Chapter 2

Lhx6 delineates a pathway mediating innate reproductive behaviors from the amygdala to the hypothalamus

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ABSTRACT

In mammals, innate reproductive and defensive behaviors are mediated by anatomically segregated connections between the amygdala and hypothalamus. This segregation poses the problem of how the brain chooses between such behaviors when faced with conflicting cues. Using genetically encoded and conventional axonal tracers, we have found that the transcription factor Lhx6 delineates the reproductive branch of this pathway. Other Lhx proteins mark neurons in amygdalar nuclei implicated in defense. We have traced parallel projections from the posterior medial amygdala, activated by reproductive or defensive olfactory stimuli, respectively, to a point of convergence in the ventromedial hypothalamus. The opposite neurotransmitter phenotypes of these convergent projections suggest a "gate control" mechanism for inhibiting reproductive behaviors by threatening stimuli. Our data therefore identify a potential neural substrate for integrating the influences of conflicting behavioral cues, and a transcription factor family that may contribute to the development of this substrate.

INTRODUCTION

Virtually all metazoan organisms exhibit innate reproductive and defensive behaviors, that are triggered by signals sensed from conspecifics or predators. Such behaviors are crucial for the survival of each species. The stereotyped nature of these behaviors suggests that their underlying neural circuits are likely to be genetically "hard-wired." At the same time, animals are frequently faced with conflicting cues in their natural environment, and therefore must make rapid decisions to engage in defensive, versus reproductive, or other appetitive behaviors (Lima and Dill, 1990). The neural mechanisms that determine which of these hard-wired behaviors will predominate when conflicting stimuli are present are poorly understood.

The basic neural pathways that mediate reproductive (such as mating or maternal) and defensive (such as aggressive or predator-avoidance) behaviors in rodents have been intensively studied. Olfactory stimuli play an important role in the release of such behaviors. These stimuli activate primary sensory neurons in the main olfactory epithelium or vomeronasal organ, which project to the main or accessory olfactory bulbs (AOB), respectively (Itaya, 1987). While recent genetic evidence increasingly suggests an important role for the main olfactory system in processing reproductive stimuli (Belluscio et al., 1998; Keverne, 2002; Leypold et al., 2002; Stowers et al., 2002), a great deal of attention has been focused on a parallel pathway involving the accessory olfactory system, which responds to pheromonal cues (Brennan and Keverne, 2004; Luo and Katz, 2004; Newman, 1999). Projection neurons in the AOB synapse in the medial amygdalar nucleus (MEA; Fig. 1A, top), which in turn projects to a series of nuclei in the medial hypothalamus (Fig. 1A, bottom) (Davis et al., 1978; Kevetter and Winans, 1981; Krettek

and Price, 1978; Scalia and Winans, 1975; Swanson, 2000; Swanson and Petrovich, 1998). The MEA also projects indirectly to the hypothalamus through the bed nucleus of the stria terminalis (BST) (Dong et al., 2001; Dong and Swanson, 2004).

The posterior portion of MEA is subdivided into dorsal and ventral subnuclei (MEApd and MEApv; Fig. 1A, top, magenta). The projections from these two subnuclei to the medial hypothalamus exhibit a striking anatomic segregation, which is thought to reflect their involvement in either reproduction or defense (Fig. 1B) (Swanson, 2000; Canteras, 2002). The dorsal portion (MEApd) is activated by reproductive stimuli (Fig. 1C) (Bressler and Baum, 1996; Fernandez-Fewell and Meredith, 1994; Heeb and Yahr, 1996; Kollack-Walker and Newman, 1997), and projects to three interconnected hypothalamic nuclei implicated in reproductive behaviors: the medial preoptic nucleus (MPN), ventrolateral part of the ventromedial hypothalamic nucleus (VMHvl), and the ventral premammillary nucleus (PMv; Fig. 1B). The ventral portion (MEApv) is activated by defensive stimuli such as predator odors (Fig. 1C) (Dielenberg et al., 2001; McGregor et al., 2004), and projects to the anterior hypothalamic nucleus (AHN) and the dorsomedial part of VMH (VMHdm), which are involved in defensive behaviors (Fig. 1B) (Swanson, 2000; Canteras, 2002). This striking anatomical and functional segregation suggests that these neural pathways for reproduction and defense are likely genetically determined, but genes that might control their wiring have not yet been identified.

Such a parallel circuit organization poses the problem of how rapid decisions between competing reproductive and defensive behaviors are made by organisms faced with conflicting cues. Such decision-making would seem to require cross-talk between

these sub-circuits, but there are very few interconnections between the reproductive and defensive hypothalamic nuclei. MEApv projects to the reproductive as well as the defensive hypothalamic nuclei (Fig. 1B), but the function of this divergent projection is not known. Some evidence suggests that suppression of reproductive behaviors by threatening stimuli may be exerted within the amygdala-hypothalamic pathway. For example, exposure of virgin female rats to newborn pups promotes defensive behaviors and inhibits maternal behavior (Numan and Sheehan, 1997; Sheehan et al., 2000). This inhibition can be overcome by lesions of the medial amygdala, and involves projections from this structure to VMH (Sheehan et al., 2001). The circuit-level mechanisms that mediate such behavioral inhibition are not understood.

The identification of genes that mark amygdalar-hypothalamic circuits might shed further light on both their functional organization and developmental specification. Here we show that different LIM homeodomain transcription factors mark neurons in different subnuclei of the medial amygdala. We have used both genetic and classical neuroanatomical tracing techniques, in conjunction with markers of neuronal activation and neurotransmitter phenotype (Jongen-Relo and Amaral, 2000; Lieberoth et al., 2003), to determine the relationship of these molecularly identified neurons to the functions and connectivity attributed to the nuclei in which they reside. Our results indicate that Lhx6 delineates a reproductive pathway that involves neurons in both MEApd and BSTpr and their projections to the three reproductive nuclei in the hypothalamic medial behavioral control column (MPN, VMHvl and PMv). Further analysis reveals, counter-intuitively, that VMHvl receives inhibitory projections from this reproductive pathway, and a convergent excitatory projection from neurons in MEApv, that are activated by a predator

odor. We suggest that this point-of-convergence may serve to "gate" (Melzack and Wall, 1965) the expression of reproductive behavior, under conditions where animals are exposed to threatening stimuli. Thus, our data identify a potential neural substrate within the hypothalamus for controlling behavioral decisions in the face of conflicting cues, and a transcription factor family that may contribute to the development of this substrate.

RESULTS

LIM homeodomain transcription factors mark different regions of medial amygdala

To begin to map connectivity within the amygdalar-hypothalamic reproductive and defensive pathways at a cellular level of resolution, we first sought to identify molecular markers for subpopulations of neurons within the medial amygdala. To do this, we took two different approaches. First, using oligonucleotide microarrays and laser-capture microdissection (Zirlinger and Anderson, 2003), we compared the gene expression profile of MEApd with that of MEApv in adult mouse brain. Second, to identify markers that might be transiently expressed during development and therefore missed in the first screen, we also examined ~100 candidate genes by *in situ* hybridization at embryonic day E14.5, a time point by which most medial amygdalar neurons are already generated (McConnell and Angevine, 1983). These candidate genes included transcription factors and cell-surface adhesion molecules, which have been implicated in regulating axonal projection patterns in other systems such as the spinal cord (Chen et al., 2001; Inoue et al., 2003; Jessell, 2000; Price et al., 2002; Shirasaki and Pfaff, 2002).

Both approaches revealed that two LIM homeodomain genes, *Lhx6* and *Lhx9*, are differentially expressed in posterior MEA (MEAp). Confocal immunofluorescent microscopy indicated that LHX6-immunoreactive cells constitute a high proportion (~80±1.5%) of all neurons in MEApd, while only a few scattered *Lhx6*$^{+}$ cells are observed in MEApv (~17±14.8%) (Fig. 2A) (Zirlinger et al., 2001). *Lhx6* mRNA was also strongly expressed in the BST principal nucleus (Fig. 2G, pr), which projects to reproductive hypothalamic nuclei, and more weakly expressed in the BST interfascicular nucleus (Fig. 6G, if), which projects to both reproductive and defensive nuclei (Dong and Swanson, 2004). In contrast, *Lhx9*-expressing cells were observed in MEApv, but not in MEApd (Fig. 2B), consistent with a recent report (Remedios et al., 2004). Double labeling (using an *Lhx6-EGFP* BAC transgenic reporter line (Gong et al., 2003)) revealed that the *Lhx9*$^{+}$ cells are distinct from the subpopulation of *Lhx6*$^{+}$ cells found within MEApv (Fig. 2J), thereby identifying at least two subpopulations within this subnucleus. The expression of *Lhx6* and *Lhx9* in MEApd/v was detected as early as E13.5 (data not shown), and persists into the adult.

The differential expression of *Lhx6* and *Lhx9* in MEAp raised the question of whether other members of the LIM homeodomain family might mark other subpopulations of neurons within the medial amygdala. *In situ* hybridization screens identified an additional family member, *Lhx5*, which is specifically expressed in a well-demarcated region within the anterior MEA (MEAa; Fig. 2F). Scattered cells expressing *Lhx6* and *Lhx9* are present in the MEAa as well (Fig. 2D, E), but double-labeling experiments indicated that the *Lhx5*$^{+}$ neurons do not co-express these markers (Fig. 2K, L). Thus, members of the LIM homeodomain family mark distinct and mutually

exclusive neuronal subpopulations within different subnuclei of the medial amygdala (Fig. 2M).

Lhx6- and *Lhx9*- expressing cells project differentially to reproductive and defensive targets in hypothalamus and BST

Based on the expression patterns of *Lhx6* and *Lhx9* and the known projections of MEA (Canteras et al., 1995), we reasoned that $Lhx6^{+}$ neurons in MEApd might project only to reproductive targets in the BST and/or hypothalamus, whereas $Lhx9^{+}$ neurons in MEApv might project to defensive and/or reproductive targets in these regions. Alternatively, one or both classes of cells could represent local interneurons. To distinguish these possibilities, we targeted, using *VelociGene* technology (Valenzuela et al., 2003), the genetically encoded axonal tracer placental alkaline phosphatase (PLAP) (Leighton et al., 2001; Shah et al., 2004) to the *Lhx6* and *Lhx9* chromosomal loci, replacing their coding regions via homologous recombination in embryonic stem cells (Fig. 3A, B). This enabled us to use PLAP expression to determine whether reproductive and defensive targets receive differential input from *Lhx6*- and *Lhx9*-expressing cells, respectively.

These experiments indicated, firstly, that $PLAP^{+}$ neurons in $Lhx6^{PLAP/+}$ mice were observed in all sites known to express *Lhx6* mRNA, arguing that the targeting construct did not grossly disrupt the transcriptional specificity of the locus. Secondly, $PLAP^{+}$ fibers were observed in all of the reproductive nuclei in the medial hypothalamus: VMHvl, PMv, and MPN (Fig. 1A and Fig. 3C-E) (Newman, 1999; Segovia and Guillamon, 1993; Simerly, 2002). Furthermore, PLAP staining was clearly excluded

from the dorsomedial part of VMH (VMHdm), and the dorsal premammillary nucleus (PMd), which are known to be part of the defensive circuit (Fig. 3C, dm; Fig. 3D, d) (Canteras, 2002). Intense PLAP staining was also observed in the BSTpr (Fig. 3E, pr), which receives projections from MEApd (Dong et al., 2001). However, as *Lhx6* mRNA is also expressed in this structure (Fig. 2G), it could not be distinguished whether this PLAP staining reflects nerve fibers, intrinsic cell bodies, or both. These data suggest that $Lhx6^{+}$ neurons project to reproductive targets in the hypothalamus, and perhaps also in the BST. All of these inferred projection sites were independently confirmed by retrograde tracing experiments (see below). In an apparent exception to this general rule, weaker PLAP staining was observed in the BSTif and AHN, two defensive behavioral targets of the MEA (Fig. 3E, F) (Canteras, 2002; Dong et al., 2001; Dong and Swanson, 2004). However, BSTif also contains a few *Lhx6*-expressing cell bodies (Fig. 2G), and retrograde tracing experiments suggest that the staining in AHN represents fibers-of-passage (see below).

