# Single-cell molecular and developmental perspectives of sexually dimorphic circuits underlying innate social behaviors

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## Highlights

- Molecular profiling of vomeronasal and olfactory sensory neurons that detect sex-specific information.
- A diversity of sexually dimorphic and non-dimorphic cell types in the amygdala and hypothalamus.
- Defining cell types with molecular, anatomical, physiological and developmental criteria.
- Transcriptional hypothesis for mammalian sexually dimorphic circuit development.

## Abstract

While single-cell transcriptomics in the brain has uncovered a vast diversity of neural cell types with unprecedented detail, it is becoming increasingly urgent to address exactly what their functional roles are in the context of circuits and behavior. In this review, we discuss the molecular profiling of cell types in circuits underlying social behaviors in mice as a prominent

case study. We first highlight key examples on the roles of molecularly identified sensory and downstream neurons involved in sexual dimorphic behaviors. We then propose future opportunities to define cell types using multimodal criteria, especially gene expression, physiology as well as developmental origin to advance our understanding of the circuits.

**The blueprint of innate social circuits at single-cell resolution**

The neural circuits underlying social behaviors drive animals' basic needs to cooperate, compete, and reproduce for the survival of individuals and species. Like other prominent neuroethological models of vertebrate behaviors [1,2], social behaviors can be triggered by well-characterized stimuli, which help establish causal links between specific neurons and robust behavioral outputs. The analysis of these behavior circuits can be further facilitated by taking advantage of genetically identified cell types. Mating, territorial aggression, and pup-directed behaviors can be triggered with little to no prior experience, indicating the genetic pre-programming of functional social circuits. These innate social behaviors therefore allow the detailed mapping of these circuits by molecular and genetic means. Understanding the structure of the innate circuits will then help identify how experience as well as hormonal changes can further modify them, for example as demonstrated in the parental behaviors of experienced mothers [3]. Moreover, many innate social behaviors are sexually dimorphic, providing an additional opportunity to interrogate the roles of sex hormones in circuit development and homeostasis. In fact, the molecular basis of sex hormone actions in the brain has been extensively studied [4], and sex hormone receptor expression has been used to label selective neurons to test their causal roles in sexually dimorphic behaviors [5].

In this short review, we highlight how single-cell molecular profiling studies of sexually dimorphic circuits have accelerated our understanding of the circuit architecture and information

processing underlying innate social behaviors. We discuss future challenges to delineate the development of sexually dimorphic circuits. Refined definition of cell types using multimodal criteria, especially with anatomical, developmental, molecular, physiological and behavioral data, should provide important foundations for future research investigating the circuits underlying social behaviors.

**Specific vomeronasal and olfactory neurons mediate social information**

Single-cell studies of vomeronasal and olfactory neurons have uncovered the organization of segregated sensory inputs carrying specific social information regarding sex, age, and hormonal states to the olfactory bulb (**Figure 1A,B**). These neurons play critical roles in the detection of pheromones that trigger multiple repertoires of innate social behaviors [6-8]. In this system, a single receptor gene is expressed in each neuron [9], allowing each neuron to be functionally classified by the receptor genes expressed. Large scale *in situ* screens have characterized nearly one hundred vomeronasal neurons activated by diverse conspecific and heterospecific chemosignals [10-12]. Importantly, these studies uncovered the remarkable specificity of each vomeronasal receptor tuned to ethologically and physiologically relevant information. For example, V2R receptors are exquisitely tuned to male, female, and predator specific ligands, and these receptor neurons are linked to the control of specific social behaviors, including lordosis, mating rejection, and pup-directed behaviors [13-15]. By comparison, specific V1R receptors recognize a variety of steroids, *e.g.*, sulfated estrogens, some of which have been linked to appetitive behaviors (**Figure 1B**) [16-18].

Moreover, important progress has been made towards identifying receptors that detect ethologically relevant volatile odorants [19-21]. In fact, thousands of odorant receptors expressed in the mouse main olfactory epithelium posed a technical challenge for identifying specific

odorant receptors that detect ethologically relevant odorants. Using phosphorylation of ribosome protein S6 as a marker for neural activity in olfactory sensory neurons, a handful of receptors that show sex-biased expression were found to be specifically activated by the male-specific chemosignals MTMT and SBT (**Figure 1B**) [21].

