

Molecular mechanisms relevant to the maintenance of locomotor control



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Declaration

I hereby declare that the work presented in this thesis is my own unless otherwise stated. This thesis has not been submitted to anywhere for any degrees or professional qualification except to The University of Oxford for the degree of Doctor of Philosophy. All the results presented are the results of my own work.

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Abstract

The striatum, consisting of enkephalin-positive (*Penk*⁺) and substance P-positive subpopulations of medium spiny neurons (MSNs), integrates and drives information and function of the basal ganglia circuits, which is crucial for maintaining normal movement. Preferential degeneration of the *Penk*⁺ MSNs has been observed at early stage of Huntington disease (HD). Recently, selective deletion of TrkB, high affinity receptor of brain derived neurotrophic factor (BDNF), specifically in the *Penk*⁺ MSNs (*Trkb*^{PENK-KO}) led to an age-dependent spontaneous hyperlocomotion, but not to neuronal loss, indicating that TrkB is essential to maintain normal locomotor control. However, the underlying molecular mechanism still remains unknown. Dissecting the molecular changes induced in the *Penk*⁺ MSNs in the context of absence of TrkB is imperative to understand mechanism of normal locomotor maintenance. We, therefore, have performed transcriptomics analysis by next generation sequencing (RNA-seq) on the purified *Penk*⁺ MSNs with and without ablation of TrkB at two ages, 3-months-old (3M) presymptomatic stage, and 8-months-old mouse (8M) when the hyperlocomotor phenotype is established. Subsequent bioinformatics analysis has revealed early energy metabolism and mitochondrial dysfunction specifically to the *Penk*⁺ MSNs, leading to the severe energy deficiency at later stage. We have then validated hits from the RNA-seq data by optimising comprehensive validation approaches. Interestingly, the bioinformatics analysis led to the discovery of dopamine dysregulation in mutants at 3M of age that could contribute to an age-dependent hyperlocomotor phenotype. Amongst the hits we validated, *Gsto2*, an enzyme involved in the glutathione metabolism, was identified as a key gene with unique expression pattern driving transcriptomic changes of the *Penk*⁺ MSNs. Furthermore, by creating a Cre-dependent *Gsto2* shRNA lentiviral vector, we selectively knocked down *Gsto2* expression in the *Penk*⁺ MSNs *in vivo* at presymptomatic age. Excitingly, this significantly rescued the hyperlocomotor phenotype of the *Trkb*^{PENK-KO} mice. These findings add knowledge to the molecular mechanisms underlying normal locomotor maintenance, and they also highlight *Gsto2* and related glutathione metabolism pathway as a potential target for the development of novel treatments to movement disorders.

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List of Abbreviations

AAO	age-at-onset
AChE	acetylcholine esterase
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AP	Anterior-Posterior
A β 42	A β peptide (1–42)
BAC	bacterial artificial chromosome
BDNF	brain-derived neurotrophic factor
BF	basal forebrain
BG	basal ganglia circuits
CA1	Cornu Ammonis area 1 of hippocampus
CaRE	Ca ²⁺ response elements
CCK	cholecystokinin
ChAT	choline acetyltransferase
CREB	Cyclic AMP responsive element binding protein
D1R	dopamine (DA) 1-receptor
D2R	DA2-receptor
DA	dopamine
DAG	diacylglycerol
DE	differential gene expression analysis
DEGs	differential expressed genes
dMSNs	direct pathway MSNs
DOPAC	3,4-Dihydroxyphenylacetic acid
DOR	δ -opioid receptor
DPRs	dipeptide repeat proteins related to C9orf72
DV	Dorsal-Ventral

EC	entorhinal cortex
EC-II	entorhinal cortex layer II
ENK	enkephalin
ERK	extracellular signal-regulated kinase
FACS	fluorescence-activated cell sorting
FRS2	fibroblast growth factor receptor substrate 2
FUS	RNA-binding protein fused in sarcoma
GAB1	GRB-associated binder 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
<i>Gcc1</i>	GRIP And Coiled-Coil Domain Containing 1
GFAP	glial fibrillary acidic protein
GO	gene ontology
GPe	globus pallidus pars externa
GPi	globus pallidus pars interna
GRB2	growth factor receptor-bound protein 2
GSEA	gene set enrichment analysis
GST	glutathione S-transferase
<i>Gsto2</i>	Glutathione S-Transferase Omega 2
HD	Huntington's Disease
HPLC	High performance liquid chromatography
<i>HTT</i>	human huntingtin gene
HVA	homovanillic acid
iMSNs	indirect pathway MSNs
MAPK	Ras-mitogen-activated protein kinase
MEK	MAPK/ERK kinase
mGluR	metabotropic glutamate receptor
<i>mHTT</i>	mutant HTT
ML	Medial-Lateral
MNs	motor neurons

MOR	μ-opioid receptor
MSNs	medium spiny neurons
NAc	nucleus accumbens
NMDA	N-methyl D-aspartate receptor
NDDs	neurodegenerative diseases
NR2B	N-methyl D-aspartate receptor subtype 2B
OF	open field test
OX-PHOS	oxidative phosphorylation
<i>Pace4/Pcsk6</i>	Proprotein Convertase Subtilisin/Kexin Type 6
PCA	primary component analysis
PCR	Polymerase chain reaction
PCs	proprotein convertase
PD	Parkinson's disease
PDPK1	3-phosphoinositide-dependent protein kinase 1
Penk	Pro-enkephalin
<i>Penk</i> -/ENK-	enkephalin negative
<i>Penk</i> + /ENK+	enkephalin positive
PFA	Paraformaldehyde
PI3K	phosphatidylinositol 3-kinase
PLCγ	phospholipase Cγ
polyQ	polyglutamine
PtdIns(4,5)P ₂	phosphatidylinositol-4,5-bisphosphate
RNA-seq	next generation sequencing/ transcriptome sequencing
RPKM	reads per kilobase of exon model per million mapped reads
RSK	ribosomal protein S6 kinase
SHP2	SRC-homology phosphatase 2
shRNA	short hairpin RNA
SIDS	sudden infant death syndrome
smFISH	single molecule fluorescence in situ hybridization

SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SODs	superoxide dismutases
SOS	son of sevenless
SP	substance P
SST	somatostatin
STN	subthalamic nucleus
STR	striatum
Tau	microtubule-associated protein tau
TCA-ETC	tricarboxylic acid cycle/Electron transport chain
TDP-43	TAR DNA-binding protein 43
TEC	transentorhinal cortex
TF	transcription factors
TGN	trans-Golgi network
TH	tyrosine hydroxylase
TrkB	Tropomyosin receptor kinase B
<i>Trkb</i> ^{PENK-KO}	cohorts of mutants carrying Trkb deletion in enkephalinergic striatal neurons
<i>Trkb</i> ^{PENK-WT}	cohorts of controls mice
VTA	ventral tegmental area
WT	Wild type
YAC	yeast artificial chromosome

Chapter 1: Introduction

1.1 The physiology underlying locomotor behaviors

1.1.1 *Basal ganglia circuits, two theoretical pathways, and the dopaminergic system*

The ability to move through the environment to obtain energy, reproduce, and escape predators is the fundamental function for animal's survival. Control of these locomotor behaviors is executed and coordinated by networks of neurons from various regions, which are phylogenetically conserved through the brain and spinal circuitry¹⁻³. From the 1970s, the basal ganglia (BG) circuits have been believed to integrate projections from diverse portions of the cortex and project this information, via the thalamus, to the motor cortex and supplementary motor area^{1,4,5}. This scheme has provided a mechanism by which movement initiation could originate from diverse functional areas, which is fundamental to understand locomotor behaviors.

The basal ganglia circuits involve a number of nuclei within the brain and their main function is to control voluntary movement. In addition, the basal ganglia has been involved not only in the locomotor function, but also in non-locomotor functions such as mood and reward-related behaviour⁶. For locomotor function *per se*, the basal ganglia are hypothesized to contribute to the selection and execution, the control of involuntary movement, but not the initiation⁷.

Canonically, the BG consists of two pathways that separate at the level of the

striatum, the main input nucleus of the BG. More specifically, distinct basal ganglia circuits forming the so-called direct and indirect pathways, originate in the dorsal striatum from distinct population of medium spiny projection neurons (MSNs)^{8 9 10}. Firstly, the striatal MSNs involved in the direct pathway (also called striatonigral pathway) receive the glutamatergic input from the cortical activation, and these GABAergic neurons then project to the substantia nigra pars reticulata (SNpr) and the globus pallidus pars interna (GPi) directly. The inhibitory input on the GABAergic neurons in SNpr and GPi leads to a disinhibition of the thalamic glutamatergic neurons, which then project to the thalamus. This chain of events collectively results in locomotor activation or movements. **(Figure 1.1.1)**

On the other hand, similar to the direct pathway, the striatal MSNs involved in the indirect pathway (also called striatopallidal pathway) also receive glutamatergic input from the cortical activation, but instead of projecting directly to the SNpr/GPi, these GABAergic neurons project indirectly to the SNpr via the globus pallidus pars externa (GPe) and the subthalamic nucleus (STN). The STN glutamatergic neurons firstly receive disinhibition from the GPe, and in turn activates the SNpr GABAergic neurons projecting to the thalamus. Consequentially, this indirect pathway decreases cortical premotor drive and inhibit movement^{9,10}. **(Figure 1.1.1)**

Parallel to the direct/indirect pathways, another key contributor involved in locomotor behaviors is the dopaminergic system (also called dopaminergic pathways or dopaminergic projections), which consist of sets of dopaminergic neurons that

synthesize and release the neurotransmitter dopamine at their projection targets¹¹. Defined by the source regions to generate dopamine projecting to the target regions, there are six major dopaminergic pathways and five other pathways, which are mainly originated from two distinct regions, the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA), formed mainly by dopaminergic neurons¹²⁻¹⁴. The two main dopaminergic pathways generated separately from these two regions, the nigrostriatal and mesolimbic pathways respectively, send dopamine input into the striatum integrating with basal ganglia circuitry to form a sequence of pathways known as the cortico-basal ganglia-thalamo-cortical loop^{15,16}.

The fuel of dopamine from VTA/SNpc boosts the activation of striatal MSNs expressing differential dopamine (DA) receptors. Moreover, MSNs can be grouped into two main subtypes based on differentially expressed DA receptors. D1 DA receptors are expressed by direct pathway MSNs (dMSNs), whereas D2 receptors are expressed by indirect pathway MSNs (iMSNs)^{17,18} (**Figure 1.1.1**). Therefore, these two subtypes of MSNs support two distinct pathways, respectively.

To summarize, these two theoretical pathways (direct/indirect pathways), regarded as canonical model composes the most important parts in the basal ganglia circuits. And by integrating with the dopaminergic and glutamatergic pathways, they established a sophisticated balance to maintain the normal locomotor behaviors⁸.

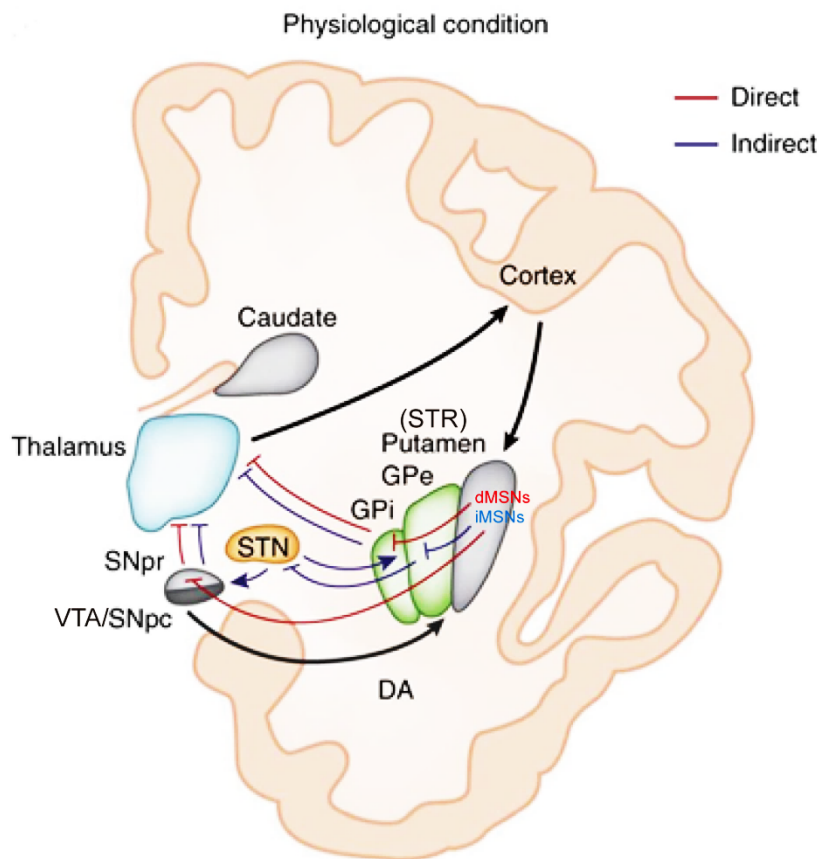


Figure 1.1.1 Diagram to indicate the canonical view of the direct/indirect pathway mode. Modified and adapted from: ⁸.

The direct striatonigral pathway activity induces disinhibition of excitatory thalamocortical projections, and facilitation of movement. The indirect striatopallidal pathway induces inhibition of thalamocortical projection neurons, which decrease cortical premotor drive and inhibit movement^{9 10}. In the physiological condition, DA arising from the VTA/SNpc is thought to activate D1-expressing striatal MSNs (dMSNs) of the direct pathway (red lines) and to inhibit D2-expressing striatal neurons (iMSNs) of the indirect pathway (blue lines). The output nuclei, GPi and SNpr, project to the thalamus, which in turn sends efferents that complete the cortico-basal ganglia-thalamo-cortical loop.

1.1.2 The striatum, and enkephalinergic MSNs

As mentioned above, the striatum gathers the glutamatergic input from the cortex and thalamus, and then projects to the downstream component nuclei of the BG. In addition, the striatum is innervated by the dopaminergic input from SNpc and VTA. Therefore, as the largest component and primary afferent structure of the BG, it has been intensively studied in both physiological and pathological conditions. In some mammals including the primates, the striatum (also known as the neostriatum) is divided into two structures, the caudate nucleus and the putamen (**Figure 1.1.1**). Actually, the anatomy of the striatum is much more complex than this. Briefly, the dorsomedial and dorsolateral regions of the striatum receive afferent projections from frontal and parietal associated cortices, and sensorimotor cortices, respectively. Whereas, the ventral striatum, largely comprised of the nucleus accumbens (NAc), receives projections from limbic structures, including the amygdala, hippocampus as well as the medial prefrontal and anterior cingulate cortices^{4,19}. This topography is then proposed to confer dissociable functions to each of the striatal subregions, allowing them to dynamically and adaptively control the flow of information to motor output systems, resulting in facilitation or inhibition of locomotor behaviors^{20,21}.

Within the organisation of the striatum, more subtle structures are identified microscopically as two neurochemically discrete compartments, the striosome (also known as the patch) and the matrix^{22,23}. Striosomes have been defined histochemically as expressing high levels of μ -opioid receptor (MOR), substance P (SP), dopamine

(DA) 1-receptor (D1R), calretinin, Nr4a1, pro-dynorphin, GAD-2, and EGR-1. The extrastriosomal matrix by contrast, is enriched with calbindin, somatostatin (SST), enkephalin (ENK), DA2-receptor (D2R), and cholinergic markers including acetylcholine esterase (AChE) and choline acetyltransferase (ChAT)²⁴. Inputs to patch neurons are mainly from the prelimbic and frontal cortices^{22,23}, while matrix neurons receive input projections from both the cortex and the thalamus²²⁻²⁴.

Interestingly, the striatum is mainly composed of GABAergic neurons, including a large population of principal cells (95%, MSNs²²) and some small interneuron population (iNs), and additionally a small population of giant cholinergic interneurons (iNs)¹⁰. The MSNs can then be further divided in two different subtypes, based on their gene expression and axonal projections^{17,25}, which give rise to the striatonigral MSNs (dMSNs) and the indirect striatopallidal MSNs (iMSNs) related pathways as mentioned above (**Figure 1.1.1**). Specifically, the dMSNs exhibit high expression of dopamine D1 receptor, muscarinic M4 receptors and substance P, and project directly to the internal globus pallidus (GPi) and substantia nigra pars reticulata (SNpr). In contrast, the iMSNs exhibit high expression of dopamine D2 receptor, adenosine A2A receptors and enkephalin, and send axons to the external globus pallidus (GPe)^{10,17,25}.

The canonical differences between the two fundamental subtypes of striatal MSNs have been summarized in **Table 1.1.2**. This orchestrated cellular and molecular differences facilitate coordinated functions of locomotor behaviors exerted by both direct and indirect pathways of BG circuits.

Table 1.1.2 Basic characteristics of the striatonigral and striatopallidal MSNs.

	Striatonigral MSNs	Striatopallidal MSNs
Neuropeptide produced	Substance P; Dynorphin	Enkephalin
Type of dopamine receptor expressed	D1 receptor	D2 receptor
Other receptors expressed	Muscarinic M4 receptor	Adenosine A2a receptor
Axonal projection	Substantia nigra pars reticulata (SNpr) and internal portion of globus pallidus (GPi)	External portion of globus pallidus (GPe)
Function	Facilitation of the initiation and execution of voluntary movement (direct pathway)	Suppression of unwanted movement (indirect pathway)
Striatal compartment	Patch and matrix	Patch and matrix

In particular, the indirect pathway MSNs selectively express the opioid peptide enkephalin (ENK) having highly conserved function across most species like birds, fish and mammals^{26,27}. The receptors for ENK are the δ -opioid receptor (DOR) and the μ -opioid receptor (MOR). Interestingly, as described before as well, ENK expression is found more enriched in the subcompartment of striatal matrix²⁸, whereas the patch compartment occupies 10%–20% of the striatal volume and expresses high levels of MOR receptor, which is relatively absent from the surrounding matrix²⁹⁻³¹. Besides, ENK is likely the primary endogenous ligand for striosomal MORs, since beta-endorphin is absent in the striatum³², and another ENK receptor DORs are uniformly expressed across the striatum. Moreover, it has been reported that ENK

suppresses inhibition onto MSNs selectively in patches, thereby disinhibiting their firing in response to cortical input³³. Therefore, it would further emphasize the important role of ENK signaling and enkephalinergic MSNs in the function of the striatum under physiological conditions. Clinically, preferential loss of these enkephalinergic MSNs decreases the amount of inhibitory control over unwanted movements leading to chorea and hyperkinesia, typically associated with basal ganglia (BG) disorders such as Huntington's Disease (HD)^{34 35}, which will be further described below.

1.2 The selective vulnerability underlying neurodegeneration

1.2.1 *Neurodegenerative diseases and cell-type specific degeneration*

The mechanisms underlying neurodegenerative diseases (NDDs), the most challenging mystery for hundreds of years, are still largely remaining unknown. Although phenotypically these NDDs appear quite heterogeneous in clinical manifestations³⁶, one fundamental feature shared commonly by NDDs is selective vulnerability of specific type of neurons. It is not clear why some specific type of neurons and brain regions are affected at the onset, whereas the neighboring cells and regions are not^{37,38}. More specifically, the two general characteristics of NDDs derived from this issue are the proteinopathy associated with the disease only affecting particular neurons of target brain region, and the proteinopathy worsening with time and involving more regions in a stereotypical and predictable fashion. Here the four canonical NDDs are briefly summarized as below to clearly demonstrate this selective vulnerability.

To begin with, Alzheimer's disease (AD) is characterized by two major pathological hallmarks: accumulation of the A β peptide (APP, the main constituent of amyloid plaques) and formation of neurofibrillary tangles (NFT, aggregates of hyperphosphorylated tau proteins)³⁹. Selective vulnerability in early AD is more closely linked to tau pathology. Three main types of neurons that are vulnerable to the

accumulation of pathological forms of tau and that are lost early in the disease include large pyramidal neurons in layer II of the entorhinal cortex (EC), the subiculum, the CA1 region of the hippocampus^{40,41}, corticopetal cholinergic neurons in the basal forebrain^{42,43}, and noradrenergic neurons in the locus coeruleus^{44,45}. As to Parkinson's disease (PD), it is primarily linked to the selective and progressive loss of dopaminergic neurons in substantia nigra pars compacta (SNpc), whereas the similar dopaminergic neurons in the ventral tegmental area (VTA) demonstrate a much milder degeneration⁴⁶. And the proteinopathy of PD is attributed to the widespread aggregation of the α -synuclein protein (encoded by the *SNCA* gene)⁴⁷. Another typical example of this selective vulnerability is Huntington's disease (HD). The mutant Huntingtin protein selectively affects medium spiny GABAergic neurons (MSNs) in the striatum (STR), while large aspiny cholinergic interneurons and other striatal interneurons are relatively spared from degeneration in HD⁴⁸⁻⁵⁰. Moreover, amongst the two subtypes of striatal MSNs, the indirect pathway MSNs expressing D2R and ENK are particularly vulnerable in HD, whereas the direct pathway MSNs expressing predominantly D1R, substance P and dynorphin are relatively spared in the early stages of HD^{34, 35,49,50}. Apart from those brain nuclei involved above, peripheral nervous system also shows this same vulnerability as manifested in amyotrophic lateral sclerosis (ALS). In ALS, motor neurons (MNs) in spinal cord and in brainstem are selectively affected, and among these MNs, fast-fatigable MNs are particularly vulnerable, while slow MNs are least vulnerable^{37,48}.

To summarize, as listed in the **Table 1.2.1**, the characteristics for these NDDs

described above clearly shows that selective vulnerability exists commonly in neurodegeneration despite the respective complex pathogenesis each NDD shows. Substantial efforts have been made to investigate the possible underlying mechanism, such as protein supersaturation/metastable subproteome, protein homeostasis, calcium homeostasis, mitochondria and energy demand, and neurotransmitters and related receptors^{37,38}. In this thesis, I will focus on HD as an example to further discuss about the pathogenesis of the disease in the next section. Overall, elucidating this fundamental question of selective vulnerability will be spectacular to understand the pathophysiology of the neurodegenerative diseases and instrumental to revolutionize the related treatments.

Diseases	Protein aggregates	Early-affected regions	Early vulnerable neurons
AD	A β 42, Tau	LC, TEC, EC, BF, HP	Pyramidal neurons in EC-II & HP-CA1; cholinergic neurons in BF, noradrenergic neurons in LC
PD	α -synuclein	OB, DMV, SNpc	Dopaminergic neurons
ALS	TDP-43, SOD1, FUS, DPRs	MNC, SC, BS	Fast-fatigable motor neurons
HD	Huntingtin	STR	MSNs

Table 1.2.1: Regions and neurons selectively vulnerable in neurodegenerative diseases at onset. Table partially adapted from ³⁸.

AD, Alzheimer's disease; PD, Parkinson's disease; ALS, Amyotrophic lateral sclerosis; HD, Huntington's disease; A β 42, A β peptide (1–42); Tau, microtubule-associated protein tau; TDP-43, TAR DNA-binding protein 43; FUS, RNA-binding protein fused in sarcoma; DPRs, dipeptide repeat proteins related to C9orf72; TEC, transentorhinal cortex; BF, basal forebrain; EC-II, entorhinal cortex layer II; CA1, Cornu Ammonis

area 1 of hippocampus.

1.2.2 Huntington's Disease, its pathogenesis, and current animal models

As mentioned above as well, HD, as a typical example of NDDs, manifests the selective vulnerability within the striatum and results in abnormal locomotor movements, which is particularly important in this thesis. Thus, I will discuss more in detail about this disease here.

1.2.2.1 General information, clinical manifestation, and current solution

HD, described initially as Huntington's chorea, a typical movement disorder, is an autosomal dominant neurodegenerative disease. It is caused by the abnormal expansion of CAG repeats of the human huntingtin gene (*HTT*), which results in the production of a mutant HTT protein (*mHTT*) with an abnormally long polyglutamine repeat⁵¹. According to this CAG repeat, it has been shown that the number of CAG repeats in the general population is normally less than 27 repeats, whilst those with greater than 39 CAG repeats are certain to develop HD, and reduced penetrance is seen between 36 and 39 repeats^{52,53}. The onset of the disease is mid-adult life, ranging between 35 and 50 years in most patients although the production of *mHTT* has been found throughout life. As to epidemiology of HD, it has a prevalence of 10.6–13.7 individuals per 100,000 in Western populations, whilst Asian regions have a much

lower incident of 1–7 per million, which may be related to the genetic differences of the *HTT* gene⁵².

Diagnosis of HD is based on a confirmed family history or positive genetic test and the onset of motor disturbance⁵², and the constitutive symptoms and signs of HD consist of 3 aspects: motor, cognitive and psychiatric disturbances⁵³, amongst which abnormal motor movement, known as chorea, is a well known characteristics of HD. Of the three main symptoms, normally the earliest symptoms shown are related to the psychiatric problems, including apathy, anxiety, irritability, depression, which often are seen prior to the onset of motor symptoms⁵⁴. Similar to the psychiatric disturbances, cognitive decline is another main sign of HD and can be present long before the first motor symptoms appear, but can also be very mild in far advanced stages of the disease, resulting in patients losing their organising skills as well as their memory⁵⁵. The most striking of the triad, and usually the last to appear is motor disturbance. This generally begins with a slightly clumsy gait; the patients later develop slowly progressive involuntary choreatic movement spreading from distal to proximal muscles. The progression of involuntary movement results in an inability to move at their own will, as well as dysarthria (difficulty in articulating words) and dysphagia (difficulty in swallowing). In the late stage of the disease, bradykinesia (slowness of movement) and akinesia (difficulty in starting movement) are present in the majority of the patients⁵³⁻⁵⁵. Collectively, progression of the disease leads to more dependency in daily life and finally death. The most common cause of death is pneumonia, followed by suicide⁵³.

The current treatment of HD still remains limited, and only optimal management of multidisciplinary approaches has been established for HD, which involves physicians, nurses, physiotherapists, dieticians and other healthcare professionals to optimize quality of life and pre-empt the changing needs of the patient as the disease progresses⁵². Thus, these pharmacological/non-pharmacological interventions go in parallel with the symptoms diagnosed of the triad in the patients as describe above. Specifically, as to the abnormal movements, chorea, one of the most prominent symptoms of HD, the only drug specifically licensed to is tetrabenazine, a synaptic vesicular amine transport inhibitor⁵⁶. This treatment also results in many side effects including sleep problems, depression, anxiety and restlessness⁵⁷. Other motor symptoms such as abnormal gait, poor balance and frequent falls are commonly treated with physiotherapy⁵².

1.2.2.2 Pathology, potential mechanisms of pathogenesis, and current animal models

Pathologically, it has been shown in the post-mortem studies of HD that diffuse atrophy of the striatum firstly degenerates at early stage of HD, whereas the globus pallidus and nucleus accumbens are affected but to a lesser extent⁵⁸. There is a classification system for HD pathology which consists of five grades (0-4) correlating with the macroscopic and microscopic criteria in ascending order of severity⁵⁹. At molecular level, neuronal dysfunction and death are caused through a number of

mechanisms by the mutant huntingtin protein (mHTT), which include direct effects from the exon 1 mHTT fragment, the propensity of mHTT to form abnormal aggregates and its effects on cellular proteostasis, axonal transport, transcription, translation, mitochondrial and synaptic function^{52,60}. The mechanisms have been illustrated in **Figure 1.2.2.2**, which is adopted from ⁵².

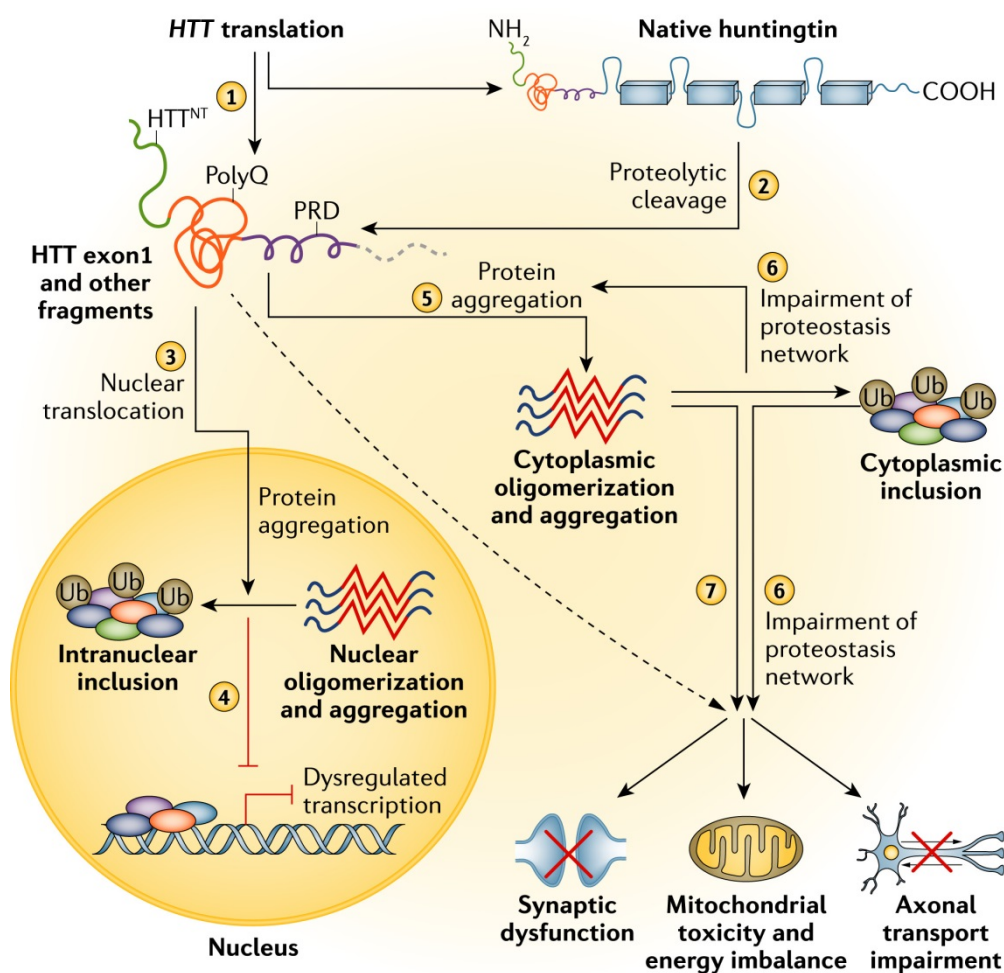


Figure 1.2.2.2 Pathogenetic cellular mechanisms in Huntington disease.

Adapted from ⁵²

Pathogenetic cellular mechanisms in Huntington disease. (1) *HTT* is translated to produce the full-length huntingtin protein as well as an amino-terminal *HTT* exon 1 fragment (the result of aberrant splicing). The length of the polyglutamine (polyQ) tract in these proteins depends on the extent of somatic instability. (2) Full-length native huntingtin is cleaved through proteolysis to generate additional protein fragments. (3) Protein fragments enter the nucleus. (4) Fragments are retained in the nucleus through self-association, oligomerization and aggregation, leading to the formation of inclusions, a process that causes transcriptional dysregulation through the sequestration of other proteins and through other incompletely defined mechanisms. (5) *mHTT* fragments oligomerize and aggregate in the cytoplasm. (6) The aggregation of *mHTT* is exacerbated through the disease-related impairment of the proteostasis network, which also leads to global cellular impairments. (7) The aberrant forms of *mHTT* result in additional global cellular impairments, including synaptic dysfunction, mitochondrial toxicity and a decreased rate of axonal transport. PRD, proline-rich domain; Ub, ubiquitin.

Interestingly, as mentioned in the previous section, HD has a typical feature of selective vulnerability in the striatum. Within the striatal atrophy, it has been found since the very beginning of HD studies that only the GABAergic striatal projection MSNs mainly degenerate whilst striatal interneurons are relatively preserved^{61,62}. Subsequently, it was shown that preferential loss of enkephalinergic striatopallidal

MSNs (iMSNs) is found in presymptomatic as well as initial and middle stages of the disease whilst the substance P-positive MSNs are less affected^{34,63-65}. More specifically, it has directly been demonstrated by using immunohistochemistry that the number of enkephalin-containing fibres, found mainly in the GPe, was drastically reduced in HD brains from all stages of the disease³⁴, whereas the number of substance P-containing axons projecting to the GPi of Vonsattel stage 2 and 3 HD brains was similar to that of controls⁶⁴. It has been also further confirmed by an *in situ* hybridisation study that a greater decline of enkephalinergic MSNs compared to substance P positive MSNs in the caudate nucleus of HD patients⁶⁵. Supported by the clinical evidence, this severe reduction of enkephalin has also been observed at mRNA levels in striatal neurons of HD mouse models⁶⁶. Moreover, based on the substructure of the striatal compartments, differential degeneration of striatal neurons within patch-matrix compartments has also been shown⁶⁷.

However, despite these clear evidence for this selective vulnerability underlying HD, the cause of this particular preferential degeneration in the early stage of HD is still largely unknown. Given that *mHTT* is ubiquitously expressed, the cell-type-specific differences in the ability to maintain proteostasis may contribute to selective vulnerability to toxic huntingtin protein^{68,69}, which may refer to lower autophagic degradation capacity in vulnerable striatal neurons than in resistant cortical and even striatal interneurons³⁸. And this vulnerability may also involve neuron-specific combinations of dysfunction in cellular stress and proteostasis pathways, which might be aggravated by age, genome, and environmental factors.

Glutamate excitotoxicity induced by activation of NMDA and AMPA receptors within the striatum has been proposed as a possible cause of MSNs vulnerability in HD^{70,71}. Previous studies have shown that the NR2B subunits of NMDA receptors are highly expressed in vulnerable MSNs, while the NR2D subunits are highly expressed in the resistant striatal interneurons^{72,73}. This differential expression pattern might explain the increased vulnerability of MSNs⁷⁴. Alterations in numerous neurotransmitter (for example, dopamine) and neurotrophic factor (for example, brain-derived neurotrophic factor or BDNF) signaling pathways occur during normal aging and may be involved in the selective vulnerability of HD, which are commonly amplified in many neurodegenerative conditions⁷⁵. Moreover, excessive dopamine may be another potential mechanism in HD. It has been hypothesized that, in early-stage HD, striatal dopamine levels are up-regulated, inducing hyperkinetic movement disorder and striatal dopamine excitotoxicity⁷⁶, which is further supported by the clinical evidence that anti-dopaminergic agents are effective suppressants of abnormal hyperkinetic movements in patients with HD⁷⁷. In a study of R2/6 transgenic HD mice, the activity of tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholamine synthesis, increased during early-stage HD and significantly decreased at a later stage⁷⁸. Another critical proposed mechanism refers to mitochondrial function and energy demand. To maintain a hyperpolarized state of MSNs requiring high amounts of energy, striatal MSNs usually remain electrophysiologically silent^{79,80}. This unique energy requirement of MSNs may contribute to their susceptibility to mitochondrial dysfunctions⁵⁰. A decreased activity in mitochondrial respiratory chain complexes II,

III, and IV has been reported in the post-mortem studies of HD brain tissue⁸¹. Intriguingly, even having higher demand of energy support, these MSNs contain low levels of antioxidant enzymes, like superoxide free radical scavengers superoxide dismutase 1 and 2 (SOD1 and SOD2), whereas cholinergic interneurons are enriched these enzymes⁸². Despite oxidative damage is rarely reported in early-stage HD, it may be involved later during disease progression⁸³.

Since the identification of the causative gene in HD, a number of models have been developed to study this disorder by introducing human *mHTT* into the genome of animals. The organisms used to study HD includes worms (*Caenorhabditis elegans*), fruitflies (*Drosophila melanogaster*), mice, rats, sheep and, pigs and monkeys⁸⁴. Different species of animals are better suited for modeling certain aspects of HD and for different applications. Therefore, the choice of species and the particular model to use will depend on the specific question of interest. At very early stage of HD studies, the HD models were achieved by applying the excitotoxic lesion, such as glutamate receptor agonists and mitochondrial toxin, within the striatum⁸⁵⁻⁸⁸. However, these models actually contrast sharply with the slow and protracted pace of HD because of the acute nature of the lesions. Genetic models are created to better replicate the HD clinical manifestations. By far the rodent is the most commonly used animals for modeling HD.

The main differences between these approaches can be classified as follow: use of full-length or only a fragment of mutated HTT; the length of the CAG repeat

incorporated into the genetic construct⁸⁴. In terms of introducing the *mHTT* fragments, R6/1 and R6/2 mice, the very first transgenic models of HD, express exon 1 of human *HTT*, truncated N-terminal fragments of *mHTT* and originally with 116 and 144 CAG repeats, respectively, under the control of the human *HTT* promoter⁸⁹. N171 mice have been generated to express a truncated *HTT* cDNA with 82 CAG repeats under the control of the mouse prion protein promoter⁹⁰. All of these truncated N-terminal fragment models exhibit a rapid onset of symptoms, including motor, cognitive and behavioural abnormalities, weight loss and a reduction in lifespan^{89,90}.

As to the full-length *mHTT* rodent models, they can be further divided into knock-in models' group and transgenic model group. In knock-in models group, the models are identical based on CAG repeat sizes ranging from 50 to 200⁹¹, such as the HdhQ111, CAG140 and HdhQ150 mice⁹²⁻⁹⁵. In the transgenic model group, the models are basically named after the techniques applied to introduce the *mHTT* and related CAG repeats. The two main techniques used for this purpose are the yeast artificial chromosome (YAC) technology and the bacterial artificial chromosome (BAC) technology, generating the transgenic models YAC128, YAC48, YAC72 mice^{96,97}, and BACHD mice and rats^{98,99}. The behavioural and neurodegenerative phenotypes of the full-length *mHTT* models tend to be more severe in the transgenic models expressing human *mHTT* than in the knock-in lines.

Since disturbances in motor function are the most apparent signs of HD as mentioned above, an ideal model for HD should recapitulate the specific HD-related

locomotor behaviors from hyperkinesia to bradykinesia in the HD clinical progression manner. However, none of the HD rodent models mentioned above can clearly show this locomotor progression. Menalled and colleagues have conducted a behavior evaluation of transgenic and knock-in HD mice mentioned above (R6/2, HdhQ111, BACHD, YAC128) to systematically examine the locomotor behavior¹⁰⁰. The open field test clearly showed that both R6/2 lines and the BACHD line were hypoactive starting from 8 months age, while BACHD lines only females showed a slight hyperactivity at 4 months. The YAC128 lines showed mild hypoactivity, whereas HdhQ111 knock-in mice did not show any related deficits¹⁰⁰. To be noticed, the age of the mice that manifest the locomotor phenotype in each model is normally much earlier and aggressive than the onset observed in human HD patients (35-50 years)⁵², that would be estimated around from 7 months of age to 15 months of age in the mouse¹⁰¹.

1.3 BDNF-TrkB signaling, and its implication in neurodegeneration

1.3.1 *BDNF*, and its *TrkB* receptor

Brain derived neurotrophic factor (BDNF) is a member of the family of growth factors and was initially purified from pig brain¹⁰². The expression of BDNF in human, rat and mouse is encoded by a single *BDNF* gene, whose transcription is regulated by several promoters¹⁰³. The transcription of *BDNF* has been reported to link with neuronal activity-dependent and regulated by membrane depolarization. The promoter of BDNF exon IV contains Ca²⁺ response elements (CaRE) – CaRE1 and CaRE3, which regulate transcription^{104,105}. Cyclic AMP responsive element binding protein (CREB), a transcription factor, binds to these CaREs, which are phosphorylated by calcium/calmodulin (CaM)-dependent protein kinases, cAMP-dependent protein kinases and MAPK, activating the promoter and resulting in Ca²⁺ dependent transcription of BDNF mRNA at exon IV¹⁰⁵. Alternate splicing terminates transcription of *BDNF* gene at two alternate polyadenylation points which shift the translation sites, giving rise to two distinct BDNF mRNA populations into specific neuronal compartments, allowing spatial and temporal translocation^{106,107}. Based on these distinct alternate BDNF mRNA transcripts, the precursor pre-pro BDNF is first to be given rise in the endoplasmic reticulum^{108,109}, then translocated to the Golgi apparatus, where the signal peptide pre-sequence is cleaved off to form pro-BDNF (30 kDa)¹¹⁰. The pro-BDNF is then further processed either intracellularly or

extracellularly, via the Golgi apparatus, into the trans-Golgi network (TGN) where the pro-domain is proteolytically cleaved off to form pro-domain and mature BDNF and is secreted into the extracellular space¹⁰⁸. During these processes, three products of BDNF are generated: the pro-domain of BDNF, the pro-BDNF, and the mature BDNF, and then interact with their respective receptors to exert their distinct physiological functions once released¹¹¹. Amongst three isoforms of BDNF, only the mature BDNF can bind with high affinity to TrkB receptor and with lower affinity to the p75 receptor^{112,113}, of which activation is responsible for the BDNF's known functions, such as neurogenesis, gliogenesis, neurite outgrowth, and enhanced neuronal survival^{114,115}.

BDNF is one of the neurotrophic factors, which are secreted proteins that promote neurite outgrowth, neuronal cell differentiation, and survival both in vivo and in vitro¹¹⁶. Nerve growth factor (NGF) is the founding and best characterized member of the neurotrophin family¹¹⁷, and then molecular cloning of the BDNF gene revealed its structural similarity to NGF¹¹⁸, leading to the concept of the neurotrophin family. Two additional members, neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5), were then identified¹¹⁹. Biological effects of each of the four mammalian neurotrophins are mediated through activation of one or more of the three members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC). In this way, NGF binds to TrkA, BDNF and NT-4/5 to TrkB, and NT-3 to TrkC. While Trk-mediated signalling usually promotes neuronal survival and growth as well as synapse modulation, p75^{NTR} activation counteracts these processes (as shown in

Figure 1.3.1).

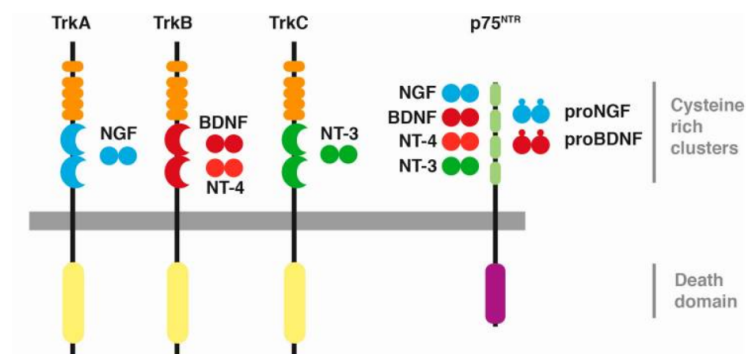


Figure 1.3.1.1 Neurotrophins and their receptors. (Adapted from Arévalo and Wu¹²⁰). Scheme showing the common structure and interactions of Trk receptors, as well as of p75^{NTR}. Trk: Tropomyosin Related Kinase; NGF: Nerve Growth Factor; BDNF: Brain-Derived Neurotrophic Factor; NT: Neurotrophin; Ig: Immunoglobulin; TM region: Transmembrane region.

