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Title

Striatal mRNA expression patterns underlying peak dose L-DOPA-induced dyskinesia in the 6-OHDA hemiparkinsonian rat.

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Abstract

L-DOPA is the primary pharmacological treatment for relief of the motor symptoms of Parkinson's disease. With prolonged treatment (≥5 years) the majority of patients will develop abnormal involuntary movements as a result of L-DOPA treatment, known as L-DOPA-induced dyskinesia. Understanding the underlying mechanisms of dyskinesia is a crucial step towards developing treatments for this debilitating side effect. We used the 6-OHDA rat model of Parkinson's disease treated with a three week dosing regimen of L-DOPA plus the dopa decarboxylase inhibitor benserazide (4 mg/kg and 7.5 mg/kg s.c., respectively) to induce dyskinesia in 50% of individuals. We then used RNA-seq to investigate the differences in mRNA expression in the striatum of dyskinetic animals, non-dyskinetic animals, and untreated parkinsonian controls at the peak of dyskinesia expression, 60 minutes after L-DOPA administration. Overall, 255 genes were differentially expressed; with significant differences in mRNA expression observed between all three groups. In dyskinetic animals 129 genes were more highly expressed and 14 less highly expressed when compared with non-dyskinetic and untreated parkinsonian controls. In L-DOPA treated animals 42 genes were more highly expressed and 95 less highly expressed when compared with untreated parkinsonian controls. Gene set cluster analysis revealed an increase in expression of genes associated with the cytoskeleton and phosphoproteins in dyskinetic animals compared with non-dyskinetic animals, which is consistent with recent studies documenting an increase in synapses in dyskinetic animals. These genes may be potential targets for drugs to ameliorate L-DOPA-induced dyskinesia or as an adjunct treatment to prevent their occurrence.

Highlights – 3-5 bullet points summarising findings

- RNA-seq used to profile gene expression changes between dyskinetic and non-dyskinetic animals in L-DOPA-induced dyskinesia.
- Dyskinetic animals have elevated expression of cytoskeletal elements, a potential mechanism for increased dendritic spines.
- Cytoskeletal elements present a potential target for treatments to prevent or ameliorate L-DOPA-induced dyskinesia.

Keywords – max 6 words or phrases

RNA-seq, dyskinesia, 6-OHDA, L-DOPA, striatum, Parkinson's disease.

1. Introduction

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder worldwide, estimated to affect 0.3% of the general population (Tanner and Aston, 2000). At the onset of motor symptoms (resting tremor, bradykinesia, rigidity, and postural instability) more than half of dopaminergic neurons have degenerated (Barbeau, 1960; Ehringer and Hornykiewicz, 1960; Barbeau et al., 1961), resulting in a loss of 80% of the dopamine in downstream nuclei, including the striatum (Ehringer and Hornykiewicz, 1960). The primary drug treatment for Parkinson's disease is replacement of endogenous dopamine with the precursor substance L-DOPA (Kaplan and Tarsy, 2013), in order to restore striatal function (Nicola et al., 2000).

L-DOPA treatment provides relief from the motor symptoms of Parkinson's disease, however the half-life of L-DOPA is only 50 - 90 minutes (Fabbrini et al., 1988; Olanow et al., 2000; Kang et al., 2010), and storage of dopamine is altered in the Parkinsonian brain (Lotharius and Brundin, 2002). Thus PD patients require multiple doses of L-DOPA during the course of a day, which can lead to a number of side effects, including dyskinesia. L-DOPA-induced dyskinesia are additional, unwanted, and often abnormal movements that develop with ongoing L-DOPA treatment. Dyskinesia may affect all areas of the body, including the head and neck, limbs, and torso; interfering with the process of everyday tasks (Barbeau, 1969).

Three major risk factors for development of L-DOPA-induced dyskinesia are: age at diagnosis of Parkinson's disease, initial L-DOPA dose, and length of treatment. A diagnosis before the age of 50 is associated with a 1.5 fold increase in dyskinesia incidence, and an initial dose of L-DOPA of more than 600 mg increases the risk of developing dyskinesia 1.4 fold (Grandas et al., 1999). Treatment with L-DOPA for a period of five years or more will almost always result in development of dyskinesia (Ahlskog and Muenter, 2001). Given the

prevalence and impact of L-DOPA-induced dyskinesia, it is critical to understand their etiology, in order to develop effective treatments to minimise dyskinesia and, in the long term, prevent them from developing altogether.

In animal models of Parkinson's disease, the development of dyskinesia is associated with an alteration in synaptic plasticity mechanisms in neurons in the striatum (Picconi et al., 2003). In a healthy animal, excitatory cortical axons that synapse onto striatal neurons can induce both an increase in synaptic transmission (long term potentiation, LTP) and a decrease in synaptic transmission (long term depression, LTD) following particular patterns of afferent stimulation (Reynolds and Wickens, 2000; Reynolds et al., 2001). Parkinsonian animals are unable to induce corticostriatal LTP without dopamine present (Picconi et al., 2003), however, only non-dyskinetic animals are able to induce de-potentiation, suggesting that the presence of dyskinesia is associated with an inability to reverse previously reinforced motor patterns. We hypothesise that changes in signalling will be accompanied with changes in gene expression.

The development of transcriptomic technologies (e.g., microarrays, RNA-seq) has facilitated large-scale investigation of the pattern of gene expression changes in the brain following treatments. Indeed several studies have investigated the changes associated with dyskinesia using this approach (Konradi et al., 2004; El Atifi-Borel et al., 2009; Heiman et al., 2014). These studies, however, only investigated gene expression in response to a large-dose challenge (El Atifi-Borel et al., 2009), or after brain levels of L-DOPA had markedly reduced (e.g. 18 hours after the last dose; Konradi et al., 2004).

Here, for the first time, we use RNA-seq technology to examine global changes in striatal gene expression between dyskinetic and non-dyskinetic animals, at a time point consistent with peak-dose dyskinesia (Kang et al., 2010). We use a model of L-DOPA-induced dyskinesia where all treated animals received the same L-DOPA dose but only half displayed dyskinesia at three weeks of treatment (Picconi et al., 2011). This provides a control group (non-dyskinetic) that has been subjected to exactly the same chronic treatment regimen as the experimental group (dyskinetic). Using these animals we examined the gene expression changes at the peak of L-DOPA levels in the blood, 60 minutes after the last L-DOPA injection (Kang et al., 2010), with the aim of identifying genes associated with peak-dose dyskinesia that could act as potential drug targets in the future.

2. Experimental procedures

2.1 Animals

18 male Wistar rats were obtained and housed in the Hercus Taieri Research Unit at the University of Otago. All rats were used in each aspect of the study. Rats were housed on a 12-hour reverse light-dark cycle with food and water *ad libitum*. All procedures were approved by the University of Otago Animal Ethics Committee.

2.2 Lesioning and behavioural testing

At 7-weeks of age rats received a unilateral lesion of the medial forebrain bundle using 6-hydroxydopamine (6-OHDA). Rats were anesthetised with ketamine (7.5 mg/kg s.c., Phoenix Pharm) and domitor (0.5 mg/kg, s.c., Orion Pharma) with atropine (0.05 mg/kg, s.c., Phoenix Pharm). Local anesthetic (bupivacaine 5%, Astra Zeneca) was injected at the incision site and the rat placed in the stereotaxic frame. The neurotoxin 6-OHDA HBr (Sigma-Aldrich) was administered to the left medial forebrain bundle via a cannula placed at +4.4 mm AP, +1.2 mm ML, and -8.3 mm DV to bregma (relative to the skull surface). 6-OHDA was administered at a concentration of 6 µg dissolved in 3 µl of 0.9% saline solution containing 0.008% L-ascorbic acid (Sigma-Aldrich) and infused at a rate of 0.3 µl/min (Parr-Brownlie et al., 2007). Noradrenergic neurons were preserved by administration of desmethylimipramine (desipramine hydrochloride, Sigma-Aldrich, 4.5mg/kg administered 30 minutes prior to surgery). Rats received pain relief (carpreive 5 mg/kg s.c., Norbrook) and anesthetic reversal (antisedan 2.5 mg/kg s.c. Orion Pharma,) before being placed in a warm fresh cage for post-operative recovery. Soft chow was provided ad libitum post-operatively to maintain weight. Five days after lesion surgery, lesion extent was examined using the adjusting step test (Olsson et al., 1995) and the forelimb use asymmetry test (Schallert et al., 2000); significance

was assessed using Excel 2011 with a paired Student's t-test for the adjusting step test and a one-way ANOVA for the forelimb asymmetry test

2.3 L-DOPA dosing and Abnormal Involuntary Movement scoring

Two weeks after lesion surgery, successfully lesioned rats (n = 18) were divided randomly into two groups, L-DOPA treated and parkinsonian untreated. The L-DOPA treated group (2/3 of the lesioned rats) received 3-4 mg/kg of L-DOPA (dissolved in saline 1 ml/kg with 7.5 mg/kg of benserazide HCl to prevent peripheral degradation) s.c. twice daily for three weeks. The remaining 1/3 of lesioned rats formed the parkinsonian untreated group and received equivalent volumes of saline plus benserazide (vehicle) s.c. on the same treatment schedule. At the end of each week dyskinesia was scored 60 minutes after an L-dopa dose using the well-established Abnormal Involuntary Movements (AIM) scoring for rodents (Winkler et al., 2002). Rats were evaluated for axial dystonia, forelimb dyskinesia, and orolingual dyskinesia; and combined to give an aggregate AIM score each week. Rats were defined as non-dyskinetic when the aggregate AIM score was zero for all three weeks of treatment.

2.4 Sacrifice, RNA extraction and sequencing

At the completion of the L-DOPA treatment regime rats were sacrificed by decapitation using a guillotine 1-hour after the final L-DOPA or vehicle injection (6 dyskinetic, 6 non-dyskinetic, and 6 untreated parkinsonian animals). Because a third of the rats were exhibiting peak-dose dyskinesia at the time of sacrifice, all animals were restrained in a decapacone™ at the time of sacrifice. Animals were previously familiarised with the decapacone daily for three days prior to sacrifice, to minimise restraint stress. The brain was rapidly removed, the lesioned striatum was dissected out and snap frozen on dry ice, and the remainder of the brain post-fixed in 4% paraformaldehyde for later confirmation of the lesion extent.

RNA extraction was performed using the Qiagen RNeasy® mini kit. RNA was quantified using the Qubit® RNA Broad Range assay kit and quality was assessed using the Agilent 2100 Bioanalyzer and the Agilent RNA 6000 Nano Kit (Agilent Technologies). To minimise differences attributable to variation between individuals RNA was pooled for RNA-seq analysis. RNA from sets of three animals was combined based on RNA concentration, resulting in two pools of 2 μg of RNA per group (dyskinetic, non-dyskinetic, and untreated parkinsonian). The RNA that was not used for the RNA-seq pools was stored at -80°C and used for subsequent RT-qPCR verification of RNA-seq results.

