A diversity of transcriptional responses to reduced dopamine in SNc and VTA in a mouse Tyrosine hydroxylase KO model of Parkinson's Disease

M. Koltun, K. Cichewicz, J.T. Gibbs[a], M. Darvas[a], J. Hirsh*

Department of Biology, University of Virginia, Charlottesville, VA 22903, USA

[a]Department of Pathology, University of Washington School of Medicine, University of Washington, Box357470, Seattle, WA 98195, USA

[*]: Corresponding author

Abstract

Decreased levels of brain dopamine are one of the early characteristics of Parkinson's Disease, a neurodegenerative disorder affecting both cognitive and motor features. To date, the majority of animal models of Parkinson's Disease are based on a selective perturbation of mitochondria, with resulting death of dopaminergic neurons due to oxidative stress. Here we use DAT:Th KO mouse model that causes a selective depletion of striatal dopamine, by inactivation of the tyrosine hydroxylase (Th) gene in dopamine transporter expressing neurons. We analyzed transcriptome responses in the pre- and postsynaptic dopaminergic brain regions of dopamine deficient animals. We find a number of dopaminergic genes differentially expressed in SNc and VTA as function of dopamine. Furthermore, we detected a diversity in responses between SNc and VTA that could reflect a difference in a variability of DA neurons in pathogenesis of PD. Neither Dorsal Striatum, nor Ventral Striatum and Prefrontal Cortex of DAT:Th KO animals shows an overall change in transcriptome. Surprisingly, half of DAT:Th KO animals that show near-normal levels of Th transcripts display an overall expression pattern more similar to wild type animals than to the remaining DAT:Th KO mice. Those animals could represent recruitment of a pathway attempting to compensate for dopamine loss.

Introduction

Midbrain dopamine (DA) 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). Through their 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; Da Cunha et al., 2002; Mura

and Feldon, 2003; Chudasama and Robbins, 2006; De Leonibus et al., 2007). Parkinson's disease (PD) is characterized by prominent degeneration of SNc neurons and presence of alpha-synuclein inclusion bodies (Lewy bodies), and only much later is there an additional 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.

The earliest changes induced by alpha synuclein accumulation are predegenerative loss of DA neurotransmission that is followed by axonopathy and later on by loss of DA neurons (Lundblad et al 2012; Luk 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 investigating 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 et al 2013; Jackson et al 2012; Zhuang et al 2005). Although these animals have severe loss of striatal DA, learning and motor deficits, they retain basic locomotor function, and are sufficiently motivated to drink and eat on their own, and have no reduced viability (Henschen et al 2013; Darvas et al 2014; Morgan et al 2015).

We used this model, referred to as DAT:Th KO, or DD Th (Dopamine Deficient Th), to investigate the transcriptome response to DA loss. Exquisite mechanisms maintain dopamine (DA) tone during normal physiology, with regulation at nearly all steps of dopamine synthesis, release and reception of the DA signal. These steps include regulation of synthesis and feedback inhibition of the rate limiting DA biosynthetic enzyme, tyrosine hydroxylase (Th), regulation of size and content of synaptic vesicles, regulation of DA reuptake via the dopamine transporter, 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, that brain physiology is altered in a manner to compensate for this loss. Both in Parkinson's Disease in animals 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 and Gross, 1998; Bezard et al., 2003; Bezard et al., 2001; 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), alterations of the serotonergic system, in addition to altered DA signaling and altered extracortical circuits (Ballanger et al., 2016).

Here we analyze transcriptome responses in SNc and VTA to DA loss using DAT:CRE driven loxP inactivation of Th. We find a surprising diversity of responses in the FLOXed animals that may represent a compensatory response to DA loss.

Results

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

We used the DAT:Th KO (DD) model to test for effects of striatal DA deficiency on the transcriptome in the regions studied. The lack of dopamine can potentially affect only subsets of cells; 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 DD male mice.

We examined general differences between brain regions and genotypes by principal component analysis (PCA). The first principal component explains the half of the variability, and clearly separates pre- and postsynaptic regions (Figure 1A). The second principal component separates SNc, VTA, DS and VS libraries from PFC. On the contrary, the 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 are localized closely together, independently of genotype, reflecting similarity in their gene expression profiles. The libraries from DS and VS create another, sparse group. The PFC samples form an independent group reflecting their separate origin. However, one sample from DD PFC (Figure 1A, datapoint indicated by an asterisk) did not localize closely to the PFC group. The library used to generate this sequencing data had a significantly lower number of sequenced reads (20% of expected yield, with 30% of reads representing poly-G) suggesting problem with a library preparation or sequencing. None of the other libraries showed these issues, so this library was eliminated from further consideration.

Th partial transcript can be detected in DD presynaptic libraries

After finding so little global variation in the DD samples on the PCA, 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 DD mice. (Figure 2A). One could imagine that inefficient removal of the Th exon 1 by Cre recombinase, or low level of Cre expression could cause the observed variation in levels of TH transcripts over the DD samples. However, we detect very few exon 1 reads in all of the DD samples (Figure 2B), even in the DD samples with high Th expression level (DD High Th) which are otherwise of normal structure (Figure 2C).

It seems likely that the TH transcripts lacking the FLOXed exon 1 in the DD mice expressing high levels of Th (DD High Th) encode a functional TH protein, since the PCA from SNc and VTA, DD High Th have overall expression pattern more similar to wild type than the DD samples with low level of Th. The WT and DD High Th samples from SNc are localized closer to the upper-left corner of the PC1 vs PC2 plot, whereas DD samples are grouped near lower-right corner (Figure 3A). Separation of the samples from VTA based on PC1 and PC2 is less clear, however both WT and DD High Th data points reside on the left side of plot, versus DD samples localized in the upper-right side (Figure 3B). Additionally, wild type samples with low level of Th localize closer to DD, yet, all data points are rather scattered and do not group into clear clusters. Considering all

those findings, we decided to separate samples from DAT:TH KO animals into two groups: DD Low Th and DD High Th, based on their Th expression level.