The TrkB receptor, a member of Tropomyosin receptor kinase family, is encoded by a single *Trkb* gene, the *NTRK2* gene encoding 24 exons located on chromosome 9q22¹²¹. The TrkB structure consists of three domains: an extracellular ligand binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. Based these fundamental domains, there are two main types of isoforms of TrkB receptor, full-length TrkB (TrkB) and a group of the truncated TrkB (TrkB-T) including two truncated TrkB (TrkB-T1) isoforms, TrkB-T2, TrkB-Shc lacking tyrosine kinase domain, and a TrkB-TK with a non-viable catalytic domain¹²².

The three TrkB splicing isoforms mentioned above share an extracellular BDNF binding domain and differ in their cytoplasmic domain (shown in **Figure 1.3.1.2**). Two splice variants encode full-length receptors, TrkB full-length (FL), that contain a tyrosine kinase domain, an SHC-binding domain and a PLC- γ -binding domain in the intracellular portion¹²³. Two isoforms encode shorter receptors, TrkB SHC, that contain only an SHC-binding domain, and the remaining isoform is a truncated receptor, TrkB-T, has lately attributed some novel functions^{124,125}.

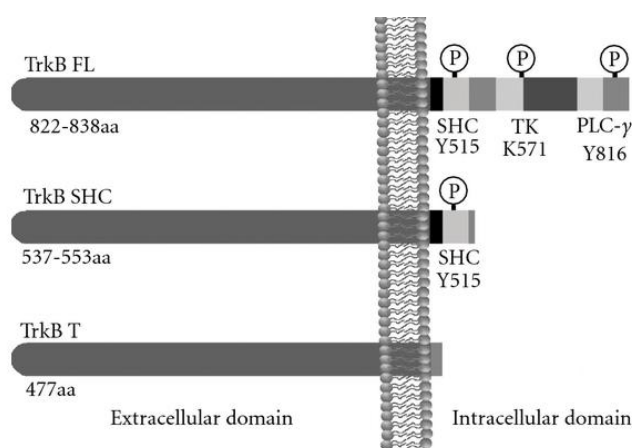


Figure 1.3.1.2 TrkB isoforms structure and functional domains (Adapted from ¹²⁶).

The extracellular portion of the receptor is conserved. The intracellular domain differs among the isoforms. TrkB-FL contains a tyrosine kinase domain, a SHC-binding domain, and a PLC- γ -binding domain. TrkB SHC contains a SHC-binding domain and TrkB T does not have any functional domain.

BDNF binds to TrkB receptors, both TrkB-T and TrkB (full length) with similar affinity, but only full length isoform can trigger the known intracellular signaling¹⁰³.

Binding of BDNF to TrkB initiates two different categories of cascades: a fast acting BDNF-TrkB cascade that excites neurons or a slow acting occurring over minutes to hours¹²⁷. Evidence suggests that the well-studied effect of BDNF-TrkB on cell survival and plasticity are mediated by TrkB full length form¹²⁸. Once BDNF binds to TrkB, ligand-mediated dimerization of the complex occurs at the cell surface, followed by autophosphorylation of specific tyrosine residues in the cytoplasmic domain, leading to activation of three interconnected intrinsic intracellular cascades^{129,130}, which is further described in detail below (**Figure 1.3.1**).

Initially, neurotrophins and their receptors were thought to be expressed only in nervous system but further studies showed they are also expressed by macrophages, endocrine cells, immune cells, smooth and striated muscle fibers^{113,131}. Recently, neurotrophins and their Trk receptors, especially BDNF and TrkB, were found to be highly up-regulated and to play a vital role in various cancers, including breast, lung, colon-rectum, pancreas, prostate, liver, myelomas and lymphoid tumors¹³². As a result of activation of the pathway cascades, this exhibits oncogenic effects by promoting cancer cell's growth, proliferation, survival, migration, epithelial to mesenchymal transition, anoikis, relapse and chemotherapeutic sensitivity¹³³. Drugs targeting these Trk receptors have been put into clinical trials for cancer therapy and promising results have been achieved with moderate side effects¹³⁴.

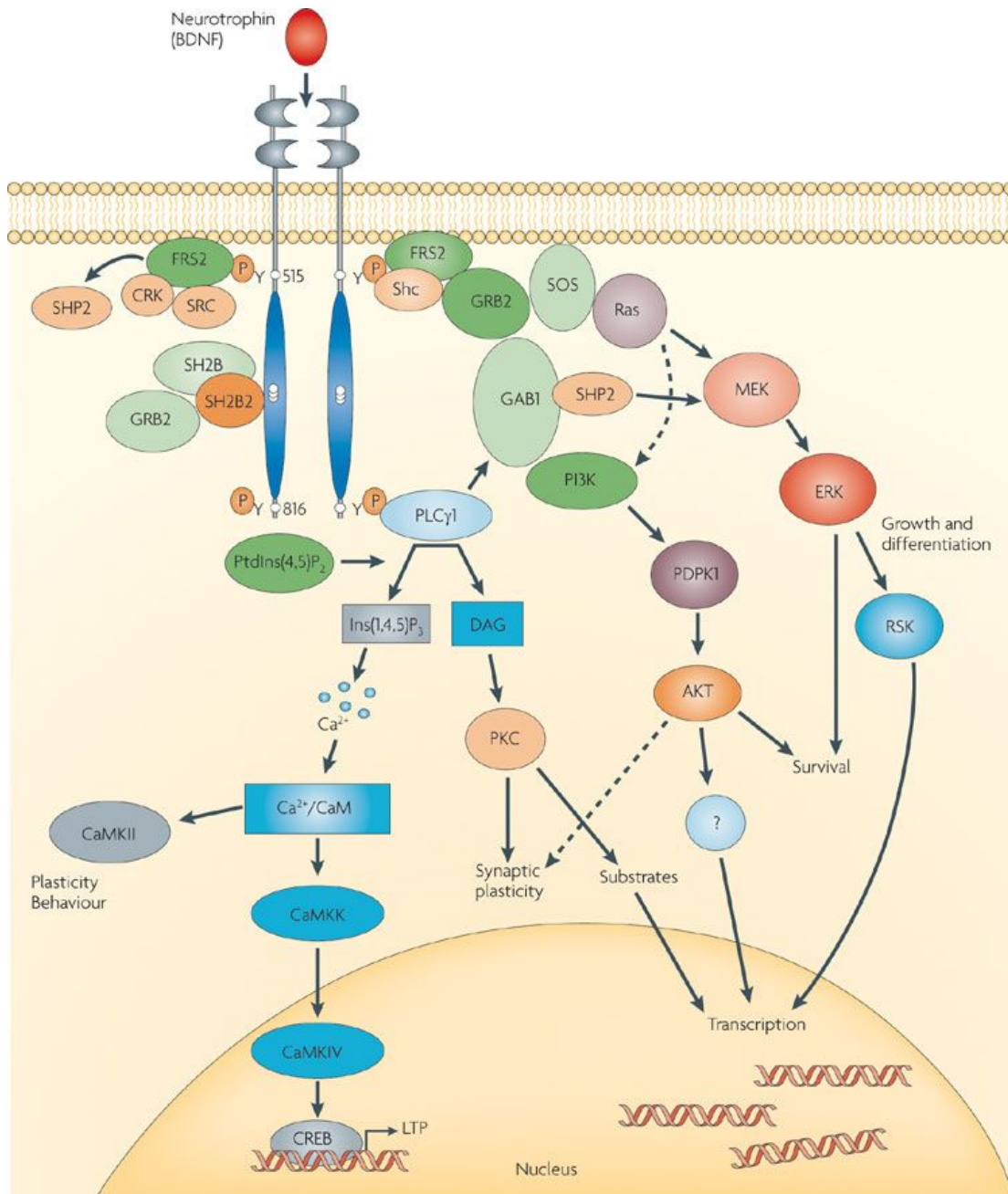


Figure 1.3.1 Major BDNF-TrkB signaling activated pathways. Adopted from ¹²⁹.

The interaction between the receptor tyrosine kinase TrkB and neurotrophins activates three main intracellular signalling pathways. Phosphorylation and recruitment of adaptors to Y515 leads to activation of the Ras–mitogen-activated protein kinase (MAPK) signalling cascade, which promotes neuronal differentiation and growth through MAPK/ERK kinase (MEK) and extracellular signal-regulated kinase (ERK), and to activation of the phosphatidylinositol 3-kinase (PI3K) cascade, which promotes survival and growth of neurons and other cells through Ras or

GRB-associated binder 1 (GAB1). Recruitment and activation of phospholipase $\text{C}\gamma 1$ ($\text{PLC}\gamma 1$) through phosphorylation of Y816 results in the generation of inositol-1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) and diacylglycerol (DAG). Whereas DAG stimulates protein kinase C (PKC) isoforms, $\text{Ins}(1,4,5)\text{P}_3$ promotes the release of Ca^{2+} from internal stores and subsequent activation of Ca^{2+} /calmodulin ($\text{Ca}^{2+}/\text{CaM}$)-dependent protein kinases (CaMKII, CaMKK and CaMKIV). Point mutation of Y816F impairs phosphorylation of CaMKII, CaMKIV and cyclic AMP-responsive element-binding protein (CREB) and synaptic plasticity. Pathways activated through the Shc adaptor protein site (Ras–MAPK and PI3K) are not impaired by Y816F mutation. $\text{PLC}\gamma 1$ is also able to associate with GAB1, which may stabilize the formation of the complex that forms downstream of TrkB upon activation. Some additional adaptors, such as the Src homology domain-containing protein SH2B and SH2B2, have also been identified to interact with TrkB.

FRS2, fibroblast growth factor receptor substrate 2; GRB2, growth factor receptor-bound protein 2; PDPK1, 3-phosphoinositide-dependent protein kinase 1; $\text{PtdIns}(4,5)\text{P}_2$, phosphatidylinositol-4,5-bisphosphate; RSK, ribosomal protein S6 kinase; SHP2, SRC-homology phosphatase 2; SOS, son of sevenless.

1.3.2 BDNF-TrkB signaling in neurodegeneration

Based on the intracellular processes described above, BDNF-TrkB signaling supports critical cellular functions such as growth and survival¹³⁵, and many important biological processes, such as energy metabolism¹³⁶, learning and memory^{137,138}. Therefore, it is expected that dysregulation of this signaling would result in cellular dysfunction. As such, affected BDNF-TrkB signaling has been involved in the pathogenesis of many neurodegenerative diseases, (Alzheimer's disease¹³⁹, Huntington disease¹⁴⁰, Parkinson's disease¹⁴¹, etc.). Substantial evidence has directly linked loss of BDNF-TrkB signalling with the pathogenesis of Alzheimer's, Huntington's and other neurodegenerative disorders. In particular, TrkB has been proposed to be a susceptibility gene contributing to Alzheimer's disease pathology¹⁴². The single nucleotide polymorphisms (SNPs) found associated with the age of onset of AD are located in areas of the gene containing sequences involved in alternative splicing and/or regulation of *Trkb*. BDNF-TrkB signalling can help the maintenance of the nigrostriatal system, and the deficiency may contribute to the progression of Parkinson's disease¹⁴³. Interestingly, TrkB activation in astrocytes drives nitric oxide production which may promote neurodegeneration in brain¹⁴⁴. BDNF-TrkB signalling is involved in the regulation of peripheral T cell apoptosis in multiple sclerosis¹⁴⁵. TrkB expression changes also indicate that it may have a role in Rett syndrome pathology¹⁴⁶. Neuroprotective functions of the BDNF-TrkB signaling may be reduced in the respiration nuclei in sudden infant death syndrome (SIDS) cases¹⁴⁷. An increase

in TrkB expression is associated with increased seizure susceptibility and epileptogenesis¹⁴⁸. In glaucoma, loss of TrkB signalling leads to preferential degeneration of RGCs^{149,150}. Transport obstruction in the optic nerve inhibits the retrograde delivery of TrkB from the brain to RGCs resulting in TrkB signalling deficit and eventual cell death^{151,152}.

Particularly in HD, BDNF-TrkB signaling appears to be indispensable for striatal cell long term survival and maintenance. Cortical neurons are the main source of striatal BDNF, which is delivered by anterograde transport through the corticostriatal afferents¹⁵³. Mutant huntingtin impairs BDNF promoter II transcriptional activity through the cytoplasmic retention of the neuron-specific transcriptional repressor REST¹⁴⁰, and can also interfere with BDNF anterograde transport from cortical neurons to striatal targets¹⁵⁴. Therefore, reduction in BDNF supply to the striatum was believed to jeopardize the long-term survival and morphology of striatal MSNs, contributing ultimately to striatal degeneration in HD. This is consistent with the reduced cortical and striatal BDNF levels observed in both HD patients and mouse models¹⁵⁵. Forebrain-specific *Bdnf* mutants recapitulate many behavioral and anatomical abnormalities seen in mouse models of HD¹⁵⁶. Similarly, mice carrying mHdh transgene, with genetic reduction of BDNF levels, exhibit advanced onset and increased severity of motor dysfunction, resulting from the degeneration of striatal ENK-MSNs¹⁵⁷. Recently, in striatal MSNs derived from HD patient fibroblast, it has been found significant decrease expression of TrkB by RNA-seq profiling, which presents cellular manifestations associated with HD¹⁵⁸.

1.3.3 Genetic models of BDNF-TrkB signaling

To facilitate the study of the role of BDNF-TrkB signaling, multiple genetically modified mouse models with altered BDNF expression or TrkB signaling have been produced. There are several distinct approaches that have been taken to genetically modify BDNF-TrkB signaling. Briefly, strategies are based on knockdown of BDNF expression or knockout of TrkB receptor from either the whole organism or from a spatially restricted region. Homozygous BDNF KO mice have severe health problems and die soon after birth¹⁵⁹, while heterozygous BDNF KO mice are vital and show their BDNF mRNA and protein levels in brain are approximately 50% of their wildtype littermates¹⁶⁰, and develop increased appetite and are usually obese^{161,162}. Spatially restricted BDNF KO mouse lines have been produced by crossing mice carrying floxed BDNF alleles with lines expressing Cre recombinase under a tissue-specific promoter. Anxiety-related behavior, increased food intake and hyperactivity have been observed in mice of conditional BDNF KO under the α -calcium/calmodulin-dependent protein kinase II (CamK) promoter, which drives expression in postmitotic neurons¹⁶³. Monteggia *et al* tested behavior on two independent lines of conditional BDNF knockout mice in which the BDNF gene is deleted selectively in forebrain by crossing with human glial fibrillary acidic protein (GFAP)-Cre mice and the other with CaMKII-Cre¹⁶⁴. On the other end, more mouse lines were generated through alteration of TrkB expression. Homozygous *Trkb* KO mice have severe developmental problems and die soon after birth¹²⁸, while more

interests arise in conditional and spatially restricted deletion of TrkB receptor to various brain regions or specific cell types. By crossing floxed *Trkb* mice with CamK-Cre mice TrkB deficiency was generated in the HC and forebrain region, normal brain morphology but impaired CA1 LTP were observed¹⁶⁵. Mice with specific TrkB deletion in adult DG neuronal progenitors (using nestin-CreERT2 mice) show decreased locomotor activity and increased anxiety-like behavior¹⁶⁶. Moreover, selective loss of TrkB signalling in cholecystokinin (CCK)-GABAergic neurons could affect HPA axis activity by inducing hypercortisolism and obesity¹⁶⁷. Of interest to this thesis, specific ablation of full length TrkB signaling from enkephalinergic striatal medium spiny neurons led to spontaneous and drug-induced hyperlocomotion without affecting neuronal survival and morphology¹⁶⁸. On the other hand, as an alternative to decreasing TrkB signaling in mice through conditional deletion of TrkB receptors, Saarelainen et al. produced transgenic mice overexpressing the truncated, dominant negative form of the TrkB receptor, and these mice display impaired long-term spatial memory¹⁶⁹.

1.4 Core questions and aim of the project

To accurately define the protective role of BDNF-TrkB signaling in enkephalinergic MSNs of the striatum, our lab has previously performed a selective deletion of the full length TrkB receptor from the enkephalinergic MSNs by generating a new mouse model¹⁶⁸. Unexpectedly, loss of TrkB in the *Penk*+ MSN did not lead to neuronal loss (in contrast to what has been shown in early cortical deficit of BDNF mouse model)¹⁷⁰, but to an age-dependent spontaneous, as well as drug-induced, hyperlocomotion, associated with increased D2R-dependent MAPK/PKC phosphorylation and reduced striatopallidal activation; demonstrating that BDNF-TrkB signaling in striatopallidal neurons contributes to the inhibitory control of locomotor behavior exerted by the indirect pathway, but is dispensable for their maintenance¹⁶⁸. These data are in agreement with a study showing that toxin-mediated ablation of striatopallidal neurons resulted in increased locomotor activity¹⁷¹, and confirm that the striatopallidal pathway exerts a basal inhibitory tone on locomotion. These results collectively indicate that the hyperlocomotor behavior is directly dependent on TrkB signaling in enkephalinergic MSNs, and therefore such signaling is essential to maintain normal locomotor function. In addition, the data show that hyperlocomotion manifests with age, or after cocaine administration in young mutant animals¹⁶⁸.

However, how the enkephalinergic MSNs dysfunction leads to the abnormal locomotor behavior in absence of TrkB signaling still remains unclear. Therefore, the

aim of this thesis is to establish the molecular mechanisms underlying enkephalinergic striatal dysfunction driving age-dependent locomotor phenotype in absence of TrkB signaling. There is no established information about how striatal circuits undergo age-dependent motor skill impairments. Moreover, the identification of the genes and associated molecular pathways relevant to maintenance of locomotor control requires a systematic, unbiased study of the aging striatal circuits, both in normal and TrkB-deficient conditions.

Hence, to address this, we have established global RNA sequencing analysis (RNA-seq¹⁷²) of purified enkephalinergic MSNs from young adult and aged mice, both in the presence and absence of TrkB signalling¹⁷³. This analysis has revealed age-dependent changes occurring at transcriptomics level in the specific MSNs subpopulation of the striatum in both control and *Trkb*^{PENK-KO} mutant mice. Therefore, in the first part of this thesis I will describe the bioinformatics analysis. By having these valuable data, we firstly conducted the bioinformatics analysis to capture transcriptomic alterations of striatal subpopulations with abnormal enriched expression. To further understand the affected functions/pathways, we employed more bioinformatic analysis, which include differential gene expression (DE), gene ontology (GO) and gene set enrichment analysis (GSEA), which helped to elucidate the pathways underlying the development of the phenotype. Collectively, this analysis led to the identification of key candidate genes with unique expression pattern specific to the *Penk*⁺ MSNs of *Trkb*^{PENK-KO} mutant mice at early stage. I will then describe the validation analysis of the bioinformatics data. We employed comprehensive validation

approaches, including optimised single molecule FISH (smFISH), quantitative PCR (Fluidigm) and immunohistology, to validate the hits from RNA-seq data. Subsequently, in the third part, I show the investigation of the dopaminergic system, in both the striatum and VTA/SNpc regions, and how it behaves in an age dependent manner, which is closely associated with the locomotor phenotype we observed in the *Trkb*^{PENK-KO} mutant mice. Last but not least, to functionally validate the key gene discovered in this analysis and its involvement in the hyperlocomotor phenotype, I will show the result we have obtained by manipulating its expression *in vivo* specifically in the *Penk*⁺ MSNs of the *Trkb*^{PENK-KO} mutant mice at early age and use the open field test (OF) as a readout to assess the consequence of the gene manipulation on the locomotor phenotype of the *Trkb*^{PENK-KO} mutant mice.

In summary, the central aim of this thesis was to understand how TrkB signaling is involved and induces aged-dependent locomotor defect in the enkephalinergic MSNs population. Thus, we have addressed this by the following:

1. Unbiasedly investigating what genes/signaling pathways have been altered with absence of TrkB signaling specifically in the enkephalinergic MSNs population.
2. Identifying the key candidate genes potentially driving the changes of molecular signature in this enkephalinergic MSNs population at presymptomatic stage.
3. Further characterizing the phenotype of the dopaminergic system in *Trkb*^{PENK-KO} mice in an age-dependent manner.
4. Functionally validating the key genes identified in the specific enkephalinergic MSNs to assess the consequence of the locomotor maintenance.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

Table 2.1.1: Chemicals used in this study. Please see in Appendix

2.1.2 Solutions

Table 2.1.2: Solutions used in this study. Please see in Appendix.

0.1M PB (pH = 7.4): 77.4 ml of 1 M Na₂HPO₄, 22.6 ml of 1 M NaH₂PO₄ for 1 litre)

PBS: 0.01 M phosphate buffer, 0.0027 M KCl and 0.137 M NaCl, pH 7.4

(prepared from Thermo Fisher Scientific PBS tablets, Cat. No. 18912014)

4% Paraformaldehyde (PFA) solution (1 litre): 40 g of paraformaldehyde

(Sigma, Cat. No. P6148) dissolved by continuous stirring in 800 ml 0.1M PB

buffer (pH 7.4) by heating, keeping the temperature below 60 °C. The solution

was cooled down to room temperature, adjusted to pH 7.4 with concentrated

NaOH, and filtered through 0.2 micron filter. Aliquots were stored at -20°C. The

aliquots were thawed slowly in a 4°C fridge when required for experiments.

Blocking buffer (fluorescence): 5% fish gelatine (Sigma, Cat. No. G7765)

and 0.5% Triton-X (Sigma, Cat. No. T8787) in PBS

Blocking buffer (DAB): 10% normal goat serum (Sigma, Cat. No. G9023), 0.3%

carrageen (Sigma, Cat. No. 22048), 0.5% TritonX-100, 0.2% sodium azide in

TBS.

Lysis buffer (Smart-seq2; per well): 0.4% Triton + RNase inhibitor (2 µl), dNTP's (10mM; 1 µl; Thermo Fisher, Cat. No. 10319879), Oligo dT30VN (10 uM; 1 µl; Nextera Kit).

Lysis buffer (microfluidic gene expression analysis; per well): CellsDirect 2x Reaction Mix (5 µl), Ambion SUPERase 12-In (0.1 µl), TE buffer (0.9 µl).

2.1.3 Reagents

Table 2.1.3: Reagents used in this study. Please see in Appendix

2.1.4 smFISH probes libraries (*Gsto2*, *Gcc1*)

Table 2.1.4.1 *Gsto2* smFISH probes library. Please see in Appendix

Table 2.1.4.2 *Gcc1* smFISH probes library. Please see in Appendix

2.1.5 Kits

Table 2.1.5: Kits used in this study. Please see in Appendix

2.1.6 Primary antibodies

Table 2.1.6: Primary antibodies used in this study.

Antibody	Isotype	Dilution	Catalogue number	Manufacturer
Tyrosine Hydroxylase (TH)	Mouse	1:400	MAB318	Chemicon
Enkephalin (ENK)	Rabbit	1:200/250	Ra14124	Neuromics
GSTO2	Rabbit	1:100/200	14562-1-AP	Proteintech
PACE4 (K-18)	Goat	1:200-500	Sc-22898	Santa Cruz

2.1.7 Secondary antibodies

Table 2.1.7: Secondary antibodies used in this study.

Antibody	Dilution	Catalogue number	Manufacturer
Alexa Fluor® 488 goat anti-chicken IgG (H+L)	1:1,000	A-11039	Molecular Probes
Alexa Fluor® 488 goat anti-mouse IgG (H+L)	1:1,000	A-11001	Molecular Probes
Alexa Fluor® 488 goat anti-rabbit IgG (H+L)	1:1,000	A-11008	Molecular Probes
Alexa Fluor® 647 goat anti-mouse IgG (H+L)	1:1,000	A-11001	Molecular Probes

2.1.8 Consumables

Table 2.1.8: Consumables used in this study. Please see in Appendix

2.1.9 Analytical software

Table 2.1.9: Analytical software used in this study.

Software	Application	Developer
R programming	bioinformatics analysis and data visualization	R community
ImageJ (version 1.49b)	Image analysis	National Institutes of Health
GraphPad Prism 8 for Windows (version 8.0.2)	Statistical analysis	GraphPad
Stellaris Probe Designer	Probe design for smFISH	Stellaris
StarSearch	smFISH analysis	Raj lab (http://rajlab.seas.upenn.edu)
Leica AF6000 software	Imaging and image analysis	Leica

2.2 Methods

2.2.1 Animals (mouse line, maintenance and genotyping)

2.2.1.1 Mouse line

All mouse strains were kept on a mixed genetic background (C57BL/6J:129). Originally, the *Trkb* floxed allele has been generated by using 129-derived ES cells¹⁶⁵. After germline transmission by crossing with C57BL/6J, the floxed allele was maintained on a C57BL/6J genetic background (60%) with some-contributions of 129/sv and CBA/J from the Flp and CRE transgenic crosses. Phenotypic analysis of mutant mice on different genetic backgrounds were not done, since previous analysis of *Trkb* null mutants (L. Minichiello and R. Klein, unpublished data 1999) had revealed no changes in phenotype when backcrossed to other inbred and outbred strains for mutations of *Trkb*. The main reason for keeping and analyzing mouse mutants on hybrid background is because, especially in behavioural tasks, it has been demonstrated that is the best way. Even if genetic background is different between mice, the use of hybrids facilitates the comparison and integration of results across experiments and perhaps among laboratories¹⁷⁴. Three lines were used in this thesis. The BAC-*Penk*-Cre transgenic line (PNKC line) was generated by pronuclear injection of circular BAC carrying Cre-recombinase under the control of pre-pro-enkephalin promoter¹⁶⁸. The Gt(ROSA)26Sore^{tm9(CAG-tdTomato)Hze} (also known as Rosa26-tdTomato, *Ai9*) is a reporter line, which contains a LoxP-flanked stop cassette in front of a cDNA for tdTomato sequence (a non-oligomerizing DsRed

fluorescent protein variant with a 12 residue linker fusing two copies of the protein (tandem dimer))¹⁷⁵. This line expresses tdTomato only when the stop cassette is removed in the presence of Cre-recombinase. The *Trkb* floxed line (*Trkb* lx/lx) has LoxP sites flanking the second exon of the *Ntrk2* gene; corresponding to the tyrosine kinase domain. Excision of the second exon by Cre-recombination leads to a disruption of *gp145^{TrkB}* receptor expression whilst expression of truncated *Trkb* isoforms is intact¹⁶⁵. Then by crossing the PNKC line with *Ai9* reporter line, control mice carrying both alleles (*Trkb^{PENK-WT}*; *Ai9*/+ as indicated as IPNK) were generated. This line expressed tdTomato fluorescence driven by the *Penk* promoter and allows also for subsequent sorting of the tdTomato positive cells. Moreover, the IPNK mouse line was then crossed to the *Trkb* floxed line (*TrKb* lx/lx), to generate cohorts of mutants carrying *Trkb* deletion in tdTomato-labelled enkephalinergic striatal neurons (*Trkb^{PENK-KO}*, *Ai9*/+; and indicated as BIPK).

2.2.1.2 Animal husbandry, maintenance and procedures

Animals were housed in the Biomedical Sciences Building (BSB), University of Oxford. Animal husbandry was performed by staff from Biomedical Services (Oxford). Mice were housed separately according to their sex. The maximum number of animals per cage was four to six. In a case of males, only littermates were housed together whereas female animals from different litters could be pooled. For breeding purposes, one male was housed with two females. The minimal age of breeding was 6

weeks for females, or 7 weeks for males. Litters were weaned after P19. An ear biopsy was collected from each mouse at P12-18 for identification purposes and for genotyping. All animal procedures were conducted under University of Oxford ethical committee guidelines and in adherence with Home Office legislation, Animals (Scientific procedures) Act 1986.

2.2.1.3 Genotyping

DNA isolation

DNA used for genotyping was isolated from ear and tail biopsies, which had been kept at -20°C until processed. Ear punches were collected between P12-18 and used for genotyping. Tail biopsies were taken from experimental animals to confirm animals' genotype at time of death. Samples were digested in 100 µL directPCR lysis reagent for mouse tail (Bioquote) with 1.5 µL PCR grade, recombinant proteinase K (16.4 mg/mL, Roche) at 56°C for a minimum of 4 hours. Heat inactivation at 85°C was then performed for 45 minutes. For each PCR reaction, 1 µL of DNA preparation was used.

Polymerase chain reaction (PCR)

Lyophilised primers were ordered from Sigma-Aldrich, dissolved in nuclease-free H₂O (Ambion) to 100 µM and stored at -80°C. PCR reaction for each target was set accordingly. All PCR reactions were set up in 0.2 mL 8-strip PCR tubes (Starlab) and run on DNAEngine Peltier Thermal Cycler (Bio-Rad).

PCR recipe of BAC-*Penk*-Cre and *Trkb* floxed (**Table 2.2.1**) can be found in

Appendix.

Agarose gel electrophoresis

Gel electrophoresis of PCR reactions was done on 2% agarose gel in 0.5x TBE buffer. The gel was prepared by melting Agarose (Sigma-Aldrich) in a microwave oven until completely dissolved. After the agarose had been allowed to cool to 55°C, ethidium bromide (10 mg/mL; Sigma-Aldrich) was added to make a final concentration of 50 ng/mL. Agarose was poured onto a gel tray where both of its ends were sealed with tape and the appropriate comb inserted. Once solidified, the gel was placed into an electrophoretic chamber containing 0.5x TBE. 5 µL KAPA universal ladder(KAPA Biosystems) and 15 µL PCR reactions (with 3 µL of loading buffer added) were loaded into each well of the agarose. Gel was applied with 170 V electricity until the bands were clearly separated. Images of the bands were taken with either Gel DocTM XR+ system (Bio-Rad) or PhotoDoc-ItTM imaging system (UVP).

2.2.2 Flow cytometry and fluorescence-activated cell sorting (FACS) and next-generation sequencing (RNA-seq)

FACS sorting and RNA-seq were collaboratively done with previous senior lab members and following the established protocol of the Minichiello's lab ¹⁷³. This protocol is briefly described below.

2.2.2.1. FACS sorting

Striatal tissue dissociation

Mice were anaesthetised as above, however instead of 0.1M PB and 4% PFA, 20 ml of artificial cerebrospinal fluid made up in sucrose was perfused. The brains were then dissected out in ice-cold platform. Sub-dissection of both striata was followed by manual chopping of the tissue. The chopped tissue was placed in an Accutase (Stem Cell Technologies, Cat. No. 07920) enzyme solution to dissociate cells, for 30 minutes at 4°C on a rotator. The digested tissue was then centrifuged for 2 min at 450 g and the pellet was resuspended in 1 ml of ice-cold Hibernate A (Brain Bits, Cat. No. HA). This was then triturated 10 times with a large-diameter (~1.3 mm) Pasteur pipette. 600 µl of the cloudy suspension containing dissociated cells was transferred to a 15 ml Falcon tube on ice. Another 600 µl of ice-cold fresh Hibernate A was added to the original Eppendorf tubes. The trituration steps were repeated two more times as above with medium-(~0.8 mm) and small-diameter (~0.4 mm) Pasteur pipettes. The last of the cells were collected from the original tube by adding 750 µl of ice-cold Hibernate A, inverting it several times and letting settle on ice. The cell suspension was then crudely filtered using 100 µm and 40 µm cell strainers.

A Percoll (Sigma, Cat. No. P1644) density gradient was set up as shown below:

Layer	Percoll (µL)	Hibernate A (µL)	1 M NaCl (µL)	Total (µL)
1 (bottom)	1,236.75	5,139	146.7	6,522.45
2	975.75	5,400	114.75	6,490.5
3 (top)	720.45	5,655	89.25	6,464.7

The cell suspension was gently placed on the top layer. The gradient was centrifuged 430 g (2400 rpm) for 5 min at 4°C (Beckman-Coulter ultracentrifuge; SW41 rotor, acceleration at max and deceleration = 0). The top layer contains cellular debris and was aspirated out. The remaining layers were gently mixed by pipetting. The mixed gradient was placed back into the centrifuge to obtain a cell pellet: 5 min at 4°C at 550 g (2700 rpm). All the supernatant was removed and the pellet was resuspended in 1 ml Hibernate A. Cell counting was performed and LIVE/DEAD viability dye (Thermo Fisher, Cat. No. L34963) was added at a concentration of 0.25 µl/1,000,000 cells and incubated on ice for 30 minutes. Dye was removed by pelleting the cells by centrifuging for 5 minutes at 550 g (at 4°C). Cells were resuspended in PBS and pelleted again to remove as much of the LIVE/DEAD dye as possible. Finally, the cells were resuspended in 1 ml Hibernate A.

FACS sorting

Pre-sort purity test: sort the DAPI-negative/TdTomato-positive fraction 100 live TdTomato-positive cells from striatum sample into a low retention 1.5 mL collection tube containing 100 µL of Hibernate A. Cells were treated with DAPI to label dead cells and sorted on a FACSAria Fusion Cell Sorter (Becton Dickson) for TdTomato signal. Wild-type striatal cells were used to calibrate signals. Cells with DAPI-negative/TdTomato-positive signals were selected. For each sample, 200 cells for each sample were collected to process to next step, as per the lab published protocol (Vatanashevanopakorn et al., 2017)¹⁷⁶.

The purity test related experiments have been done in the published protocol¹⁷⁶,

and the most related data is briefly described and quoted here. The gating strategy of FACS sorting has been described in **Figure 2.2.2.1**, and the identity of sorted cells are validated through both microscope and qPCR of specific MSNs marker genes (**Figure 2.2.2.2**)

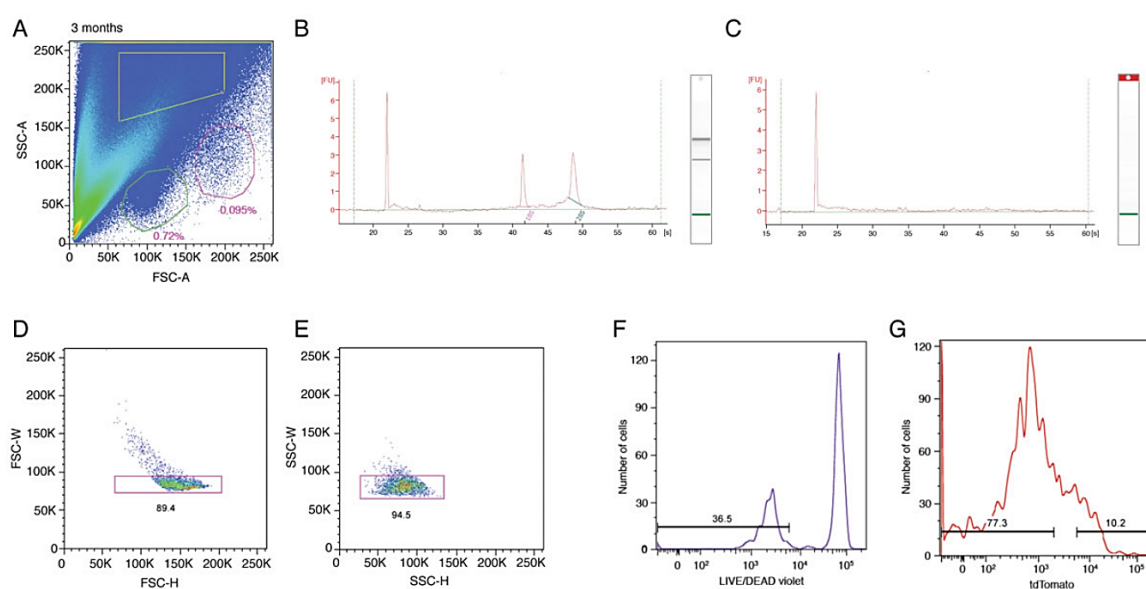


Figure. 2.2.2.1. Gating strategy for sorting. Dissociated striatal cells from 3- and 8-months old mouse brains were sorted using a FACSaria Fusion cell sorter. Adapted from ¹⁷⁶. A. Representative plot between forward scatter area (FSC-A) and side scatter area (SSC-A). The correct gate is highlighted in pink while gates containing mainly debris are highlighted in yellow and green. **B.C.** Representative electropherograms of RNA extracted from cells sorted from the correct gate (**B**, pink gate in **A**) or incorrect gate (**C**, green gate in **A**). Note the presence of 18S and 28S rRNA peaks denoting intact RNA **B**, a

feature that is absent in degraded RNA **C**. **D,E** Representative plots used for selecting only single cells during the sort for forward scatter height (FSC-H) and forward scatter width (FSC-W) **D**, and for side scatter height (SSC-H) and side scatter width (SSC-W) **E**. **F** Histogram of LIVE/DEAD violet intensity, which determines cell viability. A gate for live cells was set to exclude the very brightly stained dead cells. Staining of dead cells is typically >50-fold higher than live cells. **G** Representative histogram for tdTomato intensity within the viable cell gate, which represents enkephalinergic neurons. Gates for positive and negative populations for tdTomato were set according to wild-type control.

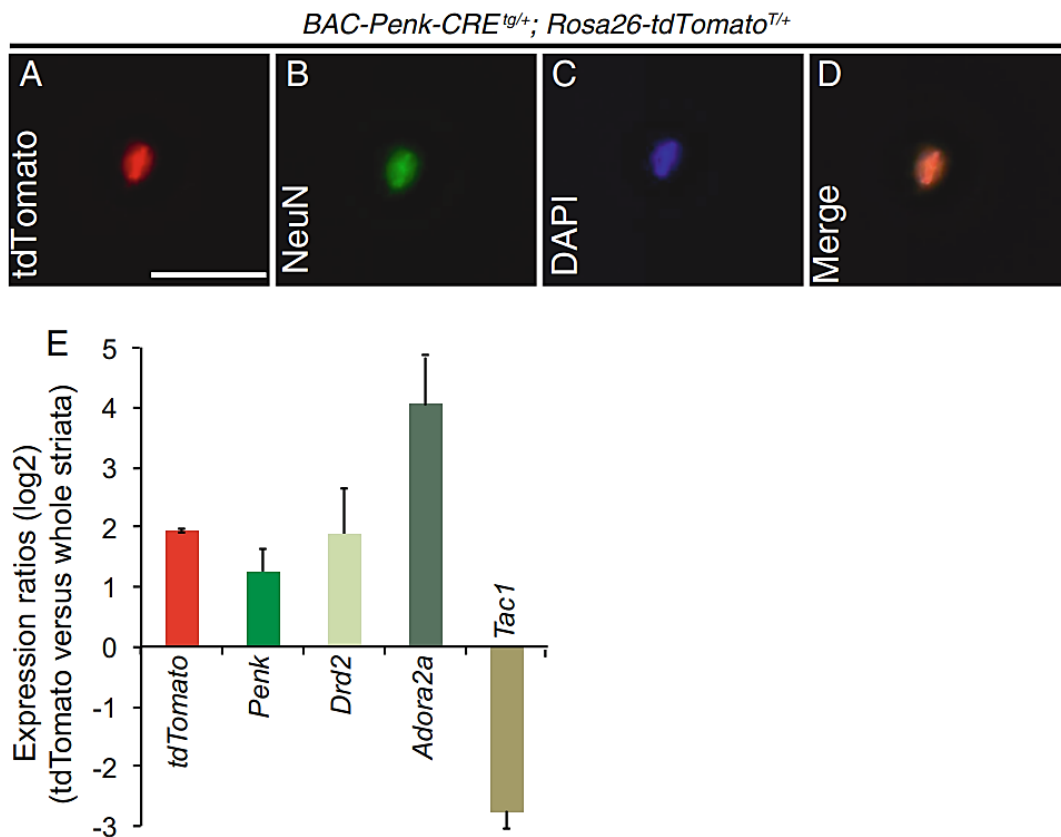


Figure 2.2.2.2 tdTomato positive sorted neurons and enrichment of

striatopallidal known markers. Adapted from ¹⁷⁶. A-D Representative immunofluorescence of sorted (3 months old) live tdTomato+ cell **A**, stained for NeuN (**B**, neuronal marker), and DAPI (**C**). **D** is the merged image of **A-C**. Scale bar, 25 μ m. (**E**) Quantitative RT-PCR of sorted tdTomato+ cells from BAC-Penk-Cre^{tg/+}; Rosa26-tdTomato^{Tg/+} mice, comparing gene expression in sorted ENK+ neurons to the total striatal cell population. The graph shows pooled data from two mice. Values are mean $\pm$ SD (n=2). Y axis is log 2 of fold difference ($-\Delta\Delta$ Ct). Note the enrichment of striatopallidal known markers (*Penk*, *Drd2*, *Adora2a*) and depletion of *Tac1*, a striatonigral marker.

2.2.2.2 Next-generation sequencing (RNA-seq)

RNA-seq was performed by a previous PhD student from the Minichiello's lab (Vatanashevanopakorn *et al.*, 2017)¹⁷⁶. Briefly, following FACS sorting, 1 μ g of RNA from sorted cells for each sample was used to construct sequencing libraries using Illumina's TruSeq RNA Sample Prep Kit according to the manufacturer's protocol. All sample dissections and RNA extractions were performed by the same individual. RNA Integrity Number (RIN) was measured by the Agilent Bioanalyzer to assess RNA quality prior to sequencing (Vatanashevanopakorn *et al.*, 2017)¹⁷⁶.

2.2.3 Transcriptomics and bioinformatics analysis (DE analysis, GO clustering analysis, and GSEA analysis)

The initial bioinformatics analysis was performed in collaboration with Dr Nikolas Barkas, Bioinformatician at the MRC Weatherall Institute of Molecular Medicine, University of Oxford. DESeq2¹⁷⁷ was used to identify the differentially expressed (DE) genes in 3 comparisons: 3M mutant ENK+ subpopulation versus with 3M control ENK+ subpopulation; 8M mutant ENK+ subpopulation versus with 8M control ENK+ subpopulation; 3M mutant ENK+ subpopulation against 8M mutant ENK+ subpopulation. Genes with adjusted P value<0.05 were considered as DEGs.

For gene ontology (GO) analysis, DAVID (the database for annotation, visualization and integrated discovery) bioinformatics resources¹⁷⁸, was used for the generated DEGs sets generated to identify enriched functions for each comparisons. More specifically, each DEGs set was then divided into up-regulated DEGs set and down-regulated DEGs set according to the log2foldchange value. The two DEGs sets were analyzed as input for DAVID separately. Functional annotations in GO biological processes with enrichment score (EASE) <0.05 (by Fisher exact test) were then considered for clustering analysis by performing DAVID clustering.

