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The Caudo-Ventral Pallium is a novel pallial domain expressing *Gdf10* and generating Ebf3-positive neurons of the medial amygdala

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ABSTRACT

In rodents, the medial nucleus of the amygdala receives direct inputs from the accessory olfactory bulbs and is mainly implicated in pheromone-mediated reproductive and defensive behaviors. The principal neurons of the medial amygdala are GABAergic neurons generated principally in the caudo-ventral Medial Ganglionic Eminence (cvMGE) and preoptic area (POA). Beside GABAergic neurons, the medial amygdala also contains glutamatergic Otp-expressing neurons generated in the lateral hypothalamic neuroepithelium and a non-well characterized Pax6-positive neuronal population. In the present work, we describe a novel glutamatergic Ebf3-expressing neuronal population distributed within the periphery of the postero-ventral medial amygdala (MePV). These neurons are generated in a pallial domain characterized by high expression of Gdf10. This territory is topologically the most caudal tier of the ventral pallium and accordingly, we named it Caudo-Ventral Pallium (CVP). In the absence of Pax6, the CVP is disrupted and Ebf3+ neurons fail to be generated. Overall, this work proposes a new model of the neural composition of the medial amygdala and unravels for the first time a new pallial population originating from the CVP and expressing the transcription factor Ebf3.

Key words: telencephalon, ventral pallium, medial amygdala, Gdf10, Ebf3, Pax6, caudo-ventral pallium

INTRODUCTION

In mammals, pheromones play a vital role in aggressive and sexual behaviors essential to respond to potential external dangers or to find a mate. One of the structures highly involved in these processes is the medial amygdala (MeA) (Sokolowski and Corbin 2012). The accessory olfactory bulbs receive pheromone information from the vomeronasal organ, and send it directly to different subdivisions of the MeA. While the dorsal portion of the postero-medial amygdala (MePD) is mainly implicated in processing reproductive information, the antero-medial amygdala (MeAa) and the ventral part of the posteroventral-medial amygdala (MePV) are involved in both, defensive and reproductive behaviors (Halpern and Martínez-Marcos 2003). GABAergic neurons in the MePV project to nuclei in the hypothalamus implicated either in defensive or reproductive behaviors (Choi et al. 2005). In addition, the MePV also contains local GABAergic interneurons and glutamatergic projecting neurons (Choi et al. 2005; Bian et al. 2008; Keshavarzi et al. 2014).

It is well accepted that the MeA forms part of the subpallial amygdala (Swanson and Petrovich 1998; Medina et al. 2004), since it is mainly composed of GABAergic neurons generated in the ganglionic eminences (GEs) and preoptic area (POA). Indeed, fate mapping studies using Dbx1, Shh and Nkx2.1 reporter mice have revealed that the majority of GABAergic neurons expressing Shh and nNOS in the MePV are generated from progenitors located in the POA (Hirata et al. 2009; Carney et al. 2010; Lischinsky et al. 2017), whereas the dorsal MeA (MePD) contains Somatostatin+ (SST) and Lhx6+ neurons generated in the Diagonal area (Dg, also called caudo-ventral MGE) (Garcia-Lopez et al. 2008; Bupesh et al. 2011a; Puelles et al. 2016b). The MePV subdomain also expresses the Paired box homeotic gene Pax6, but the origin of these neurons is not well defined. While some authors consider this neuronal population as glutamatergic generated in the thalamic eminence (TE) (Bupesh et al. 2011b; Abellán et al. 2013), another study describes this population as GABAergic produced in the caudal ganglionic eminence (CGE) (Tang et al. 2012). Interestingly, the MeA contains also neurons with extra-telencephalic origins. The lateral hypothalamus or paraventricular hypothalamic domain (PVH) gives origin to glutamatergic neurons

expressing Lhx5 and Otp that migrate to the extended amygdala, mainly to the bed nucleus of the stria terminalis and to the anterior MeA (García-Moreno et al. 2010). The only pallial neurons described in the MeA are few Lhx9+ cells localized in the most external part of the MePV and originating most probably from the ventral pallium (VP) (Garcia-Lopez et al. 2008; Bupesh et al. 2011b). The VP (also called antihem) is the most ventrolateral subdivision of the dorsal telencephalon generating different populations of glutamatergic neurons destined to the cortex and pallial amygdala (Puelles et al. 2000, 2016a; Medina et al. 2004; Bielle et al. 2005; Teissier et al. 2010, 2012). It expresses a series of morphogens and forms together with the dorsal lateral ganglionic eminence (dLGE), the pallial-subpallial boundary (PSB).

The characterization of different transgenic mice in which several transcription factors (TFs) have been inactivated has helped us to unravel the major molecular players controlling the formation of the different amygdaloid nuclei. For instance, Pax6 plays a crucial role in the generation and migration of the populations arising from the VP and dLGE, respectively (Stoykova et al. 1996; Tole 2005). In *Pax6 null* mice (*Pax6*$^{Sey/Sey}$), the VP is mis-specified acquiring subpallial features and as a consequence, the pallial amygdala is partially disrupted. In addition, Pax6+ cells normally generated in the dLGE are absent or severely reduced probably because of a migratory arrest. However, the POA and the MGE are less affected and thus, the medial amygdala, part of the subpallial amygdala, shows no obvious defects in terms of morphology (Tole 2005). In this work, we characterize a novel neural population in the MeA that, to our knowledge, has not been described so far. We found that in the amygdala, glutamatergic Ebf3+ neurons are distributed around the MePV forming a shell-like structure. These neurons are generated in a subdomain of the VP characterized by the expression of the Growth differentiation factor 10 (Gdf10), in addition to other well-known VP markers, such as Sfrp2 and Fgf15. Compared to the rest of the VP however, this Gdf10+ territory is negative for Dbx1 and positive for other markers, such as Ptch1, COUP-TFII and Mgda1. According to the gradient expression of different genes in common with the VP, and its anatomical continuation with the lateral subdivision of the thalamic eminence (LTE), we consider this novel domain as the most caudal part of the VP, and have therefore denominated it as Caudo-Ventral Pallium (CVP).

Finally, we found that Ebf3+ neurons are strongly reduced in *Pax6* mutants, while the MeA neuronal populations arising from different subpallial subdomains are not affected, which supports the origin of a Ebf3+ population originating from Pax6-expressing progenitors.