A)

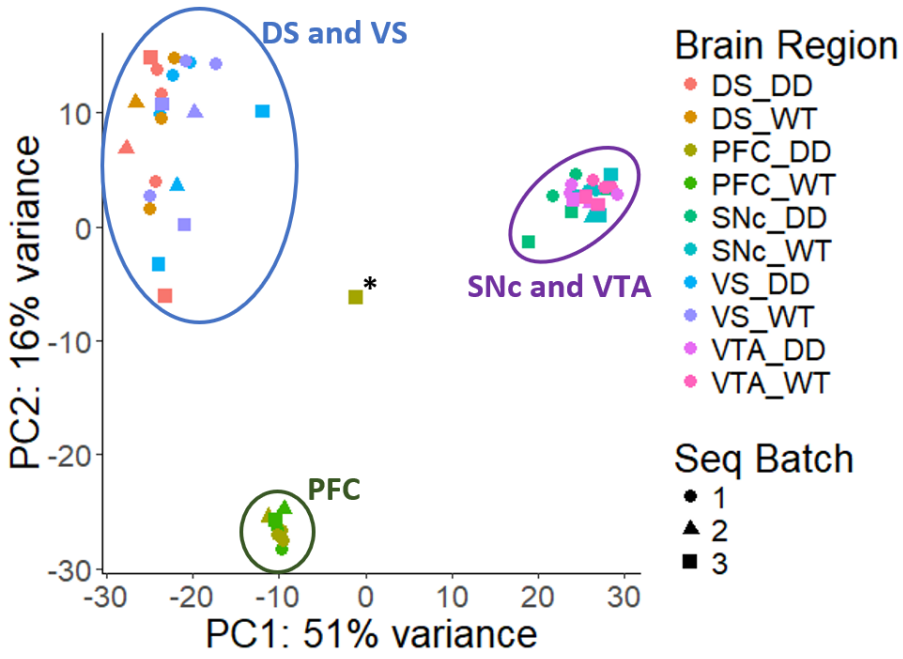

Figure 1. A) Principal Component Analysis of all sequenced samples from three independent batches of RNA-sequencing. Input data are DESeq2 normalized count values based on featureCounts output. Each data point represents an individual library, color coded based on the brain region. The asterisk marks one outlier PFC library.

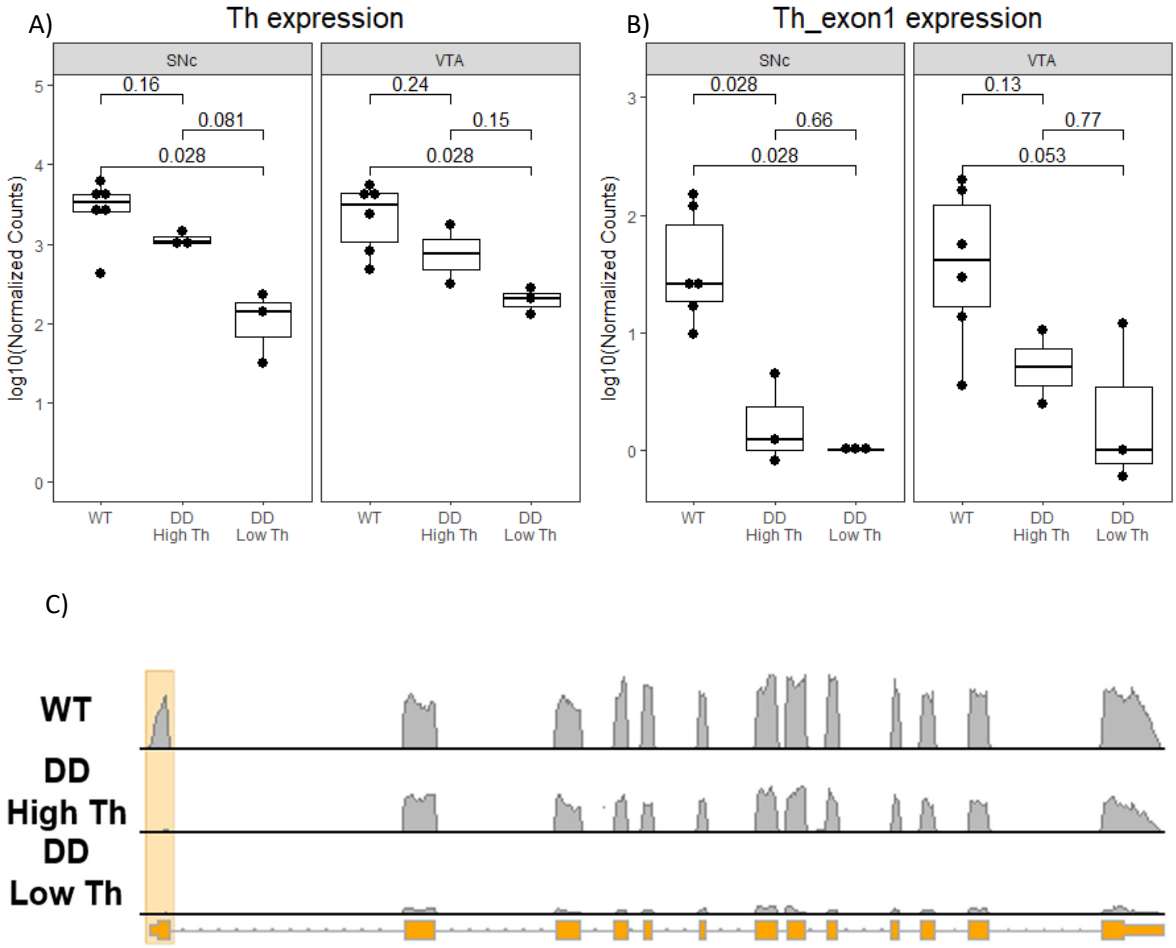

Figure 2. Th expression level in SNc and VTA samples. DAT:TH KO animals were split subjectively into two groups: DD or DD High Th, if any presynaptic library from given animal had Th expression level higher than any WT library from given brain region. DESeq2 normalized counts are based on featureCounts output. A) Normalized expression level of Th in both presynaptic regions. B) Normalized expression level of Th exon 1 is significantly lower in all DD animals in comparison to WT. Numbers over bars represent p-value from Kruskal-Wallis test. C) Representative per base coverage plot of Th. The bottom part of the plots shows known Th exons. Orange square marks the first exon, that should be removed by DAT:Cre recombinase in DD animals. Only few Th exon 1 reads are detectable in all DD samples, however the remaining exons have a high level of coverage in the DD High Th SNc and VTA samples.