For pathways enrichment analysis, Gene Set Enrichment Analysis¹⁷⁹ (GSEA analysis was used to investigate the affected pathways/signaling in the comparisons analysis. This was done in collaboration with Dr Nikolas Barkas. DEGs sets generated from DE analysis were treated as inputs for GSEA analysis. The MsigDB C2

Canonical Pathways gene sets and the MsigDB Hallmark Pathways gene sets were used to perform the enrichment analysis. Enriched pathways/signaling with FDR q-value <0.05 were considered as significantly affected pathways/signaling.

2.2.4 Immunostaining for brain tissues

Immunohistochemistry of DAB ((3'-diaminobenzidine) staining

OCT-embedded brains were transferred from -80°C freezer to the cryostat chamber (-20°C) and left to equilibrate for at least 30 minutes. Coronal sections of the brain were cut at 30 µm thickness using a Cryostat CM3030 S (Leica Biosystems). Sections were treated with 3% hydrogen peroxide (in TBS) at room temperature for 20 minutes. Three washes (10 minutes each) with TBS were done. Tissues were incubated in tissue blocking buffer 1 (10% normal goat serum, 0.3% carrageen, 0.5% Triton X-100 in TBS) at room temperature for 1 hour. Tissues were then incubated with the primary antibody (PACE4, sc-22898, 1:50, Santa Cruz) diluted in 1% normal goat serum, 0.3% carrageen, 0.5% Triton X-100 in TBS at room temperature overnight. After three washes with TBS, sections were incubated with the secondary antibody, biotinylated rabbit anti-goat (1:200, Novex) diluted in 1% normal goat serum, 0.3% carrageen, 0.5% Triton X-100 in TBS at room temperature for 2 hours. After three washes, sections were incubated in Vectastain® ABC solution (Vector laboratories) at room temperature for 30 minutes. Tissues were washed three times (15 minutes each) with TBS followed by two washes (15 minutes each) with TB.

Tissues were then incubated in DAB solution for 1-2 minutes. When colour developed, the reaction was stopped by adding cold TB. Sections were mounted on gelatin-coated slides and air-dried in a fume hood for 2 hours. Mounted sections were dehydrated in 90% and 100% ethanol, each for 1 minute before incubating in xylene for 10 minutes. Vectamount permanent mounting medium (Vector laboratories) was then applied on the slide before cover slip was placed.

Imunofluorescence

OCT-embedded brains were transferred from -80°C freezer to the Cryostat chamber (-20°C) and left to equilibrate for at least 30 minutes. Coronal sections of the brain were cut at 20 µm thickness using a Cryostat CM3030 S (Leica Biosystems).

Sections were washed with PBS at room temperature for 2X10 minutes. Sections were incubated in the blocking buffer (5% fish gelatine, 0.5% Triton in PBS) at room temperature for 1 hour. Sections were then incubated with the primary antibodies (listed in **2.1.6 Primary antibodies**) diluted in the blocking buffer at 4°C overnight.

After 4-5 washes with PBS of 0.1% Triton-X, sections were incubated with the secondary antibodies conjugated to Alexa Fluor 488 (anti-Rab, 1:1000, ThermoFisher, ThermoFisher) diluted in the blocking buffer at room temperature for 2 hours. After three washes, sections were incubated with DAPI diluted in PBS at room temperature for 10 minutes. Sections were washed three times (15 minutes each) with PBS before transferring to the slides. Vectashield mounting medium (Vector laboratories) was then applied on the slide before cover slip was placed and sealed by nail polish. Prepared

slides were ready to imaging until dry.

The sections were fluorescently imaged with an upright widefield epifluorescent microscope from Leica (DM6000B) and images acquired using a digital Color Camera (Leica, DFC310 FX). The following objectives were used for image acquisition: HCX PL APO 10x/0.40 CS, HC PL 20x/0.75 CS2 and HCX PL APO 40x/0.85 CORR CS.

2.2.5 Single molecule FISH (smFISH)

Tissue preparation

The *Trkb*^{PENK-KO} mice and respective control mice were sacrificed. The brains were then harvested, embedded in OCT embedding matrix (CellPath Ltd, Wales, United Kingdom), frozen on liquid nitrogen and stored at -80°C until used. smFISH was performed as described in Stellaris (LGC Biosearch Technologies, Petaluma, CA, USA) RNA FISH frozen tissue protocol with minor modifications. Probe libraries were designed to target coding sequences of mRNA of *Gapdh*, *Gsto2* and *Gcc1* gene and constructed by Stellaris. Each probe libraries consisted of 48 probes of length 20 bps (Probes library for *Gsto2* and *Gcc1* can be found in Appendix). *Gapdh* probes were coupled to Quasar 570 fluorophore, and *Gsto2* and *Gcc1* probes were coupled to Quasar 670 fluorophore.

Sections preparation and hybridisation

8µm cryosections was collected and directly mounted to coverslips. Then sections were fixed in 4%paraformaldehyde for 10min at room temperature, followed by 1 hour permeabilization using 70% ethonal at room temperature. Hybridization with 250 nM fluorescent label probes was carried out overnight in 37°C. DAPI dye for nuclear was counterstained during the washes. Then coverlips was mounted on slides with Vectashield antifade mounting medium (Vector Laboratories Ltd, Burlingame, CA, USA).

Imaging and analysis

Image stacks (0.2µm distance) were acquired with Leica microscope system equipped with a x100 oil-immersion objective and Leica DFC 365 FX camera using Leica AF6000 software (Leica, Wetzlar, Germany). Then image stacks was processed by 3D reconstruction of Leica AF6000, followed by image projection. 3 fields of CA3 region was imaged for each section. Quantification was done by using StarSeacher developed by Raj lab (<http://rajlab.seas.upenn.edu>) to generate the number of mRNA dots. DAPI was used to count the number of neurons in each field. The p value of comparing 3 biological replicates wildtypes and knockouts were achieved using student T test by GraphPad Prism.

2.2.6 Fluidigm quantitative PCR

FACS sorting has been described previously and subsequent Fluidigm quantitative PCR were collaboratively done with a lab colleague and following the established protocol of Minichiello's lab¹⁷³, which is briefly described as below.

After FACS sorting (described in Materials and Methods 2.2.2.1) of two biological replicates per genotype (*Trkb*^{PENK-KO} and *Trkb*^{PENK-WT}) from both 3M and 8M age. The sorted cells (50 cells for each biological replicate) were lysed in CellsDirect buffer and kept in the -80°C, then followed by the cDNA synthesis and pre-amplification. The number of pre-amplification cycles must be chosen carefully so as to be within the dynamic range, ie. ensuring that a sufficient number of cycles are selected so that enough cDNA is synthesised but not too many cycles so that too much cDNA is synthesised (which would obscure differences in gene expression levels due to saturation). Once the process is completed store the tubes at -20°C in preparation for Fluidigm Gene Expression analysis on the IFC. 17. Preparation of the cDNA samples and TaqMan assays should be exactly as stated in the Fluidigm protocol. The protocols can be found on the Fluidigm website. The chip was loaded according to the experimental plan that is appropriate for the analysis (2 technical repeats per sample), then inserted into Biomark machine and choose the appropriate gene expression analysis (GE) protocol. The expression level of each gene was normalised. This is done by normalising the expression of each gene (in each sample) to that sample's expression of a housekeeping gene (*Canx*). A couple of housekeeping

genes have been used to set up the condition of the experiment (*Canx*, *Gusb*), and two housekeeping genes have showed the consistent expression through all samples, which are qualified as housekeeping gene for normalization.

2.2.7 Stereological analysis of striatum and ventral tegmental area/substantia nigra pars compacta (VTA/SNpc)

2.2.7.1 Stereological analysis of striatal tyrosine hydroxylase optical density

Brains from both WT and mutant on each age were collected and sectioned coronally into serial sections of 30um. To cover the entire striatum(1800um, from anterior 0.345mm to anterior -1.455mm) stereologically, one in every 14 sections were selected and 4 representative sections for the striatum in total were processed to immunostaining of TH. Tyrosine hydroxylase expression levels of striatum were quantified by Leica Imaging system. Densitometric measurements by ImageJ were obtained at 4 selected anteroposterior levels for striatum. The values of staining density of each section were summed to generate the average density of striatal TH expression for each mouse. Then the values of average density of striatal TH expression was normalized to the mean of average density of control mice and the P value was calculated between control and mutants by using the one-tail Student t test.

2.2.7.2 Stereological tyrosine hydroxylase-positive cell counting in substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA)

Brains from both WT and mutant from each age analysed were collected and sectioned coronally into serial sections of 30um. To cover the entire SNpc and VTA (1400um, Bregma anterior -2.48mm to anterior -3.88mm) stereologically, one in every 14 sections were selected and 3 representative sections for SNpc and VTA in total were processed to immunostaining of TH. For tyrosine hydroxylase-positive cells counting, the region of SNpc and VTA was delineated according to the Allen Mouse brain Atlas firstly, then imageJ cell counting procedure was applied on each selected section to generate the number of TH+ cells in SNpc and VTA region. The counts from every representative section was added to estimate the number of TH+ cells for each mouse. Then the counts of TH+ cells in SNpc and VTA from each mouse was normalized to the mean number of TH+ cells in control mice and the P value was generated using the unpaired Student t test.

2.2.8 High performance liquid chromatography (HPLC)

The mobile phase was prepared with the following reagents: HPLC-grade methanol:625ml, NaH₂PO₄H₂O: 0.13M, ethylenediaminetetraacetic acid (EDTA): 0.85M, 1-octanesulphonic acid sodium salt (OSA-C₈H₁₇NaO₃S): 0.025mM,

concentrated phosphoric acid: 5ml. To prepare 5L of mobile phase, 89.694g of $\text{NaH}_2\text{PO}_4\text{H}_2\text{O}$ (MW=137.99g/mol) were diluted in 1 L of double-distilled H_2O (dd H_2O). 1.582g of EDTA (MW=372.24g/mol) were added to the solution, after which 625ml of methanol (12.5% HPLC grade) and 375 ml of dd H_2O were added. While leaving the solution to stir for a few minutes, a 10mM stock solution of OSA (MW=216.28g/mol, HPLC grade) was prepared by diluting 216.28 mg of OSA in 100 ml of dd H_2O . An additional 2750ml of dd H_2O and 12.5ml of OSA stock solution were added to the HPLC mobile phase solution. The volume was filled up to 5 L with dd H_2O and phosphoric acid (MW=98g/mol, HPLC grade) to adjust the pH to around 3.5 at 20⁰C.

To measure the levels of endogenous dopamine and related metabolites DOPAC and HVA, striatas from both *Trkb*^{PENK-KO} mice and relative control mice at both presymptomatic (3M) and symptomatic (8M) were homogenized with ultrasonicator (VibraCell, VCX 500, Sonics, USA) at AMPI: 21% for 10 sec in 200 μL of 0.092M PCA (perchloric acid), then centrifuged at 22,500 g for 15 min at 4⁰C. Samples were subsequently centrifuged at 13,000 rpm and at 4⁰C for 15 minutes. Supernatants were transferred to clean test tubes kept on ice, being particularly careful not to pipet up parts of the pellet. All samples were kept on ice at all times, unless being processed. At the beginning of experiment when brain samples were to be run, standard solutions were injected first, one at a time, at a concentration of 10⁻⁷M to calibrate the apparatus (i.e. to allow to infer initial concentrations in the samples depending on the height of the detection peak). Samples were injected 50 μL at a time in the HPLC. Each run

lasted for 20 minutes and 50 μ L of 0.092M PCA were injected between each sample, allowing at least 3 minutes for the solvent to pass through the column. In the experiment, each sample was run at least twice to assess variability in the results.

2.2.9 Stereotaxic injection of shRNA virus in the striatum

Experimental animals were first weighed prior to the procedure and then anaesthetised with vaporised 2-3% Isoflurane in 2L O₂/min (Abbott, No: B506). Each animal was placed on a standard stereotactic frame (Stoelting) by placing its teeth on the clamp and the head position fixed with ear bars and a nose clamp. Ocular lubricant (Allergan, Marlow, UK) was applied. The temperature was monitored constantly with a probe underneath the animal and an insulator on top of it to help in keeping the temperature steady at around 36.5°C. The % of the anaesthetic was usually set to 2-2.5% to sustain sufficient depth of anaesthesia at 60 breaths per min and with no pinch toe reflex and adjusted as needed during procedure. An analgesic cocktail consisting of Metacam (active substance: Meloxicam, non-steroidal anti-inflammatory and analgesic) (5mg/Kg) and Vetergesic (active substance: Buprenorphine, potent partial μ agonist analgesic) (0.1mg/Kg), was administered by intraperitoneal injection and a local injection of Marcain solution (2mg/Kg) underneath the skin of the head. The scalp was shaved with electric clippers, and the operative field was disinfected with iodine solution. The iodine application was done from the centre of the shaved part and working in circular mode outwards. A surgical stereoscope (Wild

Heerbrugg M655, Gais, Switzerland) was used for better precision in the stereotactic surgery. A 1cm long sagittal incision was made to expose the bregma and lamda points and the skull was cleaned with sterile saline from any soft tissue. Cotton buds with or without saline were used for cleaning the skull and adsorb any blood from small vessels at the site of the burr holes throughout the procedure. The skull was adjusted flat in the horizontal plane, by tweaking the height of the nose clamp and the ear bars, as measured by the coordinates of bregma and lamda and two juxtaposed lateral coordinates. The distance between bregma and lambda was measured and subsequently divided by 4.15 mm, which is the average distance between lambda and bregma for C57BL/6J mice (Allen Mouse Brain Atlas). This value was then multiplied by the predetermined stereotaxic coordinates, resulting in stereotaxic coordinates adjusted for the size of the mouse brain. The coordinates for the optimisation experiments were as follows (Allen Mouse Brain Atlas):

(Anterior injection: AP: 0.95mm,ML: 1.75mm,DV: 3.5 and 2.5mm;

Posterior injection: AP: 0.25mm,ML: 2mm,DV: 4 and 3mm) 1st

(Anterior injection: AP: 0.95mm,ML: 2.25mm,DV: 2.8 and 2.3mm;

Posterior injection: AP: 0.25mm,ML: 2.6mm,DV: 3 and 2.5mm) 2nd

(Anterior injection: AP: 0.95mm,ML: 2.00mm,DV: 2.8 and 2.3mm;

Posterior injection: AP: 0.25mm,ML: 2.3mm,DV: 3 and 2.5mm) 3rd

Burr holes of 0.8mm diameter were drilled until the dura was exposed. Drilling was done in a circular mode and not with direct force down the skull and forceps were used to assist by holding down the skull. Drilling was intermittent with small 2 second

intervals so that heat from friction is not damaging and cracking the skull. A precision 5µl syringe (Hamilton 75 RN 5uL SYR W/O NEEDLE, ESSLAB, cat.no.:7634-01) with a 34G bevelled needle (45°) with length 30mm (Hamilton RN Needle, ESSLAB, cat.no.: 207434) was used to inject any of shRNA lentivirus or scramble for the optimisation surgeries. The injectate was administered at a rate of 125nl/min using an automatic motorised micropump (Ultramicropump3 microsyringe injector with micro4 controller, World Precision Instruments). After injection, the needle was left in place for 10 minutes to prevent reflux and enable diffusion of the viral suspension, and then slowly withdrawn. The needle was retracted with a moderately fast pace from the site around 200-500µm and after 2mins of wait it was completely retracted at a steady pace. Sutures were made to close the incision with 3-4 surgical knots by using Ethicon Coated vicryl 5-0 FS3 conv 16mm 3/8c cutting (Johnson Johnson, cat.no: W9442). Each animal was observed until complete recovery from anaesthesia. The recovery was done on a pre-warmed to 30°C incubator chamber. When the animal regained its consciousness, and demonstrated it could independently move around the chamber, it was then transferred to its home cage. Each animal was closely observed for a week following the surgery and post-operative care was given by administering Metacam by intraperitoneal injection if the mouse expressed signs of pain and distress from the surgery. The animals were allowed to recover for 3-4 weeks prior of any experiments, this allowed enough time for any lentivirus to be adequately expressed in the brain.

2.2.10 Locomotor behavioral test

Behavioural experiments were done with the help of a senior postdoc in the Minichiello lab (Dr Aman Asif-Malik), and took place at 7, 8 and 12 months of the animals age post shRNA injection. All animals were tested for open field (OF) activity but only the last 4 sessions were included in the animals to eliminate behavioural effects that arise as a consequence of novelty.

Two Med Associates (Med Associates, St. Albans, VT) chambers were used, (43.2 × 43.2 cm) and a dark box insert was inserted into each chamber 30.5 cm high and a white plexiglass floor. Each chamber was contained within its own closed home cage (incl. fan-assisted ventilation and light) onto a pull-out shelf. Activity was detected by arrays of infrared light beam motion detectors (16 × 16, 2.5 cm apart) at the sides of each chamber, thus creating a detection grid. The chambers were controlled by the Activity Monitor program, version 5.10 (software settings: box size: 3; resting delay: 500 ms; resolution: 50 ms). A ventilated cover was placed on top of each chamber to prevent animals from jumping out during recording.

Each animal was placed in the centre of the open field chamber at the start of the session and behaviour was recorded for 15 minutes. The chambers were washed with water in between and then with a diluted bioclean solution at the end of each session. Animals were alternated between the two chambers for each session. Measures included ambulatory distance cm, ambulatory velocity (cm/sec), jump counts (which is a measure of the number of times the animal's body is not detectable by the motion

detectors) and resting time (sec). Measures were automatically scored by a computer using MED-PC software (Activity Monitor program version 5.10).

Chapter 3: Results

3.1 Transcriptomics and Bioinformatics analysis

3.1.1 *Transcriptomics: next-generation sequencing and bioinformatics analysis*

3.1.1.1 *Mouse lines, FACS sorting, Bulk cells deep sequencing (RNA-seq) and Quality control*

It has been shown that BDNF/TrkB signalling is essential to maintain normal locomotor function (Besusso et al. 2013)¹⁶⁸, here I began my study to uncover the underlying molecular mechanisms. The animal lines used in this thesis are described in details in **Chapter 2.2.1**, and have been well characterized by previous work¹⁶⁸. Briefly, previous work¹⁶⁸ has established successful specific deletion of TrkB signaling in enkephalin positive medium spiny neurons (MSNs). Such deletion leads to an age-dependent hyperlocomotor phenotype manifesting from 8 months of age, without causing neuronal loss or morphological changes in the first year of age. In this study, to dissect the molecular changes induced in enkephalinergic MSNs in the absence of TrkB signaling, both a control line (IPNK) (*Trkb*^{PENK-WT}; *Ai9*/+) and a mutant line (BIPK) (*Trkb*^{PENK-KO}; *Ai9*/+) at two stages, 3 months and 8 months, were used to perform global gene expression analysis by RNA-sequencing. Specifically, to address cell-specific changes, ENK+ and ENK- subpopulations from each mouse striatal tissue were purified by fluorescence activated cell sorting (FACS). In total, 38

striatal samples from 18 controls (IPNK) and 20 mutants (BIPK) at 3 M and 8M of age were used to perform RNA-seq analysis. Details of these samples can be found in the **Table 3.1.1.1**. The established FACS sorting protocol in the lab allowed us to purify the striatal subpopulations of interest from young (3M) and aged (8M) mice striatal tissue in a celltype-specific manner.

RNA-seq data was collected and data processing was done by following the methods described in **Chapter 2.2.2.2**. and ¹⁷⁶. The acquisition of RNA-seq data consists of several steps such as obtaining the raw reads, performing the read alignment and quantification. Each of these steps involves specific checks that will allow to ascertain the quality of the data. Some examples are reported in **Figure 3.1.1.1.a**, which shows an example of reads quantification. A set of housekeeping genes presenting similar distribution pattern in each sample library, indicating well prepared cDNA library and reliable sequencing process. Furthermore, when sequences were aligned against the reference genome, to evaluate the quality of reads obtained, number of mapped reads were plotted with total input reads and multiple aligned reads described in **Figure 3.1.1.1.b**, showing for each library, over 90% of input reads were mapped reads, and only a small fraction (<5%) of reads could not be aligned uniquely and would be discarded in next step.

Molecular mechanisms relevant to the maintenance of locomotor control

Samples	Symbol	Annotat ed	Group	Age	Geno-ty pe	Cell-t ype	All-mappe d reads	Normaliz ation reads
P2234_30 07_1.bam	IPNK 3007 Tm+	Control_ 8M+_A	Control_8M+	8M	Wildtype	ENK+	38659951	26096011
P2234_30 08_1.bam	IPNK 3008 Tm-	Control_ 8M-_A	Control_8M-	8M	Wildtype	ENK-	30150020	20069048
P2234_30 09_1.bam	IPNK 3009 Tm+	Control_ 8M+_B	Control_8M+	8M	Wildtype	ENK+	43730846	29773435
P2234_30 10_1.bam	IPNK 3010 Tm-	Control_ 8M-_B	Control_8M-	8M	Wildtype	ENK-	27834699	18418486
P2234_30 11_1.bam	IPNK 3011 Tm+	Control_ 8M+_C	Control_8M+	8M	Wildtype	ENK+	36882513	24697715
P2234_30 12_1.bam	IPNK 3012 Tm-	Control_ 8M-_C	Control_8M-	8M	Wildtype	ENK-	35757296	24316727
P2234_30 13_1.bam	IPNK 3013 Tm+	Control_ 8M+_D	Control_8M+	8M	Wildtype	ENK+	34672935	22878193
P2234_30 14_1.bam	IPNK 3014 Tm-	Control_ 8M-_D	Control_8M-	8M	Wildtype	ENK-	37483127	23552705
P2234_30 15_1.bam	BIPK 3015 Tm+	Mutant_8 M+_A	Mutant_8M+	8M	Mutant	ENK+	30387179	19427045
P2234_30 16_1.bam	BIPK 3016 Tm-	Mutant_8 M-_A	Mutant_8M-	8M	Mutant	ENK-	30696045	19460018
P2234_30 17_1.bam	BIPK 3017 Tm+	Mutant_8 M+_B	Mutant_8M+	8M	Mutant	ENK+	35881253	22014280
P2234_30 18_1.bam	BIPK 3018 Tm-	Mutant_8 M-_B	Mutant_8M-	8M	Mutant	ENK-	33196682	18376345
P2234_30 19_1.bam	BIPK 3019 Tm+	Mutant_8 M+_C	Mutant_8M+	8M	Mutant	ENK+	34017364	22237587
P2234_30 20_1.bam	BIPK 3020 Tm-	Mutant_8 M-_C	Mutant_8M-	8M	Mutant	ENK-	30631538	19631688
P1852_20 50.bam	BIPK 2050 Tm+	Mutant_8 M+_D	Mutant_8M+	8M	Mutant	ENK+	37695884	29660561
P1852_20 51.bam	BIPK 2051 Tm-	Mutant_8 M-_D	Mutant_8M-	8M	Mutant	ENK-	32023990	21791554
P1852_20 52.bam	BIPK 2052 Tm+	Mutant_8 M+_E	Mutant_8M+	8M	Mutant	ENK+	34937683	23505810
P1852_20 53.bam	BIPK 2053 Tm-	Mutant_8 M-_E	Mutant_8M-	8M	Mutant	ENK-	30544414	20632000
P1852_20 54.bam	BIPK 2054 Tm+	Mutant_8 M+_F	Mutant_8M+	8M	Mutant	ENK+	33739315	22649354
P1852_20 55.bam	BIPK 2055 Tm-	Mutant_8 M-_F	Mutant_8M-	8M	Mutant	ENK-	37717220	21272566
P1852_20 30.bam	IPNK 2030 Tm+	Control_ 3M+_A	Control_3M+	3M	Wildtype	ENK+	57269645	39641289

Molecular mechanisms relevant to the maintenance of locomotor control

P1852_20 31.bam	IPNK 2031 Tm-	Control_ 3M-_A	Control_3M-	3M	Wildtype	ENK-	30161228	23982627
P1852_20 32.bam	IPNK 2032 Tm+	Control_ 3M+_B	Control_3M+	3M	Wildtype	ENK+	29216476	16957031
P1852_20 33.bam	IPNK 2033 Tm-	Control_ 3M-_B	Control_3M-	3M	Wildtype	ENK-	30365941	19376684
P1852_20 34.bam	IPNK 2034 Tm+	Control_ 3M+_C	Control_3M+	3M	Wildtype	ENK+	29110081	21725443
P1852_20 35.bam	IPNK 2035 Tm-	Control_ 3M-_C	Control_3M-	3M	Wildtype	ENK-	29946036	19259812
P1852_20 36.bam	IPNK 2036 Tm+	Control_ 3M+_D	Control_3M+	3M	Wildtype	ENK+	32598680	20776347
P1852_20 37.bam	IPNK 2037 Tm-	Control_ 3M-_D	Control_3M-	3M	Wildtype	ENK-	32792069	24061641
P1852_20 56.bam	IPNK 2056 Tm+	Control_ 3M+_E	Control_3M+	3M	Wildtype	ENK+	34976572	25261370
P1852_20 57.bam	IPNK 2057 Tm-	Control_ 3M-_E	Control_3M-	3M	Wildtype	ENK-	33097396	23061402
P1852_20 38.bam	BIPK 2038 Tm+	Mutant_3 M+_A	Mutant_3M+	3M	Mutant	ENK+	30740458	19414761
P1852_20 39.bam	BIPK 2039 Tm-	Mutant_3 M-_A	Mutant_3M-	3M	Mutant	ENK-	35008633	24113811
P1852_20 40.bam	BIPK 2040 Tm+	Mutant_3 M+_B	Mutant_3M+	3M	Mutant	ENK+	33545475	21274770
P1852_20 41.bam	BIPK 2041 Tm-	Mutant_3 M-_B	Mutant_3M-	3M	Mutant	ENK-	35569896	25325029
P1852_20 44.bam	BIPK 2044 Tm+	Mutant_3 M+_C	Mutant_3M+	3M	Mutant	ENK+	40048933	24521128
P1852_20 45.bam	BIPK 2045 Tm-	Mutant_3 M-_C	Mutant_3M-	3M	Mutant	ENK-	40637310	25933389
P1852_20 46.bam	BIPK 2046 Tm+	Mutant_3 M+_D	Mutant_3M+	3M	Mutant	ENK+	29639338	19990202
P1852_20 47.bam	BIPK 2047 Tm-	Mutant_3 M-_D	Mutant_3M-	3M	Mutant	ENK-	38434885	27063661

Table 3.1.1.1 38 mice striatal samples.

Total of 38 striatal samples (19 *Penk*⁺ and 19 *Penk*⁻ from 19 mice) of both 18 controls (IPNK) and 20 mutants (BIPK) from both 3M and 8M of age were used to perform RNA-seq.

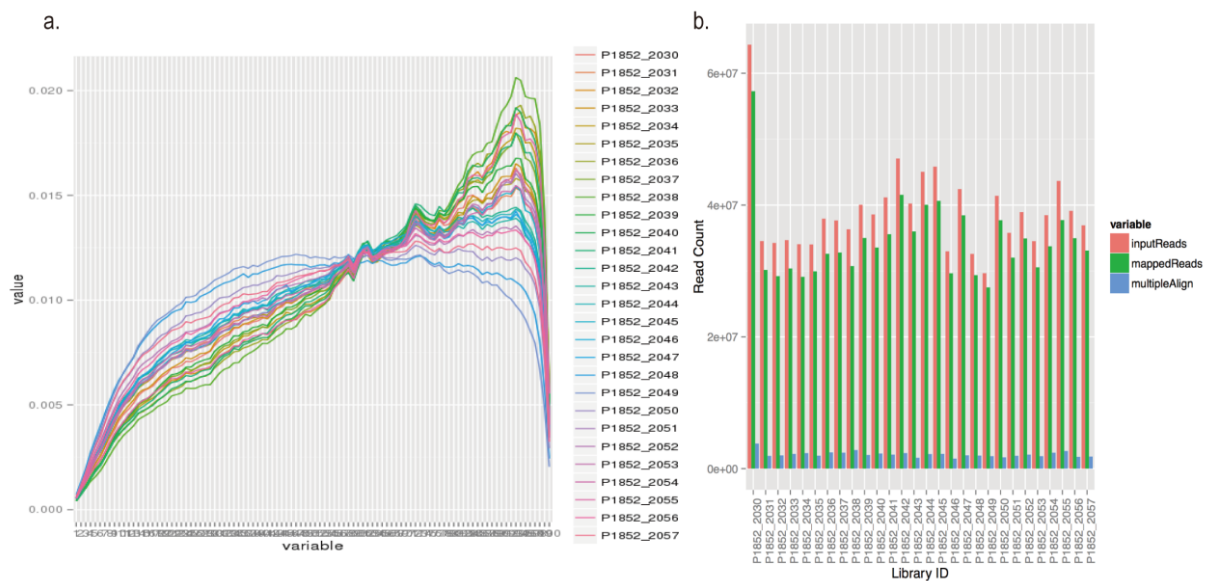


Figure 3.1.1.1 RNA-seq data process pipeline with quality control.

a. Bias of the distribution of the reads across a panel of housekeeping genes for each library. X axis represents position across aggregate of housekeeping gene and y represents proportion of reads in each position. **b.** Histogram showing the number of reads obtained and aligned for each library. For each library, three types of reads were plotted. Input reads: total reads from sample; mapped reads; and multiple aligned reads, which could not be aligned uniquely and were discarded.

3.1.1.2 RNA-seq global data properties

RNA-seq data processing quality control was followed by transcripts identification for each sample. This was estimated by raw counts onto the reference genome (mouse reference genome mm10). Raw read counts alone are not sufficient to compare expression levels among samples because these values can be affected by many factors including for instance transcript length, total number of reads, and sequencing biases. Therefore, transcript levels were quantified in reads per kilobase of exon model per million mapped reads (RPKM). This method has been previously established to appropriately quantify mammalian transcriptomes by RNAseq¹⁸⁰. The RPKM reflect the molar concentration of a transcript in the starting sample by normalizing for RNA length and for the total read number in the measurement. This allows better comparison of transcript levels both within and between samples. We then used RPKM for subsequent analysis.

To acquire an overview and to observe any alterations from whole transcriptome level across all the samples, we performed global analysis by using the expression level (RPKM) of 23004 mapped genes for each sample. Firstly, we visualized the expression level (RPKM) of 23004 mapped genes across the 38 samples by heatmap with hierarchical clustering based the expression level of those genes (**Figure 3.1.1.2.a**). This allowed us to visualize the expression pattern across all the mapped genes in each sample, moreover, by calculating Euclidean distance between sample based on the transcriptome expression levels, sample of similar expression pattern of

mapped genes would be enriched in an unbiased manner. We then performed Pearson's correlation analysis on the 38 samples to generate correlation heatmap (**Figure 3.1.1.2.b**) to evaluate the strength of association between samples. Furthermore, to convert variables into a set of values based on the whole transcriptome, primary component analysis (PCA) was applied here to visualize relation of all samples in 2D dimension (**Figure 3.1.1.2.c.d**). As shown in **Figure 3.1.1.2.a**, samples can barely be clearly clustered into assigned subpopulations based on the whole transcriptome of all mapped genes; consistent with the heatmap of correlation, no significant cluster of samples was shown by correlation analysis. This would also reflect the high quality of RNA-seq data across the well-prepared samples and reproducible workflow. Interestingly, with PCA analysis, despite the majority of subpopulations of samples had substantial overlapping pattern, vector distribution of ENK+ MSNs at 8M showed clear differences between controls and mutants (**Figure 3.1.1.2.c**). At 8M, the transcriptome of mutant ENK+ MSNs had different distribution pattern compared to the control, while at 3M this pattern was not apparent (**Figure 3.1.1.2.d**). Finally, although we have depleted TrkB signaling specifically from the ENK+MSN, this analysis showed that also the ENK negative subpopulation had an altered distribution pattern at 8M compared to controls (**Figure 3.1.1.2.c.d**).

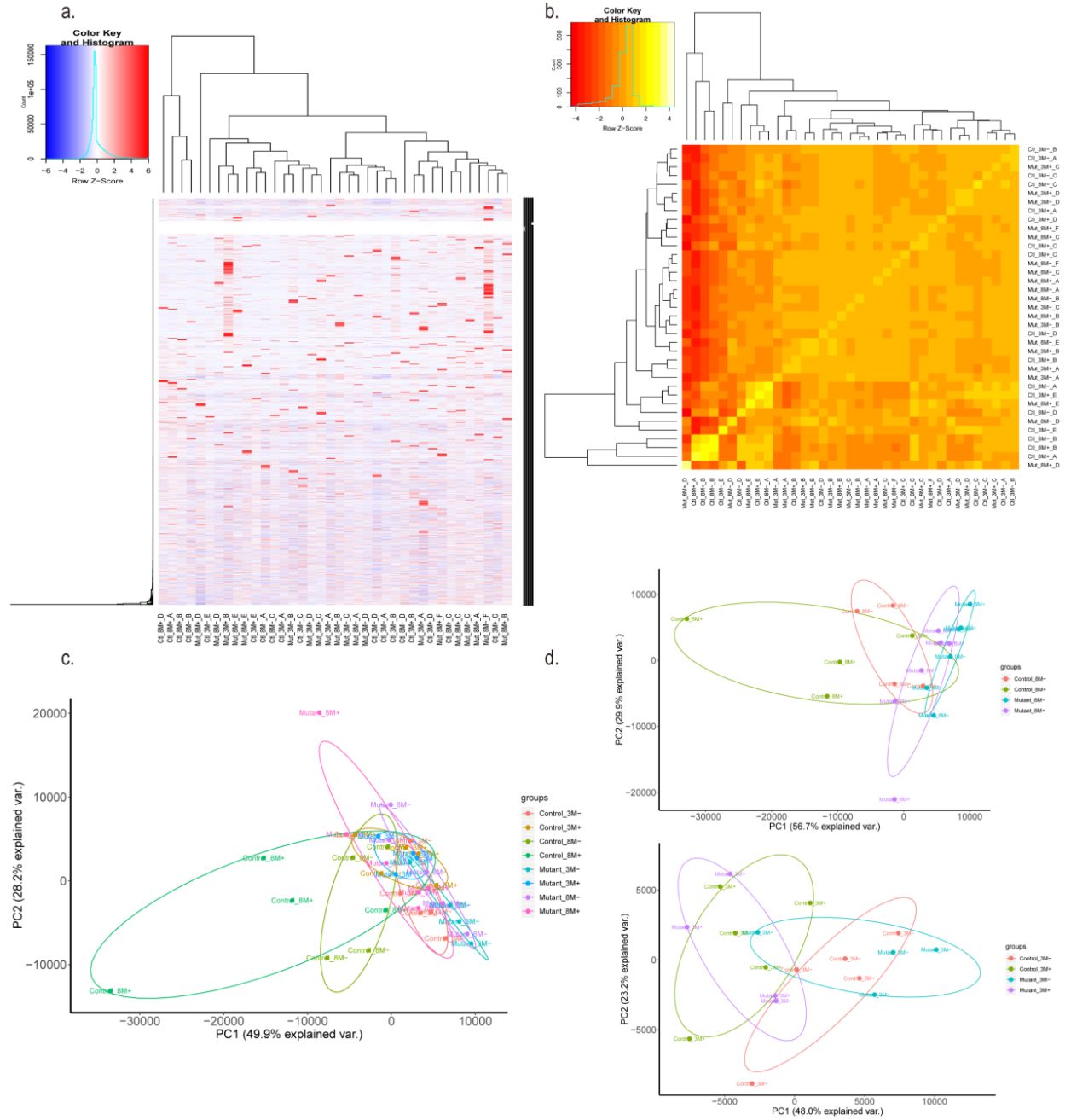


Figure 3.1.1.2 Global transcriptome analysis on 38 samples of 8 striatal subpopulations of two stages.

a. Heatmap of expression levels of 23004 mapped genes for 38 samples. Expression level (RPKM, reads per kilobase per million) is displayed as a color gradient with highly expressed genes shown in red and low ones shown in blue. Thirty-eight samples obtained by tissue dissociation and FACS sorting are grouped into 8 striatal subpopulations by 3 parameters (Genotype: Control (*Trkb*^{PENK-WT}) and Mutant (*Trkb*^{PENK-KO}); Celltype: ENK+/ENK-; Age: 3M and 8M). Alphabetic labeling is

indicating the biological replicates in each condition for both ENK+ and ENK- subpopulations (*Trkb*^{PENK-WT}-3M: 5 biological replicates; *Trkb*^{PENK-KO}-3M: 4 biological replicates; *Trkb*^{PENK-WT}-8M: 4 biological replicates; *Trkb*^{PENK-KO}-8M: 6 biological replicates). **b.** Heatmap of the correlation of all samples based on 23004 mapped genes. **c.** Primary component analysis (PCA) of the 38 samples. Values of each sample of Primary component 1 (PC1) and primary component 2 (PC2) were plotted and grouped by each subpopulation. **d.** PCA plots extracted from c. for 3M and 8M of age, respectively.

3.1.2 Transcriptomic feature of striatal cell subpopulations

3.1.2.1 Profiling of medium spiny neurons signature genes

After performing the RNA-Seq global data properties analysis and established the validity of the samples, we then looked at the expression pattern of specific signature gene sets to capture any changes related to the specific cell type (ENK+MSN), based on age and presence or absence of TrkB signaling.

Firstly, to ensure that our sorted MSN population comprises features of MSNs and to assess how these MSNs subpopulations behave in absence of *Trkb* at transcriptomic level, we compared the gene expression profiles obtained from our

striatal sample populations with that of 48 MSNs-associated genes reported in the literature¹⁸¹ using hierarchical clustering. This analysis revealed that 28 out of 48 genes (Cluster1, >58.3%), including *Penk/ Drd2/ Darpp32*, were highly enriched in tdTomato positive populations (ENK+), showing validated acquisition of ENK+ MSNs features (**Figure 3.1.2.1.a**). Moreover, by comparing the expression of the MSNs signature genes between ENK+ and ENK- subpopulations within individual genotypes, it showed significant enriched expression of the 28 Cluster1 genes in ENK+ populations compared to ENK- populations (**Figure 3.1.2.1.b**), supporting reliable acquisition of ENK+ MSNs in our conditions. Having confirmed MSNs signature (Cluster1, **Figure 3.1.2.1.a**) in our populations (ENK+), we then asked which functions involving those genes has been affected. We performed Gene ontology analysis on the gene set from cluster1. The analysis indicated that these genes were collectively associated with neuronal processes (synaptic transmission, prepulse inhibition) and critical neuronal compartments (postsynaptic membrane, synapse, axon, dendrite) (**Figure 3.1.2.1.c**). Intriguingly, the enriched biological processes (locomotory behavior, response to drug) directly reflected the phenotype observed from the previous study from our lab¹⁶⁸, and these affected processes also reinforced the functional contribution of these ENK+ subpopulations to locomotor phenotype.

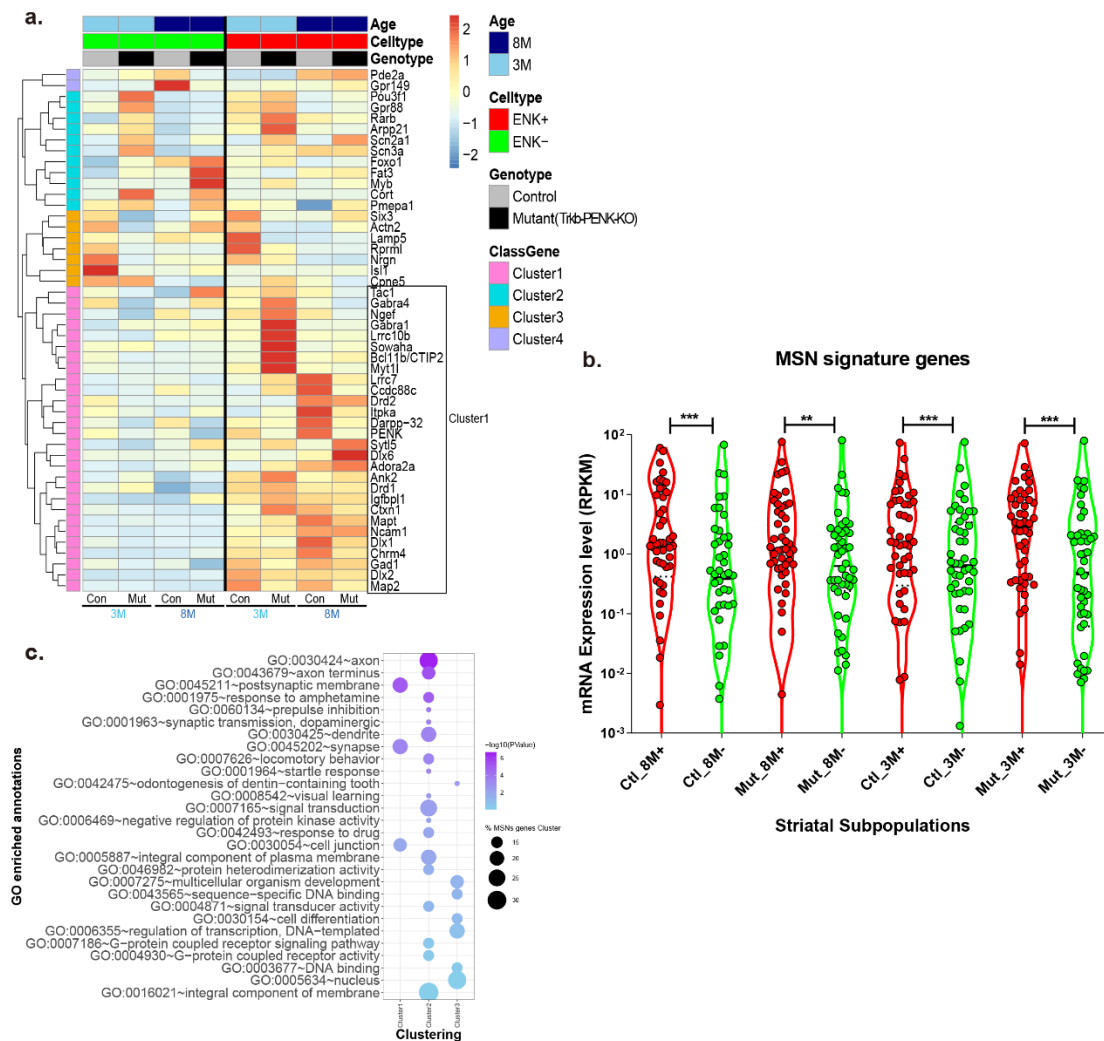


Figure 3.1.2.1 Significant enrichment of MSNs signature genes in ENK+ MSNs subpopulations.

a. heatmap profiling representing average expression for 48 MSNs signature genes in the different sorted populations (n=4-6 biological replicates for each subpopulation). The black frame highlights the largest cluster (n.1). Hierarchical clustering revealed that 28 out of 48 MSN signature genes were enriched in our populations of ENK+MSNs and varied with age. Also, this analysis revealed clear differences between presence and absence of *Trkb* at both ages compared to control populations.

b. Expression of MSNs signature genes in each subpopulation. Dots represent genes; significance values were calculated by the non-parametric Wilcoxon test; Significance values in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

c. Affected biological processes from gene ontology analysis using the enriched genes from cluster1 in a. Significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue, and the percentage of gene count is indicated as the size of bubble.

