Dual origin and multiple neuropeptidergic trajectories of hypothalamic POMC progenitors revealed by developmental single-cell transcriptomics

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Abstract (150 words)

Proopiomelanocortin (POMC) neurons of the hypothalamic arcuate nucleus are essential to regulate food intake and energy balance. However, the ontogenetic transcriptional programs that specify the identity and functioning of these neurons are poorly understood. Here, we use scRNAseq to define the transcriptomes characterizing POMC progenitors in the developing hypothalamus and their transition into mature neurons. Our data show that *Pomc*-expressing neurons originate from two independent developmental pathways expressing distinct combinations of transcription factors. The predominant cluster, featured by high levels of *Pomc* and *Prdm12* transcripts represents the precursors of canonical arcuate POMC neurons. Additional clusters of progenitors expressing lower levels of *Pomc* mature into different neuronal phenotypes characterized by distinct combinations of transcription factors, neuropeptides, processing enzymes, cell surface and nuclear receptors. We conclude that the genetic programs specifying the identity and differentiation of arcuate POMC neurons are diverse and generate a heterogeneous repertoire of neuronal phenotypes early in development.

Main

Proopiomelanocortin (POMC)-expressing neurons located in the arcuate nucleus of the hypothalamus play an essential role in the regulation of food intake by maintaining a melanocortin-dependent anorexigenic tone. The critical importance of central melanocortins, specifically in arcuate neurons, is apparent in mice lacking *Pomc* that develop hyperphagia and early-onset extreme obesity[1]. This phenotype is closely mirrored in humans carrying biallelic null-mutations in *POMC*[2]. In addition, arcuate POMC neurons release the *Pomc*-encoded opioid peptide *β*-endorphin, which is critical in mediating normal food intake and stress-induced analgesia[3].

Postmitotic POMC precursors acquire their phenotypic identity in the developing hypothalamus days earlier than any other peptidergic neurons born in the presumptive arcuate nucleus (eg. NPY/AGRP, GHRH, SST, KISSPEPTIN). *Pomc* mRNA is initially observed in the tuberal portion of the prospective mouse hypothalamus of E10.5 embryos, following the concurrent advent of the essential transcription factors (TFs) ISL1[4], NKX2-1[5] and PRDM12[6]. In fact, the early ablation of either *Isl1*, *Nkx2-1* or *Prdm12* disrupts the onset of hypothalamic *Pomc* expression in conditional mutant mouse embryos[4-6]. Given that the number of arcuate neurons coexpressing *Nkx2-1*, *Isl1* and *Prdm12* greatly exceeds that of POMC neurons in this area, it is expected that additional and still unknown TFs integrate the complete genetic program that specifies the early identity of POMC neurons.

Other molecular markers of arcuate POMC neurons such as transporters, receptors, channels and co-transmitters exhibit great variability indicating that a heterogeneous pool of diverse subpopulations of these neurons are involved in multiple circuits and functions. In fact, previous studies have suggested that subsets of arcuate *Pomc*-expressing neurons may act as precursors of terminally differentiated neurons of alternative neuropeptidergic phenotypes such

as NPY/AGRP and KISSPEPTIN/NEUROKININ-B/DYNORPHIN (KNDY) neurons in the adult hypothalamus[7, 8]. Initial attempts to characterize the transcriptome of adult POMC neurons at the single-cell level confirmed the heterogeneous nature of arcuate POMC neurons and revealed the existence of distinct clusters including those co-expressing the aforementioned neuropeptide genes[9-12]. However, the molecular signatures of the precursors of the various hypothalamic *Pomc*-expressing lineages and their transition into mature neurons within the postnatal Arc are still lacking.

Here, we track the origin and maturation of hypothalamic POMC precursors and the diverse transcriptional programs leading to alternative cellular fates in the developing and early postnatal hypothalamus by performing single-cell RNA-seq transcriptomics of fluorescently labeled cells taken from *Pomc-TdDiscomaRed-Sv40PolyA* (*Pomc-TdDsRed*) transgenic mice at different embryonic (E11.5, E13.5, E15.5 and E17.5) and early postnatal (P5 and P12) ages. Our results show that POMC precursors arise from two different transcriptional routes. Some of these precursors further develop into mature POMC neurons while others follow diverse neuropeptidergic trajectories indicating that a heterogeneous population of *Pomc*-expressing neurons is established early in the developing arcuate nucleus**.**

Results

Cluster analysis of gene expression in Pomc positive hypothalamic cells integrated across six development ages

We captured the single cell transcriptomes of hypothalamic *Pomc*-expressing cells at four key embryonic days (E11.5, E13.5, E15.5 and E17.5) and two early postnatal days (P5 and P12) when the maturing neurons already develop axons and innervate key target nuclei distal to the

Arc[13-15]. Medial basal hypothalami from mice known to express the reporter transgene *Pomc-TdDsRed* selectively in POMC cells[17,28] were pooled from multiple embryos or postnatal pups at the indicated ages. Dissociated single cell suspensions were sorted for DsRed fluorescence and used for library preparation with the 10X genomics platform (Figure 1A). After a rigorous screening procedure to remove substandard scRNA sequencing data (see Methods), we used a combination of computational platforms to analyze the transcriptomes of 13,962 cells that had *Pomc* counts of ≥ 1 unique molecular identifiers (UMI).

A Seurat analysis of all cells integrated across the six-time points revealed 11 distinct cell clusters projected onto a UMAP plot (Figure 1B). The same data are represented in a heat map corresponding to the top 30 expressed genes per cluster (Supplementary Figure 1). Clusters 1-8 (10,828 cells) were all highly enriched for a panel of three neuronal marker gene transcripts. The embedded table in Figure 1B lists the percentages of neurons of each cluster at each developmental age. The other three clusters (3,134 cells) were enriched in transcripts characteristic of either microglia, vascular and leptomeningeal cells (VLMC) or astrocytes (Figure 1C). GO ontology analyses for biological processes further confirmed the neuronal or non-neuronal identities of the 11 individual clusters (data not shown).

By definition, all cells in the neuronal and non-neuronal clusters contained *Pomc* transcripts (UMI ≥ 1), however there were significant differences in the average expression levels per cluster, ranging from 1.3 to 33.8 [exp(*log Normalized* counts) -1)]. Nomenclature of the 8 neuronal clusters is based on a combination of relatively high (>20), medium or low (<5) *Pomc* levels and high expression of one differential key feature gene as follows: no. 1, *Pomc*^{high}*/Prdm12*; no. 2 *Pomc*^{med}*/Ebf1*; no. 3, *Pomc*^{med}*/Nr4a2*; no. 4, *Pomc*^{low}*/Otp*; no. 5, *Pomc*$^{low/}$*Tac2*; no. 6, *Pomc*^{low}*/Dlx5*; no. 7, *Pomc*^{low}*/Prdm13*; and no. 8, *Pomc*^{low}*/Pitx2*. The cluster distribution and relative expression of those feature genes are represented by individual UMAP plots (Figure 1D). Average expression levels of all genes, including *Pomc*, in the 11 clusters and

complete lists of all feature genes that define the individual clusters are presented in Supplemental Tables 1 and 2, respectively.

In order to validate our experimental approach of isolating *Pomc*-expressing cells from the developing hypothalamus by FACS for the transgenic surrogate marker *Pomc-TdDsRed*, we performed an independent unsupervised cluster analysis of the transcriptomes from the 5,724 cells that had *DsRed* counts of ≥ 1 UMI (Supplemental Figure 2A-C and Supplemental Table 3). A projection analysis (Supplemental Figure 2D-F) of the differentially expressed feature genes defined 9 DsRed clusters (Supplemental Table 4) which closely corresponded to 9 of the 11 clusters based on *Pomc* counts of ≥ 1 UMI (Supplemental Table 2). The two exceptions were the smallest clusters 7. *Pomc*^{low}*/Prdm13* and Astrocytes (Supplemental Figure 2D-F). Furthermore, the percentages of cells at each developmental age from the corresponding neuronal clusters were remarkably similar in the *Pomc* UMI ≥ 1 data set compared to the *DsRed* UMI ≥ 1 data set (compare Figure 1B to Supplemental Figure 3A). The different population of DsRed fluorescent sorted cells showing UMI ≥ 1 for *Pomc* (13,962) or *DsRed* (5,724) may be due to differential half-lives of the fluorescent protein DSRED used during cell sorting and its transcript used for barcoding the RNAseq libraries.

Temporal gene expression analysis of the eight neuronal clusters at individual developmental stages

Next, we analyzed temporal gene expression patterns for each of the 8 neuronal clusters at the 6 distinct developmental ages (Supplemental Table 5). The progressive changes in feature genes across time are responsible for the observed within cluster heterogeneity on the heat map in Supplemental Figure 1.

Cluster 1. *Pomc*^{high}*/Prdm12* cells were the most abundant among the eight neuronal clusters and consistently had the highest levels of *Pomc* expression at all six developmental ages (Figure 2A). We consider them to be the direct precursors of canonical POMC neurons in the adult hypothalamus (Figure 2). Other feature genes included a set of four TFs known to be critical for the initiation and/or maintenance of Arc *Pomc* expression, *Isl1, Nkx2-1, Prdm12* and *Tbx3*[4-6, 16] (Figure 2B). They were expressed in similar developmental patterns to each other, with the exception of *Tbx3* that was significantly expressed only from day E15.5. Notably, there was a lack of the pituitary-specific *Tbx19* TF, confirming that no pituitary tissue was included in the hypothalamic dissections. Two homologues of the *Drosophila sine oculus* homeobox gene, *Six3* and *Six6,* which have not been described previously in the context of Arc POMC neuron development, were also expressed robustly at all six time points (Figure 2B). Co-expression of their mRNAs with *Pomc* by RNAScope at ages E12.5 and E16.5 confirmed the scRNAseq analysis, although the two TFs were expressed in additional hypothalamic domains and the pituitary gland (Figure 2C, D). *Vim1, Sox1, 2, 3 and 14,* and *Myc*, whose expression is typically associated with neuronal precursors or immature neurons, tended to have higher expression at the earlier embryonic ages (Figure 2B). The nuclear receptor gene *Nr5a1*, also known as steroidogenic factor 1 (*Sf1*), was a key feature gene expressed almost exclusively at ages E11.5 and E13.5 (Figure 2B), but only minimally expressed at later embryonic time points when NR5A1 is critically involved in the development of the hypothalamic ventromedial nucleus (VMN)[17]. This differential spatio-temporal presence of NR5A1 within or outside POMC neurons was confirmed by immunofluorescence on hypothalamic sections taken from E11.5 and E17.5 mouse embryos (Figure 2E). Interestingly, *Nr5a1*, *Nr0b1* (*Dax1*) and *Shh*, transcripts were also present in Cluster 1 at the two earliest ages, and are factors known to interact with the *Wnt*/β-catenin signaling pathway in early cell fate determination in the adrenal and pituitary glands[18-20].

Cluster 2. *Pomc*^{med}*/Ebf1* neurons made up the second highest abundance cluster of POMC neurons, particularly at E11.5 and E13.5. After these time points, the fraction of all

neurons constituting cluster 2 dropped by approximately two thirds from ages E15.5 through P12. In contrast, the relative percentage of neurons in clusters 3-8 increased over time with peaks at different embryonic ages (Figure 1B). Cluster 2 neurons, on average, had medium levels of *Pomc* transcripts that were divided distinctly from their lowest at ages E11.5-E13.5 to highest at later embryonic and postnatal ages (Figure 3A and B; Supplemental Tables 5). Major cluster 2 feature markers included members of the non-basic helix-loop-helix early B-cell TF family EBF (Supplemental Table 2). *Ebf1* is typically co-expressed with either *Ebf2* or *Ebf3* and pairs of these factors were shared not only in cluster 2, but also in clusters 3 and 8 (Figure 4B and F). Dual RNAScope *in situ* hybridization showed overlap of *Ebf1* and *Pomc* mRNAs in hypothalamic neurons at an early embryonic stage (E13.5), whereas at later embryonic stages (E16.5 and E17.5), POMC neurons had barely detectable *Ebf1* mRNAs (Figure 3C). A similar expression pattern was found with another cluster 2 featured gene, *Pou4f1 (POU Class 4 Homeobox 1)* (Figure 3B). Dual RNAScope *in situ* hybridization confirmed the overlap of *Pou4f1* and *Pomc* mRNAs at E13.5, but not at E17.5 (Figure 3D). These results, together, suggest that *Pomc* mRNA levels peak only after expression of *Ebf1* and *Pou4f1* decrease. Two other prominent feature genes in cluster 2 were *Crabp1* and *Crabp2*, which encode cellular retinoic acid binding proteins.

Visual examination of the UMAP plots in Figure 1D suggested that cluster 2 was composed of two compartments, one with high and one with low *Pomc* abundance. Unsupervised reclustering of the differentially expressed genes from integrated cluster 2 confirmed the existence of two subclusters (Figure 3E). Subcluster 2-1 contained two-thirds of the neurons, which were *POMC*^{high} and present only from ages E15.5-P12. Subcluster 2-2 contained the other one-third of cluster 2 neurons, which in contrast were *Pomc*^{low} and detected at ages E11.5 and E13.5. Consistently, known activating TFs for *Pomc* expression such as *Nkx2-1*, *Isl1* and *Tbx3* were limited to subcluster 2-1 while the *Ebf* and *Crabp* families were

characteristic of subcluster 2-2 (Supplemental Table 6 and Supplemental Table 7). Conspicuously absent from both subclusters was *Prdm12* mRNA.