RESULTS

Identification of a pallial domain expressing Gdf10

The growth differentiation factor Gdf10, a BMP protein (BMP-3b) known to be involved in head formation and skeletal morphogenesis (Hino et al. 1999; Zhao et al. 1999; Matsumoto et al. 2012), is expressed in the TE during development (Shimogori et al. 2010; Ruiz-Reig et al. 2017). Here, we describe for the first time that Gdf10 expression is also restricted to the telencephalon. *In situ* hybridizations (ISH) on coronal, horizontal and sagittal sections of E12.5 mouse embryos show intense *Gdf10* expression in cranial bone progenitors (CBP) (**Fig. 1A-I,** red asterisks), as previously described (Zhao et al. 1999), and in the ventricular zone of the lateral and medial TE (LTE and MTE) (**Fig 1C-H**). At mid-rostrocaudal levels, *Gdf10* is expressed very weakly in the ventricular zone (VZ) of the VP (**Fig 1B**). This expression, however, increases in intensity and extension in sections taken at more caudal levels (**Fig 1B–E**). *Gdf10* is also highly expressed in the VZ of a territory that is anatomically continuous with the *Gdf10+* LTE and medial to the caudal ganglionic eminence (CGE) (**Fig 1D,** arrowheads). Notably, *Gdf10* expression next to the LTE results more intense than in its nearby VP domain, and expands laterally in more caudal regions until the two domains join as a continuum at the most caudal part of the VP (**Fig 1E–F,** arrowheads). Horizontal sections show *Gdf10* expression in the VZ facing the lateral ventricle (**Fig 1G,** arrowhead), and in more ventral sections expression is confined to a territory described as "amygdaloid neuroepithelium" (e.g., Schambra, 2008) (**Fig 1H,** arrowhead). On sagittal sections (**Fig. 1I**), *Gdf10* is expressed in the VZ of the ventropallial amygdalopiriform area (VAPv) (as defined by the *Allen Developing Mouse Brain Atlas*).

According to the nomenclature proposed by Flames et al., 2007, the telencephalic *Gdf10+* territory was considered part of the CGE or caudo-ventral MGE (pLGE3 and pMGE3, respectively), and therefore of subpallial origin. However, its caudally curved shape and its anatomical continuation with the VP, suggests more a pallial origin. To distinguish between these two possibilities, we performed ISH and immunostaining for pallial or subpallial markers on coronal sections of the E12.5 mouse telencephalon (**Fig. 2**). First, we found that the telencephalic *Gdf10+* domain seems not to overlap with subpallial markers, such as *Gsx2* and *Ascl1* (also known as *Gsh2* and *Mash1*, respectively) (**Fig. S1**). Second, *Pax6* and *Neurog2* normally expressed in the VZ of the dorsal telencephalon with a latero-ventral^{high} medio-dorsal^{low} gradient (**Fig. 2B, C;** Medina et al., 2004; Stoykova and Gruss, 1994; Toresson et al., 2000), seem instead present in the telencephalic *Gdf10+* territory along rostro-caudal levels (**Fig. 2 A-C, A'-C',** black arrowheads). Likewise, Tbr2, a marker of cortical intermediate progenitors located in the subventricular zone (SVZ) (Englund et al. 2005; Hevner et al. 2006), is also expressed in the putative SVZ of the *Gdf10+* territory (**Fig. 2D-D'**, white arrowheads). Therefore, the telencephalic *Gdf10+* territory seems to belong to a pallial territory characterized by the expression of TFs required in pallial glutamatergic neurogenesis (Englund et al. 2005; Hevner et al. 2006). To further assess the identity of *Gdf10+* cells, ISH for *Gdf10* was combined with immunohistochemistry for pallial markers **(Fig. 2E-G).** High magnification images confirmed partial co-labeling of Pax6 protein with *Gdf10 mRNA* in VZ cells (**Fig. 2E-E'**). In addition, *Gdf10* and Tbr2 co-localize in the transition zone between VZ and SVZ (**Fig 2F-F',** black arrows). Finally, triple immunolabelling for Pax6, Tbr2 and Tbr1 in the *Gdf10+* domain reveals the characteristic neurogenic transition in the expression of these TFs, as previously reported for pallial structures (Englund et al. 2005; Hevner et al. 2006), such as Pax6 and Tbr2 co-expression in the VZ, Tbr2 and Tbr1 in the SVZ, and Tbr1 in the mantle zone (MZ) (**Fig. 2G-G''**). Taken together, these expression data strongly suggest that the telencephalic *Gdf10+* territory is a pallial domain that generates glutamatergic Tbr1-expressing neurons.

Gdf10 selectively labels the Caudo-Ventral Pallium (CVP), the caudalmost tier of the VP

At E12.5, the *Gdf10+* telencephalic domain and the VP share a similar molecular signature, since the VZ of both territories are highly positive for Pax6 and Neurog2 (**Fig. 2B, C**), but negative for *Emx1*, which is restricted to the SVZ (**Fig. S2**) (Fernandez et al. 1998; Gorski et al. 2002; Medina et al. 2004). However, *Gdf10* expression intensity differs between the telencephalic *Gdf10+* territory and the VP. To understand whether the *Gdf10+* territory could represent a subdomain of the VP, we compared on consecutive coronal sections the expression profile of *Gdf10* and three markers, such as *Fgf15*, *Sfrp2* and *Dbx1*, restricted to the VP but not present in other pallial subdivisions (Kim et al. 2001; Assimacopoulos et al. 2003; Gimeno et al. 2003; Bielle et al. 2005; Borello et al. 2008; Hirata et al. 2009; Griveau et al. 2010; Teissier et al. 2010, 2012) (**Fig. 3A-C"**). The fibroblast growth factor 15 (*Fgf15*) strongly co-localizes with the telencephalic *Gdf10+* territory (**Fig. 3A, A',** black arrowheads; **Fig. 3A'',** white arrowhead), whereas it is weakly expressed in the VP (**Fig. 3A'**, green arrowhead). The Wnt antagonist secreted frizzled-related protein 2 (*Sfrp2*) is mainly expressed in the most lateral part of the *Gdf10+* territory, the one next to the CGE (**Fig. 3B, B';** green arrowhead), even if merged images show a slight overlap of the two transcripts (**Fig. 3B";** white arrowhead). Finally, sparse cells positive for the TF *Dbx1* are not located in the telencephalic *Gdf10+* territory (**Fig. 3C, C'**). By analyzing *Fgf15*, *Sfrp2* and *Gdf10* at two different rostro-caudal levels, we noticed that their expression is flanking the CGE at rostral levels (**Fig. 3D-F, H**), whereas sections taken more caudally show a *continuum* between the VP and the telencephalic *Gdf10+* territory **(Fig. 3D'-F', H')**.

On the basis of common gene expression patterns and anatomical continuation at posterior levels, we propose that the telencephalic *Gdf10+* territory should be considered as part of the VP. This implies that the VP bends, surrounds the CGE and continues anatomically and cytoarchitectonically with the diencephalic LTE (in blue, **Fig. 3H, I**). Since the telencephalic *Gdf10+* territory represents topologically the most caudal part of the VP (**Fig. 3I**), we denominate this territory as the Caudo-Ventral Pallium (CVP). Accordingly, expression levels of *Gdf10* and *Fgf15* will be more prominent in the CVP compared to the VP, in line with their rostral^{low} to caudal^{high} expression gradient (**Fig. 3D-E', J**). However, this region is also positive for other genes not expressed in the VP, such as the Shh receptor *Ptch1* in the VZ, the IgCam,

Mdga1 in the SVZ and the TF COUP-TFII in both the VZ and the SVZ (**Fig. S3**). These characteristics, together with the lack of *Dbx1* expression, make the CVP a subdomain of the VP with special features.