3.1.2.2 Significantly enriched pattern of transcription factors relevant to Huntington disease in absence of TrkB signaling

Since TrkB signaling has been extensively implicated in neurodegenerative conditions^{182,183}, and particularly in Huntington disease, contributes as main pathogenesis¹⁸⁴ and striatal MSNs derived from HD patients shows down-regulated expression level of TrkB¹⁸¹, we then asked whether the transcriptomic changes observed in absence of TrkB signaling in the purified subpopulations would correlate with gene expression changes observed in HD¹⁸⁵.

Therefore, we examined the list of 48 transcription factors (TF) recently identified as key genes drivers of HD progression¹⁸⁵. Hierarchical clustering of expression levels of these 26 genes clearly showed that HD-related TFs expression were mainly enriched in subpopulations from *Trkb*^{PENK-KO} line compared to the control (**Figure 3.1.2.2.a**). Specifically, *Smad3*, has been validated as the core regulator of early gene expression changes in HD¹⁸⁵, we observed generally up-regulation in mutant subpopulations compared to the control (**Figure 3.1.2.2.a**). *Klf14* and *Klf1*, part of the Kruppel-like transcription factors family, highly enriched

in mutant ENK⁺ subpopulations, has been indicated as key transcription regulator associated with the pathobiology of numerous human diseases¹⁸⁶. More importantly, *KLFs* also share homology with the transcription factor *Sp1*, which is widely reported to be associated with HD pathogenesis¹⁸⁷. Moreover, this enriched pattern of TFs is supported by the significant increased expression of these HD-related TFs when comparing mutant subpopulations with its corresponding control (**Figure 3.1.2.2.b**), implying not only the affected functions in mutant mice underlying HD pathogenesis, but also suggests early priming of HD related transcription factors probably contributing to the development of the hyperlocomotor phenotype observed in the *Trkb*^{PENK-KO} mice¹⁶⁸.

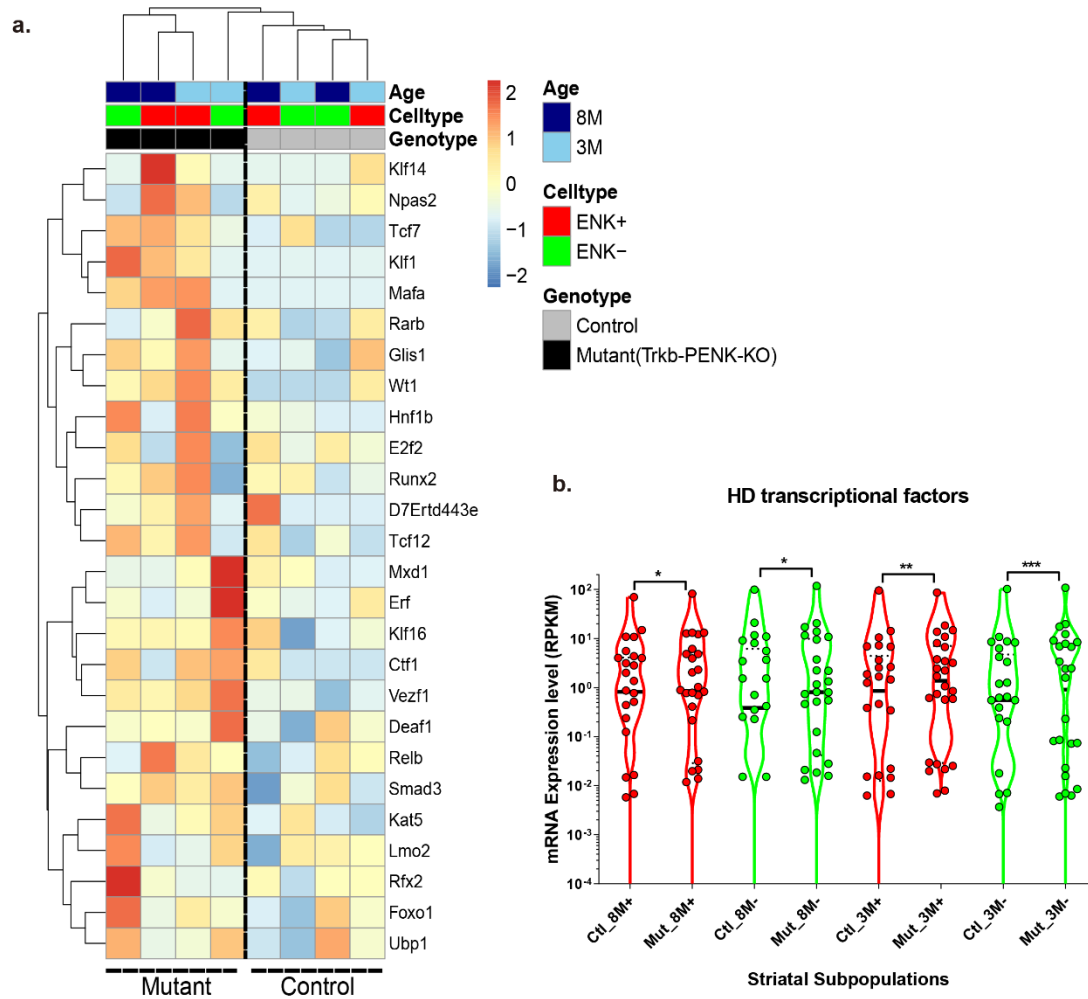


Figure 3.1.2.2 Significant enriched HD TF genes in mutant striatal populations.

a. Heatmap profiling representing average gene expression of HD related transcription factors mainly enriched in *Trkb*^{PENK-KO} populations, hierarchical clustering of average gene expression (HD related TF genes list quoted from Ament SA, et al. (2018)¹⁸⁵).

b. Expression of HD-related transcription factors in each subpopulation. Dots represent genes; significance values were calculated by the non-parametric Wilcoxon test; Significance values in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.1.2.3 Priming microglia activated genes enriched in non-enkephalinergic MSNs

Since we observed expression changes of HD related TFs in absence of TrkB signaling in the sorted striatal populations, we wondered whether other mechanism could be triggered by the conditional deletion of *Trkb* and contribute to neurodegeneration.

As neuroinflammation plays an important role in the progression of neurodegenerative diseases, especially with a prominent contribution of microglia¹⁸⁸⁻¹⁹¹, we then utilized a set of signature genes highly related to microglia priming in neurodegenerative conditions¹⁹², and examined microglia priming in ENK-sorted subpopulations that include also microglia. Analysis of the microglia priming genes (38 genes, in particular) revealed that there was substantial activation of these genes in ENK- subpopulations of *Trkb*^{ENK-KO} line compared to the control line, which also showed age progression (**Figure 3.1.2.3.a**). At stage 8M, the microglia genes were massively enriched in mutant ENK- subpopulation compared to the 8M control, including *IL1B*, *NABI*, *LGALS3BP*, the common microglia activation markers, (**Figure 3.1.2.3.b**). Although the gene set of interest was not significantly up-regulated when comparing the 3M ENK- subpopulation to the 3M control (**Figure 3.1.2.3.b**), we can clearly observe a unique subset of microglia priming genes activated at stage 3M (**Figure 3.1.2.3.a**, *CST7/ ANXA5/ CLEC7A/ SDC3/ UGCG/ CD52/ CH25H*) suggesting that priming of microglia happens in absence of TrkB

signaling at early stage leading to substantial activation at later stage, correlating with activation of microglia contributing to progressive neurodegeneration.

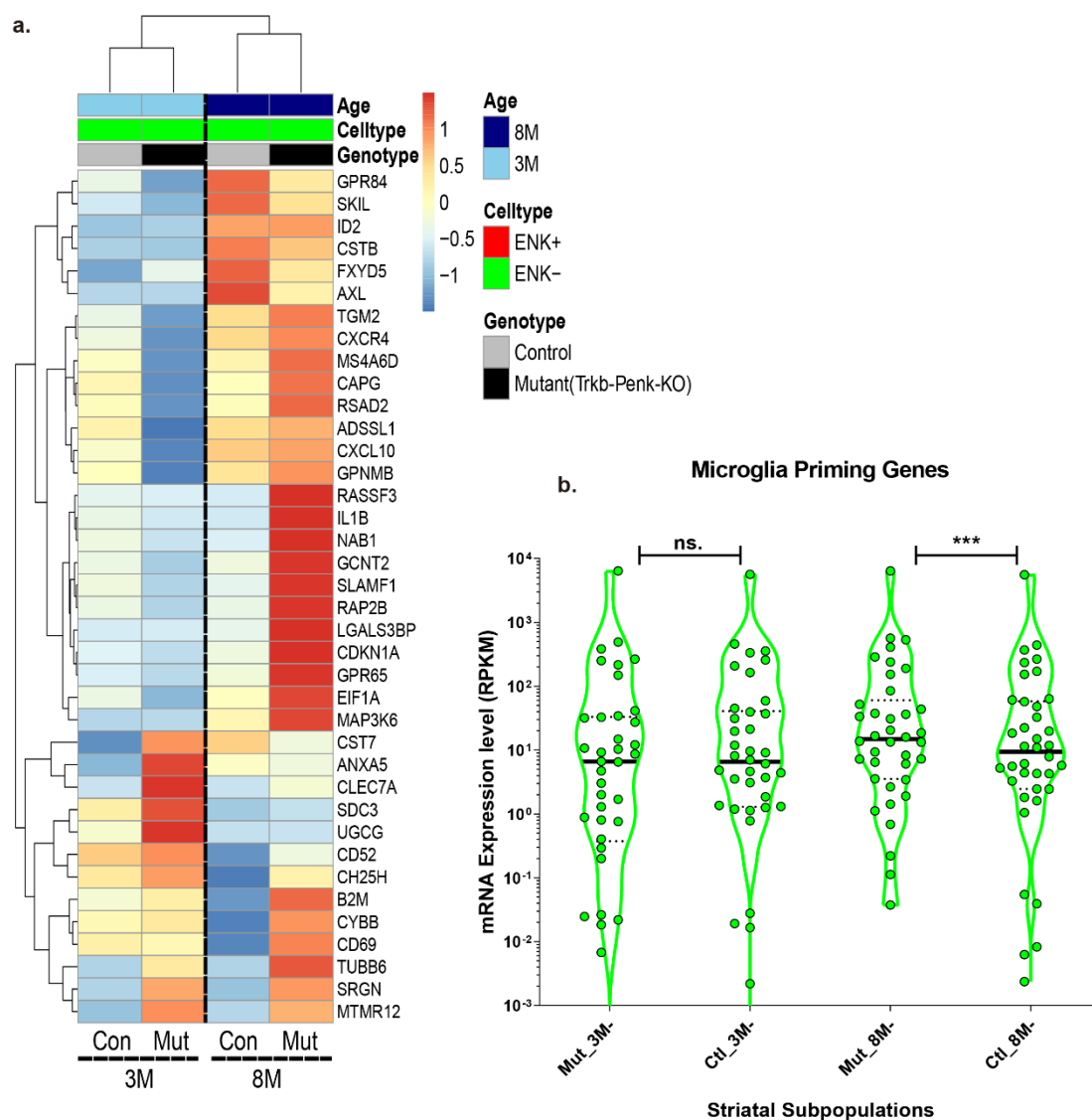


Figure 3.1.2.3 Microglia-activation genes enriched in ENK- subpopulations.

a. Heatmap profiling representing average expression of microglia activation related genes in ENK- subpopulations. Microglia priming in absence of TrkB signaling has been already starting at 3M and increasing at 8M of age. Hierarchical clustering is based on average gene expression (microglia activation related genes list quoted from Holtman IR, et al. (2015)¹⁹²).

b. Expression of microglia activation related genes in ENK- subpopulations. Dots

represent genes; significance values were calculated by the non-parametric Wilcoxon test; Significance values in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

3.1.3 Differential genes expression analysis (DEGs), gene ontology and functional enrichment analysis

3.1.3.1 Gene expression changes revealed by DEGs and overrepresented functions at 8M

To identify key genes and functions deregulated by age and TrkB-deficiency we performed differential gene expression analysis (by applying DESeq2¹⁷⁷ as described in **Chapter 2.2.3**) comparing genotype and age between the mutant ENK+ subpopulation and respective control.

Firstly, we aimed to identify the differential expressed genes that could be associated with the hyperlocomotor phenotype at 8M. We compared the control ENK+ population at 8M with the mutant ENK+ population. While 13881 genes were non significantly different between the two genotypes, only 54 genes (37 down-regulated/ 17 up-regulated) had significant differential expression ($\text{Padj} < 0.05$) (**Figure 3.1.3.1.a**). Following DGE analysis we performed gene ontology (GO) clustering analysis (described in **Chapter 2.2.3**); 107 functional annotations were

significantly enriched with EASE <0.05 . Among these 107 annotations, 35 (32.7%) were generated by the up-regulated DEG set, while 72 (67.3%) were by the down-regulated DEG set, and these annotations could be classified into 5 clusters hypergeometrically (**Supplementary Figure 3.1.3.1**, please see in Appendix). More in detail, the most enriched up-regulated cluster (21.5%) (**Figure 3.1.3.1.b**), cell death/ apoptotic process/ programmed cell death associated annotations, was significantly enriched and highest clustered in up-regulated DEGs, while among the 3 down-regulated clusters, the largest cluster (39.3%) (**Figure 3.1.3.1.c**) showed mainly RNA/phosphorus/protein metabolic processes associated annotations. These data suggest that apoptosis and cell death associated functions has been primed in absence of TrkB signaling in ENK⁺ striatal MSNs at 8M, with suppression of RNA/phosphorus/protein metabolic processes functions.

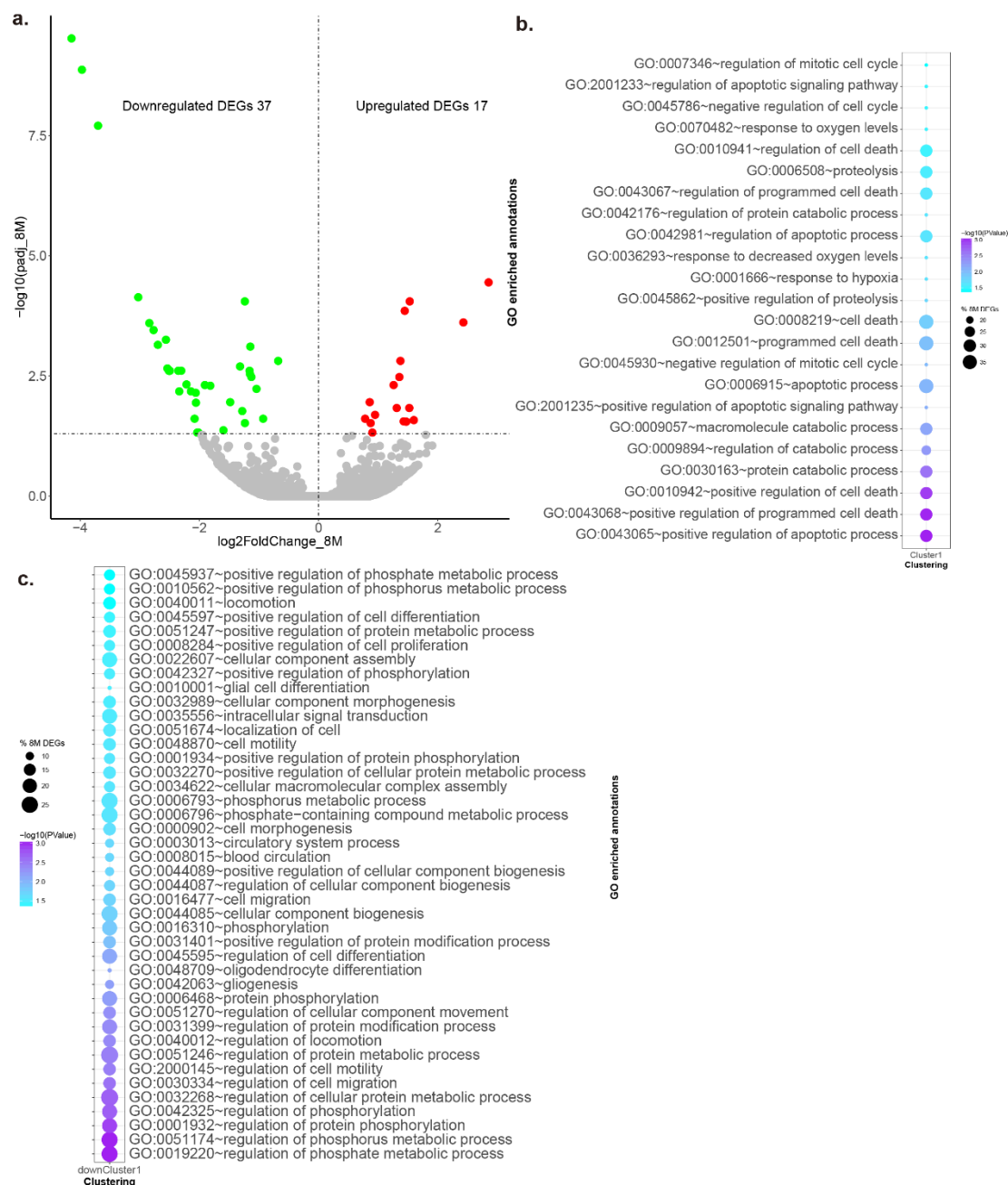


Figure 3.1.3.1 DEGs of mutant ENK+ MSNs at 8M shows primed apoptosis processes/cell deaths and suppressed metabolic processes functions.

a. Differentially expressed genes set by comparing mutant ENK+ MSNs with control at 8M. Each gene is represented by each dot and plotted by value of $\log_2(\text{foldchange})$ against respective $\log_{10}$ of P-adjusted value. Green dots represent down-regulated DEGs, red dots represent up-regulated DEGs, and grey dots represent non-significantly differential expressed genes. **b.** Largest cluster from GO clustering analysis of up-regulated DEG set at 8M. **c.** Largest cluster from GO clustering

analysis of down-regulated DEG set at 8M. X axis of histogram shows percentage of enriched DEGs for each annotation, significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue. Proportion of each cluster in total amount of enriched annotations is indicated as the size of bubble.

3.1.3.2 Gene expression changes revealed by DEGs and overrepresented functions at 3M

After examining the altered functions specific to the ENK+ subpopulation at 8M, we then wondered what genes specific to mutant ENK+ MSNs at early stage could trigger the transcriptomic changes observed at later stage and eventually contribute to developing the locomotor phenotype.

Same strategy of analysis has been applied to the DEGs set of 3M to address this issue. Firstly, while 13804 genes showed no significant differential expression between 3M mutant ENK+ MSNs and control, 162 genes (60 down-regulated/102 up-regulated) had significant differential expression ($P_{adj} < 0.05$) (**Figure 3.1.3.2.a**). GO clustering analysis then showed, intriguingly, only 45 functional annotations to be significantly enriched with $EASE < 0.05$. Among these functional annotations, most of them (42, 93.3%) were from up-regulated DEGs set forming 7 clusters, while only 3 annotations were enriched as one cluster for the down-regulated DEGs set (**Supplementary Figure 3.1.3.2**, please see in Appendix). For those up-regulated clusters, top 3 clusters formed the largest proportion (57.8%) with protein

phosphorylation processes associated functions most significantly enriched (**Figure 3.1.3.2.b**). Interestingly, the remaining clusters from up-regulated DEGs showed abnormal enriched functions specifically associated with synapse assembly/ nervous system development/ cell adhesion (**Figure 3.1.3.2.c**).

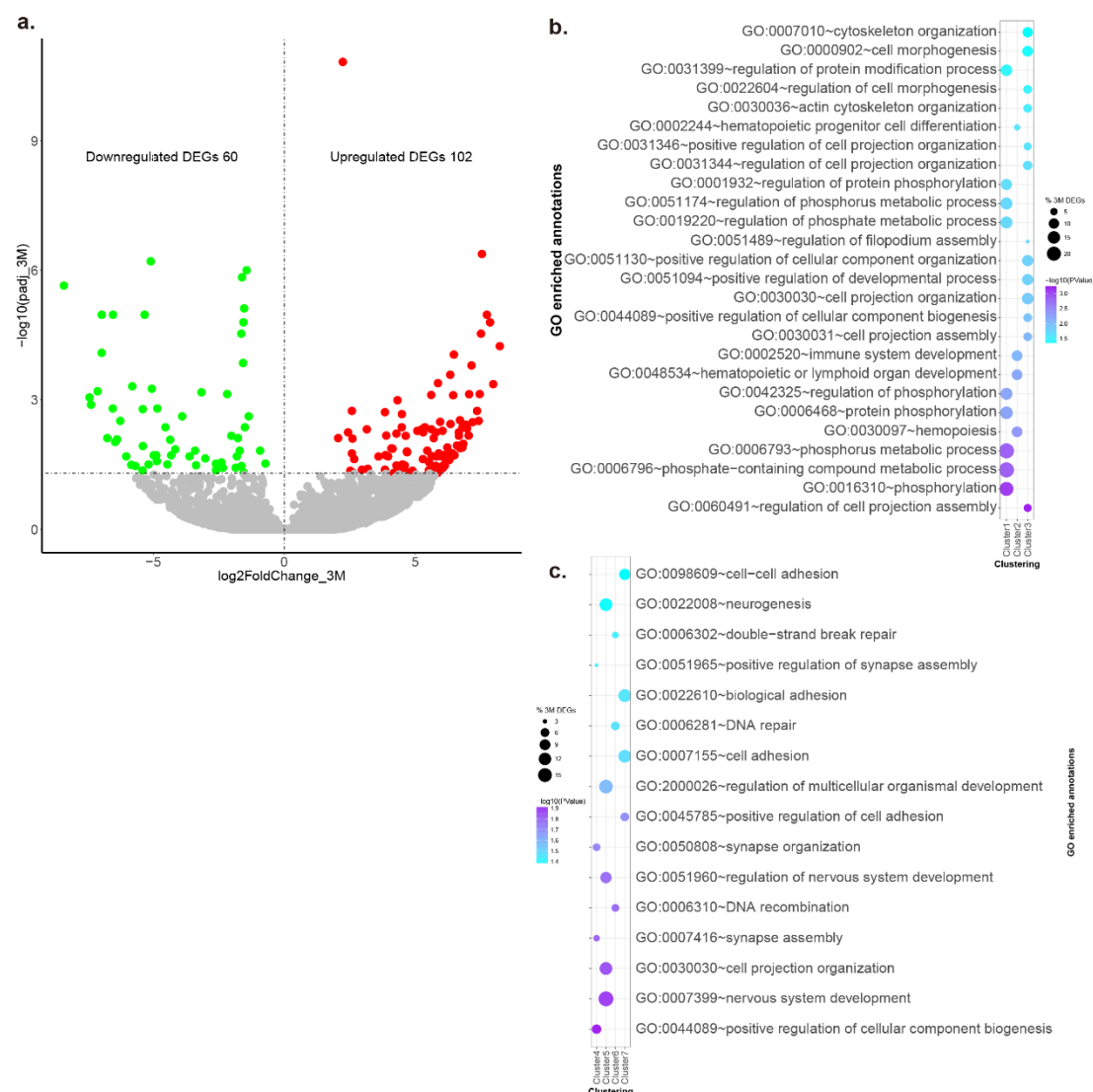


Figure 3.1.3.2 DEGs of mutant ENK+ MSNs showing altered functions at early stage.

a. Differentially expressed genes set by comparing mutant ENK+ MSNs with control at 3M. Each gene represented by each dot was plotted with value of log2foldchange against respective log10 of P-adjusted value. Green dots represent down-regulated

DEGs, red dots represent up-regulated DEGs, and grey dots represent non-significantly differentially expressed genes.

b. Top enriched clusters from GO clustering analysis of up-regulated 3M DEGs set. **c.** Other enriched clusters from GO clustering analysis of up-regulated 3M DEGs set. X axis of histogram shows percentage of enriched DEGs for each annotation, significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue. Proportion of each cluster in total amount of enriched annotations is indicated as the size of bubble.

3.1.3.3 Ageing comparison of transcriptomes: mutant Penk+ MSNs between 3M and 8M of age

Previous work has shown that age is a critical factor for developing the hyperlocomotor phenotype in Trkb-Penk-KO mice¹⁶⁸, therefore, we then sought to investigate how age could mingle with absence of TrkB signaling and contribute to the transcriptomic changes of mutant ENK+ MSNs at later stage.

We compared 3M mutant ENK+ MSNs with 8M mutant ENK+ MSNs to capture the DEGs that specifically started to be affected at early stage. By comparing to the mutant ENK+ population at 8M, while 14437 genes had no significant detectable differential expression, 159 genes (102 down-regulated/57 up-regulated) showed significant differential expression ($P_{adj} < 0.05$) (**Figure 3.1.3.3.a**). And by GO clustering analysis, 87 functional annotations were significantly enriched with $EASE < 0.05$. Only 3 annotations were from down-regulated DEGs and related to the immune response functions (not shown here). Whereas, 84 enriched annotations from the up-regulated DEGs could be hypergeometrically grouped into 3 clusters. The largest cluster (48.3%) revealed that metabolic processes and phosphorylation related functions were affected, and shared programmed cell death related annotations with cluster3 (**Figure 3.1.3.3.b**). Cluster2 (26.4%) indicated biosynthetic processes with nucleotide metabolic processes involved in abnormal development as well (**Figure 3.1.3.3.c**). Apart from cell death annotations shared with Cluster1, Cluster3 (21.8%) particularly, pointed to abnormal regulation of neuronal death processes (**Figure**

3.1.3.3.d).

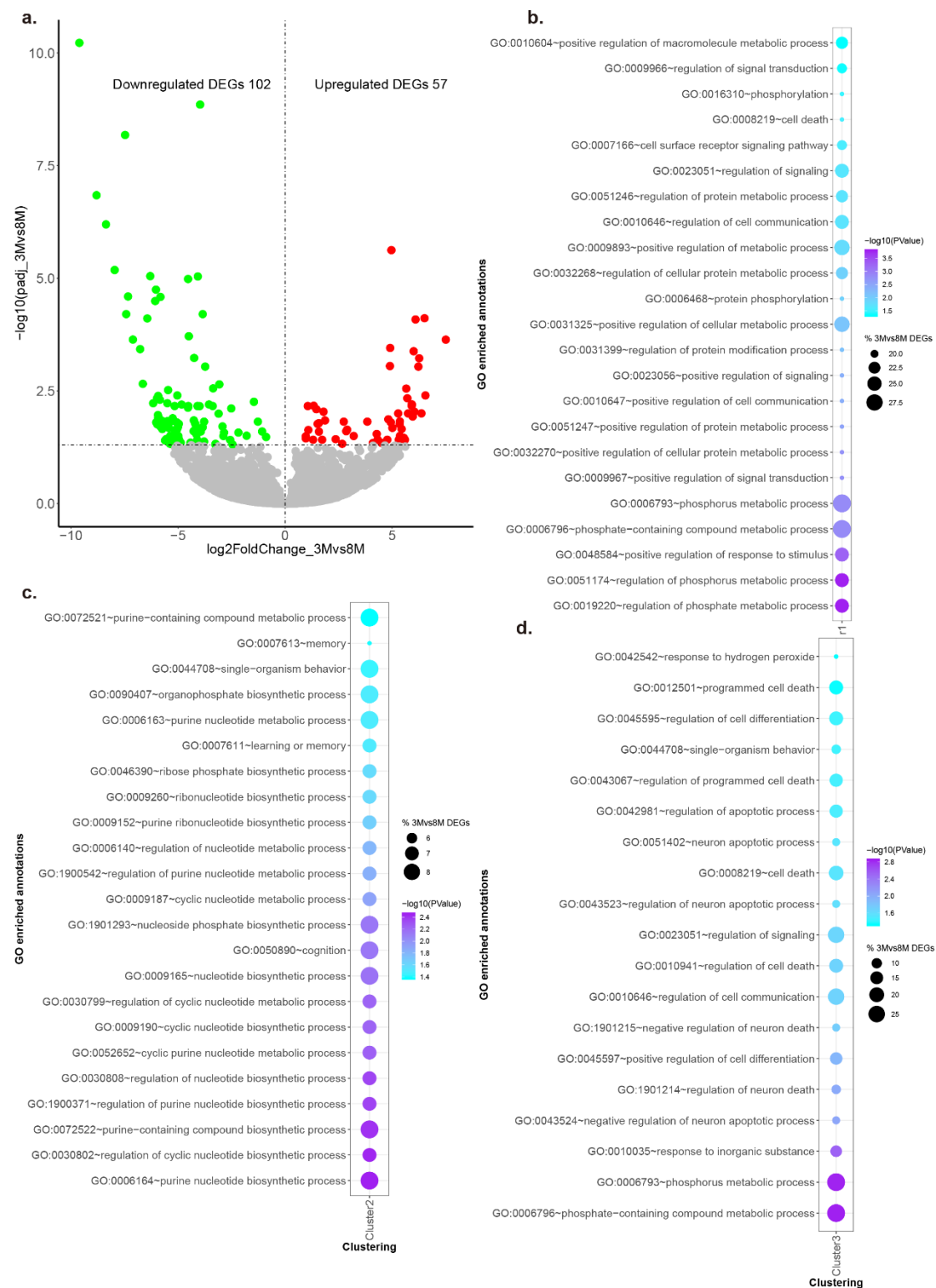


Figure 3.1.3.3 Age comparison of ENK+ subpopulations reveals functions that change during ageing.

a. Differential expressed genes (DEGs) set from comparing mutant ENK+ MSNs at 3M with mutant ENK+ MSNs at 8M. Each gene represented by each dot was plotted with value of log2foldchange against respective log10 of P-adjusted value. Green dots represent down-regulated DEGs, red dots represent up-regulated DEGs, and grey dots represent non-significantly differential expressed genes. **b-d.** Top enriched clusters from GO clustering analysis of up-regulated 3Mvs8M DEGs set. X axis of the histogram shows percentage of enriched DEGs for each annotation, significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue. Proportion of each cluster in total amount of enriched annotations is indicated as the size of bubble.

3.1.4 Discovery of key candidate genes leading to early functional changes and possibly later to the hyperlocomotor phenotype

3.1.4.1 Mining of differentially expressed gene sets revealed four genes with unique expression pattern specific to mutant enkephalinergic MSNs at 3M

Here, we continued to pursue the initial question, merely how absence of TrkB signaling in ENK+ MSNs could induce the hyperlocomotor phenotype in an age dependent manner, by asking 2 questions: 1. Which genes, leading to the transcriptomic changes observed in mutant ENK+ MSNs specifically at early stage, are the key drivers to the dysfunction observed at later stage? 2. What functions altered at early stage would contribute to the development of the phenotype observed at later stage?

To address these questions, firstly we tried to find out genes with unique expression pattern at early stage based on the DEGs analysis described above. We crossed 3 DEGs sets (3M DEGs, 8M DEGs, 3Mvs8M DEGs) to identify the genes that were not only differentially regulated in absence of *Trkb*, but were also specific to the age (**Figure 3.1.4.1.a**). Intriguingly, there were no overlapping genes between the 3 DEGs sets; whereas, 24 genes overlapped between the 3M DEGs and 3Mvs8M DEGs, 1 gene overlapped for the 8M DEGs and 3Mvs8M DEGs, and 9 genes overlapped between 3M DEGs and 8M DEGs comparison (**Figure 3.1.4.1.b**). To

further examine the specificity of these 34 overlapping genes, we then compared the expression level (RPKM) of each gene between ENK+ subpopulation and ENK- subpopulation extracted from transcriptomic analysis (**Figure 3.1.4.1.c**). Four key candidate genes, *Gsto2/Gcc1/BC048403/Zfp101*, were significantly differentially expressed in the mutant ENK+ MSNs compared to the ENK- population. *Gsto2* (Glutathione S-Transferase Omega 2) is involved in the biotransformation of inorganic arsenic and reduces monomethylarsonic acid, it is associated with the age-at-onset (AAO) of Alzheimer (AD) and Parkinson (PD) diseases¹⁹³. *Gcc1* (GRIP And Coiled-Coil Domain Containing 1) encoded protein contains a GRIP domain which is thought to be used in targeting. It may play a role in the organization of trans-Golgi network subcompartment involved with membrane transport (provided by RefSeq, Jul 2008). *BC048403* is an open reading frame protein and *Zfp101* is a zinc finger protein, both are involved in regulation of transcription processes, and have not been widely reported yet. Moreover, these 4 genes were not only significantly differentially expressed at 3M, but also specifically regulated at 3M in mutant ENK+ MSNs compared to the 8M mutant ENK+ MSNs.

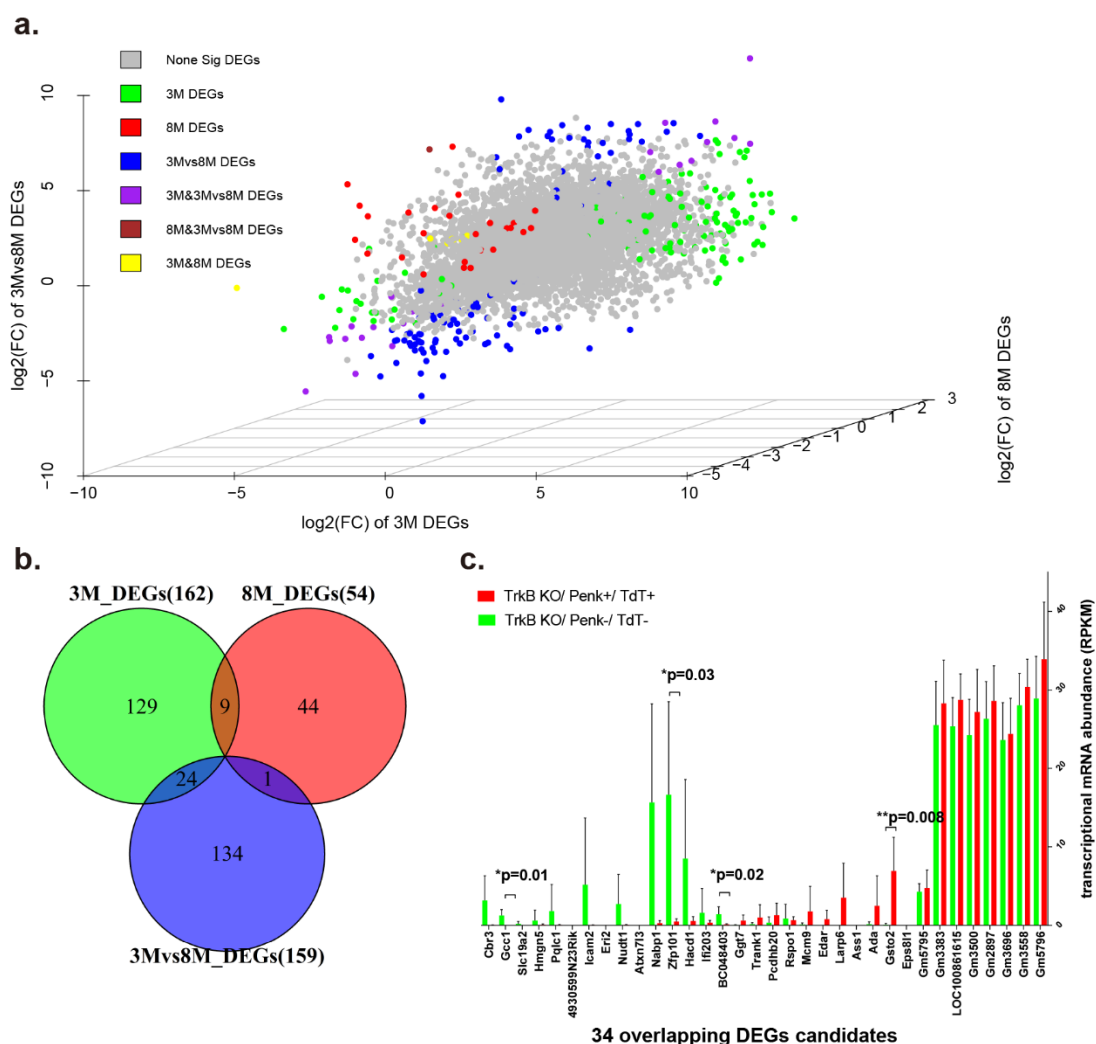


Figure 3.1.4.1 Differential gene expression analysis and comparison of DEGs identify the ENK+ MSNs specific regulated genes candidates.

a. Differential expressed genes obtained from 3 comparisons between subpopulations (*Trkb*^{PENK-KO} with Control ENK+MSNs at 3M; *Trkb*^{PENK-KO} with Control ENK+MSNs at 8M; 3Mvs8M of *Trkb*^{PENK-KO} ENK+MSNs) plotted by log₂(FoldChange) in three dimension manner (n=4-6). Each dot depicts a single gene, with the same colour scheme used in the corresponding Venn diagram of 3 DEGs sets(b). Non-significant regulated genes are depicted in grey.

b. The Venn diagram shows the number of DEGs in each comparison set and overlapping differentially-expressed genes. Totally, there are 34 overlapping DEGs from the 3 comparison sets.

c. Overlapping DEGs expression levels in ENK+/ENK- subpopulations, 4 DEGs are

significantly differentially regulated in a cell-type specific manner ($n=4-6$), $p=0.008$ (Gsto2); $p=0.02$ (BC048403); $p=0.03$ (Zfp101); $p=0.01$ (Gcc1). Unpaired t-test. RPKM, reads per kilobase per million, n -values indicate biological replicates for each subpopulation sample.

3.1.4.2 Early altered mitochondrial function and dysregulation of energy metabolism

We then answered our second question regarding what functions were altered at early stage that would contribute to the development of the phenotype at later stage. To address this issue, firstly we applied an analytical method, Gene Set Enrichment Analysis¹⁷⁹ (GSEA), to get biological insight from the gene expression data analysis we performed. For this analysis, we used the DEG sets (all differentially regulated genes including also the non-significantly different ones) generated from our previous DE analysis and investigated altered biological functions (as described in **Chapter 2.2.3**).

This analysis, based on MsigDB C2 Canonical Pathways and MsigDB Hallmark databases, revealed enrichment for quite a few pathways for each DEGs set (62 for 8M DEGs, 257 for 3M DEGs, 335 for 3Mvs8M DEGs; not shown here), interestingly only oxidative phosphorylation (OX-PHOS) and tricarboxylic acid cycle/Electron transport chain (TCA-ETC) pathways were most significantly enriched across 3 DEGs sets (**Figure 3.1.4.2.1.b**), suggesting that dysfunction of oxidative

phosphorylation caused by mitochondrial defects may be the reason underlying the early changes leading to the phenotype observed later at 8M. Oxidative phosphorylation, as metabolic processes producing ATP, directly participates into energy metabolism playing important role for cellular functions and neural activity¹⁹⁴. Moreover, it has been shown that the striatal energy metabolism deficits, particularly mitochondrial dysfunctions, are involved in HD pathogenesis based on evidence both from human and animal models¹⁹⁵. Firstly, we examined how energy metabolism pathways were affected at 8M stage, when the hyperlocomotor phenotype is manifested, from two main energy support pathways: oxidative phosphorylation and glycolysis. The GSEA analysis on 8M DEGs set showed that OX-PHOS pathway and glycolysis pathway were both significantly enriched in 8M control ENK+ MSNs (NES for OX-PHS= -2.85, NES for glycolysis= -2.09; **Figure 3.1.4.2.1.a**), indicating severe energy defects in mutant ENK+ MSNs compared to controls. And TCA-ETC pathway at 8M showed the same enrichment consistent with OX-PHOS pathway and implying the mitochondrial deficits in mutant ENK+ MSNs as potential cause for the suppression of oxidative phosphorylation processes (NES of TCA-ETC= -3.07; **Figure 3.1.4.2.1.a**), as TCA-ETC is the main energetic process executed in mitochondria. As mentioned above, OX-PHOS and its related TCA-ETC pathway were also significantly negatively enriched in 3M DEGs and 3Mvs8M DEGs (**Figure 3.1.4.2.1.b**). In other words, the suppressed oxidative phosphorylation deficit caused by mitochondrial defects had been already primed in mutant ENK+ MSNs at early stage in mutant ENK+MSNs and becomes worse at 8M stage (NES of OX-PHOS=

-3.19, NES of TCA-ETC= -3.21; **Figure 3.1.4.2.1.b**). Moreover, it also shows that OX-PHOS and TCA-ETC were negatively enriched in 3Mvs8M DEGs (NES of OX-PHS= -3.39, NES of TCA-ETC= -3.27; **Figure 3.1.4.2.1.b**), which indicates that the status of oxidative phosphorylation and mitochondrial function were even lower in 3M mutant ENK+ MSNs when compared to the 8M ones.

