2-28-2017

Embryonic transcription factor expression in mice predicts medial amygdala neuronal identity and sex-specific responses to innate behavioral cues.

Julieta E Lischinsky

Katie Sokolowski

Li Peijun

Shigeyuki Esumi

Yasmin Kamal

See next page for additional authors

APA Citation

Lischinsky, J., Sokolowski, K., Peijun, L., Esumi, S., Kamal, Y., Goodrich, M., Oboti, L., Hammond, T., Krishnamoorthy, M., Feldman, D., Huntsman, M., Liu, J., & Corbin, J. (2017). Embryonic transcription factor expression in mice predicts medial amygdala neuronal identity and sex-specific responses to innate behavioral cues.. *Elife, 6* (). http://dx.doi.org/10.7554/eLife.21012

Authors

Julieta E Lischinsky, Katie Sokolowski, Li Peijun, Shigeyuki Esumi, Yasmin Kamal, Meredith Goodrich, Livio Oboti, Timothy R Hammond, Meera Krishnamoorthy, Daniel Feldman, Molly Huntsman, Judy Liu, and Joshua G Corbin

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2 **identity and sex-specific responses to innate behavioral cues.**

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4 Lischinsky J.E.[1,2], Sokolowski K.[2], Li, P.[2], Esumi S.[2,3], Kamal Y.[2], Goodrich M.[2], Oboti L.[2],

5 Hammond T.R.[2], Krishnamoorthy, M.[2], Feldman D.[2], Huntsman M.M.[4], Liu J.[2] & Corbin J.G.[2]

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7 [1]Institute for Biomedical Sciences, The George Washington University, Washington DC;

8 [2]Center for Neuroscience Research, Children's National Medical Center, Washington, DC;

9 [3]Graduate School of Medical Sciences, Kumamoto-University, Kumamoto City, Japan;

10 [4]Department of Pediatrics, University of Colorado School of Medicine, Aurora, Colorado.

11

12   **Abstract**

13   The medial subnucleus of the amygdala (MeA) plays a central role in processing sensory cues

14   required for innate behaviors. However, whether there is a link between developmental programs

15   and the emergence of inborn behaviors remains unknown. Our previous studies revealed that the

16   telencephalic preoptic area (POA) embryonic niche is a novel source of MeA destined

17   progenitors. Here, we show that the POA is comprised of distinct progenitor pools

18   complementarily marked by the transcription factors Dbx1 and Foxp2. As determined by

19   molecular and electrophysiological criteria this embryonic parcellation predicts postnatal MeA

20   inhibitory neuronal subtype identity. We further find that *Dbx1*-derived and Foxp2+ cells in the

21   MeA are differentially activated in response to innate behavioral cues in a sex-specific manner.

22   Thus, developmental transcription factor expression is predictive of MeA neuronal identity and

23   sex-specific neuronal responses, providing a potential developmental logic for how innate

24   behaviors could be processed by different MeA neuronal subtypes.

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26   Word count: 150

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Introduction

One of the major functions of the limbic system is to integrate conspecific and non-conspecific environmental cues with social and survival salience to generate appropriate behavioral responses (Sokolowski, Corbin 2012, Stowers, Cameron & Keller 2013). The medial subnucleus of the amygdala (MeA) serves as a hub in this function, residing only two synapses away from sensory neurons in the olfactory bulb (Dulac, Wagner 2006, Sokolowski, Corbin 2012). The MeA along with bed nucleus of the stria terminalis (BNST) and multiple nuclei of the hypothalamus including the ventromedial hypothalamus, form a core limbic circuit largely dedicated to processing innate behaviors (Dulac, Wagner 2006, Gross, Canteras 2012, Choi et al. 2005). Classical studies investigating patterns of neuronal activation in response to behavioral or olfactory cues (Kollack, Newman 1992, Erskine 1993), lesioning studies and more recent optogenetic approaches (Vochteloo, Koolhaas 1987, Takahashi, Gladstone 1988, Kondo 1992, Hong, Kim & Anderson 2014) have revealed a central role for the MeA in the regulation of innate behaviors such as aggression, mating and predator avoidance.

In addition to the processing of innate cues, the MeA is one of many known sexually dimorphic regions of the brain, with differences in numbers of neurons, glia, and synaptic organization between males and females (Cooke, Woolley 2005, Johnson, Breedlove & Jordan 2008, McCarthy, Arnold 2011). The critical role that the MeA plays in regulating sex-specific behaviors is reflected in the high expression levels of steroid hormone pathway proteins such as aromatase, estrogen receptor and androgen receptor (Wu et al. 2009, Juntti et al. 2010, Unger et al. 2015). MeA neuronal subpopulations expressing different combinations of these proteins have been shown to regulate aggression or mating behaviors in male and female mice (Juntti et

57 al. 2010, Hong, Kim & Anderson 2014, Unger et al. 2015). Nonetheless, understanding how

58 developmental programs are linked to behavioral processing in the MeA remains unknown.

59   As unlearned behaviors are largely inborn, we reasoned that there must be embryonic

60 developmental programs that guide formation of sub-circuitry dedicated for different innate

61 behaviors. Previous studies of MeA development revealed that progenitors located at the

62 telencephalic-diencephalic border are a major source of MeA neuronal populations (Zhao et al.

63 2008, Hirata et al. 2009, Soma et al. 2009, Garcia-Moreno et al. 2010). Our previous work

64 revealed that one of these progenitor populations is defined by the transient expression of the

65 developmentally regulated transcription factor, *Dbx1*, which in turn generate a subclass of MeA

66 putative inhibitory projection neurons (Hirata et al. 2009). However, the MeA is also comprised

67 of diverse populations of local interneurons and both excitatory and inhibitory output neurons

68 (Bian 2013, Keshavarzi et al. 2014). This suggests the contribution of other progenitor

69 subpopulations, perhaps also originating from the POA to MeA neuronal diversity, populations

70 which may in turn play different roles in innate behavioral processing.

71   Here, we demonstrate that in addition to Dbx1 expression, a subset of MeA embryonic

72 progenitors are complementarily marked by expression of Foxp2, a forkhead transcription factor

73 implicated in the development and function of neurons and required in the motor coordinating

74 centers of the brain for the appropriate production of speech (French, Fisher 2014). We find this

75 embryonic parcellation interestingly persists into postnatal stages where *Dbx1*-derived and

76 Foxp2+ MeA neurons are separate, non-overlapping inhibitory output neuronal subpopulations.

77 Strikingly, both subpopulations are activated during specific innate behaviors in a sex-specific

78 manner. Thus, our findings link developmental patterning to innate behavioral processing and

79 further provide an embryonic developmental framework for how these behaviors may emerge.

Results

Dbx1 and Foxp2 expression segregates embryonic and postnatal MeA subpopulations

Our previous work along with the work of others revealed that the telencephalic-diencephalic border is a major source of neurons that will populate the MeA (Zhao et al. 2008, Hirata et al. 2009, Soma et al. 2009, Garcia-Moreno et al. 2010). Our previous studies (Hirata et al. 2009) revealed that the preoptic area (POA), which lies on the telencephalic side of this border (Flames et al. 2007), is a source of *Dbx1+* progenitors fated to generate a subpopulation of MeA inhibitory output neurons. Our previous studies further revealed that progenitors arising from ventral telencephalic Shh+ and Nkx2.1+ domains also contributed diverse neuronal subpopulations to the MeA (Carney et al. 2010). Thus, while a molecular map of MeA embryonic niche diversity is beginning to emerge, the diversity of mature neurons derived from this niche and whether there is a link between embryonic identity, mature identity and function remains unknown. Moreover, as these previously identified subpopulations only generate a subset of MeA neurons, we reasoned that there must be other transcription factor marked progenitor populations within the telencephalic-diencephalic niche.

Here, in addition to *Dbx1+* progenitors, we observed a progenitor population comprised of Foxp2+ cells, residing primarily in the putative subventricular zone (SVZ) of the POA **(Figure 1a-f, s)**. Interestingly, during embryogenesis, *Dbx1*-derived and Foxp2+ progenitor populations were non-overlapping **(Figure 1a-l)**. Both populations were also generally distinct from OTP+ progenitors **(Figure 1-Figure Supplement 1a-i)**, a population previously shown to define a subset of MeA-fated progenitors (Garcia-Moreno et al. 2010). We next investigated whether Foxp2+ progenitors overlapped with ventral telencephalic populations derived from *Shh* or *Nkx6.2* lineages, which also encompass the POA (Carney et al., 2010; Fogarty et al., 2007).

103   We found embryonic Foxp2+ cells were not derived from either lineage (~5% overlap) **(Figure**

104   **1- Figure supplement 2)** further expanding our knowledge of the molecular diversity of the

105   MeA niche.

106   Interestingly, this embryonic molecular parcellation persisted into adulthood as *Dbx1-*

107   derived and Foxp2+ cells remained non-overlapping across the rostro-caudal extent of the

108   postnatal MeA **(Figure 1m-r; Figure 1-Figure supplement 3).** Similarly, postnatally, the *Dbx1-*

109   derived and Foxp2+ neurons remained distinct from OTP+ cells **(Figure 1-Figure supplement**

110   **1j-x)**. Taken together, these findings reveal that *Dbx1*-derived and Foxp2+ populations, although

111   appearing to derive from the same embryonic niche, remain distinct subpopulations from

112   embryonic development to adulthood **(Figure 1t)**, a novel finding that we hypothesized has

113   implications for later subtype identity and function, explored in the next sets of experiments.

114

115   *Foxp2+ neurons in the postnatal amygdala are inhibitory*

116   Our previous work revealed that MeA *Dbx1*-derived neurons are a subclass of inhibitory

117   neurons, likely projection as opposed to local interneurons (Hirata et al. 2009). However, the

118   identity of MeA Foxp2+ neurons remains unknown. Therefore, we next examined whether adult

119   MeA Foxp2+ cells were neurons or glia. Our analysis revealed that 81%$\pm$2.6 of Foxp2+ cells

120   expressed NeuN, a pan neuronal marker (Mullen, Buck & Smith 1992) **(Figure 2a-d)**, with none

121   co-expressing the oligodendrocyte marker, CC1 (Koenning et al. 2012) **(Figure 2e-h)**. We next

122   wanted to determine if Foxp2+ neurons were excitatory or inhibitory. We found that only

123   3%$\pm$1.2 of Foxp2+ cells were derived from the *Emx1*-lineage, a broad marker of excitatory

124   neurons (Gorski et al. 2002) **(Figure 2i-l)**. We further found that 22%$\pm$6.6 of Foxp2+ cells

125   expressed the inhibitory marker Calbindin **(Figure 2m-p)**, with a smaller percentage of Foxp2+

126 cells (15%$\pm$2.5) expressing nNOS **(Figure 2q-t)**, or somatostatin (5%$\pm$0.6) **(Figure 2u-x)**,

127 inhibitory markers that mark a subset of MeA output neurons (Tanaka et al. 1997) and

128 interneurons (Petilla Interneuron Nomenclature Group et al. 2008), respectively. Collectively,

129 although we did not fully assess all putative inhibitory markers, these data reveal that Foxp2+

130 MeA are not excitatory and are likely inhibitory **(Figure 2y)**.

131 As neither *Dbx1*-derived nor Foxp2+ cells expressed OTP+, we therefore investigated the

132 identity of this population. We found that 1%$\pm$0.4 of OTP+ cells were derived from the *Emx1*-

133 lineage, and 13.5%$\pm$9.1 co-expressed CAMKIIα (Jones, Huntley & Benson 1994), both

134 excitatory markers. In contrast, we observed 56%$\pm$18.9 of OTP+ cells co-expressed calbindin,

135 while none (0%$\pm$0.1) co-expressed somatostatin (**Figure 2- Figure supplement 1**). Therefore,

136 similar to *Dbx1*-derived and Foxp2+ neurons, the majority of OTP+ cells appear to be inhibitory.

137

138 ***Dbx1-derived and Foxp2+ neurons possess distinct electrophysiological properties***

139 To determine if *Dbx1*-derived and the Foxp2+ populations are functionally distinct subclasses,

140 we next examined their electrophysiological properties. Previous studies (Bian 2013, Keshavarzi

141 et al. 2014) revealed a significant diversity in intrinsic electrophysiological properties of MeA

142 local and projection neurons. Here, we found that the majority (19/28) of *Dbx1*-derived neurons

143 were characterized by a regular, tonic spiking pattern with 3-4 spikes at rheobase **(Figure 3a)**. In

144 contrast, the majority (15/23) of Foxp2+ neurons were distinguished by a phasic firing pattern

145 and displayed a single or double spike upon repolarization after hyperpolarization, a profile

146 characteristic of inhibitory neurons (Llinas 1988) **(Figure 3b)**. *Dbx1*-derived and *Foxp2*-derived

147 neurons (confirmed Foxp2+ by immunohistochemistry) also displayed significant differences in

148 resting membrane potential, input resistance, capacitance, and action potential frequency but not

149   in rheobase **(Figure 3c-g)**. In addition, the presence of spines in Foxp2+ neurons **(Figure 3-**

150   **Figure supplement 1)** suggested that similar to *Dbx1*-derived neurons, Foxp2+ neurons are

151   projection neurons. This reveals that despite both populations being inhibitory, the *Dbx1*-derived

152   and the Foxp2+ populations possess distinct firing patterns.

153   We further analyzed spontaneous excitatory post-synaptic currents (sEPSCs), a measure

154   of excitatory inputs. *Dbx1*-derived neurons received significantly more frequent and greater

155   amplitude of EPSCs than Foxp2+ neurons **(Figure 3h-l)**. This suggests that *Dbx1*-derived MeA

156   neurons receive a greater number and/or stronger excitatory inputs than Foxp2+ neurons. In

157   summary, a combination of neuronal marker expression **(Figure 2)** and electrophysiological

158   **(Figure 3)** analyses, combined with our previous analysis (Hirata et al. 2009) revealed that

159   *Dbx1*-derived and Foxp2+ neurons are distinct subclasses of inhibitory, and likely projection

160   neurons.

161

162   ***Molecular identity of Dbx1-derived and Foxp2+ postnatal MeA cells***

163   Based on the above analyses revealing that *Dbx1*-derived and Foxp2+ neurons are separate

164   subclasses, we next wanted to determine whether these two populations express different

165   combinations of steroid pathway proteins previously associated with MeA function such as

166   estrogen receptor-alpha (ERα), aromatase and androgen receptor (AR) (Wu et al. 2009, Juntti et

167   al. 2010, Unger et al. 2015). As the MeA is a sexually dimorphic nucleus (Cooke, Woolley 2005,

168   McCarthy, Arnold 2011, Johnson, Breedlove & Jordan 2008), we characterized the expression of

169   these markers in both male and female mice **(Figure 4, Figure 4-Figure supplement 1, Figure**

170   **4-Figure supplement 2)**. We found that *Dbx1*-derived and Foxp2+ cells in males expressed

171   ERα to the same extent (28.4%$\pm$4.8 in *Dbx1*-derived cells; 24.0%$\pm$7.2 in Foxp2+ cells).

172   However, *Dbx1*-derived and Foxp2+ cells in females showed significant differences in ERα

173   expression (45%±3.4 in *Dbx1*-derived cells; 24.8%±5.8 in Foxp2+ cells) **(Figure 4a-g)**. The

174   majority of *Dbx1*-derived cells expressed aromatase both in males (61.7%±7.6) and females

175   (52.4%±5.2), which was at a significantly higher level than in Foxp2+ cells in males (0.12%±0)

176   and females (7.2%±6.0) **(Figure 4h-n)**. There were no subpopulation differences in AR

177   expression as both *Dbx1*-derived and Foxp2+ neurons in both males (26.8%±4.1 in *Dbx1*-

178   derived cells; 16.2%±3.2 in Foxp2+ cells) and females (8.4%±3.6 in *Dbx1*-derived cells;

179   7.0%±1.8 in Foxp2+ cells) co-expressed AR at the same levels **(Figure 4o-u)**. However, there

180   were sex-specific differences observed as greater percentage of *Dbx1*-derived cells in males

181   (26.8%±4.1) expressed AR than *Dbx1*-derived cells in females (8.4%±3.6).

182   We also analyzed the contribution of the *Dbx1*-derived and Foxp2+ populations to the

183   total ERα, Aromatase and AR MeA populations (**Figure 4- Figure supplement 2**). We observed

184   that both *Dbx1*-derived and Foxp2+ cells comprised between ~10 to 22% of the total ERα

185   population in male and female mice. The *Dbx1*-derived population contributed between ~30-

186   40% of the total aromatase population in males and females, which was significantly greater than

187   the contribution of the Foxp2+ population in both males and females. *Dbx1*-derived (males only)

188   and Foxp2+ cells (males and females) comprised ~20-38% of the total AR+ population. In

189   contrast, the *Dbx1*-derived population contributed to only 3% of the total AR+ population in

190   females, which was significantly less than the Foxp2+ contribution in females and less than the

191   contribution of *Dbx1*-derived cells in males. Collectively, these data reveal that *Dbx1*-derived

192   and Foxp2+ cells contributed differentially to the aromatase and AR populations, but not to the

193   ERα population.

194

Sex-specific subtype activation patterns during innate behaviors

The MeA receives direct inputs from the accessory olfactory bulb (AOB) (Scalia, Winans 1975, Martel, Baum 2009, Bergan, Ben-Shaul & Dulac 2014) and integrates this chemosensory information to regulate innate behaviors including territorial aggression, maternal aggression, mating, and predator avoidance (Dulac, Wagner 2006, Kim et al. 2015). Previous data revealed that at least aggressive and mating behaviors are controlled by MeA GABAergic neurons (Choi et al. 2005, Hong, Kim & Anderson 2014). However, whether different subsets of inhibitory neurons are activated during these behaviors, or if neuronal subtype activation is generalizable across behaviors remains unknown. To directly test these possibilities we performed well characterized aggression, mating and predator odor avoidance behavioral tests in both male and female resident mice and examined the patterns of activation of *Dbx1*-derived and Foxp2+ cells using c-fos as a readout of neuronal activity in resident mice (**Figure 5- Figure supplement 1**).