CVP-derived neurons transiently expressing Ebf3 populate the MeA

With the aim of fate mapping the CVP, we first searched for molecular markers expressed in the vicinity of the CVP. We observed strong expression of the TF *Ebf3* in an adjacent area close to the CVP (**Fig. 4 A-C,** red arrowheads). In the telencephalon, Ebf3 was previously shown to be expressed in the septum, cortical hem, VP and postmitotic Cajal-Retzius cells (CRC) (Garel et al. 1997; Yamazaki et al. 2004; Chiara et al. 2012), but its localization in the amygdala had never been reported so far. We found *Ebf3* expression next to the CVP ventricular zone and in the adjacent mantle zone, most probably corresponding to the postero-ventral nucleus of the medial amygdala (MePV) (**Fig. 4A–C**). High magnification views confirm *Ebf3* expression in a territory located just ventral to the *Gdf10*+ VZ, most probably the SVZ (**Fig. 4C1', C1''**). By combining *Ebf3* ISH with Tbr2 immunohistochemistry, we confirmed Tbr2 protein distribution in immature *Ebf3*+ neurons in the SVZ of the CVP (**Fig. 4D** red arrowhead, **4D1** black arrows). This suggests that *Ebf3*+ immature neurons might be generated from this *Gdf10*+ CVP territory. Ventrally to the CVP, *Ebf3* is also expressed in a dispersed pattern surrounding the MePV and positive for Tbr2 (**Fig. 4D-G**), while 82.6 % (±2.6) of the Ebf3+ cells co-localize with the pallial TF Tbr1 (**Fig 4H-J''**) and none with the neurotransmitter GABA (**Fig. S4A**). This strongly indicates that *Ebf3*+ cells are glutamatergic neurons originating from a pallial territory.

To further characterize *Ebf3* expression in the MePV region, we performed ISH experiments at representative prenatal and early postnatal ages, e.g. from E12.5 to P3 (**Fig. 5**). We found that *Ebf3* is highly expressed around the MePV until birth, but downregulated at early postnatal stages (**Fig. 5A–E**). While *Ebf3* is localized in the SVZ of the CVP and in the prospective amygdala at E12.5 (**Fig. 5A**, red arrowhead), its expression increases in intensity and becomes more evident around the MePV nucleus from E15.5 onwards. Coronal sections at successive rostral to caudal levels (**Fig. 5C1–C5**) clearly show *Ebf3*+ cells

surrounding the round-shaped MePV, whereas in caudalmost sections, *Ebf3+* cells cover the posterior pole of the MePV nucleus (**Fig. 5C5**). At birth, the intensity of Ebf3 expression signal decreases, and at P3 it is virtually absent (**Fig. 5E**).

Next, we aimed to characterize the nature and precise location of these Ebf3+ cells by using MeA cell-type specific markers. The MePV neurons, normally generated at around E10.5 in the mouse (Soma et al. 2009), comprise neurons co-expressing Pax6 and Tbr2, and GABAergic neurons positive for Shh and nNOS (Abellán et al. 2013; Bupesh et al. 2011b; Carney et al. 2010; Guirado et al. 2008, and see **Fig. S5**). Pax6 and Tbr2 are expressed in postmitotic neurons in this region, as evidenced by their lack of co-localization with the proliferative marker Ki67 (**Fig. S5A-A1', A1''**), but not in nNOS+ neurons (**Fig. S4B-B''**), indicating that they belong to two different neuronal populations, clearly located apart. Our staining shows that Ebf3 is expressed in the shell of the MePV but not in the core, which is instead positive for Tbr2/Pax6 and nNOS (**Fig. S4B, 5F-G**). In addition, no Ebf3 expression was detected in SST+ neurons located mainly in the adjacent MePD (**Fig. 5F).** We however noticed that while the majority of Ebf3+ neurons do not co-express Otp (**Fig. 5H,** white arrows), a subpopulation, comprising 15.5% (±1.3) of Ebf3+ neurons and mainly positioned close to the superficial layer of the medial nucleus (slMe), co-localizes with Otp (**Fig. 5H,** yellow arrows). In summary, we found that Ebf3+ neurons that populate the MeA most probably derive from the Gdf10-expressing domain that we called CVP. These neurons are positioned between the MePV and MePD dorsally, and between the MePV and the basomedial nucleus of the amygdala (BMA), laterally, to form a shell surrounding the MePV. In addition, some double Ebf3+ Otp+ neurons are located between the MePV and the slMe forming, possibly, another boundary between these two territories.

The CVP generates Tbr1+ pallial neurons of the MeA in organotypic cultures

To further support a contribution of the CVP to the MePV, we attempted to directly trace the progeny of *Gdf10+* cells of the CVP. To this end, we placed a small crystal of CellTracker^{TM} CMFDA into the CVP

of *ex vivo* organotypic cultures (n=3) taken from E12.5 mouse brains **(Fig. 6A)**. After 48 hours of incubation, CMFDA-labeled cells migrate ventrally and then get subdivided into two streams encircling an empty territory that we tentatively identified as the MePV on topographic basis (**Fig. 6B**). This pattern of migration is reminiscent of the distribution of *Ebf3+* cells around the MePV (**Fig. 6B, C**), suggesting that postmitotic cells originating from the CVP might express *Ebf3* during their migratory path, as also supported by the presence of the pallial marker Tbr1 in migrating CMFDA+ grafted cells (**Fig. 6D, D1'-D1'''**). Thus, we propose that postmitotic cells migrating from the CVP do express Tbr1 and Ebf3.

Loss of CVP-derived Ebf3+ cells in the absence of the pallial patterning gene Pax6

Since the VP contributes to form the pallial-subpallial boundary (PSB), we hypothesized that the CVP could also form part of the PSB at caudal levels, and thus delimit the boundary between the CGE and the pallium. To test this hypothesis, we first evaluated the presence of the CVP by assessing the expression of *Gdf10* in *Pax6 null* mice (*Pax6KO*), in which the PSB is altered. Previous studies showed that in *Pax6* mutant mice, the expression of *Sfrp2*, *Dbx1*, *Wnt7b* and *Tgfα* in the VP is abolished and subpallial markers shifted to dorsal territories (Stoykova et al. 1996; Kim et al. 2001; Assimacopoulos et al. 2003; Stenman et al. 2003; Carney et al. 2009; Cocas et al. 2011; Georgala et al. 2011). Accordingly, we observed a similar D-V patterning defect in E12.5 *Pax6KO* embryos, even if we noticed a dorsal shift, and not a complete loss of the VP marker *Dbx1* (**Fig. 7A, A'**). We also found that in *Pax6KO* embryos, *Gdf10* expression is downregulated in the entire VP, including the CVP (**Fig. 7B, B'**) and the TE (**Fig. 7B',** black asterisk). Since *Gdf10* expression is still maintained in CBP, its expression might be specifically abolished in the *Pax6* mutant forebrain. Interestingly, loss of *Gdf10* in the CVP is accompanied by a severe reduction of the *Ebf3+* population in the mutant amygdala region, with only residual *Ebf3* expression maintained in the MePV region (**Fig. 7C', D'**, black arrowheads). Immunostaining with Tbr2 and nNOS confirmed that these few Ebf3+ neurons are located within the MePV instead of surrounding the nucleus (**Fig 7E, E',** white arrowheads), as previously shown by the presence of nNOS+ neurons normally generated in the POA and

