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Abstract

Background: The mammalian amygdala is composed of two primary functional subdivisions, classified according to whether the major output projection of each nucleus is excitatory or inhibitory. The posterior dorsal and ventral subdivisions of the medial amygdala, which primarily contain inhibitory output neurons, modulate specific aspects of innate socio-sexual and aggressive behaviors. However, the development of the neuronal diversity of this complex and important structure remains to be fully elucidated.

Results: Using a combination of genetic fate-mapping and loss-of-function analyses, we examined the contribution and function of Sonic hedgehog (Shh)-expressing and Shh-responsive (Nkx2-1+ and Gli1+) neurons in the medial amygdala. Specifically, we found that Shh- and Nkx2-1-lineage cells contribute differentially to the dorsal and ventral subdivisions of the postnatal medial amygdala. These Shh- and Nkx2-1-lineage neurons express overlapping and non-overlapping inhibitory neuronal markers, such as Calbindin, FoxP2, nNOS and Somatostatin, revealing diverse fate contributions in discrete medial amygdala nuclear subdivisions. Electrophysiological analysis of the Shh-derived neurons additionally reveals an important functional diversity within this lineage in the medial amygdala. Moreover, inducible Gli1CreER(T2) temporal fate mapping shows that early-generated progenitors that respond to Shh signaling also contribute to medial amygdala neuronal diversity. Lastly, analysis of Nkx2-1 mutant mice demonstrates a genetic requirement for Nkx2-1 in inhibitory neuronal specification in the medial amygdala distinct from the requirement for Nkx2-1 in cerebral cortical development.

Conclusions: Taken together, these data reveal a differential contribution of Shh-expressing and Shh-responding cells to medial amygdala neuronal diversity as well as the function of Nkx2-1 in the development of this important limbic system structure.

Background

The mammalian amygdala is an aggregation of 11 to 15 nuclei, which as components of the limbic system mediate distinct aspects of emotional and behavioral processing as well as socio-sexual behaviors (reviewed in [1]). The amygdala is considered in terms of two functional subdivisions, classified according to whether the major output projection of each nucleus is excitatory or inhibitory. Based on this classification, the nuclei of the basolateral complex and cortical amygdalar nuclei, which have an excitatory output, have been hypothesized to be of a pallial origin, whereas the cortical and medial nuclei are broadly considered similar to the striatum as their output projections are primarily inhibitory. Several studies have revealed that, during embryogenesis, the emerging amygdala is generated from several forebrain embryonic domains, including the pallium, pallial-subpallial boundary, medial ganglionic eminence (MGE), preoptic area (POA) and perhaps the diencephalon [2-10].

The medial amygdala (MeA) subnuclei process pheromonal information, and regulate neuroendocrine function and socio-sexual behaviors. Anatomically, the posterior portion of the MeA is divided into dorsal...
(medial posteroventral nucleus (MePD)) and ventral (medial posteroventral nucleus (MePV)) subdivisions, which via their projections to distinct hypothalamic nuclei regulate reproductive and defensive behaviors, respectively (reviewed in [1,11]). Although not well-characterized, recent studies have revealed insight into the development of the MeA. In terms of circuit formation, the anatomical segregation of efferent projections that regulate reproductive or defensive behaviors is differentially marked by the LIM-containing homeodomain genes Lhx6 and Lhx9 [12,13]. In the embryonic subpallium, Lhx6 is expressed in tangentially migrating cortical interneurons [14-16], and is a direct transcriptional target of Nkx2-1, which is expressed in the embryonic MGE and POA [17,18]. In addition, our recent work has revealed that the embryonic telencephalic POA is a major novel source of MeA neurons [3]. Moreover, Nkx2-1-lineage cells have been shown to contribute to the anterior MeA, although their neurochemical fate was not determined [5]. Despite this developmental information, the lineages that contribute to the vast inhibitory neuronal diversity in the MeA remain largely unknown.

Based on the expression patterns of the morphogen Sonic hedgehog (Shh) as well as the Shh-responsive genes Nkx2-1 and Gli1 in the embryonic MGE and POA [19,20] and, at later developmental stages (by embryonic day 14.5 (E14.5)), expression of Shh in the MePV [2], we hypothesized that progenitor populations marked by these genes contribute to inhibitory neuronal diversity in the postnatal MeA. In this study, we find that Shh-, Nkx2-1- and Gli1-lineage cells generate inhibitory neuronal diversity in the MePD and MePV in a largely complementary manner. We further reveal a differential functional requirement for Nkx2-1 in the development of the MePD compared to the MePV, and find that Shh-lineage cells generate three functionally distinct classes of neurons in the posterior MeA as defined by electrophysiological criteria. Thus, these data provide novel insights into the lineage and genetic mechanisms that generate the amygdala nuclei that mediate socio-sexual behaviors.

Results
Embryonic expression of Nkx2-1, Shh and Gli1 and recombined cells
Progenitor pools in the embryonic telencephalon are marked by spatio-temporal diversity in gene expression patterns. Expression of these genes is typically down-regulated as cells become post-mitotic. To permanently label these transiently marked populations, we used transgenic mouse lines that express Cre recombinase under transcriptional regulation of Shh, Nkx2-1 and Gli1 [5,21,22]. The developmental expression of these genes has been well-characterized in previous studies. At E11.5 and E13.5, Nkx2-1 is expressed throughout the MGE progenitor domains (pMGE 1-5) and the progenitor domains of the dorsal (pPOA1) and ventral (pPOA2) subdivisions of the embryonic POA [19]. In addition, previous studies [5] revealed that Nkx2-1-derived cells, using the same Nkx2-1-Cre mouse line as in this study, migrate toward the developing amygdala. At E11.5, Shh is expressed in the MGE mantle zone and in the progenitor domains of the POA (pPOA1 and pPOA2) and in a small region of the septum [19,23]. Gli1 is a transcriptional target of Shh and its expression, therefore, is observed near Shh-expressing domains [24-26]. Consequently, at early developmental time points Gli1 mRNA expression is observed in the ventral POA (pPOA2) and the sulcus between the lateral ganglionic eminence (LGE) and the MGE (pLGE4 and pMGE1) [20,27].

As TauGFP mice were generated with a nuclear localization signal for LacZ [28], we used X-gal staining to visualize recombined cells. Our short-term recombination analyses at E12.5 revealed waves of putative migrating cells emanating from the ventral telencephalon to the prospective developing amygdaloid region. In the ShhCre; TauGFP line we observed intense X-gal staining in the post-mitotic regions of the MGE, POA and ventral telencephalon (Figure 1A-D). Shh-lineage cells were also observed to a lesser extent in the developing thalamus, cerebral cortex and hippocampus (Figure 1A-D). This result is also consistent with a recent study using the ROSA26 LacZ reporter mouse that showed Shh-lineage cells also in domains of the POA and MGE [29]. As MeA neurons are generated from as early as E10 [3,4], we administered a single dose of tamoxifen (TM) at E9.5 (TM E9.5) in pregnant dams from Gli1CreER(T2); TauGFP crosses to label Shh-responder cells (Gli1+) at approximately E10.5. When analyzed at E12.5, X-gal staining was observed in a number of cells in the post-mitotic region of the MGE and POA (Figure 1E-H), similar to that observed for the ShhCre line. Furthermore, Foxg1 mRNA expression (Figure 1E-H, insets) on adjacent sections showed that the basally located short-term recombined cells in the ShhCre and Gli1CreER(T2) lines were located in the telencephalon as opposed to the diencephalon.