Dbx1-derived and Foxp2+ neurons are activated during aggressive encounters

To evaluate activation of *Dbx1*-derived and Foxp2+ neurons during male conspecific aggression, we performed a territorial aggression assay in which an intruder mouse was placed in a resident cage. Concordant with previous literature (Wang et al. 2013), we found a significant increase in the number of c-fos+ cells in the MeA in comparison to naïve mice **(Figure 5a-c)**. In addition, both the number and proportion of activated *Dbx1*-derived and Foxp2+ subpopulations in males were significantly increased during territorial aggression compared to control (naïve) mice **(Figure 5d-k, x)**. We next evaluated aggression in female mice by conducting a maternal aggression assay in which pups were removed from a nursing female and a sexually naïve male intruder was introduced (Haney, DeBold & Miczek 1989). In addition to the naïve control, we

218   established a second control in which pups were removed and no intruder was presented. With

219   this control, we could compare levels of neuronal activation during maternal aggression (pups

220   removed and presence of intruder) to levels of neuronal activation in response to a strong stressor

221   (pups removed but no intruder) and to a naïve condition (with pups and without intruder). We

222   found a significant increase in the number of c-fos+ cells in the MeA in the maternal aggression

223   condition compared to both stressed and naïve controls **(Figure 5l-o)**. When examining subtype

224   specific levels of activation, we found that both the number and proportion of activated *Dbx1*-

225   derived and the Foxp2+ subpopulations significantly increased during maternal aggression in

226   comparison to the naïve condition **(Figure 5p-w, y)**. Thus, during an aggressive encounter with

227   a conspecific, both *Dbx1*-derived and Foxp2+ MeA subpopulations were activated to a greater

228   extent in both male and female mice..

229

230   *Dbx1-derived and Foxp2+ neurons are activated in a sex-specific manner during mating*

231   We next conducted male and female mating assays and monitored animals for mating (mounting

232   and intromission followed by presence of a vaginal plug). Consistent with previous studies

233   (Rowe, Erskine 1993), we observed an increase in c-fos+ cells in the MeA during both male and

234   female mating **(Figure 6a-c)**.   Intriguingly, we found subpopulation specific differences in

235   activation patterns across sexes. While the number and proportion of the activated *Dbx1*-derived

236   subpopulation was significantly increased during both male and female mating when compared

237   to naïve controls **(Figure 6d-g, l)**, the number and proportion of activated Foxp2+ cells increased

238   only during male but not during female mating **(Figure 6h-k, m)**. Thus, while *Dbx1*-derived and

239   Foxp2+ MeA subpopulations were both activated during male mating, only *Dbx1*-derived

240   neurons were activated during female mating.

11

241

Activation of Dbx1-derived and Foxp2+ neurons during predator odor exposure

We next sought to determine whether *Dbx1*-derived and Foxp2+ MeA subpopulations were activated by a strong innate stressor. To accomplish this, we exposed mice to rat odor, a well-characterized predator cue that evokes a strong aversive response in mice (Apfelbach et al. 2005, Carvalho et al. 2015). Mice were exposed to soiled bedding from a rat cage (predator) or clean bedding (benign) as a control **(Figure 7)**. Consistent with previous studies (Canteras, Pavesi & Carobrez 2015), in response to predator odor we observed a significant increase in the number of c-fos+ cells in the MeA in both male and female mice **(Figure 7a-c)**. Interestingly, the *Dbx1*-derived cells in male and female mice were not activated in response to predator odor exposure in comparison to the benign bedding **(Figure 7d-g).** In contrast, we observed a significant activation of Foxp2+ cells in comparison to controls in female mice, but not in males **(Figure 7h-k)**. When we assessed percentage of both *Dbx1*-derived (YFP+;c-fos+/total YFP+) and Foxp2+ (Foxp2+;c-fos+/total Foxp2+) cells co-labeled with c-fos over the total population there was no significant difference either subpopulation nor in male or female mice compared to control (**Figure 7m-l**). This suggests that despite an increase in the number of activated Foxp2+ cells in female mice in the presence of rat bedding, this increase might not be physiologically relevant. Therefore, to investigate which MeA subpopulation may be responding to predator odor, we next examined the activation patterns of the OTP+ population. We observed that a greater number of OTP+ cells in both males and females were activated in response to predator odor (**Figure 7- Figure supplement 1**). When analyzing the proportion of OTP+ cells activated, we found no differences between the benign and the predator avoidance groups in males, but we did find a significant increase in the OTP+ population in females. Therefore, in the female brain,

264   the OTP+ population, in contrast to *Dbx1*-derived or Foxp2+ cells, were activated at a level

265   above control in response to a strong aversive innate olfactory cue.

266   In summary, our analyses of activation patterns in response to a battery of innate

267   behavior tasks revealed that *Dbx1*-derived and Foxp2+ cells in the MeA were differentially

268   activated depending on the instinctive behavioral task **(Figure 8)**. The most striking of these

269   differences occurred during mating behaviors. While both subpopulations were activated during

270   male mating, in the female MeA only *Dbx1*-derived neurons were activated, with no activation

271   of the Foxp2+ population. In contrast, the OTP+ population in females appears more tuned to a

272   predator odor cue.   Importantly, sex differences in patterns of neuronal activation during mating

273   and predator odor were not due to overall differences in the activation of the MeA as these cues

274   activated the MeA in both sexes. Taken together with our electrophysiological and molecular

275   findings of the *Dbx1*-derived and Foxp2+ populations, our data reveal that developmental

276   parcellation of MeA progenitors predicts mature neuronal identity and sex-specific innate

277   behavioral activation patterns.

278

279   **Discussion**

280   Across a variety of species, innate behaviors such as aggression, mating and avoidance of

281   predators are initiated by sensory cues primarily detected by olfaction (Dulac, Wagner 2006,

282   Stowers, Cameron & Keller 2013). Here, focusing on the medial amygdala (MeA), a critical

283   brain region for the processing of olfactory-based sensory cues for unlearned behavior in

284   vertebrates, we took a neural developmental approach to shed light on how innate behavioral

285   information may be encoded in the male and female brain. Integrating genetic fate-mapping,

286   patch clamp electrophysiology and animal behavioral assays we uncover a fundamental link

287   between embryonic patterning and brain responses to innate behavioral cues at two levels: 1)

288   differential transcription factor expression within the embryonic MeA progenitor niche predicts

289   mature output neuronal subtype identity and 2) further predicts subpopulation sex-specific

290   responses to mating and predator odor avoidance cues. Our findings further suggest that

291   transcription factor expression at the progenitor stage may be instructive for establishment of

292   neuronal populations and sub-circuits regulating sex-specific behaviors.

293

Potential transcription factor codes for establishment of MeA neuronal diversity

295   The generation of neuronal diversity across amygdala subnuclei has been posited to occur in a

296   compartmentalized manner with amygdala inhibitory neurons generated in the subpallial

297   ganglionic eminences and excitatory neurons arising from the cortical pallial region (Swanson,

298   Petrovich 1998). In this model, the amygdala and cerebral cortex develop by a similar

299   mechanism with neurons in both structures originating in shared progenitor domains. However,

300   more recent studies have revealed that generation of amygdala neuronal diversity is more

301   complex with large populations of neurons originating in progenitor niches dedicated for limbic

302   structures (Remedios et al. 2007, Hirata et al. 2009, Soma et al. 2009, Waclaw et al. 2010,

303   Garcia-Moreno et al. 2010). One of these major niches encompasses the region at the

304   telencephalic-diencephalic border, an origin of MeA output neurons (Hirata et al. 2009, Garcia-

305   Moreno et al. 2010). Our previous studies revealed that the homedomain encoding transcription

306   factor, *Dbx1,* marks a subpopulation of progenitors within the POA, which will later generate a

307   subset of MeA inhibitory output neurons (Hirata et al. 2009). Here, we significantly extend this

308   work by revealing the presence of a complementary population of progenitors within this niche

309   marked by expression of Foxp2. Thus, our findings, combined with previous work, suggest a

310 model of MeA development in which distinct progenitor populations at the telencephalic-

311 diencephalic border defined by differential transcription factor expression (e.gs. Dbx1, Foxp2,

312 OTP) are a major source for MeA neuronal diversity.

313   The function of combinatorial sets of transcription factors in neural progenitors has been

314 shown across the neuraxis as the mechanism for the generation and specification of distinct

315 subclasses of neurons (Kepecs, Fishell 2014, Stepien, Tripodi & Arber 2010, Shirasaki, Pfaff

316 2002). In addition to specification of neuronal subtype identity, recent studies in the spinal cord

317 and globus pallidus (Dodson et al. 2015, Bikoff et al. 2016) have revealed that different

318 combinatorial codes in progenitor pools predict neuronal subtype connectivity patterns, neuronal

319 firing properties and in the case of the globus pallidus, distinct functions in regulating voluntary

320 movements (Dodson et al. 2015). Thus, transcription factor expression at the earliest stages of

321 neuronal development likely represents the beginning of an instructive continuum for the

322 establishment of not only neuronal identity, but also development of sub-circuitry regulating

323 different components of motor behaviors. Here, we show that in the MeA, complementary

324 transcription factor expression marks subsets of progenitors and predicts neuronal subtype

325 identity as defined by molecular and electrophysiological signatures. At the molecular level,

326 *Dbx1*-derived and Foxp2+ neurons express different combinations of the sex steroid hormone

327 pathway protein aromatase and estrogen receptor alpha (ERα). At the electrophysiological level,

328 these two populations possess distinct intrinsic electrophysiological profiles. Thus, our study

329 generates a novel cell-specific transcription factor-based means to predict postnatal MeA

330 neuronal identity.

331

332 **Behavioral activation of *Dbx1*-derived and Foxp2+ neurons**

333   The central importance of the MeA for processing innate behaviors such as aggression, mating

334   and predator avoidance is well-established (Dulac, Wagner 2006, Sokolowski, Corbin 2012).

335   Despite this knowledge, the question of which MeA neuronal subtypes encode instinctive

336   behavioral information has only recently begun to be addressed. Recent optogenetic

337   manipulation of the MeA revealed that glutamatergic neurons mediate repetitive self-grooming

338   behaviors while in contrast GABA-ergic neurons regulate either aggressive or mating behaviors,

339   depending on the level of neuronal activity driven by light stimulation (Hong, Kim & Anderson

340   2014). Moreover, MeA neuronal subclasses expressing different components of the stress

341   response system control appropriate behavioral responses to social cues (Shemesh et al. 2016).

342   Here, we contribute to the understanding of amygdala cell-specific regulation of behavior by

343   generating a transcription factor based map of MeA subtype responsiveness to innate-behavioral

344   cues. Thus, our findings provide a developmental molecular context in which to further dissect

345   neuronal subtype control of MeA-driven behaviors.

346   In addition to playing a central role in processing sensory information required for

347   instinctive behaviors, the MeA is one of the known sexually dimorphic structures of the brain

348   (Cooke, Woolley 2005, McCarthy, Arnold 2011, Johnson, Breedlove & Jordan 2008). Recent

349   studies employing *in vivo* recording techniques revealed that a significant number of MeA

350   neurons are dedicated to processing olfactory cues from the opposite sex rather than the same-

351   sex, thus providing a direct demonstration of sex-specific differences in sensory processing

352   (Bergan, Ben-Shaul & Dulac 2014). However, the identity of male and female MeA neurons that

353   differentially process olfactory-based sensory information has not been delineated. Here, we

354   reveal that while *Dbx1*-derived and Foxp2+ neurons are broadly activated by mating, aggressive

355   and predator cues, we found stark differences in *Dbx1*-derived, Foxp2+ and OTP+ cell-specific

356 responses in the male and female brain to mating and predator odor cues. A similar sex-specific

357 control of innate behavior has been directly demonstrated in the ventromedial hypothalamus

358 (VMH), where progesterone receptor-expressing neurons while required for male aggressive and

359 mating behaviors, are only required for female mating behavior (Yang et al. 2013). Collectively,

360 our studies in combination with previous studies point to a larger picture in which there are

361 neuronal subpopulations in the MeA and VMH that are involved in the regulation of different

362 innate behaviors in a sex-specific manner.

363 Although our data do not reveal the neuronal and/or circuit mechanisms underlying our

364 observation of sex-specific subpopulation responses to mating behavior and predator odor

365 presentation, some of our findings do provide potential insight as to how this differential

366 processing may occur. The two most straightforward and non-exclusive potential mechanisms

367 are: 1) with regard to mating, intrinsic differences in *Dbx1*-derived and Foxp2+ neurons and/or

368 2) subpopulation specific patterns of local and/or long-range connectivity.

369 Regarding the first potential mechanism, sex steroid hormones and receptors such as

370 aromatase, ERα and AR have been extensively characterized as critical for the output of distinct

371 components of male and female aggressive and mating behaviors (Yang, Shah 2014). For

372 example, deletion of AR resulted in alterations in attack duration during territorial aggression

373 and initiation of male mating (Juntti et al. 2010), while ablation of aromatase neurons led to

374 impairments in the production of distinct components of aggression in male and female mice

375 (Unger et al. 2015). Consistent with the critical role that these factors play in components of

376 innate behaviors, we found that aromatase is expressed solely in the *Dbx1*-derived lineage.

377 Across species, aromatase has a masculinizing effect (Wu et al., 2009; Balthazart et al. 2011)

378   Thus, it will be interesting to explore how *Dbx1*-derived aromatase expressing neurons may

379   control male behavioral displays such as mounting and territorial aggression.

380   Furthermore, both *Dbx1*-derived and Foxp2+ neurons possess distinct

381   electrophysiological profiles, another potential mechanism to control different components of

382   behaviors. Previous work in both vertebrates and invertebrates has revealed that the timing of AP

383   spiking is directly linked to specific behavioral actions. For example, in vertebrates timescale

384   firing differences are associated with dopamine (DA) release for the determination of reward

385   behaviors (Schultz 2007, Zhang et al. 2009). It will therefore prove interesting to explore if and

386   how firing patterns of *Dbx1*-derived and Foxp2+ MeA neurons may control different

387   components of innate behaviors across sexes.

388   The second potential and perhaps more intriguing mechanism that may account for *Dbx1*-

389   derived and Foxp2+ subtype specific male versus female patterns of neuronal activation during

390   mating are sex specific local and/or long-range patterns of connectivity. Although currently not

391   yet observed in a brain circuit in mammals, such a mechanism has recently been uncovered in *c.*

392   *elegans* in which shared male and female circuits show differences in connectivity that is

393   established during wiring (Oren-Suissa, Bayer & Hobert 2016). Consistent with this, there is

394   some suggestion of sex-specific differences in olfactory-MeA projections in rodents (Kang et al.

395   2011). Although we did not differentiate according to sex of the animal, we found differences in

396   both the amplitude and frequency of EPSCs between lineages, indicating differences in the

397   strength and/or number of inputs between *Dbx1*-derived and Foxp2+ MeA neurons. Although the

398   source of input cannot be determined from our analysis, there are direct excitatory inputs to the

399   MeA emanating from the mitral/tufted neurons of the accessory olfactory bulb (AOB) (Martel,

400   Baum 2009, Bergan, Ben-Shaul & Dulac 2014). Thus, determination of input/output wiring

401   patterns of male and female *Dbx1*-derived and Foxp2+ MeA neurons will be highly informative.

402   In summary, although the precise instructive developmental mechanisms programming

403   innate behaviors remain to be elucidated, we reveal that differential transcription factor

404   expression during development is predictive of neuronal identity based on molecular and

405   electrophysiological criteria and sex-specific patterns of neuronal activation during innate

406   behaviors.

407

408   **Methods**

409   *Animals*

410   Mice were housed in the temperature and light-controlled (12h light-dark cycle) Children's

411   National Medical Center animal care facility and given food and water *ad libitum*. All animal

412   procedures were approved by the Children's National Medical Center's Institutional Animal

413   Care and Use Committee (IACUC) and conformed to NIH Guidelines for animal use. Mice used

414   were *Dbx1cre*^{+/-} (kindly provided by A. Peirani, Institut Jacques Monod, Paris), *Shhcre*^{+/-}

415   (Jackson Labs strain B6.Cg-*Shhtm1(EGFP/cre)Cjt*/J), *Emx1cre*^{+/-} (Jackson Labs strain

416   B6.129S2-*Emx1tm1(cre)Krj*/J), *Nkx6.2cre*[+/-](Jackson Lab strain Tg *Nkx6-2-*

417   *icre)1Kess/SshiJ*), *Foxp2cre*^{+/-} (kindly provided by R. Palmiter, University of Washington)

418   (Rousso et al. 2016), all crossed to *Rosa26YFP*^{+/+}mice (Jackson lab strain R26R-EYFP). For

419   analysis of aromatase expression, we used Aromatase LacZ reporter mice (kindly provided by N.

420   Shah, University of California-San Francisco) (Wu et al. 2009). Mice were genotyped by

421   Transnetyx Inc. Genotyping Services. Adult mice were considered between 3-7 months of age.

422   Sample size was based on previous experiments and published data. No statistical methods were

423   used to determine sample sizes.

424

425   *Immunohistochemistry*

426   Postnatal mice were transcardially perfused with 4% paraformaldehyde (PFA) and brains post

427   fixed overnight at 4°C, embedded in 4% agarose (Invitrogen) and sectioned at 50 μm with a

428   vibrating microtome (Leica VT1000S). Embryos were fixed in 4% PFA overnight at 4°C

429   degrees, cryoprotected in 30% sucrose, embedded in O.C.T. compound (Tekka) and sectioned at

430   20 μm on a cryostat (Leica CM1850). For IHC, tissue sections were incubated overnight with

431   primary antibody, then washed with PBST and 10% normal donkey serum and incubated for 4

432   hours with the corresponding secondary antibodies, and mounted with DAPI Fluoromount

433   (SouthernBiotech 0100-20). Primary antibodies used were rat anti-GFP (to detect YFP

434   expression, (1:1000, Nacalai 04404-84), goat anti-Foxp2 (1:200; Santa Cruz sc-21069), rabbit

435   anti-Foxp2 (1:500; abcam ab16046), rabbit anti-OTP (1:2000; kind provided by F. Vaccarino,

436   Yale University), rabbit anti-androgen receptor (1:750; Epitomics AC-0071), rabbit anti-estrogen

437   receptor α (1:6000; Millipore 06-935); mouse anti-NeuN (1:200; Millipore MAB-377), mouse

438   anti-CC1 (1:250; Calbiochem OP80-100), goat anti-calbindin (1:200; Santa Cruz sc-7691), rat

439   anti-somatostatin (1:100; Millipore MAB354), rabbit anti-cfos (1:500; Santa Cruz sc-52), goat

440   anti-cfos (1:300; Santa Cruz sc-52G), anti-rabbit nNOS (1:8000; ImmunoStar 24287), mouse

441   anti-CAMKIIα (1:500 Biomol ARG22260.50) and chicken anti-βGal (1:2000; abcam 9361).