Cluster 3. *Pomc*^{med}*/Nr4a2* neurons displayed high expression of its major feature gene *Nr4a2* (Nuclear Receptor Subfamily 4 Group A Member 2) (Figure 4A-4C). *Nr4a2* mRNA was also expressed at relatively high levels in clusters 2 and 8. Originally known as *Nurr1*, *Nr4a2* has been implicated in the regulation of *Pomc* expression in the pituitary corticotropic cell line AtT20[21]. Furthermore, *Nr4a2* is a pioneer transcriptional regulator important for the differentiation and maintenance of meso-diencephalic dopaminergic (mdDA) neurons during development[22]. It is crucial for expression of a set of genes including *Slc6a3* (plasma membrane dopamine transporter, DAT), *Slc18a2* (synaptic vesicular monoamine transporter, VMAT), *Th* (Tyrosine hydroxylase), *Foxa1* and *Foxa2* (forkhead family TFs) and *Drd2* (dopamine receptor 2) that together define the dopaminergic neuronal phenotype[23-26]. An additional key factor involved in the generation and maintenance of mdDA neurons, *Lmx1a* (Lim- and homeodomain factor 1a)[23, 27], was also a key feature gene of clusters 3 and 8 (Figure 4B and F) suggesting that POMC and DA neurons share at least part of their genetic differentiation programs.

Similar to cluster 2, reclustering of the differentially expressed genes from cluster 3 revealed two subclusters (Figure 4D). Subcluster 3-1 constituted 72% of the main cluster 3 and was characterized by *Pomc*^{low} neurons with strong *Nr4a2* expression at all developmental ages. Feature genes for subcluster 3-1 also included *Lmx1a, Slc6a3, Slc18a2*, *Foxa2* and *Th,* all feature genes for developmental subclusters E13.5-10 and E15.5-11 derived from cluster 3 (Supplemental Figure 6) indicating that a fraction of cluster 3 neurons were dopaminergic and existed transiently during a three-day developmental window (Supplemental Tables 2, 5 and 9). In contrast, subcluster 3-2 neurons were *POMC*^{high} together with characteristic expression of the known *Pomc* transcriptional activator genes and were present only at ages P5 and P12. The dissociation of high *Pomc* expression with low *Nr4a2* expression in subcluster 3-2 suggests that

the latter is not necessary for hypothalamic *Pomc* expression, unlike its role in pituitary corticotrophs[28].

Cluster 8. *Pomc^{low}/Pitx2* is more closely related to clusters 2 and 3 than to any of the others (Figure 4E and F). In addition to *Pitx2,* major feature genes were *Barhl1, Foxa1, Foxp2, Lmx1a/b,* and *Tac1* (Supplemental Table 2). Intriguingly, virtually all of the top cluster 8 defining genes (Supplemental Table 2) are identical to those that characterize both the mesencephalic dopamine neurons and glutamatergic, non-dopaminergic, subthalamic nucleus neurons[29]. Dual RNAscope in situ hybridization for *Pitx2* and *Pomc* showed limited overlap at E12.5 (Figure 4G). Cluster 8 is the only cluster with the neuropeptide *Cck* as a feature gene at developmental ages P5 and P12 (Supplemental Figure 8).

Cluster 4. *Pomc^{low}/Otp* cells uniquely expressed *Otp* at all developmental ages (Figure 5A and B). *Pomc* expression was moderate at E11.5 and then dropped to consistently lower levels through age P12 despite continued expression of *Isl1, Nkx2-1* and *Tbx3.* Interestingly, expression of the other transcriptional modulator important for *Pomc* expression, *Prdm12*, was detected only at E11.5. Other characteristic features of Cluster 4 were increasing gradients of *Agrp, Npy* and *Calcr* expression over the course of development. Minimal co-expression of *Pomc* and *Npy* was confirmed by FISH at age E15.5 (Figure 5C). *Sst* was highly expressed in this cluster at ages E11.5, E13.5, P5 and P12 with a temporary drop off at E15.5 and E17.5. Altogether, these patterns of gene expression indicate that a subpopulation of *Pomc* neuronal progenitors differentiates into the canonical AGRP/NPY neurons located in the adult Arc as suggested previously by studies based on transgenic lineage tracing[7]. A map of gene expression in individual cells within the Cluster 4 UMAP plot demonstrated that *Npy* was expressed in virtually all *Agrp* neurons, but few *Sst* neurons. *Sst* and *Agrp* neurons were essentially separate cell populations (Figure 5D). The GABAergic marker *Slc32a1* encoding the vesicular inhibitory amino acid transporter was expressed at the second highest level in cluster

4 relative to the other clusters (Supplemental Table 1), consistent with the GABAergic phenotype of mature AGRP/NPY neurons (Supplementary Figure5).

Cluster 5. *Pomc^{low}/Tac2* represents a distinct neuronal population from Cluster 1 in its patterns of TFs associated with the development of mature POMC neurons (Figure 6A and B). In particular, *Isl1* was highly expressed only at E11.5 and *Prdm12* at E11.5 and E13.5 in cluster 5. Starting from E15.5 through P12 there was a gradual onset of expression for a set of genes including *Tac2, Ar, Esr1, Tacr3, Prlr, Pdyn,* and *Kiss1/Gm28040* isoforms. Confirmations of co-expression for *Pomc* with either *Tac2, Tacr3* and *Sox14* or TDTOMATO with ESR1 at several embryonic ages are shown in Figure 6C, D, E and F, respectively. Interestingly, *Sox14* has been reported to be necessary for expression of *Kiss1*[30]. Taken together, this ensemble of genes is characteristic of adult Arc KNDY neurons that play an essential role in the control of reproductive physiology. A previous publication based on transgene lineage tracing also concluded that a significant subpopulation of KNDY neurons is derived from POMC progenitor cells[8].

A prominent and unique characteristic of **cluster 6.** *Pomc$^{low/Dlx5}$* neurons was the expression of members of the *Dlx* family of homeobox TFs (Supplementary Figure 4A and 4B). The six family members are usually expressed in 3 linked pairs of isoforms: 1 and 2, 3 and 4 or 5 and 6. Cluster 6 was enriched in *Dlx1/2,* and *Dlx5/6* together with *Dlxos1*. The *Dlx1/2* pair is known to activate expression of *Ghrh* in the mouse Arc[31]. However, there were no transcripts for *Ghrh* expressed in cluster 6 or any of the other neuronal clusters suggesting that *Dlx1/2* are not sufficient to induce *Ghrh* expression. Moreover, it has been recently shown that *Dlx1/2* binding to *Otp* regulatory elements inhibits OTP production, subsequently reducing downstream *Agrp* expression[31]. Cluster 6 had the highest expression of the opioid prepronociceptin (*Pnoc*) compared to the other integrated clusters, but low levels of preprodynorphin (*Pdyn*), similar to cluster 5 KNDY neurons.

Cluster 6 had the highest *Gad1*, *Gad2* and *Slc32a1* (*Vgat,* vesicular inhibitory amino acid transporter) expression density of all clusters with virtually no *Slc17a6* (*Vglut2,* vesicular glutamate transporter 2) expression, suggesting that these differentiating neurons were exclusively GABAergic. A comparison of GABAergic and glutamatergic markers among the eight neuronal clusters is shown in Supplementary Figure 5. Cluster 4 neurons were also uniformly GABAergic. Cluster 1 neurons contained a mixture of glutamatergic and GABAergic markers with the exception of undetectable *Slc32a1.* However, they did express low levels of *Slc18a2* (*Vmat2*, vesicular monoamine transporter 2), which possibly functions as an alternative vesicular GABA transporter in some dopamine neurons[32]. This combination of mixed glutamatergic and GABAergic features in cluster 1 is characteristic of postnatal POMC neurons[33, 34] and was also present in cluster 2. . The remaining neuronal clusters 3, 5, 7 and 8 were all uniformly glutamatergic.

Cluster 7. *Pomc^{low}/Prdm13* contained the smallest number of cells among the eight neuronal clusters, and the cells were primarily limited to developmental ages E13.5 to E17.5 (Figure 1B). Feature genes included *Prdm13, Nr5a1, Adcypap1, Cnr1, Fam19a1, Rbp1* and *Pdyn* (Supplementary Figure 4D, E and F). *Prdm13* was reported recently[35] to also be highly expressed in E15.5 POMC cells.

We further analyzed all of the neurons and nonneuronal cells by reclustering them based on their individual developmental ages (Supplemental Figure 6) rather than for their gene expression profiles integrated across all ages. Average gene expression levels for each of these developmental subclusters are listed in Supplemental Table 8 and the feature genes defining the developmental subclusters are listed in Supplemental Table 9. Based on these data, it was then possible to trace the temporal continuity between developmental subclusters from age E11.5 to P12 relative to the original integrated cell cluster identities (Supplemental Figure 7). These data are important because they connected the dynamic nature of gene expression

profiles at each embryonic stage leading to the eventual transcriptomes present in the postnatal time points.

Comparison of transcriptional profiles for neuropeptides, neuropeptide processing enzymes, neuroendocrine secretory proteins and GPCRs in the eight neuronal POMC clusters at each developmental age

We identified 19 unique neuropeptide prohormone genes that were clearly differentially expressed features in at least one of the neuronal clusters at one or more developmental ages (Supplemental Figure 8, Supplemental Table 5). In addition to the neuropeptide genes already mentioned as characteristic features of certain clusters, additional neuropeptides were featured in other clusters primarily at postnatal ages P5 and P12. Notable examples are *Gal, Tac1, Adcyap1, Cartpt* and *Pthlh.* Unlike the mosaic of neuropeptide gene expression profiles across clusters and developmental ages, the neuroendocrine secretory proteins characteristic of dense core granules were expressed ubiquitously in all eight neuronal clusters with similar gradients across all developmental ages (Supplemental Figure 8). These included members of the chromogranin (*Chga* and *Chgb*) and secretogranin (*Scg2, Scg3* and *Scg5*) families. Among the prohormone processing enzymes, *Pcsk1* was prominently expressed only at P5 and P12 in most neuronal clusters while *Pcsk2* was expressed at both embryonic and postnatal ages in the same clusters as *Pcsk1*. *Pam* was expressed in the same cluster/developmental age pattern as *Pcsk1*. Only *Cpe* was expressed in virtually all neurons at all developmental ages.

Split plots of GPCR gene expression revealed a wide range of cluster- and age-specific patterns (Supplemental Figures 9A and 9B). *Cnr1* (Cannabinoid receptor 1) was an important feature primarily of clusters 7 and the two interrelated clusters 3 and 8. The two metabotropic GABA receptors *Gabbr1* and *Gabbr2* were strongly expressed in all eight neuronal clusters but

differed in their developmental gradients. *Gabbr1* was expressed at all developmental ages while *Gabbr2* transcripts were largely present only in more mature neurons at postnatal ages P5 and P12. The opioid receptor *Oprl1* that is selectively activated by nociception/orphanin FQ[36] stood out for its consistently strong expression in all eight neuronal clusters, particularly at postnatal days P5 and P12.

The prokineticin receptor 1 (*Prokr1*) was selectively expressed together with the GPCR accessory protein gene *Mrap2* in cluster 1 *Pomc*^{high}*/Prdm12* neurons. Although MRAP2 was initially identified as an activating modulator of melanocortin receptor signaling, consequently reducing food intake, it was subsequently shown to be promiscuous in its interaction with additional GPCRs[37]. Unlike its action on MC4R signaling, MRAP2 inhibits PROKR1 signaling to promote food intake in mice independently of its interaction with MC4R[38]. Therefore, the co-expression of *Prokr1* and *Mrap2* in anorexigenic POMC neurons suggests an additional mechanism for PROKR1's regulation of energy homeostasis by modulating the release of melanocortins from POMC neurons. Finally, the high expression of *Npy2r* (neuropeptide Y receptor 2) in both clusters 1 and 4 matches well with its expression in adult anorexigenic POMC neurons and orexigenic AGRP/NPY neurons, respectively.

Developmental relationships of gene expression patterns in the eight neuronal POMC clusters at four embryonic ages

We altered the color coding of the original UMAP plot of the neuronal clusters shown in Figure 1B to visualize the developmental age of each neuron superimposed on the integrated cluster identities (Figure 7A). An alternative depiction of the same data isolated the individual developmental ages into separate UMAP plots with color coding based on the cluster identities (Figure 7B). Although it is possible to infer the developmental relationships among the

transcriptional profiles of each neuronal cluster at selected embryonic and postnatal ages as depicted in Figure 7A and B, dimensionality reduction using a diffusion maps algorithm is an alternative method to visualize the developmental relationships in three-dimensional Euclidean space. This analysis for all neurons with *Pomc* UMIs ≥1 color-coded for the four embryonic ages is shown in Figure 7C. The image depicts a triangular beveled star polygon with rays 1 and 2 derived primarily from distinct groups of neurons at ages E11.5 and E13.5, a center vertex containing individual neurons from the intermediate ages E13.5 and E15.5 and a third ray with neurons from ages E15.5 and E17.5. The same data are alternatively color-coded for each neuron's cluster identity (Figure 7D) showing that ray 1 is primarily derived from cluster 2, ray 2 from cluster 1, the center vertex from a convergence of all clusters 1-8 and ray 3 from clusters 1, 2, 4, 5 and 6.