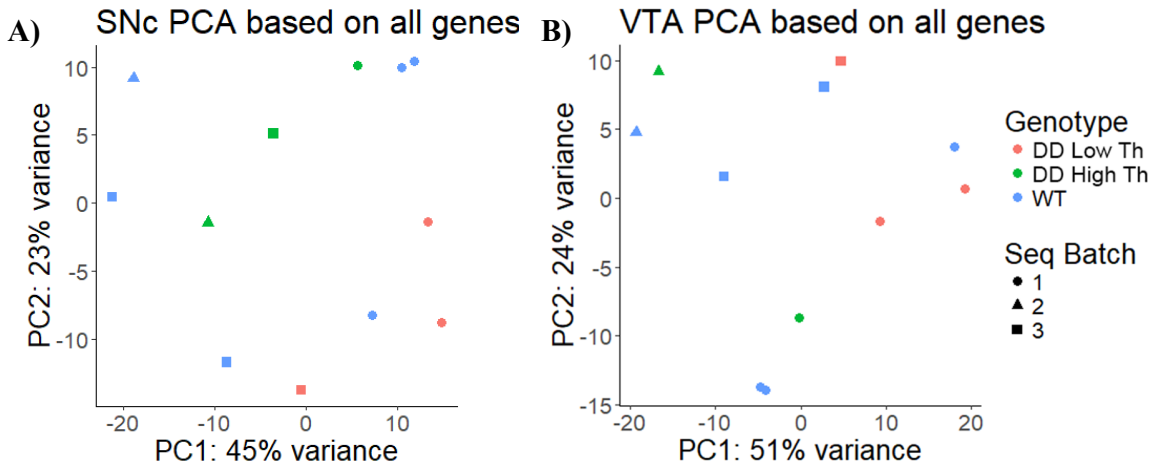

Figure 3. PCA from samples from only SNc and VTA based on detected genes represented by at least 30 normalized counts across dataset from the given brain region.

DD show reduced expression of DA related genes in SNc

To address whether the dopamine deficiency disrupted gene expression in the SNc, we compared transcriptomes from the DD and WT animals using a DESeq2 statistical analysis based on a negative binomial distribution model. We analyzed the expression level of 17,567 genes, finding 38 that are differentially expressed (DE) at a false discovery rate (FDR) <5%. All of the DE genes are downregulated in the Th FLOXed brains (Figure 4). For most genes we observed a ~16-fold expression level decrease ($\log_2$= -4) in in the absence of DA. Among the downregulated genes are transcription factors involved in DA neuron specification: Foxa2, Nr4a2, Pitx3, En1 and En2 (Figure 5A). Consistent with decreased expression of the crucial transcriptional regulators there is a reduced level the classical dopaminergic markers: Ddc, Ret, Slc18a2 (Vmat2), Slc6a3 (Dat) (Figure 5B).

The DD High Th libraries were not used during the detection of the DE genes, however when we contrasted the expression level of the DE genes from the DD High Th with the DD and the WT SNc libraries, they clustered with the WT libraries (Figure 8). Moreover, one WT sample with a relatively low level of the Th expression was grouped with the DD libraries, as all but four DE genes were also strongly downregulated in the SNc from this animal. When we directly compared the DD High Th and the WT SNc samples, none of the previously detected downregulated genes except for Lcn2 had significantly different expression levels.

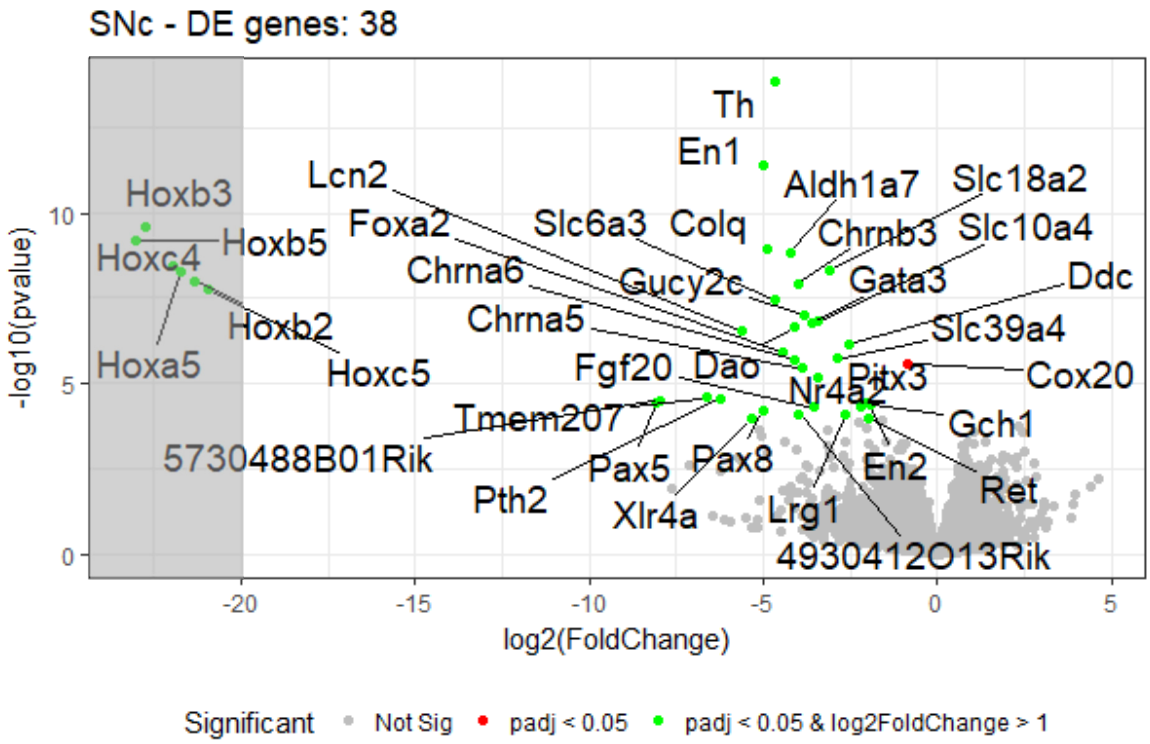

Figure 4. Volcano plot of log2 fold change versus the -log10(P-value). Data points are color coded based on their adjusted p-value at FDR <5% and fold change. Grey box is marking Hox genes omitted in subsequent analysis.

