Temporal and spatial variation among single dopaminergic neuron transcriptomes informs cellular phenotype diversity and Parkinson's Disease gene prioritization

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ABSTRACT

Parkinson's Disease (PD) causes collapse of *substantia nigra* (SN) dopaminergic (DA) neurons of the midbrain (MB), while other DA populations are relatively spared. Here, we used single-cell RNA-seq (scRNA-seq) to characterize DA neuron populations in the mouse brain at embryonic and postnatal timepoints. These data allow for the discrimination between olfactory bulb (OB), forebrain (FB), and MB DA populations as well identification of subpopulations of DA neurons in each region. We observe a longitudinal axis of MB DA development, during which specialization and heterogeneity increases. We identify three distinct subpopulations of known MB DA neurons and provide evidence of a postnatal MB DA precursor, identifying novel markers for each subpopulation. Further, we discover gene regulatory networks (GRNs) that are significantly associated with neurodegenerative diseases and highly correlated with specific DA neuron subpopulations. By integrating these data with published genome-wide association studies (GWAS), we prioritize candidate genes in all 32 PD associated loci. Collectively, our data reveal genes and pathways that may begin to explain the selective vulnerability of SN DA neurons and allow for the systematic prioritization of genes in PD GWAS loci for functional evaluation.

Parkinson's Disease (PD) is the most common progressive neurodegenerative movement disorder. Incidence of PD increases with age[1,2] affecting an estimated 1% worldwide beyond 70 years of age[3]. Although PD ultimately impacts multiple neuronal centers, preferential degeneration of the ventral midbrain (VM) dopamine (DA) neurons leading to collapse of the nigrostriatal pathway is a common theme.

Mesencephalic DA neurons and their efferent connections with the striatum are responsible for the acquisition and maintenance of fine motor control and reward pathways. In turn, motor control is largely dependent on DA neurons populating the *substantia nigra* (SN), whereas the ventral tegmental area (VTA) is responsible for reward based behaviors and satiety. Despite their shared neurotransmitter characteristic, PD compromises the viability of SN DA neurons preferentially. By contrast VTA and VM DA periaqueductal gray area (PAG) DA neurons are largely spared[4,5]. This fact has driven research interest in the genetic basis of SN vulnerability in PD compared with that of VTA/PAG DA neurons.

To date, of the more than 20 genes that have been implicated in familial PD, mutations in less than 10 have been robustly shown to explain disease expression[6,7]. Beyond rare familial cases, a recent meta-analysis of PD GWAS highlighted 32 loci associated with sporadic PD susceptibility[8]. While some GWAS loci contain genes known to be mutated in familial PD (*SNCA* and *LRRK2*)[6,7], most do not contain a known causal gene. The inability to systematically identify the causative gene/s within GWA loci establishes a roadblock to the translation of genetic findings to medical practice. This requires an understanding of the pathogenesis of the disease and a thorough characterization of the specific cell population/s affected. In PD, one can reasonably assert that a significant fraction of disease-associated variation likely mediates its influence specifically within the SN. The answers to the implicitly related question of what renders SN DA neurons more vulnerable than other DA neurons also depends on the impact of such variation on gene regulatory networks (GRNs) essential to their viability or function regardless of whether they are unique to SN or shared among DA neurons more widely.

In an effort to resolve heterogeneity among central nervous system (CNS) DA populations, we undertook single-cell RNA-seq (scRNA-seq) analyses of CNS DA neurons from discrete anatomical regions of both embryonic and postnatal mouse brains. We evaluated both MB and forebrain (FB) DA neurons at embryonic day 15.5 (E15.5) and expanded our analyses at postnatal day 7 (P7) to include DA neurons isolated from the olfactory bulb (OB), FB (posterior hypothalamus) and MB. Deeper analysis of the P7 MB allowed for refinement of DA neuronal composition including elucidation of transcriptomic differences and similarities of different anatomical DA populations, identification of novel genetic markers for the SN, and the characterization of modules of co-expressed genes in our data. The results of our analyses provide a framework within which we begin to prioritize and test hypotheses of the potential disease modulating role played by genes within PD GWAS loci.

RESULTS

Temporal scRNA-seq characterization of DA neuronal populations reveals axis of DA neuron development

To characterize DA neuronal molecular phenotypes, we undertook scRNA-seq on cells isolated from distinct anatomical locations of the mouse brain, over developmental time. To obtain DA populations, we used the Tg(Th-EGFP)DJ76Gsat BAC transgenic mouse line, expressing EGFP under the control of the tyrosine hydroxylase (*Th*) locus. We microdissected both MB and FB from E15.5 mice, extending our analyses to MB, FB, and OB in P7 mice (Figure 1a). E15.5 and P7 time points were chosen based on their representation of stable MB DA populations, either after neuron birth (E15.5) or between periods of programmed cell death (P7) (Figure 1a)[9]. We used fluorescence activated cell sorting (FACS) to retrieve single eGFP$^+$ cells from enzymatically dissociated samples (Methods).

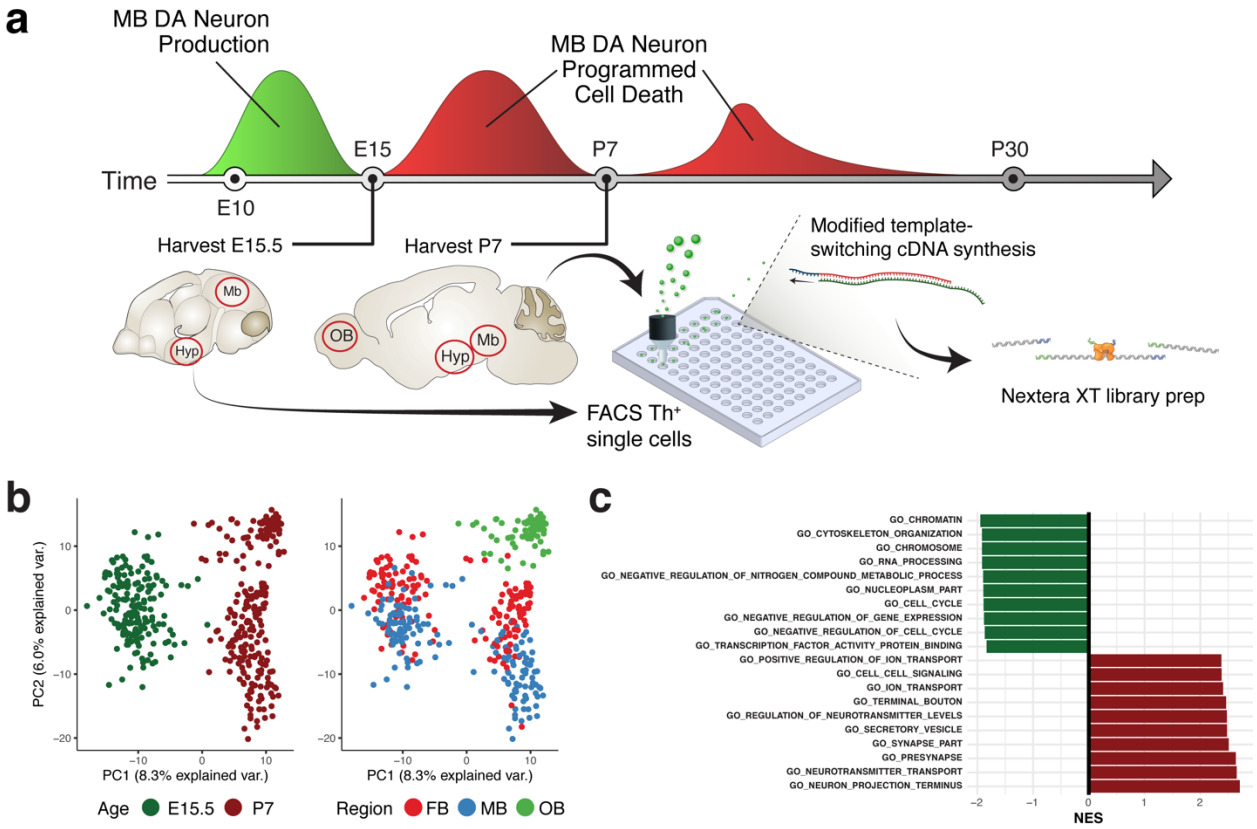

Figure 1. scRNA-seq analysis of isolated cells allows their separation by developmental time. a) Diagram of the experimental procedures. scRNA-seq of individual, GFP$^+$ cells was carried out through dissociation and FACS of GFP$^+$ cells from specific brain regions at E15.5 (MB, FB) and P7 (MB, FB, OB). GFP$^+$ cells were sorted into individual wells and cDNA was prepared via a modified Smart-Seq2 protocol and sequenced on a HiSeq 2500. E15.5 and P7 timepoints were chosen because they represent stable populations that are neither undergoing cell birth nor programmed cell death. b) Principal component analysis (PCA) on all collected cells using genes with a high biological coefficient of variation (BCV). The greatest source of variation (PC1) is explained by the time point at which the cells were collected, not the region from which the cells were collected. c) The top ten Gene Ontology

(GO) gene sets enriched in genes with positive (red) and negative (green) PC1 loadings. Analysis was carried out using Gene Set Enrichment Analysis (GSEA) PreRanked analysis. Genes with negative PC1 loadings and negative normalized enrichment scores (NES) were enriched for terms indicative of mitotically active cells such as "cell cycle," whereas genes with positive PC1 loadings and NES scores were enriched for terms expected of a more mature neuron such as "neuron projection terminus."

We sequenced RNA from single cells to an average depth of ~8.0 x 10^5 50bp paired-end fragments per cell. Using Monocle[10], we converted normalized expression estimates into estimates of RNA copies per cell. Cells were filtered based on the distributions of total mass, total number of mRNAs, and total number of expressed genes per cell (Figure S1, detailed in Methods). After QC, 410 out of 473 cells were retained. Using principal component analysis (PCA), we identified and removed 14 outliers determined to be astrocytes, microglia, or oligodendrocytes (Figure S2; Table S1), leaving 396 cells (~79 cells/timepoint-region; Figure S1d).

Following a workflow similar to the recently described "dpFeature" procedure[11], we first identified highly variant genes within the data. We then selected the PCs that described the highest percentages of variance in the data using these to represent the cells in two dimensions using t-Stochastic Neighbor Embedding (t-SNE)[12]. We called clusters of related cells within the data in an unbiased manner (see Methods). As anticipated, we observed that the greatest source of variation was between timepoints (Figure 1b). Genes associated with negative PC1 loadings (E15.5 cells) were enriched for gene sets consistent with mitotically active neuronal precursors (Figure 1c). In contrast, genes associated with positive PC1 loadings (P7 cells) were enriched for GO terms associated with mature, post-mitotic neurons (Figure 1c).

Analyses by region and timepoint reveal additional novel neuronal diversity.

Consistent with the suggestion that the embryonic cells include a less diverse progenitor population, analysis of all cells revealed that the E15.5 cells from both MB and FB cluster together (Figure 2a). By contrast, cells isolated at P7 mostly cluster by anatomical region, suggesting progressive functional divergence with time (Figure 2a). We then applied the scRNA-seq analysis workflow in a recursive manner in all regions at both timepoints to further explore heterogeneity. This revealed a total of 13 clusters (E15.5 FB, 2; MB, 2; P7 OB, 3; FB, 2; MB, 4; Figure 2b). Using known markers, we established that all clusters expressed high levels of pan-neuronal markers (*Snap25*, *Eno2*, and *Syt1*) (Figure S3). In contrast, we found weak or no, evidence of astrocyte (*Aldh1l1*, *Slc1a3*, *Aqp4*, and *Gfap*) or oligodendrocyte markers (*Mag*, *Mog*, and *Mbp*; Figure S3).

