

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

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19 **ABSTRACT**

20

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

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37 Parkinson's Disease (PD) is the most common progressive neurodegenerative movement
38 disorder. Incidence of PD increases with age^{1,2} affecting an estimated 1% worldwide beyond 70
39 years of age³. Although PD ultimately impacts multiple neuronal centers, preferential
40 degeneration of the ventral midbrain (VM) dopamine (DA) neurons leading to collapse of the
41 nigrostriatal pathway is a common theme.

42
43 Mesencephalic DA neurons and their efferent connections with the striatum are responsible for
44 the acquisition and maintenance of fine motor control and reward pathways. In turn, motor
45 control is largely dependent on DA neurons populating the *substantia nigra* (SN), whereas the
46 ventral tegmental area (VTA) is responsible for reward based behaviors and satiety. Despite their
47 shared neurotransmitter characteristic, PD compromises the viability of SN DA neurons
48 preferentially. By contrast VTA and VM DA periaqueductal gray area (PAG) DA neurons are
49 largely spared^{4,5}. This fact has driven research interest in the genetic basis of SN vulnerability in
50 PD compared with that of VTA/PAG DA neurons.

51
52 To date, of the more than 20 genes that have been implicated in familial PD, mutations in less
53 than 10 have been robustly shown to explain disease expression^{6,7}. Beyond rare familial cases, a
54 recent meta-analysis of PD GWAS highlighted 32 loci associated with sporadic PD
55 susceptibility⁸. While some GWAS loci contain genes known to be mutated in familial PD
56 (*SNCA* and *LRK2*)^{6,7}, most do not contain a known causal gene. The inability to systematically
57 identify the causative gene/s within GWA loci establishes a roadblock to the translation of
58 genetic findings to medical practice. This requires an understanding of the pathogenesis of the
59 disease and a thorough characterization of the specific cell population/s affected. In PD, one can
60 reasonably assert that a significant fraction of disease-associated variation likely mediates its
61 influence specifically within the SN. The answers to the implicitly related question of what
62 renders SN DA neurons more vulnerable than other DA neurons also depends on the impact of
63 such variation on gene regulatory networks (GRNs) essential to their viability or function
64 regardless of whether they are unique to SN or shared among DA neurons more widely.

65
66 In an effort to resolve heterogeneity among central nervous system (CNS) DA populations, we
67 undertook single-cell RNA-seq (scRNA-seq) analyses of CNS DA neurons from discrete
68 anatomical regions of both embryonic and postnatal mouse brains. We evaluated both MB and
69 forebrain (FB) DA neurons at embryonic day 15.5 (E15.5) and expanded our analyses at
70 postnatal day 7 (P7) to include DA neurons isolated from the olfactory bulb (OB), FB (posterior
71 hypothalamus) and MB. Deeper analysis of the P7 MB allowed for refinement of DA neuronal
72 composition including elucidation of transcriptomic differences and similarities of different
73 anatomical DA populations, identification of novel genetic markers for the SN, and the
74 characterization of modules of co-expressed genes in our data. The results of our analyses
75 provide a framework within which we begin to prioritize and test hypotheses of the potential
76 disease modulating role played by genes within PD GWAS loci.

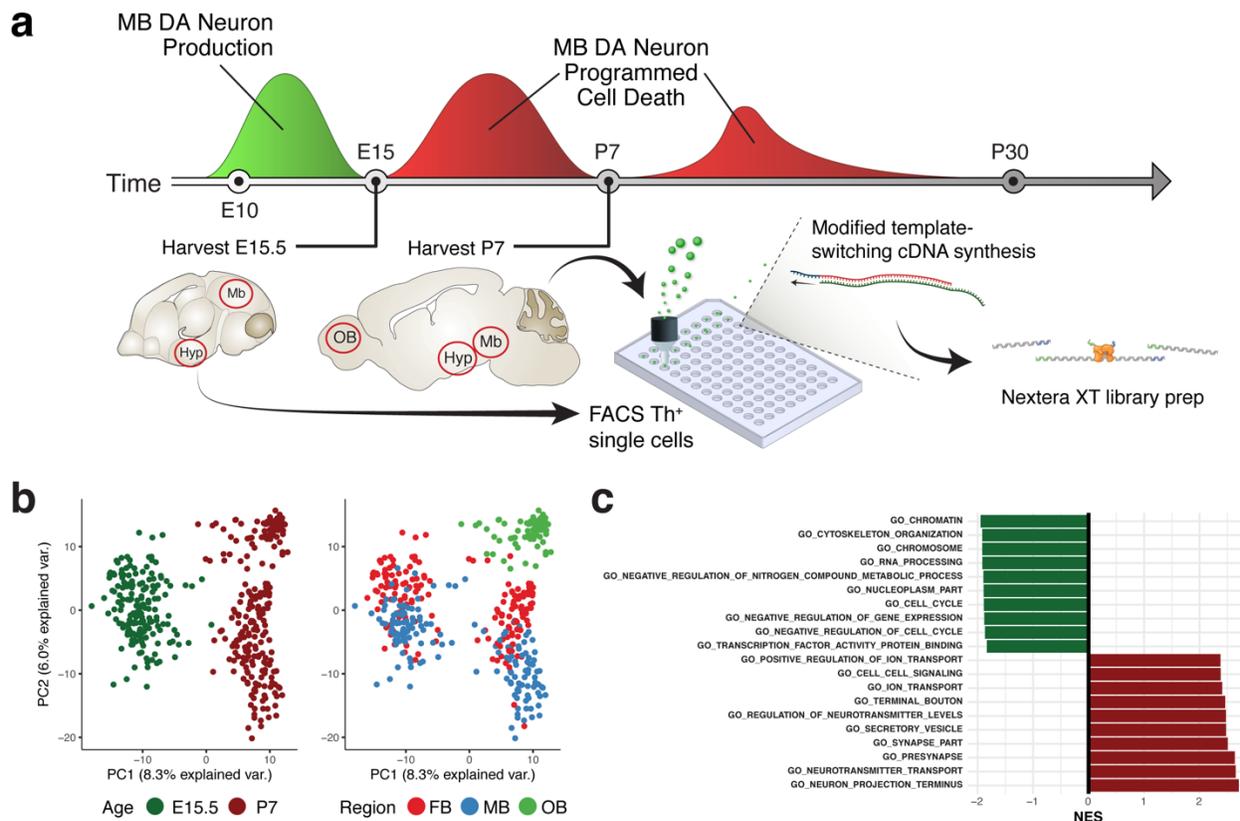
77 **RESULTS**

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

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

90



91

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

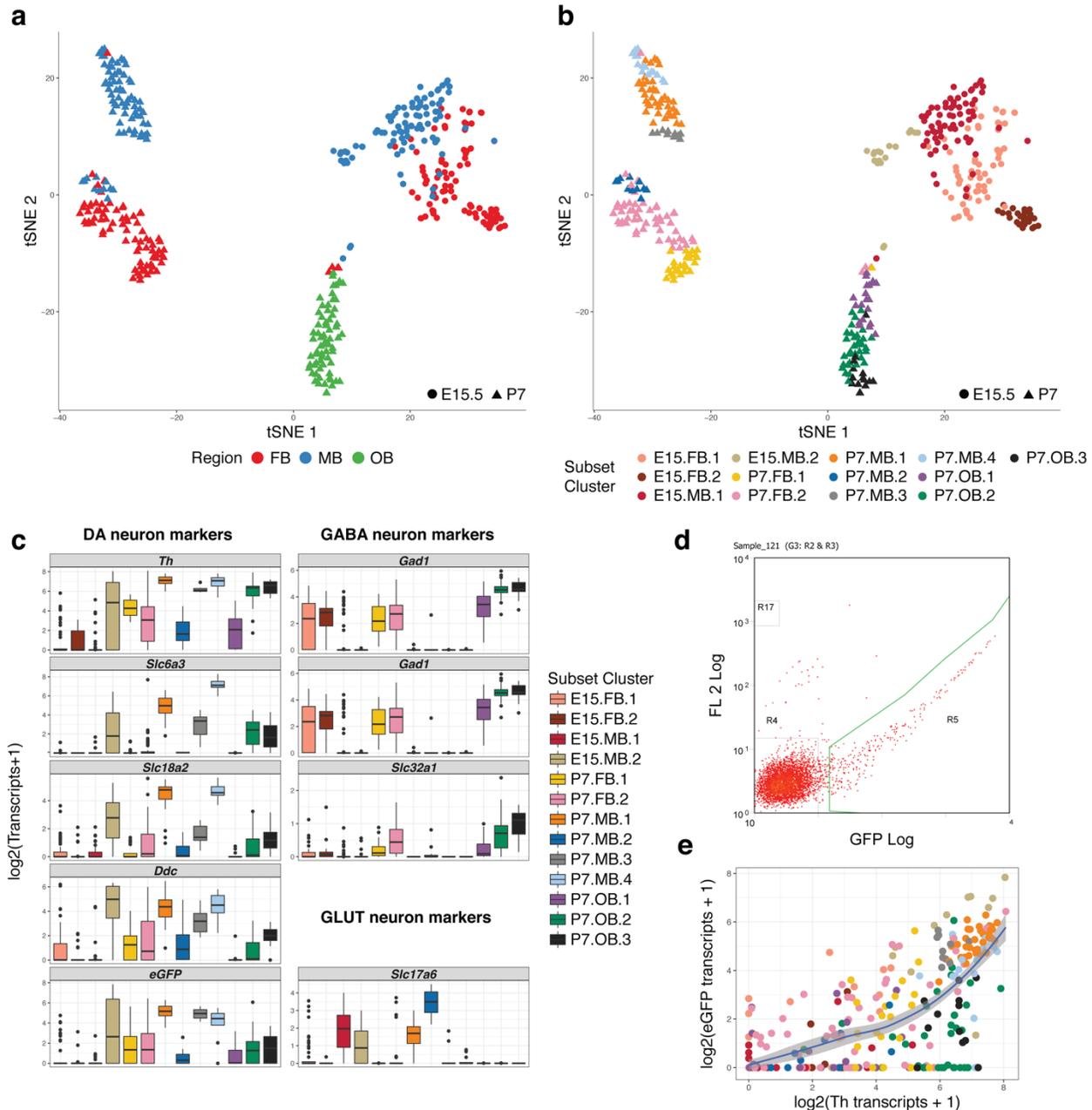
100 (GO) gene sets enriched in genes with positive (red) and negative (green) PC1 loadings. Analysis was carried out
101 using Gene Set Enrichment Analysis (GSEA) PreRanked analysis. Genes with negative PC1 loadings and negative
102 normalized enrichment scores (NES) were enriched for terms indicative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