In summary, based on our analyses and previous studies, Shh, Nkx2-1 and Gli1 are spatially and spatially present in progenitor domains known to contribute to the MeA. Therefore, we next focused our analyses on the postnatal characterization of these genetically labeled cell populations.

Nkx2-1- and Shh-lineage cells preferentially accumulate in different subdivisions of the adult posterior MeA
To determine whether Shh-, Nkx2-1- and Gli1-derived cells contribute to the dorsal and ventral subdivisions of the posterior MeA (Figure 2A), we analyzed their distribution patterns at postnatal day 22 (P22). In Nkx2-1-Cre;
Interestingly, this distribution of Shh-lineage cells appeared largely complementary to that of the Nkx2-1-lineage population. In contrast to these primarily complementary patterns of recombination, Shh-responding cells (Gli1-derived) labeled at approximately E10.5 in Gli1CreER(T2); TauCre mice (n = 2). The inducible Gli1CreER(T2) line was used to identify Shh-responding cells during a temporally restricted time window. A rostro-caudal sequence shows LacZ+ cells in the POA and vTEL (arrows). Adjacent sections, processed for Foxg1 mRNA expression (insets), show that the recombined cells are in telencephalic domains of the forebrain. Abbreviation: LGE, lateral ganglionic eminence. Scale bar: 150 μm.

Figure 1 Short-term recombination in ShhCre and inducible Gli1CreER(T2) lines. (A-D) Rostro-caudal sequence from coronal sections of E12.5 ShhCre; TauCre brains (n = 2), where LacZ staining reveals recombined cells in the region of the MGE, POA and ventral telencephalon (vTEL) (arrows) and, to a lesser extent, in the thalamus (TH), cortex (CTX) and hippocampus (HP). (E-H) A single dose of tamoxifen (TM) administered at E9.5 labeled cells at approximately E10.5 in Gli1CreER(T2); TauCre brains (n = 2). The inducible Gli1CreER(T2) line was used to identify Shh-responding cells during a temporally restricted time window. A rostro-caudal sequence shows LacZ+ cells in the POA and vTEL (arrows). Adjacent sections, processed for Foxg1 mRNA expression (insets), show that the recombined cells are in telencephalic domains of the forebrain.

Characterization of inhibitory neuronal cell types in the posterior MeA
Neuronal diversity in the MeA has not been thoroughly explored. Therefore, we first wanted to characterize the molecular/neurochemical profiles of specific cell types in both the MePD and MePV. We reasoned that this analysis was a necessary prerequisite for subsequent analysis of the fate of Nkx2-1-, Shh- and Gli1-derived populations. To this end, we immunostained P22 wild-type mice with a battery of antibodies against proteins whose expression patterns are well-characterized in other forebrain structures but have not been directly compared to or between the subdivisions of the posterior MeA. As the MeA is largely composed of GABAergic neurons (reviewed in [1]), expression of Tbr1, which is a marker of excitatory neurons [30,31], was only observed in the ventral-most portion of the MePV, adjacent to the superficial layer of the MeA (Additional file 1A, B). Conversely both the MePD and MePV were highly immunoreactive for the GABA synthesizing enzymes GAD65/67 (Additional file 1C, D). Surprisingly, we found that expression of Parvalbumin and Neuropeptide Y, which are markers of inhibitory neuronal subtypes in the cerebral cortex and striatum, were nearly absent from the posterior MeA (Additional file 1E-H).

Interestingly, other markers showed differential expression patterns between both subdivisions. Calbindin, which labels approximately half of all cerebral cortical inhibitory neurons (reviewed in [32]), was observed scattered throughout the MePV, though as previously noted the distribution of Calbindin+ cells appeared to be more concentrated near the optic tract (Figure 3A, B) [33]. Immunoreactivity to neuronal nitric oxide synthase (nNOS) labeled many medium-sized cells, which showed strong cytoplasmic staining surrounding an unstained nucleus and immunoreactive neuropil located in the MePV (Figure 3C), although similar to Calbindin, more nNOS+ cells were observed in the medial part of the MePD, bordering the optic tract (Figure 3D). On the other hand, the expression of the Forkhead box transcription factor Foxp2, which marks a subpopulation of MeA neurons of unknown profiles [34], was highly expressed in both the main body of the MePV (Figure 3E), and the MePD (Figure 3F). We also observed that the interneuron marker Somatostatin (SST), which is expressed in the MeA [35], was found in scattered cells in both subdivisions (Figure 3G, H).

Nkx2-1-, Shh- and Gli1-lineage cells contribute to inhibitory neuronal diversity in the posterior MeA
Based on the above analysis of inhibitory neuronal distribution in the posterior MeA, we focused our fate analysis on the neuronal subtypes characterized by expression of Calbindin, nNOS, Foxp2, and SST. We performed immunolabeling for these markers in combination with an anti-β-galactosidase (β-gal) antibody to visualize nuclear staining in recombined cells from Nkx2-1-Cre, ShhCre, and Gli1CreER(T2) (TM E9.5) brains (Figures 4 and 5). This analysis revealed that 69% of the β-gal+ cells in the MePD...
Figure 2  Nkx2-1- and Shh-lineage cells preferentially fate-map to complementary subdivisions of the posterior medial amygdala. (A) Schema of a caudal coronal section of an adult mouse brain showing the nuclei of the amygdala. The medial posterodorsal nucleus (MePD; red) is located lateral to the optic tract (opt; purple), and the medial posteroverentral nucleus (MePV; green) is located ventrally and is bordered by the superficial layer of the medial nucleus (slMe; blue), which is a cell sparse fiber tract. (B,E,H) LacZ staining in postnatal day 22 (P22) Nkx2-1-Cre; Tau^mGFP brains (n = 2) revealed that most recombined cells are found in the MePD (E, arrows), with fewer cells in the MePV (H, arrows). (C,F,I) Shh-producing cells revealed the opposite whereby more LacZ+ cells were observed in the MePV (I, arrows) of P22 ShhCre; Tau^mGFP brains (n = 2) than the MePD (F, arrows). (D,G,J) A single dose of tamoxifen administered at E9.5 (TM E9.5: P22; tamoxifen at E9 and survival to P22) resulted in numerous LacZ+ cells in Gli1CreER(T2); Tau^mGFP brains (n = 2). In this case, more recombined cells were observed in the MePD than MePV (GJ, arrows) of the posterior MeA. Abbreviations: BLA, basolateral amygdala nucleus; BMA, basomedial amygdala nucleus; Ce, central nucleus; HY, hypothalamus; LaDL, dorsolateral subdivision of the lateral amygdala nucleus; PIR, pinform cortex; PLCo, posterolateral cortical amygdala nucleus; PRh, perirhinal cortex; STR, striatum. Scale bar: 600 μm (B-D); 200 μm (E-J).
were from the Nkx2-1-Cre lineage as opposed to 31% (3,733 versus 1,690 out of 5,423 cells, n = 2) from the Shh-Cre lineage, which was highly statistically significant (P < 0.01). The MePV revealed the exact opposite as 69% of β-gal+ cells were derived from the Shh-Cre lineage and the Nkx2-1-Cre lineage contributed only 31% (3,132 versus 1,384 out of 4,516 cells, n = 2), which was also highly statistically significant (P < 0.01). From a single dose of tamoxifen administered at E9.5, in the inducible Gli1CreER(T2) line we observed that 61% of recombined cells (1,976 out of 3,265 β-gal+ cells, n = 2), were located in the MePD. The contribution to the MePV, however, was lower (39%; 1,289 out of 3,265 β-gal+ cells, n = 2), and significantly different (P < 0.01) from the MePD. Thus, consistent with the qualitative observations shown in Figure 2, these data reveal a significant differential distribution of Nkx2-1- and Shh-lineage cells in the MePD and MePV, respectively, with Gli1-derived cells showing a more significant contribution to the MePD.