In $Lhx9^{PLAP/+}$ mice, the pattern of PLAP expression again appeared to correctly recapitulate that of the endogenous gene. Strikingly, in the hypothalamus and BST the distribution of $PLAP^{+}$ fibers was roughly complementary to that seen in $Lhx6^{PLAP/+}$ mice. Thus, staining was detected in all of the defensive targets, including VMHdm, BSTif and AHN, but not in the reproductive targets (Fig. 3G, I, J). The complementary labeling of hypothalamic nuclei by *Lhx6*- and *Lhx9*-$PLAP^{+}$ fibers was particularly striking in VMH, where these projections were detected in the reproductive (VMHvl) and defensive (VMHdm) subdomains, respectively (Fig. 3C, G). The absence of *Lhx9* mRNA in the defensive nuclei in hypothalamus and BST (not shown) suggested that PLAP staining in

these regions represents fibers, rather than local cell bodies. No PLAP expression was observed in PMd (Fig. 3H), consistent with the fact that this defensive hypothalamic nucleus does not receive direct inputs from MEAp (Canteras et al., 1995).

Taken together, these data suggest that *Lhx6*- and *Lhx9*-expressing cells differentially project to reproductive and defensive targets in the medial hypothalamus and BST. *Lhx6*-expressing neurons preferentially project to reproductive, while those expressing *Lhx9* project to defensive, hypothalamic nuclei.

Retrograde tracing from reproductive hypothalamic nuclei labels *Lhx6*$^+$ cells in MEApd and the BST

While the foregoing data are consistent with the idea that *Lhx6*$^+$ neurons in MEApd/v and *Lhx9*$^+$ neurons in MEApv project to reproductive and defensive nuclei in the hypothalamus, respectively, they do not prove it for several reasons. First, PLAP histochemical staining does not provide sufficient resolution to distinguish nerve terminals in a given nucleus from axons passing through this structure en route to a different target, known as "fibers of passage". Second, because *Lhx6*- and *Lhx9*-expressing cell bodies are present in other parts of the brain besides MEAp, PLAP$^+$ fibers in the hypothalamus may not necessarily derive from Lhx6$^+$ or Lhx9$^+$ neurons in the MEA. To resolve these ambiguities, we performed retrograde axonal tracing using cholera toxin B subunit (CTB), injected into various targets in the BST and hypothalamus (Fig. 5A$_1$-A$_4$), and assessed the extent of co-localization of this tracer with *Lhx6*$^+$ or *Lhx9*$^+$ cells in MEA, by double-label immunofluorescence and confocal microscopy (Fig. 5A$_0$). CTB is taken up in the injection site only by nerve terminals and not by fibers of

passage to any great extent, and is retrogradely transported back to the cell bodies from which these terminals originate (Vercelli et al., 2000).

These experiments indicated that *Lhx6*$^{+}$ cells in the MEApd could be back-labeled by injection of CTB into the three reproductive medial hypothalamic nuclei: MPN, VMHvl and PMv (Fig. 4A-C, insets), consistent with the PLAP labeling observed in these structures in *Lhx6*$^{PLAP/+}$ mice. Co-expression at the single-cell level was confirmed by z-series analysis of multiple confocal optical sections (not shown). Back-labeling of *Lhx6*$^{+}$ cells in MEApd was also observed following CTB injection into BST (Fig. 4F). The majority, but not all, of the CTB-labeled cells expressed *Lhx6* (MPN: 63.5±4.4%, VMHvl: 57.3±8.6%, BST: 66±15.6%, PMv: 62.3±10.1%). Interestingly, the small population of *Lhx6*$^{+}$ cells in MEApv (Fig. 2A) was not labeled by CTB in these injections. More importantly, no labeling of *Lhx6*$^{+}$ cells, in either MEApd or MEApv, was observed when CTB was injected into defensive targets such as VMHdm and AHN (Fig. 4D, E). This latter result suggests that the PLAP^{+} fibers observed in the AHN of *Lhx6*-PLAP mice (Fig. 3F) likely represent fibers of passage, and not nerve terminals. Consistent with this interpretation, anterograde tracing data in rat indicate that axons originating from MEApd pass through the AHN to terminate in more posteriorly located hypothalamic reproductive targets (Fig. 5A$_2$) (Canteras et al., 1995).

The back-labeling of *Lhx6*$^{+}$ cells in MEApd by injection of CTB into the BST (Fig. 4F) indicates that at least some of the PLAP staining in the BST (Fig. 3E) indeed reflects projections from Lhx6-expressing cells in MEApd, and not just intrinsic *Lhx6*$^{+}$ neurons (Fig. 2G). To determine whether the intrinsic *Lhx6*$^{+}$ neurons in the BST also project to hypothalamic nuclei we injected CTB into these latter structures, and

performed double-labeling for *Lhx6* and the retrograde tracer in the BST. *Lhx6*$^+$ neurons in BSTpr were back-labeled by CTB injections into MPN, VMHvl and PMv, but not into AHN or VMHdm (Fig. 4G-K). Thus, *Lhx6*$^+$ neurons in both MEApd and the BSTpr project to reproductive, but not defensive, hypothalamic nuclei, and *Lhx6*$^+$ neurons in MEApd project to the BSTpr as well (Fig. 5B, left, purple).

Projections of Lhx9$^+$ and Lhx5$^+$ amygdalar neurons

Lhx9-PLAP$^+$ fibers were observed in defensive hypothalamic nuclei (Fig. 3G-J), and Lhx9$^+$ neurons are present in MEApv (Fig. 2B), which is known to project to these same hypothalamic nuclei (Fig. 1B) (Canteras et al., 1995). Surprisingly, however, injection of CTB into two defensive nuclei, AHN and VMHdm, labeled cells in MEApv distinct from those expressing *Lhx9* (data not shown). However, the retrograde tracer when injected in VMHdm did label *Lhx9*$^+$ cells in the paraventricular thalamic nucleus (PVT; Fig. 5B), a structure implicated in stress-related behaviors (Bhatnagar et al., 2003; Jaferi et al., 2003; Klejbor et al., 2003; Kurumaji et al., 2003). Injection of CTB into other known projection targets of MEApv, including reproductive hypothalamic nuclei, has thus far failed to label any *Lhx9*$^+$ neurons. The *Lhx9*$^+$ cells in MEApv may project to targets not yet injected with CTB, or may represent local interneurons.

Because the anterior medial amygdala (MEAa) also projects to defensive hypothalamic nuclei (Canteras et al., 1995; Gomez and Newman, 1992), we next asked whether the *Lhx5*$^+$ neurons in this structure (Fig. 2F) project to such targets. Strikingly, CTB injection into VMHdm strongly labeled the *Lhx5*$^+$ subpopulation in MEAa, and all back-labeled neurons were confined within the *Lhx5*$^+$ region (Fig. 4O). A similar

restriction to the *Lhx5*-expressing domain was obtained for neurons back-labeled from AHN (Fig. 4P). By contrast, very few *Lhx5*$^+$ neurons were labeled by injection of the tracer into MPN, a reproductive hypothalamic nucleus (Fig. 4L) (Newman, 1999; Segovia and Guillamon, 1993; Simerly, 2002). A few more neurons were labeled by injection into PMv or VMHvl (Fig. 4M, N). However, the distribution of such back-labeled neurons was only partially overlapping with the well-delineated Lhx5$^+$ domain. The partial back-labeling of Lhx5$^+$ neurons in MEAa by injection into reproductive hypothalamic nuclei could reflect partial spillover of the tracer into their neighboring defensive counterparts (PMd and VMHdm, respectively; Fig. 5A$_3$, A$_4$) (Supplemental Figure S2A-F). Consistent with this, a similar partial overlap in MEAa (~20%) was observed when two different retrograde tracers, CTB and fluorogold (FG), were injected into VMHdm and VMHvl, respectively (Fig. 4Q; cf. 4M, N). Taken together, these results suggest that Lhx5 marks a population of neurons in the anterior MEA that projects primarily, if not exclusively, to hypothalamic nuclei associated with defensive behavior (Fig. 5B, green).

Lhx6$^+$ neurons are activated by reproductive but not defensive olfactory stimuli

The foregoing observations indicate that Lhx6 is expressed by neurons that project from MEApd to regions of the hypothalamus involved in reproductive behaviors. We next sought to determine whether these Lhx6$^+$ neurons are actually activated by olfactory stimuli that release reproductive behaviors. Female urine evokes aspects of male reproductive behavior, including ultrasonic vocalizations (Nyby et al., 1977). We therefore exposed male mice to female urine, and performed double-labeling using

antibodies to LHX6 and in situ hybridization for *c-fos*, a surrogate marker of neuronal activation (Morgan and Curran, 1991). As controls, we used water, male urine, and odor from a collar worn by a domestic cat as a predator stimulus (Dielenberg et al., 2001; McGregor et al., 2004). In all cases animals were exposed to the odor stimuli for 30 minutes prior to sacrifice for analysis of *c-fos* mRNA, or for one hour for analysis of c-FOS protein (see Experimental Procedures).

The results of these experiments indicated that female urine indeed activated *c-fos* mRNA in Lhx6^{+} cells within MEApd (Fig 6D, inset) (Bressler and Baum, 1996). In contrast, water, male urine and cat odor did not activate *c-fos* expression within this region (Fig. 6A-C), although the cat odor did induce *c-fos* in MEApv (Fig. 6I), consistent with previous studies (Dielenberg et al., 2001; McGregor et al., 2004). Quantification indicated that the number of *c-fos*$^{+}$, Lhx6^{+} neurons in MEApd induced by female urine was at least 8 to 10 fold higher than that induced by any of the control stimuli (Fig. 6J). The percentage of all *c-fos*$^{+}$ cells in MEApd that expressed Lhx6 was approximately 62±2.1%. Although ~80% of MEApd cells are Lhx6^{+} as determined by single antibody-labeling experiments, when anti-Lhx6 antibody staining was combined with *c-fos* situ hybridization only 30-50% of all cells were Lhx6-immunoreactive, indicating a partial loss of Lhx6 antigenicity during the double-labeling procedure. Therefore, the percentage of *c-fos*$^{+}$ cells that co-express Lhx6 (62%) is higher than the percentage of all cells that express Lhx6 in the same experiments, suggesting that *c-fos* is preferentially (although not exclusively) induced in Lhx6^{+} cells. Female urine also activated *c-fos* within MEApv (Fig. 6H), but in only one or two cases did these *c-fos*$^{+}$ neurons express *Lhx6* (data not shown).