105
106 We sequenced RNA from single cells to an average depth of $\sim 8.0 \times 10^5$ 50bp paired-end
107 fragments per cell. Using Monocle¹⁰, we converted normalized expression estimates into
108 estimates of RNA copies per cell. Cells were filtered based on the distributions of total mass,
109 total number of mRNAs, and total number of expressed genes per cell (Figure S1, detailed in
110 Methods). After QC, 410 out of 473 cells were retained. Using principal component analysis
111 (PCA), we identified and removed 14 outliers determined to be astrocytes, microglia, or
112 oligodendrocytes (Figure S2; Table S1), leaving 396 cells (~ 79 cells/timepoint-region; Figure
113 S1d).

114
115 Following a workflow similar to the recently described “dpFeature” procedure¹¹, we first
116 identified highly variant genes within the data. We then selected the PCs that described the
117 highest percentages of variance in the data using these to represent the cells in two dimensions
118 using t-Stochastic Neighbor Embedding (t-SNE)¹². We called clusters of related cells within the
119 data in an unbiased manner (see Methods). As anticipated, we observed that the greatest source
120 of variation was between timepoints (Figure 1b). Genes associated with negative PC1 loadings
121 (E15.5 cells) were enriched for gene sets consistent with mitotically active neuronal precursors
122 (Figure 1c). In contrast, genes associated with positive PC1 loadings (P7 cells) were enriched for
123 GO terms associated with mature, post-mitotic neurons (Figure 1c).

124
125 *Analyses by region and timepoint reveal additional novel neuronal diversity.*
126 Consistent with the suggestion that the embryonic cells include a less diverse progenitor
127 population, analysis of all cells revealed that the E15.5 cells from both MB and FB cluster
128 together (Figure 2a). By contrast, cells isolated at P7 mostly cluster by anatomical region,
129 suggesting progressive functional divergence with time (Figure 2a). We then applied the scRNA-
130 seq analysis workflow in a recursive manner in all regions at both timepoints to further explore
131 heterogeneity. This revealed a total of 13 clusters (E15.5 FB, 2; MB, 2; P7 OB, 3; FB, 2; MB, 4;
132 Figure 2b). Using known markers, we established that all clusters expressed high levels of pan-
133 neuronal markers (*Snap25*, *Eno2*, and *Syt1*) (Figure S3). In contrast, we found weak or no,
134 evidence of astrocyte (*Aldh1l1*, *Slc1a3*, *Aqp4*, and *Gfap*) or oligodendrocyte markers (*Mag*, *Mog*,
135 and *Mbp*; Figure S3).

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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 be 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) marker (*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

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

153

154

155 We then evaluated the expression of known markers of DA neurons along with eGFP (Figure
156 2c). We detected consistently high levels of *Th* in cluster E15.MB.2 and all P7 clusters (Figure
157 2c) which correlated with eGFP expression (Figure 2c; Figure 2e). The inconsistent detection of
158 *Th* and eGFP in other E15.5 clusters likely reflects their low transcript abundance at this time
159 point, but sufficient expression of the eGFP reporter to permit FACS collection (Figure 2d). The
160 expression of DA neuron markers *Ddc* and *Slc18a2* correlate with *Th* expression, while *Slc6a3*
161 expression is more spatially and temporally restricted (Figure 2c).

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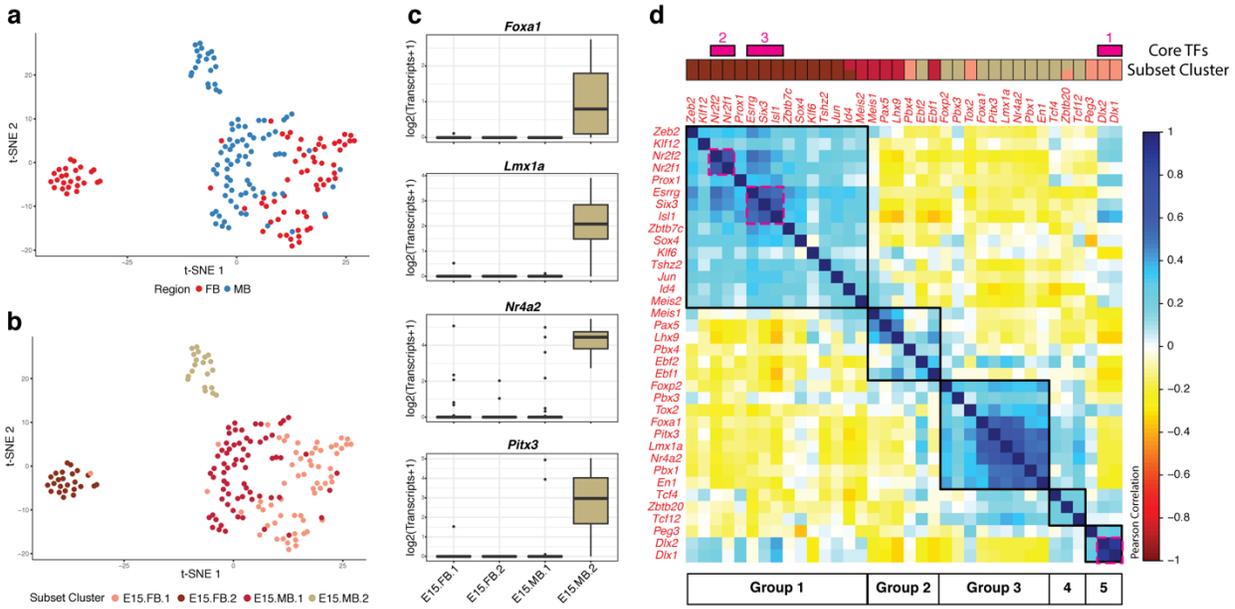
163 Multiple studies have demonstrated that *Th*-expressing neurons may also express markers
164 characteristic of other major neuronal subtypes¹³⁻¹⁵. Consequently, we evaluated expression of
165 canonical markers of other neuronal subtypes in our DA neuron subpopulations. We found co-
166 expression of *Th* with GABAergic (*Gad1/Gad2/Slc32a1*) or glutamatergic (*Slc17a6*) markers in
167 11/13 subset clusters (Figure 2c). The notable exception being two P7 MB DA neuron clusters
168 (MB3 and MB4), which exclusively expressed DA markers (Figure 2c).

169

170 *Analysis of E15.5 cells reveal regionally specialized maturing neurons*

171 Recursive analysis of E15.5 DA neuron regions revealed two distinct populations in both the MB
172 and FB. When analyzed collectively, we observe a major cluster, consisting of both MB and FB
173 cells and two smaller clusters comprising solely MB or FB cells (Figure 3a). The discrete E15.5
174 MB cluster (E15.MB.2; Figure 3b) was highlighted by specific expression of genes known to
175 mark mature MB DA neurons and have roles in MB DA neuron function (*Foxa1*, *Lmx1a*, *Pitx3*,
176 and *Nr4a2*)¹⁶ (Figure 3c; Table S2), suggesting that E15.MB.2 represents a post-mitotic,
177 maturing DA neuron population. By contrast, E15.MB.1 neurons preferentially express genes
178 including *Meis2*, *Lhx9*, *Id4*, *Ebfl*, *Pax5*, *Ephb1*, *Mir124-2hg*, and *Nrg1* (Table S2). All have
179 established roles in neuronal precursors or neuronal differentiation/maturation¹⁷⁻²⁸. Further,
180 E15.MB.1 expresses *Slc17a6*, which along with *Meis2* and *Lhx9*, were recently used to identify
181 embryonic DA neuroblasts²⁹ (Data accessed: 02/26/17; Table S11). Collectively these data
182 support E15.MB.1 as a presumptive DA precursor population.

183



184
 185 **Figure 3. Analysis of E15.5 cells reveals clusters of neurons at different stages of maturation that may be**
 186 **defined by co-correlated transcription factor programs.** a) A t-SNE plot of all neurons collected at E15.5 using
 187 high variance genes colored by regional identity. The t-SNE plot shows two distinct clusters containing only cells
 188 from one region and one cluster that contains a combination of both regions. b) The same t-SNE plot seen in Figure
 189 3a, but colored by subset cluster identity. This plot shows that iterative analysis defines two clusters in both E15.5
 190 MB and E15.5 FB. c) Boxplots showing log₂ expression in all E15.5 clusters of four genes identified as being
 191 specifically expressed in the E15.MB.2 cluster. The expression of these genes (*Foxa1*, *Nr4a2*, *Lmx1a*, and *Pitx3*) as
 192 well as others indicate that cells belonging to the E15.MB.2 cluster are post-mitotic, maturing MB DA neurons. d) A
 193 correlation plot displaying the Pearson correlation between transcription factors (TFs) that were identified as being
 194 specifically expressed in any of the four E15.5 clusters identified through iterative analysis. Above the plot is a bar
 195 labeled “Subset Cluster” indicating (by color) the cluster or clusters in which each gene was determined to be
 196 specific. Five clusters (indicated by black boxes) are identified through hierarchical clustering that primarily groups
 197 TFs by which cluster they were identified in. Sets of “Core TFs” were identified in three groups through the use of
 198 bootstrap resampling of hierarchical clustering (see Methods). Sets of core TFs are indicated by pink boxes (3)
 199 within the plot and pink blocks above the plot labeled “Core TFs.”

