Topographical organization of mammillary neurogenesis and efferent projections in the mouse brain

Authors
Yongjie Hou, Qi Zhang, Hongzhi Liu, ..., Zhuhao Wu, Ling Gong, Miao He

Correspondence
gl19870102@126.com (L.G.),
hem@fudan.edu.cn (M.H.)

In Brief
Hou et al. identify progenitor-specific expression of Fezf2 in the embryonic mammillary region. They label different temporal cohorts of neurons by an intersectional method and uncover their rostral-dorsal-lateral to caudal-ventral-medial distribution and topographical organizations of their axonal projections. Fezf2 ablation reveals that it is dispensable for mammillary neuron specification and projection.

Highlights
- Fezf2 is expressed in multipotent mammillary progenitors but not post-mitotically
- Fezf2+ progenitors produce mammillary neurons in an outside-in fashion
- Mammillary efferents from different temporal cohorts are topographically organized
- Fezf2 is dispensable for mammillary neuron specification and projection
Topographical organization of mammillary neurogenesis and efferent projections in the mouse brain

Yongjie Hou,1,4 Qi Zhang,1,4 Hongzhi Liu,1,4 Jinyun Wu,1 Yun Shi,1 Yanqing Qi,1 Mengmeng Shao,2 Zhengang Yang,1 Jiangteng Lu,2 Zhuhao Wu,3 Ling Gong,1,* and Miao He1,5,*

1Institutes of Brain Science, Department of Neurology, State Key Laboratory of Medical Neurobiology and MOE Frontiers Center for Brain Science, Zhongshan Hospital, Fudan University, Shanghai, China
2Department of Anatomy and Physiology, Shanghai Jiaotong University School of Medicine, Shanghai, China
3Department of Cell, Developmental and Regenerative Biology, Department of Neuroscience, Icahn School of Medicine at Mount Sinai, New York, NY, USA
4These authors contributed equally
5Lead contact
*Correspondence: gl19870102@126.com (L.G.), hem@fudan.edu.cn (M.H.)
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SUMMARY

The mammillary body is a hypothalamic nucleus that has important functions in memory and spatial navigation, but its developmental principles remain not well understood. Here, we identify progenitor-specific Fezf2 expression in the developing mammillary body and develop an intersectional fate-mapping approach to demonstrate that Fezf2+ mammillary progenitors generate mammillary neurons in a rostral-dorsal-lateral to caudal-ventral-medial fashion. Axonal tracing from different temporal cohorts of labeled mammillary neurons reveal their topographical organization. Unsupervised hierarchical clustering based on intrinsic properties further identify two distinct neuronal clusters independent of birthdates in the medial nuclei. In addition, we generate Fezf2 knockout mice and observe the smaller mammillary body with largely normal anatomy and mildly affected cellular electrophysiology, in contrast to more severe deficits in neuronal differentiation and projection in many other brain regions. These results indicate that Fezf2 may function differently in the mammillary body. Our results provide important insights for mammillary development and connectivity.

INTRODUCTION

The mammillary body is a diencephalic nucleus located in the ventral posterior hypothalamus. It plays important roles in spatial navigation (Dillingham and Vann, 2019; Hitier et al., 2014), learning and memory (Vann and Nelson, 2015; Roy et al., 2017), and brain oscillation (Jiang et al., 2018; Ruan et al., 2017). It is composed of lateral and medial nuclei (LM and MM). Both LM and MM receive major inputs from hippocampus through fornix and tegmental nucleus (Tg) through mammillary peduncle. Both nuclei project to anterior thalamus (AT) through mammillothalamic tract (mtt) and to Tg through mammillotegmental tract (mtg) but connect with nonoverlapping subregions (Dillingham et al., 2015). For example, LM neurons project bilaterally to anterodorsal thalamus (AD) and dorsal tegmental nucleus (DTg), whereas MM sends ipsilateral projection to anteroventral (AV) and anteromedial (AM) thalamus and ventral tegmental nucleus (VTg) (Vann and Aggleton, 2004). Studies in rats and monkeys have revealed topographic projection patterns from different MM subdomains to AT and Tg (Hayakawa and Zyo, 1989; Allen and Hopkins, 1990; Shibata, 1992; Vann et al., 2007), but the organizational principles in mouse have not been systematically investigated.

The mammillary body is composed of excitatory projection neurons (Gonzalo-Ruiz et al., 1996, 1998) generated in an “outside-in” pattern (Markakis, 2002; Szabó et al., 2015; Altman and Bayer, 1986). Temporal fate specification is an important developmental mechanism in generating neuronal diversity and specifying neuronal identity (Oberst et al., 2019; Rossi et al., 2017; Holguera and Desplan, 2018). In many brain regions including the neocortex, neuronal birthdates not only dictate the localization of cell bodies but also closely correlate with other cellular features including gene-expression and axonal projection patterns (Molyneaux et al., 2007; Woodworth et al., 2012). However, no systematic analysis has been performed in the adult mammillary body to examine the relationship between neuronal birthdates and cellular features other than their perinatal spatial distribution (Szabó et al., 2015), largely due to the lack of genetic tools. A few transcriptional factors have been shown to play important roles in regulating mammillary development or maintenance. Nkx2.1 knockout mice exhibit extensive embryonic abnormality in the forebrain including the absence of the mammillary body (Kimura et al., 1996). Lnhx5 knockout mice lack Nkx2.1 expression in the prospective mammillary area at early embryonic stage and show an absence of mammillary structure later (Miquelajuregui et al., 2015). Foxb1 knockout mice suffer perinatal degeneration of the
mammillary body (Alvarez-Bolado et al., 2000b; Klotzii et al., 2001; Raduushkin et al., 2005). However, their constitutive expression in both progenitors and postmitotic neurons precludes the possibility to perform inducible genetic mapping to label and analyze cohorts of mammillary neurons with different birthdates using recombinase driver lines targeting these genes.

The zinc finger transcription factor Fezf2 is a critical regulator in forebrain development. Cortical progenitors expressing Fezf2 are multipotent and generate excitatory neurons in all cortical layers (Guo et al., 2013). It specifies deep-layer corticofugal projection neurons and is essential for the establishment of corticospinal projections (Chen et al., 2005; Lodato et al., 2014). It also regulates the development and fate specification of neurons in amygdala (Hirata-Fukae and Hirata, 2014) and olfactory bulb (Hirata et al., 2006b; Eckler et al., 2011) and is required for the establishment of diencephalic subdivisions (Hirata et al., 2006a). In the zebrafish forebrain, the coordinated activities of Fezf2 and several other transcription factors are crucial for establishing subdomains in the mammillary area and the specification of hormonal neurons (Wolf and Ryu, 2013). Although Fezf2 expression in the developing mouse hypothalamus has been previously reported (Hirata et al., 2004, 2006a, 2006b; Shimogori et al., 2010), its dynamic expression pattern in the mammillary region has not been systematically investigated. Importantly, its function in the developing mouse hypothalamus has not been studied, and whether it regulates mammillary neurogenesis or neuron specification remains elusive.

In this study, we examined Fezf2 in the developing mammillary body and uncovered a unique pattern of restricted expression in the neural progenitors. Such an expression pattern offers us an unprecedented opportunity to fate map Fezf2+ mammillary progenitors using Fezf2CreER. Intersecting with Nkx2.1Flp further enhanced the targeting specificity. Following inductions at different embryonic time points, we analyzed the distribution of cell bodies and axonal projections from mammillary neurons with different birthdates and uncovered their topographical organization principles. We also performed whole-cell patch-clamp recordings on acute slices and discovered two electrophysiologically distinct neuronal clusters that are not dictated by birthdates. Furthermore, we analyzed mammillary phenotypes in a Fezf2 germline knockout model. Despite the smaller size, the anatomy of the mammillary body and the chronological sorting and electrophysiological features of mammillary neurons remained largely unaffected in the absence of Fezf2. Such observations are in stark contrast with the severe phenotypes in cortex, amygdala, and olfactory bulb, suggesting Fezf2 functions in a context-dependent manner and is dispensable for the formation and maintenance of the mammillary body and its axonal projections. Our results uncovered the organization framework of mammillary neurons and projections and provided critical insights about Fezf2 function in the hypothalamus and moreover established useful tools for studying the development and function of the mammillary body.