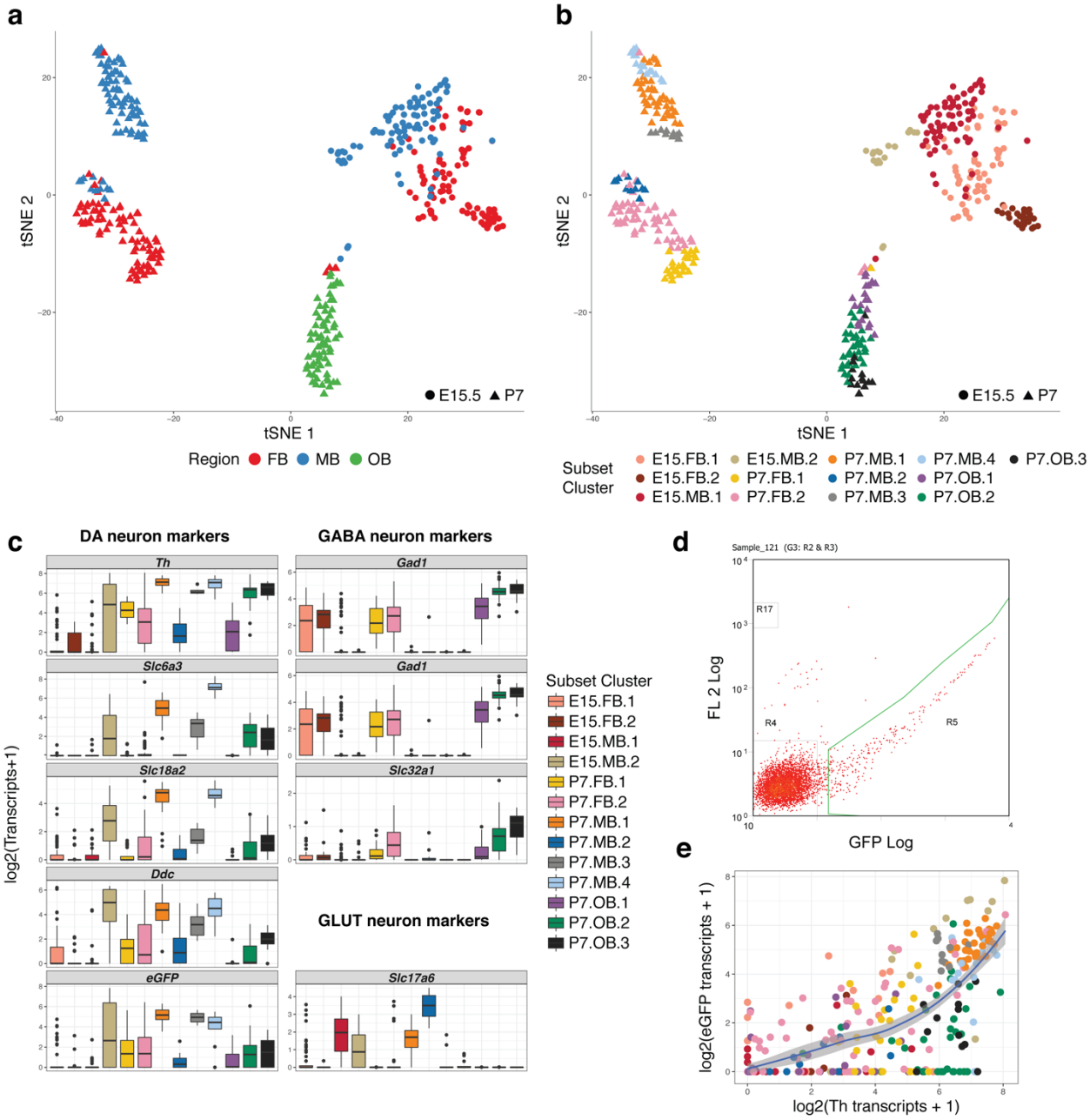

Figure 2. Subpopulations of isolated cells, identified through recursive analysis, express well-known neuronal markers. a) A t-distributed Stochastic Neighbor Embedding (t-SNE) plot of all collected cells colored by regional identity. It shows that E15.5 cells cluster together while P7 cells cluster primarily by regional identity. b) A t-SNE plot of all collected cells colored by subset cluster identity. Through iterative analysis, it was found that all timepoint-regions collected can be separated into multiple subpopulations (13 in total). Individual breakdowns of distinct subpopulations can bee seen in later figures c) Expression boxplots of well-established neuronal subtype markers including dopaminergic (DA) markers (*Th*, *Slc6a3*, *Slc18a2*, *Ddc*), GABAergic (GABA) markers (*Gad1*, *Gad2*, *Slc32a1*), and a glutamatergic (GLUT) maker (*Slc17a6*). The expression of eGFP in every cluster is also shown and mirrors the expression pattern of *Th*. d) A representative FACS plot showing the results of sorting E15.5 GFP^+ cells. A clear GFP^+ cell population was identified and that population has a wide range of GFP intensity. e) A plot of *Th* against eGFP log2 expression levels in all cells. A Loess fit regression line with a 95% confidence interval is shown, indicating that increasing levels of *Th* expression correspond to increasing levels of detected eGFP

transcripts. This plot also shows that *Th* transcript is more abundant than eGFP in the cells collected as indicated by the cells distributed along the x-axis with little to no eGFP expression.

We then evaluated the expression of known markers of DA neurons along with eGFP (Figure 2c). We detected consistently high levels of *Th* in cluster E15.MB.2 and all P7 clusters (Figure 2c) which correlated with eGFP expression (Figure 2c; Figure 2e). The inconsistent detection of *Th* and eGFP in other E15.5 clusters likely reflects their low transcript abundance at this time point, but sufficient expression of the eGFP reporter to permit FACS collection (Figure 2d). The expression of DA neuron markers *Ddc* and *Slc18a2* correlate with *Th* expression, while *Slc6a3* expression is more spatially and temporally restricted (Figure 2c).

Multiple studies have demonstrated that *Th*-expressing neurons may also express markers characteristic of other major neuronal subtypes[13–15]. Consequently, we evaluated expression of canonical markers of other neuronal subtypes in our DA neuron subpopulations. We found co-expression of *Th* with GABAergic (*Gad1*/*Gad2*/*Slc32a1*) or glutamtergic (*Slc17a6*) markers in 11/13 subset clusters (Figure 2c). The notable exception being two P7 MB DA neuron clusters (MB3 and MB4), which exclusively expressed DA markers (Figure 2c).

Analysis of E15.5 cells reveal regionally specialized maturing neurons

Recursive analysis of E15.5 DA neuron regions revealed two distinct populations in both the MB and FB. When analyzed collectively, we observe a major cluster, consisting of both MB and FB cells and two smaller clusters comprising solely MB or FB cells (Figure 3a). The discrete E15.5 MB cluster (E15.MB.2; Figure 3b) was highlighted by specific expression of genes known to mark mature MB DA neurons and have roles in MB DA neuron function (*Foxa1*, *Lmx1a*, *Pitx3*, and *Nr4a2*)[16] (Figure 3c; Table S2), suggesting that E15.MB.2 represents a post-mitotic, maturing DA neuron population. By contrast, E15.MB.1 neurons preferentially express genes including *Meis2*, *Lhx9*, *Id4*, *Ebf1*, *Pax5*, *Ephb1*, *Mir124-2hg*, and *Nrg1* (Table S2). All have established roles in neuronal precursors or neuronal differentiation/maturation[17–28]. Further, E15.MB.1 expresses *Slc17a6*, which along with *Meis2* and *Lhx9*, were recently used to identify embryonic DA neuroblasts[29] (Data accessed: 02/26/17; Table S11). Collectively these data support E15.MB.1 as a presumptive DA precursor population.

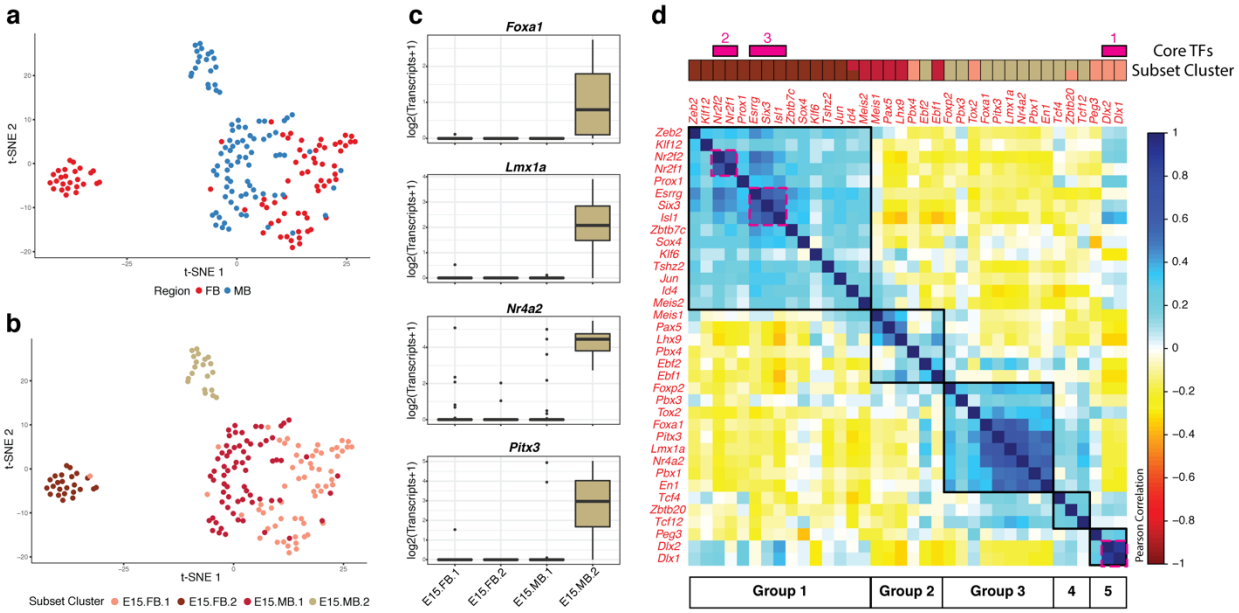

Figure 3. Analysis of E15.5 cells reveals clusters of neurons at different stages of maturation that may be defined by co-correlated transcription factor programs. a) A t-SNE plot of all neurons collected at E15.5 using high variance genes colored by regional identity. The t-SNE plot shows two distinct clusters containing only cells from one region and one cluster that contains a combination of both regions. b) The same t-SNE plot seen in Figure 3a, but colored by subset cluster identity. This plot shows that iterative analysis defines two clusters in both E15.5 MB and E15.5 FB. c) Boxplots showing log2 expression in all E15.5 clusters of four genes identified as being specifically expressed in the E15.MB.2 cluster. The expression of these genes (*Foxa1*, *Nr4a2*, *Lmx1a*, and *Pitx3*) as well as others indicate that cells belonging to the E15.MB.2 cluster are post-mitotic, maturing MB DA neurons. d) A correlation plot displaying the Pearson correlation between transcription factors (TFs) that were identified as being specifically expressed in any of the four E15.5 clusters identified through iterative analysis. Above the plot is a bar labeled "Subset Cluster" indicating (by color) the cluster or clusters in which each gene was determined to be specific. Five clusters (indicated by black boxes) are identified through hierarchical clustering that primarily groups TFs by which cluster they were identified in. Sets of "Core TFs" were identified in three groups through the use of bootstrap resampling of hierarchical clustering (see Methods). Sets of core TFs are indicated by pink boxes (3) within the plot and pink blocks above the plot labeled "Core TFs."

The markers identified for the discrete E15.FB.2 cluster, including *Six3* and *Six3os1*, are consistent with more mature FB/hypothalamic neurons[30–33]. This observation is supported by E15.5.FB.2 expression of *Sst* and *Npy*; both of which encode hormones indicative of specified, post-mitotic neurons[34]. E15.FB.1 clusters with E15.MB.1 (Figure 3b) potentially suggesting that it also represents an immature neuronal population. Indeed, the most specific marker for this population *Rnd3,* has been implicated in limiting the number of divisions of newly-fated neurons and in migration[35,36] (Table S2).

We further identified marker genes that are transcription factors (TFs) in order to define networks of TFs associated with these populations. Expression of these marker genes were correlated and hierarchical clustering was used to reveal five groups of TFs primarily defining

the different E15.5 neuron populations (Figure 3d). Core groups of TFs were also identified within two of five groups (pink outline; Figure 3d; See Methods). Two sets of core TFs were identified within "Group 1" which was primarily composed of mature FB cluster (E15.FB.2) specific genes. Both core TF sets (2 and 3) contain genes that have been previously implicated in FB neuron development[30–32,37,38] while potentially implicating a new TF in *Esrrg*. Group 5, which defined the immature E15.5 FB cluster (E15.FB.1) contained one core TF set (set #1) containing *Dlx1* and *Dlx2*, both of which have established roles in early FB neuronal development[39].

P7 neurons display regionally discrete transcriptional signatures

In contrast to E15.5, DA neurons isolated at P7 mostly cluster by anatomical region (Figure 4a). We sought to identify genes displaying region-dependent expression, identifying 54, 14 and 85 genes that defined OB, FB and MB DA neurons, respectively (Table S2).