contributing to the MePV (see also **Fig. 5F**, **S5C**). We calculated that in the absence of Pax6, the Ebf3+ population gets reduced to 78% compared to control brains (**Fig. 7F**). Furthermore, Tbr2+ neurons normally also positive for Pax6 (**Fig. S5A1'**), are located in aberrant positions in *Pax6KO* embryos (**Fig 7E, E',** yellow arrowheads). Thus, loss of Pax6 leads to specification and migratory defects of both Ebf3+ and Tbr2+ populations in the MePV. Taken together, these data obtained from the *Pax6KO* mouse line further confirm that the Gdf10+ domain in the CVP corresponds to the caudal portion of the VP delimiting the boundary between the pallium (CVP) and the subpallium (CGE) at caudal-most levels.

DISCUSSION

In this study, we have identified and characterized for the first time the expression of the growth differentiation factor Gdf10 in a telencephalic domain that, based on our detailed gene expression and anatomical analyses, we defined as a novel caudal subdivision of the ventral pallium (VP), and accordingly named it Caudo-Ventral Pallium (CVP). Fate mapping experiments revealed that the CVP is the origin of a hitherto unnoticed population of glutamatergic Ebf3+ neurons destined to the MeA.

Controversies in the definition of the Caudo-Ventral Pallium (CVP)

Our study shows that *Gdf10* is restricted to a domain of weak expression in the VZ of the VP, and to a high-expressing domain in a territory located medial to the CGE in the developing mouse telencephalon. To our knowledge, this medial territory (heretofore the CVP) has never been considered as an independent region, but instead as part of the caudal portion of the MGE or the medial CGE and therefore subpallial (Flames et al. 2007; Tang et al. 2012; Hu et al. 2017), mainly because the region ventral to the CVP, the prospective MeA and its different subdivisions, is a territory receiving massive caudal migration of several neuron subtypes generated from Nkx2.1+ progenitors in the MGE and POA (reviewed in Ruiz-Reig and Studer

2017). We consider that the mere presence of MGE and/or POA derivatives in the MeA is not a sufficient argument to establish the CVP as a subpallial territory.

On the other hand, the expression of the orphan nuclear factor COUP-TFII has likewise lead to confusion in the interpretation of the CVP. COUP-TFII is a well-known marker of CGE, caudal MGE and POA (Tripodi et al. 2004; Cai et al. 2013; Kanatani et al. 2008; Touzot et al. 2016), but is also expressed in the caudal cortex, hippocampus and postmitotic Cajal-Retzius cells (Studer et al. 2005; Flore et al. 2016; Parisot et al. 2017). Since the CVP is highly positive for COUP-TFII, some authors have considered the CVP as part of the ganglionic eminences (Tang et al. 2012; Kanatani et al. 2015; Hu et al. 2017). However, in the present study we show that COUP-TFII is co-expressed with two pallial markers, Pax6 in VZ progenitors and Tbr2 in the SVZ, thus indicating that COUP-TFII expression in this territory is of pallial and not of subpallial origin. We also demonstrate by cell tracking experiments and molecular evidence that the CVP generates glutamatergic Tbr1+ Ebf3+ neurons populating the MePV. It is noteworthy that the *Allen Brain Atlas* describes a highly expressing *Gdf10+* territory that likely corresponds to our CVP, as ventropallial amygdalopiriform area (VAPv), implying that this region is part of the VP and contributes to the neuronal populations of the amygdala.

The anatomical location of the CVP

Why does the CVP appear medial to the CGE, i.e. in a location opposite to the rest of the VP? The initial clear-cut rostro-caudal polarity of the neural tube is somehow masked in the telencephalon, which represents a huge expansion of the alar plate of the secondary prosencephalon (Puelles and Rubenstein 2003). Due to the growth of the telencephalon, the spatial relationships between its constituent elements are in certain cases difficult to define. For instance, Remedios et al. 2007 described a pallial territory located in the caudo-ventral telencephalon as part of the dorsal pallium. Another example is the cortical hem, which is situated close to the roof plate and midline in the most medial and dorsal parts of the dorsal telencephalon. Since the cortical hem folds at posterior levels (Grove et al. 1998; Ruiz-Reig et al. 2017; Yoshida et al.

2006, schematized in **Fig 3I, green region**), what seems to be ventral in coronal sections (the so-called ventral hem) is actually caudal, and thus named "caudal hem".

The VP, localized at the most latero-ventral region of the dorsal telencephalon, contributes to the subdivision between pallium and subpallium. In common with other pallial borders, the VP expresses several signaling molecules playing a key role in the establishment of the latero-medial axis of the telencephalon (Assimacopoulos et al. 2003; Subramanian and Tole 2009). One of these signaling molecules is Gdf10 (or Bmp3-b). We have shown that *Gdf10* is expressed at low levels in the VP but at higher levels in the CVP. Caudally, the VP surrounds the CGE in such a way that expression of Gdf10 and some other VP markers appears in coronal sections medial to the CGE (schematized in **Fig. 3H**). Coronal sections taken at more posterior levels reveal instead the anatomical continuity between VP and CVP. Moreover, the CVP continues anatomically with the LTE and both share the expression of several patterning genes. Therefore, these anatomical and geno-architectonic relationships with the more caudal TE make the CVP the most caudal part of the VP.

In terms of gene expression, we have also shown that *Gdf10* and *Fgf15* have rostral^{low} - caudal^{high} expression gradients in the VP and, accordingly, the intensity of their transcript signals becomes higher in the CVP than in the remaining VP. *Dbx1* is not expressed in the CVP and does not exhibit a gradient expression along the rostro-caudal axis, whereas *Sfrp2* is mainly expressed in the most medial part of the VP (schematized in **Fig. 3J**). Together with *Ptch1*, *Mgda1* and COUP-TFII, which are either not expressed or expressed at low levels in the VP, our data imply that the CVP has a molecular signature distinct from the VP. Finally, we have shown that in the *Pax6 null* mouse, in which the PSB is disorganized and VP markers absent (Stoykova et al. 1996; Kim et al. 2001; Stenman et al. 2003; Carney et al. 2009; Cocas et al. 2011; Georgala et al. 2011), *Gdf10* expression is completely abolished in the VP, including the CVP, supporting that the CVP is part of the VP. As a consequence, the *Ebf3+* population generated from the CVP is severely reduced in these mutants, confirming that *Ebf3+* neurons originate from the CVP, a territory disrupted in the absence of *Pax6*.

