Differential susceptibility to reduced dopamine in SNc versus VTA in a mouse Tyrosine hydroxylase KO model of Parkinson's Disease

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Abstract

Parkinson's Disease (PD), is a neurodegenerative disorder affecting both cognitive and motor functions. It is characterized by decreased brain dopamine (DA) and a selective and progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc), whereas dopaminergic neurons in the ventral tegmental area (VTA) show reduced vulnerability. The majority of animal models of PD are genetic lesion or neurotoxin exposure models that lead to death of dopaminergic neurons. Here we use a DAT:TH KO mouse model that by inactivation of the tyrosine hydroxylase (*Th*) gene in dopamine transporter-expressing neurons, causes selective depletion of striatal dopamine without affecting DA neuron survival. We analyzed transcriptome responses to decreased DA in both pre- and post-synaptic dopaminergic brain regions of DAT:TH KO animals. None of the post-synaptic regions analyzed - Dorsal Striatum, Ventral Striatum and Prefrontal Cortex - show an overall change in transcriptome as a function of DA deficiency. This suggests that the broad striatal transcriptional changes in neurodegeneration-based PD models are not direct effects of DA depletion, but are rather a result of DA neuronal death. However, we find a number of dopaminergic genes differentially expressed in SNc, and to a lesser extent in VTA, as a function of DA deficiency, providing evidence for a DA-dependent feedback loop. Of particular interest, expression of Nr4a2, a crucial transcription factor maintaining DA neuron identity, is significantly decreased in SNc, but not VTA, of DAT:TH KO mice. Unexpectedly, we find that half of DAT:TH KO animals show near-normal levels of *Th* transcription, and also display an overall transcript expression pattern more similar to wild type animals than to the remaining DAT:TH KO mice. These animals could represent recruitment of a pathway attempting to compensate for dopamine loss.

Keywords

ventral tegmental area, substantia nigra, dopamine, Parkinson's disease, tyrosine hydroxylase

Introduction

Midbrain dopamine neurons in the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc) are the major source of DA in the brain through their projections to the striatum, prefrontal cortex (PFC) and other limbic areas including the amygdala and hippocampus (Björklund and Dunnett 2007). Via these projections to the striatum and PFC, DA neurons play crucial roles in reward, motor and cognitive processes (Wise 2004). SNc and VTA DA neurons have different major projection fields: SNc DA neurons project primarily to the dorsal striatum whereas VTA DA neurons project to the ventral striatum and to the PFC, hippocampus and amygdala. Loss-of-function studies using neurotoxins and genetic approaches indicate that VTA and SNc DA neurons have specific involvement in reward, motor, memory and learning processes (Darvas et al. from 2008-2014, Baunez and Robbins 1999; Chudasama and Robbins 2006; Da Cunha et al. 2002; De Leonibus et al. 2007; Mura and Feldon 2003). Parkinson's disease (PD) is characterized by early degeneration of SNc neurons and the presence of alpha-synuclein inclusion bodies (Lewy bodies), yet only much later is there involvement of the VTA in PD. Clinical observations in PD patients suggest that loss of SNc DA neurons contribute to both motor and cognitive symptoms of PD (Cools et al. 2001, 2003; Sawamoto et al. 2008). The earliest changes induced by alpha synuclein accumulation are predegenerative loss of DA neurotransmission, followed by axonopathy and subsequent loss of DA neurons (Luk et al. 2012; Lundblad et al. 2012). Early changes in midbrain neurons resulting from loss of DA may be involved in the chain of pathologic processes leading to degeneration of DA neurons. We have generated a genetic model that allows investigation of the effects of DA loss on midbrain gene

expression without degeneration of midbrain DA neurons. Our model lacks tyrosine hydroxylase (TH) selectively in DA-transporter (DAT) expressing neurons, resulting from a conditional (floxed) *Th* deletion caused by expression of Cre recombinase in DAT neurons (Henschen, Palmiter, and Darvas 2013; Jackson et al. 2012; Zhuang et al. 2005). Although these animals have severe loss of striatal DA and learning and motor deficits, they retain basic locomotor function, and are normally motivated to drink and eat on their own, with normal viability (Darvas, Henschen, et al. 2014; Garrett Morgan et al. 2015; Henschen et al. 2013).

We use this model, referred to as DAT:TH KO, to investigate transcriptome changes in response to DA loss. Exquisite mechanisms maintain DA tone during normal physiology, with regulation at nearly all steps of DA synthesis, release and reception of the DA signal. These steps include regulation of synthesis and feedback inhibition of the rate limiting DA biosynthetic enzyme TH, regulation of size and content of synaptic vesicles, regulation of DA reuptake via DAT, regulation of D2-like DA presynaptic autoreceptors, and postsynaptic DA receptors. In addition, there can be direct or indirect effects of classical transmitters that act to modulate DA neurons (reviewed in Sulzer et al., 2016). With this degree of regulation in normal DA physiology, it is not surprising that in pathological conditions that cause loss of DA neurons brain physiology is altered in a compensatory manner. Both in Parkinson's Disease and in Parkinson's Disease models resulting from agents leading to death of DA neurons, motor symptoms occur only after a majority of DA neurons are lost (Ballanger et al. 2016; Golden et al. 2013).

This compensation for DA neuron loss is not well understood (Bezard et al. 2001; Bezard, Gross, and Brotchie 2003; Bezard and Gross 1998; Blesa et al. 2012; Hornykiewicz 1975; Perez et al. 2008). Although many of these reports indicate compensatory modulation of the DA system, other transmitter systems may also be involved, including striatal preproenkephalin (Bezard et al.

2001), altered cortical-basal ganglia-thalamocortical circuitry (Bezard et al. 2003; Melief et al. 2018), alterations of the serotonergic system, in addition to altered DA signaling and altered extracortical circuits (Ballanger et al. 2016).

Here we analyze transcriptome responses to DA loss in pre- and postsynaptic brain regions, using DAT:CRE driven inactivation of *Th*. We find a significant difference in responses to DA loss between SNc and VTA and a surprising diversity of responses that may represent a compensatory response to DA loss.

Results

A novel *Th* partial transcript can be detected in DAT:TH KO presynaptic libraries

We used the DAT:TH KO model to test for effects of striatal DA deficiency on the transcriptome in the regions studied. We expected that the lack of DA will affect preferentially DA producing and DA receiving parts of the brain. Therefore, we studied transcripts from five distinct brain regions, two of which are presynaptic: *Substantia nigra pars Compacta* (SNc) and Ventral tegmental area (VTA); and three postsynaptic: dorsal striatum (DS), ventral striatum (VS) and prefrontal cortex (PFC), from six wild type and six DAT:TH KO male mice. Samples from each brain region were sequenced with similar depth of 42.2 ± 4.75 million uniquely mapped reads per library. The distributions of number of reads mapped per gene in all brain regions followed the same pattern, with the majority of genes represented by over 1000 unique reads (Figure S1A). In all the following analyses we filtered out weakly expressed genes as represented by fewer than 30 reads total in all samples from a given brain region. In the final dataset, a majority of genes (14,157) was detectable in the samples from all brain regions, but there are 33 to 142 genes that are found in only one region (Figure S1B).

To validate an effective *Th* knockout in DAT expressing neurons of the DAT:TH KO animals, we examined the expression level of *Th* in the SNc and VTA samples. Unexpectedly, we detected a high level of *Th* mRNA in SNc and VTA from three DAT:TH KO mice which we designate as 'DAT:TH KO^{High}' versus 'DAT:TH KO^{Low}' (Figure 1A). Additionally, SNc and VTA samples from one WT animal have remarkably low levels of *Th* expression. To determine

if this low level of *Th* mRNA correlates with the DAT:TH KO^{Low} samples, we mark samples from this WT animal with minus sign ("-") in this and subsequent figures.

One could imagine that inefficient removal of the *Th* exon 1 by Cre recombinase by low level of Cre expression, could explain the observed variation in levels of TH transcripts over the DAT:TH KO samples. However, we detect very few exon 1 reads in all of the DAT:TH KO samples (Figure 1B), even in the DAT:TH KO samples with high *Th* expression level (DAT:TH KO^{High}) which are otherwise of normal downstream structure (Figure 1C).

We used two analytic methods, PCA and hierarchical cluster analysis, to determine whether the SNc transcriptomes of WT, DAT:TH KO^{High} and DAT:TH KO^{Low} show divergence in their overall transcriptomes. The PCA and the hierarchical cluster analysis from SNc show that DAT:TH KO^{High} have overall expression pattern more similar to wild type than to the DAT:TH KO samples with low level of *Th*. The WT and DAT:TH KO^{High} samples from SNc are localized closer to the upper-left corner of the PC1 vs PC2 plot, whereas DAT:TH KO samples are grouped near lower-right corner (Figure 2A, a cluster of samples with low level of *Th* is outlined with dashed line). This separation is even more visible on plot summarizing the hierarchical cluster analysis. DAT:TH KO samples with low level of *Th* creates one cluster with the aforementioned WT sample with the lowest level of Th ("WT-"), whereas DAT:TH KO samples with high level of Th are grouped with the remainder of the WT samples on the other branch of dendrogram (Figure 2B).

