A single-cell transcriptomic analysis of the mouse hippocampus after voluntary exercise

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Abstract

Exercise has been recognized as a beneficial factor for cognitive health, particularly in relation to the hippocampus, a vital brain region responsible for learning and memory. Previous research has demonstrated that exercise-mediated improvement of learning and memory in humans and rodents correlates with increased adult neurogenesis and processes related to enhanced synaptic plasticity. Nevertheless, the underlying molecular mechanisms are not fully understood. With the aim to further elucidate these mechanisms we provide a comprehensive dataset of the mouse hippocampal transcriptome at the single-cell level after four weeks of voluntary wheel-running. Our analysis provides a number of interesting observations. For example, the results suggest that exercise affects adult neurogenesis by accelerating the maturation of a subpopulation of *Prdm16*-expressing neurons. Moreover, we uncover the existence of an intricate crosstalk among multiple vital signaling pathways such as NF-κB, Wnt/β-catenin, Notch, retinoic acid (RA) pathways altered upon exercise in a specific cluster of excitatory neurons within the Cornu Ammonis (CA) region of the hippocampus. In conclusion, our study provides an important resource dataset and sheds further light on the molecular changes induced by exercise in the hippocampus. These findings have implications for developing targeted interventions aimed at optimizing cognitive health and preventing age-related cognitive decline.

Introduction

The positive effects of exercise in maintaining both physical and cognitive health have been widely established during the past few decades. In addition to alleviating the risk of metabolic diseases (S. A. Phillips et al., 2015; Sylow & Richter, 2019; Zanuso et al., 2010), cardiovascular disease (Gielen et al., 2015; Morris, 1994; S. A. Phillips et al., 2015) and several types of cancers (Galvão & Newton, 2005; Hojman et al., 2018), the benefits of physical exercise on brain function have also been extensively studied and reviewed (Fischer, 2016; Hillman et al., 2008; Praag, 2009; Sutoo & Akiyama, 2003; Voss et al., 2011). Numerous studies in model organisms have shown that exercise promotes significant structural and functional changes in the hippocampus, which is a region of the brain that is essential for learning and memory processes. These changes indicate an overall positive impact of exercise on hippocampal function, including protective effects against aging and neurological disorders (Ashdown-Franks et al., 2020; Brown et al., 2013; Cass, 2017; Fischer et al., 2007; Mahalakshmi et al., 2020; Sujkowski et al., 2022; Swenson et al., 2020; Zhao et al., 2020), increased adult neurogenesis (Pereira et al., 2007; Praag, Christie, et al., 1999; Praag et al., 2005; Praag, Kempermann, et al., 1999; Seri et al., 2001), enhanced synaptic plasticity and improved cognitive performance (Cotman & Berchtold, 2002; Erickson & Kramer, 2009). Multiple studies have also demonstrated these beneficial effects in humans, indicating that aerobic exercise leads to an increase in the size of the human hippocampus, as well as improvements in memory performance (Cassilhas, 2012; Erickson, 2011; Maass, 2015; Voss, 2013).

While the molecular mechanisms underlying these effects are not fully understood, exercise was shown to increase the production of several neurotrophic factors (Fabel et al., 2003; Loprinzi & Frith, 2019; Neeper et al., 1995; Trejo et al., 2001) and other signaling molecules, such as neurotransmitters and cytokines (Lin & Kuo, 2013; Małkiewicz et al., 2019; Meeusen & Meirleir, 1995; Meeusen & Piacentini, 2001; Packer et al., 2010; Pedersen, 2000). Exercise was also found to induce changes in gene expression measured via quantitative polymerase chain reaction (qPCR), microarray or RNA sequencing. For example, exercise has been found to upregulate the expression of genes that are involved in cell proliferation and differentiation , as well as genes that are involved in enhancing neuronal and synaptic plasticity (Cotman & Berchtold, 2002; Mojtahedi et al., 2013; Tong et al., 2001). Many of these studies have focused on rodent models and have used running wheels as a behavioral paradigm to test the effects of

physical exercise (Pereira et al., 2007; Praag, Christie, et al., 1999; Praag et al., 2005; Praag,

Kempermann, et al., 1999; Rendeiro & Rhodes, 2018).

Recent studies have demonstrated that single nuclei RNA sequencing (snRNA-seq), which is a

powerful next generation sequencing (NGS) technique and has several advantages over

traditional RNA sequencing methods (such as enabling transcriptomic analysis of tissues that

are difficult to dissociate or have low cell yields), enables detection of previously unknown cell

types and transcriptional subtypes (Gouwens, 2020; Habib, 2017; Lake, 2018; Mathys, 2021;

Yao, 2021). Furthermore, it can be leveraged to better understand adult neurogenesis at the

single cell level in both mice and humans (Habib, 2017; Zhou et al., 2022). Although a number

of previous studies have looked into the transcriptomic and epigenetic changes induced by

exercise on the brain (Benito et al., 2018; Buckley et al., 2022; Goldberg et al., 2021;

McGreevy et al., 2019; Miguel et al., 2021; Pan-Vazquez et al., 2015; Shah et al., 2017;

Urdinguio et al., 2021; Y. Y. Wang et al., 2023), to our best knowledge, none have investigated

hippocampal cell-type specific transcriptomic changes following four weeks of voluntary

exercise in mice without employing additional behavioral tests. In this study, we used running

wheels as a form of voluntary exercise and aimed to further elucidate exercise-related

mechanisms in the hippocampus using snRNA-seq.

Our results indicate that exercise selectively affects the proportions of cells in specific clusters

of neuronal cell-types and suggest that the transcription factor *Prdm16* may play a key role in

orchestrating the maturation of newborn dentate gyrus neurons upon exercise. In addition, our

data hint to an important role crosstalk between NF-κB, Wnt/β-catenin, Notch and retinoic acid

(RA) signaling pathways in a specific excitatory neuron cluster belonging to the Cornu

Ammonis (CA) region, which could collectively regulate and enhance synaptic plasticity in

these neurons upon exercise. In conclusion, our findings provide an important novel dataset

and help to further elucidate the molecular processes associated with improved neuronal

plasticity in response to aerobic exercise.

