

Somal and axonal translation in
Parkinsons dopamine neurons:
Sequencing the translatome



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Abstract

Nigrostriatal dopamine neurons have long, highly arborised axons, indicating a requirement for axonal translation. The axonal ‘translatome’ will reflect the neuronal response to local stressors that precedes cell death in Parkinson’s disease (PD) as well as revealing key transcriptional differences between protected and vulnerable dopaminergic populations. The translating ribosome affinity purification (TRAP) technique enables the capture of translating ribosomes specifically in dopaminergic neurons by expressing an enhanced green fluorescence protein (eGFP)-tagged ribosomal subunit driven by a cell type-specific promoter.

We have produced a novel transgenic mouse line by crossing a human alpha-synuclein overexpression model, previously generated by our lab, with a dopamine transporter (DAT)-Cre driven TRAP line. This thesis describes the collection and analysis of translating mRNA from dopaminergic cell bodies and terminals of 3 and 18-month control and Parkinsonian mice for sequencing. The expression features that best distinguished dopaminergic neurons from neighbouring cell types were first identified. This information was used to prioritise genes located within linkage regions of PD GWAS loci. Novel candidate genes were identified, including the Calcium Sensing Receptor. Comparison of axonal and somal samples demonstrated compartmental specialisation, with autophagic function, MAPK signalling, and markers of axonal homeostasis preferentially expressed in axons. Comparison between dorsal and ventral striatal axons identified a series of genes with greater dorsal expression and prior evidence of a role in axonal degeneration. These genes were considered putative markers of selective vulnerability. With age, coordinated changes in expression were observed in genes related to autophagy, oxidative stress and endocytic recycling. These changes were specific to the dopaminergic neuron and can be used to better understand the role of each gene in dopaminergic function. Results from this thesis have been collated and summarised in the form of a public dopaminergic expression atlas.

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List of Abbreviations

TRAP	Translating Ribosome Affinity Purification
-/-	Knock-out
+ or +/-	Heterozygous
+ or +/+	Homozygous
6-OHDA	6-hydroxydopamine
AD	Alzheimer's Disease
ALS	Amyotrophic Lateral Sclerosis
B-ME	Beta-mercaptoethanol
BAC	Bacterial Artificial Chromosome
bp	Base Pair
BSA	Bovine Serum Albumin
CAG	CMV early enhancer/chicken beta actin
cDNA	Complementary DNA
CNS	Central Nervous System
DAT	Dopamine Transporter
DHPC	1,2-diheptanoyl-sn-glycero-3-phosphocholine
DLB	Dementia with Lewy Bodies
L-DOPA	Levodopa
LB	Lewy Bodies
LN	Lewy Neurite
LRRK2	Leucine rich repeat kinase 2
MB	Midbrain
MPTP	1-methyl-4-phenyl-1,2,3-tetrahydropyridine
mRNA	Messenger RNA
NP-40	Nonylphenyl polyethylene glycol

List of Abbreviations

OVX	Over-expressing
PBS	Phosphate buffered saline
PCA	Principal components analysis
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PPI	Protein-protein interaction
QC	Quality Control
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
eGFP	Enhanced Green Fluorescent Protein
eGFP-L10a	eGFP-tagged RPL10a fusion protein
ER	Endoplasmic reticulum
FISH	Fluorescent in situ hybridisation
smFISH	Single molecule fluorescent in situ hybridisation
TPM	Transcript per million
fs	Floxed
GAD2	Glutamic acid decarboxylase 2
GFAP	Glial fibrillary acidic protein
GO	Gene Ontology
GWAS	Genome-wide association study
hSNCA	Human <i>a</i> -synuclein
IHC	Immunohistochemistry
IP	Immunopurified
iPSC	Induced pluripotent stem cell
IRES	Internal ribosomal entry site
kb	Kilobase
kDa	Kilo Dalton
KEGG	Kyoto Encyclopaedia of Gene and Genomes
RBD	REM sleep behaviour disorder
REM	Rapid eye movement
RIN	RNA Integrity Number

List of Abbreviations

RNA		Ribonucleic acid
RNase		Ribonuclease
ROS		Reactive oxygen species
SNARE		Soluble nsf attachment protein receptor
SNpc		Substantia nigra pars compacta
SNP		Single nucleotide polymorphism
DS		Dorsal striatum
VS		Ventral Striatum
TH		Tyrosine Hydroxylase
UTR		Untranslated region
MTA		Ventral tegmental area
AD		Alzheimer's Disease

1. Introduction

1.1 Parkinson's Disease

1.1.1 Introduction

Parkinson's Disease (PD) is the fastest growing neurological disorder in the World (Dorsey and Bloem 2018). It is estimated that the number of active cases will rise from 7 million in 2020 to 13 million by 2040 (Dorsey, Elbaz, et al. 2018). The total economic burden in the U.S. alone is estimated at \$51.9 billion, annually, due to direct medical costs and indirect costs associated with management of changes in lifestyle (Yang, Hamilton, et al. 2020). In order to develop treatments or prevention strategies for PD, the mechanisms of disease progression must be understood.

1.1.2 Clinical Presentation and Neuropathology

PD was first described over 200 years ago by James Parkinson in “An essay on the shaking palsy” (republished as (Parkinson 2002)). Clinical observations from this report form part of the diagnostic criteria of PD. Cardinal motor signs are tremor, rigidity, bradykinesia and postural instability (Gelb et al. 1999). A majority of patients are not confirmed with PD until *postmortem* confirmation of

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neuropathology, because motor symptoms can develop due to other neurological causes. Non-motor features include dementia, depression, psychosis, autonomic dysfunction, and oculomotor and olfactory deficits.

PD is characterised by the selective loss of A9 dopaminergic neurons of the substantia nigra pars compacta (SNpc) and intracellular accumulation of α -synuclein in Lewy bodies (LBs). Both features are diagnostic of PD only when found together, as LBs are detectable in other diseases, termed Lewy Body Disorders or synucleinopathies (Dickson et al. 2009; Halliday et al. 2011; McKeith et al. 1996). Gross atrophy of the brain is generally not observed, in contrast to other neurodegenerative diseases, such as Alzheimer’s Disease. In early-stage PD, loss of dopaminergic neurons is focussed to the ventrolateral substantia nigra, but spreads with age (Fearnley and Lees 1991; Damier et al. 1999). Prodromal degeneration indicates a need to identify biomarkers of early disease and modifying therapies before the development of symptoms (Dijkstra et al. 2014; Iacono et al. 2015).

LBs were first described over 100 years ago as neuronal inclusions in the brainstem of PD patients (Lewy 1912). They are spherical, eosinophilic, found in all brain regions affected by PD and are primarily composed of α -synuclein (Spillantini, Schmidt, et al. 1997; Spillantini, Crowther, et al. 1998). Insoluble cytoskeletal proteins can also be found in LBs (Galloway et al. 1992). Lewy neurites (LNs) have been described, in which α -synuclein aggregates in neuronal processes (Forno 1987). As the course of disease develops, LB pathology spreads throughout the brain (Braak et al. 2003). The Braak hypothesis stages this spread in tandem with clinical symptom progression: In prodromal disease, LB pathology is present only in the lower brainstem and olfactory bulb (stages 1 and 2), moving to the basal ganglia with the onset of motor deficits (stages 3 and 4), and finally to the cortical and limbic regions with the development of neuropsychiatric symptoms (stages 5 and 6). This hypothesis has its criticisms; neuronal degeneration and early

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toxic oligomeric α -synuclein pathology is not considered, and not all symptoms are explained (Roberts and Brown 2015; Chaudhuri et al. 2006).

The properties of LBs in disease are not clear. There are examples of familial PD patients with no LB pathology (Gaig et al. 2007; Doherty et al. 2013) and clinically normal brains with pathology (Markesbery et al. 2009). The abundance of LBs does not reliably correlate with symptoms (Colosimo 2003; Parkkinen, Kauppinen, et al. 2005; Parkkinen, Pirttilä, et al. 2008). A non-toxic role of LBs has therefore been suggested. Oligomeric α -synuclein, the precursor to the fibrillar form found in LBs, is neurotoxic and detectable in *postmortem* brains of PD patients (Winner et al. 2011; Näsström et al. 2011; Roberts and Brown 2015; Tofaris, Razzaq, et al. 2003). Attention has therefore focussed on the role of oligomeric α -synuclein in PD pathology (discussed later in this chapter).

1.1.3 Aetiology

Genetic and environmental factors contribute to the risk of PD development. The interaction of these factors with cellular and molecular biology has been the subject of intensive study, as described below.

Genetic Factors

Heritability of PD is estimated to be 27 %, with monogenic causes accounting for 5-10 % of cases (Goldman et al. 2019; Thomas and Beal 2007). Genome-wide associations studies of increasing power have detected loci with significant association to disease risk, explaining between 16 and 36 % of heritable risk (Dardiotis et al. 2013; Nalls, Blauwendraat, et al. 2019; Satake et al. 2009; Simón-Sánchez et al. 2009; Pankratz et al. 2012; Hamza et al. 2010; Nalls, et al. 2014; Chang et al. 2017). There remains a large component of heritable risk that is therefore unknown. Loci associated with disease risk are often in linkage disequilibrium with multiple “tagging” variants, as part of a haplotype block (Gabriel 2002). Tagging variants

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may span large ranges of the genome, including numerous genes that could have a disease-relevant role. Identifying or prioritising causative genes affected at these loci is a current challenge.

The A53T mutation in *SNCA*, the gene encoding α -synuclein, causes autosomal dominant PD and was the first monogenic cause identified (Polymeropoulos 1997). Additional *SNCA* mutations have since been discovered, causing familial PD or dementia with Lewy Bodies: A30P (Krüger et al. 1998); E46K (Zarranz et al. 2003); H50Q (Appel-Cresswell et al. 2013; Proukakis et al. 2013); G51D (Lesage, Anheim, et al. 2013); A53E (Ghosh et al. 2014; Pasanen et al. 2014). With particular relevance to this thesis, copy number variants of *SNCA* in the form of duplications (Chartier-Harlin et al. 2004; Ibáñez et al. 2004) and triplications (Singleton 2003; Olgiati et al. 2015) have been found to cause PD.

LRRK2 mutations are the second most common autosomal-dominant cause of PD, accounting for up to 10 % of familial cases (Lesage and Brice 2009). These include G2019S (Gilks et al. 2005), found in 1.6 % of a cohort of patients with idiopathic PD, R1441C (Zimprich et al. 2004) and I2020T (Gloeckner et al. 2005).

Parkin, phosphate and tensin homolog-induced putative kinase 1 (*PINK1*) and Daisuke-Junko-1 (*DJ-1*) are the most characterised genes associated with PD through autosomal recessive inheritance.

Parkin mutations are the most common cause of autosomal recessive PD (Klein and Lohmann 2008; Nuytemans et al. 2010; Ferreira and Massano 2016). They account for 10-20 % of cases of early onset (< 40-50 years) PD and were first identified as a cause of juvenile Parkinsonism (Kitada et al. 1998). *PINK1* mutations are a less common cause of PD, while *DJ-1* mutations are considered rare, accounting for around 1 % of early-onset cases (Nuytemans et al. 2010; Abou-Sleiman et al. 2003). The clinical features of all three mutation-driven diseases are similar, with gradual disease progression that is L-DOPA-responsive.

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Non-genetic Factors

The prevalence of PD varies by sex and ethnicity: Distinguishing between cultural exposure to environmental risk factors and genetic background is therefore difficult (Van Den Eeden 2003). Age is the major factor contributing to PD risk (Collier et al. 2011): A study of dopaminergic neurons in normal aged non-human primates indicated degenerative mechanisms in common with PD. Aged neurons have therefore been described as “pre-Parkinsonian”, with a combination of genetic and environmental risk factors accelerating the rate of degeneration (Collier et al. 2011). The “multiple hit” hypothesis posits that accumulation of toxic and genetic factors accelerates dopaminergic ageing, leading to PD (Carvey et al. 2006; Sulzer 2007). More recently, PD has been proposed to be a consequence of ageing, with the suggestion that research into ageing and PD should be coordinated together, because of a strong overlap in relevant biology (Rodriguez et al. 2015).

Selective destruction of dopaminergic neurons in the SNpc was found in users of intravenous drugs contaminated with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Williams 1984; Langston et al. 1983). The toxic metabolite of MPTP, 1-methyl-4-phenylpyridinium ion (MPP^+) is a complex I inhibitor that is structurally very similar to paraquat, a herbicide (differing only by one methyl group). Although there is heterogeneity between the findings of epidemiological studies, long-term exposure to herbicides/pesticides/insecticides that act through interference with the electron transport chain is considered a risk factor for PD (de Lau and Breteler 2006; Liou et al. 1997). Chronic rotenone exposure in rats results in nigrostriatal dopaminergic degeneration with accumulation of α -synuclein-positive intracytoplasmic inclusions (Betarbet et al. 2000).

Exposure to heavy metals is also associated with PD risk (including aluminium, dental amalgam, copper, iron, lead, manganese and zinc) (Lai et al. 2002; Powers et al. 2003).

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Cigarette smokers and coffee drinkers have a lower risk of developing PD (Hernán et al. 2002; Checkoway et al. 2002; Ritz et al. 2007; Costa et al. 2010). Proposed mechanisms of smoking-related protection from degeneration include nicotinic stimulation of dopamine release and antioxidant activity (Quik 2004). The use of the non-steroidal anti-inflammatory drug, ibuprofen is also associated with reduced risk, likely due to anti-inflammatory and antioxidant activity (Samii et al. 2009; Chaturvedi and Beal 2008).

1.1.4 Treatment

Treatment of PD is symptomatic; there are no treatments currently available to delay, stop or reverse progression. The action of most drugs licensed for PD treatment is to increase the level of striatal dopamine to ameliorate motor deficits. L-DOPA is a dopamine precursor that can be orally administered and is able to cross the blood brain barrier (Fahn 2014). It was first prescribed in the 1960's and is still in use today. In 80-90 % of patients, long-term usage leads to the development of involuntary movements, termed L-DOPA-induced dyskinesias (Ahlskog and Muentner 2001; Hauser and Zesiewicz 2007).

Monoamine oxidase B (MAO-B) inhibitors are also used as a treatment strategy, slowing the metabolism of dopamine into 3,4-dihydroxyphenylacetic acid (DOPAC) and increasing striatal DA content, as a result. MAO-B inhibitors, such as selegiline and rasagiline can be used in isolation or in combination with L-DOPA (Bonifácio et al. 2007; Riederer and Laux 2011; Fernandez and Chen 2007).

Selective dopamine receptor agonists are also in use, with lower incidence of dyskinesias reported in patients given long-acting agonists (Stocchi 2009).

Deep brain stimulation of the globus pallidus or subthalamic nucleus is an alternative method of alleviating motor symptoms of PD (Kleiner-Fisman et al. 2006; Breit et al. 2004). There has been uncertainty over the exact mechanism of action, but

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it is believed to work through modulation of oscillatory activity, synaptic plasticity and potentially neurogenesis (Eisinger et al. 2019; Dostrovsky and Lozano 2002; Vitek 2002; Benabid et al. 2002). Combination therapy allows for a lower dosage of L-DOPA to be administered, reducing dyskinetic side-effects.

Treatment of PD lacks disease-modifying agents, due to complexity of the disease and a lack of prodromal biomarkers. A continued effort to understand the mechanisms of degeneration is required in order to develop disease-modifying therapeutics.

1.2 Alpha-synuclein

There has been extensive investigation into the structural and functional properties of α -synuclein. To understand how these properties can confer PD risk or contribute to PD pathology, *SNCA* variants from familial cases have been modelled *in vitro* and *in vivo*.

1.2.1 Structure

SNCA is located on human chromosome 4q21.3-q22 and contains five coding exons, with alternative splicing of at least three 5' exons (Gámez-Valero and Beyer 2018). It is a 14 kDa protein, 140 amino acids long, although alternative splicing may result in shorter or longer forms (Yates et al. 2020). It is divided into three domains, based on structural characteristics: (1) An amphipathic N-terminal alpha-helical region; (2) a central hydrophobic, non-amyloid component; and (3) an acidic C-terminal region (Lashuel et al. 2012).

The N-terminal domain of α -synuclein contains seven 11-residue imperfect repeats, each including a consensus sequence; KTKEGV. This motif is shared with the α -helical domain of apolipoproteins, allowing α -synuclein to interact with lipids in a similar manner (Davidson et al. 1998; Bussell and Eliezer 2003; Bussell 2005).

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PD-related *SNCA* mutations, A30P, E46K, H50Q, G51D, A53T and A53E are all located within this domain. The non-amyloid component domain in the central region is responsible for α -synuclein's propensity to aggregate by forming cross β -sheets (Uéda et al. 1993; Giasson and Lee 2001; Tuttle et al. 2016). The N-terminal and non-amyloid component domain are relatively conserved between species, however the C-terminal varies considerably (Recchia et al. 2004). Phosphorylation of Serine 129 in the C-terminal of α -synuclein is associated with increased aggregation (Anderson et al. 2006; Fujiwara et al. 2002; Samuel et al. 2016), and this post-translational modification has also been reported in accumulations of α -synuclein in *postmortem* PD brains and animal models of synucleinopathies (Tenreiro et al. 2014; Oueslati 2016). Additional α -synuclein modifications include ubiquitination (Hasegawa et al. 2002; Tofaris, Layfield, et al. 2001), nitration (Giasson, Jakes, et al. 2000) and C-terminal truncation (Liu, Giasson, et al. 2005; Oueslati et al. 2010). The conformation of α -synuclein changes according to environmental conditions, interactions, subcellular localisation and the presence of sequence mutations (Fields et al. 2019). The native state of α -synuclein is disputed, with reports of an unfolded conformation or the existence of tetrameric alpha-helical oligomers, resistant to fibrillisation (Bartels et al. 2011; Wang, Perovic, et al. 2011; Fauvet et al. 2012; Burré et al. 2014; Smaldone et al. 2015).

1.2.2 Cellular Properties of α -synuclein

α -synuclein associates with synaptic vesicles in the presynaptic terminal (and is named accordingly (Iwai et al. 1995)), promoting vesicle trafficking and fusion by interacting with synaptobrevin-2 of the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) complex (Maroteaux et al. 1988; Wong and Krainc 2017). When large α -synuclein oligomers form, they preferentially bind to synaptobrevin-2, inhibiting SNARE complex assembly, disrupting vesicle motility and dopamine release, however (Burré et al. 2010; Choi, Choi, et al. 2013).

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Loss of α -synuclein results in an increase in dopamine release, suggesting that monomeric and oligomeric forms regulate dopamine release with different effects (Anwar et al. 2011; Senior et al. 2008).

α -synuclein oligomers can bind to the translocase of the outer mitochondrial membrane (TOM20) and prevent interaction with its co-receptor, TOM22, leading to inhibition of protein import, including complex I subunits (Di Maio et al. 2016). Mitochondria of A53T or A30P mutant neuroblastoma cells exhibit reduced mitochondrial transmembrane potential, cellular respiration capacity and increased reactive oxygen species (ROS) production. α -synuclein can also exert pathological effects on mitochondrial membrane fusion, fission, mitophagy, complex I activity and maintenance of membrane potential (Cole et al. 2008; Chen and Chan 2009; Devi et al. 2008).

Mutant and over-expressed forms of α -synuclein have been found to accumulate in the endoplasmic reticulum (ER) and cause ER stress (Colla et al. 2012). Mitochondrial and ER stress-induced ROS production has been found to contribute to cell death in A53T mutant cells (Smith et al. 2005). ER accumulation also results in leakage of Ca^{2+} into the cytosol; increased mitochondrial uptake potentiates ROS production (Nunnari and Suomalainen 2012; Görlach et al. 2015; Paupe and Prudent 2018).

Cell-to-cell propagation of α -synuclein may occur in a prion-like fashion: LB pathology has been demonstrated in foetal dopaminergic neuron grafts 10 years after transplantation, suggesting spread from diseased to healthy neurons (Kordower et al. 2008; Li, Englund, et al. 2008). Spread and seeded aggregation of monomeric α -synuclein has been demonstrated both *in vitro* and *in vivo* (Luk, Song, et al. 2009; Luk, Kehm, et al. 2012). In mice, intracerebral injection of brain lysates from aged, symptomatic A53T mutant mice into young, asymptomatic mice accelerated aggregation and symptom progression, even at regions far from the injection site

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(Mougenot et al. 2012). There is evidence to suggest that immunisation against the C-terminus of α -synuclein could be used to promote the clearance of toxic α -synuclein species and to reduce synaptic pathological deficits (Masliah, Rockenstein, Mante, et al. 2011; Masliah, Rockenstein, Adame, et al. 2005).

1.2.3 Transgenic Mouse Models of α -synuclein Parkinson's Disease

Transgenic rodent models often use strong heterologous promoters to drive expression of small cDNA constructs, surpassing physiological or disease-relevant levels and patterns of expression (Fernagut and Chesselet 2004). The use of promoters, such as the prion (Giasson, Duda, et al. 2002; Gispert 2003; Lee, Stirling, et al. 2002), Thy-1 (Neumann et al. 2002) and rat TH promoter (Richfield et al. 2002) has resulted in a lack of α -synuclein expression in non-dopaminergic neurons or alternatively, ectopic over-expression in cell types not typically involved in PD.

Bacterial artificial chromosomes (BACs) are large DNA vectors capable of holding the entire genomic locus of a gene, including native promoters and regulatory elements (Shizuya and Kouros-Mehr 2001; Shizuya, Birren, et al. 1992). By producing BAC-transgenic animals containing the entire locus, endogenous spatio-temporal expression is accurately reproduced (Heintz 2001). α -synuclein BAC-transgenic mice have been produced (Hansen et al. 2011; Kuo et al. 2010; Taylor et al. 2014; Yamakado et al. 2012).

α -synuclein transgenic mouse models that over-express the human wild-type, A53T or A30P have been shown to exhibit PD-like phenotypes (Visanji et al. 2016). A toxic gain-of-function mechanism is supported by the finding that expression levels of α -synuclein correlate with the severity of pathology in these models. Several clinical features of PD are recapitulated by α -synuclein transgenic models: Virally induced over-expression of α -synuclein in dopaminergic neurons results

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in neuronal death, a reduction in striatal dopamine content and motor deficits (Kirik et al. 2002).

The Wade-Martins laboratory have developed a BAC transgenic mouse model (*SNCA*-OVX) that over-expresses human α -synuclein at 1.9 times the normal level, reproducing the change in expression observed in patients with *SNCA* locus triplication (Janezic et al. 2013). *SNCA*-OVX mice exhibit selective degeneration of dopaminergic neurons of the SNpc by 18 months with motor deficits. Young mice display synaptic release abnormalities in axons of the dorsal striatum (caudate putamen). This mouse was produced on a *Snca*^{-/-} background, to avoid functional compensation or interaction between the two forms. Importantly, the *SNCA*-OVX mouse recapitulates the age-dependent progression of dopaminergic neuron loss, with disease-relevant levels of expression of α -synuclein.

1.3 Dopaminergic Axons in Parkinson’s Disease

Prevailing concepts of neurodegeneration focus on death of the neuronal soma, with less focus on the role of axon (Burke and O’Malley 2013). There is increasing evidence that axonal degeneration precedes somatic degeneration in PD and that mechanisms differ (Finn et al. 2000; Raff 2002).

Axonal Loss Before the Cell body

Striatal or specifically putaminal deficits in dopaminergic innervation may precede and be more severe than cell body loss in the SNpc: Fearnley and Lees (1991) used neuron count values regressed to clinical duration to estimate that motor symptoms develop after 31 % loss of dopaminergic neurons in the SNpc. This finding was supported by the observation that in *postmortem* tissue with incidental Lewy Bodies, mean age-adjusted dopaminergic neuronal loss was 27 %. The authors suggested that these may represent pre-clinical PD patients. Ma et al. (1997) and

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Greffard et al. (2006) performed similar age-regressed predictions and estimated a 30 % loss at the time of motor onset.

To study striatal dopaminergic terminal loss, *postmortem* neurochemical studies and functional imaging have been performed. Riederer and Wuketich (1976) used caudate dopamine content measurements from *postmortem* tissue and age of onset information to predict a 68 to 82 % decrease in caudate dopamine at the time of clinical presentation. To address concerns over the validity of *postmortem* measurement of dopamine levels, Scherman et al. (1989) measured vesicular monoamine transporter (VMAT2) binding sites using tritiated *α*-dihydrotetrabenazine and estimated a 49 % decrease in binding sites at the time of clinical onset.

In PD patients with early unilateral motor signs, PET imaging was used to determine the range of dopaminergic terminal loss within which motor symptoms develop (Lee, Samii, et al. 2000). Signs appeared between 51 and 62 % loss for a VMAT2 ligand and 56 and 71 % loss for a dopamine transporter (DAT) ligand. More recently, the same group demonstrated that the age of onset influences the percentage terminal loss required before motor onset, with 71 % loss required in younger patients (< 44 years old) (de la Fuente-Fernández et al. 2011).

Axonal Vulnerability Exposed by *α*-synuclein and MPTP in the Axon

Multiple sources of evidence indicate a role for *α*-synuclein in axonal pathology that may precede somal degeneration. A key example is the *SNCA*-OVX model in which release deficits from caudate putamen axon terminals are present at 3 months of age, before the loss of SNpc dopaminergic neurons or development of motor symptoms (Janezic et al. 2013).

Using a pre-digestion technique in paraffin-embedded tissue, the distribution of *α*-synuclein aggregates within the cell can be visualised (Kramer and Schulz-Schaeffer 2007). This technique led to the finding that aggregates were predominantly found in the neuropil and not in the cell body in brains of dementia with Lewy Body

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patients. Building upon this, Orimo et al. (2008) performed TH-, neurofilament- and α -synuclein immunostaining in cardiac sympathetic axons and ganglia of PD patients and demonstrated a distal-dominant accumulation, preceding aggregation in the neuronal somata or neurites.

In mice expressing a C-terminal truncated form of α -synuclein, loss of striatal dopamine content was observed without dopaminergic neuron loss (Tofaris 2006). A follow up investigation of these mice found a redistribution of SNARE proteins SNAP-25, syntaxin-1 and synaptobrevin-2, indicating a toxic gain of function of α -synuclein, specifically at the synapse (Garcia-Reitböck et al. 2010). In primary neuronal cultures, intraneuronal α -synuclein aggregation has been shown to initiate in the pre-synaptic terminal, with subsequent spread to the soma (Volpicelli-Daley et al. 2011).

The effects of MPP⁺ exposure have also been studied in dopaminergic neuronal axons: Aside from its role in inhibiting Complex I activity, and causing synaptic vesicle redistribution, MPP⁺ impairs microtubule assembly (Cappelletti et al. 1999a; Cappelletti et al. 1999b). By specific binding to tubulin, MPP⁺ increases the probability of microtubules shortening. Decreased neuritic trafficking of organelles (including mitochondria) results, coupled with somatic activation of Caspase 3 (Cartelli et al. 2010). The combination of microtubule trafficking defects with mitochondrial dysfunction and potentially impaired autophagy likely creates a multiple-hit scenario for dopaminergic axons that impedes homeostatic regulation and leads to stress and death as a result.

Aside from vulnerabilities exposed by α -synuclein and toxin exposure, dopaminergic axons exhibit additional functional and morphology properties that distinguish them from other axons. These distinguishing features likely contribute to their vulnerability. Dopaminergic mitochondria were compared to non-dopaminergic neurons by two independent groups; both found that they are only 40 % of the

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size of non-dopaminergic mitochondria (Kim-Han et al. 2011; Liang et al. 2007). It was also shown that they traffic almost three times slower than non-dopaminergic mitochondria. This rate of trafficking seems counterintuitive, as dopaminergic axons span massive distances through the striatum, each occupying 2.7 % of striatal volume and synapsing with approximately 75,000 neurons (Matsuda et al. 2009).

Degeneration in Dopaminergic Axons

Programmed axon degeneration (referred to as Wallerian degeneration) is a component of neurological diseases including Alzheimer’s disease, Parkinson’s disease and peripheral neuropathies (Coleman and Höke 2020). Over the past three decades, several proteins have been found to mediate or regulate the course of degeneration. Slow Wallerian Degeneration (*Wld^S*) is a chimeric protein, joining the N-terminal 70 amino acids of ubiquitination factor *Ube4b* with the full sequence of *Nmnat1* through an 18 amino acid linker region. *Wld^S* mice demonstrated ten-fold delayed Wallerian degeneration of peripheral nerve axons after axotomy and in response to a range of genetic and toxic stimuli (Coleman 2002; Feng et al. 2010; Coleman and Freeman 2010). Paradoxically, *Nmnat1* is primarily localised to the nucleus. Targeted mutations in the linker region of *Wld^S* demonstrated that this region confers a nuclear localisation signal: Weakening this signal promoted axonal survival (Beirowski et al. 2009). In the same study, native *Wld^S* was detected in axonal fractions, mitochondria and microsomes, shifting the focus away from an exclusively nuclear role of *Nmnat*-related axon protection.

Wld^S is a mutant protein, while *NMNAT2* is endogenously expressed in axons and is now known to play a critical role in blocking axonal degeneration (Conforti et al. 2014; Gerdts et al. 2016; Llobet Rosell and Neukomm 2019). Activation of Wallerian degeneration is now thought to be due to loss of axonal *NMNAT2* expression, which can occur within hours of blocking its axonal trafficking or synthesis Gilley and Coleman (2010). *NMNAT2* loss leads to an increase in the

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expression of sterile alpha and TIR motif-containing protein 1 (SARM1) which triggers axonal death.

The Wallerian pathway and mitochondrial dysfunction may share overlapping importance in PD degeneration: Pharmacological uncoupling of mitochondria causes axonal degeneration that is prevented by deletion of *Sarm1* (Loreto et al. 2015; Summers et al. 2014). *Wld^S* expression was also found to be protective against MPTP-induced dopaminergic neuron loss (HASBANI and OMALLEY 2006).

1.4 Gene Expression Profiling in Dopaminergic Neurons

To infer the functional activity of cell populations of interest, gene expression can be measured. Multiple techniques can be used to assay the abundance of a gene’s cognate transcripts or proteins, such as quantitative polymerase chain reaction, microarray, next-generation sequencing, immunochemical methods and mass spectrometry. Measures of transcript abundance are an approximation of gene expression however, due to regulatory mechanisms at the post-transcriptional, translational and post-translational level (de Sousa Abreu et al. 2009). The squared Pearson correlation coefficients between mRNA and protein abundance is ~ 0.4 (de Sousa Abreu et al. 2009; Maier et al. 2009).

Throughput, cost, result turnaround and analytical support are additional considerations. Next-generation sequencing is currently a common method used to estimate genome-wide expression from messenger RNA (mRNA). Often the primary aim of RNA sequencing studies is to compare gene expression between groups of samples from different biological conditions, such as age or genotype. “Differentially expressed” features (genes, transcripts) are used to infer changes in cellular or tissue activity.

1.4.1 Translating Ribosome Affinity Purification

A problem with measuring transcript or protein expression is variation in sample composition: If mixed cell-type samples are studied and the biological condition of interest induces a change in cellular composition, differential expression results can be dominated by that change (Oldham, Langfelder, et al. 2012).

Single-cell sequencing helps to address this issue, as distinct populations can be separated before sequencing (by fluorescence or immunocapture-based sorting) or after sequencing (by clustering). This technique presents new challenges, however: Tissue dissociation can introduce technical artifacts in expression, including the increased expression of markers of cellular stress (van den Brink et al. 2017). In addition, the composition of adult tissues, such as brain can render the isolation of rare cell types very challenging. Finally, current library preparation methods are not able to reliably capture information for the entire transcriptome, leading to stochastic detection of gene expression across cells (Kharchenko et al. 2014).

Translating ribosome affinity purification (TRAP) isolates mRNA from translating ribosomes through the expression a modified ribosomal subunit; eGFP-L10a (Figure 2.1)(Heiman, Kulicke, et al. 2014; Doyle et al. 2008). By pairing the eGFP-L10a transgene with a cell type-specific promoter, expression can be driven in distinct populations, allowing the capture of “enriched” RNA from those cells. This technique overcomes the aforementioned problems by capturing mRNA that is engaged in translation and cell type-specific. “Translatomics” have recently been found to demonstrate stronger correlation with protein abundance ($R^2 = 0.71$) (Blevins et al. 2019).

TRAP has been used to study gene expression in dopaminergic neurons in a toxic model of PD (Brichta, Shin, et al. 2015). Brains of mice given 5 daily intraperitoneal injections of either MPTP or saline were TRAP-processed to compare gene expression. Through a “master regulator inference algorithm”, transcriptional

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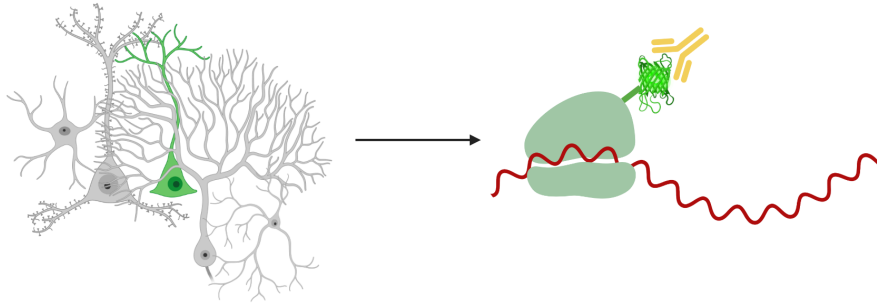


Figure 1.1: Translating Ribosome Affinity Purification: Cell type-specific expression of eGFP-L10a enables the selective immunocapture of tagged ribosomes and associated mRNA.

regulators were predicted to drive the observed expression changes due to MPTP administration. *Satb1* and *Zdhhc2* were knocked down using virally transfected short hairpin RNAs, each resulting in the selective loss of SNpc neurons, mimicking MPTP pathology. This study demonstrated a route to identifying novel genetic features important to dopaminergic neurons. As a model, this study lacked physiological progression: At the point of sample collection (9 days after starting injections), over 25 % of dopaminergic neurons were lost. The study also made no use of the additional information provided by TRAP; namely, the cell type-specificity of each gene.

The Wade-Martins Laboratory have extensively characterised and piloted the use of a novel triple transgenic, conditional TRAP-Parkinson's model (Figure 1.2) (Wagner 2017). $Rosa26^{fsTRAP} :: DAT^{IREScre}$ (TRAP) mice selectively and abundantly express eGFP-L10a in midbrain dopaminergic neurons. This was confirmed by visualising colocalisation of eGFP-L10a and the dopamine transporter, DAT (Figure 1.2). Striatal expression of eGFP-L10a could not be detected by the staining method used, however quantitative PCR of RNA obtained by performing TRAP in the striatum confirmed the enrichment of dopaminergic markers.

TRAP mice have subsequently been crossed with the *SNCA*-OVX model to enable the study of gene expression in dopaminergic cell bodies and axons in healthy

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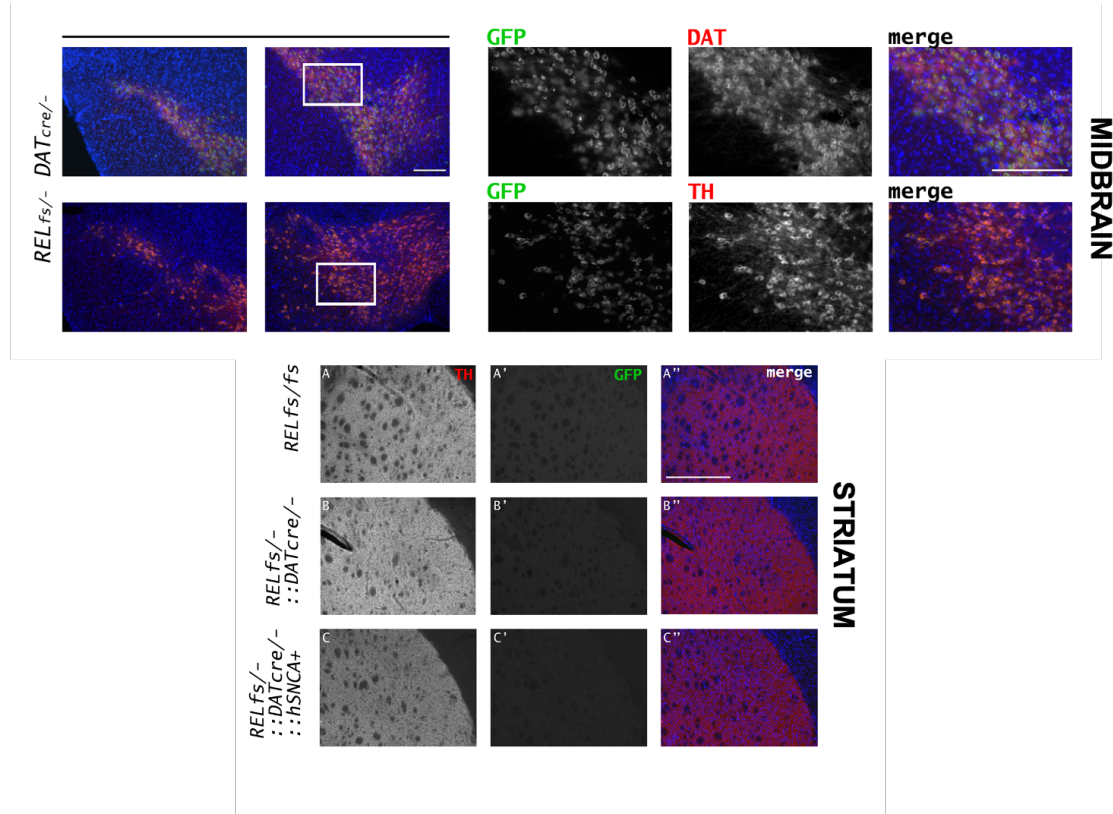


Figure 1.2: Rosa26^{fsTRAP} ::DAT^{IREScree} mice selectively express eGFP-L10a in dopaminergic neurons: A thorough characterisation of a novel TRAP model was performed by Wagner (2017). Immunohistochemical staining for GFP and DAT in the midbrain demonstrated strong expression of eGFP-L10a that colocalised with DAT. Striatal eGFP-L10a expression could not be detected by this method, however pilot TRAP experiments in striatal tissue confirmed the enrichment of dopaminergic RNA. Source: (Wagner 2017).

and Parkinsonian ageing.

1.5 Aims

The specific loss of dopaminergic neurons in PD indicates a requirement to understand the properties that uniquely distinguish this cell type from other neural populations. The unique morphology of the dopaminergic axon and potential for early degeneration warrants a comparison between somal and axonal expression. By combining the use of TRAP to isolate dopaminergic-specific mRNA with the SNCA-OVX model of PD and the process of ageing, the following aims are ad-

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dressed in this thesis:

- **To identify the expression features that specifically distinguish dopaminergic neurons from neighbouring cell types.** To use specificity of expression to prioritise candidate causal genes located within novel PD GWAS loci.
- **To identify the genes subject to localised translation in dopaminergic axons**
- **To characterise the changes in expression in dopaminergic cell bodies and axons in age and due to overexpression of human α -synuclein**

This will be described in three Chapters:

- **Building a Compartment-Specific Dopamine Neuron Transcriptomic Dataset (Chapter 3)**
- **Specific Expression Features in the Dopaminergic Neuron (Chapter 4)**
- **Compartmental and Condition-Dependent Expression in Dopaminergic Neurons (Chapter 5)**

2. Material and Methods

2.1 Animal Husbandry

2.1.1 Ethical declaration

All experiments and procedures conducted on animals were carried out in accordance with United Kingdom Home Office regulations under the Animal (Scientific Procedures) Act (1986), and were approved by the local ethical review board at the University of Oxford. All mice were housed in the University of Oxford Biomedical Services Building. Mice had access to standard food and water *ad libitum*. Mouse holding rooms were maintained at 22 °C and 60 to 70% humidity on a 12 hour light-dark cycle. All mice were bred on a C57/BL6 background. For all experiments, animals of both sexes were used unless indicated otherwise.

2.1.2 Breeding

2. 2. Material and Methods

Table 2.1: Transgenic mouse lines used in this thesis.

Referred to in text	JAX Line	JAX Stock Code	Generation Reference
Rosa26 ^{fsTRAP}	B6.129S4- <i>Gt(ROSA)26Sor^{tm1(CAG-EGFP/Rpl10a,-birA)Wtp}/J</i>	022367	(Zhou et al. 2013)
DAT ^{IREScree}	B6.SJL- <i>Slc6a3^{tm1.1(cre)Bkmn}/J</i>	006660	(Bäckman et al. 2006)
SNCA- OVX	B6.Cg- Tg(SNCA)OVX37Rwm <i>Snca^{tm1Rosl}/J</i>	023837	(Janezic et al. 2013)

Homozygous Rosa26^{fsTRAP} mice were bred with homozygous DAT^{IREScree} mice to generate heterozygous Rosa26^{fsTRAP}::DAT^{IREScree} offspring that were used for TRAP experiments. Rosa26^{fsTRAP} parents were crossed and bred to be hemizygous *hSNCA+* (*SNCA*-OVX): Offspring were a mixture of hemizygous *hSNCA+* and *hSNCA-*.

All animals were wild-type mouse *Snca*^{+/+}, due to the position of the ROSA26-eGFP-L10a locus being on the same chromosome as *Snca* (chromosome 6).

2.1.3 DNA extraction from ear tissue

To genotype mice, DNA was extracted from ear tissue by digestion in 450 μ l of lysis buffer (50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris pH 8.5, 0.45 % CA-630 (IGEPAL), 0.45 % Tween-20, ddH₂O) and 20 g/ml proteinase K for 2 hours, shaking at 55 °C. Once tissue was no longer visible, Proteinase K activity was inactivated by incubating samples at 5 °C for 20 minutes. DNA samples were stored at room temperature.

2.1.4 Genotyping PCR

To confirm the genotype of mice for study, a polymerase chain reaction (PCR) was performed on extracted DNA. The QIAGEN HotStar Taq DNA Polymerase kit was used to prepare master mixes for each primer pair in a 20 μ l reaction. 18 μ l of master mix was added to each well of a 96-well plate and 2 μ l of template DNA

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was combined. PCR was performed using a DNA Engine Tetrad 2 Peltier Thermal Cycler (Bio-Rad) using the programme described in Table 2.3. After amplification, the PCR product was separated using gel electrophoresis. A 2 % agarose gel (w/v) was prepared in 1x Tris/Borate/EDTA (TBE) buffer and 0.25 g/ml ethidium bromide was added. The Gene Ruler 1kb Plus DNA ladder was used for fragment sizing (Thermo Scientific). Samples were run at 100 V immersed in TBE buffer for 45 minutes. Gels were imaged using the GelDoc XR system (Bio-Rad).

2.2 TRAP

All reagents specified were RNase-free, treated with diethyl pyrocarbonate (DEPC) or cleaned with RNaseZap, RNase-free water or 100 % ethanol to minimise contamination. All steps were performed in 1.5 mL Eppendorf DNA-LoBind tubes, unless specified otherwise.

2.2.1 TRAP Reagents

1,2-diheptanoyl-sn-glycero-3-phosphocholine (DHPC) powder (Generon) was stored at 20 °C and thawed to room temperature for a minimum of 30 minutes before opening for reconstitution. DHPC was reconstituted to 300 mM and rotated end over end for 30 minutes. Reconstituted DHPC was stored for up to 7 days at 4 °C in its original glass container.

Table 2.2: Genotyping PCR Components

Component	Volume (μ l)
ddH ₂ O	13.5
10x Buffer	2
8 mM dNTP Mix	0.4
10 mM Primer Pair	2
HotStar Taq DNA Polymerase	0.1

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Table 2.3: Genotyping PCR Programme

Temperature (°C)	Duration	No. of Cycles
95	15 mins	1
95	30 secs	34
55	30 secs	34
72	60 secs	34
72	3 minutes	1
Room Temperature	Hold	1

Table 2.4: Genotyping PCR Primer Sequences

Primer	Forward	Reverse	Product Size (bp)
Wild-type ROSA26 Locus	AAGGGAGCTGCAGTGGAGTA	CCGAAAATCTGTGGGAAGTC	297
ROSA26-eGFP-L10a	AAGGGAGCTGCAGTGGAGTA	CGGGCCATTTACCGTAAGTTAT	284
eGFP-L10a 1	TATCATGGCCGACAAGCAGA	CACCGTCTCCAGAACTTGC	395
eGFP-L10a 2a	CAAAGACCCCAACGAGAAGC	CTCCACCGTCTCCAGAACT	227
eGFP-L10a 2b	CAAAGACCCCAACGAGAAGC	TTGGCTTCATCACAGTGCTG	361
DAT Wild-type	TGGCTGTTGGTGTAAGTGG	GGACAGGGACATGGTTGACT	264
DAT-Cre	CCGGGCTGCCACGACCAA	GGCGCGGCAACACCATTTT	445
SNCA-OVX 3' BAC Junction	GGCCTCTGTCGTTTCTTTCTCTG	ACGTGCATAGGCAGAGCTAAACT	450
SNCA-OVX 5' BAC Junction	CAC TGAGTATTGTTCTGGTAAC	TCAAACATGAGAATTGGTCG	450

Cycloheximide powder was aliquoted into 1.5 ml tubes, stored at 4 °C and dissolved on the day of use to 100 mg/ml in methanol. Solutions of cycloheximide were stored at 4 °C for a maximum of 24 hours.

Recombinant, purified Biotinylated Protein L (Fisher Scientific) was reconstituted to 1 µg/µl in water. Excess was stored at -80 °C for later use.

TRAP buffers were prepared as described below. Buffers were stored at 4 °C:

Dissection Buffer

Hank's balanced salt solution (HBSS, 10x, calcium, magnesium, no phenol red; Invitrogen), 2.5 mM HEPES-KOH (1 M, pH 7.4; Affimetrix), 35 mM glucose (Sigma), and 4 mM sodium bicarbonate (NaHCO₃; Sigma) in RNase-free water. Immediately before use, 100 g/ml CHX (100 mg/ml in MeOH; Sigma) was added to a buffer aliquot.

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Tissue-lysis buffer

20 mM HEPES KOH, 150 mM KCl (potassium chloride, 2 M, RNase-free; Applied Biosystems), and 10 mM MgCl₂ (magnesium chloride, 1 M, RNase-free; Applied Biosystems), in RNase-free water. Immediately before use, protease inhibitors (one mini tablet per 10 ml; Mini-complete EDTA-free, Roche), 0.5 mM DTT (100 mM; Superscript III kit, Invitrogen), 100 g/ml CHX (100 mg/ml in MeOH; Sigma), and 10 l/ml rRNasin (recombinant Ribonuclease Inhibitor, 20-40 U/l; Promega) and Supersasin (SUPERase In RNase Inhibitor, 20 U/l; Applied Biosystems) were added to each buffer aliquot.

Low-salt buffer

20 mM HEPES KOH (pH 7.4), 150 mM KCl, 10 mM MgCl₂, and 1% nonylphenyl polyethylene glycol (NP-40, 10%; AG scientific) in RNase-free water. Immediately before use, 0.5 mM DTT, and 100 g/ml CHX were added to a low-salt buffer aliquot. 10 l/ml rRNasin and Supersasin were only added to the low-salt buffer on TRAP day 1.

