Male pheromone–stimulated neurogenesis in the adult female brain: possible role in mating behavior

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The regulation of female reproductive behaviors may involve memories of male pheromone signatures, formed in part by neural circuitry involving the olfactory bulb and hippocampus. These neural structures are the principal sites of adult neurogenesis; however, previous studies point to their independent regulation by sensory and physiological stimuli. Here we report that the pheromones of dominant (but not subordinate) males stimulate neuronal production in both the olfactory bulb and hippocampus of female mice, which are independently mediated by prolactin and luteinizing hormone, respectively. Neurogenesis induced by dominant-male pheromones correlates with a female preference for dominant males over subordinate males, whereas blocking neurogenesis with the mitotic inhibitor cytosine arabinoside eliminated this preference. These results suggest that male pheromones are involved in regulating neurogenesis in both the olfactory bulb and hippocampus, which may be important for female reproductive success.

The reproductive success of animals involves a complex interplay of behavioral and sensory responses leading to successful mating[1,2]. Pheromones, chemical cues involved in mating and reproduction[3,4], relay information about gender, species specification, social status, health and genetic advantage[5,6]. Although the olfactory epithelium receptors for male pheromones are conserved between fish, mice, and humans[7], their roles in regulating female mating and reproductive behaviors may be best understood in rodents, where hippocampal-dependent memories are formed for social recognition[8], pregnancy block is prevented by mate-specific pheromone memories[9], and females use pheromone signatures for mate recognition and selection to provide genetic advantage for future offspring[5].

The neural circuitry mediating olfactory discrimination and the formation of olfactory memories may include the olfactory bulb and dentate gyrus of the hippocampus[8,10], which are the principal structures where neurogenesis persists in the adult mammalian brain[11,12]. In mice, new interneurons derived from progenitors of the forebrain subventricular zone (SVZ) migrate long distances along the rostral migratory stream to integrate into the olfactory bulb[13], where they appear to function in olfactory discrimination[14] and the formation of olfactory memories[15]. In the hippocampus, local neuronal progenitors of the subgranular zone give rise to new granule cells of the dentate gyrus, which have been implicated in the successful performance of certain learning and memory tasks[16,17] and in

mediating the actions of antidepressants[18]. Previous studies suggest that distinct physiological processes independently regulate olfactory bulb and dentate gyrus neurogenesis. For example, external stimuli such as running[19], enriched environments[20] and learning[21] increase dentate gyrus neurogenesis independent of olfactory bulb neurogenesis[21,22], whereas pregnancy and the act of mating increase olfactory bulb neurogenesis and leave dentate gyrus neurogenesis unaffected[23]. Surprisingly, little attention has been given to understanding why and how these two regions have evolved into the principal sites of ongoing adult neurogenesis and whether an association may exist between adult neurogenesis in both the olfactory bulb and dentate gyrus, in an ethological context.

Given the olfactory[24,25] and hippocampal[8] involvement in pheromone-dependent mating and the reproductive behaviors of female mice, we asked whether male pheromones would influence neurogenesis in these two structures of the adult female brain and whether this neurogenesis, in turn, is important in the manifestation of pheromone-dependent behavior. Here, we demonstrate that dominant-male pheromones stimulate olfactory bulb and dentate gyrus neurogenesis in the adult female brain and that this is mediated by the hormones prolactin and luteinizing hormone, respectively. Moreover, we found that disruption of olfactory bulb and dentate gyrus neurogenesis eliminates a female's natural preference for a dominant male. We conclude that adult neurogenesis may have a role in mate preference behaviors.

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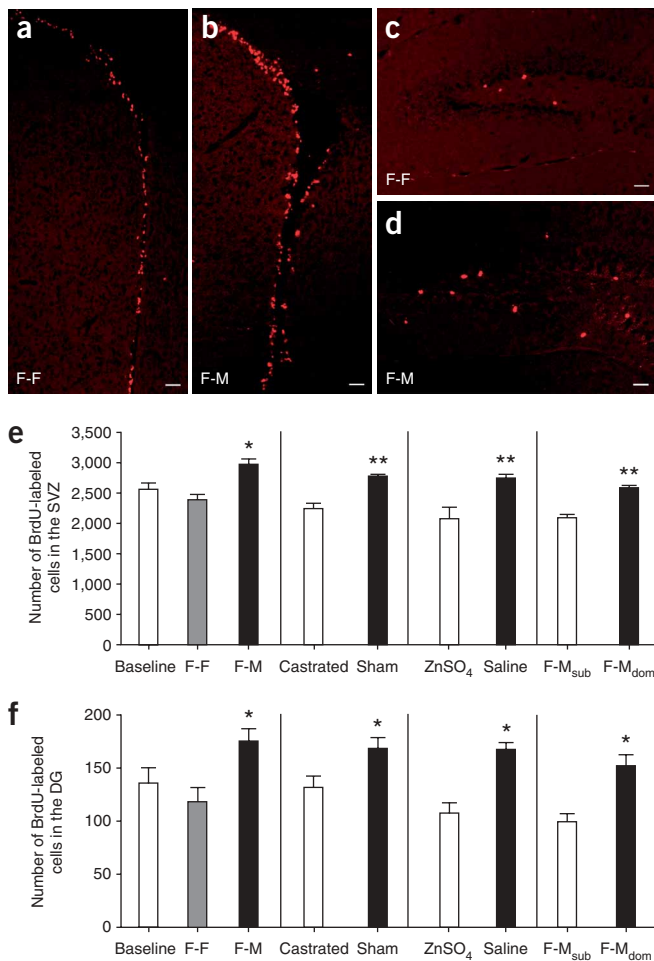
Figure 1 Male pheromones simultaneously enhance cell proliferation in the SVZ and dentate gyrus. (a–d) Fluorescence micrographs of the forebrain SVZ (a,b) and hippocampal dentate gyrus (DG) (c,d) showed increased BrdU labeling of females exposed to male pheromones (F-M) compared with females exposed to female pheromones (F-F). (e,f) Quantification (mean ± s.e.m.) showed an increase in the number of BrdU-labeled cells in the SVZ (e) and dentate gyrus (f) of F-M, which was absent when females were exposed to castrated-male odors compared with those of sham-operated males, or when females received intranasal $ZnSO_4$ (or saline) irrigation and subsequently were exposed to male odors, or when they were exposed to the subordinate-male pheromones compared with those of dominant males. In e: F-M versus F-F, $**P = 0.0016$, F-M versus baseline, $*P = 0.028$, $n = 6$ animals per group. Sham versus castrated, $*P = 0.001$, $n = 12$ animals per group. Saline versus $ZnSO_4$, $*P = 0.0093$, $n = 5$ animals per group. F-M$_{sub}$ versus F-M$_{dom}$, $**P = 0.0018$, $n = 3$ animals per group. In f: F-M versus F-F, $*P = 0.018$, F-M versus baseline, $*P = 0.048$, $n = 6$ animals per group. Sham versus castrated, $*P = 0.018$, $n = 12$ animals per group. Saline versus $ZnSO_4$, $*P = 0.010$, $n = 5$ animals per group. F-M$_{sub}$ versus F-M$_{dom}$, $*P = 0.019$, $n = 3$ animals per group. Scale bars represent 20 μm in all panels.