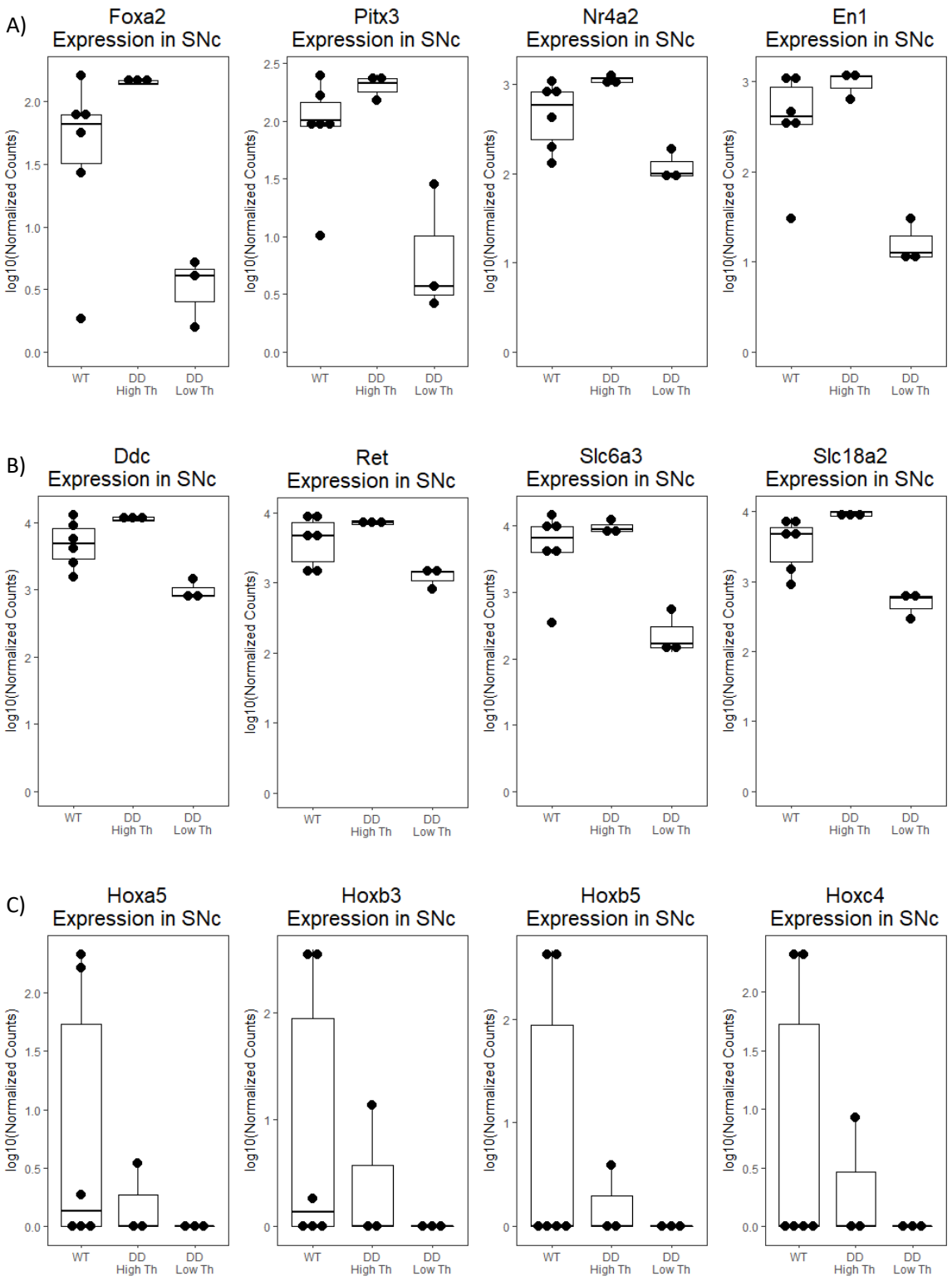

Figure 5. Representative expression level of DE genes. All plotted genes have statistically significant decreased expression level in DD at FDR <5%. A) Log10(Normalized Counts) of representative downregulated transcription factors. A) Log10(Normalized Counts) of representative downregulated dopaminergic markers. C) A group of Hox genes, with over 20-fold reduced expression

in DD SNc. This results from what appears to be artifactual overrepresentation in two WT libraries, with few to no Hox genes reads detected in the remaining samples leading us to omit these genes in subsequent analyses