High-salt buffer

20 mM HEPES KOH (pH 7.4), 350 mM KCl, 10 mM MgCl₂, and 1% NP-40 in RNase-free water. Immediately before use, 0.5 mM DTT, 100 g/ml CHX, and 10 l/ml RNasin and Supersasin were added to a HS buffer aliquot.

2.2.2 Anti-eGFP Antibody Preparation

Monoclonal anti-eGFP antibodies (Htz-GFP-19F7 and Htz-GFP-19C8: Bioreactor supernatant purity) were purchased from the Memorial Sloan-Kettering Monoclonal Antibody Facility. Upon arrival, antibodies were mixed gently, divided into experimental aliquots and stored at -80 °C. For use, aliquots were thawed

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and centrifuged at 15,000 *g* for 10 minutes at 4 °C. The supernatant was used for conjugation, as described below.

2.2.3 Streptavidin Dynabead Pre-Treatment

Streptavidin MyOne T1 Dynabeads (ThermoFisher) were washed in order to prepare them for RNA handling. Beads were resuspended by vortexing to ensure an even distribution. The required volume of beads was removed and placed in a tube for washing. Beads were collected on the sidewall of the tube using a magnet and the supernatant was removed using an aspirator. An equal volume of PBS was added, beads were resuspended by pipette mixing and left to stand for 2 minutes. This process was repeated, performing 3 PBS washes in total. Beads were subsequently washed twice in 0.1 M NaOH, 0.05 M NaCl for 2 minutes, followed by one wash in 0.1 M NaCl. Beads were finally resuspended in PBS until biotinylation (described below).

2.2.4 Biotinylation and Antibody Binding

Within each cohort, the same common source of beads, bound to anti-eGFP antibody were used, with the exception of Cohort 1 samples in which aged samples were collected 15 months after young samples (described in (Section 3.2)).

Volumes for biotinylation and antibody conjugation were maintained close to the original volume of dynabeads collected, as recommended by the manufacturer. A magnet and aspirator were used for all supernatant removal steps.

Washed streptavidin MyOne T1 dynabeads from the previous step were collected on the magnet and resuspended in an equal volume of PBS and biotinylated protein L. The mixture was rotated end over end for 35 minutes at room temperature. After biotinylation, beads were washed 5 times with PBS containing 3 % IgG and protease-free BSA (w/v). After washing, equal amounts (by mass) of each anti-eGFP antibody were added, diluted in low salt buffer. The mixture was rotated

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end over end for 1 hour. No vortexing was performed after this step. After antibody binding, beads were washed 3 times with low salt buffer and finally resuspended in 200 μ l of low salt buffer per sample aliquot (each TRAP sample received 200 μ l of prepared beads, however the number of beads per unit volume differed between somal and axonal TRAP samples, as described below). Prepared beads were used immediately or stored at 4 °C for up to 2 weeks with 0.03 % ProClin 300 added.

2.2.5 Translating Ribosome Affinity Purification

A visual guide to this protocol is shown in Figure 2.1. Numbered steps are described:

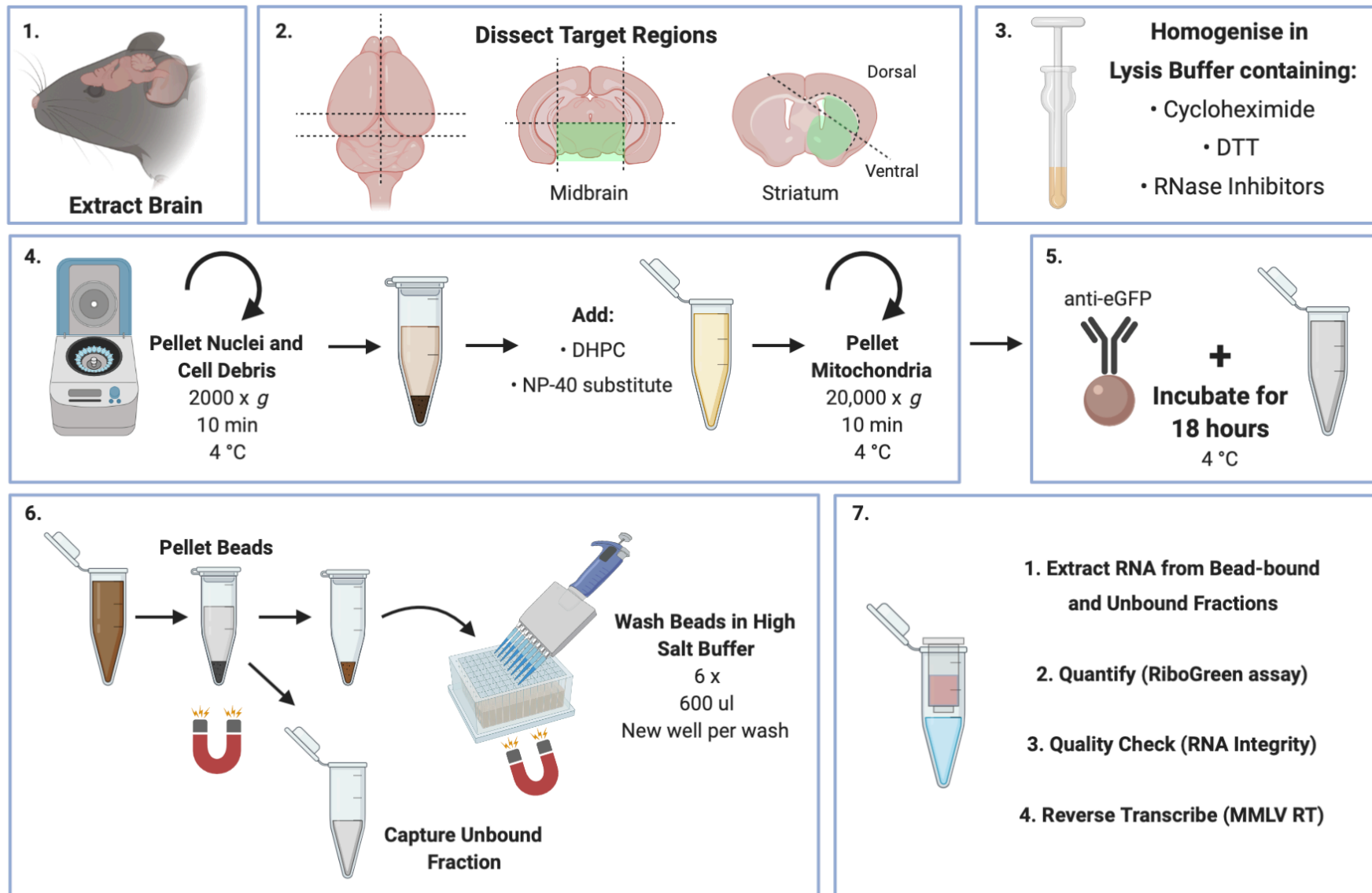


Figure 2.1: The TRAP Method

2. 2. Material and Methods

1. Mice were euthanased by cervical dislocation.
2. Brains were rapidly dissected on ice. The midbrain, dorsal and ventral striatum from each mouse was collected and placed directly in ice-cold lysis buffer.
 1. To isolate midbrain, a sagittal cut was made to divide the brain into two hemispheres. Two coronal cuts were then made at Bregma AP -2.5 mm and -4.1 mm, followed by a horizontal cut at the level of the rostral linear nucleus. Tissue ventral to this cut was taken forward.
 2. To isolate the dorsal and ventral striatum, two coronal cuts were made at Bregma AP -0.5 mm and 1.5 mm on each hemisphere, visualising the corpus callosum and lateral ventricles as a guide. The circumferential boundary of the striatum was then traced by scalpel, removing the corpus callosum, lateral ventricles and all remaining surrounding tissue. To separate the dorsal and ventral striatum, a single, angled cut was made at a 45 ° angle, as illustrated in Figure 2.1, Box 2.
3. After every fourth mouse, brains were homogenised using a 2 ml dounce homogeniser.
4. Homogenised lysates were centrifuged at 2000 x *g* for 10 minutes at 4 °C to pellet cellular debris. Meanwhile, the next four brains were collected. Lysates were held at this stage at 4 °C until all brain were collected, homogenised and cellular debris pelleted. The supernatant of each lysate was transferred to a new tube and 1/8 th volumes of DHPC and NP-40 substitute were added (equivalent to 30 mM DHPC and 1 % NP-40 substitute). Lysates were gently mixed by inversion before placing on ice for 5 minutes. Lysates were then centrifuged at 20,000 x *g* for 10 minutes at 4 °C to pellet mitochondria and any remaining debris that could interfere with bead-antibody-eGFP interaction.

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At this stage, a 20 μ l sample of lysate was taken for extraction of TOTAL RNA.

5. Beads coated in anti-eGFP antibody were added to each lysate. Samples were incubated for 18 hours at 4 °C with gentle end over end rotation.
6. After 18 hours, beads were transferred from 1.5 mL tubes to an Eppendorf DeepWell DNA LoBind 2 mL volume 96-well plate. This plate was partially submerged in a container filled with DEPC-treated water and frozen cool packs, to maintain cold. A magnet was positioned below the plate to collect the beads. From the supernatant, a 20 μ l sample of lysate was taken as an additional TOTAL RNA sample. Beads were then washed six times in high-salt buffer, transferring to a new row of the plate with each wash, to minimise carryover of lysate material. With each wash, the resuspended bead mixture was pipetted up and down 10 times with a 1 ml multichannel pipette configured to 600 μ l.
7. After the final wash, the plate was removed from the chilled bath and beads were left to warm to room temperature for 1 minute. 100 μ l of QIAGEN RLT PLUS lysis buffer + 1 % beta-mercaptoethanol (B-ME) was then added to each sample of beads. Samples were vortexed to ensure mixing within the buffer and incubated for 10 minutes. At 10 minutes, the beads were once again pelleted on the magnet and the supernatant was carried forward for RNA extraction.
8. (Not shown in Figure 2.1): To extract TRAP RNA, 100 μ l of RNA in RLT PLUS buffer + B-ME was added to a QIAGEN gDNA eliminator column for removal of genomic DNA. Columns were centrifuged immediately for 30 seconds and 8000 x g . An equal volume of 70 % ethanol was added to the flow-through, so that RNA was present in a 35 % solution. This solution was

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added to a QIAGEN RNEasy MinElute Micro column and was centrifuged at 8000 x *g* for 15 seconds to load the RNA onto the silica membrane. Columns were washed using 700 μ l of QIAGEN RW1 buffer, 500 μ l of QIAGEN RPE buffer and 500 μ l of 80 % ethanol with centrifugation at 8000 x *g* for 30 seconds after the first two washes and centrifugation at 8000 x *g* for 2 minutes after the 80 % ethanol wash. The lid of each column was then opened and columns were centrifuged at 15,000 x *g* for 5 minutes to dry the silica membrane and remove trace amounts of ethanol that would interfere with downstream RNA processing. 14 μ l of RNase-free water was applied to each silica membrane and the columns were centrifuged at 14,000 x *g* for 1 minute to collect the eluate of TRAP RNA. RNA was immediately aliquoted into a “main” component, from which samples for sequencing would be obtained and a “QC” component, from which RNA concentration and integrity could be measured. Aliquots were snap-frozen on dry-ice and stored at -80 °C. TOTAL RNA extraction from 20 μ l lysates was performed using the same steps.

2.2.6 Assessment of RNA Integrity

The RNA integrity of samples was estimated using the Agilent RNA 6000 Pico Kit (Agilent) in accordance with the manufacturer’s instructions. Reagents were stored at 4 °C between uses and were equilibrated to room temperature for 30 minutes before use. To prepare the gel matrix, 550 μ l of gel was filtered through a spin filter centrifuged at 1500 x *g* for 10 minutes. To measure up to 11 samples, the dye concentrate was vortexed, spun down and 1 μ l was added to 65 μ l of filtered gel matrix. This gel-dye mix was vortexed and centrifuged at 13,000 x *g* for 10 minutes. 9 μ l of gel-dye mix was pipetted into the loading port of the assay chip. The syringe pump was attached and pressed to the designated latch position for RNA samples. After 30 seconds, the syringe plunger was released and after 5 seconds, was slowly

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returned to a volume of 1 ml. 9 μ l of gel-dye mix was subsequently added to the two additional gel wells. 9 μ l of conditioning solution was added to the condition well. 5 μ l of RNA 6000 Pico marker was added to each sample well and the ladder well. 1 μ l of heat denatured (70 °C for 2 minutes) sample was added to each sample well and 1 μ l of ladder was added to the ladder well. The chip was horizontally shaken for 60 seconds at 2400 rpm and was then immediately processed.

All measured samples from each cohort were of good integrity (RIN $\geq$ 7 out of 10).

2.2.7 Quantification of RNA Mass from TRAP

The quantity of RNA obtained by TRAP was measured using the low-range (1-50 pg/ μ l) Quant-iT RiboGreen RNA Assay Kit (Invitrogen). All reagents were equilibrated to room temperature before use and the fluorescent dye was protected from light. A 10-step dilution series from 2 μ g/ml to 100 pg/ml was prepared in duplicate using supplied E. coli RNA. 1 μ l of each sample was diluted in 99 μ l of TE buffer and 100 μ l of working dye solution. Sample were incubated for 5 minutes before measurement on a Synergy HT plate-reader (Biotek).

2.2.8 cDNA Synthesis

To conserve RNA for sequencing, RNA was reverse transcribed according to the SMART-Seq2 protocol (Picelli et al. 2014). Template-switching reverse transcription allowed the incorporation of tailed primers and subsequent PCR amplification of cDNA. For each reaction, the following steps were performed at 4 °C, unless stated otherwise.

1. Mix 2.3 μ l of RNA with 1 μ l of 10 μ M Oligo-dT₃₀VN primer and 1 μ l of dNTP mix. Mix by flicking and spin down.
2. Incubate mix at 72 °C for 3 minutes.

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3. Add 5.3 μ l of reverse transcription mix (Table 2.5).
4. Incubate mix according to Table 2.6 to reverse transcribe RNA.
5. Add 15 μ l of PCR mix (Table 2.7) and mix.
6. Incubate mix according to Table 2.8 to amplify cDNA.
7. Incubate 25 μ l of resuspended, room temperature AmpureXP beads with 25 μ l of cDNA product for 10 minutes. Mix thoroughly by pipetting to ensure a homogenous consistency.
8. Pellet the AmpureXP-cDNA mix using a magnetic stand.
9. Aspirate the supernatant and discard. Perform 2x 500 μ l washes with 70 % ethanol without resuspending the pellet. Incubate the pellet for 30 seconds with each wash.
10. Aspirate all traces of ethanol and air-dry the pellet for 30 seconds.
11. Resuspend the pellet in 10 μ l of water and incubate for 10 minutes to elute cDNA.
12. Pellet the AmpureXP beads and remove and store the eluted cDNA for quantification on the Qubit fluorometer.

2.2.9 Quantitative PCR

500 pg minimum of cDNA was used per qPCR reaction. cDNA was combined with a qPCR mastermix (Table 2.9). Each reaction mix was pipetted into a 96-well plate including no template negative control reactions and amplified according to Table 2.10.

The primer sequences used for TRAP validation are shown in Table 2.11.

2. 2. Material and Methods

Table 2.5: SMART-Seq2 Reverse Transcription Components

Component	Volume (μ l)
SuperScript II RT (200 U μ l)	0.50
RNAse inhibitor (40 U μ l)	0.25
Superscript II first-strand buffer (5x)	2.00
DTT (100 mM)	0.50
Betaine (5 M)	2.00
MgCl ₂	0.06
Template-switching oligo (100 μ M)	0.10
Nuclease-free water	0.29

Table 2.6: SMART-Seq2 Reverse Transcription Programme

Temperature ($^{\circ}$ C)	Duration	No. of Cycles
42	90 mins	1
50	2 mins	11
42	2 mins	11
70	15 mins	1
Room Temperature	Hold	1

Table 2.7: SMART-Seq2 PCR Components

Component	Volume (μ l)
RT Product	10
KAPA HiFi HotStart ReadyMix (2x)	12.50
IS PCR primers (10 μ M)	0.25
Nuclease-free water	2.25

Table 2.8: SMART-Seq2 PCR Programme

Temperature ($^{\circ}$ C)	Duration	No. of Cycles
98	3 mins	1
98	20 secs	18
67	15 secs	18
72	6 mins	18
72	5 mins	1
Room Temperature	Hold	1

2. 2. Material and Methods

Table 2.9: qPCR Components

Component	Volume (μ l)
cDNA	5
FastSYBR Green Master Mix	12.5
Forward and Reverse Primers (10 μ M each)	0.2 each
Nuclease-free water	7.1

Table 2.10: qPCR Programme

Temperature ($^{\circ}$ C)	Duration	No. of Cycles
95	20 secs	1
95	3 secs	40
60	30 secs	40

2.2.10 RNA Sequencing

3 ng of TRAP RNA per samples in cohorts 1 and 2 were submitted to the Wellcome Trust Centre for Human Genetics for library preparation and sequencing. Samples were prepared using the SMARTer Low Input RNA kit (Clontech). cDNA libraries were sequenced on an Illumina HiSeq 4000 75 bp system.

200 pg of TRAP RNA per samples in cohort 3 was submitted to the Wellcome Trust Centre for Human Genetics for library preparation and sequencing. Samples were prepared using the SMARTer ultra-low input kit (Clontech). cDNA libraries were sequenced on a NovaSeq 6000 150 bp system.

Table 2.11: qPCR Primer Sequences

Gene	Forward	Reverse	Product length (bp)
Th	TGTTGGCTGACCGCACAT	GGCCCCAGAGATGCAAGTC	61
Slc6a3	ACACCCGCTGCTGAGTATTT	AGGTCATCAATGCCACGACT	68
Gfap	ACCGCATCACCATTCCTGTA	GCCTTCTGACACGGATTTGG	83
Gad2	TGGGTTTGAGGCACACATTG	GTGTGTTGAGGCTTCCCATC	111

2.3 Long-Read cDNA Library Preparation and Sequencing

Long-read cDNA libraries were produced for sequencing on the Oxford Nanopore Technologies MinION. The PCR-cDNA Barcoding protocol (SQK-PCB109) was followed, with amendments as described:

1. 7 ng of TRAP RNA was used per sample as input instead of the minimum of 50 ng recommended. This was due to limited availability. Pooling of 12 barcoded samples after PCR overcame the minimum number of moles required for flowcell loading.
2. RNA was added up to a volume of 9 μ l in RNase-free water to a 0.2 ml PCR tube containing 1 μ l of VN primers and 1 μ l of 10 mM dNTPs. Tubes were gently flicked to mix and spun down.
3. Samples were incubated at 65 °C for 5 minutes to allow annealing of the VN primer.
4. A mixture of 4 μ l 5x reverse transcript buffer, 1 μ l RNaseOUT, 1 μ l water and 2 μ l strand-switching primer was added to each annealed sample. Tubes were gently flicked to mix and spun down.
5. Samples were incubated at 42 °C for 2 minutes.
6. 1 μ l of Maxima H Minus Reverse Transcriptase was added (ThermoFisher). Tubes were gently flicked to mix and spun down.
7. Samples were incubated at 42 °C for 90 minutes, heat inactivated at 85 °C for 5 minutes and then held at 4 °C until the next stage.

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8. 5 μ l (one quarter) of each reverse transcribed (RT) product was used in a single PCR reaction: Four PCRs could therefore be performed from one RT product. Using a larger volume of RT product per PCR diminished amplification yield. The RT product was mixed with 1.5 μ l barcoded primer, 18.5 μ l water and 25 μ l 2x LongAmp Taq Master Mix (NEB).
9. Samples were amplified according to Table 2.12.
10. After PCR, 1 μ l of NEB exonuclease 1 was added to each tube with pipette mixing. Samples were incubated at 37 °C for 15 minutes, followed by 80 °C for 15 minutes.
11. Barcoded PCR reactions were pooled into a 1.5 mL Eppendorf DNA LoBind tube.
12. AMPure XP beads were equilibrated to room temperature for 30 minutes before use and were rigorously vortex and rotated end over end for resuspension.
13. A 0.5 x equivalent volume of AMPure XP beads was added to the volume of PCR product. This is in contrast to 0.8 X described in the SQK-PCB109 protocol. Using a lower volume enriched for longer fragments, avoiding the highly abundant, $\sim$ 200 bp fragments that were found to be detrimental to sequencing throughput.

Table 2.12: PCR Amplification of Long-Read cDNA Libraries

Temperature (°C)	Duration	No. of Cycles
95	30 secs	1
95	15 secs	18
62	15 secs	18
65	6 minutes	18
65	6 minutes	1
4	Hold	1

2. 2. Material and Methods

14. Sample-AMPure XP mixtures were rotated end over end for 20 minutes.
15. Beads were collected using a magnetic and the supernatant was removed with an aspirator.
16. Beads were washed twice in 200 μ l of 70 % ethanol. Beads were **not** resuspended between washes.
17. After the second ethanol wash, the ethanol was removed by aspiration and the tube of beads was inspected to confirm there were no visible droplets of ethanol that would interfere with library performance.
18. Before the beads dried out (a crack appears on the surface of the grouped beads when this occurs), 12 μ l of elution buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0) was added and the beads were gently resuspended. Bead-buffer mixtures were left to incubate for up to half an hour (longer than the protocol; 10 minutes) to try to maximise yield.
19. Beads were pelleted using the magnet. The mass of the cleaned-up PCR product was measured using a Qubit fluorometer (ThermoFisher). The fragment size distribution was measured using the Agilent High Sensitivity DNA Kit.
20. Based on the mass and mean fragment size, 100 fmol of PCR product was taken forward and diluted to 11 μ l in elution buffer.
21. 1 μ l of rapid adapter was added, mixed by pipetting and spun down. The sample was incubated for 5 minutes at room temperature.
22. To prime and load the MinION Mk1B flow cell, the sequencing buffer (SQB), loading beads (LB), flush tether (FLT) and flush buffer (FB) were thawed to room temperature. The SQB, FLT and FB were vortexed and spun down.

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23. The priming port cover on the flow cell was opened clockwise fully. A P1000 pipette tip was cut around 2 mm from its distal end, to create a secure fit between the tip end and the priming port of the flow cell. A P1000 pipette with a locking adjustment mechanism was used, to allow low resistance, slow turning of the volume dial.
24. The P1000 pipette was set to 200 μl and the tip was inserted into the priming port. The wheel was turned to 220-230 μl slowly, stopping once the yellow flow cell buffer could be seen entering the pipette tip. At this stage, the flow cell was checked for continuous buffer running over the sensor array and the absence of bubbles.
25. The flow cell priming mix was prepared by adding 30 μl of mixed FLT to the FB tube and mixing by vortexing.
26. 800 μl of priming mix was then loaded into the flow cell through the priming port. In contrast to the protocol, priming mix was added in gentle dropwise fashion instead of forcing the pipette contents into the port. The flow cell was left for 5 minutes after 800 μl had been delivered.
27. The LB were thoroughly mixed by pipetting and 25.5 μl were immediately added to a mixture of 12 μl DNA library and 37.5 μl SQB, making a 75 μl total volume.
28. The SpotON sample port was gently opened, carefully inspected for the introduction of bubbles.
29. 200 μl of priming mix was loaded into the flow cell through the priming port, with the SpotON port open.
30. The prepared library was pipette mixed and loading through the SpotON port in gentle, dropwise fashion.

2. 2. Material and Methods

31. The priming port was closed **fully** and the SpotON samples port cover was closed **fully**.
32. Samples were sequenced on a 48-hour run with mux scans run every 1.5 hours. Live basecalling was enabled to give translocation speed estimates.
33. If the translocation speed and associated QScore fell below accepted values, the sequencing run was paused and 250 μ l of FB was added to the SpotON port in dropwise fashion.

2.4 Computational Analysis

Data were processed up to and including read alignment using Unix-based packages. From this stage, all further analysis and the composition of this thesis was written in R (R Core Team 2020). Both phases of processing and analysis have been packaged into a snakemake pipeline for reproduction (Mölder et al. 2021). The snakemake pipeline, a full conda package list, R session info and all code for analysis and figure generation is hosted in a GitHub repository:

GitHub Repository: [THESIS_PK](#)

2.4.1 Summary of Processing

Reference Data

For short- and long-read data, GENCODE Release 25 (GRCm38) was used for mouse-related alignment, while GENCODE Release 38 (GRCh38.p13) was used for human data (Frankish et al. 2018). The cDNA sequence for the transcribed region of *eGFP-L10a*, *Cre* and all known isoforms of *SNCA* were included in all reference FASTA sequences, in order to detect reads derived from these genes.

Transcript sequences for the exact transcribed regions of the *eGFP-L10a* and *IRE5-Cre* loci could not be obtained from original sources. These sequences were therefore determined by *de novo* reconstruction: *Trinity* (Grabherr et al. 2011) was used

2. 2. Material and Methods

(with default parameters) to assemble contigs of overlapping reads from midbrain TRAP data. A string-matching search was performed on the output contigs, using manually selected regions of the Enterobacteria phage P1 genome (NC_005856.1) to detect Cre and the eGFP region of a pcDNA3-EGFP plasmid ([Addgene](#)). The two resulting FASTA sequences are included for reference on the associated GitHub page for this thesis.

Short-read Data

1. In a first stage, raw FASTQ data were:
 1. Scanned for quality control issues using *FASTQC* (Andrews 2010) (with default parameters)
 2. Trimmed using *Cutadapt* (Martin 2011b), specifying adapter sequences listed in (Table 3.4) and `--minimum-length 1`
 3. Aligned/Quantified using *Kallisto* (Bray et al. 2016) (with parameter `--bias`), *Salmon* (Patro et al. 2017) (with parameters `--validateMappings --gcBias --seqBias`) and *STAR* (Dobin et al. 2013) 2-pass alignment (with parameters `--outSAMtype BAM Unsorted --outSAMunmapped Within --quantMode GeneCounts --outFilterIntronMotifs RemoveNoncanonical --alignSJDBoverhangMin 6 --alignSJoverhangMin 6 --outFilterType BySJout --outSAMstrandField intronMotif`)
2. Trimmed FASTQ data were then subject to the above steps to determine the effect of trimming on alignment.

Long-read Data

Raw long-read data were processed into count data separately.

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1. Raw FAST5 signal was basecalled to FASTQ data using *Guppy* (Jain et al. 2016) with online barcode demultiplexing (with parameters `--config dna_r9.4.1_450bps_fast.cfg --barcode_kits PBC001`).
2. FASTQ data were mapped using *minimap2* (Li 2018) (with parameters `-ax map-ont -p 1 -N 100`)
3. Aligned data were quantified using *Salmon* (with parameters `--numGibbsSamples 20 libType U`)

Post-quantification Gene and Transcript-level Analyses

For gene-level analyses, read counts were imported using *tximeta* (Love, Soneson, Hickey, et al. 2020), summarised to gene-level counts for differential gene expression analysis and combined with relevant biological and technical sample metadata into a DESeq object using *DESeq2* (Love, Huber, et al. 2014). Size factor estimation, dispersion estimation and negative binomial Wald statistics were calculated using the DESeq function (with parameters `minReplicatesForReplace = Inf` for DESeq objects containing data from only cohorts 1 and 2 and `fitType = "local", sfType = "poscounts", minReplicatesForReplace = Inf` for DESeq objects containing data from cohort 3, to account for dropout).

For differential transcript usage analyses, read counts were imported using *tximport* (Soneson et al. 2015) (with parameter `countsFromAbundance = "dtuScaledTPM"`) and combined with relevant sample metadata into a DRIMSeq object using *DRIMSeq* (Nowicka and Robinson 2016). This object was subsequently filtered to remove low confidence features (with parameters `min_samps_gene_expr = n * 0.75, min_gene_expr = 10, min_samps_feature_expr = 10, min_feature_expr = 10, min_samps_feature_prop = 10, min_feature_prop = 0.1`).

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Deconvolution of TRAP samples

To estimate the relative cell type composition of somal and axonal TRAP samples, *CIBERSORTx* (Newman et al. 2019) was used (with parameters `perm: 500`, `rmbatchBmode: TRUE`, `QN: FALSE`). To generate a reference sample for each cell type in each reference dataset, the mean gene count was calculated across all samples of the respective cell type (Gokce et al. 2016; Zhang, Chen, et al. 2014; Brichta and Greengard 2014).

Building a Compartment-Specific Dopamine Neuron Transcriptomic Dataset

3.1 Introduction

3.1.1 Overview

This chapter describes the preparation, production and quality control workflow of a dopamine neuron-specific RNA-Seq dataset using TRAP.

TRAP is an affinity purification method that relies on specific expression of eGFP-L10a in the target cell type and selective capture of eGFP-L10a-bound RNA by performing immunopurification with anti-eGFP antibody-coated paramagnetic beads. To validate the feasibility of capturing dopaminergic RNA from dopaminergic axons in the striatum, the total content of eGFP-L10a in the midbrain and striatum of *ROSA26-eGFP-L10a::DAT-Cre* mice was determined. The selective capture of dopaminergic RNA was then demonstrated by comparing cell type marker abundance between TRAP and TOTAL samples.

In every RNA-Seq study, technical factors introduce variation in transcript abun-

3. Transcriptomic Quality Control

dance estimates. Unexplained technical variation confounds the accurate detection of biological variation. At every stage of analysis for this chapter, processing metadata were inspected for quality control and to identify sources of variation among samples. For each quality control metric, a combination of factors were assessed for their contribution:

- **Cohort:**
 - Three cohorts were collected, processed and sequenced at separate time-points using different reagents, protocols and equipment. Cohort-dependent differences were recorded to decide whether to combine data or analyse separately and combine results by meta-analysis.
- **Lane:**
 - Sequencing machines are divided into lanes, each with a fixed capacity for read generation. To obtain a sufficient quantity of reads for gene expression analysis, samples were sequenced across multiple lanes. Lane-dependent variation was assessed before pooling data across lanes.
- **Condition:**
 - Condition-dependent variation, both technical and biological (e.g. collection batch, age, genotype, region, sex) was tested. The beta coefficient for a condition effect could then be incorporated in the design of a differential expression linear model, to account for this effect.
- **Outliers:**
 - Any sample found to be more than 1.5 times the interquartile range from the 75th/25th percentile of a QC metric was recorded. Sample

3. Transcriptomic Quality Control

removal was not performed until all outlier records had been tallied and samples had been visually inspected by principal components analysis (PCA) of count data.

Before sequencing, the yield of RNA was recorded for each TRAP sample for library normalisation and to identify differences between technical or experimental groups. Once sequenced, all data for Illumina-sequenced samples were stored in FASTQ format. FASTQ files contain the read sequence, a per base quality score and additional metadata for every read generated.

To generate gene expression count data from TRAP FASTQ data, reads were trimmed, aligned and counted. This workflow is summarised in Figure 3.1.

Read “trimming” is the removal of bases at the 5’ and/or 3’ end of a sequencing read. Trimming was performed to maximise the likelihood of successful alignment to the genomic/transcriptomic reference, by removing synthetic or poor quality elements. Bases were removed due to:

- **Adapter readthrough:** The presence of part or all of an adapter sequence used in library preparation.
- **Poor base quality:** Bases with low quality scores may be incorrect and will not match the reference base.

Every trimmed sequencing read was aligned to its reference species’ genome or transcriptome in order to count the number of reads that corresponded to each gene or isoform. There are two strategies of read alignment in use in RNA-Seq analyses currently:

- **Traditional alignment** searches for the exact genomic coordinates that a read originates from. It can report mismatches, insertions and deletions. Traditional alignment of TRAP samples was performed using *STAR* (Dobin et al. 2013).

3. Transcriptomic Quality Control

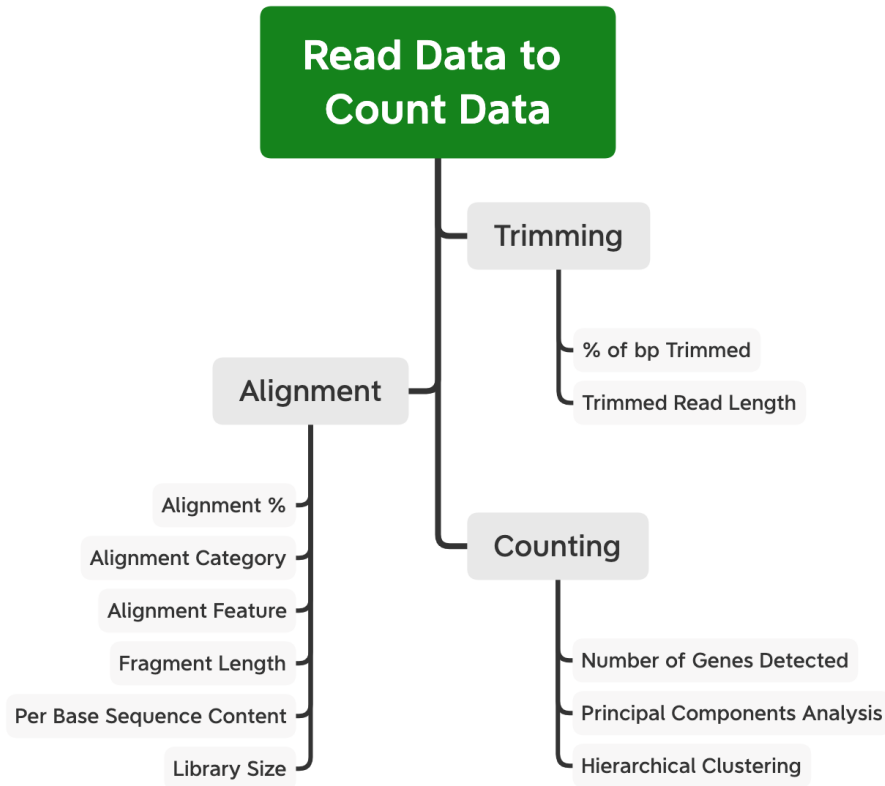


Figure 3.1: Read to Count Data Workflow. A summary of the steps taken and quality control metrics assessed to prepare gene expression count data from raw sequencing read data. Trimming removes sequence from the 5' and/or 3' end of reads. The percentage of trimmed bp per sample and the end read length were used to identify technical differences among samples. Alignment identifies the features to which reads correspond to. Alignment metrics were used to characterise biological and technical differences between samples. Counting (and normalisation) is the final step, after which count-based quality control checks were performed.

- **Pseudoalignment** determines which transcripts a read is *compatible with* (Bray et al. 2016; Patro et al. 2017). A De Bruijn graph is generated from the reference transcriptome, containing nodes/vertices, each containing a k-mer associated with one or a set of transcripts. K-mers in each read are hashed to compatible nodes of the De Bruijn graph, generating a list of transcripts the read is compatible with. Pseudoalignment of TRAP samples was performed using *Salmon* and *Kallisto*.

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Both strategies have advantages depending on the intended analysis. The difference in alignment properties between each method (and their consequences for gene expression estimation) are presented in this chapter. Differences in condition-dependent alignment properties are also presented, which may influence count estimates.

Aligned reads were then “counted”, based on the feature (gene or transcript) they mapped to. A normalisation step was then applied to the the raw count data, to adjust for technical biases:

- **Number of reads per sample:** The more reads per sample, the more reads align to each feature.
- **Feature length:** Longer features capture a greater number of reads, as more cDNA molecules are generated per feature during fragmentation for reads to be generated from.

Further count data exploration was performed, revealing differences in library complexity between cohorts and TRAP fractions. Principal components analysis was finally used to identify the strongest sources of variation (both biological and technical) in the normalised count data.

To accommodate differences in sequencing technology, raw short- and long-read data were processed separately to count data. Count data were then compared to determine the agreement between these two sequencing technologies.

3.1.2 Chapter aims

1. To demonstrate specific capture of dopaminergic RNA from the midbrain and striatum of *ROSA26-eGFP-L10a::DAT-Cre* mice.
2. To perform quality control of Illumina sequenced TRAP data to prepare for biological comparisons.

3. Transcriptomic Quality Control

3. To perform quality control of Oxford Nanopore Technologies sequenced TRAP data to prepare for biological comparisons.

3.2 Results

3.2.1 eGFP Expression in Midbrain and Striatum

To measure the expression of eGFP-L10a in *ROSA26-eGFP-L10a::DAT-Cre*, the content of eGFP-L10a in midbrain and striatum was measured by eGFP-targeted ELISA (Figure 3.2 A). Midbrain tissue contained between 450-1500 pg of eGFP per animal. Ventral striatal tissue ranged from 30-45 pg and dorsal striatal tissue from 7 to 22.5 pg; between 10 and 20-fold less than midbrain. Cortical tissue contained comparable levels of eGFP to dorsal striatal samples, indicating possible low-level ectopic expression of eGFP-L10a. eGFP was not detectable in the midbrain or striatum of *ROSA26-eGFP-L10a::fs/fs* mice.

To demonstrate the successful capture of dopamine neuron RNA by TRAP, cell type marker expression was compared between TRAP RNA and TOTAL RNA (RNA extracted directly from the dissected tissue used as input material for TRAP) using qPCR (Figure 3.2 C). *Th* and *Slc6a3* were robustly enriched in TRAP fractions from all regions. *Th* enrichment was greatest in midbrain while *Slc6a3* enrichment was greater in striatum. In midbrain, *Gad2* and *Gfap*, markers of GABAergic interneurons and astrocytes respectively were depleted in TRAP samples. In striatum, however, their relative abundance was unchanged. In control experiments performed in parallel in *ROSA26-eGFP-L10a::fs/fs* mice, RNA could not be obtained from TRAP samples (in line with eGFP being undetected by ELISA in *ROSA26-eGFP-L10a::fs/fs* tissue).

3.2.2 Cohort Design

Three cohorts of mice were collected for sequencing (Table 3.1).

- Cohort 1 was collected in 8 batches over two periods, separating young and old samples into 4 batches each. Young and old samples from this cohort are

3. Transcriptomic Quality Control

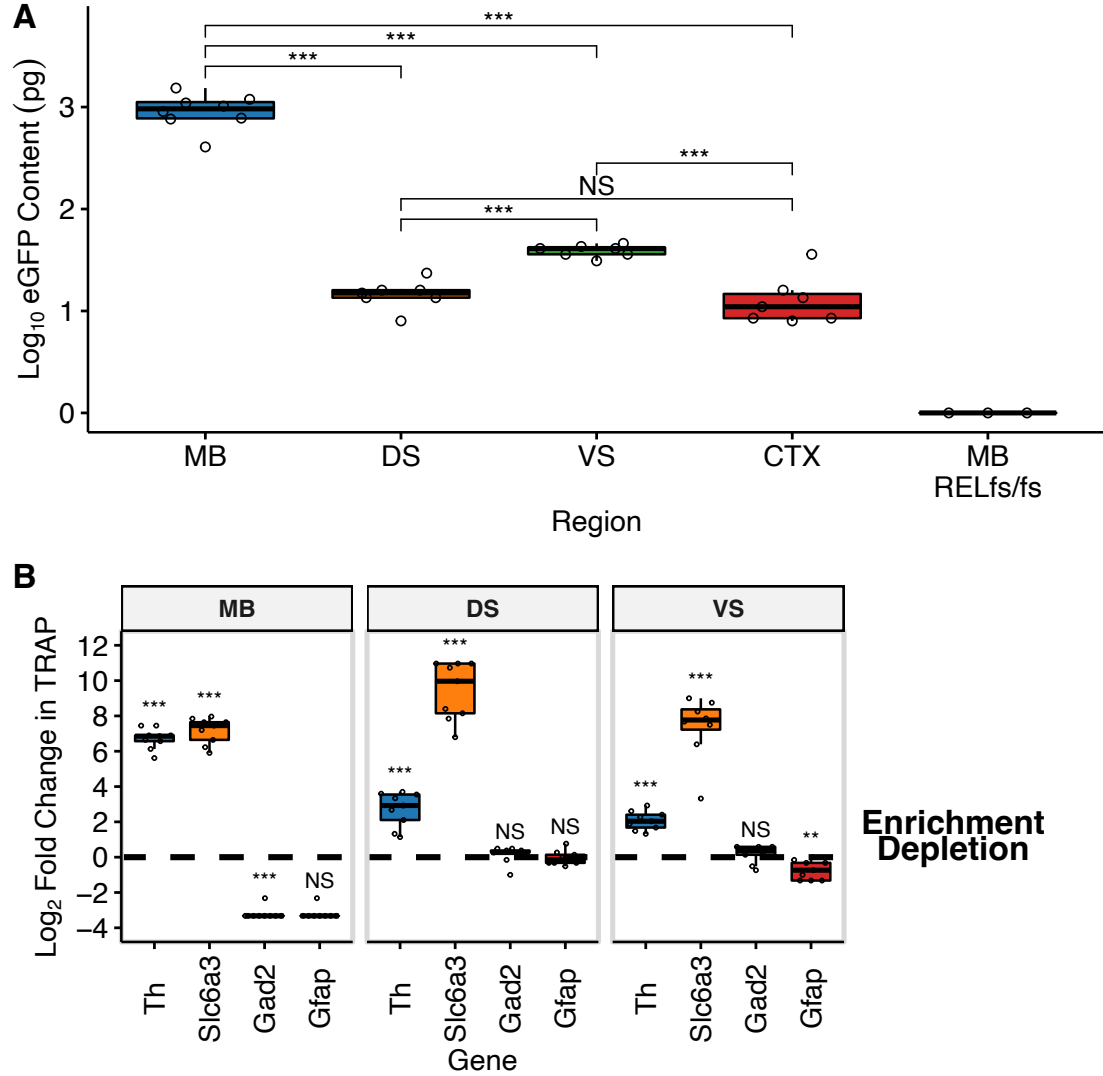


Figure 3.2: Measurement of eGFP-L10a content and confirmation of capture of dopaminergic RNA. **A:** eGFP content per midbrain, dorsal and ventral striatum measured by ELISA. There is between 10 and 20 times more eGFP content in midbrain compared to striatum. eGFP was not detectable in *ROSA26-eGFP-L10a::fs/fs* tissue. MB: Midbrain, DS: Dorsal Striatum, VS: Ventral Striatum, CTX: Cortex. Comparison by one-way ANOVA, pairwise t-testing and Benjamini & Hochberg *P* value adjustment. **B:** Fold change of cell type markers in TRAP RNA compared to TOTAL. *Th* and *Slc6a3* are robustly enriched in all TRAP regions. *Gad2* and *Gfap* are depleted in midbrain TRAP RNA, but are unchanged in striatal TRAP RNA. > 0 indicates enrichment in TRAP RNA, < 0 indicates depletion. Comparison to zero by multiple one sample t-testing and Benjamini & Hochberg *P* value adjustment. *** = *P* < 0.001, ** = *P* < 0.01, * = *P* < 0.1, NS = Not significant.

3. Transcriptomic Quality Control

from littermates.

- Cohort 2 and 3 were collected in 7 and 10 batches, respectively: In each batch, every biological condition tested was collected to avoid confounding (e.g. region, age, genotype and sex).
 - For cohort 2 striatal samples, four animals were pooled per experimental N to obtain sufficient RNA for the SMART-Seq2 library preparation method.
 - For cohort 3 striatal samples, only one animal was used per experimental N. This was necessary in order to ensure samples from every biological condition could be collected in each batch. Due to the reduced yield of RNA obtained from striatal samples, an “Ultra-low input” library preparation was used.

212 samples were next-generation sequenced; 170 TRAP and 42 TOTAL. The numbers of samples per biological condition in TRAP and TOTAL samples are shown in Table 3.2.

3.2.3 RNA yield

The yield of RNA obtained for every sequenced TRAP sample is shown in Figure 3.3.

The yield of midbrain RNA is greater in cohort 3 than cohorts 1 and 2. This may be due to modifications to the TRAP protocol that were necessary in order to

Table 3.1: Cohort design for TRAP sequencing.

Cohort	Collection	Collector	Number of mice	Comparisons	Preparation	Long-read
Cohort 1	Young mice: Q1 2017, aged mice: Q2 2018	Katharina Wagner	32	Age, Disease	SMART-Seq2	No
Cohort 2	Q4 2018	Peter Kilfeather	96	Age, Region	SMART-Seq2	No
Cohort 3	Q1 2020	Peter Kilfeather	40	Age, Region, Disease	Ultra-low	Yes

3. Transcriptomic Quality Control

Table 3.2: Numbers of TRAP and TOTAL samples sequenced for each biological condition and the number of animals used for tissue collection.

Region	Age	Genotype	N TRAP Samples	N TOTAL Samples	N Animals
MB	YOUNG	WT	27	5	27
MB	YOUNG	OVX	20	4	20
MB	OLD	WT	27	5	27
MB	OLD	OVX	20	4	20
DS	YOUNG	WT	12	4	27
DS	YOUNG	OVX	6	2	6
DS	OLD	WT	11	4	26
DS	OLD	OVX	9	2	9
VS	YOUNG	WT	12	4	27
VS	YOUNG	OVX	6	2	6
VS	OLD	WT	11	4	26
VS	OLD	OVX	9	2	9

collect 12 samples per batch (3 regions x 2 ages x 2 genotypes) (modifications discussed in Methods).

Striatal RNA yield was lower than midbrain samples. This was expected, based on measured eGFP content and due to the presence of dopaminergic axons and not cell bodies. Striatal RNA yield was lower in cohort 3 compared to cohort 2. This may be due to the reduction in input material, using one animal per experimental N in cohort 3 instead of four. It may also represent the effect of additional wash steps in the modified TRAP protocol; removing more non-specifically bound RNA. The yield from dorsal striatal samples was the lowest of all three regions. This is in agreement with the earlier finding that eGFP content in dorsal striatum is lower than ventral striatum and midbrain (Figure 3.2 A).

More RNA was obtained from aged TRAP samples compared to young. This difference is present in all three cohorts. There was no difference in yield between genotypes or collection batches of each cohort (Figure 3.3 B).

10 samples were flagged as outliers based on RNA yield, compared to their respective cohort, region and age. These are listed in Table 3.3.