RESULTS

Expression of Fezf2 during mammillary body development in the mouse

Previous studies have reported Fezf2 expression in the embryonic hypothalamus (Hirata et al., 2004, 2006a, 2006b; Shimogori et al., 2010), but its dynamic expression in the developing mammillary region has not been systematically investigated. We performed RNA in situ hybridization (ISH) for Fezf2 at 4 different developmental time points: embryonic day (E) 11.5, E13.5, E15.5, and the day of birth (P0) (Figure 1; Figures S1A–S1D). We also performed ISH for Foxb1 (Wehr et al., 1997; Alvarez-Bolado et al., 2000a) to delineate the mammillary region within the ventral hypothalamus marked by the expression of Nkx2.1 (Figure 1; Figures S1A–S1D). Fezf2 expression was similar to Foxb1 at E11.5, concentrating in the mammillary neuroepithelium (Figure 1, boxed region). At E13.5, the Fezf2 signal was prominent in the ventricular and subventricular regions (VZ/SVZ) along the ventral segment of the third ventricle (Figure 1; Figures S1A–S1C) but was nearly absent in the mammillary...
mantle region prominently stained by Foxb1 ISH and Nkx2.1 immunohistochemical (IHC) staining (Figure 1, asterisk).

In order to perform inducible expression patterns of these genes, we combined Fezf2 RNA ISH with Nkx2.1 IHC (Figure S1A). Co-localization of both signals was observed in the VZ/SVZ, but only Nkx2.1 was detected in the mantle region. We further quantitated the spatial distribution of signals from Nkx2.1 IHC and Foxb1/Fezf2 ISH to characterize their expression patterns in the mammillary region at E13.5 (Figures S1A and S1B). Signals in four evenly divided zones within a rectangular region of interest perpendicular to the ventricular wall were analyzed (Figure S1A). The Nkx2.1 IHC signal was relatively more evenly distributed across the four zones compared to the other two genes (Figure S1B), with slightly higher expression in the two lateral zones containing the mantle region than the two medial zones containing VZ/SVZ. In contrast, a much higher Fezf2 ISH signal was detected in the medial zones than the lateral zones. Foxb1 showed the opposite distribution pattern. Such spatial distribution patterns were maintained at E15.5 for all three genes (Figure S1C). At P0, Nkx2.1 was detected in both LM and MM, and Foxb1 was detected only in MM, while no Fezf2 was detected in LM or MM (Figure S1D). Immunostaining of Fezf2 protein further confirmed the expression pattern detected by RNA ISH in embryonic mammillary regions (Figures S1E and S1F).

These results suggest that mammillary Fezf2 expression is restricted to neural progenitors and is rapidly downregulated after their differentiation. This is in stark contrast to its expression pattern in other brain regions including cortex, amygdala, and olfactory bulb where it is expressed in both embryonic neural progenitors and a subset of differentiated neurons (Eckler and Chen, 2014). Moreover, we observed that, in other hypothalamic regions such as the premammillary nucleus (PM) and along the third ventricle, Fezf2 expression was sustained postnatally (Figure S1D). Therefore, such a progenitor-restricted pattern of Fezf2 expression may be unique to the mammillary body.

Inducible genetic fate mapping of Fezf2+ mammillary progenitors

The restricted expression of Fezf2 in progenitors makes it possible to perform inducible fate mapping in the mammillary body using Fezf2CreER (Figures S2A–S2C). In this strain, 2A-CreER is inserted in frame before the STOP codon of the endogenous Fezf2 gene. 2A is a self-cleavage peptide that allows CreER to co-express with Fezf2. Such a design ensures that CreER activity faithfully recapitulates endogenous Fezf2 expression without disrupting gene function (Figure S2A). Combined with a tdTomato red fluorescent protein (RFP) reporter, it allowed us to perform genetic fate mapping for the Fezf2-expressing (Fezf2+) mammillary progenitors. Oral administration of tamoxifen to a pregnant mother to induce CreER activity in E9.5 or E14.5 Fezf2CreER;Ai14 embryos successfully labeled progeny of Fezf2+ progenitors in the mammillary body with Cre-dependent RFP expression (Figures S2B and S2C). In contrast, inducing at birth failed to label any cell in the mammillary body, reconfirming the absence of Fezf2 in the postnatal mammillary body (Figures S2B and S2C). Postnatal induction-labeled neurons in cortex, hippocampus, amygdala, medial septum (MS), lateral hypothalamus (LH), ventromedial hypothalamus (VMH), and PM (Figure S3A), reconfirming Fezf2 expression in postmitotic neurons in these brain regions.

As embryonic Fezf2CreER induction also labeled cells in non-hypothalamic regions, we further bred it with Nkx2.1CreER (He et al., 2016) and the Cre/Flp-dependent intersectional reporter Ai65 (Madsen et al., 2015) to perform inducible fate mapping (Figure 2A). No RFP expression was detectable in the absence of either recombinase (Figure S2D). Such a combinatorial strategy got rid of cortical and hippocampal labeling but preserved MS and hypothalamic labeling (Figure S3B), thereby facilitating the observation of cellular morphology and neuronal projections. We first induced pregnant Fezf2CreER;Nkx2.1CreER::Ai65 mice at E9.5 (Figure 2A) and analyzed embryos at E11.5 (Figures 2B–2D) and E13.5 (Figures 2E–2G). At E11.5, RFP+ cells were observed in Nkx2.1+ ventral hypothalamic primordium (Figures 2B and 2D). Most of them were radial glia-like progenitors with emanating radial processes, residing along the ventral part of the ventricular wall and forming radial clusters (Figures 2C and 2G, arrowheads). We also observed a few axons extending caudally along the presumptive mammillotegmental tract (Figures 2D and 2G, arrowheads), indicating neuronal specification and differentiation had started at this time. At E13.5, many more differentiated cells exited the ventricular zone to enter mantle region (Figures 2E and 2F), and their axonal projections in the mammillotegmental tract became much more prominent (Figures 2G and 2H, arrowheads). We further analyzed induced brains at P56 and found that RFP+ cells showed broad distribution throughout the mammillary body including LM and MM. In addition to neurons, astrocytes were also labeled that were likely produced from the same pool of intersectionally targeted Fezf2+ progenitors after they finished neurogenesis (Figure 2H). These results suggest that Fezf2+ mammillary progenitors are multipotent at least on the population level.

Spatial and temporal patterns of mammillary neurogenesis from Fezf2-expressing progenitors

To examine the temporal profile of neurogenesis from Fezf2+ mammillary progenitors and its relation to spatial deployment of differentiated progeny in the mature mammillary body, we combined tamoxifen induction at E9 with 5-bromo-2′-deoxyuridine (BrdU) injection at E9.5, E11.5, E13.5, and E15.5 in different litters of Fezf2CreER;Nkx2.1CreER::Ai65 mice and analyzed the samples at P56 (Figures 3A–3C). Co-localization of RFP and BrdU was observed in the mammillary body when BrdU was injected prior to E15.5 (Figures 3B and 3C). Consistent with the classic view (Lengvári and Halasz, 1974) and previous report (Szabó et al., 2015), we found that BrdU+ cells were sorted in an “outside-in” gradient in the adult mammillary body (Figure S4). We further mapped RFP+ BrdU+ neurons and quantified their relative spatial distribution in the mammillary body along three axes: rostral-caudal (R-C), dorsal-ventral (D-V), and medial-lateral (M-L) (Figure 3D). The RFP+ BrdU+ cells labeled by BrdU injections at different times represent mammillary neurons with corresponding birthdates. This birthdating experiment revealed that the peak of mammillary neurogenesis was at E11.5 (Figure 3E). Importantly, clear shifts in spatial distribution were observed along all three axes for mammillary neurons with different birthdates (Figure 3F; Video S1). The
earlier-born cohort resided more rostral-dorsal-laterally, while the
later-born cohort resided more caudal-ventral-medially (Figure 3E;
Video S1). These results suggest that Fezf2+ progenitors produce
neurons throughout the entire time course of mammillary neuro-
genesis and their progeny reside in the entire territory of the
mammillary body following a rostral-dorsal-lateral to caudal-
ventral-medial pattern.