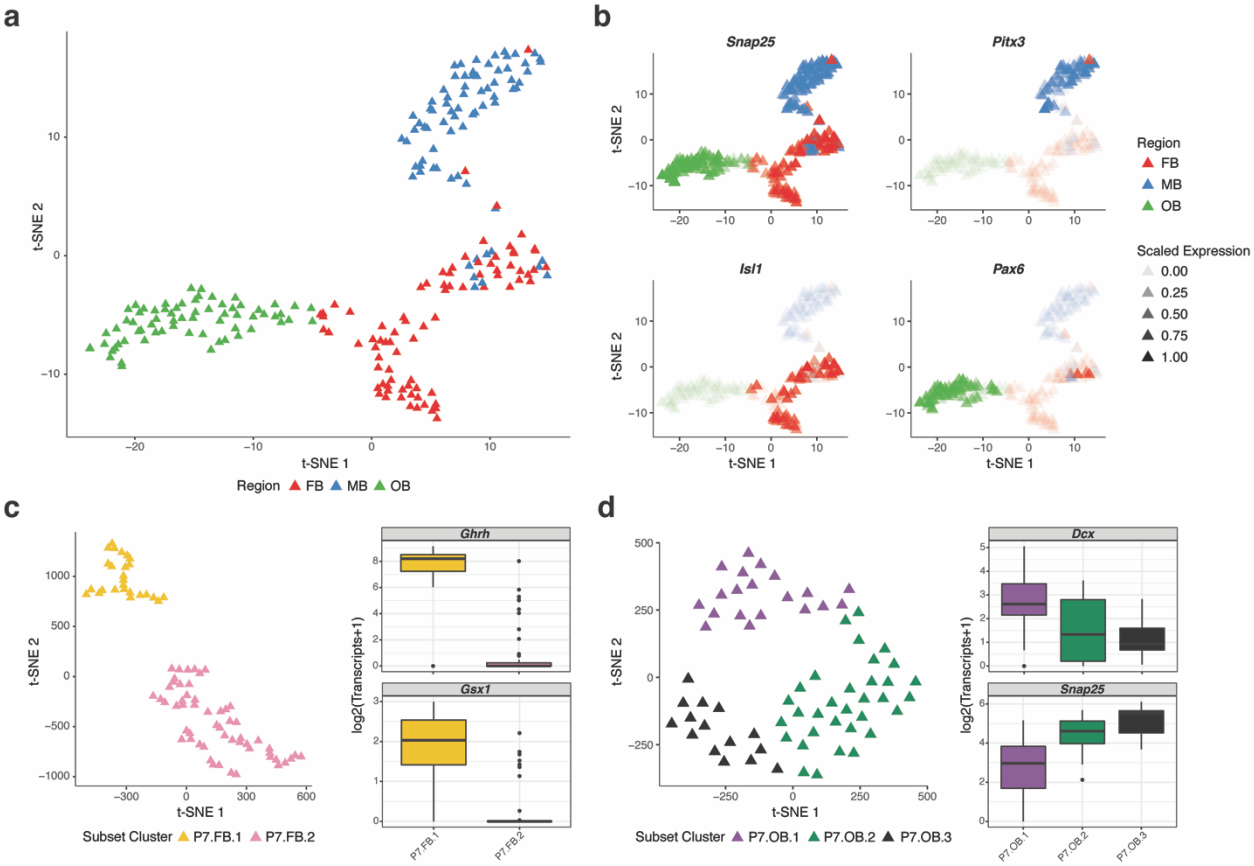

Figure 4. P7 *Th*+ neurons cluster by neuroanatomical domain, expressing markers characteristic of those regions, and further revealing regional subclusters based on iterative marker gene analyses. a) A t-SNE plot of all P7 neurons collected using high BCV genes, colored by regional identity. The neurons mostly cluster by regional identity. b) t-SNE plots showing expression scaled to max gene expression observed of a pan-neuronal gene (*Snap25*) and an example of a marker gene identified for each region at P7 (MB: *Pitx3*, FB: *Isl1*, OB: *Pax6*) in all P7 cells. c) t-SNE plot of P7 FB neurons plotted by using high BCV genes and colored by subcluster identity showing P7 FB neurons clustering into two distinct populations identified by ADPclust. Marker genes for the P7.FB.1 cluster

include *Ghrh* and *Gsx1*. Both of these genes as well as others indicate that this population of cells are tuberofundibular dopaminergic neurons located in the arcuate nucleus. d) A t-SNE plot of P7 OB neurons plotted by using high BCV genes and colored by subcluster identity. The plot shows that the P7 OB neurons cluster into three populations identified by ADPclust. Increase in expression of genes that indicate neuronal maturity (for example, *Snap25*) from P7.OB.1 to P7.OB.3 and decrease in expression of genes that indicate neuronal immaturity (for example, *Dcx*) in the same direction indicate that the clusters identified represent neuronal populations of increasing maturity.

The FB-restricted genes include markers associated with hypothalamic development and function e.g. *Isl1*[38] and *Asb4*[40] (Figure 4b; Table S2). Analyzing P7 FB Th^+ neurons alone revealed two distinct cell clusters (Figure 4c). P7.FB.1 specifically expressed the neuropeptides *Gal* and *Ghrh* and the *Gsx1* transcription factor (Figure 4c; Table S2). All three play roles in arcuate nucleus neurons[41–43] and were markers for a recently described $Th^+/Ghrh^+/Gal^+$ hypothalamic population[44]. By contrast, marker genes for P7.FB.2 did not reveal a signature or gene expression profile consistent with a known cellular phenotype (Table S2)[44,45]. However, several other arcuate nucleus markers for $Th^+/Ghrh^-$ neuronal populations were expressed in subsets of P7.FB.2 cells, including *Onecut2*, *Arx*, *Prlr*, *Slc6a3*, and *Sst* (Figure S4a)[44]. Thus, some Th^+ populations detected in other scRNA-seq analyses may be present within our data, but likely in insufficient numbers to facilitate classification here.

Of the genes whose expression defined OB Th^+ cells, many have established roles in the development or survival of OB DA neurons[46–51] (Table S2). Recursive analysis revealed three subset clusters in P7 OB (Figure 4d). In identifying marker genes for P7 OB subset clusters, we observed that P7.OB.1 expressed *Dcx* at significantly greater levels than P7.OB.2/P7.OB.3 and that *Dcx* levels decrease along a continuum towards the lowest expression in P7 OB3 (Figure 4d). *Dcx* expression diminishes with neuronal maturation with the lowest expression in adult neurons[52]. Consistent with this observation expression of the mature neuronal marker *Snap25* is anticorrelated with Dcx (Figure 4d), suggesting a progression in maturation from P7.OB.1 to P7.OB.3. This too is corroborated by concomitant increase in expression of DA neuron markers and OB DA neuron fate specification genes (Figure S4b)[53,54].

Many genes that define $eGFP^+$ MB neurons, including *Pitx3* (Figure 4b), have established roles in MB DA neuron development and biology[16]. We identified four P7 MB DA subset clusters within P7 MB DA neurons (Figure 5a). Marker gene analysis (Table S2) confirmed that three of the clusters correspond to DA neurons from the VTA (*Otx2* and *Neurod6*; P7.MB.1)[55,56], the PAG (*Vip* and *Pnoc*; P7.MB.3)[57,58], and the SN (*Sox6* and *Aldh1a7*; P7.MB.4)[55,59] (Figure 5b). These data are consistent with recent scRNA-seq studies of similar populations[29,60]. We further identify an as-yet undescribed population (P7.MB.2; Figure 5a) of Th^+ DA neurons. This population of cells display an expression signature consistent with a neuronal progenitor cell population. This postnatal population shares many markers with the progenitor-like E15.MB.1 cluster including *Fam19a2* and *Meis2* (Table S2; Figure 5b). Furthermore, P7.MB.2 markers

Meis2, *Lhx9*, and *Ldb2* have been shown to mark embryonic mouse neuroblast populations[29]. Interestingly, this P7.MB.2 population clusters with P7 FB neurons in t-SNE space (Figure 2a; Figure 2b; Figure 4a). This may be driven by lower expression of key MB DA neuron genes compared to the other P7 MB clusters, resulting in a signature more similar to both P7 FB clusters (Figure 2c).

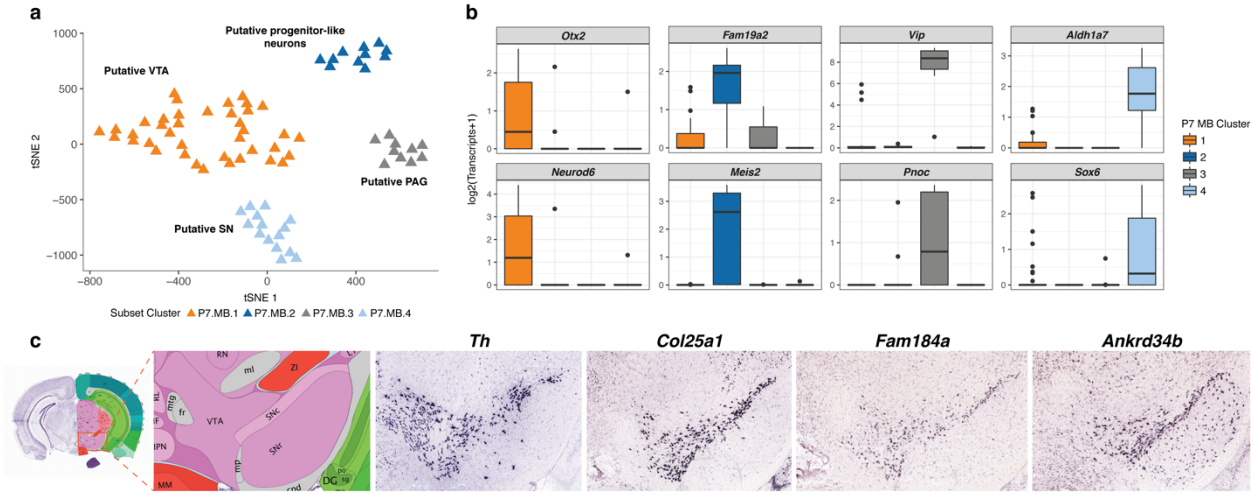

Figure 5. Identification of four distinct clusters of P7 MB *Th*$^+$ neurons based upon expression of established marker genes leads to the discovery of novel *substantia nigra* (SN) marker genes. a) t-SNE plot of P7 MB neurons colored by subcluster identity. P7 MB neurons separate into four clusters of which three can be defined using marker genes of known *Th*$^+$ populations: the ventral tegmental area (VTA), the *substantia nigra* (SN), and the periaqueductal grey area (PAG). The P7.MB.2 cluster was identified as a progenitor-like neuron primarily due to the overlap of specific markers with the immature E15.MB.1 cluster. b) Boxplots showing the expression of cluster specific genes used for identification of cluster populations. *Otx2* and *Neurod6* are established markers of VTA DA neurons. *Vip* and *Pnoc* are established markers of PAG neurons. *Aldh1a7* and *Sox6* are established markers of SN DA neurons. *Fam19a2* and *Meis2* are markers for P7.MB.2 that are shared with the E15.MB.1 cluster (See Table S2). c) Confirmation of novel SN DA neurons through the use of Allen Brain Atlas (ABA) *in situ* hybridization data (http://www.brain-map.org/). Coronal, P56 mouse *in situ* hybridization data was explored in order to confirm the expression of 37 novel SN markers. *Th* expression in P56 mice was used as an anatomical reference during analysis. Overall, 15/37 novel SN markers are confirmed to be expressed in the SN in adult mice, including *Col25a1*, *Fam184a*, and *Ankrd34b*.

We sought to ascertain the spatial distribution of P7.MB.2 DA neurons through multiplex, single molecule fluorescence *in situ* hybridization (smFISH) for *Th* (pan-P7 MB DA neurons), *Slc6a3* (P7.MB.1, P7.MB.3, P7.MB.4), and one of the marker genes identified through our analysis, either *Lhx9*/*Ldb2*/*Meis2* (P7.MB.2) (Figure 6). The ventral MB was scanned in each experiment for cells that were Th$^+$/Slc6a3$^-$ and positive for the third gene. *Th*$^+$/*Slc6a3*$^-$/*Lhx9*$^+$ cells were found scattered in the dorsal SN *pars compacta* (SNpc) along with cells expressing *Lhx9*$^+$ alone (Figure 6a, 6e). Expression of *Ldb2* was found to follow a similar pattern to *Lhx9*, with *Th*$^+$/*Slc6a3*$^-$/*Ldb2*$^+$ cells also found in the dorsal SNpc (Figure 6b, 6e). In the SNpc, *Meis2*$^+$ cells were common, however they did not display co-expression of *Th* (Figure 6d). Cells that were

Th^{+}/*Slc6a3*$^{-}$/*Meis2*$^{+}$ were found in the interpeduncular nucleus (IPN) of the ventral MB (Figure 6d, 6e). Neither *Lhx9* nor *Ldb2* were detected in the IPN (data not shown). Expression of *Lhx9*, *Ldb2*, and *Meis2* was low or non-existent in *Th*$^{+}$/*Slc6a3*$^{+}$ cells in the SNpc (Figure 6a, 6b, 6c). Importantly, cells expressing these markers express *Th* at lower levels than *Th*$^{+}$/*Slc6a3*$^{+}$ neurons (Figure 6), consistent with our scRNA-seq data (Figure 2c).

