# Prolactin-mediated restraint of maternal aggression in lactation

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**Aggressive behavior is rarely observed in virgin female mice but is specifically triggered in lactation where it facilitates protection of offspring. Recent studies demonstrated that the hypothalamic ventromedial nucleus (VMN) plays an important role in facilitating aggressive behavior in both sexes. Here, we demonstrate a role for the pituitary hormone, prolactin, acting through the prolactin receptor in the VMN to control the intensity of aggressive behavior exclusively during lactation. Prolactin receptor deletion from glutamatergic neurons or specifically from the VMN resulted in hyperaggressive lactating females, with a marked shift from intruder-directed investigative behavior to very high levels of aggressive behavior. Prolactin-sensitive neurons in the VMN project to a wide range of other hypothalamic and extrahypothalamic regions, including the medial preoptic area, paraventricular nucleus, and bed nucleus of the stria terminalis, all regions known to be part of a complex neuronal network controlling maternal behavior. Within this network, prolactin acts in the VMN to specifically restrain male-directed aggressive behavior in lactating females. This action in the VMN may complement the role of prolactin in other brain regions, by shifting the balance of maternal behaviors from defense-related activities to more pup-directed behaviors necessary for nurturing offspring.**

female aggression | lactation | prolactin | prolactin receptor | ventromedial nucleus of the hypothalamus

The mammalian postpartum period is accompanied by profound physiological adaptations and behavioral changes in a mother. One of the most dramatic switches in a mother's behavior is the lactation-specific display of aggression. This "maternal aggression" is a critical survival mechanism, enabling a mother to respond to and protect her young from dangers or perceived threats. While important, these defense activities must also be balanced with the need for the mother to prioritize offspring-directed behavior to nurture the offspring. Abnormal levels of maternal aggression have been linked to expression of postpartum anxiety and the display of hypervigilant parenting (1, 2). Specifically in rodents, high levels of intruder-directed aggression are accompanied by high anxiety–related behavior in lactating mothers (1). In humans, increased feelings of anger, including that directed toward other family members or health care providers, is higher in mothers with postpartum depression (2). This highlights the importance for fine-tuned regulation of maternal aggression during lactation, in order to support healthy and appropriate interactions of the mother with both the infant and with other individuals.

Although aggression is an innate social behavior, it is predominantly observed in males, and most of our understanding of the neural mechanisms driving aggressive behavior is derived from research undertaken in males. Within this neural circuit, multiple divisions of the amygdala and the ventromedial nucleus of the hypothalamus (VMN) have emerged as central regions governing aggressive behavior. The medial amygdala (MeA) receives vital sensory input from both the main olfactory and vomeronasal systems to elicit intermale aggression (3), with GABAergic neurons in the MeA highly active during aggressive intermale social interactions (3). More recently, a role for the posterior amygdala (PA) has also be identified, with a subpopulation of VMN-projecting estrogen receptor α (ERα)-expressing glutamatergic neurons in the PA excited during intermale aggression and chemogenetic manipulation of this population altering intermale aggression (4, 5). The VMN, downstream from both the MeA and PA, is another key site in regulating aggressive behavior in males, and optogenetic simulation of neurons in the ventrolateral VMN (VMNvl) induces male aggressive behavior toward females, whereas optogenetic silencing inhibits intermale aggression (6). Specifically with the VMN, control of aggressive behavior is restricted to ERα-expressing neurons (7), suggesting a potential role for hormonal modulation of this behavior.

In females, optogenetic and chemogenetic stimulation of VMNvl ERα-expressing neurons is sufficient to alter intruder-directed social behavior in virgin female mice that are normally nonaggressive and can induce intruder-directed aggressive behavior in mice on a Swiss Webster background (7, 8). This suggests that similar neuronal circuitry to that in males may also underlie aggression in females (9), but this system is not easily activated except during lactation. It seems likely that the expression of maternal aggression, therefore, is dependent on

## Significance

**Heightened intruder-directed aggressive behavior in female mice is displayed during lactation to enable a mother to protect her offspring. Although recent work has identified that the ventromedial nucleus of the hypothalamus plays an important role in governing aggressive behavior, it is unknown how the changing hormones of pregnancy and lactation might regulate this behavior during specific reproductive states. Here, we show that prolactin-responsive neurons are activated during aggression and project to multiple brain regions with known roles in regulating behavior. Through neuron-specific and region-specific deletion of the prolactin receptor, our data reveal that prolactin is an important modulator of maternal aggression. By acting on glutamatergic neurons in the ventromedial nucleus, prolactin restrains maternal aggression, specifically in lactating female mice.**

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sensitization or activation of this system by pregnancy and/or lactation-specific cues. During pregnancy, hormones play a key role in timing the display of other aspects of maternal behavior (10), hence we hypothesized that the changing hormones of pregnancy and lactation also act in the VMN to regulate postpartum aggressive behavior. Previously, we have reported that prolactin acting through the prolactin receptor (Prlr) in the medial preoptic area (MPOA) is required for the onset of postpartum nursing behavior in mice (11). *Prlr*s are also highly expressed in the ventrolateral region of the VMN (12, 13), and we have observed high levels of endogenous Prlr activation in the VMN during lactation (13). Therefore, we aimed to investigate whether prolactin acting in the VMN contributes to the regulation of maternal aggressive behavior in lactating mice.

## Results

**Prolactin Receptor Expression in Glutamatergic Neurons Restrains the Intensity of Maternal Aggression in Lactating Mice.** Female C57BL/6J mice exhibit the profound lactational switch in intruder-directed aggressive behavior that is observed in many mammalian species. Whereas virgin female C57BL/6J mice will rarely attack a juvenile male intruder, lactating (days 2 to 4) C57BL/6J mice show reduced latencies to attack, increased bouts of attacking, and spend a significant proportion of time attacking an intruder in a resident–intruder paradigm (Fig. 1 *A–C*; consistent with a previous report in ref. 8). As the VMN plays a key role in the display of both male and female aggression, we hypothesized that prolactin-sensitive neurons in this region might have a role in modulating maternal aggression. We have previously reported the presence of a large population of prolactin-sensitive glutamatergic neurons in the VMN (11) Fig. 1*D*). Hence, we first used VGlut2-Cre mice (14) crossed with Prlr flox (*Prlr*$^{lox/lox}$) mice (15) to conditionally delete *Prlr* from glutamatergic neurons. The Prlr flox construct is designed such that GFP expression is activated upon Cre-mediated recombination of the Prlr gene. We observed high levels of GFP expression in the VMN of VGlut2-Cre/*Prlr*$^{lox/lox}$ mice (Fig. 1 *E* and *F*), indicative of widespread deletion of Prlr from glutamatergic neurons in this region. Using pSTAT5 as a marker of functional responses to prolactin (12), we found that most endogenous pSTAT5 observed during lactation in the VMN was lost in our VGlut2-specific Prlr knockout mice, suggesting that the majority of prolactin-sensitive neurons in the VMN were glutamatergic neurons (Fig. 1*G*). In contrast to the VMN, lactation-specific increases in pSTAT5 immunoreactivity were not reduced in the anatomically closely located and highly prolactin-sensitive arcuate nucleus (ARN), and relatively few Prlr-expressing neurons in the ARN were glutamatergic (Fig. 1 *E–G*). In support of this, single-cell sequencing of the ARN suggests a number of different subpopulations of neurons in this region express the *Prlr*, many of which are GABAergic (16).