200
 201
 202 The markers identified for the discrete E15.FB.2 cluster, including *Six3* and *Six3os1*, are
 203 consistent with more mature FB/hypothalamic neurons^{30–33}. This observation is supported by
 204 E15.5.FB.2 expression of *Sst* and *Npy*; both of which encode hormones indicative of specified,
 205 post-mitotic neurons³⁴. E15.FB.1 clusters with E15.MB.1 (Figure 3b) potentially suggesting that
 206 it also represents an immature neuronal population. Indeed, the most specific marker for this
 207 population *Rnd3*, has been implicated in limiting the number of divisions of newly-fated neurons
 208 and in migration^{35,36} (Table S2).

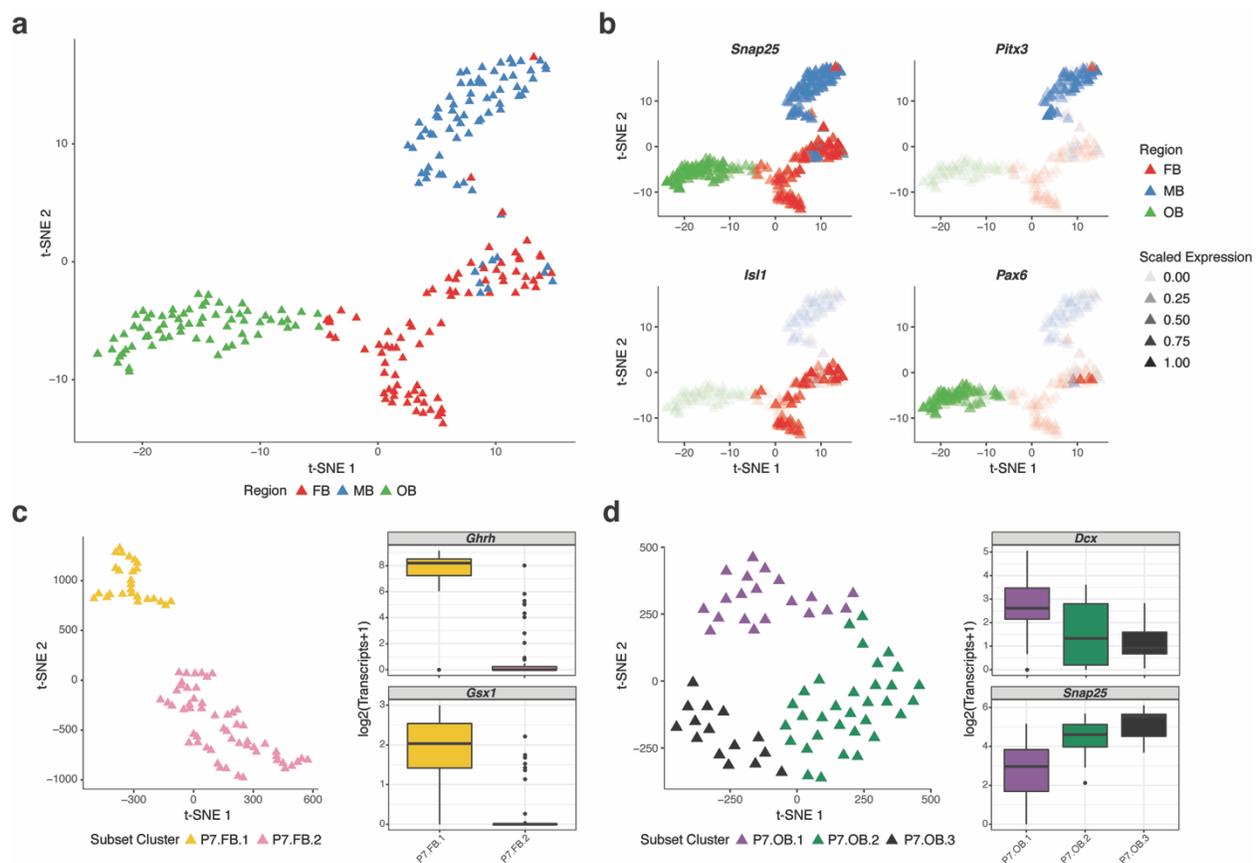
209
 210 We further identified marker genes that are transcription factors (TFs) in order to define
 211 networks of TFs associated with these populations. Expression of these marker genes were
 212 correlated and hierarchical clustering was used to reveal five groups of TFs primarily defining

213 the different E15.5 neuron populations (Figure 3d). Core groups of TFs were also identified
 214 within two of five groups (pink outline; Figure 3d; See Methods). Two sets of core TFs were
 215 identified within “Group 1” which was primarily composed of mature FB cluster (E15.FB.2)
 216 specific genes. Both core TF sets (2 and 3) contain genes that have been previously implicated in
 217 FB neuron development^{30–32,37,38} while potentially implicating a new TF in *Esrrg*. Group 5,
 218 which defined the immature E15.5 FB cluster (E15.FB.1) contained one core TF set (set #1)
 219 containing *Dlx1* and *Dlx2*, both of which have established roles in early FB neuronal
 220 development³⁹.

221

222 *P7 neurons display regionally discrete transcriptional signatures*

223 In contrast to E15.5, DA neurons isolated at P7 mostly cluster by anatomical region (Figure 4a).
 224 We sought to identify genes displaying region-dependent expression, identifying 54, 14 and 85
 225 genes that defined OB, FB and MB DA neurons, respectively (Table S2).
 226



227

228 **Figure 4. P7 *Th*⁺ neurons cluster by neuroanatomical domain, expressing markers characteristic of those**
 229 **regions, and further revealing regional subclusters based on iterative marker gene analyses.** a) A t-SNE plot of
 230 all P7 neurons collected using high BCV genes, colored by regional identity. The neurons mostly cluster by regional
 231 identity. b) t-SNE plots showing expression scaled to max gene expression observed of a pan-neuronal gene
 232 (*Snap25*) and an example of a marker gene identified for each region at P7 (MB: *Pitx3*, FB: *Isf1*, OB: *Pax6*) in all P7
 233 cells. c) t-SNE plot of P7 FB neurons plotted by using high BCV genes and colored by subcluster identity showing
 234 P7 FB neurons clustering into two distinct populations identified by ADPclust. Marker genes for the P7.FB.1 cluster

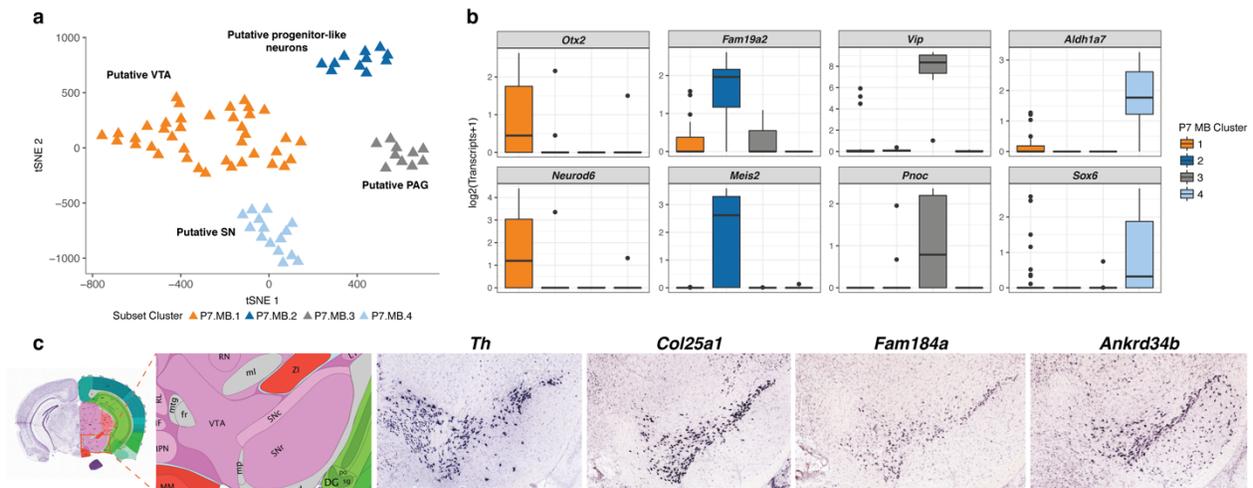
235 include *Ghrh* and *Gsx1*. Both of these genes as well as others indicate that this population of cells are
236 tuberofundibular dopaminergic neurons located in the arcuate nucleus. d) A t-SNE plot of P7 OB neurons plotted by
237 using high BCV genes and colored by subcluster identity. The plot shows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

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243
244 The FB-restricted genes include markers associated with hypothalamic development and
245 function e.g. *Isl1*³⁸ and *Asb4*⁴⁰ (Figure 4b; Table S2). Analyzing P7 FB *Th*⁺ neurons alone
246 revealed two distinct cell clusters (Figure 4c). P7.FB.1 specifically expressed the neuropeptides
247 *Gal* and *Ghrh* and the *Gsx1* transcription factor (Figure 4c; Table S2). All three play roles in
248 arcuate nucleus neurons⁴¹⁻⁴³ and were markers for a recently described *Th*⁺/*Ghrh*⁺/*Gal*⁺
249 hypothalamic population⁴⁴. By contrast, marker genes for P7.FB.2 did not reveal a signature or
250 gene expression profile consistent with a known cellular phenotype (Table S2)^{44,45}. However,
251 several other arcuate nucleus markers for *Th*⁺/*Ghrh*⁻ neuronal populations were expressed in
252 subsets of P7.FB.2 cells, including *Onecut2*, *Arx*, *Prhr*, *Slc6a3*, and *Sst* (Figure S4a)⁴⁴. Thus,
253 some *Th*⁺ populations detected in other scRNA-seq analyses may be present within our data, but
254 likely in insufficient numbers to facilitate classification here.