3. Transcriptomic Quality Control

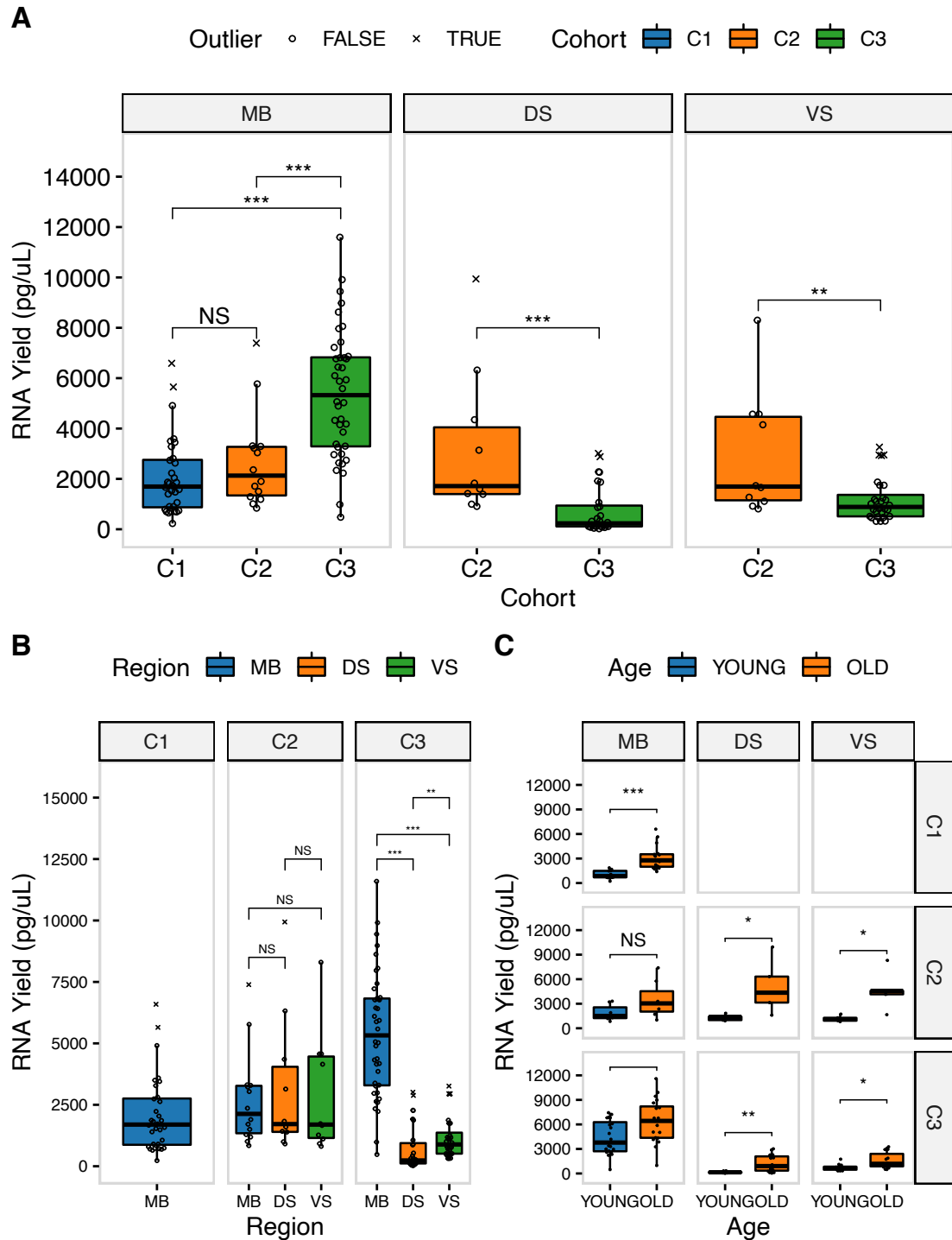


Figure 3.3: RNA yield for all TRAP samples in this study. **A:** Yield per cohort per region, coloured by cohort of origin. The yield of RNA from midbrain cohort 3 samples was significantly greater than other cohorts. The yield of striatal RNA from cohort 3 was lower than cohort 2, likely due to using 1 animal per experimental N, instead of 4 (see Cohort Design section above). **B:** Yield per region per cohort, coloured by region. Differences in yield between regions are clear in cohort 3, but an equivalent quantity of RNA was extracted from all regions in cohort 2. **C** Yield per age per region per cohort, coloured by age. A greater quantity of RNA was extracted from older samples. There were no significant differences in yield observed due to genotype or collection batch within cohorts (not shown). The Kruskal-Wallis test with post-hoc pairwise Wilcoxon rank-sum tests were used to determine significance. The Benjamini & Hochberg procedure was used to adjust for multiple comparisons. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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Table 3.3: Samples flagged as outliers due to abnormal RNA yield for their cohort, region and age.

Cohort	Sample Name	Reason
C1	MB_OLD_OVX_FEMALE_C1.3	High RNA Yield
C1	MB_OLD_OVX_FEMALE_C1.4	High RNA Yield
C2	DS_OLD_WT_MIXED_C2.2	High RNA Yield
C2	MB_OLD_WT_MIXED_C2.7	High RNA Yield
C3	DS_OLD_OVX_FEMALE_C3.4	High RNA Yield
C3	VS_OLD_OVX_MALE_C3.3	High RNA Yield
C3	VS_OLD_OVX_MALE_C3.6	High RNA Yield
C3	VS_OLD_OVX_MALE_C3.10	High RNA Yield
C3	DS_OLD_OVX_MALE_C3.3	High RNA Yield
C3	VS_OLD_OVX_MALE_C3.9	High RNA Yield

3.2.4 Trimming

In cohorts 1 and 2, no common adapter sequences were detected in the input read data. No synthetic overrepresented sequences were detected. In cohort 3, the Illumina Universal adapter was detected in ~25 % of reads, with a strong 3' positional bias (Figure 3.4 A). Additionally, the Clontech primer sequence, used in library preparation for cohort 3 was detected in 100 % of samples. To remove contaminating synthetic sequence, trimming was performed using cutadapt (Martin 2011a). The detected Illumina and Clontech sequences were manually specified as known contaminants (Table 3.4).

In cohorts 1 and 2, trimming removed a very small number of bases from input FASTA data. Removed bases were low quality and therefore may not be accurate. In cohort 3, 24 to 34 % of bases were removed from every sample (Figure 3.4). Reads were contaminated with adapter sequence, as a result of longer read length

Table 3.4: Sequences trimmed from all input reads before further processing.

Name	Sequence
Illumina adapter	AGATCGGAAGAG
Clontech primer	GCTAATCATTGCAAGCAGTGGTATCAACGCAGAGTACT

3. Transcriptomic Quality Control

(150 bp in cohort 3, compared to 75 bp in cohorts 1 and 2) and shorter mean fragment size (3.7 B).

Interestingly, midbrain samples from cohort 3 were trimmed more than striatal samples (Figure 3.4 C). This is a small effect, but may indicate a regional difference in the properties of TRAP RNA that affects library preparation.

Samples in which an unusually large percentage of bases were trimmed were flagged as outliers.

3.2.5 Alignment

Reads were aligned using Kallisto, Salmon and STAR. The percentage of reads successfully aligned is shown in Figure 3.5, faceted by cohort and aligner. The effect of trimming is evident in cohort 3; improving the alignment percentage for all samples in each aligner. Kallisto, however was able to align the largest percentage of untrimmed reads. The mean alignment percentage across all three aligners was 89.6 %, 89.4 %, and 90.3 % for cohorts 1, 2 and 3, respectively.

Of the three alignment methods, Kallisto aligned the highest percentage of trimmed reads, followed by Salmon, then STAR (Figure 3.6 A).

A smaller percentage of reads aligned by pseudoalignment in TOTAL samples, compared to TRAP samples (Figure 3.6 A). There was no difference in STAR alignment percentage, however. This suggests the difference could be due to the

Table 3.5: Trimming percentage outliers

Cohort	Sample Name	Reason
C3	DS_OLD_OVX_FEMALE_C3.4	High bp trimming percentage
C3	DS_OLD_WT_FEMALE_C3.TOTAL	High bp trimming percentage
C3	DS_YOUNG_WT_MALE_C3.TOTAL	High bp trimming percentage
C3	MB_OLD_OVX_FEMALE_C3.7	High bp trimming percentage
C3	MB_OLD_WT_MALE_C3.3	High bp trimming percentage
C3	MB_OLD_WT_MALE_C3.8	High bp trimming percentage
C3	MB_YOUNG_WT_FEMALE_C3.4	High bp trimming percentage

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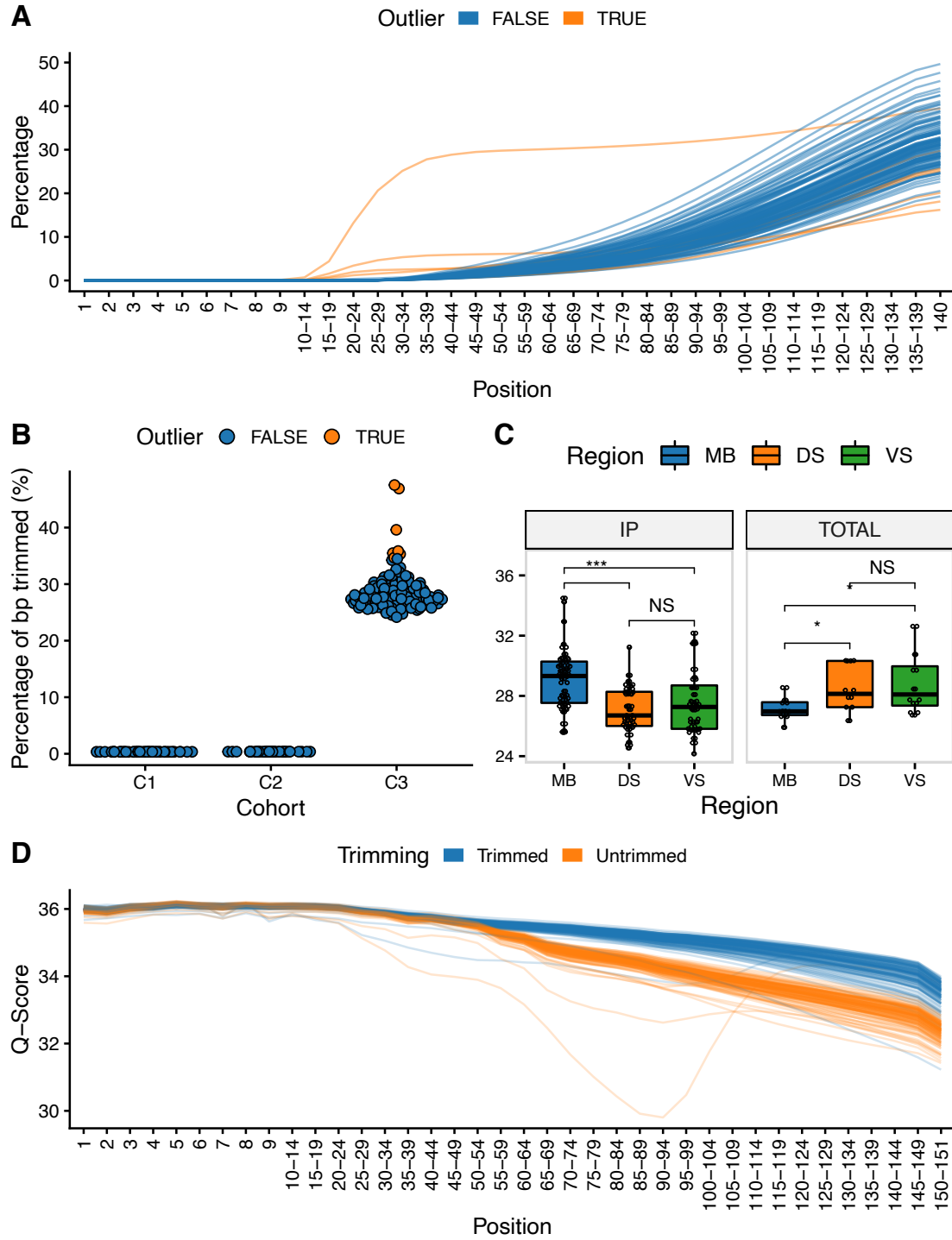


Figure 3.4: Read trimming of cohort 3 data. **A:** Adapter content per base position in samples from cohort 3. Reads are marked as outliers on the basis of percentage of bases trimmed. **B:** Cohort 3 was the only cohort to require trimming. **C:** Midbrain samples required more trimming than striatal samples (outliers removed for comparison). **D:** Trimming improves the mean Phred Q-Score from 50 to 150 bp in cohort 3. The Kruskal-Wallis test with post-hoc pairwise Wilcoxon rank-sum tests were used to determine significance. The Benjamini & Hochberg procedure was used to adjust for multiple comparisons. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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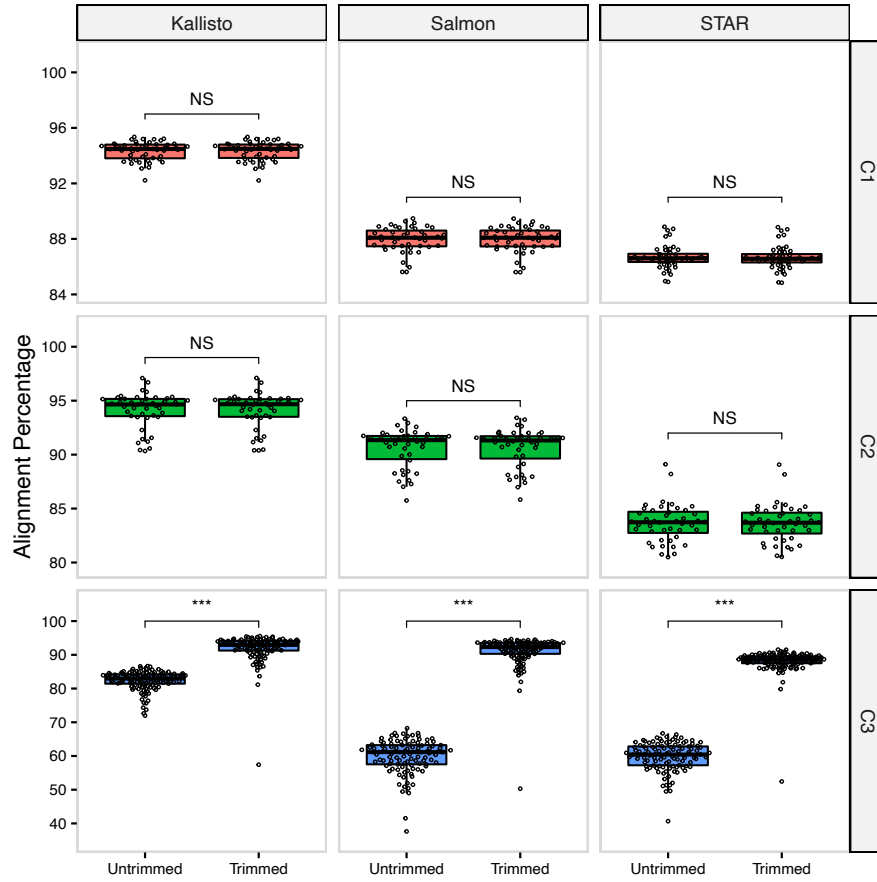


Figure 3.5: Alignment percentage before and after read trimming across three different alignment tools. Trimming does not affect alignment outcomes for cohorts 1 and 2. Cohort 3's alignment percentage is substantially improved by trimming. Kallisto appears more capable of aligning more heavily adapter-contaminated reads, compared to Salmon and STAR. Kallisto aligned the highest percentage of reads, followed by Salmon, then STAR. There was no effect of sequencing lane on alignment percentage, so data are presented as the lane averaged alignment percentage. The Wilcoxon rank-sum test was used to determine significance. The Benjamini & Hochberg procedure was used to adjust for multiple comparisons. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

Table 3.6: Samples flagged as outliers due to low alignment percentage.

Cohort	Sample Name	Reason
C3	DS_OLD_WT_FEMALE_C3.TOTAL	Low alignment percentage

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presence of splice junctions in TOTAL samples that STAR is able to resolve, but Kallisto and Salmon cannot because they are not found within reference transcriptome databases.

In Salmon and STAR-aligned striatal TRAP samples, a greater percentage of reads aligned compared to midbrain TRAP samples. This effect was not present in Kallisto-aligned samples.

Beyond alignment percentage, each aligner outputs metadata that categorises alignments based on success or cause of failure. In Figure 3.7, the proportions of reads in each mapping category is shown per sample for cohort 3, before and after trimming. The effect of trimming is to increase the proportion of uniquely mapped reads. In untrimmed samples, the unmapped reads are described as “too short”. Reads fall into this category if the number of matching bases to a reference is lower than a defined threshold (0.66x the length of the read, in this case). Adapter contamination is likely the cause of this failure.

Read data from all cohorts is paired end, allowing the original cDNA fragment size to be estimated for each alignment by counting the number of bases between read mates. In Figure 3.7 B, the estimated fragment length of every sample is shown for each aligner.

Cohort 3 has a smaller fragment length than cohorts 1 and 2. This shorter fragment length, combined with using 150 bp reads explains why there was adapter contamination. There was no difference in fragment length between TRAP fractions (not shown).

Surprisingly, there are large differences in estimated fragment size between aligners. Most notably, there is a large disparity in estimates of cohort 3 fragment size between STAR and Kallisto and Salmon. Salmon estimates of fragment length are consistently larger.

3. Transcriptomic Quality Control

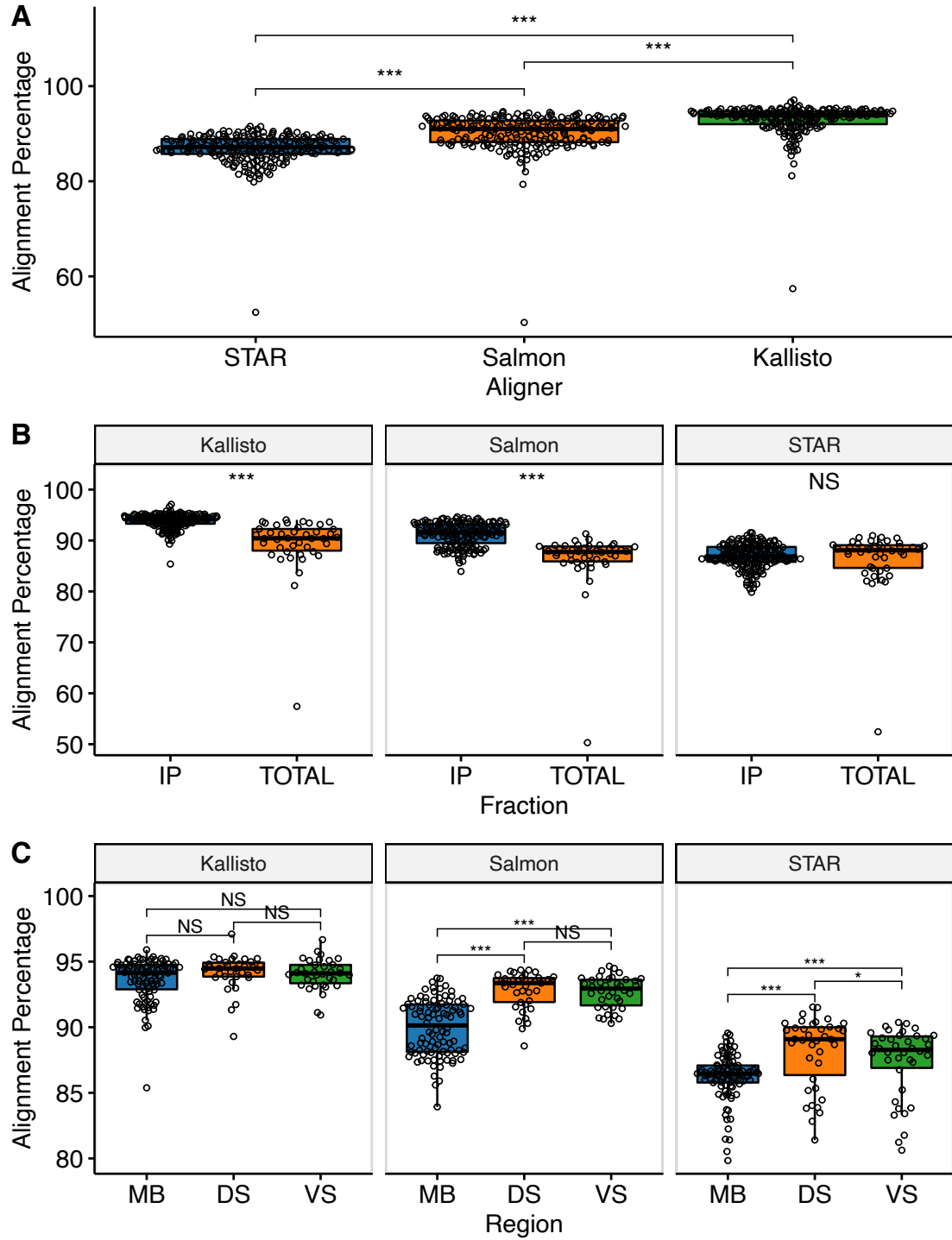


Figure 3.6: Alignment percentage by aligner, fraction and region. **A:** Alignment percentage across three alignment methods. Kallisto successfully aligned the greatest number of reads, followed by Salmon and STAR. **B:** A greater percentage of TRAP sample reads aligned compared to TOTAL samples when using pseudoalignment. This effect occurs in all three regions. **C:** In Salmon and STAR-aligned samples, striatal TRAP samples have a greater alignment percentage than midbrain TRAP samples. This effect is not present in Kallisto-aligned samples. The Kruskal-Wallis test with post-hoc pairwise Wilcoxon rank-sum tests were used to determine significance. The Benjamini & Hochberg procedure was used to adjust for multiple comparisons. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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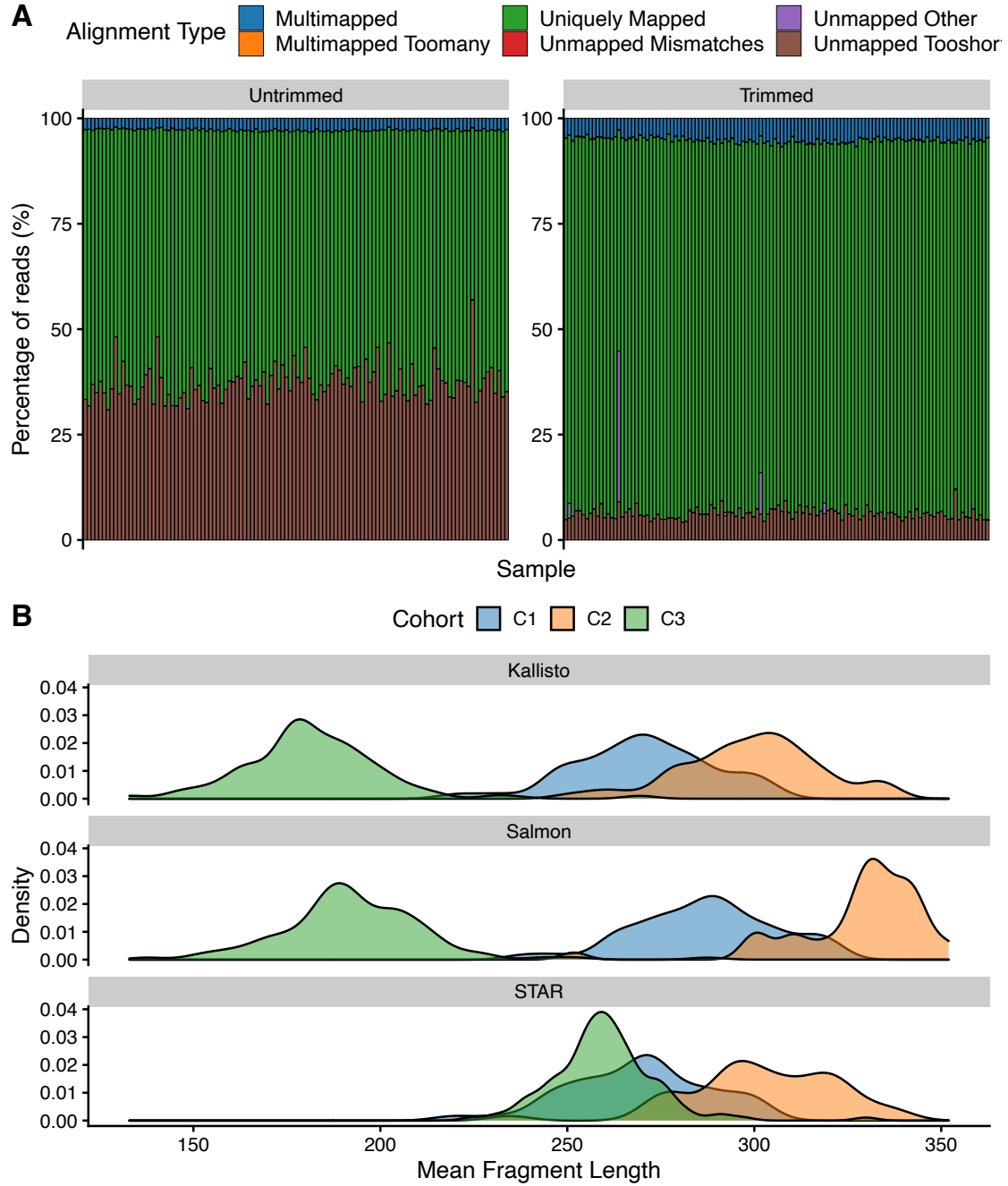


Figure 3.7: Alignment types including failures and fragment length per cohort and aligner. A: The majority of failed reads in untrimmed samples created too short an alignment (relative to total read length) to be reliably aligned. Trimming increases the relative length of alignment, above the necessary threshold to maintain reads. **B:** Estimates of cDNA fragment length per cohort per aligner. Cohort 3 is consistently estimated to be shorter than cohorts 1 and 2, however STAR’s estimate differs strongly to the pseudoalignment methods.

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The alignment feature percentage (Figure 3.8) showed a difference in the exonic/intronic alignment rate between TRAP and TOTAL samples. This likely reflects a difference in the protein-coding RNA content of TRAP samples, relative to TOTAL. As TRAP RNA is engaged with ribosomes, we would expect a greater proportion of exonic RNA. The alignment of reads to more than one possible gene (ambiguous alignment rate) did not differ between TRAP and TOTAL samples. The intergenic alignment rate (reads that align to regions between annotated genes) was lower for most TRAP samples, compared to TOTAL (this effect was smallest in cohort 1). This may be due to increased library complexity of TOTAL samples, containing RNA from unannotated, rarely expressed genes.

There were no other condition-dependent differences in alignment properties.

The causes of alignment failure were determined by examining the sequence quality and content at each read position. As a sequencing run progresses, the uncertainty of base calls declines. This is reflected in a reduced Q-Score for bases towards the 3' end of each read (Figure 3.9 A). The mean read quality of aligned reads was higher than failed reads. Additionally, from ~25 bp to the 3' end of failed reads, the quality dropped substantially. The uncertainty of base calls in this region is likely too great to accurately align. Information from these reads, therefore cannot be recovered.

Across the length of a sequencing read, we expect no positional bias in nucleotide proportion. The relative proportion of each nucleotide should reflect the composition of the genome studied. In Figure 3.9 B, the per base sequence content of aligned and failed reads from cohort 3 is presented.

In both sets of reads, there is a positional bias in the first ~20 bp. This bias is present in cohort 1 and 2 data, also. In aligned reads, the proportion of each nucleotide is then constant. In failed reads, the positional bias persists for the length of all reads. This indicates overrepresentation of certain sequences that could not be aligned to the reference.

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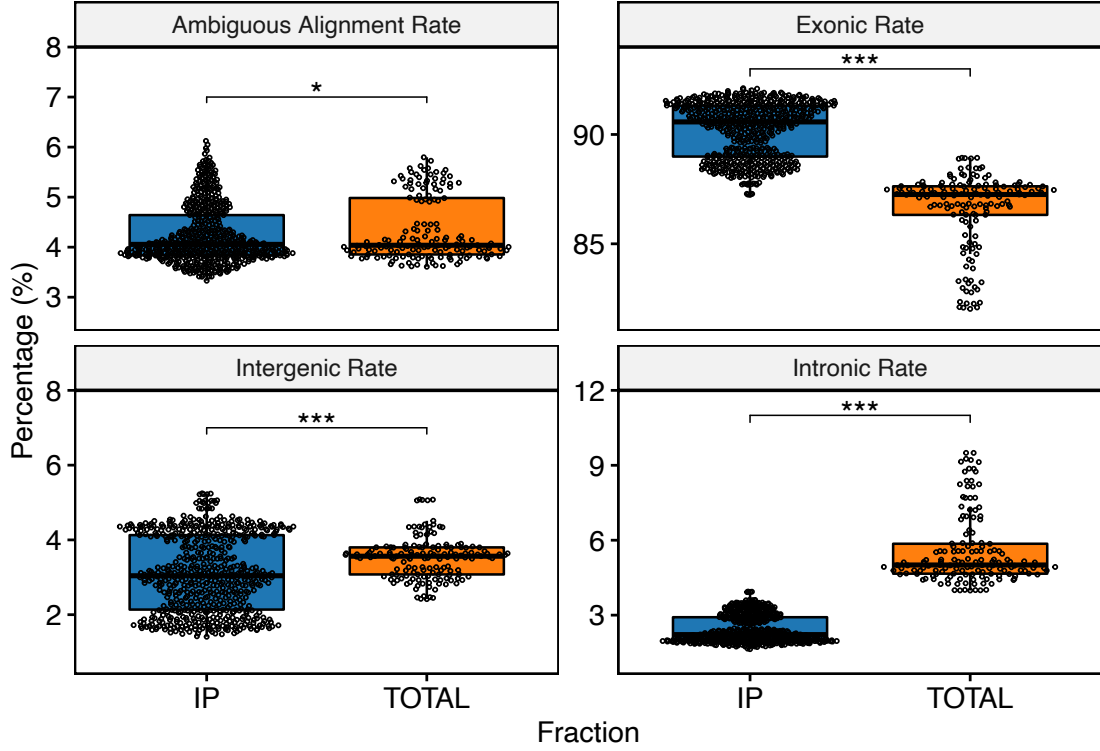


Figure 3.8: Alignment feature per TRAP fraction. The rate of exonic/intronic alignment differs between fractions, indicating an increased proportion of protein-coding mRNA in TRAP samples. The rate of alignment outside of annotated gene boundaries is greater in TOTAL samples. The rate of ambiguous alignment (alignment to multiple matching genes) does not differ between conditions. The Wilcoxon rank-sum test was used to determine significance. The Benjamini & Hochberg procedure was used to adjust for multiple comparisons. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

Given these reads only represent ~10% of the entire library, this is not a major concern and is in line with the majority of successful RNA-Seq experiments.

3.2.6 Count Data

Transcript abundance estimates from Salmon and Kallisto were summarised to gene-level estimates and imported into DESeq objects using tximport and DESeq2 (Love, Soneson, Robinson, et al. 2021; Love, Ahlmann-Eltze, et al. 2021). The number of STAR-aligned reads was counted per gene per sample using a custom bash script. Counts were integrated with sample metadata as a DESeq object.

The spearman correlation between aligners of $\log_2$ -transformed mean read counts

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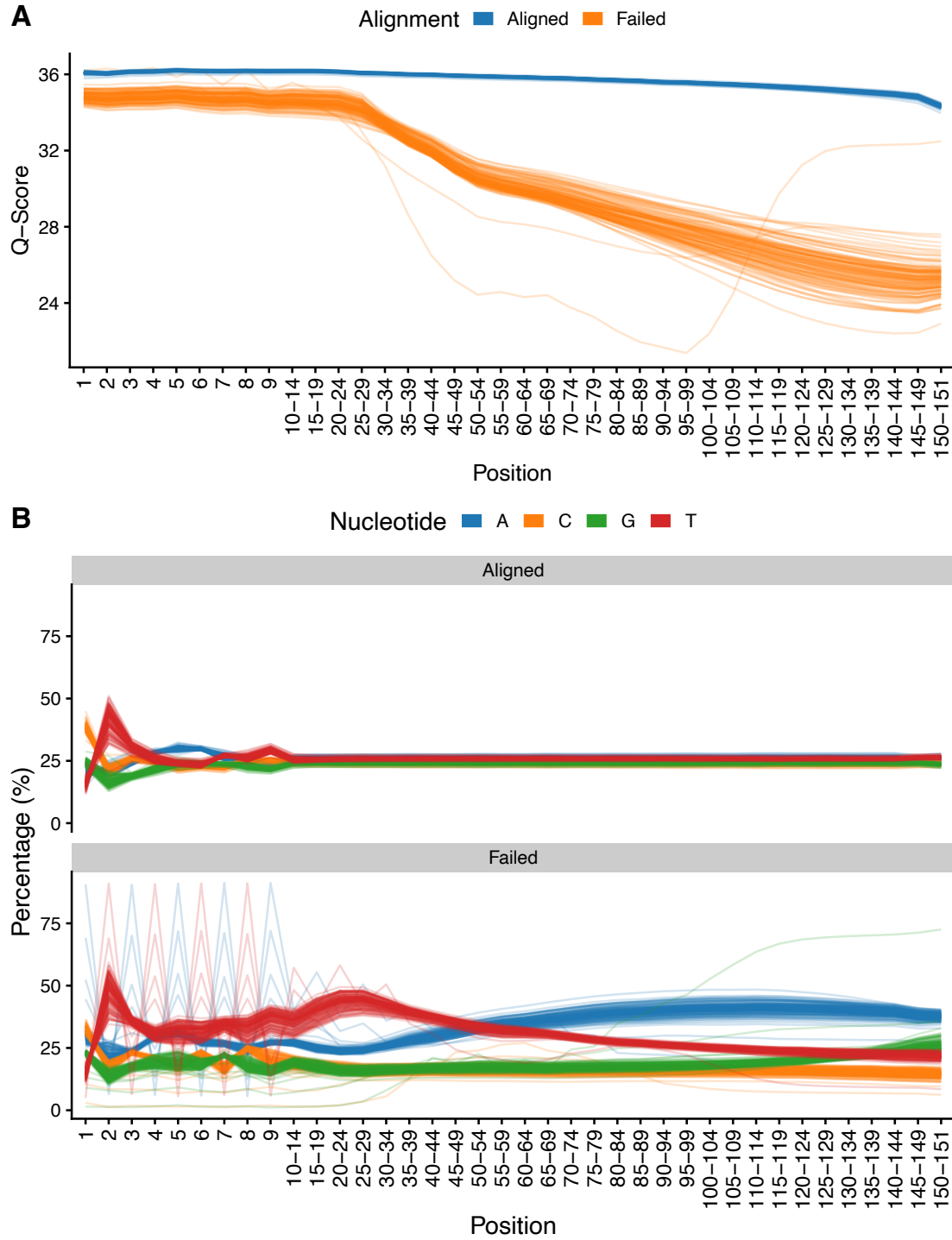


Figure 3.9: Per base sequence quality and content of aligned and failed reads from cohort 3. **A:** Read quality for failed reads is consistently lower than aligned reads and exhibits a heavy dropoff from ~25 bp to the 3' terminus. **B:** In all reads, positional bias is evident in the first 10-15 bp due to non-random transposase binding. Beyond this, aligned reads show no positional bias, whereas failed reads are heavily biased.

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was calculated (Figure 3.10). There was strong correlation between all three aligners, with Salmon the most concordant overall. Most of the variability in abundance estimates was in lowly expressed genes.

The number of genes detected per sample differed between cohorts, TRAP fractions and collection batches (in cohort 3) (Figure 3.11). Fewer genes were detected per cohort 3 sample compared to cohorts 1 and 2. Across all cohorts, a greater number of genes were detected in TOTAL samples compared to TRAP samples. In cohort 3, a greater number of genes were detected in 2 collection batches; C3.3 and C3.8. The effect of varying sequencing depth on the number of genes detected (library complexity) was tested to determine whether sequencing depths used were sufficient and to try to determine the reason for different library complexity between cohort 3 and cohorts 1 and 2. There was no significant relationship between depth and complexity in TRAP or TOTAL samples for any cohort in this study (Figure 3.12 A). Therefore, sequencing depths used in this study did not limit the detection of genes for differential expression measurement. The sample “VS_OLD_OVX_FEMALE_C3.5” from cohort 3 contains more sequencing reads than any other sample in the study and has comparable library complexity to samples with fewer than half as many reads.

Count data were downsampled to normalise for sequencing depth across cohort. Depths from 1 to 10 million total counts were tested (Figure 3.12 B). At all depths, library complexity was not saturated, however the gain in complexity per million reads added reduced rapidly until 5 million reads. In cohorts 1 and 2, there was no difference in the gain in library complexity between TRAP and TOTAL samples. Cohort 3 TOTAL samples showed a more similar profile to cohort 1 and 2 TRAP and TOTAL samples. Cohort 3 TRAP samples, however showed a much smaller gain in complexity for increasing read depth, compared to all other categories.

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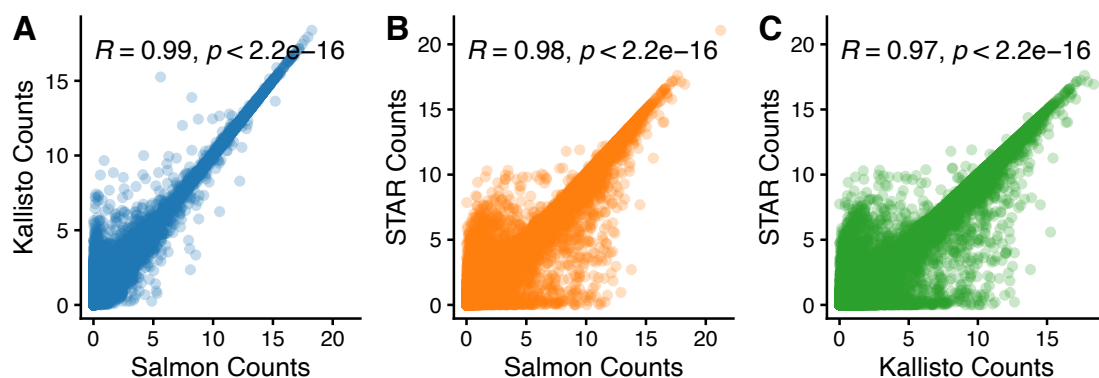


Figure 3.10: Concordance of count estimates between aligners. The correlation of log₂-transformed read counts was calculated. Each aligner had good agreement with the other two. The pseudoalignment methods showed the greatest correlation. Salmon was more closely correlated to STAR counts than Kallisto to STAR.

A large number of genes contained zero counts (termed dropouts) in random subsets of cohort 3 samples (Figure 3.13 A). The proportion of samples with dropouts differed between cohorts and fractions (Figure 3.13 B). In cohorts 1 and 2, genes with mean counts below 100 exhibited dropout, which was expected. A large number of genes fall within this category, as shown in Figure 3.13 A. The proportion of sequencing library assigned to these genes, however is very small (Figure 3.13 C), reflecting their very low abundance. In cohort 3, dropout is seen in genes with higher count values (100-1000). In TOTAL samples from cohort 3, the dropout proportion is lower than TRAP samples, but greater than cohorts 1 and 2. The proportion of sequencing library assigned to dropout genes is much greater than cohorts 1 and 2. The percentage of reads assigned to genes containing dropouts in cohort 3, was approximately equal across cohorts, suggesting that the underlying sample composition did not differ between cohorts.

Principal component analysis was performed to identify the primary drivers of variation for downstream analyses and to determine whether sample exclusion was required. Each cohort was initially analysed separately (Figure 3.14 and Figure 3.15). In cohorts 1 and 2, PCs 1 and 2 were responsible for at least 50 % of all variation. These components reflected differences between TRAP fractions,

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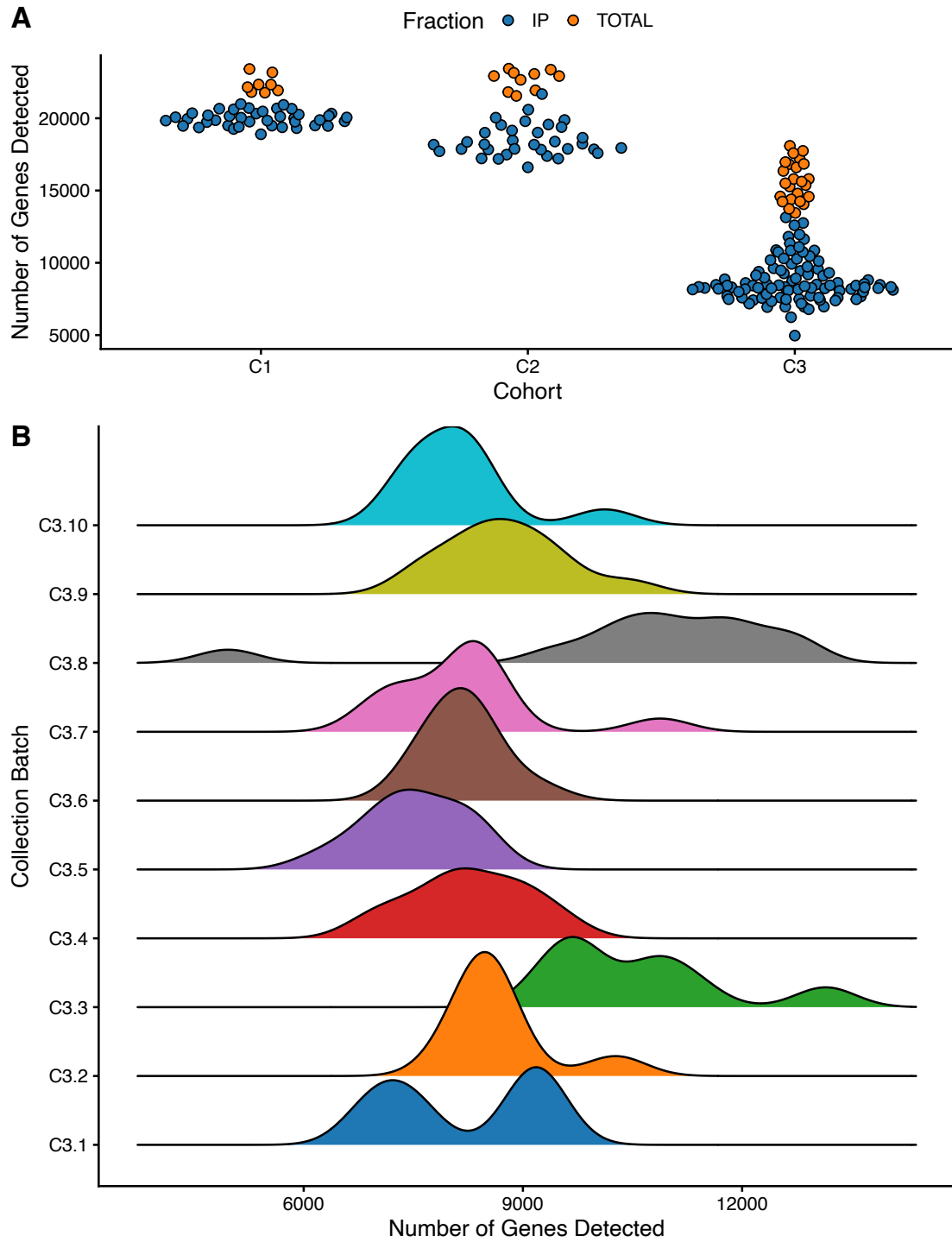


Figure 3.11: The number of genes detected per sample across cohort, fraction and collection batch. **A:** More genes were detected in cohorts 1 and 2 compared to cohort 3. In each cohort, more genes were detected in TOTAL samples than TRAP samples. **B:** The number of genes detected is greater in TRAP samples from collection batches 3 and 8 of cohort 3.

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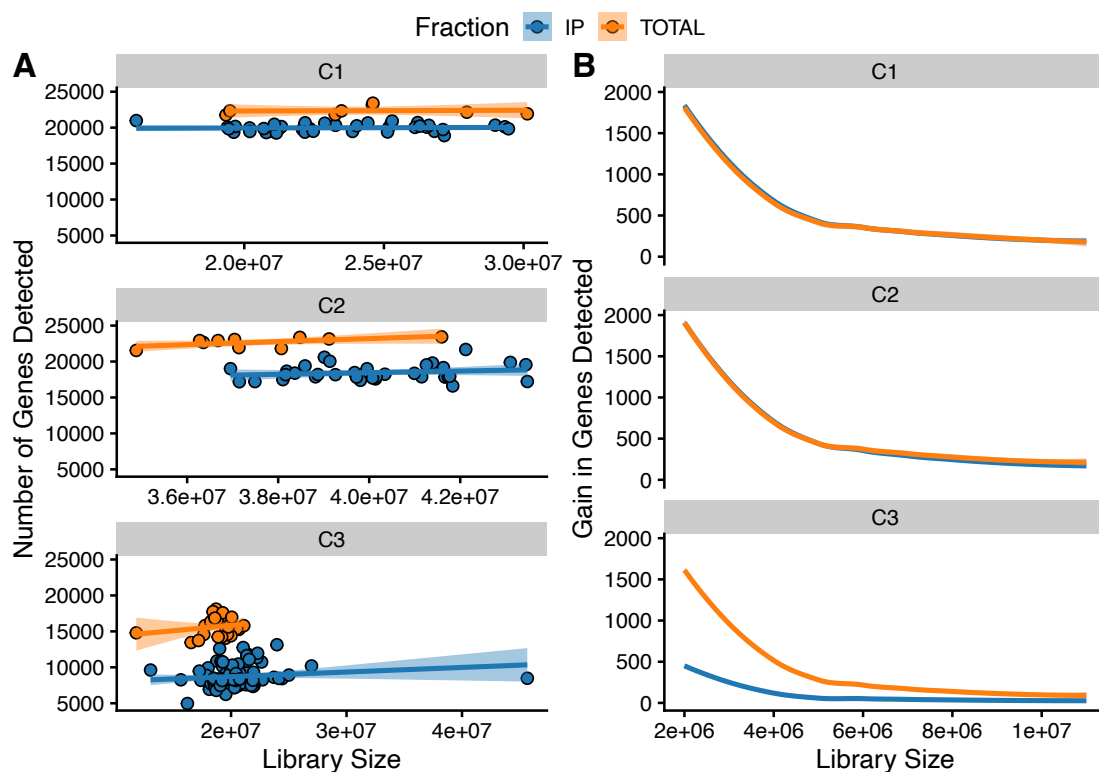


Figure 3.12: Library complexity and sequencing depth. **A:** There is no significant relationship between sequencing depth and library complexity over the range of depths used for sequencing in this study. For instance, cohort 2 samples were sequenced to almost twice the depth of cohort 1, however the number of detected genes is approximately equal. **B:** In downsampled data, the gain in library complexity for every 1 million increase in depth is similar across cohorts 1, 2 and TOTAL samples from cohort 3. Cohort 3 TRAP samples, however show a smaller gain in library complexity.

regions and ages. For PC3 onwards, the percentage of total variation explained by each component was small ($<3\%$), although some were significantly associated with biological conditions. In cohort 3, variation was distributed among principal components more evenly.