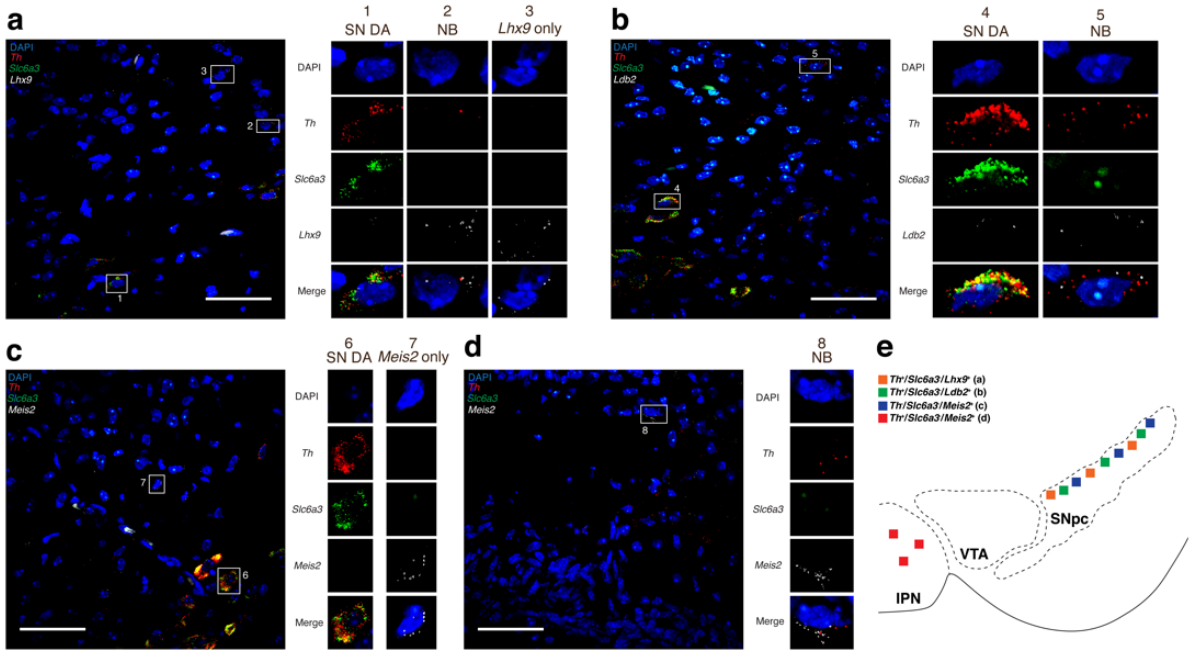

Figure 6. Single molecule fluorescent in situ hybridization (smFISH) validates the existence of a neuroblast-like P7.MB.2 cell population. Representative images of smFISH experiments analyzing four MB sections for each probe combination. a) smFISH performed with probes for *Th* (red), *Slc6a3* (green), and *Lhx9* (white). smFISH in the ventral MB reveals the presence of *Th*$^{+}$/*Slc6a3*$^{-}$/*Lhx9*$^{+}$ in the dorsal portion of the *substantia nigra* (SN) (inset 2), the presence of *Lhx9*$^{+}$ only cells in the dorsal SN (inset 3), and the lack of expression of *Lhx9* in SN DA neurons (inset 1). Scale bar, 50 uM. b) smFISH performed with probes for *Th* (red), *Slc6a3* (green), and *Ldb2* (white). smFISH in the ventral MB reveals the presence of *Th*$^{+}$/*Slc6a3*$^{-}$/*Ldb2*$^{+}$ in the dorsal portion of the SN (inset 5) with little to no expression of *Ldb2* in SN DA neurons (inset 1). Scale bar, 50 uM. c) smFISH performed with probes for *Th* (red), *Slc6a3* (green), and *Meis2* (white). smFISH in the ventral MB reveals the presence of *Meis2*$^{+}$ in the dorsal portion of the SN (inset 7) with little to no expression of *Meis2* in SN DA neurons (inset 6). Scale bar, 50 uM. d) smFISH performed with probes for *Th* (red), *Slc6a3* (green), and *Meis2* (white). smFISH in the ventral MB reveals the presence of *Th*$^{+}$/*Slc6a3*$^{-}$/*Meis2*$^{+}$ in the interpeduncular nucleus (IPN) (inset 8). Scale bar, 50 uM. e) Diagram summarizing locations of the P7 MB2 cells validated through smFISH. All images were taken at 60x magnification and processed using ImageJ (see Methods). NB, neuroblast; VTA, ventral tegmental area; SNpc, *substantia nigra* pars compacta; IPN, interpeduncular nucleus.

Furthermore, regional and subset cluster marker gene correlation analysis revealed four groups of TFs through hierarchical clustering with three groups clearly demarcating regions at P7 (Figure S5a) including seven core TF sets (three in OB, one in FB, and three in MB) (Figure S5a;

336 Table S3). To expand upon these TF networks, we performed correlation analysis with all TFs
337 that were found to be differentially expressed between regions at P7. Five out of seven (5/7) sets
338 of core TFs found with P7 marker gene analysis were recovered (Figure S5b). These core groups
339 of TFs were expanded through the addition of other differentially expressed TFs found to be
340 highly correlated with the original core TFs (Figure S5b). Two other core groups ("FB program"
341 and "SN program") were identified as containing TFs (*Isl1* and *Sox6*) known to be associated
342 with P7 FB and P7 MB SN, respectively. Additional sets of "core" TFs were also identified
343 (Table S3).
344
345 *Identification of novel SN-specific DA Neuron marker genes*
346 Motivated by the clinical relevance of SN DA neurons to PD, we set out to understand what
347 makes them transcriptionally distinct from other MB DA neurons. We postulated that genes with
348 specific expression in the P7 SN DA neuron cluster data might illuminate their preferential
349 vulnerability in PD. By hypergeometric testing, the 67 SN marker genes are enriched for GO
350 terms consistent with SN DA neuron biology (Table S4). Of the 67 SN-defining genes (Table
351 S2), three (4.5%; *Ntf3*, *Chrna6*, and *Ntn1*) were shared with P7.MB.1 (VTA) and were excluded
352 from subsequent analyses. Prior reports support the expression of 27/64 genes (42%) in
353 postnatal SN (Table S5). We then sought evidence confirming SN expression for the remaining,
354 novel 37 (58%) genes. Of these, 15/37 (~41%) were detected in adult SN neurons by *in situ*
355 hybridization (ISH) from the Allen Brain Atlas (ABA) including *Col25a1*, *Fam184a*, and
356 *Ankrd34b*. (Figure 5c, Table S6). The ABA lacks coronal ISH data on 20/37 genes; and for 2/37
357 genes ABA had relevant ISH data but lacked evidence of expression in the adult SN (*Tspan12*,
358 *Igfbp3*) (Table S6). Collectively, we identify 64 postnatal SN DA marker genes and confirm the
359 expression of those genes in the SN for 42 (65%) of them, included 15 previously undescribed
360 markers.
361
362 *Gene-coexpression modules are enriched for PD gene sets only in SN-derived data*
363 In order to explore relationships between cellular subtype identity and transcriptional programs,
364 we performed weighted gene co-expression network analysis (WGCNA)[61] on our P7 data. We
365 used all expressed genes to establish 16 co-expressed gene modules (Figure 7; Figure S6; Table
366 S7). We determined whether identified modules were enriched for Kyoto Encyclopedia of Genes
367 and Genomes (KEGG) pathways, Gene Ontology (GO) gene sets, and Reactome gene sets[62].
368 Notably, "green" and "brown" modules were significantly associated with the Parkinson's
369 Disease KEGG pathway gene set (Figure 7a) suggesting these two modules may specifically
370 contribute to PD etiopathology. Further, the brown module was significantly associated with
371 KEGG pathways that include "Cocaine addiction", while the green module was enriched for
372 genes associated with additional neurodegenerative disorders including "Alzheimer's Disease"
373 as well as "Oxidative "phosphorylation (Figure 7a). We also found the green module to be
374 significantly associated with GO gene sets including select metabolic processes and
375 mitochondrial function including sets related to the electron transport chain (Table S8-12).

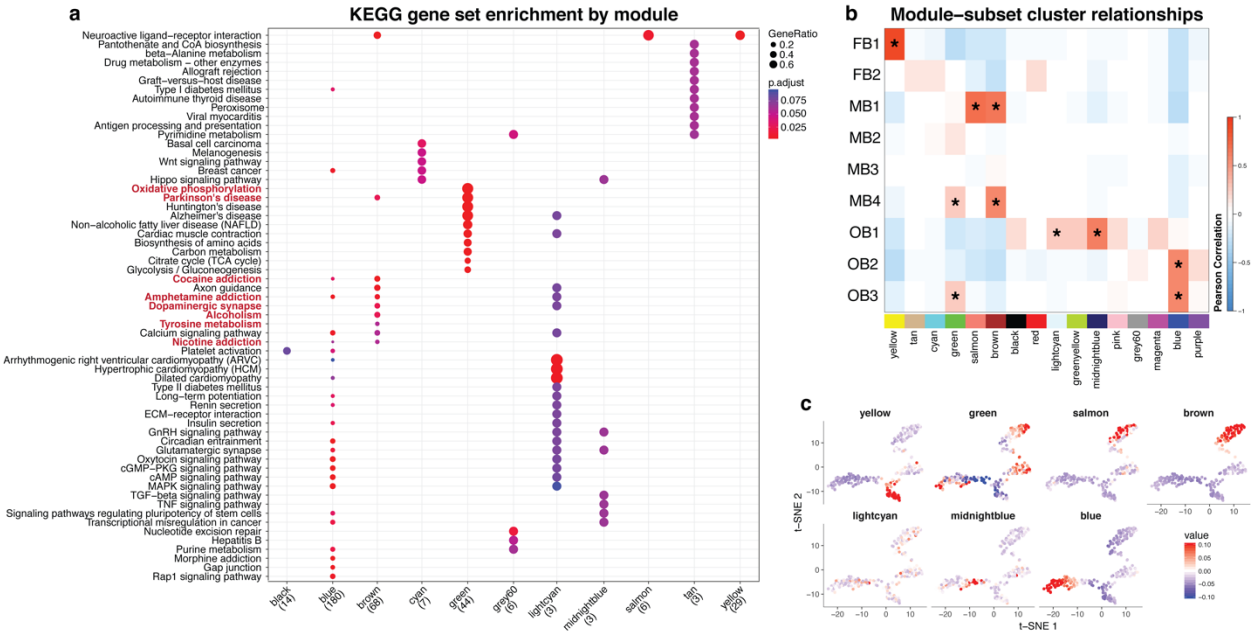

Figure 7. Weighted Gene Co-Expression Network Analysis (WGCNA) reveals gene modules that correlate with P7 *Th*$^+$ subset cluster identity and are enriched for PD gene sets. a) Enrichment for KEGG gene sets with gene modules identified through WGCNA analysis of P7 *Th*$^+$ neurons. Significance of enrichment was determined by hypergeometric test and an adjusted P-value (Benjamini & Hochberg correction) cutoff of 0.1 was used. The gene ratio (displayed by dot size) is the ratio of genes within a given module that are found in any given gene set. Notably, two identified modules ("green" and "brown") are enriched for the "Parkinson's Disease" KEGG gene set. Other notable gene sets are highlighted in red. b) Correlation heatmap of the Pearson correlation between module eigengenes and P7 *Th*$^+$ subset cluster identity. Modules are represented by their assigned colors at the bottom of the matrix. The significance (P-value) of the correlations was calculated using the Student's asymptotic test as implemented in the WGCNA R package with the function "corPvalueStudent()." Modules that had a positive correlation with a subset cluster and had a correlation P-value less than the Bonferroni corrected significance level (P-value = 4.386e-04) contain an asterisk. As shown, 7/16 modules display significant correlations with 6/9 of the P7 subset clusters including the VTA (P7.MB.1) and the *substantia nigra* (P7.MB.4). c) The eigengene value for each P7 neuron in the seven WGCNA modules shown to be significantly associated with a subset cluster overlaid on the t-SNE plot of all P7 neurons (Figure 4a). Most of these plots (6/7) show that positive eigengene values from these modules mark subsets of cells in t-SNE space corresponding to the subset clusters they are correlated with in Figure 7b. The "lightcyan" module does not seem to show the same spatial restriction, even though it is significantly correlated with P7.OB.1 identity (Figure 7b).