The logic of social inputs into the brain unraveled by these studies helps understand the role of downstream areas, including the amygdala and hypothalamus (**Figure 1C**), and the sex-specific computations performed with these inputs.

**Single-cell sequencing of sexually dimorphic circuits in the hypothalamus and amygdala**

Recent studies have characterized the diversity of cell types within the core circuitry of innate social behaviors using single-cell RNA sequencing (scRNA-seq), predominantly in the hypothalamus and amygdala (**Figure 1C**). These nuclei harbor circuits controlling sexually dimorphic behaviors such as aggression, lordosis, mounting, infanticide, and parental behaviors [22-27]. Although many of these nuclei broadly express sex hormones receptors, they indeed consist of heterogeneous populations of cells. In fact, *c-fos* induction after mating or aggression shows distinct activity patterns, uncovering the functional heterogeneity within Estrogen receptor α (Esr1) expressing neurons [23,28]. In males, *Esr1*$^+$ cells in the ventrolateral ventromedial hypothalamus (VMHvl) are required for both mating and aggression [24] (**Figure 1D**), while *Esr1*$^+$ cells in the preoptic area control parental behavior and mating [27]. Importantly, the studies of broadly classified neural populations could potentially mask the underlying complexity within local circuitry. For example, optogenetic activation of VMHvl *Esr1*$^+$ neurons induced both mating and aggression depending on photostimulation intensity [24], underscoring the importance of further characterizing potential heterogeneity within this population.

By performing scRNA-seq analysis of brain nuclei associated with social behaviors, an immense transcriptomic diversity of neurons in the medial amygdala (MeA) [29,30], preoptic region [31], and VMH [32] has already been uncovered. Collectively, these studies provided more detailed molecular classifications of neuronal subtypes within the populations originally defined by unique single gene markers, especially sex hormone receptors and neuropeptides.

For example, VMHvl neurons are hierarchically organized into four major clusters defined by the expression of *Esr1*, *Dlk1*, *Satb2*, or *Nup62cl*, within which there are seventeen transcriptional cell types, including three that are sex-specific. By profiling the neurons in combination with immediate early gene (IEG) expression (Act-Seq [30]), specific neuronal classes including *Esr1*$^{+}$ cells were activated when an animal was attacked by a dominant male, providing the molecular definition of distinct populations of VMHvl neurons involved in social fear [32,33]. Furthermore, Act-Seq in the VMHvl found a small number of *Esr1*$^{+}$ populations of neurons that distinguish IEG responses to sex-specific stimuli. Whether or not these subpopulations are functionally involved in mating or aggression remains to be determined.

Similarly, ~70 different neuronal subtypes have been identified in the preoptic region using scRNA-seq, among them are neurons expressing the neuropeptide galanin (Gal), which are implicated in parenting, feeding, and sleep [23,31]. *Gal* is expressed in ten transcriptomic cell types, including three excitatory and seven inhibitory subtypes. In addition, co-labeling of *c-fos* identified unique associations between cell types and social behaviors. For example, *Calcitonin receptor*/*Gal* double-positive cells were selectively activated during parental behavior [31], however the causal role of these neurons in parental behaviors remains to be tested. Moreover, different *Gal*$^{+}$ neurons in the medial preoptic area projecting to either the ventral tegmental area, MeA, paraventricular nucleus of the hypothalamus, or periaqueductal grey have been shown to

regulate motivational, pheromonal, hormonal, and motor-related aspects of parental behavior, respectively [34]. Therefore, the molecular profiling of projection neuron subtypes could reveal further heterogeneity within this population.

Moreover, these studies profiled both male and female brains, the comparisons of which can provide insights into the single-cell basis of sexual dimorphism. Overall, these studies found relatively minor sex differences in molecular subtypes (*e.g.*, VMHvl female- and male -specific cell clusters, with enriched expression of *Esr1* and of *Cyp19a1*, respectively [32]). The most frequent modes of sexual dimorphism appear to be the differential expression levels of small number of genes per cell [29] and the overall differences in the number of cells expressing them [31]. These observations are consistent with earlier results using bulk transcriptomic surveys of sexually dimorphic gene expression in the amygdala and hypothalamus [35]. These results therefore suggest that male and female circuits share largely invariant neuronal components and highlight the role of differential wiring among sexes and hormonal states [36,37]. Therefore, other modes of cell profiling, especially proteomics to quantify proteins associated with synapses, would prove insightful.