Neuronal organization of the MeA and MePV nuclei

We propose in this work a novel and distinct glutamatergic population generated in the CVP, in addition to the already described neuronal populations composing the MeA (see summary in **Fig. 8**). GABAergic nNOS neurons, generated in the POA from Dbx1+ progenitors, migrate caudally through the Preoptic Amygdala Stream (PAS) to reach the MePV (Hirata et al. 2009). The MeA is also composed of other GABAergic populations characterized by the expression of SST and Lhx6 and mainly localized in the dorsal portion (MePD), even if some spread cells also migrate to the ventral part (MePV). The origin of SST+ neurons has now been defined as the cvMGE, also called diagonal area (Dg) (Garcia-Lopez et al. 2008; Real et al. 2009; Bupesh et al. 2011a; Puelles et al. 2016b; Hu et al. 2017). The MeA is also composed of glutamatergic Otp+ neurons generated in the lateral hypothalamus and contributing to the MeA circuitry (García-Moreno et al. 2010). Otp+ neurons are located mainly in the MePV and in the slMe. We found a few Ebf3+ neurons co-localizing with Otp suggesting that Ebf3+ cells are a heterogenous population with some extra-telencephalic origin. However, the majority of Ebf3+ neurons express the pallial marker Tbr1 and is generated from the CVP. This population ultimately surrounds the MePV and forms the boundary between different subnuclei of the amygdala (red dots in **Fig 8A'**).

Finally, the nature and origin of the double Pax6/Tbr2+ postmitotic cells located in the MePV are not well described. Some authors propose them as GABAergic neurons generated in the CGE (Tang et al. 2012), whereas others consider them as glutamatergic neurons generated in the TE (Bupesh et al. 2011b; Abellán et al. 2013). Since this population is not GABAergic and the TE also contributes to glutamatergic Pax6/Tbr2+ neurons in the accessory olfactory bulb, we agree that the most likely origin of this population is the TE (Huilgol et al. 2013; Ruiz-Reig et al. 2017). The dLGE also generates Pax6+ postmitotic neurons to the olfactory tuberculum, but in this case these cells are GABAergic neurons. In the absence of Pax6, these two populations are affected due, most probably, to migratory defects (Tole 2005).

CVP contribution to the medial amygdala

The mammalian amygdala is a multinuclear complex composed of pallial and subpallial nuclei (Swanson and Petrovich 1998; Puelles et al. 2000; Gorski et al. 2002; Medina et al. 2004; Hirata et al. 2009; Bupesh et al. 2011b). While the amygdalar pallial nuclei are formed by principal glutamatergic projecting neurons, GABAergic projecting neurons are mainly distributed in subpallial structures (McDonald 1982; Swanson and Petrovich 1998; Cassell et al. 1999). However, the different amygdalar structures receive glutamatergic and GABAergic neurons from both pallial and subpallial domains (Swanson and Petrovich 1998; Berdel and Moryś 2000; Marin et al. 2000; Swanson 2000; Legaz et al. 2005; Carney et al. 2006, 2010; Cocas et al. 2009; Hirata et al. 2009; Waclaw et al. 2010). It has been proposed that the VP contributes to the generation of glutamatergic neurons that will colonize the basal amygdalar complex and cortical amygdala (Puelles et al. 2000, 2016a; Stenman et al. 2003; Remedios et al. 2004). However, these studies used mainly one approach, the *Dbx1-reporter* mouse line as the major tool to study VP derivatives to the amygdala.

In this work, we have instead shown that the most caudal edge of the VP, the CVP, is negative for Dbx1, and therefore, to our knowledge, no VP-derivatives could be found in the MeA. A study using a *Gdf10* reporter mouse line would allow a better understanding of the precise derivatives of the CVP not only in the MeA, but also in the cortical amygdala and other structures. Furthermore, it would be also a useful tool to understand whether cells expressing *Ebf3* represent a transitory population, or alternatively, *Ebf3* is just downregulated at postnatal stages. This situation is reminiscent of *Ebf3* expression in the transient Cajal-Retzius population, in which *Ebf3* is required for cell migration (Chiara et al. 2012; de Frutos et al. 2016). Thus, we could envisage that *Ebf3* is downregulated after cells have completed their migration and contributed to form the MeA nucleus.

Another interesting open question is the potential role of *Ebf3*+ neurons in the MeA circuitry. It is still unclear whether *Ebf3*+ neurons act as local interneurons or glutamatergic neurons projecting to the

dorso-medial part of the ventro-medial hypothalamic nucleus, as previously described by Choi et al. 2005 and Bian et al. 2008. The MeA is classically considered to form part of the subpallial amygdala, however several studies, including the present work, indicate that the glutamatergic population is larger than previously envisaged and includes projecting glutamatergic neurons resembling pyramidal neurons of the piriform cortex, most probably originating from the VP (Bian et al. 2008). This neural diversity in the MeA implies a very complex neural circuitry regulating social behavior. Activation of GABAergic neurons located in the MePD can trigger social behaviors as well as sexual mounting and aggression, whereas glutamatergic neurons promote solitary behaviors such as self-grooming. In the MePD both populations play a reciprocal inhibitory effect, whereby GABAergic neurons inhibit self-grooming and glutamatergic neurons social behaviors (Hong et al. 2014, summarized on Fig. 8C). GABAergic neurons have been classified into three different subgroups depending on their electrophysiological properties. Two of these groups (types 1 and 3) correspond to neurons projecting to the hypothalamus, whereas the types 2 are local interneurons regulating the olfactory processing circuitry. On the other hand, glutamatergic neurons in the MePV are subdivided into two different electrophysiological groups projecting to different subdivisions of the hypothalamus, and implicated in reproduction and defense (Keshavarzi et al. 2014, Fig. 8C).

Although electrophysiological studies were conducted to decipher the neural connectivity in the MeA, little was known about the neuronal origin of these populations. In the MeA, GABAergic neurons generated from the POA can be subdivided in function on their expression of Dbx1 or FoxP2 in progenitors. Both populations are activated by mating and aggressive cues, however while mating cues activate Dbx1-derived GABAergic neurons in both, male and females, FoxP2-expressing neurons can be induced only in males due to amygdala dimorphism (Fig. 8D). Otp neurons are more implicated in defensive behavior since they are activated by predator cues (Lischinsky et al. 2017, Fig. 8D). We need more work to elucidate the role of Tbr2/Pax6+ and Ebf3+ populations, since they are also part of the complex circuit implicated in processing pheromone-dependent emotional behaviors. So far, no studies have been conducted to understand their roles in the circuitry. A specific deletion of *Ebf3+* neurons in the MeA using a conditional

Pax6KO mouse line, possibly *via* a *Gdf10*^{Cre} mouse, would definitely help us in understanding the role of this population in sexual/aggressive-defensive information processing.

ABBREVIATIONS

BLA: Basolateral complex of the amygdala

BMA: Basomedial nucleus of the amygdala

C: Caudal

CBP: Cranial bone progenitors

CGE: Caudal ganglionic eminence.

C-hem: Caudal hem

CRC: Cajal-Retzius cells.

cvMGE: caudo-ventral MGE

CVP: Caudo-ventral pallium

Cx: Cortex.