We next asked whether these biologically relevant gene expression modules were engaged by distinct P7 DA neurons subtypes. WGCNA analysis establishes eigengenes for each module that can facilitate their correlation with cellular traits. We calculated the pairwise correlations between module eigengenes and P7 subset clusters. This analysis revealed 7/16 modules significantly, positively correlated (Bonferroni corrected $p < 3.5e\text{-}04$) with at least one subset cluster, including the two gene sets (brown and green) enriched for PD (Figure 7b). The majority of these significant modules (6/7) displayed strict spatial enrichment in t-SNE space (Figure 7c)

405 confirming the correlations. Strikingly, the SN (P7.MB.4) was the only P7 subset cluster
406 significantly associated with both modules enriched for PD gene sets (brown and green). The
407 identification, and subtype-specific association of these modules, reinforces their significance in
408 disease etiopathology and expands the scope of SN-associated genes identified above.
409
410 *Integration of MB DA neuron subtype specificity enables prioritization of genes within PD-*
411 *associated intervals*
412 The capacity to make informed connections between GWAS loci and causal gene/s is often
413 impeded by a paucity of expression data in biologically relevant cell populations. This is
414 particularly true of disorders in which those affected populations are difficult to isolate, as in the
415 mammalian CNS. We posited that SN DA neuron-specific genes and the broader gene co-
416 expression networks that correlate with SN DA neurons might be used to help prioritize genes
417 within loci identified in PD GWAS. Such a strategy would be unbiased and independent of genic
418 position relative to the lead SNP or prior biological evidence.
419
420 To investigate pertinent genes within PD GWAS loci, we identified all human genes within a
421 topologically association domain (TAD) encompassing each identified PD-associated lead SNP.
422 We chose to use TAD boundaries because regulatory sequences preferentially interact with
423 promoters in TADs[63]. Since data describing TAD structure of the cell types analyzed here does
424 not exist, we also examined all the genes within +/- 1 megabase of a PD GWAS SNP. We
425 selected this interval as it includes the upper bounds of reported enhancer-promoter
426 interactions[64,65]. All PD GWAS SNPs interrogated were identified by the most recent meta-
427 analysis (32 SNPs in total)[8], implicating a total of 966 unique genes. We then identified
428 corresponding mouse homologs (673/966; ~70%), primarily through the NCBI Homologene
429 database (Methods). Of the remaining 293 genes with no mouse homologs (Table S13), 62
430 (62/293, ~21%) are annotated as protein coding genes (Figure S7a). 17 loci include at least one
431 protein coding gene with no identified mouse homolog (Figure S7b).
432
433 To prioritize the genes in all 32 loci, we developed a gene-centric score that integrates gene
434 expression, differential gene expression, cluster specificity (Table S2), WGCNA module co-
435 regulation (Table S7), and evolutionary mutation tolerance. We began by intersecting the PD loci
436 genes with our scRNA-seq data, identifying 256 genes (256/673; 38%) with direct evidence of
437 expression in SN DA neurons (P7.MB.4). Each PD-associated interval contained ≥1 SN-
438 expressed gene (Table S14). Emphasizing the need for a systematic strategy, in 14/32 GWA
439 intervals (~44%), the most proximal gene to the lead SNP was not detectably expressed in the
440 mouse SN DA neuron population (Table S14; Figure S8).
441
442 Four loci (*MMP16*, *SIPA1L2, USP25*, *VPS13C*) contained only one SN-expressed gene (Figure
443 8a, Table S14): *Mmp16* (*MMP16* locus), *Tsnax* (*SIPA1L2* locus), *Hspa13* (*USP25* locus), and
444 *Rora* (*VPS13C* locus). The relevance of these candidate genes is well supported[66–71].

Furthermore, both *Mmp16* and *Rora* are detected in adult mouse SN (Figure 8c, ABA, Table S14).

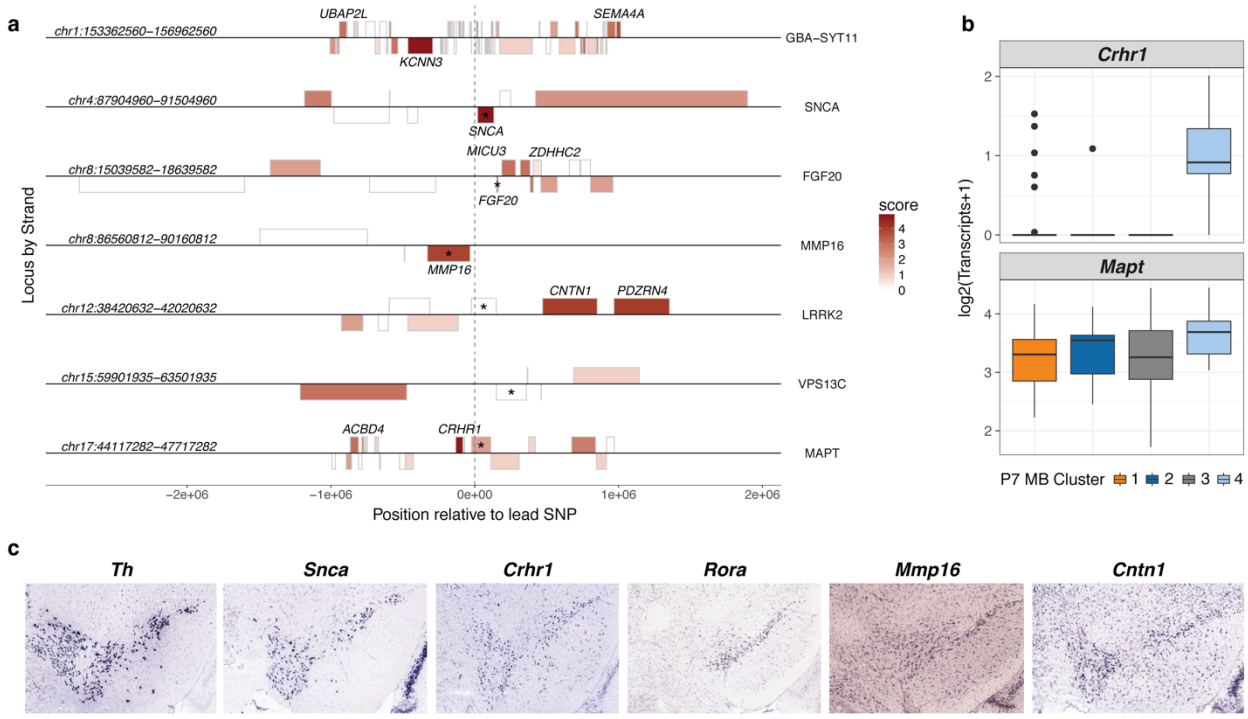

Figure 8. Potential candidate genes in PD GWAS loci can be identified and prioritized through the use of scRNA-seq data. a) A plot displaying the four megabase regions in the human genome (hg38) centered on PD GWAS SNPs in seven loci. Genes within +/- 1 Mb from the lead SNP in those loci are displayed as boxes on their appropriate strand. Genes are shaded by their prioritization score which is based on five factors: whether the gene is expressed in the P7 *substantia nigra* population (P7.MB.4); whether the gene was found to be a specific marker gene for P7.MB.4; whether the gene is differentially expressed between P7 MB clusters; whether the gene is found in any of the PD-associated WGCNA modules ("green" and "brown"); the pLI score of the gene in the ExAC database. The gene or genes with the highest scores in a given locus are considered the best candidate genes. The genes that are closest to the lead SNP are marked by an asterisk. This analysis allowed for the prioritization of all 32 PD GWAS loci in Nalls, et al[8]. b) Boxplots showing the expression of *Crhr1* and *Mapt* in identified P7 MB clusters. *Crhr1* shows specific expression in P7 SN Th^+ neurons while *Mapt* is expressed at equal levels in all P7 MB clusters. This specific expression of *Crhr1* but not *Mapt* along with *CRHR1* being identified as the highest scoring gene in the MAPT locus may help to explain why variation in this locus leads to preferential degeneration of SN Th^+ neurons in PD. c) *In situ* hybridization data from the Allen Brain Atlas (ABA) for some of the highest scoring genes in Figure 8a. The expression data for *Th* is used here for anatomical identification of the SN and for comparison purposes. As shown, the mouse homologs of high scoring genes in the *SNCA* (*Snca*), *MAPT* (*Crhr1*), VPS13C (*Rora*), MMP16 (*Mmp16*), and LRRK2 (*Cntn1*) loci show clear expression in SN DA neurons in P56 mice.

To further prioritize the remaining 28 loci, we scored on whether genes were differentially expressed between P7 MB Th^+ populations; whether they were identified as a marker gene for

471 P7.MB.4 (SN) cluster; and whether the genes were co-expressed with PD enriched gene modules
472 uncovered in WGCNA. This strategy facilitated further prioritization of one or two genes in 16
473 additional loci (Table S14; Table S15). Importantly, we prioritize the major PD gene, *SNCA* in
474 the *SNCA* locus (Figure 8a; Figure S8; Table S15). In three of these loci (*GBA-SYT11*, *LRRK2*
475 and *MAPT*), our scoring prioritizes a different gene (*Kcnn3*, *Pdzrn4*, and *Crhr1*, respectively)
476 than one previously implicated in PD phenotypes (Figure 8a, Table S15). This apparent conflict
477 is well exemplified by our observations at the *MAPT* locus. Although *MAPT* is broadly
478 implicated in neurodegeneration, we detect *Mapt* expression consistently across all assayed MB
479 DA neurons (Figure 8b). By contrast *Crhr1,* encoding the corticotropin releasing hormone
480 receptor 1*,* is specifically expressed in SN neurons (Figure 8b).
481
482 We then sought to further prioritize the SN-expressed genes in the remaining ten loci by
483 integrating the probability of being loss-of-function (LoF) intolerant (pLI) metric from the ExAC
484 database[72], due to a recent study using this metric to identify dosage sensitive genes[73]. Since
485 most GWAS variation is predicted to impact regulatory DNA and in turn impact gene
486 expression, it follows that genes in GWAS loci that are more sensitive to dosage levels may be
487 more likely to be candidate genes. With that in mind, the pLI for each gene was used to further
488 "rank" the genes within loci that were not previously prioritized. For those loci, we report a
489 group of top scoring candidate genes ($\leq$ 5) (Table S15). By integrating this step, we prioritized
490 candidates for the remaining 10/32 (31%) loci. In total, we prioritize candidates in all 32 PD
491 GWAS loci, establishing a systematic rationale for the identification of biologically pertinent
492 candidates and testable hypotheses.
493
494 **DISCUSSION**
495 Midbrain DA neurons in the *substantia nigra* have been the subject of intense research since
496 being definitively linked to PD nearly 100 years ago[74]. While degeneration of SN DA neurons in
497 PD is well established, they represent only a subset of CNS DA populations. It remains unknown
498 why nigral DA neurons are particularly vulnerable. We set out to explore this question using
499 scRNA-seq to characterize the transcriptomes of DA neuron populations from distinct regions of
500 the mouse brain over developmental time. Recently others have used scRNA-seq to characterize
501 the mouse MB including DA neurons[29]. We undertake a highly complementary strategy, making
502 several distinct and significant findings.
503
504 **Previously unknown and unappreciated aspects of SN biology are revealed through**
505 **scRNA-seq**
506 By analyzing a broad array of Th^+ neuronal populations (MB, FB, and OB), we reveal what
507 underlies their functional diversity. Perhaps most intriguing, we demonstrate that SN DA
508 neurons display no evidence of neurotransmitter or hormone co-transmission/release with
509 dopamine, unique amongst the Th^+ populations studied. Although, this observation is consistent
510 with reports suggesting co-transmission in hypothalamus and olfactory bulb[41,75] as well VTA[13]