**Uniting anatomical, developmental, molecular, and physiological definitions of cell types**

Over one hundred transcriptomic cell types identified by scRNA-seq in the amygdala and hypothalamus poses new challenges to understand their roles in controlling behavior. Indeed, the functional characterization of transcriptional cell types remains a top priority across neuroscience [38]. We propose that neurons should be classified using at least four different neural properties: 1) molecular properties, *e.g.*, ion channels, neurotransmitters, receptors to hormones and neuromodulators, and cell adhesion molecules, 2) anatomical properties: *e.g.*, projection type (local or long-range), targets and input patterns, 3) behavior and physiological processes

regulated by these neuron types, and 4) developmental origin. Thus, we must combine anatomical, developmental, molecular, physiological and behavioral readouts to functionally characterize transcriptomic cell types.

For example, the transcription factors FoxP2 and Dbx1 specify non-overlapping populations of projection neurons in the MeA during development [39]. While $FoxP2^{+}$ and Dbx1-derived neurons in the adult show differential electrophysiological properties, both populations are activated during mating and aggression in males, and it is unclear if FoxP2 and Dbx1 lineages may be involved in behavioral selectivity [39]. In the VMHvl, some transcriptomic cell types can be distinguished by the expression levels of immunoglobin I-set domain containing cell adhesion molecules [32], which play important roles in axon guidance and synaptogenesis. To further test the specific roles of these genes and cell types, gain- and loss-of-function tests will be essential.

In addition, since scRNA-seq methods do not provide the *in situ* position of profiled cells, it is difficult to uncover spatial relationships between transcriptomic cell types. To overcome this problem, several studies combined scRNA-seq and *in situ* RNA hybridization to create spatial maps of transcriptomic cell types [31,32,40]. Although only a few hundred genes can be visualized simultaneously so far, *in situ* transcriptomics generally provides more sensitive detection of RNAs than droplet-based scRNA-seq [31]. This is important because prominent sexually dimorphic genes and neuromodulator receptors, *e.g.*, *Cyp19a1* (aromatase) and *Oxtr* (oxytocin receptor), which are intimately linked to the regulation of social behaviors, are often expressed at low levels (1-10 copies per cell [31]). These methods will greatly facilitate multi-modal analysis of cell types by combining with calcium imaging [40], IEG induction [31], and long-range axonal projection mapping [41].

Finally, since activity measurements do not prove causal relationships, functional tests are essential to link transcriptionally defined neural populations to specific roles in behavior and physiology. One challenge here is that newly identified cell classes are often defined by unique combinations of multiple genes. Thus, the specific labeling of these neurons will require intersectional strategies [42], developmental lineage tracing [39], and activity-dependent labeling of neurons using IEG promoter-driven Cre recombinase [33,43], which together will allow the rigorous definition of cell types in functional circuits.

**How are neuron types associated with innate social behaviors born and wired?**

The construction of innate behavior circuits requires both the proper specification of the vast diversity of cell types and their precise wiring. Currently, our understanding of early mechanisms involved in the differentiation of individual neuron classes across the mouse brain remain incomplete. This contrasts with the full anatomical, molecular and developmental coverage of neuronal types that compose the nervous system of the nematode *C. elegans*. In the latter model, the systematic expression analysis and loss-of-function studies of hundreds of transcription factors (TFs) has revealed that all neuron types are defined by unique combinations of homeodomain (HD) type of TFs [44]. Other families of TFs have critical roles in neuronal specification but often in cooperation with HD TFs. Although, such depth of analysis is not yet achieved in the mammalian nervous system, several examples point to similar mechanisms [45,46]. A comparative analysis of the transcriptomes from 179 distinct cell populations broadly sampled from the mouse brain (including hypothalamic nuclei) suggested HD TFs combinations as the best genes to distinguish neuron types [45]. Spinal cord motor neurons can be distinguished by the combinatorial expression of LIM-HD TFs [46] and early on by Hox HD-TFs [47]. More relevant to the limbic system, using scRNA-seq several groups have generated a

comprehensive classification of hypothalamic cell types, including non-neuronal cells, and identified associated TFs, always containing HD TFs [48,49]. However, we still lack a systematic examination of the function of those TFs in generating and maintaining cellular diversity and circuit assembly.