The separation of cohort 1 samples along PC1 and PC2 due to fraction and age is shown in Figure 3.15 A and B. A component of the distinct separation of samples by age is likely due to collection of these samples at two different timepoints. Region of origin and fraction are represented along PC1 and PC2 in cohorts 2 and 3, with a strong separation between midbrain and striatal samples, particularly TRAP samples (Figure 3.15 C and E). Encouragingly, there is visible separation of dorsal

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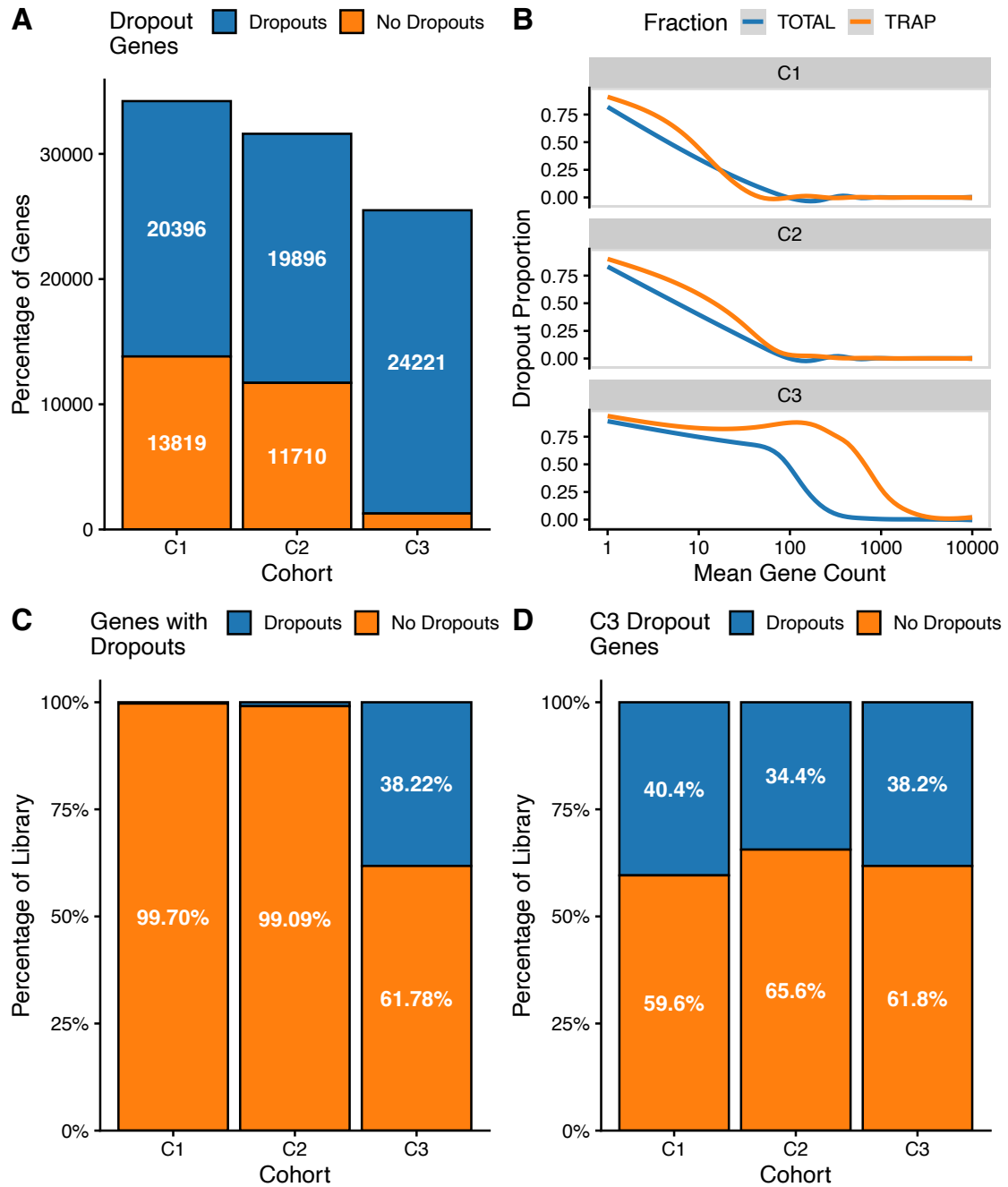


Figure 3.13: Dropout properties by cohort and TRAP fraction. In cohorts 1 and 2, genes with a mean count of greater than 100 have no dropouts in any samples. In cohort 3, there is a greater proportion of dropouts. This proportion differs between TRAP and TOTAL samples: In TRAP samples, for genes with a mean count of 1000, one quarter of all samples have a count value of zero. In TOTAL samples, there are no dropouts at this level.

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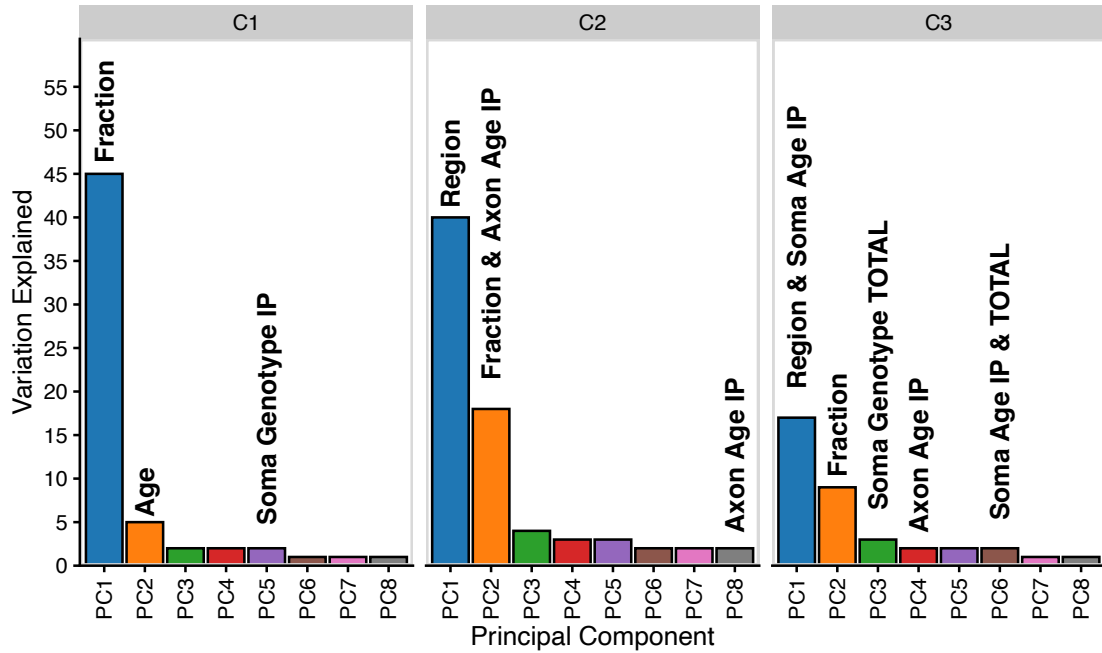


Figure 3.14: Principal components analysis: Cohort separated: Scree plots of percentage variation explained by the top 8 PCs for each cohort. Significant effects of biological conditions on PC coordinates are annotated. Beyond PC2, groups of PCs likely contribute common sources of variation.

and ventral striatal TRAP and TOTAL samples, indicating gene-level differences in expression between these regions.

Next, count data from each cohort was merged and principal component analysis repeated to interpret the relationship between cohorts. Cohort and more specifically, library complexity was associated with PC1, with lowest complexity cohort 3 TRAP samples separating from cohort 3 TOTAL, cohort 1 and cohort 2 samples (Figure 3.16 A). There was consistent separation of somal and axonal samples along PC2, regardless of fraction (Figure 3.16 C). Collections C3.3 and C3.8 appeared as intermediates between TRAP and TOTAL samples (Figure 3.16 D).

Samples from collection batches C3.3 and C3.8 were removed from downstream analysis on the basis of increased library complexity, representing samples “half-way” between TRAP and TOTAL. Collection batch variation was included in all model design formulae in subsequent expression analyses.

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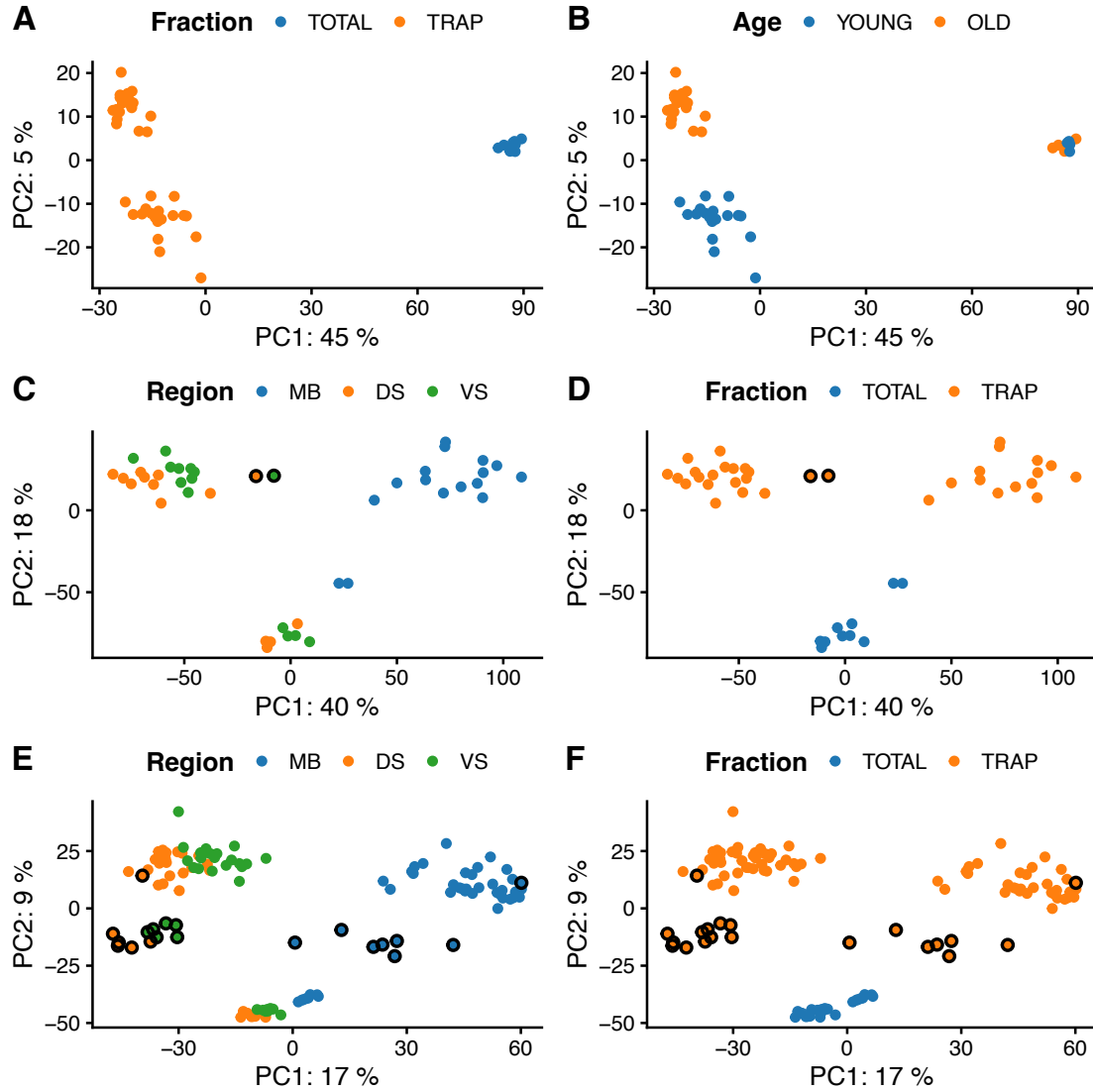


Figure 3.15: Principal components analysis: Cohort separated: Scree plots of percentage variation explained by the top 8 PCs for each cohort. Significant effects of biological conditions on PC coordinates are annotated. Beyond PC2, groups of PCs likely contribute common sources of variation.

Samples flagged as outliers due to containing either an abnormally low or high number of aligned reads are listed in Table 3.7.

3.2.7 Long-read Data Quality Control and Comparison to Short-read

Twelve somal TRAP samples from cohort 3 were sequenced on the Oxford Nanopore Technologies MinION platform. In order to improve sequencing depth, 4 flowcells

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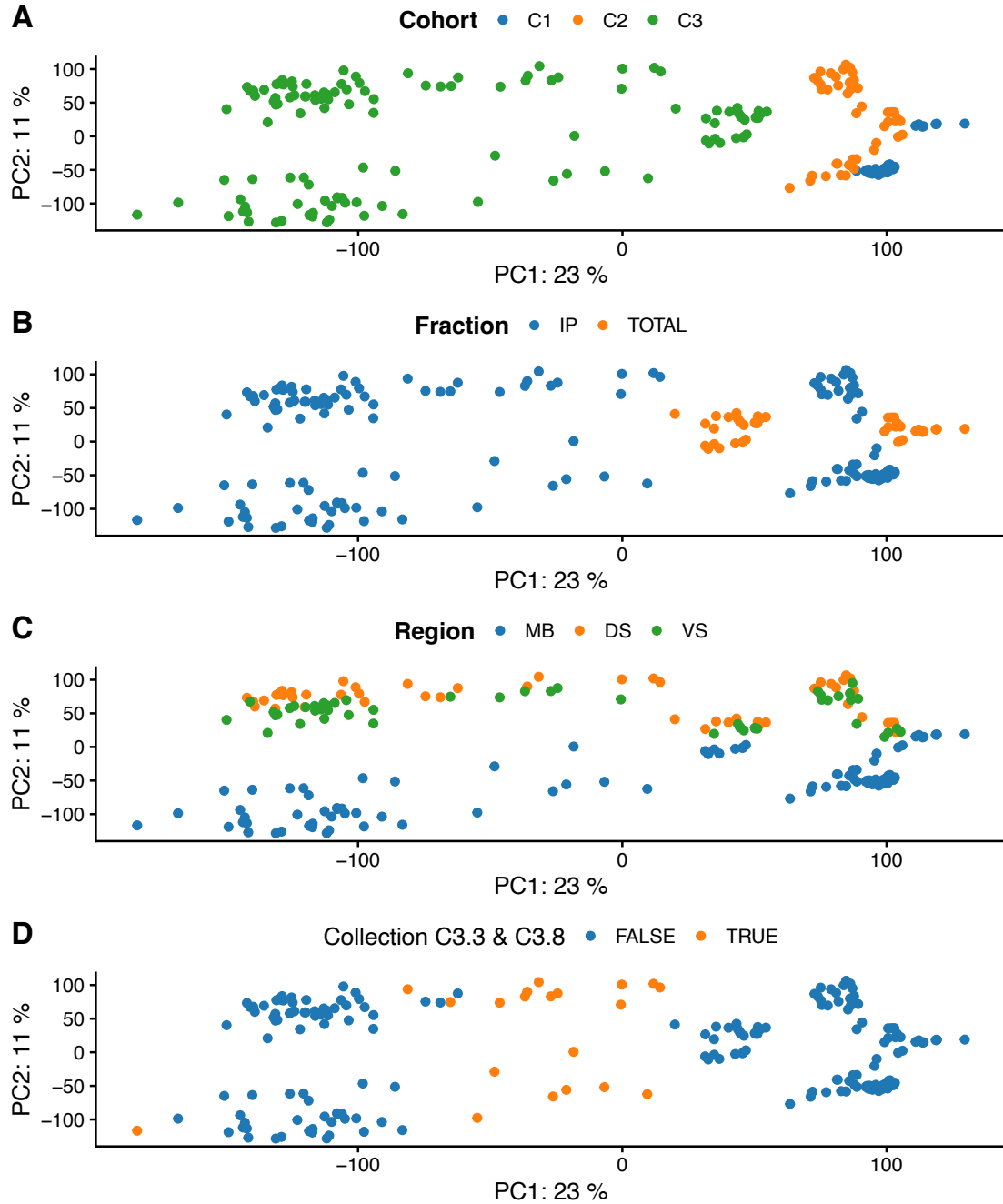


Figure 3.16: Principal components analysis: Cohorts combined. Principal components (PCs) 1 and 2 are plotted in each biplot. **A:** Cohort of origin can be distinguished from PC1. **B:** TRAP and TOTAL samples can also be distinguished from PC1, with cohort 3 TRAP samples most distinct from other groups. This suggests that library complexity is the main driver of PC1 variation. Cohort 3 TOTAL samples are situated closer to all cohort 1 and 2 data than cohort 3 TRAP samples. **C:** Samples labelled by region of origin, showing clear separation between somal and axonal driving PC2 variation (11 % of all variation). **D:** Collections C3.3 and C3.8 labelled. A greater number of genes were detected in these samples (Figure 3.11), explaining this separation pattern.

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Table 3.7: Samples flagged as outliers due to high or low read count.

Cohort	Sample Name	Reason
C3	VS_OLD_OVX_FEMALE_C3.5	High aligned read count
C3	VS_OLD_OVX_MALE_C3.8	High aligned read count

(lanes) were used. The number of reads generated by each flowcell is shown in Figure 3.17 A. Flowcells were used in order of the number of available channels for sequencing: FAL15042 only had approximately 400 channels. In the fourth run, FAL02011, barcoded samples 3, 5, 6 and 9 produced very few reads. This was due to having insufficient remaining RNA to perform library preparation.

The read length per run and per experimental condition is shown in Figure 3.17 B. There were large differences in read length between runs, that reflect a need to refine the library preparation protocol. Old, wild-type samples contained a mildly greater number of shorter length reads compared to other experimental groups (Figure 3.17 C).

To measure the concordance of the long- and short-read data, the pairwise pearson correlation between samples was calculated. A subset of genes (2666) was used that contained at least 3 reads in every sample. Due to the issue of dropout in short-read data from cohort 3, this number is far lower than would otherwise be used for cohorts 1, 2 and long-read data from cohort 3. A random set of an equal number of samples per age and genotype was taken from cohort 1 and 2 and the sample-matched cohort 3 short-read data were used. The pairwise correlation is represented as a heatmap in Figure 3.17 C. Cohort 1 and 2 short-read data showed a high pairwise correlation (greater than 0.9 in every comparison). Strikingly, Cohort 3 short-read data was much less correlated with Cohorts 1 and 2 and even with matched long-read data: Cohort 3 long-read data was well correlated with Cohorts 1 and 2 short-read (p 0.8-0.9). This was very encouraging, as it indicated that

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comparisons performed in cohorts 1 and 2 could also be performed in cohort 3 long-read data and the results combined by meta-analysis.

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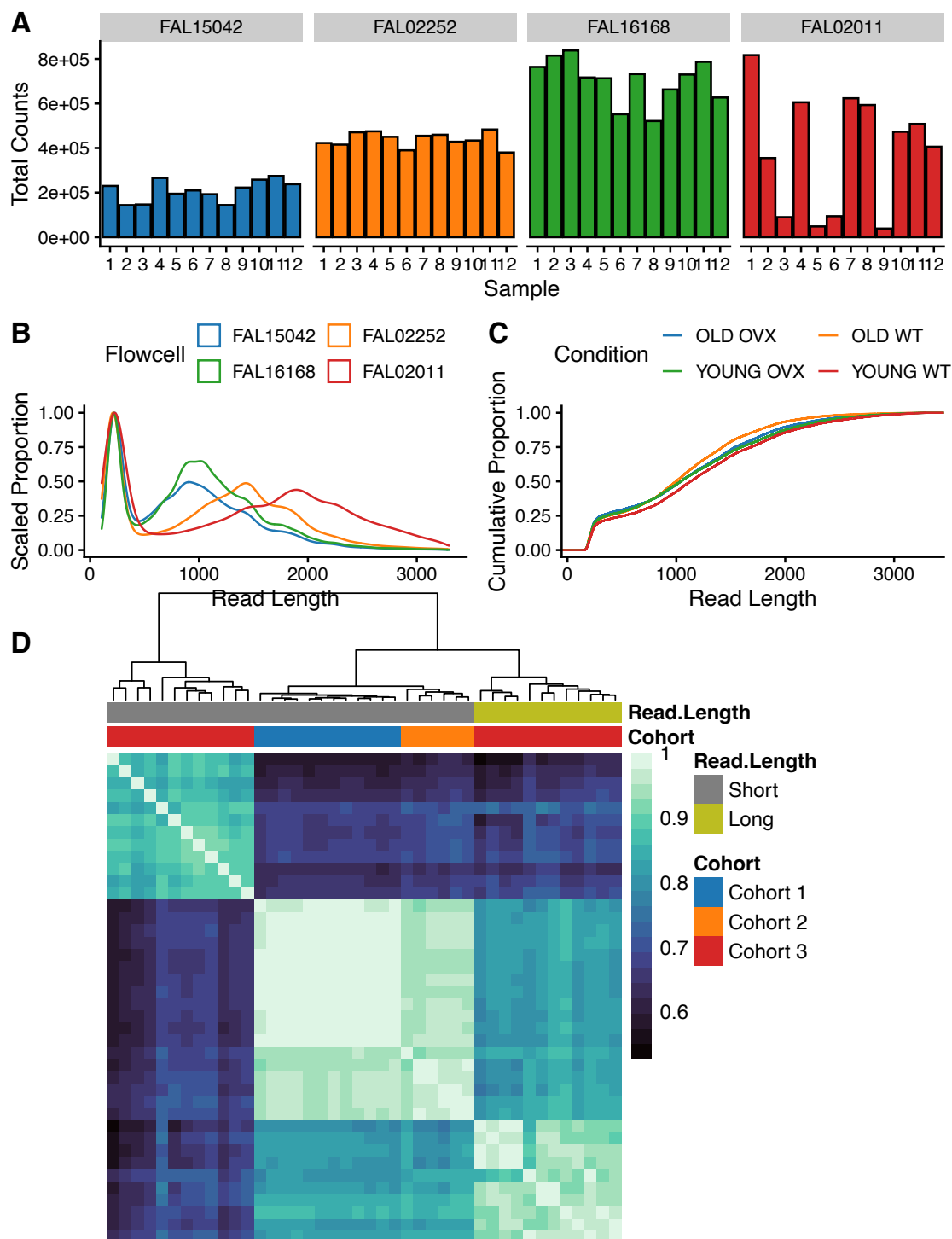


Figure 3.17: Quality Control for Long-read Sequencing Data from the Oxford Nanopore Technologies Platform. **A:** The number of aligned reads per sample, per flowcell. **B:** The read length distribution per flowcell. A substantial number of short (~200 bp) reads can be seen in all cohorts. **C:** Cumulative distribution of read length per experimental condition. **D:** The pairwise correlation between long- and short-read data from Cohorts 1, 2 and 3. Cohort 3 long-read data correlates more closely to cohort 1 and 2 short-read data than to the short-read equivalent of itself.

3.3 Discussion

Based on the findings from this chapter;

- *ROSA26-eGFP-L10a::DAT-Cre* express eGFP-L10a in the midbrain, reflecting dopaminergic cell body expression. eGFP-L10a can also be detected in the striatum, likely reflecting axonal expression, however it was detected at comparable levels in cortical tissue, suggesting a degree of expression in other cell types.
- TRAP experiments across three cohorts of young, old, wild-type and *SNCA*-OVX mice yielded sufficient high-quality RNA for next-generation sequencing and long-read sequencing.
- Choice of library preparation method and quantity of input RNA is important in determining library complexity. Cohort 3 suffers from greater dropout and dispersion, affecting differential expression analysis sensitivity.
- The TRAP fraction is the principal source of count variation, followed by brain region of origin.
- Next-generation and long-read sequencing data produce comparable estimates of transcript abundance.

This results from this chapter guided the analysis of condition-independent and -dependent cell type-specific expression in the subsequent chapters.

3.3.1 eGFP Expression in Midbrain and Striatum

TRAP, and varieties expressing an alternative ribosome modification have been extensively used to capture cell-type specific RNA from cell bodies (Brichta, Shin, et al. 2015; Dougherty, Fomchenko, et al. 2012; Dougherty, Maloney, et al. 2013;

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Heiman, Kulicke, et al. 2014; Heiman, Schaefer, et al. 2008; Nakajima et al. 2014; Shrestha et al. 2015). There has been limited use of TRAP for axonal or cell compartment-specific transcriptome profiling (Sakers et al. 2017; Shigeoka et al. 2016a).

eGFP was detected and quantified in midbrain and striatal tissue of *ROSA26-eGFP-L10a::DAT-Cre* mice, and was not detectable in *ROSA26-eGFP-L10a::fs/fs* tissue, confirming specificity of signal to the TRAP transgene. The detection of eGFP in cortical tissue indicates a degree of very low-level ectopic expression of eGFP-L10a. In all dissections, careful attention was paid to avoid including material outside of the midbrain and striatum. In previous work to establish the TRAP protocol in *ROSA26-eGFP-L10a::DAT-Cre* mice, eGFP was visible in the midbrain but not the striatum. The sensitivity and signal to background noise ratio is most likely to be responsible for this, as eGFP was detected in all striatal tissue samples by ELISA. Further IHC staining could be attempted with brighter fluorophores or alternative antigen retrieval methods could be tested.

Th and *Slc6a3* enrichment in TRAP samples relative to TOTAL samples confirmed successful capture of dopaminergic RNA. The depletion *Gad2* and *Gfap* in midbrain samples suggests that MB TRAP samples approach a “pure” dopaminergic RNA sample. The lack of depletion in striatal samples may be due to ectopic expression of eGFP-L10a in GABAergic interneurons and astrocytes, incomplete removal of non-specifically bound RNA from neighbouring cell types, or a combination of both.

3.3.2 Cohort Design

In cohort 1, young and old samples were collected one and a half years apart with different reagents. Technical variation due to collection differences confounds the detection of biological variation due to ageing. This is evidenced by “age” separation of cohort 1 samples by PCA, along PC2. In cohorts 2 and 3, age does not separate as clearly. In addition, the abundance of dopaminergic markers,

3. Transcriptomic Quality Control

Th and *Slc6a3* differs between old and young in cohort 1, whereas it did not in cohorts 2 and 3.

The reduction in number of mice used between cohorts 2 and 3 was necessary for several reasons. Processing 16 mice per batch would have increased the duration of the TRAP protocol, increasing the likelihood of sample degradation and/or handling error. Mice from different litters would need to be pooled (unevenly). Changes were made to the TRAP protocol (as described in Methods) to enable the handling of up to 12 samples, while ensuring enough RNA could be obtained from striatal samples from one animal.

The change in library preparation method in cohort 3 likely had a major effect on sample complexity and concordance with cohorts 1 and 2. Ultra-low input methods are most commonly used for single cell RNA-Seq datasets. These datasets typically have much lower library complexity than bulk RNA-Seq datasets. There is debate as to whether the reduced library complexity is due to failure of cDNA amplification from minute starting amounts of RNA or is in fact due to the stochastic nature of RNA expression in single cells (Svensson 2020). The higher complexity count data produced from matched midbrain TRAP samples using the Oxford Nanopore Technologies platform indicated that the reduced input mass used for library preparation in cohort 3 short-read samples was the cause of reduced complexity. The input mass of RNA used in the Oxford Nanopore Technologies protocol was greater and more similar to the input mass used for SMART-Seq2 preparations in cohorts 1 and 2.

3.3.3 RNA Yield

Cohort variability in RNA yield can be explained by a number of factors; for example operator handling, skill development, reagent batches. This variability is not a major concern because each cohort was prepared and sequenced separately, already creating a source of technical variation. RNA concentration is normalised for library preparation, also.

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Variation in yield due to brain region is likely explained by the relative abundance of eGFP-L10a (and of dopaminergic ribosomes, therefore) in midbrain and striatum. This is confirmed by the eGFP content in these regions.

Variation due to age, however, is unexpected. Old and young samples from cohort 1 were collected at separate time points, with different reagents, which would be the most likely explanation had this only occurred in those samples. This pattern occurs in all cohorts, however. In cohorts 2 and 3, samples were alternated between left and right sides of racks and plates during TRAP processing to minimise the likelihood of physical location influencing data variability. Biological explanations for age differences could include:

- A change in tissue composition with age that affects antibody binding efficiency.
- A change in ribosome production or stability with age.
- A change in ribosome:RNA engagement (translational efficiency) with age.

3.3.4 Trimming

As alignment tools have developed methods to “soft-clip” adapter sequences from reads, the requirement for read trimming has been disputed (Liao and Shi 2020). Read trimming adds additional time to an already lengthy data processing pipeline and is viewed by some as a redundant process. From these data, it appears that read trimming has minimal impact on alignment rates in cohort 1 and 2 data. Without trimming of cohort 3 data, however, a substantial percentage of reads would be lost, due to heavy adapter contamination.

3.3.5 Alignment

With a mean alignment percentage (after trimming) of 89.8 %, there were no further concerns about read data quality that would impair count generation. Differences

3. Transcriptomic Quality Control

in alignment feature between fractions provides some insight into the properties of TRAP samples and how they differ in complexity and splicing diversity compared to TOTAL samples. The increased exonic:intronic alignment ratio in TRAP samples suggests there may be a subset of transcripts specific to the dopamine neuron or each dopamine neuronal compartment.

The estimated fragment length is an invaluable quality control metric to identify causes of alignment failure and potential biases. The shorter fragment length of cohort 3 samples was mainly responsible for causing adapter contamination. The most reassuring finding, however, was that fragment length estimates do not differ between TRAP and TOTAL samples in cohort 3. A difference in fragment length would indicate differences in library preparation outcome for TRAP and TOTAL samples, confounding their comparison with one another.

3.3.6 Count Data

The decision to use Salmon counts for downstream count-based analyses was based on a recent finding by the package authors that when used alone, pseudoalignment can underperform in accuracy compared to conventional alignment under various experimental circumstances (Srivastava et al. 2020). The authors introduced “selective alignment”; a refinement of their original pseudoalignment algorithm that reintroduces alignment scoring to help discern between identical mapping loci along a reference. Without knowledge of the true expression levels in every sample, the accuracy of Salmon, Kallisto and STAR cannot be determined from these data. Kallisto aligned the greatest number of reads, however its count estimates were marginally less correlated to STAR counts than Salmon.

The difference in library complexity (number of genes detected) between TRAP and TOTAL samples is most likely a property of TRAP. The number of genes actively expressed in dopamine neurons will be a subset of all genes expressed in cell types of the midbrain or striatum. This reduction in library complexity could be used

3. Transcriptomic Quality Control

as a surrogate measure of TRAP success, instead of cell type marker enrichment which may be less reliable. The increased complexity of collection batches C3.3 and C3.8 may be due to insufficient removal of non-specifically bound RNA from the surface of the paramagnetic beads used during the TRAP protocol.

The lack of relationship between sequencing depth and library complexity at the range of depths used for each cohort is encouraging, as it confirms that all detectable genes are counted. The near-saturation of sequencing depth in cohort 3 samples provides some explanation for the unusual feature of dropouts in genes with higher gene counts (100-1000): With increasing depth beyond saturation, reads are assigned to existing features within the library, increasing the divide between zero and “detected”. This creates a situation where a count value of 500, for example, is not comparable to a count value of 500 in a more complex library, such as in cohorts 1, 2 or in long-read data from cohort 3.

From principal components analysis, fraction and region are the strongest drivers of variation in this dataset. This will allow the genes and transcripts that define dopamine neuron cell bodies and axons to be identified in the next chapters. Age and genotype is responsible for variation that is shared across multiple PCs. In some cases, a subset of samples separate, while others do not. Importantly, the methods used for testing differential expression in the next chapters are sensitive to these cases and will report differential expression for closer inspection.

The decision to analyse each cohort of data individually and to subsequently perform a meta-analysis is driven by the difference in library complexity between cohort 3 short read data and cohorts 1 and 2. Although PCA confirmed a similar pattern of separation according to fraction and region across cohorts, the effect of library preparation method on dispersion estimates (next chapter) would harm detection power from cohort 1 and 2 samples.

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The correlation between long-read sequencing data generated on the Oxford Nanopore Technologies platform and cohort 1 and 2 short-read data was very encouraging. The complexity of cohort 3 samples sequenced on this platform exceeded that of their short-read counterparts, closely matching the complexity of cohort 1 and 2 short read samples. Based on these findings, long-read data is included in age and disease-related comparisons in subsequent chapters.

Specific Expression Features in the Dopaminergic Neuron

4.1 Introduction

4.1.1 Overview

This chapter describes the “steady state” expression features of dopaminergic neurons in the cell body and axon.

Previous studies have aimed to characterise the fundamental expression properties of mouse or human dopaminergic neurons; using microarrays with neurons purified by laser capture microdissection (LCM) (Greene 2006; Chung et al. 2005) or fluorescence-activated cell sorting (FACS)(Poulin et al. 2014) or using next-generation sequencing with LCM (Nichterwitz et al. 2016; Aguila et al. 2021), FACS (Kramer, Risso, et al. 2018; Hook et al. 2018; Tiklová et al. 2019) or droplet-based single cell dissociation (Saunders et al. 2018; La Manno et al. 2016). These studies have provided evidence for the existence of subpopulations of dopaminergic neurons within the midbrain that are regionally localised (such as between the SNpc and VTA) and more heterogeneously mixed. Drawbacks of these investigations, however, include sensitivity to measuring gene expression, lack of a comparison

4. Specific Expression Features in Dopaminergic Neurons

of expression between dopaminergic neurons and other cell types and technical biases induced by tissue handling and cell preparation methods. The technical biases are evidenced by the substantial amount of filtering required of raw data: Zeisel et al. (2018) performed droplet-based single cell sequencing of multiple brain regions and of ~510,000 cells sequenced, ~161,000 cells were retained for cell type clustering. Similar data filtering was required in (Saunders et al. 2018; Oldham and Kreitzer 2018).

Brichta, Shin, et al. (2015) performed TRAP in young (four months old) mice in a toxic model of PD, however no comparison was performed between TRAP samples and input midbrain tissue. At the time of writing, no study has aimed to describe the local translation of transcripts in dopaminergic axons.

To identify genes that were translated in the dopaminergic cell body and axon, gene and transcript-level abundance estimates were compared between TRAP and TOTAL samples from the midbrain and striatum. The results of these comparisons were used to assess cell type specificity by grouping genes according to a database of cell type markers. Genes were ranked by enrichment, with classic dopaminergic markers ranking highly. Among this list, however were genes that do not currently have a reported association with dopaminergic biology or PD; potential roles for these genes are discussed.

Cell type specificity analysis in axonal TRAP samples indicated enrichment of dopaminergic neuron RNA, but also other cell type RNA. A deconvolution method was used to estimate the relative proportion of different cell types in axonal TRAP samples. Two additional criteria were applied to minimise the likelihood of incorrectly categorising genes as “axonally translated”. Among the most enriched genes include several known to play an important role in dopaminergic biology. The possible axonal role of less studied, but highly enriched genes is discussed.

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Splicing diversity in the dopaminergic neuron was explored by comparing transcript-level abundance estimates between TRAP and TOTAL samples. For many genes, dopaminergic neurons were found to express an alternative dominant transcript compared to neighbouring cell types. For some genes, “switching” between transcripts leads to no change in gene-level abundance, but clearly indicates specific expression properties of dopaminergic neurons. The list of genes considered “translated” by dopaminergic neurons was expanded from these results. The possible consequences of alternative dominant transcript expression are discussed with some examples.

Long-read alignment data from somal TRAP samples was used to identify putative novel transcripts. The long-read data demonstrates an impressive ability to recreate known transcript models *de novo*. Very little evidence for novelty was found however, with manual inspection of “novel” alignments revealing artefacts in long-read generation that need to be addressed.

Enrichment data were finally used to prioritise candidate genes surrounding 90 independent PD genetic risk loci (Nalls, Blauwendraat, et al. 2019). This analysis indicated an overrepresentation of dopaminergic-specific genes within regions associated with PD risk. Prioritised results were compared with quantitative trait loci (QTL) and nearest gene approaches. The Calcium Sensing Receptor (*Casr*) is proposed as a novel lead candidate gene, based on enrichment data from the soma and axon; supporting a specific role for this gene in dopaminergic biology.

Unless specified explicitly, all analyses in this chapter were conducted using all samples from both ages (3 and 18 months) and genotypes (Wild-type and *SNCA*-OVX). All analyses were divided according to cell compartment, however (somal and axonal samples were analysed separately).

4. Specific Expression Features in Dopaminergic Neurons

4.1.2 Chapter aims

1. To identify the set of genes and transcripts actively translated in the dopaminergic cell body and axon.
2. To further identify those genes that are most selectively expressed relative to the surrounding tissue.
3. To characterise splicing diversity within dopaminergic neurons and determine the dominant transcript(s) expressed in this cell type.
4. To use TRAP enrichment in the soma and axon to prioritise candidate genes in linkage regions surrounding PD GWAS loci.

4.2 Results

4.2.1 Somal Enrichment

Summary Statistics

To identify genes enriched and depleted by TRAP, somal TRAP samples were compared to their matched TOTAL samples. Both ages and genotypes were included in all comparisons, unless stated otherwise. Out of 23392 genes detected in TRAP and TOTAL somal samples of all three cohorts, 5539 were significantly enriched by TRAP (Table 4.1). 4441 were depleted and 13412 were not significantly changed. More abundant genes were more likely to be enriched, accounting for 73.7 % of each library, on average. Enrichment status was used to classify genes in terms of confidence of translation; enriched genes were considered to be translated with greatest confidence. The magnitude and significance of enrichment was used to estimate the specificity of expression to the dopaminergic neuron.

Cell Type Specificity

To assess the specificity of TRAP at the cell body, all measured genes were scored based on the magnitude and significance of fold enrichment (or depletion). These scores were combined with a database of marker genes for 178 cell types to obtain an enrichment score per cell type (Franzén et al. 2019). Dopaminergic neurons were clearly enriched in midbrain samples: 13 markers were significantly enriched, as shown in Table 4.2. This enrichment was selective, as markers of other cell types were depleted (Figure 4.1 A). Collectively, no other cell types showed robust

Table 4.1: Summary of the number of genes significantly enriched, depleted and unchanged in somal TRAP samples compared to TOTAL.

Metric	Enriched	Depleted	Unchanged
Number of Genes	5539	4441	13412
Percentage of Library	73.7 %	9.1 %	17.2 %

4. *Specific Expression Features in Dopaminergic Neurons*

enrichment (Figure 4.1 B), with the exception of adrenergic and noradrenergic neurons that share *Th*, *Slc18a2* and *Ddc* as markers.

Ontological Categorisation

To divide and characterise the large number of enriched genes into functional groups, pathway enrichment methods were used. Gene set enrichment analysis was used to identify terms overrepresented in the ranked list of enriched TRAP genes. The Gene Ontology (GO): Cellular Component, Biological Process and KEGG Pathways gene sets were used. The top 10 terms identified from each source are presented in Table 4.3.

Ribosomal cellular component and translation-related processes were the most enriched terms. Ribosomal terms are not uniformly enriched in ribosomal profiling datasets (Clarke et al. 2018; Sakers et al. 2017; Shigeoka et al. 2016b), which raises the possibility that ribosomal activity is greater in dopaminergic neurons, compared to neighbouring cell types in the midbrain. The ribosome-dependent nature of TRAP, however, confounds the interpretation of this result without further validation. Furthermore, differences in ribosomal RNA handling (for example, using ribodepletion or poly-A selection) during library preparation may account for variation in enrichment between laboratories.

Mitochondria and related biological processes then dominate, confirming that abundant expression of nuclear-encoded mitochondria-related genes is characteristic of this cell type in this brain region.

Among the KEGG Pathways tested, Parkinson’s disease was one of the most significantly overrepresented. Interestingly, other neurodegenerative conditions including Huntington’s disease, prion-related disease, Amyotrophic Lateral Sclerosis and Alzheimer’s disease all feature. This suggests that a subset of genes known to be of shared importance across neurodegenerative conditions are translated in dopaminergic neurons.

4. Specific Expression Features in Dopaminergic Neurons

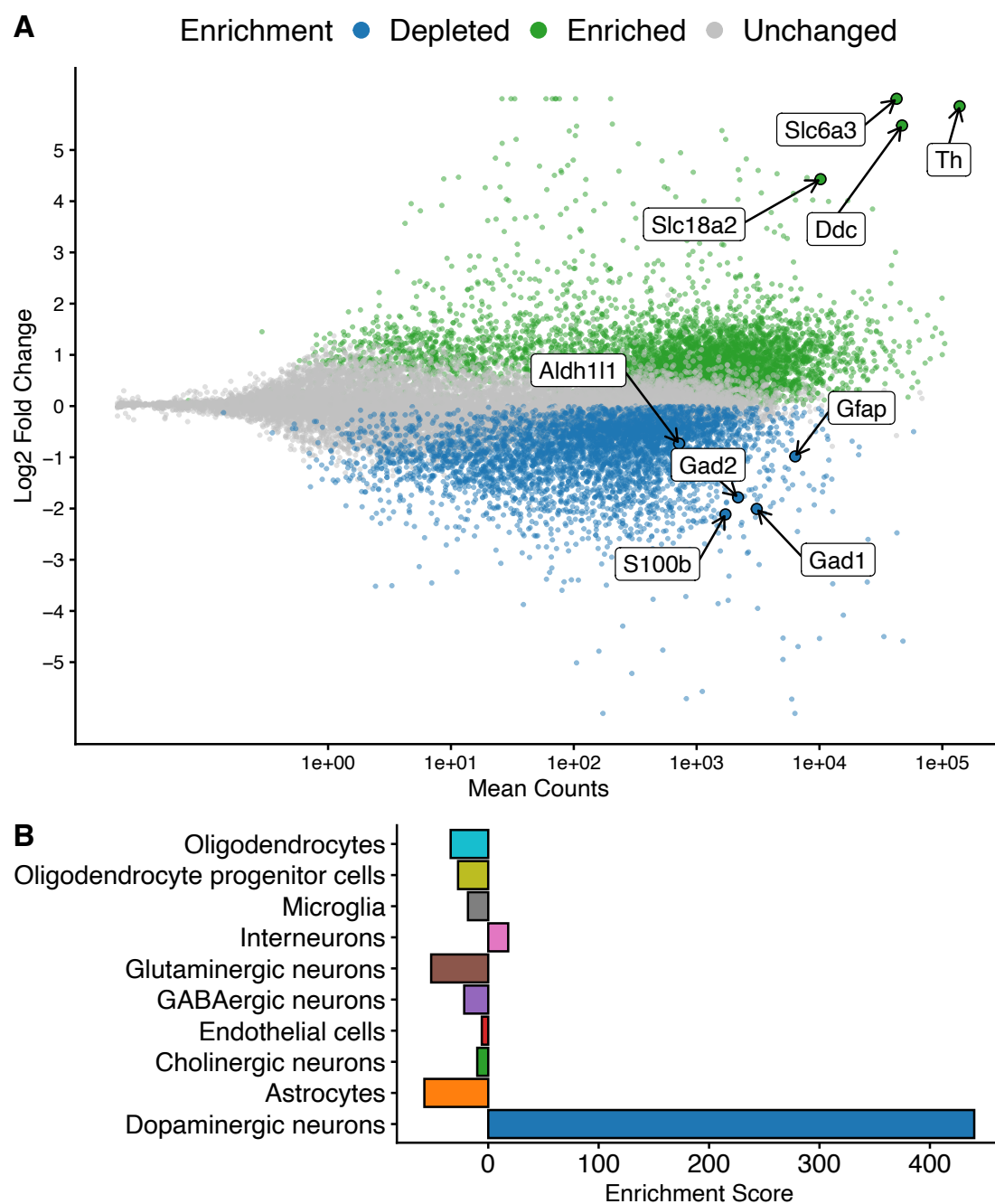


Figure 4.1: Enrichment and depletion in somal TRAP samples. A: The fold change of each gene in somal TRAP samples compared to TOTAL, plotted against the mean expression count. More abundant genes were more likely to be enriched, however this was to be expected. Markers of dopaminergic neurons were enriched and markers of neighbouring cell types depleted. **B:** The enrichment score for cell types likely to be resident within dissected midbrain tissue for TRAP. Dopaminergic neurons were the only cell type, out of 178 that showed clear enrichment.

4. Specific Expression Features in Dopaminergic Neurons

Table 4.2: Dopaminergic marker gene enrichment in somal TRAP samples.

Gene	Log ₂ Fold Change	Adjusted <i>P</i> Value
Th	5.85	5.52e-301
Ddc	4.76	5.52e-301
Slc6a3	4.28	5.52e-301
Chrna6	3.78	5.52e-301
Slc18a2	3.29	5.52e-301
Nr4a2	4.16	5.19e-286
Cadps2	3.78	2.18e-201
Foxa2	4.57	4.9e-106
Ntn1	2.48	1.74e-90
Neurod6	1.45	9.22e-18
Lmx1b	2.76	1.89e-15
Prkcg	0.351	3.95e-10
Kcnj6	1.43	7.86e-10

Gene set enrichment analysis also confirmed the underrepresentation of genes involved in non-neuronal function (Figure 4.2).

Knowledge Deficits in Highly Enriched Genes

273 genes were enriched more than 4-fold by TRAP (Figure 4.3 A). A subset of highly enriched genes were classic markers of dopaminergic neurons (e.g., *Th*, *Slc6a3*, *Slc18a2*). Interestingly, other genes did not have a reported association with dopaminergic biology.

The number of publications that associate each gene with dopamine or Parkinson’s disease-related research was parsed using the NCBI Entrez API. Synonyms and human homologs of each gene were included in the search query, as well as the Ensembl description field, with exceptions to remove spurious associations (Methods). As expected, many enriched genes have been heavily investigated and discussed in the context of dopaminergic biology and/or Parkinson’s disease (Figure 4.3 B).

Interestingly, even among the most enriched genes, several have no published association (Figure 4.3 B). The strong enrichment of these genes suggests that

4. Specific Expression Features in Dopaminergic Neurons

Table 4.3: Gene ontology terms represented by somal TRAP enrichment.

Source	Term name	Term size	Intersection size	P Value
GO: Biological Process				
GO:BP	Translation	605	194	5.58e-59
GO:BP	Peptide biosynthetic process	623	182	2.1e-57
GO:BP	Mitochondrion organization	483	178	4.12e-54
GO:BP	Peptide metabolic process	766	217	1.2e-53
GO:BP	Amide biosynthetic process	726	172	2.31e-52
GO:BP	Ribonucleoprotein complex biogenesis	408	237	4.87e-47
GO:BP	Cellular protein-containing complex assembly	931	445	1.71e-46
GO:BP	Cellular amide metabolic process	971	217	6.43e-46
GO:BP	Organonitrogen compound biosynthetic process	1430	243	1.76e-44
GO:BP	Protein-containing complex assembly	1350	353	7.13e-41
GO: Cellular Component				
GO:CC	Ribosome	229	151	4.45e-113
GO:CC	Ribosomal subunit	195	142	5.94e-112
GO:CC	Mitochondrial protein-containing complex	262	163	6.95e-105
GO:CC	Cytoplasm	9570	1520	3.76e-94
GO:CC	Protein-containing complex	4650	918	5.74e-94
GO:CC	Mitochondrion	1710	463	6e-94
GO:CC	Ribonucleoprotein complex	682	416	3.86e-86
GO:CC	Intracellular anatomical structure	11900	1620	1.59e-80
GO:CC	Large ribosomal subunit	123	92	2.37e-71
GO:CC	Cytosolic ribosome	109	77	2.68e-66
KEGG Pathways				
KEGG	Ribosome	134	114	1.08e-85
KEGG	Parkinson disease	237	128	1.09e-66
KEGG	Oxidative phosphorylation	124	78	8.4e-61
KEGG	Huntington disease	289	134	4.11e-59
KEGG	Prion disease	251	117	1.54e-50
KEGG	Amyotrophic lateral sclerosis	348	96	2.26e-48
KEGG	Pathways of neurodegeneration - multiple diseases	446	107	1.23e-47
KEGG	Alzheimer disease	345	105	6.71e-43
KEGG	Thermogenesis	219	70	8.33e-40
KEGG	Coronavirus disease - covid-19	191	80	1.43e-36

they play an important, yet understudied role in dopamine neuron function and, through “guilt by association”, Parkinson’s disease.