We then overlaid the geometric location of each neuron with the expression levels of selected feature genes from each of the eight clusters (Figure 7E). Consistent with the diffusion maps analyses, the more granular data for individual genes and neurons illustrates the essentially identical patterns of high *Pomc* expression with the two originally discovered cognate transactivating factors *Isl1* and *Nkx2-1* across embryonic development (Rays 2 and 3). However, *Isl1* was also expressed in numerous *Pomc*^{low} neurons at E11.5 and E13.5 (Ray 1). *Prdm12* and *Tbx3* exhibited complementary temporal patterns of high expression at early (Ray 2) versus late (Ray 3) embryonic ages, respectively. The combined developmental and geometric patterns of *Six3, Six6* and *Sox14* expression were all very similar to both *Pomc* and *Nkx2-1*, suggesting that all four of these TF genes might be necessary to define the cell fate of a subpopulation of hypothalamic neuronal progenitors into mature differentiated POMC neurons.

In contrast to those genes with expression patterns that defined the continuum of neuronal identity from Ray 2 to Ray 3, another set of genes including *Ebf1, Pou4f1* and *Nr4a2* was similarly highly expressed only at the two earliest embryonic ages in neurons constituting

Ray 1. The expression of *Pitx2,* which characterizes cluster 8 and a portion of cluster 3, was high in Ray 1 as it merges into the center vertex and was mostly derived from age E13.5. Finally, the temporal and geometric patterns of high expression levels for *Otp, Prdm13, Tac2* and *Dlx5* were distinct from each other and the aforementioned genes, consistent with their ontogeny into the distinct differentiated cell fates of cluster 4 AGRP/NPY/GABA, cluster 5 KNDY and cluster 6 GABA/PNOC neurons, respectively.

Projection of phenotypically distinct cell clusters identified by developmental transcriptomic profiles to clusters in the adult Arc

We next asked how the transcriptomes of developing POMC neuron progenitors were related to those of neurons in the adult Arc at age P60. The raw single cell sequencing data from Campbell, et al.[9] was transformed, filtered and then combined with our data using the standard data sets integration method from the Seurat package as detailed in the section Materials and Methods. The combined data were scaled and the top 50 principal components were selected for unsupervised clustering. Each of the eight *Pomc* neuronal clusters defined from our scRNAseq data and the 34 neuronal clusters from the adult study of Campbell *et al.* (2017)[9] were then mapped onto the combined UMAP plots to predict the cell projections between the two cluster sets (Supplemental Figure 10). A summary of the cluster projections is shown in Figure 8.

66% of cells in the *Pomc^{high}/Prdm12* neuronal cluster projected to cluster n14 *Pomc/Ttr* from the adult Arc and another 15% projected to n15 *Pomc/Anxa2* neurons. Although, neither of the adult feature genes *Ttr* or *Anxa2* was expressed significantly in any of the integrated developmental clusters, other key feature genes such as *Pomc, Nkx2-1, Tbx3, Tox2, Six3, Six6, Sox2, and Dlk1* do correlate. All together, these data indicate the continued phenotypic evolution of POMC neurons between ages P12 to P60.

93% of *Pomc^{low}/Tac2* cells projected to the adult cluster n20 *Kiss1/Tac2*, providing further evidence that at least a subpopulation of embryonic POMC progenitors are further differentiated into mature KNDY neurons. Similarly, there was a 67% correspondence of *Pomc^{low}/Otp* neurons with cluster n13 *Agrp/GM8773* neurons, while another 27% of the former projected to n23 *Sst/Unc13c* neurons. These two pairings are consistent with the developmental subclustering of *Pomc^{low}/Otp* neurons into those that co-express *Agrp* and *Npy* and those that highly express *Sst* but neither of the other two neuropeptides (Figure 5D). 72% of *Pomc^{low}/Dlx5* neurons projected to n22 *Tmem215* neurons. *Tmem215* was identified as a feature gene in combination with *Dlx5* in subcluster P12-12 from our data set (Supplemental Figure 6 and Supplemental Table 9).

78% of cluster 7 *Pomc^{low}/Prdm13* neurons projected to the adult neuronal cluster n29 *Nr5a1/Adcyap1*. In the adult hypothalamus, expression of these two genes is characteristic of the ventromedial hypothalamic nucleus instead of the Arc. Expression of the trio of *Prdm13*, *Nr5a1* and *Adcyap1* genes was a significant defining feature unique to cluster 7 *Pomc^{low}/Prdm13* neurons from our developmental data set, although *Nr5a1* and *Adcyap1* individually were also feature genes in additional clusters (Supplemental Table 2). Interestingly, the other 11% of *Pomc^{low}/Prdm13* neurons projected to cluster n20 *Kiss/Tac2.* Cluster 5 KNDY neurons had significant *Tac1* and *Sox14* expression (Figure 6 and Supplemental Table 2), which may suggest why cluster 7 also projected to the adult n20 *Kiss/Tac2* cluster.

Integrated cluster 2 *Pomc^{med}/Ebf1* neurons projected primarily to n34 unassigned2 cells, but 18% projected to n21 *Pomc/Glipr1* neurons and 8% to n14 *Pomc/Ttr* neurons. However, when cluster 2 was reclustered into two distinct components, subcluster 2-1 clearly projected to both clusters n14 *Pomc/Ttr* and n15 *Pomc/Anxa2* neurons, exactly the same as integrated developmental cluster no. 1 *Pomc^{high}/Prdm12* canonical POMC neurons. Similarly, integrated cluster 3 *Pomc^{med}/Nr4a2* neurons projected mostly to n33 unassigned1 cells but 16% of the

neurons projected to n32 *Slc17a6*/*Trhr* neurons. VGLUT2 encoded by *Slc17a6* was most prominently expressed in cluster 3 *Pomc*^{med}/*Nr4a2* neurons compared to all the other integrated clusters.

Discussion

In this study, we sought to track the genetic programs that give rise to *Pomc*-expressing neurons in the developing hypothalamus and follow their progression into terminally differentiated POMC neurons and alternative phenotypic destinies. To this end, we performed a single-cell RNA-seq study specifically of *Pomc*-expressing cells present in developing mouse hypothalami at four embryonic time points (E11.5, E13.5, E15.5, E17.5) and two early postnatal days (P5 and P12). These developmental stages were selected based on the ontogeny of *Pomc* expression in the presumptive hypothalamus[39], peak of neurogenesis of POMC neurons[4, 5], subsequent developmental maturation[7, 8] and terminal differentiation[16]. Unlike previous studies using fluorescently-labeled POMC neurons, we validated our cluster profile of sorted cells by an independent unsupervised transcriptome cluster analysis of hypothalamic cells expressing *Pomc*-driven *TdDsRed* (UMIs ≥ 1). This analysis yielded 9 clusters that closely corresponded to 9 of the 11 clusters based on *Pomc* UMIs.

Our study revealed the existence of a major group of POMC neurons (Cluster 1. *Pomc*^{high}/*Prdm12*) that showed by far the highest levels of *Pomc* UMIs relative to all other neuronal clusters at all ages. We believe these developing cells constitute the direct precursors of canonical POMC neurons in the adult hypothalamus. In addition, cluster 1 neurons were the most abundant compared to the other clusters at all six developmental ages (Fig. 1B). Cluster 1 was characterized by a unique combinatorial set of feature TFs that includes *Isl1*, *Nkx2-1* and *Prdm12*. In the last few years, our laboratories demonstrated that these three TFs are present in neurons that start expressing *Pomc* in the developing arcuate nucleus at E10.5, and their

expression continues at later developmental ages and adulthood in hypothalamic POMC neurons. Indeed, we showed that ISL1, NKX2-1 and PRDM12 are absolutely necessary to specify the identity of POMC neurons[4-6]. Moreover, we also demonstrated that adult mice lacking any of these three TFs exclusively from POMC cells display greatly reduced levels of hypothalamic *Pomc* mRNA together with increased adiposity and body weight. However, our results also indicated that the sole presence of ISL1, NKX2-1 and PRDM12 at E10.5-E11.5 is insufficient to determine the early identity of hypothalamic POMC neurons, suggesting that the combinatorial set of TFs necessary for neuronal-specific *Pomc* expression has additional early components, yet to be discovered. In this regard, our scRNA-seq study revealed several novel candidates in cluster 1 cells including *Six3*, *Six6, Sox1/2/3, Sox14* and other TFs (Figure 2). Future molecular and functional genetic studies will be needed to assess their potential contribution to Arc *Pomc* expression and the functioning of POMC neurons.

Six3 and *Six6* are two evolutionarily related TF genes known to play critical roles in the development of the eyes, forebrain and pituitary[40-42]. The expression patterns of *Six3* and *Six6* highly overlap during early embryogenesis but segregate into different territories during late embryonic development, indicating possible distinct postnatal functions[43]. Indeed, *Six3* knockout mice die at birth whereas mice lacking *Six6* exhibit decreased numbers of GNRH neurons and impaired fertility[44]. Although *Six3* and *Six6* are abundantly expressed in Arc POMC neurons across all examined developmental stages (Fig. 2), their participation in the control of *Pomc* expression and the patterning and function of POMC neurons requires further investigation. Regarding Sox genes, a family of TFs known to regulate cell fate by transactivating cell-specific genes, *in silico* analysis of conserved SOX protein binding motifs suggests the potential binding of SOX proteins including SOX1, SOX2, SOX3, and SOX14 to *Pomc* neuronal enhancers nPE1 and nPE2[35]. All four of these *Sox* genes were highly enriched in *Pomc*^{high}*/Prdm12* cluster 1 (Figure 2 and Supplementary Table 2) as well as in *Pomc*^{low}*/Tac2* cluster 5 and *Pomc*^{low}*/Prdm13* cluster 7. Furthermore, mice lacking *Sox14* exhibit a great reduction in KNDY neurons[30].

A particular feature gene present in cluster 1 was *Tbx3*, a T-box TF that has been recently reported to play a key role in Arc *Pomc* expression[16]. Unlike *Isl1*, *Nkx2.1* and *Prdm12*, which are all strictly required at the onset of *Pomc* expression (E10.5), *Tbx3* expression commences several days later (E15.5) in the Arc and, therefore, apparently does not participate in the early specification of POMC neurons. However, *Tbx3* plays an important role in the functional maturation of these neurons as a transcriptional booster of arcuate *Pomc* expression. It was recently demonstrated in mice specifically lacking *Tbx3* from POMC neurons that *Pomc* expression was diminished leading to hyperphagia and obesity[16].

An unanticipated finding of our study is that Arc *Pomc*-expressing neurons originate from two distinct genetic pathways. Neuronal precursors in cluster 1 characterized by the coexpression of feature genes *Isl1*, *Nkx2-1*, *Prdm12*, *Six3*, *Six6*, and the *Sox* genes 1, 2, 3 and 14 (Figure 2) express high *Pomc* levels and give rise to the major group of canonical POMC neurons. In contrast, cluster 2 characterized by expression of the feature genes *Ebf1* and *Pou4f1*, the absence of *Prdm12* and low levels of *Pomc,* differ essentially from cluster 1 POMC neurons at developmental ages 11.5 through 15.5. Subsequently, about two thirds of cluster 2 precursors mature into identifiable POMC neurons expressing more moderate expression levels of *Pomc* and TFs such as *Isl1*, *Nkx2-1* and *Tbx3* compared to cluster 1 (Figure 3, subcluster 2-1). The other descendants of Cluster 2 retain the expression of *Ebf* factors, *Crabp1*, *Crabp2* and *Nr4a2* and express only very low *Pomc* levels (Figure 3, subcluster 2-2). A previous study identified a novel neuronal subtype in the htypothalamus featured by *Crabp1* [10]. Both ISH and immunostaining showed the restricted expression of *Crabp1* in the Arc. Analyzing the raw data from that study, we found 11% of neurons co-expressed *Crabp1* and low-levels of *Pomc*. Collectively, these data indicate that the *Pomc*^{med}*/Ebf1* cluster contains the early progenitor cells that become adult *Crabp1* clusters in the Arc. Further studies are needed to understand the functional significance of this non-canonical population of *Pomc*-expressing neurons that originate from an alternative route.

The identification of *Pomc*^{low}*/Tac2* and *Pomc*^{low}*/Otp* clusters confirmed that some fertility-regulating KNDY neurons[8] and orexigenic AGRP/NPY neurons[7] arise from *Pomc* progenitors. Moreover, our data indicate that *Pomc* precursors may also give rise to four other less characterized neuronal populations noted as *Pomc*^{med}*/Nr4a2*, *Pomc*^{low}*/Pitx2*, *Pomc*^{low}*/Dlx5* and *Pomc*^{low}*/Prdm13*. The *Pomc*^{med}*/Nr4a2* and *Pomc*^{low}*/Pitx2* are related clusters sharing 20% of their top 150 feature genes and both of them projected to the same two heterogeneous clusters n33 and n34 in adult Arc neurons (Figure 8). Interestingly, many of the top feature genes in cluster 8 *Pomc*^{low}*/Pitx2*, including *Pitx2, Lmx1a, Nr4a2, Foxa1, Foxp2*, and *Barhl2* were reported previously as marker genes for the supramammillary nucleus (SMN) [45]. This cluster also had the most abundant *Cck* transcripts, further indicating that it may represent a group of cells from the SMN considering the distribution of *Cck*-expressing neurons in the brain[46]. Notably, POMC cells are undetectable in the SMN in adult mice, suggesting that early *Pomc* expression in these cells may disappear during postnatal growth. This holds true when we compared our datasets to Lam *et. al's* study profiling FACS sorted individual POMC-EGFP cells from adult hypothalamus[11]. None of the clusters from their study were characterized by *Pitx2*, *Lmx1a* or *Foxa1* expression. Similarly, the *Pomc*^{low}*/Prdm13* cluster failed to match any clusters from Lam *et. al's* study[11], which is not unexpected since this cluster had the most abundant *Nr5a1* transcripts and corresponded to the n29 *Nr5a1/Adcyap1* (Figure 8) cluster[9], representing a cell population in the ventromedial hypothalamus featured by *Nr5a1* (*Sf1*) expression. Immunohistochemistry showed that NR5A1 is abundantly co-localized in *Pomc*-expressing cells at early embryonic days (E11.5-15.5) but not later perinatal days (E17.5) (Figure 2E). Conversely, cluster 2 adult POMC cells (featured with *Fzd5*, *Setd4* and *4732456N10Rik*) from Lam *et.al's .* study failed to match any of the clusters in our study, indicating that this cluster may contain cells generated postnatally. The signature genes for our *Pomc*^{low}*/Dlx5* cluster including *Dlx1, Dlx2, Dlx5*, and *Dlx6* were highly enriched and specific for cluster 1 in Lam *et. al's* study, indicating that the *Pomc*^{low}*/Dlx5* cluster may become a subset of adult POMC neurons. Other than the above four

less defined clusters, the *Pomc*^{high}*/Prdm12* cluster is highly similar to cluster 4 in Lam *et. al's* study characterized by *Prdm12, S100a10, Cpne4* and *Calb1* expression, representing the classic POMC neurons in adult mice.