RESULTS

Pheromones increase SVZ and dentate gyrus cell proliferation

We exposed 8–10-week-old female mice to odors from age-matched male mice, using soiled bedding that was renewed every other day for 2- and 7-d periods (**Supplementary Fig. 1** online). Bedding was from the same male mice odor throughout the duration of the 7-d time course. Similarly, control females were exposed to female odors or clean cage bedding (baseline). Bromodeoxyuridine (BrdU) injections were given to females on the final day of odor exposure and the number of proliferating cells in the SVZ and dentate gyrus were counted (with a representative slide consisting of ten 14-μm coronal sections from the rostral to caudal portion of the lateral ventricles and hippocampus; see **Supplementary Methods** online). BrdU analysis at 2 d of exposure did not show significant differences (SVZ: F-M versus F-F, $P = 0.34$, and F-M versus baseline $P = 0.25$; dentate gyrus: F-M versus F-F, $P = 0.14$, and F-M versus baseline, $P = 0.13$; F-M, female-male; F-F, female-female) between the three groups (data not shown); however, after 7 d of exposure to male odors, the number of BrdU-labeled cells increased significantly (SVZ: F-M versus F-F, $P = 0.018$, F-M versus baseline, $P = 0.048$; dentate gyrus: F-M versus F-F, $P = 0.018$, F-M versus baseline, $P = 0.048$) in the SVZ (+23%) and in the dentate gyrus (+38%) compared with controls (**Fig. 1**). Analysis of the number of cells labeled with Ki67 (an antibody detecting an antigen expressed in dividing cells) confirmed the simultaneous increase in proliferating cells in the SVZ and dentate gyrus of females exposed to male odors, relative to controls (**Supplementary Fig. 2** online). Females exposed to novel odors of coconut or almond diluted 1:5 in PBS for 7 d did not

show increased proliferation in the SVZ or dentate gyrus compared with females exposed to male odors or PBS controls (**Supplementary Fig. 3a,b** online). We hypothesized that male pheromones, present in the urine deposited in the soiled bedding, provide the sensory stimuli responsible for increased female SVZ and dentate gyrus proliferation. Given that pheromones can be removed from urine by eliminating circulating testosterone[26], we exposed female mice to the soiled bedding of castrated and sham-operated males for 7 d, using the same methodology as above. Females exposed to castrated-male odors did not show increased cell proliferation in the SVZ or dentate gyrus compared with females exposed to sham-operated male odors (**Fig. 1e,f**). These results suggest that pheromones are the component of male odor that simultaneously increases cell proliferation in the two neurogenic regions of the adult female mammalian brain.

The main olfactory system is important in female mate recognition and sexual behaviors, as the destruction of the main olfactory epithelium with intranasal $ZnSO_4$ irrigation abolishes female responsiveness to volatile male urinary odors, decreases olfactory investigation and preference for noncastrated males, and reduces lordosis behavior, despite an intact vomeronasal system[27]. Therefore, to determine whether male pheromone–induced SVZ and dentate gyrus proliferation is mediated through the main olfactory system, we exposed $ZnSO_4$-treated females to male pheromones using the same methodology as described above (and compared them with saline-treated female controls). $ZnSO_4$-treated females exposed to male pheromones for 7 d did not show an increase in SVZ or dentate gyrus proliferation compared with saline-treated controls (**Fig. 1e,f**), thus establishing the importance of the female's main olfactory system in male pheromone–induced proliferation. Female mice not only prefer intact males over castrated males, but also show a preference for dominant-male odors[28] and prefer to mate with dominant males when permitted[29]. Given these preferences, we asked whether dominant-male pheromones specifically increase cell proliferation in the female SVZ and dentate gyrus. Using the same methodology as described above, we exposed females to a mixture of dominant and subordinate-male pheromones for 2 d (which itself does not increase neurogenesis, but allows naive females to experience dominant- and subordinate-male pheromones) and then placed them in a clean cage for 4 d. Later, we exposed these females to pheromones from a single dominant or subordinate male for 7 d and counted the number of BrdU-labeled cells in the SVZ and dentate gyrus. In comparison with pheromones from subordinate males, females exposed to dominant-male pheromones showed a 23% increase in the number of BrdU-labeled cells in
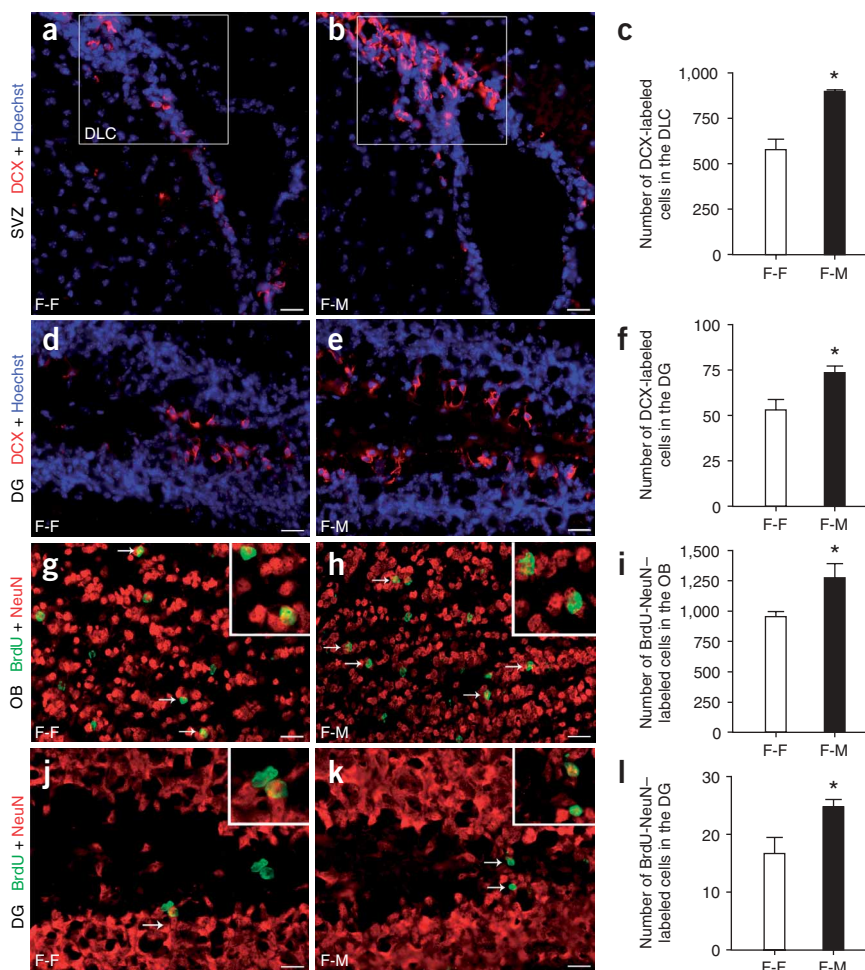
Figure 2 Male pheromones simultaneously increase neurogenesis in the olfactory bulb and dentate gyrus. (**a**–**f**) Fluorescence micrographs of the dorsolateral corner (DLC) of the SVZ (**a**,**b**) and the hippocampal DG (**d**,**e**) show an increase in doublecortin (DCX) labeling in females exposed to male pheromones (F-M) compared with females exposed to female pheromones (F-F). This increase was confirmed by quantifying (mean ± s.e.m.) the number of DCX-labeled cells in the DLC (**c**) and dentate gyrus (**f**). In **c**: F-M versus F-F, *$P = 0.019$, $n = 6$ animals per group. In **f**: F-M versus F-F, *$P = 0.031$, $n = 6$ animals per group. (**g**–**l**) Fluorescence micrographs of the olfactory bulb (OB) (**g**,**h**) and dentate gyrus (**j**,**k**) showed increased number of BrdU-NeuN double-labeled cells in F-M, 4 weeks after the final BrdU injection. Counting the number of dual-labeled cells showed an increase in the number of newly generated OB interneurons (**i**) and DG granule cells (**l**) of F-M treated females. In **i**: F-M versus F-F, *$P = 0.023$, $n = 5$ animals per group. In **l**: F-M versus F-F, *$P = 0.017$, $n = 5$ animals per group. In **a**,**b**,**d**,**e** DCX is labeled in red and Hoechst in blue. In **g**,**h**,**j**,**k** BrdU is labeled in green and NeuN in red. Scale bars represent 50 μm in all panels.