Results

snRNA-seq analysis of the mouse hippocampus reveals changes in cell-type abundance upon exercise

To understand how physical exercise affects the hippocampus, we designed an experiment where 8 wild type (WT) mice were divided into 2 equally sized groups - runners that had free and voluntary access to running wheels in their cages (the 'exercise' group), and sedentary mice that had similar wheels in their cages that were however blocked (the 'control' group) **(Supplementary Table 1)**. This voluntary exercise paradigm lasted 4 weeks, after which we isolated whole hippocampal nuclei from all mice for snRNA-seq analysis **(Fig. 1A)**. A total of 22817 nuclei across the 8 samples were recovered after quality control, doublet removal and filtering (see methods). After accounting for batch effects, clustering was performed and nuclei were grouped into 21 unique clusters. Subsequently, these clusters were assigned to major hippocampal cell types including excitatory neurons (ExN), inhibitory neurons (InN) as well as microglia, astrocytes, oligodendrocytes and oligodendrocyte precursor cells based on the expression of cell-type specific marker genes **(Fig. 1B, C Supplementary Fig. 1 and Supplementary Table 2)**. Excitatory neurons made up the majority of cells, followed by oligodendrocytes and inhibitory neurons **(Fig. 1D)**. Additionally, we also identified excitatory neuron clusters that belonged to either the CA regions (ExN2-10 and ExN12-13) or the dentate gyrus (ExN1 and ExN11) within the hippocampus, using marker genes for these regions (Cembrowski et al., 2016) **(Fig. 1C, E and Supplementary Table 2)**. Additionally, distinct marker genes for each of these clusters were determined computationally (see methods) **(Supplementary Table 3)** and gene ontology (GO) analyses of the top markers specific to each of the neuronal clusters suggested different biological functions for these neurons within the hippocampus **(Supplementary Fig. 2 and Supplementary Tables 4-6)**.

Next, we applied permutation testing to analyze if exercise would lead to detectable differences in proportions of cells originating in each cluster. Out of all clusters, InN2, ExN10 and ExN11 showed significant changes in cell-type abundance (**Fig. 1F**). Using Augur (Squair et al., 2021), we also performed cell-type prioritization analysis to detect which cell types are most perturbed in response to exercise. To measure the perturbation levels, we employed the area under the receiver operating characteristic curve (AUC). An AUC value of 0.5 indicates that cells from the exercise condition in a cluster have no significant difference in perturbation compared to cells

from the control condition (random chance). Conversely, a value of 1.0 signifies that every cell

from the exercise condition exhibits higher perturbation compared to the control condition.

Augur performs subsampling in a way that changes in relative abundances of cell-types across

conditions do not confound the analysis. Hence, if certain cell-types are found to be strongly

perturbed due to exercise, it could imply that differences in cell-type proportions appear due to

these perturbations and not the other way around. We observed that clusters InN2 and ExN11

were the only two clusters with an AUC value > 0.6, indicating changes in cell-type proportions

within these clusters upon exercising (**Fig. 1G**). More specifically, upon exercise fewer cells

were detected for cluster InN2 while more cells were detected within cluster ExN11. In

particular, the increased proportion of cells within cluster ExN11 may reflect increased adult

neurogenesis, which has been repeatedly described upon exercise (Pereira et al., 2007;

Praag, Christie, et al., 1999; Praag et al., 2005; Praag, Kempermann, et al., 1999; Seri et al.,

2001). Thus, while the changes observed for cluster ExN11 may indicate the increased number

of excitatory neurons upon neurogenesis, the changes seen within cluster InN2 are more

difficult to explain. Therefore, we decided to first analyze cluster InN2 in greater detail.

Exercise induces changes in an inhibitory neuron population expressing *Prdm16*

We identified four distinct inhibitory neuron clusters in the dataset based on the overall

expression of marker genes Gad1 and Gad2. Each cluster was furthermore characterized by

high specific expression of the following genes: Sox6 (InN1), Prdm16 (InN2), Cnr1 (InN3), and

Egfr (InN4) **(Supplementary Fig. 3)**. As indicated in Fig. 1E, we observed a selective reduction

in abundance (log2 fold difference = -2.69) of inhibitory neurons in cluster InN2 in the exercise

condition, as compared to the controls (**Fig. 2A**). Further functional analysis of the top marker

genes for InN2 cells distinguishing them from the other inhibitory clusters (i.e. differentially

expressed (upregulated) in InN2 as compared to InN1, InN3 and InN4) broadly implicated

genes involved in transmembrane transport of ions as well as dendritic spine development

(**Fig. 2B and Supplementary Table 7**). Among the top 20 of these markers, a few were more

distinctly expressed in InN2 as compared to the rest. These included *Prdm16*, *Ano1*, *Ano2*,

Zfhx3, Zic1, *Zic4*, *Zfp521* and *Ankfn1* (**Supplementary Fig. 4A**). *Prdm16* is a transcription

factor that belongs to the PRDM family of transcriptional regulators. It has been widely

characterized as an important cell-fate switch regulator in brown adipose tissue (Kajimura et

al., 2010; Seale et al., 2008, 2011), but recent studies have also identified its role in neural