Specific expression of the most enriched, underpublished genes was confirmed using mouse RNA FISH data from the Allen Brain Atlas. Representative images of midbrain expression of four genes; *Tagln3*, *Cpne7*, *Syt17* and *Fam210b* are shown in Figure 4.4. The majority of enriched genes were also confirmed as markers of dopaminergic neurons in single-cell sequencing investigations using embryonic and early postnatal mouse midbrain (Hook et al. 2018; Tiklová et al. 2019; La Manno et al. 2016; Poulin et al. 2014).

4. Specific Expression Features in Dopaminergic Neurons

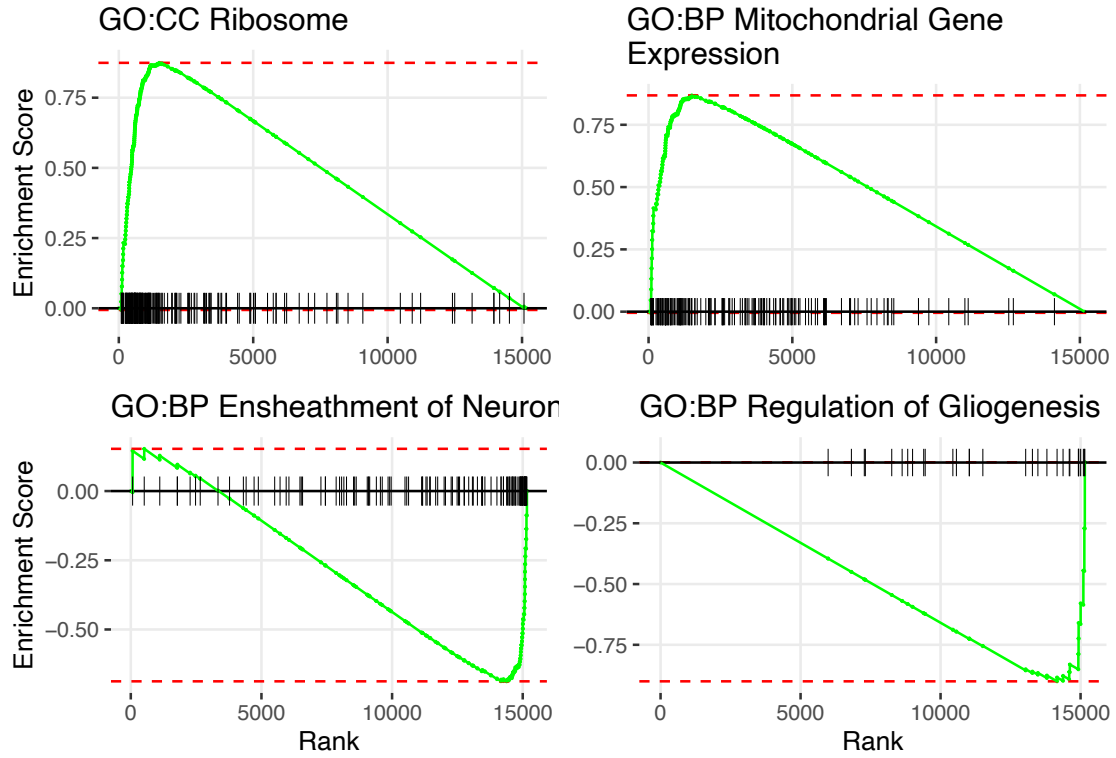


Figure 4.2: Gene set enrichment analysis: Soma TRAP. Ribosome and mitochondrial terms are most strongly represented among genes strongly enriched by TRAP. Terms related to functions of other cell types, such as neuron ensheathment and gliogenesis were depleted.

To explore the possible functional properties of enriched, underpublished genes, high-confidence protein-protein interactions were parsed from the STRING v11 database (Szklarczyk et al. 2020). Interactions were primarily driven by evidence of physical interaction in human fractions from the BioGRID database (Wan et al. 2015). Particular attention was given to interacting neighbours that were also enriched by TRAP. The potential role of these genes in dopaminergic biology and PD is discussed with a review of the surrounding literature in Section 4.3.

Transcript Usage: Diversity in the Cell Body

To find the dominant transcript(s) of genes expressed in the dopaminergic soma, transcript abundance was calculated as a proportion of total gene abundance. The dominant transcript accounted for a median of 75 % of total gene expression. The

4. Specific Expression Features in Dopaminergic Neurons

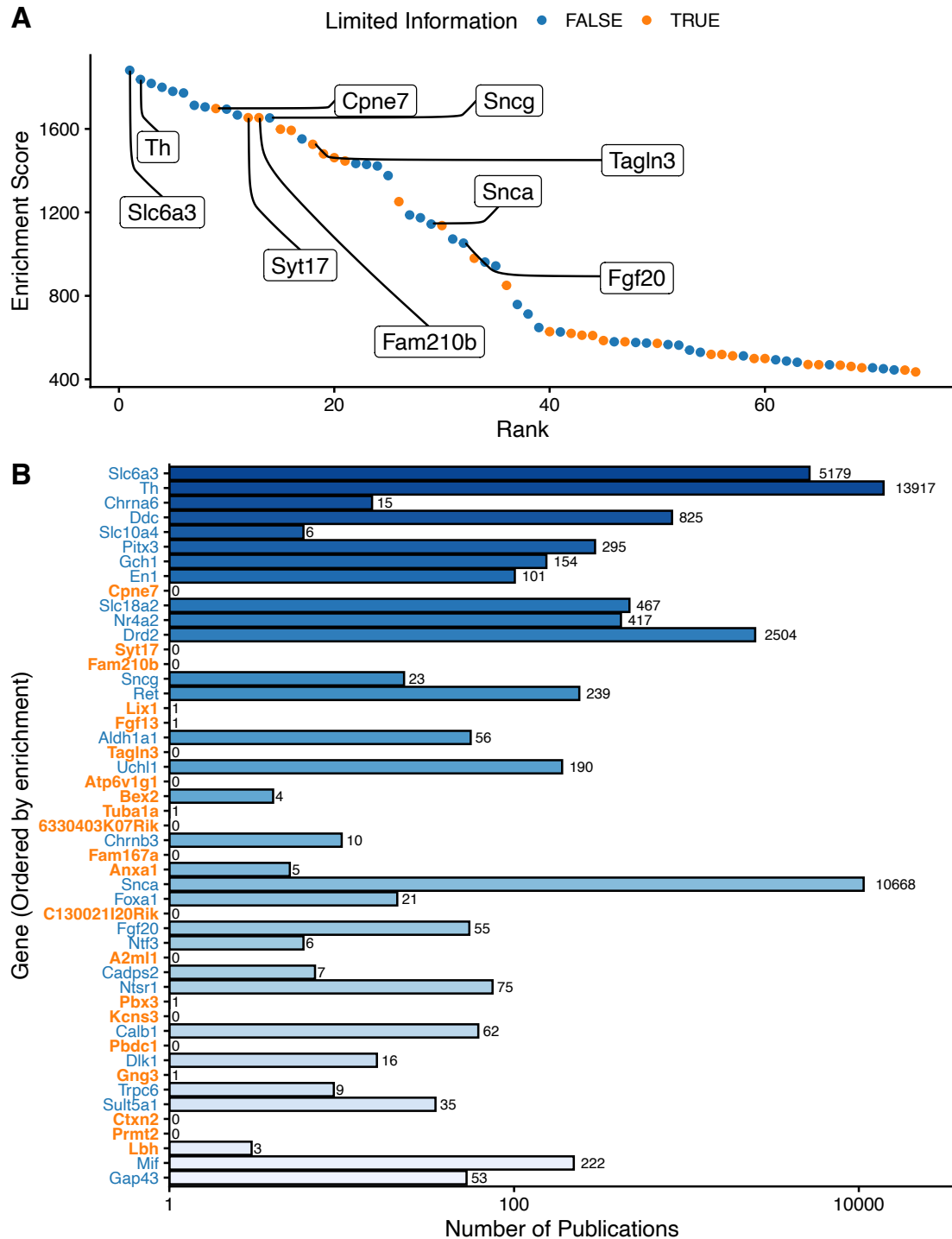


Figure 4.3: Literature deficits among genes most specific to the dopaminergic cell body. **A** The most highly enriched somal TRAP genes include many important components of dopaminergic biology and PD. **B:** The number of publications associating each enriched gene and its associated human homologs and synonyms with dopaminergic or PD-related research. The top 30 genes, ranked by fold enrichment are shown. Genes with 5 or fewer associated publications are highlighted in orange. In many cases, these genes contain 0 or 1 publication.

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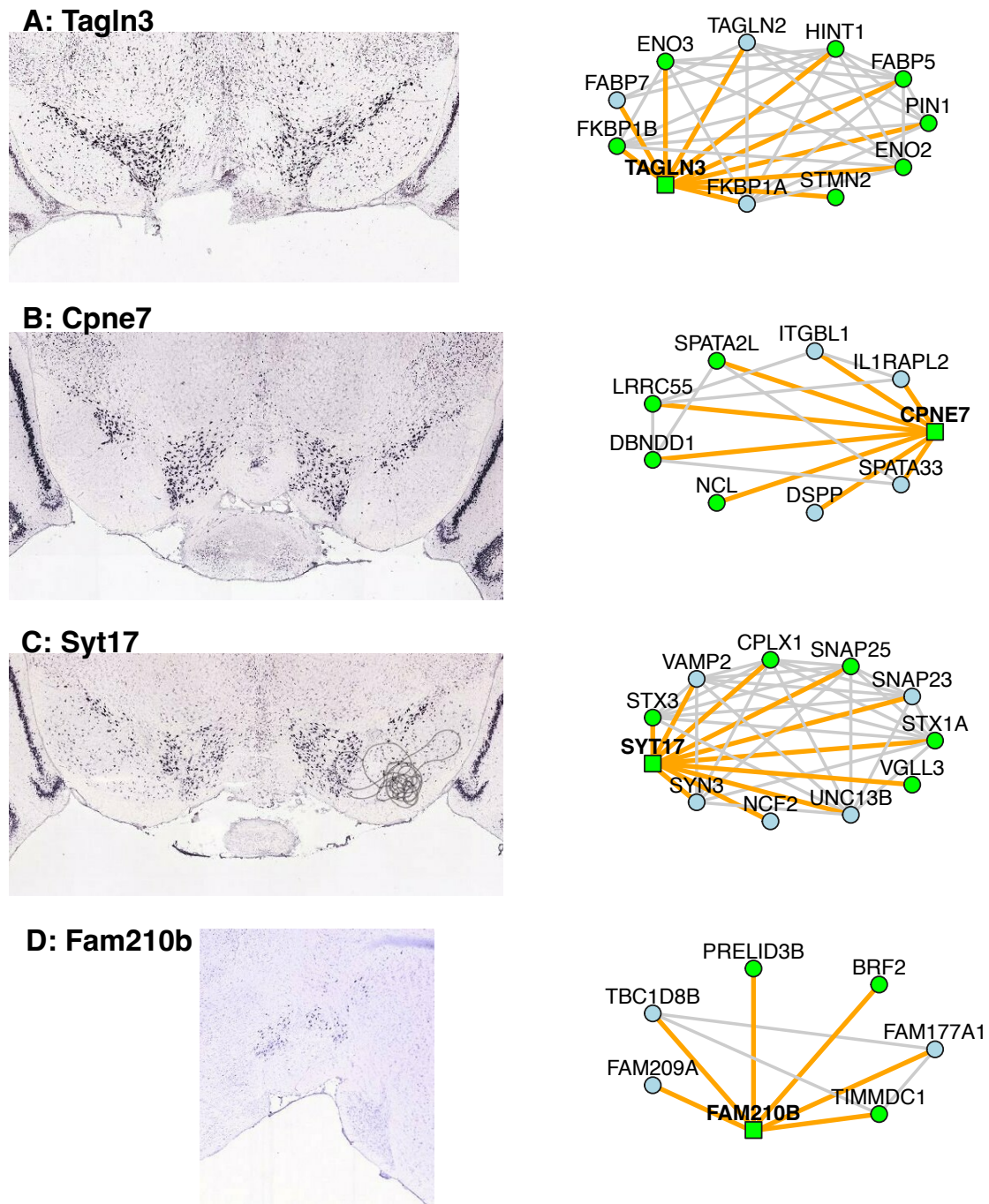


Figure 4.4: ISH Confirmation of dopaminergic specificity and the enrichment status of top interacting proteins. *Tagln3*, *Cpne7*, *Syt17* and *Fam210b* each show clear specificity of expression to dopaminergic neurons, although a difference between SNpc and VTA expression was not evident. Many interactors of each gene were also enriched in somal TRAP samples, supporting a role for each network of proteins in dopaminergic function. ISH images obtained from the Allen Brain Atlas (Sunkin et al. 2012).

4. *Specific Expression Features in Dopaminergic Neurons*

relative contribution of the dominant transcript fell as the number of annotated transcripts increased (Figure 4.5 A). Two thirds of all genes measured expressed multiple transcripts (Figure 4.5).

Transcript usage was compared between somal TRAP and TOTAL samples, to identify differential transcript usage (DTU) events, specific to the dopaminergic neuron. The primary purpose of this analysis was to highlight genes that were not enriched by TRAP at the gene-level, but showed evidence of DTU. These genes could also be considered translated in the dopaminergic neuron.

Evidence of significant DTU was found for 1617 genes, of which 817 were not enriched at the gene-level by TRAP (Figure 4.5 C). In these cases, transcript “switching” resulted in no change in gene-level expression but clear differences in transcript-specific expression, indicating dopaminergic translation. Genes that were not found to be significantly enriched at the gene-level, but showed evidence of differential transcript usage were subsequently added to the list of genes considered translated in dopamine neurons.

An example of DTU in somal TRAP samples is shown in Figure 4.5 D: *Cdc42* is a Rho-family GTPase expressed in two isoforms. Each contains a distinct 3' UTR sequence, encoding alternative post-translational modification motifs that specialise their function (Figure 4.6). Prenylated *Cdc42* (labelled ENSMUST00000030417) has recently been shown to be selectively localised and translated in the axon of dorsal root ganglion neurons (Lee, Zdradzinski, et al. 2021). siRNA targeting of the prenyl-form of *Cdc42* impaired axonal growth. Prenyl-*Cdc42* was selectively enriched in somal and axonal TRAP samples, indicating a potentially similar mode of function.

Novel Isoform Detection Using Long-Read Data

To assess whether dopaminergic neurons express novel transcripts or genes, long read alignment data were processed using TALON; a recently developed transcrip-

4. Specific Expression Features in Dopaminergic Neurons

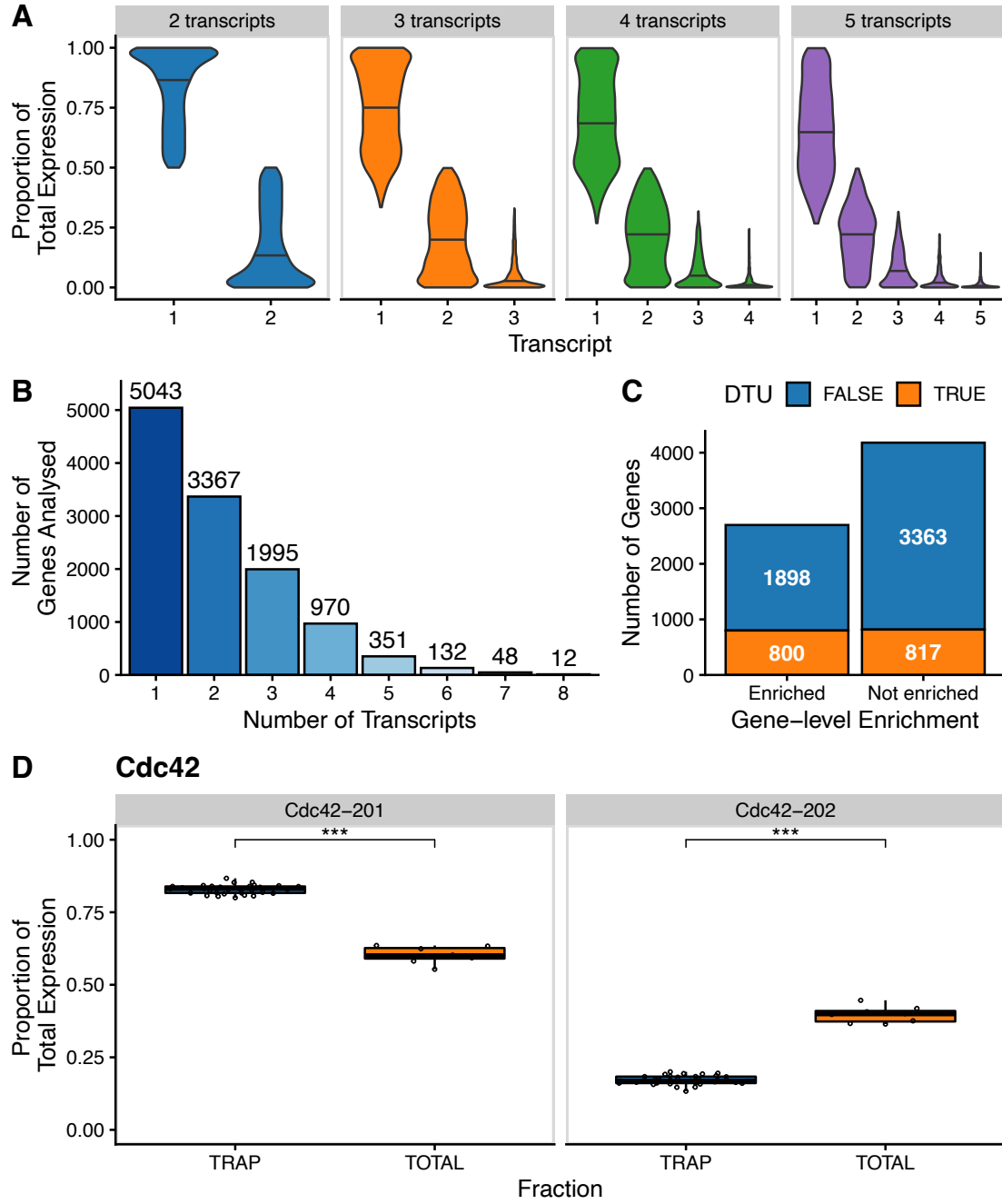


Figure 4.5: Splicing diversity in dopaminergic neurons. **A:** 75% of gene expression was mediated by the dominant transcript, with the majority of further expression being mediated by a second, but expression of more than two transcripts at once was rare. **B:** The number of genes considered for differential transcript usage analyses and the number of associated transcripts. Single-transcript genes are included for reference. **C:** Half of all genes found to exhibit differential transcript usage in somal TRAP samples were not enriched at the gene-level; expanding the scope of what is considered translated in dopaminergic neurons. **D:** Selective usage of a *Cdc42* isoform involved in axonal localisation and sustaining axonal growth. Stagewise analysis for the determination of significant differential transcript usage was performed using *stageR* (Berge 2017). Transcript-level P values are presented. *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

4. Specific Expression Features in Dopaminergic Neurons

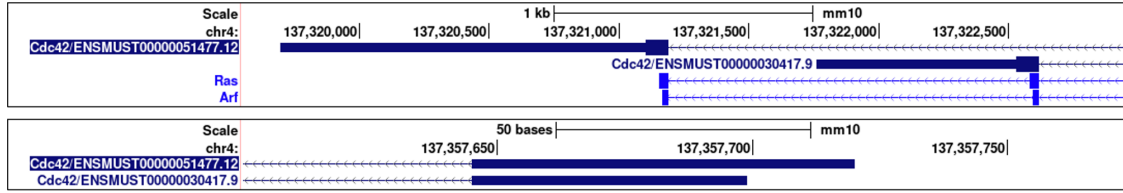


Figure 4.6: The 5' and 3' UTR Structure of Cdc42

tome assembly package (Wyman et al. 2019).

Before using alignment data to classify novel transcripts, aligned reads were subject to a series of quality control checks: Reads were tested for internal priming events by calculating the proportion of A's in a 20 bp window immediately following the end of each alignment. Internal priming could lead to spurious models of prematurely terminated transcripts, due to binding of the poly-dT reverse transcription primer at intragenic sites of high poly-A content. Secondly, reads were filtered by read length and the proportion of gene body covered (Figure 4.7 B). Notably, only around one quarter of reads passed filtering and this was primarily due to low read length: Although reads were many times longer than short-read sequencing, the majority were not classed as “full-length”, which is a requirement for this *de novo* method. Improving read length by refining the size selection step of long-read library preparation is key to maximising full length read coverage.

Passed, aligned reads were then annotated using the Gencode mouse gene annotation. Alignments that did not match with annotation entries were categorised by the type of novelty: “Incomplete splice match” (ISM) models were models that did not differ in internal exon boundaries, but either start from an unannotated position or terminate prematurely. “Novel in catalog” (NIC) models do not differ in internal exon boundaries, however the combination of exons included in the model is not annotated. “Novel not in catalog” (NNC) models differ in internal exon boundaries (for example, exon elongation or truncation events fall into this category). “Genomic” models represent alignments that include intronic segments. “Antisense” models overlap with existing models, but are in reverse orientation

4. Specific Expression Features in Dopaminergic Neurons

compared to the annotation. Differences in the start of the 5' UTR and end of the 3' UTR were not considered, due to variable read coverage at these areas. Models were only considered that were detected in every sample (12 samples in total).

Around 90 % of all models identified were annotated ("known"), however these models only accounted for around 60 % of reads (Figure 4.7 C and D). 25 % of each library aligned to ISM models. As might be expected, less than 1 % of each library aligned to NNC models: Detection of novel exons or exon boundaries in a highly studied and annotated species, such as mouse would be a rare event.

Three filtering steps followed, to select for the highest confidence novel models: CAGE-derived transcription start site (TSS) locations were obtained from the FANTOM5 database and used to measure the distance between the 5' end of each model and the nearest TSS (Abugessaisa et al. 2017). Models were classified by whether they contained a TSS within 100 bp up or downstream of the 5' terminus. Poly-A sites from the PolyASite 2.0 database were used to classify models by whether a poly-A site was located within 20 bp of the 3' terminus (Herrmann et al. 2019).

Finally, the transcript sequence of each model was scanned to identify putative peptides sequences: The longest identified peptide per model was submitted as a protein BLAST query to search for evidence of existence. Only models with TSS, poly-A and peptide-level support were retained. The proportion of models at each level of support is shown in Figure 4.7 E, F and G.

Reassuringly, models with CAGE and poly-A support also had the most coding potential support. When ISM models were further subdivided by whether they cover the 5' or 3' end of an annotated transcript (labelled "Prefix" and "Suffix" in Figure 4.7 F, respectively), there was a difference in CAGE and poly-A support: Prefix models had greater CAGE support, while Suffix models had greater poly-

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A support, suggesting that these models reflect incomplete reads, as opposed to genuinely shorter transcripts.

After filtering, approximately 8 % of the library was represented by putative novel transcript models (Figure 4.7 H). The alignment data were manually inspected and splice alignments were compared against matched short-read TRAP data. In every novel model inspected, a consistent error in read generation was identified that introduced variation of between 1 and 3 bp around splice junctions. This variation was systematic in all samples, leading to the definition of novel models, however there was no support for this variation in the matched short-read data. It was concluded that the pipeline performed well for *de novo* construction of the majority of transcript models (evidenced by the high number of “known” models), however close inspection of novel models using short-read data for confirmation is essential.

4.2.2 Axonal Enrichment

Summary Statistics

To identify axonally translated genes, striatal TRAP samples were compared to matched TOTAL samples. Out of 27050 genes detected in TRAP and TOTAL axonal samples of cohorts 2 and 3, 4726 were significantly enriched by TRAP (Figure 4.4). 3873 were depleted and 18451 were not significantly changed. Enriched genes accounted for for 77.2 % of each library, on average.

Table 4.4: Summary of the number of genes significantly enriched, depleted and unchanged in striatal TRAP samples compared to TOTAL.

Metric	Enriched	Depleted	Unchanged
Number of Genes	4726	3873	18451
Percentage of Library	77.2 %	4.94 %	17.9 %

4. Specific Expression Features in Dopaminergic Neurons

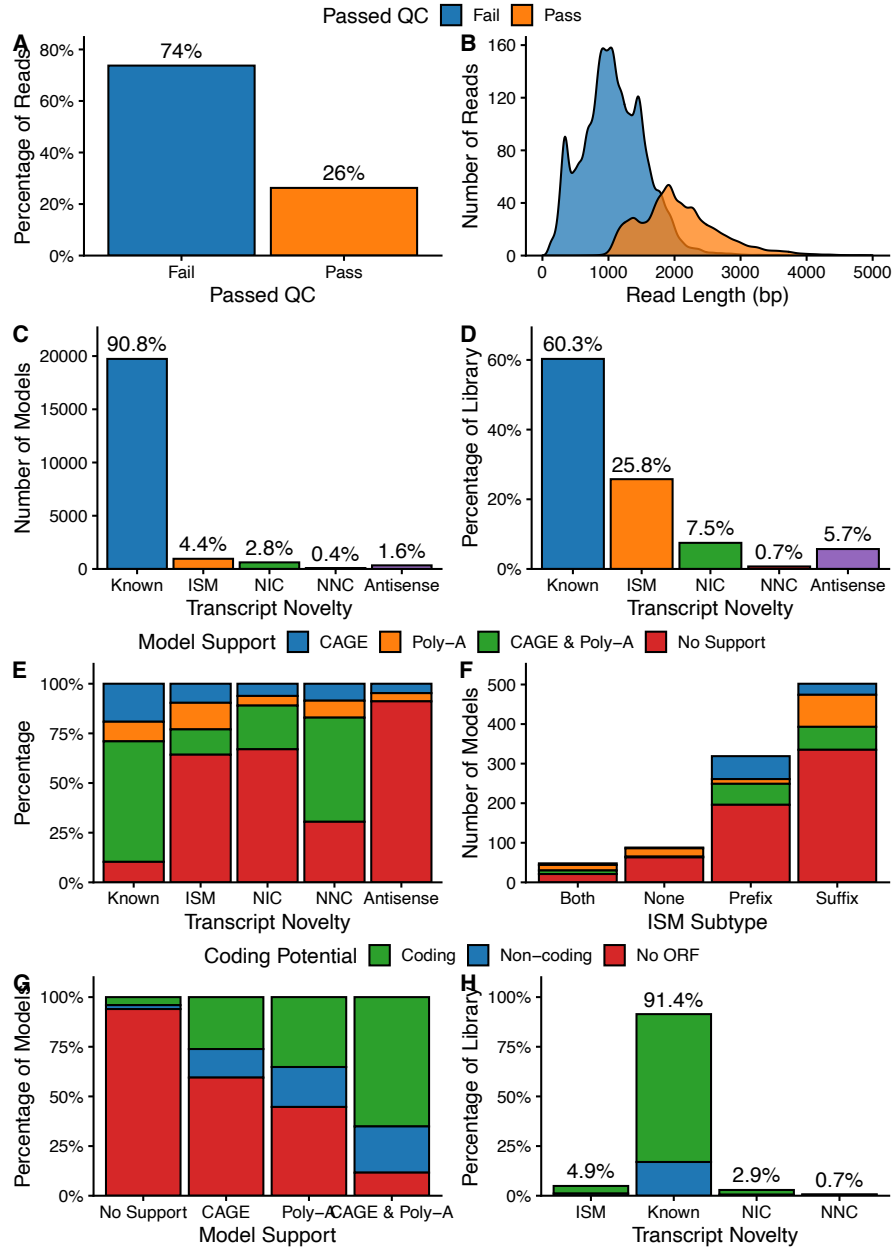


Figure 4.7: *De novo* transcript discovery using long-read sequencing data. **A and B:** Only 26 % of long-reads were used in transcript discovery, due to a stringent selection of full-length transcripts. **C and D:** The number of models (**C**) and percentage of library (**D**) accounted for by each transcript model category. Incomplete splice match (ISM) models accounted for a large percentage of library, due to read length preventing full length coverage. **E and F:** The proportion of models with each level of transcript support. “Known” models were best supported by CAGE and poly-A data. CAGE and poly-A support was greater in prefix and suffix ISM models, respectively (**F**). **G:** Coding potential among transcript models at each level of CAGE and poly-A support: Models with CAGE and poly-A support were more likely to contain an open reading frame. **H:** The proportion of library allocated to each model novelty category, after filtering.

4. Specific Expression Features in Dopaminergic Neurons

Cell Type Specificity

The cell type specificity of axonal TRAP data was carefully examined. Dopaminergic markers were robustly enriched in axonal TRAP samples (Figure 4.8 A). In contrast to somal TRAP samples, however, they were less abundant. Two explanations were considered; the translational efficiency of dopaminergic markers could be lower in the axon compared to the soma or RNA fractions from other cell types could be present in axonal TRAP samples, reducing the proportion of library assigned to these marker genes. Some markers of neighbouring striatal cell types were also enriched and were more abundant than dopaminergic markers (Figure 4.8 B).

A cell type enrichment score was calculated using the database of 178 cell type markers. Dopaminergic neurons were enriched, however there was also enrichment of markers of astrocytes, cholinergic neurons and oligodendrocytes (Figure 4.8 C). A deconvolution approach was used to estimate the proportions of different cell type RNA in axonal TRAP samples. Three datasets were tested independently, each containing expression signatures of different cell types present in the mouse striatum or midbrain. The estimated cell type proportions based on these signatures are presented in Tables 4.5, 4.6 and 4.7.

Midbrain-derived TOTAL and TRAP samples were tested for comparison: The neuronal proportion was greater in somal TRAP samples compared to TOTAL. In

Table 4.5: Sample composition estimates (% of Sample) based on the Gokce et al., 2016 single cell striatal signature matrix.

Region	Fraction	Astrocyte	Ependy-C	Macrophage	Microglia	Neuron	NSC	Oligodendrocyte
MB	TOTAL	1.77	0.51	0.39	0.00	86.10	0.67	10.55
MB	TRAP	0.74	0.34	0.05	0.00	96.81	0.26	1.80
DS	TOTAL	1.81	0.00	0.15	0.01	94.13	0.00	3.89
DS	TRAP	2.68	0.03	0.15	0.08	93.31	0.00	3.74
VS	TOTAL	1.35	0.29	0.08	0.01	93.92	0.00	4.35
VS	TRAP	2.21	0.34	0.10	0.04	93.55	0.00	3.75

4. Specific Expression Features in Dopaminergic Neurons

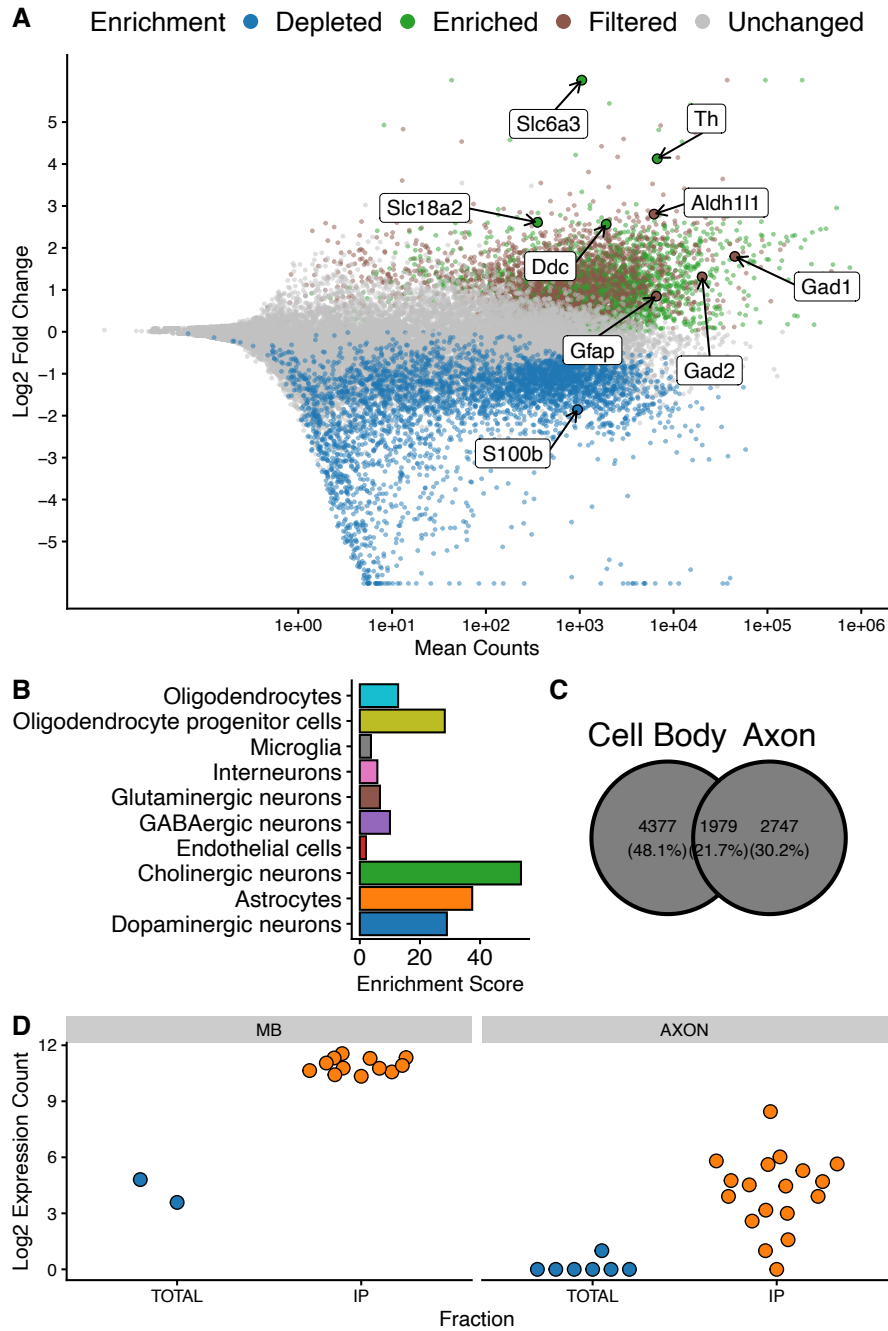


Figure 4.8: Enrichment and depletion in axonal TRAP samples. **A:** The fold change of each gene in axonal TRAP samples compared to TOTAL, plotted against the mean expression count. Dopaminergic markers were enriched, however their abundance was lower than in somal TRAP samples. Markers of other cell types were also enriched. **B:** The enrichment score for cell types likely to be resident within dissected striatal tissue for TRAP. Dopaminergic neurons, cholinergic neurons, astrocytes and oligodendrocytes were enriched. **C:** The number of genes enriched in somal and axonal TRAP samples, with the intersection of both lists used to define axonally translated genes. **D:** Cre expression in midbrain and striatal TRAP and TOTAL samples. Cre expression was higher in midbrain and enriched by TRAP. Cre was virtually undetectable in striatal TOTAL samples, but was enriched in TRAP samples.

4. Specific Expression Features in Dopaminergic Neurons

Table 4.6: Sample composition estimates (% of Sample) based on Chen et al., 2014 sorted brain cell types.

Region	Fraction	Astrocyte	Microglia	Neuron	NFO	OPC
MB	TOTAL	0.00	1.48	71.28	25.25	0.00
MB	TRAP	0.00	0.47	95.81	3.70	0.02
DS	TOTAL	2.73	1.30	79.22	12.82	2.35
DS	TRAP	10.14	1.74	68.01	20.01	0.09
VS	TOTAL	1.07	1.29	79.69	12.42	3.32
VS	TRAP	7.19	1.74	71.87	18.50	0.70

Table 4.7: Sample composition estimates (% of Sample) based on a combination of TRAP/Ribotag datasets.

Region	Fraction	Astrocyte	D1 MSN	D2 MSN	Midbrain Dopamine Neuron	Oligodendrocyte
MB	TOTAL	11.22	0.00	3.96	42.29	42.53
MB	TRAP	1.04	3.64	0.32	94.09	0.90
DS	TOTAL	7.90	0.00	42.28	29.28	20.54
DS	TRAP	4.21	0.00	60.01	18.50	17.28
VS	TOTAL	9.63	0.00	37.10	32.63	20.64
VS	TRAP	4.75	0.00	58.37	19.61	17.27

the third dataset, the estimated dopaminergic proportion was $\sim 94\%$. Accordingly, the estimated proportions of astrocytes and oligodendrocytes fell to almost 0% .

In striatum-derived samples, the estimated neuronal proportion ranged from $\sim 68\%$ – 94% , depending on the dataset. The estimated dopaminergic neuronal proportion in axonal TRAP samples was lower than TOTAL samples. This was judged to likely be artefactual, as enrichment of dopaminergic markers had been confirmed and over 60% of each axonal TRAP library was found to contain reads that aligned to genes expressed by dopaminergic neurons (see next chapter). The estimated D2 medium spiny neuron proportion increased in axonal TRAP samples.

In pilot TRAP experiments using *ROSA26-eGFP-L10a::fs/fs* mice that do not express eGFP-L10a, no RNA was obtained. It was therefore considered unlikely that non-specific binding of RNA from neighbouring cell types was a main contributor to the cell type proportions seen in axonal TRAP samples. Additionally, markers of other cell types were enriched, which indicated that RNA from specific cell types

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were bound. Cre expression was quantified by realigning reads from all TRAP and TOTAL samples to the Cre locus (NC_005856.1). Cre was not detected in all but one striatal TOTAL sample, however it was enriched in axonal TRAP samples (Figure 4.8 D). Unless there is a requirement for axonal translation of Cre, this indicates low-level ectopic Cre expression in some cell type(s) in striatum.

4.2.3 Axon-specific Genes

Genes enriched in axonal TRAP samples were filtered to retain those that were also enriched in somal TRAP samples. 1979 genes remained (Figure 4.8 C).

To identify genes most specific to the axon relative to surrounding striatal tissue, axonal TRAP samples were compared to axonal TOTAL samples (identification of genes most specific to the axon relative to the soma is described in the next chapter). Enriched, retained genes were ranked by the magnitude of enrichment. To further prioritise genes most specific to the axon, striatal “expression energy” values from the Allen Mouse Brain Atlas were parsed. The expression energy was calculated as a function of the signal intensity from coronal and sagittal ISH experiments for each gene. The expression energy of *Th*, *Slc6a3* and *Gad2* for comparison were 0.42, 0.06 and 18.38, respectively. 497 enriched genes with expression energy values of 0.5 or below were therefore considered specific.

The top 30 most enriched and specific genes are presented in Table 4.8. Many genes have an established role in dopaminergic axonal biology: Autophagy-related 7 is a key regulator of autophagy in dopaminergic axons (Friedman et al. 2012). There is a large amount of evidence to support cholinergic receptor subunit translation in dopaminergic axons; in particular *Chrna6* (Meyer et al. 2008; Rice and Cragg 2004; Cragg 2006). *Slc6a3*, *Th* and *En1* also ranking within the top 15 enriched genes adds confidence to this approach of gene prioritisation.

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Table 4.8: The top 30 genes enriched by axonal TRAP, ranked by enrichment and filtered for low striatal expression energy from the Allen Mouse Brain Atlas. All genes shown are also enriched in the cell body.

Gene	Description	Log ₂ Fold Change	Striatal Expression Energy
Atg7	Autophagy related 7	8.11	0.07
En1	Engrailed 1	6.90	0.07
Slc6a3	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	6.83	0.06
Cspg4	Chondroitin sulfate proteoglycan 4	5.44	0.48
Rnf111	Ring finger 111	5.41	0.12
Chrna6	Cholinergic receptor, nicotinic, alpha polypeptide 6	4.57	0.16
Yy1	Yy1 transcription factor	4.53	0.41
Lrguk	Leucine-rich repeats and guanylate kinase domain containing	4.22	0.11
Th	Tyrosine hydroxylase	4.12	0.42
Nufip2	Nuclear fragile x mental retardation protein interacting protein 2	3.34	0.08
Uba1	Ubiquitin-like modifier activating enzyme 1	2.94	0.03
Uba6	Ubiquitin-like modifier activating enzyme 6	2.94	0.11
Tpp2	Tripeptidyl peptidase ii	2.84	0.15
Kif3a	Kinesin family member 3a	2.82	0.37
Slc5a11	Solute carrier family 5 (sodium/glucose cotransporter), member 11	2.68	0.15
Gmps	Guanine monophosphate synthetase	2.64	0.04
Atic	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/imp cyclohydrolase	2.61	0.11
Slc18a2	Solute carrier family 18 (vesicular monoamine), member 2	2.61	0.13
Ddc	Dopa decarboxylase	2.56	0.09

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4. Specific Expression Features in Dopaminergic Neurons

Table 4.8: The top 30 genes enriched by axonal TRAP, ranked by enrichment and filtered for low striatal expression energy from the Allen Mouse Brain Atlas. All genes shown are also enriched in the cell body. (*continued*)

Gene	Description	Log ₂ Fold Change	Striatal Expression Energy
AW551984	Expressed sequence aw551984	2.54	0.16
Copb2	Coatomer protein complex, subunit beta 2 (beta prime)	2.53	0.31
Ncoa4	Nuclear receptor coactivator 4	2.52	0.05
Gch1	Gtp cyclohydrolase 1	2.48	0.06
Foxa1	Forkhead box a1	2.46	0.09
Dcaf12l1	Ddb1 and cul4 associated factor 12-like 1	2.45	0.04
Pgd	Phosphogluconate dehydrogenase	2.43	0.17
Serpinb9	Serine (or cysteine) peptidase inhibitor, clade b, member 9	2.40	0.08
Chrn3	Cholinergic receptor, nicotinic, beta polypeptide 3	2.38	0.07
Naa35	N(alpha)-acetyltransferase 35, nate auxiliary subunit	2.38	0.14
Dmd	Dystrophin, muscular dystrophy	2.36	0.03

4.2.4 GWAS Prioritisation

Candidate gene prioritisation in genome-wide association studies (GWAS) of complex traits is challenging: Often the defined variant is in high linkage disequilibrium with neighbouring variants, overlapping multiple genes. Functional resources such as tissue-specific transcriptomic data from genotyped patients are increasingly used to aid prioritisation (Kichaev et al. 2014).

A recent meta-analysis of PD GWAS identified 38 novel and 42 previously reported, independent loci (Nalls, Blauwendraat, et al. 2019). The study mined single cell mouse RNA sequencing data for signatures relevant to PD risk; gene expression specific to substantia nigra dopaminergic neurons was the most significant. Methylation and expression data were taken from brain region (GTEx) and blood-derived

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datasets to identify QTL's with significant association to PD risk. Over half of all genes within linkage regions of the PD loci were found to be significant. In a majority of these cases, more than one significant gene was present and the nearest gene to the lead variant was selected. In loci without any QTL association, the nearest gene to the lead variant was selected. This “nearest gene” approach has been shown to have limited sensitivity for detecting causal genes (Pers et al. 2015). Somal and axonal enrichment data were used to re-prioritise candidate genes. The same starting set of 278 genes were used as in Nalls, Blauwendraat, et al. (2019). These genes overlap any variant in linkage disequilibrium ($r^2 > 0.5$ or $D' > 0.5$) with a lead variant within +/- 500 kb. Axonal genes were only considered that were retained after filtering based on expression intensity values from the Allen Brain Atlas, as described previously.

233 mouse homologs were mapped from human genes and detectable in TRAP samples (Figure 4.10 A). The majority of unmapped genes were not associated with a variant and were generally not protein coding or annotated (Figure 4.10 B). All significantly enriched and depleted genes from the candidate list were ranked by magnitude to compare the relative number of enriched genes compared to depleted (Figure 4.9). There were approximately 45 % more somal enriched genes than depleted. This supports the findings from Nalls, Blauwendraat, et al. (2019) that dopaminergic neuron gene expression is of primary importance in understanding the effects PD associated genetic variation.

Genes that were depleted or not significantly enriched in either somal or axonal TRAP samples were classed as “Not Dopaminergic” and removed from consideration. In 39 loci in which only a single gene was present, 22 were classed as “Not Dopaminergic”, including *LRRK2* (Figure 4.10 C). In 61 loci containing multiple candidate genes, 12 did not contain any dopaminergic genes and were not considered further.

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From 49 remaining loci, the most enriched gene from the somal and/or axonal compartment was selected. In two-thirds of loci, somal TRAP samples were responsible for candidate gene selection (Figure 4.10 D). In 17 loci, enrichment values were available from both compartments. In only 3 of these cases, the same candidate gene was identified by both compartments (Figure 4.10 E). The resulting selected genes were compared to the selections made by Nalls, Blauwendraat, et al. (2019): The same gene was selected in only $\sim 20\%$ of loci, however only 25 % of genes selected by Nalls, Blauwendraat, et al. (2019) were enriched in dopaminergic neurons.

The Calcium sensing receptor, *CASR* was one of the most enriched genes identified in both somal and axonal TRAP samples (Figure 4.9). *CASR* was selected as the lead gene at the novel rs55961674 locus: The nearest gene and QTL selection methods instead selected *KPNA1* as the lead gene, however there is no evidence of dopaminergic expression from TRAP data.

Table 4.9: Candidate genes compared between somal and axonal TRAP and Nalls, 2019 selections. Axonal genes were filtered for expression intensity in the Allen Brain Atlas. Missing values are where a selection was not possible by that method.