Next, we analyzed the percentage co-expression of the markers Calbindin, nNOS, FoxP2 and SST in recombined cells in all three Cre lines. Calbindin was expressed by almost half of the recombined cells in the MePD of Nkx2-1-Cre (43 ± 13%, n = 740 β-gal+ cells; Figure 4A, A'), Shh-Cre (46 ± 16%, n = 350 β-gal+ cells; Figure 4B, B') and Gli1CreER(T2) brains (39 ± 4%, n = 476 β-gal+ cells; Figure 4C, C'). As mentioned previously, nNOS is expressed in a relatively low number of cells in the MePD compared to the MePV. Interestingly, for the MePD, the contributions of the Nkx2-1 lineage (20 ± 10%, n = 1,200 β-gal+ cells; Figure 4D, D') and the Shh-lineage (22 ± 3%, n = 432 β-gal+ cells; Figure 4E, E') to the nNOS population were remarkably similar. This likely reflects the contribution of the preoptic domains pPOA1 and pPOA2 in which Nkx2-1 and Shh are co-expressed [19] and is consistent with previous work from our laboratory showing that Dbx1+ cells from the POA generate nNOS+ cells in the MeA [3]. Furthermore, from early-labeled Gli1CreER(T2) (TM E9.5) brains we found that MePD nNOS+ cells also derive from this Shh-responing population (15 ± 6%, n = 621 β-gal+ cells; Figure 4F, F'). In addition, FoxP2+ cells were derived from both Nkx2-1 (37 ± 10%, n = 903 β-gal+ cells; Figure 4G, G') and Shh (27 ± 12%, n = 477 β-gal+ cells; Figure 4H, H') lineages in the MePD, although this difference was not statistically significant (P = 0.48). Gli1-derived cells co-expressing FoxP2 were abundant in the MePD (23 ± 6%, n = 450 β-gal+ cells; Figure 4I, I'), showing that progenitor cells that respond to Shh signaling from an early developmental stage generate this inhibitory cell type in the posterior MeA. Interestingly, in the MePD we found a statistically significant (P < 0.01) difference in the generation of SST-positive cells from the Nkx2-1-lineage cells (10 ± 1%, n = 1116 β-gal+ cells; Figure 4J, J') versus the Shh-lineage cells (2 ± 2%, n = 431 β-gal+ cells; Figure 4K, K'). SST+ cells were also significantly generated from Gli1-expressing progenitors (20 ± 10%, n = 590 β-gal+ cells; Figure 4L, L'), which has also been observed for the cerebral cortex [36].

In the MePV, we also found that recombined cells from all three Cre lines showed high co-expression of Calbindin, indicating an inhibitory neuronal phenotype. As expected, a large number of recombined cells from the Nkx2-1 line co-expressed Calbindin (60 ± 14%, n = 282 β-gal+ cells; Figure 5A, A'), although the percentage values determined from ShhCre (46 ± 11%, n = 761 β-gal+ cells; Figure 5B, B') and Gli1CreER(T2) (TM E9.5) brains (48 ± 9%, n = 300 β-gal+ cells; Figure 5C, C') were slightly lower, but similar to the MePD values. As shown earlier,
nNOS expression between the two nuclei is disproportionately higher in the MePV (Figure 6), which is preferentially attributed to the Shh-lineage cells. Accordingly, we found that Shh\textsuperscript{Cre} recombined cells had high co-expression with nNOS (68 ± 11%, n = 884 β-gal\textsuperscript{+} cells; Figure 5E, E'), whereas Nkx2-1-lineage cells generated a much lower proportion of nNOS\textsuperscript{+} cells (13 ± 6%, n = 331 β-gal\textsuperscript{+} cells; Figure 5D, D'), which was statistically significant (P < 0.05) between both groups. The Shh-responding cell population also co-expressed nNOS (29 ± 11%, n = 461 β-gal\textsuperscript{+} cells; Figure 5F, F') in the MePV. Similar to the MePD, all recombined cells from both genetic lineages co-expressed FoxP2 in the MePV. Likewise, a higher percentage of co-localization was found in the Nkx2-1-Cre (32 ± 10%, n = 357 β-gal\textsuperscript{+} cells; Figure 5G, G') than the Shh\textsuperscript{Cre} brains (27 ± 13%, n = 940 β-gal\textsuperscript{+} cells; Figure 5H, H'), although this did not reach statistical significance. A similar percentage co-expression was observed in the Gli1\textsuperscript{CreER(T2)} brains (24 ± 10%, n = 310 β-gal\textsuperscript{+} cells; Figure 5I, I'). Finally, we quantified the occurrence of β-gal/SST double positive cells in the MePV. Interestingly, we also found a preferential bias for the Nkx2-1 lineage to generate SST\textsuperscript{+} cells in the MePV (23 ± 4%, n = 414 β-gal\textsuperscript{+} cells; Figure 5K, K'). Gli1-derived cells also had high co-expression with SST (24 ± 10%, n = 310 β-gal\textsuperscript{+} cells; Figure 5L, L').

In summary, these data revealed that: Nkx2-1- and Shh-lineage cells show a complementary distribution between the dorsal and ventral subdivisions in the adult posterior MeA - in contrast, Gli1-derived neurons contribute more predominantly to the MePD but heavily to both subdivi-

### Figure 4
Characterization of fate-mapped Nkx2-1- and Shh-lineage and Shh-responder cells in the postnatal medial posteroventral nucleus.

(A-L) P22 coronal sections of the MePD from Nkx2-1-Cre; Tau\textsuperscript{mGFP} (A,D,G,J), Shh\textsuperscript{Cre}; Tau\textsuperscript{mGFP} (B,E,H,K) and Gli1\textsuperscript{CreER(T2)}; Tau\textsuperscript{mGFP} (TM E9.5: P22) (C,F,I,L) brains showing dual immunofluorescence for β-galactosidase (β-gal\textsuperscript{+}) recombined cells (green) and the expression of the inhibitory neuronal markers Calbindin (top row), neuronal nitric oxide synthase (nNOS; second row), FoxP2 (third row) and Somatostatin (last row). (A’-L’) High-power images from corresponding boxed regions in (A-L) also show DAPI nuclear counterstaining (blue) in single optical sections from confocal images. Co-expressing cells (arrows), β-gal\textsuperscript{+}/marker\textsuperscript{-} (closed arrowheads) and β-gal\textsuperscript{-}/marker\textsuperscript{+} (open arrowheads) cells are indicated. (M-O) Pie charts depicting the percentage value of β-gal\textsuperscript{+} cells in each Cre line that co-expressed the corresponding neuronal marker. The images were taken from either the rostral or caudal portions of the posterior MeA in which the analysis was performed. Abbreviations: opt, optic tract. Scale bar: 200 μm (A-L); 100 μm (A’-L’).
neurons; nNOS+ cells, which are preferentially localized in the MePV, are primarily derived from Shh-lineage cells; Nkx2-1-lineage cells generated a higher proportion of SST+ cells in the MePD and MePV than Shh-lineage cells; Nkx2-1-derived cells showed a higher co-expression with FoxP2 than Shh-lineage cells, although within both lineages there was no discrimination between the MePV and the MePD; and early-generated Gli1+ progenitor cells generated inhibitory neuronal cells in proportions similar to those derived from the Nkx2-1 lineage.