511 DA neurons, we see no evidence supporting co-transmission in SN DA neurons. This result
512 raises the question of whether this sole neurotransmitter phenotype of SN DA neurons may
513 contribute to their selective vulnerability in PD.
514
515 We further reveal SN marker genes and GRN components that more fully characterize the
516 unique biology of these neurons. Several genes, including SN marker gene *Prr16*, play roles in
517 oxidative phosphorylation and mitochondrial function, consistent with established SN neuronal
518 biology[76,77]. We also identify new SN marker genes that encode secreted proteins and cell-
519 surface proteins that further define how SN DA neurons may interact with their environment. For
520 example, we identify *Fam19a4* as being specifically expressed in the SN at P7. *FAM19A4*
521 encodes a secreted protein that has been shown to be expressed in the brain and act as a chemo-
522 attractant and activator of macrophages through the binding of *FPR1*[78,79]. *FAM19A4* expression
523 has also been found to be upregulated in immune cells upon lipopolysaccharchide (LPS)
524 treatment, a model of neuroinflammation[79]. This finding potentially links SN DA specific gene
525 expression and protein secretion to the role of inflammation in PD[80] and the specific
526 vulnerability of SN DA neurons to degeneration caused by inflammation[81].
527
528 **A novel postnatal MB *Th*$^+$ cell type is a putative progenitor-like MB DA neuron**
529 Our analysis of embryonic and postnatal MB *Th*$^+$ neurons revealed a population of neurons,
530 present at both embryonic and postnatal timepoints (E15.MB.2 and P7.MB.2), that share
531 expressed genes indicative of MB DA neuron progenitors. While progenitor cell populations in
532 the ventral MB have been previously characterized at embryonic timepoints[29], the existence of a
533 postnatal MB progenitor neuron population has not been noted in previous scRNA-seq
534 studies[29,60]. Notably, previous studies characterized postnatal neurons marked by transgenes
535 under *Slc6a3* regulatory control. Given that we demonstrate this marker to be absent from
536 P7.MB.2 cluster, it follows that this population would likely have been overlooked. By contrast,
537 our use of *Th* left this population available for discovery. We show that specific markers for this
538 population place it in the dorsal portion of the SN or the IPN at P7. The existence of markers of
539 this population in different ventral MB sites potentially indicates that this cluster of cells
540 represents a specific cell state reflecting neuronal immaturity instead of a reflection of spatial
541 arrangement.
542
543 One may speculate regarding the function of a postnatal MB progenitor population. While
544 beyond the scope of this paper, some clues may be found in the literature about *Th*$^+$ neuron
545 development. Studies of SN DA neuron development in mice have shown that there are two
546 periods of programmed cell death with peak apoptosis occurring at P2 and P14 (Figure 1a)[82].
547 Paradoxically, even though there are high levels of cell death at these points, the actual number
548 of *Th*$^+$ neurons in the mouse SN does not decrease[82,83]. It has been shown that this can be
549 explained by increasing levels of *Th* in cells over time, leading to "new" neurons appearing that
550 are increasingly able to be immunostained[82]. These results have led to the suggestion that there is

551 a "phenotypic maturation" of MB DA neurons during the early postnatal time period[82]. This very
552 well may explain the presence of our "progenitor-like" MB DA neurons at P7, which display
553 much lower levels of *Th* than other populations.
554
555 **Prioritization of genes within PD GWAS loci identifies genes that may contribute to**
556 **common PD susceptibility**
557 The majority of variants identified in GWAS are located in non-coding DNA[84]. They are
558 enriched for characteristics denoting regulatory DNA[84,85], and have been shown to modulate
559 tissue-dependent elements[84–87]. Despite this evidence, in practice, the gene closest to the lead
560 SNP identified within a GWAS locus is frequently treated as the prime candidate gene, often
561 without considering tissue-dependent context. In an effort to more systematically identify and
562 prioritize gene candidates from GWAS, our study integrates layers of orthogonal genetic and
563 genomic data. We posit that genes pertinent to PD are likely expressed within MB DA neurons,
564 specifically within the SN. We conservatively define an interval of interest (TAD boundary/2
565 Mb) around each lead SNP and ask which genes therein are expressed in the SN. We
566 systematically move from intervals that reveal one primary candidate, by harboring only one SN-
567 expressed gene, to those with many candidates, requiring a cumulative body of biological
568 evidence to prioritize genes for functional inquiry.
569
570 Supporting our strategy, we prioritize one gene in each of three PD loci (*Snca*, *Fgf20*, *Gch1*), that
571 have been directly associated with PD, MB DA development, and MB DA function. *SNCA* is
572 mutated in autosomal dominant versions of PD; it is a pro-aggregation component of Lewy
573 Bodies, a pathognomonic hallmark of PD neuronal degeneration (OMIM: 163890). *Fgf20* is
574 expressed preferentially in the SN, contributes to DA neuron differentiation in cell culture, and
575 protects against DA neuron degeneration[88]. Additionally, SNPs within *Fgf20* have been reported
576 to modulate PD risk[88]. Finally, *Gch1* encodes the rate-limiting enzyme in tetrahydrobiopterin
577 synthesis (GTP cyclohydrolase I). Tetrahydropterin is an important cofactor for many enzymes
578 including *Th*, the rate limiting enzyme of dopamine synthesis. Consistent with these data,
579 mutations in *GCH1* cause dopa-responsive dystonia that often presents with parkinsonian
580 symptoms (OMIM: 128230).
581
582 While our method successfully prioritized one familial PD gene (*Snca*), we do not prioritize
583 *Lrrk2,* another familial PD gene harbored within a PD GWAS locus. *Lrrk2* is not prioritized
584 simply because it is not expressed in our SN DA neuronal population. This is expected as
585 numerous studies have reported little to no *Lrrk2* expression in Th^+ MB DA neurons both in mice
586 and humans[89,90]. Instead, our method prioritizes *PDZRN4* within the *LRRK2* locus, based upon
587 differential expression and the finding that it is co-expressed with identified PD gene modules.
588 Whether *PDZRN4* should now be considered a novel alternative PD candidate independent of or
589 in addition to *LRRK2* requires functional evaluation.
590

591 This strategy also reveals genes that may be biologically relevant but are overlooked due to the
592 presence of prior candidates. *MAPT*, for example, is known to play a significant role in the broad
593 neurodegenerative pathology of Alzheimer's disease, and has additionally been associated with
594 susceptibility to PD (OMIM: 168600). Our data confirms that *Mapt* is expressed at consistent
595 levels throughout MB DA neurons, including the SN. However, we prioritize *Crhr1* because it is
596 specifically expressed in the SN compared to the other MB DA populations. Although prior data
597 demonstrated *Crhr1* to be expressed in MB DA neurons[91], it is noteworthy that the MB DA
598 neuroprotective activity of the urocortin (Ucn) neuropeptide in PD animal models is mediated
599 through its interaction with Crhr1[92–95]. Recently, Ucn-Crhr1 binding was shown to improve DA
600 neuron differentiation *in vitro,* data supported by reports linking it to a role in MB DA neuron
601 development[96]. We do not believe that these results contradict the clear connection between
602 genes in these loci and PD risk. Rather, we propose that other genes in these loci may also
603 contribute to PD susceptibility, possibly in combination with other genes in the locus. These data
604 set the stage for a new generation of independent and combinatorial functional evaluation.
605
606 By extending our ranking of candidate genes from exclusive or preferential expression in the SN
607 to include, co-regulation with WGCNA identified modules implicated in PD and ultimately the
608 inference of dosage sensitivity through the pLI (ExAC) metric, we establish a rank order of
609 candidate genes within every one of 32 major GWAS-implicated PD loci.
610
611 Despite this success, we should acknowledge several notable caveats. First, not all genes in PD-
612 associated human loci have identified mouse homologs. Thus, it remains possible that we
613 overlooked genes whose biology is not comprehensively queried in this study. Secondly, we
614 assume that identified genetic variation acts in a manner that is at least preferential, if not
615 exclusive, to SN DA neurons. Lastly, by prioritizing expressed genes, we assume that PD
616 variation affects genes that are normally expressed in the SN. We readily acknowledge that
617 regulatory variation may require stress/insult to reveal its relevance.
618
619 **CONCLUSIONS**
620 In summary, our study of DA neurons in the developing mouse brain using scRNA-seq allowed
621 for further definition of *Th*$^{+}$ neuron signatures at both embryonic and postnatal ages. These data
622 facilitated definition of a SN DA neuron signature as well as revealed previously undescribed
623 markers of this important neuronal population. This data also provides the first demonstration of
624 a postnatal progenitor-like MB neuron and its characteristic molecular signature. Finally, we use
625 the totality of our data to provide the first comprehensive candidate gene prioritization of GWAS
626 loci for a major common disease trait. Collectively these data establish a platform from which
627 the next generation exploration of PD genetics can more effectively proceed.

628 **METHODS**
629
630 **Data availability**
631 Raw data will be made available on Sequence Read Archive (SRA) and Gene Expression
632 Omnibus (GEO) prior to publication. Summary data is available where code is available below
633 (https://github.com/pwh124/DA_scRNA-seq).
634
635 **Code Availability**
636 Code for analysis, for the production of figures, and summary data is deposited at
637 https://github.com/pwh124/DA_scRNA-seq
638
639 **Propagation of Th:GFP BAC transgenic mice.**
640 The Th:EGFP BAC transgenic mice (Tg(Th-EGFP)DJ76Gsat/Mmnc) used in this study were
641 generated by the GENSAT Project and were purchased through the Mutant Mouse Resource &
642 Research Centers (MMRRC) Repository (https://www.mmrrc.org/). Mice were maintained on a
643 Swiss Webster (SW) background with female SW mice obtained from Charles River
644 Laboratories (http://www.criver.com/). All work involving mice (husbandry, colony
645 maintenance and euthanasia) were reviewed and pre-approved by the institutional care and use
646 committee.
647
648 The Tg(Th-EGFP)DJ76Gsat/Mmnc line was primarily maintained through matings between
649 Th:EGFP positive, hemizygous male mice and wild-type SW females (dams). Timed matings for
650 cell isolation were similarly established between hemizygous male mice and wild-type SW
651 females. The observation of a vaginal plug was defined as embryonic day 0.5 (E0.5).
652
653 **Dissection of E15.5 brains.**
654 At 15.5 days after the timed mating, pregnant dams were euthanized and the entire litter of
655 embryonic day 15.5 (E15.5) embryos were dissected out of the mother and immediately placed
656 in chilled Eagle's Minimum Essential Media (EMEM). Individual embryos were then
657 decapitated and heads were placed in fresh EMEM on ice. Embryonic brains were then removed
658 and placed in Hank's Balanced Salt Solution (HBSS) without Mg^{2+} and Ca^{2+} and manipulated
659 while on ice. The brains were immediately observed under a fluorescent stereomicroscope and
660 $EGFP^{+}$ brains were selected. $EGFP^{+}$ regions of interest in the forebrain (hypothalamus) and the
661 midbrain were then dissected and placed in HBSS on ice. This process was repeated for each
662 $EGFP^{+}$ brain. Four $EGFP^{+}$ brain regions for each region studied were pooled together for
663 dissociation.
664
665 **Dissection of P7 brains.**
666 After matings, pregnant females were sorted into their own cages and checked daily for newly
667 born pups. The morning the pups were born was considered day P0. Once the mice were aged to

668 P7, all the mice from the litter were euthanized and the brains were then quickly dissected out of
669 the mice and placed in HBSS without Mg^{2+} and Ca^{2+} on ice. As before, the brains were then
670 observed under a fluorescent microscope, $EGFP^+$ status for P7 mice was determined, and $EGFP^+$
671 brains were retained. For each $EGFP^+$ brain, the entire olfactory bulb was first resected and
672 placed in HBSS on ice. Immediately thereafter, the $EGFP^+$ forebrain and midbrain regions for
673 each brain were resected and also placed in distinct containers of HBSS on ice. Five $EGFP^+$
674 brain regions for each region were pooled together for dissociation.
675
676 **Generation of single cell suspensions from brain tissue.**
677 Resected brain tissues were dissociated using papain (Papain Dissociation System, Worthington
678 Biochemical Corporation; Cat#: LK003150) following the trehalose-enhanced protocol reported
679 by Saxena, et. al, 2012[97] with the following modifications: The dissociation was carried out at
680 37°C in a sterile tissue culture cabinet. During dissociation, all tissues at all time points were
681 triturated every 10 minutes using a sterile Pasteur pipette. For E15.5 tissues, this was continued
682 for no more than 40 minutes. For P7, this was continued for up to 1.5 hours or until the tissue
683 appeared to be completely dissociated.
684 Additionally, for P7 tissues, after dissociation but before cell sorting, the cell pellets were passed
685 through a discontinuous density gradient in order to remove cell debris that could impede cell
686 sorting. This gradient was adapted from the Worthington Papain Dissociation System kit.
687 Briefly, after completion of dissociation according to the Saxena protocol[97], the final cell pellet
688 was resuspended in DNase dilute albumin-inhibitor solution, layered on top of 5 mL of albumin-
689 inhibitor solution, and centrifuged at 70g for 6 minutes. The supernatant was then removed.
690
691 **FACS and single-cell collection.**
692 For each timepoint-region condition, pellets were resuspended in 200 μL of media without serum
693 comprised of DMEM/F12 without phenol red, 5% trehalose (w/v), 25 μM AP-V, 100 μM
694 kynurenic acid, and 10 μL of 40 U/μl RNase inhibitor (RNasin® Plus RNase Inhibitor, Promega)
695 at room temperature. The resuspended cells were then passed through a 40 uM filter and
696 introduced into a Fluorescence Assisted Cell Sorting (FACS) machine (Beckman Coulter MoFlo
697 Cell Sorter or Becton Dickinson FACSJazz). Viable cells were identified via propidium iodide
698 staining, and individual neurons were sorted based on their fluorescence (EGFP+ intensity, See
699 Figure 2d) directly into lysis buffer in individual wells of 96-well plates for single-cell
700 sequencing (2 μL Smart-Seq2 lysis buffer + RNAase inhibitor, 1 μL oligo-dT primer, and 1 μL
701 dNTPs according to Picelli et al., 2014[98]. Ninety-five cells of each type were collected along
702 with a control blank well. Upon completion of a sort, the plates were briefly spun in a tabletop
703 microcentrifuge and snap-frozen on dry ice. Single cell lysates were subsequently kept at -80°C
704 until cDNA conversion.
705
706 **Single-cell RT, library prep, and sequencing.**

