Prolactin action in the medial preoptic area is necessary for postpartum maternal nursing behavior

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Pregnancy hormones, such as prolactin, sensitize neural circuits controlling parental interactions to induce timely activation of maternal behaviors immediately after parturition. While the medial preoptic area (MPOA) is known to be critical for maternal behavior, the specific role of prolactin in this brain region has remained elusive. Here, we evaluated the role of prolactin action in the MPOA using complementary genetic strategies in mice. We characterized prolactin-responsive neurons within the MPOA at different hormonal stages and delineated their projections in the brain. We found that MPOA neurons expressing prolactin receptors (Prlr) form the nexus of a complex prolactin-responsive neural circuit, indicating that changing prolactin levels can act at multiple sites and thus, impinge on the overall activity of a distributed network of neurons. Conditional KO of Prlr from neuronal subpopulations expressing the neurotransmitters GABA or glutamate within this circuit markedly reduced the capacity for prolactin action both in the MPOA and throughout the network. Each of these manipulations, however, produced only subtle impacts on maternal care, suggesting that this distributed circuit is robust with respect to alterations in prolactin signaling. In contrast, acute deletion of Prlr in all MPOA neurons of adult female mice resulted in profound deficits in maternal care soon after birth. All mothers abandoned their pups, showing that prolactin action on MPOA neurons is necessary for the normal expression of postpartum maternal behavior in mice. Our data establish a critical role for prolactin-induced behavioral responses in the maternal brain, ensuring survival of mammalian offspring.

Significance

Prolactin-responsive neurons in the medial preoptic area project widely throughout the brain. After targeted deletion of prolactin receptors in the preoptic area of adult female mice, mice were able to get pregnant and give birth normally. However, mothers lacking prolactin receptors in the medial preoptic area abandoned their litters soon after birth, establishing a critical role for prolactin/placental lactogen action in this area for establishment and maintenance of normal parental care.

Maternal care is critical to survival of dependent offspring in mammals. Seminal work from Rosenblatt (1), published 50 y ago, showed that maternal behavior can be exhibited in ovariectomized, hypophysectomized rats, suggesting an underlying neural basis that was not dependent on hormonal inputs. Subsequent studies have characterized a complex neural circuitry controlling parental interactions (2, 3), with distributed sites mediating different components of the behavior (4). While the neural circuit controlling maternal behavior is not thought to be dependent on hormonal inputs, it is clear that pregnancy hormones, particularly rising levels of estradiol, oxytocin, prolactin, and placental lactogen, coupled with an abrupt decrease in progesterone, can sensitize the underlying circuitry to induce timely activation of maternal behaviors immediately after parturition (5). The medial preoptic area (MPOA) forms a critical nexus (2, 3), integrating a range of hormonal and sensory inputs into the maternal circuit.

Within the complex hormonal milieu of pregnancy, the specific role of prolactin in maternal behavior has remained elusive. This is largely because prolactin and the related placental lactogen have an obligate role in sustaining ovarian progesterone production during pregnancy in rodents (6), meaning that traditional approaches, such as pharmacological inhibition of prolactin secretion and antagonism or KO of prolactin receptors (Prlr), will terminate the pregnancy, making study of postpartum maternal behavior impossible. Circumvention of this issue has usually necessitated investigation of the effects of prolactin on maternal behaviors in artificial, nonpregnant models. For example, virgin Prlr KO mice (Prhr−/−) show a deficit in pup-induced maternal care (7), but these mice are infertile because of the absence of luteotrophic support of the ovary (8), preventing investigation of normal postpartum maternal behavior. Similarly, the key evidence showing that prolactin is involved in the initiation of maternal care has come from studies administering hormones to nonpregnant rats (9, 10). Importantly, these studies have identified the MPOA as a major region where prolactin action can facilitate the onset of maternal behavior in nonpregnant females (10).