Nkx2-1 mutant analysis demonstrates a greater functional role for Nkx2-1 in the MePD compared to the MePV

Having shown that the Nkx2-1 lineage contributes to neural diversity in the posterior MeA, we next sought to determine whether there is a functional requirement for Nkx2-1 in the generation of these cell types. To this end, we analyzed the expression of genes that mark the above-described inhibitory neurons in Nkx2-1 mutant mice [37]. This analysis was accomplished at E18.5, the latest stage possible due to perinatal lethality. First, we used GAD67 mRNA expression as a pan-inhibitory neuronal marker, which in controls is abundantly expressed throughout the whole telencephalon, including the hippocampus, cortex, striatum and amygdala (Figure 6A). In agreement with prior studies [18,38,39], we observed a major reduction in GAD67 mRNA expression throughout the Nkx2-1 mutant telencephalon, including the posterior MeA (Figure 6B). In particular, the MePD displayed intense GAD67 mRNA expression in the control (Figure 6A), which was reduced in the absence of Nkx2-1 (Figure 6B). The mutant MePV also showed a clear reduction in

Figure 5 Characterization of fate-mapped Nkx2-1- and Shh-lineage and Shh-responder cells in the postnatal medial posteroventr.
Later explained by the demonstration that directly regulates MeA that expressed were surprised to find a subset of cells in the posterior (Figure 6D). These ‘remnant’ Lhx6 expression was observed in the MePD than the MePV in control brains (Figure 6A, B). Next, we examined the expression of GAD67, a pan-inhibitory marker, is severely reduced in Nkx2-1 mutant mice (B) compared to controls (A). This is particularly evident in the MePD of the mutant (B, asterisk) as GAD67 mRNA expression appears stronger in the MePD in controls (A). (C,D) The Lhx6 mRNA expression in controls (C) is similar to that observed for GAD67, being notably stronger in the MePD than MePV. Although it is known that Nkx2-1 directly regulates Lhx6 expression, remnant Lhx6 mRNA expression was observed in the mutant posterior MeA, particularly around the MePD (D, asterisk). (E,F) The same was observed for Sst mRNA, whereby the expression in the control MePD (E) was significantly reduced in the mutant (F), although some remnant expression of Sst was observed in the MePD (F, asterisk). n = 4 for both control and Nkx2-1 mutant for each gene. Abbreviations: AMY, amygdala; CTX, cortex; HP, hippocampus; HY, hypothalamus; PIR, piriform cortex. Scale bar: A-F: 500 μm.

GAD67 mRNA expression compared to control (Figure 6A, B). Next, we examined the expression of Lhx6, which, similar to GAD67, exhibited notably more intense expression in the MePD than the MePV in control brains (Figure 6C). Prior studies have described a complete loss of Lhx6 in the Nkx2-1 mutant telencephalon [18,40], which was later explained by the demonstration that Nkx2-1 directly regulates Lhx6 transcription [17]. Therefore, we were surprised to find a subset of cells in the posterior MeA that expressed Lhx6 mRNA in the Nkx2-1 mutant (Figure 6D). These ‘remnant’ Lhx6 mRNA-expressing cells were located in the MePD, whereas the MePV was devoid of Lhx6 expression (Figure 6D). Subsequently, we examined Sst mRNA expression. In control, there were numerous Sst mRNA-expressing cells in the MePD, whereas the MePV contained only a few positive cells (Figure 6E). However, similar to Lhx6, we observed a number of remaining Sst mRNA-expressing cells that were primarily observed in the MePD (Figure 6F). This is in interesting contrast to other areas of the telencephalon, such as the cerebral cortex and hippocampus, that display a complete absence of Sst mRNA-expressing cells. A large number of Sst mRNA-expressing cells in the central nucleus was also maintained in the absence of Nkx2-1 (Figure 6F).

This analysis demonstrates that Nkx2-1 plays an important role in the specification of inhibitory neuronal subtypes in the posterior MeA. Surprisingly, however, we noticed that a population of cells that expressed Lhx6 and Sst mRNA persisted in the MePD in the Nkx2-1 mutant. Therefore, in interesting contrast to the cerebral cortex, a small population of posterior MeA Lhx6+ and Sst+ cells is not dependent on Nkx2-1 function.

To further explore the potential differential function of Nkx2-1 in the MePD and MePV, we examined the expression of genes that specifically mark the MePV, such as Cck, Shh, Lhx9 and nNOS in Nkx2-1 mutants (Figure 7). In the absence of Nkx2-1, there was no noticeable change in Cck mRNA expression in the mutant MePV compared to control (Figure 7A, B). Next, we analyzed Lhx9, which is expressed in the embryonic and postnatal MePV [2,12,41]. Previous studies have shown that Lhx9 mRNA expression is restricted to the superficial layer of the posterior MeA and the MePV [41]. In our hands, a prolonged exposure (up to 48 hours) revealed a signal, albeit weak, in the body of the MePV in both control and mutant brains. When compared to Lhx9 mRNA expression in the control MePV (Figure 7C), expression in the mutant was present but appeared weak (Figure 7D), possibly indicating a subtle effect by the absence of Nkx2-1. We next examined the expression of Shh, which is expressed in the embryonic MePV [2]. Previously it was shown that at earlier developmental stages in the Nkx2-1 mutant, most telencephalic expression of Shh mRNA is lost, with the exception of a small domain caudally that corresponds to the prospective amygdala [23]. When examined at E18.5, the expression of Shh mRNA that is observed in the control MePV (Figure 7E) is maintained in the mutant MePV (Figure 7F), although the expression domain is significantly smaller. Lastly, we examined the expression of nNOS, where expression is notably higher in the MePV than the MePD [33]. Permanent immunohistochemistry for nNOS showed strong expression in the control MePV (Figure 7G), which, interestingly, does not appear to be significantly altered in the Nkx2-1 mutant (Figure 7H).
In summary, the \textit{Nkx2-1} mutant analysis at E18.5 suggests a functional role for this gene in both the MePD and MePV, with primary effects on the MePD. This is consistent with the fate-mapping analysis (Figure 2) in which \textit{Nkx2-1}-lineage cells show a major contribution to the MePD.