707 Library preparation and amplification of single-cell samples were performed using a modified
708 version of the Smart-Seq2 protocol[98]. Briefly, 96-well plates of single cell lysates were thawed to
709 4°C, heated to 72°C for 3 minutes, then immediately placed on ice. Template switching first-
710 strand cDNA synthesis was performed as described above using a 5'-biotinylated TSO oligo.
711 cDNAs were amplified using 20 cycles of KAPA HiFi PCR and 5'-biotinylated ISPCR primer.
712 Amplified cDNA was cleaned with a 1:1 ratio of Ampure XP beads and approximately 200 pg
713 was used for a one-quarter standard sized Nextera XT tagmentation reaction. Tagmented
714 fragments were amplified for 14 cycles and dual indexes were added to each well to uniquely
715 label each library. Concentrations were assessed with Quant-iT PicoGreen dsDNA Reagent
716 (Invitrogen) and samples were diluted to ~2 nM and pooled. Pooled libraries were sequenced on
717 the Illumina HiSeq 2500 platform to a target mean depth of ~8.0 x 10^5 50bp paired-end
718 fragments per cell at the Hopkins Genetics Research Core Facility.
719
720 **RNA sequencing and alignment.**
721 For all libraries, paired-end reads were aligned to the mouse reference genome (mm10)
722 supplemented with the Th-EGFP$^+$ transgene contig, using Hisat2[99] with default parameters
723 except: -p 8. Aligned reads from individual samples were quantified against a reference
724 transcriptome[100] (GENCODE vM8) supplemented with the addition of the eGFP transcript.
725 Quantification was performed using cuffquant with default parameters and the following
726 additional arguments: --no-update-check –p 8. Normalized expression estimates across all
727 samples were obtained using cuffnorm[101] with default parameters.
728
729 **Single-cell RNA data analysis.**
730 *Expression estimates.*
731 Gene-level and isoform-level FPKM (Fragments Per Kilobase of transcript per Million) values
732 produced by cuffquant[101] and the normalized FPKM matrix from cuffnorm was used as input for
733 the Monocle2 single cell RNA-seq framework[102] in R/Bioconductor[103]. Genes were annotated
734 using the Gencode vM8 release[100]. A CellDataSet was then created using Monocle (v2.2.0)[102]
735 containing the gene FPKM table, gene annotations, and all available metadata for the sorted
736 cells. All cells labeled as negative controls and empty wells were removed from the data.
737 Relative FPKM values for each cell were converted to estimates of absolute mRNA counts per
738 cell (RPC) using the Monocle2 Census algorithm[10] using the Monocle function "relative2abs."
739 After RPCs were inferred, a new cds was created using the estimated RNA copy numbers with
740 the expression Family set to "negbinomial.size()" and a lower detection limit of 0.1 RPC.
741
742 *QC Filtering.*
743 After expression estimates were inferred, the cds containing a total of 473 cells was run through
744 Monocle's "detectGenes" function with the minimum expression level set at 0.1 transcripts. The
745 following filtering criteria were then imposed on the entire data set:
746

747 i. Number of expressed genes - The number of expressed genes detected in each cell in the
748 dataset was plotted and the high and low expressed gene thresholds were set based on
749 observations of each distribution. Only those cells that expressed between 2,000 and 10,000
750 genes were retained.
751
752 ii. Cell Mass - Cells were then filtered based on the total mass of RNA in the cells calculated by
753 Monocle. Again, the total mass of the cell was plotted and mass thresholds were set based on
754 observations from each distribution. Only those cells with a total cell mass between 100,000 and
755 1,300,000 fragments mapped were retained.
756
757 iii. Total RNA copies per cell - Cells were then filtered based on the total number of RNA
758 transcripts estimated for each cell. Again, the total RNA copies per cell was plotted and RNA
759 transcript thresholds were set based on observations from each distribution. Only those cells with
760 a total mRNA count between 1,000 and 40,000 RPCs were retained.
761
762 A total of 410 individual cells passed these initial filters. Outliers found in subsequent, reiterative
763 analyses described below were analyzed and removed resulting a final cell number of 396. The
764 distributions for total mRNAs, total mass, and number of expressed, can be found in Figure S1.
765
766 *Log distribution QC.*
767 Analysis using Monocle relies on the assumption that the expression data being analyzed follows
768 a log-normal distribution. Comparison to this distribution was performed after initial filtering
769 prior to continuing with analysis and was observed to be well fit.
770
771 **Reiterative single-cell RNA data analysis.**
772 After initial filtering described above, the cds was then broken into subsets based on "age" and
773 "region" of cells for recursive analysis. Regardless of how the data was subdivided, all data
774 followed a similar downstream analysis workflow.
775
776 *Determining number of cells expressing each gene.*
777 The genes to be analyzed for each subset iteration were filtered based on the number of cells that
778 expressed each gene. Genes were retained if they were expressed in > 5% of the cells in the
779 dataset being analyzed. These are termed "expressed_genes." For example, when analyzing all
780 cells collected together (n = 410), a gene had to be expressed in 20.5 cells (410 x 0.05 = 20.5) to
781 be included in the analysis. Whereas when analyzing P7 MB cells (n = 80), a gene had to be
782 expressed in just 4 cells (80 x 0.05 = 4). This was done to allow include genes that may define
783 rare populations of cells that could be present in any given population.
784
785 *Monocle model preparation.*

786 The data was prepared for Monocle analysis by retaining only the expressed genes that passed
787 the filtering described above. Size factors were estimated using Monocle's
788 "estimateSizeFactors()" function. Dispersions were estimated using the "estimateDispersions()"
789 function.
790
791 *High variance gene selection.*
792 Genes that have a high biological coefficient of variation (BCV) were identified by first
793 calculating the BCV by dividing the standard deviation of expression for each expressed gene by
794 the mean expression of each expressed gene. A dispersion table was then extracted using the
795 dispersionTable() function from Monocle. Genes with a mean expression > 0.5 transcripts and a
796 "dispersion_empirical" >= 1.5*dispersion_fit or 2.0*dispersion_fit were identified as "high
797 variance genes."
798
799 *Principal component analysis (PCA).*
800 PCA was then run using the R prcomp function on the centered and scaled log2 expression
801 values of the "high variance genes." PC1 and PC2 were then visualized to scan the data for
802 obvious outliers as well as bias in the PCs for age, region, or plates on which the cells were
803 sequenced. If any visual outliers in the data was observed, those cells were removed from the
804 original subsetted cds and all filtering steps above were repeated. Once there were no obvious
805 visual outliers in PC1 or PC2, a screeplot was used plot the PCA results in order to determine the
806 number of PCs that contributed most significantly to the variation in the data. This was manually
807 determined by inspecting the screeplot and including only those PCs that occur before the
808 leveling-off of the plot.
809
810 *t-SNE and clustering.*
811 Once the number of significant PCs was determined, t-Distributed Stochastic Neighbor
812 Embedding (t-SNE)[12] was used to embed the significant PC dimensions in a 2-D space for
813 visualization. This was done using the "tsne" package available through R with "whiten =
814 FALSE." The parameters "perplexity" and "max_iter" were tested with various values and set
815 according what seemed to give the cleanest clustering of the data.
816
817 After dimensionality reduction via t-SNE, the number of clusters was determined in an unbiased
818 manner by fitting multiple Gaussian distributions over the 2D t-SNE projection coordinates using
819 the R package "ADPclust"[104] and the t-SNE plots were visualized using a custom R script. The
820 number of genes expressed and the total mRNAs in each cluster were then compared.
821
822 *Differential expression Analyses.*
823 Differential expression analysis was performed using the "differentialGeneTest" function from
824 Monocle that uses a likelihood ratio test to compare a vector generalized additive model

825 (VGAM) using a negative binomial family function to a reduced model in which one parameter
826 of interest has been removed. In practice, the following models were fit:
827
828 "~kmeans_tSNE_cluster" for timepoint-region datasets
829 "~region" for timepoint datasets
830
831 Genes were called as significantly differentially expressed if they had a q-value (Benjamini-
832 Hochberg corrected p-value) < 0.05.
833
834 *Cluster/Region Specific marker genes.*
835 In order to identify differentially expressed genes that were "specifically" expressed in a
836 particular cluster or region, R code calculating the Jensen-Shannon based specificity score from
837 the R package cummerbund[105] was used similar to what was described in Kelly et. al[106].
838
839 Briefly, the mean RPC within each cluster for each expressed gene as well as the percentage of
840 cells within each cluster that express each gene at a level > 1 transcript were calculated. The
841 ".specificity" function from the cummRbund package was then used to calculate and identify the
842 cluster with maximum specificity of each gene's expression. Details of this specificity metric can
843 be found in Cabili, et al[107].
844
845 To identify cluster/region specific genes, the distribution of specificity scores for each
846 region/cluster was plotted and a specificity cutoff was chosen so that only the "long right tail" of
847 each distribution was included (i.e. genes with a specificity score above the cutoff chosen). For
848 each iterative analysis, the same cutoff was used for each cluster or region. Once the specificity
849 cutoff was chosen, genes were further filtered by only retaining genes that were expressed in >=
850 40% of cells within the cluster the gene was determined to be specific for.
851
852 **Transcription Factor Correlation.**
853 For transcription factor (TF) correlation analysis, aggregated lists of mouse genes (whether
854 specific or differentially expressed) were intersected with the Animal Transcription Factor
855 Database[108] (Data accessed: 04-04-2017; http://www.bioguo.org/AnimalTFDB/) in order to
856 identify genes within those lists that were TFs. Pairwise correlation of log2(RPC + 1) for the TFs
857 were calculated and plotted using the "corrplot" function from the "corrplot" R package with the
858 following settings: order = "hclust", hclust.method = "ward.D2", cor.method = "pearson",
859 method = "color". After plotting, the "corrplot" function option "addrect" was used to identify
860 groups of TFs based on hierarchical clustering. "addrect" was set to a number that best fit the
861 data. "Core transcription factors" within the larger groups were identified by using multiscale
862 bootstrap resampling of hierarchical clustering using the R package 'pvclust' (v2.0-0)[109]. Again,
863 log2(RPC + 1) for each group of TFs were used in these analyses. The analysis was carried out
864 using the function 'pvclust()' with the following settings: nboot = 1000, method.dist =