442   Secondary antisera used were donkey anti-rat or anti-goat Alexa 488 (1:200; Life Technologies),

443   anti-rabbit or anti-goat Cy5 (1:1000; Jackson ImmunoResearch), anti-rabbit or anti-mouse Cy3

444   (1:1000; Jackson ImmunoResearch), anti-mouse dylight 649 (1:500; Jackson ImmunoResearch),

445   or anti-chicken dylight 549 (1:500; Jackson ImmunoResearch).

446

447   ***Microscopy***

448   Fluorescent photographs were taken using an Olympus FX1000 Fluoview Laser Scanning

449   Confocal Microscope (1um optical thickness)

450

451   ***Quantification***

452   *Molecular marker analysis*

453   For embryonic analysis, the average of 2-3 sections encompassing the POA were imaged and

454   quantified **(Figure 1; Figure 1-Figure supplement 1-2)**. For adult analyses **(Figures 1-7;**

455   **Figure 1- Figure supplement 1-2; Figure 2- Figure supplement 1; Figure 4-Figure**

456   **supplement 1; Figure 5- Figure supplement 1; Figure 7-Figure supplement 1)**, every 6th

457   serial coronal section encompassing the anterior to posterior extent of the MeA (Bregma -1.30 to

458   -1.90, see **Figure 1- Figure supplement 3**) was immunostained with antibodies to Foxp2, YFP

459   and markers of interest. Quantification was done by counting single and double-labeled cells

460   encompassing the entire domain of expression within the POA or MeA **(Figures 1-2, 4; Figure**

461   **1- Figure supplement 1-2; Figure 2- Figure supplement 1; Figure 4-Figure supplement 1)**.

462

463   *Neuronal activation*

464   For analyses of neuronal activation **(Figures 5-7, Figure 7- Figure supplement 1)**, a single

465   MeA section with highest number of c-fos+ cells corresponding with the presence of *Dbx1-*

466   derived, Foxp2+ or OTP+ cells was chosen for quantification. c-fos, YFP and Foxp2 triple

467   immunostaining was conducted on the same section and single and double labeled cells counted.

468   c-fos and OTP double immunostaining was conducted and single and double cells counted.

469

470   *Statistical Evaluation*

471   Unless otherwise stated, data was analyzed using GraphPad 6 statistical software. We first tested

472   the distribution of the data with the Shapiro-Wallis test for normality. Data that was normally

473   distributed was analyzed using an unpaired two-tailed *t*-test for analysis of experiments involving

474   two groups **(Figure 1-Figure supplement 1; Figure 1- Figure supplement 2; Figure 5x;**

475   **Figure 6c females, k, m, l; Figure 7c, g males, k, l, m Foxp2+ subpopulation; Figure 7-**

476   **Figure supplement 1c, d, h, i)** and a one-way ANOVA **(Figure 5o, w, y Foxp2+**

477   **subpopulation)** followed by Tukey-Kramer multiple comparison test was used for analysis of

478   experiments involving three or more groups. Data with a non-normal distribution was analyzed

479   by using the non-parametric test Mann-Whitney when comparing two groups **(Figure 5c, g, k, y**

480   ***Dbx1*-derived subpopulation; Figure 6c males, g; Figure 7g females, m *Dbx1*-derived**

481   **subpopulation; Figure 7-Figure supplement 1j, k)** and Kruskal-Wallis with Dunn's post-hoc

482   corrections for data with three groups **(Figure 5s)**. For analysis of data shown in **Figure 4** and

483   **Figure 4- Figure supplement 1** we performed the following statistical analysis: when the data

484   met the normality assumption or could be transformed to meet the normality assumption,

485   generally two-way analysis of variance models were implemented to evaluate the evidence of

486   differences in mean effects of the two experimental factors on cell activation **(Figure 4 g, u;**

487   **Figure 4-Figure supplement 2 a, b)**. In the situation where no data transformation could be

488   found to achieve an acceptable level of normality, quantile regression was performed, which

489   does not require the normality assumption, to evaluate comparable differences in median effects

490　**(Figure 4 n; Figure 4-Figure supplement 2c)**. In each case, the initial models included a cross-

491　products term to assess evidence of effect modification or interaction. When there was no

492　interaction, it was taken as evidence of the absence of effect modification and the cross-products

493　term was removed leaving only a model that assessed independent effects of the each factor

494　separately, while holding constant any effects of the other factor. Depending on the underlying

495　model, either mean or median effects +/- 95% confidence intervals were derived to reflect

496　differences that were consistent with statistically meaningful differences in the final

497　model. Under consideration of protecting the experiment-wise error rate, as long as the

498　evaluation of differences focused only on identifying the nature of effects deemed statistically

499　meaningful in the final model, there was no correction made for multiple comparisons. Analysis

500　of data meeting the normality assumption was based on GraphPad Prism 6 and analysis based on

501　quantile regression was implemented in Stata 13. As mice had to be sacrificed after each

502　behavioral assay in order to conduct c-fos immuno-analysis, technical repeats were not available.

503　Measurements from different mice were considered biological repeats to determine sample size.

504　Data points were considered outliers and excluded if they were two standard deviations away

505　from the mean.

506

507　*Electrophysiology and biocytin filling*

508　Mice (P25-40) were anaesthetized with isoflurane and sacrificed. Brains were removed and

509　immediately immersed in ice-cold oxygenated (95% O2/5% CO_2) sucrose solution (234 mM

510　sucrose, 11 mM glucose, 26 mM $NaHCO_3$, 2.5 mM KCl,1.25 mM $NaH_2PO_4.H_2O$, 10 mM

511　$MgSO_4$ and 0.5 mM $CaCl_2$). Coronal slices of 300μm in thickness were cut. Slices with

512　amygdala were collected and placed in oxygen-equilibrated artificial cerebral spinal fluid

513 (ACSF) as previously described (Hirata et al. 2009). Either *Dbx1*-derived or *Foxp2*-derived

514 neurons were then visualized using a fluorescent lamp (Nikon) with a 450-490λ filter. Whole-

515 cell patch-clamp recordings from YFP-positive fluorescent cells were performed at room

516 temperature with continuous perfusion of ACSF (Multiclamp 700A, DigiDATA1322, Molecular

517 Devices). Intracellular solution (in mM): 130 Kgluconate, 10 KCl, 2 MgCl2, 10 HEPES, 10

518 EGTA, 2 Na2-ATP, 0.5 Na2-GTP. All measurements of intrinsic and synaptic properties were

519 analyzed off-line using Clampfit Software (V.10.2, Molecular Devices) and graphing software

520 (OriginPro 9.1). At the end of each recording, biocytin (1%) was injected with the depolarizing

521 current (1nA) for post-hoc morphology analysis. All slices were then fixed with

522 paraformaldehyde overnight at 4°C and processed for Fluorescein-conjugated Avidin-D (1:200,

523 Vector Laboratories), YFP IHC (*Dbx1*-derived and Foxp2+ recordings) or Foxp2 IHC (for

524 Foxp2+ recordings) as described above.

525

526 ***Neuronal Reconstruction***

527 Neurons were filled with biocytin and imaged using an Olympus FX1000 Fluoview Laser

528 Scanning Confocal Microscope (0.5um optical thickness). VIAS software was used to align

529 confocal images taken at 40x and 60x in the same plane (x,y,z). Neurons were then traced using

530 neuTube software, which uses fixed radii small tubes to estimate the dendritic branches length

531 and thickness (Feng, Zhao & Kim 2015).

532

533 ***Behavioral assays***

534 *Dbx1cre$^{+/-}$;Rosa26YFP$^{+/+}$* male and female mice 3-7 months old were used for the behavioral

535 assays. One week prior to testing, animals were single housed. Testing was performed between

536   the hours of 18:00 and 20:00 corresponding to the beginning of the dark cycle for all assays

537   except the maternal aggression assay which took place from 13:00 to 15:00pm corresponding to

538   the light cycle.

539

540   *Mating*

541   Sexually naïve hormonally primed females were analyzed during female mating. Mating was

542   assessed by placing a mouse of the opposite sex inside the resident's cage and checking for plugs

543   every 30 minutes. When a plug was observed, noting successful intromission, female or male

544   mate was removed from the cage and the experimental animal was left inside the cage for an

545   additional 30 minutes before being sacrificed. Females with no plugs were excluded from the

546   analysis as no mating occurred.

547

548   *Territorial and maternal aggression*

549   Male territorial aggression was assessed by performing a resident-intruder assay in which an

550   unfamiliar male mouse ('intruder') was placed inside the resident's cage for 10 minutes. During

551   this period, the homecage male displayed typical aggressive behaviors including attacking and

552   biting. The intruder was removed and the after an additional 50 minutes the resident male was

553   sacrificed. For the maternal aggression assay, female mice were single-housed after a plug was

554   observed. The following experimental conditions were run: 1) for maternal aggression pups age

555   between P5-P8 were removed and 2 minutes later a sexually naïve male was introduced into the

556   cage for 10 minutes, 2) for 'no pups' condition, pups were removed but no intruder presented

557   and 3) for naïve control, the cage was undisturbed in which pups were not removed and no

558   intruder presented. In conditions 1) & 2) the female mice were sacrificed after 50 minutes after

559 pup removal. If female mice did not actively attack the intruder at least two times, they were

560 excluded from the analysis. All male mice attacked the intruder for the 10 minute period.

561

562 *Predator avoidance*

563 Predator avoidance was assessed by introducing a petri dish containing rat bedding to the

564 homecage for 1 hour. The control predator group was presented with a petri dish containing

565 clean mouse bedding (benign). Mice were sacrificed after 1 hour presentation of rat bedding or

566 benign bedding. Mice that did not show escaping responses after the presentation of the petri

567 dish were excluded.

581   **References**

582   Apfelbach, R., Blanchard, C.D., Blanchard, R.J., Hayes, R.A. & McGregor, I.S. 2005, "The effects of
583   predator odors in mammalian prey species: a review of field and laboratory studies", *Neuroscience*
584   *and biobehavioral reviews,* vol. 29, no. 8, pp. 1123-1144.

585   Bale, T.L. & Epperson, C.N. 2015, "Sex differences and stress across the lifespan", *Nature neuroscience,*
586   vol. 18, no. 10, pp. 1413-1420.

587   Balthazart, J., Charlier, T.D., Cornil, C.A., Dickens, M.J., Harada, N., Konkle, A.T.M., Voigt, C. & Ball,
588   G.F. 2011. "Sex differences in brain aromatase activity: genomic and non-genomic controls",
589   *Frontiers in Endocrinology,* vol. 2, no. 34.

590   Bergan, J.F., Ben-Shaul, Y. & Dulac, C. 2014, "Sex-specific processing of social cues in the medial
591   amygdala", *eLife,* vol. 3, pp. e02743.

592   Bian, X. 2013, "Physiological and morphological characterization of GABAergic neurons in the medial
593   amygdala", *Brain research,* vol. 1509, pp. 8-19.

594   Bikoff, J.B., Gabitto, M.I., Rivard, A.F., Drobac, E., Machado, T.A., Miri, A., Brenner-Morton, S.,
595   Famojure, E., Diaz, C., Alvarez, F.J., Mentis, G.Z. & Jessell, T.M. 2016, "Spinal Inhibitory
596   Interneuron Diversity Delineates Variant Motor Microcircuits", *Cell,* vol. 165, no. 1, pp. 207-219.

597   Canteras, N.S., Pavesi, E. & Carobrez, A.P. 2015, "Olfactory instruction for fear: neural system analysis",
598   *Frontiers in neuroscience,* vol. 9, pp. 276.

599   Carney, R.S., Mangin, J.M., Hayes, L., Mansfield, K., Sousa, V.H., Fishell, G., Machold, R.P., Ahn, S.,
600   Gallo, V. & Corbin, J.G. 2010, "Sonic hedgehog expressing and responding cells generate neuronal
601   diversity in the medial amygdala", *Neural development,* vol. 5, pp. 14-8104-5-14.

602   Carvalho, V.M., Nakahara, T.S., Cardozo, L.M., Souza, M.A., Camargo, A.P., Trintinalia, G.Z., Ferraz,
603   E. & Papes, F. 2015, "Lack of spatial segregation in the representation of pheromones and
604   kairomones in the mouse medial amygdala", *Frontiers in neuroscience,* vol. 9, pp. 283.

605   Choi, G.B., Dong, H.W., Murphy, A.J., Valenzuela, D.M., Yancopoulos, G.D., Swanson, L.W. &
606   Anderson, D.J. 2005, "Lhx6 delineates a pathway mediating innate reproductive behaviors from the
607   amygdala to the hypothalamus", *Neuron,* vol. 46, no. 4, pp. 647-660.

608   Cooke, B.M. & Woolley, C.S. 2005, "Sexually dimorphic synaptic organization of the medial amygdala",
609   *The Journal of neuroscience : the official journal of the Society for Neuroscience,* vol. 25, no. 46,
610   pp. 10759-10767.

611   Dodson, P.D., Larvin, J.T., Duffell, J.M., Garas, F.N., Doig, N.M., Kessaris, N., Duguid, I.C., Bogacz, R.,
612   Butt, S.J. & Magill, P.J. 2015, "Distinct developmental origins manifest in the specialized encoding
613   of movement by adult neurons of the external globus pallidus", *Neuron,* vol. 86, no. 2, pp. 501-513.

614   Dulac, C. & Wagner, S. 2006, "Genetic analysis of brain circuits underlying pheromone signaling",
615   *Annual Review of Genetics,* vol. 40, pp. 449-467.

616 Erskine, M.S. 1993, "Mating-induced increases in FOS protein in preoptic area and medial amygdala of
617   cycling female rats", *Brain research bulletin,* vol. 32, no. 5, pp. 447-451.

618 Feng, L., Zhao, T. & Kim, J. 2015, "neuTube 1.0: A New Design for Efficient Neuron Reconstruction
619   Software Based on the SWC Format", *eNeuro,* vol. 2, no. 1, pp. 10.1523/ENEURO.0049-14.2014.
620   eCollection 2015 Jan-Feb.

621 Flames, N., Pla, R., Gelman, D.M., Rubenstein, J.L., Puelles, L. & Marin, O. 2007, "Delineation of
622   multiple subpallial progenitor domains by the combinatorial expression of transcriptional codes",
623   *The Journal of neuroscience : the official journal of the Society for Neuroscience,* vol. 27, no. 36,
624   pp. 9682-9695.

625 Fogarty, M., Grist, M., Gelman, D., Marin, O., Pachnis, V., & Kessaris, N. 2007, "Spatial genetic
626   patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult
627   cortex", *Journal of Neuroscience,* vol 27, no. 41, pp. 10935-10946.

628 French, C.A. & Fisher, S.E. 2014, "What can mice tell us about Foxp2 function?", *Current opinion in*
629   *neurobiology,* vol. 28, pp. 72-79.

630 Garcia-Moreno, F., Pedraza, M., Di Giovannantonio, L.G., Di Salvio, M., Lopez-Mascaraque, L.,
631   Simeone, A. & De Carlos, J.A. 2010, "A neuronal migratory pathway crossing from diencephalon to
632   telencephalon populates amygdala nuclei", *Nature neuroscience,* vol. 13, no. 6, pp. 680-689.

633 Gorski, J.A., Talley, T., Qiu, M., Puelles, L., Rubenstein, J.L.R. & Jones, K.R. 2002, "Cortical excitatory
634   neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage.", *The*
635   *Journal of neuroscience,* vol. 22, no. 15, pp. 6309-6314.

636 Gross, C.T. & Canteras, N.S. 2012, "The many paths to fear", *Nature reviews.Neuroscience,* vol. 13, no.
637   9, pp. 651-658.

638 Haney, M., DeBold, J.F. & Miczek, K.A. 1989, "Maternal aggression in mice and rats towards male and
639   female conspecifics.", *Aggressive Behavior,* vol. 15, pp. 443-453.

640 Hirata, T., Li, P., Lanuza, G.M., Cocas, L.A., Huntsman, M.M. & Corbin, J.G. 2009, "Identification of
641   distinct telencephalic progenitor pools for neuronal diversity in the amygdala.", *Nature*
642   *Neuroscience,* vol. 12, no. 2, pp. 141-149.

643 Hong, W., Kim, D. & Anderson, D. 2014, "Antagonistic Control of Social versus Repetitive Self-
644   Grooming Behaviors by Separable Amygdala Neuronal Subsets", *Cell,* vol. 158, no. 6, pp. 1348-
645   1361.

646 Johnson, R.T., Breedlove, S.M. & Jordan, C.L. 2008, "Sex differences and laterality in astrocyte number
647   and complexity in the adult rat medial amygdala", *The Journal of comparative neurology,* vol. 511,
648   no. 5, pp. 599-609.

649 Jones E.G., Huntley, G.W., Benson, D.L. 1994. "Alpha calcium/calmodulin-dependent protein kinase II
650   selectively expressed in a subpopulation of excitatory neurons in monkey sensory-motor cortex:
651   comparison with GAD-67 expression", *Journal of Neuroscience,* vol 14, pp.611-629.

652 Juntti, S.A., Tollkuhn, J., Wu, M.V., Fraser, E.J., Soderborg, T., Tan, S., Honda, S., Harada, N. & Shah,
653   N.M. 2010, "The Androgen Receptor Governs the Execution, but Not Programming, of Male Sexual
654   and Territorial Behaviors", *Neuron,* vol. 66, no. 2, pp. 260-272.

655 Kang, N., McCarthy, E.A., Cherry, J.A. & Baum, M.J. 2011, "A sex comparison of the anatomy and
656   function of the main olfactory bulb-medial amygdala projection in mice", *Neuroscience,* vol. 172,
657   pp. 196-204.