stem cell homeostasis (including maintenance of the neural stem cell pool), as well as
development, differentiation and positional specification of neurons (Aguilo et al., 2011;
Chuikov et al., 2010; Leszczyński et al., 2020; Shimada et al., 2017; L. Su et al., 2020; Turrero
García et al., 2020). *Ano1* and *Ano2* are calcium-activated chloride channels (CaCCs) of the
anoctamin protein family. CaCCs play crucial roles in regulating the excitability of smooth
muscle cells and also some types of neurons (Hawn et al., 2021). Specifically, *Ano1* has been
shown to contribute to the process maturation of radial glial cells during cortex development
(Hong et al., 2019), while *Ano2* modulates action potential waveforms in hippocampal neurons,
along with the integration of excitatory synaptic potentials (Huang et al., 2012). Moreover, *Ano2*
has been implicated in calcium-dependent regulation of synaptic weight in GABAergic inhibition
in the cerebellum, hence regulating ionic plasticity (Zhang et al., 2015). *Zfhx3* is a large
transcription factor with 23 zinc fingers and 4 homeodomains (Morinaga et al., 1991), which
has been shown to promote neuronal differentiation during neurogenesis in development and
primary cultures (Jung et al., 2005; Miura et al., 1995). *Zic1* and *Zic4* are also transcription
factors containing zinc finger domains, which are involved in nervous system development
(Gaston-Massuet et al., 2005; Inoue et al., 2007), specifically by regulating neuron
differentiation and maintaining neural precursor cells in an undifferentiated state (Inoue et al.,
2007). *Zfp521* encodes another zinc-finger protein that regulates many genes involved in
neural differentiation. Studies have shown that *Zfp521* is essential and sufficient for driving the
intrinsic neural differentiation of mouse embryonic stem cells (Kamiya et al., 2011), and is also
sufficient for converting adult mouse brain-derived astrocytes into induced neural stem cells
(Zarei-Kheirabadi et al., 2019). Little is known about *Ankfn1* but it has been genetically linked to
cannabis dependence (Agrawal et al., 2011).

In order to further understand what makes the InN2 inhibitory neurons distinct, we used the
SCENIC workflow (Aibar et al., 2017) to identify regulons (sets of transcription factors and their
putative targets) that were specifically active in the InN2 cluster (**Fig. 2C and Supplementary
Table 8**). A functional enrichment analysis of the collective list of genes comprising the top 5
InN2-specific regulons (*Prdm16*, *Rfx2*, *Gtf2ird1*, *Dlx1* and *Zfp941*) indicated significant
enrichment of gene ontology (GO) terms for regulation of synaptic signaling and
transmembrane ion transport, membrane depolarization and response to acetylcholine, and
interestingly, regulation of learning and memory (**Fig. 2D and Supplementary Table 9**).
Among these top 5 regulons, the transcription factor *Prdm16* and its 17 target genes comprised
the most specific active regulon in this cluster. This was substantiated by the highly specific

expression in InN2 of both *Prdm16* and the combined expression of the 17 regulon genes (**Fig.**
2E). The observed reduction of exercise cells in InN2 was subsequently indicated in the stark
upregulation of Prdm16 in the control cells (**Fig. 2F**). 5 candidates from the *Prdm16* regulon
(*Prdm16*, *Ankfn1*, *Meis2*, *Myo5b*, *Zfhx3*, *Zfp521*) are also shared with the top 20 InN2 marker
genes (differentially expressed in InN2 as compared to InN1, InN3 and InN4) (**Supplementary**
Fig. 4), most of which have been implicated in regulating neuronal differentiation (Agoston et
al., 2014; Jung et al., 2005; Kamiya et al., 2011; Y. Liu et al., 2013; Miura et al., 1995; L. Su et
al., 2020; Turrero García et al., 2020). Additionally, functional analysis specifically on the
Prdm16 regulon genes suggests enrichment of GO terms such as rhythmic process (*Ankfn1*,
Hs3st2, *Rorb*, *Zfhx3*) and regulation of bone morphogenetic protein (BMP) signaling (*Prdm16*,
Gpc3, *Zfp423*), which is known to modulate neuronal maturation and adult neurogenesis (Bond
et al., 2012) (**Fig. 2G, 2H and Supplementary Table 10**).

Among the other top regulons, the gene candidates within the *Dlx1* regulon indicated that *Dlx1*
itself positively regulates *Prdm16*. *Dlx1* is a TF that, along with its related gene *Dlx2*, is involved
in regulating neuronal migration (of newborn neurons, away from the proliferative zone) as well
as the functional longevity of GABAergic interneurons in the hippocampus (Cobos et al., 2005).
Moreover, a recent study indicated that the expression of the *Meis2* gene driven by *Dlx1/2*
promoted the fate determination of striatal neurons in mice (Z. Su et al., 2022). Since *Meis2* is
regulated by *Prdm16* (as seen in the *Prdm16* regulon), and it is also one of the top markers of
the InN2 cluster which is comprised mostly of control cells, these findings collectively suggest
that exercise leads to repression of *Prdm16* in specific hippocampal neurons (which are
characterized by high *Prdm16* expression). Although we identified these neurons initially as
cluster InN2, it is possible that these cells represent a specific developmental stage during
adult neurogenesis that is only captured in control mice in our dataset, since exercise is known
to increase proliferation but also maturation of adult newborn neurons (Ambrogini et al., 2013;
Lattanzi et al., 2022; Wu et al., 2008; Yang et al., 2015). In conclusion, these data may suggest
that decreased expression of *Prdm16* could be an important regulatory step during adult
neurogenesis that is affected by exercise.

Exercise affects a subpopulation of excitatory neurons linked to enhanced adult
neurogenesis

Following the cell-type abundance and perturbation analyses (Fig. 1E and F), we also sought
to take a deeper look into the ExN11 cluster belonging to the dentate gyrus. These cells
showed an increase in abundance (log2 fold difference = 0.79) in the exercise condition, as
compared to the controls. This increase can also be seen in a small fraction of cells from
ExN11 that seem to integrate into the mature granule cell cluster ExN1 of the dentate gyrus (as
indicated in Fig. 3D by expression of GC markers *Prox1* (Cembrowski et al., 2016; Erwin et al.,
2020; Kempermann et al., 2015) and *Calb1* (Kempermann et al., 2004), which shows an
overall low but relatively higher expression in ExN1), with the number of these integrating cells
increasing more than two-fold after exercise (**Fig. 3A**).

In order to understand the underlying gene expression patterns of ExN11 cells, we looked at
the top computationally detected marker genes for this cluster. Most of these markers were not
specific to ExN11, and in fact were also highly expressed in either ExN1 neurons, or
oligodendrocyte lineage cells (ODC and OPC clusters). This becomes more evident after
plotting the combined expression of the top 50 genes differentially expressed between ExN11
and ExN1 (**Supplementary Fig. 5A and Supplementary Table 11**), and the top 50 genes
differentially expressed between ExN11 and the oligodendrocyte lineage clusters (ODC and
OPC) (**Supplementary Fig. 5B and Supplementary Table 12**). Functional enrichment
analysis of these two lists of differentially expressed markers indicates enrichment of GO terms
related to ensheathment of neurons, regulation of synaptic plasticity, neurogenesis and
neuronal migration (**Fig. 3B and Supplementary Table 13**), suggesting that increased
neurogenesis upon exercise results in more excitatory neurons in ExN11.