RNA-seq was performed using the Illumina HiSeq 2000 platform (service provided by BGI, Shenzhen, China). Each sample (pool) produced 12 million single end reads of 50 base pairs. Tarazona et al. (2011) demonstrated that this depth of sequencing was able to detect transcripts from approximately 18,000 genes in human brain samples, and thus provides good coverage of the transcriptome. It is possible that low abundance RNAs as well as smaller RNAs may be missed by this approach, although RNA-seq has a many-fold greater dynamic range than microarrays, so its ability to quickly and accurately assess cellular transcription is in general excellent (Wilhem et al. 2009). Relative to microarrays, RNA-seq has the advantage of not pre-selecting for known genes or genes of interest. That is, as more genomic elements are discovered/annotated, the alignment process can be rerun using the existing sequence data and updated genome annotation – there is no requirement to generate new RNA samples, as would be the case with microarrays.

Bioinformatic analysis was performed using CLC Genomics Workbench version 6 (CLC bio) (Cameron et al., 2013). Adaptor sequences, contamination and low quality reads were

removed by the sequence service provider. Reads were mapped to the annotated rat genome (RGSC v 3.4.65) using the RNA-seq tool with an average mapping rate of 87%. In total the tool mapped reads against 29,516 annotated rat genes and resulted in positive reads (at least 1 read) for 18,919 genes. Non-specific matches (more than two mismatches per read) were discarded, as were multiply mapped reads. Expression values were calculated based on reads that uniquely mapped to a gene (unique gene counts). Samples underwent quantile normalisation before being combined within groups. The correlation between the two pools which combined made up a treatment group was $R^2 = 0.99$ for all three treatment groups indicating a high degree of similarity between the two pools. Differences in gene expression between treatment groups (dyskinetic, non-dyskinetic, and untreated parkinsonian) were assessed using the Baggerly test (Baggerly et al., 2003) with the Benjamini and Hochberg correction (Benjamini and Hochberg, 1995) used to control for multiple testing. The Baggerly test is similar to a two sample t-test but the test statistic is weighted by the number of reads in each sample (Baggerly et al., 2003). An adjusted p-value of 0.05 was used as the threshold for significance (Cameron et al., 2013). A subset of genes were selected for further examination, based on their role as transcription factors and genes known to be activated soon after dopamine receptor stimulation (Cole et al., 1992; Moratalla et al., 1992; Berke et al., 1998). These genes: *Arc, Egr1, Fos, Junb, and Nr4a1,* were examined using TaqMan gene expression probe sets (Life Technologies) to confirm gene expression patterns observed in the RNA-seq dataset. This was conducted using pooled RNA that had been conserved prior to construction of the RNA-seq libraries. Gene expression quantification was conducted using a Roche LightCycler 480 and expression normalised to two standards *Tbp* and *Slc35f2* based on a pilot study of reference genes in this tissue (data not shown). Differences between groups were analysed using a one-way ANOVA with a Turkey post-hoc test, with a p-value threshold of <0.05.

Gene set cluster analysis was performed using the online Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (Huang da et al., 2009) with the full expressed gene set as a background and a Fisher's exact test limit of 0.1. This analysis identified sets of genes with similar functions that are overrepresented in the sample. The top ten gene set categories are shown in the results section.

2.5 Confirmation of lesion extent using tyrosine hydroxylase immunohistochemistry

Brain slices were cut on a vibratome in 50 μm sections, and every second section containing the substantia nigra was stained for tyrosine hydroxylase immunoreactivity. The primary antibody was Millipore rabbit anti-tyrosine hydroxylase (AB152) diluted 1:1600 and applied for 24 hours at 4 °C. The Vectastain Elite ABC Rabbit IgG kit (Vector Laboratories) was then used. Immunoreactivity was visualised with 3,3'-diaminobenzidine reacted for 5 minutes. Slides were visualised using a Zeiss Axioskop light microscope at 5x and 10x magnifications. Images were photographed at 10x with an Olympus DP71 camera and processed using the Olympus micro DP controller, before being saved as JPEG files. JPEG files were processed for cell counts using MBF-Image J software as previously described (Clements et al., 2012). Three sections were examined per brain; with the substantia nigra pars compacta outlined in Image-J prior to cell count procedures. Cell counts are presented as percentage of the non-lesioned hemisphere and differences between hemispheres were calculated using a paired Student's t-test (Excel 2011).

3. Results

3.1 Analysis of lesion extent

The extent of the dopaminergic lesion was estimated pre-mortem and post-mortem (Figure 1). Pre-mortem estimates used the adjusting step test and forelimb use asymmetry test (Olsson et al., 1995; Schallert et al., 2000; Tseng et al., 2005; Parr-Brownlie et al., 2007). Successfully lesioned animals used the affected right paw for less than 5% of movements in both the step test (Figure 1 A; $p < 0.001$, $F = 259$) and the asymmetry test (Figure 1B; $p < 0.001$, $F = 899$). Following all experimental procedures, lesion extent was confirmed post-mortem by immunohistochemical analysis of the substantia nigra pars compacta (SNc; Figure 1 G). In the lesioned hemisphere less than 5% of dopaminergic neurons remained in the SNc compared with the intact hemisphere; $p<0.05$ (Figure 1 H; $p < 0.001$, $F = 177$).

3.2 Rating of L-DOPA-induced dyskinesia

L-DOPA-induced dyskinesia status was evaluated one hour after L-DOPA injection using the rat AIM rating scale (Winkler et al., 2002). All dyskinetic animals showed a positive score in each of the three categories examined (axial dystonia - Figure 1 C, forelimb dyskinesia – Figure 1 D, and orolingual dyskinesia – Figure 1 E), with an average total score of 7.1 ± 2.5 (mean ± S.D.; Figure 1 F). Non-dyskinetic animals were defined as animals that scored zero in all categories examined (Figure 1 F), with an overall score of zero; similarly, untreated parkinsonian animals showed no signs of abnormal involuntary movements. The difference between groups was statistically significant ($p < 0.001$, $F = 38$). It was noted during AIMs analysis that non-dyskinetic animals displayed an improvement in overall locomotor ability, however this was not specifically tested and thus not quantified.

3.3 RNA-seq profiles

Transcriptomic analysis was performed on total RNA samples isolated from the lesioned striatum of dyskinetic, non-dyskinetic, and untreated parkinsonian animals 1-hour following the final L-DOPA or vehicle injection. This allowed examination of the striatial gene expression profiles of dyskinetic, non-dyskinetic, and untreated parkinsonian animals at the peak L-DOPA effectiveness. Significant differences in gene expression were observed between all three groups (Figure 2); 267 genes were differentially expressed in total (Figure 2 A; Table 1), with 129 more highly expressed and 14 less highly expressed in L-DOPA treated dyskinetic animals and non-dyskinetic animals (Figure 2 B and 2 C; total genes enclosed within complete topmost circles). In L-DOPA treated animals (both dyskinetic and non-dyskinetic) 42 genes were more highly expressed and 95 less highly expressed when compared with untreated parkinsonian controls (Figure 2 B and 2 C; total genes enclosed within both of the lowermost circles). A subset of genes was selected for further examination, based on their role as transcription factors and immediate early genes, known to be activated in response to dopamine receptor stimulation (Cole et al., 1992; Moratalla et al., 1992; Berke et al., 1998). Expression was examined in RNA from the individual rats in the RNA-seq experiment using RT-qPCR to verify patterns observed in the RNA-seq dataset and the results compared with the RNA-seq counts (Figure 3). There was a positive correlation ($R^2 = 0.834$) between the RNA-seq and qPCR data. The data points that did not conform may be due to the effect of pooling RNA in RNA-seq samples compared with individual animal RNA examined with RT-qPCR or the sensitivity associated with qPCR.

3.4 Gene ontology analysis

Gene ontology analysis revealed changes between all groups (Figure 4). One interesting change was a decrease in expression of genes involved in DNA-binding, transcription, and regulation of transcription in L-DOPA-treated dyskinetic and non-dyskinetic animals

compared with their untreated parkinsonian controls (Figure 4 B and C). In particular immediate early genes and transcription factors were decreased in dyskinetic animals compared with untreated parkinsonian controls (*Atf3* -6.91 fold, FDR 0.04; *Fos* -7.33 fold, FDR 1.52E-158; *Fosb* -3.98 fold, FDR 9.03E-29; *Egr1* -2.93 fold, FDR 2.72E-94; *Egr2* -6.65 fold, FDR 1.63E-06; *Egr3* -1.59 fold, FDR 5.8E-06; *Egr4* -2.31 fold, FDR 4.08E-43; *Junb* -3.55 fold, FDR 6.61E-26; *Nr4a1* -2.62 fold, FDR 2.65E-41; *Nr4a3* -2.96 fold, 1.97E-60).

Gene enrichment analysis revealed dyskinetic animals exhibit a distinct increase in expression of genes involved in cytoskeletal organisation, cytoplasmic vesicles, endocytosis, and phosphoproteins compared with the non-dyskinetic animals (Figure 4 A). In particular dynein complex genes (*Dnaaf3* 2.31 fold, FDR 0.01; *Dnah1* 3.01 fold, FDR 0.01; *Dnah2* 2.12 fold, FDR 5.11E-09; *Dnah11* 2.3 fold, FDR 7.23E-09; *Dnah10* 1.94 fold, FDR 0.04), integrin (*Itgb4* 2.74 fold, FDR 0.04), actin (*Acta1* 1.62 fold, FDR 0.03), and activity related cytoskeletal genes (*Arc* 1.51 fold, FDR 9.8E-03) were increased in dyskinetic animals compared with non-dyskinetics. This indicates a potential change in cytoskeletal structure and signalling in dyskinetic animals.

Phosphoproteins that were increased in dyskinetic animals include aurora kinase B (*Aurkb* 7.81 fold, FDR 2.96E-08), calcyon neuron-specific vesicular protein (*Caly* 1.2 fold, FDR 0.02), protein phosphatase 1 regulatory subunit 15a (*Ppp1r15a* 1.34 fold, FDR 3.9E-04), and regulator of g-protein signalling 14 (*Rgs14* 1.56 fold, FDR 1.28E-03).

4. Discussion

L-DOPA remains the gold standard treatment for alleviating symptoms in PD patients, however the majority of patients will develop dyskinesia with prolonged L-DOPA treatment.