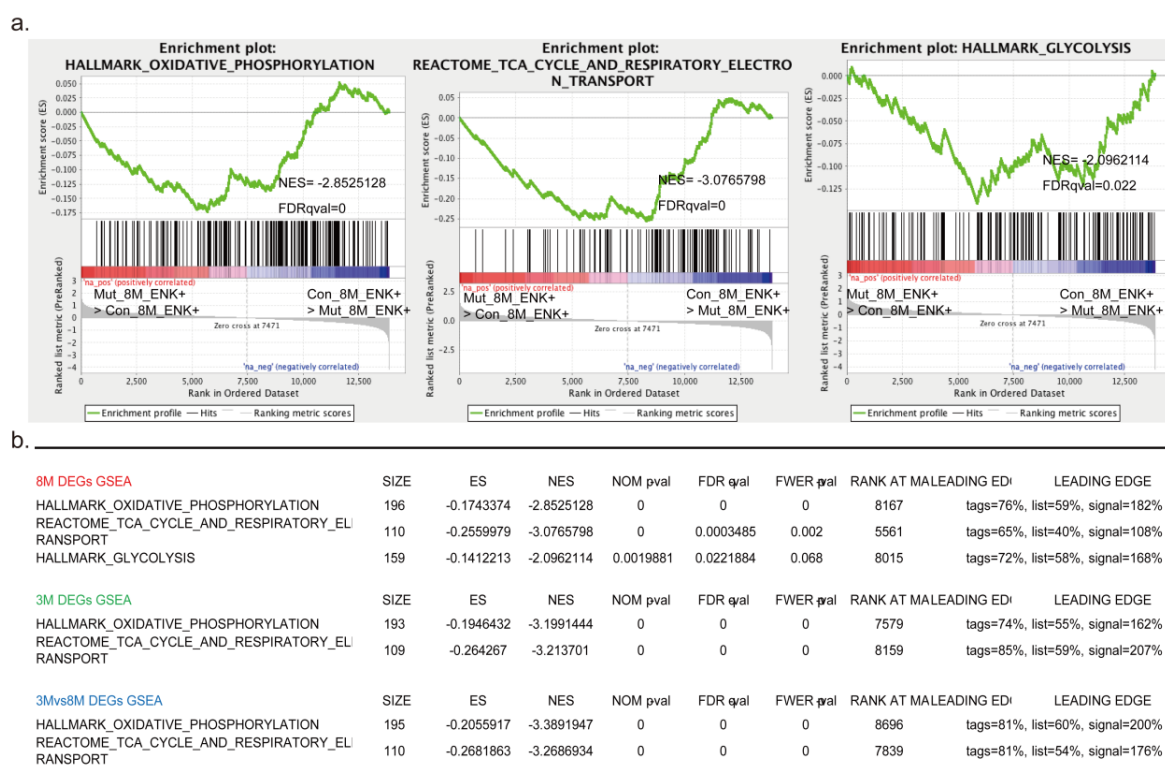


Figure 3.1.4.2.1 Gene set enrichment analysis (GSEA) of DEGs reveals specific energy support deficiency in ENK+ MSNs in absence of TrkB signaling.

a. Representative Gene-set-enrichment analysis on DEGs set of ENK+MSNs at 8M stage, showing results for 3 energy support related genes sets (Oxidative Phosphorylation, TCA cycle and respiratory electron transport, Glycolysis). NES, normalized enrichment score.

b. Table showing details of GSEA analysis on each DEGs set of 3 energy support related pathways, Oxidative Phosphorylation, TCA cycle/respiratory electron transport

and Glycolysis pathway at different stages (3M and 8M) and 3Mvs8M comparisons.

Since energy metabolism related pathways have been highlighted by the above analysis as potential early changes leading to the behavioural phenotype developed around 8M (hyperlocomotor behavior¹⁶⁸), we then investigated more in detail by bioinformatics how these two main pathways, OX-PHOS and glycolysis, would be affected and behave in each striatal subpopulation.

The expression gene sets for OX-PHOS and glycolysis pathways from the GSEA MsigDB Hallmark database were compared to the transcriptome data of each subpopulations in our analysis (using RPKM), and then visualized by hierarchical clustering (**Figure 3.1.4.2.2.a**). This global view would not only reflect what we have observed already from the GSEA analysis on 3 DEGs sets, more interestingly, it has also clearly shown specific enriched patterns of energy supportive pathways in age and cell-type specific manner. Moreover, to statistically evaluate these enriched patterns based on the gene expression levels, we compared each striatal subpopulation by both genotype (control/mutant) and cell-type (ENK+/ENK-) specific manner to dissect how energy metabolism pathways respectively were affected (**Figure 3.1.4.2.2.b&c**). Firstly, these two main cellular energetic pathways, OX-PHOS and glycolysis, were both in higher demand at later stage, especially in ENK+ MSNs, and

extremely suppressed in the mutant populations compared to the 8M control populations, which is consistent with previous findings from the GSEA analysis. Whereas at 3M stage, only OX-PHOS was down-regulated in both mutant populations (ENK+ & ENK-), while glycolysis, comparing control with mutant, was not evident at this stage. Even more interestingly, when comparing cell-type (ENK+/ENK-) within genotype, as shown above, at 8M stage, ENK+ MSNs were more energetically demanding both within control and mutant, suggesting naturally these neurons require more energy support from OX-PHOS and glycolysis at this stage. However, at 3M stage there was no difference between cell-type (ENK+/ENK-) on these two energy support pathways between the control populations, while mutant ENK+ MSNs up-regulated the glycolysis pathway but not OX-PHOS at this stage. Together, with hierarchical clustering (**Figure 3.1.4.2.2.a**), these data has shown that at 8M stage ENK+ MSNs require substantial energy support, while compared to the control, mutant striatal populations (both ENK+ and ENK-) had severe energy deficiency of both OX-PHOS and glycolysis, which could contribute to dysfunction of the mutant striatum; whereas at 3M stage, only OX-PHOS was suppressed in the mutant striatal populations, and to compensate this insufficient energy support, probably mutant ENK+ MSNs activated complementary glycolysis pathway to fuel the cellular functions compared to the mutant ENK- subpopulation.

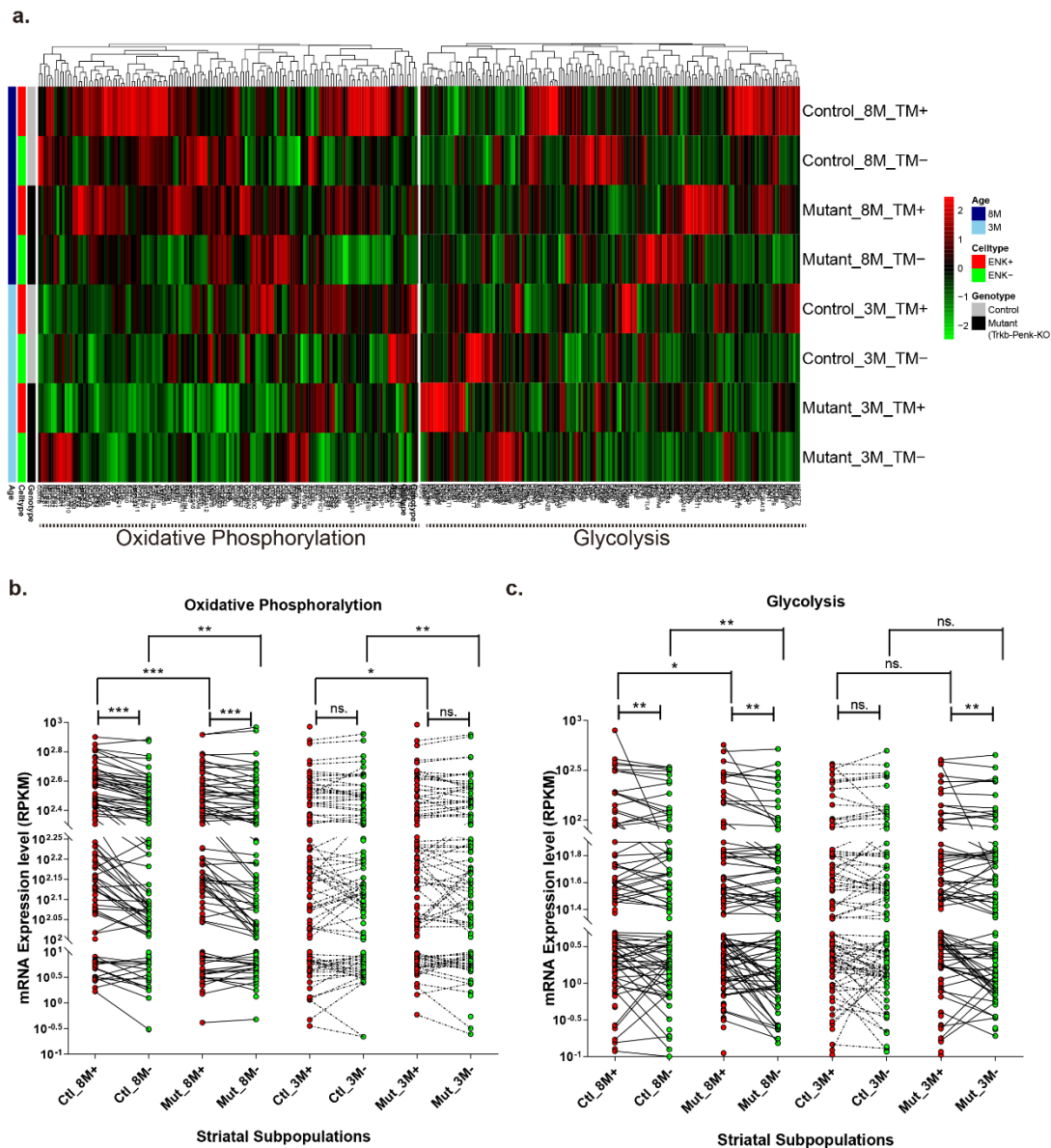


Figure 3.1.4.2.2 Affected energy metabolism pathways at early stage and cell-type specific dysfunction.

a. Heatmap representing average expression levels (RPKM) of genes from two energy support related pathways in samples of subpopulations ($n=4-6$ biological replicates for each subpopulation). **b.** Expression plot of oxidative phosphorylation related genes in each subpopulation. Each dot represents each gene in the oxidative phosphorylation pathway. **c.** Expression plot of glycolysis related genes in each subpopulation. Each dot represents each gene in the glycolysis pathway. Significant values were calculated by the non-parametric Wilcoxon test; Significant values in this figure: $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

Based on the analysis above and the GSEA analysis as well, OX-PHOS was shown as the main energy defect in the mutant mice manifested from 3M to 8M stage, to gain better understanding of how OX-PHOS has been affected, we then took closer look at the main pathway involved in OX-PHOS, Tricarboxylic acid cycle/Electron transport chain (TCA-ETC), indicated as mitochondria function.

Same analysis was applied on the set of genes involved in TCA-ETC pathway (**Figure 3.1.4.2.3.a**), and consistent with our findings from the GSEA analysis, TCA-ETC showed the same enrichment pattern along with OX-PHOS, which was severely suppressed in the mutant populations (both ENK+ and ENK-) relative to the control at both 3M and 8M stage (**Figure 3.1.4.2.3.b**). For TCA-ETC, in contrast to OX-PHOS at 3M stage, ENK+ MSNs had higher expression of TCA-ETC than ENK- population in control mice, whereas, this advantage was not detected in the mutant ENK+ MSNs that showed even lower expression compared to the ENK- population although not significant.

To carry out the process of TCA-ETC, major execution relies on the subunits complex I, II, III, IV and V embedded in the mitochondrial inner membrane. We then examined the set of genes associated with these subunits complex of ETC chain to test if the dysfunction of TCA-ETC was generated by abnormality of these subunits complex (**Figure 3.1.4.2.3.c**). At 8M stage when mutant mice are suffering the severe energy deficiency, consistently these subunits complex showed the clearly significant inhibition in mutant subpopulations relative to the control, and higher demand in

ENK⁺ MSNs, which reflected the defects observed above for TCA-ETC and OX-PHOS (**Figure 3.1.4.2.3.c**). More intriguingly, at 3M stage, in addition to higher enrichment of these complexes associated genes in ENK⁺ MSNs in control mice, the TCA-ETC and OX-PHOS were affected only in mutant ENK⁺ MSNs but not in mutant ENK⁻ subpopulation compared to the relative control.

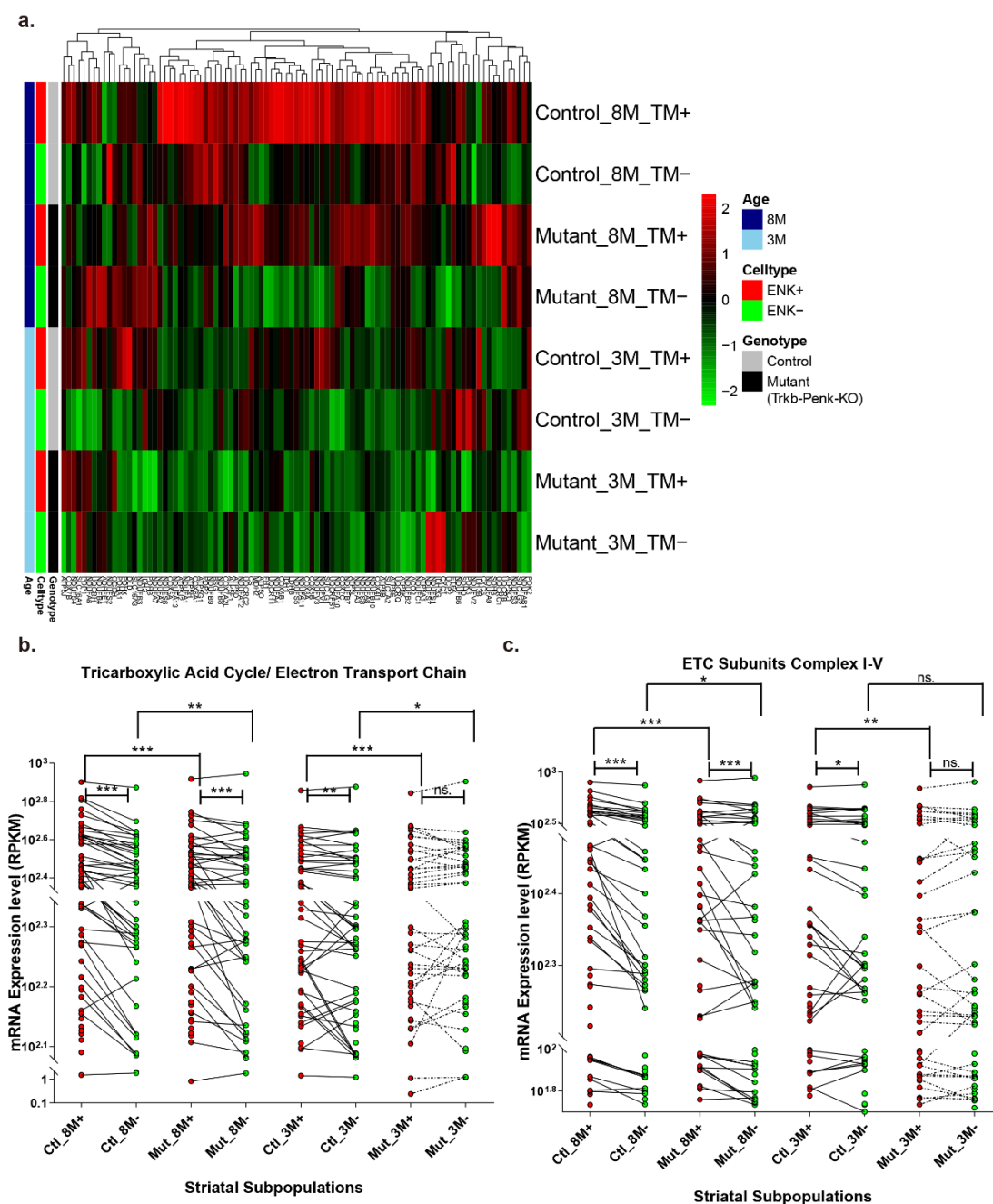


Figure 3.1.4.2.3 Mitochondrial defects of ETC complex in ENK+ MSNs leading to OX-PHOS dysfunction.

- a. Heatmap representing average expression levels (RPKM) of genes from Tricarboxylic acid cycle/Electron transport chain (TCA-ETC) pathway in samples of subpopulations (n=4-6 biological replicates for each subpopulation).
- b. Expression plot of TCA-ETC related genes in each subpopulation. Each dot represents each gene in TCA-ETC pathway.

c. Expression plot of ETC subunit complex genes in each subpopulation. Each dot represents each gene. Significance values were calculated by the non-parametric Wilcoxon test; Significance values in this figure: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$.

Collectively, this analysis has shown that despite ENK+ MSNs required more energy support relative to the ENK- subpopulation, at 8M stage the mutant populations of both ENK+ and ENK- was suffering from the severe energy deficiency of both OX-PHOS and glycolysis pathways. Whereas, at 3M stage when the behavioural phenotype has not manifested yet, only the OX-PHOS, but not the glycolysis pathway, was decreased in mutant striatal populations compared to the control. To compensate for this energy defect, mutant ENK+ MSNs then specifically tuned up glycolysis relative to the ENK- population. Consistent with OX-PHOS and glycolysis behaviour at 8M stage, the TCA-ETC of mitochondria and its inner membrane subunits complex I to V showed the same down-regulation in the mutant population. Moreover, this TCA-ETC, as the main mitochondrial function fueling OX-PHOS, was also decreased in mutant population relative to the control at 3M stage just as OX-PHOS. However, the specific advantage of up-regulated TCA-ETC in 3M ENK+ MSNs compared to the ENK- subpopulation in control subpopulations was not observed in the mutant subpopulations, which correlated with the up-regulation of glycolysis in this mutant ENK+ MSNs to probably regain the sufficient energy support. More intriguingly, as the main executive mitochondrial function, the ETC subunits complex were specifically down-regulated in the mutant

ENK+ MSNs, but not ENK- population, compared to the related controls. These data suggest that the defect observed in ETC complex I to V exclusively primed in mutant ENK+ MSNs potentially causes the mitochondrial dysfunction of both mutant striatal populations (ENK+ and ENK-) at 3M stage, leading to the development of severe energy deficiency.

3.2 Validation of transcriptomic data

3.2.1 Single molecule FISH (smFISH) optimisation and validation of specific selected genes

3.2.1.1 Establishment and optimisation of single molecule FISH technique

To validate the RNA-seq data we used different techniques. First, we employed quantitative RNA hybridisation technique, single molecule fluorescence in situ hybridization (Single molecule FISH, smFISH)¹⁹⁶. We applied it to selected genes from the data set analysis based on their differential gene expression. This technique not only allows to visualise the spatial distribution of the mRNA of the selected gene in the particular brain region, but also quantitatively examines the mRNA copy number to verify gene expression from the RNA-seq data.

To establish this smFISH technique, we firstly adopted from Stellaris RNA FISH Protocols, (<https://www.biosearchtech.com/support/resources/stellaris-protocols>), LGC Biosearch Technologies, and compared with the original version from Lyubimova A, *et al*¹⁹⁶ to optimise conditions. The customised experimental procedures has been described in detail in **Chapter 2.2.5**, and briefly shown in the workflow (**Figure 3.2.1.1.1**). Overall, the customised smFISH experiment was performed by following the flow of Stellaris RNA FISH protocol for fresh frozen mouse brain tissue with some modifications, which can be briefly summarized into four stages (**Figure**

3.2.1.1.1). For the sample preparation, we managed to use 8µm cryosections from fresh frozen brain tissue. Instead of using TEA (Triethanolamine) buffer treatment and gradient ethanol permeabilization, we treated sections with 70% ethanol 1 hour at room temperature for better permeabilization effect and lower background noise signal. By following the overnight hybridization with the designed probes library, sections were then counterstained with DAPI, washed with washing buffer and then equilibrated with GLOX buffer before mounting on the slides, rather than gradient dehydration with ethanol. As shown in **Figure 3.2.1.1.2**, two protocols, a commercial one from Stellaris RNA FISH and a customised one with modifications, were compared by applying Quasar-570 coupled *Gapdh* probe on the brain tissue sections. The customised protocol allowed to generate a better and more robust signal of mRNA dots (**Figure 3.2.1.1.2.c.d**), and hybridised *Gapdh* mRNA distributed appropriately around the nucleus with detectable signal, despite that there was slightly higher background noise compared to the commercial methods, which can be solved by applying deconvolution and projection method of imaging software (**Figure 3.2.1.1.1 image acquirement**).

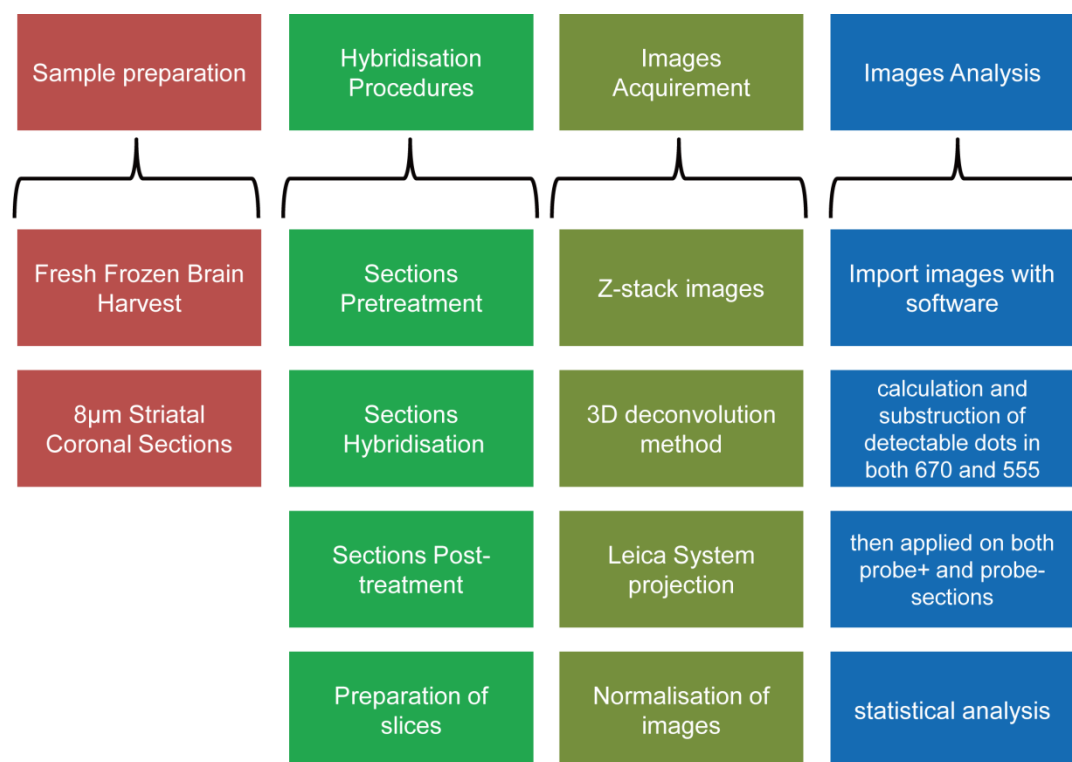


Figure 3.2.1.1.1 Workflow of customised single molecule FISH technique

The experimental procedures can be summarized into four stages: sample preparation, hybridization procedures, images acquirement and images analysis.

Trkb^{PENK-KO} and respective control mice were sacrificed. The brains were then harvested, frozen in liquid nitrogen and stored at -80°C until used. Probe libraries were designed to target coding sequences of mRNA of selected gene and constructed by Stellaris. Probe libraries consisted of 48 probes of length 20 bps. 8µm cryosections was collected and directedly mounted to coverslips. Then sections were fixed in 4%paraformaldehyde for 10min at room temperature, followed by 1-hour permeabilization using 70% ethonal at room temperature. Hybridization with 250 nM fluorescent label probes was carried out overnight in 37°C oven. DAPI dye for nuclear was counterstained during the washes. Then coverlips was mounted on slides with Vectashield antifade mounting medium. 8µm image stacks (0.2µm distance) were acquired with Leica microscope system equipped with a x100 oil-immersion objective and Leica DFC 365 FX camera using Leica AF6000 software. Then, image stacks were processed by 3D reconstruction using a Leica AF6000, followed by image projection. Three to five random fields from the dorsal striatum were imaged for each

section. Quantification was done by using StarSearch developed by Raj lab (<http://rajlab.seas.upenn.edu>) to count the number of mRNA dots. DAPI was used to identify the number of cells in each field. The p value of comparing the mRNA counts of 3 biological replicates for both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} were achieved by using the student t test.

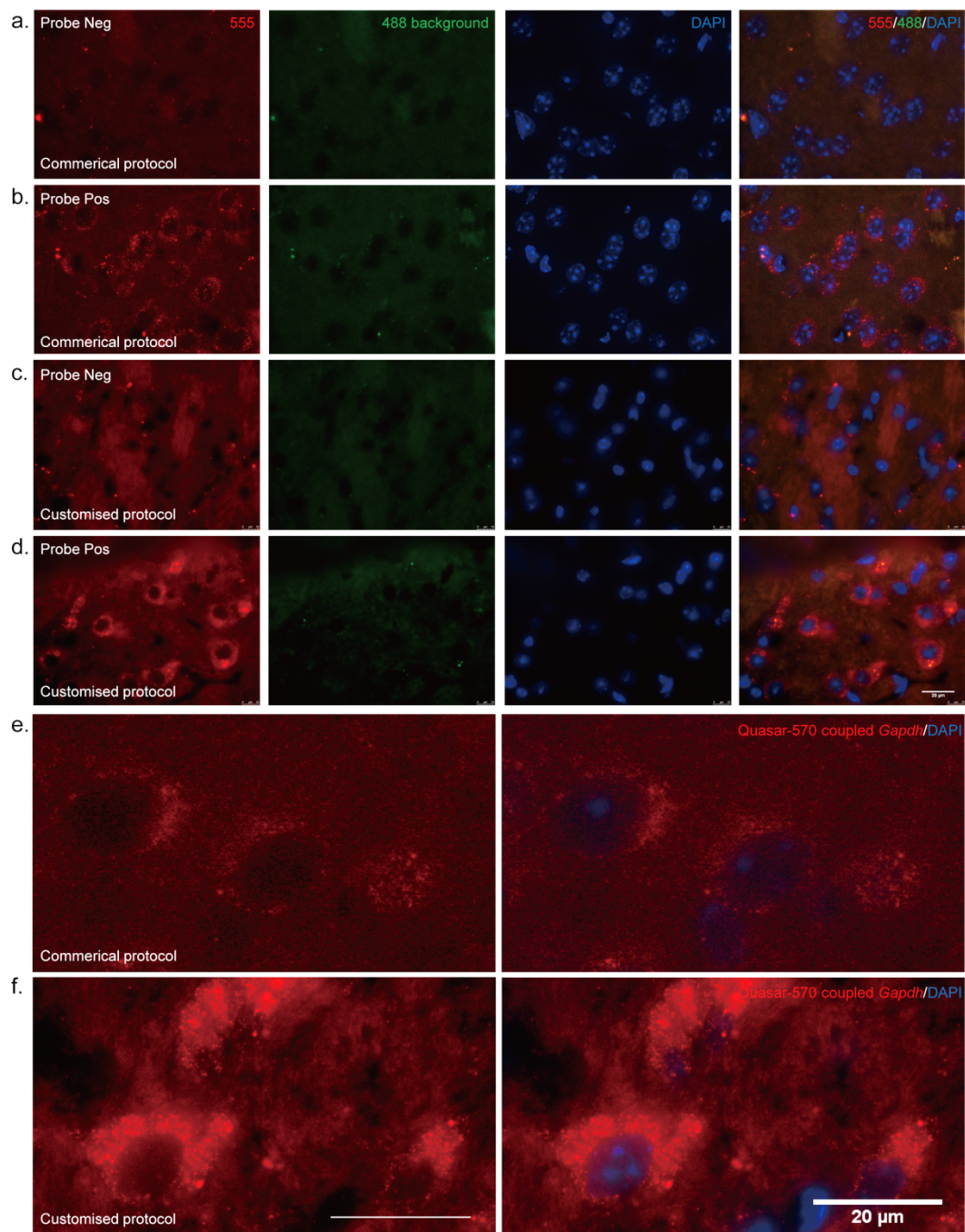


Figure 3.2.1.1.2 Establishment and optimisation of single molecule FISH technique by Quasar-570 coupled *Gapdh* probe showing robust *Gapdh* mRNA

dots with our customised protocol.

a.b. rows showing sections treated with (Probe Pos)/without (Probe Neg) Quasar-570 coupled *Gapdh* probe by commercial protocol. mRNA of *Gapdh* was shown in 555 channel, and 488 channel shows the tissue background.

c.d. rows showing sections treated with/without Quasar-570 coupled *Gapdh* probe by customised protocol, with same channels arrangement as **a.b.**

e.f. Higher magnification of comparison of *Gapdh* smFISH by commercial and customised protocols. Our customised protocol shows better and more robust signal than the commercial one. All the scales are 20µm.

3.2.1.2 Validation of mRNA expression of selected genes (*Gsto2*, *Gcc1*) from the RNA-seq analysis by smFISH

Following the optimization of sample preparation and hybridisation procedures, we then customised and designed the strategy for image acquisition and analysis in the context of our laboratory setting for the purpose of RNA-seq data validation.

As for the image acquirement stage (**Figure 3.2.1.1.1**), 8µm image stacks (0.2µm distance) were acquired with the Leica microscope system to assemble comprehensive information of signal from every focus of the section, followed by default 3D deconvolution and projection method to calibrate background noise and reconstruct the images. For each section, 3-5 random fields of dorsal striatum were acquired for image analysis (**Supplementary Figure 3.2.1.2.1**, please see in Appendix). Quantification of the number of mRNA dots from the reconstructed images was done

by using StarSearch developed by the Raj lab (<http://rajlab.seas.upenn.edu>, who is involved in the development of the original version of smFISH as well¹⁹⁶). DAPI was used to identify the number of cells in each field. Importantly, while setting up conditions, we noticed that in older brain tissue some background dots were detected on the sections without treatment with Quasar-570 coupled *Gapdh* probe (**Figure 3.2.1.1.2**). This is apparently an unavoidable signal that may be attributed to: 1. Either lipofuscin accumulated in the tissue with age, 2. or unspecific signal from treatments of the sections. To minimise this issue, we firstly labeled the probes generated for *Gsto2* and *Gcc1* with Quasar-670 to avoid the broad spectrum (400-650nm) of lipofuscin¹⁹⁷. And we then applied the same quantification on both 555 channel and 670 channel of the images from both the sections treated with/without the probe (Probe Pos/Neg) (**Supplementary Figure 3.2.1.2.1**, please see in Appendix). By using the strategy of subtraction¹⁹⁸, the counts of 670 channel were subtracted from the counts of 555 channel to eliminate the trespassed lipofusion signal, and the 670 counts of Probe Pos were then subtracted with the 670 counts of Probe Neg to minimise the unspecific signals from the sections treatments. Sections were also counterstained with DAPI to count the number of cells in the fields and calculate the single mRNA molecules per cell (**Figure 3.2.1.1.1 image analysis**).

We chose *Gsto2* and *Gcc1* from DEGs of the RNA-seq analysis for smFISH validation. *Gsto2* (Glutathione S-Transferase Omega 2) is involved in the biotransformation of inorganic arsenic and reduces monomethylarsonic acid, it is associated with the age-at-onset (AAO) of Alzheimer's (AD) and Parkinson's (PD) diseases¹⁹³. *Gcc1* (GRIP And Coiled-Coil Domain Containing 1) encoded protein contains a GRIP domain and is thought to contribute to membrane traffic at the Golgi apparatus¹⁹⁹. Our RNA-seq analysis has shown that *Gsto2* is highly up-regulated at 3M age in the *Trkb*^{PENK-KO} compared to the *Trkb*^{PENK-WT} (log2foldchange: 7.55; PadjustValue: 4.18E-07), while *Gcc1* is highly decreased in the *Trkb*^{PENK-KO} at 3M age (log2foldchange: -7.44; PadjustValue: 0.00087666).

Firstly, to perform *Gsto2* smFISH analysis, we designed Quasar-670 coupled *Gsto2* probes library, which contained 36 probes of 20 nucleotides in length to cover the coding sequence of *Gsto2* mRNA. *Gsto2* smFISH analysis clearly showed that *Gsto2* mRNA was sporadically expressed in cells of the dorsal striatum from *Trkb*^{PENK-WT} (**Figure 3.2.1.2.1.a.c**), whereas, more *Gsto2* mRNA copies were apparent in the *Trkb*^{PENK-KO} sections (**Figure 3.2.1.2.1.b.d**), which was significantly increased compared to the *Trkb*^{PENK-WT} (**p<0.0001, 2.166 foldchange of *Trkb*^{PENK-WT}). As to *Gcc1*, we performed smFISH by designing Quasar-670 coupled *Gcc1* probes library containing 44 probes. As shown in Figure 2.1.2.2, *Gcc1* mRNA counts in the dorsal striatum was significantly (48%) decreased in *Trkb*^{PENK-KO} at 3M stage (**p=0.0027, 0.5279 foldchange of *Trkb*^{PENK-WT}).

Furthermore, given that DEGs of previous RNA-seq analysis were generated based on the *Penk*⁺ MSNs by FACS sorted tdTomato labeled population, we unfortunately had to abandon the tdTomato reporter to avoid the strong red signal trespassing into the 670 probe channel. To further verify smFISH validation in the *Penk*⁺ MSNs' manner, we therefore combined the smFISH technique with immunostaining of Penk protein, which is exclusively expressed by *Penk*⁺ MSNs in dorsal striatum (**Figure 3.2.1.2.3** for *Gsto2* and **Figure 3.2.1.2.4** for *Gcc1*). This combinatorial method further confirms the increased expression of *Gsto2* mRNA and decreased *Gcc1* mRNA in *Penk*⁺ MSNs.

To summarise, we have managed to establish and optimise smFISH analysis in our laboratory setting, and to validate 2 crucial DEGs, *Gsto2* and *Gcc1*, at early stage validating the RNA-seq analysis. Moreover, by identifying *Penk*⁺ MSNs, smFISH further confirmed this validation in *Penk*⁺ MSNs.

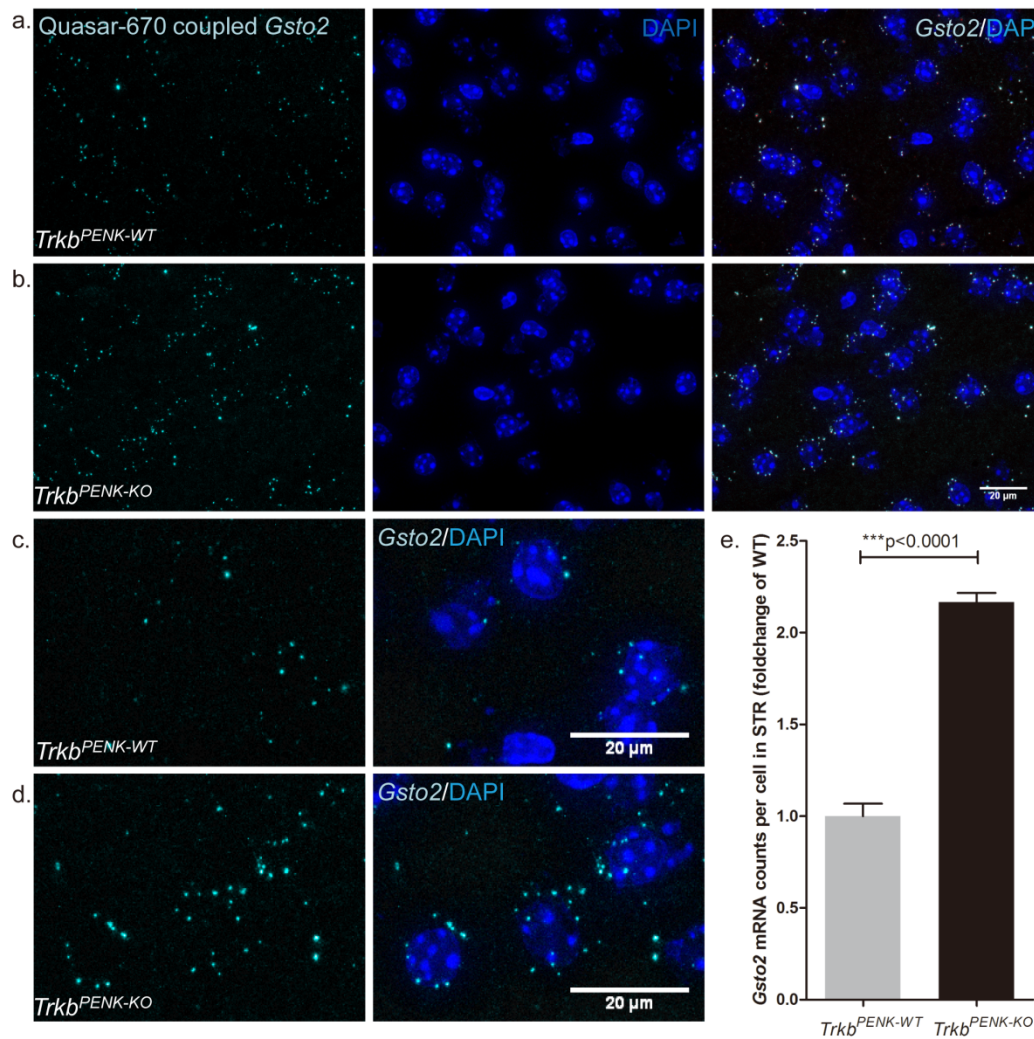


Figure 3.2.1.2.1 Gsto2 smFISH analysis at 3M in the dorsal striatum shows up-regulation of Gsto2 expression in *Trkb*^{PENK-KO}.

a. Representative Gsto2 smFISH images of the dorsal striatum for *Trkb*^{PENK-WT}, Gsto2 mRNA in Cyan channel. **b.** Representative Gsto2 smFISH images of the dorsal striatum for *Trkb*^{PENK-KO}, same channels arrangement as in **a**.

c. Higher magnification of Gsto2 smFISH for *Trkb*^{PENK-WT}. **d.** Higher magnification of Gsto2 smFISH for *Trkb*^{PENK-KO}. All scale bars are 20 μ m.

e. Gsto2 mRNA counts from the dorsal striatum comparing *Trkb*^{PENK-WT} with *Trkb*^{PENK-KO} showing that expression of Gsto2 mRNA is significantly higher in *Trkb*^{PENK-KO} at 3M stage. ***p<0.0001, number of biological replicates for each genotype is 3, values presented are mean $\pm$ s.d.

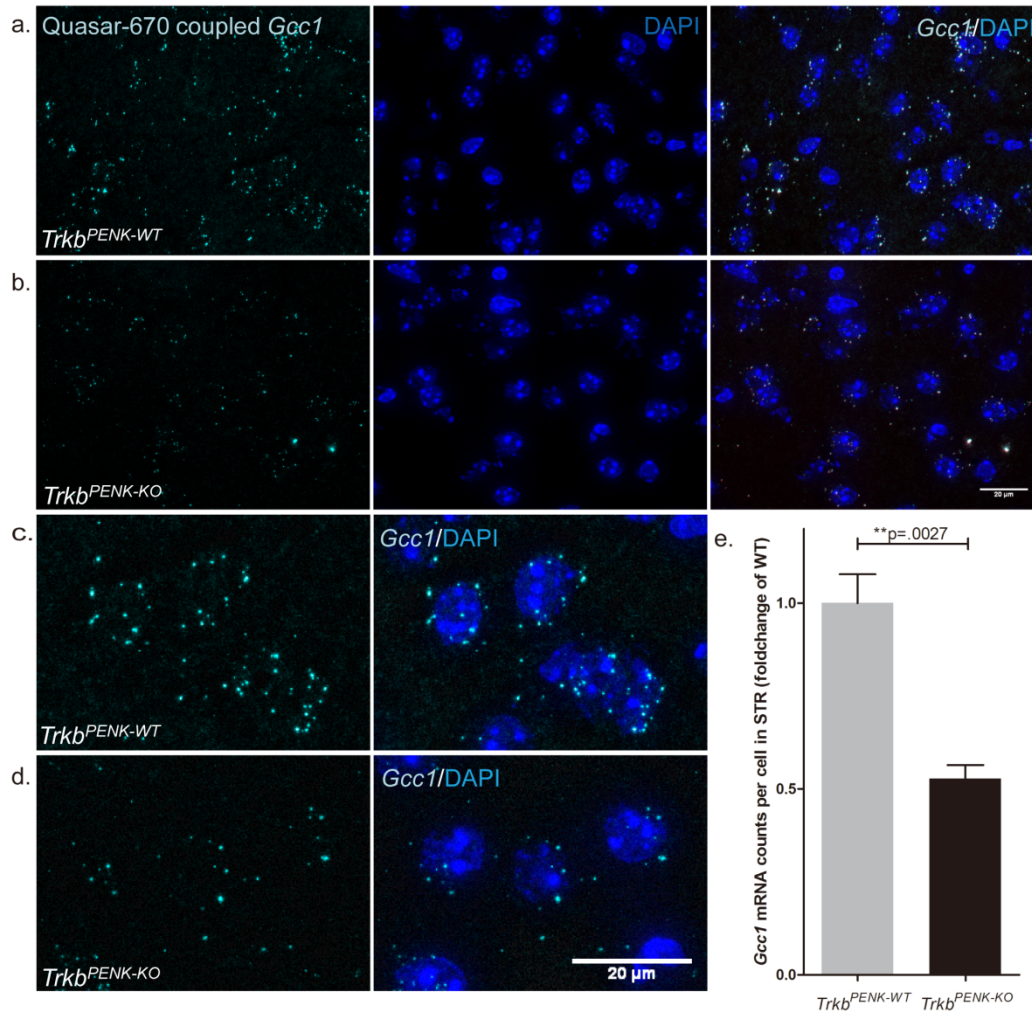


Figure 3.2.1.2.2 *Gcc1* smFISH analysis reveals the decreased expression levels of *Gcc1* comparing to *Trkb*^{PENK-WT}

a. Representative *Gcc1* smFISH images of the dorsal striatum for *Trkb*^{PENK-WT}, *Gcc1* mRNA in Cyan channel. **b.** Representative *Gcc1* smFISH images of the dorsal striatum for *Trkb*^{PENK-KO}, same channels arrangement as in **a**.

c. Higher magnification of *Trkb*^{PENK-WT} for *Gcc1* smFISH. **d.** Higher magnification of *Trkb*^{PENK-KO} for *Gcc1* smFISH. All scale bars of images above are 20 μm.

e. *Gcc1* mRNA counts from the dorsal striatum comparing *Trkb*^{PENK-WT} with *Trkb*^{PENK-KO} showing that expression of *Gcc1* mRNA is significantly decreased in *Trkb*^{PENK-KO} at 3M stage. **p=0.0027. Number of biological replicates for each genotype is 3. Values presented are mean ± s.d.