These data indicate that MEApd, which contains Lhx6$^+$ neurons that project to the reproductive hypothalamus (Fig. 4A-C, 5B), also contains Lhx6$^+$ neurons that are activated by a reproductive (but not a defensive) olfactory stimulus. To directly test whether Lhx6$^+$ neurons in MEApd activated by female urine also project to reproductive hypothalamic nuclei, we retrogradely labeled Lhx6$^+$ neurons in MEApd by injection of CTB into VMHvl, allowed the animals to recover from surgery, and then exposed them to female urine. Triple labeling for Lhx6, CTB and *c-fos* in MEApd indicated that some Lhx6$^+$ neurons that project to VMHvl are indeed activated by female urine (Fig. 6E, arrowheads). Since only a relatively small subset of neurons in MEApd (9±0.06%) are activated by female urine (Fig. 6D), and since the Lhx6$^+$ population contains not only neurons that project to VMHvl, but also neurons projecting to other reproductive targets (e.g., MPN, PMv and BSTpr; Fig. 4), it is not surprising that only a few neurons were co-labeled by *c-fos*, Lhx6 and CTB in these experiments. Nevertheless, the results demonstrate that at least some of the Lhx6$^+$ neurons that project to reproductive hypothalamic targets can also be activated by a reproductive olfactory stimulus.

We also asked whether Lhx5$^+$ neurons in MEAa, which project to known defensive hypothalamic targets, could be activated by cat collar odor. No *c-fos* activation was elicited in MEAa by this olfactory stimulus (data not shown). However, some Lhx5$^+$ neurons in MEAa were activated in male mice engaged in aggressive encounters with conspecifics (Supplemental Figure S1C).

Lhx6$^+$ neurons in MEApd that project to reproductive targets are inhibitory

To gain more insight into the function of the $Lhx6^+$ neurons in MEApd that project to reproductive regions of the hypothalamus, we next asked whether these neurons are excitatory or inhibitory. Double-labeling for Lhx6 and the GABAergic marker GAD67 (Kaufman et al., 1991) indicated that most or all $Lhx6^+$ neurons are $GAD67^+$ (Fig. 6F). By contrast, *in situ* hybridization for the glutamatergic marker Vglut2 (Fremeau et al., 2001) revealed scattered cells in MEApd, that did not overlap with Lhx6 (Fig. 6G). These data suggest that the $Lhx6^+$ neurons that project to the medial hypothalamus are likely to be inhibitory. However the $GAD67^+Lhx6^+$ population in MEApd may also include local inhibitory interneurons. Taken together with the *c-fos* labeling experiments, these data suggest that $Lhx6^+$ neurons in MEApd that are activated by reproductive olfactory stimuli send inhibitory projections to reproductive hypothalamic nuclei.

Cat collar odor activates distinct populations of MEApv neurons projecting to reproductive or defensive subnuclei of VMH

As mentioned earlier, cat collar odor activates *c-fos* in MEApv (Dielenberg et al., 2001; McGregor et al., 2004). Our results confirm this (Fig. 6I), but also indicate that female urine, a reproductive stimulus, activates neurons in this subnucleus as well (Fig. 6H). Because MEApv projects to both defensive and reproductive hypothalamic nuclei (Canteras et al., 1995), we sought to determine which type of olfactory stimulus activates the neurons that project to these two hypothalamic cell groups, and whether these neurons are the same, or different. To address this question, we injected CTB into the reproductive (vl) or defensive (dm) regions of VMH, allowed the animals to recover from

surgery, exposed them to female urine or cat collar odor, and then performed double-label *in situ* hybridization for *c-fos* with antibody staining for CTB.

As expected, cat collar odor induced *c-fos* mRNA expression in MEApv neurons that project to VMHdm (Fig. 7A). By contrast, such projection neurons were distinct from the population activated by female urine (Fig. 7C, red cells). Surprisingly, MEApv neurons that project to the reproductive portion of VMH (VMHvl) were also activated by cat collar odor (Fig. 7B), and not by female urine (Fig. 7D). Thus, cat collar odor activated MEApv neurons that project to both the defensive and reproductive portions of VMH, while female urine activated MEApv neurons that did not project to either hypothalamic target.

The foregoing results could be explained by a collateral projection of individual MEApv neurons activated by cat collar odor to both subdomains of VMH. To test this possibility, we performed double-label retrograde tracing by injection of CTB and a second retrograde tracer, FG, into VMHdm and VMHvl, respectively. The results indicated that neurons in MEApv projecting to these reproductive and defensive nuclei were distinct, but intermingled (Fig. 7F). Quantification indicated that approximately 85% of the CTB^+ neurons in this structure were negative for FG (Fig. 7L, MEApv, dm+vl). Positive-control experiments indicated that ~70% of CTB^+ neurons were FG^+ when both tracers were injected (separately) into VMHdm (Fig. 7E, L, MEApv, dm+dm). These data indicate that MEApv contains two separate populations of neurons that project to VMHdm and VMHvl (Fig. 7F), both of which are activated by a defensive, but not by a reproductive, olfactory stimulus.

Cat collar odor activates excitatory projections from MEApv to VMHvl

The foregoing observations presented a paradox: why should a defensive olfactory stimulus activate neurons in MEApv that project to a reproductive hypothalamic target? One possibility is that these projections might be inhibitory, while projections from MEApv to defensive nuclei would be excitatory; the fact that distinct neurons in MEApv project to these two classes of hypothalamic targets (Fig. 7F) would permit such a scenario, in principle. To address this question, we asked whether MEApv neurons projecting to VMHdm or VMHvl were GABAergic (inhibitory), or glutamatergic (excitatory), by combining retrograde tracing with *in situ* hybridization for markers of these two classes of neurons: VGLUT2 (Fremeau et al., 2001) and GAD65 (Kaufman et al., 1991), respectively. These experiments indicated that the neurons in MEApv that project to VMHvl (the reproductive target) are glutamatergic (Fig. 7H), but not GABAergic (Fig. 7J), and therefore excitatory. The same was true for the neurons that project to VMHdm (the defensive target; Fig. 7G, I).

Taken together with the results obtained from the analysis of $Lhx6^+$ neurons, the foregoing experiments indicated that VMHvl, a reproductive hypothalamic cell group, receives two types of projections from the medial amygdala (Table I). One projection derives from $Lhx6^+$ neurons in MEApd that are activated by a reproductive, but not a defensive, olfactory stimulus; these projections are inhibitory. The second projection derives from neurons in MEApv that are, conversely, activated by the defensive but not the reproductive stimulus; these projections are excitatory. Thus, tracing of parallel circuits for reproduction and defense from the medial amygdala identifies a point of

convergence of these circuits in VMHvl. The complementary neurotransmitter phenotypes of these synaptic inputs raised the question of whether they might exert opposing influences on inhibitory neurons. Consistent with this idea, *in situ* hybridization with *Gad67* revealed that the reproductive portion of VMH (the lateral-most portion of VMHvl, and immediately adjacent regions of the tuberal (TU) nucleus) contains GABAergic cell bodies (Fig. 7N, arrowheads). By contrast, the defensive, dorsomedial portion of VMH is essentially devoid of such GABAergic neurons, and rich in *Vglut2*$^{+}$ neurons (Fig. 7M). Thus, the convergent inputs to VMHvl from the reproductive (Lhx6^{+}) and defensive regions of posterior MEA synapse in a region containing GABAergic neurons, as well as glutamatergic neurons. By contrast, the neighboring defensive region of VMH, which receives input from only the defensive region of MEA, lacks such inhibitory neurons.

DISCUSSION

Olfactory stimuli that trigger innate reproductive and defensive behaviors are relayed from the medial amygdala to the hypothalamus, both directly and indirectly via the BST (Canteras, 2002; Dong et al., 2001; Newman, 1999; Petrovich et al., 2001; Segovia and Guillamon, 1993; Simerly, 2002). The pathways mediating reproduction and defense are anatomically segregated (Petrovich et al., 2001), suggesting that they are likely to be genetically specified. We have found that the LIM homeodomain transcription factor Lhx6 delineates the reproductive branch of this pathway. Other LIM proteins mark different medial amygdalar subgroups, possibly involved in defense or aggression. The parallel reproductive and defensive amygdalar projections converge in

the reproductive hypothalamic subnucleus, VMHvl. The opposite neurotransmitter phenotypes of these convergent projections, and the complementary olfactory stimuli that activate them, suggest a potential neural substrate for suppressing reproductive behaviors when threatening stimuli are present.

Lhx6 marks an amygdalar-hypothalamic pathway for reproductive behavior

LIM homeodomain transcription factors comprise a combinatorial code that marks the columnar identity of motoneurons in the spinal cord, and determines the topography of their projections to limb muscle (Jessell, 2000; Shirasaki and Pfaff, 2002). We have found that in the amygdala, Lhx6 marks neurons in MEApd, a nucleus implicated in reproductive behaviors (Newman, 1999; Segovia and Guillamon, 1993; Simerly, 2002), while Lhx9 (Remedios et al., 2004) and Lhx5 mark other nuclei in the medial amygdala, including some implicated in defensive behaviors (Canteras, 2002; Canteras et al., 1995).

We have investigated the relationship between the neurons marked by these transcription factors, and the functions and connectivity attributed to the nuclei in which they reside (Canteras 2002). Lhx6 is expressed by neurons in MEApd that are activated by reproductive olfactory stimuli, and that project to regions of the hypothalamus involved in reproduction. While $Lhx6^{+}$ cells are not the only cells in MEApd that project to reproductive portions of the BST and medial hypothalamus, they project only to reproductive and not to defensive targets, and are activated only by reproductive and not by defensive olfactory stimuli. Lhx6 also marks neurons in the BSTpr that project to these same hypothalamic nuclei. In apparent exception to this rule, $Lhx6^{+}$ cells in

MEApv did not project to reproductive targets injected with CTB. These cells may be local interneurons, or may project to as-yet unidentified targets. The expression of Lhx6 by neurons in both MEApd and their projection target in BSTpr (Fig. 5B, purple) may be coincidental, or may suggest that Lhx6 participates in "transcriptional matching" of pre- and post-synaptic partners within neural circuits, as suggested previously for Ets domain and paired homeodomain transcription factors (Chen et al., 2001; Lin et al., 1998). In any case, these data indicate that Lhx6 delineates a pathway for reproductive behavior, comprising the MEApd and BSTpr and their projections to the hypothalamus (Fig. 5B).

These data raise the question of whether *Lhx6* functions in the development of this pathway. Our *Lhx6*$^{-/-}$ animals died before projections from the amygdala to the hypothalamus were established. Conditional mutations will therefore be required to determine whether LHX6 is essential for this pathway. Nevertheless, there is considerable evidence from *Drosophila,* chick, and mice to indicate that LIM homeodomain proteins control aspects of motoneuron projection specificity, as well as neurotransmitter identity (Jessell, 2000; Shirasaki and Pfaff, 2002; Thor et al., 1999; Thor and Thomas, 1997). Our data suggest that the concept of "LIM codes" as determinants of nuclear/columnar identity and projection topography may generalize to a complex region of the brain involved in processing innate social behaviors.

Lhx9 and Lhx5 also marked subpopulations of medial amygdalar neurons. Unlike Lhx6^{+} cells, these neurons do not project to reproductive targets in the hypothalamus or BST, and are not activated by female urine or by cat collar odor. Lhx5^{+}neurons were, however, activated during inter-male aggression (Supplemental Figure S1C). Lhx9^{+} neurons also project to defensive hypothalamic nuclei, but these neurons are located in

the paraventricular nucleus of the thalamus (PVT), a structure implicated in stress- and anxiety-related behaviors (Bhatnagar et al., 2003; Jaferi et al., 2003; Klejbor et al., 2003; Kurumaji et al., 2003). Thus, while the behavioral relevance of the Lhx5- and Lhx9-expressing neurons in MEA is not yet clear, the data are consistent with the notion that their projections mediate some aspects of defense or aggression.