658 Kepecs, A. & Fishell, G. 2014, "Interneuron cell types are fit to function", *Nature,* vol. 505, no. 7483, pp.
659   318-326.

660 Keshavarzi, S., Sullivan, R.K., Ianno, D.J. & Sah, P. 2014, "Functional properties and projections of
661   neurons in the medial amygdala", *The Journal of neuroscience : the official journal of the Society for
662   Neuroscience,* vol. 34, no. 26, pp. 8699-8715.

663 Kim, Y., Venkataraju, K.U., Pradhan, K., Mende, C., Taranda, J., Turaga, S.C., Arganda-Carreras, I., Ng,
664   L., Hawrylycz, M.J., Rockland, K.S., Seung, H.S. & Osten, P. 2015, "Mapping social behavior-
665   induced brain activation at cellular resolution in the mouse", *Cell reports,* vol. 10, no. 2, pp. 292-
666   305.

667 Koenning, M., Jackson, S., Hay, C.M., Faux, C., Kilpatrick, T.J., Willingham, M. & Emery, B. 2012,
668   "Myelin gene regulatory factor is required for maintenance of myelin and mature oligodendrocyte
669   identity in the adult CNS", *The Journal of neuroscience : the official journal of the Society for
670   Neuroscience,* vol. 32, no. 36, pp. 12528-12542.

671 Kollack, S.S. & Newman, S.W. 1992, "Mating behavior induces selective expression of Fos protein
672   within the chemosensory pathways of the male Syrian hamster brain", *Neuroscience letters,* vol. 143,
673   no. 1-2, pp. 223-228.

674 Kondo, Y. 1992, "Lesions of the medial amygdala produce severe impairment of copulatory behavior in
675   sexually inexperienced male rats", *Physiology & Behavior,* vol. 51, no. 5, pp. 939-943.

676 Llinas, R.R. 1988, "The intrinsic electrophysiological properties of mammalian neurons: insights into
677   central nervous system function", *Science (New York, N.Y.),* vol. 242, no. 4886, pp. 1654-1664.

678 Martel, K.L. & Baum, M.J. 2009, "A centrifugal pathway to the mouse accessory olfactory bulb from the
679   medial amygdala conveys gender-specific volatile pheromonal signals", *The European journal of
680   neuroscience,* vol. 29, no. 2, pp. 368-376.

681 McCarthy, M.M. & Arnold, A.P. 2011, "Reframing sexual differentiation of the brain", *Nature
682   neuroscience,* vol. 14, no. 6, pp. 677-683.

683 Mullen, R.J., Buck, C.R. & Smith, A.M. 1992, "NeuN, a neuronal specific nuclear protein in vertebrates",
684   *Development (Cambridge, England),* vol. 116, no. 1, pp. 201-211.

685 Oren-Suissa, M., Bayer, E.A. & Hobert, O. 2016, "Sex-specific pruning of neuronal synapses in
686   Caenorhabditis elegans", *Nature,* vol. 533, no. 7602, pp. 206-211.

687 Petilla Interneuron Nomenclature Group, Ascoli, G.A., Alonso-Nanclares, L., Anderson, S.A.,
688   Barrionuevo, G., Benavides-Piccione, R., Burkhalter, A., Buzsaki, G., Cauli, B., Defelipe, J., Fairen,
689   A., Feldmeyer, D., Fishell, G., Fregnac, Y., Freund, T.F., Gardner, D., Gardner, E.P., Goldberg, J.H.,
690   Helmstaedter, M., Hestrin, S., Karube, F., Kisvarday, Z.F., Lambolez, B., Lewis, D.A., Marin, O.,
691   Markram, H., Munoz, A., Packer, A., Petersen, C.C., Rockland, K.S., Rossier, J., Rudy, B.,
692   Somogyi, P., Staiger, J.F., Tamas, G., Thomson, A.M., Toledo-Rodriguez, M., Wang, Y., West, D.C.
693   & Yuste, R. 2008, "Petilla terminology: nomenclature of features of GABAergic interneurons of the
694   cerebral cortex", *Nature reviews.Neuroscience,* vol. 9, no. 7, pp. 557-568.

695 Remedios, R., Huilgol, D., Saha, B., Hari, P., Bhatnagar, L., Kowalczyk, T., Hevner, R.F., Suda, Y.,
696   Aizawa, S., Ohshima, T., Stoykova, A. & Tole, S. 2007, "A stream of cells migrating from the
697   caudal telencephalon reveals a link between the amygdala and neocortex", *Nature neuroscience,* vol.
698   10, no. 9, pp. 1141-1150.

699 Rousso, D.L., Qiao, M., Kagan, R.D., Yamagata, M., Palmiter, R.D. & Sanes, J.R. 2016, "Two Pairs of
700   ON and OFF Retinal Ganglion Cells Are Defined by Intersectional Patterns of Transcription Factor
701   Expression", *Cell reports,* vol. 15, no. 9, pp. 1930-1944.

702 Rowe, D.W. & Erskine, M.S. 1993, "c-Fos proto-oncogene activity induced by mating in the preoptic
703   area, hypothalamus and amygdala in the female rat: role of afferent input via the pelvic nerve",
704   *Brain research,* vol. 621, no. 1, pp. 25-34.

705 Scalia, F. & Winans, S.S. 1975, "The differential projections of the olfactory bulb and accessory olfactory
706   bulb in mammals", *The journal of comparative neurology,* vol. 161, no. 1, pp. 31-55.

707 Schultz, W. 2007, "Multiple dopamine functions at different time courses", *Annual Review of*
708   *Neuroscience,* vol. 30, pp. 259-288.

709 Shemesh, Y., Forkosh, O., Mahn, M., Anpilov, S., Sztainberg, Y., Manashirov, S., Shlapobersky, T.,
710   Elliott, E., Tabouy, L., Ezra, G., Adler, E.S., Ben-Efraim, Y.J., Gil, S., Kuperman, Y., Haramati, S.,
711   Dine, J., Eder, M., Deussing, J.M., Schneidman, E., Yizhar, O. & Chen, A. 2016, "Ucn3 and CRF-
712   R2 in the medial amygdala regulate complex social dynamics", *Nature neuroscience, .*

713 Shirasaki, R. & Pfaff, S.L. 2002, "Transcriptional codes and the control of neuronal identity", *Annual*
714   *Review of Neuroscience,* vol. 25, pp. 251-281.

715 Sokolowski, K. & Corbin, J.G. 2012, "Wired for behaviors: from development to function of innate
716   limbic system circuitry", *Frontiers in molecular neuroscience,* vol. 5, pp. 55.

717 Sokolowski, K., Esumi, S., Hirata, T., Kamal, Y., Tran, T., Lam, A., Oboti, L., Brighthaupt, S.C.,
718   Zaghlula, M., Martinez, J., Ghimbovschi, S., Knoblach, S., Pierani, A., Tamamaki, N., Shah, N.M.,
719   Jones, K.S. & Corbin, J.G. 2015, "Specification of select hypothalamic circuits and innate behaviors
720   by the embryonic patterning gene dbx1", *Neuron,* vol. 86, no. 2, pp. 403-416.

721 Soma, M., Aizawa, H., Ito, Y., Maekawa, M., Osumi, N., Nakahira, E., Okamoto, H., Tanaka, K. &
722   Yuasa, S. 2009, "Development of the mouse amygdala as revealed by enhanced green fluorescent
723   protein gene transfer by means of in utero electroporation", *The Journal of comparative neurology,*
724   vol. 513, no. 1, pp. 113-128.

725 Stepien, A.E., Tripodi, M. & Arber, S. 2010, "Monosynaptic rabies virus reveals premotor network
726   organization and synaptic specificity of cholinergic partition cells", *Neuron,* vol. 68, no. 3, pp. 456-
727   472.

728 Stowers, L., Cameron, P. & Keller, J.A. 2013, "Ominous odors: olfactory control of instinctive fear and
729   aggression in mice", *Current opinion in neurobiology,* vol. 23, no. 3, pp. 339-345.

730 Swanson, L.W. & Petrovich, G.D. 1998, "What is the amygdala?", *Trends in neurosciences,* vol. 21, no.
731   8, pp. 323-331.

732 Takahashi, L.K. & Gladstone, C.D. 1988, "Medial amygdaloid lesions and the regulation of sociosexual
733   behavioral patterns across the estrous cycle in female golden hamsters", *Behavioral neuroscience,*
734   vol. 102, no. 2, pp. 268-275.

735 Tanaka, M., Ikeda, T., Hayashi, S., Iijima, N., Amaya, F., Hisa, Y., Ibata, Y. 1997, "Nitrergic
736   neurons in the medial amygdala project to the hypothalamic paraventricular nucleus of the
737   rat" *Brain Research,* vol 777, pp. 13-21.

738 Unger, E.K., Burke, K.J.,Jr, Yang, C.F., Bender, K.J., Fuller, P.M. & Shah, N.M. 2015, "Medial
739   amygdalar aromatase neurons regulate aggression in both sexes", *Cell reports,* vol. 10, no. 4, pp.
740   453-462.

741 Vochteloo, J.D. & Koolhaas, J.M. 1987, "Medial amygdala lesions in male rats reduce aggressive
742   behavior: interference with experience", *Physiology & Behavior,* vol. 41, no. 2, pp. 99-102.

743 Waclaw, R.R., Ehrman, L.A., Pierani, A. & Campbell, K. 2010, "Developmental origin of the neuronal
744   subtypes that comprise the amygdalar fear circuit in the mouse", *The Journal of neuroscience : the*
745   *official journal of the Society for Neuroscience,* vol. 30, no. 20, pp. 6944-6953.

746 Wang, Y., He, Z., Zhao, C. & Li, L. 2013, "Medial amygdala lesions modify aggressive behavior and
747   immediate early gene expression in oxytocin and vasopressin neurons during intermale exposure",
748   *Behavioural brain research,* vol. 245, pp. 42-49.

749 Wu, M.V., Manoli, D.S., Fraser, E.J., Coats, J.K., Tollkuhn, J., Honda, S., Harada, N. & Shah, N.M.
750   2009, "Estrogen masculinizes neural pathways and sex-specific behaviors", *Cell,* vol. 139, no. 1, pp.
751   61-72.

752 Yang, C.F. & Shah, N.M. 2014, "Representing sex in the brain, one module at a time.", *Neuron,* vol. 82,
753   no. 2, pp. 261-278.

754 Yang, C.F., Chiang, M.C., Gray, D.C., Prabhakaran, M., Alvarado, M., Juntti, S.A., Unger, E.K., Wells,
755   J.A. & Shah, N.M. 2013, "Sexually dimorphic neurons in the ventromedial hypothalamus govern
756   mating in both sexes and aggression in males", *Cell,* vol. 153, no. 4, pp. 896-909.

757 Zhang, L., Doyon, W.M., Clark, J.J., Phillips, P.E. & Dani, J.A. 2009, "Controls of tonic and phasic
758   dopamine transmission in the dorsal and ventral striatum", *Molecular pharmacology,* vol. 76, no. 2,
759   pp. 396-404.

760   Zhao, T., Szabo, N., Ma, J., Luo, L., Zhou, X. & Alvarez-Bolado, G. 2008, "Genetic mapping of Foxb1-
761   cell lineage shows migration from caudal diencephalon to telencephalon and lateral hypothalamus",
762   *The European journal of neuroscience,* vol. 28, no. 10, pp. 1941-1955.

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765

766   **Figure Legends**

767   **Figure 1. Embryonic and postnatal segregation of *Dbx1*-derived and Foxp2+ cells.**

768   Minimal co-localization of *Dbx1*-derived (green) and Foxp2+ (red) labeled cells in coronal

769   sections at the level of the POA at E11.5 (a-f) and E13.5 (g-l). As shown at E11.5 (s) Foxp2+

770   precursors in the VZ (arrows) are observed putatively migrating to the SVZ. The segregation of

771   *Dbx1*-derived and Foxp2+ cells persists into adulthood (m-r). Summary schematic of spatial

772   segregation of *Dbx1*-derived and Foxp2+ cells in the embryonic POA and postnatal MeA (t).

773   Venn diagram depicting the overlap of the total number of *Dbx1*-derived and Foxp2+ cells in 2-3

774   sections/embryo or 5-8 sections/adult brain. The scale bars represent 200μm (a-c, g-i, s), 100μm

775   (m-o) and 25μm (d-f, j-l, p-r). Abbreviations: MeA, medial amygdala; MePD, medial amygdala-

776   posterior dorsal; MePV, medial amygdala posterior ventral; POA, preoptic area; SVZ,

777   subventricular zone; V, ventricle; VZ, ventricular zone. n=3 embryonic brains; n=5 postnatal

778   brains. Data are mean $\pm$ s.e.m. n is the number of animals.

779

780   **Figure 2. Identity of Foxp2+ medial amygdala neurons.**

781   Dual immunofluorescence of Foxp2 with NeuN (n=3 mice) (a-d), CC1 (n=3 mice) (e-h), YFP

782   (in *Emx1^{cre};RYFP* mice) (n=5 mice) (i-l), calbindin (n=3 mice) (m-p), nNOS (n=6 mice) (q-t),

783   and somatostatin (n=4 mice) (u-x) (arrows show double labeled cells). Quantification of co-

784   localization with each marker (y). Data are mean $\pm$ s.e.m. n is the number of animals. The scale

785   bar represents 200μm (a, e, i, m, q, u) and 25μm (b-d, f-h, j-l, n-p, r-t, v-x).

786

787   **Figure 3. *Dbx1*-derived and Foxp2+ MeA neurons possess distinct electrophysiological**

788   **properties.**

789   Typical firing patterns of *Dbx1*-derived (a) and Foxp2+ (b) neurons with current injections at -

790   60pA, +20pA and +60pA. Significant differences across populations in resting membrane

791   potential (Rm) (c), voltage at rest (Vrest) (d), capacitance (e), and action potential (AP) firing

792   patterns (f) but not rheobase (g) are observed (two-tailed *t*-test, (c) p=0.0006, t=3.676, df=49; (d)

793   p=0.036, t=2.159, df=49; (e) p=0.002, t=3.1987, df=49; (f) p=0.016, t=3.1988, df=49; (g)

794   p=0.610, t=0.514, df=49; *n*=28 *Dbx1*-derived cells and *n*=23 Foxp2+ cells). Excitatory post-

795   synaptic currents (EPSCs) are observed in both *Dbx1*-derived (h) and Foxp2+ (i) neurons, with

796   significant differences in sEPSC frequency (j), amplitude (k) and decay (l) (two-tailed *t*-test, (j)

797   p=<0.0001, t=3.041, df= 34; (k) p=0.006, t=2.949, df=34; (l) p=0.005, t=3.041, df=34; *n*=28

798   *Dbx1*-derived neurons, *n*=23 Foxp2+ neurons). Data are mean $\pm$ s.e.m. *n* is the number of cells.

799   p<0.05 (*), p<0.01 (**), and p<0.001 (***), n.s.; not significant.

800

801   **Figure 4. Expression of sex hormone pathway markers in *Dbx1*-derived and Foxp2+ cells.**

802   Dual immunofluorescence for YFP (green) or Foxp2 (red) with the sex steroid hormone pathway

803   markers (white): estrogen receptor α (ERα) (a-g), aromatase (h-n) or androgen receptor (AR) (o-

804   u) (arrows show double labeled cells). Subpopulations of both *Dbx1*-derived (a-c, g) and Foxp2+

805   cells express ERα (d-g). Quantification reveals a greater percentage of *Dbx1*-derived cells

806   expressing ERα compared to Foxp2+ cells in females (two-way ANOVA with no interaction

807   between subpopulation and sex, but with main effect for subpopulation p=0.0433,

808   F(1,10)=5.352; *n*=4 and x=45.38 for *Dbx1*-derived cells in female mice, *n*=4 and x=24.77 for

809   Foxp2+ cells in female mice, *n=3* and x=28.43 for *Dbx1*-derived cells in male mice, *n=3* and

810   x=24 for Foxp2+ cells in male mice). The majority of *Dbx1*-derived cells express aromatase in

811   both males and females (h-j, n). In contrast, only a small percentage of Foxp2+ cells in both

812   males express aromatase (two quantile regression analysis for non-normal distributions shows no

813   interaction for sex and subpopulation but a main effect for subpopulation p=0.000 with a 95%

814   confidence interval of 31.27 and 69.53, *n=3* for *Dbx1*-derived cells in male mice, *n=3* and for

815   Foxp2+ cells in male mice, *n=3* for mice for *Dbx1*-derived cells in female mice, *n=3* and for

816   Foxp2+ female mice). A greater percentage of *Dbx1*-derived neurons in males express AR in

817   comparison to *Dbx1*-derived cells in female mice (o-q, u). No differences in percentages of

818   Foxp2+ male or female subpopulations expressing AR (r-u) nor differences across

819   subpopulations (two-way ANOVA with no interaction between subpopulation and sex, but with

820   main effect for sex p=0.0166, $F_{(1,8)}$=9.118; *n=3* and x=10.07 for *Dbx1*-derived cells in female

821   mice, *n=3* and x=7.133 for Foxp2+ cells in female mice, *n=3* and x=24.3 for *Dbx1*-derived cells

822   in male mice, *n=3* and x=16.27 for Foxp2+ cells in male mice). Data are mean $\pm$ s.e.m. *n* is the

823   number of animals and x is the mean. The scale bar represents 50μm. p<0.05 (*) and p<0.01

824   (**).

825

826   **Figure 5. *Dbx1*-derived and Foxp2+ neurons are activated during aggressive encounters.**

827   Significant increases in the number of c-fos+ cells (white) compared to controls is observed in

828   the MeA during both male territorial aggression (a-c) (two tailed Mann-Whitney test, U=0,

829   p=0.0025, *n=5* naïve mice, *n=7* territorial aggression) and maternal aggression (one-way

830   ANOVA, p=<0.0001, $F_{(2, 18)}$=19.05, *n=7* naïve mice, *n=6* 'no pup' condition, *n=8* maternal

831   aggression) (l-o). Dual immunofluorescence for c-fos (white) and YFP (green) or Foxp2 (red)

832   after male territorial aggression reveals an increase in the number of *Dbx1*-derived cells (two-

833   tailed Mann-Whitney test, U=4, p=0.0238, *n*= 6) (d-g) and Foxp2+ cells (two-tailed Mann-

834   Whitney test, U=0, p=0.0023 *n=7*) expressing c-fos in comparison to naïve control mice (*n=6*)

835   not exposed to an intruder (h-k). In female mice, an increase in the number *Dbx1*-derived cells

836   expressing c-fos during maternal aggression (*n*=4) in comparison to naïve (*n*=5), but not in the

837   'no pups' conditions (*n*=4), is observed (Kruskal-Wallis test, $X^2(3,13)$=7.124 p=0.0164) (p-s).