Next, we generated a partition-based graph abstraction (PAGA) graph with velocity directed
edges constructed from RNA velocity measurements (Bergen et al., 2020; Wolf et al., 2019),
after subsetting the dataset to analyze our clusters of interest: InN2, ExN11 and ExN1 (**Fig.
3C**). The graph indicates a potential connectivity between these three clusters, and a transition
from ExN11 to ExN1, which supports our findings so far suggesting that ExN11 represents
newborn excitatory neurons that eventually integrate into the dentate gyrus. Moreover, we see
that the proportion of cells coming from ExN11 and InN2 combined, taken among the total cells
from the three clusters of interest (ExN1, ExN11 and InN2), decreases in exercise, while the
proportion of mature granule cells (ExN1) increases in the exercise condition as compared to
controls (**Fig. 3D**). This suggests that exercise leads to faster maturation of developing or
immature neurons (InN2 and ExN11) to mature granule cells (ExN1).

We also looked at the expression of markers corresponding to different stages of neurogenesis
in this cluster, in order to assign these cells to a specific stage of adult neurogenesis (Bohlen
Und Halbach, 2007) (**Fig. 3E**). However, we observed either no expression (*Neurod*, *Tbr2*,
Tubb3) or very low expression (*Gfap*, *Nestin*, *Pax6*) of many canonical markers, possibly due
to dropout effects in the sequencing data. As compared to other neurons, ExN11 cells show
higher expression of *GLAST* (*Slc1a3*), which is a marker for radial glia cells, but at the same
time we also observed low expression of Dcx and TUC-4 (*Dpysl3*) and high expression of
Prox1 which could suggest that these are transiently amplifying (or transit amplifying)
progenitor cells (Potten & Loeffler, 1990; Torii et al., 1999). This latter cell-type is known to
show transient expression of markers: initially expressing glial or stem cell markers like *Gfap*
and *Nestin*/*Sox2*, and later expressing granule cell markers such as *Prox1*. ExN11 cells also
express stathmin (*Stmn1*) at relatively higher levels. Stathmin is a microtubule destabilizing
protein that is considered to be an immature neuron marker, since it has been implicated in
controlling the transition from dividing neuronal precursors to postmitotic neurons in the
subgranular zone of the DG during adult neurogenesis (Boekhoorn et al., 2014). *Bdnf (brain-*
derived neurotrophic factor), which is known to be involved in exercise-mediated neurogenesis
in the hippocampus (Isackson et al., 1991; P. Z. Liu & Nusslock, 2018; Neeper et al., 1995;
Sleiman et al., 2016; Vaynman et al., 2004), showed very low expression throughout the
dataset, which made it difficult to reliably quantify any overall changes in its expression levels
in the exercise condition as compared to the sedentary controls. However, it did have relatively
higher expression levels in ExN11, although we found no significant differences between the
exercise and control conditions in this cluster.

We further analyzed the gene regulatory landscape for ExN11 using the SCENIC workflow to
identify regulons which are specifically active in this cluster, and found the *Meis1* regulon to be
the most specific out of the top 5 regulons (**Fig. 3F and Supplementary Table 14**). *Meis1* is a
transcription factor that has been shown to play a role in promoting neuronal differentiation and
possibly also neurogenesis (Barber et al., 2013; Mojsin & Stevanovic, 2009; Owa et al., 2018).
Other top transcription factors include *Cebpd (CCAAT enhancer binding protein delta)* which
has been implicated in hippocampal neurogenesis and regulation of learning and memory
(Banerjee et al., 2019; Powell et al., 2017), *Jun* which is an immediate early gene and
transcriptional regulator involved in cell proliferation (K. H. Schlingensiepen et al., 1994; K.-H.
Schlingensiepen et al., 1993; Weston & Davis, 2007), *Zfhx2* which is involved in neuronal
differentiation (Komine et al., 2012), and *Foxo1* which regulates the long-term maintenance of

adult neural stem cells, and belongs to the FoxO family that plays an important role in the
maintenance of autophagic flux and neuronal morphogenesis in adult neurogenesis (Ro et al.,
2013; Schäffner et al., 2018). A functional enrichment analysis of the collective list of genes
comprising these top 5 ExN11-specific regulons indicated significant enrichment of gene
ontology (GO) terms for gas transport (*Car2*, *Car14* - carbonic anhydrases that play a role in
neuronal excitability and signaling (Ruusuvuori & Kaila, 2014)), regulation of neural
development, neurogenesis, and also glial cell differentiation, which is in line with our previous
observation of glial markers in this cluster (**Fig. 3G and Supplementary Table 15**). We also
observed that some of the TFs from the top regulons of ExN11 and InN2 clusters shared
downstream target genes, and also regulated each other, such as *Rfx2* regulating *Jun* as well
as *Prdm16*. These common gene-regulatory relationships between our two clusters of interest
are highlighted in **Fig. 3H**. Building on our previous hypothesis, these results further suggest
that the observed loss of *Prdm16*-expressing neurons upon exercise might represent a
molecular snap shot of enhanced adult neurogenesis and especially the maturation of new
born neurons. In this scenario, ExN11 neurons could be a consequence of their differentiation
into and possible switch to an excitatory neuron subtype.