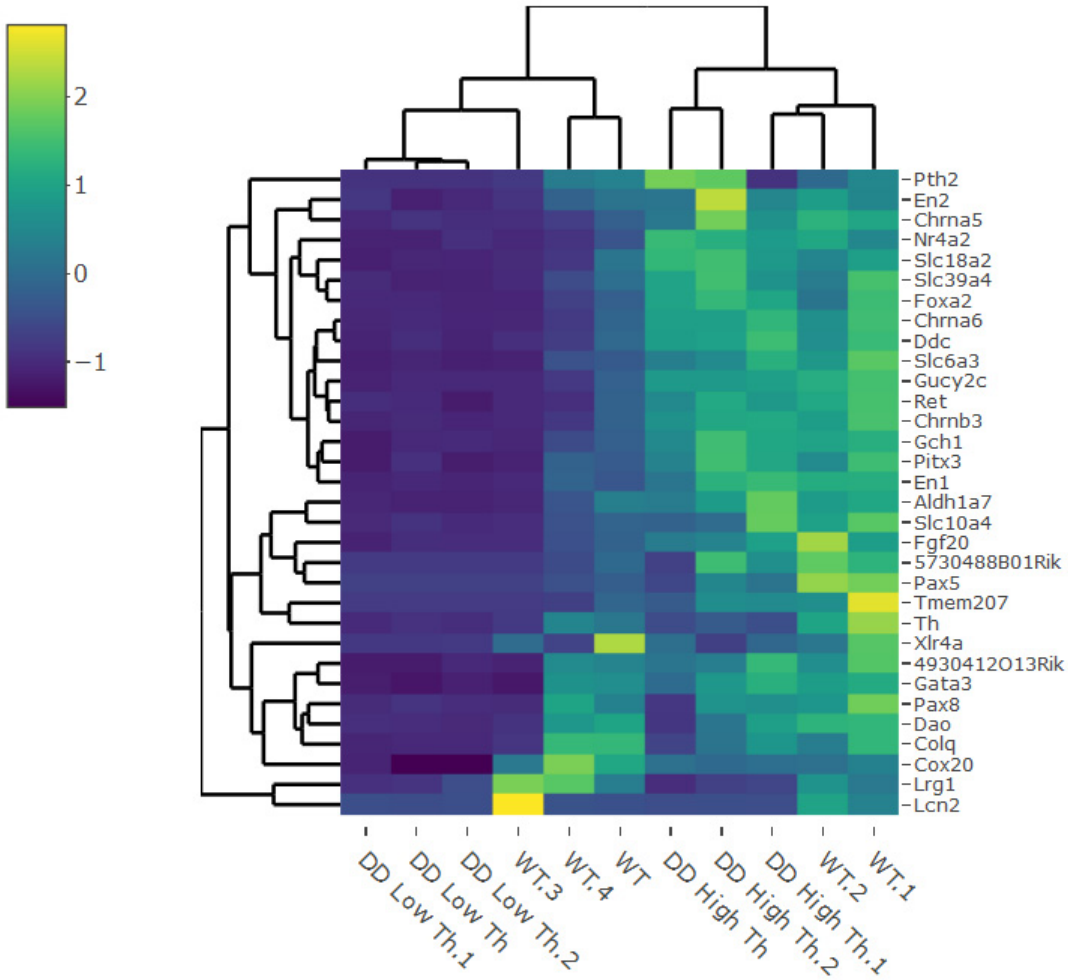

Figure 6. Heatmap representing hierarchical clustering of the SNc libraries based on detected DE genes. Color scale represent Z-score, with yellow marking high expression level.

VTA responds differently than SNc to Dopamine Deficiency

We compared expression levels of 17,244 genes detected in VTA in the DD Low Th and WT samples. We detected 18 DE genes at FDR <5%, half of them upregulated and the other half downregulated in the dopamine deficient background (Figure 7). Six genes were significantly downregulated in DD SNc: Chrnb3, Chrna5, Dao, En1, Pitx3, Slc6a3 (Figure 8). To further examine potential similarities between the dopamine dependency in SNc and VTA we looked at the Gene Ontology terms enriched in detected DE genes. In both presynaptic regions, 'dopamine biosynthetic process' and 'dopaminergic neuron differentiation' were terms with the highest enrichment level among downregulated genes suggesting similar responses to decrease in striatal dopamine level (Figure 10).

The difference in the expression level of upregulated genes was smaller, with an approximately 2-fold upregulation in the absence of dopamine (Figure 8). Moreover, none of these genes are annotated with dopamine related GO terms (Figure 10).

A hierarchical cluster analysis based on the DE genes again shows that all DD Low Th samples are grouped together, whereas DD High Th are more similar to WT (Figure 9). When we compared expression level across all genes, only Rmb3 was significantly upregulated in DD High Th VTA, while none of the DE genes detected between DD and WT had a statistically significant difference in expression level between DD High Th and WT VTA.

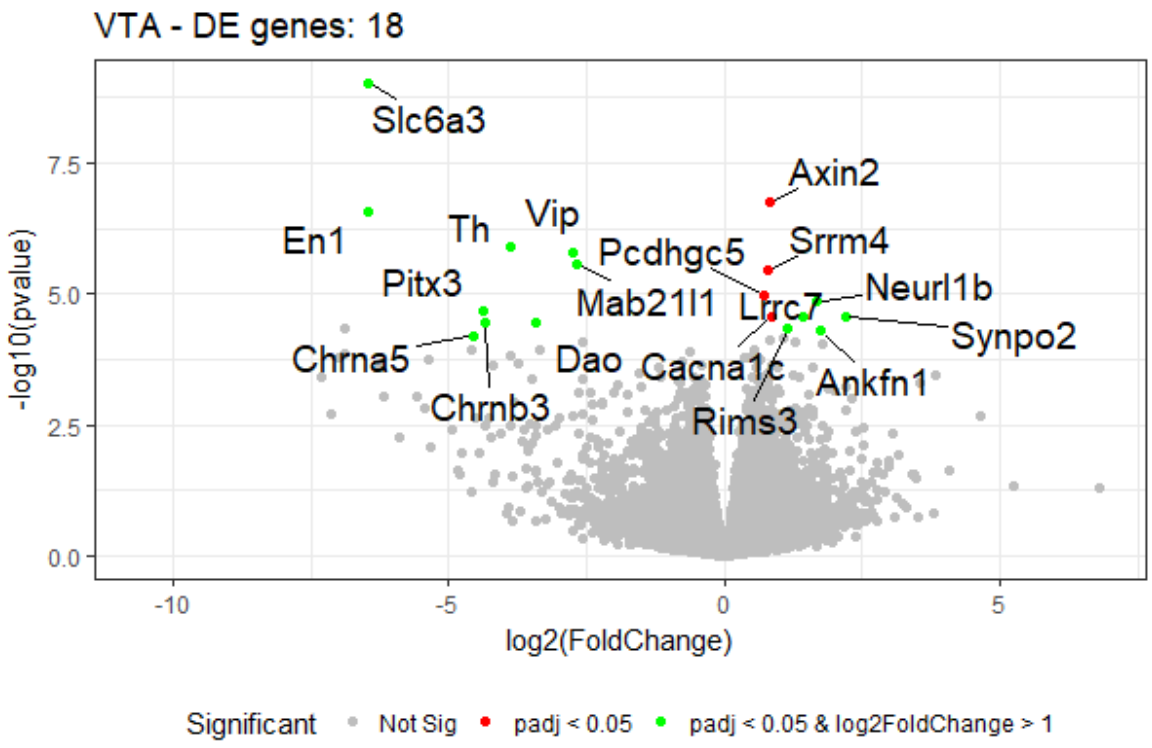

Figure 7. Volcano plot of log2 fold change versus the -log10(P-value). Data points are color coded based on their adjusted p-value at FDR <5% and fold change threshold.