Intruder-directed aggression was assessed on day 3 of lactation in *Prlr*$^{lox/lox}$/VGlut2-Cre and Cre-negative control *Prlr*$^{lox/lox}$ mice. The mechanisms driving aggression that are specific to lactation are not known, and we initially hypothesized that prolactin might be involved in promoting maternal aggression and predicted that there would be reduced aggression in mice lacking Prlr in the VMN. However, compared to control *Prlr*$^{lox/lox}$ mice, *Prlr*$^{lox/lox}$/VGlut2-Cre mice exhibited a hyperaggressive phenotype toward a male juvenile intruder. Intruder-directed aggression was markedly increased in lactating *Prlr*$^{lox/lox}$/VGlut2-Cre mice, with very short latencies to attack and increased time spent attacking compared to control *Prlr*$^{lox/lox}$ mice (Fig. 1 *H–J*). Furthermore, whereas lactating C57BL/6 mice will rarely exhibit aggressive behavior toward an adult male intruder (17) (Fig. 1 *H–J*; control *Prlr*$^{lox/lox}$ data), lactating *Prlr*$^{lox/lox}$/VGlut2-Cre mice (on C57BL/6J background) also showed significantly reduced latencies to attack an adult male, increased number of attacking bouts, and a significantly longer time spent attacking the male (Fig. 1 *H–J*). This increased aggression in lactating *Prlr*$^{lox/lox}$/VGlut2-Cre mice was specifically targeted toward male intruders, with no change in aggressive behavior observed toward adult or juvenile female intruders (Fig. 1 *H–J* and *SI Appendix*, Fig. 1). Furthermore, pup-targeted aggression was never observed in these studies, with no difference in litter survival and growth in *Prlr*$^{lox/lox}$/VGlut2-Cre mice compared to control *Prlr*$^{lox/lox}$ mice (*SI Appendix*, Fig. 2). In support of a specific effect on maternal aggression, we have previously reported normal pup retrieval behavior in lactating *Prlr*$^{lox/lox}$/VGlut2-Cre mice (11), and when tested for anxiety in an elevated plus maze (EPM), there were no differences in time spent in the open arms or distance traveled in the EPM between lactating *Prlr*$^{lox/lox}$/VGlut2-Cre and control *Prlr*$^{lox/lox}$ mice (*SI Appendix*, Fig. 3). This hyperaggression in *Prlr*$^{lox/lox}$/VGlut2-Cre mice was restricted to lactation, with no intruder-directed aggressive behavior observed in virgin *Prlr*$^{lox/lox}$/VGlut2-Cre or control virgin *Prlr*$^{lox/lox}$ mice (Fig. 1 *H–J*). Investigative behavior was also assessed during interactions with intruders, and a corresponding decrease in investigative behavior was observed in lactating *Prlr*$^{lox/lox}$/VGlut2-Cre mice only when exposed to a juvenile male intruder, but not in virgin *Prlr*$^{lox/lox}$/VGlut2-Cre mice or in response to a novel object or female and adult male intruders (*SI Appendix*, Figs. 1 and 4).

To confirm that this hyperaggressive maternal phenotype was specific to the loss of prolactin signaling in glutamatergic neurons, *Prlr*$^{lox/lox}$ mice were crossed with VGat-Cre mice (14) to conditionally delete Prlr from GABAergic neurons (*Prlr*$^{lox/lox}$/VGat-Cre mice) (15), and intruder-directed behavior was assessed in virgin and lactating female mice. We have previously reported the presence of large subpopulations of Prlr-expressing GABAergic neurons throughout the forebrain, particularly in the bed nucleus of the stria terminalis (BNST) and MeA, but notably not in the VMN (11, 15). Intruder-directed aggressive behavior was not different in either virgin or lactating female *Prlr*$^{lox/lox}$/VGat-Cre mice compared to Cre-negative control *Prlr*$^{lox/lox}$ mice (Fig. 1 *H–J* and *SI Appendix*, Fig. 1). These data reveal a role for prolactin acting on glutamatergic neurons to restrain male-directed aggressive behavior during lactation.

**Prolactin's Role in Restraining the Display of Maternal Aggression in Lactating Mice Is Mediated through Action on the VMN of the Hypothalamus.** In addition to the VMN, *Prlr*s are expressed in multiple other populations of glutamatergic neurons, with expression in the MPOA being most notable for the regulation of maternal nursing behaviors (11). To confirm that the role of prolactin in modulating maternal aggressive behavior is mediated by the VMN, *Prlr*s were specifically deleted from the VMN of adult mice using an adeno-associated virus (AAV) to bilaterally deliver Cre recombinase into the VMN of adult *Prlr*$^{lox/lox}$ mice (Fig. 2). AAV-Cre injection into the VMN of *Prlr*$^{lox/lox}$ mice resulted in high levels of GFP expression, indicating successful recombination of the *Prlr* gene, and completely removed functional prolactin responses (as indicated by loss of pSTAT5 immunoreactivity) from this region (Fig. 2 *A* and *B*). Two control groups were generated: a "Cre-only" using AAV-Cre administration in C57BL/6J mice and "Cre negative" using AAV-mCherry administration into *Prlr*$^{lox/lox}$ mice. Both showed an absence of GFP immunoreactivity (indicating no recombination of the *Prlr* gene had taken place) and normal pSTAT5-labeling (Fig. 2 *A* and *B*). Fourteen days following injection, animals were mated and monitored throughout pregnancy. Replicating that seen in *Prlr*$^{lox/lox}$/VGlut2-Cre mice, the lactating *Prlr*$^{lox/lox}$/AAV-Cre mice displayed a hyperaggressive maternal phenotype. Compared to lactating control groups, *Prlr*$^{lox/lox}$/AAV-Cre mice were very