Lack of striatal dopamine does not alter the overall pattern of gene expression

Upon finding variation between the DAT:TH KO samples, we examined general differences between brain regions and genotypes by two techniques, principal component analysis (PCA)

(Figure 3A) and hierarchical cluster analysis (Figure 3B). The first principal component in PCA explains over the half of the variability, and clearly separates pre- and postsynaptic regions. The second principal component separates SNc, VTA, DS and VS from PFC. On the contrary, WT vs DAT:TH KO genotype is not recognizable from this analysis, indicating that the lack of DA does not trigger global transcriptome changes. All samples from both presynaptic regions, SNc and VTA, are closely localized, independent of genotype, reflecting similarity in their gene expression profiles. The libraries from DS and VS create another sparse group, and the PFC samples form an independent group, reflecting their separate origin. However, one sample from DAT:TH KO PFC (Figure 3A, datapoint indicated by a hollow square) did not localize closely to the PFC group. The library used to generate this datapoint had only 20% of the expected read number, with 30% of reads representing poly-G, suggesting a problem with library preparation or sequencing. None of the other libraries showed these issues, so this library was eliminated from further consideration. Similar strong grouping by a region of origin is visible in a heatmap representing results of hierarchical cluster analysis (Figure 3B). Two main branches of the dendrogram separate pre- and postsynaptic samples, with a further division into samples from PFC and striatum. As with the PCA, the DAT:TH KO and WT samples do not show clustering. Moreover, we do not detect any strong batch affect and samples from three different sequencing batches are clustered in alternating order (color coded in the leaves of the dendrogram in Figure 3B).

Lack of DA results in small transcriptomic effects in postsynaptic brain regions

To address whether DA deficiency affects gene expression in the DS, VS and PFC, we compared transcriptomes from the DAT:TH KO^{Low} and WT animals using a DESeq2 statistical analysis based on a negative binomial distribution model. We analyzed the expression level of 14,648,

14,694 and 14,692 genes per brain region, respectively. DS samples have the strongest observed response to the decreased DA level among the postsynaptic brain parts, with 7 differentially expressed (DE) genes at a false discovery rate (FDR) < 0.05. We detected only 3 downregulated: *Nefm*, *Pdyn* and *Sntg2* (Figure 4A); and only 4 upregulated genes in DS: *Anxa3*, *Fam43a*, *Slc7a8*, and *Wdfy* (Figure 4B). Only one of those genes, *Sntg2*, is also differentially expressed in the ventral striatum of DAT:TH KO^{Low} animals. Additionally, *Gpx6* has a significantly higher level of expression and *Shisa8* is significantly downregulated in DAT:TH KO^{Low} VS samples. Finally, we do not detect any DE genes in DAT:TH KO PFC samples.

DAT:TH KO^{Low} show reduced expression of DA related genes in SNc

We next asked whether DA deficiency leads to differential gene expression in the SNc. Comparing expression level of 15,282 genes from the DAT:TH KO^{Low} and WT animals, we find 34 that are DE at a false discovery rate (FDR) < 0.05 (Figure 5A). The DAT:TH KO^{High} libraries were not used during the detection of the DE genes since earlier analyses show that these libraries most resemble WT. However, when we examined the expression level of the DE genes from the DAT:TH KO^{High} with the DAT:TH KO^{Low} and the WT SNc libraries, DAT:TH KO^{High} cluster with the WT samples (Figure 5B), indicating that TH expression level is a better predictor of clustering than genotype. When we directly compared the DAT:TH KO^{High} and the WT SNc samples, none of the previously detected downregulated genes except for *Lcn2* and *Lrg1* had significant DE.

All, but one of the DE genes are downregulated in the DAT:TH KO^{Low} SNc samples (Figure 5A). The upregulated gene, 2900052N01Rik, encodes a long intergenic noncoding RNA with no known function. For most genes we observed a ~10-fold expression level decrease (with median log2 FoldChange of -3.47 and mean log2 FoldChange of -3.2) in in the absence of DA. Among

the downregulated genes are several transcription factors involved in DA neuron specification: *Foxa2*, *Gata3*, *Nr4a2*, *Pax5*, *Pitx3*, *En1* and *En2* (Figure 6A). Consistent with decreased expression of the crucial dopaminergic transcriptional regulators, there is a reduced level of classical dopaminergic markers: *Dao*; *Ddc*; *Ret*; *Gch1*; *Slc6a3* (codes DAT); *Slc18a2* (VMAT2) (representative expression plots are collected on Figure 5B). Supplemental file 1: Table S1 reports the complete results of this analysis, providing full information about DE genes, with the values of log2FoldChange, log2foldchange Standard Error, Wald statistic, p-value and FDR adjusted p-value.

VTA and SNc Transcriptomes Respond Distinctly to DA Deficiency

Comparing expression levels of 15,187 genes detected in VTA in the DAT:TH KO^{Low} and WT samples, we detected 89 DE genes at FDR < 0.05. In contrast to the general directionality of the DE observed in SNc, the majority of DE genes were upregulated in DAT:TH KO^{Low} VTA samples (55 upregulated vs 34 downregulated genes; Figure 7A). Moreover, the magnitude of observed changes is smaller than in SNc. The majority of the DE is down- or upregulated less than two-fold (60 out of 89 DE genes, with median absolute value of log2 Fold Change of 0.7 and mean of 1.09). However, the hierarchical cluster analysis based on the DE genes shows again that all DAT:TH KO^{Low} samples are clustered together, whereas DAT:TH KO^{High} are more similar to WT. Moreover, the “WT-” sample with a relatively low level of the *Th* expression is clustered with the DAT:TH KO^{Low} libraries, showing an intermediate pattern of DE (Figure 7B). Furthermore, when we compared DE across the entire transcriptome, none of the DE genes detected between DAT:TH KO^{Low} and all WT samples have a statistically significant difference in expression level between DAT:TH KO^{High} and WT VTA.

In addition to *Th*, only five DE genes from DAT:TH KO^{Low} SNc were also significantly downregulated in VTA samples: *Chrnb3*, *Cox20*, *En1*, *Pitx3* and *Slc6a3* (Figure 8A). Among the rest of the downregulated genes in VTA only additional two are known transcription factors: *Foxa1* and *Pbx3*. According to this, no additional classical dopaminergic markers were downregulated in VTA.

To further examine potential differences between the dopamine dependency in SNc and VTA we looked at the Gene Ontology (GO) terms enriched in detected DE genes, with top enriched Biological Process (BP) GO terms are collected in Table 1. The full results of the GO enrichment analyses are collected in the Additional file 2 Table S2. As expected, the top ten most enriched terms in genes downregulated in SNc are connected to DA metabolism and to dopaminergic neurons and their functions (20 out of 34 enriched categories). Results of the enrichment analysis from the genes downregulated in the VTA are not as explicit. Only 5 categories were significantly enriched after correction using Benjamini–Hochberg procedure. While none of them directly connect to DA, one term, functionally related to DA, the GO:0007626 (locomotory behavior), is also enriched in DE genes in SNc. The other two related categories are connected to mitochondria. In contrast to the downregulated, the genes upregulated in VTA show high level of enrichment in 85 BP categories. This difference could stem from the greater number of genes with increased level of expression in DAT:TH KO^{Low} VTA samples. However, it could also suggest stronger and active upregulation of particular pathways. The top ten enriched terms revolve around synaptic transmission and glutamatergic signaling. Other terms of particular interest point to the regulation of calcium and GABA-ergic signaling. These results suggest that while the decrease level of DA in SNc may cause loss of dopaminergic identity, the neurons in VTA seem to have more synaptic plasticity and react by remodeling of synaptic signaling.

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Biological Process GO term enriched in DE genes in SNc

ID	Description	Count	p-value	p.adjust	Genes
GO:0009713	catechol-containing compound biosynthetic process	7	1.97E-14	1.12E-11	*Ddc/Slc6a3/Gch1/Gata3/ Nr4a2/Dao/Th*
GO:0042423	catecholamine biosynthetic process	7	1.97E-14	1.12E-11	*Ddc/Slc6a3/Gch1/Gata3/ Nr4a2/Dao/Th*
GO:0071542	dopaminergic neuron differentiation	6	5.81E-11	9.45E-09	*En1/Pitx3/Nr4a2/Foxa2/ Pax5/En2*
GO:0009636	response to toxic substance	10	2.28E-10	2.88E-08	*Ddc/Slc6a3/Gch1/ Slc18a2/Lcn2/Nr4a2/ Th/ Gucy2c/ Chrnb3/Chrna6*
GO:0098700	neurotransmitter loading into synaptic vesicle	4	3.94E-09	4.08E-07	*Ddc/Slc18a2/Slc10a4/Th*
GO:0007626	locomotory behavior	8	1.96E-08	1.86E-06	*En1/Slc6a3/Sncg/Pitx3/ Slc18a2/Nr4a2/Foxa2/Th*
GO:1901617	organic hydroxy compound biosynthetic process	7	5.64E-08	4.94E-06	*Ddc/Slc6a3/Gch1/Gata3/ Nr4a2/Dao/Th*
GO:0097164	ammonium ion metabolic process	6	3.94E-07	2.99E-05	*Ddc/Slc6a3/Gch1/Nr4a2/ Dao/Th*
GO:0001505	regulation of neurotransmitter levels	8	4.71E-07	3.35E-05	*Ddc/Slc6a3/Colq/Sncg/ Gch1/Th/Chrnb3/Chrna6*

Biological Process GO term enriched in downregulated genes in VTA

ID	Description	Count	p-value	p.adjust	Genes
GO:0007005	mitochondrion organization	8	3.07E-07	0.000205	*Cox20/Dcn/Prdx3/ Ndufaf4/Tomm5/Ndufab1/ Higd1a/Apoo*

GO:0033108	mitochondrial respiratory chain complex assembly	3	1.81E-05	0.006042	*Cox20/Ndufaf4/ Ndufab1*
GO:0007626	locomotory behavior	5	0.000154	0.034539	*En1/Slc6a3/Pitx3/Pbx3/Th*
GO:0046500	S-adenosylmethionine metabolic process	2	0.000235	0.039340	*Mat2a/Mat2a*
GO:0051591	response to cAMP	3	0.000328	0.043891	*Slc6a3/Mat2a/Mat2a*