255
256 Of the genes whose expression defined OB *Th*⁺ cells, many have established roles in the
257 development or survival of OB DA neurons⁴⁶⁻⁵¹ (Table S2). Recursive analysis revealed three
258 subset clusters in P7 OB (Figure 4d). In identifying marker genes for P7 OB subset clusters, we
259 observed that P7.OB.1 expressed *Dcx* at significantly greater levels than P7.OB.2/P7.OB.3 and
260 that *Dcx* levels decrease along a continuum towards the lowest expression in P7 OB3 (Figure
261 4d). *Dcx* expression diminishes with neuronal maturation with the lowest expression in adult
262 neurons⁵². Consistent with this observation expression of the mature neuronal marker *Snap25* is
263 anticorrelated with *Dcx* (Figure 4d), suggesting a progression in maturation from P7.OB.1 to
264 P7.OB.3. This too is corroborated by concomitant increase in expression of DA neuron markers
265 and OB DA neuron fate specification genes (Figure S4b)^{53,54}.

266
267 Many genes that define eGFP⁺ MB neurons, including *Pitx3* (Figure 4b), have established roles
268 in MB DA neuron development and biology¹⁶. We identified four P7 MB DA subset clusters
269 within P7 MB DA neurons (Figure 5a). Marker gene analysis (Table S2) confirmed that three of
270 the clusters correspond to DA neurons from the VTA (*Otx2* and *Neurod6*; P7.MB.1)^{55,56}, the
271 PAG (*Vip* and *Pnoc*; P7.MB.3)^{57,58}, and the SN (*Sox6* and *Aldh1a7*; P7.MB.4)^{55,59} (Figure 5b).
272 These data are consistent with recent scRNA-seq studies of similar populations^{29,60}. We further
273 identify an as-yet undescribed population (P7.MB.2; Figure 5a) of *Th*⁺ DA neurons. This
274 population of cells display an expression signature consistent with a neuronal progenitor cell
275 population. This postnatal population shares many markers with the progenitor-like E15.MB.1
276 cluster including *Fam19a2* and *Meis2* (Table S2; Figure 5b). Furthermore, P7.MB.2 markers

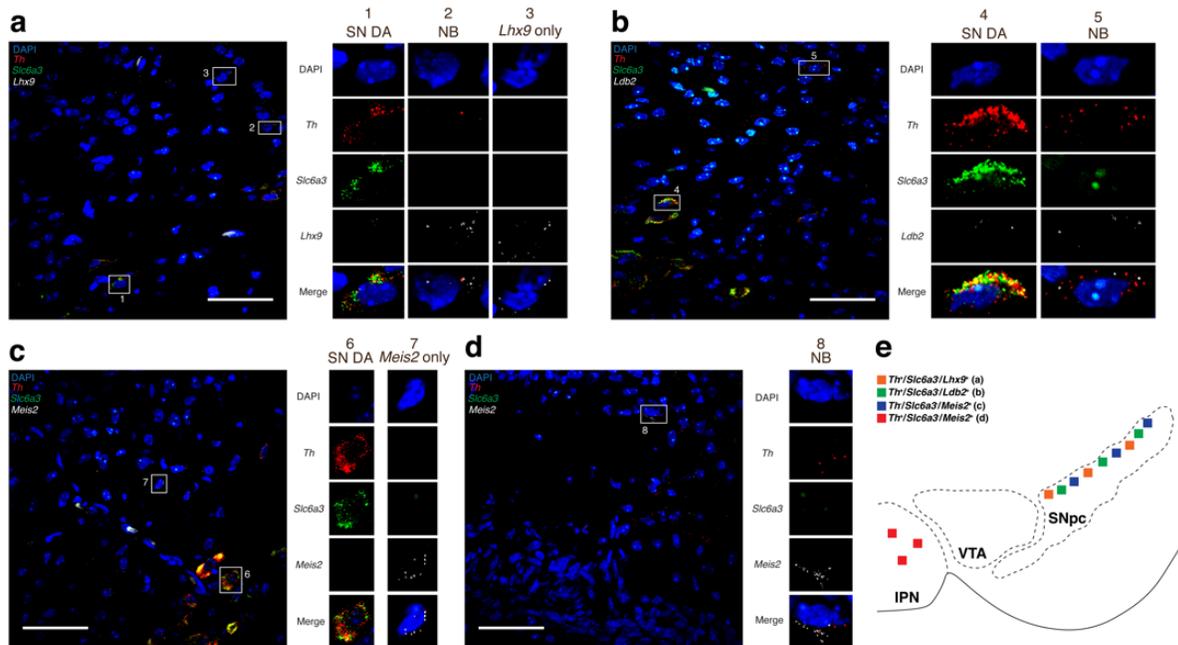
277 *Meis2*, *Lhx9*, and *Ldb2* have been shown to mark embryonic mouse neuroblast populations²⁹.
 278 Interestingly, this P7.MB.2 population clusters with P7 FB neurons in t-SNE space (Figure 2a;
 279 Figure 2b; Figure 4a). This may be driven by lower expression of key MB DA neuron genes
 280 compared to the other P7 MB clusters, resulting in a signature more similar to both P7 FB
 281 clusters (Figure 2c).
 282



283
 284 **Figure 5. Identification of four distinct clusters of P7 MB Th^+ neurons based upon expression of established**
 285 **marker genes leads to the discovery of novel *substantia nigra* (SN) marker genes.** a) t-SNE plot of P7 MB
 286 neurons colored by subcluster identity. P7 MB neurons separate into four clusters of which three can be defined
 287 using marker genes of known Th^+ populations: the ventral tegmental area (VTA), the *substantia nigra* (SN), and the
 288 periaqueductal grey area (PAG). The P7.MB.2 cluster was identified as a progenitor-like neuron primarily due to the
 289 overlap of specific markers with the immature E15.MB.1 cluster. b) Boxplots showing the expression of cluster
 290 specific genes used for identification of cluster populations. *Otx2* and *Neurod6* are established markers of VTA DA
 291 neurons. *Vip* and *Pnoc* are established markers of PAG neurons. *Aldh1a7* and *Sox6* are established markers of SN
 292 DA neurons. *Fam19a2* and *Meis2* are markers for P7.MB.2 that are shared with the E15.MB.1 cluster (See Table
 293 S2). c) Confirmation of novel SN DA neurons through the use of Allen Brain Atlas (ABA) *in situ* hybridization data
 294 (<http://www.brain-map.org/>). Coronal, P56 mouse *in situ* hybridization data was explored in order to confirm the
 295 expression of 37 novel SN markers. *Th* expression in P56 mice was used as an anatomical reference during analysis.
 296 Overall, 15/37 novel SN markers are confirmed to be expressed in the SN in adult mice, including *Col25a1*,
 297 *Fam184a*, and *Ankrd34b*.
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300 We sought to ascertain the spatial distribution of P7.MB.2 DA neurons through multiplex, single
 301 molecule fluorescence *in situ* hybridization (smFISH) for *Th* (pan-P7 MB DA neurons), *Slc6a3*
 302 (P7.MB.1, P7.MB.3, P7.MB.4), and one of the marker genes identified through our analysis,
 303 either *Lhx9/Ldb2/Meis2* (P7.MB.2) (Figure 6). The ventral MB was scanned in each experiment
 304 for cells that were $Th^+/Slc6a3^-$ and positive for the third gene. $Th^+/Slc6a3^-/Lhx9^+$ cells were
 305 found scattered in the dorsal SN *pars compacta* (SNpc) along with cells expressing *Lhx9^+* alone
 306 (Figure 6a, 6e). Expression of *Ldb2* was found to follow a similar pattern to *Lhx9*, with
 307 $Th^+/Slc6a3^-/Ldb2^+$ cells also found in the dorsal SNpc (Figure 6b, 6e). In the SNpc, *Meis2^+* cells
 308 were common, however they did not display co-expression of *Th* (Figure 6d). Cells that were

309 $Th^+/Slc6a3^-/Meis2^+$ were found in the interpeduncular nucleus (IPN) of the ventral MB (Figure
 310 6d, 6e). Neither $Lhx9$ nor $Ldb2$ were detected in the IPN (data not shown). Expression of $Lhx9$,
 311 $Ldb2$, and $Meis2$ was low or non-existent in $Th^+/Slc6a3^+$ cells in the SNpc (Figure 6a, 6b, 6c).
 312 Importantly, cells expressing these markers express Th at lower levels than $Th^+/Slc6a3^+$ neurons
 313 (Figure 6), consistent with our scRNA-seq data (Figure 2c).
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 316 **Figure 6. Single molecule fluorescent in situ hybridization (smFISH) validates the existence of a neuroblast-**
 317 **like P7.MB.2 cell population.** Representative images of smFISH experiments analyzing four MB sections for each
 318 probe combination. a) smFISH performed with probes for Th (red), $Slc6a3$ (green), and $Lhx9$ (white). smFISH in the
 319 ventral MB reveals the presence of $Th^+/Slc6a3^-/Lhx9^+$ in the dorsal portion of the *substantia nigra* (SN) (inset 2), the
 320 presence of $Lhx9^+$ only cells in the dorsal SN (inset 3), and the lack of expression of $Lhx9$ in SN DA neurons (inset
 321 1). Scale bar, 50 μ M. b) smFISH performed with probes for Th (red), $Slc6a3$ (green), and $Ldb2$ (white). smFISH in
 322 the ventral MB reveals the presence of $Th^+/Slc6a3^-/Ldb2^+$ in the dorsal portion of the SN (inset 5) with little to no
 323 expression of $Ldb2$ in SN DA neurons (inset 1). Scale bar, 50 μ M. c) smFISH performed with probes for Th (red),
 324 $Slc6a3$ (green), and $Meis2$ (white). smFISH in the ventral MB reveals the presence of $Meis2^+$ in the dorsal portion of
 325 the SN (inset 7) with little to no expression of $Meis2$ in SN DA neurons (inset 6). Scale bar, 50 μ M. d) smFISH
 326 performed with probes for Th (red), $Slc6a3$ (green), and $Meis2$ (white). smFISH in the ventral MB reveals the
 327 presence of $Th^+/Slc6a3^-/Meis2^+$ in the interpeduncular nucleus (IPN) (inset 8). Scale bar, 50 μ M. e) Diagram
 328 summarizing locations of the P7 MB2 cells validated through smFISH. All images were taken at 60x magnification
 329 and processed using ImageJ (see Methods). NB, neuroblast; VTA, ventral tegmental area; SNpc, *substantia nigra*
 330 *pars compacta*; IPN, interpeduncular nucleus.