Electrophysiological characterization reveals three distinct functional classes of \textit{Shh}-lineage neurons in the posterior MeA
The overlapping pattern of \textit{Shh} and \textit{Dbx1} expression in the embryonic POA [19], and the remarkable similarity in distribution and high percentage of nNOS co-localization between \textit{Dbx1}-derived [3] and \textit{Shh}-derived neurons (Figure 5) raised the question as to whether there were functional differences in these two lineages. To examine this we performed an electrophysiological characterization of \textit{Shh}-lineage populations in the posterior MeA. In order to visualize the recombined cells, we crossed \textit{Shh}\textsubscript{Cre} mice with the \textit{RYFP} reporter line, which our group has previously found to facilitate electrophysiological analysis [3,8]. Importantly, the distribution of recombined \textit{Shh}\textsubscript{Cre} cells in the \textit{RYFP} reporter line was identical to that of the \textit{Tau}\textsubscript{mGFP} reporter line (Additional file 2A, B). We carried out whole-cell patch clamp recordings of recombinant YFP\textsuperscript{+} neurons from \textit{Shh}\textsubscript{Cre}; \textit{RYFP} mice at P17 to P23. Each recorded neuron was filled with biocytin and slices were subsequently fixed and immunostained for nNOS and FoxP2. From this analysis, we could discriminate three populations of YFP\textsuperscript{+} neurons that exhibited distinct responses to a hyperpolarizing current injection, and also differed in their firing pattern and immunohistochemical profile (Figure 8; Additional file 3).

When hyperpolarized to -100 mV by injecting a step current (-40 to 120 pA, 1,000 ms), the first class of YFP\textsuperscript{+} neurons (class I; 9 out of 28 neurons) was characterized by clear depolarizing sag current (5.5 ± 2 mV; \( n = 9 \)), usually resulting in a single rebound spike upon repolarization (\( n = 7 \) out of 9; Figure 8A). A small depolarizing step current at rheobase value (10 to 40 pA, 1,000 ms) typically induced a series of three to four spikes separated by relatively large and slow rising afterhyperpolarization (amplitude = -11.2 ± 2.5 mV; decay half-time = 50.7 ± 14.7 ms). Larger depolarizing current steps (100 to 200 pA, 1,000 ms) were injected to determine their maximal discharge frequency (24 ± 5 Hz) and showed accommodating firing patterns (accommodation ratio = 0.46 ± 0.08).

Post hoc immunohistochemical analysis showed that all neurons in this class were nNOS\textsuperscript{+}/FoxP2\textsuperscript{+} (Figure 8B), with the exception of one cell that was immunonegative for both markers. Interestingly, both the electrophysiological and immunohistochemical profile of this first class of YFP\textsuperscript{+} neurons made them indistinguishable from nNOS\textsuperscript{+} MeA neurons generated from the \textit{Dbx1}-derived progenitors previously described by our laboratory [3].

A second class of YFP\textsuperscript{+} neurons (class II; 8 out of 28 neurons) could be distinguished by their inability to repetitively fire even when large depolarizing currents were injected (100 to 300 pA, 1,000 ms) (Figure 8C). These neurons exhibited a single or dual spike discharge
Figure 8 Electrophysiological characterization reveals three functionally distinct classes of Shh-lineage cells in the posterior medial amygdala. (A-F) Representative examples of three classes of Shh\textsuperscript{Cre}; RYFP\textsuperscript{+} neurons recorded by patch-clamp in current-clamp mode (A,C,E), filled with biocytin (B,D,F, green) and immunostained for nNos (B,D,F, red) and FoxP2 (B,D,F, blue). (B’-B’’’, D’-D’’’,F’-F’’’) Single optical sections from high-power confocal imaging of biocytin, nNOS or FoxP2 immunohistochemistry. Each neuron was recorded at its resting membrane potential, as given at the left of each group of traces. Neurons were subjected to a hyperpolarizing step (A,C,E, black) to test for the presence of sag current and rebound firing, a small depolarizing step corresponding to the rheobase of the neuron (A,C,E, red) and the minimal depolarizing step able to induce firing at a maximum frequency (A,C,E, grey). Current injection step values are indicated in the bottom of each recording and accordingly colored. The fraction and percentage of Shh\textsuperscript{Cre}; RYFP\textsuperscript{+} neurons corresponding to each class are indicated on the top of each group of traces. Scale bar: 80 μm (A-H); 40 μm (B’-E’’’).
upon depolarization. At rheobase value (20 to 80 pA), the discharge systematically rode on a slow-depolarizing envelope (amplitude = 18 ± 8 mV), suggesting the presence of a low-threshold Ca++ current (I_T). These neurons were exempt of sag currents when hyperpolarized to -100 mV but would exhibit a slow-depolarizing envelope upon repolarization, confirming the likely presence of large I_T currents in these cells. Interestingly, all of these neurons were FoxP2+ and most were also nNOS+ (6 out of 8 cells; Figure 8D).

The third class of YFP+ neurons (class III; 11 out of 28 neurons) was characterized by an irregular firing (frequency 5 to 20 Hz) in response to a large depolarization (Figure 8E). At rheobase value (20 to 80 pA), these neurons displayed a single early discharge of one to three spikes riding a small depolarizing envelope (10 ± 5 mV) and exhibited sharp fast-rising afterhyperpolarization (amplitude = -13.2 ± 1.5 mV; decay half-time = 15.4 ± 4.2 ms). In response to larger depolarizing current steps (100 to 200 pA, 1,000 ms), these cells exhibited a very variable maximal discharge frequency (11 ± 5 Hz) ranging from a single initial burst of three to five spikes to a series of two or three bursts of three to five spikes each. When hyperpolarized to -100 mV, these cells exhibited no or a minimal sag current (1.5 ± 0.8 mV) and small rebound I_T-like currents were observed (10 ± 5 mV). In most cells (9 out of 11 cells) these currents were not large enough to induce a rebound spike. All cells in this third class of cells were immunonegative for both nNOS and FoxP2 (Figure 8F).

Taken together, the results from the electrophysiological and post hoc immunohistochemical analyses revealed that Shh-lineage neurons generate functionally distinct inhibitory neuronal classes in the posterior MeA. We find that in addition to the class I neurons that are similar to neurons derived from the Dbx1+ lineage [3], at least two other classes of neurons have distinct electrophysiological and molecular profiles. Based on their expression of nNOS, a marker of MeA inhibitory projection neurons, as well as their morphology, we speculate that the classes I and II are inhibitory projection neurons. Our electrophysiological analysis shows that our Shh-lineage class III neurons that lack expression of both nNOS and FoxP2 exhibit electrophysiological characteristics that partially reflect a glutamatergic phenotype in the posterior MeA, which has been suggested by a prior study [42]. However, the restriction of these neurons to a Tbr1-negative region indicates that they may possess inhibitory character. Interestingly, we found that Shh-lineage cells gave rise to a subset of deep layer neurons in the cerebral cortex with a pyramidal morphology (data not shown), indicating that these progenitors can give rise to a subset of glutamatergic neurons. However, the vast majority of Shh-lineage cells in the amygdala are likely to be inhibitory because the MeA is primarily GABAergic in neuronal output, as shown by intense expression of GAD65 and GAD67 ([1] and this study) and because nNOS [43] and Calbindin, which are expressed in most Shh-lineage neurons, are markers of inhibitory neurons.