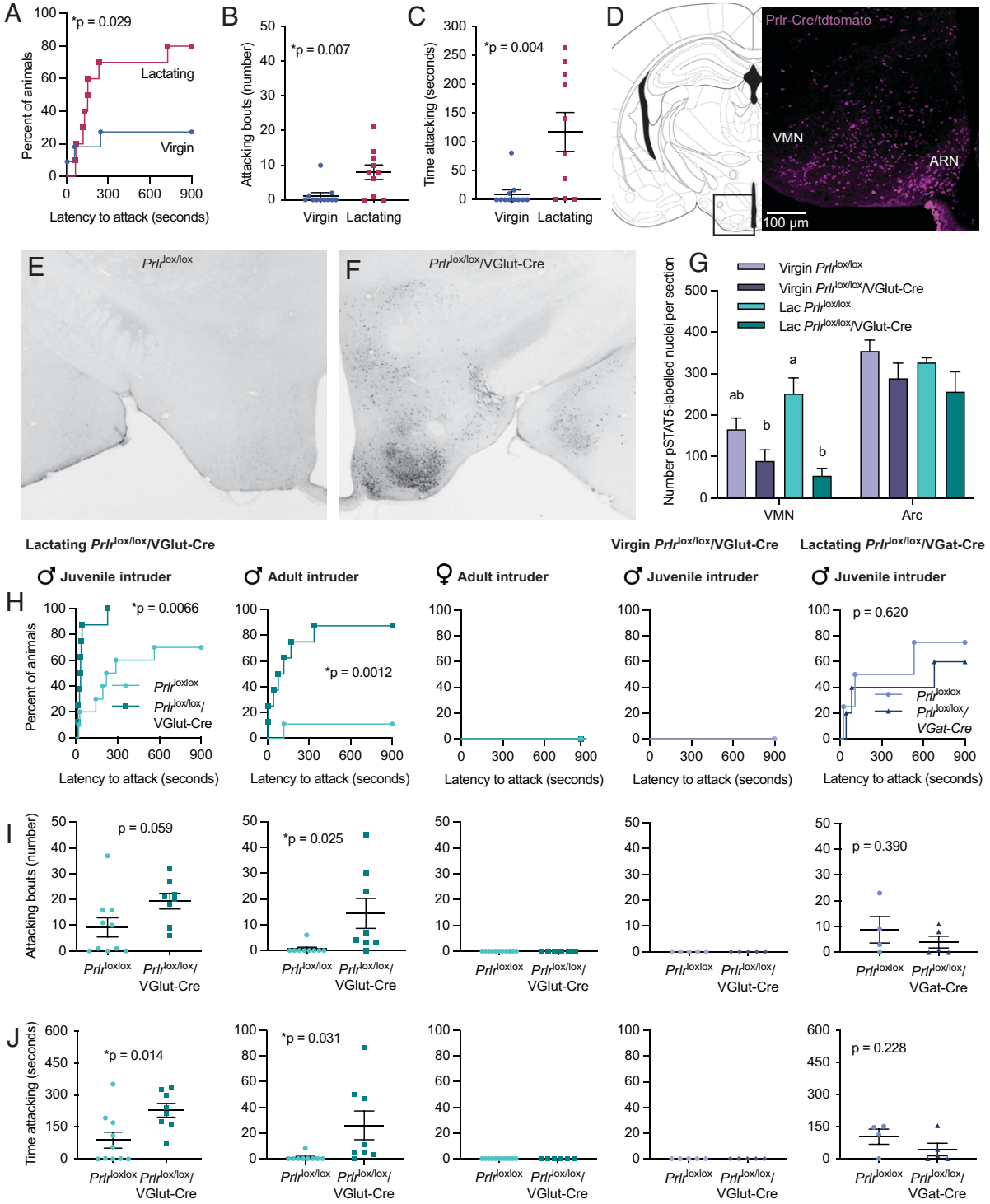


**Fig. 1.** Glutamatergic-specific deletion of *Prlr* induces hyperaggressive intruder-directed behavior in lactating mice. (*A* and *B*) Intruder-directed aggression in virgin and lactating C57BL/6J female mice. Few virgin females display aggressive behavior (*A–C*) and spend little time attacking a juvenile male intruder (*C*). In comparison, lactating (days 2 to 4) C57BL/6J mice show reduced latencies to attack, more bouts of attacking, and spend significantly longer attacking a intruder. (*D*) tdtomato expression in Prlr-Cre/tdtomato mice, driven by the prolactin receptor (Prlr) promoter. There are large numbers of Prlr-expressing cells (indicated by tdtomato expression) in the VMN and ARN of female mice. (*E* and *F*) Expression of eGFP (black nuclear staining) in the VMN of glutamatergic neuron-specific ($Prlr^{lox/lox}$/VGlut-Cre) mice is indicative of the targeting construct and deletion of the *Prlr* gene. (*G*) Prolactin-induced pSTAT5 in the VMN is significantly reduced during lactation in $Prlr^{lox/lox}$/VGlut-Cre mice compared to Cre-negative controls ($Prlr^{lox/lox}$). A lack of change in pSTAT5 immunolabeled-nuclei in the Arc, suggests few Prlr-expressing neurons in the Arc are glutamatergic. (*H–J*) Intruder-directed aggressive behavior in lactating $Prlr^{lox/lox}$/VGlut-Cre, virgin $Prlr^{lox/lox}$/VGlut-Cre mice, and in lactating mice with Prlr conditionally deleted from GABAergic neurons ($Prlr^{lox/lox}$/VGat-Cre). Compared to lactating control $Prlr^{lox/lox}$ mice, lactating $Prlr^{lox/lox}$/VGlut-Cre mice show reduced latencies to attack (*H*), more bouts of attacking (*I*), and spent significantly more time attacking (*J*) C57BL/6J male but not female intruders. In contrast, deletion of Prlr from GABAergic neurons ($Prlr^{lox/lox}$/VGat-Cre mice), had no effect on maternal aggression during lactation. Virgin $Prlr^{lox/lox}$ and $Prlr^{lox/lox}$/VGlut-Cre mice did not display any intruder-directed aggressive behavior (*H–J*). Different letters represent statistically different groups ($P < 0.05$).

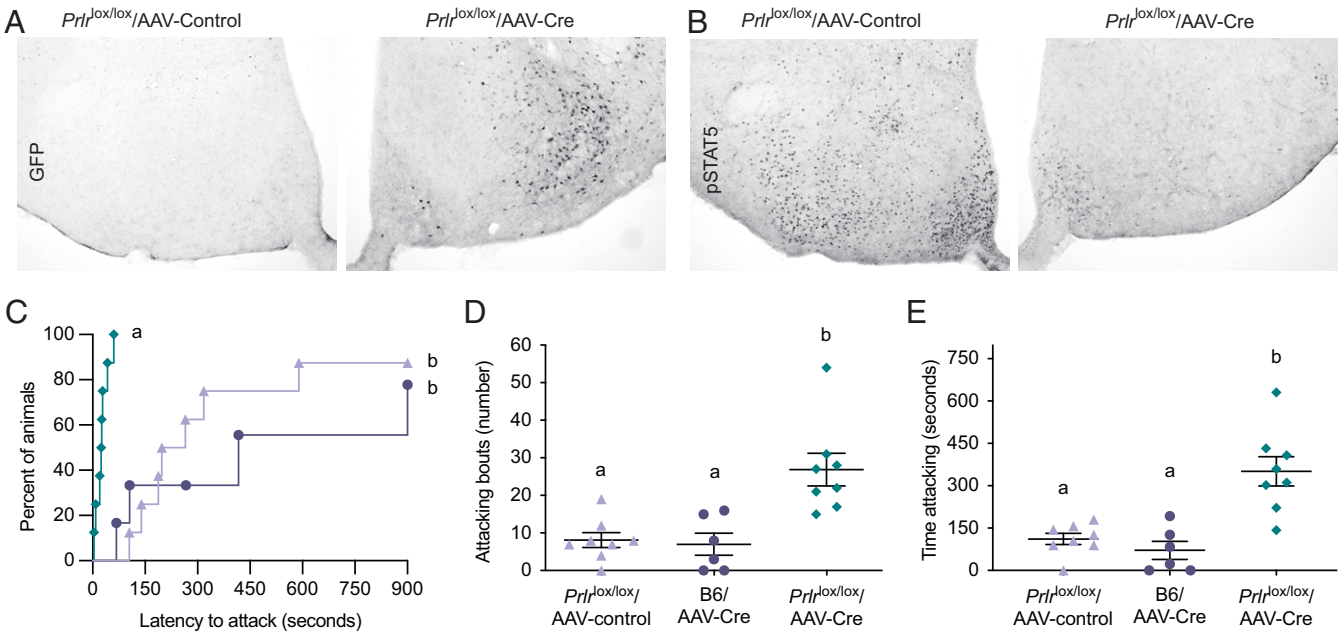


**Fig. 2.** VMN-specific deletion of Prlr induces hyperaggressive intruder-directed behavior in lactating mice. (*A* and *B*) Bilateral administration of AAV/DJ-CMV-mCherry-iCre into the VMN of adult *Prlr*$^{lox/lox}$ female mice resulted in extensive GFP [black nuclear staining; (*A*)] and complete loss of pSTAT5 labeling [black nuclear staining; (*B*) in lactating animals], indicating successful deletion of the *Prlr* gene. GFP labeling was absent, and high levels of pSTAT5 labeling were observed in *Prlr*$^{lox/lox}$ mice receiving the control AAV/DJ-CMV-mCherry (*Prlr*$^{lox/lox}$/AAV-control). (*C–E*) Intruder-directed aggressive behavior in lactating mice with a VMN-specific deletion of *Prlr* (*Prlr*$^{lox/lox}$/AAV-Cre), compared to control groups of wild-type C57BL/6J mice that received AAV-Cre (B6/AAV-Cre) or *Prlr*$^{lox/lox}$ mice that received control AAV-mCherry (*Prlr*$^{lox/lox}$/AAV-control). *Prlr*$^{lox/lox}$/AAV-Cre mice showed very short attack latencies, more bouts of attacking, and spent significantly more time attacking than control groups. Different letters represent statistically different groups ($P < 0.05$).

quick to attack a juvenile male intruder, had increased bouts of attacking, and spent significantly more time attacking (Fig. 2 *C–E* and *SI Appendix*, Fig. 5 and Movie S1). There was no effect of AAV-Cre administration on time taken to get pregnant or litter survival on day 3 of lactation (*SI Appendix*, Fig. 5). Collectively, these data suggest that prolactin acts through glutamatergic neurons in the VMN to restrain levels of maternal aggression.