A potential neural substrate for gating reproductive behavior by threatening stimuli

Animals faced with conflicting cues in their environment must often choose between mutually incompatible behaviors, such as reproduction or defense. Typically, threatening stimuli, such as predators or aggressive conspecifics, will suppress reproductive behavior. Thus, for example, cat odor can inhibit maternal behavior in rats (N. Canteras, personal communication). Although amygdalar-hypothalamic pathways mediating reproduction and defense have been mapped extensively using anterograde tracers (Canteras et al., 1995; Coolen and Wood, 1998; Dong et al., 2001; Gomez and Newman, 1992; Kevetter and Winans, 1981), there is, so far, no clear model to suggest where and how decisions between these competing behaviors might be controlled within this pathway.

The fact that medial amydgdalar nuclei activated by defensive stimuli (MEAa and MEApv) project to reproductive, as well as defensive, hypothalamic targets (Fig. 1B), suggests a potential neural substrate by which such stimuli might inhibit reproductive behaviors. However, the nature of the neurons involved in these projections, and the stimuli that activate them, have not been clear. Indeed, we find that reproductive (female urine) as well as defensive (cat odor) stimuli specifically activate neurons in MEApv.

Furthermore, we have shown that separate subpopulations of MEApv neurons project to distinct reproductive (VMHvl) and defensive (VMHdm) hypothalamic targets. This convergence and divergence makes it difficult to dissect circuits without relating projection specificity to stimulus selectivity, at the level of resolution of single cells. By performing double-labeling for retrograde tracers and markers of neuronal activation, or of neurotransmitter phenotype, we have discovered that, unexpectedly, the MEApv neurons that project to the reproductive hypothalamic target VMHvl are activated by cat collar odor, and not by female urine. Moreover, these projections are excitatory. These results are counter-intuitive: naively, one might have anticipated that excitatory neurons projecting from MEApv to a reproductive target would be activated by reproductive, rather than by defensive stimuli.

These observations can be rationalized when taken together with our finding that the $Lhx6^{+}$ projections to VMHvl from MEApd, which are activated by a reproductive stimulus, are inhibitory. Thus, VMHvl receives convergent inputs, of opposite "signs," from different subpopulations of medial amygdalar neurons that are activated by reproductive or defensive stimuli, respectively (Fig. 8). Such a circuit organization suggests that these convergent inputs function antagonistically to regulate output from this hypothalamic nucleus involved in reproductive behavior. What is perhaps surprising is that the "signs" of the inputs are reversed, relative to what one might have expected: the reproductive input to VMHvl is inhibitory, while the defensive input is excitatory.

Why should these potentially antagonistic inputs be assigned their neurotransmitter phenotypes in this manner? There are two classes of circuit model that could explain this observation. In one model, the inhibitory projections from $Lhx6^{+}$

neurons in MEApd would release reproductive behavior by inhibiting inhibitory interneurons in VMHvl (Fig. 8A, B). In that case, the glutamatergic projections from MEApv, which are activated by predator odors, could excite these same interneurons, thereby suppressing output from VMHvl (Fig. 8A). In another variant of this model, the convergent reproductive and defensive projections from MEApd and MEApv might synapse onto separate sub-populations of inhibitory interneurons, which later converge onto common glutamatergic output neurons (Fig. 8B). Consistent with this class of models, the reproductive portion of VMH (the lateral-most portion of VMHvl and the immediately adjacent regions of the tuberal (TU) nucleus) contains GABAergic neurons (Fig. 7N) and synapses (Commons et al., 1999), while the defensive portion (VMHdm) does not. This type of synaptic "gate control" has a precedent in the dorsal spinal cord, where a similar mechanism was originally proposed to explain why high-threshold (Aß) cutaneous mechanosensory input (i.e., vigorous rubbing of the skin) can suppress painful signals transmitted by nociceptors (Wall, 1980).

A second class of model does not invoke the involvement of local circuit inhibitory interneurons to mediate suppression of reproductive behaviors by defensive stimuli. In this model, the inhibitory $Lhx6^{+}$ projections from MEApd would synapse directly onto glutamatergic output neurons in VMHvl (Fig. 7M), suppressing their activity (Fig. 8C). Dis-inhibition of reproductive behavior would be mediated by the parallel, double-negative projection, involving the BST (Swanson, 2000). In such a circuit arrangement, specific presynaptic excitation of the direct inhibitory projection from MEApv by the glutamatergic predator odor-driven projection from MEApv, would

enhance inhibition of the glutamatergic output neurons, thereby suppressing reproductive behavior (Fig. 8C).

Clearly, more complicated circuit arrangements combining aspects of both models are possible. Tests of these models will require combining site-directed pharmacologic or genetic manipulations of GABAergic and glutamatergic synaptic transmission, with detailed anatomical analysis of synaptic connectivity within VMHvl. The important point is that our data suggest a potential site for the "gating" of reproductive behavior by threatening stimuli in the hypothalamus. This does not exclude the possibility that such antagonistic control of reproductive behavior may occur instead, or in addition, at other sites in the circuit. For example, the convergence of parallel reproductive and defensive projections from MEApd/v may also occur in other reproductive hypothalamic nuclei, such as MPN or PMv. Furthermore, there are known reciprocal connections between MEApd and MEApv (Canteras et al., 1995), which could in principle also mediate such antagonism.

If VMHvl receives opposing inputs from both reproductive and defensive amygdalar nuclei, what determines which behavior predominates in a situation where the organism is faced with conflicting cues? More importantly, the projections from the MEA to the hypothalamus exhibit an overall asymmetry: the "reproductive" portion of MEA, MEApd, projects only to reproductive hypothalamic nuclei, whereas the "defensive" portion of MEA, MEApv, projects to both reproductive and defensive hypothalamic nuclei (Fig. 1B). If the projection of MEApv to reproductive nuclei indeed serves to inhibit reproductive behaviors, then this asymmetry implies a dominance of defensive over reproductive behaviors. In other words, priority has evidently been given

to interrupting reproductive behaviors if threatening stimuli are present, rather than the other way around. From an evolutionary standpoint, this makes sense: animals abrogating aggressive encounters or predator defenses to engage in reproductive behavior, despite the continued presence of the threat, are less likely to achieve reproductive success.

Finally, the notion that threatening stimuli repress reproductive behaviors is consistent with the observation that mice lacking *TrpC2*, which is required for VNO function, display reduced inter-male aggression and increased male-male mounting (Leypold et al., 2002; Stowers et al., 2002). While this phenotype has been interpreted to primarily reflect a defect in gender recognition (but see Pankevich et al., 2004), it may in addition involve a dis-inhibition of mating behavior, as a secondary consequence of an inability to detect aggression-promoting cues. Consistent with this notion, female *TrpC2*$^{-/-}$ mice also show reduced maternal aggression (Leypold et al., 2002). Although cat collar odor does not elicit aggressive behavior per se, it is thought to be detected by the VNO (McGregor et al., 2004) and suppresses ultrasonic vocalization evoked by female urine (G.C. and D.J.A., unpublished observations). It is possible that chemosensory stimuli that signal threats from a variety of sources simultaneously suppress reproductive behaviors in order to give priority to fight or flight responses.

EXPERIMENTAL PROCEDURES

The generation of gene-targeted PLAP mice was accomplished using *VelociGene* technology (Valenzuela et al., 2003). *In situ* hybridization, immunohistochemical

staining, and retrograde tracing experiments were performed using established procedures. Further experimental details can be found in the supplemental data.

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FIGURE LEGENDS

Figure 1. Processing of chemosensory stimuli through the VNO pathway.

(A) Schematic diagram of a transverse section through MEAp (top panel) and a flat map of diencephalon (bottom panel). Reproductive nuclei (red) and the defensive nuclei of the hypothalamic behavioral control column are indicated (adopted from Swanson, 2000). See Supplementary Material and Supplementary Table 1 for list of abbreviations. (B) Topographic organization of the projections from the accessory olfactory bulb (AOB) to the MEA, to the nuclei in the hypothalamic behavior control column (modified from Swanson, 2000). (C) Convergence and divergence in the activation and projections of MEAp subnuclei. MEApv is activated by both reproductive (this study) and defensive (Dielenberg et al., 2001) stimuli, and projects to both reproductive ("R") and defensive ("D") hypothalamic nuclei.

Figure 2. Expression of LIM homeodomain genes in the subnuclei of the MEA

(A-I) In situ hybridizations were performed with the indicated probes on serially adjacent transverse sections of an adult male mouse through MEAp (A-C), MEAa (D-F), or BST (G-I). Abbreviation: opt = optic tract, sm = stria medullaris, fx = fornix. (J) Double label immunofluorescence staining with anti-GFP and anti-Lhx9 antibodies on coronal sections of MEApv from $Lhx6^{EGFP/+}$ mice. (K-L) Coronal sections through MEAa hybridized with Lhx5 cRNA probes, combined with immunofluorescence detection of Lhx6 (K) or Lhx9

(L). (M) Schematic diagram showing that MEA subnuclei express different members of LIM homeodomain family.

Figure 3. PLAP staining of reproductive and defensive nuclei in Lhx6- and Lhx9-PLAP mice.

(A-B) Generation of Lhx6-PLAP and Lhx9-PLAP mice. A PLAP/loxp/PGK neomycin cassette replaced the Lhx6 (A) or Lhx9 (B) coding region. (C-J) Transverse sections through the indicated brain regions stained for PLAP activity from postnatal (P1-P7) $Lhx6^{PLAP/+}$ (C-F) or $Lhx9^{PLAP/+}$ (G-J) mice. Abbreviation: dm=VMHdm, vl=VMHvl, d=PMd, v=PMv, pr=BSTpr, if=BSTif.

Figure 4. Retrograde tracing experiments from reproductive and defensive nuclei of hypothalamus and BST

(A-P) Double immunofluorescence staining with anti-CTB and anti-Lhx6 antibodies (A-K) or with anti-CTB and anti-Lhx5 antibodies (L-P) on transverse sections through MEApd (A-F), BST (G-K), or MEAa (L-P). Mice were injected with CTB in the brain regions indicated on the left. (Q) Double immunofluorescence staining with anti-CTB antibody and anti-FG antibody on coronal section through MEAa from mouse injected with CTB in VMHdm and FG in VMHvl. Abbreviations: dm= VMHdm, vl=VMHvl.

Figure 5. Summary of retrograde tracing experiments

(A) Lhx6+ cells in MEApd could be back-labeled from BST (A_2), MPN (A_2), VMHvl (A_3), and PMv (A_4). Axons originating from MEApd pass through AHN (A_2) to

terminate in more posteriorly located VMHvl and PMv. (B) Schematic diagram showing that Lhx6$^+$ cells in MEApd project to all the reproductive nuclei, where as Lhx5$^+$ cell in MEAa and Lhx9$^+$ cells in PVT project to defensive nuclei.

Figure 6. Lhx6$^+$ cells in MEApd are activated by reproductive stimuli

(A-D) Double immunofluorescence staining of transverse sections though MEApd with anti-Lhx6 antibody, and either anti-c-fos antibody (C), or a *c-fos* cRNA *in situ* hybridization probe (A-B, D). Male mice were exposed to olfactory stimuli as indicated. (E) Mice injected with CTB in VMHvl, and exposed to female urine one week later. Thirty minutes after exposure, animals were sacrificed and transverse sections were hybridized with cRNA probes for *c-fos*, and then immunostained with anti-CTB and anti-Lhx6 antibodies. Arrowheads indicate triple-positive cells. (F-G) Coronal sections through MEApd hybridized with cRNA probes for GAD67 (F) or for Vglut2 (G), combined with immunofluorescence detection of Lhx6. (H- I) Quantification (mean +/- STDEV) of *c-fos* activation in MEApv by reproductive (H) or defensive (I) stimuli. (J) Quantification (mean +/- STDEV) of c-fos activation in Lhx6$^+$ neurons in MEApd by olfactory stimuli. Abbreviations: MU=male urine, FU=female urine, control C = control collar, Cat C= cat collar.