A challenging but critical question, especially relevant for innate social behaviors, is how the stereotyped circuit becomes reproducibly assembled in a sexually dimorphic fashion. As pioneering examples, the studies of *Drosophila* TF *fruitless* and *doublesex*/*dmrt* (doublesex *mab-3* related transcription factor) in *Drosophila* and *C*. *elegans* illustrate several key mechanisms for the developmental programming of sexually dimorphic circuit assembly. $Fru^M$ (male-specific isoform) and Dmrts are required for critical steps in the sex determination, differentiation and function of sex-specific circuits, including sex-specific cell death and survival, neurogenesis (**Figure 2A**), neurotransmitter identity specification (**Figure 2B**), synaptic fine-tuning and neurite arborization (**Figure 2C**) [50-55]. Specifically, $Fru^M$ marks key interconnected neurons in every layer of the male circuit including sensory, central and motor components [51,56]. Furthermore, it regulates dimorphic connectivity configurations of sex-shared neurons [57] and the expression of the axon-guidance molecule robo1 to sculpt sex-specific wiring [58].

In contrast, TFs sufficient to orchestrate the specification of sexually dimorphic circuits have not yet been found in mammals. Instead, we speculate three potential mechanisms for the development of mammalian sexually dimorphic circuit (described as models A-C in **Figure 3**). First, conserved TFs responsible for neuronal identity specification, may play a role in sexual differentiation of the brain circuitry (**Figure 3A**). In fact, Dsx belongs to the conserved family of *Dmrt* genes involved in mammalian gonadal sex-determination [59], and *Dmrt* genes are expressed in the mammalian nervous system [59]. Second, it is well-established that specific

combinations of TFs drive the expression of axon guidance molecules and receptors in a genetically preprogrammed fashion (**Figure 3B**). For example in the spinal cord, LIM HD proteins mediate axon guidance through the regulation of Ephrin signaling [60].

Finally, in mammals, fetal sex hormone production plays an essential role in the development of sexually dimorphic behaviors [61]. Remarkably, many of the roles of sex hormone receptors, most of which are TFs, are to regulate analogous processes to those described for Fru and Dsx including cell death, neurite outgrowth, dimorphic connectivity and synaptogenesis [22,61]. In addition, the phagocytic activity of microglia to prune astrocytes is regulated by androgens and critical for producing male-typical behavior [62]. Therefore, although not all sexual brain dimorphisms can be explained by the action of sex hormones [61], they have far reaching effects on circuit formation, not only through their direct effects on neurons but also on non-neuronal cells. One outstanding question in this respect is the regulatory logic of hormone receptor gene expression, *i.e.*, through *cis*-regulatory elements, which could provide an entry point to elucidate TFs critical for the specification of these circuits (**Figure 3C**).

However, many questions remain unsolved. For example, sex hormone receptors are expressed in many of the brain areas associated with innate social behaviors, but not in all neurons in these nuclei [31,32]. Therefore, single-cell developmental trajectories of adult cell types may help identify additional TFs and axon guidance molecules that are involved in specific wiring of these circuits. Developmental history could add another important classifier to the molecular definition of adult cell types.

## Concluding remarks

We are approaching an exciting era of neuroscience where maps of hardwired neural circuits, with detailed molecular descriptions will soon become available. This circuit map, similarly to the comprehensive map of metabolic pathways, will allow us to capture the logic of information flow within this circuitry. A future priority will then be to overlay the molecular and developmental information with physiological and behavioral data and to define dynamic interactions between circuit nodes. These combined efforts will provide systematic insights on how these circuits operate during social behaviors.