**Acute Manipulation of Prolactin Does Not Induce Rapid Changes in VMN Neuronal Activity or in Aggressive Behavior.** To begin to assess how prolactin might be acting on VMN neurons to influence maternal aggression, we next aimed to assess whether prolactin could acutely regulate the activity of VMN neurons or alter intruder-directed aggressive behavior. Elsewhere in the brain, including the arcuate nucleus, prolactin can acutely stimulate the activity of specific subpopulations of Prlr-expressing neurons (18–21). Here, we measured prolactin-induced (250 nM) changes in intracellular calcium in ex vivo VMN brain slices of female Prlr-iCre/GCaMP6f mice (methods described previously in ref. 20), where changes in fluorescence are indicative of changes in intracellular calcium in Prlr-expressing cells (Fig. 3*A*). Heterogenous responses were observed in Prlr-expressing VMN cells, with some cells being excited (virgins, 8/64 cells; lactating, 16/81 cells), and a few inhibited (4/81 cells, only observed in lactating females), but the majority of cells did not respond acutely to bath application of prolactin (Fig. 3 *B* and *C*).

The effect of acute changes in prolactin on intruder-directed aggressive behavior was assessed in both virgin and lactating female mice in response to a juvenile male intruder. Exogenous prolactin (5 mg/kg, intraperitoneally [i.p.]) administration to virgin female mice (low endogenous prolactin) (12), failed to induce aggressive behavior; however, it did significantly reduce the time spent investigating the intruder (Fig. 3 *D–E* and *SI Appendix*, Fig. 6). During lactation, effects of exogenous prolactin administration are unlikely to be observed as prolactin levels are already chronically elevated to support milk production. Therefore, endogenous prolactin secretion was pharmacologically suppressed in lactating mice using the dopamine D2 receptor agonist, bromocriptine, to examine the effect of acute reduction of circulating prolactin on intruder-directed behavior in these mice. Similar to virgin mice, acute manipulation of prolactin had no effect on aggressive behavior, with equivalent levels of maternal aggression observed between vehicle- and bromocriptine-administered lactating females (Fig. 3 *G–I*). This finding that acute prolactin modulation does not alter aggressive behavior is consistent with the lack of acute prolactin-induced changes in neuronal activity (Fig. 3 *A–C*), and with the concept that prolactin (with the exception of its negative feedback actions on tuberoinfundibular dopamine neurons in the arcuate nucleus) (18, 19, 22) predominantly acts through a longer-term transcriptional pathway (18, 20).

**Prolactin-Receptor–Expressing Neurons in the VMN Are Activated in Response to an Intruder; However, Acute Chemogenetic Activation Does Not Alter Aggressive Behavior.** We next sought to determine whether prolactin-sensitive neurons in this region were activated during expression of maternal aggression. Male juvenile intruders were introduced into the home cages of Prlr-iCre (23) female mice crossed with a tdtomato reporter mouse to label Prlr-expressing neurons (Prlr-iCre/tdtomato mice; Fig. 4*C*). Immunolabeling for cFos was used as a marker for neuronal activation, with low basal levels of cFos immunolabeling observed in control virgin and lactating animals. Exposure to an intruder induced significant cFos immunolabeling in the VMN of both virgin and lactating female Prlr-iCre/tdtomato mice, with approximately one-third of these activated cells colabeled with tdtomato (Fig. 4 *D–F*), indicating that a significant proportion of intruder-activated cells in the VMN express the *Prlr*. Although exposure to an intruder induced cFos expression in virgin animals in the absence of aggressive behavior, this is