The experiments presented here examined the gene expression profile associated with L-DOPA-induced dyskinesia at the peak of L-DOPA dose with the aim of finding patterns of gene expression changes related to the most common form of dyskinesia in humans, so-called peak-dose dyskinesia. This is a unique timepoint; previous studies have examined gene expression changes that are not attributable to the peak of L-DOPA concentration. This has left us with a dearth of understanding about changes occurring at the peak of L-DOPA concentration in the brain that might underlie the chronic behavioural characteristics related to dyskinesia. According to Jenner "it is still unclear how striatal output contributes to dyskinesia … this makes improving our understanding difficult: in both MPTP-treated primates and 6-OHDA-lesioned rats, the task of distinguishing the causes and consequences of dyskinesia is a major challenge" (Jenner, 2008). Such understanding is required for the development of therapies that might ameliorate the debilitating effects of dyskinesia or prevent their development.

We found 267 genes to be significantly differentially expressed between dyskinetic, non-dyskinetic, and/or untreated parkinsonian controls at the peak of L-DOPA dose. Of those, almost half (143) of the differences were observed between the dyskinetic and non-dyskinetic groups. The majority of differentially expressed genes were more highly expressed in the dyskinetic group compared to the non-dyskinetic and untreated parkinsonian groups. qPCR validation of the RNA-seq gene expression patterns confirmed the data with a strong positive association identified ($R^2 = 0.834$) and the non-conforming genes were typically those with small fold changes.

Gene ontology analysis showed enrichment in cytoskeletal-associated genes between dyskinetic and non-dyskinetic rats. Recent microscopy studies examining differences in the

spines of striatal neurons found an increase in the number of cortico-striatal synapses in L-DOPA-treated rats compared with untreated parkinsonian controls (Zhang et al., 2013; Fieblinger et al., 2014). Changes in synapse number or size require changes to the underlying architecture of the neuron, the cytoskeletal frame. Interestingly, we observed changes in the genes underlying cytoskeletal architecture; namely *dynein complex*, *ephrin b*, *cadherin*, and *integrin* genes were more highly expressed in dyskinetic rats compared to both untreated parkinsonian controls and non-dyskinetic animals. Cadherins and catenins, along with Arc, are known to interact with and alter the actin cytoskeleton structure (Tanaka et al., 2000; Drees et al., 2005); Figure 5). We propose that this increase in transcription may indicate a mechanism for the previously observed increase in the number of synapses in dyskinetic animals (Zhang et al., 2013; Fieblinger et al., 2014). However, our changes were observed on a timescale consistent with peak-dose dyskinesia, whereas Zhang et al (2013) made these observations 72 hours after the last dose. There is evidence that spine morphology changes can occur within a period of <30 minutes (Maletic-Savatic et al., 1999; Nikonenko et al., 2002). Hence, it is possible that the changes in gene expression we observed may underlie changes in the cytoskeleton in response to acute L-DOPA dosing, although a systematic comparison with changes in these genes, their protein products, and microscopy to highlight subcellular changes in cytoskeletal networks on a chronic timescale would be needed to draw this conclusion. If this association occurs at the protein level then cytoskeletal elements represent a potential drug target area for treatment of L-DOPA-induced dyskinesia. Pharmacological agents that modify cytoskeletal elements could be administered in conjunction with L-DOPA/benserazide in order to decrease the expression of selected cytoskeletal genes and prevent the increase in synaptic number. However, given the ubiquitous nature of cytoskeletal architecture across the brain and indeed the body, any

potential drugs will need to be thoroughly tested for side-effects, or drugs targeted specifically to the striatum, when such approaches become readily available.

Parkinsonian animals show impairment in the ability to induce synaptic potentiation in response to electrical stimulation, which is overcome with dopamine treatment. However, only non-dyskinetic animals are able to show subsequent depotentiation (Picconi et al., 2003). Our results showed enrichment for 37 phosphoproteins in dyskinetic animals compared with non-dyskinetic animals. The increase in expression of genes encoding phosphoproteins observed in this study is consistent with previous findings that dyskinetic animals have increased phosphorylated DARPP32 (Picconi et al., 2003), ERK (Pavon et al., 2006; Santini et al., 2007; Westin et al., 2007), and MEK (Santini et al., 2007) resulting in increased potentiation at synapses and impaired depotentiation. Drugs that reverse protein phosphorylation (phosphatases) represent a potential treatment avenue that could be used to reduce the phosphorylation of phosphoproteins we have identified as being expressed and potentially rescue depotentiation at synapses in dyskinetic animals. Previous studies have shown that blocking an inhibitor of protein phosphatase 1 in dyskinetic animals rescues the ability to depotentiate (Picconi et al., 2003); our paper suggests further targets for protein phosphatase treatment. However, these drugs will need to be specifically targeted to the brain region and specific phosphoprotein to minimise off-target effects given that phosphorylation is a key part of normal cell-signalling.

In contrast to the signalling changes observed between dyskinetic and non-dyskinetic animals, differences in mRNA expression between L-DOPA treated and untreated parkinsonian controls were primarily genes associated with DNA binding, transcription, and regulation of transcription. Untreated parkinsonian controls did not receive L-DOPA at any point in the

study, thus this is a result of one group of animals receiving an intervention (L-DOPA) and the controls receiving no effect from the vehicle injection. In addition, the changes identified between the dyskinetic and non-dyskinetic animals were different to those of untreated parkinsonian controls, and thus not a reflection of a failed L-DOPA injection (non-dyskinetic).

This is the first study to use RNA-seq analysis of the transcriptome to compare the overall profile of gene expression in the striatum of dyskinetic, non-dyskinetic, and untreated parkinsonian animals at the height of L-DOPA-induced dyskinesia expression. Our results imply that genes and proteins involved in cytoskeletal processes may be a potential target for drugs to treat L-DOPA-induced dyskinesia. However, our results have been obtained using an analysis of tissue RNAs, which will show changes over all cell types. Recent studies have shown that a cell-type specific analysis can better differentiate functional changes in protein phosphorylation and gene expression (Heiman et al., 2014). Given the influence that small populations of neurons such as cholinergic interneurons have with respect to dyskinesia (Ding et al., 2011; Won et al., 2014), extensive further study is required, focussing on the contribution of single populations of cells to the overall gene expression pattern.

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Figures Legends

Figure 1. Pre-mortem assessment of lesion extent and dyskinesia score, and post-mortem confirmation of the extent of lesion. Pre-mortem behavioural tests indicate successful lesion; the affected right paw makes < 5% of the steps the unaffected left paw makes in the adjusting step test **A** and the cylinder-based forelimb use asymmetry test **B**. Animals were assessed for dyskinetic behaviour after three weeks of L-DOPA dosing. Three categories were assessed: axial dystonia **C**, forelimb dystonia **D**, and orolingual dystonia **E**, to give a final dyskinesia extent score out of 12 **F**. Animals who received L-DOPA but scored a zero total dyskinesia score at week three were classified as non-dyskinetic. Animals who did not receive L-DOPA did not show any signs of dyskinesia during assessment. A dyskinetic animal was one who scored at least 1 in the total dyskinesia score at week three. At the conclusion of the experiment post-mortem tyrosine hydroxylase immunohistochemistry confirmed >95% loss of dopamine neurons (Example brain slice **G,** quantification **H)**. Total dyskinesia scores for untreated parkinsonian and non-dyskinetic animals depict the absence of any dyskinesia signs in any animals but the dyskinetic group **F**. All data are shown as mean ± standard deviation. * indicates a p-value of < 0.05 denoting significance, which was assessed using a Student's t-test for A and H and a one-way ANOVA for B, C, D, E and F.

Figure 2. RNA-seq analysis of differential gene expression between groups 1-hour after L-DOPA or vehicle treatment (Untreated parkinsonian control). Overall expression changes are shown (A), those where expression was highest in the first group of the comparison (B) and those where expression was lowest in the

first group of the comparison (C). Overall there were 267 differentially expressed genes (found by the summation of the numbers in A). Differences between groups were assessed using a Baggerly test (Baggerly et al., 2003) and the threshold for a significant difference was a Benjamini and Hochberg adjusted p-value of <0.05.

Figure 3. Concordance of changes in gene expression between RNA-seq and qPCR experiments. Comparison was made between the RNA-seq counts and qPCR results for five genes of interest. For qPCR all genes were normalised to *Tbp* and *Slc35f2* and all results were normalised to the untreated parkinsonian group. There was a positive correlation between the RNA-seq counts and qPCR results (R^2 value 0.83).

Figure 4. DAVID Gene ontology analysis conducted to identify gene set enrichment. Genes ontology terms enriched in the comparison between dyskinetic and non-dyskinetic **A**, dyskinetic and untreated parkinsonian **B**, and non-dyskinetic and untreated parkinsonian **C**. Gene ontology terms are listed in order of significance, as assessed with Fisher's exact test (bottom axis, black line) and show the number of genes identified as significantly changed (top axis, green bars) that fit into a classification group.

Figure 5. Schematic of postsynaptic protein interactions thought to be altered in dyskinetic L-DOPA-treated animals. Activation of NMDA and dopamine D1 receptors induces changes in MAPK signalling, phosphorylating the transcription factor ERK. The dynein protein complex (purple) then transports transcription factors (e.g. ERK) down microtubules to the dendritic shaft and cell soma to induce changes in gene transcription. Kinesin (brown) transports proteins such as ARC to the dendritic spine. ARC interacts with: (i) actin and actin binding proteins to induce activity-related changes in spine shape and structure (Tanaka et al., 2000; Drees et al., 2005); (ii) AMPA receptors to induce internalisation of these receptors, resulting in increased glutamate activation of NMDA receptors; and (iii) integrin to stabilise the spine structure in its altered state. Figure based on gene expression changes observed in this study, which are indicated with green arrows, as well as from Tai et al. (2008) and Dalva et al. (2007).

References

Baggerly KA, Deng L, Morris JS, Aldaz CM. 2003. Differential expression in SAGE: accounting for normal between-library variation. Bioinformatics 19:1477-1483.

Barbeau A. 1960. Preliminary observations on abnormal catecholamine metabolism in basal ganglia diseases. Neurology 10:446-451.

Barbeau A. 1969. L-dopa therapy in Parkinson's disease: a critical review of nine years' experience. Can Med Assoc J 101:59-68.

Barbeau A, Murphy GF, Sourkes TL. 1961. Excretion of dopamine in diseases of basal ganglia. Science 133:1706-1707.

Benjamini Y, Hochberg Y. 1995. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B (Methodological) 57:289-300.

Berke JD, Paletzki RF, Aronson GJ, Hyman SE, Gerfen CR. 1998. A complex program of striatal gene expression induced by dopaminergic stimulation. J Neurosci 18:5301-5310.

Cameron RC, Duncan EJ, Dearden PK. 2013. Biased gene expression in early honeybee larval development. BMC genomics 14:903.