the dentate gyrus (**Fig. 2g**–**l**), where NeuN is a marker for mature neurons. These increases were not a result of decreased apoptosis, as no differences in TUNEL labeling were detected. Furthermore, BrdU/NeuN double-labeled cells were not detected in the accessory olfactory bulb (data not shown). Therefore, these findings suggest that male pheromones, acting through the main olfactory system, simultaneously increase both cell proliferation and the production of new neurons in the adult female olfactory bulb and dentate gyrus.

PRL and LH receptors in the SVZ and dentate gyrus

Male pheromones elicit endocrine changes in recipient females, leading to behavioral and physiological changes, such as the initial proestrus increase in estrogen that induces a surge in luteinizing hormone (LH) and prolactin (PRL)[2,24,25,32,33]. In our study, we found that females were at random stages of the estrus cycle before pheromone exposure, but synchronized to proestrus on the seventh day of exposure to male pheromones; females exposed to female pheromones or clean cage bedding remained at random stages of the estrus cycle during the 7-d exposure (data not shown). Nevertheless, despite being at random points in their estrus cycle, we detected little variance in their baseline neural proliferation. Given that we previously found PRL to be a mediator of olfactory neurogenesis in pregnant females[23], we asked whether PRL or LH mediates pheromone-induced neurogenesis.

We first determined whether receptors for PRL and LH were expressed in both the SVZ and dentate gyrus (**Fig. 3**). Previously, we have found that the PRL receptor (PRLR) is expressed in the choroid plexus and dorsolateral corner of the SVZ in the adult female forebrain[23]. This was confirmed by detecting PRLR immunoreactivity in the choroid plexus and dorsolateral corner of the SVZ (**Fig. 3a**); however, the same antibody did not detect immunoreactivity in the dentate gyrus (**Fig. 3c**). RT-PCR also showed PRLR expression in the SVZ (sample includes both SVZ and choroid plexus) through

the SVZ and a 29% increase in the dentate gyrus (**Fig. 1e**,**f**). Taken together, these findings suggest that dominant-male pheromones selectively stimulate proliferation in the female SVZ and dentate gyrus.

Pheromone-induced olfactory and dentate gyrus neurogenesis

Newly generated cells of the SVZ die, generate glia, or become neuroblasts that migrate to the olfactory bulb and differentiate into granule and periglomerular interneurons[11,30], whereas those of the SGZ migrate into the hippocampal dentate gyrus and differentiate into granule cells[30]. We used the neuronal progenitor marker doublecortin (DCX), a microtubule-associated phosphoprotein[31], to determine whether a 7-d exposure to male pheromones would increase the production of neuronal precursors in the female SVZ and dentate gyrus. There was a 55% increase in the number of DCX-labeled cells in the SVZ and a 38% increase in the dentate gyrus of females exposed to male pheromones compared with controls (**Fig. 2a**–**f**). Furthermore, the increases in the numbers of DCX-labeled cells in both structures of the female brain were significantly less when the mice were exposed to the odors of castrated males compared with sham-operated controls (**Supplementary Fig. 4a**–**f** online). We then assessed the fate of the newly generated cells in the SVZ and dentate gyrus of females exposed to male pheromones for 7 d, by examining their phenotypic properties 4 weeks after a 10-h series of BrdU injections. In females exposed to male pheromones, there were 38% more BrdU/NeuN double-labeled cells in the olfactory bulb and 47% more double-labeled cells in
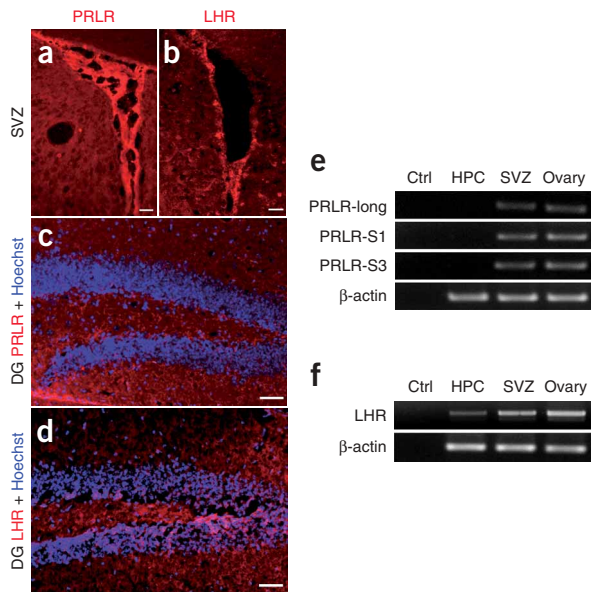
Figure 3 PRLR is expressed in the SVZ and choroid plexus, whereas LHR is expressed in both the SVZ and dentate gyrus of female mice. (**a–d**) Fluorescence micrographs showed PRLR immunoreactivity (**a**) in the choroid plexus and dorsolateral corner of the forebrain SVZ; however, LHR immunoreactivity is only detected along the length of the SVZ (**b**). Moreover, although PRLR immunoreactivity is absent in the DG (**c**), there is clear evidence for LHR immunoreactivity (**d**). (**e,f**) RT-PCR of ovary (positive control), SVZ/choroid plexus (SVZ), hippocampus (HPC) and water control (Ctrl) demonstrated expression of the PRLR-long, PRLR-S1 and PRLR-S3 subunits in only the ovary and SVZ (**e**), whereas LHR expression was detect in the ovary, SVZ and HPC (**f**). Scale bars represent 20 µm in all panels.

the detection of the PRLR-long, PRLR-S1 and PRLR-S3 isoforms, none of which were detected in the dentate gyrus (**Fig. 3e**). We then examined LH receptor (LHR) expression in the SVZ and dentate gyrus with an antibody against LHR. We detected LHR immunoreactivity along the length of the SVZ (**Fig. 3b**), but unlike PRLR, LHR immunoreactivity was also detected in the dentate gyrus (**Fig. 3d**). The expression of LHR in both the SVZ and dentate gyrus was confirmed by detecting the appropriate (by sequence analysis) 703-kb RT-PCR product in the SVZ, dentate gyrus and ovary control tissue (**Fig. 3f**).