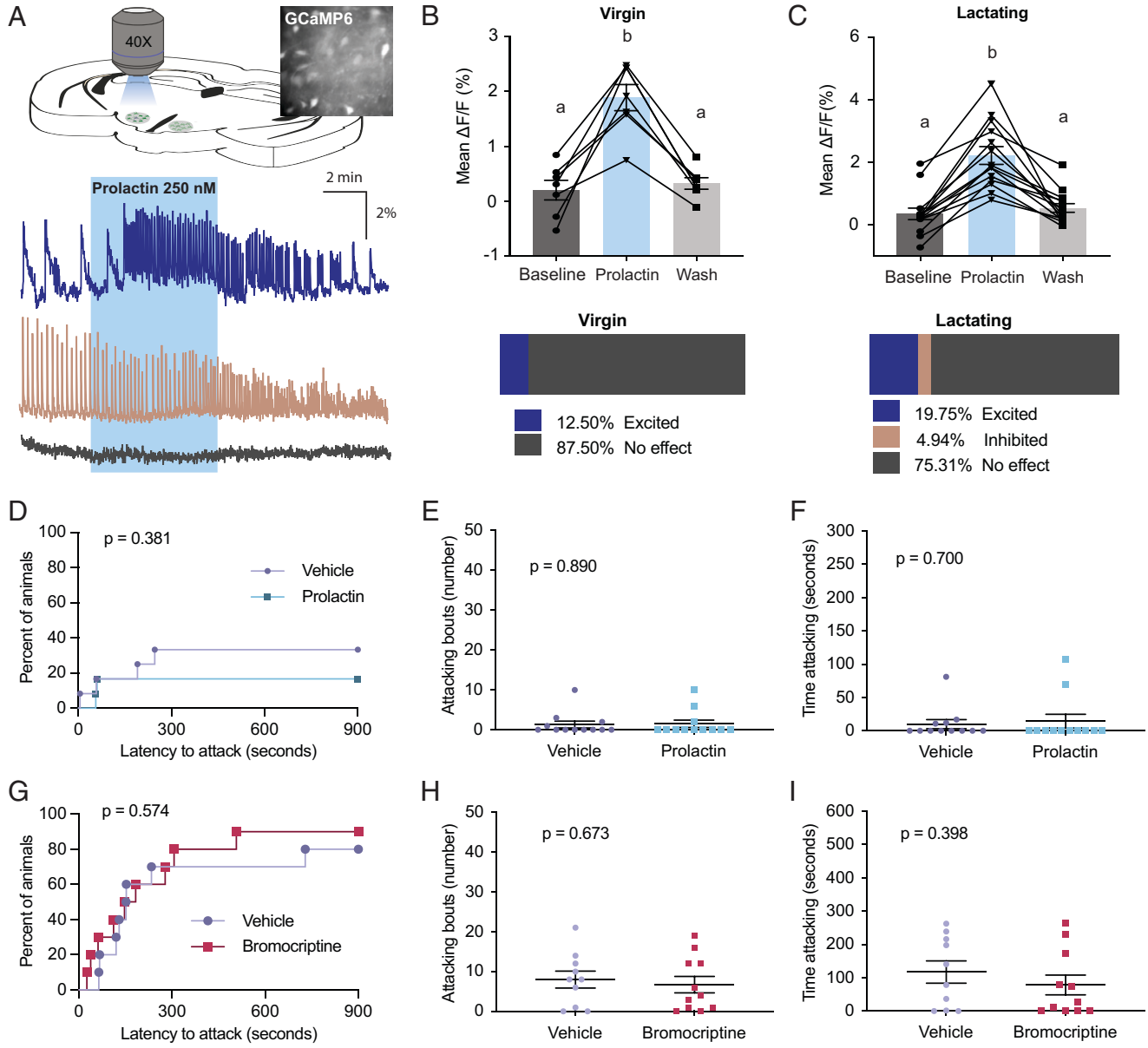


**Fig. 3.** The majority of prolactin-sensitive neurons in the VMN do not show acute changes in activity in response to prolactin, and acute prolactin manipulation does not alter aggressive behavior. (*A–C*) Prolactin-induced (250 nM) changes in intracellular calcium in ex vivo VMN brain slices of female Prlr-iCre/GCaMP6f mice. Changes in fluorescence are indicative of changes in calcium [$Ca^{2+}$] in Prlr-expressing cells. (*A*) Representative examples of prolactin-induced excited, inhibited, and nonresponding Prlr-expressing VMN cells. (*B* and *C*) Changes in fluorescence before, during, and following bath application of prolactin of Prlr-expressing cells that showed a prolactin-induced excitation. (*Bottom*) Proportion of Prlr-expressing VMN cells that were excited, inhibited, or showed no effect in response to prolactin in ex vivo brain slices from virgin female and lactating mice. (*D–I*) Intruder-directed aggressive behavior in virgin C57BL/6J female mice receiving either vehicle or prolactin (5 mg/kg, i.p., 45 min prior to testing) administration. (*G–I*) Intruder-directed aggressive behavior in day 2- to 4-lactating mice receiving either vehicle or a D2-receptor agonist, bromocriptine, to supress endogenous prolactin release (5 mg/kg, i.p., 2 h prior to testing). Different letters represent statistically different groups ($P < 0.05$).

consistent with prolactin affecting investigative behavior in virgin females (*SI Appendix*, Fig. 6) and with the role of the VMN in regulating multiple aspects of social behavior.

To determine whether activation of Prlr-expressing neurons in the VMN could influence intruder-directed maternal aggression, we used a chemogenetic strategy to activate these cells in female mice. Prlr-iCre mice were stereotaxically injected with a Cre-dependent AAV2-hSyn-DIO-hM3D(Gq)-mCherry (Addgene) into the VMN, to target expression of the stimulatory Designer Receptor Exclusively Activated by Designer Drugs (DREADD), hM3D, exclusively into Prlr-expressing cells in the VMN. In ex vivo VMH brain slices, bath application of the DREADD agonist, clozapine-n-oxide (CNO, 5 μM), resulted in increased firing of Prlr-expressing neurons (Fig. 4*D*), and in vivo administration of CNO (1.5 mg/kg; i.p.) induced extensive cFos immunoreactivity in the VMN of Prlr-Cre mice injected with AAV2-hSyn-DIO-hM3D(Gq)-mCherry but not with a control AAV2-hSyn-DIO-mCherry (Fig. 4 *E* and *F*). Intruder-directed behavior was assessed following acute CNO and vehicle administration in virgin and lactating female mice. Similarly to acute manipulations of prolactin, chemogenetic activation of Prlr-expressing VMN

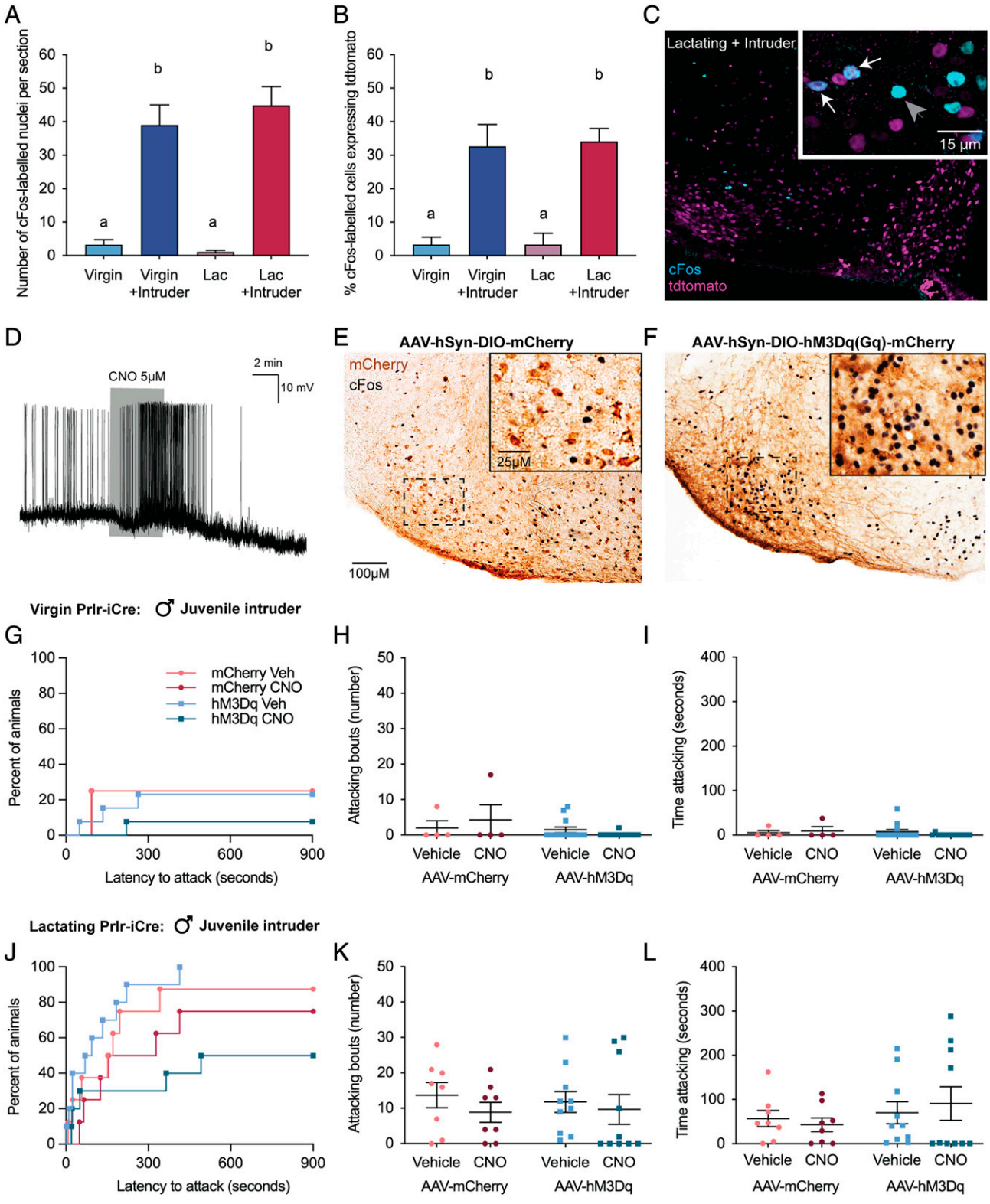


**Fig. 4.** Presence of an intruder activates prolactin-sensitive neurons in the VMN but acute chemogenetic activation does not stimulate intruder-directed aggressive behavior in female mice. (*A–C*) The introduction of a juvenile male intruder induced significant activation (indicated by cFos immunoreactivity, cyan) of Prlr-expressing neurons (tdtomato, magenta) in the VMN of both virgin and lactating Prlr-iCre/tdtomato mice. (*D*) Prl-iCre mice injected with AAV5-hSyn-DIO-hM3Dq(Gq)-mCherry into the VMN to drive Cre-dependent expression of the stimulatory hM3Dq show increased firing following application of DREADD agonist, CNO (5 µM) in ex vivo brain slices. (*E* and *F*) Dual-label immunohistochemical labeling for mCherry (brown cytoplasmic labeling) and cFos (black nuclear staining) in the VMN of control AAV-hSyn-DIO-mCherry (*E*)- and AAV-hSyn-DIO-hM3Dq(Gq)-mCherry (*F*)-injected Prlr-iCre mice following in vivo CNO administration (1.5 mg/kg; i.p.). (*G–L*) There was no significant difference in intruder-directed aggressive behavior between hM3Dq-injected and control mCherry-injected mice following CNO administration, either in virgin (*G–I*) or lactating (*J–L*) Prlr-iCre mice. Different letters represent statistically different groups ($P < 0.05$).

neurons did not induce intruder-directed aggressive behavior in virgin animals and did not further increase aggression in lactating mice (Fig. 4 *G–L*). In lactating mice, CNO administration did, however, induce an increase in time spent investigating the male intruder (*SI Appendix*, Fig. 7).