SNP	Soma	Axon	Nearest	QTL
rs2904880	Sult5a1	Sult5a1	CD19	NFATC2IP
rs10748818	Pitx3		GBF1	CUEDC2
rs12147950	Foxa1	Foxa1	MIPOL1	
rs11158026	Gch1	Gch1	GCH1	GCH1
rs55961674	Casr	Casr	KPNA1	KPNA1
rs114138760	Kcnn3		PMVK	
rs35749011	Kcnn3		KRTCAP2	
rs76763715	Kcnn3		GBAP1	GBAP1
rs666463	Dnah17		DNAH17	DNAH17
rs17686238	Itga2b	Grn	MAP3K14	
rs2269906	Itga2b	Grn	UBTF	UBTF
rs850738	Itga2b	Grn	FAM171A2	GRN
rs34311866	Cplx1		TMEM175	TMEM175
rs873786	Cplx1		GAK	GAK
rs12456492	Rit2		RIT2	
rs9261484	Ppp1r11		TRIM40	TRIM40

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4. Specific Expression Features in Dopaminergic Neurons

Table 4.9: (continued)

SNP	Soma	Axon	Nearest	QTL
rs11950533	Pcbd2	Txndc15	C5orf24	TXNDC15
rs1293298	Fdft1		CTSB	CTSB
rs77351827	Crls1		CRLS1	
rs11150601	Bcl7c	Hsd3b7	SETD1A	SETD1A
rs12497850	Wdr6	Slc25a20	IP6K2	IP6K2
rs10221156	Tox3		CHD9	CYLD
rs3104783	Tox3		CASC16	CYLD
rs61169879	Brip1	Ints2	BRIP1	MED13
rs75859381	Rps12		RPS12	
rs62333164	Sh3rf1		CLCN3	NEK1
rs12600861	Zbtb4		CHRNA1	CHRNA1
rs12951632	Mlx	Naglu	RETREG3	RETREG3
rs1450522	B3galnt1		SPTSSB	NMD3
rs11557080	Nucks1		RAB29	RAB29
rs823118	Nucks1		NUCKS1	NUCKS1
rs11578699	Vamp4		VAMP4	VAMP4
rs1867598	Ndufaf2	Elovl7	ELOVL7	ELOVL7
rs6476434	Ube2r2		UBAP2	PRSS3
rs199351	Klhl7		GPNMB	GPNMB
rs3742785	Fcf1		RPS6KL1	RPS6KL1
rs11658976	Crhr1	Wnt3	WNT3	WNT3
rs199453	Crhr1	Wnt3	NSF	
rs7221167	Crhr1	Wnt3	MAPT- AS1	
rs7225002	Crhr1	Wnt3	KANSL1	
rs26431	Gin1		PAM	PAM
rs2042477	Mrps5		KCNIP3	KCNIP3
rs76949143	Sbds		GS1- 124K5.11	LINC00174
rs10513789	Dcun1d1		MCCC1	MCCC1
rs117896735	Tial1		INPP5F	SEC23IP
rs72840788	Tial1		BAG3	BAG3
rs1941685	Nol4		ASXL3	
rs2280104		Ccar2	BIN3	BIN3
rs7134559		Scaf11	SCAF11	

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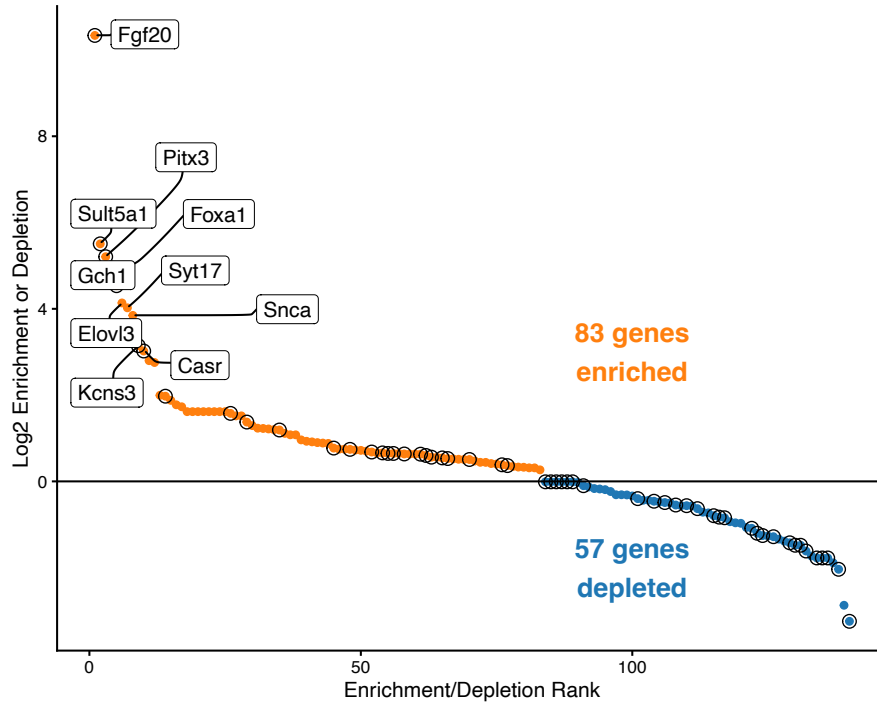


Figure 4.9: Overrepresentation of dopaminergic-specific genes among PD GWAS loci: Genes located within linkage regions of PD GWAS loci with significant evidence of enrichment or depletion are shown: More genes were enriched than depleted, suggesting that many genes with significant genetic association to PD risk are specific to dopaminergic neurons.

4.3 Discussion

4.3.1 Somal and Axonal Enrichment

Based on the findings from this chapter;

- Dopaminergic neurons translate thousands of genes at one time.
- Hundreds of these genes are expressed with specificity to the soma and axon.
- Around half of all translated multi-isoform genes express an alternative dominant isoform to the tissue average. Existing annotations of splicing diversity in the mouse are largely complete.
- Enrichment data can be used to prioritise candidate genes within PD GWAS loci

4. Specific Expression Features in Dopaminergic Neurons

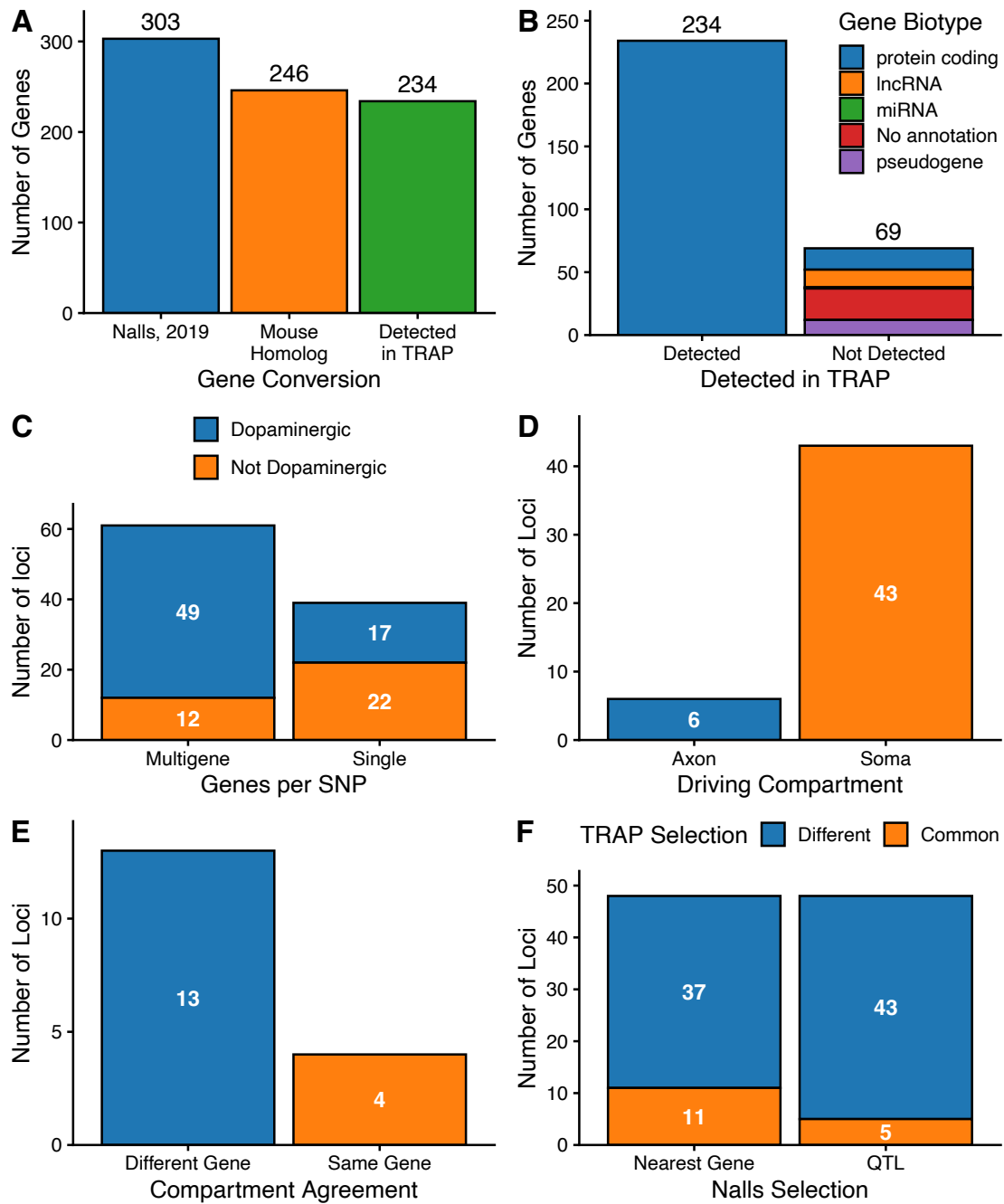


Figure 4.10: GWAS candidate gene selection: Summary information. **A:** Most genes within range of PD GWAS loci have mouse homologs that are detectable in mouse TRAP data. **B:** Genes without mouse homologs were primarily noncoding or not fully annotated. **C:** Loci containing only one gene were not considered further. The majority of multi-gene loci contain at least one enriched gene. **D:** Somal TRAP samples were responsible for the classification of lead genes from two-thirds of loci. **E:** In loci containing genes enriched in both neuronal compartments, there is low agreement over which is the most enriched and therefore the lead gene. **F:** Only ~ 20 % of genes selected by Nalls, Blauwendraat, et al. (2019) were in common with genes selected by TRAP.

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This specificity information provides a framework to prioritise results of condition-dependent comparisons, such as age, genotype and cell region, as performed in the next chapter.

The composition of TRAP samples from the soma and axon differed: Somal TRAP samples were estimated to contain 80-90 % dopaminergic transcripts, while the estimated dopaminergic fraction in axonal TRAP samples was lower. The library composition differences between samples is considered in more detail in the next chapter. By considering somal TRAP enrichment and using orthogonal information from striatal ISH experiments, approximately 500 highest confidence axonal genes were identified. This filtering may be overly conservative: Genes that are translated in both the axon and neighbouring cell types will be excluded due to lower enrichment values and greater striatal expression intensity from ISH data. In addition, genes or transcripts that are translated in the dopaminergic axon may not be translated in the cell body, so these may be missed. An *in vitro* study of axonal gene expression in motor neurons did find a reduction in the number of expressed genes compared to the cell body, however 3-3500 genes were still expressed (Nijssen et al. 2018). For these reasons, no gene is excluded from further analyses, but is considered in light of the evidence from this chapter.

Ectopic expression of eGFP-L10a in non-dopaminergic neurons could contribute to “background” contamination of axonal TRAP samples. This could be due to DAT expression in other cell types, either transiently in development or persistently. Low-level readthrough of the floxed eGFP-L10a cassette is less likely, due to the finding that eGFP-L10a was not detectable in *ROSA26-eGFP-L10a::fs/fs* mice and RNA could not be obtained by performing TRAP. A recent investigation into local translation in astrocytes has considered a role for RNA granule movement between neurons and astrocytes across the synaptic cleft (Sakers et al. 2017).

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Axon-localised translation has been demonstrated in retinal ganglion cell axons *in vivo* (Shigeoka et al. 2016b). In this case, genes known to be involved in synaptic transmission, endocytic recycling and calcium handling were found to be consistently enriched in ribotag (TRAP equivalent) samples. Closely related processes, however, were more enriched in somal samples, such as the regulation of neuron projection development. It is not clear, therefore, why genes with closely related function differ in expression between compartments. They may serve additional compartment-specific functions or these differences may be due to technical error in compartmental translomic studies: Shigeoka et al. (2016b) confirmed the specific expression of the Ribotag transgene to the cell body and axonal compartment of retinal ganglion neurons using a LacZ reporter line. They reported non-specific binding of RNA to the beads used for pulldown, however, confounding the reliable detection of compartment-specific transcripts. High resolution spatial transcriptomics may enable the detection of subcellular patterns of expression in non-transgenic animals, avoiding the need to generate reporter models of cell types or compartments and removing a substrate of non-specific contamination.

A critical next stage of this investigation will be to confirm the axonal expression of these genes using single molecule ISH (smFISH). smFISH is able to resolve individual transcripts and it is compatible with protein co-staining: The aim will be to confirm colocalisation of axonally enriched transcripts (such as prenyl-Cdc42) with TH or, signal permitting, eGFP.

Many genes were found to be highly specific to somal and axonal TRAP samples, but lacked any published report of involvement in dopaminergic function. Some of these genes were ranked as lead candidate genes within significant PD GWAS loci. The existing literature surrounding some of these genes is discussed below.

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Calcium-Sensing Receptor (*Casr*)

The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor that is highly expressed in the kidney and parathyroid and among many other functions, plays a role in divalent cation homeostasis (Riccardi and Brown 2010). Changes in extracellular calcium concentration activate the receptor, leading to changes in parathyroid hormone release and secretion; also directly regulated by CaSR. Variation around the *CASR* locus is significantly associated with hypercalcaemic disorders and hypocalciuria (Hannan and Thakker 2013): Recently, chronic renal dysfunction and proteinuria have been identified as independent risk factors for PD development in older adults (Nam et al. 2019).

Casr is highly enriched in somal and axonal TRAP samples and was identified as the lead candidate gene of the novel rs55961674 locus. Recently the CaSR has been found to interact with tropomyosin receptor kinase B (TrkB) to mediate neurite growth through glycogen synthase kinase phosphorylation, supporting a role for this receptor in the brain and in axonal biology (Markworth et al. 2019).

Modulators of CaSR function, including therapeutics used for patients with secondary hyperparathyroidism are available that could be used to test for the involvement of this receptor in calcium homeostasis in dopaminergic neurons (Riccardi and Valenti 2016).

Copine VII (*Cpne7*)

Copines are calcium-dependent phospholipid binding proteins that contain two C2 domains, also found in protein kinase C, phospholipase C and several synaptotagmins (Rizo and Südhof 1998; Nalefski and Falke 1996; Perestenko et al. 2010). They also contain a von Willebrand factor A domain, which modulates their calcium sensitivity and intracellular targeting. Copine-7 is preferentially localised to the nucleus in human embryonic kidney cell line 293 cells and rapidly translocates

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to the nucleus and plasma membrane in response to ionomycin or methacholine-application (Perestenko et al. 2010).

Copine-7 induces odontoblastic differentiation from mesenchymal stem cells by binding to nucleolin and translocating to the nucleus (Oh et al. 2015). Odontoblastic cells are post-mitotic and accumulate lipofuscin with age. Copine-7 induces autophagic activity in mature odontoblasts, promoting clearance of lipofuscin (Park et al. 2021). Furthermore, an increase in the expression of TAU and NESTIN proteins was observed as Copine-7 induced process elongation in odontoblasts. Based on these findings, it could be speculated that Copine-7 plays an important role in autophagy and axonal/dendritic extension in the dopaminergic neuron. Cell-penetrating Copine-7-derived peptides have been developed for the purposes of stimulating odontoblast function (Lee, Park, et al. 2019). These peptides could be used to study the role of Copine-7 in dopaminergic neurons.

Nucleolin, which binds Copine-7 is also enriched in somal TRAP samples and interacts with α -synuclein and DJ-1 (Jin et al. 2007). The expression of nucleolin has been found to be reduced in human PD brain tissue and *in vitro* SiRNA targeting of nucleolin in a rotenone PD model resulted in additional generation of oxidative stress and proteasomal inhibition (Caudle et al. 2009).

Copine-7 has also been shown to regulate rapid eye movement sleep (REMS) in mice, however the authors did not report any movement related findings that could be of relevance to REM sleep behaviour disorder and PD.

The highly specific expression of *Cpne7*, combined with multiple lines of evidence for it playing an important role in mechanisms known to be of importance in dopaminergic neurons (e.g. autophagy), indicates that it may be a worthwhile candidate for further investigation.

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Transgelin-3 (*Tagln3*)

Transgelin-3 is an actin-binding protein that is robustly enriched in somal TRAP samples. It shares amino acid sequence homology with Transgelin-1, Transgelin-2 (Lees-Miller et al. (1987)) and Calponin-1 to 3 (Takahashi and Nadal-Ginard (1991)). These family members were all depleted in somal TRAP samples, indicating a specific requirement for Transgelin-3. Allen Brain Atlas data confirmed an expression pattern resembling the substantia nigra parts and ventral tegmental area (Figure 4.4 A). *Tagln3* expression increases at defined time points in rat brain development, with greatest expression during synaptogenesis (Depaz and Wilce 2006). It is believed to be important for maintenance of neuronal morphology in the adult, with a homogenous distribution inside the cell. Several interacting proteins were identified, most of which were also enriched in somal TRAP samples. Of the interactors, *FKBP1B* and *FABP5* have a reported role in neuronal ageing and Parkinson's disease respectively: *FKBP1B* is a ryanodine receptor-binding protein that when disrupted causes an ageing phenotype in hippocampal neurons (Gant et al. 2011). Co-overexpression of *FABP5* and *a-synuclein* in Neuro-2A cells resulted in reduced viability upon exposure to rotenone (Wang, Shinoda, et al. 2021). The interaction of *TAGLN3* in these mechanisms has not yet been explored.

Synaptotagmin 17 (*Syt17*)

Synaptotagmin 17 supports neurite outgrowth and axonal regrowth after injury in hippocampal neurons (Ruhl et al. 2019). It was found to be localised to the Golgi complex and to not play a role in exocytosis, unlike other more commonly studied synaptotagmins. Loss of *Syt17* in hippocampal neurons resulted in disrupted endocytic trafficking and overexpression of AMPA receptors postsynaptically, impairing synaptic plasticity.

No experimental interaction evidence for *Syt17* was available in the STRING database. Putative interactions between *Syt17* and *Cplx1*, *Snap25*, and *Stx1a* (all

4. *Specific Expression Features in Dopaminergic Neurons*

enriched in somal TRAP samples) were predicted on the basis of *Syt1* interaction, however these are to be interpreted with caution given the evidence that Syt17 does not have an exocytotic function.

Two variants, rs11343 and rs6497339 are located in a gene body intron and 3' UTR of Syt17 and are significant risk loci for PD (Chang et al. 2017; Nalls, Blauwendraat, et al. 2019). Even after including surrounding genes in linkage regions of these variants, Syt17 was still the lead selected gene.

Family With Sequence Similarity 210 Member B (Fam210b) and Nuclear receptor coactivator 4

Fam210b is generally understudied, with only three publications associated in the NCBI Gene database at the time of writing (Kondo, Fujiwara, et al. 2016; Sun et al. 2017; Yien et al. 2018). It is localised to the inner mitochondrial membrane in maturing erythroblasts, where it facilitates mitochondrial iron uptake for heme, iron-sulfur cluster and haemoglobin synthesis and also activates ferrochelatase (FECH) activity.

FAM210B shares a GATA-1/GATA-2 transcription factor binding site with *SNCA* and *FECH* (Kondo, Fujiwara, et al. 2016; Scherzer et al. 2008). GATA-2 has been reported to be expressed in human substantia nigra dopaminergic neurons, however in somal TRAP samples it is depleted: *Gata1* is strongly enriched, however. The interaction between FAM210B and α -synuclein has not been studied beyond mRNA co-expression, but it may be very important for iron handling in dopaminergic neurons, due to the specificity of expression.

Nuclear receptor coactivator 4, *Ncoa4*, is very specifically enriched in the dopaminergic axon. It mediates selective autophagy of ferritin (ferritinophagy). Disruption in ferritinophagy leads to systemic iron homeostasis deficits, affecting oxidative stress regulation and erythropoiesis. α -synuclein has been found to impair ferritinophagy,

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not by direct interaction with NCOA4, but through disrupting trafficking of lysosomal hydrolases (Baksi and Singh 2017).

These findings, together with age and *SNCA*-OVX-induced changes in the next chapter point to iron homeostasis as being specifically important in dopaminergic function.

Engrailed-1 (in the axon)

The axonal translation of *En1*, a transcription factor important for dopaminergic neuron survival may be an example of retrograde signalling to the nucleus to drive transcriptional change in response to local cues (Ji and Jaffrey 2013). In addition to binding DNA however, *En1* and *En2* (also enriched in somal TRAP samples) are also secreted and internalised between cells, regulating local translation to guide axonal growth cone development (Brunet, Di Nardo, et al. 2007; Stettler et al. 2012; Brunet, Weinl, et al. 2005). This could indicate an unexplored role for Engrailed-1/2 in dopaminergic axonal function.

4.3.2 GWAS Prioritisation

Of the loci analysed in this chapter, only 25 % of the lead candidate genes selected by Nalls, Blauwendraat, et al. (2019) showed evidence of dopaminergic expression in TRAP data. This explains why only 20 % of selected lead candidates were in common between their approaches (nearest gene selection and QTL analysis) and TRAP: Cell type-specificity information is given priority and genes not enriched in either somal or axonal samples were not considered at all. Using cell-type specificity, genes such as *SNCA* and *CPLX1* were confirmed. On the other hand, genes that were not abundantly expressed in dopaminergic neurons, such as *LRRK2* were missed.

A refinement to this analysis could be to threshold the magnitude of enrichment: If a locus only contained genes enriched below a set value (e.g. 2-fold) then no gene

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would be considered a lead and additional information would need to be sought. Another improvement would be to use public cell type-specific transcriptomic data from other cell types to assess whether the number of enriched candidate genes is lower than dopaminergic neurons. Other cell type-data could be used to ask whether loci and candidate genes that were discarded due to lack of dopaminergic enrichment were enriched within that cell type. Other TRAP or affinity purification-based datasets of different cell types present within the midbrain could be used to calculate a specificity index of expression for each gene, to further select the most specific genes to dopaminergic neurons (Dougherty, Schmidt, et al. 2010).

Compartmental and Condition-Dependent Expression in Dopaminergic Neurons

5.1 Introduction

5.1.1 Overview

This chapter describes the effects of cell compartment, ageing and over-expression of *a*-synuclein on gene abundance in TRAP samples.

The functional activity of dopaminergic neurons can be estimated by identifying expression changes that occur with ageing, disease or between subcellular compartments. The sensitivity and accuracy of detection depends upon technical factors, however, such as library composition.

To identify genes and transcripts that were differentially localised or expressed between conditions, TRAP samples from each cohort were compared. Differences in library composition due to experimental factors, healthy ageing and the interaction of ageing and *a*-synuclein overexpression needed to be accounted for and the steps taken for each comparison are described at the start of this chapter. In addition, the likelihood of axonal measurements deriving from dopaminergic

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neurons was considered for each gene, using enrichment information obtained from the previous chapter.

To prioritise gene expression changes most specific to dopaminergic neurons, matched TOTAL samples were compared in parallel. The results of TRAP comparisons were flagged if the same result could be detected at a tissue-level.

Greater expression of a gene or transcript was interpreted to reflect greater demand. Functional descriptions were based on this assumption. Analysis was not restricted to the set of genes enriched by TRAP in either the soma or axon, however results were subsequently filtered by these criteria.

Unless specified explicitly, all analyses in this chapter were conducted using all samples from both ages (3 and 18 months) and genotypes (Wild-type and *SNCA*-OVX). All analyses were divided according to cell compartment, however (somal and axonal samples were analysed separately).

5.1.2 Chapter aims

- To identify dopaminergic genes and transcripts most specifically localised to the axonal compartment of dopaminergic neurons.
- To identify genes more specifically expressed in dorsal striatal dopaminergic axons compared to central striatal axons.
- To describe the changes that occur with age in both cell compartments.
- To describe the changes that occur due to overexpression of human *a*-synuclein and how this interacts with ageing.

5.2 Results

5.2.1 Interpretation of Expression Measurements from Axonal TRAP Samples

In this chapter, comparisons were made between neuronal compartments, age groups and disease genotypes. For comparisons between somal TRAP samples, the interpretation of results was straightforward: Over 80 % of somal TRAP libraries were represented by genes enriched by the TRAP protocol in this brain region (Figure 5.1 A). These samples were exclusively enriched in dopaminergic cell markers. Results were therefore filtered to retain only those genes that were enriched or exhibited DTU and were therefore considered translated by the dopaminergic neuron.

Interpretation of results from comparisons involving axonal TRAP samples required extra consideration. While ~ 77 % of each axonal TRAP library was represented by genes enriched by TRAP, markers of other striatal cell types were enriched and therefore included in this category. The proportion of library originating from dopaminergic neurons was not known.

To further characterise the dopaminergic specificity of axonal TRAP, genes were categorised based on compartmental enrichment:

- Genes exclusively enriched in somal TRAP samples accounted for ~ 39 % of each somal library. In axonal samples, however, these genes accounted for ~ 15 % of each library. This category accounted for the largest number of genes (Figure 5.1).
- Genes exclusively enriched in axonal TRAP samples accounted for ~ 22 % of each axonal library and 8 % of somal libraries. With a small number of exceptions, measurements of these genes were not considered to originate from dopaminergic neurons.

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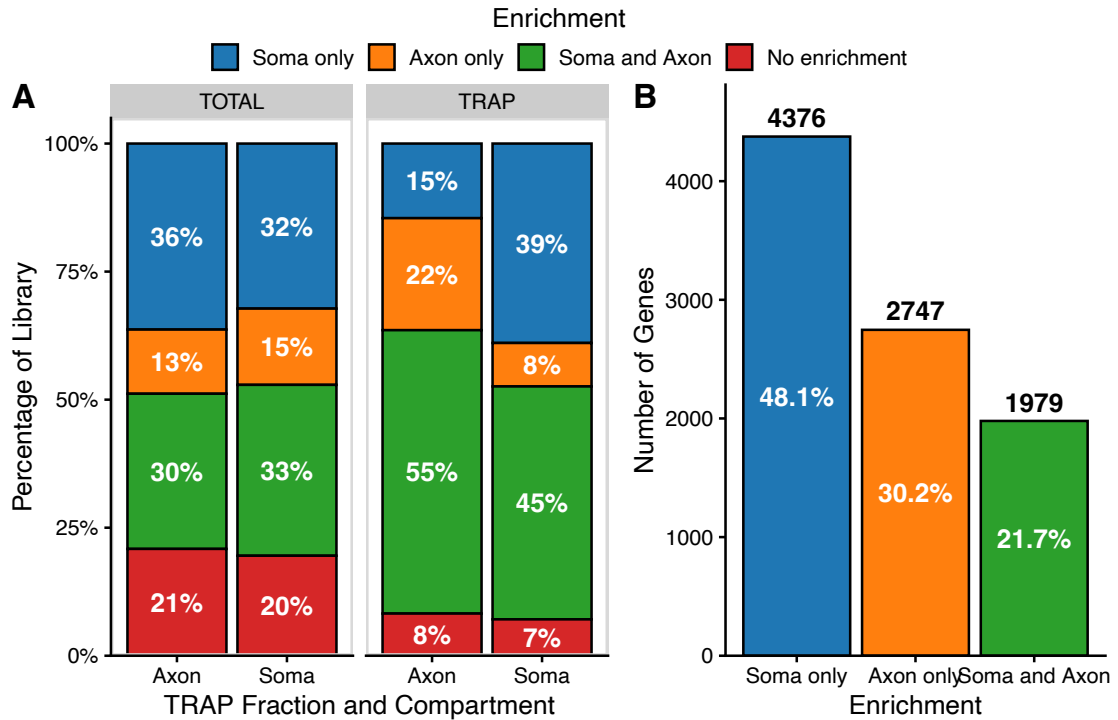


Figure 5.1: Library composition of somal and axonal TRAP samples by enrichment category. **A:** The percentage of somal and axonal libraries represented by genes of each category of enrichment. Somal TRAP samples contain a greater percentage of reads corresponding to enriched genes and axonal TRAP samples contain a larger percentage of reads that correspond to genes exclusively enriched in axonal TRAP samples. **B:** The number of genes within each enrichment category. Exclusively soma-enriched genes represent the largest number of genes, however they are the lowest represented enriched group in axonal TRAP libraries.

- ~ 45 % of each somal TRAP library was represented by genes enriched in both compartments. In axonal samples, this subset accounted for over half of each library. This was the smallest subset of enriched genes, however.

Jointly enriched genes were initially presumed to represent the most reliable subset for expression comparisons involving axonal TRAP samples. The smaller number of genes within this subset could reflect reduced diversity of gene expression in the axon: In an *in vitro* transcriptomic study of motor neuron gene expression, axons were found to express a subset of somal transcripts (Nijssen et al. 2018). The abundance of jointly enriched genes was greater than “Soma-only” genes in both somal and axonal TRAP samples.

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Measurements from this subset of genes were found to not be purely dopaminergic in origin, however. Analysis of gene abundance in aged axonal TRAP samples revealed a split expression pattern.

In a comparison between aged and young axonal TRAP samples (described in further detail later in this chapter), 406 genes were differentially abundant. The genes were labelled by enrichment group which illustrated a divided expression pattern (Figure 5.2). The subset of genes exclusively enriched in axons increased in abundance, while exclusively soma-enriched genes decreased. It was assumed that most of the observed differences in abundance were not true changes in expression, but instead reflected changing cell type proportions due to age. The changes could be due to a reduction in dopaminergic neuron number, axonal density or RNA content with age. An increase in relative cell marker abundance of astrocytes and oligodendrocytes supported this possibility (Figure 5.2 C).

Jointly enriched genes in aged axonal TRAP samples did not follow the same pattern of *en masse* down-regulation as “Soma only” genes. The majority of genes were in fact more abundant. This was unexpected, as it indicated shared expression of these genes between dopaminergic neurons and other cell types of the striatum. A subset of these genes could represent upregulated expression from remaining dopaminergic axons, however this cannot be distinguished without confirmation by an alternative method, such as fluorescence *in situ* hybridisation (FISH).

Genes in each enrichment category (including exclusively axon-enriched genes) that were less abundant in aged axonal TRAP samples were classed as putatively dopaminergic. For every comparison in this chapter, exclusively soma-enriched genes were prioritised from results, followed by jointly enriched, exclusively axon-enriched and genes with no evidence of enrichment.

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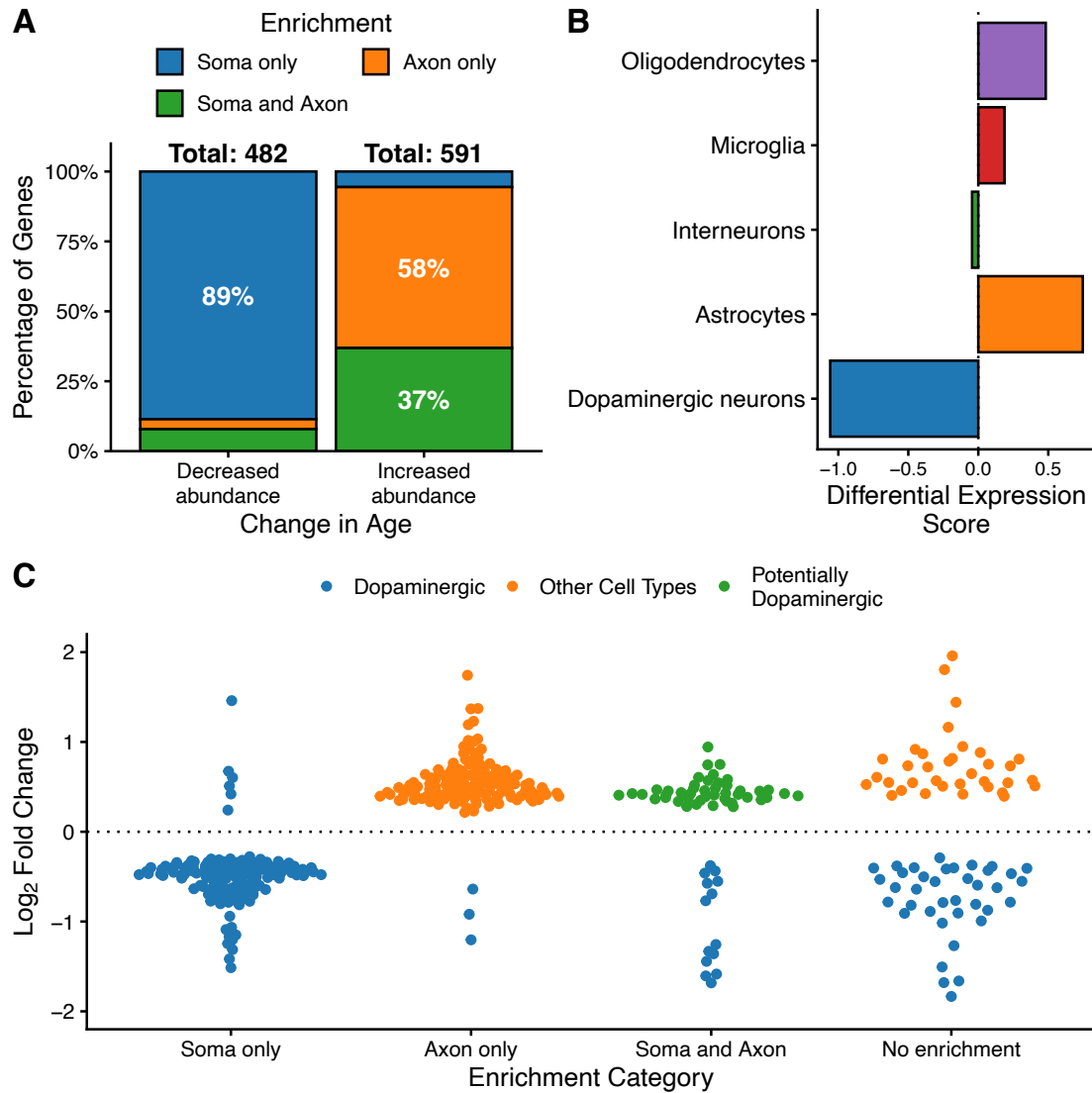


Figure 5.2: Library composition changes in aged axonal TRAP samples. **A:** A breakdown of differentially abundant genes in aged axonal TRAP samples by enrichment category. The majority of exclusively soma-enriched genes were less abundant, while levels of genes from other enrichment categories were elevated. **B:** The shift in cell type marker abundance in aged axonal TRAP samples, reflecting a reduction in dopaminergic proportion, countered with an increase in astrocyte and oligodendrocyte representation. **C:** Genes with lower abundance in aged axonal TRAP samples were labelled as putatively dopaminergic for further comparisons.

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5.2.2 Library Composition Across Cohorts in Aged Somal TRAP Samples

The expression of dopaminergic markers (*Th*, *Slc6a3*, *Slc18a2*) and non-dopaminergic markers (*Gfap*, *S100b*, *Gad2*) was compared between age groups of somal TRAP samples from each cohort (Figure 5.3 A). The abundance of dopaminergic markers was greater in aged samples from cohort 1: The purity of these samples was greater than matched young samples, likely due to use of different reagents. The abundance of non-dopaminergic markers was reduced. These differences appeared subtle, however a gene expression comparison between ages in this cohort resulted in the detection of thousands of genes as differentially abundant: Those that were more abundant in old samples were also found to be enriched by TRAP (dopaminergic transcripts), while those that were less abundant were depleted (non-dopaminergic transcripts). This effect is summarised in Figure 5.3 B: The percentage of library occupied by genes enriched by TRAP increased with age.

In cohorts 2 and 3, the dopaminergic proportion was reduced in aged samples. This was a milder change than in aged axonal TRAP samples. Young and aged samples were collected together using the same reagents, so it is likely that the reduced dopaminergic proportion was due to differences in dopaminergic neuron abundance, dopaminergic RNA content, dopaminergic eGFP-L10a expression or tissue lysate properties affecting the capture of eGFP-L10a ribosomes.

By combining cohorts, the effects of library composition variation could be minimised, preserving changes in gene abundance that were consistent across datasets. In this light, the artefactual increase in dopaminergic proportion in cohort 1 helped to screen and filter changes in gene abundance in cohorts 2 and 3 that were not true changes in expression.

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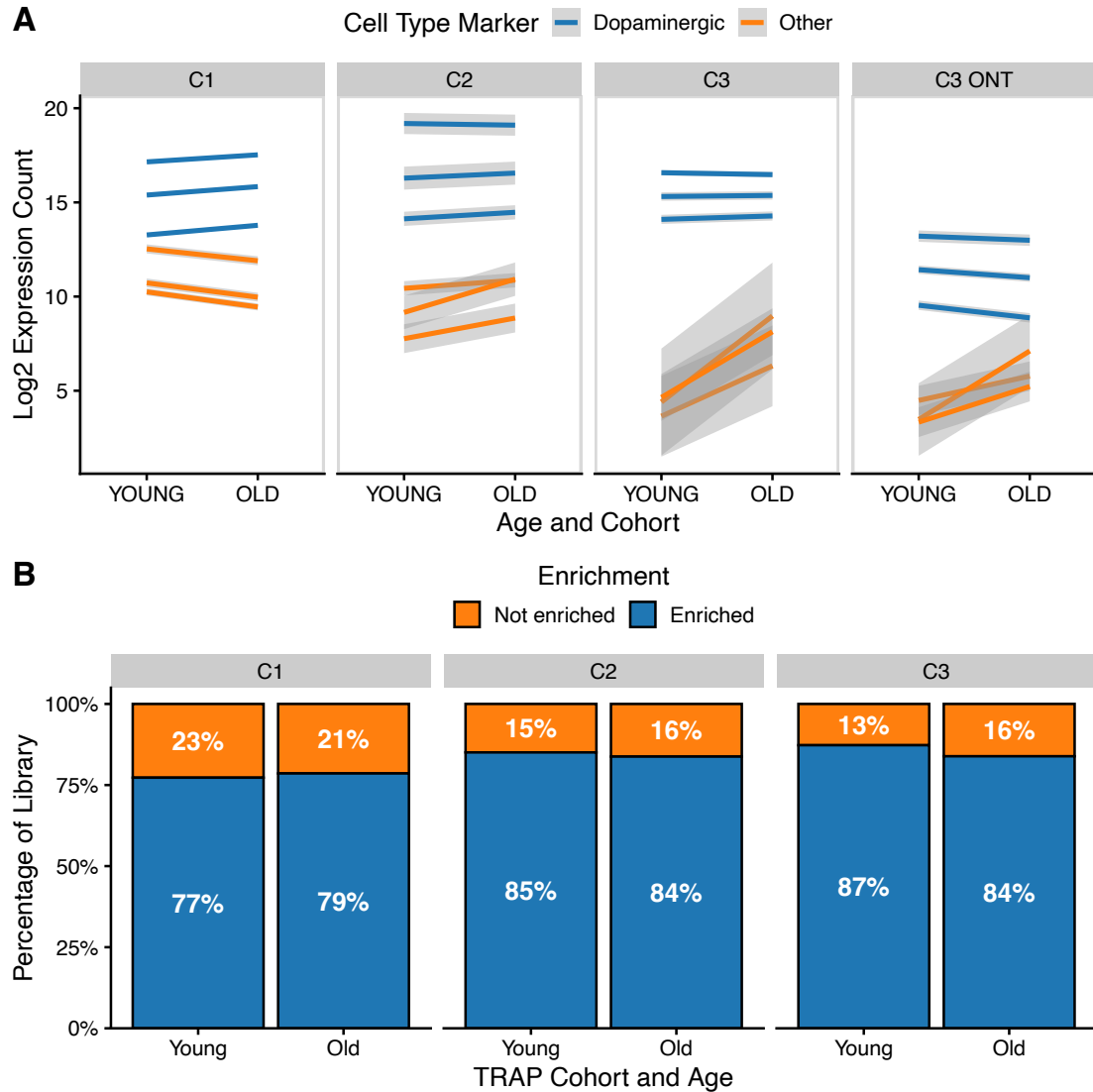


Figure 5.3: Library composition changes in aged somal TRAP samples. A: The abundance of dopaminergic markers (*Th*, *Slc6a3*, *Slc18a2*) and markers of other cell types (*Gfap*, *S100b*, *Gad2*). In cohort 1, dopaminergic marker abundance increase in aged samples, while other cell type markers decrease. The opposite effect is seen in cohorts 2 and 3. **B:** The percentage of library represented by enriched genes is different between ages in each cohort. Increased dopaminergic content in aged samples from cohort 1 is contrasted with decreased content in cohorts 2 and 3.

5.2.3 Local Translation: The Axon and Cell Body

Gene-level differences between the Axon and Cell Body

Based on earlier PCA and library composition analyses (Figure 3.15 and Figure 5.1), axonal and somal TRAP samples were known to differ at the gene-level. PCA showed clear separation by cell compartment along the first two principal components of variation in each cohort (Figure 3.15). In addition, the library composition of axonal TRAP samples was different: Canonical markers of dopaminergic neurons (e.g. *Th*, *Slc6a3*, *Slc18a2*) were much more abundant in somal TRAP samples, whilst other genes, such as *Atg7* and *Ubr3* were found to be extremely highly expressed in axonal TRAP samples. Axon-localised proteomics have recently been performed in mouse dopaminergic neurons *in vivo*: *Atg7* was found to be in significantly greater abundance compared to the cell body, confirming this finding from TRAP data.

Differences in abundance between the cell body and axon were only interpreted when a gene or feature was more expressed in the axon. It was not possible to identify soma-exclusive features, because the dopaminergic proportion in somal TRAP samples was greater, confounding this analysis.

All detectable genes in common between datasets from each cohort were analysed to provide the largest number of genes for normalisation. Results were filtered to include genes that were enriched in somal TRAP samples alone or jointly with axonal TRAP samples.

10806 genes were differentially abundant between somal and axonal TRAP samples, of which 4502 were retained based on enrichment criteria. This number was much larger than expected. The majority of genes were in greater abundance in the soma; this was due to filtering results for genes that are significantly enriched in this region (Figure 5.4 A).

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946 genes were more abundant in axonal TRAP samples, of which 742 were retained after removing results also observed in matched TOTAL samples. In a subset of cases, gene expression patterns opposed between TRAP and TOTAL samples, providing stronger evidence of cell type-specific expression. Among the genes most significantly more abundant in the axon were Autophagy-related 7 (*Atg7*) (Figure 5.4 B), Prosaposin (*Psap*), Kinesin-like Protein 3A (*Kif3a*), Doublecortin-like Kinase 1 (*Dclk1*), Heterogeneous Nuclear Ribonucleoprotein A2/B1 (*Hnrnpa2b1*) and FERM Domain Containing Kindlin 2 (*Fermt2*). Each gene is associated with multiple lines of evidence supporting a role in axonal and/or dopaminergic function and examples are discussed at the end of this chapter.

Encouragingly, gene set enrichment analysis confirmed a significant axonal enrichment of genes required for organisation of neuronal projections ([G0:0106027](#), BH $P = 7.82\text{e-}3$). Genes at the leading edge of this result were *Camk2b*, *Kalrn*, *Epha4*, *Lzts3*, *Dhx36*, *Homer1*, *Sipa1l1*, *Actr2*, *Dnm1l*, *Lgmn* and *Arhgap33*.

Transcript-level differences between the Axon and Cell Body

Alternative splicing (including alternative polyadenylation) is particularly important in the study of axonal gene expression, as the inclusion or exclusion of sequence motifs can guide transcript localisation and translation among many other functions (Mayr 2018). Transcript-level abundance estimates were compared between axonal and somal TRAP samples to identify instances of differential transcript usage (DTU). 25 % of genes were filtered due to expression of only a single transcript or due to insufficient read coverage of each non-dominant transcript (Figure 5.5 A). DTU was detected in 1925 genes out of 7628 that were retained. The equivalent comparison was run using TOTAL samples, to distinguish dopaminergic-specific alternative splicing events and each gene was categorised by evidence of axonal translation (Figure 5.5 B). Due to obtaining very small amounts of TRAP RNA from axonal samples, this DTU analysis was performed using short-read data. It

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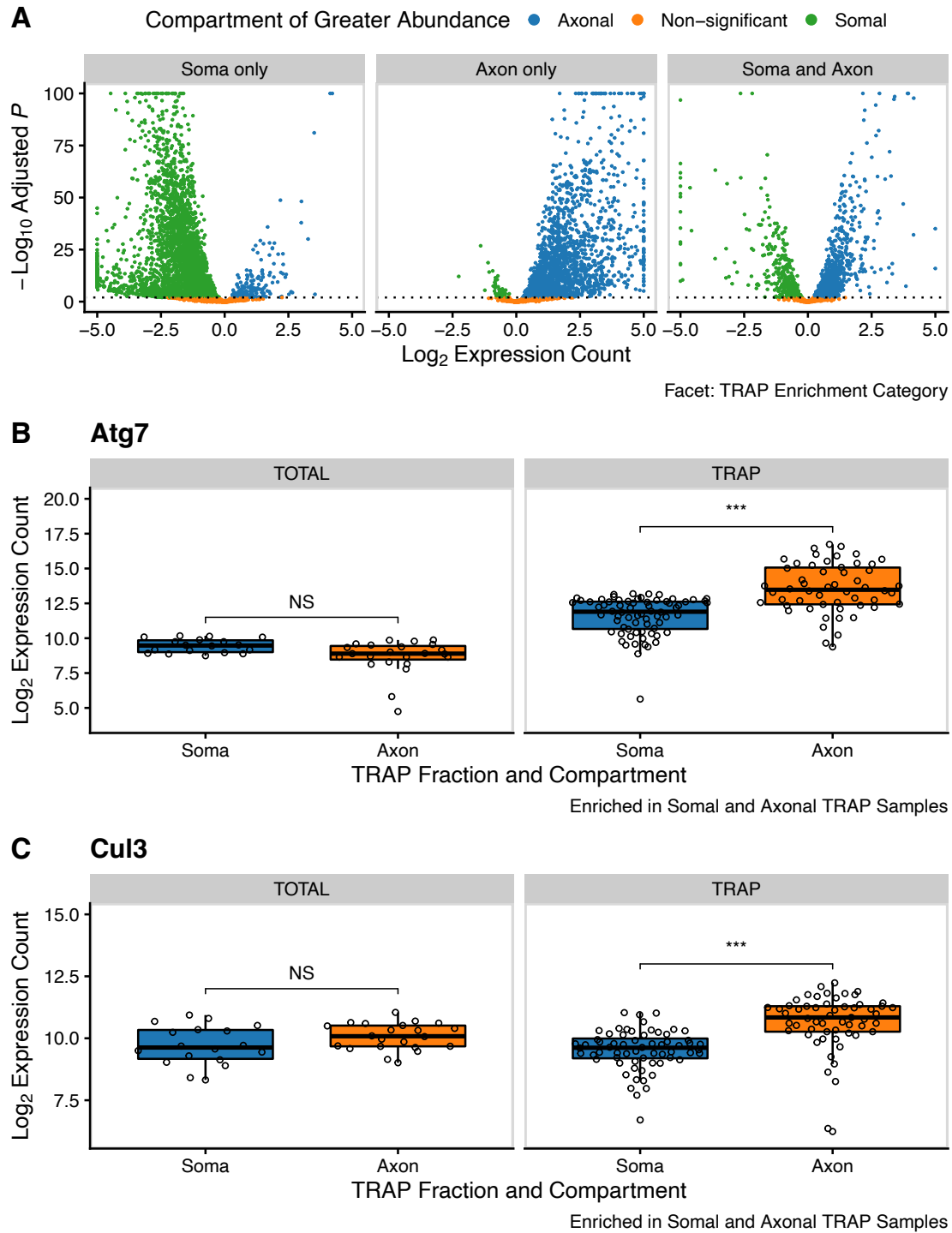


Figure 5.4: Compartmental Gene Expression: Axon vs Soma. **A:** Volcano plots to demonstrate the dependency of compartmental abundance on TRAP enrichment category. Genes within the “Soma and Axon” enriched category were prioritised and “Axon only” genes were filtered from results. **B:** *Atg7* expression in TRAP and TOTAL samples. *Atg7* is more abundant in the axonal compartment. This difference is specific to dopaminergic neurons. **C:** *Cul3* expression in TRAP and TOTAL samples. *Cul3* is also more abundant in the axonal compartment, while whole striatal expression of *Cul3* does not differ in comparison to the midbrain compartment. P values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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is likely, therefore that the full extent of splicing diversity between the cell body and axon is greater than presented.