Figure 7. Separate populations of MEApv neurons project to reproductive and defensive domains of VMH, but are both activated by a defensive stimulus.

(A-D) Transverse sections through MEApv in mice retrogradely labeled in either VMHdm (A, C) or VMHvl (B, D), and subsequently exposed to cat odor (A, B) or female

urine (C, D), were double-labeled with a *c-fos* cRNA probe and anti-CTB antibody. (E-F) Double immunofluorescence staining for CTB and FG on transverse sections through MEApv in mice co-injected with CTB and FG in VMHdm (E), or with CTB in VMHdm and FG in VMHvl (F). See (L) for quantification. (G-J) Transverse sections through MEApv in mice retrogradely labeled from either VMHdm (G, I) or VMHvl (H, J) were hybridized with Vglut2 (G-H) or GAD65 (I-J) cRNA probes and immunostained with anti-CTB antibody. (K) Quantification (mean +/- STDEV) of *c-fos* induction by cat odor vs. female urine among neurons back-labeled by CTB from VMHdm or VMHvl. Abbreviations: dm=CTB injection in VMHdm, vl=CTB injection in VMHvl, FU=female urine, Cat=cat odor. (L) Quantification (mean +/- STDEV) of overlap between neurons in MEAa and MEApv back-labeled by dual injection of CTB and FG into VMHdm and/or VMHvl. Abbreviations: dm+dm: CTB and FG both injected in VMHdm; dm+vl: CTB and FG injected in VMHdm and VMHvl, respectively. (M and N) Transverse sections through VMH hybridized with Vglut2 (M) or GAD67 (N) cRNA probes. Filled arrowhead indicate $GAD67^+$ neurons in the capsular part of the VMHvl; open arrowhead indicate $GAD67^+$ neurons in the adjacent tuberal nucleus. Abbreviations: vl=VMHvl, TU=tuberal nucleus.

Figure 8. Models for "gate control" of reproductive behavior in the hypothalamus

Schematic illustrating possible mechanisms by which the convergent inputs to the reproductive hypothalamic nucleus VMHvl might antagonistically control reproductive behaviors. "+" indicates excitatory (glutamatergic) neurons; "-" indicates inhibitory (GABAergic) neurons. (A, B) In one class of model, projections from MEApd and

MEApv synapse onto local inhibitory interneurons in VMHvl, which in turn inhibit firing of excitatory output neurons. In (A) the amygdalar projections synapse onto common inhibitory interneurons (*); in (B) the axons synapse onto distinct interneurons, which in turn converge onto output neurons (*). (C) In a different class of model, antagonism is not mediated by local inhibitory interneurons. Rather, the level of excitatory output from VMHvl is controlled by a balance between direct inhibition (from MEApv), versus indirect dis-inhibition (via BSTpr) (see Swanson, 2000). The input from MEApv shifts the balance in favor of inhibition, by pre-synaptically exciting the direct inhibitory projection from $Lhx6^{+}$ neurons in MEApd. Note that VMHdm, which controls defensive behaviors, does not receive any input from the reproductive portion of MEApd (see also Fig. 1B), and does not contain any inhibitory interneurons (Fig. 7N).

Table 1. Summary table showing the axonal termination sites of various MEAp neuronal populations, forms of stimuli used to activate these neurons, and transmitters they use.

Origin of Projections	Termination Site	c-fos induction		Transmitter	
		Female Urine	Cat odor	GAD65	Vglut2
MEApd	VMHvl	+	–	+	–
MEApv	VMHvl	–	+	–	+
	VMHdm	–	+	–	+

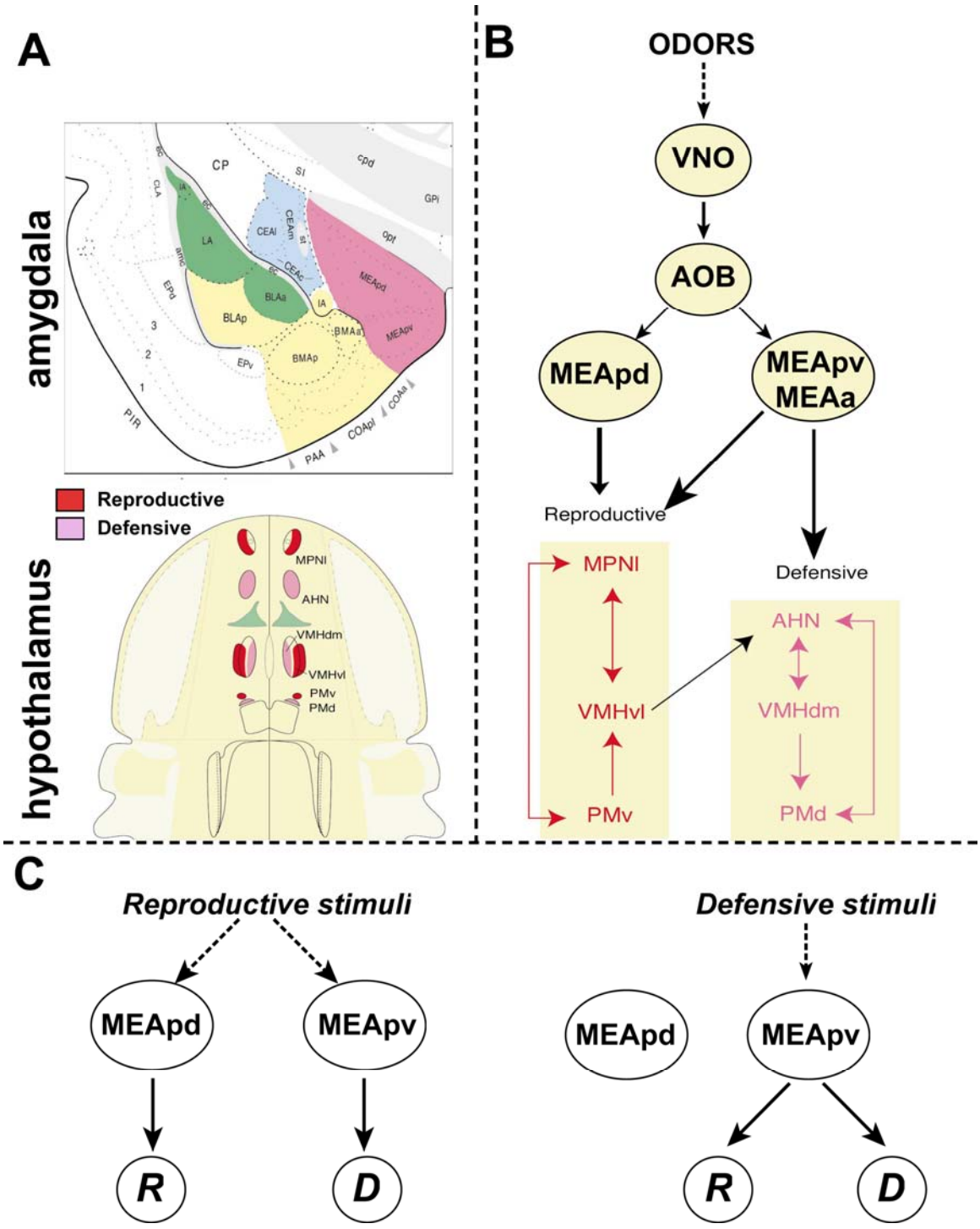


Figure 1. Processing of chemosensory stimuli through the VNO pathway.