865 “correlation”; method.hclust = “ward.D2”; and r = seq(0.5,1.4, by=.1). The distance metric and
866 hclust method matched those used in the “corrplot” analysis described above. Significant clusters
867 of TFs were identified using the ‘pvpick()’ function with the following settings: pv = ‘au’; alpha
868 = 0.90; max.only = F; and type = ‘geq’. The clusters of TFs were deemed significant if the
869 approximate unbiased (AU) p-value was greater than or equal to 90% (alpha = 0.90). Since
870 “max.only” was set to FALSE, many smaller significant clusters were encompassed by larger
871 clusters that were also significant. In those cases, the larger cluster was kept. Also, in the case
872 where groups were identified by through the ‘addrect’ option of the ‘corrplot’ analysis above, if
873 any significant cluster identified through bootstrap analysis was identical to the larger groups, it
874 was not considered a “core” TF group.
875
876 **Gene Set Enrichment Analyses.**
877 Gene set enrichment analyses were performed in two separate ways depending upon the
878 situation. A Gene Set Enrichment Analysis (GSEA) PreRanked analysis was performed when a
879 ranked list (e.g. genes ranked by PC1 loadings) using GSEA software available from the Broad
880 Institute (v2.2.4)[110,111]. Ranked gene lists were uploaded to the GSEA software and a
881 “GSEAPreRanked” analysis was performed with the following settings: ‘Number of
882 Permutations’ = 1000, ‘Collapse dataset to gene symbols’ = true, ‘Chip platform(s)’ =
883 GENE_SYMBOL.chip, and ‘Enrichment statistic’ = weighted. Analysis was performed against
884 Gene Ontology (GO) collections from MSigDB, including c2.all.v5.2.symbols and
885 c5.all.v5.2.symbols. Top ten gene sets were reported for each analysis (Table S1). Figures and
886 tables displaying the results were produced using custom R scripts.
887
888 Unranked GSEA analyses for lists of genes was performed using hypergeometric tests from the
889 R package ‘clusterProfiler’ implemented through the functions ‘enrichGO’, ‘enrichKEGG’, and
890 ‘enrichPathway’ with ‘pvalueCutoff’ set at 0.01, 0.1, 0.1, respectively with default settings[62].
891 These functions were implemented through the ‘compareCluster’ function when analyzing
892 WGCNA data.
893
894 **Weighted Gene Co-Expression Network Analysis (WGCNA).**
895 WGCNA was performed us in R using the WGCNA package (v1.51)[112,113] following established
896 pipelines laid out by the packages authors (see
897 https://labs.genetics.ucla.edu/horvath/CoexpressionNetwork/Rpackages/WGCNA/ for more
898 detail). Briefly, an expression matrix for all P7 neurons containing all genes expressed in >= 20
899 cells (n = 12628) was used with expression counts in log2(Transcripts + 1). The data were
900 initially clustered in order to identify and remove outliers (n = 1) to leave 223 total cells (Figure
901 S6). The soft threshold (power) for WGCNA was then determined by calculating the scale free
902 topology model fit for a range of powers (1:10, 12, 14, 16, 18, 20) using the WGCNA function
903 “pickSoftThreshold()” setting the networkType = “signed”. A power of 10 was then chosen
904 based on the leveling-off of the resulting scale independence plot above 0.8 (Figure S6).

905 Network adjacency was then calculated using the WGCNA function "adjacency()" with the
906 following settings: power = 10 and type = "signed." Adjacency calculations were used to then
907 calculate topological overlap using the WGCNA function "TOMsimilarity()" with the following
908 settings: TOMtype = "signed." Distance was then calculated by subtracting the topological
909 overlap from 1. Hierarchical clustering was then performed on the distance matrix and modules
910 were identified using the "cuttreeDynamic" function from the dynamicTreeCut package[114] with
911 the following settings: deepSplit = T; pamRespectsDendro = FALSE, and minClusterSize = 20.
912 This analysis initially identified 18 modules. Eigengenes for each module were then calculated
913 using the "moduleEigengenes()" function and each module was assigned a color. Two modules
914 ("grey" and "turquoise") were removed at this point. Turquoise was removed because it
915 contained 11567 genes or all the genes that could not be grouped with another module. Grey was
916 removed because it only contained 4 genes, falling below the minimum set module size of 20.
917 The remaining 16 modules were clustered (Figure S6) and the correlation between module
918 eigengenes and subset cluster identity was calculated using custom R scripts. Significance of
919 correlation was determined by calculated the Student asymptotic p-value for correlations by
920 using the WGCNA "corPvalueStudent()" function. Gene set enrichments for modules were
921 determined by using the ClusterProfiler R package[62].The correlation between the t-SNE position
922 of a cell and the module eigengenes was calculated using custom R scripts.
923
924 **Prioritizing Genes in PD GWAS Loci.**
925 *Topologically Associated Domain (TAD) and Megabase Gene Data.*
926 The data for human TAD boundaries were obtained from human embryonic stem cell (hESC)
927 Hi-C data[115] and converted from human genome hg18 to hg38 using the liftOver tool from
928 UCSC Genome Browser. PD GWAS SNP locations were then intersected with the TAD
929 information to identify TADs containing a PD GWAS SNP. The data for +/- 1 megabase regions
930 surrounding PD GWAS SNPs was obtained by taking PD GWAS SNP locations in hg38 and
931 adding and subtracting 1e+06 from each location. All hg38 UCSC RefSeq genes that fall within
932 the TADs or megabase regions were then identified by using the UCSC Table Browser. All
933 genes were then annotated with PD locus and SNP information. Mouse homologs for all genes
934 were identified using the NCBI Homologene database (Date accessed: 03/06/2017) and manual
935 annotation. The TAD and megabase tables were then combined to create a final PD GWAS
936 locus-gene table.
937
938 *PD GWAS Loci Gene Scoring.*
939 Genes within PD GWAS loci were initially scored using four gene lists: Genes with an average
940 expression ≥0.5 transcripts in the SN cluster in our data (points = 2); Genes that were
941 differentially expressed between P7 MB clusters (points = 1); Genes found to be "specifically"
942 expressed in the P7 MB SN cluster (points = 1); Genes found in the WGCNA modules that are
943 enriched for PD (points = 1). Expression in the SN cluster was considered the most important
944 feature and was weighted as such. Furthermore, a piece of external data, pLI scores for each gene

945 from the ExAC database[72], were added to the scores in order to rank all loci. pLI scores
946 (fordist_cleaned_exac_r03_march16_z_pli_rec_null_data.txt) were obtained from
947 http://exac.broadinstitute.org/ (Date dowloaded: March 30, 2017).
948
949 **In situ hybridization.**
950 *In situ* hybridization data was downloaded from publically available data from the Allen Institute
951 through the Allen Brain Atlas (http://www.brain-map.org/). The image used in Figure 5 was
952 obtained from the Reference Atlas at the Allen Brain Atlas (http://mouse.brain-
953 map.org/static/atlas). URLs for all Allen Brain Atlas in situ data analyzed and downloaded for
954 *substantia nigra* marker genes (Figure 5c) are available in Table S6. Data for *substantia nigra*
955 expression *in situ* data for PD GWAS genes (Figure 8c) were obtained from the following
956 experiments: 1056 (*Th*), 79908848 (*Snca*), 297 (*Crhr1*), 77371865 (*Rora*), 72129224 (*Mmp16*),
957 and 414 (*Cntn1*). Data accessed on 03/02/17.
958
959 **Single molecule in situ hybridization (smFISH).**
960 For *in situ* hybridization experiments, untimed pregnant Swiss Webster mice were ordered from
961 Charles River Laboratories (Crl:CFW(SW); http://www.criver.com/). Mice were maintained as
962 previously described. Pups were considered P0 on the day of birth. At P7, the pups were
963 decapitated, the brain was quickly removed, and the brain was then washed in 1x PBS. The intact
964 brain was then transferred to a vial containing freshly prepared 4% PFA in 1x PBS and incubated
965 at 4°C for 24 hours. After 24 hours, brains were removed from PFA and washed three times in 1x
966 PBS. The brains were then placed in a vial with 10% sucrose at 4°C until the brains sunk to the
967 bottom of the vial (usually ~1 hour). After sinking, brains were immediately placed in a vial
968 containing 30% sucrose at 4°C until once again sinking to the bottom of the vial (usually
969 overnight). After cryoprotection, the brains were quickly frozen in optimal cutting temperature
970 (O.C.T.) compound (Tissue-Tek) on dry ice and stored at -80°C until use. Brains were sectioned
971 at a thickness of 14 micrometers and mounted on Superfrost Plus microscope slides
972 (Fisherbrand, Cat. # 12-550-15) with two sections per slide. Sections were then dried at room
973 temperature for at least 30 minutes and then stored at -80°C until use.
974
975 RNAscope *in situ* hybridization (https://acdbio.com/) was used to detect single RNA transcripts.
976 RNAscope probes were used to detect *Th* (C1; Cat No. 317621), *Slc6a3* (C2; Cat No. 315441-
977 C2), *Lhx9* (C3; Cat No. 495431-C3), *Ldb2* (C3; Cat No. 466061-C3), and *Meis2* (C3; Cat No.
978 436371-C3). The RNAscope Fluorescent Multiplex Detection kit (Cat No. 320851) and the
979 associated protocol provided by the manufacturer were used. Briefly, frozen tissues were
980 removed from -80°C and equilibrated at room temperature for 5 minutes. Slides were then
981 washed at room temperature in 1x PBS for 3 minutes with agitation. Slides were then
982 immediately washed in 100% ethanol by moving the slides up and down 5-10 times. The slides
983 were then allowed to dry at room temperature and hydrophobic barriers were drawn using a
984 hydrophobic pen (ImmEdge Hydrophobic Barrier PAP Pen, Vector Laboratories, Cat. # H-4000)

985 around the tissue sections. The hydrophobic barrier was allowed to dry overnight. After drying,
986 the tissue sections were treated with RNAscope Protease IV at room temperature for 30 minutes
987 and then slides were washed in 1x PBS. Approximately 100 uL of multiplex probe mixtures (C1
988 - *Th*, C2 - *Slc6a3*, and C3 - one of *Lhx9*, *Ldb2*, or *Meis2*) containing either approximately 96 uL
989 C1: 2 uL C2: 2 uL C3 (*Th*:*Slc6a3*:*Lhx9*) or 96 uL C1: 0.6 uL C2: 2 uL C3 (*Th*:*Slc6a3*:*Ldb2* or
990 *Th*:*Slc6a3*:*Meis2*) were applied to appropriate sections. Both mixtures provided adequate *in situ*
991 signals. Sections were then incubated at 40°C for 2 hours in the ACD HybEZ oven. Sections
992 were then sequentially treated with the RNAscope Multiplex Fluorescent Detection Reagents kit
993 solutions AMP 1-FL, AMP 2-FL, AMP 3-FL, and AMP 4 Alt B-FL, with washing in between
994 each incubation, according to manufacturer's recommendations. Sections were then treated with
995 DAPI provided with the RNAscope Multiplex Fluorescent Detection Reagents kit. One drop of
996 Prolong Gold Antifade Mountant (Invitrogen, Cat # P36930) was then applied to each section
997 and a coverslip was then placed on the slide. The slides were then stored in the dark at 4°C
998 overnight before imaging. Slides were further stored at 4°C throughout imaging. Manufacturer
999 provided positive and negative controls were also performed along side experimental probe
1000 mixtures according to manufacturer's protocols. Four sections that encompassed relevant
1001 populations in the P7 ventral MB (SN, VTA, etc.) were chosen for each combination of
1002 RNAscope smFISH probes and subsequent analyses.
1003
1004 **Confocal Microscopy.**
1005 RNAscope fluorescent *in situ* experiments were analyzed using the Nikon A1 confocal system
1006 equipped with a Nikon Eclipse Ti inverted microscope running Nikon NIS-Elements AR 4.10.01
1007 64-bit software. Images were captured using a Nikon Plan Apo λ 60x/1.40 oil immersion lens
1008 with a common pinhole size of 19.2 μM, a pixel dwell of 28.8 μs, and a pixel resolution of 1024
1009 x 1024. DAPI, FITC, Cy3, and Cy5 channels were used to acquire RNAscope fluorescence.
1010 Positive and negative control slides were used in order to calibrate laser power, offset, and
1011 detector sensitivity, for all channels in all experiments performed.
1012
1013 **Image Analysis and processing.**
1014 Confocal images were saved as .nd2 files. Images were then processed in ImageJ as follows.
1015 First, the .nd2 files were imported into ImageJ and images were rotated in order to reflect a
1016 ventral MB orientation with the ventral side of the tissue at the bottom of the image. Next the
1017 LUT ranges were adjusted for the FITC (range: 0-2500), Cy3 (range: 0-2500), and Cy5 (range:
1018 0-1500) channels. All analyzed images were set to the same LUT ranges. Next, the channels
1019 were split and merged back together to produce a "composite" image seen in Figure 6. Scale bars
1020 were then added. Cells of interest were then demarcated, duplicated, and the channels were split.
1021 These cells of interest were then displayed as the insets seen in Figure 6.
1022
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AUTHOR CONTRIBUTIONS

PWH, ASM, and LAG designed the study and wrote the paper. PWH, SAM, WDL and GAC performed the experiments. PWH and LAG implemented the computational algorithms to process the raw data and conduct analyses thereof. PWH, LAG, and ASM analyzed and interpreted the resulting data. LAG contributed novel computational pipeline development. Correspondence to ASM (andy@jhmi.edu) and LAG (loyalgoff@jhmi.edu).

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The authors declare no competing financial interests.

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