Differential gene expression and gene regulatory network analyses reveal broad
transcriptional changes linked to synaptic plasticity

To get a better understanding of the broad transcriptomic changes mediated by exercise, we
looked at differential gene expression between cells from the exercise and control conditions in
all other clusters apart from InN2 and ExN12 (complete list of differentially expressed genes is
given in **Supplementary Table 16**). Most clusters revealed either no significant changes in
gene expression or very few deregulated genes across the two conditions, with the exception
of ExN1, ExN2, ExN6, InN1 and ODC (**Fig. 4A** (top) and **4B** (top)). The top significant GO
(biological process) terms enriched for these deregulated genes indicate broad processes
involved in specific clusters upon exercise (**Fig. 4A** (bottom) and **4B** (bottom), **Supplementary**
Tables 17-18). In particular, ExN6 shows a distinctly large number of both up- and
downregulated genes, with the former being enriched for dendrite morphogenesis and synaptic
signaling pathways and the latter for postsynapse organization and synapse assembly
processes (**Supplementary Tables 19-20**). UpSet plots in Fig. 4A and 4B identified certain
genes that are commonly up- or downregulated upon exercise across multiple clusters.
Vps13a, for example, is upregulated in exercise in 8 out of the 21 clusters and also
downregulated in 2 excitatory neuron clusters, suggesting a role of chorein (the protein

encoded by *Vps13a*) which is a powerful regulator of cytoskeletal architecture and cell survival

in many cell types (Föller et al., 2012). Moreover, *Vps13a* mutations lead to chorea-

acanthocytosis, a rare disorder that also affects the brain and reduced chorein levels have

been linked to Alzheimer's disease (Lazarczyk et al., 2016). *Plxna4*, which has been shown to

regulate synaptic plasticity (Tan et al., 2019) and dendrite morphogenesis in the hippocampus

(Yamashita et al., 2016), is upregulated in ExN1 and ExN6. *Zbtb16*, upregulated in

oligodendrocytes and ExN7, is involved in neural progenitor cell proliferation and neuronal

differentiation during development (Usui et al., 2021). *Fkbp5* is a glucocorticoid receptor (GR)

binding protein, which negatively regulates GR, and is interestingly upregulated upon exercise

only in oligodendrocytes and microglia. It has been associated with mental disorders as well as

in regulating neuronal synaptic plasticity (Qiu et al., 2019). Among the downregulated genes,

Ddx5 is an RNA helicase which has been shown to regulate neurogenesis and neuronal

differentiation, likely by inhibiting reprogramming to pluripotency (H. Li et al., 2017; Suthapot et

al., 2022). *Tcf4*, downregulated in ExN1 and InN1, has been linked to neurodevelopment

(Forrest et al., 2014), regulation of dendritic spine morphology (Crux et al., 2018) and

regulation of neuronal migration through *Bmp7* (Chen et al., 2016). Altered *Tcf4* function has

also been linked to schizophrenia and mice in which *Tcf4* is overexpressed develop cognitive

impairment, which is attenuated by exercise (Badowska et al., 2020).

We further identified regulons that significantly differ in their activity across the exercise and

control cells in certain clusters (**Fig. 4C**). Functional enrichment analysis of these regulon

genes suggests that these TFs regulate a variety of biological processes in selected cells upon

exercise (**Supplementary Fig. 6A and Supplementary Table 21**). Since ExN6 clearly exhibits

a larger number of regulated genes as well as regulons with differential activity upon exercise,

we sought to characterize these neurons by looking at the specific transcription factors active in

this cluster, using the SCENIC workflow. The top 5 ExN6-specific regulons included *Hes1*, *Klf8*,

Nr2f2, *Fosl2* and *Sox5* (**Fig. 4D and Supplementary Table 22**), and the top 3 regulons (*Hes1*,

Klf8 and *Nr2f2* regulons) also showed significant upregulation upon exercise in the differential

regulon activity analysis as shown in Fig. 4C, further corroborated by increased overall

expression of these regulon genes in the ExN6 exercise cells (**Fig. 4E and Supplementary**

Fig. 6B). The functional enrichment analysis of the combined list of genes comprising the top 5

regulons suggested regulation of timing of neuron differentiation (mediated by *Hes1* and *Sox5*)

as the term with the highest enrichment (**Fig. 4F and Supplementary Table 23**). Additionally,

Hes1 is an important effector gene of the Notch signaling pathway, which has been linked to

learning and memory by regulating synaptic plasticity in mature neurons (Costa et al., 2003; Y.

Wang et al., 2004). Interestingly, *Hes1* was also induced in the early (0-1h) time window upon

acute exercise in skeletal muscle tissue (Amar et al., 2021), and another study showed that

inactivation of *Hes1* in excitatory neurons resulted in abnormal fear and anxiety behaviors

(Matsuzaki et al., 2019). *Klf8* is a TF playing critical roles in various biological processes such

as regulation of cell cycle progression. Elevated protein levels of *Klf8* were shown to induce the

activation of Wnt/β-catenin signaling pathway, by promoting the expression and stabilization of

β-catenin which further interacts with and inhibits NF-κB to regulate synaptic plasticity and

cognition (Yi et al., 2014). Moreover, Wnt/β-catenin signaling has been strongly implicated in

the regulation of synaptic assembly, neuroprotection, neurotransmission as well as synaptic

plasticity, while disruption or deregulation of Wnt signaling has been linked to several

neurodegenerative diseases such as AD (Oliva et al., 2013; Yi et al., 2014). *Nr2f2* (or COUP-

TFII) belongs to the COUP-TF transcription factor family which is involved in regulating

neuronal differentiation and migration of neural progenitors (Fuentealba et al., 2010), and was

additionally observed to be a member of the *Klf8* regulon from the ExN6 SCENIC analysis.

Interestingly, the regulon for retinoic acid (RA) receptor *Rarb* showed decreased activity in

exercise cells, along with the regulons for *Lhx6*, *Foxp2* and *Pbx3* (as seen in Fig. 4C). *Rarb* is a

direct transcriptional target of RA signaling, and this suggests its possible role in regulating

neuronal plasticity upon exercise, which could be linked to the already-established roles of RA

signaling in neuronal differentiation (Janesick et al., 2015).

These findings suggest a pattern of crosstalk between NF-κB, Wnt/β-catenin, Notch, RA and

other signaling pathways, which could collectively regulate and enhance synaptic plasticity

specifically in ExN6 neurons upon exercise. These relationships are visualized and highlighted

in the ExN6 gene regulatory network (**Fig. 4G**).