Clements K, Smith L, Reynolds J, Overton P, Thomas J, Napper R. 2012. Early Postnatal Ethanol Exposure: Glutamatergic Excitotoxic Cell Death During Acute Withdrawal. Neurophysiology 44:376-386.

Cole AJ, Bhat RV, Patt C, Worley PF, Baraban JM. 1992. D1 dopamine receptor activation of multiple transcription factor genes in rat striatum. J Neurochem 58:1420-1426.

Dalva MB, McClelland AC, Kayser MS. 2007. Cell adhesion molecules: signalling functions at the synapse. Nat Rev Neurosci 8:206-220.

Ding Y, Won L, Britt JP, Lim SA, McGehee DS, Kang UJ. 2011. Enhanced striatal cholinergic neuronal activity mediates L-DOPA-induced dyskinesia in parkinsonian mice. Proc Natl Acad Sci U S A 108:840-845.

Drees F, Pokutta S, Yamada S, Nelson WJ, Weis WI. 2005. Alpha-catenin is a molecular switch that binds E-cadherin-beta-catenin and regulates actin-filament assembly. Cell 123:903-915.

Ehringer H, Hornykiewicz O. 1960. [Distribution of noradrenaline and dopamine (3-hydroxytyramine) in the human brain and their behavior in diseases of the extrapyramidal system]. Klin Wochenschr 38:1236-1239.

El Atifi-Borel M, Buggia-Prevot V, Platet N, Benabid AL, Berger F, Sgambato-Faure V. 2009. De novo and long-term l-Dopa induce both common and distinct striatal gene profiles in the hemiparkinsonian rat. Neurobiol Dis 34:340-350.

Fabbrini G, Mouradian MM, Juncos JL, Schlegel J, Mohr E, Chase TN. 1988. Motor fluctuations in Parkinson's disease: central pathophysiological mechanisms, Part I. Ann Neurol 24:366-371.

Fieblinger T, Graves SM, Sebel LE, Alcacer C, Plotkin JL, Gertler TS, Chan CS, Heiman M, Greengard P, Cenci MA, Surmeier DJ. 2014. Cell type-specific plasticity of striatal projection neurons in parkinsonism and L-DOPA-induced dyskinesia. Nat Commun 5:5316.

Grandas F, Galiano ML, Tabernero C. 1999. Risk factors for levodopa-induced dyskinesias in Parkinson's disease. J Neurol 246:1127-1133.

Heiman M, Heilbut A, Francardo V, Kulicke R, Fenster RJ, Kolaczyk ED, Mesirov JP, Surmeier DJ, Cenci MA, Greengard P. 2014. Molecular adaptations of striatal spiny

projection neurons during levodopa-induced dyskinesia. Proc Natl Acad Sci U S A 111:4578-4583.

Huang da W, Sherman BT, Lempicki RA. 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc 4:44-57.

Jenner P. 2008. Molecular mechanisms of L-DOPA-induced dyskinesia. Nat Rev Neurosci 9:665-677.

Kang KS, Wen Y, Yamabe N, Fukui M, Bishop SC, Zhu BT. 2010. Dual beneficial effects of (-)-epigallocatechin-3-gallate on levodopa methylation and hippocampal neurodegeneration: in vitro and in vivo studies. PLoS One 5:e11951.

Kaplan S, Tarsy D. 2013. Initial treatment of Parkinson's disease: an update. Curr Treat Options Neurol 15:377-384.

Konradi C, Westin JE, Carta M, Eaton ME, Kuter K, Dekundy A, Lundblad M, Cenci MA. 2004. Transcriptome analysis in a rat model of L-DOPA-induced dyskinesia. Neurobiol Dis 17:219-236.

Lotharius J, Brundin P. 2002. Pathogenesis of Parkinson's disease: dopamine, vesicles and alpha-synuclein. Nat Rev Neurosci 3:932-942.

Maletic-Savatic M, Malinow R, Svoboda K. 1999. Rapid dendritic morphogenesis in CA1 hippocampal dendrites induced by synaptic activity. Science 283:1923-1927.

Moratalla R, Robertson HA, Graybiel AM. 1992. Dynamic regulation of NGFI-A (zif268, egr1) gene expression in the striatum. J Neurosci 12:2609-2622.

Nicola SM, Surmeier J, Malenka RC. 2000. Dopaminergic modulation of neuronal excitability in the striatum and nucleus accumbens. Annu Rev Neurosci 23:185-215.

Nikonenko I, Jourdain P, Alberi S, Toni N, Muller D. 2002. Activity-induced changes of spine morphology. Hippocampus 12:585-591.

Olanow W, Schapira AH, Rascol O. 2000. Continuous dopamine-receptor stimulation in early Parkinson's disease. Trends Neurosci 23:S117-126.

Olsson M, Nikkhah G, Bentlage C, Bjorklund A. 1995. Forelimb akinesia in the rat Parkinson model: differential effects of dopamine agonists and nigral transplants as assessed by a new stepping test. J Neurosci 15:3863-3875.

Parr-Brownlie LC, Poloskey SL, Flanagan KK, Eisenhofer G, Bergstrom DA, Walters JR. 2007. Dopamine lesion-induced changes in subthalamic nucleus activity are not associated with alterations in firing rate or pattern in layer V neurons of the anterior cingulate cortex in anesthetized rats. The European journal of neuroscience 26:1925-1939.

Pavon N, Martin AB, Mendialdua A, Moratalla R. 2006. ERK phosphorylation and FosB expression are associated with L-DOPA-induced dyskinesia in hemiparkinsonian mice. Biol Psychiatry 59:64-74.

Picconi B, Bagetta V, Ghiglieri V, Paille V, Di Filippo M, Pendolino V, Tozzi A, Giampa C, Fusco FR, Sgobio C, Calabresi P. 2011. Inhibition of phosphodiesterases rescues striatal long-term depression and reduces levodopa-induced dyskinesia. Brain 134:375-387.

Picconi B, Centonze D, Hakansson K, Bernardi G, Greengard P, Fisone G, Cenci MA, Calabresi P. 2003. Loss of bidirectional striatal synaptic plasticity in L-DOPA-induced dyskinesia. Nat Neurosci 6:501-506.

Reynolds JN, Hyland BI, Wickens JR. 2001. A cellular mechanism of reward-related learning. Nature 413:67-70.

Reynolds JN, Wickens JR. 2000. Substantia nigra dopamine regulates synaptic plasticity and membrane potential fluctuations in the rat neostriatum, in vivo. Neuroscience 99:199-203.

Santini E, Valjent E, Usiello A, Carta M, Borgkvist A, Girault JA, Herve D, Greengard P, Fisone G. 2007. Critical involvement of cAMP/DARPP-32 and extracellular signal-regulated protein kinase signaling in L-DOPA-induced dyskinesia. J Neurosci 27:6995-7005.

Schallert T, Fleming SM, Leasure JL, Tillerson JL, Bland ST. 2000. CNS plasticity and assessment of forelimb sensorimotor outcome in unilateral rat models of stroke, cortical ablation, parkinsonism and spinal cord injury. Neuropharmacology 39:777-787.

Tai CY, Kim SA, Schuman EM. 2008. Cadherins and synaptic plasticity. Curr Opin Cell Biol 20:567-575.

Tanaka H, Shan W, Phillips GR, Arndt K, Bozdagi O, Shapiro L, Huntley GW, Benson DL, Colman DR. 2000. Molecular modification of N-cadherin in response to synaptic activity. Neuron 25:93-107.

Tanner CM, Aston DA. 2000. Epidemiology of Parkinson's disease and akinetic syndromes. Curr Opin Neurol 13:427-430.

Tarazona, S., Garcia-Alcalde, F., Dopazo, J., Ferrer, A., & Conesa, A. (2011). Differential expression in RNA-seq: A matter of depth. Genome Research, *21*(12), 2213–2223.

Tseng KY, Kargieman L, Gacio S, Riquelme LA, Murer MG. 2005. Consequences of partial and severe dopaminergic lesion on basal ganglia oscillatory activity and akinesia. The European journal of neuroscience 22:2579-2586.

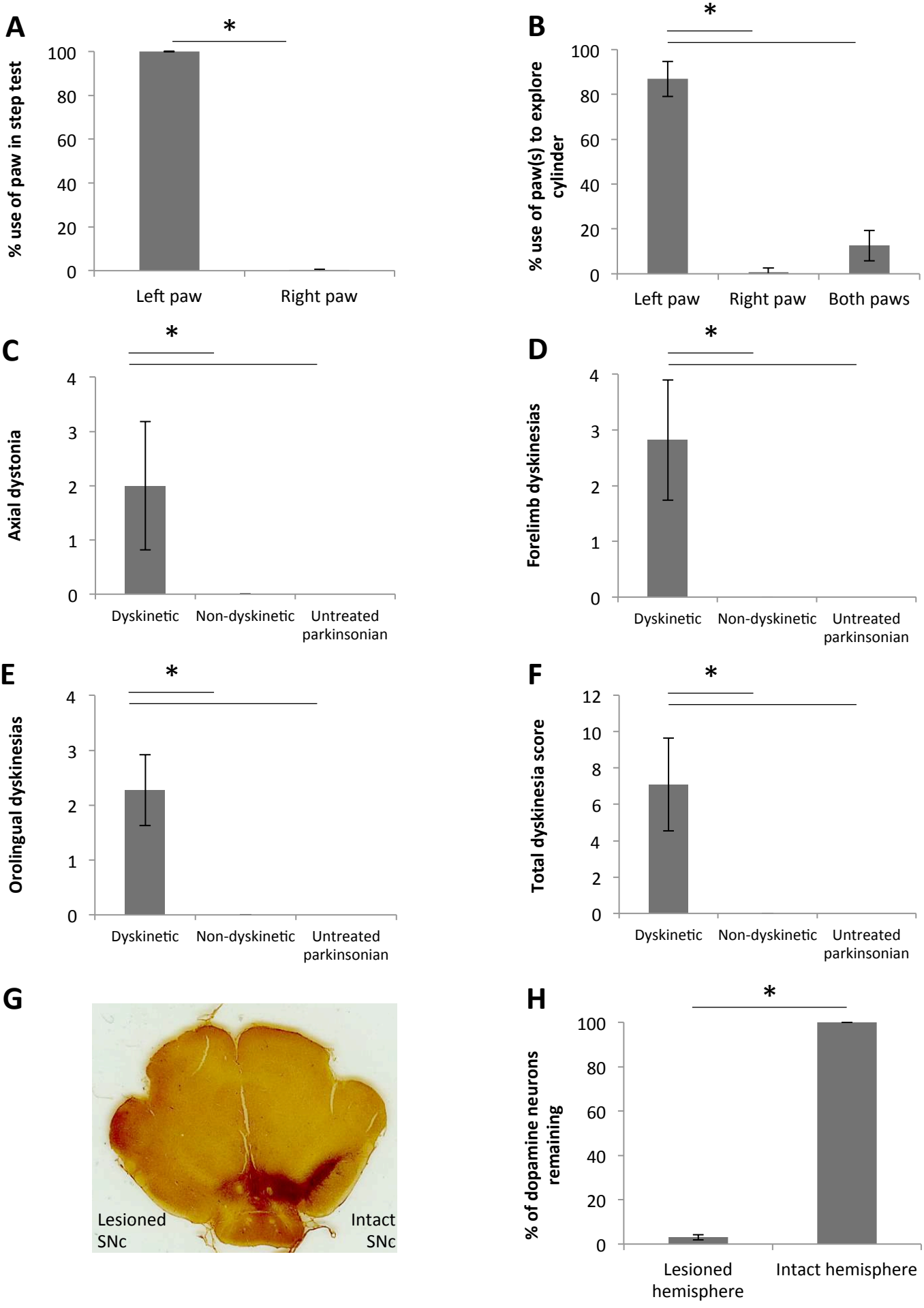
Westin JE, Vercammen L, Strome EM, Konradi C, Cenci MA. 2007. Spatiotemporal pattern of striatal ERK1/2 phosphorylation in a rat model of L-DOPA-induced dyskinesia and the role of dopamine D1 receptors. Biol Psychiatry 62:800-810.