Examples of differential transcript usage of *Rabgap1l* and *Oxr1* are presented in Figure 5.5 C and D. Their structure and potential roles in dopaminergic neuronal biology are discussed at the end of this chapter.

Gene-level differences between Axons of the Dorsal and Ventral Striatum

Axonal TRAP samples were collected from dissected dorsal and ventral striatum separately to compare expression: Dorsal striatal tissue is innervated by axons originating from A9 dopaminergic neurons of the SNpc whereas ventral striatal tissue receives input from A10 dopaminergic neurons of the VTA. Comparing expression between these two populations provides a route to identifying differences in their activity or requirements that may confer susceptibility to degeneration.

1671 genes were differentially abundant between the two regions (Figure 5.6 A). The distribution of enrichment categories differed by the direction of change: Genes that were more abundant in ventral striatal samples were enriched in somal samples, while the opposite was true for more dorsally abundant genes. eGFP-L10a expression was found to be higher in ventral striatal tissue (Figure 3.2 A) and the estimated dopaminergic proportion in ventral axonal TRAP samples from deconvolution was also subtly larger (Figure 4.7). It was noted therefore that the difference in abundance may be partially due to library purity. Results of this analysis were filtered to include only genes enriched in somal TRAP samples that were more abundant in dorsal striatal TRAP samples.

232 genes were retained and their expression was compared with matched TOTAL samples to identify dopaminergic-specific changes. The majority of genes shared the same expression pattern with TOTAL samples (Figure 5.6 B). This was not expected, as it was estimated that the dopaminergic proportion of striatal TOTAL RNA was very small and therefore that dopaminergic-specific changes would not

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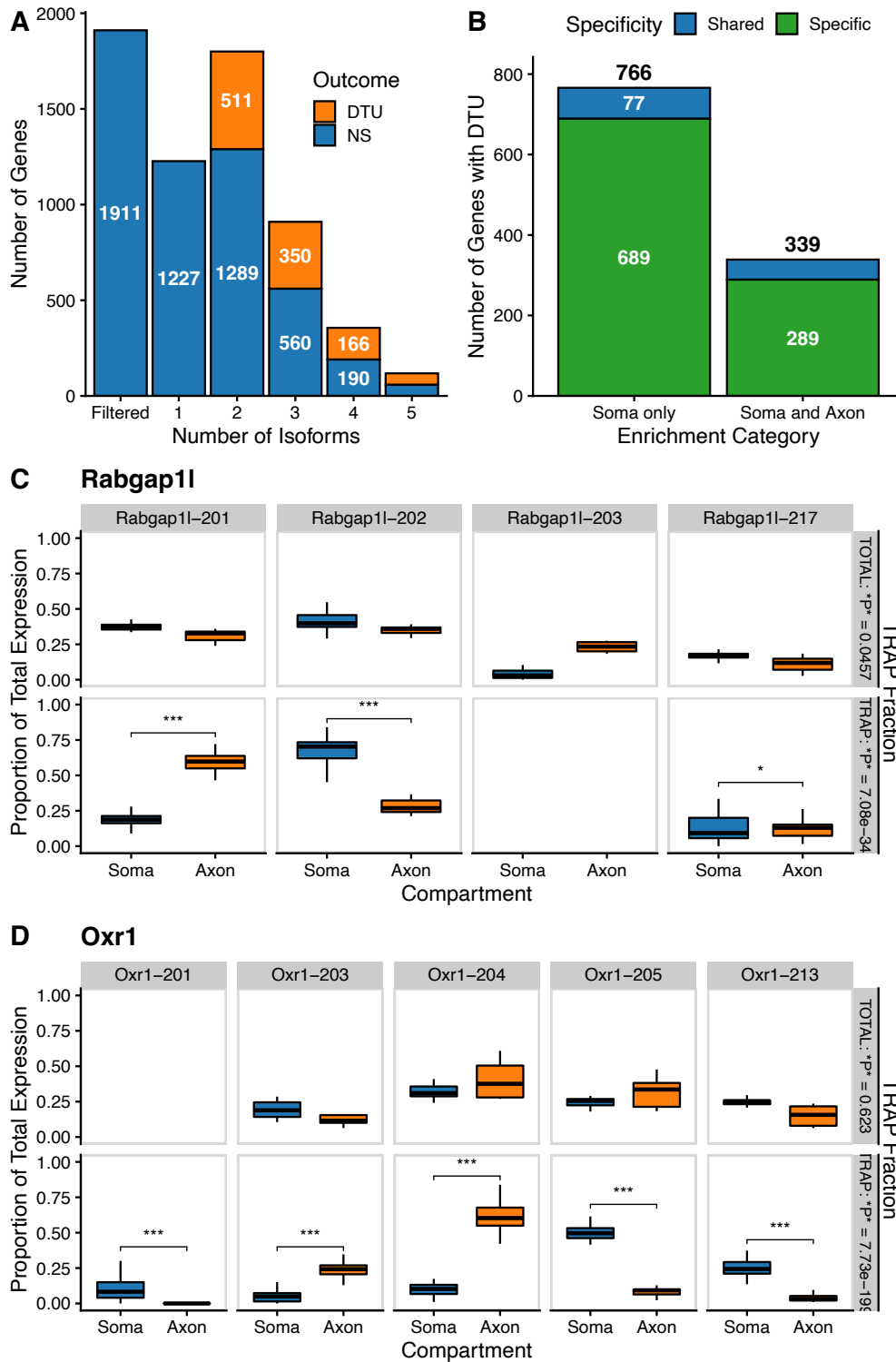


Figure 5.5: Compartmental gene expression: Transcript usage between the soma and axon. **A:** The number of genes filtered and retained, coloured by significance. (NS = Not significant). **B:** Most DTU events were not detected in the equivalent comparison using TOTAL samples. **C and D:** DTU of functional, long (axonal) and short (somal) isoforms of *Rabgap1l* and *Oxr1*; two genes with an established role in neuronal function and neurodegeneration. Blank panels represent isoforms filtered from comparison due to low abundance. Gene-level P values are shown aside TRAP fraction labels. Transcript-level P values were calculated using *stageR* (Berge 2017), applying the Benjamini & Hochberg procedure for post-hoc correction at an alpha of 0.05.

5. Compartmental and Conditional Expression

be observed at the tissue level. It is possible that shared changes reflect cell type-independent activity. Alternatively (and not exclusively) these changes may specifically derive from another cell type that is more abundant in the striatum and expresses DAT/Cre/eGFP-L10a.

Many retained genes have prior support for dopaminergic and/or axonal expression, such as *Micu1*, *Oxr1* (also found to be alternatively spliced in the axon) and subunits of the nicotinic acetylcholine receptor (Figure 5.6 C): *Chrna5* and *Chrn3*; two subunits of the nicotonic acetylcholine receptor are within this filtered set of genes, for example (Wen et al. 2016). *Chrn3* expression has been reported to concentrate in the rostral SNpc and caudolateral VTA in midbrain tissue, which would support the finding that it is more abundant in dorsally projecting axons in this dataset (Chakrabarty et al. 2012).

The relevance of these genes, including others that may share an expression pattern with TOTAL samples is discussed at the end of this chapter. Through guilt by association, genes within this more dorsally abundant set may represent important yet understudied components that distinguish this group of neurons from their more resilient ventral neighbours.

5.2.4 Ageing

Ageing in the Cell Body

Somal TRAP samples were compared between aged (18 - 22 months old) and young mice (3 - 5 months old). 667 genes were differentially expressed in aged mice. A breakdown of the enrichment category of these genes is shown in Figure 5.7 A. The majority of differentially expressed genes that were enriched were upregulated. Dopaminergic and other cell type markers were not significantly changed, indicating that library composition differences were not driving these changes (Figure 5.2.2). Soma-enriched genes were retained and compared with TOTAL samples. Most gene expression changes were TRAP-specific, as shown in

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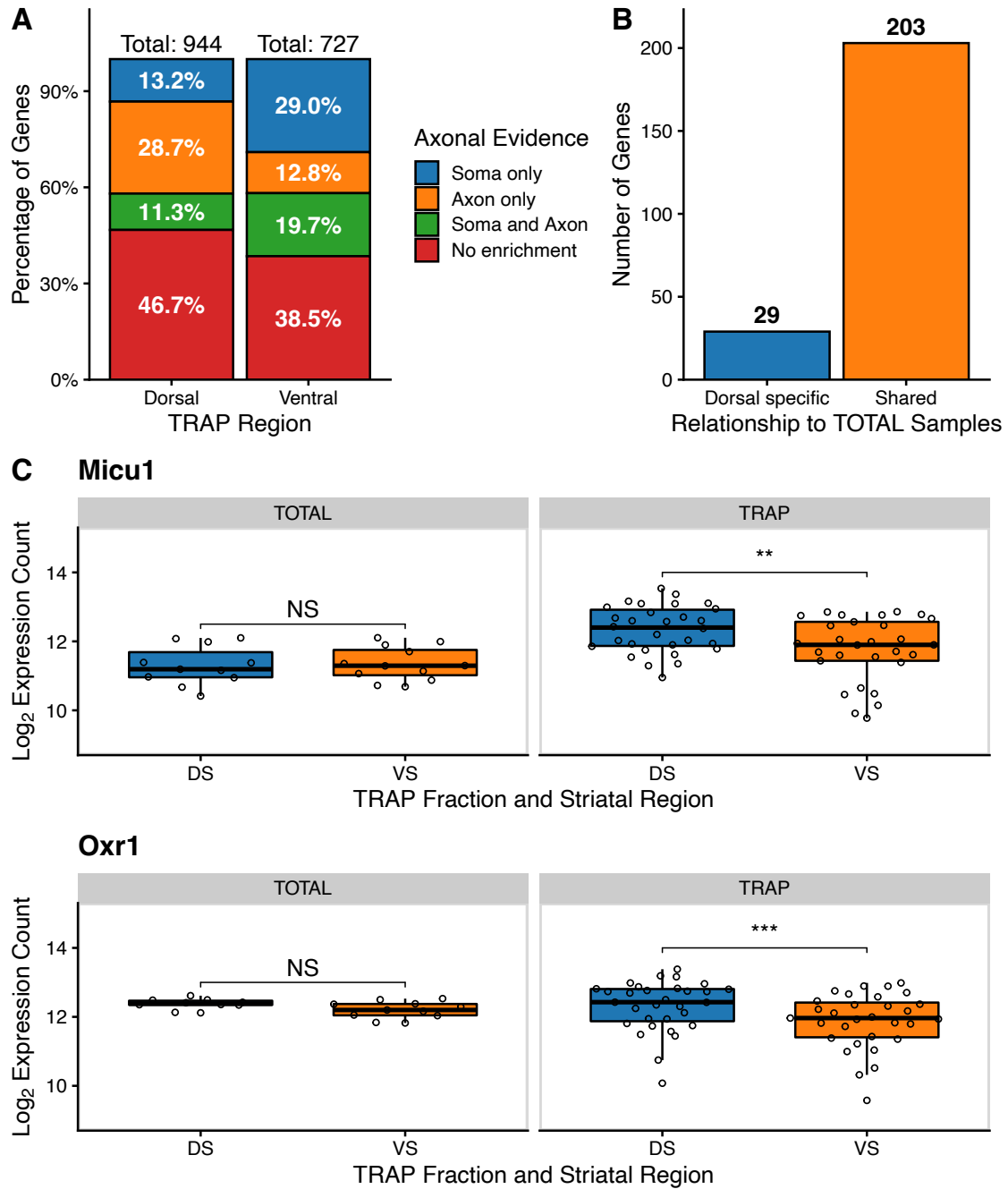


Figure 5.6: Regional expression differences in striatum. **A:** A breakdown of the axonal enrichment categories for differentially abundant genes. Ventrally abundant genes are associated with stronger evidence of axonal translation. This difference in confidence between regions could indicate a difference in library composition. **B:** The specificity of differential abundance in striatum. Genes that were not enriched by somal TRAP (lower confidence of axonal translation) are not shown. **C:** *Micu1* and *Oxr1* expression is greater in dorsal axonal TRAP samples and this difference is specific to TRAP samples. *P* values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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Figure 5.7 B and C. A small number of cases of opposing expression were detected, such as upregulation of the Ca^{2+} binding protein Secretagranin 2 (*Scg2*) while tissue-level expression is reduced.

To determine pathway-level changes that occur with age in dopaminergic neurons, the STRING database was used to extract high confidence (confidence > 0.8) interactions between retained genes. A confidence score of 0.8 means that approximately 20 % of labelled interactions will be a false positive. Around one third of genes were not associated with evidence for functional interaction (Figure 5.7 C). Local communities in the resulting network were identified using a fast greedy modularisation algorithm (Figure 5.7 D), which resulted in the formation of 13 clusters of related genes. The expression of each gene in young and old somal TRAP samples from cohort 1 is presented in Figure 5.7 F. Genes listed had the highest degree within each cluster (termed “hub genes”). The genes within each cluster, ranked by degree are shown in Table 5.1.

Clustered genes have well documented roles in lysosomal function, autophagy, clathrin mediated endocytosis, regulation of oxidative stress, Ca^{2+} mediated exocytosis, intracellular transport and iron handling. Most genes are up-regulated, which may indicate an increased requirement for these activities. This increased requirement may be due to greater demand for a function (e.g. acidification of lysosomes) or it could represent a growing inefficiency in transcription, transcript handling or translation. Interestingly, certain genes with shared roles among clusters were downregulated, such as Beclin 1, a key regulator of autophagy. The roles for a subset of age-dependent genes are discussed at the end of this chapter.

Table 5.1: Ageing gene expression changes in somal TRAP samples. Genes are divided into clusters based on evidence of protein interaction. The most densely interacting gene is highlighted in bold in each cluster.

Gene	Description	Aged expression
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Cluster 1

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Atp6v0c	ATPase, H+ transporting, lysosomal V0 subunit C	Up
Anxa1	Annexin a1	Up
Atp6ap2	ATPase, H+ transporting, lysosomal accessory protein 2	Up
Gaa	Glucosidase, alpha, acid	Up
Atp6v0d1	ATPase, H+ transporting, lysosomal V0 subunit D1	Up
Gnaq	Guanine nucleotide binding protein, alpha q polypeptide	Up
Atp6ap1	ATPase, H+ transporting, lysosomal accessory protein 1	Up
Psap	Prosaposin	Up
Atp6v1e1	ATPase, H+ transporting, lysosomal V1 subunit E1	Up
Ntsr1	Neurotensin receptor 1	Up
Cd47	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	Up
Plcb4	Phospholipase c, beta 4	Up
Oprk1	Opioid receptor, kappa 1	Up
Cluster 2		
Ap2m1	Adaptor-related protein complex 2, mu 1 subunit	Down
Ap2a2	Adaptor-related protein complex 2, alpha 2 subunit	Up
Wasl	Wiskott-Aldrich syndrome-like (human)	Up
Dnajc6	DnaJ heat shock protein family (Hsp40) member C6	Up
Picalm	Phosphatidylinositol binding clathrin assembly protein	Up
Synj1	Synaptojanin 1	Up
Ap2b1	Adaptor-related protein complex 2, beta 1 subunit	Up
Amph	Amphiphysin	Up
Snap91	Synaptosomal-associated protein 91	Up
Pacsin1	Protein kinase c and casein kinase substrate in neurons 1	Up
Ap1b1	Adaptor protein complex ap-1, beta 1 subunit	Up
L1cam	L1 cell adhesion molecule	Up
Syng	Synergism, gamma	Up
Pi4ka	Phosphatidylinositol 4-kinase alpha	Up
Cntn1	Contactin 1	Up
Cluster 3		
Hsp90aa1	Heat shock protein 90, alpha (cytosolic), class a member 1	Up
Pafah1b1	Platelet-activating factor acetylhydrolase, isoform 1b, subunit 1	Up
Ntn1	Netrin 1	Up
Canx	Calnexin	Up
Ago2	Argonaute 2 catalytic subunit 2	Up
Elmo1	Engulfment and cell motility 1	Down
Ahl1	Abelson helper integration site 1	Up
Tsn	Translin	Up
Ganab	Alpha glucosidase 2 alpha neutral subunit	Up
Becn1	Beclin 1, autophagy related	Down
Hsp90ab1	Heat shock protein 90 alpha (cytosolic), class b member 1	Up
Ndel1	Nude neurodevelopment protein 1 like 1	Down

Cluster 4

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Sos1	SOS Ras/Rac guanine nucleotide exchange factor 1	Up
Rhof	Ras homolog family member f (in filopodia)	Down
Bcr	BCR activator of RhoGEF and GTPase	Up
Gab2	Growth factor receptor bound protein 2-associated protein 2	Up
Fgfr1op2	FGFR1 oncogene partner 2	Down
Gfra1	Glial cell line derived neurotrophic factor family receptor alpha 1	Up
Rasgrf2	RAS protein-specific guanine nucleotide-releasing factor 2	Up
Srgap1	SLIT-ROBO Rho GTPase activating protein 1	Up
Stk25	Serine/threonine kinase 25 (yeast)	Down
Cluster 5		
Syt1	Synaptotagmin i	Up
Apba1	Amyloid beta (a4) precursor protein binding, family a, member 1	Up
Nrxn3	Neurexin iii	Up
Rims1	Regulating synaptic membrane exocytosis 1	Up
Syp	Synaptophysin	Up
Epb41l1	Erythrocyte membrane protein band 4.1 like 1	Up
Cplx2	Complexin 2	Up
Cluster 6		
Prkar2a	Protein kinase, camp dependent regulatory, type ii alpha	Up
Prkce	Protein kinase c, epsilon	Up
Irs4	Insulin receptor substrate 4	Up
Bad	BCL2-associated agonist of cell death	Down
Cluster 7		
Thy1	Thymus cell antigen 1, theta	Up
Sco2	SCO2 cytochrome c oxidase assembly protein	Up
Lsamp	Limbic system-associated membrane protein	Up
Cluster 8		
Kpnb1	Karyopherin (importin) beta 1	Up
Nup98	Nucleoporin 98	Down
Kpna6	Karyopherin (importin) alpha 6	Up
Cluster 9		
Cul3	Cullin 3	Up
Uba6	Ubiquitin-like modifier activating enzyme 6	Up
Rlim	Ring finger protein, lim domain interacting	Up
Cdc26	Cell division cycle 26	Down
Fbxw2	F-box and WD-40 domain protein 2	Down
Dvl1	Dishevelled segment polarity protein 1	Up

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Cluster 10		
Cds2	CDP-diacylglycerol synthase (phosphatidate cytidyltransferase) 2	Up
Mboat7	Membrane bound o-acyltransferase domain containing 7	Up
Pld3	Phospholipase d family, member 3	Up
Cluster 11		
Taf6	TATA-box binding protein associated factor 6	Down
Usp22	Ubiquitin specific peptidase 22	Up
Taf13	TATA-box binding protein associated factor 13	Down
Cluster 12		
Sart1	Squamous cell carcinoma antigen recognized by t cells 1	Up
Hnrnpa0	Heterogeneous nuclear ribonucleoprotein a0	Up
Sf3b2	Splicing factor 3b, subunit 2	Up
Pcbp2	Poly(rc) binding protein 2	Up
Cluster 13		
Kifap3	Kinesin-associated protein 3	Up
Kif21a	Kinesin family member 21a	Up
Kif3a	Kinesin family member 3a	Up

Ageing in the Axon

The dopaminergic proportion in aged axonal TRAP samples was reduced, leading to the finding that markers of dopaminergic neurons were in lower abundance while markers of other cell types increased. To identify genes up-regulated by dopaminergic neurons, the results of this comparison were filtered to remove genes enriched exclusively in axonal TRAP samples and genes without evidence of enrichment (Figure 5.8 A).

6 genes were up-regulated that were enriched exclusively in somal TRAP samples. All changes were not observed in TOTAL samples. 47 genes were up-regulated that were enriched in both TRAP compartments. 6 of these genes were also up-regulated in somal TRAP samples, supporting their detection from dopaminergic neurons.

Nicotinamide mononucleotide adenylyltransferase 2 (*Nmnat2*) was up-regulated in both neuronal compartments (Figure 5.8 C). NMNAT2 is critical for axon

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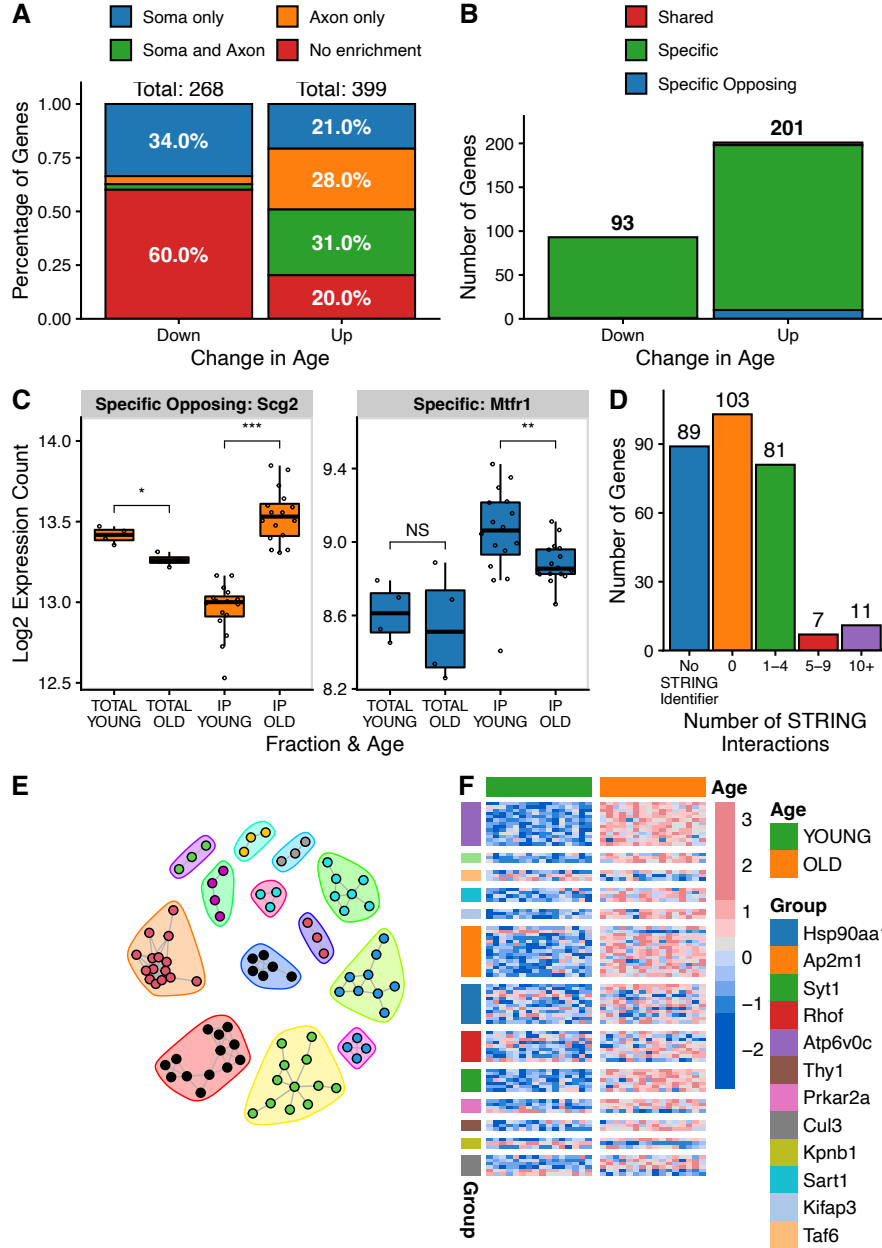


Figure 5.7: Ageing in the cell body. **A:** The enrichment status of differentially expressed genes. Most down-regulated genes were either not enriched or were exclusively enriched in somal TRAP samples. **B:** Ageing changes in somal TRAP samples were not observed in TOTAL samples in most cases. **C:** Examples of “Specific” and “Specific Opposing” cases of differential expression: Mitochondrial fission regulator 1 (*Mtfr1*) expression is reduced in aged samples, while Secretagranin 2 (*Scg2*) expression is increased. **D:** The number of high confidence protein-protein interactions associated with each differentially expressed gene. **E:** 13 Clusters of interacting genes were formed. **F:** Expression heatmap of all genes across the 13 clusters. With some exceptions, most clusters are up-regulated with age. The gene with highest degree is annotated. *P* values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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maintenance and loss of this protein leads to Wallerian degeneration (Gilley and Coleman 2010). Sterile alpha and TIR motif containing 1 (SARM1) is required for NMNAT2-deficient axonal degeneration. Interestingly, *Sarm1* expression appeared to increase specifically in dorsal striatal axonal TRAP samples, although this result was not significant (Figure 5.8 D).

Prostaglandin E2 (*Ptges2*/PGE₂) expression increased in both axonal compartments, while remaining constant in the cell body (Figure 5.8 E). Cyclooxygenase-2 (*Ptgs2*/COX-2) is known to contribute to neuronal degeneration through induction of PGE₂ production: Administration of a COX-2 selective inhibitor to Neuro-2a and SH-SY5Y cells treated with 6-OHDA reduced the cytotoxic effects of 6-OHDA administration (Kang, Qiu, et al. 2017). The expression of *Ptgs2* was over 4-fold greater in axonal TRAP samples compared to somal TRAP samples, while it was enriched by TRAP in both compartments. This suggests that COX-2/PGE₂ activity may contribute specifically to axonal vulnerability of dopaminergic neurons with age.

Copine-6 (*Cpne6*/CPNE6) was upregulated in the cell body and axonal compartment with age (Figure 5.8 F). More is known about the role of *CPNE6* in neuronal biology than *CPNE7* (discussed in the previous chapter for its extreme dopaminergic neuron-specific expression). It is localised to presynaptic terminals and binds to synaptobrevin2 and other SNARE proteins, blocking spontaneous neurotransmission in a Ca²⁺-dependent manner (Liu, Khvotchev, et al. 2018). Interestingly, copine-4 is also up-regulated in the cell body with age, suggesting that copines may have an as yet unexplored but important role in this process.

5.2.5 Overexpression of Human α -Synuclein

SNCA-OVX mice overexpress human α -synuclein protein at 1.9 times the level of endogenous mouse α -synuclein (Janezic et al. 2013). *SNCA* expression was higher in *SNCA*-OVX mice and enriched in somal and axonal samples by TRAP, confirming

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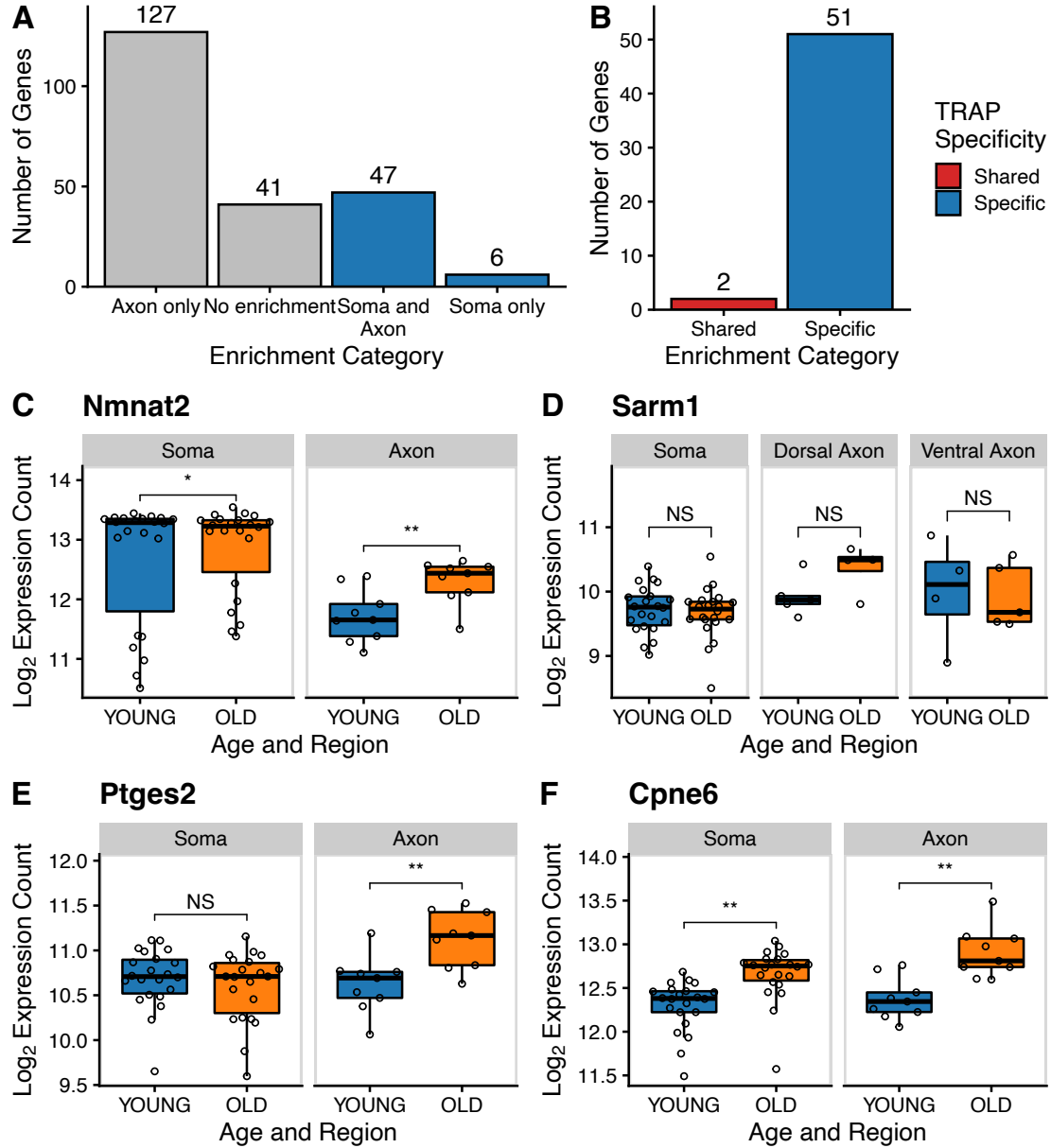


Figure 5.8: Ageing in the axon. **A:** Breakdown of differentially expressed genes by enrichment category: Only genes with evidence of somal enrichment were interpreted, to identify changes most specific to dopaminergic neurons. **B:** Most age-related changes were not detected in matched TOTAL samples. **C and D:** An increase in *Nmnat2* expression occurs in aged axonal samples, indicating an increased requirement for NMNAT2-mediated regulation of axonal integrity. In dorsal axonal TRAP samples, a trending increase in *Sarm1* expression was observed. **E:** *Ptges2* (PGE₂) expression increases in axonal TRAP samples with age. Strong compartment enrichment of *Ptgs1* and *Ptg2* (COX-1 and COX-2) is seen in axonal TRAP samples, indicating an important role for these enzymes in axonal function. **F:** *Cpne6* expression increases across the cell body and axon in age. *P* values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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transgene expression at the level of RNA. There are 17 isoforms of *SNCA* and aberrant splicing can play a role in PD (Fu et al. 2013). Long-read sequencing was performed on 6 *SNCA*-OVX somal TRAP samples, allowing the splicing profile of these mice to be compared (Figure 5.10). Every isoform of *SNCA* was detected and importantly, mouse *Snca* expression was not detected as increased in *SNCA*-OVX samples, indicating that read alignment was specific to each gene.

The splicing profile of *SNCA* was compared between *SNCA*-OVX samples and human LCM-derived and iPSC-derived dopaminergic neurons (Nichterwitz et al. 2016; Zambon et al. 2019). *SNCA*-203, -204, -205 and in particular, -217 were the dominant transcripts in *SNCA*-OVX samples. In human iPSC-derived neurons, *SNCA*-201, -203, -205 and -211 were dominant. In LCM neurons, a similar profile was observed, with the addition of *SNCA*-203 and *SNCA*-217. *SNCA*-217 is the longest of all transcripts, containing two additional exons. The dominance of this transcript and *SNCA*-OVX and differences in the relative abundance of others transcripts in *SNCA*-OVX samples could be due to the lack of human splicing factors in mouse dopaminergic neurons.

In young somal *SNCA*-OVX TRAP samples, there were no significant differences in gene expression compared to wild-type samples (with the exception of *SNCA*) (Figure 5.11 A). This finding is in keeping with the original characterisation of *SNCA*-OVX mice, in which pathology was age-dependent (with the exception of a dopamine release deficit in the striatum of young mice: See axonal findings below). In aged somal TRAP samples, 8 genes were significantly differentially abundant between genotypes. These genes were not enriched by somal TRAP. Initially, it was considered that this reflected a greater importance of non-dopaminergic cell types in *SNCA*-OVX pathology. Thousands of genes were subtly, but non-significantly different in abundance between these groups, however (Figure 5.11 A). At a higher false discovery rate of 10 %, 50 were differentially abundant (Figure

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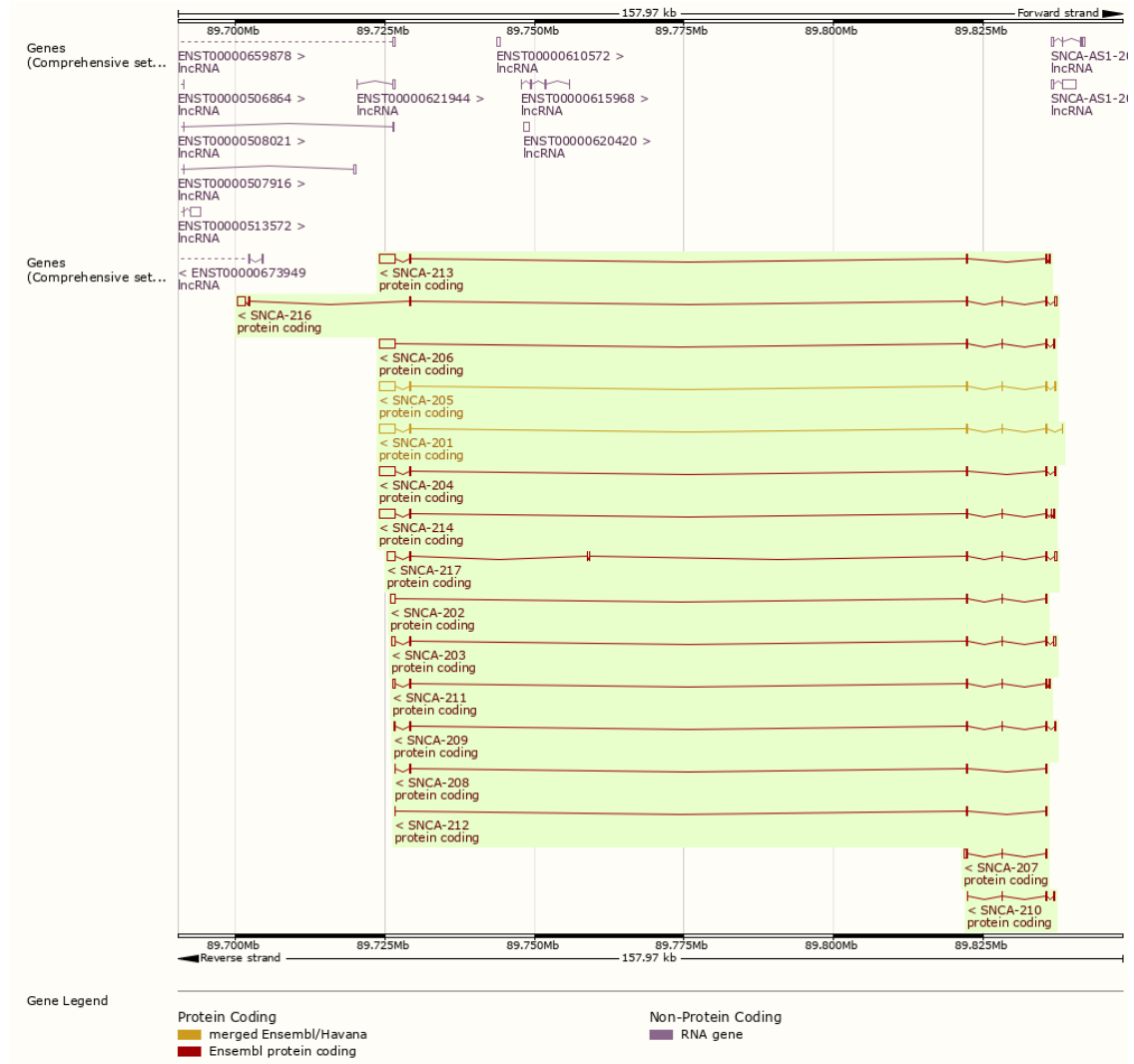


Figure 5.9: Human SNCA Isoforms, as described in this chapter.

5.11 B). The majority of these genes were still more abundant in *SNCA*-OVX samples and not enriched by somal TRAP. Those that were less abundant were enriched by somal TRAP. These findings resembled library composition differences observed with ageing.

Somal TRAP samples were divided into separate datasets by genotype and the effect of age on gene abundance was reanalysed. Due to the effect of collection strategy in cohort 1, analysis of the interaction between ageing and *SNCA* overexpression was restricted to cohort 3 in which samples were collected together. The number of differentially abundant genes was much larger in *SNCA*-OVX samples

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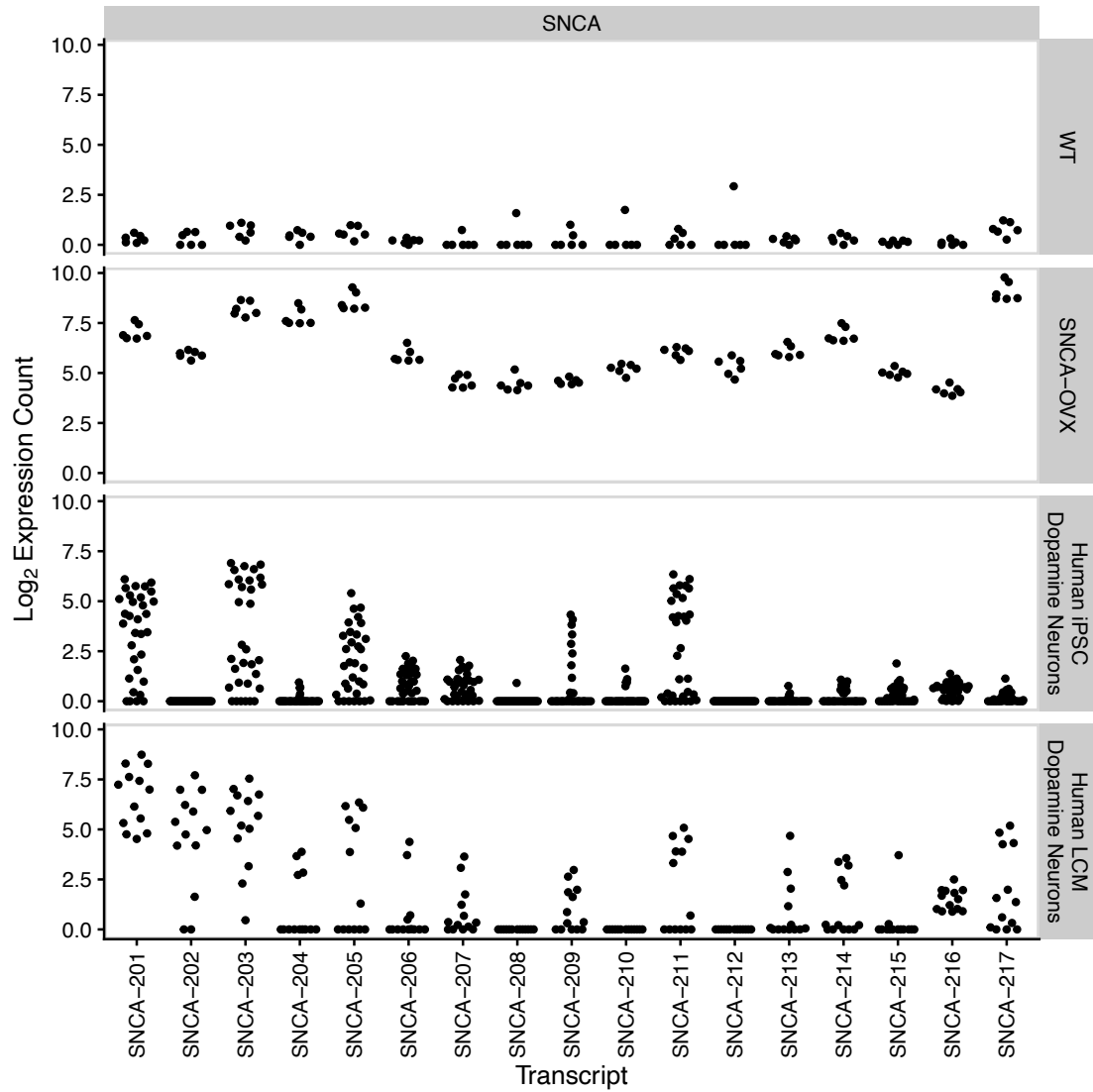


Figure 5.10: Splicing profile of *SNCA* in SNCA-OVX mice: Read alignment successfully distinguished *SNCA* and *Snca* transcripts. *SNCA* in SNCA-OVX mice is most represented by SNCA-203, -204, -205 and -217. In human cells, SNCA-201, -203, -205, -211 and -217 are expressed. Differences in relative sequencing depth and expression of *SNCA* are a limitation of this comparison.

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(Figure 5.11 C). The magnitude of commonly detected changes was greater also (Figure 5.11 D). It was concluded that overexpression of human α -synuclein induced a more severe reduction in dopaminergic proportion in aged TRAP samples. This confirmed the original finding that *SNCA*-OVX mice display an age-dependent loss of dopaminergic neurons of the SNpc (Janezic et al. 2013).

Due to the change in library proportion, analysis of dopaminergic-specific gene expression was restricted to soma-enriched genes that were more abundant in *SNCA*-OVX samples. 1 gene was significantly up-regulated in aged somal *SNCA*-OVX TRAP samples at an FDR of 0.1, compared to matched wild-type samples; Zfp672, a zinc finger protein of unknown function (Figure 5.11 E).

Axonal *SNCA*-OVX TRAP samples were collected in cohort 3 only. In young, dorsal and ventral axonal TRAP samples analysed separately, over 100 genes were classed as significantly differentially abundant due to genotype. Due to the high dropout percentage and resulting dispersion observed in cohort 3 data (described in 3.2) only 3 genes were retained after manual inspection (Figure 5.12). Of these genes, Casein Kinase 1a has been identified as a physiological kinase regulator of LRRK2 (Chia et al. 2014). Constitutive phosphorylation by CK1a modulates the recruitment of LRRK2 to TGN46-positive Golgi-derived vesicles. If dorsal axonal wild-type levels of *Csnk1a1* reflect an increased requirement for Ck1a activity, dorsal axonal *SNCA*-OVX samples fail to meet this demand, likely impacting LRRK2's functional interaction with the trans-Golgi network. Dual specificity phosphatase 8 (*Dusp8*) is an inhibitor of mitogen activated protein kinases (MAPKs) Jnk, Erk and p38 (Patterson et al. 2009). MAPK signalling is important for regulation of many processes relevant to dopaminergic and PD biology including apoptosis and autophagy (Obergasteiger et al. 2018). Interestingly, over 20 kinases were significantly more abundant in axonal TRAP samples than somal TRAP samples.