The *Pomc*^{med}*/Nr4a2* cluster, particularly subcluster 3-1, is the second cluster enriched with *Pitx2, Lmx1a,* and *Foxa1* expression and also has the most abundant *Nr4a2*, *Pitx3*, and *Th* transcripts (Supplemental Table1). The early B cell factors (*Ebf1-3*) were also present in this subgroup of cells in a temporally restricted expression pattern (Figure 4B). Since the *Ebfs* control differentiation or migration of MbDA subpopulation in a temporal-dependent manner[47], it is reasonable to infer that *Pomc*^{med}*/Nr4a2* subcluster 3-1 (Figure 4) may define a subpopulation of immature MbDA. Indeed, *Nr4a2* is expressed in 18.5% of cells in cluster 4 of adult POMC cells from the Lam *et.al.* study[11].

It is worth mentioning that despite some extent of similarities between embryonic and adult POMC cells, the majority of cells profiled in our study are distantly related to mature adult POMC neurons. For example, most adult POMC cells express proprotein convertase PC1/3 (*Pcsk1*) and leptin receptor (*Lepr*), whereas only 22% and 2.7% of cells from our datasets express the two genes, respectively, indicating dramatic transcriptional signaling changes in POMC neurons during postnatal maturation after age P12.

Concluding remarks

In summary, this study is the first to comprehensively characterize transcriptomes of POMC cells during embryonic and early postnatal development. This dataset extends our understanding of the diversification of early POMC cells and provides a valuable resource for further elucidating the regulatory mechanism for *Pomc* expression and neuronal maturation. The

identification of new marker genes in each subpopulation as mentioned above and listed in Supplementary Table 2 will potentially lead to new directions for future functional studies.

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Author Contributions

H.Y., M.R., and M.J.L. jointly conceived the study, designed the experiments, analyzed data and drafted the manuscript. H.Y. conducted the experiments. All of the authors edited and approved the final manuscript.

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Competing Interests

The authors declare no competing interests.

Accession Codes

All raw data have been deposited in the Gene Expression Omnibus under accession number GSE154153. No custom code was used in this study. The detailed analytical methods were described in the methods.

Reporting Summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Methods

Mice

All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan and followed the Public Health Service guidelines for the humane care and use of experimental animals. Mice were housed in ventilated cages under controlled temperature and photoperiod (12-h light/12-h dark cycle, lights on from 6:00 AM to 6:00 PM), with free access to tap water and laboratory chow (5L0D, LabDiet). Breeding mice were fed with the breeder chow diet (5008, LabDiet). Transgenic mice expressing the fluorescent protein Tdimer-Discosoma red (TdDsRed) in POMC neurons were generated previously[17]. The vast majority of POMC-TdDsRed cells were validated as authentic POMC neurons based on co-localization of ACTH immunostaining with the POMC-TdDsRed fluorophore [17].

Generation of single cell (sc) suspension for scRNAseq

Adult male POMC-TdDsRed/+ mice were bred with female POMC-TdDsRed/+ mice and the day of copulation plug detection was counted as embryonic day 0.5 (E0.5). Embryonic hypothalami were dissected at E11.5, E13.5, E15.5, and E17.5. Postnatal hypothalami were isolated from

pups at postnatal day 5 (P5) and P12. In each dissection, hypothalami from at least 6 pups were pooled together to acquire enough cells for fluorescence-activated cell sorting (FACS). Pooled tissue samples were digested in papain solution supplemented with DNAse for 20 mins (E11.5 and E13.5), 30 mins (E15.5), 40 mins (E17.5) or 1hr (P5, and P12) with gentle agitation based on a published protocol[14]. The isolated cells were stained with DAPI as an indicator for cell viability and sorted by FACS for the transgenic red fluorophore (Supplemental Figure 11). The collected cells were counted and subjected to scRNA-seq library preparation and sequencing (10x Genomics).

Data processing

A total of 37,053 single cells (E11.5: 5,329, E13.5: 7,051, E15.5: 5,148, E17.5: 2,488, P5: 7,551 and P12: 9,486) from six different developmental stages were processed using the 10X Genomics Chromium system. The libraries were sequenced on Illumina HiSeq 4000 and NovaSeq platforms. We obtained a total of over 2.6 billion reads with an average of 70,7896 reads per cell. Over 85% of reads mapped confidently to the mouse genome across all six developmental stages. Raw reads were processed with Cell Ranger (version 2.2 and version 3.0). Seurat package (version 3.1.5)[48] was used for downstream analysis. Since the number of genes and UMI counts varied across developmental stagewe applied different criteria at each stage to filter out possible doublets and low quality cells. Specifically, at E11.5, we removed outlier cells that had UMI counts < 5,000 or >60,000 and gene counts <1,000 or >8,000 (determined by the visualization of UMI counts and gene count distributions). At E13.5, we removed outlier cells that had UMI counts <2,500 or >30,000 and gene counts >1,500. At E15.5, we removed outlier cells that had UMI counts >40,000 and gene counts <1,000 or > 6,000. At E17.5, we removed outlier cells that had UMI counts >25,000 and gene counts <1.000 or > 5,000. At P5, we removed outlier cells that had UMI counts <1,500 or >60,000 and gene

counts <1,500 or >8,000. At P12, we removed outlier cells that had UMI counts<2,000 or >60,000and gene counts <1,500 or > 8000. Moreover, cells with high proportions of mitochondrial genes (>10%) or hemoglobin genes (>10%) were filtered out. Finally, we removed all the cells without any *Pomc* transcript UMI counts. A total of 13,953 cells passed the criteria (E11.5: 1,498, E13.5: 1,796, E15.5: 2,078, E17.5: 1,139, P5: 3,909 and P12: 3,533) for downstream analysis.

The gene expression level for each cell was normalized by the total expression, multiplied by a scaling factor of 10,000 followed by log-transformation[48]. The top 2,000 most variable genes were selected from each age and 60 integration anchors were used to combine all the datasets together. After scaling and centering the integrated dataset, we performed principal components (PC) analysis on the data matrix and the top 50 PCs were selected based on JackStraw and Elbow plots from Seurat Package[19] for data visualization using Uniform Manifold Approximation and Projection (UMAP) technique. We next constructed a Shared Nearest Neighbor (SNN) graph by setting an expected number of neighbors to 50. In order to cluster the cells, a SNN modularity optimization technique within a function FindClusters was used to group cells together with a resolution parameter 0.1. The identity for each cluster was assigned based on the level of *Pomc* transcripts and prior knowledge of marker genes. The similar analysis was applied to define *TdDsRed* cell clusters (Supplementary Figure 2 and Supplementary Figure 3). In addition to the above criteria applied to remove unwanted cells, we chose cells with at least one *TdDsRed* transcript UMI count and used the top 2,000 highly variable genes for principal components analysis. The top 30 PCs were selected for unsupervised clustering with a resolution of 0.3.

Unsupervised cell clustering from each developmental age (Supplementary Figure 6) was conducting using the following PC numbers (E11.5: 25; E13.5:24, E15.5: 26, E17.5: 19, P5: 40, P12: 34) with their corresponding resolutions (E11.5: 0.2; E13.5: 0.6, E15.5: 0.5, E17.5: 0.5, P5: 0.5, P12: 0.5. The UMAP plots, violin plots, feature plots and dot plots were all

generated in the Seurat Package. The average expression levels for each gene within each developmental subcluster (Supplemental Table 8) were obtained from Seurat and calculated by the average of [*exp(logNormalized counts) -1*]. The differentially expressed genes in each identified cluster was identified by the comparison of gene expression levels in a specific cluster to all the other clusters.

Cluster identity comparisons

We took the following steps to compare cluster identities from developmental POMC cells to 34 adult ARC neuronal clusters[9] (Figure 8 and Supplementary Figure 10). 1) We obtained the adult ARC single cell sequencing data from https://hemberg-lab.github.io/scRNA.seq.datasets/mouse/brain[49] and transformed the dataset from SingleCellExperiment format to Seurat object format. 2) We normalized adult ARC data and acquired the top 2000 most variable gene for further analysis. 3) The standard datasets integration method from Seurat package was performed with 50 integration anchors and used to combine the two datasets. 4) The integrated data were scaled and the top 50 PCs were selected for unsupervised clustering implemented in Seurat. 5) We chose 50 transfer anchors to predict the developmental POMC cells' identities based on adult ARC cell clusters. A similar approach was applied to compare cluster identities between POMC cells and DTDSRED cells (Supplementary Figure 2) with the top 50 anchors for data integration and 50 anchors to transfer cell identities from POMC cells to DSRED cells. We also applied this approach to conduct age-pairwise comparisons (Supplementary Figure 6) with default setting from Seurat V3.1.5 package.

Cell lineage construction

Cell lineage was constructed using the Destiny (version 2.14.0) package, which implements the formulation of diffusion maps[50]. Diffusion maps are a spectral method for non-linear dimension reduction, which is especially suitable for analyzing single-cell gene expression data from different time-courses. We removed VLMC, glial cells and astrocytes (cluster 9, 10 and 11 in Figure1) and kept only neurons at embryonic stages (E11.5, E13.5, E15.5 and E17.5), resulting in a total of 6,005 cells for this analysis. The log-transformed normalized data from Seurat data slot and the corresponding annotation information were imported to construct an expression matrix. The diffusion maps were constructed under the default setting for the Gaussian kernel width sigma (σ) and 300 nearest neighbors. The first two Diffusion Components (DCs) were used to visualize the results. The 3D plots were produced using rgl package (version 0.100.50) with the top three DCs.

Tissue collection and Immunofluorescence staining

Depending on developmental stage, embryos at E10.5 – E17.5 were fixed in 4% paraformaldehyde in phosphate buffer solution (PBS) at 4 °C for a various period of time. Specifically, embryos at E10.5 to E12.5 were fixed for 1h; heads from embryos at E15.5-E16.6 and E17.5 were fixed for 2 h and 4 h respectively. Tissues were stabilized in 10% sucrose/10% gelatin in PBS at 37 °C for 30 min prior to embedding in O.C.T. compound as described previously[9]. Embryonic brains were sectioned sagittally on a cryostat (Leica CM1950) at 20 μm thickness. For dual immunofluorescence, after washing excess O.C.T. compound with PBS, a heat-induced antigen retrieval process was performed using citrate buffer (10 mM anhydrous citric acid and 0.05% Tween-20, pH 6.0) at 80 °C for 30 min followed by two washes in PBS. Sections were then blocked with 3% normal goat serum and 0.1% Triton X-100 for 1 hour and incubated with primary antibodies at room temperature overnight. After PBS washes, sections were incubated with goat secondary antibodies for 2 hours at room temperature. Nuclei were

stained with DAPI (1 mg/l) for 10 min and the slides washed five times with PBS before mounting with ProLong™ Gold Antifade Mountant (ThermoFisher). Sections were imaged on a Nikon 90i fluorescence microscope with NIS-Elements software. Information on the sources and dilution of antibodies are listed in Supplementary Table 10.

In situ **hybridization**

Fluorescence *in situ* hybridization (FISH) was performed using RNAscope® Multiplex Fluorescent V2 Assay (Advanced Cell Diagnostics) according to the manufacturer's instructions with slight modifications. Specifically, after washing with PBS, sections were post-fixed with 4% paraformaldehyde for 20 minutes, and dehydrated in a series of ethanol solutions (50%, 70% and 100%). After drying, dehydrated sections were incubated in RNAscope® Hydrogen Peroxide solution for 10 mins followed by the protease treatment using RNAscope® Protease IV for 20 mins. Sections were then hybridized to target RNA probes for 2 hours in the HybEZ™ II hybridization oven. Hybridized probes were amplified using a cascade of signal amplification solutions (AMP 1-3) followed by standard signal developing protocol as described in the manufacturer’s brochure. Detailed information on RNA probes and dilutions are listed in Supplementary Table10. Confocal images were obtained using a Nikon Instruments A1 Confocal Laser Microscope with NIS-Elements software.

