigenetic regulation in two models of birth at timepoints where the mother is experiencing increased levels of oxytocin exposure, whether endogenous (natural birth) or exogenous (induced labor). This common birth intervention results in a change in the regulatory state of *Oxtr*. Birth following intervention, then, may be occurring at a time of non-optimal maternal *Oxtr* regulation. Understanding better the role of the natural birth experience and differences following a birth with interventions is critical to better understanding factors contributing to maternal outcomes.

Supplementary Information

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Supplementary Material 1. [70]

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Authors' contributions
A.M.P., W.M.K., J.R.Y., C.F.C., C.S.C., and J.J.C. were responsible for designing the research. A.M.P., W.M.K., J.R.Y., and T.S.L. conducted experiments. A.M.P., R.E.W, and J.J.C. analyzed and interpreted the data. A.M.P. and J.J.C. wrote the manuscript. All authors read and approved the final manuscript.

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Data availability
The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
All procedures in the term pregnant and unmanipulated vaginal birth model were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at Indiana University, Bloomington (protocol #15 – 002). All procedures in the labor induction model were reviewed and approved by the IACUC at Northeastern University (protocol #13–0409).

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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