838   There is also an increase in the number of Foxp2+ cells (*n*=8) expressing c-fos during maternal

839   aggression in comparison to the naïve (*n*=7) and 'no pups' control conditions (*n*=6) (one-way

840   ANOVA p=<0.0001, F(2,18)=16.93) (t-w). Analyses of the percentage of both *Dbx1*-derived

841   (YFP+ and c-fos+ /total YFP+) and Foxp2+ cells (Foxp2+ and c-fos+/ total Foxp2+) expressing

842   c-fos in male mice reveals an increase in both the *Dbx1*-derived subpopulation (*n*=6) (two-tailed

843   *t*-test, p=0.0009, t=4.685, df=11) and the Foxp2+ subpopulation (*n*=7) (two-tailed *t*-test,

844   p=0.001, t=4.295, df=11) in comparison to naïve controls (*n*=6) (x). In females, the percentage of

845   *Dbx1*-derived cells expressing c-fos during maternal aggression is higher in comparison to the

846   naïve condition but not to the 'no pups' condition   (Kruskal-Wallis test, $X^2(3,13)$=9.461

847   p=0.0005, *n*=5 naïve, *n*=4 'no pups' and *n*=4 aggression) (y). The percentage of Foxp2+ cells

848   expressing c-fos during maternal aggression is also higher during maternal aggression (*n*=8) in

849   comparison to both the naïve (*n*=7) and 'no pups' (*n*=6) controls (one way ANOVA, p=<0.0001,

850   F(2,18)=24.16) (y). Data are mean $\pm$ s.e.m. *n* is the number of animals. Arrows show double

851   labeled cells. p<0.05 (*), p<0.01 (**), and p<0.001 (***). The scale bar represents 50µm.

852

853   **Figure 6. Sex-specific subpopulation responses during mating.**

854   A significant increase in the number of c-fos+ cells (white) in the MeA during mating compared

855   to naïve control is observed in both male (two tailed Mann-Whitney test, U=0, p=0.0079, *n*=5

856   naïve, *n*=5 mating) and female brains (two-tailed *t*-test, p=0.0038, t=4.022, df=8, *n*=6 naïve, *n*=4

857   mating) (a-c). Double immunofluorescence for c-fos (white) and YFP (green) or Foxp2 (red)

858 after mating reveals an increase in the number of *Dbx1*-derived neurons expressing c-fos in male

859 (two tailed Mann-Whitney test, U=2, p=0.0152, *n*=6 naïve, *n*=5 mating) and female (two tailed

860 Mann-Whitney test, U=1, p=0.0476, *n*=6 naïve, *n*=3 mating) brains (d-g). A significant increase

861 in the number of Foxp2+ cells expressing c-fos is only observed in male mice during mating

862 (two-tailed *t*-test, p=0.009, t=3.331, df=9, *n*=6 naïve, *n*=5 mating) but not in female mice during

863 mating (two tailed *t*-test, p=0.8993, t=0.1312, df=7, *n*=6 naïve, *n*=3 mating) as compared to

864 naïve controls (h-k). A significant increase in the percentage of *Dbx1*-derived cells expressing c-

865 fos (YFP+ and c-fos+ /total YFP+) is observed in males during mating in comparison to the

866 naïve controls (two-tailed *t*-test, p=0.0067, t=3.507, df=9, *n*=6 naïve, *n*=5 mating) (m). A

867 significant increase in the percentage of Foxp2+ cells expressing c-fos (Foxp2+ and c-fos+ /total

868 Foxp2+) in male brains during mating is also observed in comparison to naïve controls (two-

869 tailed *t*-test, p=0.0001, t=6.477, df=9, *n*=6 naïve, *n*=5 mating) (m). An increase in the percentage

870 of *Dbx1*-derived cells expressing c-fos (two-tailed *t*-test, p=0.0131, t=3.302, df=7, *n*=6 naïve,

871 *n*=3 mating) is observed in female brains during mating (l). In contrast, no increase is observed

872 in the Foxp2+ population (two-tailed *t*-test, p=0.0905, t=1.96, df=7, *n*=6 naïve, *n*=3 mating) in

873 female brains during mating (l). Data are mean ± s.e.m. *n* is the number of animals. p<0.05 (*),

874 p<0.01 (**), and p<0.001 (***), n.s.; not significant. Arrows show double labeled cells. The

875 scale bar represents 50μm.

876

877 **Figure 7. *Dbx1*-derived and Foxp2+ MeA subpopulation activation patterns in response to**

878 **predator odor.**

879 A significant increase in the number of c-fos+ cells (white) in the MeA in both male (two-tailed

880 *t*-test, p=0.0344, t=0.3.153, df=4, *n*=3 benign, *n*=3 predator avoidance) and female (two-tailed *t*-

881 test, p=0.001, t=4.778, df=9, *n*=6 benign, *n*=5 predator avoidance) brains is observed in the

882 presence of rat bedding compared to benign unsoiled bedding (a-c). Double immunofluorescence

883 for c-fos (white) and YFP (green) or Foxp2 (red) after predator odor exposure reveals no

884 increase in the number of *Dbx1*-derived cells (two-tailed *t*-test, p=0.687, t=0.35, df=4, *n*=3

885 benign, *n*=3 predator avoidance) (d-g) or Foxp2+ cells (two-tailed *t*-test, p=0.703, t=0.70, df=4,

886 *n*=3 benign, *n*=3 predator avoidance) (h-k) expressing c-fos in male brains as compared to

887 control. In the female brain, there is also no increase in the number of *Dbx1*-derived cells during

888 predator odor exposure as compared to control (two tailed Mann-Whitney, U=5, p=0.1349, *n*=5

889 benign, *n*=5 predator avoidance) (d-g). In contrast, an increase the number of Foxp2+ cells (two-

890 tailed t-test, p=0.036, t=2.47. df=9, *n*=6 benign, *n*=5 predator avoidance) in female brains is

891 observed (h-k). Analysis of the percentage of *Dbx1*-derived (YFP+ and c-fos+ /total YFP+) and

892 Foxp2+ cells (Foxp2+ and c-fos+/ total Foxp2+) expressing c-fos in both male and female brains

893 revealed no increases in either population (*Dbx1*-derived population in male mice: two-tailed *t*-

894 test, p=0.2318, t=1.408, df=4, naïve *n*=3, predator avoidance *n*=3; Foxp2+ population in male

895 mice: two-tailed *t*-test, p=0.1023, t=2.11, df=4, naïve *n*=3, predator avoidance *n*=3; *Dbx1*-

896 derived population in female mice: two-tailed Mann-Whitney, U=5, p=0.1349, naïve *n*=5,

897 predator avoidance *n*=5; Foxp2+ population in female mice: two-tailed *t*-test, p=0.2299, t=1.288,

898 df=9, naïve *n*=6, predator avoidance *n*=5). Data are mean ± s.e.m. *n* is the number of animals.

899 Arrows show double labeled cells. p<0.05 (*), p<0.01 (**), and p<0.001 (***). The scale bar

900 represents 50µm.

901

902 **Figure 8. Summary of findings.**

903   Schematic summarizing our findings in which embryonic segregation of *Dbx1*-derived (green)

904   and Foxp2+ (red) cells in the embryonic brain predicts postnatal intrinsic electrophysiological

905   profiles and differential sex and subpopulation-specific neuronal activation patterns (neurons

906   indicate activation and X's indicate no activation) in response to aggression (territorial and

907   maternal), mating and predator avoidance.   The OTP+ population is shown responding to

908   predator odor in female brains.   The male predator odor responsive population remains to be

909   determined.

910

911   **Supplemental Figures**

912

913   **Figure 1- Figure supplement 1. Embryonic and postnatal distribution of OTP+ cells.**

914   Dual immunostaining of coronal sections at the telencephalic-diencephalic border at E11.5

915   shows very little overlap between *Dbx1*-derived (green) (*n*=3) and the Foxp2+ (red) (*n*=3)

916   progenitor populations with the OTP+ population (white) (a-f).   Quantification of overlap of

917   OTP with the *Dbx1*-derived and Foxp2+ populations (YFP+ and OTP+/ total YFP+ population

918   for *Dbx1*-derived cells; Foxp2+ OTP+/ total Foxp2+ population for Foxp2+ cells) (g). Venn

919   diagrams depicting the overlap of the total number of *Dbx1*-derived (h) and Foxp2+ cells (i) with

920   the OTP+ population (2-3 sections/embryo). Dual immunostaining of postnatal coronal sections

921   also revealed very little overlap of *Dbx1*-derived population (*n*=3) or Foxp2+ populations (*n*=4)

922   with OTP   (j-u). Quantification of overlap of OTP with the *Dbx1*-derived and Foxp2+

923   populations (YFP+ and OTP+/ total YFP+ population for *Dbx1*-derived cells; Foxp2+ OTP+/

924   total Foxp2+ population for Foxp2+ cells) (v). Venn diagram depicting the overlap of the total

925   number of *Dbx1*-derived (w) and Foxp2+ cells (x) with the marker OTP+ (average of MeA A-P

926   extent across 5-8 sections). p<0.05 (*). The scale bar represents 100 μm (a-f, j-l, p-r) and 100 μm

927   (m-o, s-u). Data are mean $\pm$ s.e.m. *n* is the number of animals.

928

929   **Figure 1- Figure supplement 2. Foxp2+ cells are not derived from the *Shh*- or *Nkx6.2***

930   **lineages.**

931   Dual immunostaining of the amygdala primordium in horizontal sections at E11.5 reveals minor

932   contribution of either the *Nkx6.2*-lineage (*n*=3) (white) (a-d, i) or the *Shh*-lineage (*n*=3) (white)

933   (e-h, i) to the Foxp2+ population (red). Data are mean $\pm$ s.e.m. *n* is the number of animals.

934   p<0.05 (*). The scale bar represents 200μm (a, e) and 25 μm (b-d, f-h).

935

936   **Figure 1- Figure supplement 3. Localization of *Dbx1*-derived and Foxp2+ cells in the adult**

937   **MeA.**

938   Double immunofluorescence staining of adult coronal sections at the level of the MeA shows

939   patterns of localization of *Dbx1*-derived (green) and Foxp2+ (red) cells across the anterior to

940   posterior extent of the MeA (a-i). Bregma levels correspond to the K. Franklin and G. Paxinos

941   Mouse Brain in Stereotaxic Coordinates Atlas. The scale bar represents 200μm.

942

943   **Figure 2- Figure supplement 1. Identity of OTP+ medial amygdala neurons.**

944   Dual immunofluorescence of Foxp2 with YFP (in *Emx1^{cre}*;*RYFP* mice) (*n*=3) (a-d), CAMKIIα

945   (*n*=3) (e-h), calbindin (*n*=3) (i-l) and somatostatin (*n*=3 mice) (m-p) (arrows show double

946   labeled cells). Quantification reveals that most Foxp2+ are not Emx1-derived, nor express

947   somatostatin, express to some extent CAMKIIα, but are mainly Calbindin+ (q) Data are mean $\pm$

948   s.e.m. *n* is the number of animals. The scale bar represents 100μm (a, e, i, m) and 25μm (b-d, f-h,

949   j-l, n-p),.

950

951   **Figure 3- Figure supplement 1. Foxp2+ neurons possess projection neuronal morphology.**

952   Biocytin filling depicting the morphology of a typical Foxp2+ neuron possessing dendritic spines

953   (a-b, arrow). Representative 3-D reconstruction of several Foxp2+ neurons (c-e). The scale bars

954   represent 100μm (a) and 10μm (b).

955

956   **Figure 4- Figure supplement 1. Patterns of MeA expression of sex steroid hormone**

957   **markers.**

958   Schematic of adult brain shows MeA (red box) (a). Immunostaining for ERα (b), aromatase (c)

959   or AR (d) shows patterns of localization in the MeA. The scale bar represents 100μm.

960

961   **Figure 4- Figure supplement 2. Percent contribution of *Dbx1*-derived and Foxp2+ cells to**

962   **sex steroid hormone marker populations.**

963   The percent contribution of *Dbx1*-derived cells and Foxp2+ cells in male and female brains to

964   the ERα, Aromatase+ and Androgen Receptor+ populations are as follows: _ERα+ population:_

965   _Dbx1_-derived contribution: 18%+4.2 (male *n*=3, x=18.37), 21%+3.59 (female *n*=4, x=20.75).

966   Foxp2+ contribution: 11%+2.72 (male *n*=3, x=10.89); 21%+3.0 (female *n*=4, x=19.48). No

967   subpopulation or sex differences were observed (two-way ANOVA with no interaction between

968   subpopulation and sex and no main effects ($F_{(1,10)}$=0.8261 p=0.3848) (a). _Aromatase+_

969   _population:_ *Dbx1*-derived contribution: 37%+9.89 (male *n*=3, x=37), 34%+6.05 (female *n*=3,

970   x=33.6). Foxp2+ contribution: 0%+0.3 (male *n*=0.3), 10%+5.49 (female *n*=3, x=9.667) (b). This

971   difference in expression was significant across subpopulations (two-way ANOVA with no

972   interaction between subpopulation and sex, with a main effect for subpopulation p=0.0015,

973    $F_{(1,8)}=22.33$). _Androgen Receptor+ population:_ _Dbx1_-derived contribution: 36.7%+16.71 (male

974   _n_=3, x=31.81), 3%+2.0 (female _n_=3, x=2.11), Foxp2+ contribution: 27%+4.05 (male _n_=3,

975   x=26.45), 23%+0.85 (female _n_=3, x=22.69) (c). A two-way ANOVA revealed no interaction

976   between subpopulation and sex; however the value was trending (p=0.0660), with a main effect

977   for sex within the _Dbx1_-derived subpopulation (p=0.0107, $F_{(1,8)}=10.95$). As the interaction was

978   trending, we also analyzed subpopulation differences and found significant increases in the

979   percent of AR+ expressing Foxp2+ cells in comparison to the _Dbx1_-derived subpopulation in

980   females (p=0.0308, $F_{(1,8)}=6.85$) (c). _n_ is the number of animals and x is the mean. p<0.05 (*),

981   p<0.01 (**), and p<0.001 (***).

982

983   **Figure 5- Figure supplement 1. c-fos expression in the posterio-medial (MeApd) and**

984   **posterio-ventral (MeApv) subnuclei during different innate behavioral assays.**

985   Immunofluorescence staining for c-fos after innate behavioral assays: territorial (c-d) and

986   maternal (g-h) aggression, mating (g-h), predator avoidance (k-l) and their respective native (a-b)

987   and benign (i-j) controls.

988

989   **Figure 7- Figure supplement 1. Sex-specific activation of OTP+ cells during predator**

990   **avoidance.**

991   A significant increase in number of c-fos+ cells (white) in the MeA in males (two-tailed _t_-test,

992   p=0.0004, t=5.435, df=9, _n_=6 benign, _n_=5 predator avoidance) (a-c) and in females (two-tailed _t_-

993   test, p=0.0010, t=5.08, df=8, _n_=5 benign, _n_=5 predator avoidance) (a-b, c) in the presence of rat

994   bedding compared to benign unsoiled bedding. Double immunofluorescence for c-fos (white)

995   and OTP (pink) after predator odor exposure reveals an increase in number of cells expressing c-

996   fos in the male OTP+ subpopulation (two-tailed t-test, p=0.023, t=2.727, df=9, n=6 benign, n=5

997   predator avoidance) (e-h) and in the female OTP+ subpopulation (two-tailed t-test, p<0.0001,

998   t=11.09, df=8, n=5 benign, n=5 predator avoidance). There is no difference in the percentage of

999   OTP+ cells expressing c-fos (OTP+c-fos+/total OTP) between benign (n=6) and predator

1000   avoidance (n=6) conditions in males (two tailed Mann-Whitney test, U=9, p=0.3290) (j). In

1001   contrast, there is a significant increase in the percent of OTP+ cells expressing c-fos between

1002   benign (n=5) and predator avoidance (n=5) conditions in female brains (two tailed Mann-

1003   Whitney test, U=0, p=0.0079, n=5 benign, n=5 predator avoidance condition). p<0.05 (*),

1004   p<0.01 (**), and p<0.001 (***). Arrows show double labeled cells. The scale bar represents