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 333 Furthermore, regional and subset cluster marker gene correlation analysis revealed four groups
 334 of TFs through hierarchical clustering with three groups clearly demarcating regions at P7
 335 (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

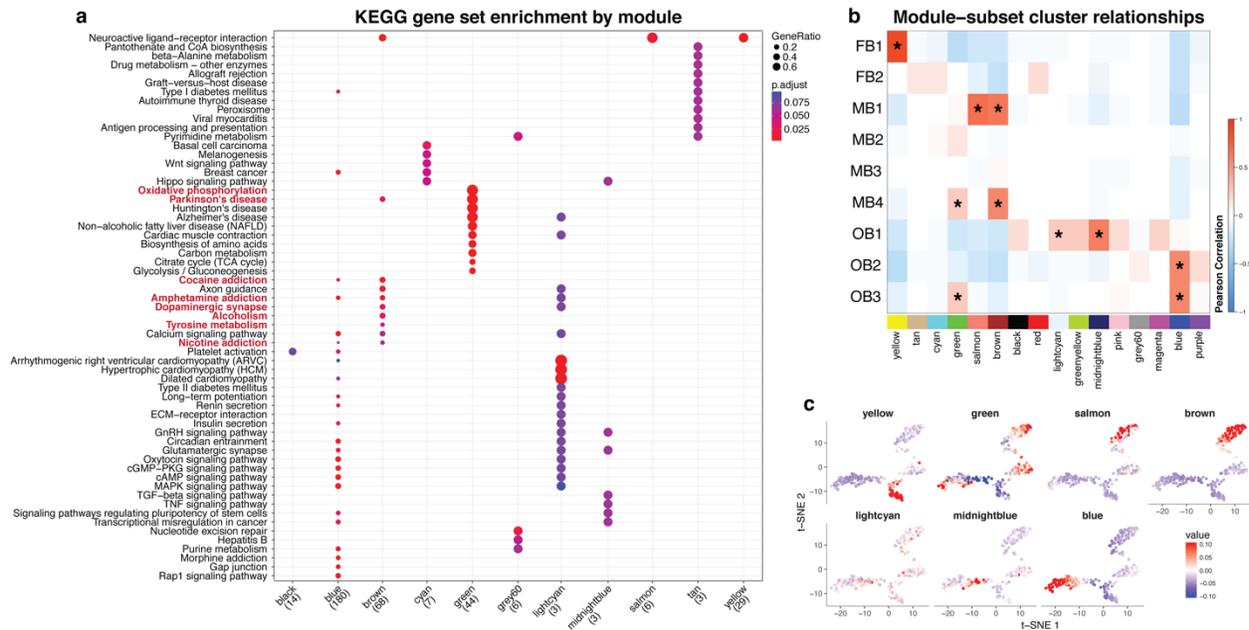
346 Motivated by the clinical relevance of SN DA neurons to PD, we set out to understand what
347 makes them transcriptionally distinct from other MB DA neurons. We postulated that genes with
348 specific expression in the P7 SN DA neuron cluster data might illuminate their preferential
349 vulnerability 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 sets⁶².
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).

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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 $p < 3.5e-04$) 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 TADs⁶³. 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).

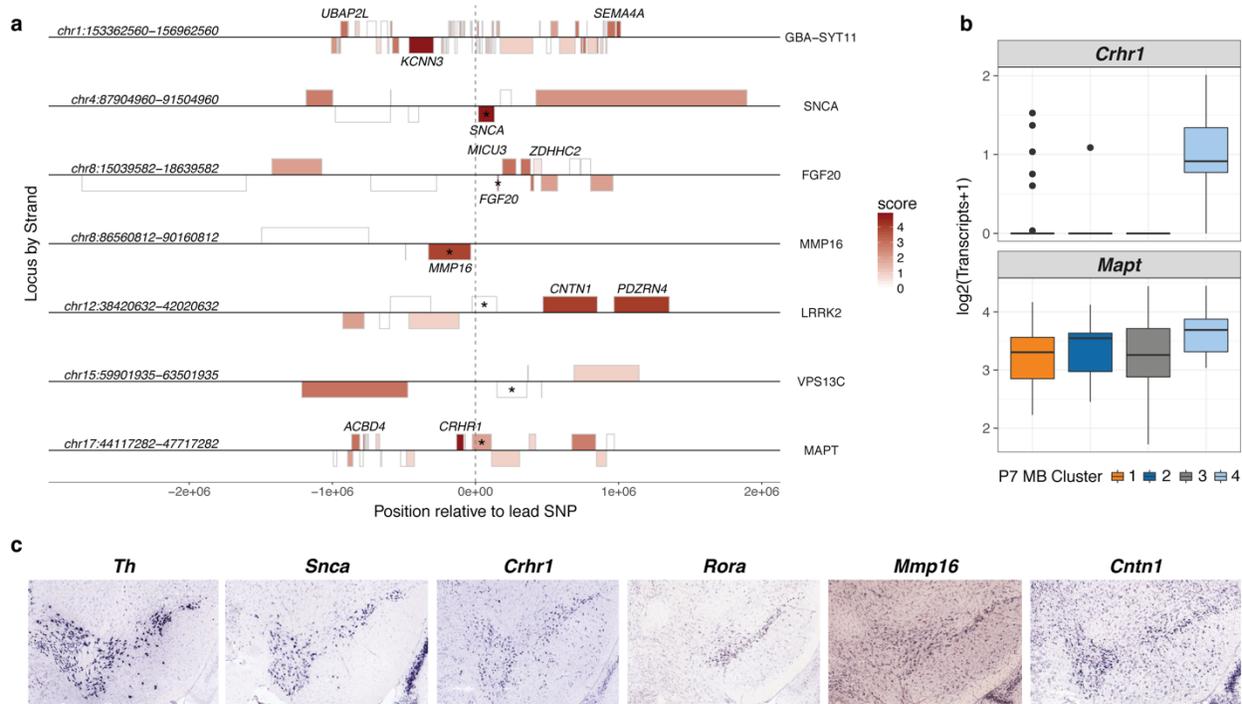
432

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 supported⁶⁶⁻⁷¹.

445 Furthermore, both *Mmp16* and *Rora* are detected in adult mouse SN (Figure 8c, ABA, Table
 446 S14).
 447



448
 449
 450 **Figure 8. Potential candidate genes in PD GWAS loci can be identified and prioritized through the use of**
 451 **scRNA-seq data.** a) A plot displaying the four megabase regions in the human genome (hg38) centered on PD
 452 GWAS SNPs in seven loci. Genes within +/- 1 Mb from the lead SNP in those loci are displayed as boxes on their
 453 appropriate strand. Genes are shaded by their prioritization score which is based on five factors: whether the gene is
 454 expressed in the P7 *substantia nigra* population (P7.MB.4); whether the gene was found to be a specific marker
 455 gene for P7.MB.4; whether the gene is differentially expressed between P7 MB clusters; whether the gene is found
 456 in any of the PD-associated WGCNA modules (“green” and “brown”); the pLI score of the gene in the ExAC
 457 database. The gene or genes with the highest scores in a given locus are considered the best candidate genes. The
 458 genes that are closest to the lead SNP are marked by an asterisk. This analysis allowed for the prioritization of all 32
 459 PD GWAS loci in Nalls, et al⁸. b) Boxplots showing the expression of *Crhr1* and *Mapt* in identified P7 MB clusters.
 460 *Crhr1* shows specific expression in P7 SN *Th*⁺ neurons while *Mapt* is expressed at equal levels in all P7 MB
 461 clusters. This specific expression of *Crhr1* but not *Mapt* along with *CRHR1* being identified as the highest scoring
 462 gene in the MAPT locus may help to explain why variation in this locus leads to preferential degeneration of SN *Th*⁺
 463 neurons in PD. c) *In situ* hybridization data from the Allen Brain Atlas (ABA) for some of the highest scoring genes
 464 in Figure 8a. The expression data for *Th* is used here for anatomical identification of the SN and for comparison
 465 purposes. As shown, the mouse homologs of high scoring genes in the *SNCA* (*Snca*), *MAPT* (*Crhr1*), *VPS13C*
 466 (*Rora*), *MMP16* (*Mmp16*), and *LRRK2* (*Cntn1*) loci show clear expression in SN DA neurons in P56 mice.