We further performed whole-cell patch-clamp recordings in
adult MM to probe whether Fezf2+ progenitor produce electro-
physiologically heterogeneous neuronal subpopulations. No significant difference
was detected between RFP+ and RFP− cells, and thereby we grouped them
together to perform unsupervised hierarchical clustering analysis. Resting mem-
brane potential was excluded from this analysis as a substantial proportion of
mammillary neurons showed sponta-
neous firing, which may render them incomparable to the silent neurons for
this feature. Two distinct clusters were re-
vealed by this analysis, which differ
significantly in 6 out of the 9 passive and
active features we have quantified (Fig-
ure S5A; Table S1). Cluster 1 is character-
ized with higher input resistance (Rin),
larger membrane capacitance (Cm),
larger action potential (AP) half-width,
longer AP rise time and decay time, and
slightly higher AP threshold than cluster
2 (Figure S5B). No difference was detected between RFP+ and
RFP− cells within either cluster, but most of the between-cluster
differences hold true within RFP+ or RFP− group (Figure S5B; Ta-
ble S1). These results demonstrate that mammillary neurons are elec-
physiologically heterogeneous and Fezf2+ progenitors are capable of generating both subpopulations. Interestingly, the spatial distribution of neurons in the two clusters were largely segregated. Compared to cluster 2, cluster 1 neurons resided more ventrolaterally (Figure SSC). This spatial segregation is
not absolute. Closely residing neurons could still fall into different clusters. But it is worth noting that the distribution bias of electrophysiological clusters does not match the dorsolateral to ventromedial sorting pattern determined by birthdates. This disagreement suggests that birthdate may not be a deterministic factor for the specification of the two electrophysiologically distinct subpopulations. In other words, neurons produced by the same pool of progenitors on the same day may still differ in their passive and active features and thereby may be able to respond differently to the same set of stimuli.

Topographic organization of mammillary efferents
Based on the chronological arrangement of mammillary neurons generated by Fez 

Figure 3. Spatial and temporal profiles of mammillary neurogenesis from Fezf2+ progenitors
(A) Scheme of BrdU birth dating. Mammillary neurons produced from Fezf2+ progenitors are labeled by tamoxifen (TMX) induction at E9.5 (red arrowhead) using Fezf2CreER::Nkx2.1Flp::Ai65. BrdU injections were performed at the indicated dates (green arrowheads), and colocalization analysis was performed at P66. (B) Representative images. (C) High-magnification images of the boxed regions in (B). No colocalization between RFP and BrdU was observed in E15.5 injected samples. Closed arrowheads: RFP cells with BrdU colocalization. Open arrowheads: RFP only and BrdU only cells. Scale bars: 200 μm in (B) and 50 μm in (C). (D) RFP cells with BrdU colocalization were registered into a reference mammillary body atlas for 3D visualization. Only representative E9.5, E11.5, and E13.5 samples were presented as no BrdU colocalization was observed with E15.5 injection. (E) The temporal profile of mammillary neurogenesis from Fezf2+ progenitors, with a peak at E11.5. (F) Quantitative analysis of normalized spatial distribution of BrdU-labeled RFP+ neurons in the mammillary body along the rostral-caudal (R-C), dorsal-ventral (D-V), and medial-lateral (M-L) axes. The blue plane in the top panels indicates the reference plane for binning. Along each axis, the mammillary body was evenly divided into 10 bins. The number of RFP+BrdU cells in each bin was counted and divided by the total number of RFP+BrdU in each mammillary body to calculate the percentage (%). n = 3 for each time point. Data are shown as mean ± SEM. The distribution was fitted with nonlinear Gauss curves (bottom panels). Cells born later distributed more caudal-ventral-medially. Dots represent data from individual sample. **p < 0.01, ***p < 0.001. For more statistical details, please refer to Table S3. See also Figure S4, Table S1, and Video S1.
the D-V and M-L axes (Figure 4D). Consistent with the absence of Fezf2 expression in the postnatal mammillary body, postnatal induction failed to label any neuron in the mammillary body but labeled tanycytes along the third ventricle (Figure 4E).

High levels of tdTomato expression from Ai65 reporter enabled anatomical tracing of axonal projections of labeled neurons (Figure S6). We performed whole-mount immunolabeling with volume imaging using a modified Adipo-Clear method (Chi et al., 2018) and achieved three-dimensional visualization of the entire path of mammillary efferents in a cleared and intact adult mouse brain for the first time (Figures S6B–S6D; Video S3). In addition to the prominent mammillothalamic and mammilotegmental tracts, we also observed a stream of axonal collaterals that bifurcated ventrally from the mtg and then ran caudo-rostrally and terminated in the reticular tegmental nucleus (RtTg), forming a third tract in the shape of a backward “L” (Figure S6B, arrowheads). Most prominent axon projections were observed in the AT, which was covered with very dense networks of beaded terminal arborizations (Figures S6C and S6D). The absence of axon projections to AT, Tg, or RtTg in postnatal-induced samples (data not shown) reassured that the axons we observed in these areas in the embryonic-induced samples were from mammillary neurons.

To verify the existence of synaptic contact, we performed vesicular glutamate transporter 2 (vGluT2) immunostaining to identify glutamatergic presynaptic terminals in the target brain regions. Abundant vGluT2+ puncta on RFP+ axons were observed in AT, RtTg, DTg, and VTg. Some of the RFP+vGluT2+ varicosities were closely opposing the NeuN+ cell bodies, indicating the establishment of perisomatic innervations (closed arrowheads, Figure S6E). However, many other RFP+vGluT2+ varicosities were not in contact with neuronal cell bodies (open arrowheads, Figure S6E), which could be innervating dendrites. We further quantified the size of RFP+ boutons and detected significant differences among different target regions. Boutons in the AT were the largest, followed by those in RtTg, DTg, and VTg (Figure S6F).

To further validate the existence of synaptic contacts, we performed channelrhodopsin2 (ChR2)-assisted circuit mapping (Figures 5A–5D). Following AAV2/9-hSyn-ChrR2-mCherry (AAV-ChrR2-mCherry) injection in the mammillary body (Figure 5A), we performed whole-cell voltage-clamping recordings in AT...
Figure 5. Topographic organizations of mammillary efferent projections

(A–D) Optogenetic confirmation of monosynaptic connections between the mammillary body and downstream targets. (A) AAV2/9-hSyn-ChR2-mCherry (AAV-ChR2-mCherry) was injected into the mammillary body. Fluorescence was more intense in the medial mammillary body (MM), but the high-magnification image of the boxed region confirmed that the lateral mammillary bodies (LM) were also infected. (B) Representative images for projections in anterior thalamus (AT) and representative traces of postsynaptic responses recorded in anterodorsal, anteroventral, and anteromedial thalamus (AD, AV, and AM). (C) Representative images for projections and representative traces of postsynaptic responses recorded in dorsal and ventral tegmental nuclei (DTg and VTg). (D) Quantification of response latency after laser stimulation. n = 9, 11, 21, 13, and 9 for AD, AM, AV, DTg, and VTg. 7 mice were injected and recorded.

(E–I) Axonal projections from mammillary neurons labeled by tamoxifen induction at different embryonic dates to the AT and Tg. Only in E9.5 and E11.5-induced samples were there projections to AD and DTg. (E) Representative images for AT projections. (F) Binarized and pseudocolored images of the boxed area in (E) to better illustrate the spatial patterns of mammillothalamic projections. (G) AT projection patterns of different temporal cohorts of mammillary neurons were

(legend continued on next page)
(Figure 5B) and Tg (Figure 5C) upon light activation of ChR2+ mammillary axons. Both the short latency of post-synaptic responses after laser stimulation (Figure 5D) and the preservation of synaptic responses in the presence of tetrodotoxin (TTX) and 4-aminoypyridine (4-AP) proved the existence of monosynaptic contacts (Figures 5B and 5C). Mammillary projections to RtTg was very restricted and relatively weak (Figure 5E; Video S3), making it difficult to recognize in acute brain slice. Therefore, we were not able to perform the recording in this region.

We further analyzed the organization pattern of axonal projections from different temporal cohorts of mammillary neurons to different downstream brain regions. The projection pattern of earlier-born cohorts can be deduced by subtracting the projection patterns of later inductions from those of early inductions. In general, the projections in E9.5 and E11.5-induced samples were indistinguishable from each other, but the projections in E13.5 and E15.5-induced samples were much more restricted (Figures 5E–5I). LM provides driver inputs to AD (Petrof and Sherman, 2009) and also projects to DTg. Consistent with the absence of LM neuron labeling in the E13.5 and E15.5-induced samples, projections to AD (Figure 5E) and DTg (Figure 5H) were only present in the E9.5 and E11.5-induced samples (Figure 5I). Clear trends of spatial shifts were also observed in AV/AM, RtTg, and VTg. Projections to AT followed an early-lateral to late-medial pattern in both AV and AM (Figures 5F and 5G). Projections to RtTg were concentrated in the dorsomedial part of its pericentric region (RtTgP), with later-born neurons projecting to a more restricted diagonal stratum surrounded by the projection domain of earlier-born neurons (Figure 5H). Projections to VTg followed an early-medial to late-lateral pattern. Together with the neuronal somata distribution data, our results uncovered the topographic organization principles of axonal projections from different temporal cohorts of MM neurons. Early-born MM neurons reside more rostral-dorsal-laterally and project to lateral AT and medial VTg, whereas late-born MM neurons reside more caudal-ventral-medially and project to dorsomedial AT and lateral VTg (Figure 5J).