LH and PRL mediate enhanced neurogenesis
Because LH is elevated at proestrus and its receptor is expressed in both neurogenic regions, we hypothesized that LH was mediating male pheromone–induced neurogenesis in females. To test this, we delivered LH subcutaneously for 2 d into 8–10-week-old female mice via osmotic pump (16 µg per d, 2 d). LH induced a 29% and 53% increase in the number of BrdU-labeled cells in the SVZ and dentate gyrus, respectively (**Fig. 4a–f**), mimicking the simultaneous increase in proliferation observed when females were exposed to male pheromones for 7 d. LH given to ovariectomized female mice also resulted in increased SVZ and dentate gyrus proliferation, supporting a direct action of LH in the dentate gyrus, rather than indirectly through estrogen release (data not shown). Similar to male pheromones, LH increased the numbers of DCX-labeled cells in the SVZ (+43%: LH infused, 1575 ± 321; vehicle infused, 1103 ± 191; $n = 4$, $P = 0.016$) and dentate gyrus (+26%: LH infused, 67 ± 7; vehicle infused, 53 ± 4; $n = 4$, $P = 0.024$) and the number of new neurons in the olfactory bulb (+41%: LH infused, 1025 ± 120; vehicle infused, 726 ± 98; $n = 4$, $P = 0.0042$) and dentate gyrus (+33%: LH infused, 24 ± 2; vehicle infused, 18 ± 2; $n = 4$, $P = 0.044$). These increases were not a result of apoptosis, as no differences in TUNEL were detected between LH- and vehicle-infused groups (data not shown). These findings strongly support LH as the candidate hormone mediating simultaneous male pheromone–induced neurogenesis in the female olfactory bulb and dentate gyrus.

We next hypothesized that female mice with a targeted disruption in the gene encoding LHR[34] would fail to show a simultaneous increase in neurogenesis when exposed to male pheromones. Female $Lhcgr^{+/+}$ and $Lhcgr^{-/-}$ mice were exposed to the soiled bedding of age-matched wild-type males for 7 d using the same procedure as the initial exposure

experiment. $Lhcgr^{+/+}$ and $Lhcgr^{-/-}$ females exposed to either wild-type female odors or clean cage bedding (baseline) served as controls. Both $Lhcgr^{+/+}$ and $Lhcgr^{-/-}$ females exposed to male pheromones showed a significant (+22%) increase in the number of BrdU-labeled cells in the SVZ compared with their respective baseline controls (**Fig. 4g**). However, $Lhcgr^{+/+}$ females exposed to male pheromones showed an increase (+30%) in the number of BrdU-labeled cells in the dentate gyrus, whereas $Lhcgr^{-/-}$ females did not (**Fig. 4h**). These findings did not support our hypothesis of LH mediating simultaneous male phero- mone–induced olfactory bulb and dentate gyrus neurogenesis. Rather, the result suggests that LH specifically mediates enhanced neurogenesis in the dentate gyrus. However, such a conclusion does not agree with the results of our 2-d LH subcutaneous infusion, which increased both olfactory bulb and dentate gyrus neurogenesis. Therefore we asked whether a dose-dependent effect of LH exists, given that our infusion concentration may significantly exceed physiological levels associated with pheromone detection. To test this, we infused 10% of the original dose (subcutaneous LH, 1.6 µg per d for 2 d) and examined cell proliferation in the SVZ and dentate gyrus. BrdU numbers in the SVZ of low-dose LH-infused females were not different from those treated with vehicle (LH infused: 2814 ± 84, $n = 4$; vehicle infused: 2578 ± 130, $n = 4$, $P = 0.093$), although a 35% increase in the number of BrdU-labeled cells in the dentate gyrus was detected (LH infused: 360 ± 26, $n = 4$; vehicle infused: 267 ± 21, $n = 4$, $P = 0.016$). Taken together, these results suggest that pheromone-induced neurogenesis in the dentate gyrus appears to be mediated by LH.

As male pheromones elevate both LH and PRL, we asked whether PRL mediates the female olfactory bulb component of male phero- mone–induced neurogenesis. Infusions of PRL (subcutaneous, 16 µg per d for 2 d) increased the number of BrdU-labeled cells in the SVZ, but not in the dentate gyrus (**Fig. 4c,f**), as previously reported[23]. We then exposed female $Prlr^{+/+}$ and $Prlr^{-/-}$ mice to age-matched male pheromones for 7 d. Female $Prlr^{+/+}$ and $Prlr^{-/-}$ mice exposed to wild- type female odors or clean bedding (baseline) served as controls. $Prlr^{+/+}$ females exposed to male pheromones showed a significant increase in the number of BrdU-labeled cells in the SVZ, whereas similarly exposed $Prlr^{-/-}$ females did not ($P = 0.007$; **Fig. 4i**). Both $Prlr^{+/+}$ and $Prlr^{-/-}$ females exposed to male pheromones showed a significant increase in the number of BrdU labeled cells in the dentate gyrus ($P = 0.001$; **Fig. 4j**). These finding suggest that PRL mediates the olfactory bulb component of male pheromone–induced neurogenesis.