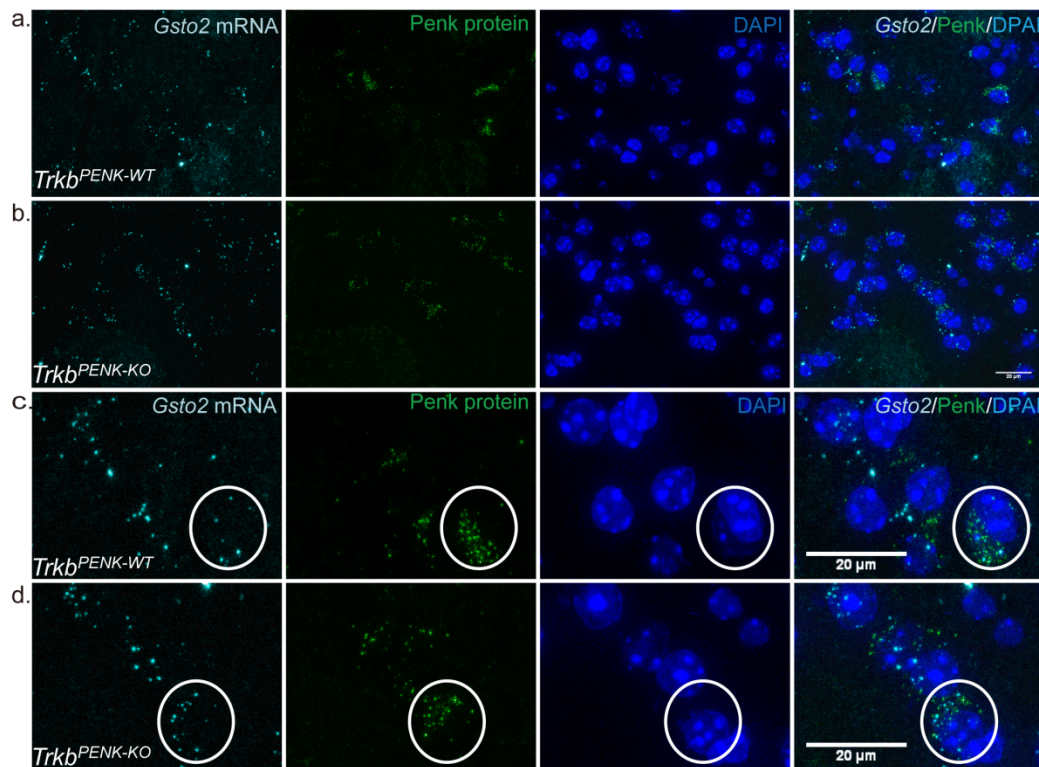


Figure 3.2.1.2.3 Colocalisation of *Gsto2* mRNA with Penk protein in dorsal striatum to further show the up-regulation in *Penk*+ MSNs of *Trkb*^{PENK-KO}.

a. row shows *Trkb*^{PENK-WT} *Gsto2* mRNA colocalised with Penk protein, *Gsto2* mRNA in Cyan channel and Penk protein in 488 channel. **b.** row shows *Trkb*^{PENK-WT} *Gsto2* mRNA with Penk protein colocalisation, same channels arrangement as in **a.**

c.d. Higher magnification of *Gsto2* mRNA colocalisation with Penk protein in *Trkb*^{PENK-WT} and *Trkb*^{PENK-KO}, the circles highlight the example of one *Penk*+ MSN in each channel. All scale bars are 20 μm.

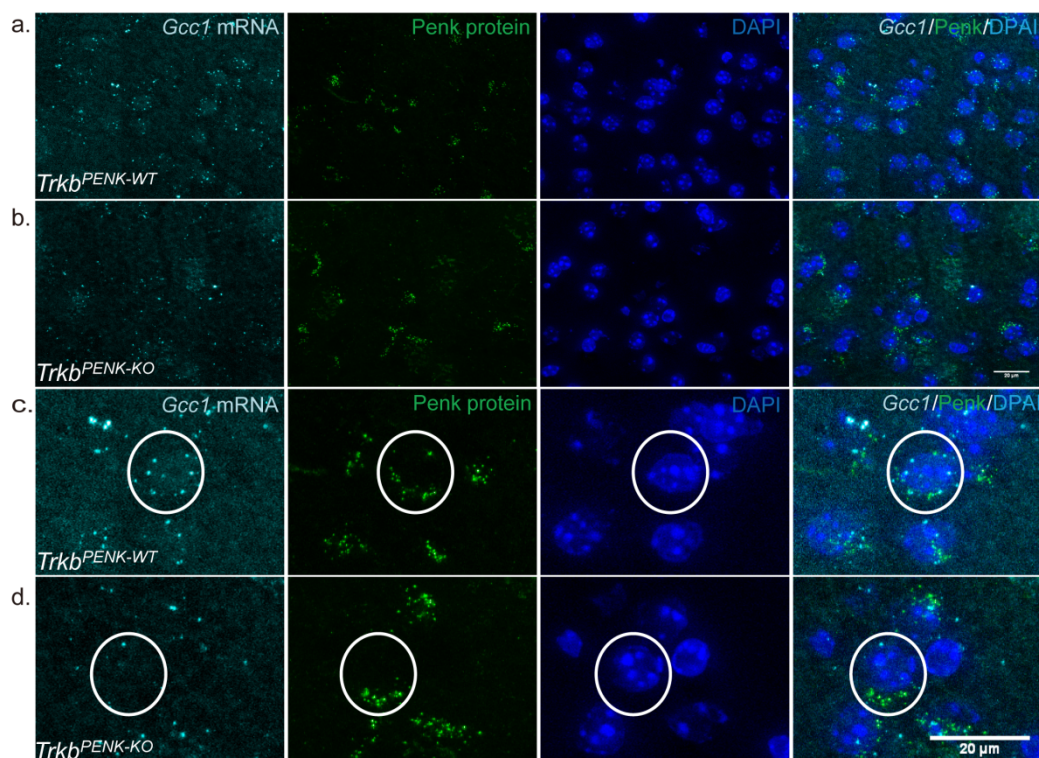


Figure 3.2.1.2.4 *Gcc1* mRNA colocalisation with Penk protein in dorsal striatum to verify the decreased expression in *Penk*+ MSNs of *Trkb*^{PENK-KO}.

a. row shows *Trkb*^{PENK-WT} *Gcc1* mRNA colocalised with Penk protein, *Gcc1* mRNA in Cyan channel and Penk protein in 488 channel. **b.** row shows *Trkb*^{PENK-WT} *Gcc1* mRNA with Penk protein colocalisation, same channels arrangement as in **a.**

c.d. Higher magnification of *Gcc1* mRNA colocalisation with Penk protein in *Trkb*^{PENK-WT} and *Trkb*^{PENK-KO}, the circles highlight the example of one *Penk*+ MSN in each channel. All scale bars are 20 μm.

3.2.2 Fluidigm qPCR validation of selected DEGs at both 3M and 8M age

With smFISH analysis we were able to show that the two selected genes, *Gsto2* and *Gcc1*, have consistent modulation *in vivo*. To extend the validation in a high throughput manner, with the help of another PhD student in the Minichiello lab (Arup Nath), we employed the fluidigm quantitative PCR to examine selected DEGs at both 3M and 8M stage. This would allow us to follow the exact same cell sorting procedure involved in RNA-seq, and to confirm the expression of the genes we are interested in.

In total we selected 14 DEGs from the bioinformatics analysis (7 genes from 3M age, 7 genes from 8M age). Striata from single mice were treated as a biological replicate, they were collected from both genotypes at two stages (3M and 8M) to obtain bulk cells samples (50 cells each), and tested for their expression of the genes of interest (GOI) by fluidigm analysis. At 3M age, expression of 7 selected genes (*Apln*; *Pcsk6*; *Gsto2*; *Cdkn2b*; *BC048403*; *Gcc1*; *Trip10*) were examined (RNA-seq data attached as below).

3M DEGs	<i>Apln</i>	<i>Pcsk6</i>	<i>Gsto2</i>	<i>Cdkn2b</i>	<i>BC048403</i>	<i>Gcc1</i>	<i>Trip10</i>
log2foldchange	8.233133 861	7.9812 64277	7.550178 3	6.075053 022	-4.3564356 45	-7.4386960 79	-8.417298 896
PadjustValue	5.71E-05	0.0004 34026	4.18E-07	0.033094 689	0.0084024 32	0.0008766 6	2.26E-06

Figure 3.2.2.a shows that at 3M age *Trkb*^{PENK-KO} had extensively up-regulated expression of DEGs (*Apln*/*Pcsk6*/*Gsto2*/*Cdkn2b*), while there was little expression of

those genes in *Trkb*^{PENK-WT}. For down-regulated DEGs (*BC048403/Gcc1/Trip10*), fluidigm analysis same tendency with consistent difference between *Trkb*^{PENK-KO} and control (*Trkb*^{PENK-WT}). As to the 7 genes of 8M DEGs (*Hace1*; *Ankrd40*; *Psen2*; *Clu*; *Enpp2*; *Ttr*; *Gstm2*), **Figure 3.2.2.b** shows the same results similar to what has been observed at 3M age except for the lower foldchange, which was also reflected by the lower log2foldchange from the RNA-seq data (RNA-seq data attached as below).

8M DEGs	<i>Hace1</i>	<i>Ankrd40</i>	<i>Psen2</i>	<i>Clu</i>	<i>Enpp2</i>	<i>Ttr</i>	<i>Gstm2</i>
log2foldchange	2.430294 441	1.598943 045	1.4468 50174	-1.90763 4309	-2.0615180 78	-2.1415254 59	-2.696438 949
PadjustValue	0.000243 779	0.026269 774	0.0001 39976	0.004900 326	0.0071036 74	0.0066284 84	0.000712 768

In summary, utilizing fluidigm gene expression analysis offered us a direct validation of the data obtained by RNAseq analysis as allowed to follow the exact same procedure to collect the bulk cells samples, and further to validate the expression of a set of selected DEGs from both 3M and 8M age. The results obtained with the fluidigm analysis has confirmed what has been observed in the RNA-seq data.

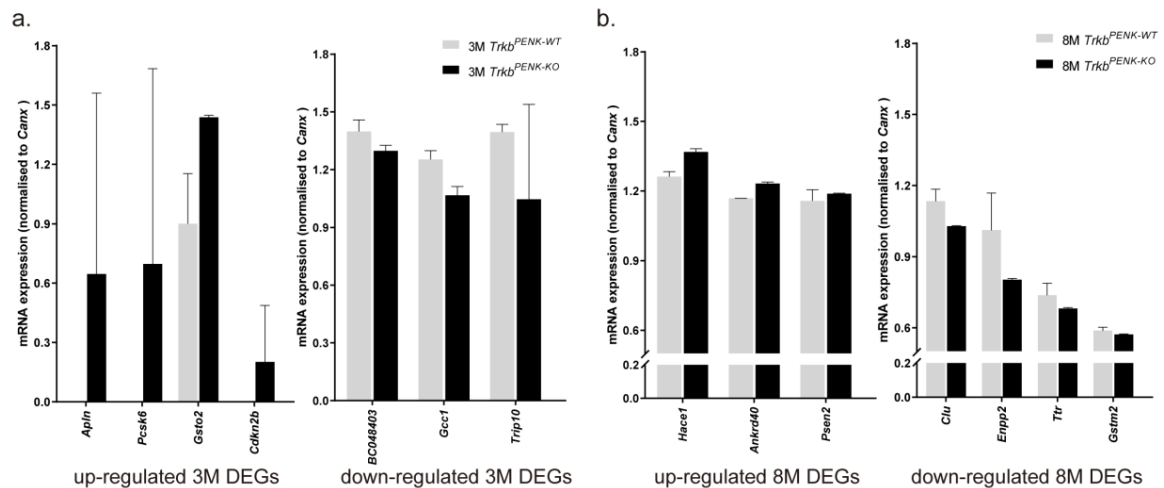


Figure 3.2.2 Fluidigm gene expression analysis shows the consistent results of selected DEGs with RNA-seq analysis.

a. shows fluidigm gene expression analysis on selected genes of both up-regulated and down-regulated DEGs at 3M age. Analysis shows similar difference to the RNAseq results when comparing *Trkb^{PENK-KO}* with *Trkb^{PENK-WT}*. **b.** fluidigm analysis of 8M DEGs with same experimental arrangement as **a.** The expression of all genes was normalised to the housekeeping gene *Canx*. Values are mean $\pm$ s.d. of 2 biological replicates.

3.2.3 Immunohistochemical validation of specific selected genes (*Gsto2* and *Pace4*)

To further confirm and validate the RNAseq data with a method that analyse the protein more than the gene expression, the best is to perform immunochemical analysis. However, this is not always possible as it involves the availability of good antibodies against the proteins of interest.

Here we focused on the early stage (3M) and applied this method to two genes. *Pcsk6* (also known as *Pace4*) encodes a member of the subtilisin-like proprotein convertase family (Proprotein Convertase Subtilisin/Kexin Type 6). This gene was extremely highly expressed in *Trkb*^{PENK-KO} compared to the *Trkb*^{PENK-WT} at 3M age (log2foldchange: 7.981264277; PadjustValue: 0.000434026). The activity of proprotein convertase (PCs) has been implicated in several diseases such as Alzheimer's disease²⁰⁰, endocrinopathies²⁰¹ and cancer²⁰². Research has also shown *Pcsk6* gene associated with neurodevelopmental issues^{203,204}. We managed to set up the condition for DAB staining with Pace4 antibody. The results show brown dots like staining in the dorsal striatum of *Trkb*^{PENK-KO} at 3M age (**Figure 3.2.3.a.b**), which is consistent with data obtained from the RNAseq and fluidigm analyses.

Next, we tested another extremely highly expressed gene *Gsto2* (log2foldchange: 7.5501783; PadjustValue: 4.18E-07) by immunofluorescence analysis. Also this gene has been validated by both smFISH analysis and fluidigm qPCR. Clinical data has shown that *GSTO2* level is associated with age-at-onset of PD and AD²⁰⁵, and could

confer AD risk in older age²⁰⁶. Moreover, as shown by our bioinformatics analysis, this gene is one of the four genes (*Gsto2/Gcc1/BC048403/Zfp101*) with unique expression pattern specific to enkephalinergic MSNs of the *Trkb*^{PENK-KO} at 3M age.

Figure 3.2.3.c.d shows barely detectable staining of *Gsto2* in *Trkb*^{PENK-WT} dorsal striatum, while intensive expression of *Gsto2* can be observed in *Trkb*^{PENK-KO} section.

Overall, the immunochemical analysis above further reinforce the validation of the RNA-seq data, and moreover, shows that *Pcsk6* and *Gsto2* were both up-regulated in the dorsal striatum of *Trkb*^{PENK-KO} at 3M age not only at transcriptional level, but also at protein level.

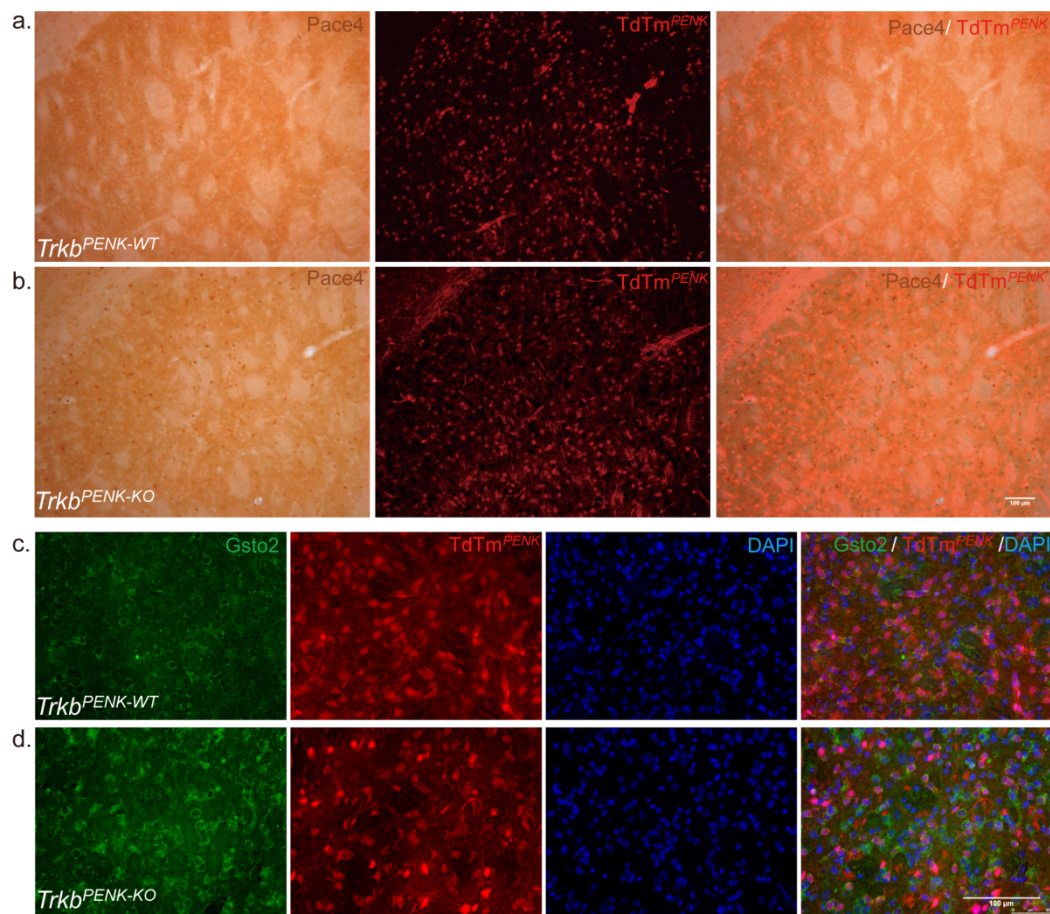


Figure 3.2.3 *Pace4* (*Pcsk6*) and *Gsto2* immunostaining show increased protein expression in the dorsal striatum at early stage (3M).

a.b. DAB staining for *Pace4* (*Pcsk6*), Dorsal striatal sections from both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} at 3M age were stained with DAB, imaged, and merged with tdTomato channel. More brown staining of *Pace4* was seen in the dorsal striatum of *Trkb*^{PENK-KO}.

c.d. Immunofluorescence staining against *Gsto2* protein in 488 channel on dorsal striatal sections from both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} at 3M age. More intense *Gsto2* staining can be observed in the dorsal striatum of *Trkb*^{PENK-KO}. All scale bars are 100μm.

3.3 Absence of TrkB signaling in enkephalinergic MSNs causes hyperdopaminergia at early stage

3.3.1 *Striatal stereological analysis of TH expression shows increased TH density upon deletion of Trkb*

Previous work from our lab has shown that absence of TrkB signaling in enkephalinergic MSNs triggers a time dependent dopamine D2 receptor-mediated increase of striatal protein kinase C and MAP kinase activation and affects intrinsic activation of striatal enkephalinergic neurons¹⁶⁸. Our current RNA-seq and bioinformatics analysis pointing to alteration in the oxidative phosphorylation pathway, therefore mitochondrial dysfunctions further supported a defect in the *Penk*+ MSNs in absence of TrkB signaling. Moreover, recent data also suggest that dopamine can influence mitochondrial physiology in the striatal neurons²⁰⁷. Together, this has suggested a possible altered dopaminergic system in *Trkb*^{PENK-KO} conditional knockout model. Dopaminergic pathways are extremely important for brain activity and dysfunction is directly involved in the pathogenesis of neurodegenerative disorders, such as PD and HD. To comprehensively assess if the dopaminergic system was altered in the striatum of mice carrying absence of TrkB signaling, we, firstly, analysed the striatum by immunostaining of tyrosine hydroxylase (TH), a crucial enzyme in the synthesis of dopamine, in a stereological manner. TH-immunostaining is commonly used to determine the dopamine signal in the striatum^{208,209}. We systematically selected four 30µm sections spanning the entire dorsal striatum (3

biological replicates per genotype for each age) across the four time points (1-2-3 and 8M) (described in **Chapter 2.2.7.1**). Sections were then immunostained using fluorescence with TH and imaged to obtain TH optical density per pixel. The area of the striatum was also calculated to rule out tissue atrophy, which has also been demonstrated in previous work¹⁶⁸. As shown in Figure3.1, we managed to capture the significant increase of dorsal striatal TH expression in *Trkb*^{PENK-KO} at 3M age (1.114 foldchange of *Trkb*^{PENK-WT}, $p=0.0031$) (**Figure 3.3.1.c.g**). This difference was not present at earlier stage 2M (1.036 foldchange of *Trkb*^{PENK-WT}, $p= 0.7748$) (Figure3.1.b.f.) or 1M (1.008 foldchange of *Trkb*^{PENK-WT}, $p= 0.9203$) (**Figure 3.3.1.a.e**), while at 8M age, the expression of dorsal striatum TH tends to decrease even though not significantly (0.966 foldchange of *Trkb*^{PENK-WT}, $p=0.3395$) (**Figure 3.3.1.d.h**). And as to the striatal area, there was no significant difference observed between the genotypes across these 4 time points (1M: $p= 0.4053$; 2M: $p= 0.6195$; 3M: $p= 0.2821$; 8M: $p= 0.3658$).

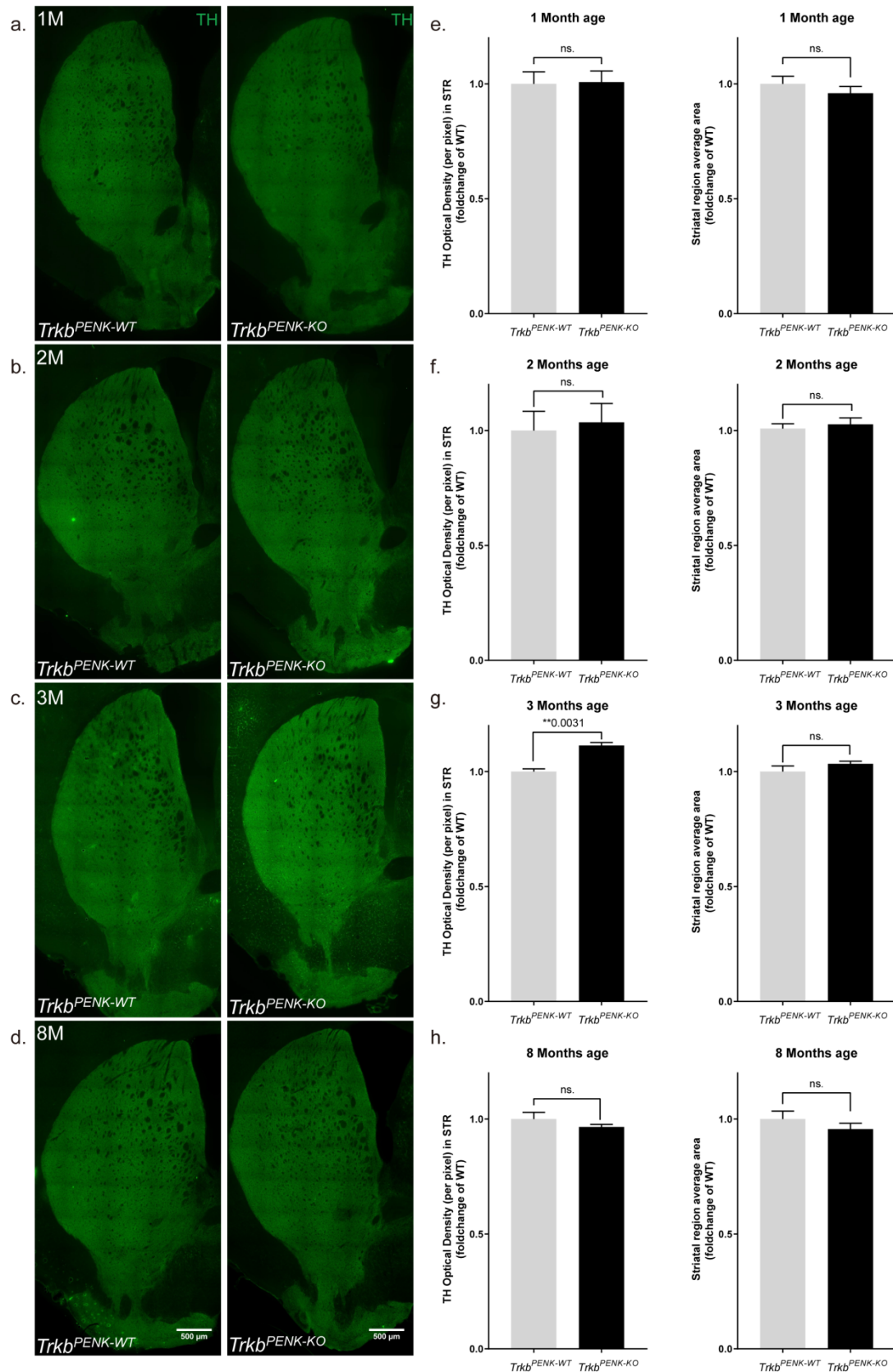


Figure 3.3.1 Stereological analysis reveals increased TH striatal levels specifically at 3M age.

a.b.c.d. rows show representative coronal striatal section of TH immunofluorescence for both *Trkb*^{PENK-WT} and *Trkb*^{PENK-KO} at each stage, respectively. **e.f.g.h.** rows show unpaired t test statistics comparing striatal TH optical density and estimated striatal area from the stereological analysis between genotypes at each stage. ** $p < 0.005$, ns. indicates non-significant ($p > 0.05$), number of biological replicates for each genotype and each age is 3, values are mean $\pm$ s.d.

3.3.2 Stereological immunostaining of TH positive cells in the SNpc/VTA area reveals increased number of dopaminergic neurons specifically in *Trkb*^{PENK-KO} at 3M age

As shown above, increased TH expression was observed in the striatum of *Trkb*^{PENK-KO} specifically at 3M age. We next examined two regions of enriched dopamine projection neurons, substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA), involved in dopaminergic pathways projection to the striatum, which have been extensively reported in many movement disorders¹⁸, particularly in HD²⁰⁸. To perform this analysis, we systematically selected 3 representative sections spanning through SNpc/VTA region to identify dopaminergic neurons in this area by TH positive immunostaining (described in **Chapter 2.2.7.2**). Respectively, we examined the number of TH positive neurons at 3 time points, 2M, 3M and 8M, according to the preceding striatal TH analysis (**Figure 3.3.2.a.b.c**). Coincidentally, we observed a significant increased number of TH positive cells in SNpc/VTA area of *Trkb*^{PENK-KO} specifically at 3M age (**** $p < 0.0001$, mean of *Trkb*^{PENK-WT} is 4583 and

mean of $Trkb^{PENK-KO}$ is 6173, 1.35 foldchange of $Trkb^{PENK-WT}$), but not at earlier stage ($p= 0.1906$, mean of $Trkb^{PENK-WT}$ is 4713 and mean of $Trkb^{PENK-KO}$ is 4908), while at 8M age, tendency of a decreased number of TH positive cells was shown in SNpc/VTA of $Trkb^{PENK-KO}$ but not significantly ($p= 0.1315$, mean of $Trkb^{PENK-WT}$ is 4720 and mean of $Trkb^{PENK-KO}$ is 4515) (**Figure 3.3.2.d.e.f**). Overall, this stereological analysis of SNpc/VTA shows that at 3M age absence of $Trkb$ causes increased number of dopaminergic neurons in SNpc/VTA area projecting to the striatum that correlates with the increased striatal TH expression.

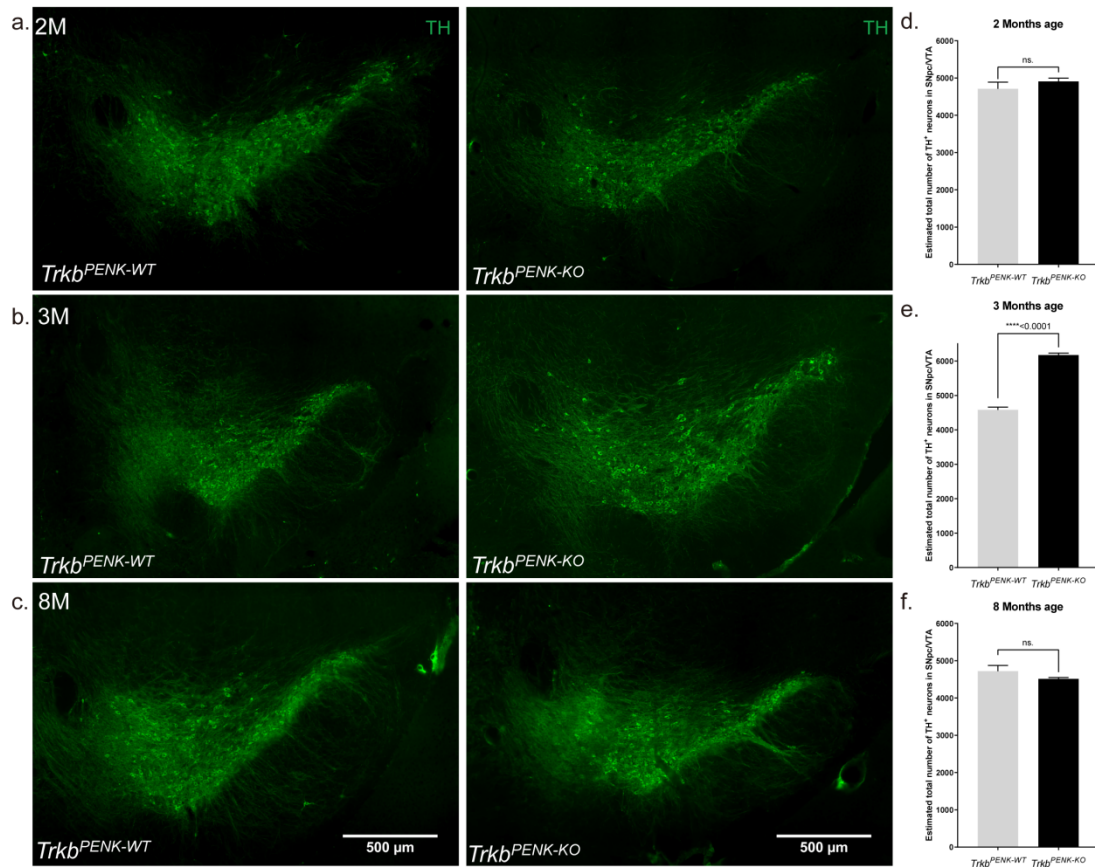


Figure 3.3.2 Number of dopaminergic neurons in SNpc/VTA showing corresponding increase specifically in $Trkb^{PENK-KO}$ at 3M age.

a.b.c. rows show representative TH immunostaining of coronal SNpc/VTA sections of both *Trkb*^{PENK-WT} and *Trkb*^{PENK-KO} at different stage, respectively. **d.e.f.** rows shows unpaired t test statistics from comparing the number of TH positive cells within SNpc/VTA region by stereological analysis between mutants and controls. **** $p < 0.0001$, ns. indicates non-significant ($p > 0.05$), number of biological replicates for each genotype/age is 3, values are mean $\pm$ s.d.

3.3.3 HPLC analysis demonstrates the increased levels of striatal dopamine and related metabolites in *Trkb*^{PENK-KO} at 3M age

As tyrosine hydroxylase (TH) is involved in the critical step of synthesizing dopamine in the biological processes, to further demonstrate our hypothesis that increase of striatal TH expression could directly lead to increase account of dopamine, which is well known as active modulator for striatal function underlying locomotor activity, we then employed high performance liquid chromatography (HPLC) to analyse the amount of dopamine (DA) and its two major metabolites, 3,4-Dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), in striatal tissue of *Trkb*^{PENK-KO} and relative controls at both 3M and 8M stage. The results and the statistical analysis of the HPLC data has been presented in detail in **Figure 3.3.3.a**. We found that the striatal amount of dopamine was significantly increased in *Trkb*^{PENK-KO} at 3M (* $p = 0.0385$, mean of *Trkb*^{PENK-WT} is 80.47 and mean of *Trkb*^{PENK-KO}

is 109.4, 1.36 foldchange of *Trkb*^{PENK-WT}) and consistent increase was also observed in its metabolites, DOPAC and HVA (DOPAC: ***p*=0.0032, mean of *Trkb*^{PENK-WT} is 38.96 and mean of *Trkb*^{PENK-KO} is 59.30, 1.52 foldchange of *Trkb*^{PENK-WT}; HVA: ***p*=0.0368, mean of *Trkb*^{PENK-WT} is 15.61 and mean of *Trkb*^{PENK-KO} is 20.27, 1.29 foldchange of *Trkb*^{PENK-WT}) (**Figure 3.3.3.b**), whereas at 8M age, no significant difference could be recognised in either dopamine (*p*=0.2135, mean of *Trkb*^{PENK-WT} is 56.53 and mean of *Trkb*^{PENK-KO} is 65.87) or its metabolites (DOPAC: *p*=0.2403, mean of *Trkb*^{PENK-WT} is 34.96 and mean of *Trkb*^{PENK-KO} is 42.93; HVA: *p*=0.3470, mean of *Trkb*^{PENK-WT} is 23.92 and mean of *Trkb*^{PENK-KO} is 29.30) (**Figure 3.3.3.c**). Moreover, to be noticed, the amount of dopamine was sharply reduced comparing 8M age with 3M age (8M DA: mean of *Trkb*^{PENK-WT} is 56.53 and mean of *Trkb*^{PENK-KO} is 65.87; 3M DA: mean of *Trkb*^{PENK-WT} is 80.47 and mean of *Trkb*^{PENK-KO} is 109.4), but not evident in DOPAC and HVA. In conclusion, HPLC result demonstrated that increased amount of dopamine and its metabolites, DOPAC and HVA, in striatum of *Trkb*^{PENK-KO} compared to the relative controls at 3M, but not 8M age, supporting the consistent preceding findings of elevated striatal TH expression and increased number of TH positive cells in SNpc/VTA region.

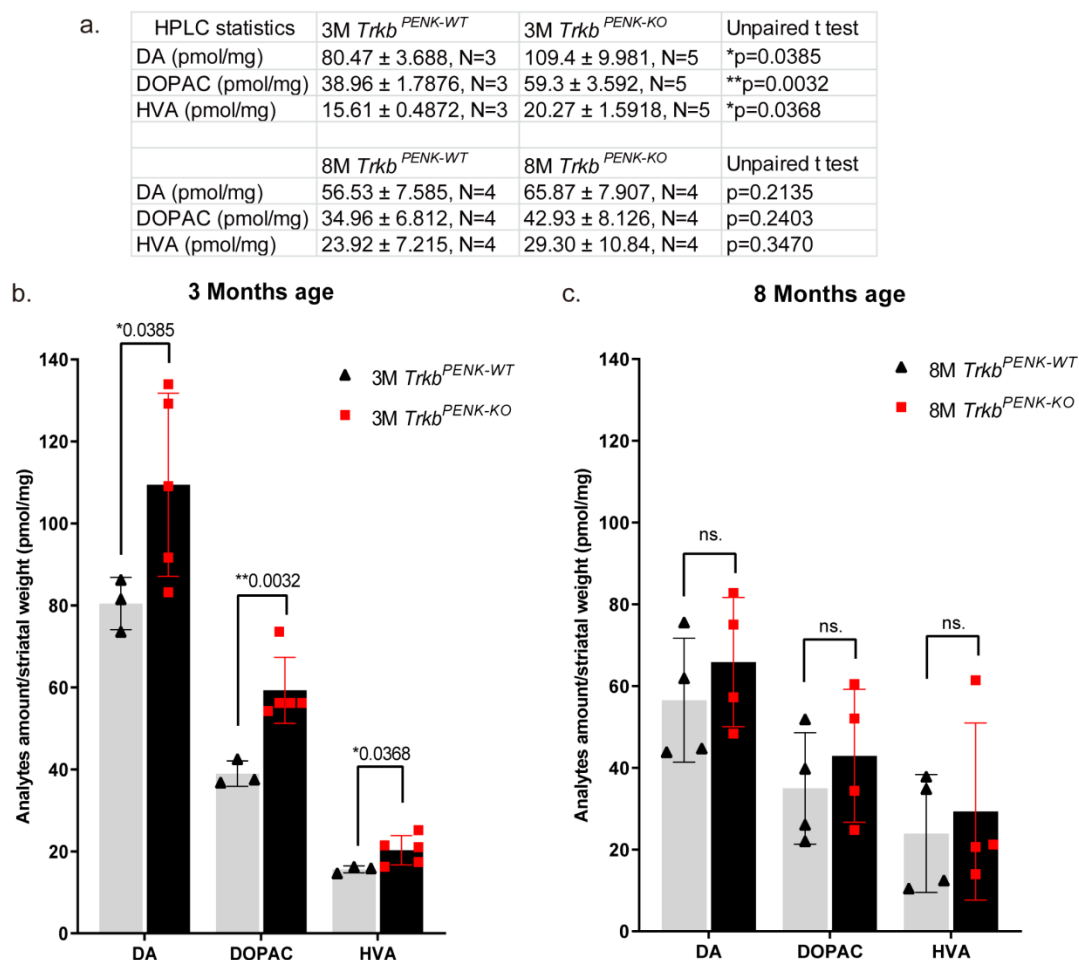


Figure 3.3.3 Amount of dopamine and related metabolites in striatum increased consistently at 3M, not 8M age.

a. Statistical analysis table of HPLC data for DA and related metabolites, DOPAC and HVA. N= sample size. Unpaired t test is applied to the comparisons between the genotypes for each analyte. Number of analytes were presented in pmol calibrated with weight of tissue (pmol/mg).

b. Graphical representation at 3M age, amount of DA, DOPAC and HVA were increased in striatum of *Trkb*^{PENK-KO} compared to *Trkb*^{PENK-WT}. c. DA, DOPAC and HVA levels were not different between *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} at 8M age. *p<0.05, **p<0.005, ns. indicates non-significant (p>0.05), values are mean ± s.d.

3.4 *In vivo* validation of *Gsto2* alteration by shRNA intracranial injections

3.4.1 Stereotaxic injection of *Gsto2* shRNA lentivirus on STR

Combining the findings from the bioinformatics analysis discovering a set of four DEGs (*Gsto2/Gcc1/BC048403/Zfp101*) with unique expression pattern specific to enkephalinergic MSNs of the *Trkb*^{PENK-KO} at 3M age, the critical time point when elevated striatal TH/dopamine expression and increased TH+ dopaminergic neurons in SNpc/VTa were observed, and then diminished at 8M age, we wondered if the four DEGs may be involved in driving the molecular signature of *Trkb*^{PENK-KO} associated with abnormal hyperdopaminergia at early stage leading to the hyperlocomotor phenotype at later stage. Moreover, to be noticed, *Gsto2* is the only DEGs up-regulated specifically in enkephalinergic MSNs of the *Trkb*^{PENK-KO} at 3M age amongst the 4 genes (the other 3 are down-regulated). Therefore, we further investigated the consequences of *Gsto2* knockdown specifically in *Penk*+ MSNs at early stage (3M) on the progression of hyperlocomotor phenotype at later stage.

Here, firstly we modified a commercially available viral construct of *Gsto2* short hairpin RNA (shRNA) in a Cre-dependent construct labeled with *Gfp* reporter to tracing the spreading. Briefly, a stop cassette flanked by two loxP sites, which can only be removed by Cre recombinase, was inserted under human U6 promoter before *Gsto2* or Scramble shRNA sequence, while *eGfp* was directly driven by the original promoter of the vector (**Figure 3.4.1.a**). Therefore, this construct would activate the

Gfp expression in any successfully transduced cell, but only express the shRNA to silence *Gsto2* or the Scramble shRNA in presence of Cre recombinase. In this case, the Cre recombinase is expressed in enkephalinergic MSNs under the Penk promoter, as explained in **Chapter 2.2.1**, in addition to tdTomato reporter. By prepared lentivirus with the modified shRNA construct, we then established the conditions of stereotaxic injection to target the dorsal striatum appropriately. The detailed procedures were described in **Chapter 2.2.9**. To maximize the covered area, we injected 2500nl lentivirus in total per hemisphere by dispensing 1250nl for each anterior position, which included two depth injection (625nl for each depth) (**Figure 3.4.1.b.c**). The first starting Coordinates 1 (Anterior injection: AP: 0.95mm, ML: 1.75mm, DV: 3.5 and 2.5mm; Posterior injection: AP: 0.25mm, ML: 2mm, DV: 4 and 3mm) was chosen according to literature (A/P: -0.93 mm, M/L: +1.8 mm, D/V: -3.46 mm)²¹⁰. As being examined in **Figure 3.4.1.e**, the coronal section at Bregma 0.545mm shows the unsatisfactory injection position and the optimised coordinates accordingly. As shown in the **Figure 3.4.1.d** table, we kept the AP (Anterior-Posterior) position intact and adjusted the ML (Medial-Lateral) and DV (Dorsal-Ventral) to eventually result in an appropriate stereotaxic injection of the dorsal striatum (**Figure 3.4.1.e** Coordinates 3), which was then applied in the following experiments.