Discussion

Genetic fate-mapping is a powerful tool to elucidate the contribution of progenitor populations marked by individual genes to the cellular diversity found within the adult mammalian forebrain. Using this approach, we correlated the neurochemical phenotypes in the postnatal amygdala with defined domains of gene expression in the embryonic forebrain. With regard to the amygdala, genetic fate-mapping thus far has shown that excitatory neurons in the basolateral complex are derived from the pallial Emx1 lineage and the ventral pallial Dbx1 lineage [3,8,44]. In addition, pan-subpallial fate-mapping using the Dlx5/6 enhancer shows substantial recombination in the medial and central nuclei as well as the basolateral complex [45], consistent with subpallial sources of amygdala local interneurons and inhibitory projection neurons. Genes with more restricted expression patterns have shown that the subpallial embryonic POA, which expresses Dbx1 and Nkx5-1, contributes to neural diversity in the MeA [3,46]. Here, in agreement with a prior study [5], we show that cells expressing the transcription factor Nkx2-1, which is expressed in the embryonic MGE and POA [18,19], also contributes to inhibitory neuronal diversity in the MeA. Importantly, we significantly extend this knowledge by showing: 1, that Nkx2-1-lineage cells have a biased contribution to the dorsal subdivision of the posterior MeA (MePD); 2, consistent with this, Nkx2-1 functions to a greater degree in the development of the MePD as opposed to the MePV; 3, Shh-lineage cells generate complementary patterns of cellular diversity in the ventral subdivision of the posterior MeA, with a greater bias toward generation of MePV neurons; 4, Shh-responding (Gli1CreER(T2)) progenitor cells contribute to posterior MeA neural diversity from approximately E10.5 (Figure 9); and 5, neurons derived from the Shh-lineage are a functionally diverse group consisting of at least three classes of neurons as defined from unique combinations of electrophysiological and neurochemical profiles.

Shh- and Nkx2-1-lineage cells generate distinct classes of inhibitory neurons in the posterior MeA

Shh is the most studied of the Hedgehog family of secreted glycoproteins and is a potent morphogen that plays a crucial role in ventral patterning of many tissues, including the forebrain, midbrain, cerebellum, spinal cord and the limb (reviewed by [47-52]). Here, we used a Cre line under the transcriptional regulation of Shh [22] to indel-
Therefore, this subpopulation likely derives from the ventral subdivision of the POA (pPOA2), which expresses Dbx1 [3]. Thus, similar to the generation of midbrain dopaminergic neurons [53], Shh-lineage cells appear to give rise to diverse neural subtypes in the telencephalon.

The co-expression of FoxP2 with nNOS in the class II Shh-derived neurons is also quite intriguing. FoxP2 has been identified as essential for development of brain circuitry involved in speech in humans and vocalization in other vertebrates. Mutations in FoxP2 have also been implicated in both language disorders and autism, the latter of which is characterized by both language and amygdala-based impairments (reviewed in [54,55]). Therefore, it is possible that the electrophysiological and marker diversity of Shh-lineage neurons underlies important differences in both pathways of connectivity and function of MeA output neurons. For example, as the MeA is a central component of circuitry that regulates innate behaviors such as feeding, reproduction, aggression and maternal bonding, perhaps these different Shh-lineage populations differentially modulate these behaviors. Developmentally, it is also possible that transcription factor genes that show discrete and overlapping expression domains within the POA, such Dlx1, Nkx6-2, Nkx5-1 or Lhx2 [19,46], may selectively or combinatorially generate the class I to III electrophysiological subtypes that we describe here. In addition, as Shh and Dbx1 are expressed in the embryonic hypothalamus [23,56,57], which itself has been speculated to generate cells destined for the amygdala [4,6], further complexity or refinement of neural populations may be related to more precise origins within the developing forebrain.

We also find that, in contrast to Shh-lineage neurons, Nkx2-1-derived cells preferentially fate-map to the MePD, a nucleus that regulates reproductive behaviors. A previous study showed that Nkx2-1 lineage cells generate inhibitory cells in the MeA [5], in addition to the known and recently identified roles for Nkx2-1 in generating inhibitory cells for the cerebral cortex [5,18,58] and globus pallidus [29,59], respectively. We find that analogous to the cerebral cortex, a number of SST+ cells are Nkx2-1-derived, although another subset are derived from Shh-lineage progenitor cells. Nkx2-1-lineage cells show high co-expression with Calbindin and FoxP2 in both the MePD and MePV. Whereas Shh-lineage cells generate the vast majority of nNOS+ cells in the MePV, Nkx2-1-lineage cells generate an equivalent portion of nNOS+ cells within both nuclei. Therefore, this study reveals that inhibitory neural diversity in the posterior MeA is generated from Shh-expressing and Shh-responding (Nkx2-1-CRE+ and Gli1CREERT2) components of the Shh signaling pathway (see Figure 9 for schematic).
Function of Nkx2-1 in medial amygdala patterning
The transcription factor Nkx2-1 plays a major role in the generation of inhibitory neurons in the forebrain [5,18,60] and the expression of Nkx2-1 is largely dependent on Shh [61]. The notable exception to this is at the level of the prospective amygdala, where a small focal expression domain of Shh remains in Nkx2-1 mutants [23]. Interestingly, as previously shown, this remnant expression is coincident with the persistence of markers of the oligodendrocyte lineage [23]. Here, we show that Shh-lineage cells generate inhibitory neurons as shown by the differential expression of Calbindin, nNOS, FoxP2 and SST. Our gene expression analysis in Nkx2-1 mutant embryos revealed that Nkx2-1 has a significant functional role in the development of the MePD, where we observed major decreases in GAD67, Lhx6 and Sst mRNA gene expression. This finding is consistent with previous studies that have described a complete loss of Lhx6 mRNA expression in the Nkx2-1 mutant telencephalon [18,40]. Surprisingly, we observed a sparing of a subset of these GAD67+, Lhx6+ and Sst+ neurons in the MeA. As Nkx2-1 has more recently been shown to directly regulate Lhx6 transcription [17], our results suggest that in at least a subpopulation of amygdala neurons, this might not be the case. Indeed, it is likely that in this spared population, the remnant Shh expression domain in the embryonic caudal telencephalon in Nkx2-1-mutant mice is sufficient to directly specify these inhibitory neurons directly.

Fate-mapping of embryonic Gli1+ cells reveal the precocious generation of inhibitory neural diversity in both subdivisions of the posterior MeA compared to other forebrain structures
Shh signaling is mediated through the Gli family of transcription factors, which are homologous to the Drosophila zinc finger transcription factor cubitus interruptus, which mediates all Hedgehog signaling in the fly [62]. Of the three mammalian Gli family members, Gli1 and Gli2 primarily act as activators whereas Gli3 functions as a repressor [63-65]. Analysis of loss of function mutant mice has shown that Shh signaling is required for the initial transcriptional activation of endogenous Gli1, but not Gli2, in the forebrain [24]. Here, by administering a single tamoxifen dose at E9.5, we were able to label Gli1+ Shh-responder cells born from approximately E10.5 in the Gli1CreER(T2) line, a readout of Shh activity [21]. From this analysis, we show that cells that respond to Shh signaling also contribute to inhibitory neural diversity in the posterior MeA. Co-expression was observed with all inhibitory markers used in this study: Calbindin, nNOS, FoxP2 and SST. Furthermore, numerous LacZ+ recombined cells in the MePD and MePV in Gli1CreER(T2); TaucGFP (TM E9.5) brains lend credence to prior observations that indicate that neurons of the MeA are born earlier in development than those destined for other amygdala nuclei, such as those of the basolateral complex [3,4,7,8].