A role for neurogenesis in adult female mate preference
Females prefer the odors of dominant males and selectively mate with dominant males[28,29]. Thus, we hypothesized that neurogenesis induced by dominant-male pheromones may have a role in this female preference behavior. Females were pre-exposed to a mixture of male pheromones for 2 d and then exposed to either dominant- or subordinate-male pheromones for 7 d, as described above. Females were habituated to a test cage (adapted from references[29,30]), which
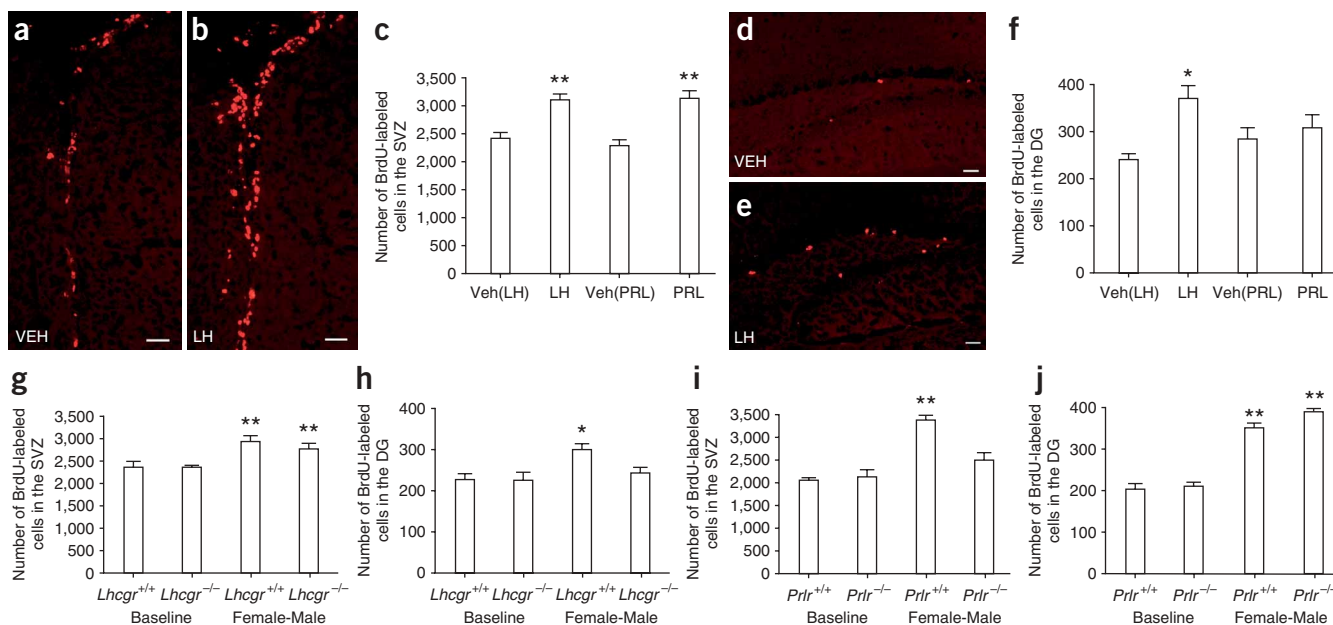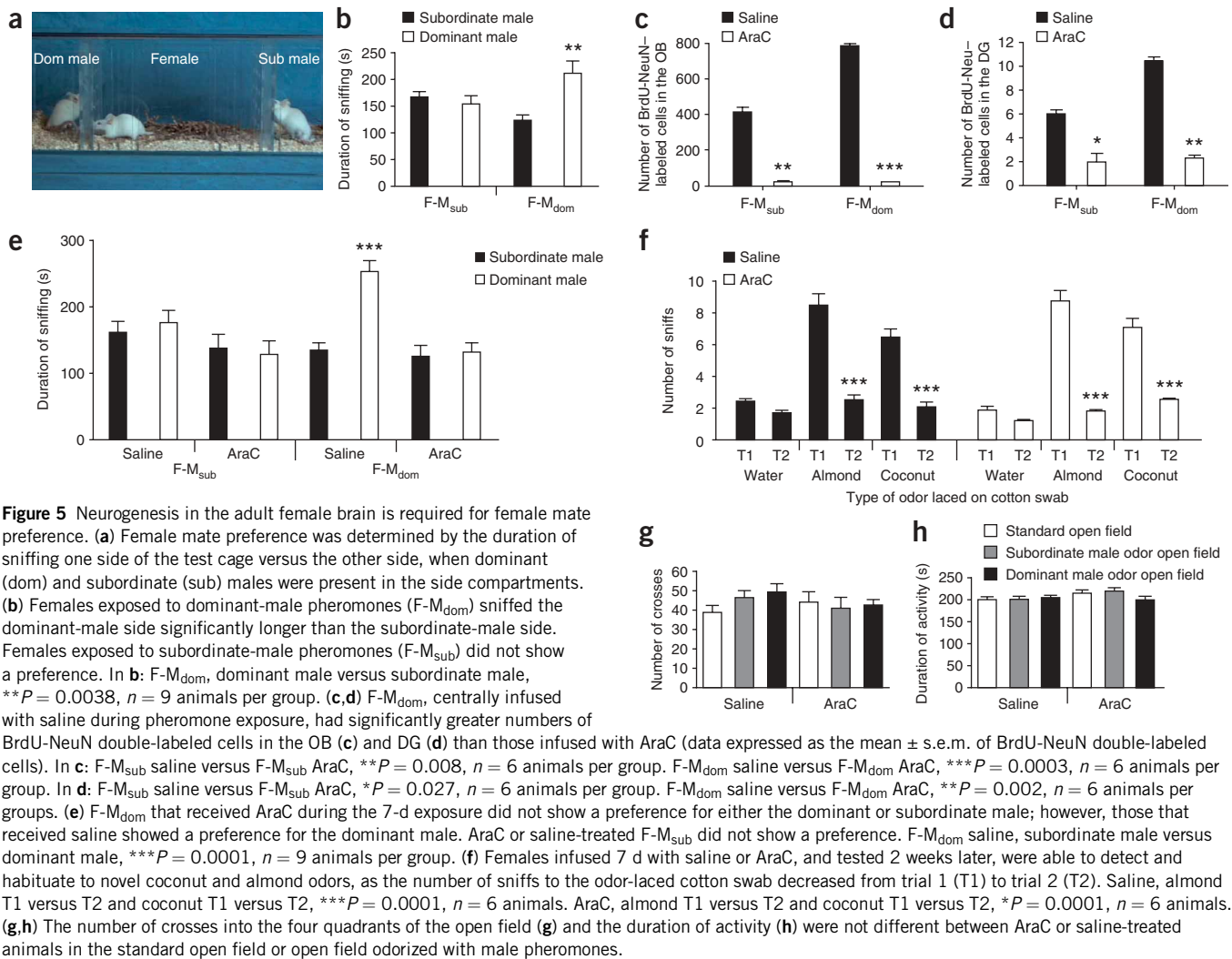
Figure 4 LH and PRL independently mediate male pheromone–induced cell proliferation in the DG and SVZ, respectively. (a–f) Fluorescence micrographs of the forebrain SVZ (a,b) and hippocampal DG (d,e) showed an increase in the number of BrdU-labeled cells in mice given subcutaneous infusions of LH compared with vehicle. Quantifying (mean ± s.e.m.) the number of BrdU-labeled cells in the SVZ showed an increase in LH- and PRL-infused animals compared with controls (c), whereas only LH-infused animals showed an increase in the DG (f). In c: LH versus Veh(LH), $**P = 0.006$, PRL versus Veh(PRL), $**P = 0.003$, $n = 4$ animals per group. In f: LH versus Veh(LH), $*P = 0.013$, $n = 4$ animals per group. (g,h) BrdU-labeled cells counted in the SVZ (g) showed an increase in both $Lhcgr^{-/-}$ and $Lhcgr^{+/+}$ females exposed to male pheromones, whereas BrdU-labeled cells counted in the DG (h) showed an increase in $Lhcgr^{+/+}$ females only. In g: $Lhcgr^{-/-}$ F-M versus baseline $Lhcgr^{-/-}$, $**P = 0.008$, $Lhcgr^{+/+}$ F-M versus baseline $Lhcgr^{+/+}$, $**P = 0.001$, $n = 6$ animals per group. In h: $Lhcgr^{+/+}$ F-M versus baseline $Lhcgr^{+/+}$, $*P = 0.003$, $Lhcgr^{+/+}$ F-M versus $Lhcgr^{-/-}$ F-M, $*P = 0.01$, $n = 6$ animals per group. (i,j) On the other hand, BrdU-labeled cells in the SVZ (i) increased only in $Prlr^{+/+}$ females exposed to male pheromones compared with baseline, whereas both $Prlr^{-/-}$ and $Prlr^{+/+}$ females showed an increase in the number of BrdU-labeled cells in the DG (j) when exposed to male pheromones. In i: $Prlr^{+/+}$ F-M versus baseline $Prlr^{+/+}$, $**P = 0.001$, $Prlr^{+/+}$ F-M versus $Prlr^{-/-}$ F-M, $**P = 0.007$, $n = 3$ animals per group. In j: $Prlr^{-/-}$ F-M versus baseline $Prlr^{-/-}$, $**P = 0.001$, $Prlr^{+/+}$ F-M versus baseline $Prlr^{+/+}$, $**P = 0.001$, $n = 3$ animals per group. Scale bars represent 20 μm in all panels.

allows exchange of olfactory stimuli, but prevents physical contact through perforated dividers, 2 weeks after the odor exposure (**Fig. 5a**). To test preference, females were individually placed in the center compartment, and a dominant and subordinate male, whose pheromones the female had originally been pre-exposed to, were placed randomly in one of the side compartments. Female preference was determined by the duration of time spent sniffing the side compartments. Females exposed to dominant-male pheromones, and therefore with increased olfactory bulb and dentate gyrus neurogenesis, preferred the dominant male, whereas females exposed to subordinate-male pheromones did not show a preference (**Fig. 5b**). Furthermore, females pre-exposed to a mixture of male pheromones for 2 d and then exposed to dominant-male pheromones for 2 d (which does not increase neurogenesis; see **Supplementary Fig. 5a,b** online) did not show a preference when tested 2 weeks later (**Supplementary Fig. 5c**). Likewise, $Prlr^{-/-}$ females exposed to dominant-male pheromones for 7 d (using the method described above) did not show a preference for the dominant or subordinate male when tested 2 weeks later (**Supplementary Fig. 6** online). However, $Prlr^{+/+}$ females exposed to dominant-male pheromones did show a preference for the dominant male.