**Glutamatergic Neurons in the VMN Contribute to a Prolactin-Sensitive Neural Network Controlling Parental Behaviors.** We have shown that Prlr signaling in the VMN has a profound effect on the intensity of aggressive behavior displayed during lactation toward male intruders. However, the downstream target regions of these neurons are unknown. To identify projections of Prlr-expressing neurons in the VMN, female Prlr-iCre/tdtomato mice were stereotaxically injected with a recombinant AAV encoding Cre-dependent YFP into the VMNvl (Fig. 5*A*). YFP is anterogradely transported within neuronal processes, thereby labeling axons of the Cre-expressing cells and enabling visualization of brain regions receiving inputs from Prlr-expressing neurons in the VMN. Prlr-expressing VMN neurons project to ∼20 areas throughout the forebrain and hindbrain in female mice, with the strongest projection to the BNST (medial division, posteromedial part) and MPOA (Fig. 5*B* and *SI Appendix*, Fig. 8). Many of these regions have previously been shown to be involved both in maternal offspring–directed behavior, such as retrieval and nursing, and in maternal aggressive behavior (3, 24) (Fig. 5*B*). In addition to these regions, fiber labeling from Prlr-expressing VMN neurons was also observed in the anteroventral periventricular hypothalamic nucleus (AVPV), MeA, paraventricular nucleus (PVN), and periaqueductal gray (PAG) (Fig. 5 *C–E*), regions that have all previously been documented to play a role in regulating female aggressive behavior. Interestingly, a number of these regions that received significant projections from Prlr-expressing VMN neurons, including the AVPV, BNST and MeA, showed high levels of tdtomato expression in cell bodies, indicating that prolactin can also act directly on cell bodies in these regions. Mapping the projections of Prlr-expressing cells in the VMN has revealed that these neurons are part of an overall parental circuit that was first identified from the MPOA, with prolactin regulation of the VMN specifically influencing aggressive behavior. The diverse projections of prolactin-sensitive VMN neurons also raises the possibility that these neurons may be involved in regulating other aspects of maternal behavior not typically associated with the VMN.

## Discussion

These data provide insights into how neural systems controlling aggression can be modulated by pregnancy/lactation-specific factors, such as chronically elevated prolactin. Maternal aggression, which enables a mother to protect her offspring in response to a perceived threat, is a necessary component of a successful reproductive strategy and increases the likelihood that offspring survive and reach reproductive maturity. With virgin females in many species not innately displaying aggressive behavior, a switch is required during lactation to enable this behavior to occur. Although we initially hypothesized that prolactin might induce maternal aggression, our data have revealed a role for this hormone in restraining aggressive behavior. It is becoming increasingly apparent that in addition to the requirement for factors to induce aggressive behavior, unchecked or abnormally high levels of maternal aggression are also detrimental for both mother and offspring (1, 2). The presence of a hormonal cue to restrain the investment of energy and time into excessive defensive or aggressive behaviors may be adaptive to ensure sufficient maternal investment into offspring-directed behaviors.

The neural regulation of aggression has been the focus of much research in recent years and has led to significant progress into determining the underlying neural circuit governing aggressive behavior in both males and females. Much of this has relied on the use of optogenetics to drive the activity of specific neuronal populations within the VMN. Specifically within the VMN, ERα-expressing neurons appear to be important regulators of female aggression, with optogenetic inhibition of these neurons blocking intruder-directed maternal aggression in lactating mice of both Swiss Webster and C57 backgrounds (8) and optogenetic stimulation increasing aggression in already aggressive lactating, but not virgin, C57BL/6N mice (7, 8). Examination of ERα immunolabeling in Prlr-expressing cells in the VMN in the present study revealed that the majority of Prlr-expressing neurons in the VMN coexpress ERα (*SI Appendix*, Fig. 9). Therefore, the inability of chemogenetic activation of Prlr-expressing neurons to induce aggressive behavior in virgin C57BL/6J mice is consistent with these previous reports. However, it is important to note the presence of many additional ERα-expressing neurons in the VMN that did not express the Prlr (*SI Appendix*, Fig. 9), suggesting that we have targeted only a subset of ERα-expressing neurons in this region. This may account for our observation that acute chemogenetic stimulation of VMN Prlr-expressing neurons did not further increase aggressive behavior in lactating females, as seen when the whole ERα-expressing neuronal population is targeted. Single-cell sequencing has identified 29 neuronal subtypes in the VMN, 17 of these within the VMNvl, including 7 different transcriptomic ERα-expressing populations (25). The expression of *Prlr* mRNA was not reported in these specific subpopulations (25) and further technical refinement would be required to identify the specific subpopulations prolactin is acting on to reduce maternal aggressive behavior within this heterogenous and sexually dimorphic (25, 26) brain region.

Our data revealing the presence of hormonal cues that act in the VMN to restrain excessive maternal aggression suggest that a strong promaternal aggressive cue is required during lactation to enable the display of intruder-directed aggression at this time. In the Lee et al. and Hashikawa et al. studies (7, 8), the expression of ERα was used as a marker of a neuronal population that directed female aggression, but there was no manipulation of estrogen signaling itself in the VMN. In females, circulating estradiol has been well documented to play a role in facilitating maternal aggression in rats (27), but whether this is mediated by the VMN remains to be determined. In rodents, circulating levels of estradiol rise throughout pregnancy before rapidly declining at parturition (28–30). Differing from rats, maintaining elevated estradiol in parturient mice actually suppressed maternal aggression (31). Therefore, if estradiol is involved in promoting maternal aggression in mice it may be acting during pregnancy to prime neural circuitry to respond differently during lactation in the presence of an intruder. With evidence in males for a requirement for estrogen receptor α signaling in the VMN for the display of intermale aggression (32), further investigation into whether estrogen may have an analogous role in females is warranted. A combination of estradiol and prolactin hormonal cues combined with the suckling stimulus (33, 34) may be required to achieve an optimal balance of aggression behaviors, with prolactin serving to limit aggression and promote appropriate levels of social interactions.

It is clear from our data that prolonged Prlr signaling is necessary for prolactin-induced effects on maternal aggression to be observed. Acute manipulation of circulating prolactin failed to alter maternal aggression, with the majority of VMN neurons showing a lack of change in activity in response to acute prolactin administration ex vivo. This is consistent with what we have previously reported, where in most brain regions investigated, prolactin does not induce rapid changes in electrical activity of cells (18, 20). The arcuate nucleus is the exception to this. This region plays a vital role in the short-loop feedback regulation of