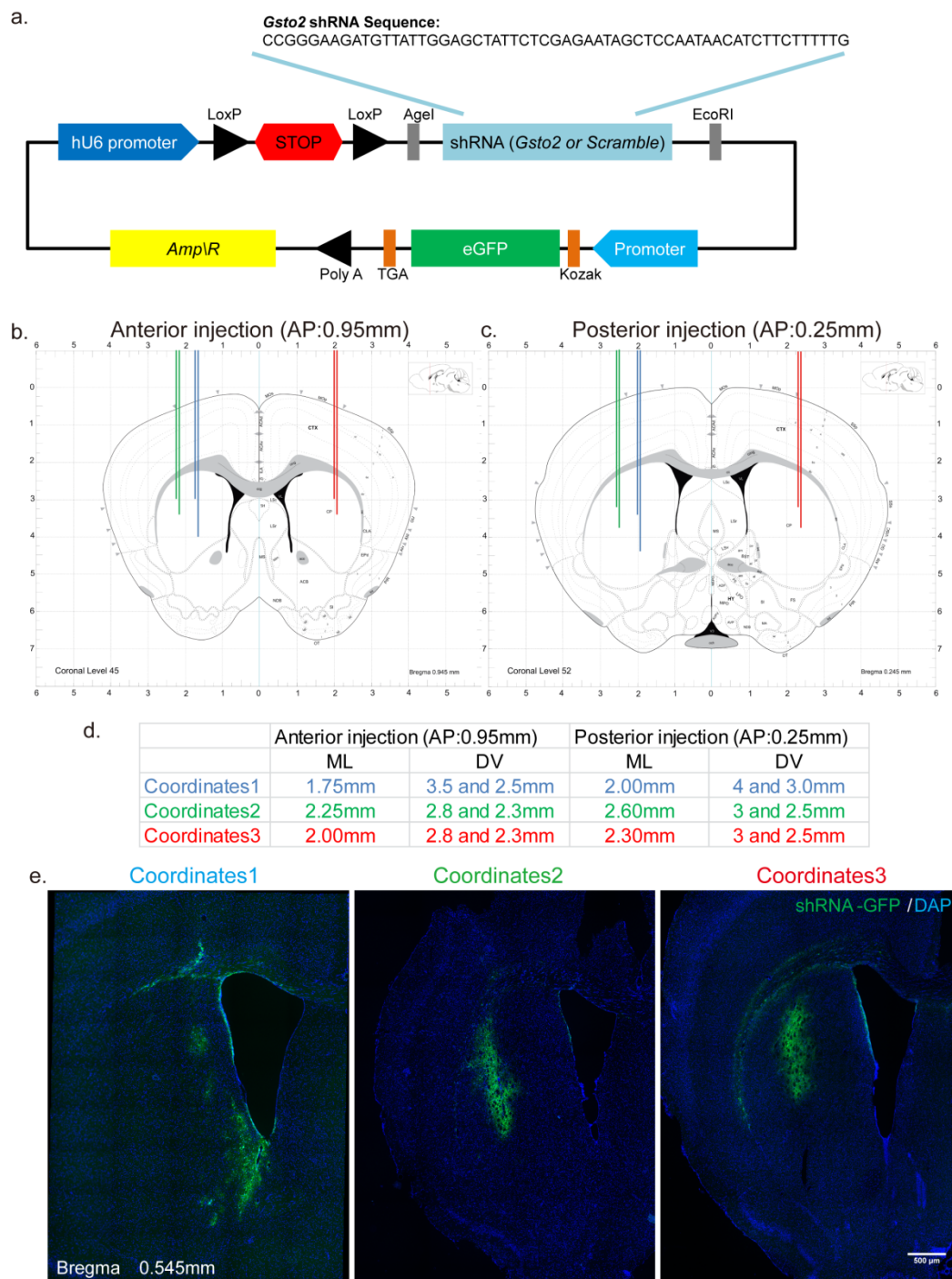


Figure 3.4.1 Coordinates optimisation of intracranially striatal injection of shRNA virus showing shRNA virus targeting dorsal striatum properly with GFP labeling.

a. Scheme of modified shRNA plasmid construct in a Cre dependent manner.

b.c. Two depths for two sites of injection per hemisphere as shown in respective Atlas images (Atlas images: Paxinos and Franklin. The Mouse Brain in Stereotaxic Coordinates.). The lines of three colors indicates the estimated injected position

respectively.

b. Anterior injection at Bregma 0.95mm with 3 coordinates **c.** Anterior injection at Bregma 0.25mm with 3 coordinates.

d. Three coordinates detail presented in the table showing setting of AP, DV and ML. Same colors highlight positions in **b**.

e. Respective coronal sections of the three coordinates for shRNA injected striatum showing improved spreading pattern with modified coordinates. Scale bars of images are 500µm.

3.4.2 Examination of striatal spreading pattern and the effect of knock down on shRNA^{Gsto2} lentivirus injection

As the conditions of shRNA injection were established, at first, we systematically examined the striatal spreading pattern to make sure the injection appropriately spanned through the dorsal striatum with decent coverage of transduced enkephalinergic MSNs. Both shRNA^{Scramble} and shRNA^{Gsto2} group were followed the stereotaxic conditions established above with Coordinates3. As shown in **Figure 3.4.2.1.a**, one representative set of serial sections from one shRNA^{Gsto2} injected *Trkb*^{PENK-KO} brain spanning through the two injected AP positions (AP 0.95mm, AP 0.25mm) with 100µm interval shows that the shRNA lentivirus spreading situated decently within the dorsal striatum in each level.

We then measured the coverage of lentivirus spreading by systematically calculating the ratio of the number of GFP positive cells with tdTomato positive cells in both shRNA^{Scramble} and shRNA^{Gsto2} injected *Trkb*^{PENK-KO} brains. Results reveals that shRNA^{Scramble} and shRNA^{Gsto2} injected *Trkb*^{PENK-KO} brains had similar number of *Penk*⁺ MSNs (mean shRNA^{Scramble} group is 3563 and mean of shRNA^{Gsto2} group is 3522) and similar number of GFP⁺ cells (mean shRNA^{Scramble} group is 585.8 and mean of shRNA^{Gsto2} group is 623.6) on average level, showing no difference in lentivirus coverage by GFP⁺/*Penk*⁺ ratio (mean shRNA^{Scramble} group is 16.42% and mean of shRNA^{Gsto2} group is 17.73%) (**Figure 3.4.2.1.b**). Furthermore, the GFP labeling that allows us to visualise the spreading pattern of shRNA lentivirus and the

coverage of lentiviral transduction, combined with tdTomato labeling of *Penk*⁺ MSNs, permits to score the successfully transduced enkephalinergic MSNs by examining the colocalisation of GFP and tdTomato. By systematically counting the GFP⁺ and *Penk*⁺ colocalised MSNs (in the stereological manner as described in **Chapter 2.2.7**), we estimated the efficiency of successful transduced enkephalinergic MSNs. In shRNA^{*Gsto2*} group, there were 294.7±49.5 MSNs transduced with shRNA among the total transduced cells (GFP⁺ 623.7±72.8), while 261.2±43.4 MSNs transduced with shRNA among the 585.8±76 total transduced cells in shRNA^{Scramble} group. Both shRNA^{Scramble} and shRNA^{*Gsto2*} injected group show the acceptable efficiency of lentivirus transduction on *Penk*⁺ MSNs similarly (mean shRNA^{Scramble} group is 44.5% and mean of shRNA^{*Gsto2*} group is 47%), which is also close to the results reported in the literatures^{211,212}. To further validate the knockdown effect of shRNA for *Gsto2* in enkephalinergic MSNs, we then immunostained the shRNA^{*Gsto2*} injected *Trkb*^{PENK-KO} brain sections with the antibody against *Gsto2* under the condition we established in **Chapter 3.2.3**. As shown in **Figure 3.4.2.2**, *Gsto2* is expressed in the *Penk*⁺ MSNs with no viral transduction (GFP negative), whereas in the colocalised MSNs, *Gsto2* expression can be hardly detected.

Overall, the above analysis demonstrates that the stereotaxic shRNA lentiviral injection of the striatum we established allows knockdown of *Gsto2* expression specifically in enkephalinergic MSNs with appropriate coverage of viral spreading in dorsal striatum and considerable transduction efficiency.

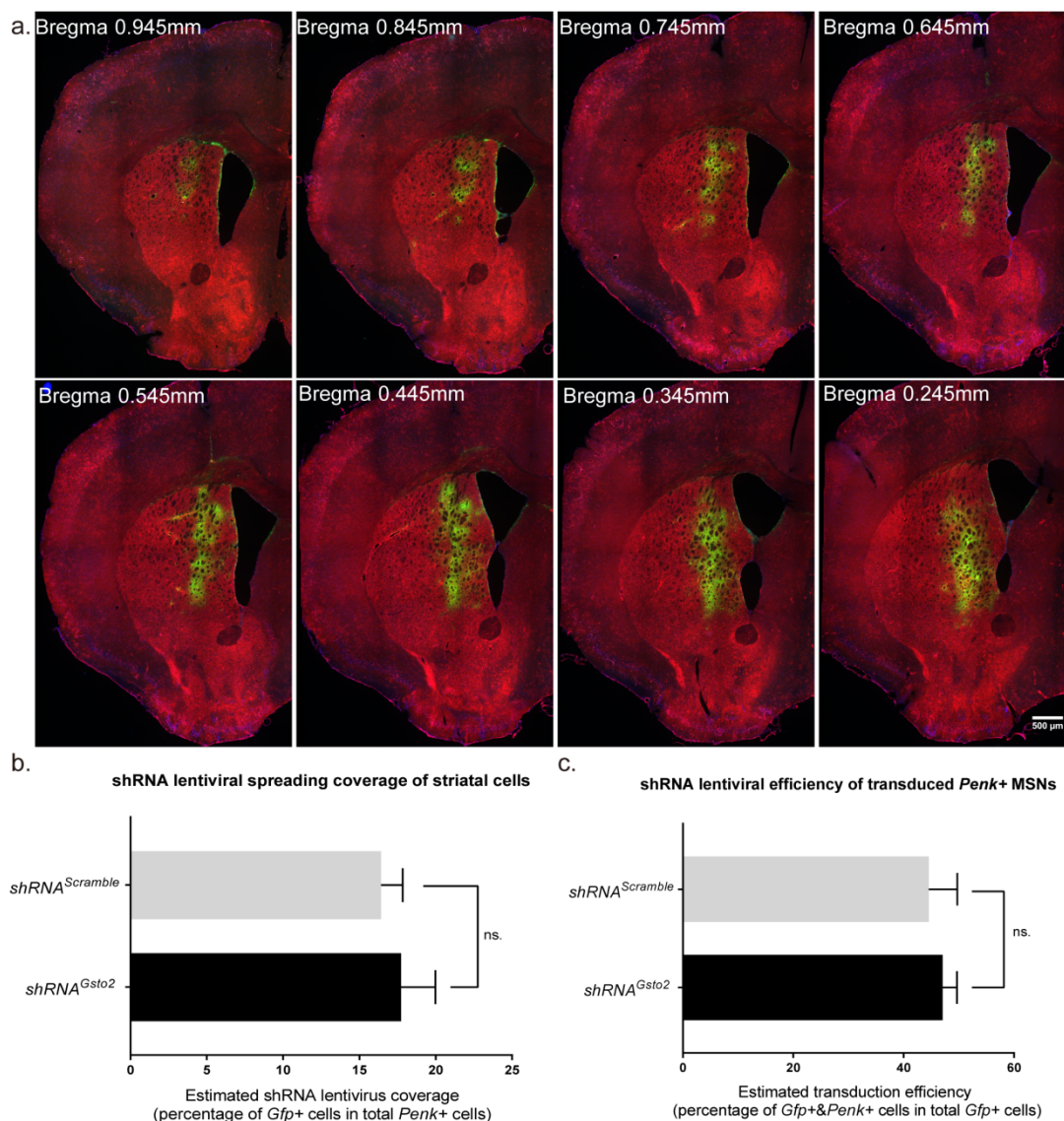


Figure 3.4.2.1 Evaluation of shRNA striatal injection showing viral spreading pattern with appropriate coverage and transduction efficiency in enkephalinergic MSNs.

a. panel represents serial coronal sections of shRNA injected *Trkb*^{PENK-KO} striatum from Bregma 0.95mm to Bregma 0.25mm. Scale bars are 500 μ m.

b. Examination of shRNA viral spreading coverage of striatal cells by comparing number of GFP+ cells with number of total tdTomato cells, showing no difference between *shRNA*^{Scramble} and *shRNA*^{Gsto2} group.

c. Transduction efficiency of *Penk*+ cells was estimated by comparing the number of GFP and tdTomato colocalised cells with total GFP+ cells. Unpaired t test was applied to examined the difference between *shRNA*^{Scramble} injected group and *shRNA*^{Gsto2}

injected group. ns. indicates non-significant ($p>0.05$), values are mean $\pm$ s.d. Number of biological replicates is 3 for each injected condition.

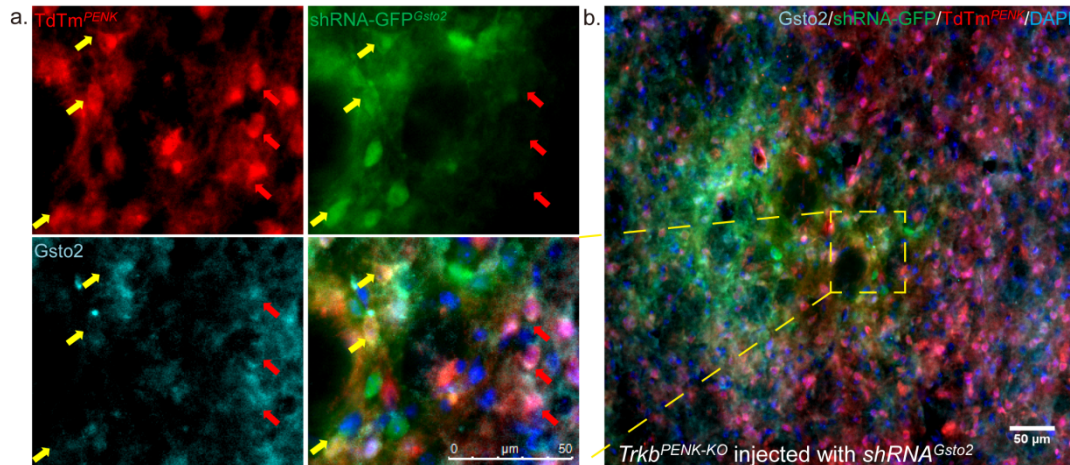


Figure 3.4.2.2 Gsto2 immunostaining to examine striatal injection of shRNA^{Gsto2} knockdown effect on *Penk*+ MSNs.

a. High magnification of shRNA^{Gsto2} injected *Trkb*^{PENK-KO} striatum showing the knockdown effect of Gsto2 protein in transduced *Penk*+ MSNs. Red channel shows the enkephalinergic MSNs with genetic labeling, green channel shows the GFP of shRNA indicating the transduced cells, and Cyan channel shows the Gsto2 immunostaining. The red arrows highlight the *Penk*+ MSNs with no transduction of shRNA, while the yellow arrows highlight the transduced *Penk*+ MSNs.

b. Gsto2 immunostaining of shRNA^{Gsto2} injected *Trkb*^{PENK-KO} striatum overview. Yellow box highlights the area presented in the **a**. All scales of images above are 50μm.

3.4.3 Locomotor behavior by the Open Field (OF) test as a readout for the effect of the intracranial injected shRNA^{Gsto2}

With the help of a senior postdoc in the Minichiello lab (Aman Asif-Malik), we performed behavioural analysis to assay the effect of *Gsto2* knockdown by shRNA. As shown in our previous work¹⁶⁸, the hyperlocomotor phenotype of *Trkb*^{PENK-KO} mice develops at later stage, however, the gene expression changes, in particular, for *Gsto2* are evident already at 3M before the hyperactivity develops. Therefore, to functionally examine the consequences of *Gsto2* knockdown specifically in *Penk*+ MSNs at early stage (3M) and check on the progression of the hyperlocomotor phenotype, we planned our shRNA injection of both shRNA^{Scramble} and shRNA^{Gsto2} in *Trkb*^{PENK-KO} mice at 3M stage. This was then followed by the open field test (OF) to assay the locomotor behavior at different stage: 7M, 8M and 12M (**Figure 3.4.3.a**).

As shown in **Figure 3.4.3.b**, we observed that the *Trkb*^{PENK-KO} mice injected with shRNA^{Scramble} developed the spontaneous hyperlocomotor activity compared to the *Trkb*^{PENK-WT} mice group and consistent with our previous results¹⁶⁸. However, since we have used only females for this experiment as they can be better managed in experiments involving craniotomy, we also observed that the hyperlocomotor phenotype of the *Trkb*^{PENK-KO} mice was already apparent around 7M of age and persisted until 12M. Whereas the *Trkb*^{PENK-KO} mice injected with shRNA^{Gsto2} at 3M age did not develop the phenotype and showed consistently comparable locomotor activity with the *Trkb*^{PENK-WT} mice group across the time from 7M to 12M age.

Specifically, the Two-way ANOVA analysis has shown the significant difference within the three groups ($F_{(2,22)} = 7.362$; $p = 0.0036$), and then by Tukey's *post hoc* multiple comparisons test, the *Trkb*^{PENK-KO} mice injected with shRNA^{Scramble} group shows the significant difference with both the *Trkb*^{PENK-KO} mice injected with shRNA^{Gsto2} and the control *Trkb*^{PENK-WT} mice respectively, but no difference was observed between the *Trkb*^{PENK-KO} mice injected with shRNA^{Gsto2} and the control *Trkb*^{PENK-WT} mice (**Figure 3.4.3.b**).

Overall, the shRNA injection at early stage followed by the locomotor behavioral test at later stage demonstrates that the knockdown of abnormally increased *Gsto2* specifically in enkephalinergic MSNs at 3M stage would maintain the normal locomotor activity of the *Trkb*^{PENK-KO} mice, which otherwise would develop into the hyperlocomotor behavior with absence of TrkB signaling in *Penk*+ MSNs.

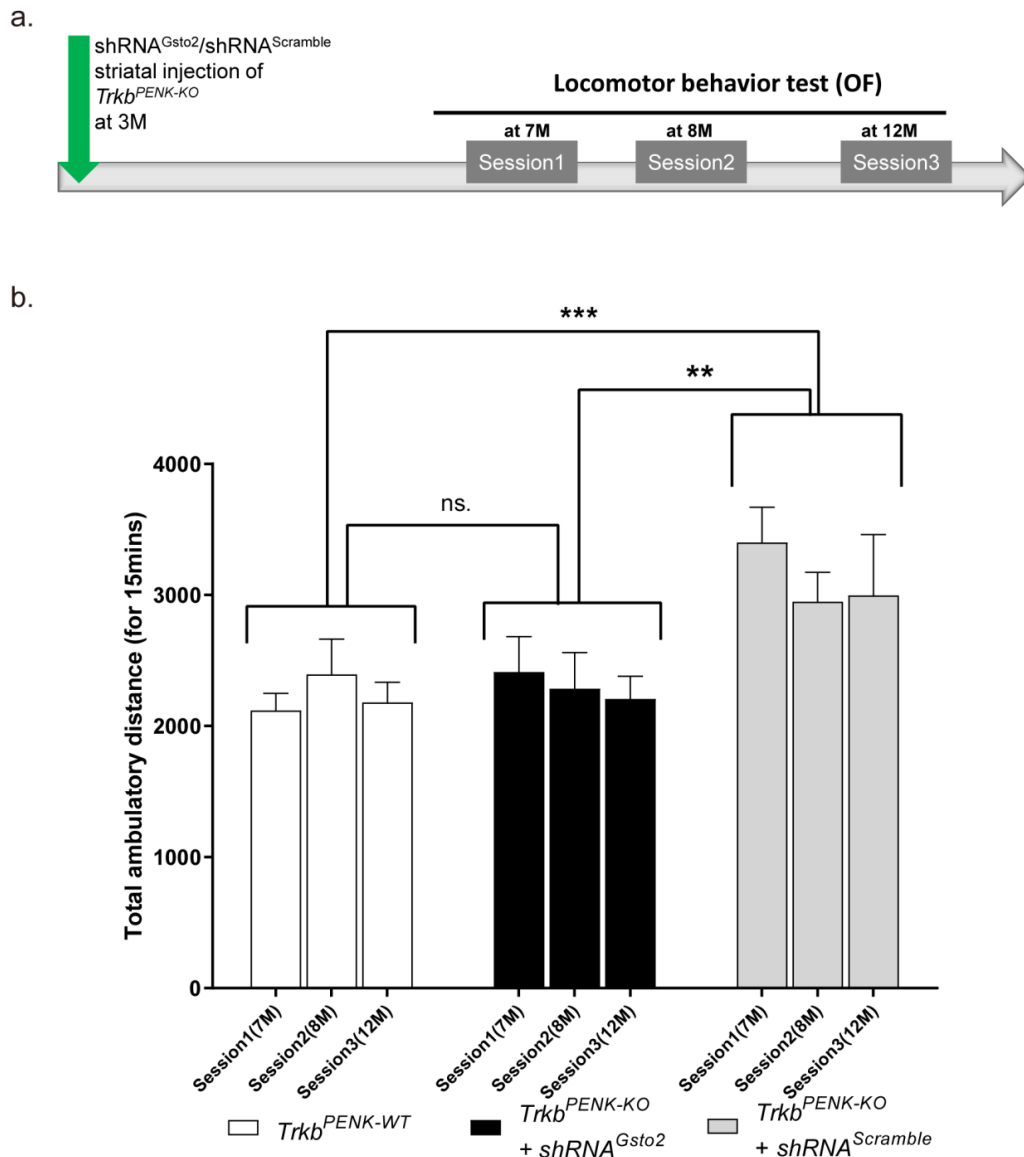


Figure 3.4.3 The hyperlocomotor phenotype is rescued by shRNA^{Gsto2} striatal injection at 3M age.

a. shRNA striatal injection and locomotor behavior test experimental scheme. Following intracranially striatal injection of shRNA^{Gsto2} (two sites per hemisphere) at 3M age, locomotor behavior test (Open Field test) was assigned at 7M, 8M and 12M accordingly.

b. shRNA^{Scramble} injected *Trkb*^{PENK-KO} group developed hyperlocomotor phenotype from 7M age comparing to the control *Trkb*^{PENK-WT} group, whereas shRNA^{Gsto2} injection at 3M rescued the *Trkb*^{PENK-KO} group hyperlocomotor phenotype across the sessions. Two-way ANOVA statistical analysis was applied with Tukey post hoc of multiple

comparisons. N of $Trkb^{PENK-WT}$ group is 10, N of shRNA^{Gsto2} injected $Trkb^{PENK-KO}$ group is 8 and N of shRNA^{Scramble} injected $Trkb^{PENK-KO}$ group is 7. ** $p=0.0035$, *** $p=0.0009$, ns. indicates non-significant ($p=0.9138$), values are mean $\pm$ S.E.M.

Chapter 4: Discussion

4.1 Transcriptomics and Bioinformatics analysis

4.1.1 Purified subpopulations of striatal MSNs showing specific transcriptome features in absence of TrkB signaling.

To further understand the underlying molecular mechanism of the locomotor phenotype observed in mice carrying deletion of *Trkb* in *Penk*+ MSNs¹⁶⁸, firstly we have successfully applied the protocols established in our laboratory^{173,176} to purify the enkephalinergic MSNs from both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} mice from young (3M) and aged (8M) stage, and to perform deep transcriptome analysis. This allowed us to capture the transcriptomic differences between age and genotype from a limited number of striatal neurons. Our results, we believe, are the first to apply deep sequencing on striatal subpopulations to investigate the molecular consequence of the absence of TrkB signaling in an age-dependent manner. From the global view of transcriptomes across all 38 samples of both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} mice (**Figure 3.1.1.2.a**), visualization of 23004 mapped genes expression showed a similar distribution and the correlation heatmap has further confirmed this similarity with homogeneous matrix (**Figure 3.1.1.2.b**). Therefore, with global transcriptome analysis tool we have not observed particular clusters from the gene expression or evident correlation pattern of the transcriptomes. Likewise, recent work on bipolar disorder has also shown a similar pattern when comparing the transcriptome of dorsal

striatum of bipolar patients and control subject²¹³. Thus, global analysis, which is supposed to identify outliers from the samples, has supported the high quality of our RNA-seq data across the well-prepared samples and reproducible workflow of our experiment. Furthermore, it might also suggest that the transcriptome from absence of TrkB signaling might suffer from more subtle molecular changes, which was not manifested in the global view. To be noticed, PCA analysis tend to capture the trend of differences (**Figure 3.1.1.2.c.d**). Intriguingly, the PCA vector distribution that indicates similarity of the striatal subpopulations showed evident differences between *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} at older age (8M), while at 3M the difference of vector distribution was found between cell types (*Penk*⁺ and *Penk*⁻). This might indicate that the difference of the global transcriptome is mainly attributed to the phenotype-associated transcriptomic features from absence of TrkB signaling at 8M age, whereas at 3M the cell-type-specific transcriptome features might be more dominant in the global transcriptome.

To capture the transcriptome changes more precisely, we zoomed into specific signature genes sets from global view to look at the expression pattern of striatal subpopulations. First, to confirm the identity of the sorted cells, we selected a set of 48 medium spiny neurons (MSNs) molecular signature genes reported in literature¹⁸¹ (**Figure 3.1.2.1.a**). This analysis clearly showed that the expression of those 48 MSNs signature genes was significantly enriched in our tdTomato sorted subpopulations (**Figure 3.1.2.1.b**). Furthermore, the most enriched cluster out of the MSNs signature genes (**Figure 3.1.2.1.a**, cluster1), were particularly associated with functions related

phenotype observed (locomotory behavior, response to drug) (**Figure 3.1.2.1.c**). This result further reinforced the functional contribution of these ENK+ subpopulations to locomotor phenotype observed from our previous study¹⁶⁸. Of interest is also the fact that, apart from the 28 most enriched MSNs signature genes of *Penk*+ MSNs, there was also considerable expression of genes (41.7%) enriched in the *Penk*- MSNs subpopulations, which might be attributed to the heterogeneity of MSNs. Moreover, for each *Penk*+ MSNs subpopulations of *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT} mice from both 3M and 8M age, there was expression of MSNs genes showing specific pattern at the different stages. For instance, 6 genes (*Tac1*/ *Gabra4*/ *Lrrc10b*/ *Sowaha*/ *Bcl11b*/ *Myt1l*) were extremely enriched in the *Penk*+ MSNs of *Trkb*^{PENK-KO} at 3M. This might suggest that actually the expression of these MSNs signature genes would be affected by the conditions of age and presence of TrkB signaling as well. It has been shown in our previous work that there was decreased Enkephalin expression in the *Penk*+ MSNs of *Trkb*^{PENK-KO} at 8M¹⁶⁸, which is also consistent with the transcriptomic data showing that expression of *Penk* in *Trkb*^{PENK-KO} *Penk*+ MSNs was much lower compared to the *Trkb*^{PENK-WT} *Penk*+ MSNs.

Furthermore, we next examined whether the transcriptomes of the striatal populations analysed displayed any alterations associated with the pathogenesis of neurodegenerative diseases. TrkB signaling has been extensively implicated in neurodegenerative conditions^{182,183}, and particularly in the pathogenesis of HD¹⁴⁰. Therefore, we compared the transcriptomes of each striatal subpopulations obtained from our analysis with key genes associated with HD from the literature¹⁸⁵. We then

observed that 26 transcription factors (TF), identified as key drivers of HD progression¹⁸⁵, were consistently enriched in *Trkb*^{PENK-KO} subpopulations compared to *Trkb*^{PENK-WT} controls starting from 3M (**Figure 3.1.2.2.a**). These results are in agreement with the affected locomotor function observed in mutant mice in an age-dependent manner underlying HD pathogenesis, but also suggest early transcriptional changes possibly leading to the development of the hyperlocomotor phenotype observed in the *Trkb*^{PENK-KO} mice¹⁶⁸. Interestingly, this significantly increased expression of the HD related TFs (such as *Klf1/ Mafa/ Smad3*) was observed in both mutant striatal subpopulations (*Penk*⁺ and *Penk*⁻) compared to its corresponding control (**Figure 3.1.2.2.a**). This would also indicate that affecting TrkB signaling in one population within the striatum would interfere with the cell-cell communication between *Penk*⁺ and *Penk*⁻ MSNs or other cell types, which is critical to the functions of striatal connectivity²¹⁴, therefore, further indicating that the *Penk*⁻ MSNs subpopulation and/or other *Penk*⁻ cell types participates to the development of the phenotype despite the intact presence of their TrkB signaling.

Additionally, since microglia activation has been regarded as the prominent contributor in the progression of neurodegenerative diseases¹⁸⁸⁻¹⁹¹, we employed a set of signature genes highly related to microglia priming in neurodegenerative conditions¹⁹², and examined microglia priming in the *Penk*⁻ MSNs subpopulations. This analysis clearly showed that at 8M microglia signature genes were massively enriched in the *Trkb*^{PENK-KO}, while this increased activation was not significant at 3M age (**Figure 3.1.2.3.b**). However, we can still clearly observe a unique subset of

microglia priming genes were already activated in *Trkb*^{PENK-KO} at 3M (**Figure 3.1.2.3.a**), which indicates that priming of microglia happens in absence of TrkB signaling at early stage leading to substantial activation at later stage.

4.1.2 DEGs mining uncovers the key drivers of transcriptome and the functional changes at early stage potentially leading to the later phenotype

To identify the genes exclusively altered at presymptomatic stage and possibly triggering the later phenotype, we performed differential gene expression analysis¹⁷⁷ on the transcriptomic data. This analysis was based on two important factors, genotype (*Trkb*^{PENK-KO} and *Trkb*^{PENK-WT}) and age (3M and 8M). We here firstly conducted three comparisons of *Penk*⁺ MSNs based on genotype and age to generate the respective DEGs sets (8M DEGs, 3M DEGs and 3Mvs8M DEGs), and then used these differential expressed genes as input for gene ontology analysis²¹⁵ to see what functions may be affected in each context. As shown in **Chapter 3.1.3**, intriguingly, the size of DEGs sets and number of significantly enriched gene functions turned out to be in contrast with each other at 3M and 8M stage. At 8M age, 54 genes (37 down-regulated/ 17 up-regulated) were significantly differential expressed (Padj<0.05) (**Figure 3.1.3.1.a**), whereas 162 genes (60 down-regulated/ 102 up-regulated) were found significantly changed (**Figure 3.1.3.2.a**). As to the gene functions of these DEGs, by integrating with GO analysis, 107 functional

annotations were significantly enriched with EASE <0.05 at 8M (**Supplementary Figure 3.1.3.1**), while only 45 functional annotations were significantly enriched (**Supplementary Figure 3.1.3.2**). This might reflect the different contribution of absence of TrkB signaling to *Penk*⁺ MSNs transcriptomic changes at each stage, which has also been implicated in the vector distribution of the PCA analysis (**Figure 3.1.1.2.d**). More specifically, at 8M age, we observed that the most enriched up-regulated cluster (21.5%) of 107 annotations was associated with apoptotic process/ programmed cell death functions (**Figure 3.1.3.1.b**), although it has been proven there is no cell death of MSNs at this stage¹⁶⁸, which suggest the ongoing threat of priming cell death may devastate functions of the *Penk*⁺ MSNs maintaining normal locomotion. At 3M age, most of annotations (42 of 45, 93.3%) enriched by DEGs up-regulated in *Trkb*^{PENK-KO}, shows mainly protein phosphorylation processes associated functions (**Figure 3.1.3.2.b**) and synapse assembly/ nervous system development (**Figure 3.1.3.2.c**). Protein phosphorylation processes has been intensively involved in pathogenesis of neurodegeneration^{216,217}, and TrkB signaling is imperative for synaptic assembly²¹⁸ and neuronal development²¹⁹. This data is the first to show these altered functions in absence of TrkB signaling at early stage when even no phenotype was observed. Moreover, our data of comparing 3M with 8M *Penk*⁺ MSNs in *Trkb*^{PENK-KO} further showed that metabolic processes and phosphorylation related functions (**Figure 3.1.3.3.b**), and biosynthetic processes (**Figure 3.1.3.3.c**) were affected in *Penk*⁺ MSNs with absence of TrkB signaling during ageing, correlating with abnormal regulation of neuronal death processes

(Figure 3.1.3.3.d).

Given that we have obtained DEGs specific to each stage, the functions of these genes were observed separately in each comparison by this conventional analysis. We then decided to mine the DEGs sets in a more integrated way by following questions: what functions altered at early stage would contribute to the development of the phenotype observed at later stage, and which genes are the key drivers leading to the early transcriptomic changes.

We employed the Gene Set Enrichment Analysis¹⁷⁹ (GSEA) to get biological insight from the DEGs analysis. Despite having observed quite a few enriched pathways for each DEGs set (62 for 8M DEGs, 257 for 3M DEGs, 335 for 3Mvs8M DEGs; not shown here), only oxidative phosphorylation (OX-PHOS) and tricarboxylic acid cycle/Electron transport chain (TCA-ETC) pathways were significantly enriched across all 3 DEGs sets (**Figure 3.1.4.2.1.b**). They were both suppressed in *Trkb*^{PENK-KO} at 3M and 8M of age respectively compared to the relative controls. TCA-ETC executes electron transportation fundamentally in mitochondrial function to support oxidative phosphorylation, and it has been intensively shown in neurodegenerative conditions²²⁰, particularly in HD¹⁹⁵. Defects in energy metabolism, particularly mitochondrial function, represent a common pathogenesis in humans and animal models. By further looking into the suppressed OX-PHOS and TCA-ETC in each subpopulations, we found that gene expression of striatal subpopulations briefly behaved with same pattern in these two canonical pathway (OX-PHOS: **Figure**

3.1.4.2.2.b ; TCA-ETC : **Figure 3.1.4.2.3.b**). However, in 3M *Trkb*^{PENK-WT}, *Penk*⁺ MSNs elevated the TCA-ETC of mitochondrial function to maintain the normal OX-PHOS status compared to the *Penk*⁻ subpopulation, while this elevation was abolished in *Trkb*^{PENK-KO} *Penk*⁺ MSNs. In addition, as the main components of TCA-ETC pathway, the ETC subunits complex I, II, III, IV and V showed corresponding expression pattern as TCA-ETC pathway (**Figure 3.1.4.2.3.c**). Intriguingly, despite both *Penk*⁺ MSNs and *Penk*⁻ subpopulation in *Trkb*^{PENK-KO} were decreased in OX-PHOS and TCA-ETC compared to the *Trkb*^{PENK-WT}, only the ETC subunits complexes in *Trkb*^{PENK-KO} *Penk*⁺ MSNs were significantly repressed, but not the *Penk*⁻ subpopulation, which indicates the specific deficit of the ETC subunits complexes happened in *Penk*⁺ MSNs of *Trkb*^{PENK-KO} at 3M age, contributing to the inhibited mitochondrial functions of OX-PHOS and TCA-ETC.

Moreover, we found that the two main cellular energetic pathways, OX-PHOS and glycolysis, were both in higher demand at later stage (8M), especially in *Penk*⁺ MSNs, and were both highly suppressed in the mutant subpopulations compared to the relative controls (**Figure 3.1.4.2.2**), which might explain the cellular dysfunctions at later stage triggering the phenotype. To be noticed, the demand of high energy metabolism in *Penk*⁺ MSNs of *Trkb*^{PENK-WT} compared to the *Penk*⁻ cells was not observed at 3M age (**Figure 3.1.4.2.2**). More intriguingly, at 3M only OX-PHOS of both *Penk*⁺ and *Penk*⁻ populations in the *Trkb*^{PENK-KO} was suppressed compared to the *Trkb*^{PENK-WT}, but not glycolysis. However, the *Penk*⁺ MSNs in the *Trkb*^{PENK-KO} at this stage showed the elevated glycolysis relative to the *Penk*⁻ cells. These two findings

might explain in a complementary way that at 3M, when no phenotype is manifest, the energy metabolism seems to be maintained in the *Penk*⁺ MSNs by increasing glycolysis pathway to compensate for the suppressed OX-PHOS with absence of TrkB signaling. It has been suggested that activity of neurons is powered by oxidative phosphorylation, not glycolysis¹⁹⁴. Here, our data suggests that at early stage, OX-PHOS was highly affected in the *Trkb*^{PENK-KO}, while *Penk*⁺ MSNs were trying to maintain the energy support elevating the glycolysis pathway. This idea of metabolic reprogramming process has recently been just aroused in the way that astrocytes of HD striatum adapt by metabolically reprogramming their mitochondria to use endogenous, non-glycolytic metabolites as an alternative fuel, which contributes to neuronal susceptibility in HD mice^{221,222}. In our case here, we observed severe energy deficits from both suppressed OX-PHOS and glycolysis at 8M stage, this might be attributed to the dysfunctions of energy metabolism at early stage (3M). At early stage, to maintain the normal energy support in absence of TrkB signaling, *Penk*⁺ MSNs elevated the glycolysis pathway to compensate for the suppressed OX-PHOS and TCA-ETC caused by the specific defects of ETC subunits complexes compared to the *Penk*⁻ subpopulation at 3M. Our data is the first to suggest this reprogramming of energy support caused by mitochondrial deficit in a cell-type specific manner. This severe dysfunction of energy support at later stage could potentially be responsible for the hyperlocomotor phenotype.

Apart from the functional alterations observed above, we have managed to identify the candidate genes that might be the key drivers leading to the early

transcriptomic changes. These key genes would be further investigated in the following validation and functional experiments. By crossing 3 DEGs sets we have generated (**Figure 3.1.4.1.a.b**), 34 DEGs were shown to overlap, suggesting they were not only differentially regulated in absence of *Trkb*, but were also specific to the age. Amongst 34 DEGs, only four genes, *Gsto2/Gcc1/BC048403/Zfp101*, were significantly differentially expressed in the *Trkb*^{PENK-KO} *Penk*⁺ MSNs compared to the *Penk*⁻ subpopulation (**Figure 3.1.4.1.c**). Amongst these 4 genes, *Gsto2* gene, the only up-regulated DEG, is extremely interestingly to us, since it is associated with the age-at-onset (AAO) of AD and PD^{193,206}, and has been reported recently to play an important role in neuroprotective effects in *non-rodent* model^{223,224}. Moreover, this gene may potentially regulate the mitochondrial Complex V activity in neurodegenerative conditions²²⁵. Interestingly, our data might contrarily suggest that absence of TrkB signaling may trigger the abnormal increase of *Gsto2* specifically in *Penk*⁺ MSNs at early stage, which could contribute to the defects of mitochondrial function, causing the dysfunction of energy metabolism.

4.2 Validation of transcriptomic data

4.2.1 Establishment and application of smFISH technique validates the altered expression of selected DEGs (*Gsto2* and *Gcc1*) at early stage

To validate the RNA-seq data *in situ* in a quantitative fashion, here we adapted single molecule FISH¹⁹⁶, the advanced version of classic RNA in situ hybridisation technique to quantitatively validate expression of selected genes and visualize the mRNA spatial distribution. By combining the original version from Lyubimova A, *et al*¹⁹⁶ with a commercial protocol from Stellaris, RNA FISH Protocols from LGC Biosearch Technologies, we managed to establish the smFISH method (**Figure 3.2.1.1.1**) in our laboratory. I used the Quasar-570 coupled *Gapdh* probe on fresh frozen brain tissue sections and successfully established conditions as shown in this thesis (**Figure 3.2.1.1.2.c.d**). The main issue was to minimise the background and unspecific signal from the tissue. We refined both tissue preparation and sections pre-treatment procedures, and optimised the image processing and analysis procedures^{196,198} (**Figure 3.2.1.1.1**). This optimised protocol allowed us to validate *Gsto2* and *Gcc1* identified by DEGs analysis following RNA-seq analysis. We decided to use Quasar-670 to label short oligonucleotides library for each gene to avoid broad spectrum (400-650nm) of lipofuscin¹⁹⁷. The smFISH analysis showed significantly increased expression of *Gsto2* and decreased expression of *Gcc1* in the striatum of *Trkb*^{PENK-KO} consistent with the RNA-seq data. To be noticed,

unfortunately we had to abandon the tdTomato reporter to avoid the strong red signal interfering the 670 signal. Therefore, we combined the smFISH with immunochemistry of Penk protein to further validate cell specificity of the signal in the *Penk*⁺ MSNs (**Figure 3.2.1.2.3, Figure 3.2.1.2.4**). This analysis was consistent with the RNA-seq data as it revealed that the Penk positive MSNs had increased *Gsto2* mRNA and less *Gcc1* mRNA compared to the Penk negative cells (**Figure 3.2.1.2.3, Figure 3.2.1.2.4**). These data further confirm the power of the RNAseq analysis but also uncover and validate novel results.

4.2.2 Validation of RNA-seq DEGs by Fluidigm qPCR and Immunocytochemistry

To further validate the RNA-seq data in a larger scale, we then employed the fluidigm quantitative PCR and examined the expression level of 14 selected DEGs from the bioinformatics analysis (7 genes from 3M age, 7 genes from 8M age) (**Figure 3.2.2**).

The fluidigm analysis²²⁶ methods we used was optimized also in our lab¹⁷³. We sorted the striatal subpopulations of interest as per the established methods¹⁷⁶ and confirmed the expression of the selected genes by fluidigm analysis (**Figure 3.2.2**). Among the DEGs at 8M, we validated up-regulated expression of *Hace1*, *Ankrd40* and *Psen2*, and down-regulated expression of *Clu*, *Enpp2*, *Ttr* and *Gstm2*. Of note,

Hace1 has been reported to regulate the oxidative stress by interacting with NRF2²²⁷ and mitochondrial functions, directly associated with the HD phenotype²²⁸. Interestingly, both *Hace1* and *Ankrd40* have the Ankyrin repeat domain, which is commonly involved in protein-protein interaction²²⁹. This domain has been suggested to mediate interaction with *mHTT* associating with its inclusion formation and toxicity²³⁰. For the down-regulated DEGs, *Clu* encodes glycoprotein clusterin, which has proapoptotic function²³¹ and it has also been implicated as risk gene in neurodegenerative conditions^{232,233}. *Ttr*, which encodes a carrier protein for thyroxine and retinol that has been associated with amyloid diseases²³⁴, and *Enpp2*, encodes ectonucleotide pyrophosphatase/ phosphodiesterase 2, are both involved in neurogenesis of adult subventricular zone²³⁵. *Gstm2* belongs to the family of glutathione S-transferases, which also include *Gsto2* in 3M DEGs. For the 3M stage, we validated up-regulated expression of *Apln*, *Pcsk6*, *Gsto2* and *Cdkn2b*, and down-regulated expression of *BC048403*, *Gcc1* and *Trip10*. Apart from the four genes we uncovered with specific expression pattern in the *Penk*⁺ MSNs (*Gsto2/Gcc1/BC048403/Zfp101*), *Pcsk6*, also known as *Pace4*, one of secretory proprotein convertases, may be involved in neuroinflammation, contributing to the pathogenesis of neurodegeneration²³⁶. Interestingly, despite the consistent difference of expression level of DEGs between fluidigm and RNA-seq data, the foldchange of RNA-seq data seems not be reproduced in fluidigm analysis. This could be attributed to several reasons as outlined below. Firstly, number of collected cells was differed between the two methods (200 cells per sample for RNA-seq, 50 cells per sample for

Fluidigm), which might affect the abundance of transcripts in the collected sample as evaluated also in a recent report²³⁷. This could also be coupled with the different amplification steps in the two methods, which collectively contribute to different amplitude of detected reads.

Moreover, for two genes differentially expressed at early stage, *Pcsk6* and *Gsto2*, we complemented the validation analysis by immunohistochemical analysis, and further confirmed that *Pcsk6* and *Gsto2* were both up-regulated in the dorsal striatum of *Trkb*^{PENK-KO} at 3M of age not only at transcriptional level, but also at protein level (**Figure 3.2.3**). In particular, *Gsto2* was validated through the three methods we have employed here. Therefore, it was extremely appealing to us to follow up this gene by functional analysis.