Conclusions
In this study, we have shown that Nkx2-1- and Shh-lineage cells preferentially fate-map to the dorsal and ventral subdivisions of the posterior MeA, with differential contributions to both local and projection inhibitory neurons. Previous tracing studies have identified that MePD efferent neurons project to three interconnected nuclei that are involved in reproductive behaviors: the medial preoptic nucleus, the ventrolateral part of the ventromedial hypothalamic nucleus and the ventral premammillary nucleus. In contrast, projections from the MePV involved in defensive behaviors terminate in the anterior hypothalamic nucleus and the dorsomedial portion of the ventromedial hypothalamic nucleus [12,66]. In a behavioral context, the reproductive and defensive actions are closely interrelated, and upon the appearance of threatening behaviors, a ‘gate-control’ mechanism ensures the rapid shut-down of reproductive behaviors to aid survival. The use of different Lhx transcription factors that delineate these projections to their hypothalamic targets may potentially serve as a neural substrate to integrate conflicting reproductive and defensive behavioral cues [12]. Here, we show the inhibitory neural diversity of these nuclei is generated from Shh-expressing and Shh-responsive cells, implicating the Shh-pathway component of MeA development. Therefore, the data from the current study provide novel insights into the gene network complexity and genetic mechanisms involved in the development of the MeA.

Materials and methods
Animal use
Mouse lines used in this study were: Swiss-Webster (SW; Taconic Farms, Albany, NY, USA), Nkx2-1 mutant [18], ShhCre [22], ROSA-YFP [67], TauGFP [28], Gli1CreER(T2) [21] and Nkx2-1-Cre [5]. Mice were maintained according to protocols approved by the Animal Welfare and use committee at Children’s National Medical Center, Washington DC, National Institutes of Health, Bethesda, MD, and NYU School of Medicine, NY. Nkx2-1 mutant mice were maintained on a SW background. The Nkx2-1-Cre and ShhCre lines were maintained on a C57BL/6 background, the Gli1CreER(T2) mice were kept on a SW background and the reporter mice on C57BL/6 × SW mixed backgrounds.

Animal crosses and genotyping
For staging of the embryos, midday of the day of vaginal plug detection was considered as E0.5. The day of birth was considered P0. To avoid potential sexual dimorphic differences in the MeA, only males were used for postna-
tal analyses. Mice were transcardially perfused with 4% paraformaldehyde (PFA) and brains were post-fixed in 4% PFA for at least 6 hours to overnight. Pregnant females from Gli1CreERT2; TauGFp crosses were administered a single dose (100 mg/kg in corn oil) of tamoxifen (Sigma, St Louis, MO, USA) by oral gavage at E9.5, and embryos collected at E12.5 or pups were perfused at P22. For early embryonic analysis (E10.5 to E13.5) pregnant dams were euthanized by CO2 inhalation and the embryos were harvested by Caesarian section and whole heads were fixed for 2 hours (for X-gal staining) or overnight in 4% PFA at 4°C. Pregnant dams of E18.5 litters were anaesthetized with Nembutal and the embryos were transcardially perfused. The isolated brains were post-fixed overnight at 4°C. Genomic DNA for genotyping was isolated by phenol:chloroform extraction. Mice for fate-mapping and electrophysiological analyses were identified by PCR for Cre [68] and yellow fluorescent protein (YFP) [69] for transgenic and reporter lines, respectively.

**Tissue preparation and histology**

After rinsing in PBS, postnatal brains were embedded in 4% agarose and sectioned coronally at a thickness of 50 μm using a Vibroslicer (Leica VT1000S, Leica, Nussloch, Germany). Embryonic brains were cryoprotected by graded sucrose immersion (10%, 20% then 30% overnight) and embedded in Tissue-Tek OCT Compound (Sakura Finetek USA Inc., Torrance, CA, USA). Coronal sections at a thickness of 20 (E10.5 to E13.5) or 30 μm (E18.5) were collected on glass slides, air-dried and stored at -20°C.

**Non-radioactive dioxygenin-labeled RNA in situ hybridization**

mRNA in situ hybridization was carried out as described previously [68] with the exception that the reaction product was visualized by NBT/BCIP (Roche) diluted in AP buffer (10 μl/100 mM NaCl, 100 mM Tris pH 9.5, 50 mM MgCl2). The following cDNA probes were used in this study: Cck, Foxg1, GAD67, Gli1, Lhx6, Lhx9, Nkx2-1, Shh and Sst.

**Immunofluorescence**

Free-floating adult vibratome sections were rinsed in PBS prior to blocking in 10% normal serum (NS) in PBS with Triton 0.03% (PBST; Sigma) for 1 hour at room temperature (RT), then overnight incubation in the primary antibodies diluted in PBST with 1% NS at RT for free-floating or 4°C for mounted tissue. The sections underwent three 10-minute rinses in PBS and were incubated in the appropriate donkey anti-Cy3, Cy5 (1:200; Jackson Immunoresearch, West Grove, PA, USA) or donkey anti-Alexa 488 (Invitrogen, Carlsbad, CA, USA) secondary antibodies, diluted in PBST with 1% normal donkey serum (NDS) for 2 hours at RT. The sections were rinsed twice then incubated in DAPI (1:1,000 in PBS; Sigma) for 10 minutes. The sections were mounted on Superfrost slides and coverslipped using Gel Mount aqueous mounting media (Sigma). The primary antibodies used in distinct combinations were: goat anti-β-gal (1:500; Biogen, Cambridge, MA, USA), goat anti-FoxP2 (1:500; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), goat anti-GFP (1:1,000; Novus Biologicals, Littleton, CO, USA), rabbit anti-β-gal (1:1,000; ICM Pharmaceuticals Inc, Costa Mesa, CA, USA), rabbit anti-Calbindin (1:1,000; Calbiochem, La Jolla, CA, USA), rabbit anti-nNOS (1:1,000; Sigma), rat anti-GFP (1:1,000; Nacalai USA, San Diego, CA, USA) and rat anti-SST (1:250; Millipore, Billerica, MA, USA).

**Permanent immunohistochemistry**

Free-floating adult vibratome sections and E18.5 cryostat sections were rinsed in PBS and incubated in PBS:methanol:30% H2O2 (Sigma) in an 8:1:1 ratio for 20 minutes at RT to quench endogenous peroxidases. The sections underwent further rinses in PBS prior to blocking of non-specific binding sites using 10% NS in PBST. The primary antibodies were incubated overnight at RT. Further rinses in PBS preceded and followed incubations for 1 hour at RT in biotinylated antibodies (1:500; Vector Labs, Burlingame, CA, USA) then extravidin-peroxidase (1:2,000; Sigma), all diluted in 1% NS in PBST. The reaction product was visualized using a DAB kit (Vector Labs) according to the manufacturers’ instructions. The reaction was stopped in Tris-buffered saline pH7.5 and the sections were mounted and/or coverslipped using permanent mounting media (Sigma). The primary antibodies used were as follows: goat anti-FoxP2 (1:1,000), goat anti-GFP (1:1,000), mouse anti-Parvalbumin (1:1,000; Sigma), rabbit anti-Calbindin (1:2,000), rabbit anti-nNOS (1:3,000), rabbit anti-Tbr1 (1:1,000; kind gift of R Hevner), rabbit anti-Neuropeptide Y (1:1,000; Immunostar, Hudson, WI, USA), rabbit anti-GAD65/67 (1:1,000; Millipore), and rat anti-SST (1:250).