Normal production and specification of mammillary neurons in the absence of Fezf2

Fezf2 plays important roles in cell-type specification and axonal development in many other forebrain regions and has also been implicated in regulating mammillary developmental and neuron specification in zebrafish. Therefore, we wondered whether it also participates in regulating mammillary development in the mouse brain. We generated a Fezf2 knockout allele (Fezf2<sup>Δ/Δ</sup>) (Figures 6A and 6B). Consistent with previous report (Chen et al., 2005), Fezf<sup>Δ/Δ</sup> mice produced Fezf2<sup>-/-</sup> offspring at a Mendelian ratio; Fezf2<sup>-/-</sup> mice were viable and fertile, exhibiting lower body and brain weight with smaller hippocampus and absence of the corpus callosum.ISH confirmed the absence of Fezf2 mRNA in embryo (Figure 6C, left panels) and adult (Figure 6F). To rule out possible compensatory expression of the other Fez family member Fez1 in the absence of Fezf2, we also performed RNA ISH for this gene. In the control Fezf2<sup>Δ/WT</sup> mice, Fez1 was expressed in the developing amygdala (Figure 6C, arrowheads in right panels) but not in hypothalamus. This expression pattern was maintained in the Fezf2<sup>-/-</sup> mouse brain.

Interestingly, the mammillary body remained largely normal in the absence of Fezf2. The anatomical division of LM and MM and the expression of Foxb1 expression in the MM were unaffected (Figure 6D). The size of the mammillary body was reduced (Figures 6E and 6F), but the extent of reduction was not significant if normalized to total brain size (Figure 6F). A concordant increase of neuronal density was observed, suggesting that neuronal production is not affected (Figure 6F). Consistent with this observation, E12.5 BrdU birthdating experiments showed that the spatial distribution pattern determined by birthdate was unaffected (Figure 6G). Therefore, despite its expression in the mammillary progenitors, Fezf2 seems to be dispensable for mammillary neurogenesis.

Fezf2 has been shown to play critical role in regulating cortico-spinal projections (Chen et al., 2005). To check whether it also participates in regulating mammillary projections, we performed Nissl staining and anterograde viral tracing using AAV. Sagittal view of Nissl-stained brain sections revealed that the mammillo-thalamic tract was still prominent in the mutant brain (Figure 6H). We further performed anterograde tracing using the AAV2/9-CAG-Gcamp6S virus. AAV was injected into the medial mammillary body, and the mammillary efferent was labeled by green fluorescence from Gcamp6S (Figures 6I and 6J). Prominent projections to AV, AM, RTtG, and VTg were observed, and quantification of axon density showed no significant difference between the two genotypes (Figures 6I and 6J). To prove whether Fezf2 ablation may affect topographic organizations of axonal projections despite the normal birthdate-dependent neuronal sorting and preservation of major axonal projections, we injected CTB into subregions of AD, AV, and AM and traced neuronal cell bodies in the mammillary body (Figure 6J). Comparable retrograde patterns were observed in the two genotypes. Both Fezf2<sup>Δ/WT</sup> and Fezf2<sup>-/-</sup> showed bilateral retrograde labeling from AD to LM, and ipsilateral retrograde labeling from AV/AM to MM. Consistent with the anterograde tracing results from genetically labeled temporal cohorts of MM neurons (Figures 5E–5J), lateral injections in both AV and AM retrogradely labeled MM neurons in the dorsolateral domain, while medial injections labeled neurons in the ventromedial domain. Therefore, the
topographic organization of mammillothalamic projections remained unaffected by Fezf2 ablation. These results suggest that Fezf2 is not essential for the establishment and maintenance of major mammillary efferents.

We further examined whether Fezf2 knockout affects electrophysiological properties of MM neurons by whole-cell patch-clamp recordings in acute slices of Fezf2KO/WT and Fezf2KO/KO brains (Figure 7A; Table S2). Only 2 out of the 9 parameters we measured showed mild but significant differences between the two genotypes (Table S2). When we performed the same kind of unsupervised cluster analysis for recorded neurons as in the genetic fate-mapping samples, we got very similar results: two distinct clusters were identified (Figure 7B), which differ significantly in Rin, Cm, and most of the AP features (Figures 7C and 7D; Table S2). Among them, the differences in Rin, Cm, AP half-width, and AP decay time consistently existed between clusters in both genotypes: cluster 1 is featured with higher Rin, smaller Cm, larger AP half-width, and longer AP decay time than cluster 2. The segregated spatial distribution of neurons in these two clusters was also evident in both genotypes: cluster 1 neurons resided more ventrolaterally compared to cluster 2 neurons (Figure 7E). Within each cluster, different sets of features were affected by Fezf2 knockout. Cluster 1 neurons showed a mild increase in AP half-width in the absence of Fezf2, while...
changes in cluster 2 neurons were responsible for the rest of the changes observed between the two genotypes except for Rin. These data suggest that knocking out Fezf2 does not affect the specification of two electrophysiologically distinct neuronal sub-populations residing in different spatial domains but affects different aspects of their intrinsic parameters. However, it is not clear whether these changes were due to a mammillary-autonomous effect of Fezf2 knockout or were secondary to the abnormalities in other brain regions caused by Fezf2 knockout. For example, the major input of the mammillary body is the hippocampus (Vann and Nelson, 2015), which is severely affected by Fezf2 knockout.

Taken together, our data suggest that Fezf2 is dispensable for the formation of the mammillary body and its major anatomical divisions, the production and specification of mammillary neurons, and the establishment of its major efferent projections. Intrinsic features of MM neurons are mildly affected by Fezf2 knockout, but it remains to be tested whether these effects are cell or brain region autonomous and if such changes may lead to circuit dysfunctions.

**DISCUSSION**

The mammillary body is a highly conserved brain structure from rodents to human and is arguably the first brain region to be

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**Figure 7. Electrophysiological properties of mammillary neurons were largely unaffected by Fezf2 knockout**

(A) Representative infra-red image of the recording site with one neuron (arrow head) patched by a glass pipette. Scale bar: 300 μm in the low-magnification image and 10 μm in the high-magnification image.

(B) Unsupervised hierarchical cluster analysis based on 8 out of 9 electrophysiological parameters listed in Table S2 identified 2 distinct clusters (clusters 1 and 2) from 49 Fezf2KO/WT neurons and 50 Fezf2KO/KO neurons recorded at P46–50. The x axis represents individual neurons, and the y axis corresponds to the linkage distance between cells measured by squared Euclidean distance, with smaller distance representing higher similarity. The limit between clusters (dashed blue line) was determined based on Thorndike method.

(C) Action potential (AP) sample traces. Insets: shorter timescale highlights the difference in AP half-width between the two clusters. Cluster 1 cells in both genotypes exhibited wider AP half-width than those in cluster 2.

(D) Only one feature of cluster 1 and two features of cluster 2 neurons showed mild but significant changes. Data are shown as mean ± SEM. Squares represent data from individual cell. NS p > 0.05; "p < 0.05; ""p < 0.01; """"p < 0.001. For more statistical details, please refer to Table S2.

(E) Manual registration of recorded cells revealed spatial segregation of neurons in the two clusters in the medial mammillary body in both genotypes. Recorded cells on the left half was mirror-imaged onto the right half to better illustrate the relative positions. Cluster 1 neurons tend to reside in the ventrolateral domain, while cluster 2 neurons tend to reside in the dorsomedial domain. See also Figure S5.
associated with memory (Vann and Nelson, 2015). Despite its functional importance, relatively few studies have been performed to genetically dissect its development and connectivity compared to other memory related structures like hippocampus. Here, we identified Fezf2 as a molecular marker for mammillary neuroprogenitors and utilized its unique expression pattern to perform genetic fate-mapping experiments. Unlike previously reported mammillary progenitor-expressing transcriptional factors including Nkx2.1 (Kimura et al., 1996), Foxb1 (Alvarez-Bolado et al., 2000b; Kloetzli et al., 2001; Radyushkin et al., 2005), and Lhx5 (Heide et al., 2015; Miquelajauregui et al., 2015), which continue to express in postmitotic mammillary neurons, Fezf2 expression is not retained in differentiated neurons. Therefore, it provided us with a unique opportunity to develop an intersectional fate-mapping approach and label different temporal cohorts of mammillary neurons by timed induction at different embryonic stages. The bright labeling by fluorescent reporter allowed us to not only quantify the spatial distribution of neuronal somata in the adult mammillary body and record their intrinsic electrophysiological features but also trace the trajectory of axonal projections and analyze the distribution of axon terminals in target brain regions. It is worth noting that although the somata distribution, downstream axon distribution, and birthdates were tightly coupled with one another, the intrinsic electrophysiological features were not. In order to examine the potential role Fezf2 played in mammillary body development, we generated a Fezf2 knockout model and analyzed the size, anatomical division, axonal projection, and cellular electrophysiology of the mammillary body. Interestingly, the mammillary body is largely normal except for reduced size and several mildly changed intrinsic features in the absence of Fezf2, indicating it is largely dispensable for mammillary neurogenesis, specification, and projection. This result is in stark contrast to that from zebrafish in which Fezf2 plays an essential role in the establishment of the mammillary area subdomains and the specification of two distinct neuron types (Wolf and Ryu, 2013).