1005   50μm.
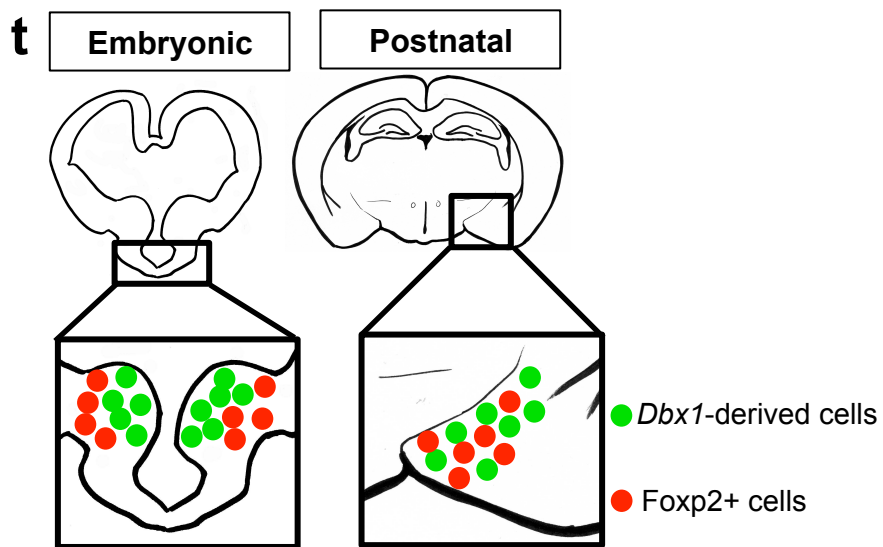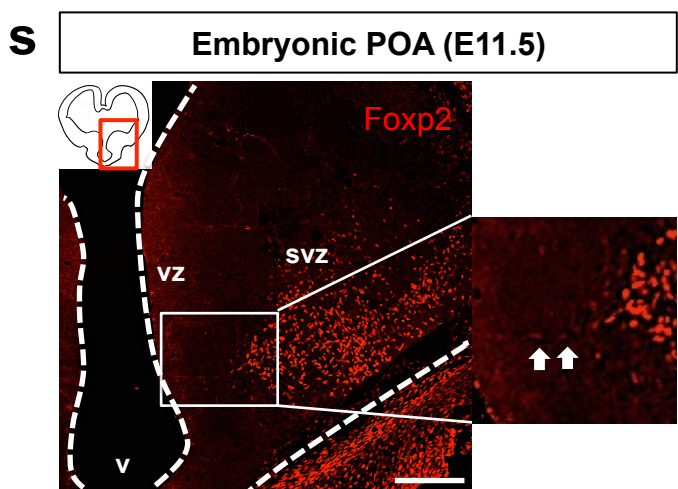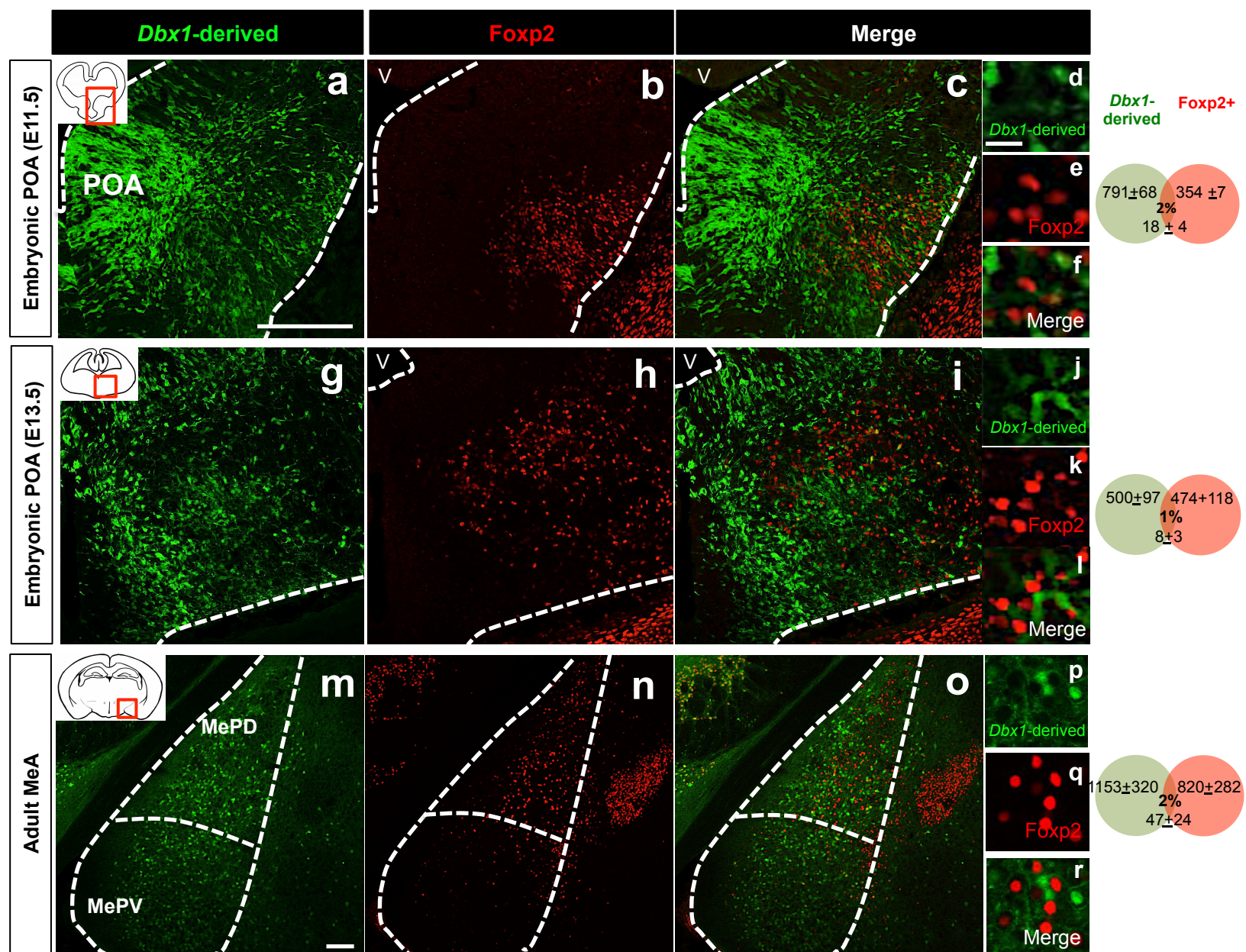
1006

Figure 1

Figure 1- Figure supplement 1

Embryonic POA (E11.5)

Nkx6.2Cre; Rosa26YFP

a — POA, Amygdala primordium, *Nkx6.2*-derived, Foxp2

b — *Nkx6.2*-derived

c — Foxp2

d — Merge

ShhCre; Rosa26YFP

e — POA, Amygdala primordium, *Shh*-derived, Foxp2

f — *Shh*-derived

g — Foxp2

h — Merge

i

% co-localization

$\dfrac{Nkx6.2\text{-derived+Foxp2+}}{Nkx6.2\text{-derived+}}$ $\dfrac{Shh\text{-derived+Foxp2+}}{Shh\text{-derived+}}$
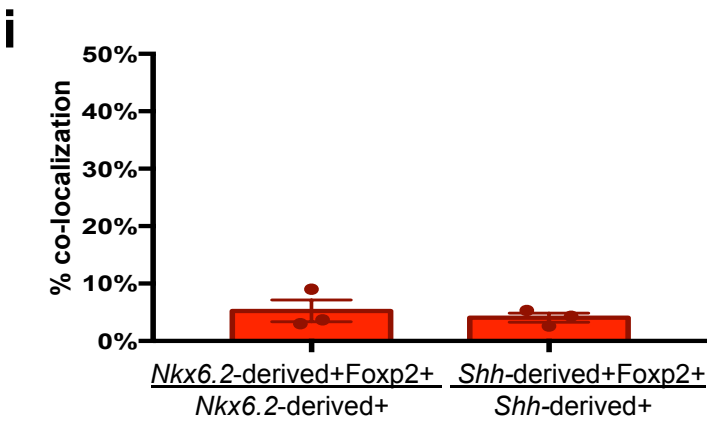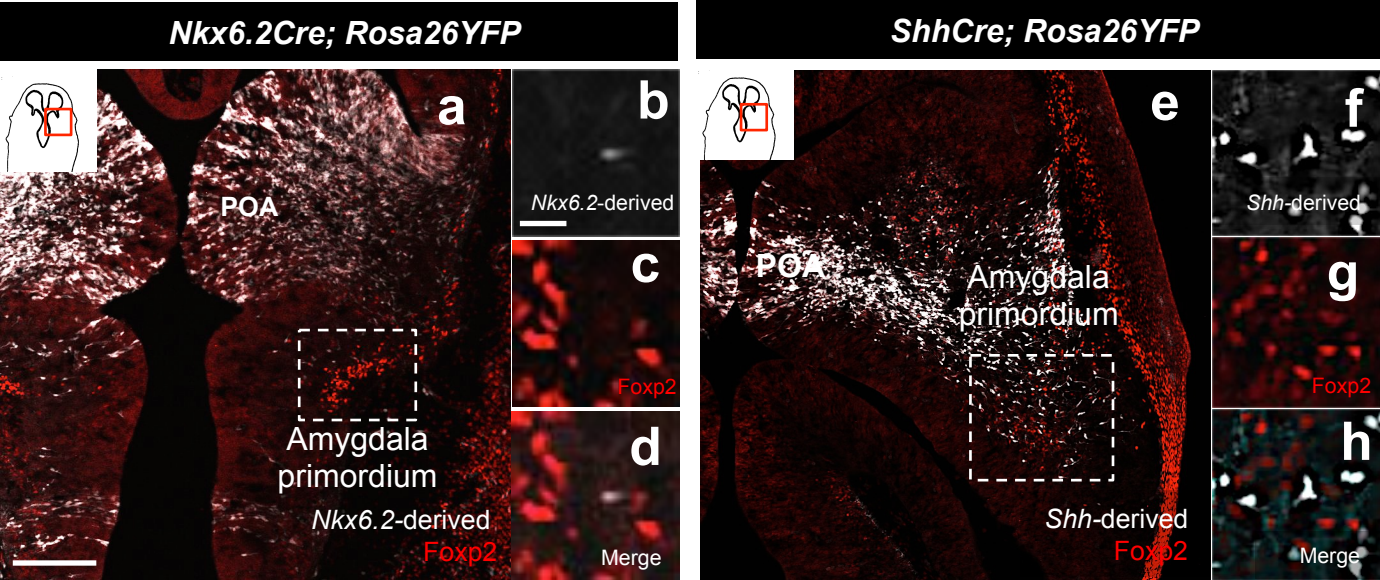
Figure 1- Figure supplement 2

Dbx1Cre; Rosa26YFP

a — *Dbx1*-derived/Foxp2 — Bregma-1.30mm
b — Bregma-1.40 mm
c — Bregma-1.50 mm
d — Bregma-1.60 mm
e — Bregma-1.70 mm
f — Bregma-1.80 mm
g — Bregma-1.90 mm
h — Bregma-2.00 mm
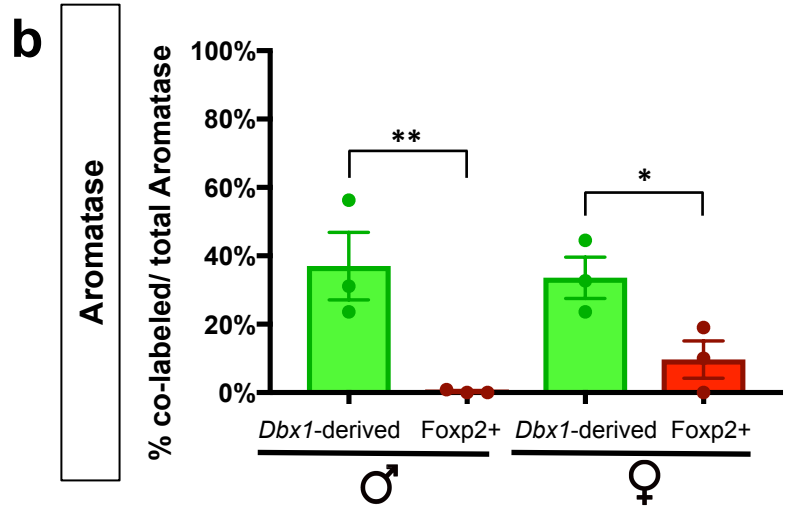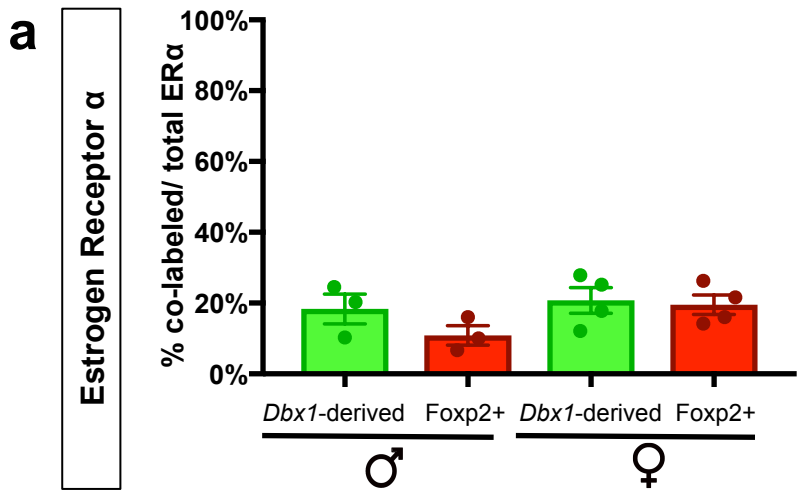i — Bregma-2.10 mm

SI, opt, MePD, MePV, AC
EP, st, opt, MePD, MePV
cp, opt, sox, MePD, MePV

Figure 1- Figure supplement 3

NeuN/Foxp2

a b NeuN c Foxp2 d Merge

CC1/Foxp2

e f CC1 g Foxp2 h Merge

***Emx1Cre;Rosa26YFP*/Foxp2**

i j *Emx1*-derived k Foxp2 l Merge

Calbindin/Foxp2

m n Calbindin o Foxp2 p Merge

nNOS/Foxp2

q opt r nNOS s Foxp2 t Merge
MeApd
MeApv

Somatostatin/Foxp2

u v Somatostatin w Foxp2 x Merge

y

% co-localization with Foxp2

100%
80%
60%
40%
20%
0%

NeuN CC1 *Emx1*-derived Calbindin nNOS Somatostatin

Figure 2

Figure 2- Figure supplement 1

Intrinsic electrophysiological properties

Dbx1-derived

a

Foxp2+

b

+60pA
+20pA
-60pA

200ms 20mV

+60pA
+30pA
-50pA

200ms 20mV

c *** Rm(MΩ)

d * Vm(mV)

e ** Capacitance(pF)

Dbx1-derived   Foxp2+

f * AP Frequency(Hz)

g n.s. Rheobase(pA)

Dbx1-derived   Foxp2+

Extrinsic electrophysiological properties

Dbx1-derived

h

Foxp2+

i

20pA
10sec

20pA
10sec

10pA
5ms

10pA
5ms

j ** sEPSC Frequency(Hz)

k ** sEPSC Amplitude(pA)
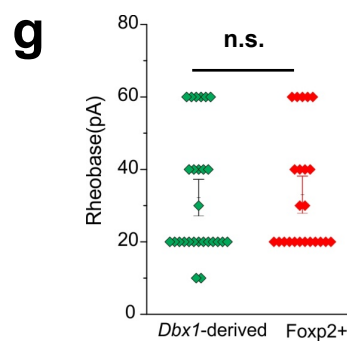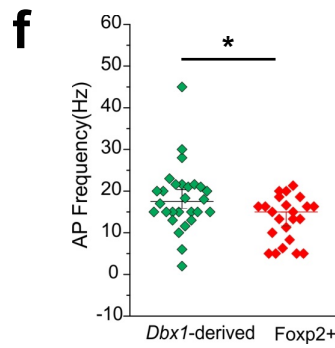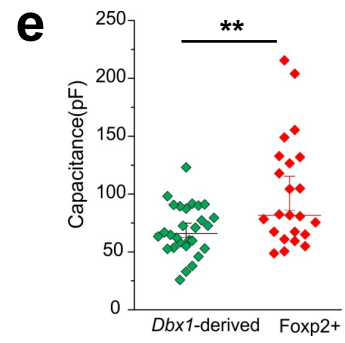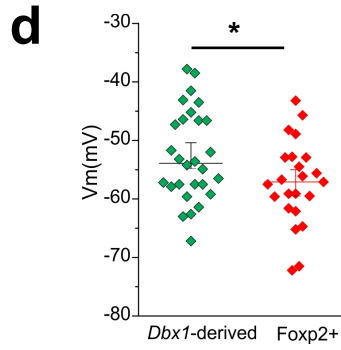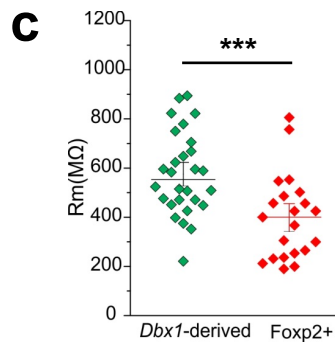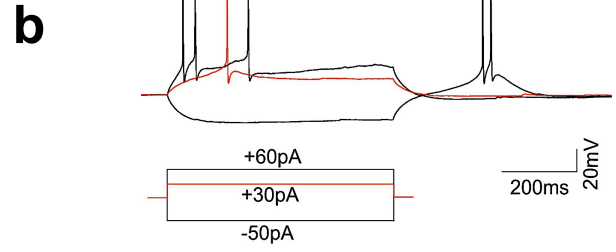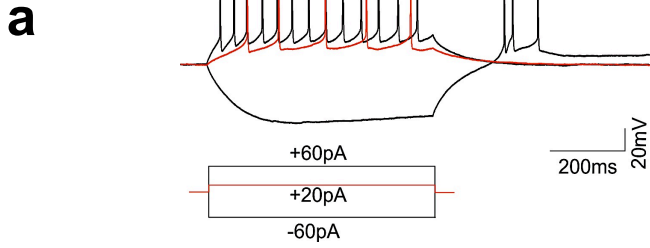
l ** sEPSC Decay Time(ms)
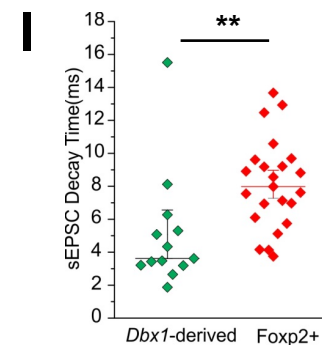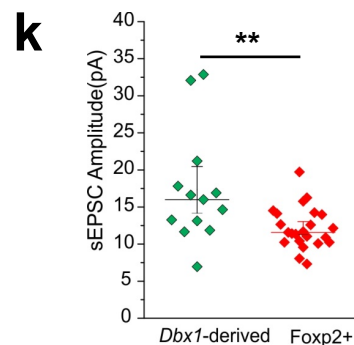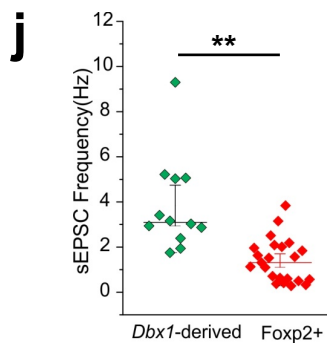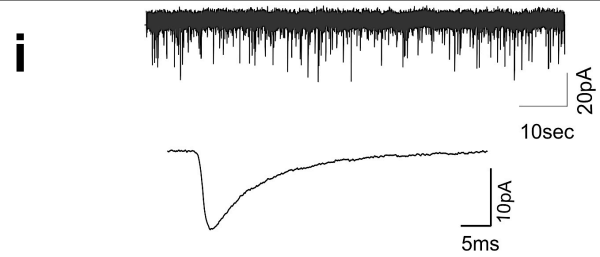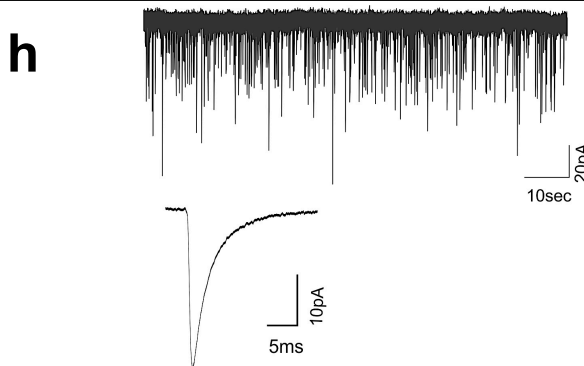
Dbx1-derived   Foxp2+