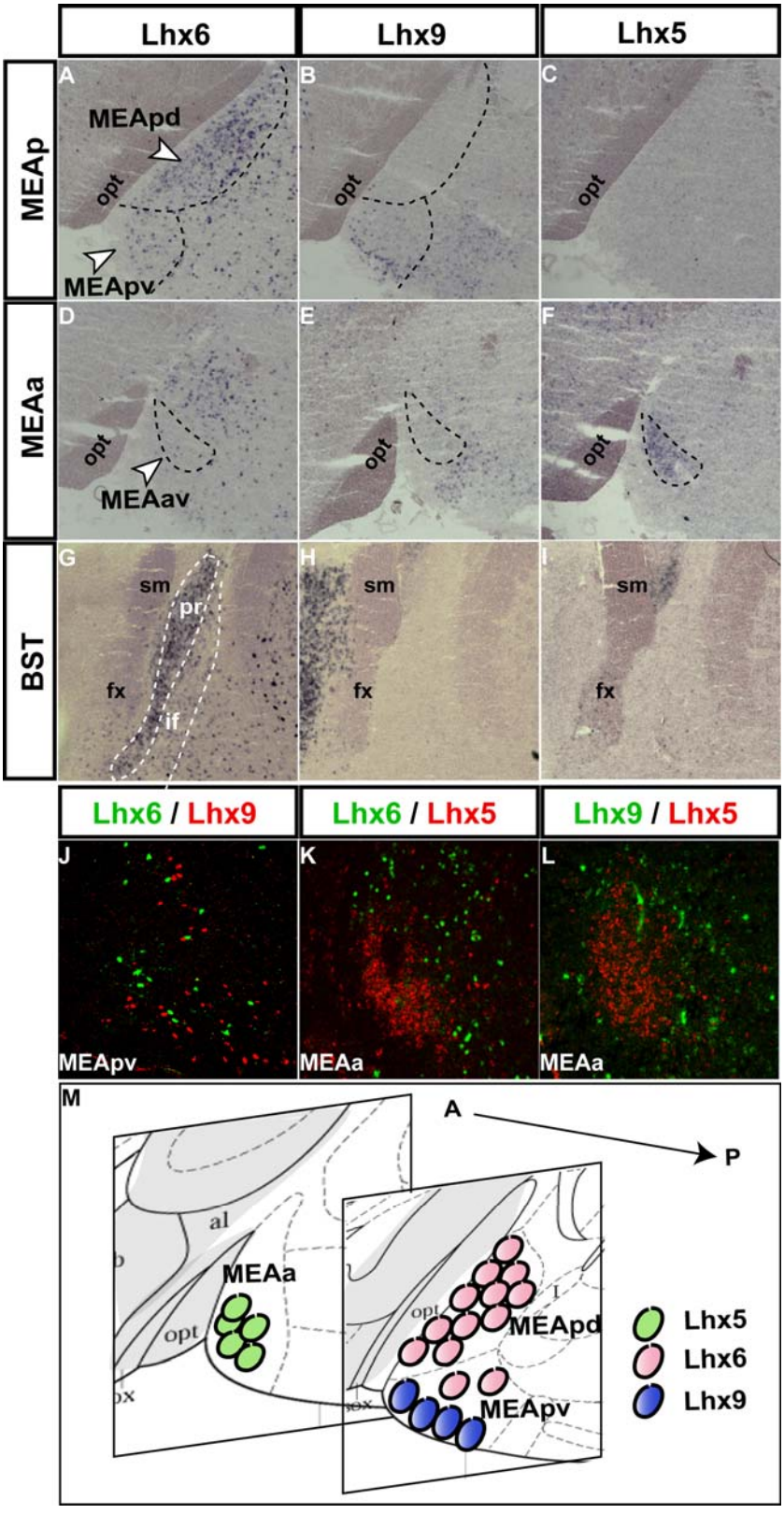


Figure 2. Expression of LIM homeodomain genes in the subnuclei of the MEA

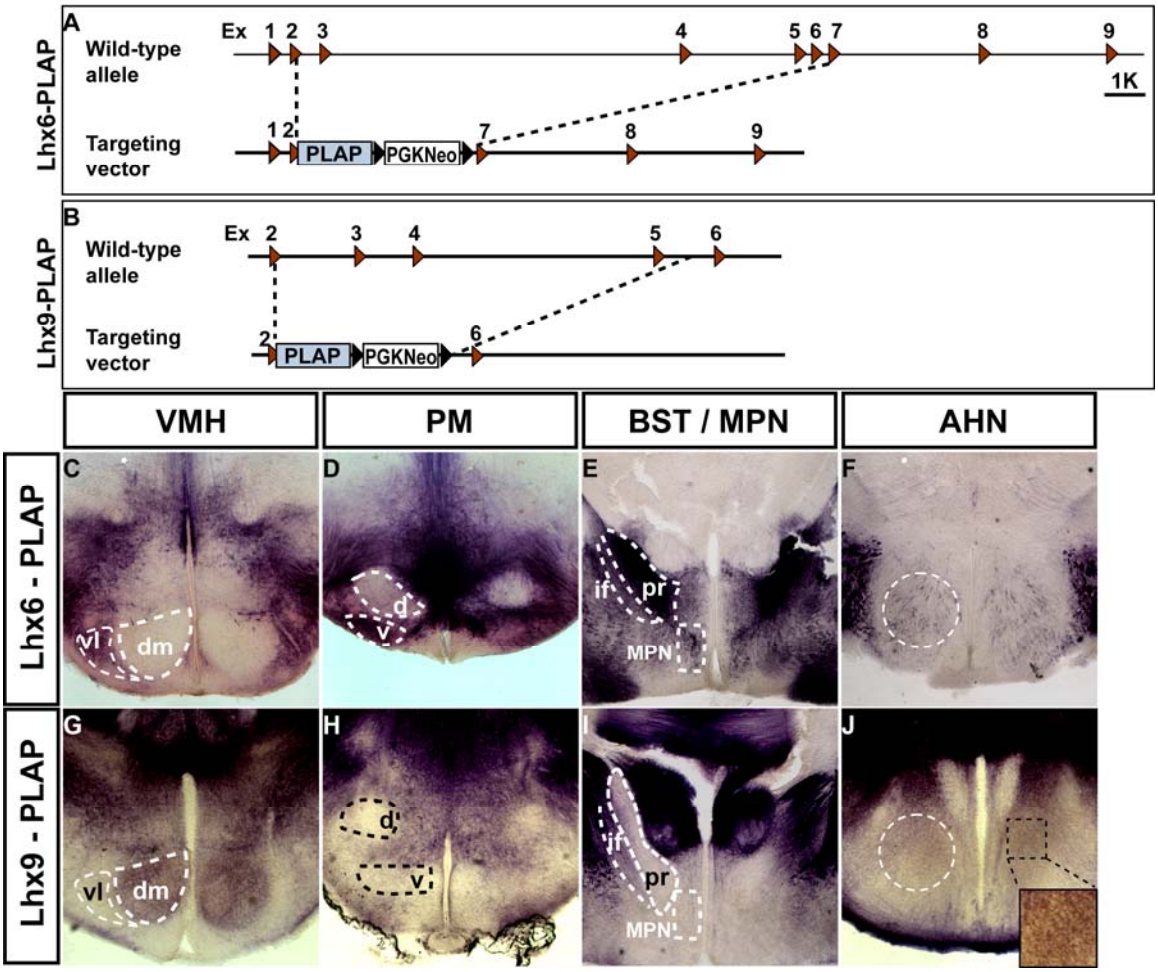


Figure 3. PLAP staining of reproductive and defensive nuclei in Lhx6- and Lhx9-PLAP mice.

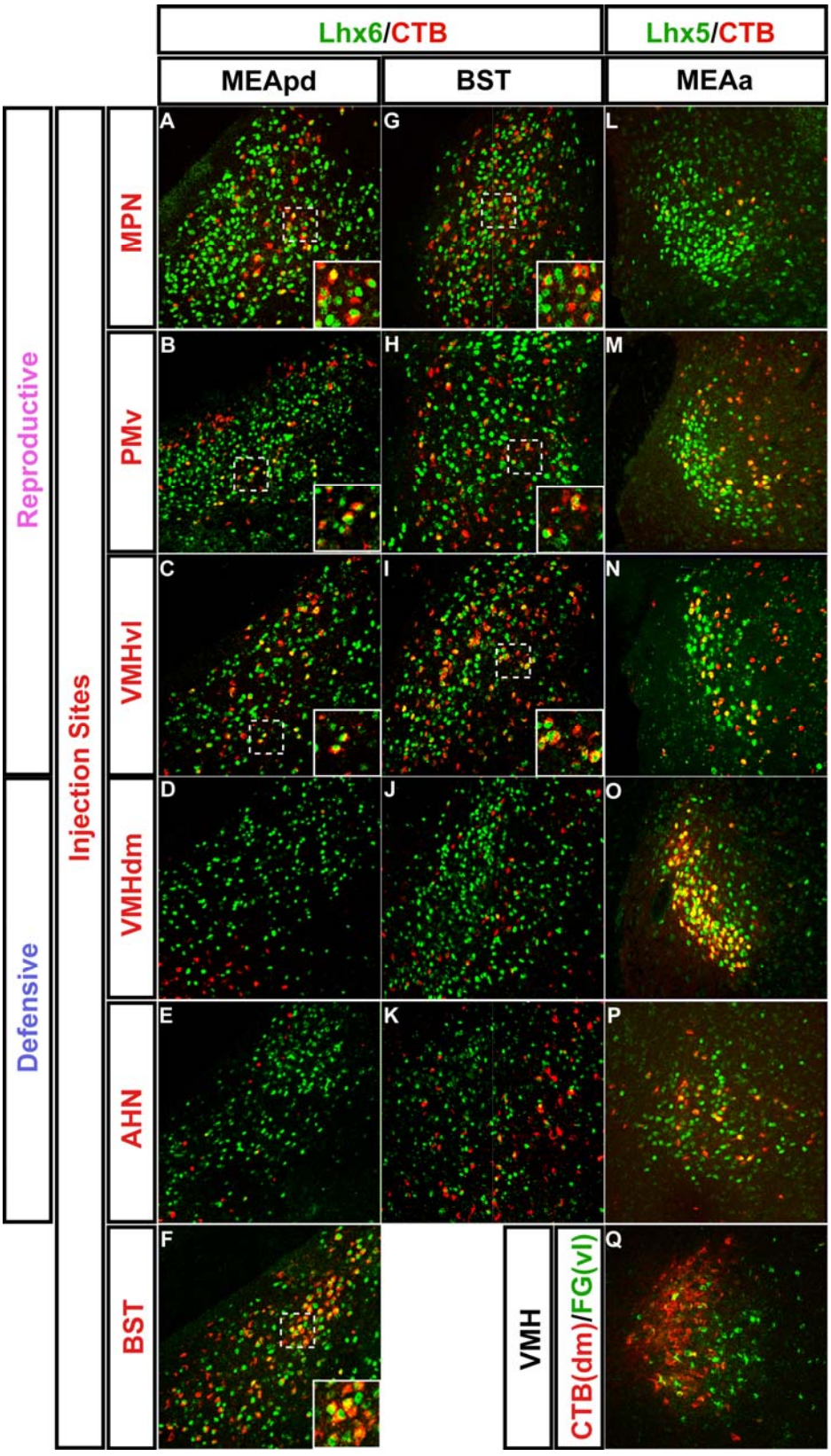


Figure 4. Retrograde tracing experiments from reproductive and defensive nuclei of hypothalamus and BST