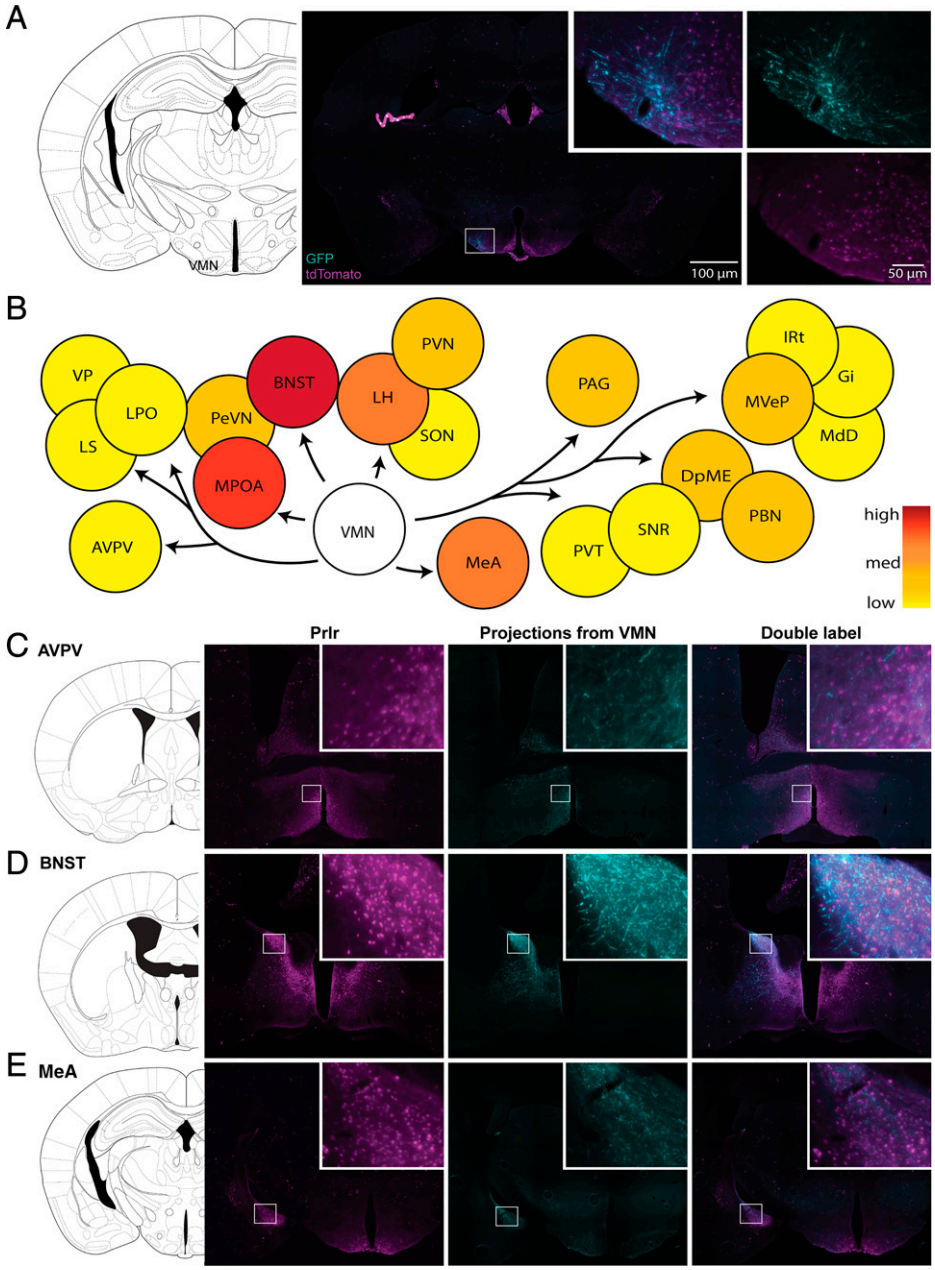


**Fig. 5.** Prlr-expressing neurons in the VMN contribute to an extensive prolactin-sensitive neural network. (*A*) Prlr-iCre/tdtomato mice (Prlr-expressing neurons express tdtomato, magenta) received a unilateral AAV2-EF1a-DIO-hChR2(H13R)-eYFP-WPRE into the VMN to drive Cre-dependent expression of YFP (GFP immunolabeling, cyan) specifically in the VMN. (*B*) Schematic representation of GFP-labeled projections from the VMN in the sagittal plane, with the heat map indicating relative amount of YFP-labeled fibers present in each brain region. AVPV, anteroventral periventricular region; BNST, bed nucleus of the stria terminalis; DpME, deep mesencephalic nucleus; Gi, gigantocellular reticular nucleus; IRt, intermediate reticular nucleus; LH, lateral hypothalamic area; LPO, lateral preoptic area; LS, lateral septal nucleus; MPOA, medial preoptic area; MdD, medullary reticular nucleus; MeA, medial amygdala; MVeP, medullary vestibular nucleus; PAG, periaqueductal gray; PBN, parabrachial nucleus; PeVN, periventricular hypothalamic nucleus; PVN, paraventricular nucleus; PVT, paraventricular thalamic nucleus; SON, supraoptic nucleus; SNR, substantia nigra, reticular part; VMN, ventromedial nucleus; VP, ventral pallidum. (*C–E*) Representative sections illustrating the distribution of tdtomato labeling showing Prlr expression (magenta), YFP-positive projections (cyan), and the composite images through the forebrain at the level of the AVPV (*C*), BNST (*D*), and MeA (*E*), showing low, high, and medium levels of fiber labeling, respectively.

prolactin secretion, and rapid responses to circulating levels of prolactin are necessary to maintain low basal levels of prolactin in nonpregnant and nonlactating individuals (35). The requirement for chronic, as opposed to acute, action of prolactin to alter behavior is likely an optimal arrangement, meaning short-term fluctuations in prolactin such as induced by stress or during the estrous cycle, would not have a major effect on behavior, whereas chronic elevations in prolactin during pregnancy and lactation could exert these behavioral changes. We suggest that chronically elevated prolactin is likely to be acting through a transcriptional pathway in the VMN to alter the transcriptional signature of neuronal populations during specific reproductive periods when prolactin is elevated, such as during pregnancy and lactation. Future examination of transcriptional changes in prolactin-sensitive populations of neurons across different reproductive states could provide profound insight into how lactation-specific behaviors are

elicited in response to the presence of offspring or other social stimuli. In the VMN, we hypothesize that prolactin to some extent is countering the normal pregnancy and/or lactation-induced changes that enable maternal aggression to be displayed.

As prolactin-sensitive VMN neurons project to several other key regions in the regulation of maternal behavior, notably the MPOA, BNST, and the MeA, prolactin could be acting in the VMN to influence multiple aspects of maternal behavior. Our data showing that prolactin-sensitive VMN neurons that modulate maternal aggression project to the BNST and MeA provides key evidence in support of the model of a female aggressive circuit proposed by Chen and Hong (3). Although, in the regulation of aggressive behavior, the VMN is typically thought to lie downstream of the MeA (3), our data suggest that the VMN may be involved in reciprocal modulation of activity with the MeA. Indeed, the fact that hyperaggression was only seen in the presence of a male but not female intruder in mice with a VGlut2 neuron-specific deletion of the Prlr, suggests processing of olfactory or hormonal cues from intruders (36), likely involving the MeA and BNST, are influencing VMN prolactin-sensitive neurons.

The most well-characterized role for prolactin in regulating behavior is the requirement for prolactin action in the MPOA to facilitate the onset of postpartum nursing behavior (11, 37, 38). We propose that the role for prolactin demonstrated here, in acting on glutamatergic neurons in the VMN to restrain maternal aggression, is consistent with an overall role for prolactin in promoting offspring-directed maternal behaviors. Through actions in multiple aspects of the maternal neural network, prolactin is able to both limit the display of nonoffspring-directed maternal behavior and promote increased mother–offspring interactions.

## Methods

**Animals.** The generation and genotyping of *Prlr*$^{lox/lox}$ and Prlr-iCre mice have previously been reported (15, 23), with the genetic crosses used in this study detailed elsewhere (*SI Appendix, SI Methods*). Groups of wild-type C57BL/6J female mice were used to assess the acute effect of prolactin on intruder-directed interactions and as an additional control group in the VMN-specific Prlr deletion experiment. All female mice were used as adults (8 to 16 wk) and were group housed prior to mating or behavioral testing. Animals used as virgins were individually housed 1 wk prior to behavior testing. To generate lactating groups of animals, females were individually housed with a stud wild-type C57BL/6J male mouse. Successful mating was confirmed by the presence of a vaginal plug, the males were removed, and day of parturition was recorded as day 1 of lactation. All animals were housed under conditions of controlled temperature (22 °C) and lighting (12-h light and 12-h dark cycles with lights on at 06:00 h), with ad libitum access to food and water. All animal experiments were approved by the University of Otago Animal Ethics Committee.