Biological Process GO term enriched in upregulated genes in VTA

ID	Description	Count	p-value	p.adjust	Genes
GO:0097060	synaptic membrane	14	1.37E-10	2.49E-08	*Epha4/Grm1/Atp2b1/Adcy8/ Adgrb1/Kcnb1/Lrrc7/Gabbr2/ Grin3a/Rims3/Cacna1c/Prkcg/ Gabrg3/Iqsec2*
GO:0099699	integral component of synaptic membrane	10	5.02E-09	4.56E-07	*Epha4/Grm1/Atp2b1/Adcy8/ Adgrb1/Lrrc7/Gabbr2/Grin3a/ Cacna1c/Gabrg3*
GO:0099240	intrinsic component of synaptic membrane	10	1.09E-08	6.60E-07	*Epha4/Grm1/Atp2b1/Adcy8/ Adgrb1/Lrrc7/Gabbr2/Grin3a/ Cacna1c/Gabrg3*
GO:0098984	neuron to neuron synapse	1q	1.97E-08	7.44E-07	*Epha4/Grm1/Ptk2b/Adcy8/ Adgrb1/Syngap1/Lrrc7/Grin3a /Cacna1c/Prkcg/Iqsec2*
GO:0098978	glutamatergic synapse	13	2.04E-08	7.44E-07	*Epha4/Grm1/Atp2b1/Ptk2b/ Adcy8/Adgrb1/Syngap1/Lrrc7 /Gabbr2/Grin3a/Rims3/ Cacna1c/Iqsec2*
GO:0050804	modulation of chemical synaptic transmission	13	1.16E-09	7.75E-07	*Epha4/Grm1/Jph4/Ptk2b/ Adcy8/Adgrb1/Syngap1/ Cacna1b/Kcnb1/Grin3a/Rims3 /Prkcg/Iqsec2*
GO:0099177	regulation of trans-synaptic signaling	13	1.19E-09	7.75E-07	*Epha4/Grm1/Jph4/Ptk2b/ Adcy8/Adgrb1/Syngap1/ Cacna1b/Kcnb1/Grin3a/Rims3 /Prkcg/Iqsec2*
GO:0014069	postsynaptic density	11	1.06E-07	2.98E-06	*Epha4/Grm1/Ptk2b/Adgrb1/ Syngap1/Lrrc7/Grin3a/ Cacna1c/Prkcg/Prkcg/Iqsec2*
GO:0032279	asymmetric synapse	10	1.15E-07	2.98E-06	*Epha4/Grm1/Ptk2b/Adgrb1/ Syngap1/Lrrc7/Grin3a/ Cacna1c/Prkcg/Iqsec2*
GO:0099055	integral component of postsynaptic membrane	7	2.16E-06	3.93E-05	*Epha4/Grm1/Adgrb1/Lrrc7/ Gabbr2/Grin3a/Cacna1c*
GO:0097060	synaptic membrane	14	1.37E-10	2.49E-08	*Epha4/Grm1/Atp2b1/Adcy8/ Adgrb1/Kcnb1/Lrrc7/Gabbr2/ Grin3a/Rims3/Cacna1c/Prkcg/ Gabrg3/Iqsec2*

Discussion

The majority of animal models of PD are based on neurotoxins or neuropharmacological agents targeting mitochondria, in particular the complex I of respiratory chain (Gubellini and Kachidian 2015). Although mitochondrial disfunction is well described in the pathogenesis of PD (Dawson and Dawson 2003; Gubellini et al. 2010), the neurodegeneration induced in those models is more rapid and acute than observed in PD. The strong cellular and behavioral phenotypes are observable in animals just weeks after a treatment with 6-OHDA or MPTP (Jackson-Lewis et al. 1995; Ungerstedt 1968; Ungerstedt and Arbuthnott 1970), whereas PD develops slowly, and the first locomotor symptoms could occur years after initiation of neurodegeneration (Halliday et al. 2008; Postuma et al. 2012). In this study we used a DAT:TH KO mouse model, generated by DA transporter driven CRE (DAT-CRE) to inactivate the *Th* gene, with loxP sites flanking *Th* exon 1, which encodes the *Th* translation startsite. This approach results in animals that lack 95% of a normal level of DA in the striatum, however the number of DA neurons is otherwise unaffected (Darvas, Henschen, et al. 2014; Garrett Morgan et al. 2015). This allows observation of transcriptome changes in response to DA loss, especially in SNc and VTA, that could have been missed in models based on the loss of DA neurons. Furthermore, as the DAT:TH KO model used here does not induce neuronal death, we could separate effects of the DA deficiency and a decreased amount of DA projections into striatum that are difficult to discriminate in the lesion-based models. Previously published comparisons between these two approaches show that more severe symptoms of PD, such as bradyphrenia, cognitive rigidity and spatial learning deficits, are dependent on both loss of DA and DA neuronal loss in striatum (Darvas, Henschen, et al. 2014). However, cognitive flexibility, working memory and cue-dependent associative learning can be

caused solely by decrease in striatal DA, independent of depletion of nigrostriatal DA neurons (Garrett Morgan et al. 2015).

Based on the previously described distinct phenotype of DAT:TH KO animals, we hypothesized that potential transcriptome changes might affect only subpopulations of cells that are the most sensitive to dysregulation of DA homeostasis. We focused on the two midbrain regions, SNc and VTA, as they are the major source of DA in the brain, and the three postsynaptic regions, VS, DS and PFC that are innervated by VTA and SNc neuronal projections. This approach increased sensitivity of our RNA-Seq analysis, allowing us to assess genes expressed specifically in these brain regions, that could be overlooked in whole brain samples.

Surprisingly, we detected near normal levels of *Th* transcripts in half of the DAT:TH KO samples (Fig. 1A). However, almost no reads were mapped to the FLOXed *Th* exon 1 in either of the DAT:TH KO samples, eliminating the possibility that this results from inefficient Cre recombinase function (Fig. 1B and C). The few *Th* exon 1 reads in DAT:TH KO samples could come from DA neurons that do not express DAT (Darvas, Henschen, et al. 2014).

The unusual *Th* transcript that is detectable in the DAT:TH KO^{High} samples lacks the first exon that encodes the normal translation startsite, yet the remaining transcript has the same structure as native *Th* mRNA. The normal N-terminal domain, encoded by exon 1, is involved in regulation of TH enzymatic activity, with several phosphorylatable serines involved in TH activation (Tekin et al. 2014), though deletion of this region can stabilize the TH protein (Nakashima et al. 2005). To date, only one isoform of *Th* was characterized in mouse, however, there are four alternative splicing forms of *TH* in humans that differ in structure at the 5' end of the transcript, resulting in proteins with different N-terminal regulatory segments (Tekin et al. 2014). The *Th* transcripts detected in DAT:TH KO^{High} animals could potentially represent a

novel mouse *Th* isoform that is differentially regulated relative to wildtype *Th*. The regional PCA and the hierarchical cluster analysis based on SNc samples show that DAT:TH KO^{High} cluster more strongly with WTs than with DAT:TH KO^{Low} animals (Fig. 2), supporting this hypothesis. Therefore, we decided to treat the samples from those animals as a separate group, that could represent potential DA-compensatory mechanism. Interestingly, when we compared the expression levels of the genes, detected as differentially expressed between DAT:TH KO^{Low} and WT, the samples from DAT:TH KO^{High} resemble WT animals. In the hierarchical cluster analysis (Fig. 5 and 7) all DAT:TH KO^{High} samples clustered between WT samples. Additionally, WT samples with lower level of Th, and thus presumably lower level of DA, are clustered closer to the DAT:TH KO^{Low} animals. Those results support our hypothesis that the alternative Th transcripts from DAT:TH KO^{High} animals could rescue the DAT:TH KO^{Low} phenotype. However, further studies will be needed to confirm if DAT:TH KO^{High} animals exhibit more wildtype-like behavioral phenotype and whether they express normal levels of striatal DA.

The PCA and hierarchical cluster analysis of all samples clearly reflect differences in transcriptomes of pre- and postsynaptic brain areas (Fig. 3A and B). The samples of those two origins are strongly separated on the PC1 axis, whereas the PC2 additionally creates cluster of the PFC datapoints. However, we could not observe the clear separation of DAT:TH KO samples in any of tested regions based on the two first PC nor in the results of hierarchical cluster analysis. These finding suggest that previously described broad transcriptional changes in striatum in neurodegeneration-based PD models are induced by decrease in the number of DA neurons, and not only by the decrease in DA level (Chin et al. 2008; Patel et al. 2008; Pattarini et al. 2008; Singh et al. 2010).

In our study we detected nine genes DE in the postsynaptic regions, seven in DS and three in VS, with one gene downregulated in both parts of striatum. We confirmed the dopaminergic regulation of dynorphin expression in DS described previously in DA deficient models based on both complete inactivation of *Th* and 6-OHDA-lesions (Sivam 1996; Zhou and Palmiter 1995). Dynorphin belongs to an endogenous opioid peptide family, involved in regulation of DA release, motor behavior, and learning (Carey et al. 2009; Gerfen and Steiner 1998; Reid et al. 1990; You, Herrera-marschitz, and Terenius 1999). The transcriptional activity of *Pdyn*, the gene encoding prodynorphin, is dependent on signaling through the DA D1 receptor, and works in a regulatory loop suppressing excessive activation of the striatal neurons (Gerfen and Steiner 1998; Xu et al. 1994). We detected a decreased expression level of a related protein, syntrophin gamma 2, encoded by *Sntg2*, in both DS and VS DAT:TH KO^{Low} samples. Syntrophin gamma 2 belongs to the family of cytoplasmic peripheral membrane proteins that binds to the C-terminal of dynorphin and act as a adaptor proteins by its PDZ domain (Alessi et al. 2006; Piluso et al. 2000). However, target proteins associated with dynorphin-syntrophin scaffold complex are currently unknown.

Subsequently, we focused on the SNc and VTA, as inactivation of Th directly affect neurons from those areas.