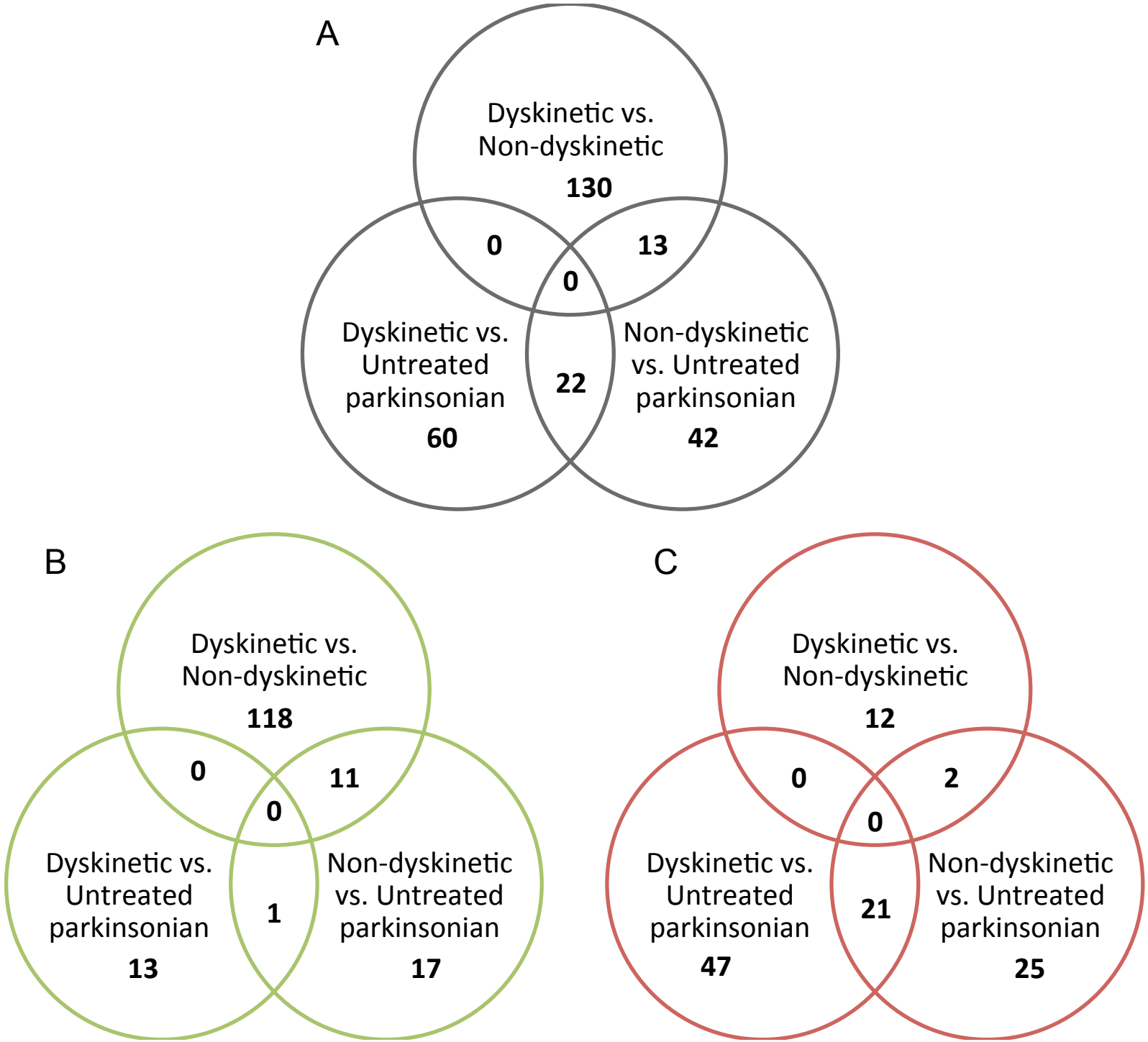
Wilhelm, B. T. & Landry, J. R. 2009. RNA-Seq quantitative measurement of expression through massively parallel RNA-sequencing. Methods **48**, 249-257.

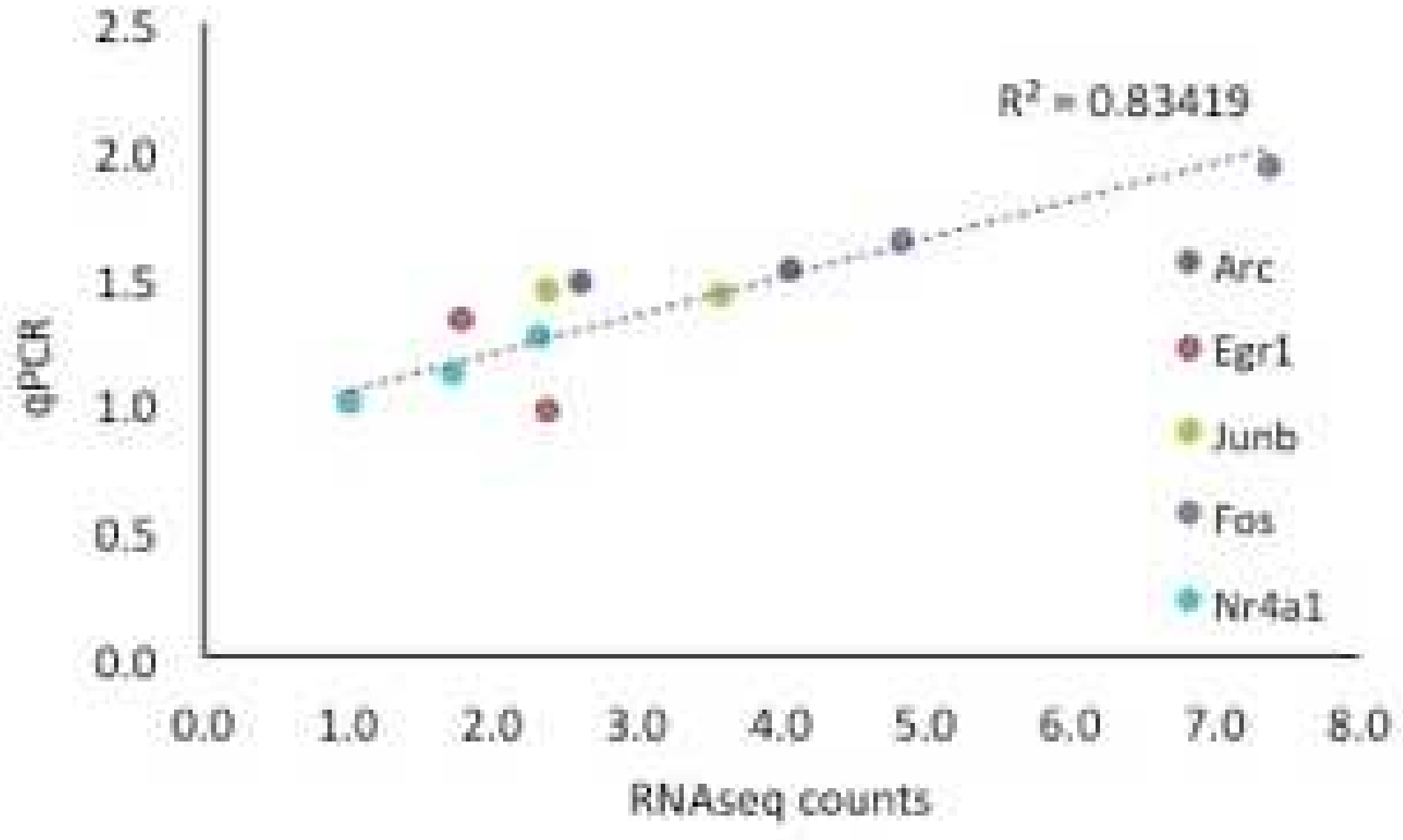
Winkler C, Kirik D, Bjorklund A, Cenci MA. 2002. L-DOPA-induced dyskinesia in the intrastriatal 6-hydroxydopamine model of parkinson's disease: relation to motor and cellular parameters of nigrostriatal function. Neurobiol Dis 10:165-186.

Won L, Ding Y, Singh P, Kang UJ. 2014. Striatal cholinergic cell ablation attenuates L-DOPA induced dyskinesia in Parkinsonian mice. J Neurosci 34:3090-3094.

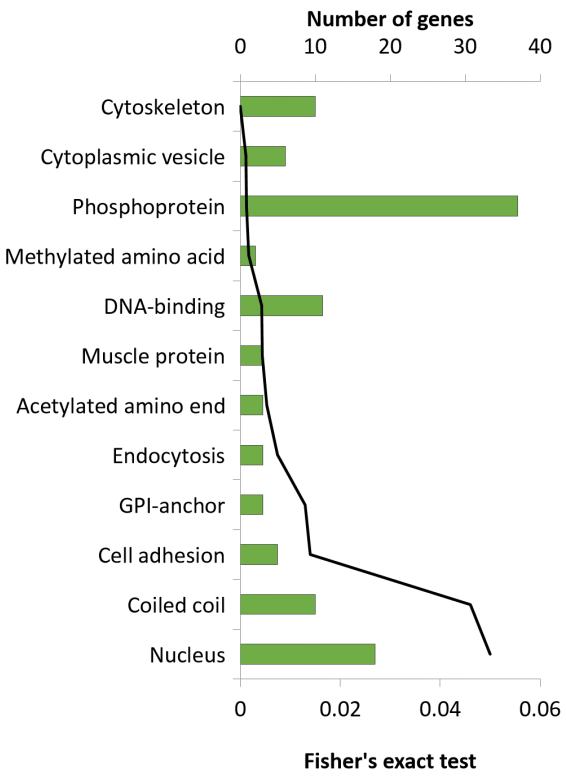
Zhang Y, Meredith GE, Mendoza-Elias N, Rademacher DJ, Tseng KY, Steece-Collier K. 2013. Aberrant restoration of spines and their synapses in L-DOPA-induced dyskinesia: involvement of corticostriatal but not thalamostriatal synapses. J Neurosci 33:11655-11667.