467
 468
 469 To further prioritize the remaining 28 loci, we scored on whether genes were differentially
 470 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 database⁷², due to a recent study using this metric to identify dosage sensitive genes⁷³. 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 (≤ 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 ago⁷⁴. 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 neurons²⁹. 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 VTA¹³

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 neuroinflammation⁷⁹. This finding potentially links SN DA specific gene
525 expression and protein secretion to the role of inflammation in PD⁸⁰ and the specific
526 vulnerability of SN DA neurons to degeneration caused by inflammation⁸¹.

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 timepoints²⁹, 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 immunostained⁸². These results have led to the suggestion that there is

551 a “phenotypic maturation” of MB DA neurons during the early postnatal time period⁸². 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 DNA⁸⁴. They are
558 enriched for characteristics denoting regulatory DNA^{84,85}, and have been shown to modulate
559 tissue-dependent elements⁸⁴⁻⁸⁷. 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 degeneration⁸⁸. Additionally, SNPs within *Fgf20* have been reported
576 to modulate PD risk⁸⁸. 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 *GCHI* 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 neurons⁹¹, 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 development⁹⁶. 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, 2012⁹⁷ 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 protocol⁹⁷, 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 μ L of media without serum
693 comprised of DMEM/F12 without phenol red, 5% trehalose (w/v), 25 μ M AP-V, 100 μ M
694 kynurenic acid, and 10 μ L of 40 U/ μ L RNase inhibitor (RNasin® Plus RNase Inhibitor, Promega)
695 at room temperature. The resuspended cells were then passed through a 40 μ M 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 μ L Smart-Seq2 lysis buffer + RNAase inhibitor, 1 μ L oligo-dT primer, and 1 μ L
701 dNTPs according to Picelli et al., 2014⁹⁸). 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 protocol⁹⁸. 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 10⁵ 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 Hisat2⁹⁹ with default parameters
723 except: -p 8. Aligned reads from individual samples were quantified against a reference
724 transcriptome¹⁰⁰ (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 cuffnorm¹⁰¹ 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 cuffquant¹⁰¹ and the normalized FPKM matrix from cuffnorm was used as input for
733 the Monocle2 single cell RNA-seq framework¹⁰² in R/Bioconductor¹⁰³. Genes were annotated
734 using the Gencode vM8 release¹⁰⁰. 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 algorithm¹⁰ 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 ($410 \times 0.05 = 20.5$) 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 ($80 \times 0.05 = 4$). 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" $\geq 1.5 * \text{dispersion_fit}$ or $2.0 * \text{dispersion_fit}$ 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 cummerbund¹⁰⁵ was used similar to what was described in Kelly et. al¹⁰⁶.

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 al¹⁰⁷.

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 \geq
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 Database¹⁰⁸ (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 $\log_2(\text{RPC} + 1)$ 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 $\log_2(\text{RPC} + 1)$ 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 settings⁶².
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 $\log_2(\text{Transcripts} + 1)$. 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 package¹¹⁴ 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 package⁶². 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 data¹¹⁵ 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 database⁷², 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 downloaded: 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-](http://mouse.brain-map.org/static/atlas)
953 [map.org/static/atlas](http://mouse.brain-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 μ M, a pixel dwell of 28.8 μ s, 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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1027

1028 **AUTHOR CONTRIBUTIONS**

1029 PWH, ASM, and LAG designed the study and wrote the paper. PWH, SAM, WDL and GAC
1030 performed the experiments. PWH and LAG implemented the computational algorithms to
1031 process the raw data and conduct analyses thereof. PWH, LAG, and ASM analyzed and
1032 interpreted the resulting data. LAG contributed novel computational pipeline development.
1033 Correspondence to ASM (andy@jhmi.edu) and LAG (loyalgoff@jhmi.edu).

1034

1035 **FINANCIAL INTERESTS STATEMENT**

1036 The authors declare no competing financial interests.

1037 **REFERENCES**

1038

- 1039 1. de Rijk, M. C. *et al.* Prevalence of parkinsonism and Parkinson's disease in Europe: the
1040 EUROPARKINSON Collaborative Study. European Community Concerted Action on the
1041 Epidemiology of Parkinson's disease. *J Neurol Neurosurg Psychiatry* **62**, 10–15 (1997).
1042 2. Pringsheim, T., Jette, N., Frolkis, A. & Steeves, T. D. The prevalence of Parkinson's
1043 disease: a systematic review and meta-analysis. *Mov Disord* **29**, 1583–1590 (2014).
1044 3. Savitt, J. M., Dawson, V. L. & Dawson, T. M. Diagnosis and treatment of Parkinson
1045 disease: molecules to medicine. *J Clin Invest* **116**, 1744–1754 (2006).
1046 4. Paulus, W. & Jellinger, K. The neuropathologic basis of different clinical subgroups of
1047 Parkinson's disease. *J Neuropathol Exp Neurol* **50**, 743–755 (1991).
1048 5. Brichta, L. *et al.* Identification of neurodegenerative factors using translome-regulatory
1049 network analysis. *Nat. Neurosci.* **18**, 1325–33 (2015).
1050 6. Puschmann, A. Monogenic Parkinson's disease and parkinsonism: clinical phenotypes and
1051 frequencies of known mutations. *Park. Relat Disord* **19**, 407–415 (2013).
1052 7. Klein, C. & Westenberger, A. Genetics of Parkinson's disease. *Cold Spring Harb*
1053 *Perspect Med* **2**, a008888 (2012).
1054 8. Nalls, M. a *et al.* Large-scale meta-analysis of genome-wide association data identifies six
1055 new risk loci for Parkinson's disease. *Nat. Genet.* **56**, 1–7 (2014).
1056 9. Barallobre, M. J. *et al.* DYRK1A promotes dopaminergic neuron survival in the
1057 developing brain and in a mouse model of Parkinson's disease. *Cell Death Dis.* **5**, e1289
1058 (2014).
1059 10. Qiu, X. *et al.* Single-cell mRNA quantification and differential analysis with Census. *Nat*
1060 *Methods* **14**, 309–315 (2017).
1061 11. Qiu, X. *et al.* Reversed graph embedding resolves complex single-cell developmental
1062 trajectories. *bioRxiv* (2017). doi:10.1101/110668
1063 12. Van Der Maaten, L. & Hinton, G. Visualizing Data using t-SNE. *J. Mach. Learn. Res.* **9**,
1064 2579–2605 (2008).
1065 13. Morales, M. & Margolis, E. B. Ventral tegmental area: cellular heterogeneity,
1066 connectivity and behaviour. *Nat Rev Neurosci* **18**, 73–85 (2017).
1067 14. Everitt, B. J., Hökfelt, T., Wu, J. Y. & Goldstein, M. Coexistence of tyrosine hydroxylase-
1068 like and gamma-aminobutyric acid-like immunoreactivities in neurons of the arcuate
1069 nucleus. *Neuroendocrinology* **39**, 189–191 (1984).
1070 15. Asmus, S. E. *et al.* Increasing proportions of tyrosine hydroxylase-immunoreactive
1071 interneurons colocalize with choline acetyltransferase or vasoactive intestinal peptide in
1072 the developing rat cerebral cortex. *Brain Res* **1383**, 108–119 (2011).
1073 16. Arenas, E., Denham, M. & Villaescusa, J. C. How to make a midbrain dopaminergic
1074 neuron. *Development* **142**, 1918–36 (2015).
1075 17. Hou, P. S. *et al.* LHX2 regulates the neural differentiation of human embryonic stem cells
1076 via transcriptional modulation of PAX6 and CER1. *Nucleic Acids Res.* **41**, 7753–7770
1077 (2013).
1078 18. Agoston, Z. *et al.* Meis2 is a Pax6 co-factor in neurogenesis and dopaminergic
1079 periglomerular fate specification in the adult olfactory bulb. *Development* **141**, 28–38
1080 (2014).
1081 19. Agoston, Z. & Schulte, D. Meis2 competes with the Groucho co-repressor Tle4 for
1082 binding to Otx2 and specifies tectal fate without induction of a secondary midbrain-