**Figures**

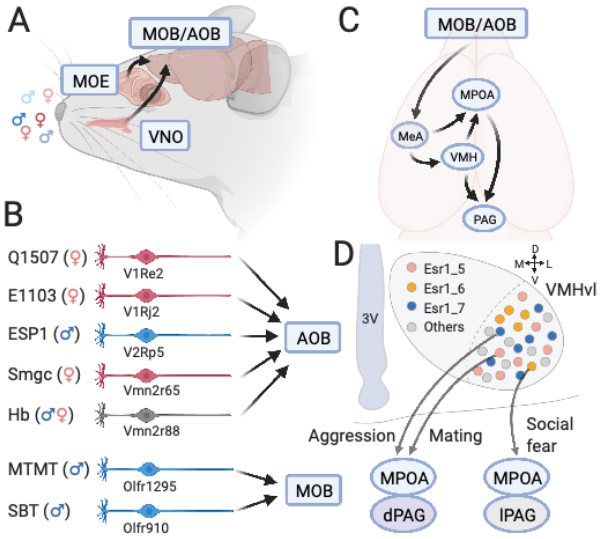


**Figure 1. Molecular definition of the circuit components underlying the processing of pheromonal information in mice.**
**(A)** The vomeronasal organ (VNO) and the main olfactory epithelium (MOE) mediate pheromone detection in rodents and relay their inputs to the accessory olfactory bulb (AOB) and the main olfactory bulb (MOB). **(B)** Specific ligand–receptor pairs carry social information to the AOB and MOB. Neurons that detect sex-specific ligands, indicated by male and female symbols, are colored – male (in blue) and female (in pink). **(C)** A simplified circuit diagram of the core circuitry underlying innate social behaviors. Note that this diagram depicts only brain areas and connections relevant for this article and omits many critical connections. **(D)** Schematics illustrating the specificity of *Esr1*$^{+}$ subtypes and their projection targets in the ventrolateral ventromedial hypothalamus (VMHvl) for the regulation of mating, aggression and social fear.

Colored dots represent molecularly distinguishable *Esr1*$^{+}$ subtypes defined in [32]. Other cell types in this area include non-*Esr1*$^{+}$ cells. dPAG: dorsal periaqueductal grey, lPAG: lateral PAG.

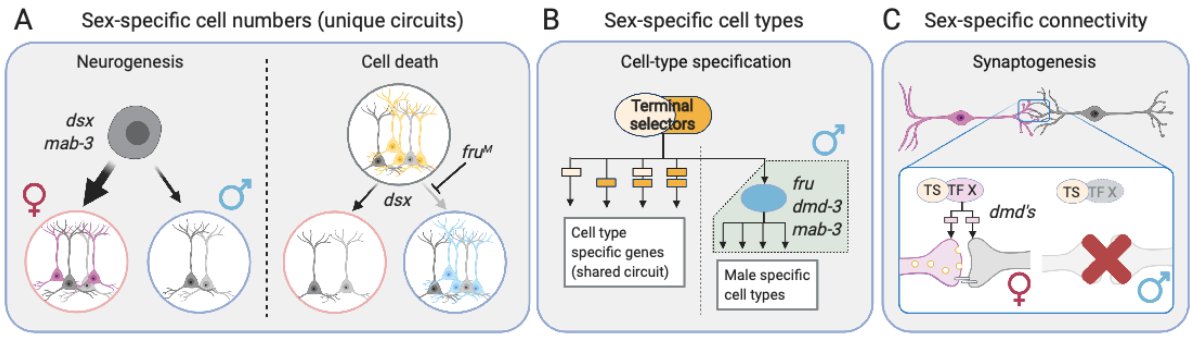


**Figure 2. Conserved logic of sexually dimorphic circuit formation in invertebrates.** Different ways to generate sex-specific circuits: **A)** Sex-specific neurogenesis or cell death to generate differential cell numbers in homologous brain nuclei [50]. **B)** Generation of sex-specific cell types. Terminal selectors (TS), a special class of TFs, specify and maintain a battery of cell type specific genes, including sex-specific transcription factors (TFs) (in green box), which then generate sex-specific cell types [63]. Small rectangles indicate direct TS regulation for target genes. **C)** Modulation of sex-specific synaptogenesis and pruning by TFs [55,61]