A
% use of paw in step test
100
80
60
40
20
0
*
Left paw
Right paw
B
% use of paw(s) to explore cylinder
100
80
60
40
20
0
*
Left paw
Right paw
Both paws
C
Axial dystonia
4
3
2
1
0
*
Dyskinetic
Non-dyskinetic
Untreated parkinsonian
D
Forelimb dyskinesias
4
3
2
1
0
*
Dyskinetic
Non-dyskinetic
Untreated parkinsonian
E
Orolingual dyskinesias
4
3
2
1
0
*
Dyskinetic
Non-dyskinetic
Untreated parkinsonian
F
Total dyskinesia score
12
10
8
6
4
2
0
*
Dyskinetic
Non-dyskinetic
Untreated parkinsonian
G
Lesioned SNc
Intact SNc
H
% of dopamine neurons remaining
100
80
60
40
20
0
*
Lesioned hemisphere
Intact hemisphere

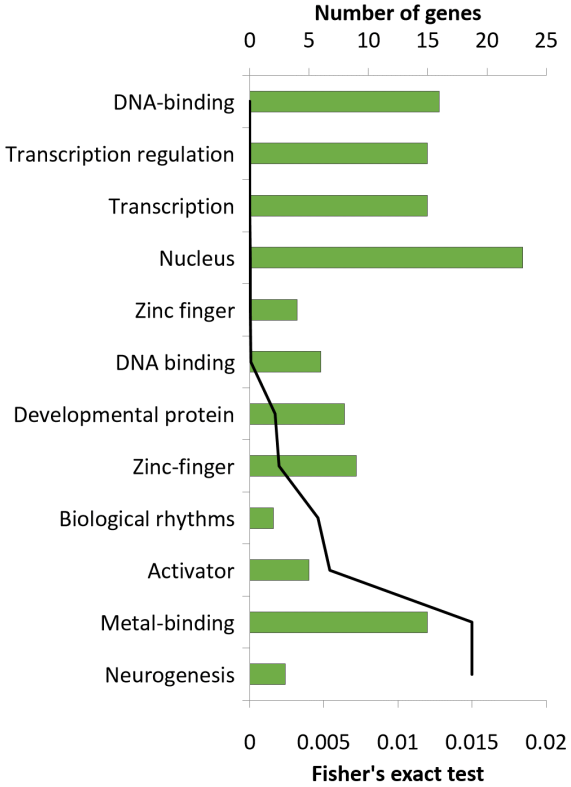

A
Dyskinetic vs.
Non-dyskinetic
130
0
13
0
Dyskinetic vs.
Untreated
parkinsonian
60
22
Non-dyskinetic
vs. Untreated
parkinsonian
42
B
Dyskinetic vs.
Non-dyskinetic
118
0
11
0
Dyskinetic vs.
Untreated
parkinsonian
13
1
Non-dyskinetic
vs. Untreated
parkinsonian
17
C
Dyskinetic vs.
Non-dyskinetic
12
0
2
0
Dyskinetic vs.
Untreated
parkinsonian
47
21
Non-dyskinetic
vs. Untreated
parkinsonian
25


2.5
2.0
1.5
1.0
0.5
0.0
qPCR
R^2 = 0.83419
Arc
Egr1
Junb
Fos
Nr4a1
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
RNAseq counts

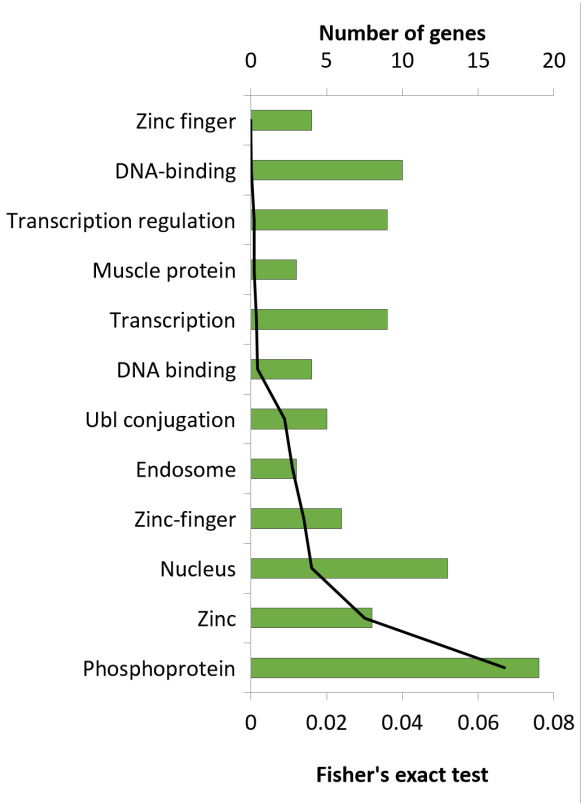
A


Dyskinetic v Non-dyskinetic
Number of genes
0
10
20
30
40
Cytoskeleton
Cytoplasmic vesicle
Phosphoprotein
Methylated amino acid
DNA-binding
Muscle protein
Acetylated amino end
Endocytosis
GPI-anchor
Cell adhesion
Coiled coil
Nucleus
0
0.02
0.04
0.06
Fisher's exact test

B


Non-dyskinetic v Untreated parkinsonian
Number of genes
0
5
10
15
20
25
DNA-binding
Transcription regulation
Transcription
Nucleus
Zinc finger
DNA binding
Developmental protein
Zinc-finger
Biological rhythms
Activator
Metal-binding
Neurogenesis
0
0.005
0.01
0.015
0.02
Fisher's exact test

C


Dyskinetic v Untreated parkinsonian
Number of genes
0
5
10
15
20
Zinc finger
DNA-binding
Transcription regulation
Muscle protein
Transcription
DNA binding
Ubl conjugation
Endosome
Zinc-finger
Nucleus
Zinc
Phosphoprotein
0
0.02
0.04
0.06
0.08
Fisher's exact test

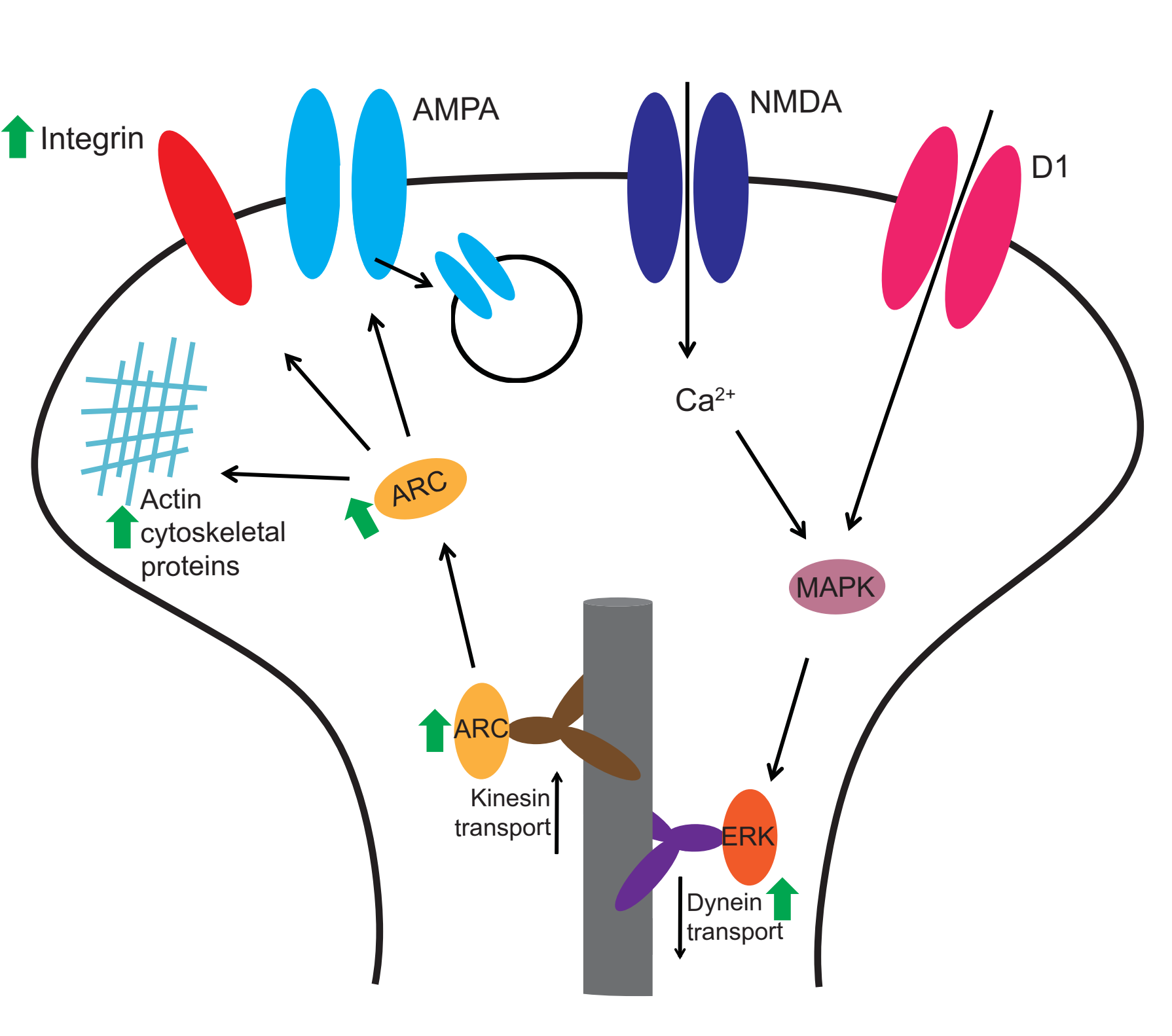

Integrin
AMPA
NMDA
D1
Ca2+
Actin
cytoskeletal
proteins
ARC
MAPK
ARC
Kinesin
transport
ERK
Dynein
transport

Table 1. Summary table of all gene expression changes identified. D – dyskinetic, N – non-dyskinetic, and P – untreated parkinsonian control.