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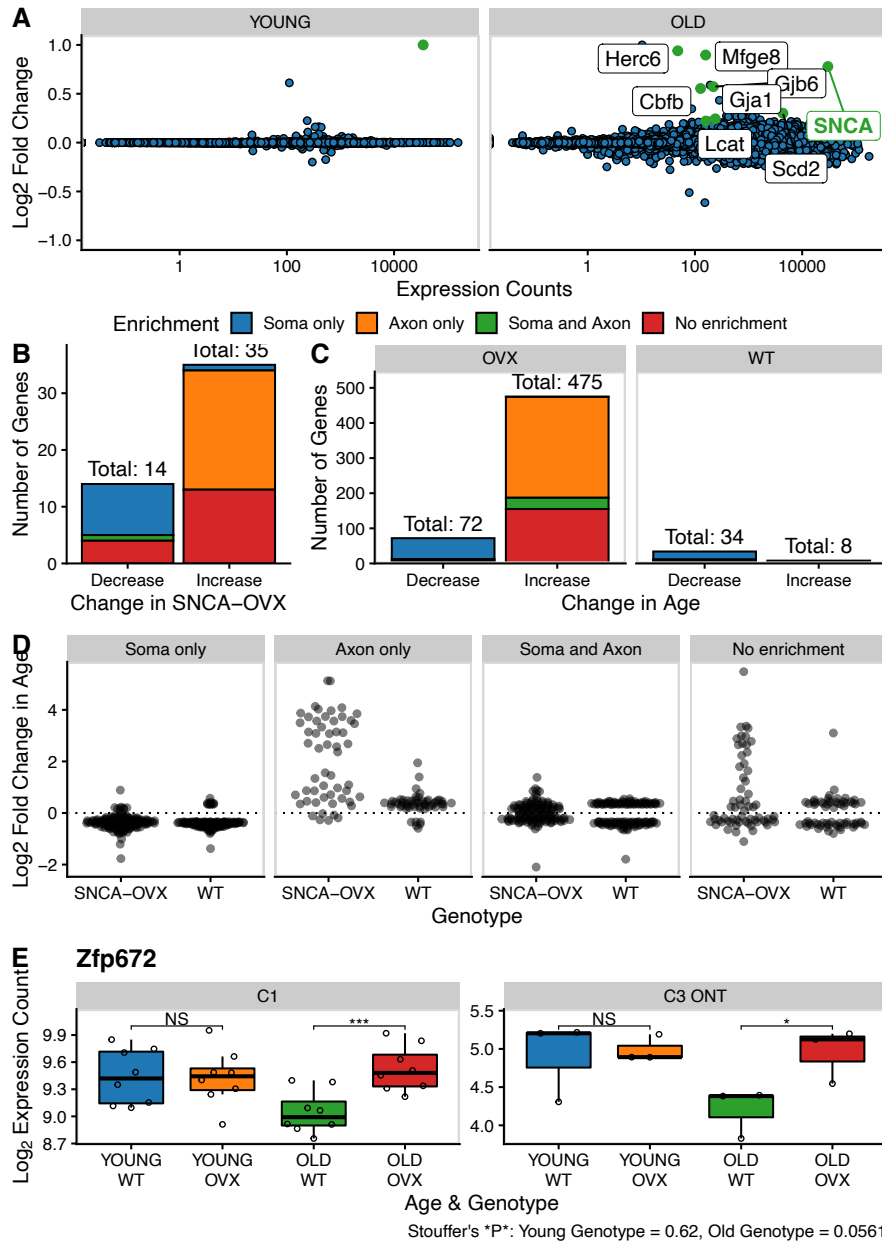


Figure 5.11: *SNCA*-OVX in the cell body: **A:** There were no significantly differentially expressed genes in young somal *SNCA*-OVX TRAP samples compared to wild-type. At 18 months, differential abundance was detected, however up-regulated genes were not enriched in somal TRAP samples. **B:** The enrichment status of differentially abundant genes from **A: OLD**. “Axon only” genes were up-regulated while “Soma only” genes were down-regulated: The library composition was different. **D:** Results from comparing old and young somal TRAP samples, split by genotype: A greater number of genes are differentially abundant due to *SNCA*-OVX-induced changes to library composition (neuronal loss). **D:** The magnitude of age-induced change was greater in *SNCA*-OVX samples for genes exclusively enriched in axons or with no evidence of enrichment. **E:** *Zfp672*, a zinc finger protein of unknown function is differentially expressed in old samples. *Zfp672* expression is reduced in aged wild-type samples, but appears to be at young levels in *SNCA*-OVX samples. *P* values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

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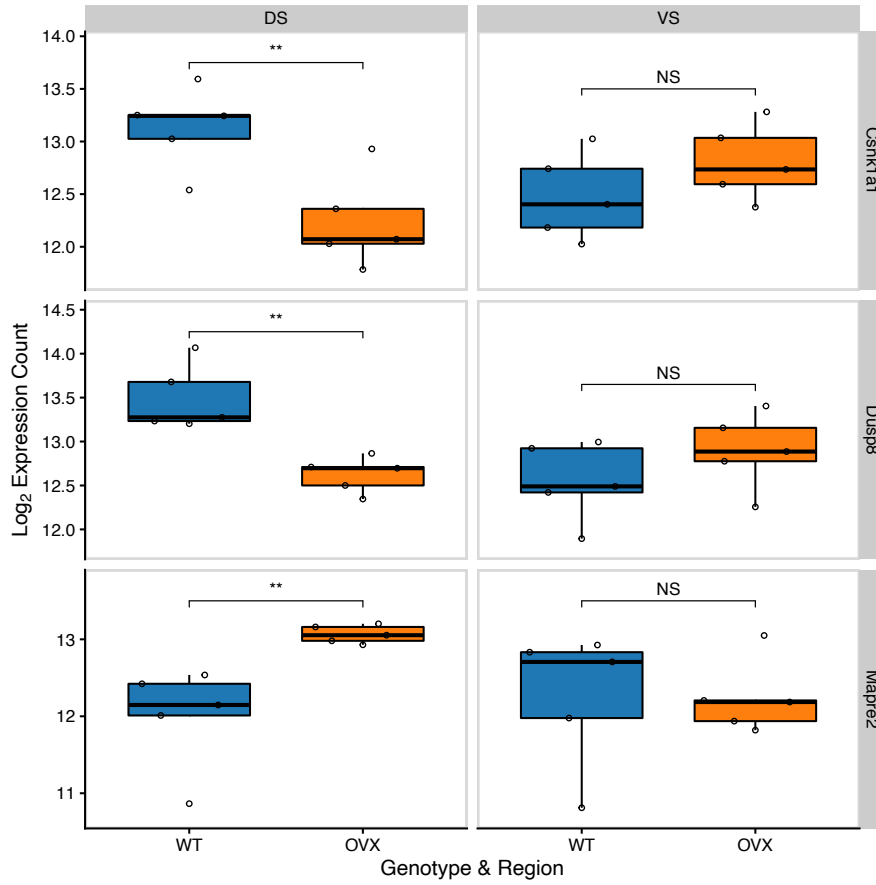


Figure 5.12: *SNCA*-OVX expression in young axonal TRAP samples: Three genes identified as differentially expressed in young dorsal axonal TRAP samples compared to all other samples. A role for *Csnk1a1* in phosphorylating LRRK2 has been reported (Chia et al. 2014). *P* values presented are derived from the Wald test using *DESeq2* (Love, Ahlmann-Eltze, et al. 2021). *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.1$, NS = Not significant.

5.3 Discussion

Based on the findings from this chapter;

- The library composition of TRAP samples differs between biological conditions (age and genotype), affecting interpretability of gene expression comparisons.
- A subset of genes are more abundantly expressed in the axonal compartment, compared to the cell body.

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- Groups of interacting genes are differentially expressed in dopaminergic neurons with age. These genes are involved in a variety of pathways, as described below.
- The transcriptome of *SNCA*-OVX dopaminergic neurons is subtly different compared to wild-type neurons, regardless of age. This indicates either very subtle effect of overexpression of human α -synuclein that may require greater sampling frequency along the ageing time course, or it may indicate a role of α -synuclein pathology in cell types other than dopaminergic neurons.

5.3.1 Local Translation

Over 200 genes were differentially expressed between dorsal and ventral striatal TRAP samples, revealing a potentially valuable profile of the differences between A9 and A10 dopaminergic axons. Many of the differences were also observed in TOTAL samples, however. This could be partially explained by expression that is not cell type specific but rather region specific. Alternatively, the fraction of striatal RNA derived from dopaminergic axons could be large enough for expression differences to be detectable.

For many genes that were more abundant in the axon or in dorsal striatal TRAP samples, there is surrounding evidence for a role in axonal biology or disease. A subset of these genes are discussed below.

Alpha 2 catalytic subunit of AMP-activated protein kinase (AMPK)

Prkaa2, the alpha 2 catalytic subunit of AMP-activated protein kinase (AMPK) has a regulatory role in many functions directly relevant to PD, including macroautophagy (Moors et al. 2017), mitochondrial quality control (Zhang and Lin 2016), redox homeostasis (Jeon et al. 2012), inflammatory signalling (Pilon et al. 2004) and the regulation of cellular energy metabolism (Curry et al. 2018). AMPK has

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received attention as a potential therapeutic target, most notably through the use of insulin sensitisers, such as metformin. Activation of AMPK has been shown to facilitate clearance of *a*-synuclein by promoting macroautophagy, however there may be a narrow therapeutic window (Bobela et al. 2017): There are *in vitro* and *in vivo* sources of evidence to show that activation of AMPK can lead to dopaminergic cell death, likely through excessive macroautophagic activity (Xilouri and Stefanis 2011; Kang, Zhang, Liu, Manfredsson, He, et al. 2017). The elevated expression of AMPK in dorsal striatal axonal TRAP samples may reflect a greater (and healthy) local requirement for one or many of the functions of AMPK compared to ventral axons. If this functioning were compromised, A9 dopaminergic neurons could become more vulnerable to degeneration. Alternatively, with the accumulation of cellular stressors over time, the requirement for AMPK activity could grow to a level at which it becomes detrimental to the neuron. *Prkaa2* abundance was not greater in aged dorsal axonal TRAP samples, however. In this case, the demand for AMPK may not have increased with age, however the deleterious consequences of lifelong elevated AMPK activity may start to become apparent.

Krüppel-like factor-13 (KLF13) *Klf13* (Krüppel-like factor-13, KLF13) is a transcriptional repressor of axon growth in central nervous system neurons (Moore et al. 2009). It is part of the Basic Transcription Element Binding (BTEB) subfamily of KLFs. Recently, BTEB KLFs were found to bind c-Jun N-terminal kinase 3 (JNK3) (Apara et al. 2017). Interference with this interaction prevented neurite growth suppression *in vivo* and promoted axonal regeneration in an *in vivo* model of optic nerve injury. Independent studies have demonstrated a role for JNK13 in mediating the effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and pesticide neurotoxicity (Choi, Abel, et al. 2010; Pan et al. 2009). The finding that *Klf13* expression is greater in the axonal compartment and greatest in dorsal

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axonal TRAP samples may indicate a repressed regenerative capacity in these neurons due to KLF13-JNK3 interaction.

Actin-filament associated protein 1-like 1 Actin-filament associated protein 1L1 (*Afap1l1*) is robustly more abundant in dorsal axonal TRAP samples. Immunohistochemical staining for AFAP1L1 in mouse Purkinje neurons showed greater signal in segments extending away from the cell body, indicating axonal localisation (Snyder et al. 2011). AFAP1L1 was found to interact with cortactin, an adaptor protein found at sites of podosome formation. Cortactin is a substrate of histone deacetylase-6 (HDAC6) and is essential for HDAC6-dependent F-actin network assembly at protein aggregates (Lee, Koga, et al. 2010; Wang, Tan, et al. 2018). Failure of F-actin assembly impairs autophagosome-lysosome fusion. Overexpression of the A53T mutant of α -synuclein or administration of MPP⁺ induces HDAC6 expression and autophagic degradation (Su et al. 2011). The role of AFAP1L1 in HDAC6-dependent autophagy has not been studied, however the elevated expression in dorsal axonal TRAP samples suggests an important role for this protein and potentially for HDAC6-dependent autophagy in the axon.

Downstream of tyrosine kinase/docking protein 5 (Dok5) Downstream of tyrosine kinase/docking protein 5 (*Dok5*) is a substrate of the c-Ret receptor tyrosine kinase expressed in neuronal tissues (Grimm et al. 2001). Interaction between *Dok5* and c-Ret is required for ligand-induced axonal outgrowth. *Dok5* is also a substrate for tropomyosin-related kinase (Trk)A and TrkB signalling, enhancing mitogen-activated protein kinase (MAPK) activity in response to brain-derived neurotrophic factor (BDNF) or neurotrophin-3 (NT-3) administration (Shi et al. 2006; Li, Shi, et al. 2010). BDNF/TrkB signalling promotes dopaminergic neuronal survival but is directly inhibited by α -synuclein (Kang, Zhang, Liu, Manfredsson, Benskey, et al. 2017). The elevated expression of *Dok5* in

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dorsal axonal TRAP samples suggests a greater requirement for this signalling pathway. Inhibition under conditions such as α -synuclein overexpression could place additional stress on the axon.

Nervous system nuclear protein induced by axotomy (NNA1) Nervous system nuclear protein induced by axotomy (NNA1), also referred to as ATP/GTP-Binding protein 1 (AGTPBP1) and cytosolic carboxypeptidase 1 (CCP1) regulates tubulin polyglutamylation and was found to be more abundant in dorsal axonal TRAP samples. As the name describes, it was originally characterised by rapid induction in motor neurons in response to sciatic nerve injury (Harris et al. 2000). Mice lacking functional NNA1 develop adult-onset ataxia with degeneration of Purkinje neurons, retinal photoreceptors, olfactory bulb mitral neurons and thalamic neurons (Fernandez-Gonzalez 2002). Dopaminergic neuronal death was not examined. Excessive polyglutamylation due to loss of NNA1 lead to impaired axonal transport, organelle accumulation, axonal swelling and cytoplasmic densification in myelinated hippocampal neurons (Magiera et al. 2018). Loss of function variants in NNA1 have since been identified, with patients presenting with infantile-onset neurodegeneration (Shashi et al. 2018). Immunohistochemical staining for glutamylated and unmodified 2-tubulin in *postmortem* hippocampal tissue from patients with Alzheimer’s Disease found increased intensity of the glutamylated form in CA3 mossy fibers and apical dendrites of pyramidal neurons (Vu et al. 2017). NNA1 was identified and confirmed as the most significantly coexpressed interaction partner of chromosome 9 open reading frame 72 (C9orf72), the most commonly mutated gene implicated in frontotemporal dementia and amyotrophic lateral sclerosis (FTD/ALS).

Regulator of G protein signalling 6 (RGS6) Regulator of G protein signalling 6 (RGS6) is a regulator of D₂R-G_{i/o} signalling and it is selectively expressed

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SNpc dopaminergic neurons, providing orthogonal confirmation of its higher expression in dorsal axonal TRAP samples (Luo et al. 2019). RGS6 loss is associated with age-dependent loss of SNpc dopaminergic neurons, α -synuclein accumulation, D₂R hyperactivity and locomotor deficits.

Kinesin family member 3A (KIF3A) KIF3A is a member of the kinesin-2 family that was first described as a fast anterograde axonal motor protein (Kondo, Sato-Yoshitake, et al. 1994). It forms a heterotrimeric complex with KIF3B and a cargo-binding adaptor, KAP3 (both enriched in axonal TRAP samples) (Hirokawa et al. 2010; Scholey 2013). In axon development, this complex binds and transports TRIM46 (also enriched in axonal TRAP samples) to the developing axon initial segment, where TRIM46 guides neuronal polarity by building parallel, stabilised microtubules (Ichinose et al. 2019). This process is spatially regulated; a phosphorylation event prevents TRIM46 binding in the somatodendritic compartment. The greater abundance of *Kif3a* and associated transcripts in the axon suggests that there is a role for this process in the distal axon. Transport of the relevant mRNA and local translation may be a more energy efficient means. *Kif3a* expression was up-regulated with age (discussed in Ageing, below).

Prostaglandin-endoperoxide synthase 2 (COX-2) *Ptgs2* (COX-2) is ~ 2.5 times more abundant in dorsal axonal TRAP samples compared to ventral and more than 4 times more abundant in the axonal compartment compared to the cell body. COX-2 expression has been shown to be increased in dopaminergic neurons of PD patients and MPTP-treated mice (Teismann et al. 2003). The authors demonstrated that COX-2 inhibition prevented formation of reactive oxygen species (ROS), including dopamine-quinone and found that cell death occurred in the absence of evidence of microglial activation from PGE₂. They argued that COX-2 mediated damage was due to ROS formation, while COX-1 was responsible for

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most PGE₂ production and sequent stimulation of an inflammatory response. *Ptgs1* (COX-1) and *Ptgs2* (PGE₂) are also robustly enriched by TRAP and *Ptgs1* is also more abundant in axonal samples compared to somal. Increased *Ptgs2* abundance in dorsal striatal axonal TRAP samples could indicate a potentiation of COX-2 activity contributing to selective vulnerability in this region.

Heterogeneous nuclear ribonucleoprotein A2/B1 (HnRNP A2/B1) *HnRNP* A2/B1 is more abundant in the axonal compartment and is a member of the heterogeneous nuclear ribonucleoprotein particle protein family, which also includes TDP-43 (enriched in axonal TRAP samples) and FUS/TLS. Trafficking of RNAs containing A2 response elements into distal neurites relies on binding of *HnRNP* A2/B1 and is calcium-dependent (Shan et al. 2003; Muslimov et al. 2014). A transcriptome-wide analysis of *HnRNP* A2/B1 binding showed that it primarily binds to 3' UTRs, while also regulating alternative polyadenylation (Martinez et al. 2016). For identifying the 3' UTR elements that confer localisation of transcripts to the dopaminergic axon, *HnRNP* A2/B1 could be a worthwhile candidate for RNA immunoprecipitation.

Alternative Splicing in the Axon

Rabgap1l and *Oxr1* are high confidence candidates for axonal translation in dopaminergic neurons. They both represent examples of alternative exon usage and alternative polyadenylation.

Rab GAP 1-like (RABGAP1L) RABGAP1L is a TBC (Tre2, Bub2 and Cdc16) domain-containing Rab GAP (Popovic et al. 2012). It is recruited by ankyrin-B (AnkB) to inactivate Rab22A and promote the polarised trafficking of phosphatidylinositol 3-phosphate (PI3P)-positive endocytic cargo to the leading edge of migrating fibroblasts (Qu et al. 2016). AnkB, through an interaction with

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dynactin, has been found to promote fast axonal transport of organelles and to promote axonal growth (Lorenzo et al. 2014). This provides evidence to support a requirement for RABGAP1L translation in axons, however the role for differential transcript usage is less clear.

Rabgap1l-201 is more expressed in the axonal compartment, while *Rabgap1l*-202 is dominant in the soma. Both isoforms are protein coding. They contain alternative exons and 5' and 3' UTRs, encoding proteins 815 and 370 amino acids long, respectively.

The TBC domain of RABGAP1L contains a catalytic site required for inactivation of *Rab22A* (Itoh et al. 2006; Frasa et al. 2012). TBC domain-containing Rab GAPs including RABGAP1L have also been found to bind directly to ATG8 modifiers and colocalise with LC3-positive autophagy membranes (Popovic et al. 2012). *Rabgap1l*-201 (more abundant in the axon) contains this domain, whereas *Rabgap1l*-202 does not. They also contain alternative 3' UTRs which may guide the trafficking of *Rabgap1l*-201 to the axon while retaining *Rabgap1l*-202 in the soma.

There is evidence for a role for RABGAP1L in neurodegeneration: Expression was found to be reduced in *postmortem* hippocampal and medial septal tissue from AD patients (de Yebra et al. 2004). At the very least, the finding that the axonal dominant form of *Rabgap1l* supports autophagy while the somal dominant form does not adds to the evidence that autophagy is of high priority in axons.

Oxidation resistance 1 (OXR1) Oxidation resistance 1 is regarded as an essential protein in regulating the sensitivity of neurons to oxidative stress, with *Oxr1*^{-/-} displaying cerebellar degeneration (Oliver et al. 2011). The dominant form in axons (*Oxr1*-204) encodes an full length (866 amino acid) form, whilst the dominant somal form (*Oxr1*-205) encodes a C-terminal fragment. Both forms contain a TLDC domain required for their functional role in reducing oxidative stress. Gross ISH of short and long forms of *Oxr1* did not show a difference in

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localisation to different brain regions, however the consequences of this splicing event on subcellular localisation have not been investigated. There is increasing evidence to support the functional role of longer 3' UTRs in guiding transcript localisation to the axon (Miura et al. 2013). The *Oxr1*-204 isoform may be an example of this process.

5.3.2 Ageing

Human dopaminergic neuronal properties change with age, although there is limited consensus as to how these changes are reflected in the mouse: In humans, the number of nigral dopaminergic neurons falls at a rate of ~4.7 % per decade (McGeer et al. 1977; Fearnley and Lees 1991). A total reduction in neuron number of up to 35 % has recently been reported in C57Bl/6 mice, aged over 100 weeks (6 months older than aged mice used in this investigation) (Noda et al. 2020). Greatest neuron loss was found in the VTA, however, while there was not a significant loss of SNpc neurons. Osterburg et al. (1981) reported no significant change in dopaminergic neuron number in 21 - 30 month old mice: A subtle downward trend in striatal dopamine content was observed. Noda et al. (2020) also reported halving of mitochondrial area in aged SNpc neurons, with increased fragmentation. The latency to fall in the accelerating rotarod test was reduced in these mice, indicating the development of locomotor deficits.

Based on the pattern of change in gene abundance between aged and wild type TRAP samples, it was estimated that dopaminergic neuron loss had occurred. Stereological analysis is now underway to confirm this. The artefactually greater dopaminergic proportion in aged somal TRAP samples from cohort 1 was fortunate in that it served to “cancel out” the effects of the ageing-induced reduced dopaminergic proportion in samples from cohorts 2 and 3. This allowed a characterisation of both up- and down-regulated genes.

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The majority of genes found to be differentially expressed were up-regulated. This could reflect increased demand for the proteins represented, perhaps due to pathway-specific conditions (e.g. a greater burden of defective mitochondria requiring up-regulation of autophagy-related genes), or it could represent a response to a more general reduction or compromise in translational efficiency. A slowing of translation has been reported to occur with age Rattan (1996), and the finding that many elements of autophagic function are up-regulated could reflect raised transcriptional demand for a process that is not being met by the reduced translational capacity in the cell.

Cullin 3 (*Cul3*) was up-regulated with age. In a complex with *Keap1* and *Nedd8* (both enriched in somal TRAP samples) it plays an important role in regulating the transcriptional activity of nuclear factor erythroid 2 related factor (*Nrf2*) through degradation by the ubiquitin proteasome system. Overexpression of *Cul3* in breast cancer tissue has been shown to deplete *Nrf2* levels, increasing susceptibility to oxidative stress (Loignon et al. 2009). Reactive oxygen species (ROS) can be generated from multiple sources in dopaminergic neurons: Increased *Cul3* expression with age may increase their susceptibility to ROS-induced damage. *Cul3* was also enriched and more abundant in the axonal compartment compared to the cell body: This could indicate an increased susceptibility to oxidative stress in the axon.

Argonaute 2 (*Ago2*) expression increased in somal TRAP samples while tissue-level expression decreased. This suggests an increased requirement for miRNA processing with age. Interestingly, a role for autophagy in regulating miRNA homeostasis has been described (Gibbins et al. 2013). In cells lacking key autophagy components, such as ATG7, ubiquitinated AGO2 and DICER1 enzymes accumulate. Additionally it was shown that autophagy was necessary for AGO2-miRNA binding.

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Secretagranin 2 was not clustered due to a limited number of protein-protein interactions in the STRING database. There is evidence that *Scg2* could play one or more specialised roles in dopaminergic function, however: It was robustly differentially expressed between ages and it was alternatively spliced between somal and axonal compartments. *Scg2* has been shown to be up-regulated in hypoxic conditions in skeletal muscle and in myocardium (Egger et al. 2007; Røsjø et al. 2012). Through cleavage of its protein into secretoneurin it exerts anticardio-protective properties. Secretoneurin also enhances dopamine release in a Ca^{2+} -dependent manner (Saria et al. 1993). This suggests at least a dual role for *Scg2* in protecting dopaminergic neurons from accumulating oxidative damage and supporting dopamine release with age.

5.3.3 Overexpression of Human α -Synuclein

To its merit, the *SNCA*-OVX model provides a subtle perturbation that causes selective, enhanced and progressive dopaminergic neuron loss with age. This subtlety however may be an explanation for the lack of robust differential expression findings.

With approximately 15,000 dopaminergic neurons in the SNpc, loss of 30 % by 18 months would require an average daily loss of 10 neurons per day or 0.06 - 0.08 % of the population (Ip et al. 2017; Nair-Roberts et al. 2008). Each neuron will exist along an axis of degeneration; some may function normally while others are close to death. To capture the expression of most affected cells requires single cell resolution or a more aggressive model.

Another consideration is cell subpopulation specificity: Findings from cohort 3 axonal data indicate that there may be *SNCA*-OVX-induced expression differences in dorsal striatal samples of young mice that could help to explain early release deficits. No changes were observed in ventral striatal samples. These samples were regionally separated, along vulnerable neurons to be characterised. At the

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level of the cell body, RNA from SNpc and VTA neurons was mixed, so any distinctive responses to human *a*-synuclein overexpression could not be determined. To address this, and to provide spatial context to future comparisons, a spatially mapped transcriptomic dataset is being generated of midbrain and striatal tissue from *SNCA*-OVX and wild type, young and aged mice.

The genetic background of *SNCA*-OVX mice used in this study was different to the original *SNCA*-OVX mouse model. *SNCA*-OVX mice, as described in Janezic et al. (2013) were pure C57BL/6J bred on a mouse *a*-synuclein-null background. This was intended to avoid interaction between endogenous and human *a*-synuclein. The ROSA26-eGFP-L10a locus is located on the same chromosome as mouse *a*-synuclein (chromosome 6). To avoid unexpected genetic or phenotypic complications, *SNCA*-OVX mice were backcrossed onto a wild-type mouse *a*-synuclein background. Therefore it is possible that the presence of mouse *a*-synuclein limited the effect of human *a*-synuclein overexpression on neuronal function.

6.1 Confirmation of Results

Stereological and densitometric characterisation of the effect of ageing and overexpression of human α -synuclein

The abundance of dopaminergic markers decreased in aged TRAP samples, while non-dopaminergic markers increased. In *SNCA*-OVX TRAP samples, the abundance was reduced further. This may be due to a change in relative cell type proportions, caused by dopaminergic cell loss. To confirm this, stereological cell counting of TH-positive dopaminergic neurons will need to be performed in 3- and 18-month SNpc from Rosa26^{fsTRAP}::DAT^{IREScre} and Rosa26^{fsTRAP}::DAT^{IREScre}::*SNCA*-OVX mice. Stereological counting has been performed in 18-month old *SNCA*-OVX mice (bred on a *Snca*^{-/-} background) (Janezic et al. 2013): There was a 30 % reduction in the number of TH positive neurons in the SNpc compared to mice expressing normal levels of human α -synuclein. In 3-month old mice, there was not a significant difference between genotypes. 3- and 18-month old animals expressing normal levels of human α -synuclein were not directly compared.

A reduction in the number of eGFP-positive, TH-positive neurons has previously been reported in the SNpc and VTA of 12-month old mice from an alternative

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TRAP model (Wagner 2017). Stereological cell counting of TH-positive neurons was also performed in these animals: No significant difference was observed between age groups, however. Count estimates were variable (2-fold variation between the largest and smallest values) and it is likely that by 12-months, mice were not old enough to detect a robust effect. This could indicate a reduction in expression of eGFP-L10a with age or selective vulnerability of eGFP-L10a-expressing neurons to degeneration. eGFP-L10a-expressing neurons accounted for less than 10 % of all TH-positive neurons in this TRAP model, so specific loss of this subpopulation may explain the lack of difference observed in stereological count estimates.

Dopaminergic innervation of the striatum will also be measured by TH-positive densitometry. This will quantify the change in innervation due to age and overexpression of human *α* -synuclein. Dorsal and ventral regions of the striatum will be considered separately. If the change in innervation with age, genotype or region is greater than the change in neuronal number in the midbrain, results from axonal TRAP data will need to be interpreted with respect to this. For example, expression of *Nmnat2* increased in somal and axonal TRAP samples with age: This could indicate a greater requirement for protection from axonal degeneration. A reduction in striatal innervation in excess of cell body loss would support this hypothesis.

Ultrastructural evaluation of eGFP-L10a expression in striatum

The presence of ribosomes in the axoplasm of striatal dopaminergic neurons has not been visually confirmed. eGFP signal in cell bodies or axons of the striatum was not detectable by immunohistochemical staining during the original characterisation of the *ROSA26-eGFP-L10a::DAT-Cre* mouse model (Wagner 2017). Ultrastructural analysis of striatal dopaminergic neurons is now underway. A double-labelling approach will be used to confirm the presence of eGFP-L10a-containing ribosomes

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in TH-positive axons. This will also permit an assessment of the presence of eGFP-L10a in TH-negative axons.

Visualisation of transcript localisation to the axon by single molecule fluorescent *in situ* hybridisation

Single molecule fluorescent *in situ* hybridisation (smFISH) can be used to label individual transcripts, using probes directed at specific regions of a gene, such as exons, UTRs and introns (Yang, Titlow, et al. 2017). Dopaminergic neurons were found to change their expression profile according to cell compartment, age and genotype, including the expression of alternative dominant transcripts. Examples presented in 6 demonstrated compartmental transcript usage in *Rabgap1l* and *Oxr1*. The axonal specification of these, and other genes needs to be confirmed through visualisation. smFISH sections can be immunolabelled in parallel: Probes targeted at the axon-dominant 3' UTR of *Rabgap1l* or *Oxr1* can be combined with anti-eGFP labelling to test for colocalisation of these transcripts to the ribosome of axons. smFISH is a quantitative method. The age-dependent expression of genes with clear axonal importance can be verified, such as in the case of increased *Nmnat2* expression with age.

6.2 Expansion of Results

Functional characterisation of dopaminergic-specific genes

Enrichment analysis of somal and axonal TRAP samples revealed many genes that are expressed by dopaminergic neurons with high specificity. This information guided the selection of candidate causal genes found within windows of linkage disequilibrium with known PD GWAS variants. For a selection of these genes, there is little to no association of their function with dopaminergic neurons, yet evidence for their role in cell types in other regions of the body or diseases draws close

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parallels with PD biology. A particular example is the calcium sensing receptor, CASR, as highlighted in 4.

To define a role for the calcium sensing receptor in dopaminergic neurons, *Casr* expression and function need to be modulated. A mouse model has been developed to conditionally knockout *Casr* in cell types expressing Cre recombinase (Toka et al. 2012). In *Casr^{lox}* mice, *loxP* sites flank exon 3, which is contained within all four transcripts expressed in this dataset, confirming complete targeting. *Casr^{lox}* mice could be crossed with DAT^{IRES^{cre}} mice to conditionally knockout expression in dopaminergic neurons. Depending on the investigative approach, a triple transgenic line could be generated containing the Rosa26^{fsTRAP} allele to enable the transcriptomic study of gene expression in dopaminergic neurons as a result of loss of CASR. Alternatively, or in combination, the *Casr^{lox}::DAT^{IRES^{cre}}* mice could be crossed with a transgenic model of PD, such as the *SNCA*-OVX line, to study the pathological effects of overexpression of human α -synuclein without CASR activity. There is evidence to support a pore-forming role for α -synuclein, allowing the influx of Ca²⁺ and causing Ca²⁺-mediated excitotoxicity (Zaichick et al. 2017; Angelova et al. 2016; Danzer et al. 2007; Di Scala et al. 2016).

The process of generating and studying the effects of conditional knockout of a dopamine-relevant gene has been demonstrated by our laboratory, through conditional knockout of *Atg7* in mouse dopaminergic neurons (Hunn et al. 2019). ATG7 is an essential protein in the induction of macroautophagy. Conditional knockout of *Atg7* in *SNCA*-OVX mice enabled the study of the interaction between impaired macroautophagy and human α -synuclein overexpression. Mice convincingly demonstrated a loss of SNpc dopaminergic neurons, a reduction in dopamine uptake in the caudate putamen and accumulation of protein aggregates. *Atg7* was one of the most abundant genes in axonal TRAP samples, with greater expression compared to the cell body and elevated expression in age, indicating an important

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role for ATG7 in the axon. Such a role has been demonstrated in Purkinje neurons, where loss of ATG7 expression induced axonal swelling and degeneration of axon terminals (Komatsu et al. 2007). This illustrates (in reverse) how the use of cell type specificity data can be used to inform candidate gene selection for further investigation, as in the case of *Casr*.

Ultimately, the investigation of dopaminergic-specific genes needs to translate into human models. Induced pluripotent stem cells (iPSC) have become a valuable tool for modelling PD (Caiazza et al. 2020). By reprogramming patient fibroblasts into iPSCs, the effects of disease-relevant mutations can be studied and compared with healthy controls. A range of phenotypic assays can be used to discern the cellular components and mechanisms affected. In this example, the role of CASR in dopaminergic Ca^{2+} handling could be studied. To modulate the activity of CASR, positive and negative allosteric modulators could be used. Calcimimetics, such as cinacalcet and calcilytics, such as Calhex-231 could be administered and the effects on organelle-specific Ca^{2+} flux imaged using genetically encoded calcium indicators (Suzuki et al. 2016). To study the effects of transcriptional repression of *CASR*, CRISPR interference could be used (Gilbert et al. 2014). This platform can be knock down arrays of target genes, enabling parallel phenotypic screening. Each selected candidate GWAS gene from 4 could be tested across a series of assays relevant to neuronal function.

Spatial transcriptomics

While transcriptomic methods enable the measurement of thousands of different genes in parallel, spatial contextual information is lost. For expression studies in tissue containing mixed cell populations, this creates a problem of averaged expression and variable cell type proportions between samples. Methods, such as TRAP and single cell RNA sequencing were developed to overcome these limitations, yet

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without spatial information, there is still uncertainty over whether a measurement derives from a known healthy or diseased region or cell type.

This uncertainty is a limitation of the present work: SNpc and VTA dopaminergic neurons were not separable and therefore expression measurements from somal TRAP samples represent an averaged mixture of both populations.

Spatial transcriptomics uses arrays of location-barcoded reverse transcription primers to synthesise cDNA from tissue sections *in situ* (Ståhl et al. 2016). After reverse transcription, the cDNA is cleaved from the array, amplified by PCR and sequenced as a standard RNA sequencing library. Using barcode information, the original location of each sequenced read can be traced to its origin, building a cell/population/region-specific map of expression.

Spatial transcriptomic sequencing of brains from young and aged, *SNCA*-OVX and wild-type mice is now underway. This method will overcome the spatial limitations of TRAP data and will provide sufficient resolution to not be affected by changes in cell type proportion due to age or genotype.

6.3 Collation

Public exploration of dopaminergic expression properties

To maximise the utility and reach of the data from this study, results were collated and summarised for presentation on a website. Users may query the expression status of a gene or genes of interest. The expression measurements are dynamically plotted for each comparison and the estimated fold change and P value are supplied. To enable comparison with other datasets relevant to PD, results from RNA sequencing experiments in iPSC-derived dopaminergic neurons are also presented (Lang et al. 2019; Zambon et al. 2019; Sandor et al. 2017). Over time, this website will present complementary data from follow up investigations, such as the results of spatial transcriptomic sequencing.

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The results or the interpretation of the data from this study may develop over time. For example, in the somal TRAP comparison of ageing, over 80 genes were not included in a network analysis of protein-protein interactions, because there was no available evidence. Members of this group are still likely to be important in ageing, as in the example of Secretogranin-2 upregulation. Over time, interaction data will improve and become more specific to the tissue or cell type studied. An aim for this website is to continually integrate novel interaction data.

6.4 Considerations

Specificity of eGFP-L10a expression in the striatum

The challenge of capturing of dopaminergic eGFP-L10a in striatal tissue was signal-to-noise ratio. Measurement of striatal eGFP content confirmed expression of eGFP-L10a, however the level was 10-20 times lower than in the midbrain (Figure 3.2 A). eGFP was also detected in neighbouring cortical tissue at comparable levels to the dorsal striatum. This indicated a potential mixture of sources of TRAP RNA. In somal TRAP samples, there may be an equivalent level of ectopic eGFP-L10a expression, however the greater abundance of eGFP-L10a in the cell bodies of dopaminergic neurons increased the signal to noise ratio.

Dopaminergic marker genes were consistently enriched and represented in axonal TRAP samples, confirming (indirectly) eGFP-L10a expression in the axons of these neurons. Some markers of non-dopaminergic cells were mildly enriched in line with expected values for non-rare cell types, however (Dougherty, Schmidt, et al. 2010). In keeping with this, nearly one quarter of axonal libraries were composed of genes exclusively enriched by TRAP in the striatum. It is conceivable that axons may uniquely translate genes not required in the cell body, however this percentage was larger than expected.

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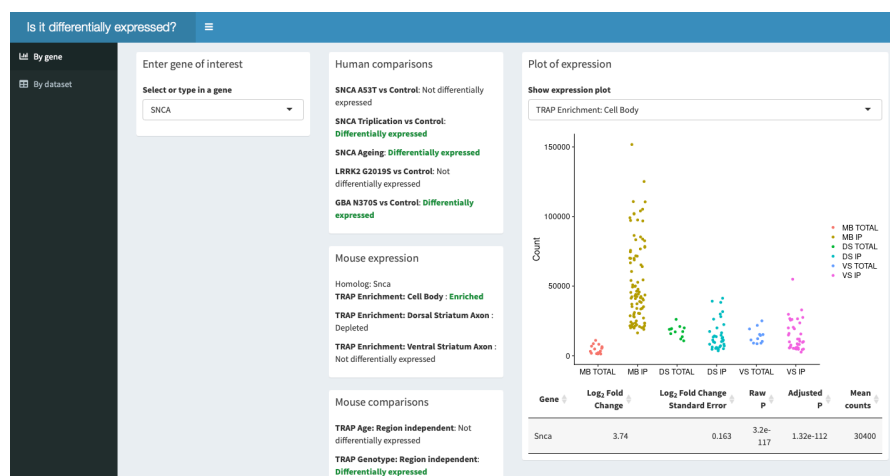


Figure 6.1: A resource for the exploration of dopaminergic gene expression data. Users may query TRAP data to identify the conditions in which a gene of interest is differentially expressed or all genes that are differentially expressed in a comparison.

Cre transcripts were detectable and enriched in axonal TRAP samples. Cre transcript enrichment in somal TRAP samples was expected, as this compartment includes the nucleus as the site of Cre action. In the absence of evidence for an axonal role for Cre recombinase activity however, striatal expression can only be assumed to derive from cell bodies. The detection of Cre transcript enrichment in axonal TRAP samples suggests constitutive regional low-level expression in adult mice. Transient Cre expression during development or in the germline could also contribute to the ectopic expression of eGFP-L10a in adults (Song and Palmiter 2018). Shigeoka et al. (2016a) used two Cre-responsive reporter mice to confirm restricted expression of the RiboTag (*HA-rpL22*) allele to retinal ganglion cell axons. Rosa26-StopLox-LacZ mice label the cell bodies of Cre-expressing cells and their progeny, while Rosa26-StopLox-TauLacZ mice label the axons. Because of the dense axonal bush formed by dopaminergic neurons in the striatum, cell body labelling using Rosa26-StopLox-LacZ mice may be best suited to visualising the cell type specificity of Cre expression.

If developmental Cre expression is responsible for ectopic eGFP-L10a expression in the striatum, viral induction could be performed as an alternative. Adminis-

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tration of a Cre-dependent adeno-associated virus expressing eGFP-L10a has been successfully used to isolate TRAP RNA from dopaminergic neurons of the VTA (Nectow, Moya, et al. 2017; Nectow, Ekstrand, et al. 2015). The authors also demonstrated successful administration of a retrograde tracing virus that labelled projection-specific inputs. Use of a retrograde tracing virus in the dorsal or ventral striatum of *DAT-Cre* mice could improve regional selectivity, circumventing the need to manually dissect tissue. The dopaminergic expression of eGFP-L10a in viral-TRAP may be lower than in *ROSA26-eGFP-L10a::DAT-Cre* mice, however. Nectow, Moya, et al. (2017) reported yields of ~ 9 ng per sample which is 3-5 times less than obtained from somal TRAP samples in this study.

DAT-Cre-driven reporter expression in clusters of non-dopaminergic neurons has recently been described (Papathanou et al. 2019). Reporter expression was identified within the amygdala, mammillary subnuclei, septum and habenula. Projections were traced from the amygdala to the nucleus accumbens core and the bed nucleus of the stria terminalis 6.2. Cre-activated reporters colocalised with markers of glutamatergic and GABAergic neurons. These findings, coupled with the greater detected eGFP content in ventral striatal samples, suggests that amygdaloid glutamatergic or GABAergic neurons are a source of ectopic eGFP-L10a expression in *ROSA26-eGFP-L10a::DAT-Cre* mice.

Library Preparation

The library preparation method used for cohort 3 was necessary to reverse transcribe RNA from the minimal quantities (< 0.2 -1 ng) obtained in each striatal TRAP sample. This “ultra-low input” (ULI) technique is regularly used in droplet-based single cell RNA sequencing. A larger quantity of RNA was used with the Smart-Seq2 library preparation method for cohorts 1 and 2 and an equivalent template-switching method developed by Oxford Nanopore Technologies was used

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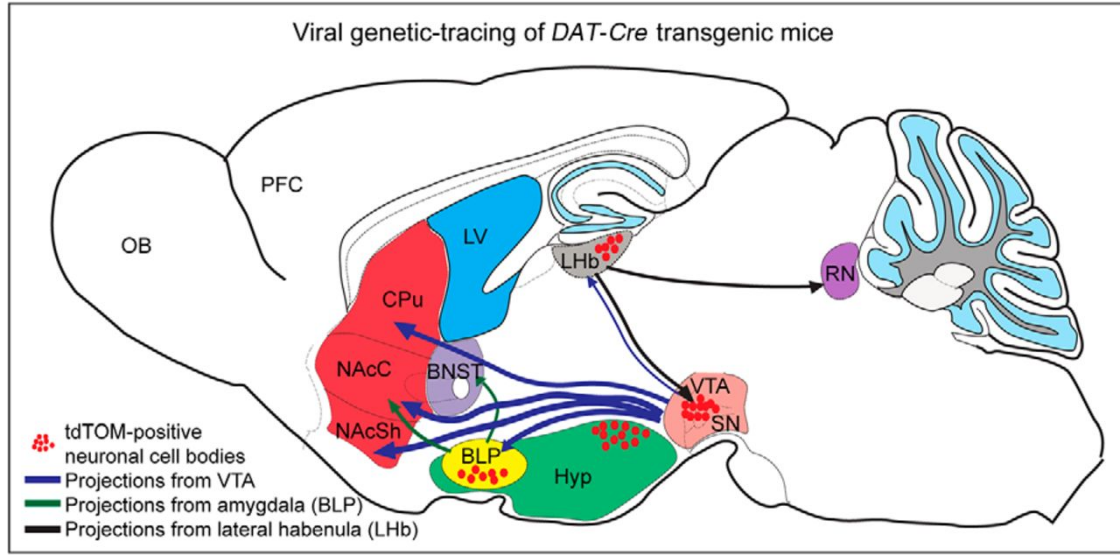


Figure 6.2: Schematic of projections from amygdala (BLP) and lateral habenula (LHB) identified by viral tracing in DAT-Cre mice. VTA injections confirmed projection to the striatum. It is likely that eGFP-L10a expression in projections from the amygdala contribute to axonal TRAP RNA. CPu, caudate putamen; LV, lateral ventricle; NAcSh, NAc shell; OB, olfactory bulb; PFC, prefrontal cortex; RN, raphe nucleus; SN, substantia nigra Hyp, Hypothalamus; NAcC, NAc core. Source: (Papathanou et al. 2019).

for long-read library preparation. Dropout in ULI samples affected all but around 2500 genes, increasing dispersion estimates and hampering sensitivity.

The actual library complexity of TRAP RNA from cohort 3 was not different to samples from cohorts 1 and 2. This was demonstrated by the matched preparation of long-read libraries from 12 somal TRAP samples. It was remarkable that gene expression estimates from long-read sequenced data correlated more strongly with cohort 1 and 2 samples than with their matched ULI samples from cohort 3.

A recent study compared three protocols used for low input RNA sequencing library preparation (Wang, Rieder, et al. 2019). They found that libraries produced from over 1000 cells were consistent between technical replicates and library preparation methods. Inputs of 100 cells were highly variable, with poor correlation to 1000 cell samples of the same population ($R^2 = 0.6$; similar to the values obtained when short- and long-read libraries of somal TRAP samples from cohort 3 were compared). The ULI preparation method advises a minimum input of 10 pg RNA.

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The expected yield of RNA from a single cell is typically ~10 pg (Wang and Navin 2015). 200 pg was used for library preparation in cohort 3 samples.

Dropouts are an endemic property of current single cell RNA sequencing datasets and strategies that attempt to account for this technical variation have been developed (Tracy et al. 2019; Duò et al. 2020; Saelens et al. 2019; Risso et al. 2018). A recent approach has resorted to converting expression matrices from single cell datasets to binary measurements for clustering purposes (Qiu 2020).

In future, pooling small quantities of RNA to reach the equivalent RNA content of ~1000 cells (1 ng) would be recommended; at the cost of replicate number, but gain of detection sensitivity.

Cell type specificity as an estimator of disease relevance and a conclusion

This study was designed to identify expression features that are most specific to dopaminergic neurons. This aim was guided by the hypothesis that genes or transcripts most specific to the dopaminergic neuron are more likely to play a causal role in PD.

In a large meta-analysis of tissue specific gene expression, preferential tissue-level expression of causal genes in disease was found in a third to half of over 1000 diseases tested, including PD (Lage et al. 2008). The study of “heritability enrichment” has gained increasing interest with the availability of public, genotyped, tissue-specific datasets (Finucane et al. 2018).

Tissue-specific enrichment analysis of the GWAS loci identified by Nalls, Blauwendraat, et al. (2019) found that many loci were heavily enriched with genes expressed preferentially in the brain. Dopaminergic neurons were the most significantly implicated cell type from a meta-analysis of single cell datasets in the same study. A single-nuclei sequencing dataset has since applied linkage disequilibrium score regression and a method for gene-set analysis of GWAS data to identify the cell types with greatest association to PD risk (Agarwal et al. 2020): Dopaminergic

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neurons were significantly associated. Tissue-specific datasets are now giving way to single-cell or single-nuclei datasets that facilitate more specific profiling of individual cell types (Bryois et al. 2020).

TRAP datasets may have a very considerable advantage over tissue-specific and single cell datasets for these investigations; sensitivity. *CASR* was robustly enriched in somal and axonal TRAP samples across all three datasets, providing confidence that it is translated by dopaminergic neurons. It is not highly expressed, however and approaches the limits of detection in TOTAL samples. The levels of expression of genes such as *CASR* are currently too low for reliable detection in single-cell datasets.

6.5 Conclusions

The work described in this thesis has demonstrated how cell type-specific profiling can provide highly sensitive measures of gene expression that are specific to the target cell population. It has provided an insight into the expression properties of the axonal translome, with examples of compartmental specialisation, based on gene and transcript-level expression.

Through the use of novel PD GWAS loci, several putative novel PD genes were identified. They will require functional follow-up which will inform on the validity of somal and axonal TRAP data going forward. The collation of TRAP results into a public access dataset will hopefully aid researchers to ask straightforward questions about the role of a gene or transcript in dopaminergic function.

Appendices

.1 Session Info

```
## R version 4.1.1 (2021-08-10)

## Platform: x86_64-pc-linux-gnu (64-bit)

## Running under: Ubuntu 20.04.3 LTS

##

## Matrix products: default

## BLAS:   /usr/lib/x86_64-linux-gnu/blas/libblas.so.3.9.0

## LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.9.0

##

## locale:

##  [1] LC_CTYPE=en_GB.UTF-8      LC_NUMERIC=C
##  [3] LC_TIME=en_GB.UTF-8      LC_COLLATE=en_GB.UTF-8
##  [5] LC_MONETARY=en_GB.UTF-8  LC_MESSAGES=en_GB.UTF-8
##  [7] LC_PAPER=en_GB.UTF-8     LC_NAME=C
##  [9] LC_ADDRESS=C             LC_TELEPHONE=C
## [11] LC_MEASUREMENT=en_GB.UTF-8 LC_IDENTIFICATION=C

##

## attached base packages:

## [1] stats      graphics  grDevices  datasets  utils      methods    base

##

## other attached packages:

##  [1] cowplot_1.1.1    ggpubr_0.4.0    kableExtra_1.3.4 forcats_0.5.1
##  [5] stringr_1.4.0    dplyr_1.0.7     purrr_0.3.4      readr_1.4.0
##  [9] tidyr_1.1.3      tibble_3.1.2    ggplot2_3.3.4    tidyverse_1.3.1
## [13] knitr_1.33

##

## loaded via a namespace (and not attached):
```

## [1]	nlme_3.1-152	fs_1.5.0	lubridate_1.7.10	RColorBrewer_1.1-2
## [5]	webshot_0.5.2	httr_1.4.2	rprojroot_2.0.2	tools_4.1.1
## [9]	backports_1.2.1	utf8_1.2.1	R6_2.5.0	vipor_0.4.5
## [13]	mgcv_1.8-36	DBI_1.1.1	colorspace_2.0-1	withr_2.4.2
## [17]	gridExtra_2.3	tidyselect_1.1.1	curl_4.3.1	compiler_4.1.1
## [21]	cli_2.5.0	rvest_1.0.0	xml2_1.3.2	labeling_0.4.2
## [25]	bookdown_0.22	scales_1.1.1	ggridges_0.5.3	systemfonts_1.0.2
## [29]	digest_0.6.27	foreign_0.8-81	rmarkdown_2.9	svglite_2.0.0
## [33]	rio_0.5.27	pkgconfig_2.0.3	htmltools_0.5.1.1	dbplyr_2.1.1
## [37]	rlang_0.4.11	readxl_1.3.1	rstudioapi_0.13	farver_2.1.0
## [41]	generics_0.1.0	jsonlite_1.7.2	zip_2.2.0	car_3.0-10
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