**Electrophysiology**

Electrophysiological analysis of Shh-lineage cells in the posterior MeA was performed on male mice at P17 to P21 from ShhCre; ROSA26-YFP crosses. Mice were anesthetized with isoflurane, decapitated, and their brains were removed. Brain hemispheres were rapidly dissected on ice and cut into 300-μm thick coronal sections on a Leica VT1005 vibratome in an ice-cold oxygenated solution consisting of (in mM) 87 NaCl, 2.5 KCl, 1.25
composition (in mM): 124 NaCl, 3 KCl, 2.5 CaCl2, 1.3 MgSO4, 26 NaHCO3, 1.25 NaHPO4, 15 glucose; saturated with 95% O2/5% CO2 at room temperature (20 to 25°C).

Slices were transferred to the recording chamber and perfused with artificial cerebrospinal fluid at a rate of 1 to 2 ml/s. Cells were visualized using an upright Olympus BX51W microscope equipped with infrared and fluorescent illumination, Normasky optics, and infrared camera (all from Olympus). Cells were only chosen based on their YFP fluorescence and apparent viability (clear membrane, nucleus not visible) without bias in terms of location within the MePD or MePV, cell size or morphology. Patch electrodes had resistances between 3 and 6 MΩ when filled with the intracellular solution of the following composition (in mM): 130 K-glucerate, 10 NaCl, 2 Mg-ATP, 0.3 Na-GTP, 10 HEPES, 0.6 EGTA, biocytin 5 mg/ml, solution adjusted to pH 7.2, 275 mOsm (junction potential = 13 mV). Whole-cell recordings were obtained using a Multiclamp 700B (Molecular Devices, Sunnyvale, CA, USA) and recordings were monitored via a PC running pClamp 9.2 (Molecular Devices) and Mini Analysis (Synaptosoft Inc., Fort Lee, NJ, USA). In all experiments, data were filtered at 10 kHz during capacitance compensation and 5 kHz during subsequent data recording. The traces were digitized at 10 kHz. All voltage measurements and steps were corrected for a junction potential offset.

Data analysis
Analysis of in situ hybridization experiments was performed using bright-field microscopy (Olympus BX51, Olympus, Center Valley, PA, USA) and high-resolution digital images were captured under a 4x objective using an Olympus D570 camera. For fluorescence, digital photographs were obtained from epifluorescence microscopy (Olympus BX61) and selected for further analysis using a Zeiss LSM 510 META confocal microscope (Thornwood, NY, USA). For confocal analysis, each fluorophore was scanned sequentially and confocal images are presented as individual optical sections. Figures were prepared using Adobe Photoshop CS and Adobe Illustrator CS software (Adobe Systems, San Jose, CA, USA). Adjustments to contrast were applied across each image as a whole and equally to control and mutant brains. For anatomical considerations we included the embryonic expression domain terminology of Flames and colleagues [19]. For the adult anatomy we used the atlas of Franklin and Paxinos [70]. General anatomical consultations were made from prior publications on the posterior MeA [2,4,33,41]. All n values for immunohistochemistry, in situ hybridization and electrophysiology are detailed in each corresponding figure legend.

Cell quantification
To assess the co-expression of immunohistochemical markers in recombined cells from fate-mapping analysis in adults from the three Cre lines, we performed cell counts in the MePD and MePV from two rostro-caudal levels of the posterior MeA, corresponding to Bregma levels -1.46 mm and -1.82 mm. Cells were counted in a 400 μm x 400 μm boxed area through 10 adjacent sections (1-μm steps) of each Z-stack using the LSM510 software. For the fate-mapping analysis we used the TauGFP reporter mouse, which was designed with a nuclear localization signal for LacZ [28]. Therefore, we used an anti-β-gal antibody for nuclear staining of recombined cells, which, along with DAPI counterstaining, facilitated quantification of cells expressing other makers that were also primarily nuclear. The numerical data from the fate-mapping analysis are presented as the average percentage and standard deviation of recombined β-gal+ cells that co-expressed the second marker. Statistical significance was determined using an unpaired t-test (alpha value was set at 0.05) and was calculated using online GraphPad software (GraphPad Software Inc., La Jolla, CA, USA).

Additional material

Additional file 1 Expression patterns of excitatory and inhibitory neuronal markers in the MePD and MePV (A-H)
Permanent immunohistochemistry in P22 wild-type brains (n = 3) showing the expression of known excitatory and inhibitory neuronal markers in the MePD (A,C,E,G) and MePV (B,D,F,H) of the posterior MeA (A,B) Tbr1 (arrows) expression is largely devoid in these nuclei, which have primarily a GABAergic neuronal projection output, as indicated by intense GAD65/67 expression (C,D, arrows). Interestingly, classic inhibitory markers such as Parvalbumin (E,F, arrows) and Neuropeptide Y (NPY; G,H, arrows) are also sparsely observed in the MePD and MePV. Abbreviations: Tbr, intercalated nuclei of the amygdala; opt, optic tract; slME, superficial layer of the medial nucleus. Scale bar: 200 μm (A-H).

Additional file 2 ShhCre recombination in the Ryfp reporter mouse line (A,B) Permanent immunohistochemistry for GFP in ShhCre; RYFP brains (n = 2) shows that this reporter mouse shows the same distribution of Shh-lineage cells in the adult MePD (A, arrows) and MePV (B, arrows) as the TauGFP reporter line used in this study. Abbreviations: opt, optic tract; slME, superficial layer of the medial nucleus. Scale bar: 200 μm (A-H).

Additional file 3 Intrinsic electrophysiological properties of Shh-lineage cells in the posterior medial amygdala
Intrinsic electrophysiological properties of Shh-lineage cells in the posterior medial amygdala.

Abbreviations
β-gal: β-galactosidase; E: embryonic day; LGE: lateral ganglionic eminence; MeA: medial amygdala; MePD: medial posterior dorsal nucleus; MePV, medial posteroverentral nucleus; MGE: medial ganglionic eminence; nNOS: neuronal nitric oxide synthase; NS: normal serum; P: postnatal day; PBS: phosphate-buffered...
ered saline; PBST: PBS with Triton 0.03%; PFA: parafomaldehyde; pPOA: prefrontal area; POA: preoptic area; RT: room temperature; Shh: Sonic hedgehog; SST: Somatostatin; SW: Swiss-Webster; TM: tetroxifene; YFP: yellow fluorescent protein.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
RSEC and JGC devised the study. RSEC performed all of the experiments with the exception of the electrophysiology, and was the primary contributor to the design, data analysis and figure preparation. J-MM performed the electrophysiology experiments and analysis. RM provided further technical assistance, and along with LH, VHS and RPM provided preliminary data. LH and RPM generated the Gli3(loxp/lox) and Nkx2.1-Cre tissue, respectively. VG, SA and GF provided reagents and gave intellectual input to the study. RSEC wrote the majority of the manuscript with minor contributions from J-M (electrophysiology methods and results) and JGC. VG, SA and GF further edited and all authors approved the manuscript.

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