Genetic targeting of the mammillary body

Very few methods exist for genetic labeling of mammillary progenitors and mammillary neurons. Some have employed Foxb1 to drive LacZ (Alvarez-Bolado et al., 2000b; Kloetzli et al., 2001; Szabó et al., 2011) or Cre expression (Zhao et al., 2008; Szabó et al., 2011, 2015; Heide et al., 2015; Zhang et al., 2017). However, the constitutive expression of Foxb1 in both progenitors and postmitotic MM neurons precludes its application in inducible fate mapping of neurons born at different times. Despite the limited selection of genetic targeting methods, most studies relied on anatomical approaches to identify the mammillary body and trace its efferent projections. An in utero transfection technique was developed to target mammillary progenitors by injecting plasmids directly in the third ventricle and electroporating at specific angle (Szabó et al., 2015; Haddad-Tóvolli et al., 2013), which is relatively technically demanding. Dye injection (Alpeeva and Makarenko, 2009; Makarenko, 2014) and virus injection were often used to label mammillary axons. Although feasible, the small size and the deep anatomical location of the mammillary body still present great challenges for precise labeling, especially for targeting subregions and for developmental studies. Therefore, it is desirable to develop more genetic methods that allow specific and precise labeling of mammillary progenitors and neurons to facilitate developmental, anatomical, and functional studies. Here, we combined Fezf2CreER with Nkx2.1Py to label mammillary progenitors with high specificity and performed inducible fate mapping and axonal tracing with high spatial resolution. With the ever-expanding resource of intersectional reporters (Madsen et al., 2015; He et al., 2016; He and Huang, 2018), this approach may also support other applications such as optogenetic and chemogenic activity modulation, or transynaptic viral tracing.

Expression and function of Fezf2 in the mammillary body

Fezf2 has been extensively studied in many other forebrain structures, especially in the cortex where it plays essential roles in regulating cell-fate specification and axonal projections of corticofugal neurons (Chen et al., 2008). In the cortex, Fezf2 is expressed in both progenitors (Guo et al., 2013) and postmitotic deep-layer neurons. Some studies suggest it functions as a strong selector gene instructing the acquisition of specific cell fate (De la Rossa et al., 2013; Lodato et al., 2014). In other brain regions including basolateral amygdala (Hirata-Fukae and Hirata, 2014) and olfactory bulb (Hirata et al., 2006b; Eckler et al., 2011), it is also expressed in both the progenitors and certain populations of postmitotic neurons. When Fezf2 is absent, neurogenesis, neuronal specification, and even neuronal survival were severely affected in these brain regions. In contrast, Fezf2 expression in the mammillary body seems to be restricted into progenitors. No Fezf2 mRNA was detected in the postnatal mammillary body. Peri- or post-natal inductions also failed to label any mammillary neurons but did label the brain regions mentioned above, as well as several other hypothalamic regions that also express Fezf2 embryonically. Therefore, the expression pattern of Fezf2 in the mammillary body seems to be quite unique in the sense that it is only enriched in progenitors. Interestingly, the mammillary phenotypes caused by Fezf2 knockout were rather mild compared to other brain regions. No drastic change in mammillary anatomy, birthdate-dependent neuronal sorting, axonal projection, or specification of the two electrophysiological clusters was observed. These data suggest the function of Fezf2 may be context dependent, and its selector function may require sustained expression in postmitotic neurons.

Neuronal specification in the mammillary body

Temporal patterning is an important developmental mechanism for orchestrating the production, specification, and wiring of neurons (Kohwi and Doe, 2013; Telley et al., 2019). In the neocortex, projection neurons with distinct laminar distribution and axonal projection pattern are generated in a stereotyped birth order (Molyneaux et al., 2007; Leone et al., 2008). Similarly, our birth-dating and fate-mapping experiments corroborated a previous report (Szabó et al., 2015) but provided a more comprehensive and quantitative view and demonstrated that the birth order dictates distribution of mammillary neurons generated from multiple Fezf2+ progenitors in a rostral-dorsal-lateral to caudal-ventral-medial fashion. In addition, our axonal tracing experiments revealed that projections from successive temporal cohorts of mammillary neurons were topographically organized.
Therefore, temporal patterning seems to play critical roles in regulating the production, specification, and wiring of mammillary neurons. Neurons in the mammillary body were traditionally regarded as only one cell type, despite substantial heterogeneity in their morphology including cell body size (Rose, 1939; Veazey et al., 1982; Seki and Zyo, 1984) and certain aspects of dendritic features (Allen and Hopkins, 1988). A recent study performed single-cell RNA sequencing (RNA-seq) and discovered extensive molecular heterogeneity in the mammillary body (Mickelsen et al., 2020). Our slice recording results revealed another dimension of mammillary neuron heterogeneity. Importantly, the unsupervised hierarchical clustering analysis identified two distinct clusters that reside in spatially segregated domains in the MM. Although whether they represent bona-fide subtypes requires further analysis of other cellular features such as molecular signatures and morphological features, the fact that their distribution patterns were independent of those determined by birthdate suggests that there may be other mechanisms at play in specifying mammillary neuronal identity in addition to temporal patterning. Whether and how the differences in spatial distribution, electrophysiological properties, gene-expression profiles, and projection targets correlate with one another, which one(s) may be the deterministic features for neuronal subtypes, and what are the functional significances remain elusive. The genetic tools developed and the organization rules discovered in this study laid solid foundation for future inquiries, but dedicated studies incorporating other techniques that have higher spatial resolution and molecular specificity will be required to address these important questions.

Organization principles of mammillary circuitry
Inducible genetic targeting allowed us to label different temporal cohorts of mammillary neurons residing in different spatial domains and trace their axonal projections to downstream targets. Whole-mount immunostaining and imaging of cleared brain allowed us to achieve a complete view of the entire trajectory of efferent mammillary axons. There are two major branches stemming out of principle mammillary tract, mtt, and mtg. We observed a third branch bifurcating out of mtg, running ventrally then turning rostrally to innervate RtgP. Obvious differences in the density of axonal terminals and size of covered area in different target regions were also observed in this dataset. Closer inspection of immunostained brain sections further revealed differences in the size of presynaptic boutons. The large bouton size at perisomatic sites in AT corroborates with the idea that the MB-AT pathway provides driver inputs (Petrof and Sherman, 2009). Whether the projections to Tg also provide driver inputs remains elusive. Besides, we discovered that the topographic schemes of projection from the mammillary body to different targets also differ. The projections to AT seem to follow a lateral-to-lateral, medial-to-medial rule, while the projections to VTg seem to follow an opposite rule.

In summary, the present study elucidates the expression pattern of Fezf2 during mammillary body development and demonstrates that it is largely dispensable for mammillary neurogenesis, neuronal specification, and major projections. The intersectional inducible genetic labeling strategy presented in this study not only allowed us to uncover the organization principle of mammillary neurons and projections but also provides powerful tools for studying mammillary body development connectivity and function.

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SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2021.108712.