D: Dorsal

Dg: Diagonal area.

dLGE: dorsal LGE.

DP: Dorsal pallium.

GE: Ganglionic eminences

HC: Hippocampus

ISH: *In situ* hybridization

L: Lateral

LGE: Lateral ganglionic eminence.

LP: Lateral pallium.

LTE: Lateral thalamic eminence.

M: Medial

MeA: Medial nucleus of the amygdala.

MeAa: Antero-medial Amygdala

MePD: Posterodorsal medial Amygdala

MePV: Posteroventral medial amygdala

MGE: Medial ganglionic eminence.

MP: Medial pallium.

MTE: Medial thalamic eminence

MZ: Mantle zone

OB: Olfactory bulb.

PAS: Preoptic amygdala stream

POA: Preoptic area.

PSB: Pallial-subpallial boundary.

PVH: Paraventricular hypothalamic domain

pTh: Prethalamus

R: Rostral

SlMe: Superficial layer of the medial nucleus

SVZ: Subventricular zone.

TE: Thalamic eminence.

TF: Transcription factor.

Th: Thalamus

V: Ventral

VAPv: Ventropallial amygdalopiriform area

VP: Ventral pallium.

VZ: Ventricular zone

ZLI: Zona limitants intrathalamica

MATERIALS AND METHODS

Animals

Wild type C57BL/6J and ICR mice were maintained in the animal facilities of Universidad Miguel Hernández, San Juan de Alicante, Spain, whereas *Pax6 KO* mice were maintained in the animal facility of the Institut of Biology Valrose, University of Nice, France. These mice were obtained from Dr. Ahmed Mansouri, Max Planck Institute, Göttingen, Germany and maintained in a C57BL/6J background as heterozygotes, due to postnatal lethality (St-Onge et al. 1997). The day of vaginal plug was considered as embryonic day 0.5 (E0.5). The care and handling of animals prior or during the experimental procedures followed European Union (2010/63/UE) and were approved by the Animal Care and Use Committees of Spain and France.

Immunohistochemistry and in situ hybridization (ISH)

Embryos were fixed in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4, at room temperature (RT) for 2hrs for immunohistochemistry or at 4ºC overnight for ISH, and rinsed in PBS for 2 hrs. For thick sections (80 µm), embryonic brains were embedded in 4% agarose diluted in PBS and cut on a Vibratome (Leica VT1000S). For thinner sections, brains were cryoprotected in 30% sucrose in 0.1M PB under agitation at 4ºC overnight and included in optimum cutting temperature (O.C.T.) medium (Cryomatrix™, Thermo) before being frozen. Thin sections (10 µm) were obtained with the help of a Leica cryostat. Immunohistochemical staining was performed as previously described (Touzot et al. 2016) on 80 µm-thick Vibratome sections. Briefly, vibratome sections were blocked in 4% bovine serum albumin (BSA), 3% normal goat serum (NGS), 0.2% Triton X-100 in PBS at room temperature (RT) for 1h and incubated with the primary antibodies diluted in blocking solution at 4ºC overnight. The incubation with the secondary antibodies lasted for 2hrs. Primary antibodies used were: rabbit anti-Tbr1 (Millipore, 1:500); chicken anti-Tbr2 (Millipore, 1:500); rabbit anti-COUP-TFII (1:500); Mouse anti-Pax6 (Developmental Studies Hybridoma Bank (DSHB), 1:500); Rabbit anti-Pax6 (Milliore, 1:500); Rabbit anti-Ki67 (AbCam

1:100); Rabbit anti-GABA (Sigma, 1:1000); Mouse anti-Ebf3 (Sigma 1:500); Rabbit anti-nNOS (ImmunoStar, 1:1000); Goat anti-SST D-20 (Santa Cruz, 1:200); Rabbit anti-Otp (provided by Flora Vaccarino, 1:500). Different combinations of AlexaFluor secondary antibodies (Invitrogen, 1:500) were used. After immunohistochemistry the section were incubated with DAPI 100 µM for 5 min. Sections were mounted on microscope slides and covered with AF2 (Citifluor Ltd., London, UK) for immunofluorescence. Each experiment was repeated on at least 3 independent animals.

ISH was performed as described (Martinez-Lopez et al. 2015) on cryostat sections. For fluorescent ISH, the reaction was revealed using TSA Plus Cyanine 3 System (Perkin Elmer). The following riboprobes were used: *Dbx1* (Lu et al. 1994), Fgf15 (McWhirter et al. 1997), *Gdf10* (Zhao et al. 1999), *Gsx2* (Hsieh-Li et al. 1995), *Ascl1* (Guillemot and Joyner 1993), *Neurog2* (Gradwohl et al. 1996), *Pax6* (Walther and Gruss 1991), *Sfrp2* (Kim et al. 2001), *Ebf3* (Garel et al. 1997), *Ptch1* (Goodrich et al. 1996), *Mdga1* (Litwack et al. 2004), *Shh* (Echelard et al. 1993), *Emx1* (Simeone et al. 1992). If immunohistochemistry was due after ISH, slides were incubated in a solution of 3 % hydrogen peroxide in PBS for 30 min to inhibit the endogenous peroxidase. Immunohistochemistry was performed, as for immunofluorescence, by using biotinylated secondary antibody diluted in blocking solution. To reveal the immunohistochemical staining, the tissue was incubated with ABC (Avidin-Biotin-Peroxidase, Vector Labs) for 2hrs and the reaction was developed in 0.05% 3,3'- diaminobezidine (DAB)/0.01% hydrogen peroxide in PBS. Each experiment was performed on at least 3 independent animals.

Slice cultures and CMFDA injections

Timed pregnant dams were killed by cervical dislocation. Uterine horns were removed and isolated in cold Krebs solution. E12.5 brain embryos were embedded in 4% low melting point agarose (Sea Plaque Agarose, Cambrex) in PBS. 300µm thick coronal sections were carefully cut on a Leica vibratome (Frequency=8,

Speed=3). The sections were transferred to polycarbonate culture membranes (Millicell-CM® Low Height Culture Plate Inserts, 0.4 µm pore size, Millipore) that had been previously placed in 6-well culture plastic dishes (Nunc, Thermo Scientific) containing 1 ml of DMEM-F12 supplemented with N2 supplement (5 µl/ml), L-glutamine (2 mM), glucose (6 mg/ml), Penicillin–Streptomycin (500 U/ml) and 10% fetal bovine serum (all these reagents provided by Invitrogen). Slices were maintained at 37ºC in 5% CO2 in a standard sterile incubator for 1h. Next, resin beads (Bio-Rad), previously soaked in CellTracker CMFDA (Molecular Probes – Invitrogen) were placed in the CVP. DMEM-F12 medium was replaced by pre-warmed Neurobasal medium supplemented with B27 supplement (1X), L-glutamine (2 mM), glucose (6 mg/ml) and Penicillin–Streptomycin (500 U/ml) (all these reagents provided by Invitrogen). The slices were kept in the incubator at 37ºC and 5% CO2 for 48 hours. Slices were then fixed in 4% PFA in PBS for 2 hrs and prepared for immunostaining. This experiment was repeated three times.