Discussion

In this study, we aimed to investigate the effects of physical exercise, in the form of voluntary wheel running, on the mouse hippocampal transcriptome at the single-cell level. Voluntary wheel running is an established approach in rodent models to test the effects of aerobic exercise on the brain (Pereira et al., 2007; Praag, Christie, et al., 1999; Praag et al., 2005; Praag, Kempermann, et al., 1999; Rendeiro & Rhodes, 2018). We decided to investigate the hippocampal transcriptomic changes at the 4 week time point after exercise, since this experimental duration has commonly been employed in similar studies and has been shown to be sufficient in enhancing neuronal plasticity and would moreover allow us to capture specific states of adult neurogenesis (Kodali et al., 2016; Miguel et al., 2021; Pan-Vazquez et al., 2015).

Through our analysis, we first observed that 4 weeks of exercise training leads to changes in the proportions of specific cell-types, most significant and pronounced in two clusters: a reduction in cells in the inhibitory cluster InN2 and an increase in cells in the excitatory cluster ExN11. Further investigation into the gene expression patterns in InN2 indicated that these cells, almost all originating from the control (sedentary) samples, showed high expression of the TF *Prdm16*. *Prdm16* has already been studied in the context of cell-fate switch regulation in brown adipose tissue, but interestingly, many recent studies have implicated its potential role in neural development and differentiation, particularly in stem cell homeostasis and maintenance and positional specification of neurons (Aguilo et al., 2011; Chuikov et al., 2010; Leszczyński et al., 2020; Shimada et al., 2017; L. Su et al., 2020; Turrero García et al., 2020). For example, one study showed that *Prdm16* in mouse medial ganglionic eminence (MGE) progenitors plays an essential role in controlling the timing of maturation of forebrain GABAergic interneurons, with *Prdm16* expression promoting the proliferation of interneurons and repressing maturation (Turrero García et al., 2020). Expression of other top gene markers for InN2 indicated genes such as CaCCs *Ano1* and *Ano2* regulating ion transport, neuronal excitability and plasticity, and zinc-finger protein encoding genes *Zfhx3*, *Zic1*, *Zic4* and *Zfp521* involved in neuronal and stem-cell differentiation. We further looked into the transcriptional regulation of InN2 by identifying highly active regulons in this cluster, with the results again implicating *Prdm16* and its target genes, which showed enrichment of GO terms involved in neuronal maturation and adult neurogenesis, such as rhythmic process (*Ankfn1*, *Hs3st2*, *Rorb*, *Zfhx3*) and regulation of BMP signaling (*Prdm16*, *Gpc3*, *Zfp423*). This was substantiated by the highly specific

expression of the *Prdm16* gene in InN2, suggesting that the observed loss of exercise cells in

InN2 is linked to the high expression of *Prdm16* in cells from the control samples. Moreover,

Dlx1, another highly active InN2 regulon that plays a role in regulating the functional longevity

of GABAergic interneurons in the hippocampus, regulates *Prdm16* and drives the expression of

Prdm16-regulated Meis2 gene. Interestingly, a recent study also indicated that high *Prdm16*

expression is essential in promoting the disappearance of radial glia and the ending of cortical

neurogenesis in the postnatal mouse brain (J. Li et al., 2023). Collectively, these findings,

supported by the existing literature, allowed us to hypothesize that InN2 cells characterized by

high *Prdm16* expression could represent a specific developmental stage during adult

neurogenesis captured only in cells from sedentary mice, possibly as a result of faster

maturation of these *Prdm16*-high neurons upon exercise. These data also suggest that snRNA-

seq analysis upon exercise could be a suitable approach to detect distinct molecular stages of

adult neurogenesis.

This view is further supported when we investigated the NeuN-positive cluster ExN11 that

showed an increase in the number of cells after exercise. Through RNA velocity measurements

and PAGA graph generation, we observed a potential cell-type transition (in the context of

trajectory inference) from the ExN11 cluster to the mature DG cluster ExN1, suggesting that

ExN11 represents newborn excitatory neurons that eventually integrate with the mature

granule cells in the DG. ExN11 also seemed to express both neuronal and glial gene markers,

which is consistent with studies that characterized radial glia-like precursor cells with astrocytic

as well as neural stem cell-like properties during neurogenesis (Kempermann et al., 2015;

Steiner et al., 2004). At the same time, ExN11 cells were *Dcx*-negative and had high

expression of *Prox1*, suggesting that these could be transit amplifying progenitor cells that

show transient expression of markers: from glial/stem cell markers to granule cell markers,

while high stathmin (*Stmn1*) expression in these cells could also implicate them as postmitotic

immature neurons (Boekhoorn et al., 2014). Upon exploring the gene regulatory landscape for

ExN11, we further identified specifically active regulons such as *Meis1*, *Cebpd*, *Jun*, *Zfhx2* and

Foxo1, all of whom are involved in neurogenesis, neuronal as well as glial differentiation,

morphogenesis and development. It was interesting to note some common gene-regulatory

relationships between ExN11 and InN2 clusters, such as *Rfx2* regulating *Jun* as well as

Prdm16.

On this basis, we suggest that ExN11 neurons indicate increased adult neurogenesis upon

exercise. Although the relevance of adult neurogenesis in humans is a topic that remains under

debate and is still associated with inconsistencies, with some studies claiming that there was
no evidence of neurogenesis past youth and in adulthood (Sorrells et al., 2018), there is also
much evidence supporting adult hippocampal neurogenesis in humans (Eriksson et al., 1998)
including studies implicating a decline of the latter with age (Boldrini et al., 2018; Spalding et
al., 2013; Vecchio et al., 2018). Moreover, research has indicated that adult-born hippocampal
neural stem cells can be induced to produce distinct neuronal populations, and these neurons,
despite losing their proliferative capacity and showing age-related inactivity, may be reactivated
through certain mechanistic stimuli such as running (Katsimpardi et al., 2014; Katsimpardi &
Lledo, 2018; Katsimpardi & Rubin, 2015; Lugert et al., 2010; Villeda et al., 2014).