Figure 1. Single cell sequencing of *Pomc*^{DsRed} cells from developing mouse hypothalami at E11.5, E13.5, E15.5, E17.5, P5 and P12 reveals eight distinct neuronal clusters and three non-neuronal clusters. (A) Schematic diagram showing the overall experimental procedure. (B) UMAP (Uniform Manifold Approximation and Projection) plot showing the distribution of major cell clusters and their corresponding cell% at each developmental stage. (C) Violin plots showing the expression of three signature genes and *Pomc* for each cluster. (D) UMAP plots showing the enrichment of feature genes in each cluster. Genes representing each cluster were colored and highlighted to show the cluster-specific enrichment. The color intensity corresponds to the normalized gene expression from Seurat analysis, where gene expression for each cell was normalized by the total expression, multiplied by a scale factor of 10,000, and then log-transformed. VLMC, vascular and leptomeningeal cell.

Figure 2. Temporal gene expression patterns of the *Pomc*^{high}*/Prdm12* cluster. (A) UMAP plot highlights cells from the *Pomc*^{high}*/Prdm12* cluster. (B) Violin plots showing the expression of signature genes at each developmental stage. Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Six6* (red) (C), and the co-localization of *Pomc* (green) and *Six3* (red) (D) at indicated developmental stages. (E) Immunofluorescence showing the co-localization TDTOMATO (red) NR5A1 (green) at the indicated developmental stages. Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 µm. Image orientation: left, posterior(P); right, anterior(A). RP: Rathke's pouch; Pit: pituitary gland. Arc: arcuate nucleus; VMH: ventromedial hypothalamus.

Figure 3. Temporal gene expression patterns of the *Pomc*^{med}*/Ebf1* cluster. (A) UMAP plot highlights cells from *Pomc*^{med}*/Ebf1* cluster. (B) Violin plots showing the expression of signature genes at each developmental stage. (C) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Ebf1* (red). (D) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Pou4f1* (red). (E) Re-clustering cells from the *Pomc*^{med}*/Ebf1* cluster reveals two sub-clusters denoted as 2-1 *Pomc*^{high} and 2-2 *Pomc*^{low}. Violin plots showing the signature gene expression in 2-1 *Pomc*^{high} and 2-2 *Pomc*^{low} clusters. Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 µm. Image orientation: left, posterior(P); right, anterior(A). Pit: pituitary gland. Mes: mesenchyme.

Figure 4. Temporal gene expression patterns of the *Pomc*^{med}*/Nr4a2* and *Pomc*^{low}*/Pitx2* clusters. (A) UMAP plot highlights cells from the *Pomc*^{med}*/Nr4a2* cluster. (B) Violin plots showing the expression of signature genes in the *Pomc*^{med}*/Nr4a2* cluster at each developmental stage. (C) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Nr4a2* (red). (D) Re-clustering cells from the *Pomc*^{med}*/Nr4a2* cluster reveals two sub-clusters denoted as 3-1 *Pomc*^{low} and 3-2 *Pomc*^{high} clusters. Violin plot showing the signature gene expression in 3-1 *Pomc*^{low} and 3-2 *Pomc*^{high} clusters. (E) UMAP plot highlights cells from the *Pomc*^{low}*/Pitx2* cluster. (F) Violin plots showing the expression of signature genes at each developmental stage in the *Pomc*^{low}*/Pitx2* cluster. (G) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Pitx2* (red). Scale bar: 50 µm. Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 µm. Image orientation: left, posterior (P); right, anterior (A). RP: rathke's pouch; Pit: pituitary gland.

Figure 5. Temporal gene expression patterns of the *Pomc*^{low}*/Otp* cluster. (A) UMAP plot highlights cells from the *Pomc*^{low}*/Otp* cluster. (B) Violin plots showing the expression of signature genes at each developmental stage. (C) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Npy* (red) at E15.5. Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. (D) UMAP plots showing cells in the *Pomc*^{low}*/Otp* cluster expressing *Sst*, *Agrp*, *Npy*, *Sst*/*Agrp*, or *Sst*/ *Npy*. Cells expressing *Sst* and cells expressing both *Agrp*/*Npy* are from two distinct cell populations. Scale bar: 50 µm. Image orientation: left, posterior; right, anterior; Pit: pituitary gland

Figure 6. Temporal gene expression patterns of the *Pomc*^{low}*/Tac2* cluster. (A) UMAP plot highlights cells from the *Pomc*^{low}*/Tac2* cluster. (B) Violin plots showing the expression of signature genes at each developmental stage. Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Tac2* (red) (C), and the co-localization of *Pomc* (green) and *Tacr3* (red) (D) at the indicated developmental stages. (E) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Sox14* (red). (F) Immunofluorescence showing the co-localization of TDTOMATO (red) and ESR1 (green) at the indicated developmental stages. Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 µm. Image orientation: left, posterior; right, anterior; Pit: pituitary gland.

Figure 7. Diffusion maps analysis illustrating the developmental trajectories of hypothalamic neuronal clusters. (A) UMAP plot showing the distribution of cells in each cluster from their corresponding developmental stages. (B) UMAP plots showing the splitting of neuronal clusters by developmental stages. Clusters were dash-dot circled to show the emerging / evolving progress through development. (C-D) Diffusion map showing the cell lineages of POMC neurons during embryogenesis. Cells are colored on the basis of their developmental stage (C) or based on their previously defined clusters (D). (E) Diffusion maps showing the expression of selected genesaccording to previous characterization of the feature genes representing each cluster. The color intensity corresponds to the normalized gene expression from Seurat analysis.

Figure 8. Projection of embryonic POMC^{DsRed} cell subtypes to adult arcuate nucleus neuronal clusters. Projected cell clusters from the *Campbell et al.* study [14] were labeled with the same color as the POMC^{DsRed} cells clusters. The adult ARC neuronal clusters highlighted in bold font indicate a higher percentage of cells projected to this cluster from the corresponding developmental clusters. The detailed cell embedding and percentage of cells projecting to adult Arc neuronal clusters are shown in Supplemental Figure 10.

Supplemental Data

Figure S1

Heat map showing the expression of the top 30 marker genes defining each cluster from Figure 1. M: microglia; V: vascular and leptomeningeal cells; A: astrocytes.

Figure S2

Analysis of POMC cells with *DsRed* transcripts reveals 9 distinct cell clusters. (A) UMAP plot showing the distribution of major cell clusters. (B) Violin plots showing signature gene expression in each cell type. Three feature genes were chosen to represent each cell type. (C) UMAP plots showing the enrichment of feature genes in each cluster. Genes representative of each cluster were colored and highlighted to show the cluster-specific enrichment. The color intensity corresponds to the normalized gene expression from Seurat analysis. (D) Identification of similar cell clusters between POMC cells with *DsRed* transcripts and POMC cell clusters. Red: POMC cell clusters are from Figure 1. Blue: POMC cells with *DsRed* transcripts from Supplemental Figure 2A. (E) Venn diagram showing the number of cells expressing *Pomc* transcripts, *DsRed* transcripts or both *Pomc* and *DsRed* transcripts. Pie chart showing the percentage of POMC cells expressing *DsRed* transcripts at each developmental stage. (F) Vertical bar-chart showing the percentage of cells with *DsRed* transcripts projected to corresponding POMC cell clusters. VLMC, vascular and leptomeningeal cell.

Figure S3

Overview of cell clusters from POMC cells with *DsRed* transcripts. (A) UMAP plot showing the distribution of major cell clusters and their corresponding cell% from each developmental stage. (B) UMAP plots showing the number of cells at each developmental stage. (C) UMAP plots showing the splitting of clusters by developmental stages.

Figure S4

Temporal gene expression patterns of the *Pomc^{low}/Dlx5* and *Pomc^{low}/Prdm13* clusters. (A) UMAP plot highlights cells from *Pomc^{low}/Dlx5* cluster. (B) Violin plots showing the expression of signature genes at each developmental stage in the *Pomc^{low}/Dlx5* cluster. (C) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Dlx5* (red). (D) UMAP plot highlights cells from the *Pomc^{low}/Prdm13* cluster. (E) Violin plots showing the expression of signature genes at each developmental stage in the *Pomc^{low}/Prdm13* cluster. (F) Fluorescence *in situ* hybridization showing the co-localization of *Pomc* (green) and *Prdm13* (red). Insets are magnified views of the indicated boxes. Arrows indicate co-expressing neurons in the merged panels. Scale bar: 50 µm. Image orientation: left, posterior; right, anterior; Pit: pituitary gland.

Figure S5

Distribution of GABAergic and glutamatergic neurons in POMC^{DsRed} cell clusters (A) UMAPs plot showing the distribution of cells with *Gad1* or *Vglut2* gene expression. (B) Violin plots showing expression of the GABAergic or glutamatergic marker genes in each neuronal cluster.

Figure S6

UMAP plots showing the distribution of major cell clusters at each developmental stage. (A) E11.5, (B) E13.5, (C) E15.5, (D) E17.5, (E) P5 and (F) P12. The developmental subclusters derived from each primary cluster are colored to corresponding cells in the UMAP plots.

Figure S7

POMC^{DsRed} developmental cell subclusters shown in Supplemental Figure 6 are projected to the original integrated cell clusters from Figure 1. The projections were based on the Seurat analysis and manual comparisons of feature genes in each subcluster (Supplemental Table 2

and supplemental Table5). The clusters from individual stages leading to the same final integrated clusters are labeled with the same color. Question marks indicate uncertainties of individual projections.

Figure S8

Overview of neuropeptide, secretory granule and processing enzyme gene expression patterns across clusters and developmental ages. The size of dots indicates the percentage of cells expressing the specific transcripts. The color intensity corresponds to the levels of normalized gene expression.

Figure S9 A and B

Overview of G-protein coupled receptor (GPCR) gene expression patterns across clusters and developmental ages. The size of dots indicates the percentage of cells expressing the specific transcripts. The color intensity corresponds to the levels of normalized gene expression. The list of genes was acquired from https://www.guidetopharmacology.org/targets.jsp and Gene Ontology (GO:0005576).

Figure S10

Identification of similar clusters between the neuronal $POMC^{DsRed}$ developmental clusters and the neuronal clusters derived from adult arcuate nucleus[9]. (A) UMAP plots showing the integration of $POMC^{DsRed}$ clusters and adult arcuate nucleus neuronal clusters. (B) The percentage of $POMC^{DsRed}$ cells projecting to corresponding adult arcuate nucleus neuronal clusters.

Figure S11

Fluorescence-activated cell sorting (FACS) strategy for collecting the single POMC-TdDsRed cells used for scRNAseq.

Table S1 (corresponding to Figure 1)

Average gene expression in each defined POMC^{DsRed} clusters. Average gene expression was obtained from Seurat analysis, which was calculated by the average of *exp(logNormalized counts) -1* in each gene within each cluster.

Table S2 (corresponding to Figure 1)

Differentially expressed genes in each identified POMC^{DsRed} cluster. For each cluster, the fold-change values for each gene was calculated by the comparison of expression level in a specific cluster to the rest of other clusters. The data was sorted by average_logFC > 0.25. pct.1: the percentage of cells expressed the gene in the defined cluster; pct.2: the percentage of cells expressed the gene in other clusters; p-val: p-value; p_val_adj: adjusted p-value. Wilcox test was performed to identify differentially expressed genes in each cluster.

Table S3 (corresponding to Supplemental Figure S2)

Average gene expression in each defined DsRed clusters. Average gene expression was obtained from Seurat analysis, which was calculated by the average of *exp(logNormalized counts) -1* in each gene within each cluster.

Table S4 (corresponding to Supplemental Figure S2)

Differentially expressed genes in each identified DsRed cluster. For each cluster, the fold-change values for each gene was calculated by the comparison of expression level in a specific cluster to the rest of other clusters. The data was sorted by average_logFC > 0.25. pct.1: the percentage of cells expressed the gene in the defined cluster; pct.2: the percentage of cells expressed the gene in other clusters; p-val: p-value; p_val_adj: adjusted p-value. Wilcox test was performed to identify differentially expressed genes in each cluster.

Table S5 (corresponding to Figures 2 –6)

Average gene expression at individual developmental ages for each integrated cluster. Average gene expression was obtained from Seurat analysis, which was calculated by the average of *exp(logNormalized counts) -1*for each gene within each cluster.

Table S6 (corresponding to Figures 3 and 4)

Average gene expression in subclusters 2-1, 2-2, 3-1 and 3-2. Average gene expression was obtained from Seurat analysis, which was calculated by the average of *exp(logNormalized counts) -1* in each gene within each cluster.

Table S7 (corresponding to Figures 3 and 4)

Differentially expressed genes in subclusters 2-1, 2-2, 3-1 and 3-2 derived from integrated clusters 2 and 3, respectively. The data were sorted by average_logFC > 0.25. pct.1: the percentage of cells expressed the gene in the defined cluster; pct.2: the percentage of cells expressed the gene in other clusters; p-val: p-value; p_val_adj: adjusted p-value. Wilcox test was performed to identify differentially expressed genes in each cluster.

Table S8 (corresponding to Supplemental Figure 6)

Average gene expression in each defined cluster from the individual developmental ages. Average gene expression was obtained from Seurat analysis, which was calculated by the average of [*exp(logNormalized counts) -1*] for each gene within each cluster.

Table S9 (corresponding to Supplemental Figure 6)

Differentially expressed genes in each identified cluster from the individual developmental ages. For each cluster, the fold-change values for each gene was calculated by the comparison of

expression level in a specific cluster to the rest of other clusters. The data was sorted by average_logFC > 0.25. pct.1: the percentage of cells expressed the gene in the defined cluster; pct.2: the percentage of cells expressed the gene in other clusters; p-val: p-value; p_val_adj: adjusted p-value. Wilcox test was performed to identify differentially expressed genes in each cluster.

Table S10

Table of key resources.