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AUTHOR CONTRIBUTIONS
M.H. conceived and supervised the study. M.H., Y.H., and L.G. wrote the manuscript. Y.H. performed genetic labeling, BrdU birthdating, RNA ISH, and most of the immunostaining. Q.Z. performed virus and CTB tracing and
contributed immunostaining. H.L. performed slice recording and ChR2-assisted circuit mapping. J.W. performed RT-qPCR. L.G., H.L., Q.Z., M.S., Y.S., and J.L. contributed data analysis, figure production, and manuscript editing. Y.Q. contributed immunostaining. Z.W. performed whole-brain immunostaining and imaging. Z.Y. contributed RNA ISH.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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## STAR METHODS

### KEY RESOURCES TABLE

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**Experimental models: organisms/strains**

| Mouse: Ai14: B6.Cg-Gt(ROSA) 26Sor1tm1(CAG-tomato) Hey/J | The Jackson Laboratory | Jax 007914 |
| Mouse: Ai65: B6;129S-Gt(ROSA) 26Sor1tm5.1(CAG-tomato) Hey/J | The Jackson Laboratory | Jax 021875 |
| Mouse: Fezf2CreER | Z. Josh Huang (Matho et al., 2020) | N/A |
| Mouse: Fezf2KO | This paper | N/A |
| Mouse: Nkx2.1Fbp: Nkx2-1tm2.1(Fbp)2/J | The Jackson Laboratory | Jax 028577 |

**Oligonucleotides**

| Fezf2-koF: GCAAGGTGAGGCCTA CGGTTC | This paper | N/A |
| Fezf2-koR: GCCCCGAGGCTACAAT TTACCTC | This paper | N/A |
| Fezf2-F: CGTCACCGGCCACTTCCCAT | This paper | N/A |
| Fezf2-R: CATTGAACACCTTGCCGCAC | This paper | N/A |
| Foxb1-F: CCATCCCAGTGCCCATCAAG | This paper | N/A |
| Foxb1-R: ATCTTTAGAAGCCCGGGAGG | This paper | N/A |
| Fezf1-F: TGATGATCCCTTTTGTCCC | This paper | N/A |
| Fezf1-R: TACCGAAGTGCGTCTTG | This paper | N/A |
| Fezf2-qPCR-F: ACCACGTTCCTATCCCAT | This paper | N/A |
| Fezf2-qPCR-R: CTTTGCGCCTCTG | This paper | N/A |
| Actin-qPCR-F: TTTGCAGCTCCTTGCTTG | This paper | N/A |
| Actin-qPCR-R: CCCATCGCTCCACCATAC | This paper | N/A |

**Software and algorithms**

| GraphPad Prism 7.0 | GraphPad Software | http://www.graphpad.com/scientific-software/prism/ |
| Neurolucida | MBF Bioscience | https://www.mbfbioscience.com/neurolucida |
| Photoshop CS6 | Adobe | https://www.adobe.com |
| IMARIS | Bitplane | https://imaris.oxinst.com |
| OrinPro 8.5 | OrinLab | https://www.originlab.com |
| MATLAB | Mathworks | https://www.mathworks.com |
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Miao He (hem@fudan.edu.cn).

Materials availability
All unique/stable reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement with reasonable compensation by requestor for their processing and shipping.

Data and code availability
All software used is available either commercially or as freeware. All other data and code are available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

All mouse studies were carried out in strict accordance with the guidelines of the Institutional Animal Care and Use Committee of Fudan University. All husbandry and experimental procedures were reviewed and approved by the same committee. Experiments were conducted as sex unbiased and both male and female mice were used. Age/developmental stages of analysis were marked in the figure and figure legends. Nkx2.1\textsuperscript{Flp} (Jax 028577) (He et al., 2016), Ai65 (Jax 021875) (Madisen et al., 2010), Ai14 (Jax 007914) (Madisen et al., 2010) have been reported previously. The Fez2\textsuperscript{CreER} mouse line was generated and characterized by Miao He in Z. Josh Huang’s lab at Cold Spring Harbor Laboratory. T2A-CreER was inserted in frame with Fez2 gene before the STOP codon by homologous recombination in C57/black6 ES cell line. Heterozygous male founders were generated by tetraploid complementation using targeted ES clones. More details are available in a preprint manuscript (Matho et al., 2020). The Fez2\textsuperscript{KO} allele was generated by inserting a splicing acceptor (SA) -nLacZ-T2A-tTA cassette in the second intron of Fez2 gene using homologous recombination. Gene targeting vector was generated using PCR-based cloning approach as described before (He et al., 2016). More specifically, a 4.5 kb 5’ homology arm, a 5.1 kb 3’ homology arm, a SA-LacZ-2A-pA gene trap cassette, and a loxP flanking Neo positive selection cassette followed by exon2, were sequentially cloned into a building vector containing the DTA negative selection cassette to generate the targeting vector. Targeting vector was linearized and transfected into a C57/black6 ES cell line. ES clones were first screened by genomic PCR, then confirmed by Southern blotting using appropriate DIG-dUTP labeled probes. One positive ES cell clone was used for blastocyst injection to obtain male chimera mice carrying the modified allele following standard procedures. Chimera males were bred with C57BL/6J females to confirm germline transmission. Heterozygous F1 siblings were bred with one another to establish the colony. Targeting vector construction, ES cell transfections and screening, blastocyst injections and chimera breeding were performed by Biocytogen Co, China.

METHOD DETAILS

Tamoxifen induction
Tamoxifen (SIGMA T5648-5Q) was prepared by dissolving in corn oil (20 mg/ml) at room temperature with constant rotation overnight, and administered to pregnant mice by gavaging under isoflurane anesthesia at a dose of 0.05mg per gram body weight at appropriate stages of pregnancy.

BrdU administration
BrdU was prepared by dissolving in 0.9% saline (10mg/ml) and intraperitoneally injected to pregnant mice at a dose of 0.1mg per gram body weight, at appropriate stage of pregnancy.

Genomic PCR
Genomic DNA was prepared from mouse tails. Tissue was lysed by incubation in tail lysis buffer (Viagen, 102-T) with 0.1 mg/ml proteinase K overnight at 55°C followed by 45 min at 90°C in an air bath to inactivate proteinase K. The lysate was cleared by centrifugation at maximum speed (21,130 G) for 15 min in a table-top centrifuge. Supernatant containing genomic DNA was used as the PCR template for amplifying DNA products. The following primers were used:

- \textit{Fez2-kF}: 5' - GCAAGGTGAGGCCTACGGTTC - 3'
- \textit{Fez2-kR}: 5' - GCCCGGAGGCTACAATTTACCTC - 3'

RT-qPCR
RNA was extracted usingTRIZol (Invitrogen 15596018), and cDNA was synthesized using SuperScriptIII reverse transcriptase (Invitrogen 18080093). SYBR green qPCR mix (TQ green Premix Ex TaqII,Takara RR820A)was used to perform qPCR (Applied Bio-
systems, 7300 Real Time PCR System) according to the manufacturer’s instructions. Expression of Fezf2 was normalized to that of Actin using the ΔCt method. The following primers were used to detect Fezf2 and Actin:

Fezf2-qPCR-F: 5'- ACCACGTTCTTATCCCAT – 3'
Fezf2-qPCR-R: 5'- CTTGCGCAGCTGGTACAC – 3'
Actin-qPCR-F: 5'- TTTGAGCTCCTTGCGTG-3'
Actin-qPCR-R: 5'- CCATTCCCACCACATCAC-3'