Next, we inhibited neurogenesis through intracerebroventricular infusions of the mitotic blocker cytosine arabinoside (AraC), which prevents SVZ neural progenitor cell division[35] and inhibits endogenous adult hypothalamic neurogenesis and weight regulation[36]. Females that received AraC for 7 d, during the 7-d exposure to dominant- or subordinate-male pheromones, had significantly reduced (olfactory bulb: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, $P = 0.008$, F-M$_{dom}$ saline

versus F-M$_{dom}$ AraC, $P = 0.0003$; dentate gyrus: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, $P = 0.027$, F-M$_{dom}$ saline versus F-M$_{dom}$ AraC, $P = 0.002$) numbers of BrdU/NeuN double-labeled cells in the olfactory bulb (–96% F-M$_{dom}$ and –95% F-M$_{sub}$; **Fig. 5c, Supplementary Fig. 7a–d** online) and dentate gyrus (–77% F-M$_{dom}$ and –67% F-M$_{sub}$; **Fig. 5d, Supplementary Fig. 7e–h**) compared with those receiving control saline infusions, thus confirming the efficacy of AraC. Notably, we found that females exposed to dominant-male pheromones while receiving AraC did not show a subsequent preference for dominant or subordinate males, whereas those receiving saline showed dominant-male preference (**Fig. 5e**). Furthermore, females receiving AraC for 2 d during the 7-d exposure to dominant- or subordinate-male pheromones also showed significantly reduced (olfactory bulb: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, $P = 0.0001$, F-M$_{dom}$ saline versus F-M$_{dom}$ AraC, $P = 0.001$; dentate gyrus: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, $P = 0.020$, F-M$_{dom}$ saline versus F-M$_{dom}$ AraC, $P = 0.0003$) numbers of BrdU/NeuN double-labeled cells in the olfactory bulb (–97% F-M$_{dom}$ and F-M$_{sub}$; **Supplementary Fig. 8a** online) and the dentate gyrus (–68% F-M$_{dom}$ and –57% F-M$_{sub}$; **Supplementary Fig. 8b**). Once again, the attenuation of neurogenesis in females exposed to dominant-male pheromones with a short 2-d infusion of AraC resulted in a loss of preference for the dominant male (**Supplementary Fig. 8c**). Notably, females treated with AraC or saline both detected and habituated to novel odors, as demonstrated by an initial increase in the number of sniffs when presented with almond- or coconut-laced cotton swabs and a subsequent decline during the second presentation[37] (**Fig. 5f, Supplementary Fig. 9** online).

Figure 5 Neurogenesis in the adult female brain is required for female mate preference. (**a**) Female mate preference was determined by the duration of sniffing one side of the test cage versus the other side, when dominant (dom) and subordinate (sub) males were present in the side compartments. (**b**) Females exposed to dominant-male pheromones (F-M$_{dom}$) sniffed the dominant-male side significantly longer than the subordinate-male side. Females exposed to subordinate-male pheromones (F-M$_{sub}$) did not show a preference. In **b**: F-M$_{dom}$, dominant male versus subordinate male, **$P = 0.0038$, $n = 9$ animals per group. (**c**,**d**) F-M$_{dom}$, centrally infused with saline during pheromone exposure, had significantly greater numbers of BrdU-NeuN double-labeled cells in the OB (**c**) and DG (**d**) than those infused with AraC (data expressed as the mean ± s.e.m. of BrdU-NeuN double-labeled cells). In **c**: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, **$P = 0.008$, $n = 6$ animals per group. F-M$_{dom}$ saline versus F-M$_{dom}$ AraC, ***$P = 0.0003$, $n = 6$ animals per group. In **d**: F-M$_{sub}$ saline versus F-M$_{sub}$ AraC, *$P = 0.027$, $n = 6$ animals per group. F-M$_{dom}$ saline versus F-M$_{dom}$ AraC, **$P = 0.002$, $n = 6$ animals per groups. (**e**) F-M$_{dom}$ that received AraC during the 7-d exposure did not show a preference for either the dominant or subordinate male; however, those that received saline showed a preference for the dominant male. AraC or saline-treated F-M$_{sub}$ did not show a preference. F-M$_{dom}$ saline, subordinate male versus dominant male, ***$P = 0.0001$, $n = 9$ animals per group. (**f**) Females infused 7 d with saline or AraC, and tested 2 weeks later, were able to detect and habituate to novel coconut and almond odors, as the number of sniffs to the odor-laced cotton swab decreased from trial 1 (T1) to trial 2 (T2). Saline, almond T1 versus T2 and coconut T1 versus T2, ***$P = 0.0001$, $n = 6$ animals. AraC, almond T1 versus T2 and coconut T1 versus T2, *$P = 0.0001$, $n = 6$ animals. (**g**,**h**) The number of crosses into the four quadrants of the open field (**g**) and the duration of activity (**h**) were not different between AraC or saline-treated animals in the standard open field or open field odorized with male pheromones.

Presenting a water-soaked cotton swab before the novel odors shows that increased investigation was not a result of the novelty of the cotton swab (**Fig. 5f**). Additionally, AraC (that gained weight[36])- and saline-treated females showed normal levels of activity during standard open field analysis, as no differences were detected in the number of crosses into the four quadrants of the open field and the duration of ambulatory activity (**Fig. 5g,h**). Similarly, the number of crosses and the duration of activity between AraC- and saline-treated females in an open field environment odorized by either dominant or subordinate-male pheromones was not significantly different (saline-treated females, dominant versus subordinate: number of crosses, $P = 0.33$, duration of activity, $P = 0.32$; AraC-treated females, dominant versus subordinate: number of crosses, $P = 0.41$, duration of activity, $P = 0.18$; **Fig. 5g,h**).

DISCUSSION

Male pheromone signatures are used by female mice to identify prospective mates and olfactory memories of these differing signatures may be formed, in part, by neural circuitry involving the olfactory bulb and hippocampus[8]. A female mouse prefers to mate with the dominant male in her territory, and thus processing his pheromonal signature promotes endocrine changes associated with mating[5,28,32]. Our study establishes an association between female mating preference elicited by dominant-male pheromones and neurogenesis in the olfactory bulb

and dentate gyrus, whose enhanced stimulation is mediated by the independent actions of PRL and LH, respectively.