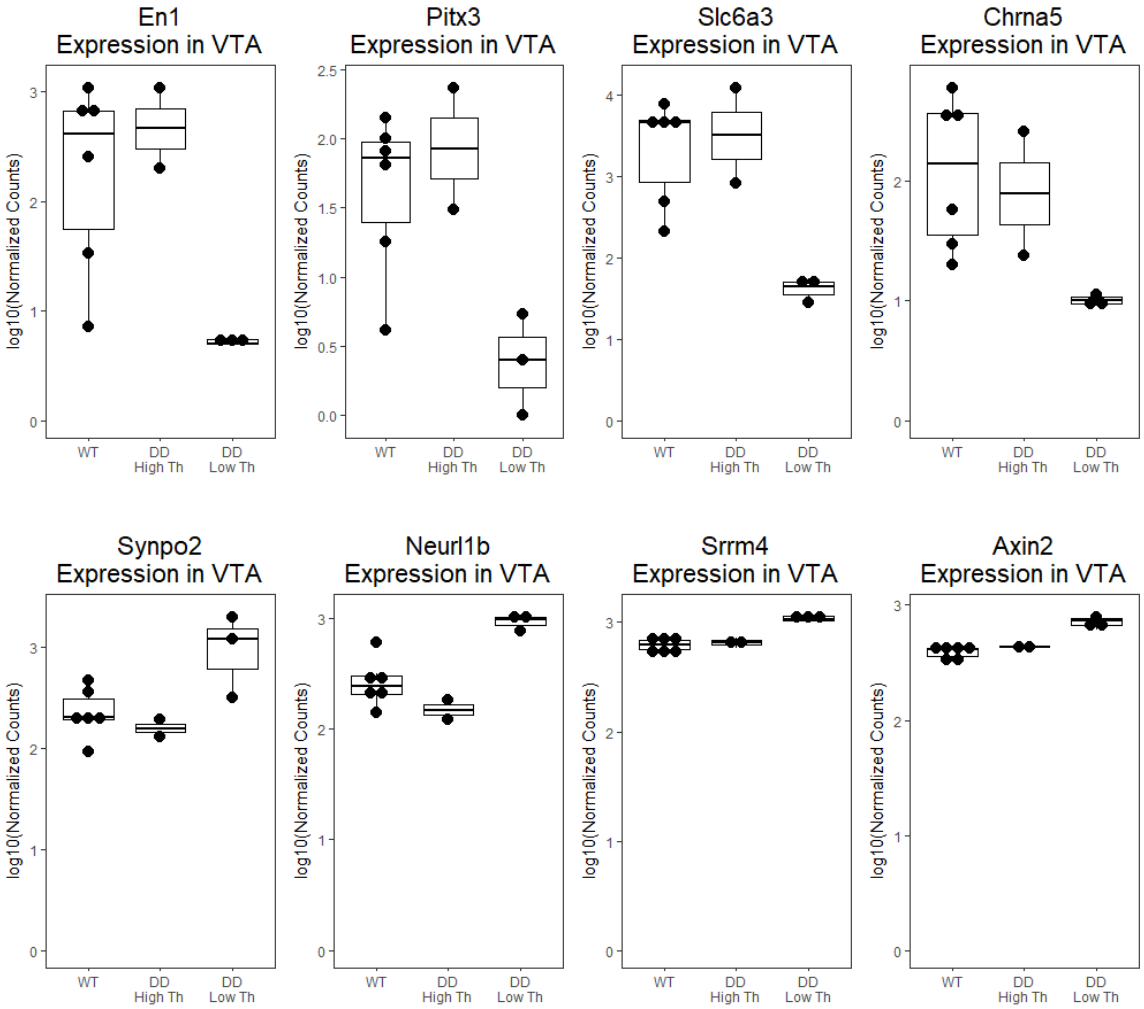

Figure 8. Representative downregulated and upregulated genes in VTA at FDR <5%.

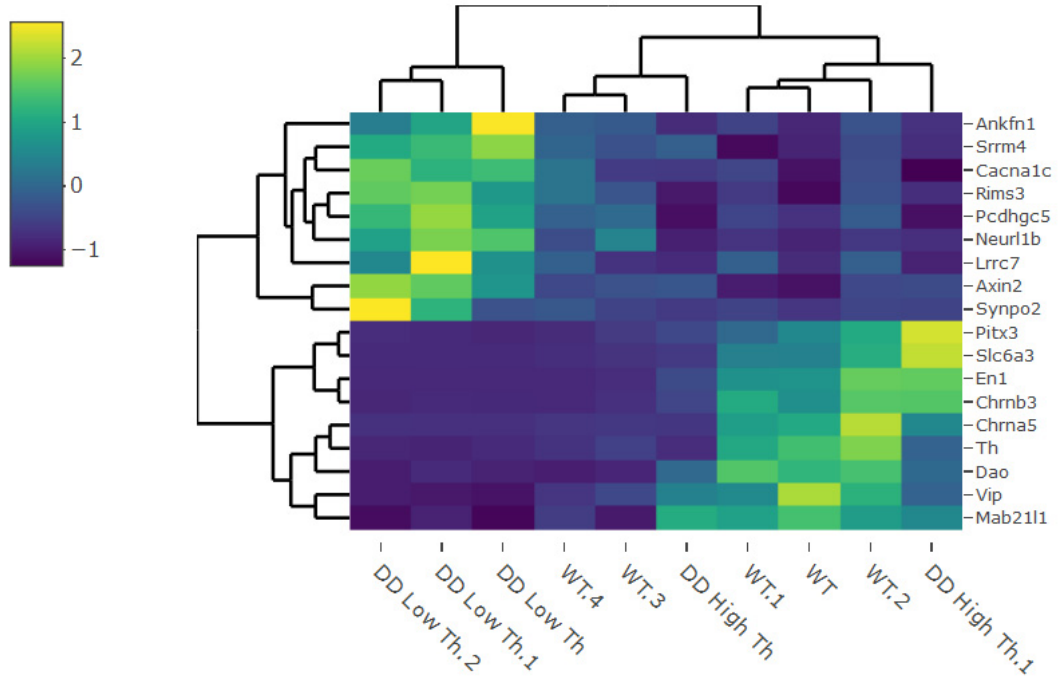

Figure 9. Heatmap representing hierarchical clustering of the VTA libraries based on detected DE genes. Color scale represent Z-score, with yellow marking high expression level.