Additionally, we propose that the observed loss of InN2 neurons and higher number of ExN11
neurons could potentially result from an inhibitory to excitatory switch of *Prdm16*-expressing
developing GABAergic neurons after exercising. It has already been shown that the polarity
switch of GABAergic responses from depolarization to hyperpolarization could be a
physiological hallmark of neuronal maturation and that this dual role of GABAergic transmission
is closely linked to neuronal development (Ben□Ari et al., 1989; Kim et al., 2012; Mueller et al.,
1984; Owens & Kriegstein, 2002; Pozzi et al., 2020). At the same time, these developmental
changes imply the existence of structural and functional changes in GABAergic transmission in
the adult brain as compared to neonatal development. Therefore, even though the inferences
from our results suggest a polarity switch in the opposite direction (inhibitory to excitatory), we
believe this provides new insights into the involvement of GABA in neuronal differentiation and
maturation in the context of exercise-induced neurogenesis and changes in plasticity in the
adult hippocampus.

In summary, these data may indicate that downregulation of *Prdm16* in maturing new born
hippocampal neurons could be an important regulatory step by which exercise enhances the
process of adult neurogenesis. It could therefore be interesting to test strategies that reduce
Prdm16 function in the hippocampus as a therapeutic approach to improve cognitive function.

Finally, we also looked at the broad gene expression patterns in the dataset that differed
between exercise and sedentary conditions in specific cell-types. Although most clusters only
showed very mild gene expression changes between the two conditions, ExN6 (belonging to
CA excitatory neurons) exhibited a markedly high number of regulated genes as well as
regulons with differential activity upon exercise, prompting us to explore it further. The most
differentially active as well as specific regulon in ExN6 was *Hes1*, an important effector gene of

the Notch signaling pathway, which itself regulates learning and memory by regulating synaptic
plasticity in mature neurons (Costa et al., 2003; Y. Wang et al., 2004). GO term analysis
indicated a role of *Hes1* in regulating the timing of neuron differentiation, mediated along with
another specific ExN6 regulon *Sox5*. Multiple studies have shown that oscillating *Hes1*
expression regulates the maintenance and proliferative capacity of neuronal progenitor cells,
acting as a repressor of the commitment of the latter to a neuronal fate, which in turn influences
the number of neurons produced, the timing of neurogenesis as well as overall brain
morphogenesis (Nakamura et al., 2000; Ochi et al., 2020; N. E. Phillips et al., 2016; Shimojo et
al., 2011; Sueda & Kageyama, 2020). Interestingly, *Hes1* was also induced in the early (0-1h)
window upon acute exercise in skeletal muscle tissue (Amar et al., 2021), further indicating the
possibility of an oscillating pattern of Notch signaling activation upon exercise. Our findings
suggest that *Hes1* upregulation (and subsequent Notch activation) in ExN6 at the 4-week
exercise time point could mark a late maturation state of adult neurogenesis in adjacent DG
neurons, potentially through lateral inhibition of neuronal fate commitment of newborn cells.
This is in line with our previous hypothesis that *Prdm16* upregulation in InN2 neurons at this
time could also be associated with an important maturation step in adult neurogenesis in the
DG, and that ExN11 neurons represent a maturing state of excitatory cells that are past the
neurogenesis phase. Analysis of other top gene markers and regulons of ExN6 (*Klf8*, *Nr2f2*,
Fosl2 and *Sox5*) suggested involvement of pathways regulating neuronal and synaptic
plasticity, such as Notch signaling, retinoic acid (RA) signaling and Wnt/β-catenin signaling,
which is further linked to inhibition of NF-κB to regulate synaptic plasticity and cognition.
Together, these findings point to a unique pattern of crosstalk between these various signaling
pathways, suggesting that they act collectively to regulate and enhance synaptic plasticity in
ExN6 neurons upon exercise..

This study of course comes with its own limitations, such as technical drop-outs in the snRNA-
seq dataset that limit gene expression analyses. We also look solely at a fixed snapshot of
changes occurring at the 4-week time-point after exercise, and it would be beneficial to
consider a longer range and/or multiple time-points for temporal changes that occur with these
experimental paradigms, especially when it comes to gene expression, which would give a
better insight into the dynamic processes we highlight with our study. Additionally, the different
neuronal clusters/subtypes and their interactions could be more deeply characterized through
functional studies in the future. Despite the fact that our findings are currently limited to
interpretations from bioinformatic analyses and a single snRNA-seq dataset, we believe that

they encourage further research focusing on functional interpretation and validation of these
results, and probe important questions in the field of aerobic exercise and its impact on brain
health.

In conclusion, our study provides a useful single-cell sequencing resource for exploring
exercise-induced transcriptomic changes in the adult mouse hippocampus. Some of the
observed changes allow for a deeper insight into the molecular processes that control adult
hippocampal neurogenesis. For example, we provide evidence that a cluster of neurons
characterized by high *Prdm16* expression may represent a specific state in the maturation of
adult born hippocampal neurons and our data suggest that a decrease of *Prdm16* levels is
critical to eventually promote the maturation of newborn neurons in the adult brain. We also
identified a cluster of excitatory CA neurons exhibiting pronounced gene expression changes
leading to regulation and enhancement of synaptic plasticity in the hippocampus upon
exercise. Since adult hippocampal neurogenesis is impaired during aging and
neurodegenerative diseases such as Alzheimer's diseases, targeting *Prdm16* could be a novel
approach to enhance plasticity in the diseased brain.

Methods

Animal cohort

The experiments were performed according to the ethical guidelines of the national animal protection law and were approved by the responsible ethical committee of the State of Lower Saxony, Germany. Male C57BL/6J mice were purchased from Janvier and housed individually in an animal facility with a 12-h light–dark cycle at constant temperature (23 °C) with *ad libitum* access to food and water. Animals were subjected to voluntary running at 3 months of age. Mice were allowed free access to the running wheel 24 h/day (Klinker et al., 2017). In the control groups, the running wheel was blocked.