Feature ID	D v N		D v P		N v P	
	Fold change	FDR p-value	Fold change	FDR p-value	Fold change	FDR p-value
Acta1	1.62	0.03	-1.09	1.00	1.48	0.15
Ankrd34c	-2.53	0.01	1.80	0.63	-1.40	0.93
Apold1	1.80	0.02	-4.10	9.58E-09	-2.27	5.84E-07
Arc	1.51	9.84E-03	-4.02	3.12E-18	-2.66	1.08E-18
Arnt2	1.21	0.02	-1.05	0.98	1.15	0.93
Arpp21	1.29	5.11E-09	-1.10	0.51	1.17	0.34
Atp6ap1l	1.45	7.23E-09	-1.05	0.99	1.38	7.09E-03
Atp6v1a	1.15	2.56E-04	-1.10	0.09	1.05	0.93
Aurkb	7.81	2.96E-08	-1.28	1.00	6.10	0.93
Blcap	1.19	0.01	-1.09	0.85	1.10	0.79
Btg1	1.30	8.95E-03	-1.31	0.32	-1.01	1.00
Cadm3	1.20	2.93E-08	-1.05	1.00	1.14	0.93
Calm1	1.12	1.46E-54	-1.00	1.00	1.12	0.00
Caly	1.20	0.02	1.03	1.00	1.23	3.63E-05
Cbr3	1.98	2.30E-03	-1.22	0.94	1.62	0.87
Ccdc108	9.23	4.84E-03	-1.07	1.00	8.63	0.93
Ccdc135	3.76	6.17E-04	-1.17	1.00	3.22	0.83
Ccdc146	2.38	5.39E-03	-1.00	1.00	2.38	0.93
Ccdc151	3.99	2.52E-03	1.26	0.94	5.01	2.77E-05
Ccdc153	5.01	4.18E-09	1.13	1.00	5.66	0.93
Ccdc162	4.22	5.66E-05	1.15	1.00	4.87	0.93
Ccdc176	1.48	0.02	-1.10	0.95	1.35	0.59
Ccdc19	3.48	0.03	-1.17	1.00	2.97	0.93
Ccdc37	1.86	0.02	-1.14	1.00	1.63	0.93
Ccdc39	1.24	6.45E-03	-1.04	1.00	1.20	0.93
Ccdc50	-1.32	0.02	1.11	0.94	-1.19	0.59
Ccdc78	3.40	0.02	1.03	1.00	3.49	0.67
Cd24	1.71	1.14E-09	-1.11	0.94	1.55	8.34E-04
Cdca7	5.25	6.22E-09	-1.50	0.95	3.49	0.93
Cdh2	1.18	8.43E-03	-1.14	0.15	1.04	0.97
Cdh29	7.79	6.34E-07	1.02	1.00	7.97	0.93
Celsr1	1.74	2.26E-04	-1.27	0.61	1.37	0.39
Cntnap3	1.83	2.90E-04	-1.18	0.94	1.56	0.59
Cpne6	1.52	5.30E-04	-1.14	0.94	1.34	0.82
Cyp7b1	1.46	2.09E-04	-1.28	0.01	1.14	0.93
Cyr61	1.90	0.02	-14.66	3.84E-20	-7.70	1.78E-04
Dbi	-2.06	3.35E-03	1.49	0.69	-1.39	0.69
Dlx1	2.54	0.02	-1.51	0.94	1.68	0.89
Dnaaf3	2.31	0.01	1.32	0.94	3.05	9.67E-06
Dnah1	3.01	0.01	-1.00	1.00	3.00	0.93
Dnah10	1.94	0.04	-1.14	1.00	1.70	0.95
Dnah11	2.30	7.23E-09	-1.07	1.00	2.15	0.76
Dnah2	2.12	5.11E-09	-1.03	1.00	2.05	0.93
Dnah6	6.48	1.01E-03	-1.17	1.00	5.52	0.91
Dos	1.14	2.52E-03	-1.13	0.02	1.02	1.00
Drc1	2.15	3.14E-03	-1.54	0.94	1.40	0.95
Dusp1	1.45	0.01	-4.60	6.38E-43	-3.18	5.32E-11
Dynlrb2	3.28	1.01E-04	1.13	1.00	3.69	0.40
Efnb1	1.60	0.04	-1.18	0.94	1.35	0.69
Egr1	1.32	9.36E-05	-2.39	2.72E-94	-1.81	8.87E-12
Egr4	1.27	5.05E-06	-2.31	4.08E-40	-1.82	2.35E-16
Enkur	1.92	6.03E-07	-1.19	1.00	1.61	0.93
Erf	1.23	0.01	-1.28	5.78E-03	-1.05	0.99
Fam214a	1.16	7.43E-03	-1.16	0.45	-1.00	1.00
Fam81b	1.26	2.18E-03	-1.06	0.94	1.19	0.13
Fhad1	3.92	0.01	-1.31	1.00	2.99	0.93

Folr1	9.75	0.04	-1.41	1.00	6.92	0.93
Fos	1.48	2.69E-13	-7.33	1.52E-158	-4.96	3.29E-86
Fosb	1.47	2.72E-03	-3.98	9.03E-29	-2.72	1.31E-08
Gad2	1.20	1.57E-04	-1.11	0.25	1.08	0.24
Gpr149	1.65	5.71E-03	-1.05	0.99	1.57	0.04
Grb10	1.21	6.43E-03	-1.10	0.76	1.10	0.80
Gtf2ird1	1.18	0.03	1.00	1.00	1.18	0.43
Hcn2	-1.23	6.34E-07	1.24	0.56	1.01	1.00
Hnrph1	1.10	0.02	-1.13	6.65E-04	-1.03	0.93
Hrh3	1.12	0.04	1.04	1.00	1.17	0.93
Hydin	3.18	8.58E-05	1.38	1.00	4.37	0.93
Iiig9	2.51	0.02	1.14	1.00	2.87	0.93
Iqca1	1.67	0.04	1.05	1.00	1.75	0.93
Iqcg	2.47	0.02	1.09	1.00	2.68	0.13
Itga1	-1.91	0.03	1.40	0.94	-1.37	0.83
Itgb4	2.74	0.04	1.23	1.00	3.37	0.93
Junb	1.47	0.02	-3.55	6.61E-26	-2.41	3.84E-09
Kcnk16	7.54	1.68E-03	-1.23	1.00	6.11	0.93
Klf5	1.50	7.69E-03	-1.71	1.62E-03	-1.14	0.93
Kmt2a	1.09	0.02	1.01	1.00	1.11	0.93
Kpna3	1.16	0.04	-1.16	0.37	1.00	1.00
Krt8	9.83	0.04	1.07	1.00	10.50	0.93
Ldb2	1.34	1.89E-03	-1.02	1.00	1.31	8.25E-06
LOC679641	4.08	0.01	1.06	1.00	4.31	0.93
Lpl	1.37	7.57E-03	-1.06	1.00	1.29	0.93
Lrrc34	2.06	0.04	-1.06	1.00	1.94	0.93
Lrriq1	2.86	5.05E-06	-1.38	0.94	2.07	0.87
Map2	1.06	0.02	-1.04	1.00	1.02	1.00
Mapk15	2.72	2.34E-05	-1.11	1.00	2.46	0.63
Mki67	2.34	8.95E-03	-1.39	0.95	1.69	0.93
Mllt11	1.15	0.01	-1.15	0.40	-1.00	1.00
Mns1	1.53	0.04	1.11	1.00	1.69	0.76
Mtnd4l	1.07	2.84E-52	-1.07	1.42E-52	-1.00	1.00
Mylk	-1.57	2.34E-05	1.09	0.96	-1.44	2.17E-03
Nptx1	1.99	0.01	-1.23	0.99	1.62	0.93
Nr4a1	1.45	6.22E-17	-2.62	2.65E-41	-1.80	1.08E-10
Nr4a3	1.39	8.03E-11	-2.96	1.97E-60	-2.12	4.33E-22
Nrsn2	1.19	0.04	-1.02	1.00	1.17	0.16
Nup210l	1.63	0.04	-1.28	0.94	1.27	0.93
Obscn	1.93	0.01	-1.61	0.25	1.20	0.99
P2rx6	2.07	2.59E-03	-1.01	1.00	2.05	0.93
Pcmtd2	1.14	0.02	-1.10	0.33	1.04	0.93
Pfkl	1.19	0.04	-1.07	0.99	1.12	0.93
Pitpnm2	1.17	7.61E-03	1.02	1.00	1.19	0.30
Ppap2b	1.13	0.03	-1.09	0.07	1.04	0.93
Ppfia2	1.19	0.01	1.00	1.00	1.19	0.02
Ppp1r15a	1.34	3.94E-04	-2.11	5.43E-20	-1.58	2.32E-05
Ppp1r15b	1.21	0.03	-1.21	0.61	-1.00	1.00
Prkg1	1.57	3.30E-03	-1.17	0.94	1.34	0.93
Prr13	-1.68	9.48E-03	1.17	0.99	-1.44	0.73
Psma8	16.00	0.04	-1.43	0.96	11.17	0.28
Ptplad1	1.18	1.38E-03	-1.10	0.55	1.07	0.93
Resp18	1.30	1.36E-03	-1.14	0.34	1.15	0.61
Rgs14	1.56	1.28E-03	-1.30	0.46	1.20	0.76
Rgs22	4.83	3.06E-03	-1.35	0.94	3.57	0.53
Rsph4a	4.48	2.74E-03	-1.12	1.00	4.00	0.93
Rtn4rl2	5.56	0.04	-1.85	0.94	3.00	0.80
Scg5	1.11	0.03	-1.04	0.94	1.07	0.73
Scml4	2.15	0.03	-1.12	1.00	1.92	0.93
Sdcbp	1.24	0.01	-1.22	3.58E-04	1.02	1.00