- 1083 hindbrain boundary organizer. *Development* **136**, 3311–3322 (2009).
- 1084 20. Rétaux, S., Rogard, M., Bach, I., Failli, V. & Besson, M. J. Lhx9: a novel LIM-
1085 homeodomain gene expressed in the developing forebrain. *J. Neurosci.* **19**, 783–793
1086 (1999).
- 1087 21. Hoekstra, E. J. *et al.* Lmx1a Encodes a Rostral Set of Mesodiencephalic Dopaminergic
1088 Neurons Marked by the Wnt/B-Catenin Signaling Activator R-spondin 2. *PLoS One* **8**, 1–
1089 12 (2013).
- 1090 22. Peukert, D., Weber, S., Lumsden, A. & Scholpp, S. Lhx2 and Lhx9 determine neuronal
1091 differentiation and compartment in the caudal forebrain by regulating Wnt signaling. *PLoS*
1092 *Biol.* **9**, (2011).
- 1093 23. Yun, K., Mantani, A., Garel, S., Rubenstein, J. & Israel, M. a. Id4 regulates neural
1094 progenitor proliferation and differentiation in vivo. *Development* **131**, 5441–5448 (2004).
- 1095 24. Bedford, L. *et al.* Id4 is required for the correct timing of neural differentiation. *Dev. Biol.*
1096 **280**, 386–395 (2005).
- 1097 25. Yin, M. *et al.* Ventral mesencephalon-enriched genes that regulate the development of
1098 dopaminergic neurons in vivo. *J. Neurosci.* **29**, 5170–82 (2009).
- 1099 26. Hegarty, S. V., Sullivan, A. M. & O’Keeffe, G. W. Midbrain dopaminergic neurons: A
1100 review of the molecular circuitry that regulates their development. *Developmental Biology*
1101 **379**, 123–138 (2013).
- 1102 27. Makeyev, E. V., Zhang, J., Carrasco, M. A. & Maniatis, T. The MicroRNA miR-124
1103 Promotes Neuronal Differentiation by Triggering Brain-Specific Alternative Pre-mRNA
1104 Splicing. *Mol. Cell* **27**, 435–448 (2007).
- 1105 28. Mei, L. & Xiong, W. C. Neuregulin 1 in neural development, synaptic plasticity and
1106 schizophrenia. *Nat Rev Neurosci* **9**, 437–452 (2008).
- 1107 29. La Manno, G. *et al.* Molecular Diversity of Midbrain Development in Mouse, Human, and
1108 Stem Cells. *Cell* **167**, 566–580.e19 (2016).
- 1109 30. Gestri, G. *et al.* Six3 functions in anterior neural plate specification by promoting cell
1110 proliferation and inhibiting Bmp4 expression. *Development* **132**, 2401–2413 (2005).
- 1111 31. Lagutin, O. V *et al.* Six3 repression of Wnt signaling in the anterior neuroectoderm is
1112 essential for vertebrate forebrain development. *Genes Dev* **17**, 368–379 (2003).
- 1113 32. Lavado, A., Lagutin, O. V & Oliver, G. Six3 inactivation causes progressive caudalization
1114 and aberrant patterning of the mammalian diencephalon. *Development* **135**, 441–450
1115 (2008).
- 1116 33. Geng, X., Lavado, A., Lagutin, O. V., Liu, W. & Oliver, G. Expression of Six3 Opposite
1117 Strand (Six3OS) during mouse embryonic development. *Gene Expr. Patterns* **7**, 252–257
1118 (2007).
- 1119 34. Kelsom, C. & Lu, W. Development and specification of GABAergic cortical interneurons.
1120 *Cell Biosci* **3**, 19 (2013).
- 1121 35. Pacary, E. *et al.* Proneural transcription factors regulate different steps of cortical neuron
1122 migration through Rnd-mediated inhibition of RhoA signaling. *Neuron* **69**, 1069–1084
1123 (2011).
- 1124 36. Pacary, E., Azzarelli, R. & Guillemot, F. Rnd3 coordinates early steps of cortical
1125 neurogenesis through actin-dependent and -independent mechanisms. *Nat Commun* **4**,
1126 1635 (2013).
- 1127 37. Naka, H., Nakamura, S., Shimazaki, T. & Okano, H. Requirement for COUP-TFI and II in
1128 the temporal specification of neural stem cells in CNS development. *Nat Neurosci* **11**,

- 1129 1014–1023 (2008).
- 1130 38. Lee, B., Lee, S., Lee, S.-K. & Lee, J. W. The LIM-homeobox transcription factor Isl1
1131 plays critical roles in development of multiple arcuate nucleus neurons. *Development*
1132 (2016). doi:10.1242/dev.133967
- 1133 39. Petryniak, M. A., Potter, G. B., Rowitch, D. H. & Rubenstein, J. L. Dlx1 and Dlx2 control
1134 neuronal versus oligodendroglial cell fate acquisition in the developing forebrain. *Neuron*
1135 **55**, 417–433 (2007).
- 1136 40. Li, J. Y. *et al.* Arcuate nucleus transcriptome profiling identifies ankyrin repeat and
1137 suppressor of cytokine signalling box-containing protein 4 as a gene regulated by fasting
1138 in central nervous system feeding circuits. *J. Neuroendocrinol.* **17**, 394–404 (2005).
- 1139 41. Björklund, A. & Dunnett, S. B. Dopamine neuron systems in the brain: an update. *Trends*
1140 *Neurosci* **30**, 194–202 (2007).
- 1141 42. Li, H., Zeitler, P. S., Valerius, M. T., Small, K. & Potter, S. S. Gsh-1, an orphan Hox
1142 gene, is required for normal pituitary development. *EMBO J* **15**, 714–724 (1996).
- 1143 43. McNay, D. E., Pelling, M., Claxton, S., Guillemot, F. & Ang, S. L. Mash1 is required for
1144 generic and subtype differentiation of hypothalamic neuroendocrine cells. *Mol Endocrinol*
1145 **20**, 1623–1632 (2006).
- 1146 44. Campbell, J. N. *et al.* A molecular census of arcuate hypothalamus and median eminence
1147 cell types. *Nat Neurosci* **20**, 484–496 (2017).
- 1148 45. Romanov, R. A. *et al.* Molecular interrogation of hypothalamic organization reveals
1149 distinct dopamine neuronal subtypes. *Nat Neurosci* **20**, 176–188 (2017).
- 1150 46. Kohwi, M., Osumi, N., Rubenstein, J. L. & Alvarez-Buylla, A. Pax6 is required for
1151 making specific subpopulations of granule and periglomerular neurons in the olfactory
1152 bulb. *J. Neurosci.* **25**, 6997–7003 (2005).
- 1153 47. Hack, M. a *et al.* Neuronal fate determinants of adult olfactory bulb neurogenesis. *Nat.*
1154 *Neurosci.* **8**, 865–872 (2005).
- 1155 48. Ninkovic, J. *et al.* The transcription factor Pax6 regulates survival of dopaminergic
1156 olfactory bulb neurons via crystallin ??A. *Neuron* **68**, 682–694 (2010).
- 1157 49. Waclaw, R. R. *et al.* The zinc finger transcription factor Sp8 regulates the generation and
1158 diversity of olfactory bulb interneurons. *Neuron* **49**, 503–516 (2006).
- 1159 50. Miller, T. E. *et al.* Lgr5 marks post-mitotic, lineage restricted cerebellar granule neurons
1160 during postnatal development. *PLoS One* **9**, (2014).
- 1161 51. Chen, M. *et al.* Wnt-responsive Lgr5⁺ globose basal cells function as multipotent olfactory
1162 epithelium progenitor cells. *J. Neurosci.* **34**, 8268–76 (2014).
- 1163 52. Francis, F. *et al.* Doublecortin is a developmentally regulated, microtubule-associated
1164 protein expressed in migrating and differentiating neurons. *Neuron* **23**, 247–256 (1999).
- 1165 53. Agoston, Z. *et al.* Meis2 is a Pax6 co-factor in neurogenesis and dopaminergic
1166 periglomerular fate specification in the adult olfactory bulb. *Development* **141**, 28–38
1167 (2014).
- 1168 54. Vergaño-Vera, E. *et al.* Nurr1 blocks the mitogenic effect of FGF-2 and EGF, inducing
1169 olfactory bulb neural stem cells to adopt dopaminergic and dopaminergic-GABAergic
1170 neuronal phenotypes. *Dev Neurobiol* **75**, 823–841 (2015).
- 1171 55. Panman, L. *et al.* Sox6 and Otx2 control the specification of substantia nigra and ventral
1172 tegmental area dopamine neurons. *Cell Rep.* **8**, 1018–1025 (2014).
- 1173 56. Viereckel, T. *et al.* Midbrain Gene Screening Identifies a New Mesoaccumbal
1174 Glutamatergic Pathway and a Marker for Dopamine Cells Neuroprotected in Parkinson's

- 1175 Disease. *Sci Rep* **6**, 35203 (2016).
- 1176 57. Kozicz, T., Vigh, S. & Arimura, A. The source of origin of PACAP- and VIP-
1177 immunoreactive fibers in the laterodorsal division of the bed nucleus of the stria terminalis
1178 in the rat. *Brain Res.* **810**, 211–219 (1998).
- 1179 58. Darland, T., Heinricher, M. M. & Grandy, D. K. Orphanin FQ/nociceptin: A role in pain
1180 and analgesia, but so much more. *Trends in Neurosciences* **21**, 215–221 (1998).
- 1181 59. Cai, H., Liu, G., Sun, L. & Ding, J. Aldehyde Dehydrogenase 1 making molecular inroads
1182 into the differential vulnerability of nigrostriatal dopaminergic neuron subtypes in
1183 Parkinson's disease. *Transl. Neurodegener.* **3**, 27 (2014).
- 1184 60. Poulin, J. F. *et al.* Defining midbrain dopaminergic neuron diversity by single-cell gene
1185 expression profiling. *Cell Rep* **9**, 930–943 (2014).
- 1186 61. Waldegger, S., Barth, P., Raber, G. & Lang, F. Cloning and characterization of a putative
1187 human serine/threonine protein kinase transcriptionally modified during anisotonic and
1188 isotonic alterations of cell volume. *Proc Natl Acad Sci U S A* **94**, 4440–4445 (1997).
- 1189 62. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing
1190 biological themes among gene clusters. *OMICS* **16**, 284–7 (2012).
- 1191 63. Krijger, P. H. *et al.* Cell-of-Origin-Specific 3D Genome Structure Acquired during
1192 Somatic Cell Reprogramming. *Cell Stem Cell* **18**, 597–610 (2016).
- 1193 64. Lettice, L. A. *et al.* A long-range Shh enhancer regulates expression in the developing
1194 limb and fin and is associated with preaxial polydactyly. *Hum. Mol. Genet.* **12**, 1725–1735
1195 (2003).
- 1196 65. Benko, S. *et al.* Highly conserved non-coding elements on either side of SOX9 associated
1197 with Pierre Robin sequence. *Nat. Genet.* **41**, 359–364 (2009).
- 1198 66. Yong, V. W., Power, C., Forsyth, P. & Edwards, D. R. Metalloproteinases in biology and
1199 pathology of the nervous system. *Nat Rev Neurosci* **2**, 502–511 (2001).
- 1200 67. Li, Z., Wu, Y. & Baraban, J. M. The Translin/Trax RNA binding complex: clues to
1201 function in the nervous system. *Biochim Biophys Acta* **1779**, 479–485 (2008).
- 1202 68. Broer, L. *et al.* Association of heat shock proteins with Parkinson's disease. *Eur J*
1203 *Epidemiol* **26**, 933–935 (2011).
- 1204 69. Zhu, Y., McAvoy, S., Kuhn, R. & Smith, D. I. RORA, a large common fragile site gene, is
1205 involved in cellular stress response. *Oncogene* **25**, 2901–2908 (2006).
- 1206 70. Boukhtouche, F. *et al.* Human retinoic acid receptor-related orphan receptor alpha1
1207 overexpression protects neurones against oxidative stress-induced apoptosis. *J Neurochem*
1208 **96**, 1778–1789 (2006).
- 1209 71. Hamilton, B. A. *et al.* Disruption of the nuclear hormone receptor RORalpha in staggerer
1210 mice. *Nature* **379**, 736–739 (1996).
- 1211 72. Lek, M. *et al.* Analysis of protein-coding genetic variation in 60,706 humans. *Nature* **536**,
1212 285–291 (2016).
- 1213 73. Doan, R. N. *et al.* Mutations in Human Accelerated Regions Disrupt Cognition and Social
1214 Behavior. *Cell* **167**, 341–354.e12 (2016).
- 1215 74. Parent, M. & Parent, A. Substantia nigra and Parkinson's disease: a brief history of their
1216 long and intimate relationship. *Can J Neurol Sci* **37**, 313–319 (2010).
- 1217 75. Liu, S., Plachez, C., Shao, Z., Puche, A. & Shipley, M. T. Olfactory bulb short axon cell
1218 release of GABA and dopamine produces a temporally biphasic inhibition-excitation
1219 response in external tufted cells. *J Neurosci* **33**, 2916–2926 (2013).
- 1220 76. Winklhofer, K. F. & Haass, C. Mitochondrial dysfunction in Parkinson's disease. *Biochim*