Looking more closely at differences between animals with expected low level of *Th* mRNA and WT controls, we detect 33 strongly downregulated genes in SNc (Fig. 5). These genes include dopaminergic markers, such as *Aldh1a7*, *Ddc*, *En1*, *En2*, *Foxa2*, *Nr4a2* (*Nurr1*), *Pitx3*, *Slc18a2* (VMAT2), *Slc6a3* (DAT) and *Th* (Domanskyi et al. 2014; Jacobs et al. 2011; Tekin et al. 2014). Significant 1-2 log decreases in expression of those genes could be potentially explained by a depletion of DA neurons in the SNc in DAT:TH KO animals. However we did not detect a

downregulation of antiapoptotic genes or an upregulation of canonical genes connected to cell death, genes that are usually differentially expressed in PD and PD models, such as genes from *Bcl2* family, *Mapk8ip3*, *Lrpprc* and *Nfrkb* or *Casp8* (Elmore 2007; Simunovic et al. 2009). The lack of evidence for cell death is consistent with the previous descriptions of this DAT:TH KO model (Darvas, Henschen, et al. 2014; Garrett Morgan et al. 2015). Nevertheless, downregulation of the DA related genes in SNc of DAT:TH KO^{Low} animals is in accordance with changes in PD and the lesion-based PD animal models. However, here for the first time we show that decreased level of expression of dopaminergic genes in SNc and VTA could be a direct effect of decreased DA as opposed to a result of death of dopaminergic neurons. Additionally, we detected downregulation of genes associated with PD, such as the neuroprotective gene *Fgf20* (Gao et al. 2008; Sleeman, Boshoff, and Duty 2012; Van Der Walt et al. 2004); and *Gch1*, of which variants are connected with decreased risk of PD (Kalia and Lang 2015). We also found that two different subunits of Nicotinic Acetylcholine Receptor (*Chrna6*, *Chrnb3*) are also downregulated in DAT:TH KO SNc. Nicotinic receptors involved in regulation of DA release and are proposed as potential targets for PD treatment (Koranda et al. 2014; Leino et al. 2018; Quik and Wonnacott 2011). Among the DE genes in SNc are also ones previously unconnected to PD, yet of particular interest: *Dao*, D-amino acid oxidase, that may interact with *Aldh1a7* in alternative pathway of GABA synthesis, that was also implicated in alternative pathway for DA biosynthesis (Kawazoe et al. 2007; J.-I. Kim et al. 2015); *Slc10a4*, a relatively less known vesicular monoaminergic and cholinergic associated transporter, that plays a role in the regulation of DA homeostasis (Larhammar et al. 2015; Melief et al. 2016). Another potentially important transcription factor that is involved in the development of DA neurons, *Pax5* (Simon et al. 2003), is also significantly downregulated in DAT:TH KO^{Low} SNc. That

suggests, that some of the observed changes could be connected to decreased striatal DA level during development. The downregulation of the postmitotic selector *Gata3*, involved in differentiation of SNc and VTA GABAergic neurons indicate developmental effect of DAT:TH KO as well (Haugas et al. 2016; Moriguchi 2006).

The results of differential gene expression analysis of DAT:TH KO^{Low} and WT VTA samples highlight the important differences between VTA and SNc. We not only detect more DE genes, but moreover the majority of them are upregulated in the absence of DA. This distinct response could reflect a differential variability of DA neurons in SNc and VTA in the PD (Brichta and Greengard 2014), as only four DA genes have significantly lower level in DAT:TH KO^{Low} VTA (*Th*, *Slc6a3*, *Pitx3* and *En1*; **Error! Reference source not found.**). Among the genes downregulated in SNc, but not in VTA, *Nr4a2*, gene encoding the highly conserved transcription factor Nurr1, is of particular interest. In PD patient brains, Nurr1 is downregulated in regions affected by α-synuclein inclusions and variants of *Nr4a2* are associated with cases of PD (Grimes et al. 2006; Le et al. 2003; Xu et al. 2002; Zheng, Heydari, and Simon 2003). Nurr1 is expressed in the early postmitotic neurons, is necessary for a proper differentiation and is maintained in midbrain DA neurons in the adult brain (Perlmann and Wallén-Mackenzie 2004; Zetterström et al. 1997). Nurr1 null mice die within 24h after birth and conditional knockout of *Nr4a2* in DA neurons in adult animals caused decreased expression of DA markers, including *Th* and *Slc6a3*, followed by progressive loss of DA (Castillo et al. 1998; Kadkhodaei et al. 2009, 2013). Six additional DA-related transcription factors are downregulated in SNc (*Foxa2*, *Gata3*, *Pax5*, *Pitx3*, *En1* and *En2)*, but only *Pitx3* and *En1* are also downregulated in VTA of DAT:TH KO^{Low} animals. Together, our data suggests that DA is involved in maintaining expression of the

DA-related genes required for DA biosynthesis and DA signaling by a novel regulatory loop with different susceptibility in SNc and VTA.

One of the genes downregulated in both SNc and VTA is *Chrnb3*, that encodes the nicotinic acetylcholine receptor. These results strengthen the role of the nicotinic acetylcholine receptors in the development of PD. However, the observed downregulation in both regions could actually represent the counter reaction of brain to decreased DA, since mice with α5 nicotinic receptor subunit gene knockout have less severe phenotype after treatment with 6-OHDA (Leino et al. 2018).

Another gene downregulated in both presynaptic regions encodes the cytochrome c oxidase assembly protein COX20. This finding could indicate that DA deficiency contributes to mitochondrial dysfunction involved in pathogenesis of PD, since COX20 is required for Cytochrome c oxidase assembly, required for normal mitochondrial function (Bourens et al. 2014; Elliott, Saracco, and Fox 2012).

Furthermore, we observe downregulation of five additional genes involved in assembly and proper function of the mitochondrial respiratory chain: *Ndufaf4* and *Ndufab1* connected to mitochondrial complex I, with *Ndufab1* downregulated in PD patients (Baertling et al. 2017; Marcus et al. 2013; Saada et al. 2009; Simunovic et al. 2009; Triepels et al. 1999; Zurita Rendón and Shoubridge 2012); *Apoo*, involved in complexes assuring stability of cristae (Ott et al. 2015); *Higd1a* regulating cytochrome c oxidase (Hayashi et al. 2015); and *Tomm5*, a part of the translocase complex of the mitochondrial outer membrane (Kato and Mihara 2008). However, the decrease in expression of these particular genes does not necessarily implicate mitochondrial dysfunction, but could also be an effect of a decrease in mitochondrial burden in the absence of DA-related oxidative stress. A study utilizing the 6-OHDA PD mouse model showed that the DA

depletion does not elevate markers of oxidative stress in SNc, and moreover another study indicates that decreased DA suppresses oxidative stress caused by ectopic DAT overexpression (Chen et al. 2008; Smith and Cass 2007). This hypothesis is additionally supported by our data, as we observed a decreased level of *Prdx3* in DAT:TH KO VTA, the gene encoding the only mitochondrial member of peroxiredoxin family enzymes that plays a protective antioxidant role by its peroxidase activity (Jiang et al. 2017; Liu et al. 2016).

The majority of genes upregulated in DAT:TH KO VTA are annotated with GO terms connected to the regulation of trans-synaptic signaling and function of glutamatergic synapses. Reciprocal signaling between DA and glutamate is well documented. Decreased levels of midbrain DA resulting from unilateral 6-OHDA lesions, bilaterally increases levels of glutamate in striatum and rostromedial tegmental nucleus, with parallel changes in expression of glutamate receptors (Chang et al. 2019; Lindefors and Ungerstedt 1990; Rodríguez-Puertas et al. 1999). There are additional levels of crosstalk between glutamate and dopamine-glutamate neurons and their local synapses within the midbrain DA regions (Dobi et al. 2010; Morales and Root 2014; Viereckel et al. 2016; Wang and Morales 2009; Yamaguchi et al. 2015). In our study we detect increased levels of metabotropic glutamate receptor 1 and ionotropic glutamate receptor NMDA 3A. Increased glutamate signaling may play a role in a tuning of the VTA, since activation of glutamate receptors modulates firing of dopaminergic neurons and regulates extracellular DA levels (Ferrada et al. 2017; Floresco et al. 2003; Lodge and Grace 2006; Oakman et al. 1995).

Here we show data that suggests that DA is required for maintained expression of dopaminergic genes in SNc, and to a lesser degree in VTA, suggesting the existence of a feedback loop responsible for the maintenance of DA signaling. Furthermore, our study implicates an

unexpected diversity in responses to DA loss between individual animals. Future studies should show whether the unusual class of animals expressing near-normal levels of the FLOXed Th mRNA represent a compensatory response, partly compensating for the DA loss.

Acknowledgements:

The laboratory of J.H. was supported by NIH 5R01GM084128.

Materials and methods

Animals

All animal use procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. Mice were housed under a 12-h, light–dark cycle in a temperature-controlled room with food and water available ad libitum. Mice with inactivation of the Th gene in DAT-expressing neurons (DAT:TH KO) were generated by crossing mice with two floxed *Th* alleles ($Th^{lox/lox}$) with mice that have one deleted *Th* allele ($Th^{/+}$) and one allele with Cre recombinase expressed under the control of the *Slc6a3* gene encoding DAT ($Slc6a3^{Cre/+}$). DAT:TH KO mice had the genotype $Th^{/lox}$-$Slc6a3^{Cre/+}$. C57Bl/6 were used as wild type (WT) control animals.