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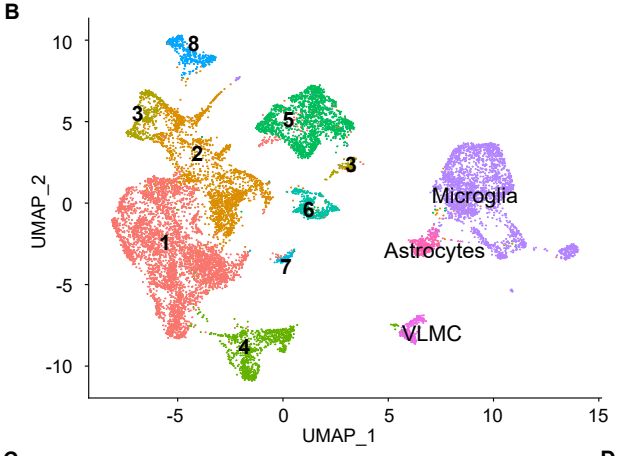
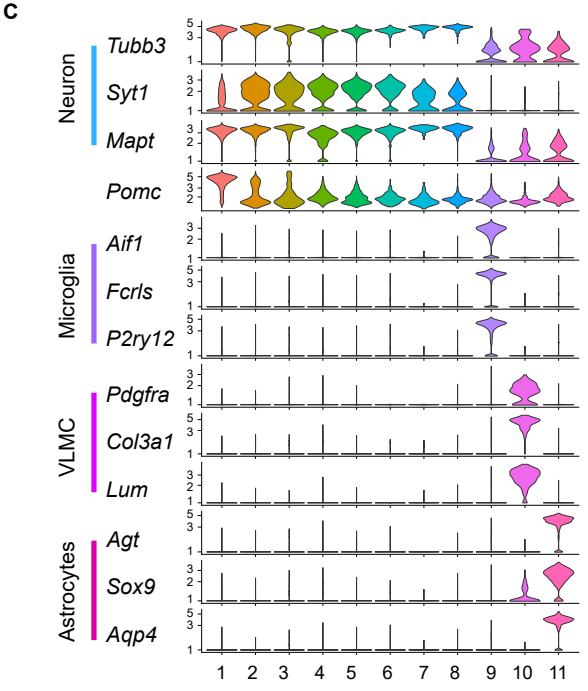
1019

Figure 1A



Percentage of Total Neurons

	E11.5	E13.5	E15.5	E17.5	P5	P12
1. *Pomc*^{high}/*Prdm12*	50.8%	40.6%	34.7%	33.3%	44.1%	47.2%
2.*Pomc*^{med}/*Ebf1*	43.4%	31.1%	11.8%	13.7%	13.8%	14.4%
3.*Pomc*^{med}/*Nr4a2*	0.5%	3.4%	5.9%	1.1%	5.6%	9.1%
4.*Pomc*^{low}/*Otp*	2.4%	6.0%	20.7%	20.9%	2.8%	4.2%
5.*Pomc*^{low}/*Tac2*	1.2%	3.3%	10.7%	24.6%	29.1%	16.0%
6.*Pomc*^{low}/*Dlx5*	0.5%	1.0%	6.2%	4.9%	3.9%	5.7%
7.*Pomc*^{low}/*Prdm13*	0.1%	1.4%	3.3%	1.1%	0.2%	0.4%
8.*Pomc*^{low}/*Pitx2*	1.0%	13.2%	6.8%	0.4%	0.6%	2.9%



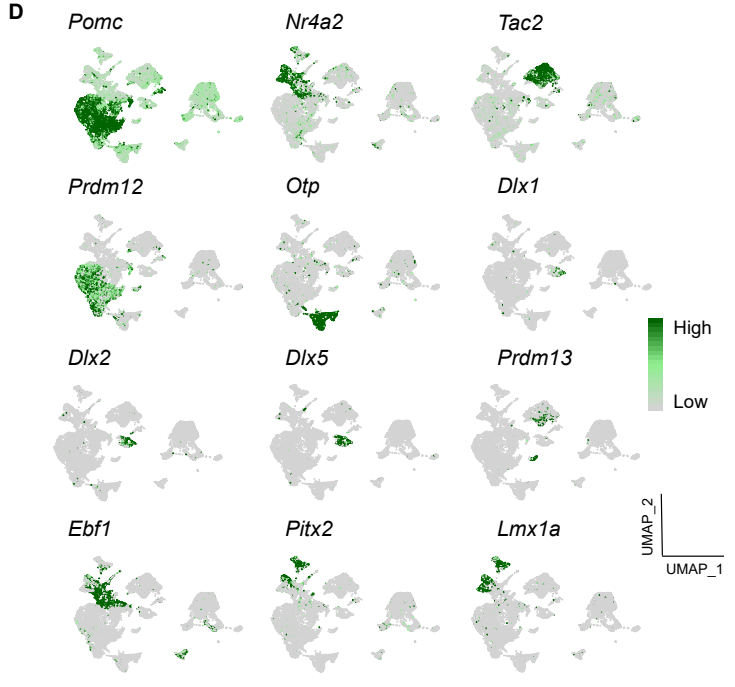


Figure 2

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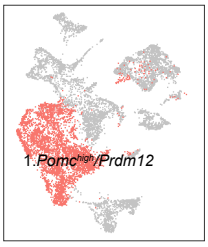

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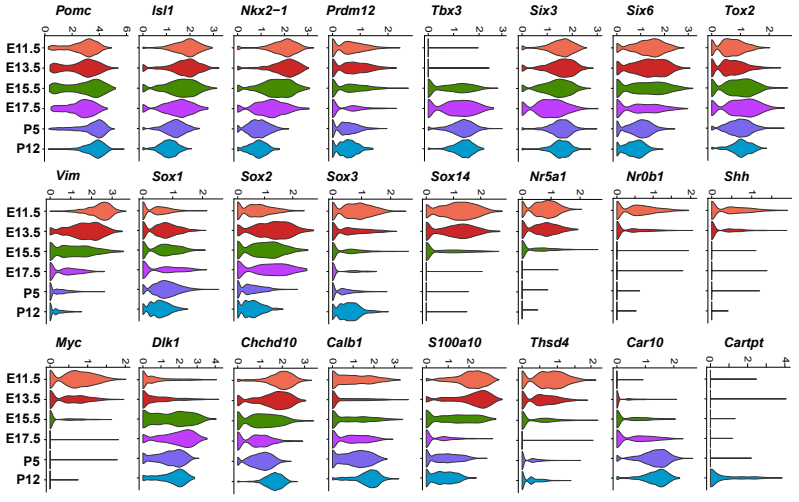

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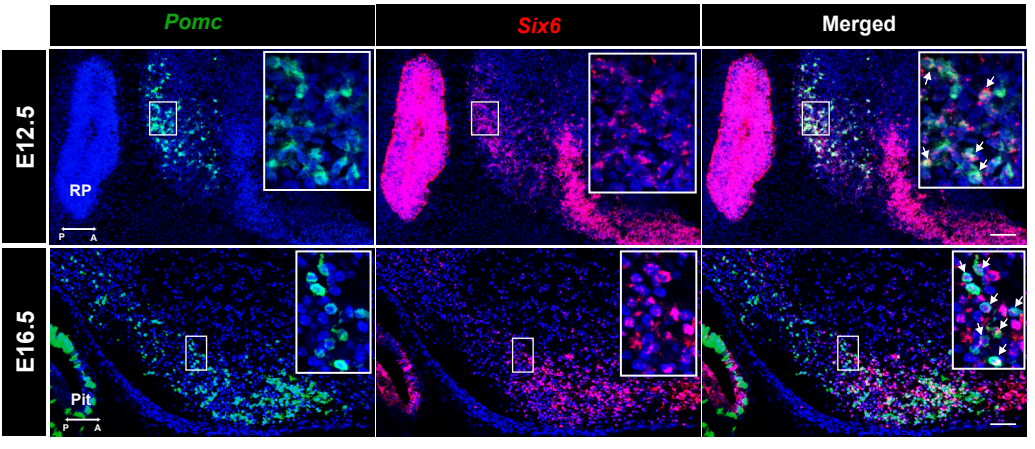

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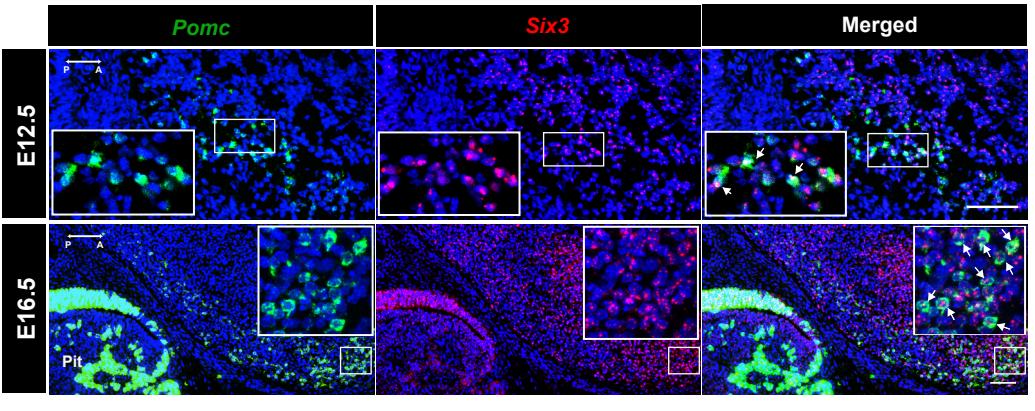

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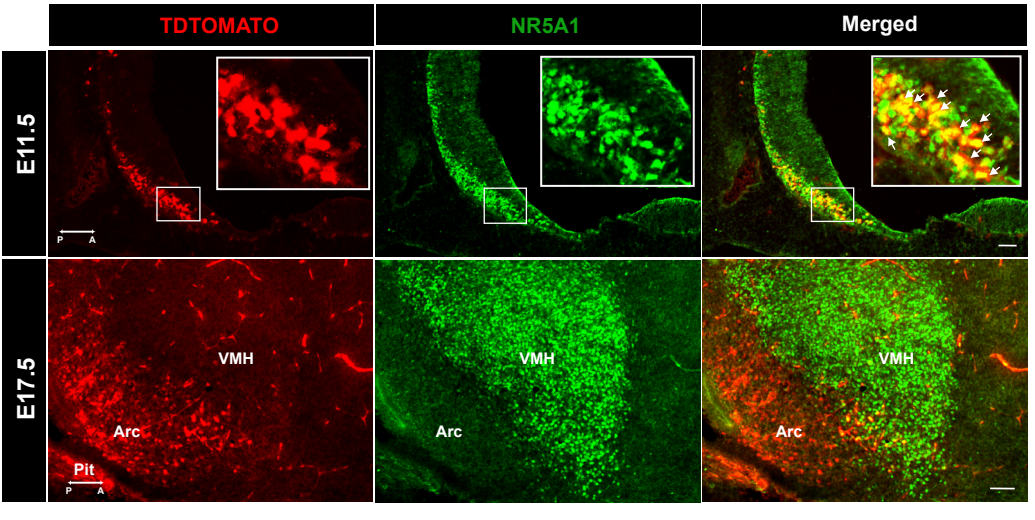
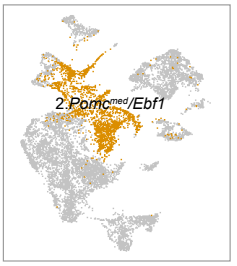


Figure 3 A



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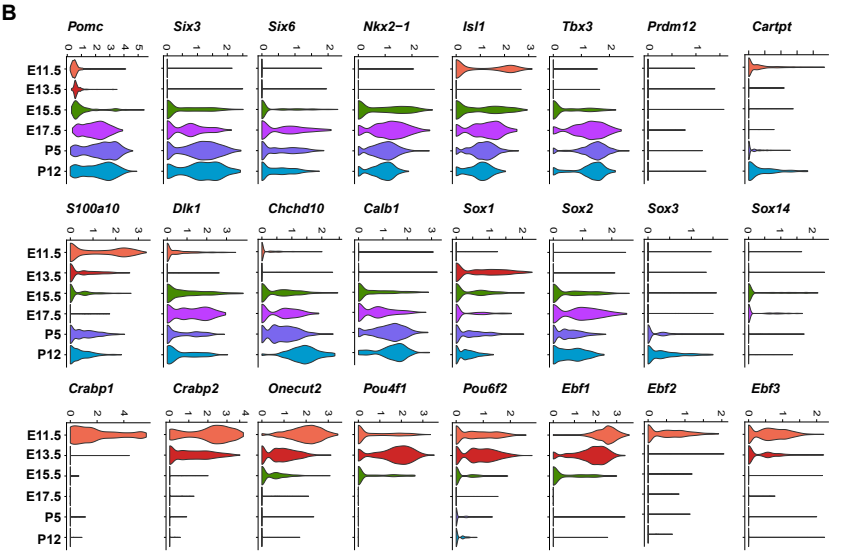