To specifically evaluate the role of prolactin action in the MPOA in maternal behavior in the context of a normal pregnancy, we used complementary genetic strategies in mice. We characterized prolactin-responsive neurons within the MPOA at different hormonal stages and delineated their projections in the brain. Most (75%) of the MPOA neurons expressing c-Fos during maternal behavior are GABAergic (3), and we have previously observed extensive Prlr expression on GABAergic neurons in this region (11). In addition, this region is also rich in glutamatergic neurons (although only a small proportion of these express c-Fos during maternal behavior) (3). Hence, we next generated conditional KO animals and deleted Prlr in GABA and/or glutamate neurons in the brain, while retaining Prlr expression in peripheral reproductive tissues. This allowed us to evaluate maternal behavior in the context of a normal pregnancy but with markedly reduced capacity of the maternal circuits to sense prolactin. Finally, we specifically evaluated the role of Prlr in the MPOA using a viral approach to acutely ablate Prlr specifically within this region in the adult brain, while leaving Prlr expression in other parts of the maternal circuit intact.

Results

The MPOA Forms the Nexus of a Prolactin-Sensitive Neural Network.

The MPOA contains a large number of neurons expressing Prhr mRNA (12). Prolactin typically activates transcriptional responses through the JAK/STAT signal transduction pathway mediated by


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phosphorylation of STAT5 (pSTAT5) (13). We found that the number of MPOA neurons showing prolactin-induced pSTAT5 was relatively low in nonpregnant mice but markedly increased during lactation (Fig. 1A). These data suggest considerable plasticity in the MPOA during lactation, with enhanced transcriptional responses to prolactin, although there is no change in Prlr mRNA expression in this region at this time (14).

To analyze the role of prolactin-responsive neurons specifically in the MPOA, we next developed a mouse line in which Cre recombinase is expressed under the control of the Prlr promoter using an internal ribosome entry site (IRES) (15). We inserted the IRES-Cre construct immediately downstream of exon 10 of the Prlr gene, resulting in Cre expression in cells that express the long form of the Prlr (Prlr-iCre). We then crossed the Prlr-iCre animals with mice expressing a Cre-dependent τGFP reporter (ROSA26-CAGS-τGFP; eR26-τGFP) (16) to fluorescently label prolactin-responsive neurons. Because τGFP associates with microtubules, it effectively labels neuronal processes of Prlr neurons as well as their cell bodies. We found that τGFP expression was restricted to areas known to express Prlr in the adult brain (12). In particular, we detected high levels of τGFP-positive cell bodies in the MPOA, bed nucleus of the stria terminalis (BNST), paraventricular nucleus (PVN), ventromedial hypothalamic nucleus (VMH), arcuate nucleus (ARN), and medial amygdala (MeA) (Fig. 1 and Fig. S1), all regions that have previously been implicated in the neural circuitry governing parental behavior (4, 5). We also detected τGFP-positive fibers throughout the brain, including a number of areas, such as the supramammillary region and ventral tegmental area (VTA), that were devoid of Prlr-expressing cell bodies (12).

To specifically trace the projections of MPOA Prlr neurons, we stereotaxically injected a recombinant adeno-associated virus (AAV) encoding Cre-dependent mCherry into this brain area. mCherry is anterogradely transported within neuronal processes labeling axons of the Cre-expressing cells. After unilateral AAV injection into the MPOA of Prlr-iCre/eR26-τGFP mice, we found that a subset of the Prlr neurons (green in Fig. 1B) expressed mCherry (red in Fig. 1B). While mCherry-positive cell bodies were restricted to the side of the injection, we identified extensive mCherry-positive projections from the MPOA Prlr neurons (Fig. 1), predominantly but not exclusively on the ipsilateral side (quantified in Table S1). Many of the major projections connected to regions that also showed high levels of τGFP in cell bodies, indicating prolactin-responsive neurons, such as the BNST, PVN, ARN, and VMH (Fig. 1, Figs. S1 and S2, and Table S1). Importantly, few of these areas showed mCherry-positive staining in the cell bodies, which would have been indicative of retrograde transport of the marker, suggesting that the observed fiber staining was localized almost exclusively in projections from the MPOA. Taken together,

![Fig. 1](image_url)