- 1221 *Biophys Acta* **1802**, 29–44 (2010).
- 1222 77. Perier, C. & Vila, M. Mitochondrial biology and Parkinson's disease. *Cold Spring Harb*
1223 *Perspect Med* **2**, a009332 (2012).
- 1224 78. Tom Tang, Y. *et al.* Tafa: a novel secreted family with conserved cysteine residues and
1225 restricted expression in the brain. *Genomics* **83**, 727–734 (2004).
- 1226 79. Wang, W. *et al.* FAM19A4 is a novel cytokine ligand of formyl peptide receptor 1 (FPR1)
1227 and is able to promote the migration and phagocytosis of macrophages. *Cell Mol Immunol*
1228 **12**, 615–624 (2015).
- 1229 80. Beal, M. F. Mitochondria, oxidative damage, and inflammation in Parkinson's disease.
1230 *Ann N Y Acad Sci* **991**, 120–131 (2003).
- 1231 81. Kim, W. G. *et al.* Regional difference in susceptibility to lipopolysaccharide-induced
1232 neurotoxicity in the rat brain: role of microglia. *J Neurosci* **20**, 6309–6316 (2000).
- 1233 82. Jackson-Lewis, V. *et al.* Developmental cell death in dopaminergic neurons of the
1234 substantia nigra of mice. *J Comp Neurol* **424**, 476–488 (2000).
- 1235 83. Lieb, K. *et al.* Pre- and postnatal development of dopaminergic neuron numbers in the
1236 male and female mouse midbrain. *Brain Res Dev Brain Res* **94**, 37–43 (1996).
- 1237 84. Maurano, M. T. *et al.* Systematic Localization of Common Disease-Associated Variation
1238 in Regulatory DNA. *Science (80-.)*. **337**, 1190–1195 (2012).
- 1239 85. Farh, K. K. *et al.* Genetic and epigenetic fine mapping of causal autoimmune disease
1240 variants. *Nature* **518**, 337–343 (2015).
- 1241 86. Emison, E. S. *et al.* A common sex-dependent mutation in a RET enhancer underlies
1242 Hirschsprung disease risk. *Nature* **434**, 857–863 (2005).
- 1243 87. Praetorius, C. *et al.* A polymorphism in IRF4 affects human pigmentation through a
1244 tyrosinase-dependent MITF/TFAP2A pathway. *Cell* **155**, 1022–1033 (2013).
- 1245 88. Itoh, N. & Ohta, H. Roles of FGF20 in dopaminergic neurons and Parkinson's disease.
1246 *Front Mol Neurosci* **6**, 15 (2013).
- 1247 89. Galter, D. *et al.* LRRK2 expression linked to dopamine-innervated areas. *Ann Neurol* **59**,
1248 714–719 (2006).
- 1249 90. Higashi, S. *et al.* Expression and localization of Parkinson's disease-associated leucine-
1250 rich repeat kinase 2 in the mouse brain. *J Neurochem* **100**, 368–381 (2007).
- 1251 91. Sauvage, M. & Steckler, T. Detection of corticotropin-releasing hormone receptor 1
1252 immunoreactivity in cholinergic, dopaminergic and noradrenergic neurons of the murine
1253 basal forebrain and brainstem nuclei - Potential implication for arousal and attention.
1254 *Neuroscience* **104**, 643–652 (2001).
- 1255 92. Abuirmeileh, A., Harkavyi, A., Lever, R., Biggs, C. S. & Whitton, P. S. Urocortin, a CRF-
1256 like peptide, restores key indicators of damage in the substantia nigra in a
1257 neuroinflammatory model of Parkinson's disease. *J. Neuroinflammation* **4**, 19 (2007).
- 1258 93. Abuirmeileh, A. *et al.* The corticotrophin-releasing factor-like peptide urocortin reverses
1259 key deficits in two rodent models of Parkinson's disease. *Eur J Neurosci* **26**, 417–423
1260 (2007).
- 1261 94. Abuirmeileh, A., Harkavyi, A., Kingsbury, A., Lever, R. & Whitton, P. S. The CRF-like
1262 peptide urocortin greatly attenuates loss of extracellular striatal dopamine in rat models of
1263 Parkinson's disease by activating CRF1 receptors. *Eur. J. Pharmacol.* **604**, 45–50 (2009).
- 1264 95. Huang, H. Y. *et al.* Urocortin modulates dopaminergic neuronal survival via inhibition of
1265 glycogen synthase kinase-3 β and histone deacetylase. *Neurobiol. Aging* **32**, 1662–1677
1266 (2011).

- 1267 96. Huang, H. Y. *et al.* Epigenetic regulation contributes to urocortin-enhanced midbrain
1268 dopaminergic neuron differentiation. *Stem Cells* **33**, 1601–1617 (2015).
- 1269 97. Saxena, A. *et al.* Trehalose-enhanced isolation of neuronal sub-types from adult mouse
1270 brain. *Biotechniques* **52**, 381–385 (2012).
- 1271 98. Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat. Protoc.* **9**,
1272 171–181 (2014).
- 1273 99. Kim, D., Langmead, B. & Salzberg, S. L. HISAT: a fast spliced aligner with low memory
1274 requirements. *Nat. Methods* **12**, 357–60 (2015).
- 1275 100. Mudge, J. M. & Harrow, J. Creating reference gene annotation for the mouse C57BL6/J
1276 genome assembly. *Mamm Genome* **26**, 366–378 (2015).
- 1277 101. Trapnell, C. *et al.* Differential gene and transcript expression analysis of RNA-seq
1278 experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–78 (2012).
- 1279 102. Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed by
1280 pseudotemporal ordering of single cells. *Nat. Biotechnol.* **32**, 381–6 (2014).
- 1281 103. Huber, W. *et al.* Orchestrating high-throughput genomic analysis with Bioconductor. *Nat*
1282 *Methods* **12**, 115–121 (2015).
- 1283 104. Wang, X. F. & Xu, Y. Fast clustering using adaptive density peak detection. *Stat Methods*
1284 *Med Res* (2015). doi:10.1177/0962280215609948
- 1285 105. Trapnell, C. *et al.* Differential analysis of gene regulation at transcript resolution with
1286 RNA-seq. *Nat. Biotechnol.* **31**, 46–53 (2013).
- 1287 106. Burns, J. C., Kelly, M. C., Hoa, M., Morell, R. J. & Kelley, M. W. Single-cell RNA-Seq
1288 resolves cellular complexity in sensory organs from the neonatal inner ear. *Nat Commun*
1289 **6**, 8557 (2015).
- 1290 107. Molyneaux, B. J. *et al.* DeCoN: genome-wide analysis of in vivo transcriptional dynamics
1291 during pyramidal neuron fate selection in neocortex. *Neuron* **85**, 275–288 (2015).
- 1292 108. Zhang, H. M. *et al.* AnimalTFDB: a comprehensive animal transcription factor database.
1293 *Nucleic Acids Res* **40**, D144–9 (2012).
- 1294 109. Suzuki, R. & Shimodaira, H. Pvcust: an R package for assessing the uncertainty in
1295 hierarchical clustering. *Bioinformatics* **22**, 1540–1542 (2006).
- 1296 110. Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for
1297 interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545–
1298 15550 (2005).
- 1299 111. Mootha, V. K. *et al.* PGC-1alpha-responsive genes involved in oxidative phosphorylation
1300 are coordinately downregulated in human diabetes. *Nat Genet* **34**, 267–273 (2003).
- 1301 112. Langfelder, P. & Horvath, S. WGCNA: an R package for weighted correlation network
1302 analysis. *BMC Bioinformatics* **9**, 559 (2008).
- 1303 113. Langfelder, P. & Horvath, S. Fast R Functions for Robust Correlations and Hierarchical
1304 Clustering. *J Stat Softw* **46**, (2012).
- 1305 114. Langfelder, P., Zhang, B. & Horvath, S. Defining clusters from a hierarchical cluster tree:
1306 the Dynamic Tree Cut package for R. *Bioinformatics* **24**, 719–720 (2008).
- 1307 115. Dixon, J. R. *et al.* Topological domains in mammalian genomes identified by analysis of
1308 chromatin interactions. *Nature* **485**, 376–380 (2012).
- 1309