RNA Extraction

Mice, 3-5-month-old, were sacrificed by decapitation after brief exposure to CO2; the VS, DS, SNc, VTA and PFC from each mouse were dissected from the brain. Tissues were immediately frozen in liquid nitrogen and stored at −80 °C. Tissues were processed, and subsequently cDNA

libraries from isolated RNA were prepared in three independent batches; first one with samples from 3 DAT:TH KO and 3 WT animals; second with samples from one mouse per group; third with 2 DAT:TH KO and 2 WT animals. Each time tissues were homogenized using Bullet Blender with 0.5 mm RNase Free Zirconium Oxide Beads (Next Advance) in RLT buffer from RNeasy Plus Micro Kit (Qiagen). Total RNA was isolated using the RNeasy Plus Micro Kit (Qiagen). The quality and quantity of RNA from each sample was determined using an Agilent 2100 Bioanalyzer with Agilent RNA 6000 Pico Chips. Messenger RNA was then obtained from at least 100 ng of total RNA using NEBNext® Poly(A) mRNA Magnetic Isolation Module. RNA-seq libraries were prepared using NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina® following manufacturer's protocol. The library quality and insert length were checked using the Agilent 2100 Bioanalyzer with DNA High Sensitivity DNA Kit. The first two sets of 30 and 10 libraries were sequenced using Illumina HiSeq 2000 (BGI), third set of 20 libraries was sequenced using Illumina HiSeq 2500 (UCLA).

The quality of obtained fastq files was examined using FASTQC (S. 2010). The reads were aligned to the mouse reference genome (GRCm38) using HISAT2 v2.1.0 (D. Kim, Langmead, and Salzberg 2015) aligner with --rna-strandness RF parameter. Aligned reads were then summarized with featureCounts from the SourceForge Subread package (Liao, Smyth, and Shi 2014). Summarizing, we obtained over 2.3 billion uniquely mapped 100 bp paired-end reads (42206643± 4754672 uniquely mapped reads per library). A differential expression analysis was performed with R/Bioconductor package DESeq2 including the batch variable into the model design (Love, Huber, and Anders 2014). Multiple testing was corrected for using the Benjamini-Hochberg procedure. Sequencing data have been deposited in the Sequence Read Archive under accession code PRJNA550435.

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Figures legends

Figure 1. Th expression level in SNc and VTA samples.

DAT:TH KO animals were split into two groups: DAT:TH KO^{High} or DAT:TH KO^{Low}. DAT:TH KO animals were assigned to DAT:TH KO^{High} group if they had *Th* expression level higher than any WT animal in a given brain region. In all following analyses all samples from the same animal are grouped into the same DAT:TH KO$^{Low\ or\ High}$ group. DESeq2 normalized counts are based on featureCounts output. A) Normalized expression level of Th in both presynaptic regions. The normalized counts are shown on log10 scale. B) Normalized expression level of Th exon 1 is significantly lower in all DAT:TH KO animals in comparison to WT. Numbers over bars represent p-value from Kruskal-Wallis test. Datapoints from "WT-" mouse are marked with "-". C) Representative per base coverage plot of Th. The bottom part of the plots shows known Th exons in orange and yellow rhombuses mark loxP sites flanking exon 1. Yellow square marks the first exon, that should be removed by DAT:Cre recombinase in DAT:TH KO animals. Only few Th exon 1 reads are detectable in all DAT:TH KO samples, however the remaining exons have a high level of coverage in the DAT:TH KO^{High} SNc and VTA samples.

Figure 2. Clustering analyses of SNc samples.

A) SNc PCA based on genes represented by at least 30 normalized counts across all samples. The datapoint from "WT-" mouse is marked with "-". The dashed ellipse outlines a cluster of samples with low level of *Th*. B) Hierarchical cluster analysis of SNc samples. Dendrogram and heatmap were created based on the maximum distance between samples. Blue color indicates

large distance, with red diagonal line representing identity from comparison of the same sample. Each row and column represent an individual library, with row/column labeling by color coded boxes, following the same pattern as PCA plot. Leaves of dendrogram are color coded by sequencing batch. Blue squares with "-" mark the sample form "WT-" mouse.

Figure 3. Cluster analyses.

Input data for all analyses are DESeq2 normalized count values based on featureCounts output. A) Principal Component Analysis of all sequenced samples from three independent batches of RNA-sequencing, coded by the shape of datapoints. Each data point represents an individual library, color coded based on the brain region and genotype. The hollow square marks one outlier PFC library. B) Hierarchical cluster analysis of all sequenced samples from three independent batches of RNA-sequencing. Dendrogram and heatmap were created based on the Euclidean distance between samples. On heatmap blue color indicate large distance, with red diagonal line representing identity from comparison of the same sample. Each row and column represent an individual library, with row/column labeled by color coded boxes based on the brain region, following the same pattern as PCA plot. Leaves of dendrogram are color coded by sequencing batch: 1st – red, 2nd – yellow, 3rd – light blue.

Figure 4. Expression level of DE genes in postsynaptic brain regions.

All plotted genes have statistically significant DE in DAT:TH KO^{Low} at FDR <0.05. All figures follow color coding from figure A. Datapoints from WT mouse with low level of *Th* mRNA are marked by "-" sign. The lower number of WT samples in DS is due to a loss of 2 libraries during preparation. A) Genes downregulated in DS. B) Genes upregulated in DS. C) DE genes in VS.

Figure 5. DE genes in SNc.

A) Volcano plots of DE analysis between WT and DAT:TH KO^{Low} samples. Data points are color coded based on their adjusted p-value at FDR <0.05 and fold change. DE DA-related genes are labeled. B) Heatmap representing hierarchical clustering of the SNc libraries based on detected DE genes. Each column represents an expression patter of one DE gene. Color scale represent Z-score, with red marking high expression level. Leaves of dendrogram and row labels are by color coded based on the genotype and expression level of Th. WT sample with low level of *Th* mRNA is marked by "-" sign.

Figure 6. Representative expression level of genes DE in SNc.

All plotted genes have statistically significant decreased expression level in DAT:TH KO^{Low} vs WT samples at FDR <0.05. All figures follow color coding from figure B. Datapoints from WT mouse with low level of *Th* mRNA are marked by "-" sign. A) $\log_{10}$(Normalized Counts) of DA-related downregulated transcription factors. B) $\log_{10}$(Normalized Counts) of representative downregulated dopaminergic markers.

Figure 7. Genes DE in VTA.

A) Volcano plots of DE analysis between WT and DAT:TH KO^{Low} VTA samples. Data points are color coded based on their adjusted p-value at FDR <0.05 and fold change threshold. DE DA-related genes are labeled. B) Heatmap representing hierarchical clustering of the VTA samples based on detected DE genes. Each column represents an expression patter of one DE gene. Color scale represent Z-score, with red marking high expression level. Leaves of dendrogram and row labels are by color coded based on the genotype and expression level of *Th*. WT sample with low level of *Th* mRNA is marked by "-" sign.

Figure 8. Expression levels of genes downregulated in both DAT:TH KO presynaptic regions across VTA samples.

All plotted genes have statistically significant decreased expression level in DAT:TH KO^{Low} SNc and VTA at FDR <5%. Datapoints from WT mouse with low level of *Th* mRNA are marked by "-" sign.

Supplementary Figure 1. RNA-Seq Samples profiles

Input data for all analyses are DESeq2 normalized count values based on featureCounts output. A) Histograms representing the distributions of uniquely mapped reads per gene in examined brain regions. B) Venn diagram showing numbers of genes detected uniquely in the individual brain regions or in the intersections of two or more regions. Genes represented by fewer than 30 reads total were filtered out.

Table 1. Results of the GO enrichment analysis based on the DE genes in SNc and VTA at FDR < 0.05.

P-adjusted values were corrected using Benjamini–Hochberg procedure. This table shows only up to the top ten terms enriched based on the DE gene in SNc and VTA, from the total number of 33 and 85 significantly enriched categories.

Supplemental Information

SUPPLEMENTAL FILE 1 SF1.xlsx

Spreadsheet with the detailed results of DE analysis.

SUPPLEMENTAL FILE 2 SF2.xlsx

Spreadsheet with the results of GO analysis.