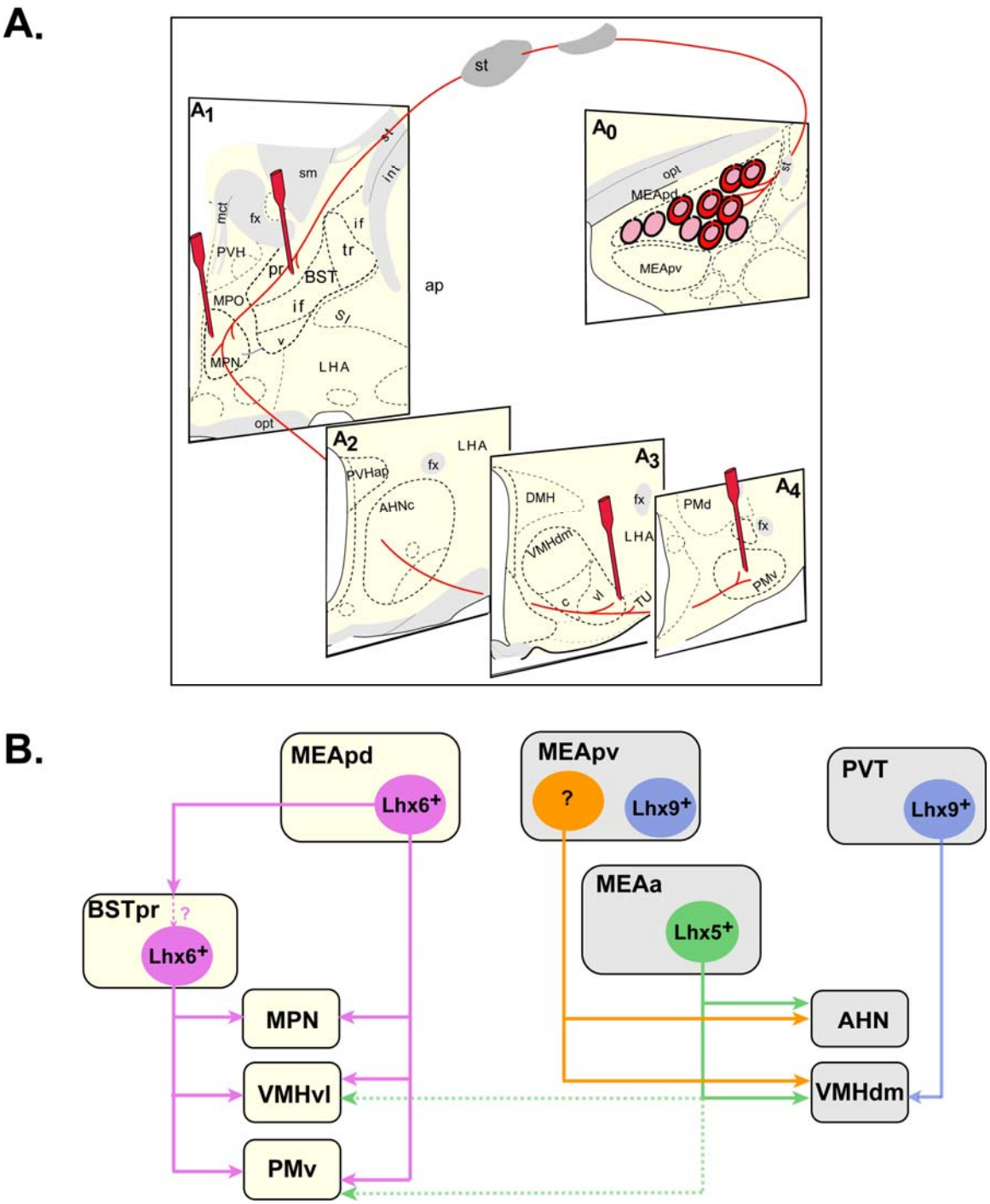


Figure 5. Summary of retrograde tracing experiments

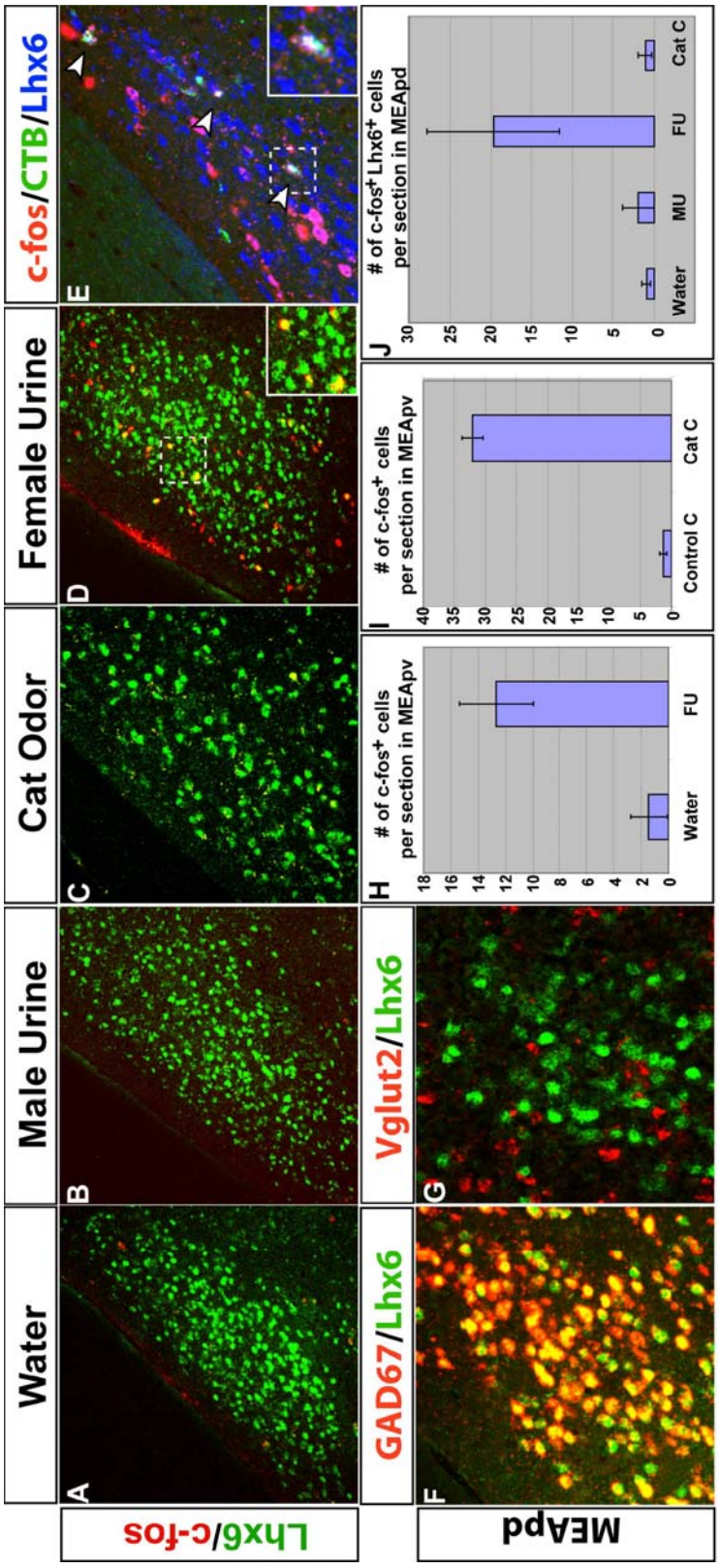


Figure 6. Lhx6^{+} cells in MEApd are activated by reproductive stimuli

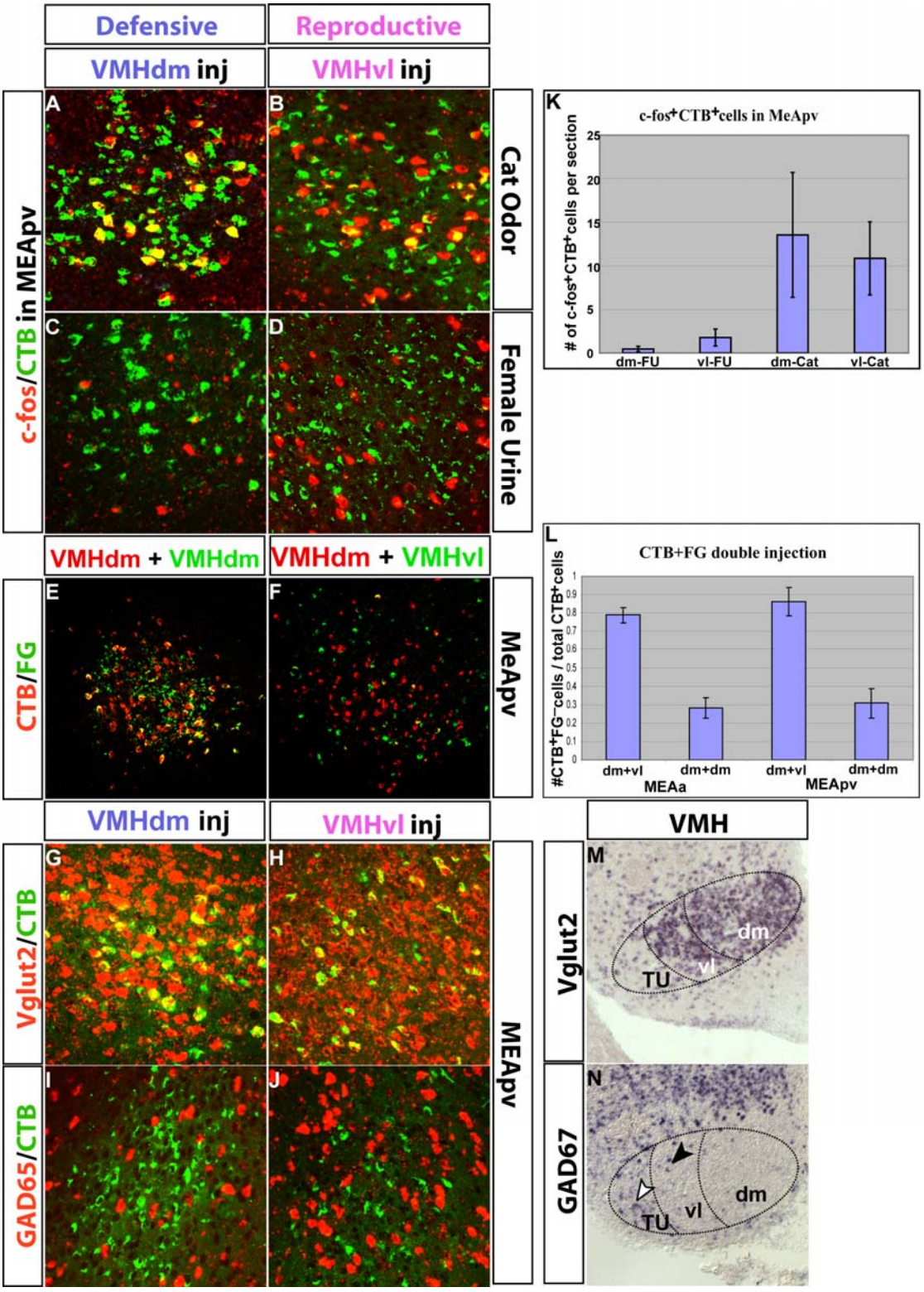


Figure 7. Separate populations of MEApv neurons project to reproductive and defensive domains of VMH, but are both activated by a defensive stimulus.

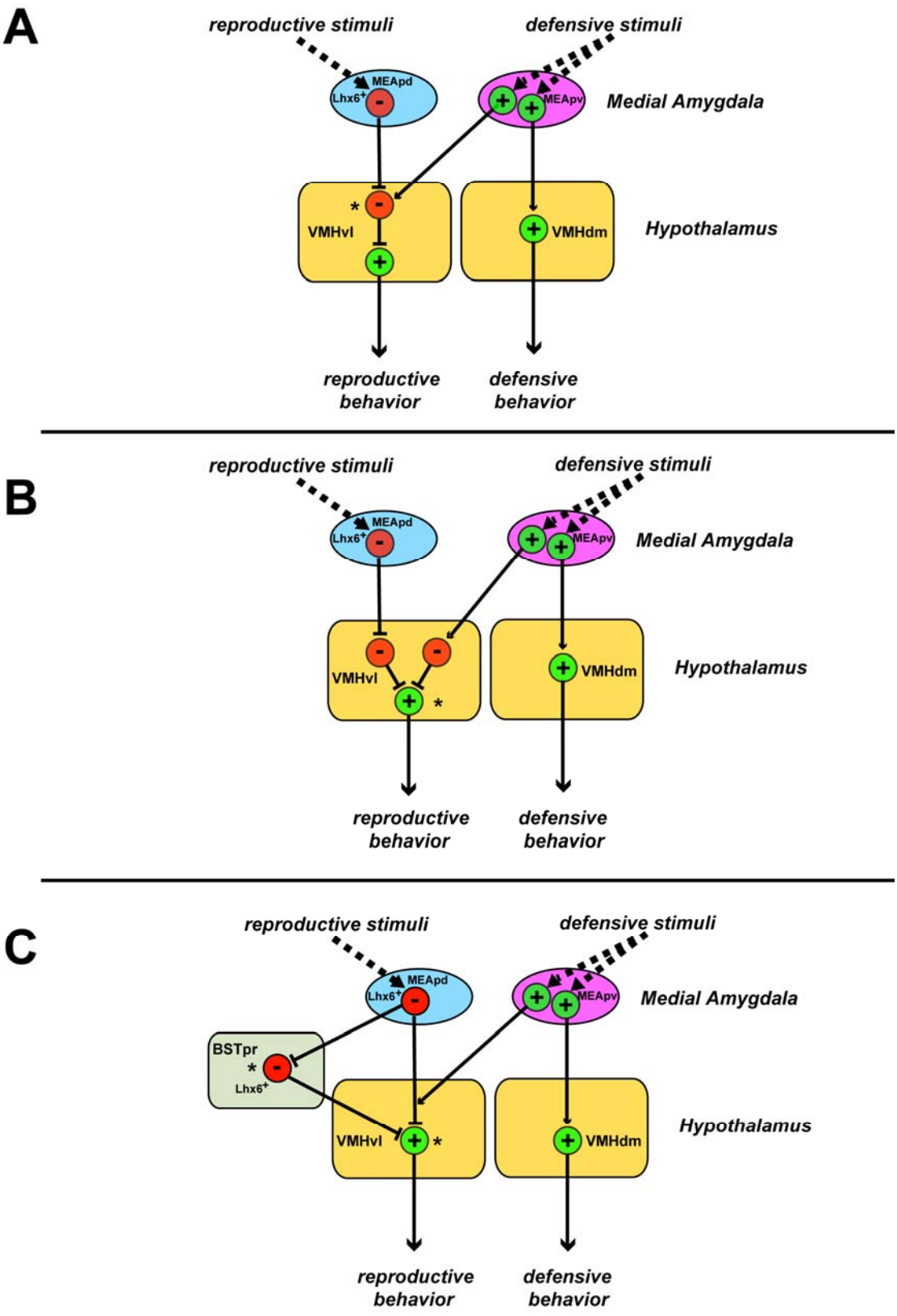


Figure 8. Models for "gate control" of reproductive behavior in the hypothalamus

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Generation of PLAP mice

VelociGene technology was used to generate a deletion and exchange of Lhx6 and Lhx9 coding regions with placental alkaline phosphotase (PLAP) reporter gene as well as a neomycin-selection cassette (Valenzuela et al., 2003). Chimeric male mice resulting from such manipulation were then bred to C57BL/6 females to generate F1 mice or embryos.