Fig. 1. A prolactin-sensitive neural network centered on the medial preoptic area (MPOA). (A) Changes in prolactin-induced signal transduction in Prlr-expressing MPOA neurons. Photomicrographs show immunohistochemical labeling of pSTAT5 (black nuclear staining) after prolactin administration in diestrus nonlactating mice and day 7 lactating mice compared with vehicle-treated controls. Prolactin induces widespread and intensive pSTAT5 labeling in lactating mice but not in diestrus mice. (B–F) Distribution of projections from Prlr-expressing neurons in the MPOA. Prlr-iCre/eR26-τGFP mice received a unilateral injection of AAVS-EF1a-DIO-HChr2(1H34R)-mCherry-WPRE-pA into the MPOA to drive Cre-dependent expression of mCherry specifically in the MPOA. Images were captured using a 10× objective using a Zeiss Axio Imager2 microscope with a motorized stage, with multiple images combined to form a composite image using the Mozaix module in the Axiovision software. (B) Site of the injection into the MPOA, with Prlr-expressing cells being labeled with τGFP (green) and a subset of Prlr-expressing neurons in the MPOA on one side expressing mCherry (red). (C) Schematic representation of mCherry-labeled projections from the MPOA in the sagittal plane, with regions containing Prlr-expressing cells colored in green and regions lacking Prlr-expressing cell bodies in white. ac, anterior commissure; ARN, arcuate nucleus; BNSTpnr, bed nucleus of the stria terminals principle nucleus; ls, lateral septum; MeA, medial amygdala; ox, optic chiasm; PVN, paraventricular nucleus; rPOA, rostral preoptic area; SON, supraoptic nucleus; vBNST, ventral bed nucleus of the stria terminals; VTA, ventral tegmental area. (D–F) Representative sections illustrating the distribution of τGFP labeling showing Prlr expression (green), mCherry-positive projections of MPOA prolactin receptor-expressing cells (red), and the composite images through the forebrain (Figs. S1 and S2 and Table S1).
these data suggest that MPOA Prlr-expressing neurons form the nexus of a complex prolactin-responsive neural circuit, indicating that changing prolactin levels may significantly impact on the overall activity of a distributed network of neurons.

**Conditional KO Mice Lacking Prolactin Receptors in Glutamate and/or GABA Neurons Display Mild Perturbations of Maternal Behaviors.** To investigate the functional role of prolactin in GABAergic and/or glutamatergic neuronal populations, we deleted the Prlr gene from GABA and/or glutamate neurons using VGat-Cre and VGlut2-Cre mouse strains (17) in combination with Prlrlox/lox animals (11). In Prlrlox/lox/VGlut2-Cre mice, there was a 25% loss of prolactin-induced pSTAT5 in the MPOA (P = 0.028), confirming successful deletion of Prlr in glutamate neurons in this area (Fig. 2A and B). In addition, these animals showed an almost complete loss of prolactin-induced pSTAT5 in the PVN and VMH, suggesting that all prolactin-responsive neurons in these regions are glutamatergic. Conditional deletion of Prlr in GABA neurons (Prlrlox/lox/VGlut-Cre) resulted in a 50% decrease in prolactin-induced pSTAT5 in the MPOA (P = 0.038) (Fig. 2C and D). These mice also had an almost total loss of pSTAT5 in the MeA as well as reduced STAT5 activation in the ARN and BNST. The overall change in numbers of neurons responding to prolactin in each of the prolactin-responsive nuclei in the maternal circuit is illustrated schematically in Fig. S3. Both conditional KO strains were fertile and able to carry a pregnancy to term, although an increased number of the animals abandoned their litters (two or fewer pups alive on day 3 of lactation) compared with controls. While this difference for Prlrlox/lox/VGlut2-Cre mice (3 of 12 abandoned litters) was not significantly different from controls (1 of 18; P = 0.274), Prlrlox/lox/VGat-Cre mice showed higher levels of litter loss (7 of 20; P = 0.045) (Fig. 2E). Nevertheless, the majority of animals in each genotype were able to maintain their litters. In mice with surviving litters, we completed a pup retrieval experiment both in the home cage on day 3 postpartum and in a novel cage [a slightly more rigorous test of maternal behavior (18)] on day 5 postpartum. The effect of the conditional deletion of Prlr in either GABA or glutamate neurons on pup retrieval in the home cage of postpartum female mice (Fig. 2F and H) was not significantly different from controls (P = 0.784) (Fig. 2E). There was no significant difference in pup retrieval time in either GABA or glutamate neurons in the novel cage compared with controls (P = 0.274). Mothers were separated from their pups, then three pups were placed into the cage, and the time taken to retrieve pups was recorded. Note the difference in scale on the y axis, with retrieval taking much longer in the novel cage. As there was no preformed nest in the novel cage, all mice took significantly longer to retrieve their pups and crouch over them in a newly constructed nest. Prlrlox/lox/VGlut2-Cre mice were not significantly different from control animals in performing this task in the novel cage. In contrast, while Prlrlox/lox/VGat-Cre exhibited normal retrieval of the pups, they took significantly longer to crouch over the pups and exhibit full maternal behavior (P = 0.041) (Fig. 2G and I).