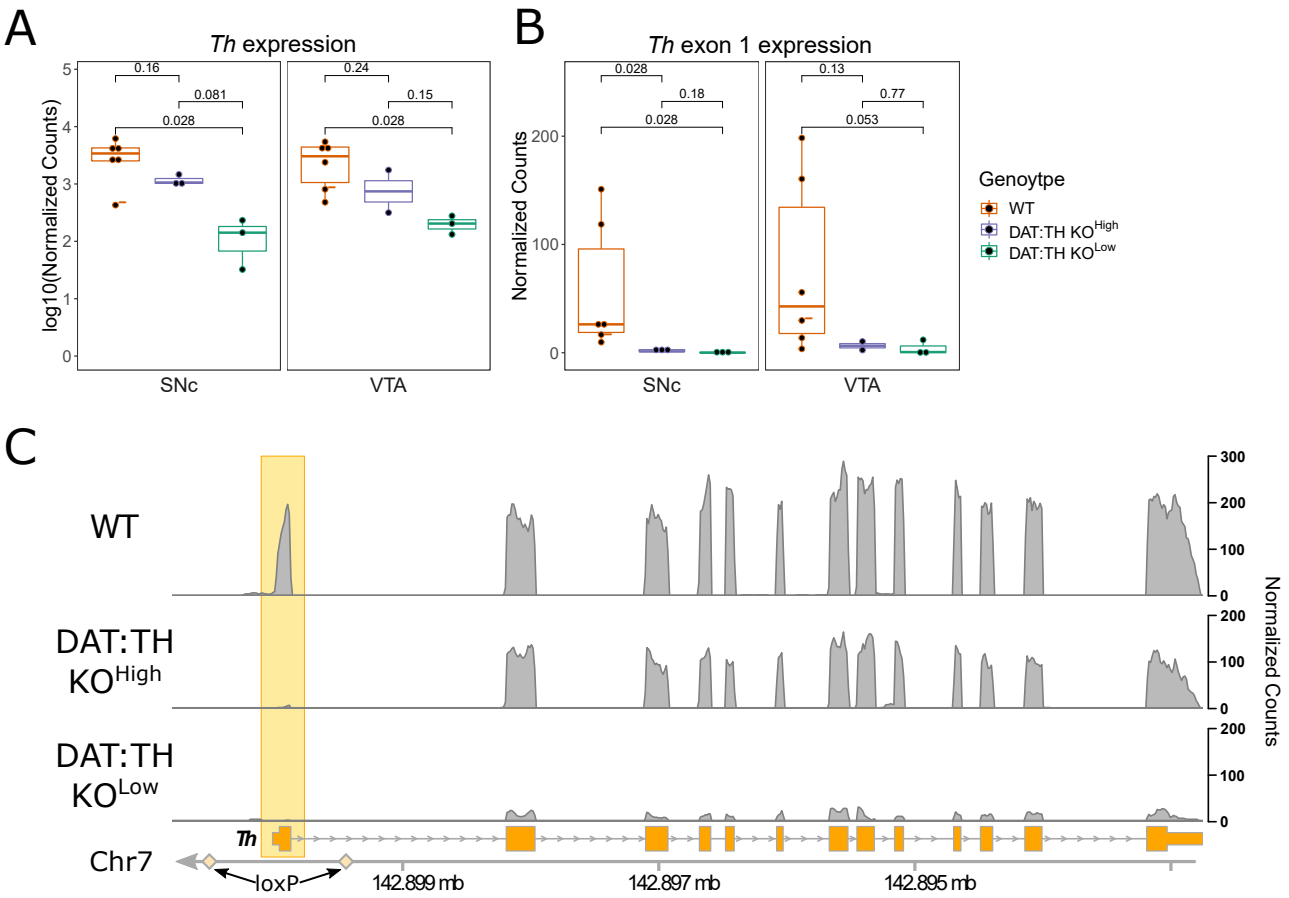

A
Th expression
log10(Normalized Counts)
0.16
0.081
0.028
0.24
0.15
0.028
SNc
VTA
B
Th exon 1 expression
Normalized Counts
0.028
0.18
0.028
0.13
0.77
0.053
SNc
VTA
Genoytpe
WT
DAT:TH KO High
DAT:TH KO Low
C
WT
DAT:TH KO High
DAT:TH KO Low
Normalized Counts
Th
Chr7
loxP
142.899 mb
142.897 mb
142.895 mb

Figure 1

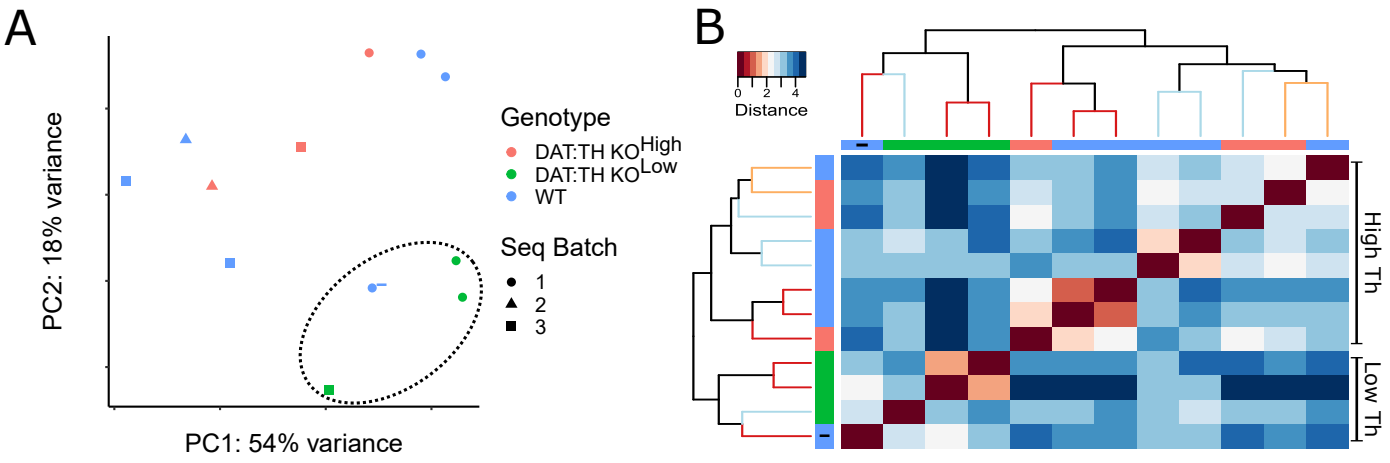

A
PC2: 18% variance
PC1: 54% variance
Genotype
DAT:TH KO High
DAT:TH KO Low
WT
Seq Batch
1
2
3
B
0
2
4
Distance
High Th
Low Th

Figure 2

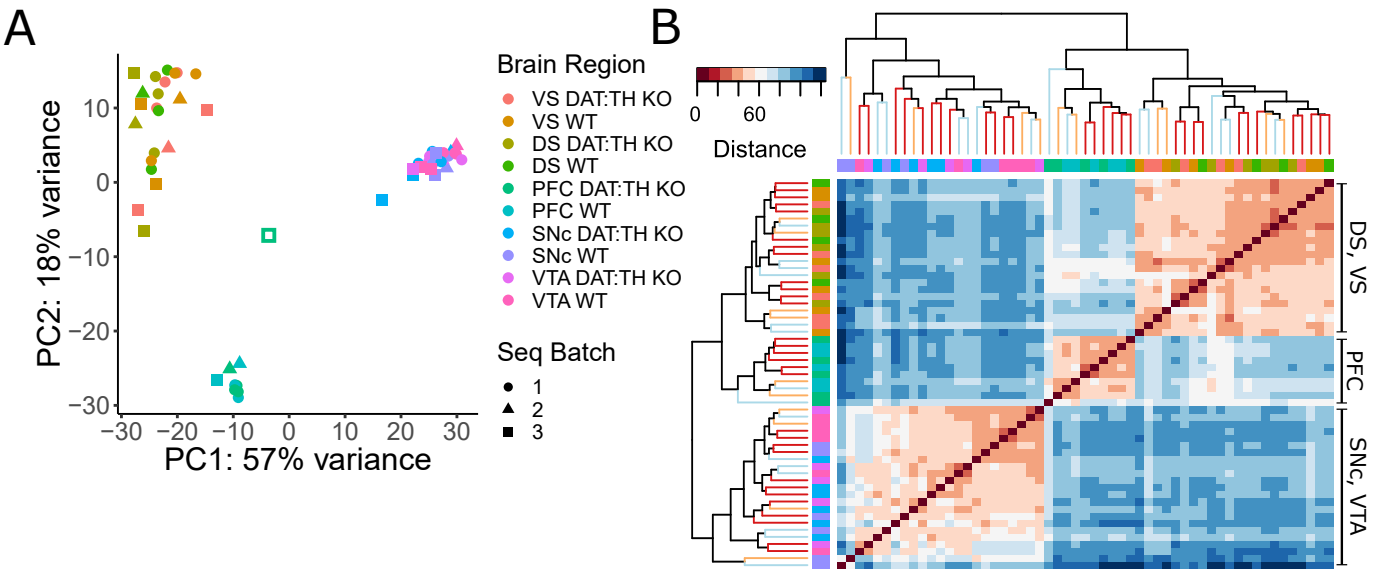

A
PC2: 18% variance
PC1: 57% variance
10
0
−10
−20
−30
−30 −20 −10 0 10 20 30
Brain Region
VS DAT:TH KO
VS WT
DS DAT:TH KO
DS WT
PFC DAT:TH KO
PFC WT
SNc DAT:TH KO
SNc WT
VTA DAT:TH KO
VTA WT
Seq Batch
1
2
3
B
0
60
Distance
DS, VS
PFC
SNc, VTA