In Situ Hybridization and PLAP Staining

Nonradioactive in situ hybridization was performed as previously described (Zhou et al., 2000). The following mouse genes were used: Lhx6, Lhx9, Lhx5, GAD65, VGLUT2, and c-fos.

For fluorescent in situ hybridization, digoxygenin- or fluorescein labeled probes were detected with horseradish peroxidase-conjugated antibodies. The signal was amplified with the avidin-biotin complex system (ABC Elite Kit, Vector Laboratories, Burlingame, CA) and subsequently developed with fluorochrome-conjugated tyramide (Cy3- or FL-Tyramide, NEN, Boston, MA). For double fluorescent in situ hybridization, the first peroxidase-conjugated antibody was inactivated at 85°C. The second probe was detected in the essentially the same way as the first.

PLAP staining was performed as previously described (Shah et al., 2004).

Immunohistochemistry

The following primary antibodies were used: rabbit anti-Lhx6 (1:1000, kind gift of Dr. Vassilis Pachnis), rabbit anti-Lhx2/Lhx9 (1:1000, kind gift of Dr. Thomas M. Jessell), rabbit anti-Lhx5 (1:2000, kind gift of Dr. Thomas M. Jessell), goat anti-CTB (1:1000,

List), rabbit anti-FG (1:1000, Chemicon), and goat anti-c-fos (1:1000, Santa Cruz Biotechnology), chicken anti-GFP (1:1000, Aves Labs). The fluorophore-conjugated secondary antisera used are: Cy5 donkey anti-rabbit (1:300, Jackson ImmunoResearch), Alexa 488 donkey anti-rabbit (1:250, Molecular Probes), Alexa 488 donkey anti-goat (1:250, Molecular Probes), Alexa 568 donkey anti-goat (1:250, Molecular Probes), and Alexa 568 Goat anti-rabbit (1:250, Molecular Probes).

Retrograde Tracing Experiments

Eight week old, sexually naïve C57BL/6 male mice from Jackson received bilateral injections of 0.5% cholera toxin B subunit (CTB; low salt; List, Campbell, CA) or/and 2% Fluoro-Gold (FG; Fluorochrome, Englewood, CO) in the following brain areas: MPN, AHN, VMHdm, VMHvl, PMv, and BST. The tracers were delivered with a positive-pulsed current of 5 μA for 2 minutes. After surviving for 7 days, the animals were anesthetized with a mixture of ketamine and xylazine and perfused transcardially with 5 ml of PBS followed by ice-cold 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were carefully dissected out, cryoprotected in 15% sucrose, and sectioned at 20 μm. The sections were immunostained with anti-CTB (see below) or/and ani-FG (see below) antibody and subsequently incubated with fluorescent Nissl staining reagent, Neurotrace Green (1:100, Molecular Probes).

Behavioral Assays

Retired C57BL/6 males from Harlan Sprague-Dawley were used for all behavioral assays. For the behavioral assays following retrograde tracing experiments, animals were allowed to recover for a minimum of one week after the surgery. For cat-odor exposure experiments, animals were exposed for 30 minutes to pieces of a cat collar, which was

worn for two weeks by a domestic cat. The control odor group was exposed to equivalent pieces from a collar that had not been worn by a cat. For urine exposure experiments, males were exposed to either distilled water, urine from C57BL/6 males, or C57BL/6 females of various ages. For resident-intruder assays, C57 males were allowed to interact with no animal (left alone), DBA2 females, or DBA2 males for 10 minutes. 30 minutes (for c-fos mRNA detection) or one hour (for c-fos protein detection) after the introduction of stimulus, animals were processed for in situ hybridization and/or immunohistochemistry as described above.

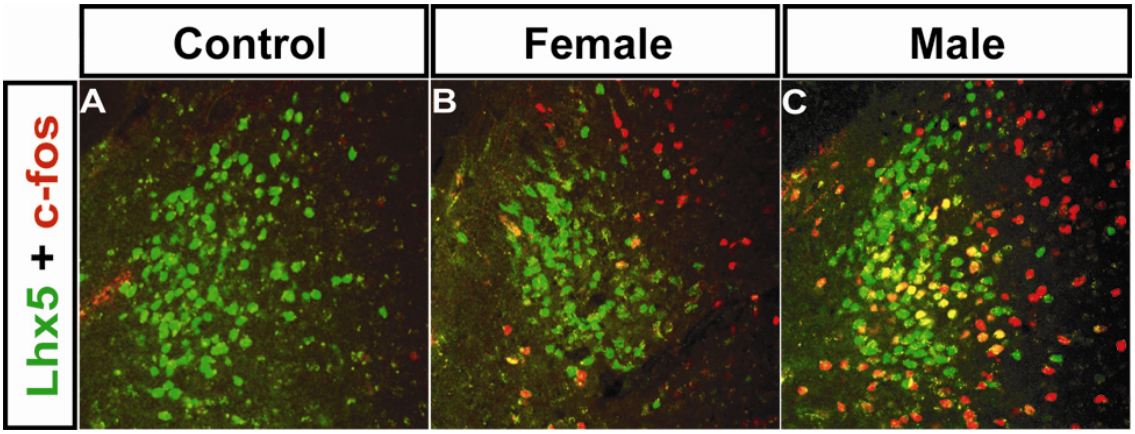
Quantification

Images (~1 μm optical thickness) of MEApd, MEApv or MEA from each animal were counted to quantify the number of c-fos$^+$ cells, c-fos$^+$Lhx6$^+$ cells, or c-fos$^+$CTB$^+$ cells. The total number of such cells was obtained by analyzing all MEA sections at every 120 μm apart. The number of cells per section was obtained by averaging the total number by the number of the sections analyzed for each animal. For CTB and FG double injection experiments, the number of CTB$^+$FG$^-$ or the number of total CTB$^+$ cells were counted in the same manner.

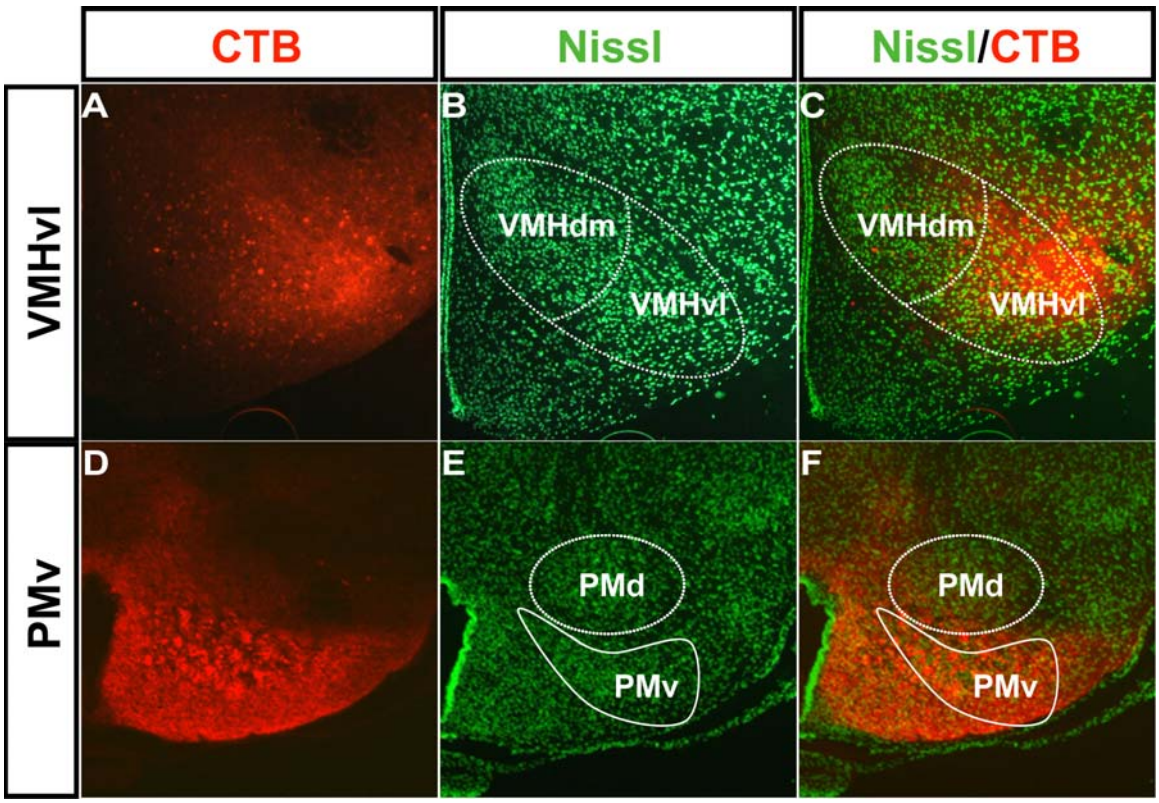
SUPPLEMENTAL INFORMATION FOR FIGURE 1A

Abbreviations: AHN = anterior hypothalamic nucleus; BLAa,p = basolateral amygdala, anterior or posterior part; BMAa,p = basomedial amygdala, anterior or posterior part; CEAc,l,m = central amygdala, capsular, lateral, or medial part; LA = lateral amygdala; MEApd,pv = medial amygdala, posterior dorsal or posterior ventral part; MPNI = medial preoptic nucleus; PMd = dorsal premammillary nucleus; PMv = ventral premammillary nucleus; VMHdm,vl = ventromedial hypothalamic nucleus, dorsomedial or ventrolateral part.

SUPPLEMENTAL FIGURES



Supplemental Figure 1. Lhx5^{+} neurons are activated during aggressive encounters. (A-C) Transverse sections through MEAa of C57 males with no animal (A), DBA2 female (B) or DBA2 male (C), immunostained with anti-Lhx5 and anti-c-fos antibody.



Supplemental Figure 2. Injection sites into the PMv and VMHvl.

(A-C) Transverse section through VMH from the mouse injected with CTB in VMHvl immunostained with anti-CTB antibody and followed by fluorescent Nissl staining. (D-F) Transverse section through PM from the mouse injected with CTB in PMv immunostained with anti-CTB antibody and followed by fluorescent Nissl staining.

SUPPLEMENTAL TABLE 1

AOB	Accessory Olfactory Bulb
AHN	Anterior Hypothalamic Nucleus
BSTif	Bed Nucleus of the Stria Terminalis, interfascular division
BSTpr	Bed Nucleus of the Stria Terminalis, principle division
MEAa	Medial Amygdala, anterior division
MEApd	Medial Amygdala, posterior dorsal division
MEApv	Medial Amygdala, posterior ventral division
MPN	Medial Preoptic Nucleus
PMv	Pre-mammillary nucleus, ventral portion
PMd	Pre-mammillary nucleus, dorsal portion
PVT	Paraventricular thalamic nucleus
TU	Tuberal hypothalamic nucleus
VMHdm	Ventromedial Hypothalamic Nucleus, dorso-medial
VMHvl	Ventromedial Hypothalamic Nucleus, ventro-lateral
VNO	Vomeronasal Organ