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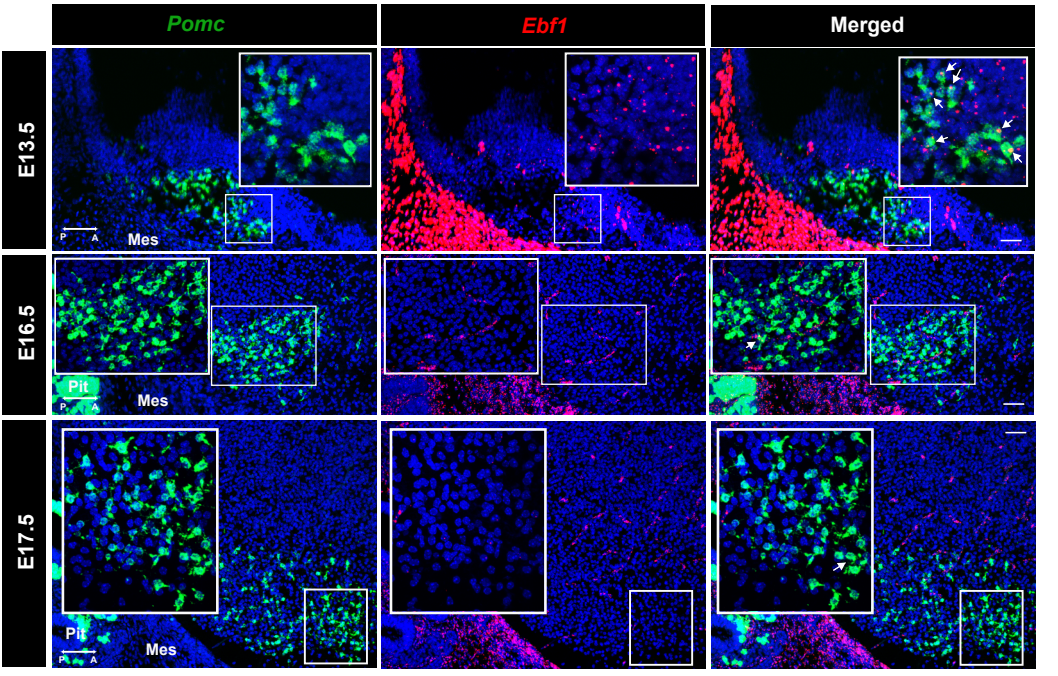
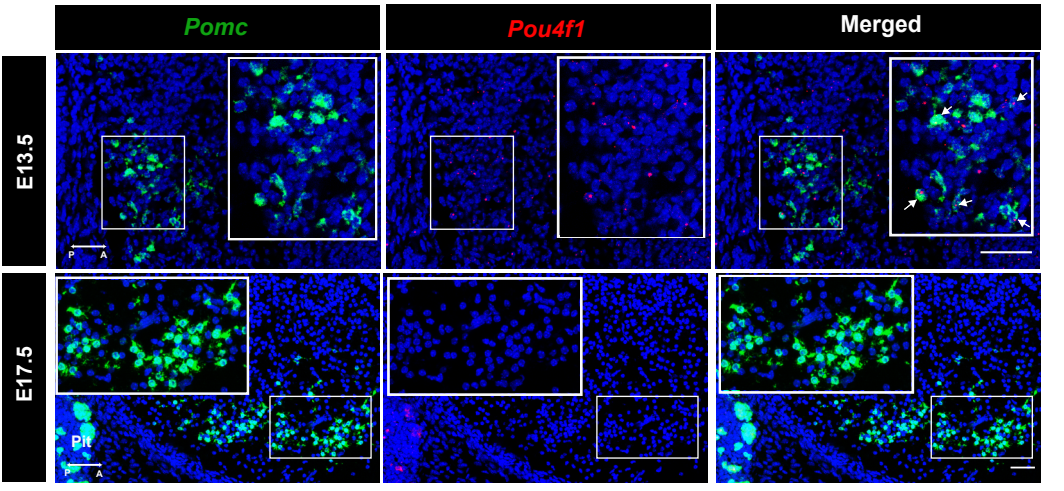
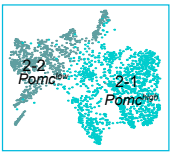


D



E

Subcluster
2. Pomc^med/Ebf1



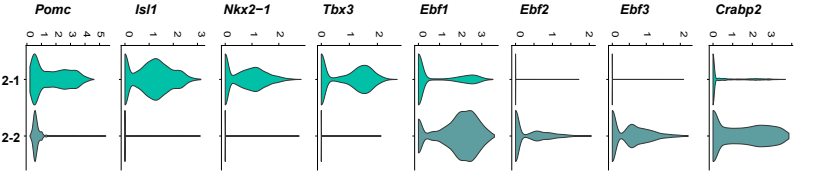
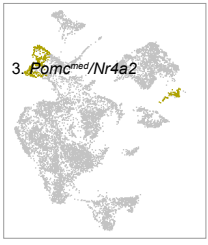
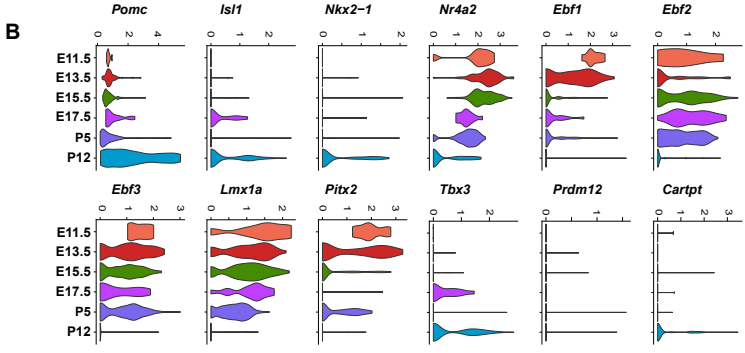
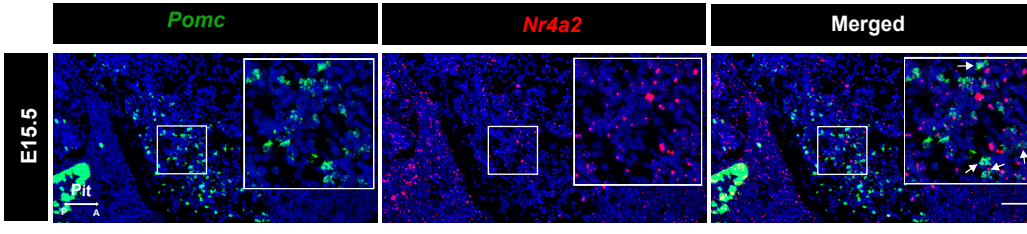
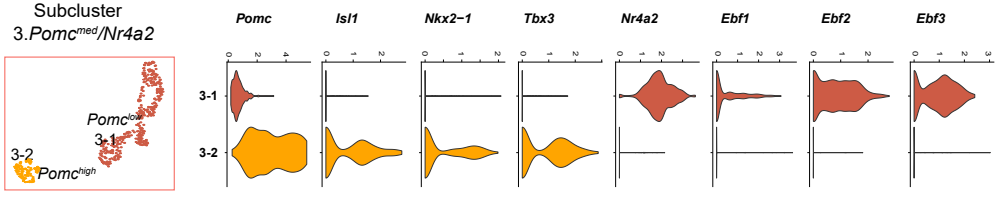


Figure 4 A

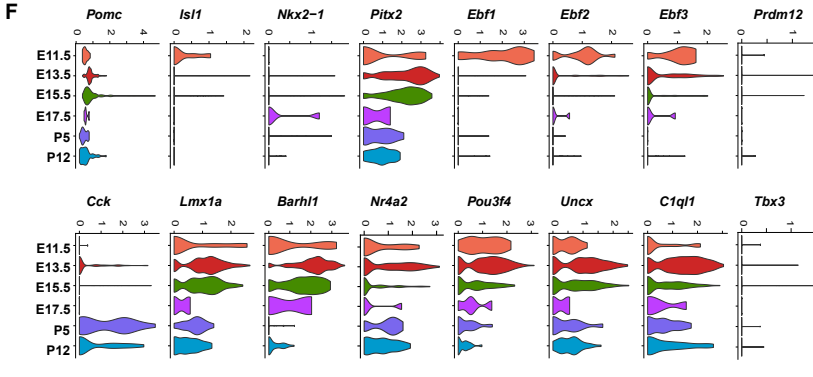

3.Pomc^med/Nr4a2


B
Pomc
Isl1
Nkx2−1
Nr4a2
Ebf1
Ebf2
Ebf3
Lmx1a
Pitx2
Tbx3
Prdm12
Cartpt
E11.5
E13.5
E15.5
E17.5
P5
P12


C
Pomc
Nr4a2
Merged
E15.5
Pit
P
A


D
Subcluster
3.Pomc^med/Nr4a2
Pomc^low
3-1
3-2
Pomc^high
Pomc
Isl1
Nkx2−1
Tbx3
Nr4a2
Ebf1
Ebf2
Ebf3

E
8.Pomc^low/Pitx2


F
Pomc
Isl1
Nkx2−1
Pitx2
Ebf1
Ebf2
Ebf3
Prdm12
Cck
Lmx1a
Barhl1
Nr4a2
Pou3f4
Uncx
C1ql1
Tbx3
E11.5
E13.5
E15.5
E17.5
P5
P12

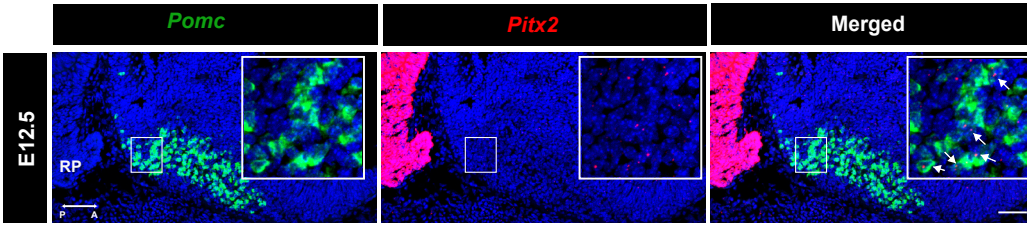
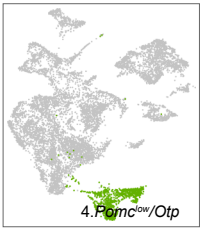
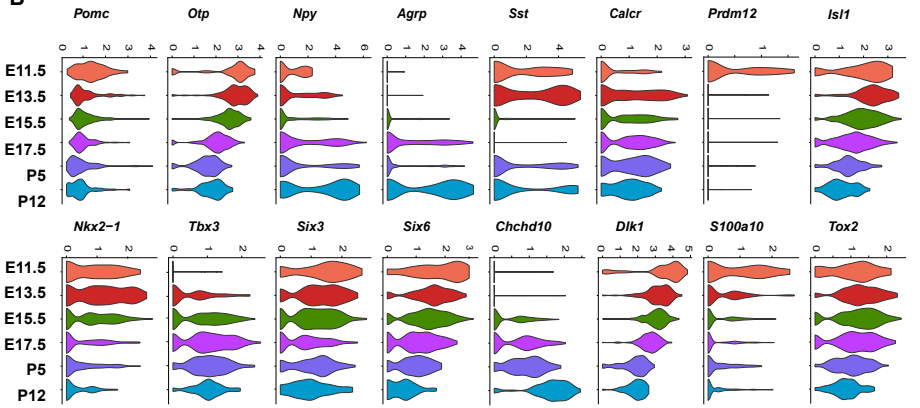

G
Pomc
Pitx2
Merged
E12.5
RP
P
A

Figure 5

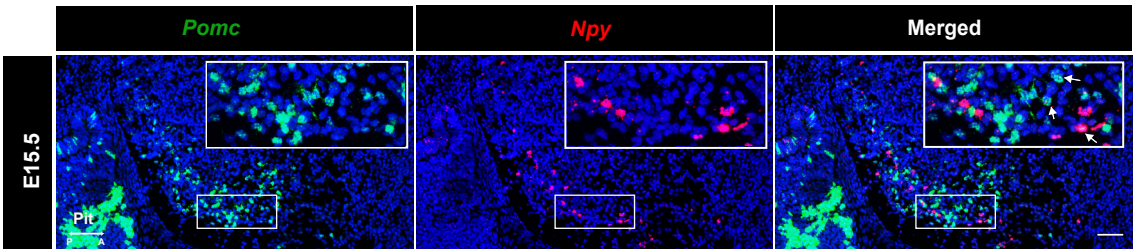
A



B



C



D

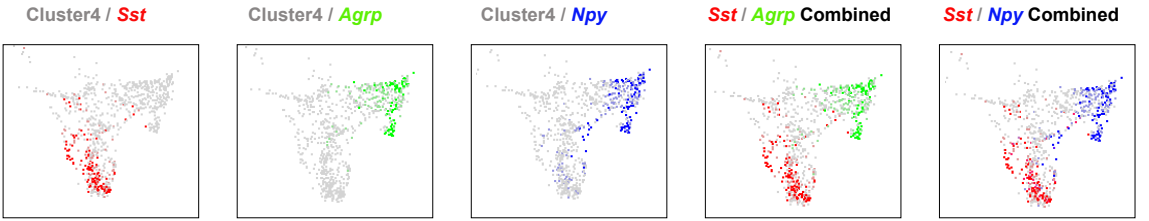
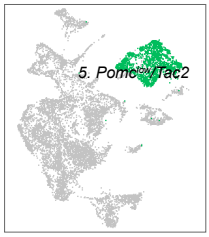
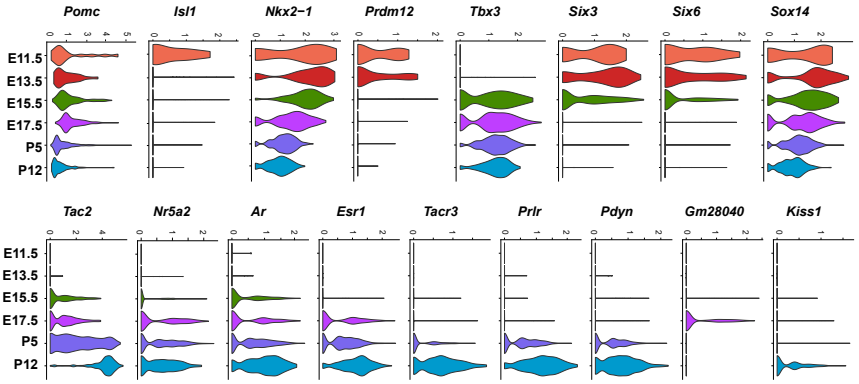