Combining both Cre alleles to generate mice lacking Prlr in both GABA and glutamate neurons resulted in approximately additive effects in terms of loss of prolactin responses. These animals had markedly reduced prolactin-induced pSTAT5 in the MPOA (~75%; P = 0.004) as well as almost total loss of STAT5 responses in the PVN, VMH, and MeA (Fig. 3). Despite this, the animals were no worse off in terms of maternal behavior, and most of them were able to care for their litters. Importantly, however, none of the manipulations above generated a complete loss of Prlr throughout the circuit, raising the possibility that the few remaining prolactin-responsive neurons in the MPOA were sufficient to sustain maternal behavior that was only mildly impaired compared with controls.
Acute Deletion of Prolactin Receptors from the MPOA in Adults Abolishes Maternal Nursing Behavior. To test this hypothesis and specifically investigate the role of prolactin action in postpartum maternal behavior, we used an AAV to deliver Cre recombinase into the MPOA of adult female Prl/Cre mice (11). The Prl/Cre construct is designed such that Cre-mediated recombination will activate GFP expression and thus, report successful Prlr deletion (Fig. 4).

AAV-Cre injection into the MPOA of Prl/Cre mice completely removed functional prolactin receptors from this region (Fig. 4). The effect was highly localized, as there was little or no expression of GFP in the nearby PVN, and prolactin-induced pSTAT5 was unaffected in that and other nuclei (Fig. S4). Three days after injection, animals were mated and monitored throughout pregnancy. There was no effect of treatment on the time taken to get pregnant or the duration of pregnancy (Fig. S4). Animals gave birth to normal-sized litters and initiated the normal pup-directed activities (19), retrieving the pups to a nest, cleaning and grooming the pups, and eating the placenta. Having initiated these behaviors, however, the animals failed to establish full maternal care defined as crouching over the pups in a nest and assuming an arched back posture (kyphosis) to facilitate nursing. The animals failed to crouch over the pups, and over the course of the next 24 h, all of the mothers lacking Prlr in the MPOA abandoned their litters, resulting in death of all pups (Fig. 4).

There was no evidence that the Prl/Cre/AAV-Cre animals ever suckled their young after birth. When examined in the first 6 h after birth (in the cross-fostering study; see below), the pups showed no signs of milk feeding or matted fur around the nipples, the normal signs of suckling seen in the controls. By contrast, control dams did not abandon their litters, and the pups suckled after birth. When examined in the first 6 h after birth (in the cross-fostering study; see below), the pups showed no signs of milk feeding or matted fur around the nipples, the normal signs of suckling seen in the controls. By contrast, control dams did not abandon their litters, and the pups suckled after birth. When examined in the first 6 h after birth (in the cross-fostering study; see below), the pups showed no signs of milk feeding or matted fur around the nipples, the normal signs of suckling seen in the controls. By contrast, control dams did not abandon their litters, and the pups suckled after birth.
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MPOA-specific Prlr deletion (Prlrlox/lox/AAV-Cre; n = 4) compared with control groups of AAV-Cre-injected WT C57BL/6J mice (B6/AAV-Cre; n = 2) and Prlrlox mice (n = 4), with pups scattered and dams spending no time at the nest or with pups. Different letters represent statistically different groups (P < 0.01).