Figure 3

Foxp2+ Neuronal Morphologies
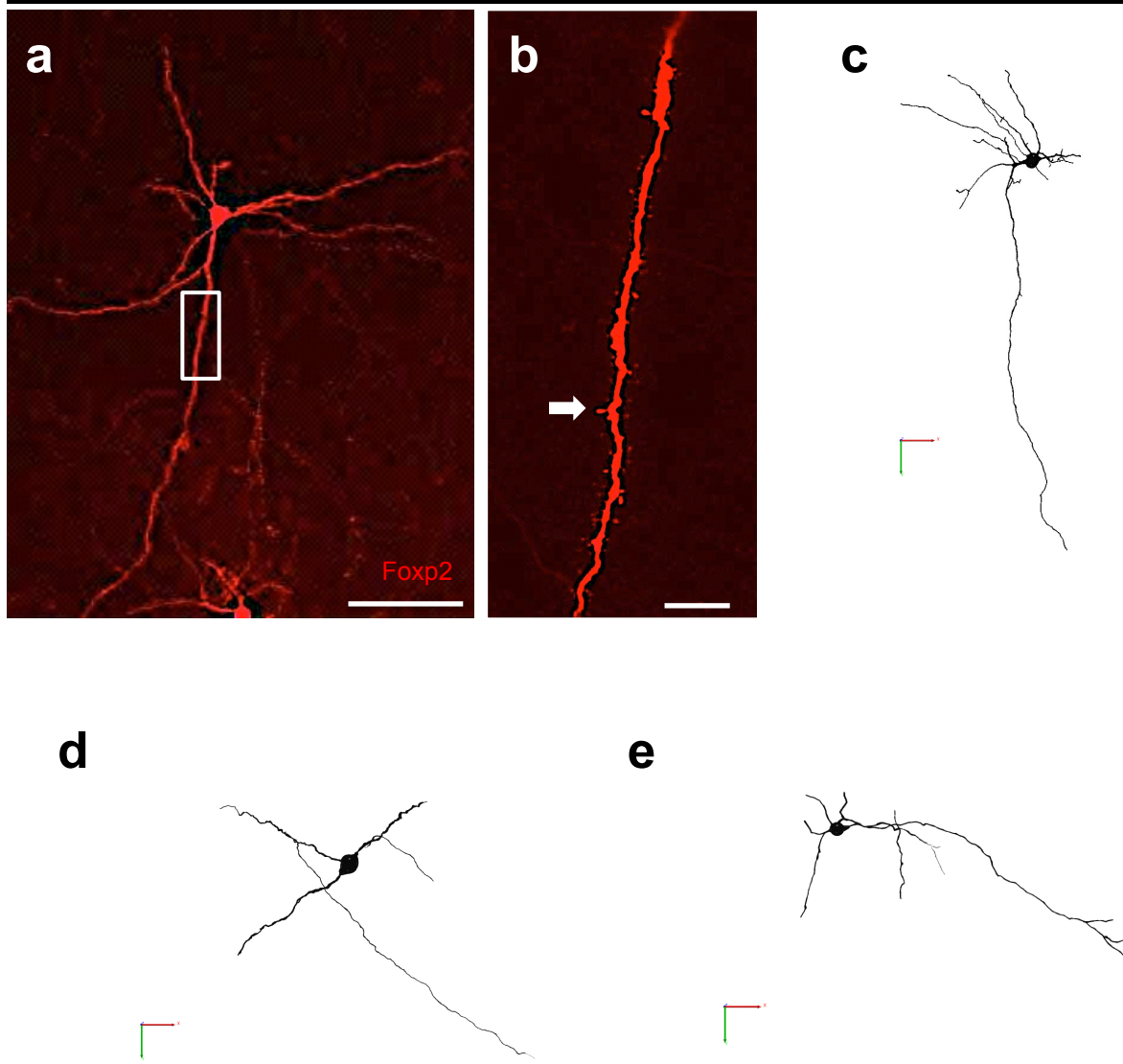
a

b Foxp2

c

d

e

Figure 3- Figure supplement 1

Figure 4

a

ERα

b

Aromatase

c

AR

d

Figure 4- Figure supplement 1

a Estrogen Receptor α

b Aromatase

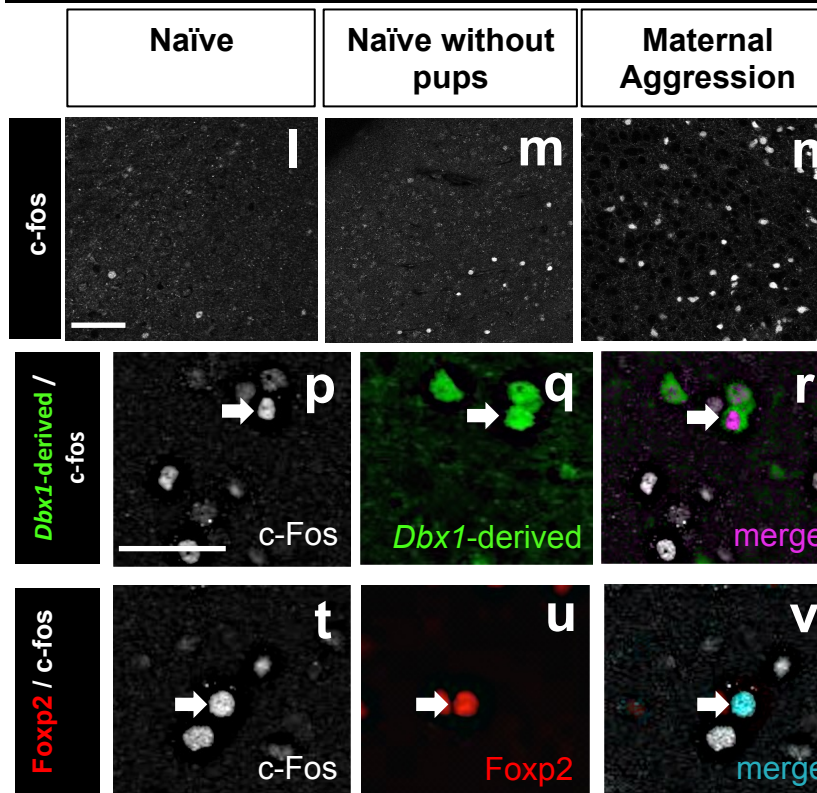**c** Androgen Receptor
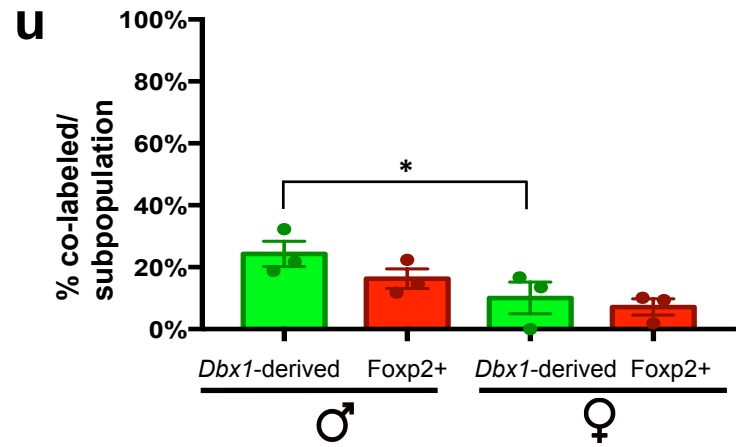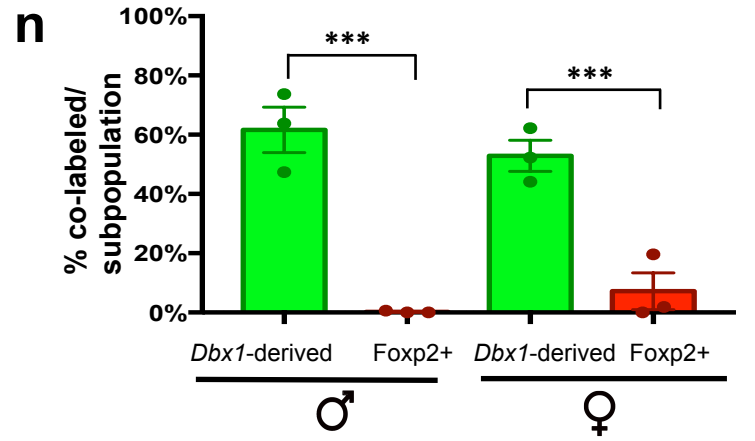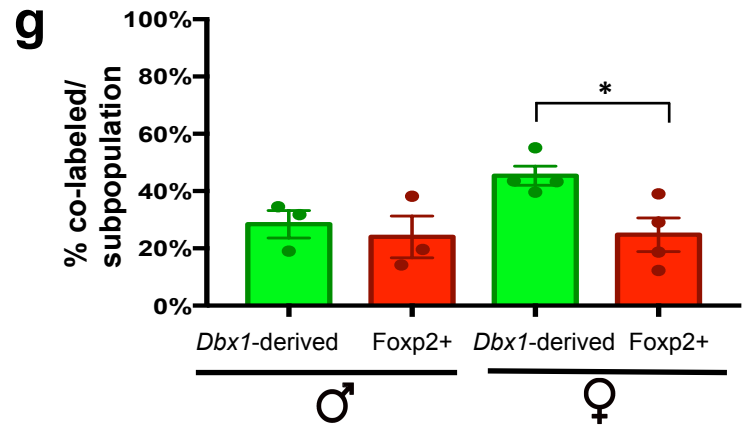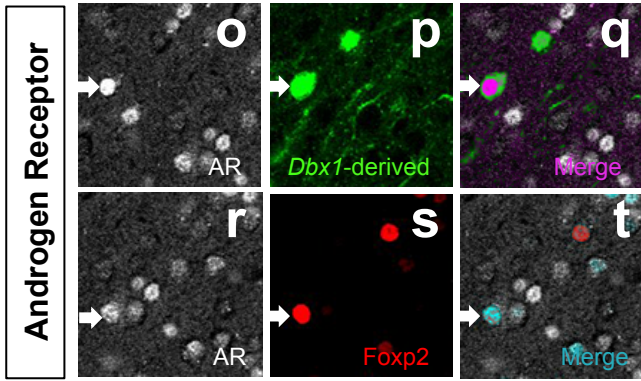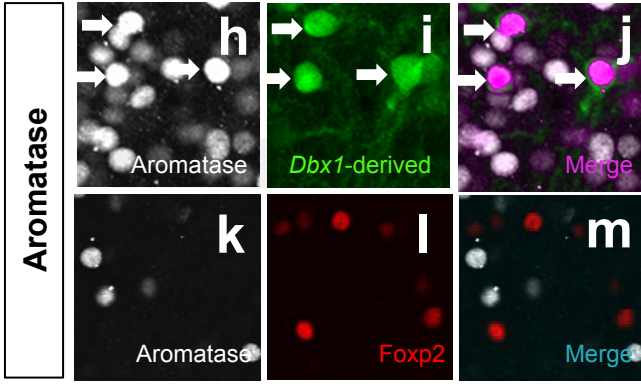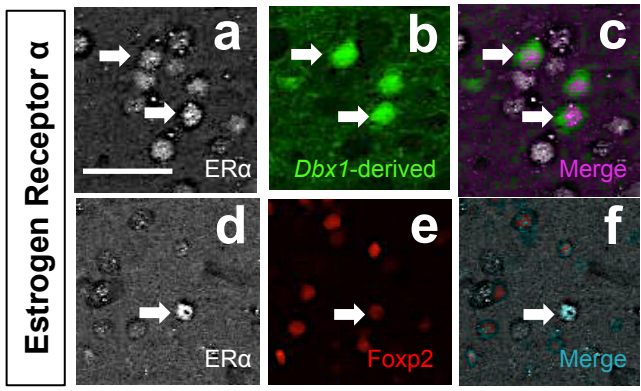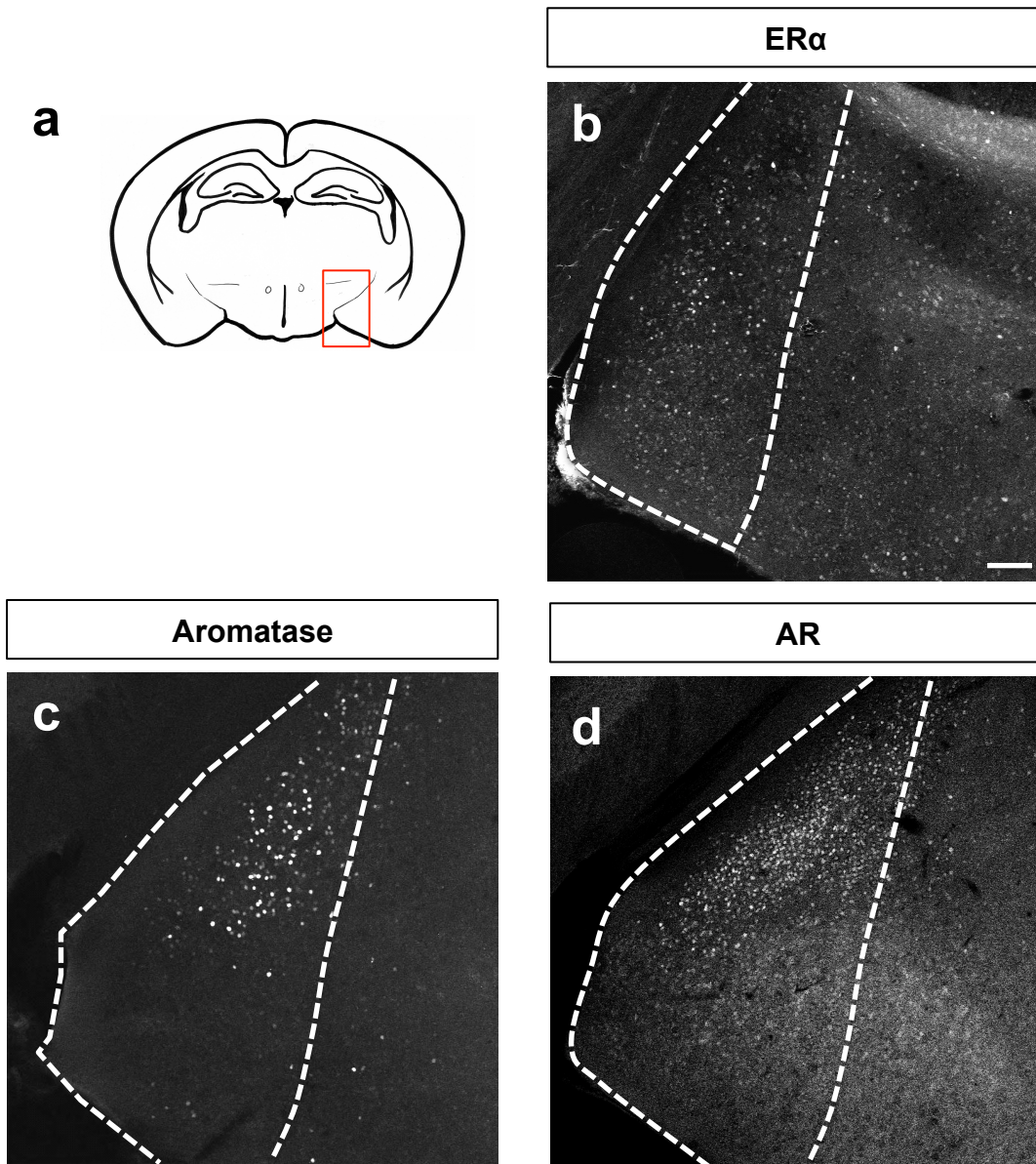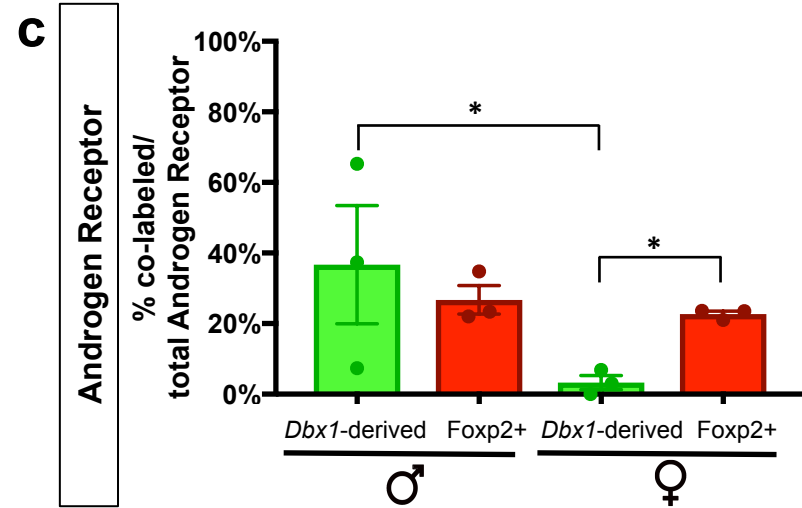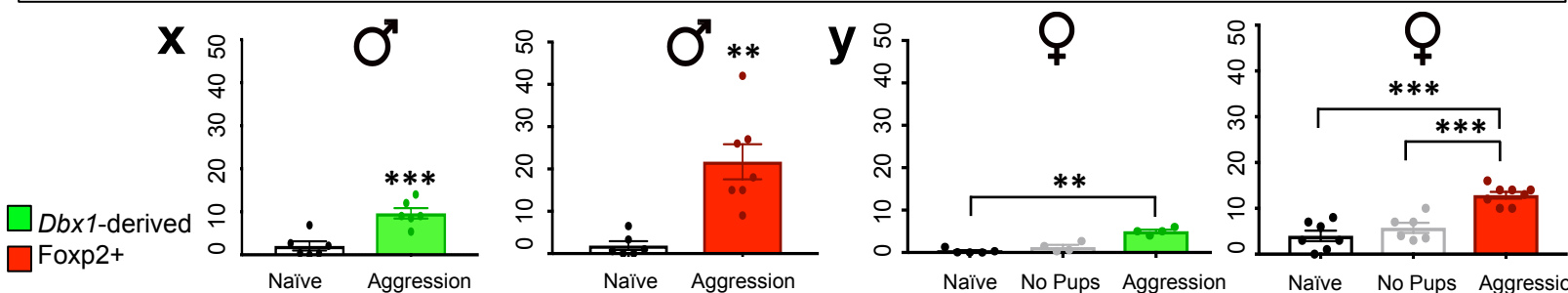
Figure 4- Figure supplement 2