Imaging and data analysis

The sections were imaged using Leica TCS SP2 AOBS and Zeiss 780 for confocal microscopes and Leica MZ APO stereomicroscope. Images were acquired using the LAS AF or ZEN software and processed with Adobe Photoshop CS5 and Adobe Illustrator CS5. A minimum of 3 animals and 3 slices of each animal were used for all the analyses and quantifications. All quantifications were presented as the mean ± standard error mean. Minimal statistical significance was fixed at $p<0.05$ for the results of the Student's t test and are represented in the figure 7 as $**p < 0.01$.

FIGURE LEGENDS

Figure 1. *Gdf10* expression pattern in the telencephalon. (A-F) *In situ* hybridization (ISH) for *Gdf10* transcript on adjacent coronal sections of E12.5 wild type embryos showing weak *Gdf10* expression in the ventral pallium (VP), and in a telencephalic territory medial to the CGE and anatomically continued with the lateral thalamic eminence (LTE) (black arrowheads). In the diencephalon, *Gdf10* is expressed in both domains of the thalamic eminence (TE), the medial and the lateral ones (MTE and LTE, respectively). ISH for *Gdf10* in horizontal **(G-H)** and sagittal sections **(I)** of E12.5 wild type embryos. Red asterisks indicate *Gdf10* expression in cranial bone progenitors. Scale bars: 100 µm.

Figure 2. The telencephalic Gdf10+ territory express pallial markers. (A-C, A'-C') *In situ* hybridization (ISH) on adjacent coronal sections of E12.5 wild type embryos comparing the expression of *Gdf10*, *Pax6* and *Neurog2* in the telencephalic *Gdf10+* territory (black arrowheads) at two different rostro-caudal levels. **(D, D')** Tbr2 immunolabeling in coronal sections of an E12.5 embryo showing Tbr2 expression in the SVZ of the telencephalic Gdf10+ territory (white arrowhead). **(E, E')** Fluorescent ISH for *Gdf10* combined with immunofluorescence for Pax6 protein showing double-positive cells in the VZ. **(F, F')** High magnification views of the telencephalic *Gdf10+* territory labeled by *Gdf10* transcript (in blue) and Tbr2 protein (in brown). (**G**) Details taken in the region boxed in D' and showing triple immunofluorescence for Pax6, Tbr2 and Tbr1 in the telencephalic *Gdf10*+ territory. Insets in **G'** and **G''** illustrate a detail of the neuron indicated by the white arrows and co-localizing both markers. The radial distribution of the three markers perfectly reproduces the pallial organization (Englund et al. 2005; Hevner et al. 2006). Scale bars: (A-D, A'-D') 100 µm; (E-G) 50 µm.

Figure 3. The telencephalic Gdf10+ territory or Caudo-Ventral Pallium (CVP) is part of the VP. (A-C') *In situ* hybridization (ISH) for *Gdf10*, *Fgf15*, *Sfrp2* and *Dbx1* in adjacent coronal sections of the same

brain in E12.5 wild type embryos showing the correspondence between *Gdf10* expression and VP markers in the telencephalon. The green arrowheads indicate the VP and the black arrowheads indicate the telencephalic *Gdf10*+ territory, named as Caudo-Ventral Pallium (CVP). **(A'''-C''')** Overlay of *Gdf10* and VP markers from adjacent sections obtained in false color with ImageJ and Photoshop. **(D-G')** ISH for VP markers in coronal sections at two different rostro-caudal levels of E12.5 wild type embryos. Note that *Gdf10*, *Fgf15* and *Sfrp2* expression is situated flanking the CGE in more rostral sections (D-F), whereas in more caudally sections the expression of these genes extend forming a *continuum* (D'-F'). **(H, H')** Schematic coronal sections taken at levels indicated in I. **(I)** Schematic illustration of a telencephalic vesicle showing the position of the ventral pallium (VP-pink-violet band) at the most latero-ventral part of the pallium. The VP and the lateral ganglionic eminences (LGE-represented in yellow) form the PSB. At posterior caudal levels, the VP bends, surrounds the CGE and continues anatomically with the LTE. The caudal portion of the VP is the CVP, colored in dark violet. **(J)** Expression gradients of VP markers represented as in a dorsal view. *Gdf10* and *Fgf15* have a rostral (R)-low caudal (C)-high expression gradient. Thus, the expression intensity of both genes in CVP is always higher compared to the remaining of the VP. *Dbx1* is represented in a "salt and pepper" manner in the VP, but this expression stops at the level of the CVP. *Sfrp2* is expressed in the most medial (M) part of the VP Scale bars: (A'-D') 100 µm; (D-G') 100 µm.

Figure 4. *Ebf3* expression in the amygdala. (A-C) *In situ* hybridization (ISH) of *Ebf3* on coronal sections at three rostro-caudal levels of an E12.5 wild type embryo. The black arrowheads indicate the SVZ of the CVP. **(C1'-C1'')** High magnification views of the boxed area in C showing *mRNA* expression of *Gdf10* and *Ebf3* in two consecutive thin sections of the same brain. *Gdf10* expression delimits the VZ of the CVP, whereas *Ebf3* is expressed in the corresponding SVZ and in ventrally dispersed cells that most probably migrate toward the MeA. (**D-G**) Immunohistochemistry for Tbr2 and ISH of *Ebf3* in the CVP showing double+ neurons for both markers in the lower SVZ (red arrowhead in D and black arrows in D1). **(H)**

Double immunofluorescence for Ebf3 and Tbr1 in the MePV. **(J-J'')** High magnifications of the MePV region showing Ebf3+neurons co-expressing the pallial marker Tbr1 in orthogonal confocal views. Scale bars: (A, C1, D, H) 100 μm, (D1, H') 50 μm.

Figure 5. Temporal and spatial Ebf3 expression pattern in the shell of the MePV nucleus. (A-E) *In situ* hybridization (ISH) on consecutive coronal sections showing *Ebf3* expression in the shell surrounding the MePV at the stages indicated above. The red arrowhead points to *Ebf3* expression in the SVZ**. (C1-C5)** *Ebf3* ISH in five consecutives sections taken from rostral to caudal at E15.5. **(F-H)** Immunofluorescence for Ebf3, SST, nNOS, Pax6 and Otp illustrating the anatomical localization of Ebf3+ neurons (in red) with respect to other neural populations in the MeA. Insets correspond to high magnification views of the areas indicated in the box and showing a detail of cells labeled for Ebf3 and other markers. The white arrow in H indicates Ebf3+ cells not co-localizing with Otp, yellow arrows indicates Ebf3 neurons co-expressing Otp. Scale bars: 100 μm.