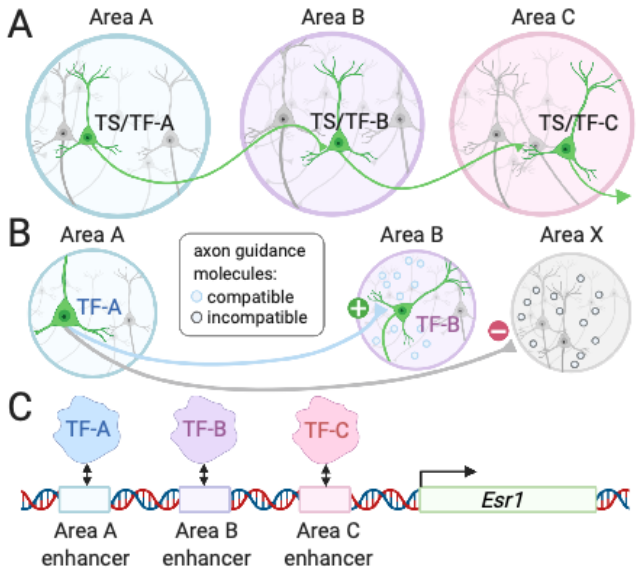


**Figure 3: Hypothetical mechanisms of early events for innate social behavior circuit formation in mammals.**

**Model A** postulates that conserved terminal selectors (TS, as defined in Figure 2) commonly expressed in connected neurons in the social behavior circuits act in concert with region specific transcription factors (TFs) to organize the development of the circuits. **Model B** describes a classical axon targeting strategy using axon guidance molecules and their receptors to establish the specificity of the wiring. **Model C** considers that the expression of hormone receptors could be independently controlled by region specific TFs (*e.g.*, hypothetical TF-A, B, or C) by their binding to specific enhancers.

**Declaration of interest**

None

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**Highlighted references of interest**

• special interest

•• outstanding interest

••Chen et al. 2018

This study uncovered the sexually dimorphic gene expression in GABAergic neurons in the medial amygdala by scRNA-seq. The authors did not find sex specific cell types but found quantitative gene expression differences in GABAergic cells.

••Kim et al. 2019

This study used scRNA-seq and spatial transcriptomics (seqFISH) to uncover 40 neural transcriptomic cell types in the ventromedial hypothalamus (VMH). The authors identified 17 cell types, including sex-specific clusters, in the VMHvl, which have been previously shown to regulate aggression, mating and social fear. This study found that out of these mixed transcriptomic types in this area, only a few of which display behavior-specific activation and specific connectivity.

••Moffitt et al. 2018

This study uncovered ~70 transcriptomic cell types of the preoptic region by scRNA-seq and spatial transcriptomics (MERFISH). This dataset gave new insights on the spatial relationships of the reported cell types, for example, in processes underlying signaling of gonadal hormones. In addition, this study identified key cell types that are linked to the reproductive behaviors of males and females as well as parenting.

•Inoue et al. 2019

This study identified that the connection between VMHvl cells expressing the progesterone receptor, and the anteroventral periventricular nucleus (AVPV) is cyclically strengthened and weakened during the estrous cycle. This strengthening of synapses between VMHvl and AVPV was shown to be essential for female receptivity, which coincides with estrus.

••Lischinsky et al. 2017

This study maps two distinct lineages of medial amygdala cells originating from the progenitor pools in the telencephalic preoptic area, marked by Dbx1 and FoxP2. These cells differ by electrophysiological properties and are found to be activated during mating and aggression.

•Stockinger et al. 2005

The authors show that many if not all of the neurons that express $Fru^M$ are interconnected in a circuit that is directly and specifically involved in male sexual behavior. By inserting GAL4 into the *fruitless* locus, both male and female neurons can be visualized and they show little sexual dimorphism in location and number of neurons in the two sexes.

••Sugino et al. 2019

The authors show that homeobox transcription factors distinguish more than 99% of neuronal cell types in the mouse nervous system. They provide an extensive resource for investigation of mouse neuronal cell types by linking transcriptional identity to genetic strains and anatomical atlases. They profile 179 genetically and anatomically identified populations of neurons and 15 populations of non-neuronal cells. Additionally, they claim that neuronal effector genes, such as channels and cell adhesion molecules, contribute to neuronal diversity.

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