Our findings reveal that PRL signaling, which is known to have a fundamental role in reproductive functions and behaviors[23,38], specifically regulates female olfactory neurogenesis in response to male pheromones. In contrast, LH signaling, demonstrated to be under the control of pheromone-mediated neural circuitry in the olfactory bulb and a known mediator of social and reproductive behaviors[24,25,39], specifically regulates the female dentate gyrus neurogenic response to male pheromones. Not surprisingly, both $Prlr^{-/-}$ and $Lhcgr^{-/-}$ female mice have multiple abnormalities impairing their ability to mate and reproduce. The physiological consequences $Prlr^{-/-}$ and $Lhcgr^{-/-}$ females endure include infertility, underdeveloped mammary glands, irregular cycling or inability to cycle, and delayed vaginal opening[33,38,39]. Therefore, the physiological abnormalities resulting from the absence of PRLR or LHR impair the reproductive success of females by directly affecting the anatomy and function of their reproductive system. As such, the lack of mate preference that we observed in $Prlr^{-/-}$ female mice are not without caveats, and must be interpreted with caution. However, the paramount role for LH and PRL signaling in mating and reproductive success is further exemplified here, by their importance in regulating plasticity in the female CNS through the increased generation of new neurons in response to dominant-male pheromones.

The specific roles for LH and PRL in male pheromone–induced neurogenesis adds to our knowledge of the complex interplay between the olfactory bulb, hypothalamus, pituitary and limbic areas (including the hippocampus) in the control of various aspects of reproductive and mating behaviors[40]. Neurons in the olfactory bulb that express gonadotropin-releasing hormone (also known as LH-releasing hormone) receive signals from pheromones and send projections to the hypothalamus, which results in the release of LH and follicular-stimulating hormone (FSH) by the anterior pituitary[24,25]. Together, LH and FSH control the release of steroid hormones, such as estrogen, that influence sexual behavior, ovulation and estrus cycling[1,9,32]. Estrogen, in turn, is known to promote the release of PRL and provides a regulatory feedback loop for LH and FSH release[1,32]. Although null mutants for *Prlr* and *Lhcgr* appear to simplify the link between pheromones and enhanced neurogenesis, it is clear that multiple endocrine interactions are likely involved in mediating both neurogenesis and the ultimate physiological responses associated with mate preference behavior.

Olfactory and hippocampal neurogenesis are evolutionarily well conserved, and a number of environmental and physiological factors increase neurogenesis in the adult mammalian brain[30]. Recent studies suggest olfactory neurogenesis has a role in olfactory discrimination[14] and perhaps in the formation of olfactory memories[15]. In the hippocampus, neurogenesis increases in response to exercise[19] and environmental enrichment[20], but the functional contribution of neurogenesis in response to running has not been investigated and is not required for enhanced spatial memory or a decline in anxiety-like behaviors due to enriched environments[16]. One study, using low doses of the antimitotic agent methylazoxymethanol acetate, found that hippocampal neurogenesis was required for the formation of trace memories[17]. However, another report suggests that the use of this agent may be limited by side effects that result from the doses needed to significantly disrupt neurogenesis[41]. A recent study used both focal X-irradiation and generalized, genetic ablation of neuronal progenitors to demonstrate the involvement of hippocampal neurogenesis in contextual learning and memory[42]; however, both approaches required 2 months of post-treatment recovery before behavioral testing could be initiated. Moreover, irradiation may reduce body weight in animals[43] and thus may be susceptible to some of the same issues related to the use of antimitotic drugs. Recently, a functional correlate of adult neurogenesis was established by centrally administering AraC to inhibit ciliary neurotrophic factor (CNTF)-induced hypothalamic neurogenesis, which prevented the CNTF-induced weight loss in obese mice[36]. The use of AraC is not without its complications, as it also is an anti-mitotic agent like methylazoxymethanol acetate; however, we have previously found that AraC can rapidly and reversibly block adult neurogenesis[14]. We found that AraC-treated mice showed normal motor activity in the presence of subordinate, dominant or standard open-field environments, despite gaining[36] rather than losing[41] weight, suggesting that their general health was not compromised. Moreover, general olfactory behavioral responses, including detecting and habituating to novel, enriched odors, were unaffected by AraC treatment. Notably, the female preference for a dominant male was not established if olfactory bulb and dentate gyrus neurogenesis was attenuated using either a 2- or 7-d treatment with AraC, nor in *Prlr*$^{-/-}$ females devoid of male pheromone–stimulated olfactory neurogenesis, suggesting that neurogenesis is important in mate selection and perhaps increases in females to support the discrimination and memory formation of dominant-male odor signatures.

Although our study suggests that dominant-male pheromone–induced neurogenesis in both principal adult neurogenic structures may have a role in female mate preference behavior, we do not claim that this pheromone-dependent ethological behavior establishes a link between olfactory bulb and dentate gyrus neurogenesis. In fact, given that AraC inhibits neurogenesis in both the olfactory bulb and dentate gyrus, the independent contribution of each to female mating behavior has not been addressed and awaits further investigation. Partitioning out the individual roles of olfactory bulb and dentate gyrus neurogenesis in mating preference behavior, let alone the functional roles of the specific changes in neurogenesis that are hormonally regulated, is extremely challenging and may require the development of conditional mutant mice that specifically, selectively and reversibly impair neurogenesis in a temporally useful manner. Creating such mice is beyond the scope of the current study. However, our study does propose an ethologically relevant function for newly generated neurons in the adult female brain in response to male pheromones. Further, this may provide a foundation for future investigations that will partition out the relative roles of resident neurons in the olfactory bulb and dentate gyrus to those that are newly arriving and integrating into the established circuitry, in the manifestation of a critical reproductive behavior.

Although the influence of pheromones on female human reproduction and behavior is a matter of ongoing debate[44], fMRI scans show that exposure to a synthetic derivative of male axillary secretion activates hypothalamic areas of the female brain[45]. Moreover, families of trace amine-associated–receptor genes specific to the detection of male pheromones are present in the human olfactory epithelium[7], and pheromones are implicated in processes such as menstrual cycle synchrony in women living in close proximity and the modulation of mood and pulsatile LH secretion in women when exposed to male axillary extracts[46]. Notably, given the evidence supporting hippocampal and olfactory neurogenesis in adult humans[47], it seems reasonable to speculate that pheromones and their hormonal mediators may also be involved in plasticity at the level of neurogenesis in the adult human CNS.

METHODS

All methods are further detailed in the **Supplementary Methods** with corresponding references.

Odor exposure. We used 8–10–week-old female and male mice throughout the study. CD1 and C57BL6/J mice were obtained from Charles River (Laval). Dominant-male pheromone–induced neurogenesis and the female preference behavior shown by females exposed to dominant-male pheromones were replicated in C57BL6 mice to demonstrate that our results are not strain specific. *Lhcgr* and *Prlr* mutant mice were maintained on a C57BL6/J background[34,38]. Animals were handled in accordance with the institutions animal care policies of the University of Calgary and the University of Turku. Animals were maintained on a 12-h light/dark cycle with food and water *ad libidum*.