of the animals to give birth normally suggests at least some level of function of the oxytocin system (20), meaning that milk ejection would likely have occurred had the animals suckled. In addition to maternal behavior, the MPOA has a number of important homeo-

critical role in linking the multiple regions involved in maternal behavior and in integrating the various hor-

moral and sensory inputs that influence the behavior (4, 5). A number of studies have traced connections from the MPOA to other structures involved in the maternal circuit, and have dem-

strated a major output of the system to midbrain effector regions, such as the VTA (and nucleus accumbens) (23, 24). Our data show that some of these projections are derived from prolactin-responsive cells. After deletion of Prfr in the MPOA in adult females, the mothers initially showed appropriate pup-directed behavior, grooming the pups after birth and consuming the placent.

However, these interactions were not sustained, and the mothers never exhibited full maternal care and abandoned their pups within 24 h of birth. Thus, it seems that prolactin action in the MPOA is necessary for the normal function of the maternal system, working, or possibly altering the key output of the network to regions that reinforce the behavior (25). This might be mediated by prolactin itself but also by the closely related placental lactogens that act on the Prlr and are elevated in the blood throughout the second half of pregnancy (25). Importantly, unlike some recent reports that have used cell ablation approaches (26, 27) or blockade of neurotransmitter production (28, 29) to identify critical cell types mediating maternal behavior, in this model, the neuronal circuitry is completely intact. The profound deficit arises specifically from the lack of appropriate hormonal input into these circuits.

Methods

Animals. The generation and genotyping of Prlr-iCre and Prlrlox/lox mice (11) are detailed elsewhere (SI Methods). Prlr-iCre mice were crossed with ROSA26-CAGS-tGFP reporter animals (16), generating mice that express tGFP specifically in Prlr-expressing neurons. VGat- and VGlut2-IRES-Cre mice, originally developed by Brad Lowell (17), were purchased from Jackson Labs [Sancar et al. (20)] (stock no. 028862) and [stock no. 028863], respectively. These mice were crossed with our Prlrlox/lox mice. As Cre-mediated inversion deletes the Prfr gene and knocks in EGFP in its place (11), EGFP expression in the brain was used as a marker for both successful recombination and for the normal pattern of receptor expression. Finally, double-Cre animals were generated initially by breeding male VGat-Cre +/- mice with female VGlut-Cre +/- mice. After a number of double-Cre males had been produced, the breeding strategy was changed to cross double-Cre male mice with homozygous Prlrlox/lox females (SI Methods). Groups of WT C57BL/6J (B6) mice were used as additional control groups in some experi-

ments. All mice were used as adults (8–14 wk) and were group-housed under conditions of controlled temperature (22 °C ± 1 °C) and lighting (12:12-light and 12-h dark cycles with lights on at 0600 hours), with ad libitum access to food and water. All animal experimental protocols were approved by the University of Otago or the University of Saarland animal ethics committee.

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Stereotoxic Injections of AAV. Adult mice (8–12 wk old) were anesthetized with isoflurane and placed in a stereotaxic apparatus. For tract tracing experiments, PrlR-Cre/Lox/Lox females received unilateral 1-L injections of AAVS-F1a-Dio-HcHR2(H134R)-mCherry-WPRE-pA (5 × 1012; UNC Vector Core) into the MPOA. For gene KO experiments, PrlR/Lox/Lox animals received bilateral 1-L injections of AAV2-CMV-PLC-cre-RbG (2.27 × 1013; Penn Vector Core) into the MPOA. All injections were given at a rate of 100 nL/min, and the syringes were left in situ for 3 min before and 10 min after injections. Coordinates for the MPOA were 0.2 mm anterior to Bregma and 0.3 mm lateral to midline, and depth was adjusted for age (8 wk: 4.7-mm depth; 10 wk: 4.9-mm depth; 12 wk: 5.0-mm depth). A control group of WT C57BL/6J mice received bilateral AAV-Cre injections, and a control group of PrlR/Lox/Lox mice received saline injections.