Sept3	1.14	7.69E-03	-1.01	1.00	1.13	0.33
Serinc2	2.54	7.38E-04	-1.04	1.00	2.44	0.93
Slc2a3	1.31	9.48E-03	-1.18	0.46	1.11	0.93
Sort1	-1.18	0.04	1.07	0.61	-1.10	0.77
Srebf2	-1.15	0.04	1.04	0.94	-1.10	0.15
Stpg1	3.65	2.34E-05	-1.03	1.00	3.55	0.93
Stx6	-1.20	8.43E-03	1.18	0.04	-1.02	1.00
Sv2c	1.24	1.54E-07	-1.02	1.00	1.22	0.69
Synpo2	-1.40	0.02	1.11	0.94	-1.27	0.45
T2	5.42	3.57E-03	-1.17	1.00	4.62	0.93
Tagln	-1.49	0.04	-1.12	0.98	-1.67	1.59E-05
Tmem130	1.20	6.03E-04	-1.07	0.94	1.12	0.59
Tmsb10	1.19	0.02	-1.03	1.00	1.15	0.93
Tnnt1	1.52	0.01	1.03	1.00	1.57	4.53E-03
Top2a	2.48	3.15E-03	-1.79	0.92	1.39	0.99
Tpbg	1.52	3.27E-03	-1.01	1.00	1.51	0.23
Tril	-1.28	0.02	1.03	1.00	-1.24	0.11
Ttc25	3.17	9.39E-06	-1.00	1.00	3.16	0.93
Ttll10	6.40	0.04	1.52	1.00	9.75	0.93
Vopp1	1.18	5.66E-03	-1.02	1.00	1.16	0.04
Vwa5b1	2.47	3.43E-03	-1.03	1.00	2.39	0.93
Wdr34	1.52	0.03	-1.11	0.94	1.37	0.39
Wdr52	4.94	8.43E-03	1.02	1.00	5.02	0.92
Wdr63	3.36	1.78E-03	1.57	0.94	5.27	0.10
Wdr96	2.58	3.57E-03	-1.12	1.00	2.30	0.93
Yipf1	-1.28	0.04	1.13	0.96	-1.13	0.93
Ywhab	1.06	0.02	-1.03	0.69	1.03	0.93
Akap8l	1.11	0.92	1.02	1.00	1.13	6.11E-05
Aldoc	-1.10	0.52	1.12	3.72E-03	1.02	1.00
Arf4	1.11	0.31	-1.19	8.31E-03	-1.07	0.93
Arhgap31	-1.31	0.05	-1.04	1.00	-1.36	0.02
Arl4d	1.37	0.15	-2.19	2.91E-07	-1.59	2.17E-06
Atf3	1.60	0.92	-6.91	0.04	-4.31	0.13
Atp1a2	-1.08	0.92	-1.04	1.00	-1.12	9.18E-21
B3gnt2	1.08	1.00	-1.21	9.81E-04	-1.12	1.00
Bhlhe40	1.08	0.92	-1.38	7.58E-03	-1.28	0.04
Blnk	-1.19	0.92	-1.37	0.94	-1.63	4.96E-03
Btg2	1.59	0.14	-5.75	1.21E-12	-3.61	1.59E-08
Cacng1	-8.22	0.70	12.56	0.03	1.53	0.94
Cadps2	1.55	0.92	-1.81	0.03	-1.17	1.00
Cbln1	-1.33	1.00	-4.35	0.03	-5.77	0.93
Ccnl1	1.04	1.00	-1.23	1.89E-03	-1.18	0.78
Ccrn4l	1.19	0.24	-1.41	5.79E-05	-1.18	0.51
Cdc42ep3	1.36	0.14	-1.67	4.68E-10	-1.23	0.93
Celf4	1.22	0.05	-1.08	0.94	1.12	0.02
Cited2	1.12	0.84	-1.28	0.01	-1.15	0.80
Clgn	1.32	0.76	1.37	0.73	1.81	0.04
Clmp	-1.24	0.81	-1.38	0.69	-1.72	0.02
Cnot8	1.13	0.92	-1.20	0.04	-1.06	1.00
Col4a4	-1.67	0.38	-1.02	1.00	-1.71	0.02
Coq10b	1.10	0.92	-1.69	2.24E-06	-1.53	1.35E-03
Cpsf6	1.08	0.63	-1.14	0.04	-1.05	0.93
Cspg4	-1.21	0.96	-1.18	1.00	-1.43	3.52E-05
Ctgf	1.26	0.95	-1.53	0.04	-1.22	1.00
Cygb	-1.22	0.38	1.26	0.01	1.03	1.00
Cyth4	-1.31	0.92	-1.15	1.00	-1.50	0.03
Dclk1	1.12	0.92	-1.09	4.12E-03	1.03	1.00
Ddit3	-1.05	0.99	-1.44	1.45E-03	-1.51	6.87E-05
Dlx5	1.25	0.92	-1.29	0.03	-1.03	1.00

Dusp5	1.47	0.92	-3.18	0.08	-2.15	8.47E-03
Dynlt3	-1.05	0.92	-1.14	0.42	-1.19	0.03
Egr2	1.48	0.57	-6.65	1.63E-06	-4.49	8.48E-90
Egr3	1.19	0.87	-1.59	5.80E-06	-1.34	0.19
Eif4e	1.13	0.35	-1.19	0.04	-1.05	0.93
Elp5	1.05	0.94	-1.26	6.44E-03	-1.20	0.13
F8	-1.17	0.71	1.33	3.16E-03	1.14	0.87
Fam115a	1.08	0.66	1.08	0.59	1.16	8.15E-03
Fbxl3	-1.13	0.57	-1.14	0.63	-1.30	1.98E-03
Fxyd7	1.55	0.08	1.07	1.00	1.65	0.02
Gadd45g	1.70	0.42	-3.73	5.08E-24	-2.19	0.81
Gldn	-1.11	0.92	1.27	0.03	1.15	0.93
Gltscr2	-1.11	0.79	1.24	0.01	1.12	0.69
Golim4	-1.11	0.81	-1.16	0.49	-1.28	4.30E-03
Gpr98	1.75	0.19	1.10	1.00	1.93	8.15E-03
Herc2	-1.01	1.00	-1.17	0.05	-1.17	0.04
Ip6k2	1.11	0.66	-1.17	0.03	-1.05	0.96
Kif17	1.39	0.06	1.02	1.00	1.41	0.03
Klf2	1.42	0.44	-1.96	0.03	-1.38	0.93
Klf4	1.15	0.94	-1.99	0.03	-1.73	1.48E-03
Lrmp	1.19	0.89	1.38	0.07	1.65	1.14E-04
Lrrc23	1.58	0.59	1.30	0.94	2.05	0.04
Lyn	1.05	1.00	-1.42	0.03	-1.35	0.15
Mrpl15	1.15	0.57	1.12	0.91	1.29	0.01
Nab2	1.18	0.92	-1.37	2.46E-04	-1.16	0.98
Nckap1l	-1.10	1.00	-1.34	0.94	-1.48	0.02
Nefl	-1.19	0.23	-1.03	1.00	-1.22	0.04
Nfkbid	1.27	0.92	-3.03	2.11E-04	-2.39	0.04
Nlrc3	2.42	0.12	-3.69	9.91E-03	-1.52	0.93
Nop58	1.15	0.92	-1.22	0.02	-1.06	1.00
Npas4	1.91	0.72	-5.58	0.01	-2.92	0.38
Nsmce2	1.11	0.92	1.13	0.94	1.26	5.65E-03
Ntrk2	1.04	0.92	-1.12	1.55E-09	-1.07	0.93
Nupl1	-1.05	0.98	-1.17	0.71	-1.23	0.03
Pcdh8	-1.41	0.92	1.50	5.03E-03	1.07	1.00
Pde10a	-1.09	1.00	1.12	5.81E-06	1.04	1.00
Pdlim1	-1.34	0.78	-1.40	0.94	-1.88	8.15E-03
Per1	1.17	0.30	-1.54	1.84E-14	-1.31	0.13
Polr2a	-1.13	0.92	-1.01	1.00	-1.14	0.03
Ppm1d	1.22	0.37	-1.33	3.47E-03	-1.09	0.93
Ptms	1.03	0.84	1.05	0.48	1.08	4.63E-03
Retsat	-1.14	0.26	-1.27	1.20E-03	-1.44	2.59E-10
Rgs2	1.31	0.24	-1.50	4.79E-04	-1.14	0.93
Rgs5	-1.18	0.96	-1.24	0.96	-1.47	2.52E-06
Rif1	1.24	0.39	-1.59	2.02E-04	-1.28	0.16
Rnf111l	-1.25	0.40	1.38	0.04	1.10	0.93
Rpl17	1.20	0.94	-1.36	5.49E-03	-1.13	1.00
Rtn3	1.03	1.00	-1.09	6.97E-04	-1.06	0.94
Sec62	1.07	0.97	-1.19	0.04	-1.11	0.93
Sertad1	1.03	1	-1.93	0.03	-1.87	0.69
Sfrs11	1.10	0.92	-1.12	0.01	-1.02	1.00
Sgsm1	1.14	0.59	1.11	0.77	1.26	1.84E-05
Sh3bgrl	1.03	0.98	-1.15	0.03	-1.11	0.63
Sik1	1.52	0.7	-2.53	4.77E-03	-1.67	0.37
Sik2	1.09	0.69	-1.29	1.79E-04	-1.19	0.12
Ska3	1.20	0.49	1.06	0.97	1.28	0.04
Slc25a25	1.29	0.06	-2.03	2.49E-09	-1.57	1.61E-06
Slc25a31	1.28	0.92	-2.42	0.03	-1.88	0.34
Slc6a6	-1.10	0.97	1.20	1.08E-04	1.09	0.97
Slx4	-1.15	0.42	1.29	9.05E-04	1.12	0.68

Spin1	1.35	0.92	-1.58	3.30E-07	-1.16	1.00
Spred1	1.13	0.96	-1.29	1.50E-03	-1.14	0.98
Sprr1a	1.20	1.00	3.81	0.03	4.59	0.01
Ssfa2	-1.10	0.92	-1.14	0.85	-1.25	0.04
Stat4	-1.27	0.77	-1.46	0.46	-1.87	3.06E-03
Syt5	1.18	0.49	1.02	1.00	1.20	2.08E-04
Tac1	1.06	1.00	-1.59	3.46E-16	-1.50	0.93
Tiparp	1.07	0.99	-2.04	8.86E-08	-1.92	1.81E-10
Tmem47	1.15	0.05	-1.16	3.18E-04	-1.01	1.00
Tmem65	-1.14	0.74	-1.11	0.94	-1.26	8.15E-03
Tns1	-1.21	0.87	-1.10	0.99	-1.34	3.59E-03
Tp53inp2	-1.38	0.80	-1.06	1.00	-1.46	0.03
Tpm1	1.21	0.28	1.03	1.00	1.25	0.03
Trak1	-1.05	0.98	-1.18	3.02E-03	-1.12	0.93
Trim9	-1.15	0.97	1.11	7.15E-04	-1.03	1.00
Txnl4b	1.25	0.74	1.24	0.81	1.55	0.02
Tyw5	1.39	0.05	-1.46	3.61E-04	-1.05	1.00
Zbtb16	1.09	1.00	-1.52	3.69E-04	-1.39	0.93
Zdbf2	1.07	0.99	-1.50	1.96E-05	-1.41	0.15
Zfp36	1.43	0.34	-1.98	0.03	-1.38	0.93