Figure 3

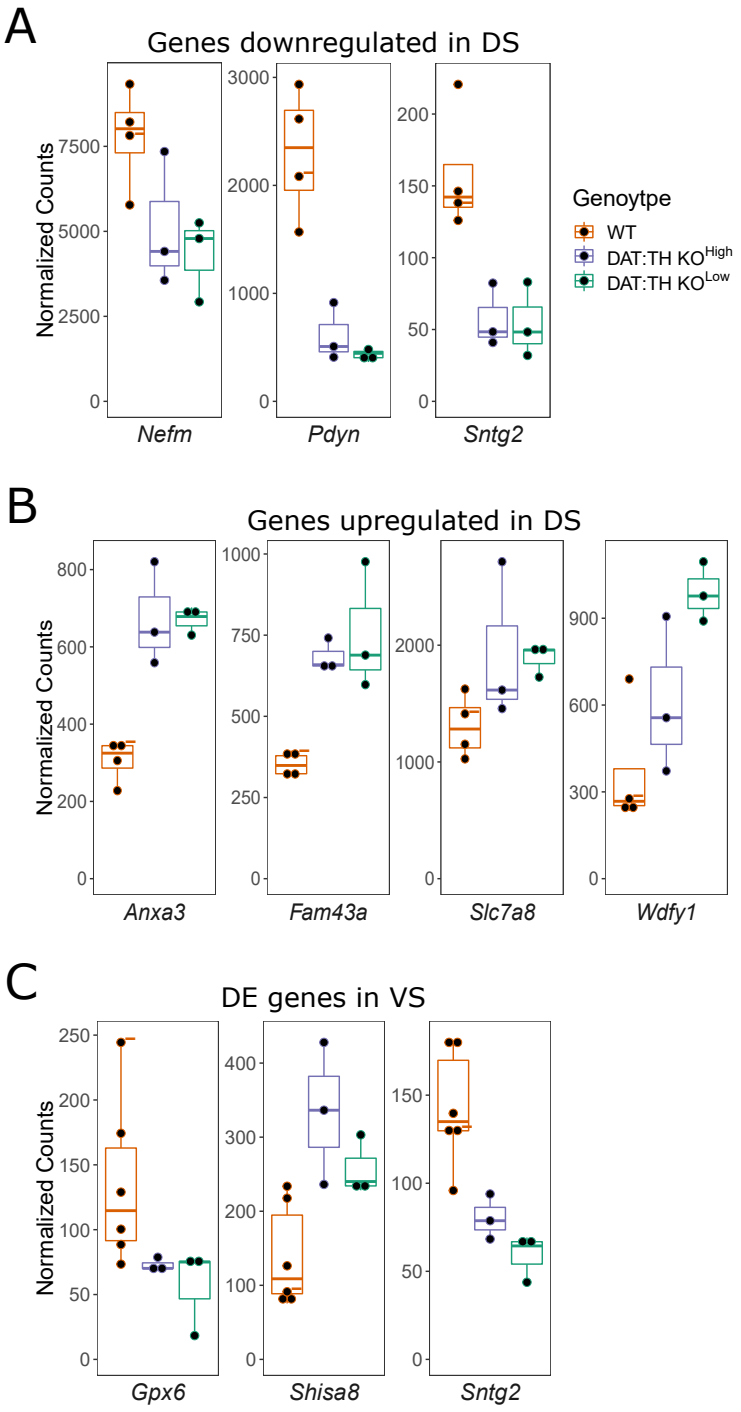
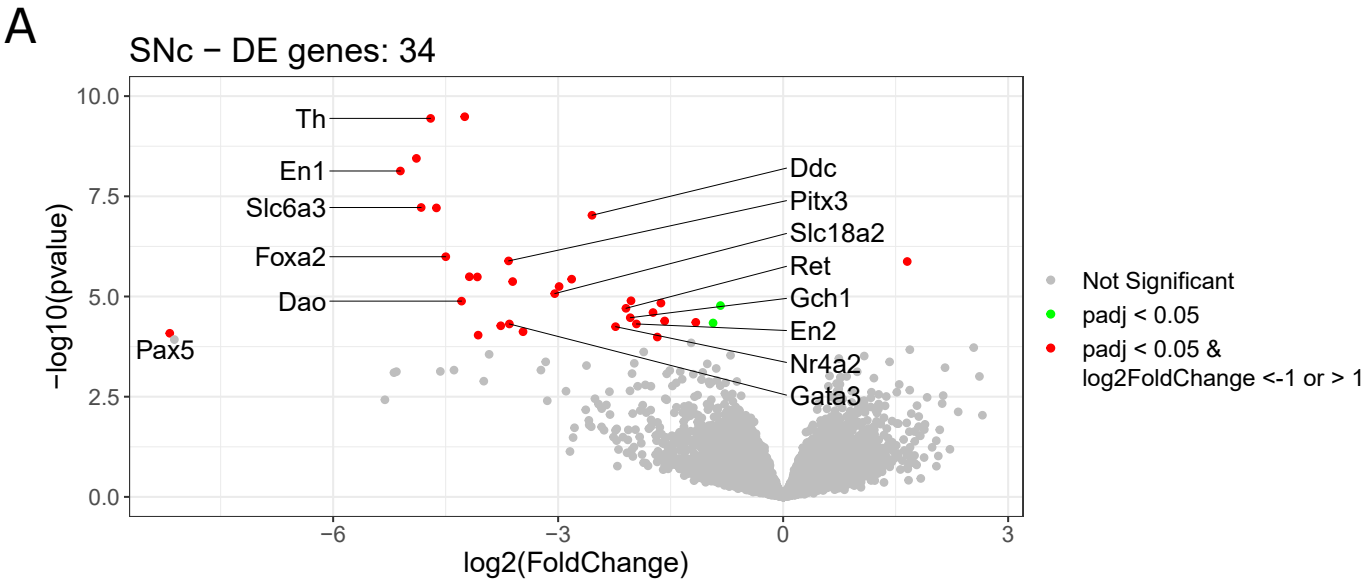

A
Genes downregulated in DS
Normalized Counts
Nefm
Pdyn
Sntg2
Genoytpe
WT
DAT:TH KO High
DAT:TH KO Low
B
Genes upregulated in DS
Normalized Counts
Anxa3
Fam43a
Slc7a8
Wdfy1
C
DE genes in VS
Normalized Counts
Gpx6
Shisa8
Sntg2

Figure 4


A
SNc − DE genes: 34
10.0
7.5
5.0
2.5
0.0
−log10(pvalue)
−6
−3
0
3
log2(FoldChange)
Th
En1
Slc6a3
Foxa2
Dao
Pax5
Ddc
Pitx3
Slc18a2
Ret
Gch1
En2
Nr4a2
Gata3
Not Significant
padj < 0.05
padj < 0.05 &
log2FoldChange <-1 or > 1

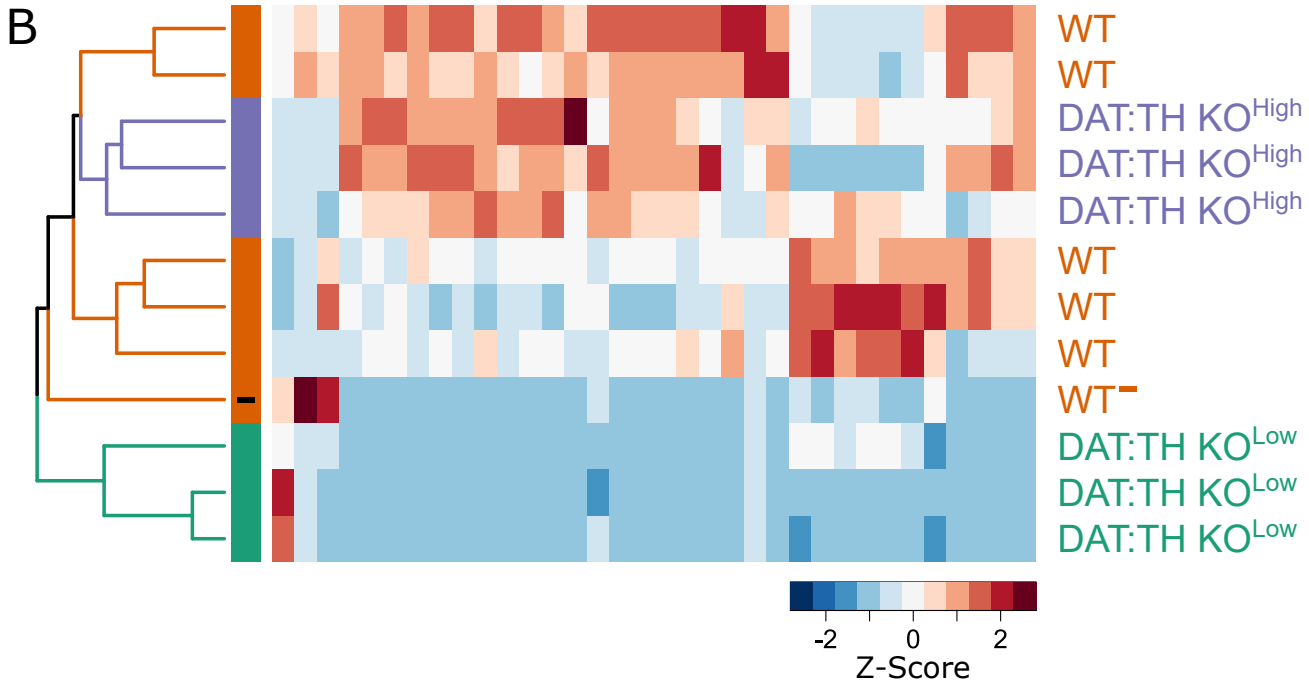

B
WT
WT
DAT:TH KO High
DAT:TH KO High
DAT:TH KO High
WT
WT
WT
WT
DAT:TH KO Low
DAT:TH KO Low
DAT:TH KO Low
-2
0
2
Z-Score