4.3 Absence of TrkB signaling in enkephalinergic MSNs causes hyperdopaminergia at early stage

The dopaminergic system, which forms one of the most important brain circuits, have multiple functions from the control of movement to modulation of cognition and desire, and as a result it is not surprising that it is implicated in a variety of psychiatric and neurological diseases, such as schizophrenia, attention-deficit/hyperactivity disorders, drug addiction, PD and HD²³⁸⁻²⁴⁰. Therefore, considering our previous work¹⁶⁸ showing that absence of TrkB in enkephalinergic MSNs triggers a time dependent dopamine D2 receptor-mediated increase of striatal protein kinase C and MAP kinase activation and affects intrinsic activation of striatal enkephalinergic neurons, this bioinformatic analysis and the literature²⁰⁷, we reasoned that the *Penk*+ MSNs in absence of TrkB signaling could affect dopamine signaling. Thus, we systematically examined if the dopaminergic pathways were affected with/without absence of TrkB signaling across different age points. First, we observed that the striatal density of tyrosine hydroxylase (TH) was increased in *Trkb*^{PENK-KO} specifically at 3M age, but not at earlier stage (1M or 2M) and decreased at later stage (8M) in mutants (**Figure 3.3.1**), this also correlated with dopamine levels and its two major metabolites, DOPAC and HVA, in striatal tissue (**Figure 3.3.3**). Analysis of the substantia nigra pars compacta (SNpc) and ventral tegmental area (VTA) of mesolimbic and nigrostriatal pathways, the two main pathways sending dopaminergic projections and terminating in the striatum¹⁸, showed alterations consistent with

TH/dopamine increase in the striatum, namely an increased number of dopaminergic cells in these two regions specifically at 3M age (**Figure 3.3.2**). This data correlate with the hyperdopaminergia in the striatum and SNpc/VTA observed in a transgenic rat model of HD²⁰⁸, which shows progressive neurodegeneration and chorea. Moreover, our data are consistent with the early increase of dopamine levels that has been shown in human HD²⁴¹, then followed by the late decrease²⁴². The decreased dopamine has also been replicated in the most widely used transgenic mice models, R6/2 and YAC128²⁴³⁻²⁴⁵. In addition, both striatal TH density and number of TH positive dopaminergic cells from our data decrease at 8M relative to the mutants at 3M, but it is not significantly different from the controls at this stage (**Figure 3.3.1.d.h. Figure 3.3.2.c.f.**). And to be noticed, the hyperlocomotor phenotype of our conditional knockout model at 8M is much less severe compared to that developed by the transgenic mice models, R6/2 and YAC128 used in the literature²⁴³⁻²⁴⁵, which is more similar to the hyperkinetic unwanted movement observed in human HD^{101,246}. This is suggesting that addition of the *mHTT* in absence of TrkB signaling in these cells would trigger a more aggressive effect. Overall, the use of our unique model has allowed to dissect the contribution of TrkB signaling to maintain normal homeostasis in enkephalinergic MSNs. We show that absence of TrkB signaling is sufficient to cause the early hyperdopaminergia with elevated striatal dopamine level and TH expression and increased number of dopaminergic cells in SNpc/VTA regions observed in human HD and the HD rat model.

4.4 *In vivo* validation of *Gsto2* alteration by shRNA intracranial injections

4.1 *Stereotaxic injection of shRNA lentivirus on STR selectively knocks down *Gsto2* expression in *Penk*+ MSNs*

The bioinformatics analysis allowed us to capture the key candidate DEG, *Gsto2*, showing specific expression pattern in the *Penk*+ MSNs. The *in vivo* functional analysis of this gene specifically in the *Penk*+ MSNs allowed to evaluate whether *Gsto2* would potentially contribute to the hyperlocomotor phenotype developed in our model. To our knowledge, the *in vivo* function of *Gsto2* has not been reported yet, except for the research been done in *Drosophila* and *Apis cerana cerana*²²³⁻²²⁵ and suggested functions of risk variants from clinical screening showing it is associated with onset-of-age of AD²⁰⁶. To facilitate the *in vivo* manipulation of *Gsto2*, we modified a commercial construct of *Gsto2* shRNA to allow its expression in a Cre-dependent manner (**Figure 3.4.1.a**). By referring to the well-established striatal injection in the literature²¹⁰, we then optimised the injection coordinates (**Figure 3.4.1.b.c.d**), which allowed for a reasonable spreading pattern targeting the dorsal striatum (**Figure 3.4.1.e, Figure 3.4.2.a**). Despite that SNpc/VTA dopaminergic neurons projection to both the dorsal and ventral striatum, these two regions of the striatum have similar composition in terms of MSNs²⁴⁷. While the ventral striatum and the nucleus accumbens, in particular, primarily mediates reward, cognition, and reinforcement, the dorsal striatum primarily mediates motor function, certain

executive functions (e.g., inhibitory control and impulsivity), and stimulus-response learning^{16,248-250}, which is more related to the hyperlocomotor behavior we observed. It has been shown that restoration of dopamine signaling in the dorsal striatum is sufficient to restore feeding and locomotion^{251,252}, which reinforces the important function of the dorsal striatum in locomotor behavior. By systematically examining the spreading coverage and transduction efficiency of shRNA lentivirus (**Figure 3.4.2.1.b.c.**), we showed that both shRNA^{Scramble} and shRNA^{Gsto2} injected groups show good coverage of lentiviral spreading (mean shRNA^{Scramble} group is 16.5% and mean of shRNA^{Gsto2} group is 17.8%) and quite considerable efficiency of lentiviral transduction in *Penk*+ MSNs (mean shRNA^{Scramble} group is 44.5% and mean of shRNA^{Gsto2} group is 47%), which is close to the results of transduction efficiency reported in the literatures^{211,212}.

4.2 Striatal injected shRNA lentivirus for *Gsto2* at early stage rescued the hyperlocomotor phenotype developed at later stage

As a readout of gene function manipulation, we employed the same locomotor behavioral assay, open field test, which has been applied in previous work as well¹⁶⁸, to measure general activity of the mice and evaluate how *Gsto2* shRNA at early stage would affect the hyperlocomotor phenotype developed at later stage. In the **Chapter 3.4.3**, knockdown of *Gsto2* gene specifically in the *Penk*⁺ MSNs results in a prevention of developing the hyperlocomotor phenotype of *Trkb*^{*Penk*} mice at later stage. This *Gsto2* gene has been shown by bioinformatics analysis to have a unique expression pattern specifically to the *Penk*⁺ MSNs with absence of TrkB signaling. Our data further demonstrates the function of *Gsto2* gene in the *Penk*⁺ MSNs contributing to the hyperlocomotor behavior. Currently, to our knowledge, this is the first *in vivo* work to elucidate the function of *Gsto2* gene in mammalian, while it has been reported by genome association studies that both *Gsto1* and *Gsto2* show linkage to AD and PD^{193,253}, clinical data has directly indicated that it may confer AD risk in older age²⁰⁶. *Gsto2* gene encodes an omega class glutathione S-transferase (GST), Gsto2, which execute xenobiotic detoxification through glutathione conjugation²⁵⁴. This detoxification function of GSTO protein has been involved in cellular defense as an antioxidant enzyme and glutathione metabolism. The research been done in the *Drosophila* model system has suggested that it plays an important role in

neuroprotective effects^{223,224}, and this gene may potentially regulate the Complex V activity in neurodegenerative conditions²²⁵. In our case, we did actually observe the abnormally enriched glutathione metabolism pathway in the *Penk*⁺ MSNs of *Trkb*^{PENK-KO} (data not shown here, MetaCore analysis done by collaborators), and suppressed OX-PHOS and mitochondrial functions (**in Chapter 3.1.4.2**), which might be attributed to this overreacted cellular defense led by overexpression of *Gsto2*. As a consequence, these *Penk*⁺ MSNs have to compensate for the energy deficiency of OX-PHOS and activate the energy alternative pathway of glycolysis. Collectively, this abnormal energy metabolism may maintain the function of these cells temporarily at early stage, whereas the demand of energy support from these *Penk*⁺ MSNs is growing compared to the *Penk*⁻ MSNs during ageing, the glycolysis pathway would be depleted as well and this would result in the severe energy deficiency observed at 8M of age. Thus, by interfering with the overexpression of *Gsto2* at early stage, this might alleviate the energy burden of the glycolysis pathway and unlock the suppressed OX-PHOS and mitochondrial function, which further help the *Penk*⁺ MSNs to maintain the normal function in the long term. These results not only explore the unknown functions of *Gsto2* and glutathione metabolism in the *Penk*⁺ MSNs contributing to locomotor behavior, but also offers further potential development for the treatment of movement disorders.

Chapter 5: Overall summary, conclusions and future directions

5.1 Overall summary and conclusions

The striatum integrates and drives information and function of the basal ganglia circuits, which maintain normal movement behavior²⁵⁵. Preferential degeneration of enkephalinergic MSNs has been observed at early stage of HD and driving the abnormal involuntary movement³⁴. The studies presented in this thesis investigate molecular mechanisms of TrkB signaling related to the locomotor maintenance. The core question we asked is how absence of TrkB signaling in ENK+ MSNs can induce an hyperlocomotor phenotype in an age dependent manner. To address this question, we have combined FACS sorting of adult and aged striatal neurons^{173,176} (here in particular the enkephalinergic MSNs in presence and absence of TrkB signaling¹⁶⁸) with RNA-seq, bioinformatics and functional analysis. Together, this has allowed us to start addressing our original question related to the specific striatal populations. The transcriptomes of striatal subpopulations from both *Trkb*^{PENK-WT} and *Trkb*^{PENK-KO} at presymptomatic stage (3M) and at symptomatic age (8M) allowed to uncover the affected genes expression pattern implicating the striatum-related dysfunctions (**Figure 3.1.2.1**). In particular, the absence of TrkB signaling caused abnormally enriched HD-related TF genes in the *Trkb*^{PENK-KO} striatum of both *Penk*⁺ and *Penk*⁻ subpopulations. Specifically, microglia-activation genes were progressively and evidently enriched in the *Penk*⁻ subpopulations, and extremely significant enrichment

was found at symptomatic age (8M). All these transcriptomic features might contribute to degeneration of the striatum underlying the developing hyperlocomotor phenotype, which can be barely captured by a simple global transcriptomic view (**Figure 3.1.1.2**).

Therefore, first, we used differential genes expression analysis to generate three DEGs sets for 3 specific comparisons (3M, 8M, 3Mvs8M) to identify the key genes and functions deregulated by age and TrkB-deficiency in enkephalinergic population (**Figure 3.1.4.1**). GO analysis on these DEGs sets showed that at 3M age 162 DEGs only significantly enriched 45 functional annotations (**Supplementary Figure 3.1.3.2**), whereas a smaller set of DEGs (54 genes) involved many more functions affected (107 annotations) at 8M age (**Supplementary Figure 3.1.3.1**). Moreover, further clustering of GO enriched annotations revealed that apoptosis and cell death associated functions have been primed in *Penk*⁺ MSNs of *Trkb*^{PENK-KO} at 8M with suppression of RNA/phosphorus/protein metabolic processes functions (**Figure 3.1.3.1**), even though it has been previously shown no cell death happens at this stage (8M)¹⁶⁸. At 3M of age, absence of TrkB signaling in the *Penk*⁺ MSNs tends to abnormally up-regulate the protein phosphorylation processes specifically associated with synapse assembly/ nervous system development/ cell adhesion to maintain cellular functions (**Figure 3.1.3.2**).

GSEA analysis next uncovered that only OX-PHOS and mitochondrial related TCA-ETC pathways were significantly enriched across all DEGs sets (**Figure 3.1.4.2.1.b**), and the *Penk*⁺ MSNs of *Trkb*^{PENK-KO} were under severe energy defects of

both OX-PHOS and glycolysis at 8M age compared to controls (**Figure 3.1.4.2.1.a**). This led us to further dissect the specific enriched patterns of energy support pathways in each striatal subpopulation with age and cell-type specific manner. We found that the early defect observed in TCA-ETC of mitochondria and its inner membrane subunits complex I to V caused the mitochondrial dysfunctions in both mutant striatal populations (*Penk*⁺ and *Penk*⁻) at 3M stage (**Figure 3.1.4.2.3**). These deficits were compensated by abnormal increased glycolysis specifically by the *Penk*⁺ MSNs, then leading to the development of severe energy deficiency of both OX-PHOS and glycolysis at later phenotypic stage (**Figure 3.1.4.2.2**).

Furthermore, in addition to the energy metabolism deficiency, we have identified four key gene candidates, *Gsto2/Gcc1/BC048403/Zfp101*, with unique expression pattern specifically to the *Penk*⁺ MSNs of *Trkb*^{PENK-KO} at early stage (3M) (**Figure 3.1.4.1**). While the overall bioinformatic analysis has been crucial to start to get a clue about what is driving the hyperlocomotor dysfunction in the *Trkb*^{PENK-KO} mutants, it is only through validation and functional analysis of these results that will help to reach firm conclusions. Thus, we first validated the key genes by using the optimised smFISH technique, in combination with fluidigm and immunochemical analysis. In summary, we managed to quantitatively validate the expression of two genes, *Gsto2* and *Gcc1* (**Figure 3.2.1.2.1&2**), and more DEGs were then successfully validated by utilizing fluidigm gene expression analysis to further confirm the RNA-seq data (**Figure 3.2.2**). Two extremely highly expressed genes, *Gsto2* and *Pace4*, were also examined by immunohistochemical analysis, consistently showing up-regulation at 3M

age of *Trkb*^{PENK-KO} not only at transcriptional level, but also at protein level (**Figure 3.2.3**).

By using the bioinformatic data, our previous results and the literature, we discovered that the hyperlocomotor phenotype developed around 8M of age in *Trkb*^{PENK-KO} is preceded by the development of hyperdopaminergia at an earlier stage (3M) (**Figure 3.3.1&3**). This increase in dopamine and TH expression observed in the striatum correlated with increased number of dopaminergic neurons in SNpc/VTA region at same time point (**Figure 3.3.2**), collectively showing hyperdopaminergia of *Trkb*^{PENK-KO} at early stage of 3M potentially contributing to the development of the hyperlocomotor phenotype at later stage.

Finally, since the preceding bioinformatics analysis has discovered 4 DEGs with unique expression pattern specific to enkephalinergic MSNs of the *Trkb*^{PENK-KO} at 3M age, we then manipulated the only up-regulated gene of 4 DEGs, *Gsto2* specifically in *Penk*⁺ MSNs at early stage (3M) by intracranial injection of modified shRNA viral construct (**Figure 3.4.1**) allowing knockdown of *Gsto2* expression specifically in *Penk*⁺ MSNs with decent coverage of viral spreading in dorsal striatum and considerable transduction efficiency (**Figure 3.4.2**). This resulted in rescue of the hyperlocomotor phenotype normally developed in *Trkb*^{PENK-KO} mice around 8M of age (**Figure 3.4.3**).

Overall, this thesis discusses methods and experiments that helped to start dissecting the molecular mechanisms underlying normal locomotor maintenance and

how absence of TrkB signaling in specific striatal neurons may induce hyperlocomotor phenotype, relevant to movement disorders. Moreover, excitingly the target *Gsto2* we have found and described in this thesis seems to be crucial to the process of locomotor behavior and further studies could benefit the potential development of novel treatments for movement disorders.

5.2 Future directions

5.2.1 RNA-seq Profiling of shRNA^{Gsto2} injected mice

Since we have successfully validated the function of *Gsto2* gene in the *Penk*+ MSNs (Chapter 3.4), as a follow-up study, to further understand what molecular changes have been reversed to maintain the normal locomotor function in the *Trkb*^{PENK-KO} mice injected with shRNA^{Gsto2}, we will perform gene expression profiling of the *Penk*+ MSNs of these shRNA^{Gsto2} injected mice. The same profiling would also be applied on the *Penk*+ MSNs of the shRNA^{Scramble} injected mice as control. Comparing these two transcriptomes can give us insight about which molecular signature has been normalized in these treated neurons. It may be that this treatment could rescue the MSNs from degeneration caused by absence of TrkB signaling. Moreover, by further integrating these profiling data with the RNA-seq data presented in this thesis, we can then have a more comprehensive picture of molecular signatures underlying manipulation of locomotor phenotype relevant to absence of TrkB signaling. This would be such valuable information to help understanding the molecular mechanism contributing to the locomotor behavior through specific neurons such as the *Penk*+ MSNs. This has huge implication for the development of potential treatment to the movement disorders.

5.2.2 Cell-type specific energy metabolic reprogramming

The bioinformatics data (**Chapter 3.1.4**) have pointed to the energy metabolism reprogramming from deficiency of OX-PHOS and mitochondria TCA-ETC to the abnormal increase of glycolysis occurring specifically in the *Penk*⁺ MSNs at early stage, which eventually may contribute to the development of hyperlocomotor phenotype. This idea of metabolic reprogramming has recently been suggested for astrocytes of the striatum of HdhQ(150/150) mice which adapt by metabolically reprogramming their mitochondria to use endogenous, non-glycolytic metabolites as an alternative fuel, which contributes to neuronal susceptibility in HD mice²⁵⁶. And by using Phasor-FLIM (fluorescence lifetime imaging microscopy), it further suggests that metabolic dysfunction in HD shifts to increased glycolysis leading to oxidative stress and cell death in HEK293 cell lines and in Htt transgenic *Drosophila*²²². Here the transcriptomic profiling data from specific striatal populations has led us to the observation of energy dysfunction of reprogramming in a cell-type specific manner. It will be exciting to verify this concept with functional experiments by applying the Phasor-FLIM technique. Moreover, since this energy metabolic reprogramming happened in a cell-type specific manner, by taking advantage of our transgenic reporter line and combining with FACS, we could test the separated striatal subpopulations to validate this concept functionally. Collectively, this work will be fundamental for understanding the vulnerability of *Penk*⁺ MSNs in the pathogenesis of HD.

5.2.3 Potential development of treatment targeting *Gsto2* gene:

In the studies presented in this thesis, bioinformatics analysis and functional validation led us to discover abnormal overexpression of this key gene, *Gsto2*, and shRNA knockdown of *Gsto2* gene specifically in the *Penk*⁺ MSNs at early stage successfully rescues the hyperlocomotor phenotype developed at the later stage. These findings may encourage the developments of potential treatment based on *Gsto2* gene for movement disorders, particularly HD.

Applying shRNA^{*Gsto2*} interference on the HD mouse model

In order to further facilitate the development of novel therapies for HD, it will be exciting to see how our findings will correlate in other animal models of HD, such as R6/2, HdhQ111, BACHD, YAC128. It should be taken into consideration that the locomotor manifestations vary in these models, and the interference by shRNA^{*Gsto2*} need to follow the cell-type specific fashion to target the enkephalinergic MSNs. This would further confirm the contribution of *Gsto2* gene to the behavioral phenotype of HD in the *Penk*⁺ MSNs with the presence of mutant huntingtin.

Appendix

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Table 2.1.1: Chemicals used in this study.

Chemical	Formula	Catalogue number	Manufacturer
Agarose		A9539-500G	Sigma-Aldrich
Buffer solution pH4.0 (20°C)		33665-500ML	Fluka
Buffer solution pH7.0 (20°C)		33666-500ML	Fluka
Buffer solution pH10.0 (20°C)		33668-500ML	Fluka
Citric acid monohydrate	$C_6H_8O_7 \cdot H_2O$	C1909-25G	Sigma-Aldrich
4',6-diamidino-2-phenylindole (DAPI)	$C_{22}H_{27}N_5O_6$	D3571	Molecular Probes
Ethanol (EtOH)	C_2H_6O	20821.330	vWR
Ethidium bromide	$C_{21}H_{20}BrN_3$	E1510-10ML	Sigma-Aldrich
Gelatin, from cold water fish skin		G7765-250ML	Sigma-Aldrich
Hydrogen peroxide solution (≥30%)	H_2O_2	95321	Fluka
Isopentane (2-methylbutane)	C_5H_{12}	M32631- 500ML	Sigma-Aldrich
Isopropanol	C_3H_8O	20842.330	vWR
2-mercaptoethanol (2-ME)	C_2H_6OS	M6250-100ML	Sigma-Aldrich
Paraformaldehyde (PFA)	$OH(CH_2O)_nH$ (n=8-100)	P6148-1KG	Sigma-Aldrich
Phosphate buffered saline (PBS) tablet		P4417-50TAB	Sigma-Aldrich

Sodium citrate tribasic dihydrate	$C_6H_5Na_3O_7 \cdot 2H_2O$	C8532-1KG	Sigma-Aldrich
Sodium hydroxide	NaOH	S5881-500G	Sigma-Aldrich
Sodium pentobarbital	$C_{11}H_{17}N_2NaO_3$	P3761-5G	Sigma-Aldrich
Sodium phosphate dibasic dihydrate	$Na_2HPO_4 \cdot 2H_2O$	71643-1KG	Sigma-Aldrich
Sodium phosphate monobasic monohydrate	$NaH_2PO_4 \cdot H_2O$	A429146347	Merck
Tris base	$(CH_2OH)_3CNH_2$	BP152-1	Fisher Scientific
Triton X-100	$C_{14}H_{22}O(C_2H_4O)_n$ (n=9-10)	T8787-250ML	Sigma-Aldrich
Tween 20	$C_{58}H_{114}O_{26}$	P1379-500ML	Sigma-Aldrich

Table 2.1.2: Solutions used in this study.

Cell blocking buffer	10% normal goat serum, 0.3% TritonX-100, 0.2% sodium azide in PBS
Phosphate buffer (PB)	100 mM PB, pH 7.4 (7.74% of 1 M Na_2HPO_4 , 2.26% of 1 M NaH_2PO_4)
4% paraformaldehyde	40 g paraformaldehyde in PB, pH 7.4
TB	50 mM Tris, pH 8.3
10x TBE	890 mM Tris, 890 mM boric acid, 20 mM EDTA, pH 8.3
TBS	50 mM Tris, 150 mM NaCl, pH 7.5
Tissue blocking buffer 1 (DAB)	10% normal goat serum, 0.3% carrageen, 0.5% TritonX-100, 0.2% sodium azide in TBS

Tissue blocking buffer 10% normal goat serum, 0.3% TritonX-100, 0.2%
2 sodium azide in TBS

Table 2.1.3: Reagents used in this study.

Reagent	Catalogue number	Manufacturer
DirectPCR lysis reagent (mouse tail)	102-T	Viagen Biotech
Normal goat serum	S-1000	Vector labs
OCT mounting media	361603E	VWR
Proteinase K, recombinant, PCR grade	03115844001	Roche
SIGMAFAST™ 3,3'diaminobenzidine tablets	D4168-5SET	Sigma-Aldrich
Vectamount™ mounting medium	H-5000	Vector Laboratories
Vectashield® mounting medium	H-1000	Vector Laboratories

Table 2.1.4.1 *Gsto2* smFISH probes library.

PROBE #	PROBE (5'→ 3')	PROBE POSITION *	PERCENT GC
1	ctcacatcctactgggaatc	26	50.00%
2	aatacagcagataaaccgccg	109	50.00%
3	gacaggctggatggttaagac	164	55.00%
4	aggctcacaggcaactact	211	50.00%
5	cactttccactgcagcagat	233	50.00%
6	aacagtcgacagcactcttg	276	50.00%
7	ctccagacccaaactgaatt	300	45.00%
8	taccggaaagctcacagtcg	354	55.00%
9	cggacatcaatctccagtta	383	45.00%
10	tttcccaagcatcttgacaa	408	40.00%
11	catgctgtagattcggatca	463	45.00%
12	tgtgcgagtaggggcagaac	485	60.00%
13	ctttggccttgagaaccagg	512	55.00%
14	ttgatcactcatgcctgat	534	40.00%
15	aggcttgcttttcaggtaa	556	40.00%
16	ggatgctttgtatagtacca	579	40.00%
17	ctccaagacaggaatttggc	604	50.00%
18	gattcatagaccagctgaca	633	45.00%
19	ccaggctactcacaagcaatg	656	50.00%
20	tacggaaacagctttcttcc	690	45.00%
21	accttacagaatagctccaa	744	40.00%
22	gacattccttgcttaaaggc	767	45.00%
23	cagatccgtacagtctcttc	805	50.00%
24	aatctcttccatgttgacaca	847	40.00%
25	aggtagtgttctgatattca	869	35.00%
26	gatatacagctccgccgaa	891	50.00%
27	gccagacgaggtaataatc	914	50.00%
28	tatacgtccaggcgctcaaa	939	50.00%
29	ggtgtgattcacgcagtcag	967	55.00%
30	ttatcagtgatgcagagcaca	1035	45.00%
31	ttcaagaagcccaggaagac	1059	50.00%
32	agcccaaaatcaaaggcaca	1101	45.00%
33	gcaatgcctggattatcgta	1135	45.00%
34	atcagaatgaccgagagtc	1158	50.00%
35	caagatgacccgtgatgcag	1180	55.00%
36	catatccatatttattagg	1233	35.00%

Table 2.1.4.2 *Gcc1* smFISH probes library.

PROBE #	PROBE (5'-> 3')	PROBE POSITION *	PERCENT GC
1	aattcatcccaaacttctcc	3	40.00%
2	ccaagagatccttcttactt	36	40.00%
3	cttttctgtgtctcgatgg	59	45.00%
4	cttgcttggtactggagaag	82	55.00%
5	tttataggcacgcaccacat	110	45.00%
6	tcgtgggataccgacagtac	172	55.00%
7	tgtcctcactgtgagtggag	261	55.00%
8	tatccaagctagtggcagtt	288	45.00%
9	gacacttgaagactggctg	311	50.00%
10	tcatcttcaaccccaaattc	337	40.00%
11	ttcagactttgggggaagtg	374	50.00%
12	tactaacgccactctctgac	405	50.00%
13	tctttgtccatctcactac	452	40.00%
14	caactgagtcttcagctgat	476	45.00%
15	ccaaggagctagtcaaagtg	498	50.00%
16	gacttttctgagtgactgt	520	45.00%
17	caggtaagacgcctccatac	542	55.00%
18	ctgcttcactcttcttctat	566	35.00%
19	ctttttattggcatcctcta	590	35.00%
20	atcgtgctgtgtgtaatga	686	45.00%
21	atcaaggcatggtcactctg	718	50.00%
22	tgcaagagcttctgaagctc	745	50.00%
23	gacctgatcagcgcatatg	836	55.00%
24	tgcttggtctgcagttcaaa	862	45.00%
25	aatgggttgagctcacctt	908	45.00%
26	tctggtcggttcttctcatc	931	50.00%
27	aatggcttttaagacgggca	981	45.00%
28	catttcttgctgcaattggg	1007	45.00%
29	tgctcttctacctgagactg	1063	50.00%
30	caagggaggaccggctagag	1236	65.00%
31	agactggattcgtcgccatg	1261	55.00%
32	ttgtccttcaagacattgat	1285	35.00%
33	tagcttctgagcttctcca	1307	45.00%
34	cttttctagctgcaacctg	1330	50.00%
35	ggagttggccattatctcag	1385	50.00%
36	aacttgggctcgcacttat	1484	45.00%
37	tttgggttcttgcttttga	1508	35.00%
38	cgcagggagatatacttgc	1585	50.00%
39	acagaacgtagttgctccag	1807	50.00%
40	ctggcaaccagaggacaag	1830	60.00%
41	caagctatctgaggaggctg	1907	55.00%
42	tttctgcttctcagagatg	2012	45.00%

43	ccctcatattcttttcgatg	2124	40.00%
44	ttgagatattccagggtggc	2161	45.00%

Table 2.1.5: Kits used in this study.

Kit	Catalogue number	Manufacturer
RNase-free DNase set	79254	Qiagen
Vectastain[®] ABC kit (standard)	PK-4000	Vector Laboratories

Table 2.1.8: Consumables used in this study.

Consumable	Catalogue number	Manufacturer
Combitips advanced[®], 0.1 mL, Eppendorf Biopur[®]	0030089618	Eppendorf
Combitips advanced[®], 0.2 mL, Eppendorf Biopur[®]	0030089626	Eppendorf
13 mm cover glass	631-0149	vWR
Falcon[®] 60 mm polystyrene tissue culture dish	353004	Becton Dickinson
plates, 12-well, flat bottom		Dickinson
Falcon[®] polystyrene tissue culture multiple well plates, 24-well, flat bottom	353047	Becton Dickinson
1.5 mL microcentrifuge tubes	S1615-5599	Starlab
0.2 mL 8-strip PCR tubes	I1402-2908	Starlab
Costar[®] 5 mL Stripette[®] serological pipettes	4487	Corning
Costar[®] 10 mL Stripette[®] serological pipettes	4488	Corning
50 mL pipettes	FB55486	Fisher Scientific

Corning® 15 mL polypropylene centrifuge tubes, conical bottom	430790	Corning
Corning® 50 mL polypropylene centrifuge tubes, conical bottom	430829	Corning
Purple nitrile gloves, medium size	90627	Kimberly-Clark Professional
RNaseZap® solution	AM9780	Ambion
SigmaClean® water bath treatment	S5525-4OZ	Sigma-Aldrich
1,000 µL tips	F167104	Gilson
200 µL tips	F167103	Gilson
10 µL tips	F167101	Gilson
150 mm unplugged glass Pasteur pipettes	FB50251	Fisher Scientific

Table 2.2.1 PCR recipe of BAC-*Penk*-Cre and *Trkb* floxed lines

BAC-*Penk*-Cre PCR

Primers:

LM40 5' - AGG CAC CCG GGG AGG TTG TT - 3'

LM41 5' - CCA CGA CCG GCA AAC GGA CA - 3'

LM42 5' - CTC CTG CCA CGC ACC TTC CG - 3'

Reaction contained:

1 µL gDNA

2.5 µL 10x buffer

2.5 µL MgCl₂ (25 mM stock)

0.625 µL dNTPs (10 mM stock, 2.5 mM for each dNTP)

0.5 µL LM40 primer (10 µM stock)

0.5 µL LM41 primer (10 µM stock)

0.5 µL LM42 primer (10 µM stock)

0.05 µL Taq polymerase (Sigma-Aldrich)

14.325 µL nuclease-free H₂O

Total reaction volume: 25 µL

Cycling conditions: Denaturation for 2 minutes at 94°C

35 cycles of 15 seconds at 94°C

15 seconds at 64°C

1 minute at 72°C

Final extension for 5 minutes at 72°C

Product size:

Wild type 371 bp

Mutant 527 bp

***Trkb* floxed PCR**

Primers:

LM11 5' - TGA AGG ACG CCA GCG ACA ATG CAC G - 3'

LM12 5' - CCA AGG TGA TCA ACA GCC CAA GTC - 3'

Reaction contained:

1 µL gDNA

2.5 µL 10x buffer

2.5 µL MgCl₂ (25 mM stock)

0.625 µL dNTPs (10 mM stock, 2.5 mM for each dNTP)

0.5 µL LM11 primer (10 µM stock)

0.5 µL LM12 primer (10 µM stock)

0.1 µL Taq polymerase (Sigma-Aldrich)

17.275 µL nuclease-free H₂O

Total reaction volume: 25 µL

Cycling conditions: Denaturation for 2 minutes at 94°C

40 cycles of 15 seconds at 94°C

15 seconds at 68°C

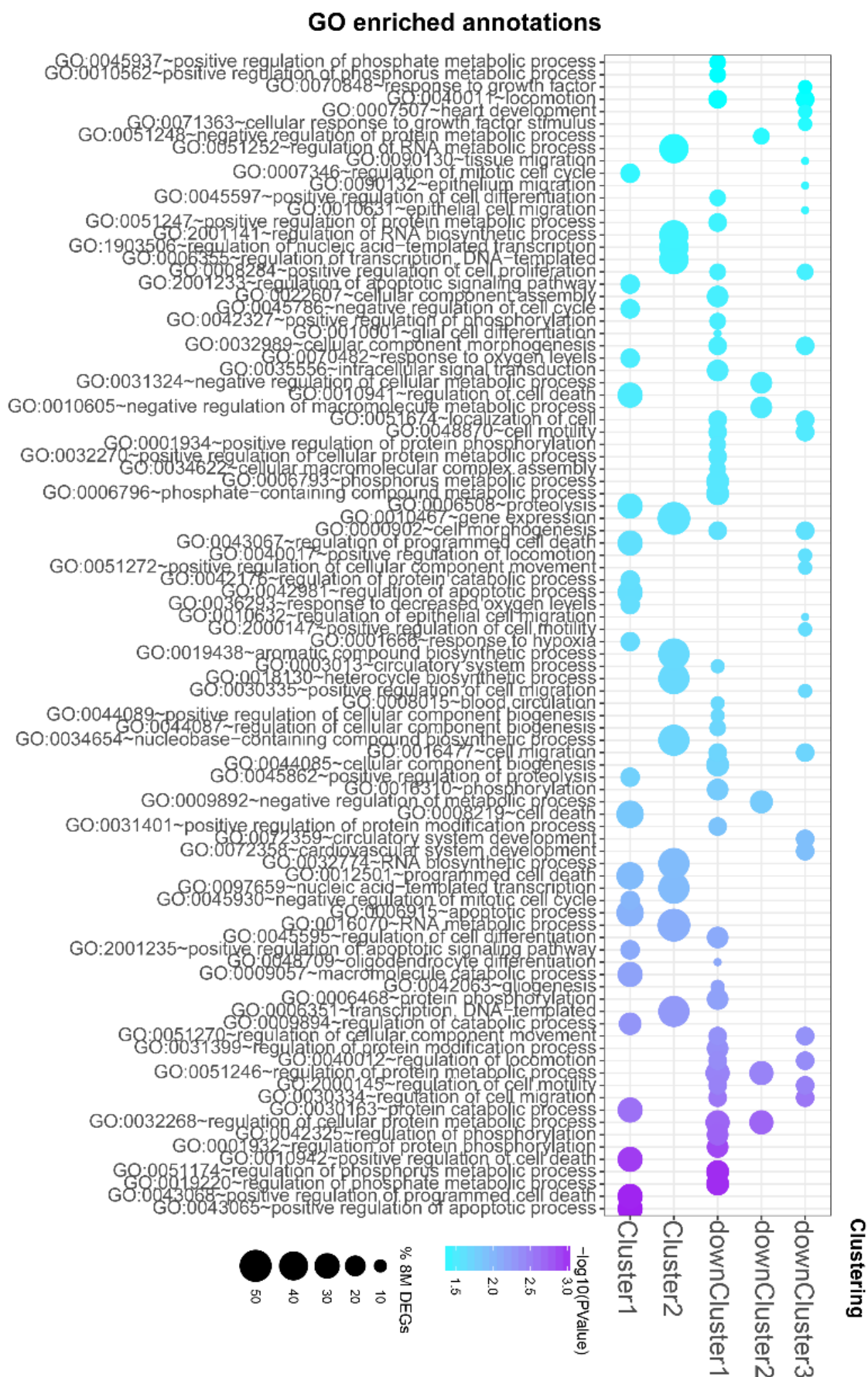
1 minute 30 seconds at 72°C

Final extension for 5 minutes at 72°C

Product size:

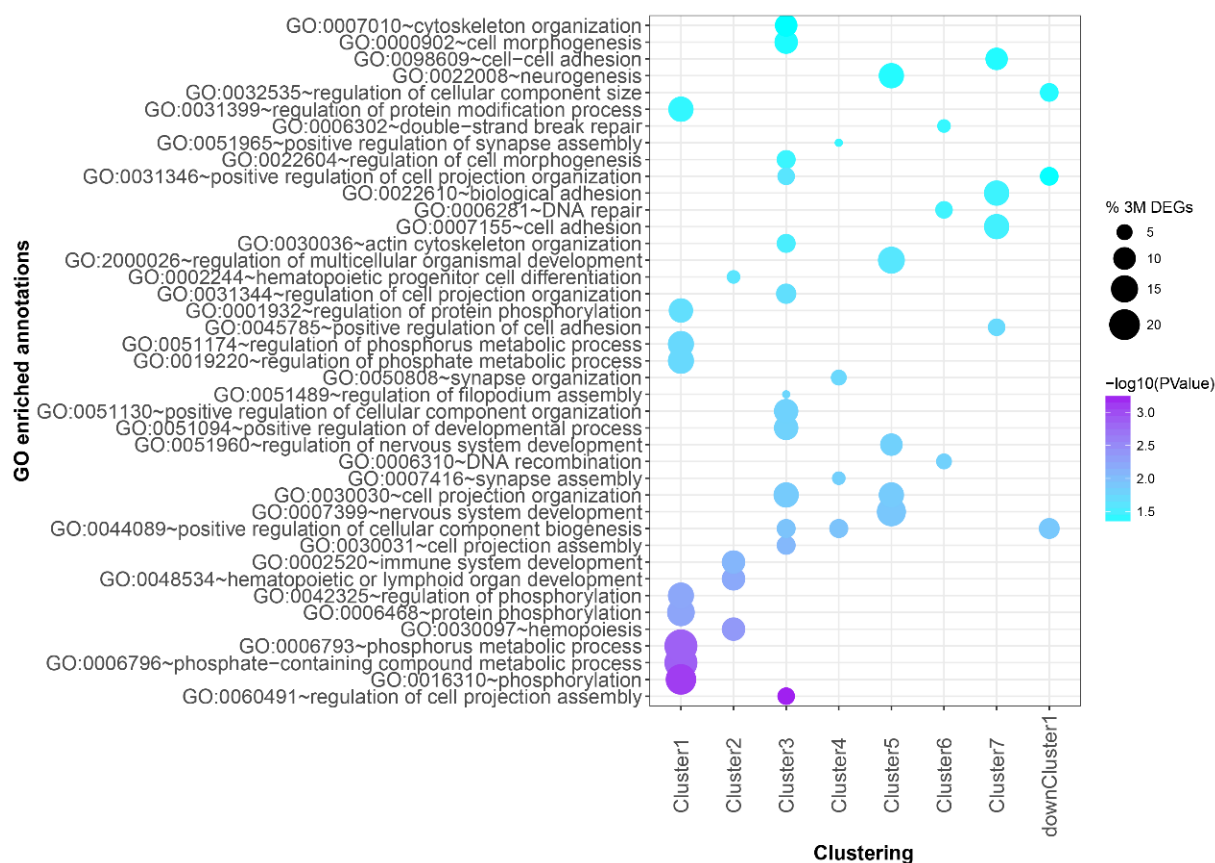
Wild type 857 bp

Mutant 913 bp



Supplementary Figure 3.1.3.1 GO biological processes of 8M DEGs.

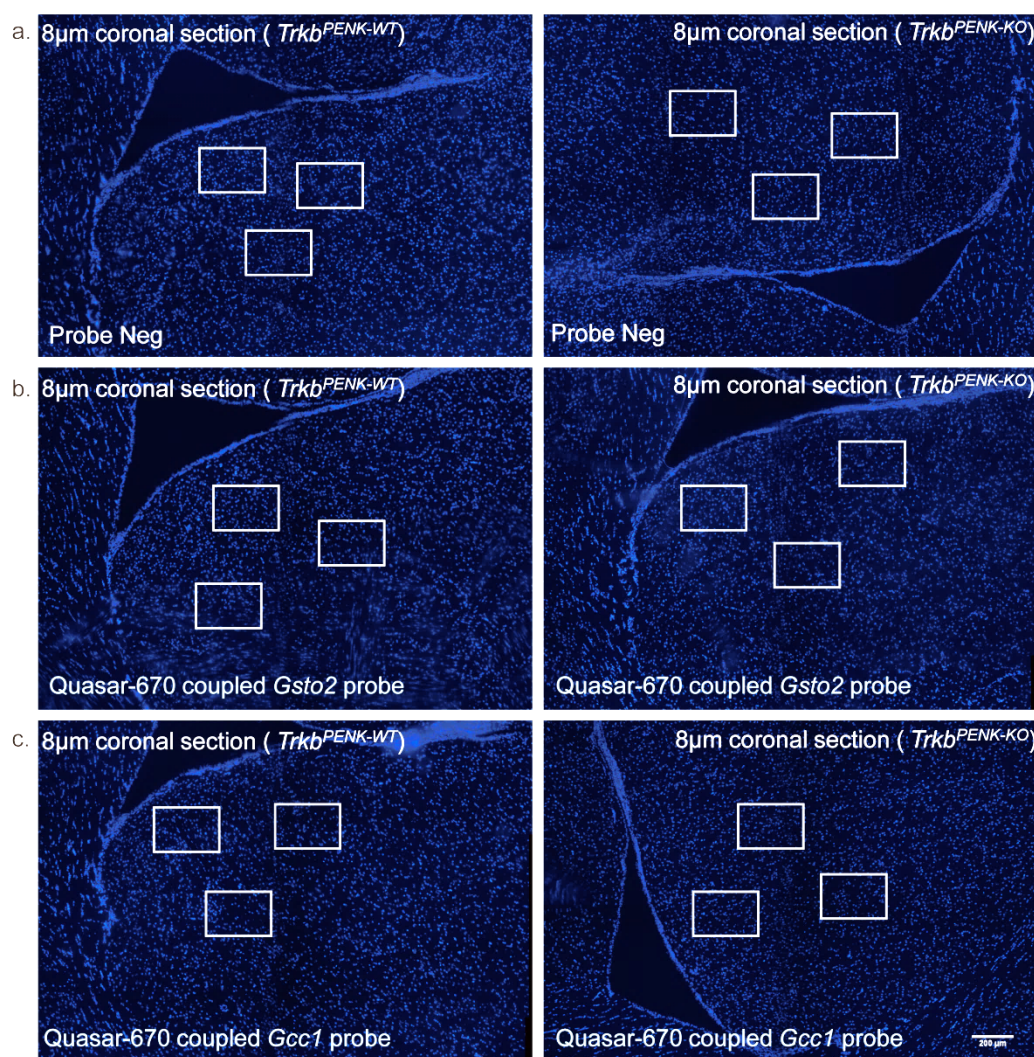
Gene ontology analysis of DEGs from 8M comparison DEGs set, hypergeometric clustering performed on enriched annotations (EASE score<0.05) of 8M up-regulated DEGs and down-regulated DEGs separately. Y axis of histogram shows percentage of enriched DEGs for each annotation. Significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue. Proportion of each cluster in total amount of enriched annotations is indicated as the size of bubble.



Supplementary Figure 3.1.3.2 GO biological processes of 3M DEGs.

Gene ontology analysis of DEGs from 3M comparison DEGs set, hypergeometric clustering performed on enriched annotations (EASE score<0.05) from 3M up-regulated DEGs and 3M down-regulated DEGs. The y axis of the histogram shows

percentage of enriched DEGs for each annotation; significance of annotation enrichment is displayed as a color gradient with highly significant enriched terms shown in purple and low ones shown in sky blue. Proportion of each cluster in total amount of enriched annotations is indicated as the size of bubble.



Supplementary Figure 3.2.1.2.1 Sections layout and design of *Gsto2* and *Gcc1* smFISH experiments.

a. Representative probe negative control sections group (Probe Neg) to control for the unspecific signals from the section treatments.

b. Quasar-670 coupled *Gsto2* probe treated striatal sections for both *Trkb*^{PENK-KO} and

Trkb^{PENK-WT}.

c. Quasar-670 coupled *Gcc1* probe treated striatal sections for both *Trkb*^{PENK-KO} and *Trkb*^{PENK-WT}.

All images above are at scales 200µm. Squares highlight the examples of 3 random fields imaged from the dorsal striatum for each section.

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