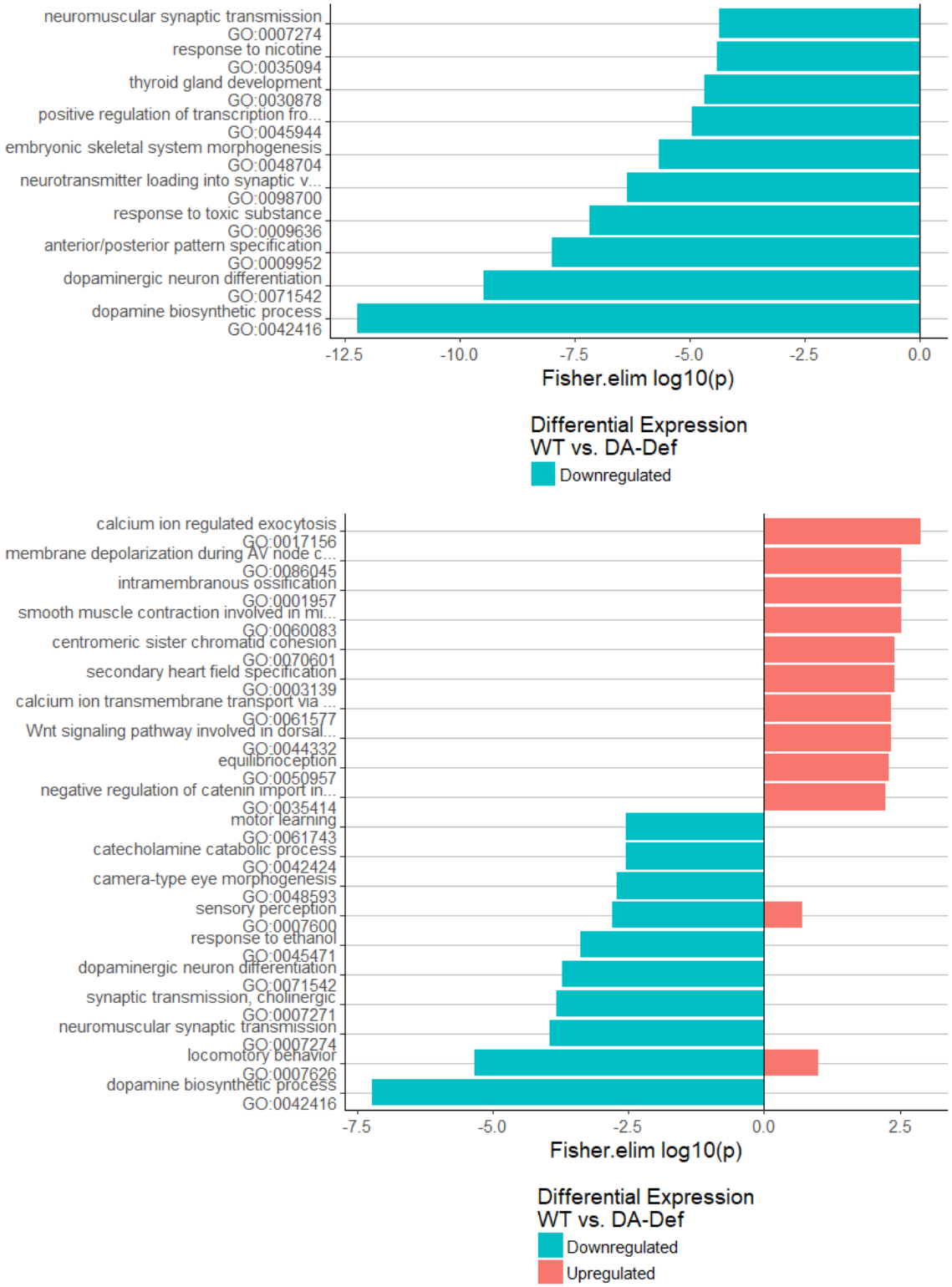

Figure 10. GO terms enriched DE genes in SNc (top) and VTA (bottom).

Discussion

The majority of animal models of Parkinson's disease (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. 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 occurs years after the neurodegeneration has begun (Halliday et al. 2008; Postuma et al. 2012). In this study we used a DD mouse model, generated by DA transporter driven CRE (DAT-CRE) to FLOX the Th gene, with loxP sites flanking Th exon 1, encoding the Th translation startsite. This approach results in the animals that lack 95% of a normal level of DA in striatum, however the number of DA neurons is otherwise unaffected (Garrett Morgan et al. 2015; Henschen, Palmiter, and Darvas 2013). This allows as to observe changes in transcriptome that could have been missed in models based on the depletion of DA neurons. Previously published comparisons between these two approaches shows that more severe symptoms of PD, such as bradyphrenia, cognitive rigidity and spatial learning deficits, are dependent on both loss of DA and DA neurons in striatum. However, cognitive flexibility, working memory and cue-dependent associative learning could be affected by decrease in striatal DA, independent of a depletion of nigrostriatal DA neurons (Garrett Morgan et al. 2015).