Females were continuously exposed to male odors for 2 and 7 d through the use of soiled bedding, renewed every second day from the same males. A social interaction component was not part of this study. Females exposed to female odors or clean cage bedding (baseline) served as controls. *Lhcgr*$^{-/-}$, *Lhcgr*$^{+/+}$, *Prlr*$^{-/-}$ and *Prlr*$^{+/+}$ female mice were exposed to wild-type male pheromones in the same manner. To identify pheromones as the environmental stimulus, females were exposed to castrated or sham-operated male (surgery described in **Supplementary Methods**) odors for 7 d using the same methodology. To determine whether the main olfactory system is important for pheromone-induced neurogenesis, we exposed female mice given $ZnSO_4$ lesions (surgery described in **Supplementary Methods**) to male pheromones for 7 d using the same methodology as described above and compared with saline controls. Exposure to dominant or subordinate-male pheromones involved identifying dominant and subordinate males within a group of three using the mouse social ethogram[48]. Female mice were then exposed to male odors (consisting of dominant- and subordinate-male odors) for 2 d and placed into a clean cage

for 4 d. Females were then exposed to either the single dominant- or subordinate-male odors for 7 d.

Female mate preference task. Females were exposed to dominant or subordinate males as outlined above and left for 2 weeks before testing. A testing chamber (25 inches × 5 inches × 9 inches), designed using previous female preference studies[28,29], was constructed with removable dividers to allow for tertiary compartmentalization. Dividers consisted of two perforated panels separated by 0.5 inches and offset so that only odors could be transferred. Females were habituated to the test cage before testing. On test day, individual dominant and subordinate males were placed randomly in either side compartment along with their soiled bedding. A female was then placed in the center of the test cage and observed for 10 min. The 10-min trial was recorded and analyzed blind and double-blind by an individual who did not have knowledge of this experiment. The amount of time spent sniffing each side of the test cage was recorded, where a preference was defined by a greater amount of time spent sniffing one side of the cage versus the other. To assess dominant or subordinate preference in females that received AraC infusions, females were treated with either AraC or saline for 2 or 7 d of male pheromone exposure.

Olfactory habituation assay. This assay is adapted from methods described previously[37]. Females were habituated to the test cage before testing. On test day, females were individually presented with a water-soaked cotton swab for 2 min. The number of sniffs was recorded during the 2-min period and then repeated for another 2 min. The two trials were separated by 1 min. Females were then presented with an odor-laced cotton swab (coconut or almond scent) and the number of sniffs over a 2-min period was recorded. This was repeated twice, separated by 1 min between trials, and followed by the introduction of a different odor (coconut or almond scent). Data are expressed as the average number of sniffs per trial.

Hormone infusions and osmotic pump implantation. Female CD1 mice were anesthetized with sodium pentobarbital (120 mg per kg of body weight, intraperitoneal) and implanted with an osmotic pump (Alzet 1007D, Alza), placed dorsal subcutaneously. LH and PRL (mouse recombinant, National Hormone & Peptide Program) were dissolved in 0.9% saline containing 1 mg ml^{-1} mouse serum albumin (Sigma) at the concentrations stated in the text. Each animal was infused for two consecutive days. Control mice were implanted with an osmotic pump in the same manner and duration containing 1 mg ml^{-1} mouse serum albumin (Sigma). For central administration of AraC, female CD1 or C57BL6/J mice were anesthetized as described above and implanted with an osmotic pump, placed dorsal subcutaneously, where the contents of the osmotic pump were channeled though tubing attached to a cannula that was implanted into the lateral ventricles of the brain +0.2 mm anterior/posterior, –0.8 mm medial/lateral lateral, –2.5 mm dorsal/ventral from bregma. A 2% solution of AraC (Sigma) was made in 0.9% saline, whereas control mice received saline only.

BrdU labeling and immunohistochemistry. At the end of a particular odor exposure, after 2 d of subcutaneous LH or PRL infusions, or after 7 d of AraC infusions, mice were given BrdU (Sigma, 120 mg per kg, intraperitoneal, dissolved in 0.007% NaOH in phosphate buffer) every 2 h for 10 h, and killed 0.5 h (or longer) after the final injection. To detect BrdU-labeled cells in the olfactory bulb or dentate gyrus 4 weeks later, we administered BrdU on the seventh day of odor exposure, on the second day of hormone infusion or on the seventh day of AraC treatment. Immunostaining was carried out on 14-μm sections using the following primary antibodies: rat monoclonal anti-BrdU (1:200, Oxford Biotechnology), rabbit polyclonal anti-Ki67 (1:500, Novacastra), goat polyclonal doublecortin (1:200), mouse monoclonal NeuN (1:200, Santa Cruz Biotechnology), mouse monoclonal anti–prolactin receptor (1:100, Affinity Bioreagents), rabbit polyclonal anti-CGR (1:100, supplied by A.T. Fazleabas, University of Illinois). Primary antibodies were enhance with donkey or goat biotinylated secondary antibodies (all at 1:200, Jackson ImmunoResearch), and subsequently conjugated with streptavidin-Cy3 (1:1,500, Jackson ImmunoResearch) and Hoechst 3358 (0.015 mg ml^{-1} stock solution diluted to 0.001 mg ml^{-1}, Sigma) or detected with secondary antibodies labeled with Rhodamine or FITC (Jackson ImmunoResearch).

TUNEL staining was carried out using the Roche *In Situ* Cell Death Kit (FITC). Slides were viewed with a Zeiss Axiophot fluorescence microscope.

RT-PCR. A PCR of 40 cycles at an annealing temperature of 62 °C was used for the detection of LHR, PRLR-S1 and PRLR-S3, whereas a PCR of 35 cycles at an annealing temperature of 59 °C and 61 °C was used for PRLR-S2 and PRLR-long, respectively. The sense primer for LHR corresponded to nucleotides 176–195 (5′-CTCTCACCTATCTCCCTGTC-3′) and the antisense primer to nucleotides 878–858 (5′-TCTTTCTTCGGCAAATTCCTG-3′) of the mouse LHR cDNA[49]. PRLR expression was determined using a common extracellular domain primer corresponding to nucleotides 610–632 (5′-AAGCCAGAC CATGGATACTGGAG-3′), and specific primers for PRLR-long, PRLR-S1, PRLR-S2 and PRLR-S3 corresponding to the first 23 nucleotides of the unique coding region of the different receptor splice forms (nucleotides 841–863): PRLR-long (5′-AGCAGTTCTTCAGACTTGCCCTT-3′), PRLR-S1 (5′-AACTG GAGAATAGAACACCAGAG-3′), PRLR-S2 (5′-TCAAGTTGCTCTTTGTT GTGAAC-3′) and PRLR-S3 (5′-TTGTATTTGCTTGGAGAGCCAGT-3′)[50]. The β-actin gene (*Actb*) was used as a control: β-actin forward primer (5′-CGTGG GCCGCCCTAGGCACCA-3′) and β-actin reverse primer (5′-TTGGCC TTAGGGTTCAGGGGGG-3′).

Statistics. Analysis of significant differences was carried out using Student's *t*-test when an experiment only had two groups to compare, or ANOVA followed by Tukey's *post hoc* analysis for experiments with more than two groups. *P* value was set at 0.05 for significance.

Note: Supplementary information is available on the Nature Neuroscience website.

AUTHOR CONTRIBUTIONS
G.K.M. conducted all of the experiments and co-authored the manuscript. E.E.K. and C.G. contributed to the planning and interpretation of the pheromone- and hormone-stimulated neurogenesis studies, T.P., M.P. and I.H. collaborated on studies of LH actions on neurogenesis, LHR immunochemistry and pheromone-stimulated neurogenesis in *Lhcgr* mice, and S.W. supervised the project and co-authored the manuscript.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

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