**Stereotaxic Injections of AAV.** Adult mice (8 to 12 wk old) were anesthetized with isoflurane and placed in a stereotaxic apparatus. For VMN-specific deletion of the Prlr gene, groups of *Prlr*$^{lox/lox}$ mice ($n$ = 8/group) and a wild-type C57BL/6J group ($n$ = 6) received bilateral 0.5-μL injections of AAV/DJ-CMV-mCherry-iCre (titer 1.4 × $10^{13}$ genomic copies/mL; Vector Biosystems Inc.) or control AAV/DJ-CMV-mCherry (titer 3.7 × $10^{13}$ genomic copies/mL; Vector Biosystems Inc.) into the VMN. For chemogenetic experiments, Prlr-iCre female mice received bilateral 0.5-μL injections of AAV2-hSyn-DIO-hM3D(Gq)-mCherry (titer 1.4 × $10^{13}$ genomic copies/mL, Addgene No. 44361; $n$ = 10) or control 0.5 μL AAV5-hSyn-DIO-mCherry (titer 1.4 × $10^{13}$ genomic copies/mL; Addgene No. 50459; $n$ = 7) into the VMN. For tract tracing experiments, Prlr-iCre/tdtomato females received unilateral 0.3-μL injections of AAV5-EF1a-DIO-hChR2(H13R)-eYFP-WPRE (titer 1.0 × $10^{13}$ genomic copies/mL; Addgene No. 20298; $n$ = 3) into the VMN. All injections were given at a rate of 100 nL/min, and syringes were left in situ for 3 min before and 5 min after injections. Coordinates for the VMN were 1.1 mm posterior to Bregma, 0.6 mm lateral to midline, and 5.4 mm deep.

**Immunohistochemistry.** 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 brain from each animal were cut using a sliding microtome.

To evaluate Prlr expression in the VMN and arcuate nucleus of *Prlr*$^{lox/lox}$/VGlut2-Cre mice and in AAV-Cre or AAV-control injected *Prlr*$^{lox/lox}$ and C57BL/6J mice, one series of brain tissue each was used to examine EGFP immunoreactivity (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) by chromogenic and fluorescent immunohistochemistry, respectively (*SI Appendix, SI Methods*). A third set of brain sections from *Prlr*$^{lox/lox}$/VGlut2-Cre mice was used to examine GFP and ERα immunolabeling by dual-label fluorescent immunohistochemistry (*SI Appendix, SI Methods*). To assess AAV-hM3Dq transfection, groups of AAV-mCherry/Prlr-iCre and AAV-hM3Dq/Prlr-iCre mice were administered 1.5 mg/kg CNO (saline/0.5% dimethyl sulfoxide [DMSO]; i.p.) 45 min prior to collection of brains. One series of tissue was used to label mCherry (as a marker of successful AAV transfection) and cFos (as a marker of CNO-induced cellular activation in AAV-hM3Dq administered mice) by dual label chromogenic immunohistochemistry (*SI Appendix, SI Methods*). To assess intruder-induced activation of Prlr-expressing neurons, groups of Prlr-iCre/tdtomato female mice ($n$ = 5 to 9/group) had a wild-type juvenile male mouse (19 to 25 d old) introduced to the home cage for 15 min at 10:00 h. Separate control groups of virgin and lactating mice were generated where mice were not exposed to an intruder. Mice were anesthetized 90 min following introduction of intruder and brains processed for cFos immunoreactivity (*SI Appendix, SI Methods*). To identify fiber projections from Prlr-expressing VMN cells, brains were collected from day-3 lactating Prlr-iCre/tdtomato mice injected with AAV5-EF1a-DIO-hChR2(H13R)-eYFP-WPRE. Brains were processed for GFP immunoreactivity to label YFP-expressing cell bodies and fibers (*SI Appendix, SI Methods*).

**Resident–Intruder Test.** To assess social interactions with an intruder, female aggression was tested using weaned juvenile male and female C57BL/6J mice (age 19 to 25 d old), and adult male and female C57BL/6J mice (age 3 to 6 mo). The intruder or a novel object was introduced into the home cage of individually housed virgin and lactating female mice for 15 min during the light phase (between 09:00 and 12:00 h). All test females were individually housed for at least 1 wk prior to testing, and if used as dams, the resident–intruder test was undertaken on days 2 to 3 of lactation (*SI Appendix, SI Methods*). Investigation was defined as active nose contact to any parts of the body of the intruder by the resident mouse. Attacks were defined by a collection of behaviors initiated by the resident toward the intruder, including pushes, lunges, bites, tumbling, and chasing. For investigating and attacking behavior, latency to first behavioral incident, total number of bouts, and total time spend were analyzed.

**hM3Di-Mediated Neuronal Activation.** For activating Prlr-expressing VMH neurons during lactation, Prlr-iCre female mice (8 to 12 wk) were mated with stud males 14 d following injection with either AAV2-hSyn-DIO-hM3D(Gq)-mCherry ($n$ = 10) or control AAV5-hSyn-DIO-mCherry ($n$ = 8) as reported above. On day 2 of lactation, mice were randomly assigned to receive either CNO (1.5 mg/kg in saline/0.5% DMSO; i.p.) or vehicle (saline/0.5% DMSO; i.p.). Resident–intruder tests were undertaken 45 min following CNO or vehicle administration. On day 4 of lactation, mice received the opposite treatment and were tested again in the resident–intruder test.

**Acute Manipulation of Prolactin.** To assess the effect of acute manipulation of circulating prolactin on intruder-directed behavior, groups of wild-type virgin and lactating C57BL/6J female mice were used. To test whether acute prolactin administration could induce lactation-like intruder-directed behavior, virgin mice were randomly assigned to receive 5 mg/kg ovine prolactin (phosphate buffered saline/130 mM NaCl; i.p.) or vehicle ($n$ = 12). Resident–intruder tests were undertaken 45 min following prolactin or vehicle administration. Two weeks later, mice received the opposite treatment and were tested again in the resident–intruder test. To assess whether acute suppression of endogenously high prolactin in lactating mice could abolish lactation-specific intruder-directed behavior, groups of day 2- to 4-lactating mice ($n$ = 10 to 11/group) were administered with either 100 μg bromocriptine (saline/5% ethanol; subcutaneously [s.c.]) or vehicle (saline/5% ethanol; s.c.) 2 h prior to resident–intruder testing. Bromocriptine is a D2 receptor agonist and is known to inhibit prolactin secretion from the pituitary gland and abolishes all prolactin-induced pSTAT5 immunoreactivity in the brain (18).

**Ex Vivo Brain Slice Electrophysiology and Calcium Imaging.** Intracellular calcium imaging and electrophysiological recordings were made from adult (10 to 20 wk) Prlr-iCre/GCaMP6f and Prlr-iCre/AAV-hM3Dq mice, respectively.

Coronal brain slices (200 μm) were illuminated through a 40× immersion objective, and epifluorescence images were (495 nm long pass and emission 500 to 520 nm) collected using a Hamamatsu ORCA-ER digital charged-coupled device camera (*SI Appendix, SI Methods*). For whole-cell recordings (*SI Appendix, SI Methods*), electrophysiological signals were recorded using a Multiclamp 700B amplifier connected to a Digidata 1440A digitizer (Molecular Devices). Signals were low-pass filtered at 2 kHz and digitized at a rate of 10 kHz. Signal acquisition was carried out with pClamp 10 (Molecular Devices). All pharmacological compounds were bath applied for 5 min following a 2-min baseline period (*SI Appendix, SI Methods*). For calcium imaging recordings, the responsiveness of fluorescent cells was tested by bath application of 20 mM KCl at the end of each recording. Relative changes in fluorescence (ΔF/F) for each region of interest were calculated, where F is the mean baseline fluorescence intensity. The fluorescence change was represented as a percentage.

**Data Availability.** All study data are included in the article and supporting information.

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