Figure 6

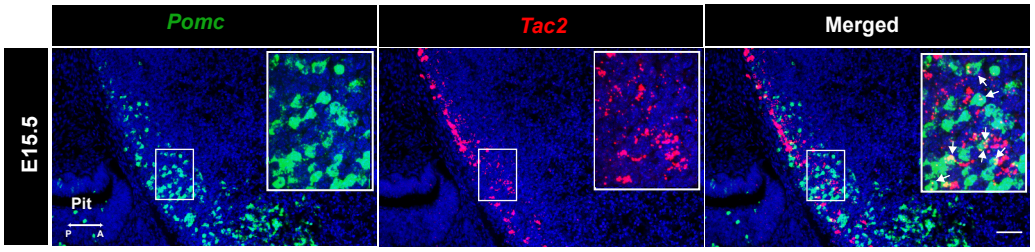
A



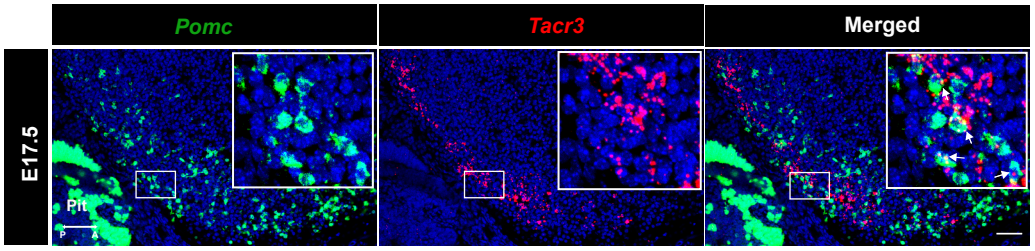
B



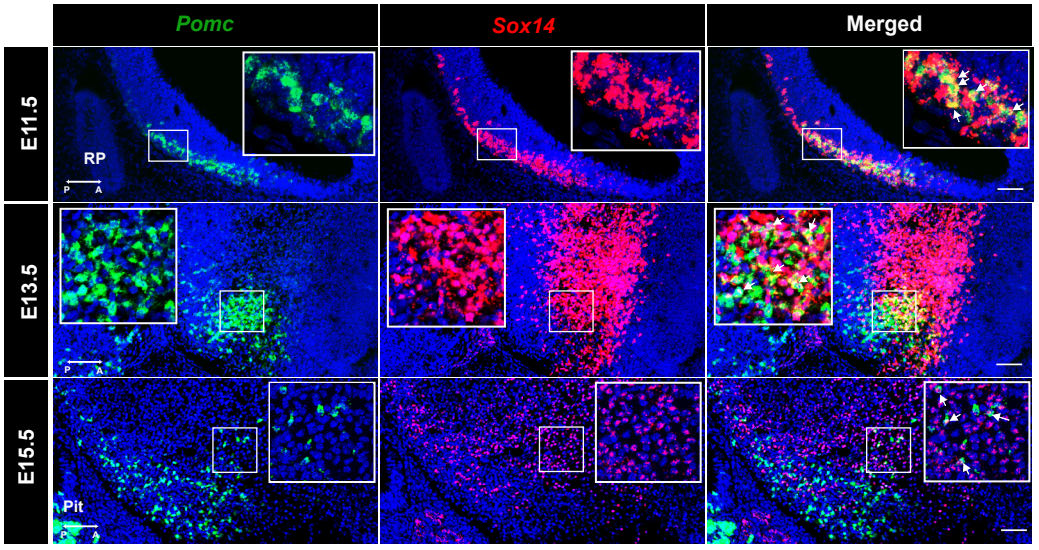
C



D



E



F

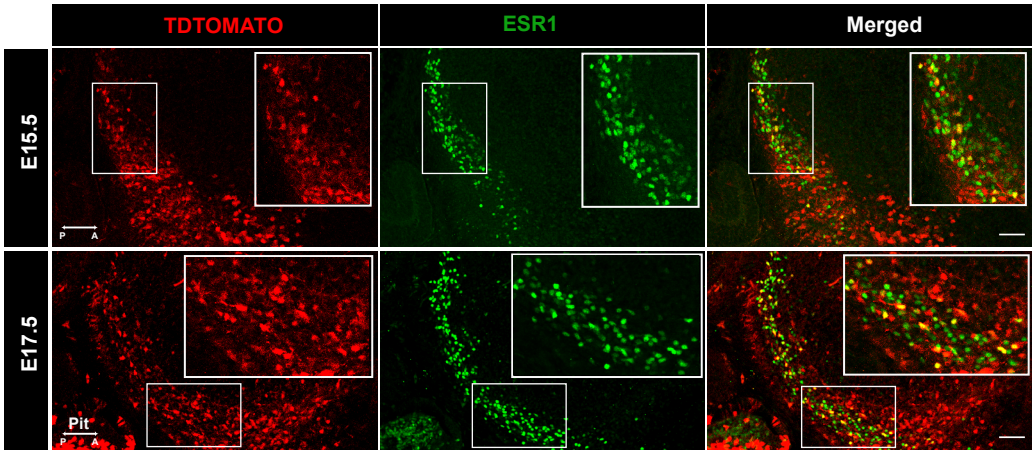
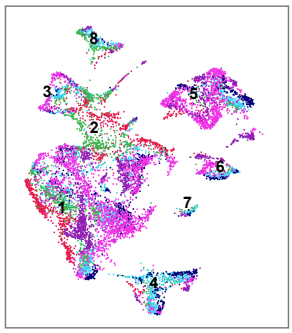
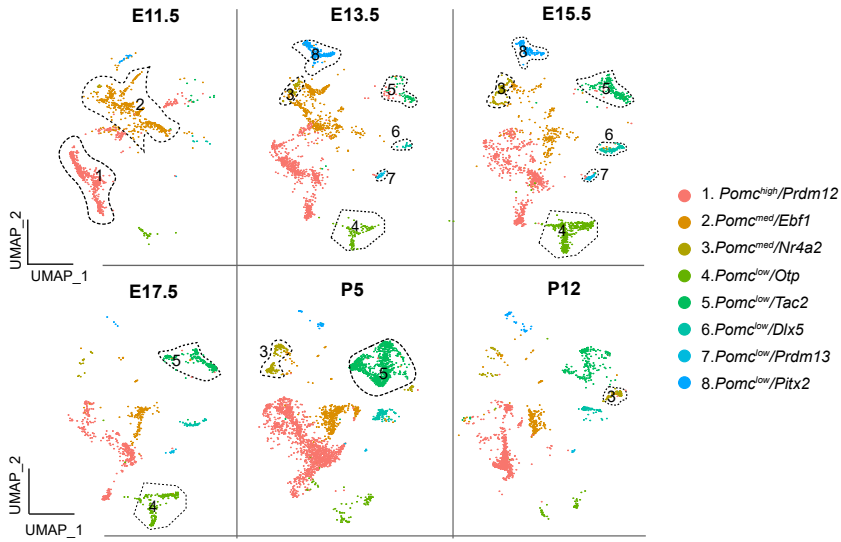


Figure 7A

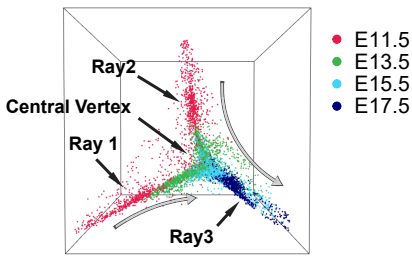


	Cell #		Cell #
E11.5	1286	E17.5	1133
E13.5	1622	P5	3449
E15.5	1964	P12	1365

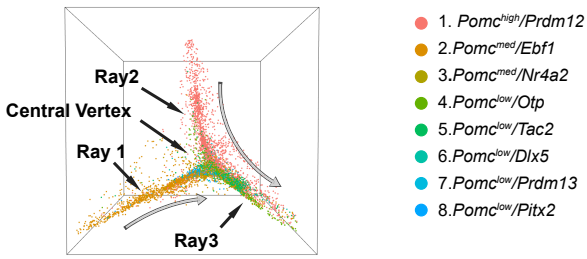
B



C



D



E

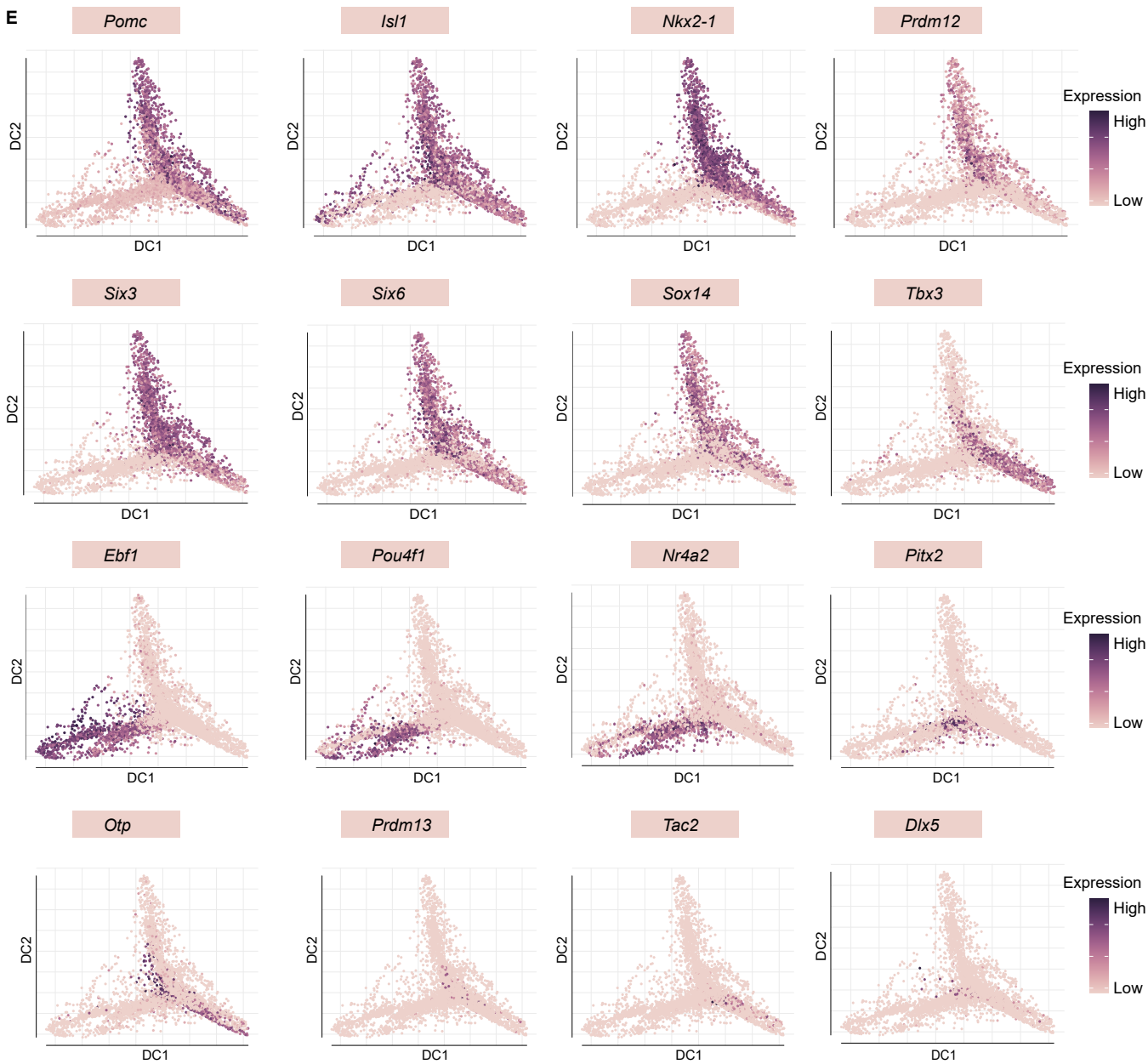


Figure 8

Developmental POMC^{+} Cells		Adult ARC Neurons
1.Pomc^{high}/Prdm12	›››	**n14. *Pomc/Ttr*** n15. *Pomc/Anxa2*
2.Pomc^{med}/Ebf1--1	›››	**n14. *Pomc/Ttr*** **n15. *Pomc/Anxa2***
2.Pomc^{med}/Ebf1--2	›››	**n34. unassigned2** n03. *Th/Sst*
3.Pomc^{med}/Nr4a2--1	›››	**n33. unassigned1** n34. unassigned2
3.Pomc^{med}/Nr4a2--2	›››	**n14. *Pomc/Ttr*** n15. Pomc/Anxa2
4.Pomc^{low}/Otp	›››	**n13. *Agrp/Gm8773*** n23. *Sst/Unc13c*
5.Pomc^{low}/Tac2	›››	**n20. *Kiss1/Tac2***
6.Pomc^{low}/Dlx5	›››	**n22. *Tmem215*** n34. unassigned2
7.Pomc^{low}/Prdm13	›››	**n29. *Nr5a1/Adcyap1*** n20. *Kiss1/Tac2*
8.Pomc^{low}/Pitx2	›››	**n34. unassigned2** n32. *Slc17a6/Trhr*