Figure 5

Territorial Aggression
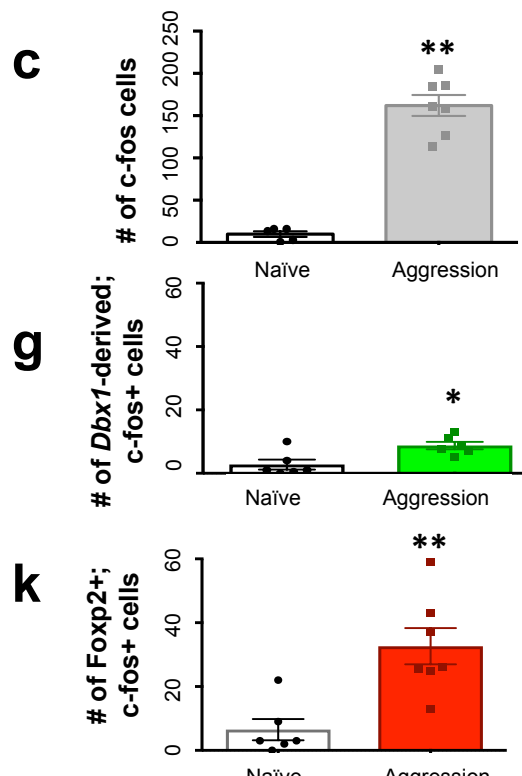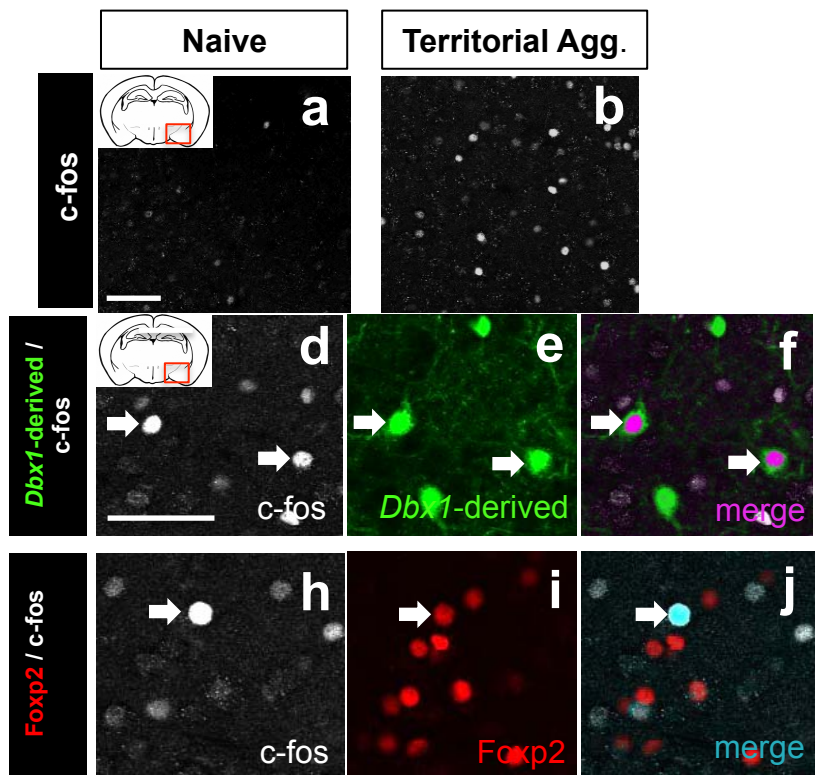
Maternal Aggression
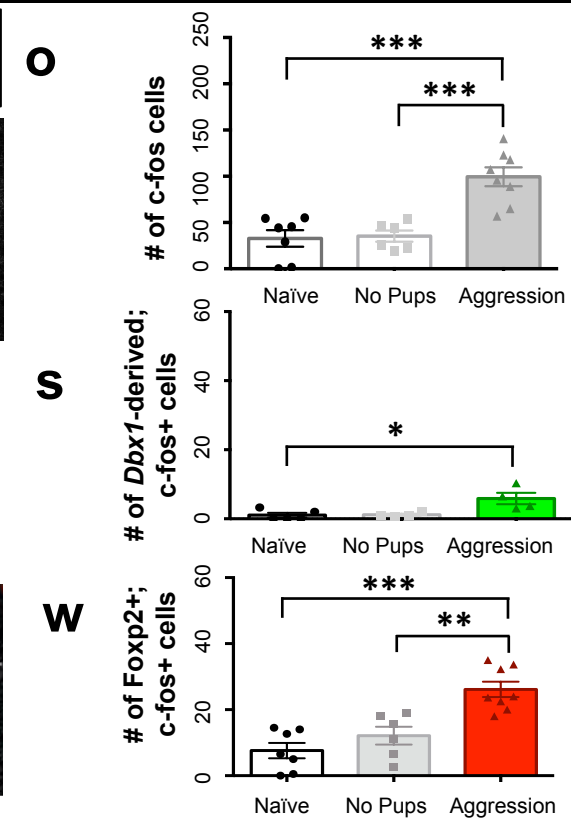
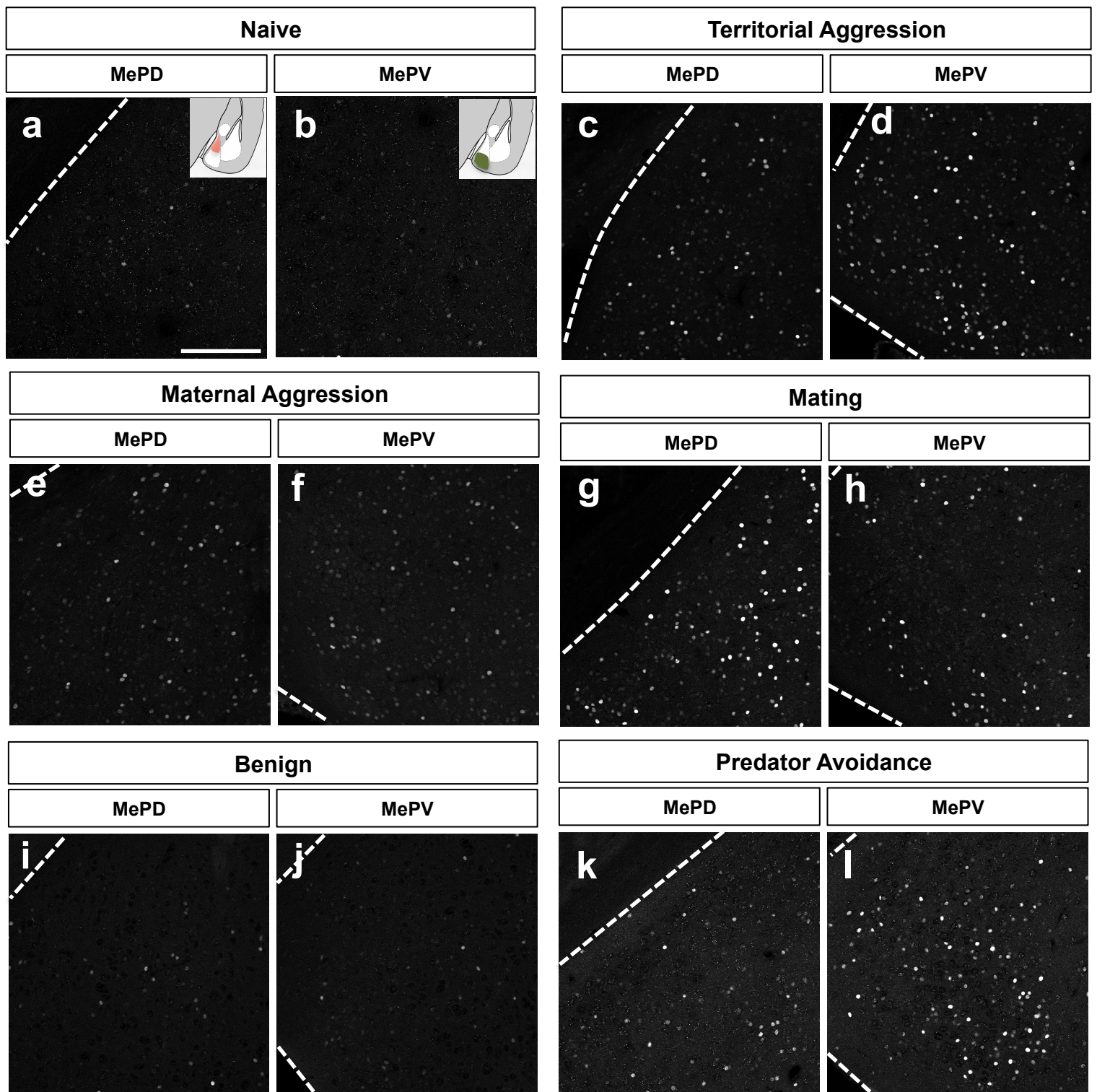**% of *Dbx1*-derived or Foxp2 cells co-labeled with c-fos/total *Dbx1*-derived or Foxp2 cells**
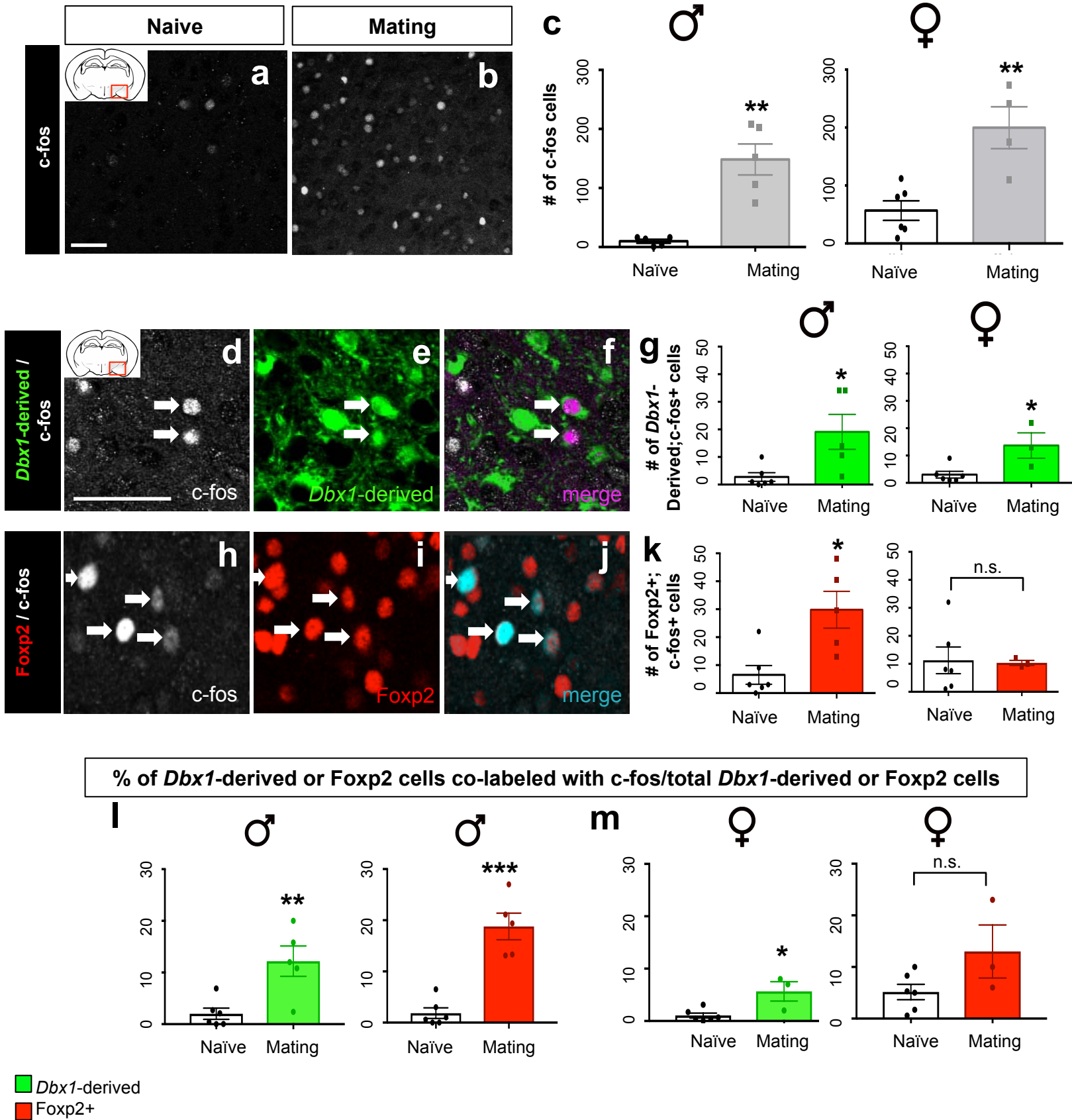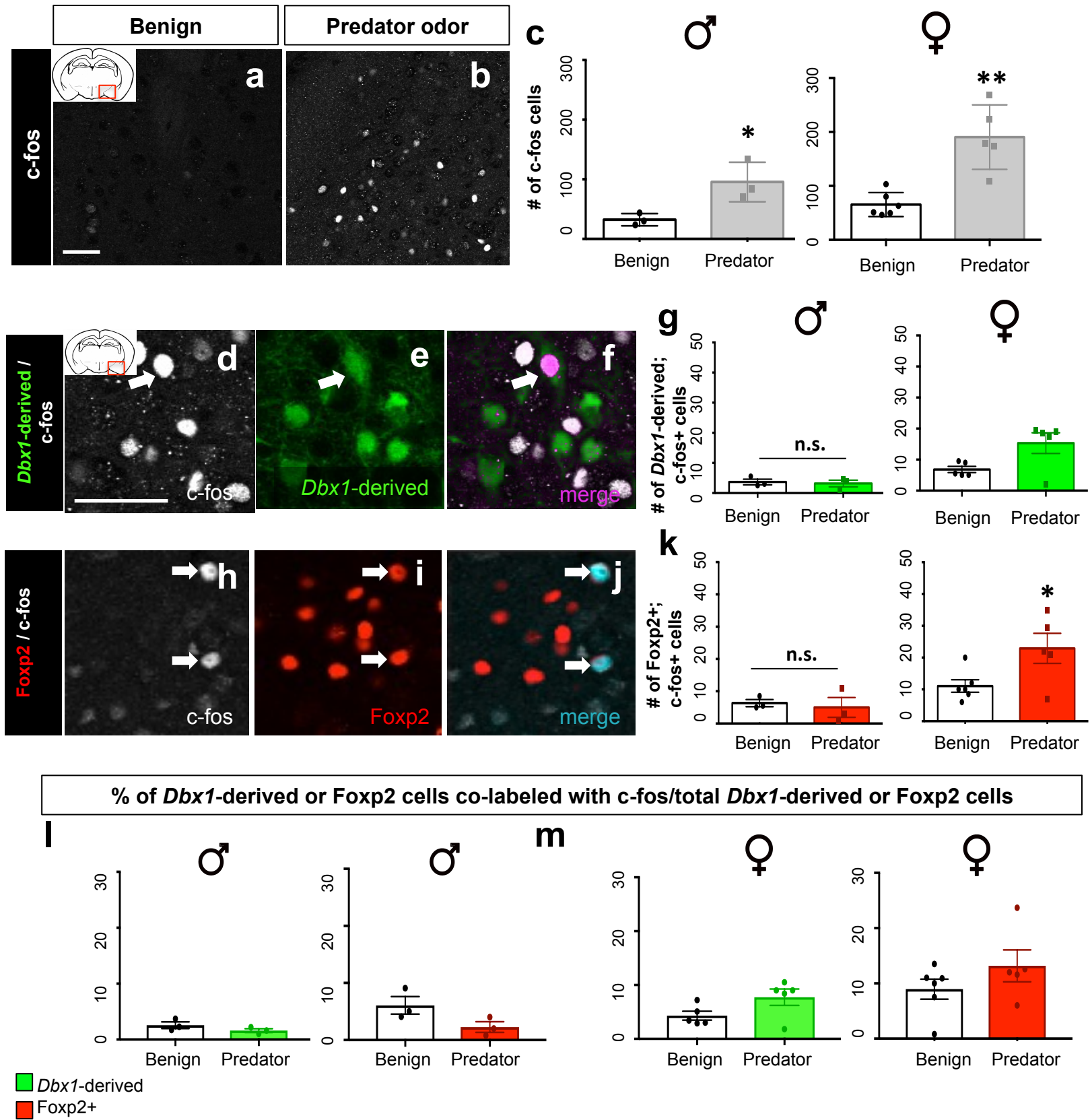
Naive		Territorial Aggression	
MePD	MePV	MePD	MePV
a	b	c	d

Maternal Aggression		Mating	
MePD	MePV	MePD	MePV
e	f	g	h

Benign		Predator Avoidance	
MePD	MePV	MePD	MePV
i	j	k	l

Figure 5- Figure supplement 1

Figure 6

Figure 7

Figure 7- Figure Supplement 1