Figure 6. The CVP generates Tbr1+ cells that migrate tangentially around the MePV nucleus. (A) Schematic image showing the localization of the CMFDA tracker in the VZ of the CVP and the distribution of the cells generated from this region after two days of incubation. **(B)** Coronal section of organotypic cultures labeled with CMFDA (green) and showing labeled cells migrating toward the MePV. (**C**) *In situ* hybridization for *Ebf3* in a coronal section showing the localization of *Ebf3*+ cells around the MePV. Note that CMFDA grafted cells (B) and *Ebf3*+ cells (C) have a similar distribution around the MePV (white and black arrowheads, respectively). **(D)** Coronal section of organotypic cultures labeled with CMFDA (green) and Tbr1. **(D', D'')** High magnification views of the square D' showing CMFDA+ cells co-expressing the pallial postmitotic marker Tbr1. Insets represent a detail of a neuron co-labelled with CFMDA and Tbr1. Scale bars: (B-D) 100 μm; (D'-D") 50 μm.

Figure 7. ***Gdf10*** **expression is lost in** ***Pax6 null*** **mice (*****Pax6 KO*****). (A, A', B, B')** *Dbx1* and *Gdf10 in situ* hybridization (ISH) on coronal sections of E12.5 control and *Pax6KO* littermate embryos. In *Pax6KO* embryos, *Gdf10* expression is lost in the forebrain, including the CVP and the TE (black asterisk points to the TE), but maintained in cranial bone progenitors (CBP). Dbx1 expression is displaced in the VP (black arrows), but it is unaltered in the POA. (**C, C', D, D'**) Coronal sections of control and *Pax6KO* littermates showing *Ebf3* expression in the MeA at E13.5 and E15.5**. (E, E')** Triple immunolabeling for Tbr2, nNOS and Ebf3 showing the distribution of the different MeA neural populations in control and mutant mice at E15.5. White arrowheads point to the few Ebf3+ neurons in mutant mice, yellow arrowheads indicate the aberrant position of Tbr2+ neurons in mutant condition. **(F)** Histogram showing the number of Ebf3+ cells in control and mutant MeA. Bars indicate the mean ± standard error mean. Statistical significance: $^{**}p < 0.01$. Scale bars: (A-D, A'-D') 100 µm; (E, E') 50 µm.

Figure 8. The Medial Amygdala is composed of several neural populations with different origins and functions. (A) Schematic illustration of a caudal coronal section of a P0 mouse telencephalon. **(A')** Close up illustration from the box indicated in A showing the distribution of the different neural population in the MePD and the MePV. The violet area and dots stand for SST+ neurons localized mainly in the MePD, but also spread in the MePV. The light blue represents nNOS+ Shh+ neurons in the core of the MePV. Yellow dots are for Otp+ neurons in the SlMe and MePV, red dots for Ebf3+ neurons and green dots for Pax6/Tbr2+ neurons. Some neurons localized close to the slMe co-express Ebf3 and Otp and are represented in orange color. **(B)** Table summarizing the different neural populations that contribute to the MeA, indicating markers and neurotransmitters (NT) they express, the origin of each population, and the studies referring to their description. **(C)** Schema representing the neural connectivity of the MeA populations classified according the neurotransmitter they express and their electrophysiological properties. **(D)** Schema

representing activation of the different MeA neural populations by different cues and classified according to their origin and markers.

SUPPLEMENTARY FIGURE LEGENDS

Figure S1. The telencephalic Gdf10 territory does not belong to the ganglionic eminences. (A-C') *In situ* hybridization for *Ascl1*, *Gsx2* and *Gdf10* in coronal sections taken at two different rostro-caudal levels of E12.5 wild type brains. The telencephalic *Gdf10+* territory is negative for *Ascl1* and *Gsx2*, two markers expressed in the ganglionic eminences Scale bar: 100 µm.

Figure S2. *Emx1* is not expressed in the VZ of the telencephalic *Gdf10*+ territory. (A, B) *In situ* hybridization for *Gdf10* and *Emx1* in consecutives coronal sections of the same E12.5 wild type brain. **(A', B')** High magnification views of the boxes in A and B showing that at this stage the *Gdf10+* territory in the VZ is negative for *Emx1*, which is restricted to the SVZ. Scale bar: 100 µm.

Figure S3. Additional markers located in the CVP. (**A-A', B-B'**) *In situ* hybridization for *Ptch1* and *Mdga1* in coronal sections at two different caudo-rostral levels of E12.5 wild type brains. *Ptch1* is expressed in the VZ, while Mdga1 expression is restricted to the SVZ of the telencephalic Gdf10+ territory (or CVP as indicated by black arrowheads). Green arrowheads point to the VP. (**C, D**) Triple immunofluorescence for two pallial markers, Tbr2 and Pax6, and COUP-TFII in coronal sections **(C)** and horizontal sections **(D)** of E12.5 wild type brains. **(C'-D')** High magnification views of the boxes in C and D showing co-expression of Pax6 and COUP-TFII in the VZ, and Tbr2 and COUP-TFII in the SVZ of the CVP. Scale bars: (A-D) 100 µm; (C'- D') 50 µm.

Figure S4. Ebf3-, nNOS- and Tbr2-expressing cells form three independent neural populations in the MePV nucleus. (A) Double immunofluorescence for GABA and Ebf3 in a coronal section of P0 wild type

brains. **(A', A'')** High magnification views of the shell of the MePV showing lack of Ebf3+ (red) cells co-localization with GABA+ (green) neurons (white arrowheads). **(B)** Triple immunofluorescence for Tbr2, Ebf3 and nNOS in a coronal section of E15.5 wild type brains. **(B', B'')** High magnification views of the core of the MePV showing no co-localization between Tbr2+ (green) and nNOS+ (blue) cells, which are located as a separate population with respect to Ebf3+ cells (red). Scale bars: (A, B) 100 µm; (A'-B'') 50 µm.

Figure S5. The MePV contains Pax6+Tbr2+ cells in addition to Shh+ and nNOS+ neural populations. **(A)** Triple immunofluorescence for Tbr2, Pax6 and Ki67 in a coronal section of E12.5 wild type brain. **(A1', A1'')** High magnification views of the square in **A** showing in yellow cells co-localizing with Pax6 and Tbr2 but negative for Ki67 in the core of the MePV. Green Tbr2+ single cells are most probably intermediate progenitors. A1" display the cells positive for Pax6. (**B**) *In situ* hybridization of *Shh* in a coronal section of E12.5 wild type brain shows expression in the MePV region. **(C)** Immunofluorescence for nNOS in a coronal section of E15.5 wild type embryo indicates high expression in the MePV region. Scale bars: (A-C) 100 µm; (A1'-A1'') 50 µm.

ETHICAL STATEMENT

I. Compliance with ethical standards

The authors state that the present manuscript presents original research that has not been previously published and is not being considered for publication elsewhere.

II. Funding

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III. Conflict of interest

The authors declare that they have no conflict of interest.

IV. Ethical approval

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. The care and handling of mice prior or during the experimental procedures followed European Union (2010/63/UE) and were approved by the Animal Care and Use Committees of Spain and France.

V. Informed consent

Informed consent was obtained from all individual participants included in the study.

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