**Brain slice immunostaining and imaging**

Postnatal mice were anaesthetized by intraperitoneal injection of 1.5% sodium pentobarbital (0.09 mg/g body weight) then transcardially perfused with saline followed by 4% paraformaldehyde (PFA) in 0.1 M PB. Brains were dissected out and post-fixed overnight in 4% PFA at 4°C and sectioned at 50 μm using a vibratome (Leica 1000S); or transferred into 30% sucrose in 0.1 M PB for cryoprotection, OCT embedded, and sectioned at 20 μm using a cryostat (Leica CM1950). For Fezf2 immunostaining, brains were post-fixed for 0.5 hr in 4% PFA at room temperature; antigen retrieval of 20 μm cryosections was performed prior to blocking by boiling for 3 min in 10 mM citrate buffer (pH6.0). Embryonic brains were directly dissected out, post-fixed overnight, cryoprotected, OCT embedded and sectioned at 12-18 μm. For BrdU immunostaining, sections were postfixed overnight, blocked for 0.5 hr, then incubated with the following primary antibodies in the blocking solution at room temperature overnight: Nkx2.1 (rabbit polyclonal antibody, 1:500, Santa Cruz Biotechnology, SC-13040), RFP (goat polyclonal antibody, 1:2000, SIGEN AB0081-200; rabbit polyclonal antibody, 1:2000, Rockland 600-401-379), vGluT2 (Guinea pig polyclonal antibody, 1:500, Millipore, ab22521), NeuN (mouse monoclonal antibody, 1:500, Millipore, MAB377), GFP (chicken polyclonal antibody, 1:1000, Aves Labs, GFP-1020), BrdU (rat polyclonal antibody, 1:1000, Accurate Chemical OBT0030), Fezf2 (rabbit polyclonal antibody, 1:200, Immuno-Biological Laboratories, 18997). Sections were then incubated with appropriate Alexa Fluor dye-conjugated IgG secondary antibodies (1:500, Thermo Fisher Scientific; 1:400, Jackson Lab) or CF dye-conjugated IgG secondary antibodies (1:250, Sigma). Samples were dehydrated with methanol gradient series (80%, 60%, 40%, 20%) in B1n buffer, 30 min for each step. Samples were then washed in B1n buffer for 1 hr, 2 hr, and reverse methanol gradient series (20%, 40%, 60%, 80%) in B1n buffer, 4 mL for each brain, 1 hr for each step; then 100% methanol for 1 hr; then overnight incubation in 2:1 mixture of DCM:methanol and a 1.5 hr incubation in 100% DCM the following day; then 100% methanol for 3 x 1 hr, and reverse methanol gradient series (80%, 60%, 40%, 20%) in B1n buffer, 30 min for each step. Samples were then washed in B1n buffer for 1 hr and overnight. The above procedures were done at room temperature with rocking to complete delipidation. The delipidated samples were then blocked in PTxwH buffer (PBS/0.1% Triton X-100/0.05% Tween 20/0.1% DMSO and 0.3 M glycine for 3 hr and overnight at 37°C, then washed with PTxwH for 1 hr, 2 hr, and overnight at room temperature. For staining, brain samples were incubated in primary antibody (rabbit polyclonal antibody, 1:400, Rockland 600-401-379) diluted in PTxwH for 14 days at 37°C. After primary antibody incubation, samples were washed in PTxwH for 1 hr, 2 hr, 4 hr, overnight, then 3 x 1 day, and then incubated in secondary antibody (Alexa Fluor 647 Donkey-anti-Rabbit IgG, 1:200, Thermo Fisher Scientific A-31573,) diluted in PTxwH for 10 days. Samples were then washed in PTxwH for 1 hr, 2 hr, 4 hr, overnight, then 3 x 1 day. Samples were finally washed in PBS for one day, then proceeded for clearing with iDISCO+ (Renier et al., 2016). Samples were dehydrated with methanol gradient with water, then 100% methanol, DCM/methanol mixture overnight, and 100% DCM for 1 hr twice the next day. Brains were finally cleared for 4 hr in dibenzyl ether and then stored in a fresh tube of dibenzyl ether before imaging with a LaVision Ultramicroscope II lightsheet fluorescence microscope using 4 x objective. The brain was scanned in horizontal orientation from both dorsal and ventral ends. The scans met in the middle of the sample. Volume imaging datasets were processed using Imaris x64 software (version 8.0.1, Bitplane) to generate the whole brain projection views and three-dimensional (3D) visualizations.

**RNA in situ hybridization**

In situ hybridization was performed using Digoxigenin (DIG)-labeled RNA probes on 12-20 μm cryosections as previously described (Zhang et al., 2016). Briefly, the sections were postfixed in 4% PFA for 20 min and washed in PBS three times, then treated with 2 μg/ml proteinase K in 50mM Tris (5mM EDTA) for 10 min, transferred to 4% PFA for 10 min, and washed in PBS for 5 min three times. Subsequently, sections were acetylated for 10 min (1.3% triethanolamine, 0.25% acetic anhydride, 17.5mM HCl) and blocked for 1 hr at room temperature in blocking buffer (50% formamide, 5 x SSC, 0.25mg/ml tRNA, 5 x Denharts solution, 0.5mg/ml herring sperm DNA) in a hybridizing chamber. DIG-labeled RNA probes were heated to 85°C for 5 min, cooled in ice, and added to prewarmed (62°C) hybridization solution to a final concentration of 100-200ng/ml. 200 μL of hybridization solution containing the appropriate
Following RNA in situ Double-labeling of RNA in situ/C14 Article and AAV2/9-hSyn-ChR2-mCherry Virus and CTB injection fitting were performed using Origin 8.5.

mamillary body from each brain were measured for data normalization and binning. Distribution plots and nonlinear Gauss curve identified for the following brain regions: mammillary body for wild-type animals and tactic injections were performed via rodent stereotax (RWD Life Science 68025; Narishige SR-5M-HT). Bregma coordinates were Medical Biotech. Mice were anesthetized by intraperitoneal injection of 1.5% sodium pentobarbital (0.09 mg/g body weight). Stereo-

Fezf2-F: 5’-CGTACCGGCACTTCTAAA –3’
Fezf2-R: 5’-CATGAAACACTTGCCGCAC –3’
Foxb1-F: 5’-CCATCCCATGTCCCATCAAG –3’
Foxb1-R: 5’-ATCTTAAAGCAGCCGGGAGG –3’
Fezf1-F: 5’-TGCAATGCCCTTTGTTCCC –3’
Fezf1-R: 5’-TTACACAGAACTGGTCTCG –3’

Double-labeling of RNA in situ hybridization and immunohistochemistry
Following RNA in situ hybridization, brain sections were rinsed with 0.5% H2O2 in PBS for 1 hr at RT, then rinsed three times with PBS for 10 min each, blocked in PBS containing 0.05% Triton and 5% normal donkey serum for 1 hr, and then incubated with rabbit anti-Nkx2.1 (1:500; Santa Cruz Biotechnology, SC-13040) in blocking buffer at 4°C overnight. The next day, brain sections were rinsed with PBS for 10 min three times, incubated with Biotin conjugated secondary antibody (donkey anti rabbit, 1:500, Jackson Immuno Research, 711-065-152) for 3 hr at room temperature, washed with PBS for 10 min three times, and then staining with VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, PK-6100) and DAB Peroxidase Substrate Kit (Vector Laboratories, SK-4100) according to the manufacturer’s instruction. Reactions were terminated by rinsing with distilled water for 30 min, then dehydrated with a series of ethanol gradient at 30, 75, 95, and 100% for 5 min each, cleared with dimethylbenzene for 15 min, and coverslipped with neutral balsam.

Nissl staining
Sagittal brain slices at 100 μm were sectioned using a vibratome (Leica VT1000s), mounted on positively charged glass sides and air-dried for 2-3 hr, then fixed with 4% PFA for 1 hr. After rinsing with PBS, the sections were dehydrated with a series of ethanol gradient at 30, 75, 95, and 100% for 2 min each and rehydrated with a series of ethanol gradient at 100, 95, 75, 30% for 2min each. Preheated 0.5% cresyl violet (BBI Life Sciences, 10510-54-0) (in 0.25% v/v glacial acetic acid buffer) at 37°C were added onto the sections for 10 min, and reaction was terminated by incubation in distilled water. Stained sections were dehydrated with a series of ethanol gradient at 30, 75, 95, and 100% for 2 min each, cleared with dimethylbenzene for 30 min, and coverslipped with neutral balsam.

Visualization and quantification of labeled neurons in mammillary body
The 3D reference mammillary body template was generated by tracing the contours of mammillary body in serial coronal sections of adult mouse brain using Neurolucida (MBF Bioscience) software. RFP* BrdU* neurons or RFP* cells were then manually registered into this reference template. After the registration was completed, 3D visualization animations were made following standard protocol provided by the software and edited with Adobe Premiere. For each brain, coronal sections were numbered rostral-caudally with the section containing the rostral end of mammillary body designated as section 1. The rostral-caudal coordinates for all mammillary RFP* cells in any section were set as the thickness of brain section multiplied by the number of that section. The total length of mammillary body from each brain was calculated by multiplying thickness of brain section with total number of slices from rostral end to caudal end of mammillary body for data normalization and binning. For each section, the medial-lateral coordinate and dorso-ventral coordinate of each RFP* cell was set as the distances measured from cell body to the medial-lateral midline and the dorso-ventral midline of the mammillary body using custom-written MATLAB (MathWorks) scripts. The maximum width and height of mammillary body from each brain were measured for data normalization and binning. Distribution plots and nonlinear Gauss curve fitting were performed using Origin 8.5.