Tract Tracking Experiments. Two to 4 weeks after viral injection, mice were anesthetized with ketamine and xylazine and perfused transcardially with 4% paraformaldehyde, and brains were prepared for immunohistochemistry for mCherry and gEFP (SI Methods). Nuclei were visualized by Hoechst staining. Sections were imaged using the Axiol Image M2 microscope with AxioVision software (Zeiss) and then ImageJ. Images were split into single channels, regions of interest were assigned to individual brain regions, and percentages of each area occupied by mCherry-positive signal above threshold, as calculated by the Triangle method (30), were obtained (Table S1).

Monitoring of Maternal Behavior. Seventy-two hours after AAV-Cre injection, females were individually housed with a WT C57BL/6J male mouse. Successful mating was confirmed by the presence of a vaginal plug, the male was removed, and day of parturition was recorded as day 1 of lactation. Pup survival was monitored daily, and total number of live pups was recorded on day 3 of lactation. Maternal behavior (on the home cages with their own pups) was recorded 6-24 h after parturition in groups of PrlR/Lox/Lox (n = 8), B6aAV-Cre (n = 2), and PrlR/Lox/Lox/AAV-Cre (n = 4) mice. The percentages of time that the dam spent over the pups in the nest, elsewhere in the cage, and eating were calculated during both the light and dark periods. The percentage of time that pups were gathered in a nest was also calculated. To evaluate whether any deficit in maternal care was caused by pup-related factors, pups from two PrlR/Lox/Lox/AAV-Cre mothers were cross-fostered onto PrlR/Lox/Lox control dams, with control pups placed with the KO mothers. Pups were collected as soon as possible after birth (within 6 h) and were checked for general health and the presence of milk bands in their stomach. At the same time, the mothers were assessed for evidence of suckling (matted fur around the nipples and/or elongated nipples). Pups were then placed with the new mother and observed for the next 48 h. Pup retrieval behavior was tested in home cages on day 3 of lactation (SI Methods).

Immunohistochemistry. To evaluate PrlR expression in the MPOA of PrlR/Lox/Lox mice, brains were processed for the presence of EGFP immunoactivity (to indicate Cre-dependent recombination in the PrlR/Lox/Lox mice and therefore, represent cells that expressed PrlR before recombination) and prolactin-induced pSTAT5 (a sensitive and reliable marker of activated PrlR (12)). Pups were removed from dams on days 7–10 of lactation at 0900 hours, and at 1215 hours, dams were administered with ovine prolactin (5 mg/kg injection i.p.). Mice were anesthetized with sodium pentobarbital and perfused transcardially 45 min after prolactin administration with 4% paraformaldehyde. Brains were removed, postfixed for 1 h in the same fixative, and cryoprotected in 30% sucrose overnight. Three sets of 30-μm-thick coronal sections through the forebrain from each animal were cut using a sliding microscope. One series of tissue each was used to examine pSTAT5 and EGFP expression by chromoimmunohistochemistry as previously described (11, 12) (SI Methods). Quantification of pSTAT5 labeling in the MPOA of lactating PrlR/Lox/Lox (n = 4–8 mice) was undertaken by counting the total number of pSTAT5-labeled nuclei in two sections per animal. Sections were anatomically matched between different experimental animals. Images were collected with a Zeiss Plan-Apochromat 10x/0.45 (for the inset figures in Fig. 1 D–F) using the Axio Imager M2 microscope with an AxioVision software (Zeiss) and then ImageJ. Images were split into single channels, regions of interest were assigned to individual brain regions, and percentages of each area occupied by mCherry-positive signal above threshold, as calculated by the Triangle method (30), were obtained (Table S1).