Figure 5

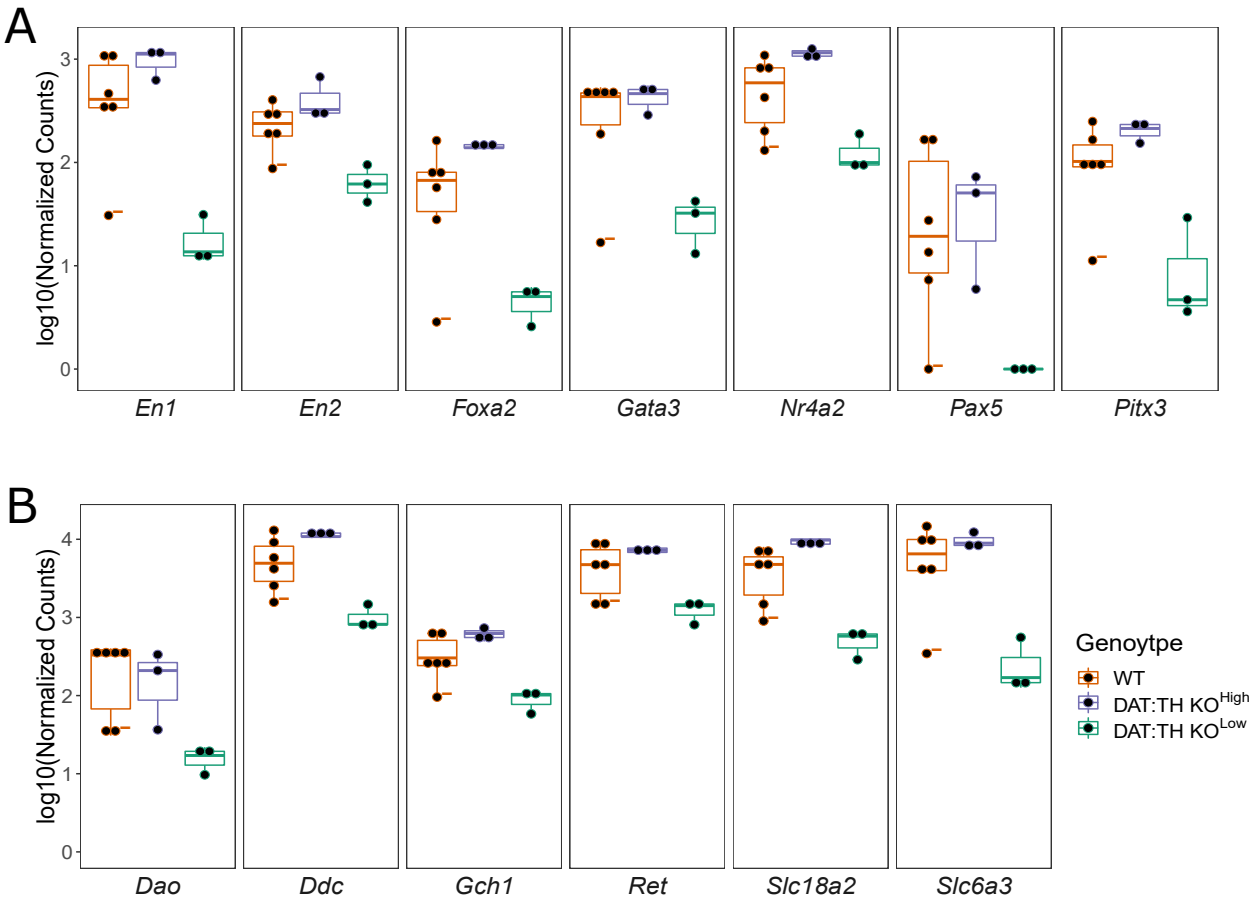

A
log10(Normalized Counts)
0
1
2
3
En1
En2
Foxa2
Gata3
Nr4a2
Pax5
Pitx3
B
log10(Normalized Counts)
0
1
2
3
4
Dao
Ddc
Gch1
Ret
Slc18a2
Slc6a3
Genoytpe
WT
DAT:TH KO High
DAT:TH KO Low

Figure 6

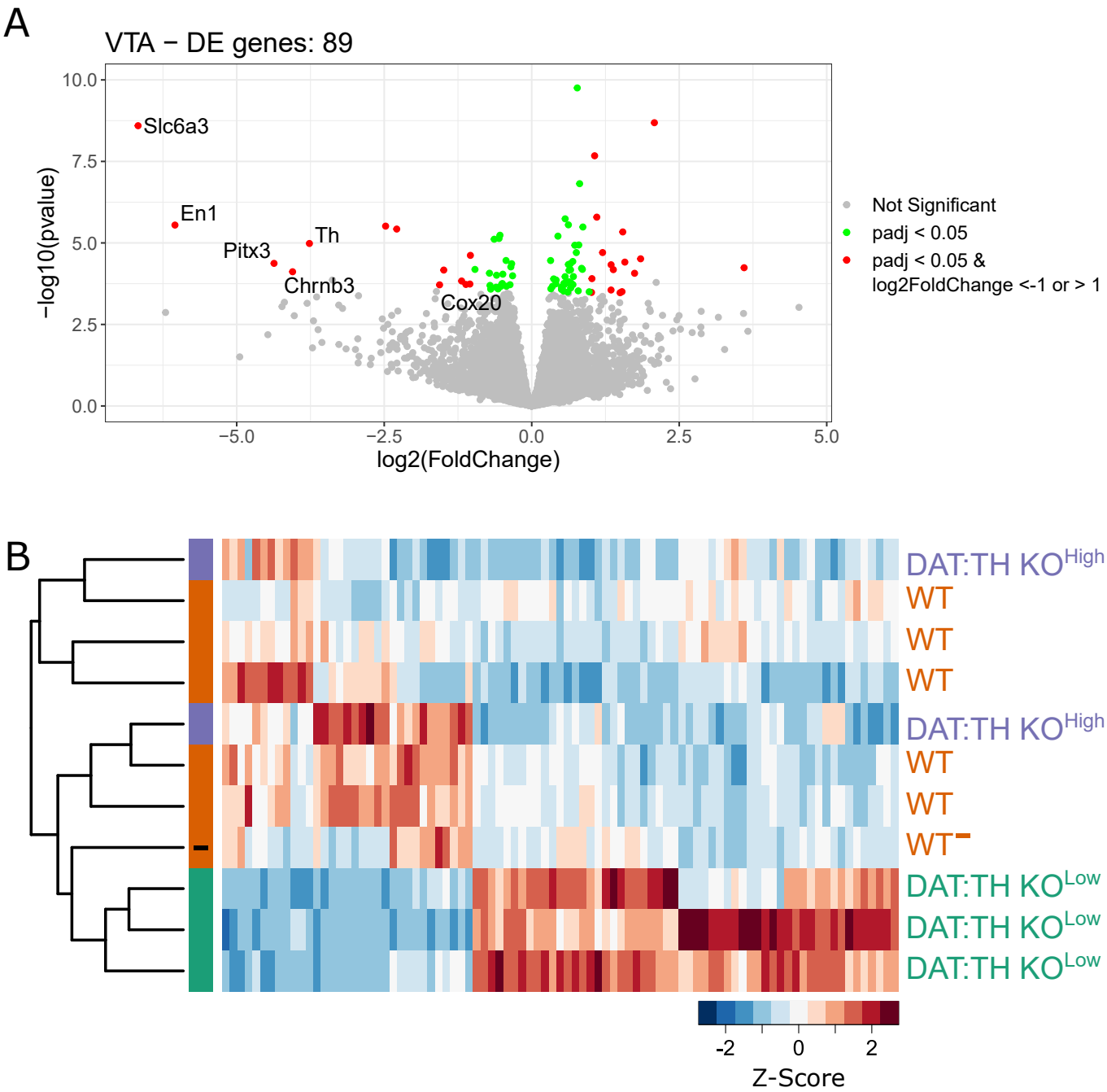

A
VTA − DE genes: 89
Slc6a3
En1
Pitx3
Th
Chrnb3
Cox20
−log10(pvalue)
log2(FoldChange)
Not Significant
padj < 0.05
padj < 0.05 &
log2FoldChange <-1 or > 1
B
DAT:TH KO High
WT
WT
WT
DAT:TH KO High
WT
WT
WT
DAT:TH KO Low
DAT:TH KO Low
DAT:TH KO Low
Z-Score

Figure 7

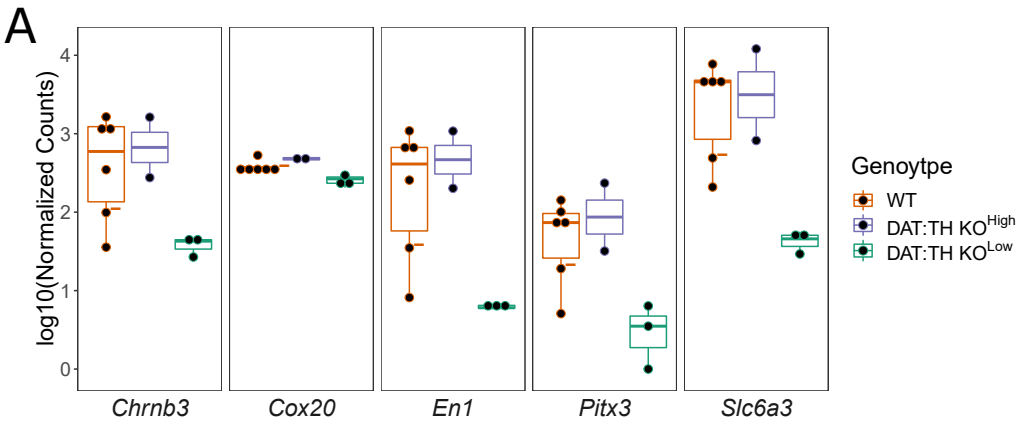
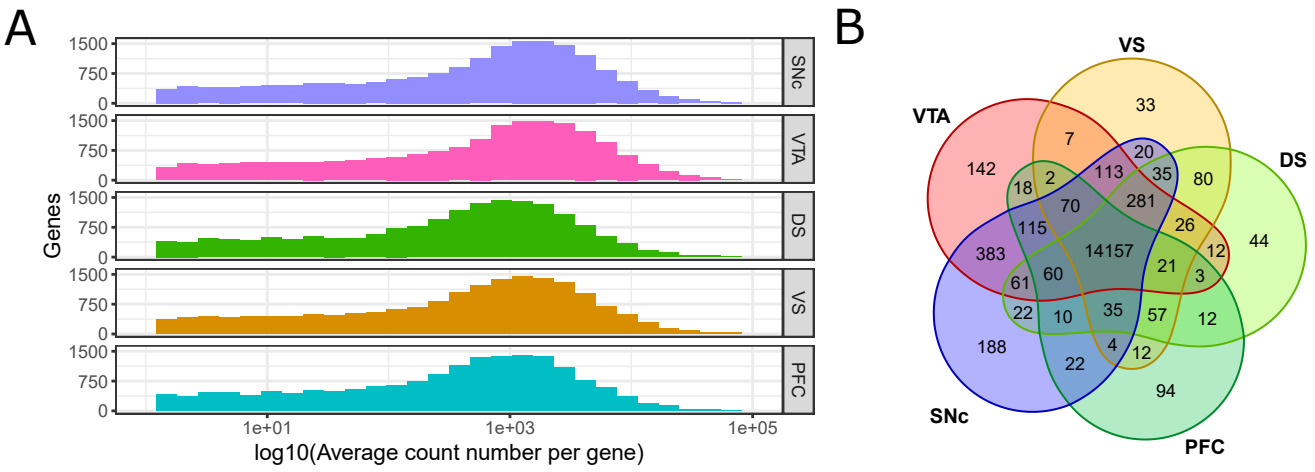

A
log10(Normalized Counts)
4
3
2
1
0
Chrnb3
Cox20
En1
Pitx3
Slc6a3
Genoytpe
WT
DAT:TH KO^High
DAT:TH KO^Low

Figure 8



Supplementary Figure 1