Virus and CTB injection
AAV2/9-hSyn-Chr2-mCherry and AAV2/9-CAG-Gcamp6s were purchased from Shanghai Tailtool Bioscience and S&E Shanghai Medical Biotech. Mice were anesthetized by intraperitoneal injection of 1.5% sodium pentobarbital (0.09 mg/g body weight). Stereotactic injections were performed via rodent stereotax (RWD Life Science 68025; Narishige SR-5M-HT). Bregma coordinates were identified for the following brain regions: mammillary body for wild-type animals and Fezf2KO/WT (Antero-posterior, A/P: -2.06--2.56mm; medio-lateral, M/L: 0.0–0.2 mm; dorso-ventral, D/V: 4.7–5.07 mm depth from the pial surface) and for Fezf2KO/KO (A/P -2.4--2.6 mm; M/L: 0.0–0.15 mm; D/V: 4.9–5.1); AD for Fezf2KO/WT (A/P -0.5--0.55 mm; M/L: 0.45–0.5 mm; D/V: 3.05–3.1 mm)
and for Fez2KO/KO (A/P −0.45−0.5 mm; M/L: 0.45−0.5 mm; D/V: 3.2−3.25); AV for Fez2KO/WT (A/P −0.75−0.85 mm; M/L: 0.65−1 mm; D/V: 2.9−3.45 mm) and for Fez2KO/KO (A/P −0.3−0.7 mm; M/L: 0.7−0.9 mm; D/V: 3.25−3.45 mm); AM for Fez2KO/WT (A/P −0.5−0.6 mm; M/L: 0.5−0.8 mm; D/V: 3.2−3.4 mm) and for Fez2KO/KO (A/P −0.3−0.6 mm; M/L: 0.65−0.85 mm; D/V: 3.4−3.45 mm). An incision was made over the scalp, a small burr hole was drilled into the skull using a dental drill (Strong 102Z), and brain surface was exposed. A glass micropipette (tip size 10−30 µm) containing virus or 2% CTB was lowered below the pial surface to the specified coordinates. 200 nL of virus or 50 nL of CTB was injected per animal were delivered using Nanoject 2 (Drummond) or Nanoject 2010 (WPI) at a rate of 10 nl/min. Virus injected mice were analyzed 2-6 weeks post injection. CTB injected mice were analyzed 7-14 days post injection.

**Analysis of axonal coverage, projection pattern and density**

Fluorescent images from coronal brain sections were acquired using identical microscope settings across all samples. On each image, two 500 by 500-pixel regions containing no axonal projections were manually identified for fluorescence measurement using ImageJ, and the average fluorescence intensity was used for background subtraction. To quantify axonal coverage, rectangular regions containing the target brain area were cropped out and binarized, and the contours of mammillary projections were manually traced by linking the positive pixels in the periphery. The area of axonal coverage was measured using ImageJ. Relative axonal coverage was calculated by first normalizing the area of axonal coverage by the area of the entire coronal section, and then dividing the values from each time point by the average value of E9.5. To facilitate the visualization of AT projection patterns from different induction time, representative coronal images at the same coordinates were chosen, aligned with one another, background-subtracted, down-sized at 50 pixels, and binarized using ImageJ. The resulted images were pseudo-colored for display. To deduce projection patterns of different temporal cohorts of mammillary neurons, downsized and binarized images of earlier induction time points were subtracted by later time points using custom-written MATLAB scripts, and the resulted images were pseudo-colored in different shades of brown and then merged for display. To compare the axonal density in projection targets between Fez2KO/WT and Fez2KO/KO, background subtracted images were binarized. Region of interest (ROI) were identified and drawn manually based on DAPI staining with reference to “The mouse brain in stereotactic coordinates” (Franklin and Paxinos, 2013). Axon density was calculated by dividing positive valued pixels by total pixels in the ROIs.

**Cortical slice preparation, in vitro electrophysiology and ChR2-assisted circuit mapping**

Mice were anaesthetized with isoflurane, and transcardially perfused with ice-cold oxygenated (95% O₂, 5% CO₂) cutting solution (pH 7.3-7.4, 300-305 mOsm) containing the following (in mM): 93 N-methyl-D-glucamine, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na-ascorbate, 2 thiourrea, 3 Na-pyruvate, 10 MgCl₂, 0.5 CaCl₂, and 12 N-acetyl-cysteine. Cortical slices were sectioned at 300 µm with a vibratome (Leica V1200S). Slices were incubated in an chamber filled with cutting solution at 34 °C for 10 min, then transferred to oxygenated HEPES solution (pH 7.3-7.4, 300-305 mOsm) containing the following (in mM): 94 NaCl, 2.5 KCl, 1.2 NaH₂PO₄, 30 NaHCO₃, 20 HEPES, 25 glucose, 5 Na-ascorbate, 2 thiourrea, 3 Na-pyruvate, 2 MgCl₂, 2 CaCl₂, and 6 Na-Acetyl-cysteine. After 30 min incubation at room temperature, the slices were transferred to ACSF (pH 7.3-7.4, 300-305 mOsm) containing the following (in mM): 118 NaCl, 2.5 KCl, 26 NaHCO₃, 1 NaH₂PO₄, 22 D-glucose, 2 MgCl₂, 2 CaCl₂. After 1 hr incubation at room temperature, slices were transferred to a recording chamber of an upright microscope (Olympus BX51) and submerged in ACSF. Whole-cell patch-clamp was performed in the mammillary body. A Sutter P1000 puller was used to pull patch pipettes from borosilicate glass capillaries with filament (1.5 mm outer diameter and 0.86 mm inner diameter, Sutter BF150-86-10) with a resistance of 3-6 MΩ when filled with internal solution and measured in ACSF. The patch pipettes were filled with the following solution (in mM): 120 K-gluconate, 16 KCl, 2 MgCl₂, 10 HEPES, 0.2 EGTA, 2.5 MgATP, 0.5 Na₃GTP, 10 Na-phosphocreatine, pH 7.3. Passive membrane properties were measured in current clamp mode. For cells that did not spontaneously fire, resting membrane potential was recorded immediately after obtaining whole-cell configuration with no current applied (I = 0 mode) for 1 min. For cells that fired spontaneously, resting membrane potential was measured in the first resting phase between firing. Input resistance was measured as the slope of the regression line fitted to the input-output curve with increasing hyperpolarization current steps of 30 pA (500 ms) from −120 pA to −300 pA. Membrane time constant was measured with −30 pA hyperpolarization current injection. Membrane capacitance was calculated by dividing the membrane time constant with input resistance. For cells did not exhibit spontaneous firing, properties of the single action potential were measured from the first spike fired at rheobase. Rheobase was determined with increasing depolarization current steps of 5 pA (500 ms). For cells that exhibit spontaneous firing, properties of the single action potential were measured from the spontaneous spikes. For ChR2-assisted circuit mapping, AAV2/9-hSyn-hChR2(H134R)-mcherry (Shanghai Taitool Bioscience, S0312-9-H20) was injected in the mammillary body to label axonal projections to downstream brain regions, 2-6 weeks post injection, monosynaptic responses initiated from ChR2 + axons were recorded in postsynaptic neurons. Regions with axonal projections in each target brain area were identified under epifluorescence and postsynaptic responses were downsized at 50 pixels, and binarized using ImageJ. The resulted images were pseudo-colored for display. To deduceprojection patterns of different temporal cohorts of mammillary neurons, downsized and binarized images of earlier induction time points were subtracted by later time points using custom-written MATLAB scripts, and the resulted images were pseudo-colored in different shades of brown and then merged for display. To compare the axonal density in projection targets between Fez2KO/WT and Fez2KO/KO, background subtracted images were binarized. Region of interest (ROI) were identified and drawn manually based on DAPI staining with reference to “The mouse brain in stereotactic coordinates” (Franklin and Paxinos, 2013). Axon density was calculated by dividing positive valued pixels by total pixels in the ROIs.
Multiclamp 700B (Molecular Devices, Digidata 1550A). Data were analyzed using pClamp 10.6. All recordings were performed at temperature 29-31°C with the chamber perfused with oxygenated ACSF. To construct the dendrogram, unsupervised hierarchical cluster analysis was performed in SPSS with the within-groups linkage method using squared Euclidean distances based on 8 electrophysiological parameters that were standardized with Z scores: input resistance, membrane capacitance, action potential threshold, action potential amplitude, action potential half-width, action potential rise time, action potential decay time and after-hyperpolarization amplitude.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism version 7.0 was used for statistical calculations. No statistical method was used to predetermine sample sizes, but our sample sizes are similar to those reported in previous publications. Data collection and analysis were performed blind to the experimental conditions whenever possible. No animals or data points were excluded from the analysis. Equal variances were assessed using the Levene’s test. Normality was assessed using the Kolmogorov-Smirnov test. Statistical significance was tested using two-tailed unpaired t test and one-way ANOVA with Tukey’s Post hoc analysis, or using Kolmogorov-Smirnov test and Kruskal-Wallis test if the datasets were not normally distributed or have unequal variance. Data are presented as mean ± SEM p < 0.05 was considered significant. Significance is marked as *p < 0.05; **p < 0.01 and ***p < 0.001. Statistical values for all experiments, including all post hoc test p values, are shown in Tables S1–S3.