Based on the previously described distinct phenotype of DD animals, we hypothesized that potential changes in transcriptome might affect only subpopulations of cells that are the most sensitive to a 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 innervate by VTA and SNc neurons 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. The PCA clearly reflects those differences in transcriptomes of pre- and postsynaptic brain areas (Fig. 1A). The samples of those two origins are strongly separated on the PC1 axis, whereas the PC2 additionally creates cluster of the PFC datapoints. The remaining postsynaptic samples from DS and VS are grouped into a larger, sparse cluster. Similar variability was already described in human samples by Parkes et al., (2017) as they observed that the main source of variance in the samples from ventral, dorsal and caudal parts of striatum is transcriptional variability that is common to all three subregions (Parkes et al. 2017). In contrary, the libraries from both regions enriched in DA neurons - SNc and VTA, create a compact cluster. However, we could not observe the clear separation of DD samples in any of tested regions based on the two first PC. Moreover, a closer examination of all postsynaptic regions did not reveal any DE genes as a function of DA (data not shown). These finding suggest, that previously described transcriptional changes in stratum in neurodegeneration-based PD models are induced by decrease in the number of DA neurons, and not only by the relative decrease in DA level (Chin et al. 2008; Patel et al. 2008; Pattarini et al. 2008; Singh et al. 2010). Subsequently, we focused on the SNc and VTA, as inactivation of Th directly affect neurons form those areas.

Surprisingly, we detected high, near to normal, levels of Th transcript in the half of the DD samples (Fig. 2A). However, almost no reads were mapped to FLOXed Th exon 1 in either of the DD samples, eliminating possibility of an inefficient removal of the targeted region by Cre recombinase (Fig. 2B and C). A few remaining Th exon 1 reads in DD samples could come from the DA neurons that do not express DAT (Henschen et al. 2013).

The unusual Th transcript that is detectable in the DD High Th samples lacks first exon that encodes the normal translation startsite, however the remaining part 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), thought 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 segment (Tekin et al. 2014). The Th transcripts detected in DD High Th animals could potentially represent a novel mouse Th isoform that is differentially regulated relative to wildtype Th. The regional PCAs based SNc or VTA samples show that DD High Th cluster more strongly with WTs than with DD Low Th animals (Fig. 3), supporting this hypothesis. Therefore, we decided to treat the samples from those animals as a separate group, that could represent a potential DA-compensatory mechanism.

Looking more closely at differences between animals with expected low level of Th mRNA and WT controls, we detect 32 strongly downregulated genes in SNc (Fig. 4). 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 logs decrease in expression of those genes could be potentially explained by a depletion of DA neurons in the SNc in DD animals. However, we did not detect a downregulation of antiapoptotic genes or an upregulation of canonical genes connected to cell death 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 DD model (Garrett Morgan et al. 2015; Henschen et al. 2013). Nevertheless, downregulation of DA related genes in SNc of DD Low Th animals is in accordance with changes in PD. Additionally, we have detected downregulation of genes associated with PD, such as neuroprotective Fgf20 (Gao et al. 2008; Sleeman, Boshoff, and Duty 2012; Van Der Walt et al. 2004); and Gch1, which variants are connected with decreased risk of PD (Kalia and Lang 2015). We also found that three different subunits of Nicotinic Acetylcholine Receptor (Chrna5, Chrna6, Chrnb3) are downregulated in DD SNc. Nicotinic receptors are involved in regulation of DA release and are 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 dopamine 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); and precursor of parathyroid hormone 2, Pth2 that interact with Nr4a2, one of the main DA

transcription factor (Kim et al. 2011; Zierold, Nehring, and Deluca 2007). Another potentially important transcription factor that is involved in the development of DA neurons, Pax5 (Simon et al. 2003), is also significantly downregulated in DD Low Th 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 DD as well (Haugas et al. 2016; Moriguchi 2006).

The results of differential gene expression analysis of DD Low Th and WT VTA samples highlight the important differences between VTA and SNc. We not only detect a fewer DE genes, but moreover the half 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 DD Low Th VTA (Th, Slc6a3, Pitx3 and En1; Fig. 7). We also observe a downregulation of the two nicotinic acetylcholine receptor genes, Chrna5 and Chrnb3, that were also downregulated in SNc. 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 the decreased level of DA, as it was described that mice with α5 nicotinic receptor subunit gene knockout have less severe phenotype after treatment with 6-OHDA (Leino et al. 2018).

Another gene of a particular interest, Vip, encoding vasoactive intestinal peptide, has a potential protective activity in PD (Tunçel et al. 2012), also shows decreased expression in DD VTA, in the contrast to our expectations. The next gene that was DE in the direction opposite to anticipated is Rims3, encoding factor regulating synaptic membrane exocytosis. Rims3 is downregulated in PD (Simunovic et al. 2009), however, in our dataset it has over two-times higher expression in DD animals, suggesting that decreased level of this gene in PD could be connect to depletion of DA neurons. Similarly, we observe upregulation of Axin2, a negative regulator of neuroprotective Wnt/βcatenin pathway signaling (Singh et al. 2018; Stephano et al. 2018; Wu et al. 2018). Increased expression of these two, both presumably negative factors, could reflect early changes in PD. Otherwise, we detect one upregulated gene, Cacna1c, that could be connected to an active compensatory or protective mechanism. Variants of Cacna1c, which codes for the L-type calcium channel $Ca_v1.2$, are known as risk factors for psychiatric disorders including schizophrenia and bipolar disorder (Diaries et al. 2013; Working Group of the Psychiatric Genomics Consortium 2014). Furthermore, L-type calcium channel $Ca_v1.2$ is involved in behavioral responses to DA in VTA (Terrillion et al. 2017).

The DD High Th samples were omitted in the differential gene expression analysis. Interestingly, when we compared the expression levels of the genes, detected as differentially expressed between DD Low Th and WT, the samples from DD High Th resemble WT animals. In the hierarchical cluster analysis (Fig. 6 and Fig. 9) all DD High Th 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 DD Low Th animals. Those results support our hypothesis that the alternative Th transcripts from DD High Th animals could rescue DD Low Th phenotype.

However, further studies will be needed to confirm if DD High Th animals exhibit more wildtype-like behavioral phenotype and whether they have more normal levels of striatal DA.

Here we show transcriptome analyses that show an unexpected diversity in response to DA loss between individual animals. Further 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:

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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 (DD) 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 ($Slc6a3^{Cre/+}$). DD 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 DD and 3 WT animals; second with samples from one mouse per group; third with 2 DD 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 (Love, Huber, and Anders 2014). Multiple testing was corrected for using the Benjamini-Hochberg procedure.

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