Sample preparation, single nuclei isolation, library preparation and sequencing

For RNA sequencing analysis 4 runners and 4 control mice at the age of 3 months were assessed. Sample preparation, isolation of nuclei and library preparation for sequencing were performed according to previously published protocols (Kettwig et al., 2021). After 4 weeks in cages with or without running wheels, mice were deeply anesthetized with ketamine and medetomidine and transcardially perfused with phosphate-buffered saline (PBS, pH 7.4). After cervical dislocation, the whole brain was removed from the skull and the preparation of the hippocampus was performed in a petri dish filled with ice cold PBS buffer under a binocular. Tissue samples from two hemispheres of different mice from the same behavioral cohort were pooled and immediately frozen on liquid nitrogen and stored at –80 °C until further processing. Nuclei from frozen mouse brain tissues were isolated according to the previously published protocol with certain modifications (Sakib et al., 2021). Briefly, frozen tissues were Dounce homogenized in 500 µl EZ prep lysis buffer (Sigma NUC101) supplemented with 1:200 RNAse inhibitor (Promega, N2615) for 45 times in a 1.5 mL Eppendorf tube using micro pestles. The volume was increased with lysis buffer up to 2000 µl and incubated on ice for 5 min. Lysates were centrifuged for 5 min at 500 × g at 4 °C and supernatants were discarded. The pellet was resuspended into 2000 µl lysis buffer and incubated for 5 min on ice. After 5 min of centrifugation (500 × g at 4 °C), the resulting pellet was resuspended into 1500 µl nuclei suspension buffer (NSB, 0.5 % BSA, 1:100 Roche protease inhibitor, 1:200 RNAse inhibitor in 1×PBS) and centrifuged again for 5 min (500 × g at 4 °C). The pellet was finally resuspended into 500 µl NSB and stained with 7AAD (Invitrogen, Cat: 00-6993-

592 50). Single nuclei were sorted using BD FACS Aria III sorter. Sorted nuclei were counted in
593 Countess II FL Automated Counter. Approximately 4000 single nuclei per sample were used
594 for GEM generation, barcoding, and cDNA libraries according to 10X Chromium Next GEM
595 Single Cell 3☐ Reagent v3.1 protocol. Pooled libraries were sequenced in Illumina NextSeq
596 550 in order to achieve >50,000 reads/nuclei.

Preprocessing and exploratory analysis of snRNA sequencing data

The raw sequencing data generated from the snRNA-seq libraries were demultiplexed with
cellranger mkfastq using CellRanger software v.4.0.0 (10XGenomics). Reads were aligned to
the mm10 genome (NCBI:GCA_000001635.8) (GRCm38.p6) to obtain gene counts. The
cellranger count pipeline was used with default options to generate a gene-count matrix by
mapping reads to the pre-mRNA reference to account for unspliced nuclear transcripts.

All following downstream analyses were performed R (version 4.1.0). The feature-barcode
matrices generated for all 8 samples by CellRanger were converted to sample-wise Seurat
objects using the Run10X() and CreateSeuratObject() functions from the Seurat package
(version 4.0.2) (Hao, 2021). For each sample, the object was filtered to remove nuclei where
less than 200 genes and/or 500 reads and/or greater than 50000 reads were detected. Genes
that were expressed in fewer than 10 nuclei were filtered out. Moreover, any nuclei expressing
more than 0.1% mitochondrial gene counts ('percent.mt') were also removed. Normalization
was done using the SCTransform() function from the Seurat and sctransform (version 0.3.2)
packages (Hafemeister & Satija, 2019), with the arguments method = "glmGamPoi" and
vars.to.regress = "percent.mt". PCA embeddings were calculated using the RunPCA() function,
and RunHarmony() function from the harmony package (version 0.1.0) (Korsunsky et al., 2019)
was used to remove confounding effects due to sequencing batches. Leiden clustering (Traag
et al., 2019) was performed using the FindNeighbors() and FindClusters() functions in Seurat
with 50 principal components and a resolution parameter of 0.2. Uniform Manifold
Approximation and Projection (UMAP) (McInnes et al., 2018) was used for visualization of
clusters with the following arguments as parameters: umap.method = "umap-learn", metric =
"correlation", n.neighbors = 30, n.components = 2, min.dist = 0.3, spread = 0.5, reduction =
"harmony", dims = 1:50. For each sample object, the DoubletFinder package (version 2.0.3)
(McGinnis et al., 2019) was used to detect and remove any potential doublets. All individual
sample objects were then merged into one Seurat object, which was re-normalized, integrated
and re-clustered using the SCTransform, RunPCA(), RunHarmony(), FindNeighbors() and

FindClusters() functions, with the same parameter values as before. UMAP was again used for

visualization of clusters in the final merged object. Cell-type annotation of clusters was done

using the expression of selected marker genes (as listed in **Supplementary Table 1**). Three

small clusters (22, 23 and 24) identified after the initial clustering (**Supplementary Fig. 1A**)

were removed from the dataset before further analysis, since they did not show specific

expression of any cell-type markers and subsequently could not be assigned to any known cell-

type.

Cell-type abundance and prioritization analyses

The differences in the proportion of exercise and control cells in each cluster were calculated

using the scProportionTest R package (https://github.com/rpolicastro/scProportionTest)

(version 0.0.0.9), which calculates the p-value of the magnitude difference of abundance for

each cluster using a permutation test (no. of permutations = 1000), and generates a confidence

interval for the same using bootstrapping. Clusters with an observed fold difference magnitude

of at least 1.5 with an FDR threshold of 0.05 were considered to have significant changes in

cell-type proportions between exercise and control conditions.

To identify clusters (cell-types) in which the response to perturbation because of the given

stimulus, i.e. exercise, is prioritized, we used the Augur R package (version 1.0.3) (Skinnider et

al., 2021; Squair et al., 2021). This tool trains a machine-learning classifier specific to each

cluster to predict whether the cells originate from exercise or control samples, based on the

hypothesis that the separability between cells "responding" to exercise and the ones that don't

can be quantified by seeing how readily the experimental sample labels already associated

with each cell (exercise or control) might be predicted from their underlying molecular

measurements. The accuracy of the classifier is further subjected to cross-validation to

strengthen the predicted results.

Differential gene expression and regulon activity analyses

The top gene markers for each cluster, as well as genes differentially expressed between

exercise and control cells in each cluster, were identified using the FindMarkers() function in

Seurat with the following parameters: logfc.threshold = 0.1, test.use = "MAST", latent.vars =

c("batch","percent.mt"), assay = "RNA". We adopted the single-cell regulatory network

inference and clustering (SCENIC) workflow (Aibar et al., 2017; Sande et al., 2020) to construct

gene regulatory networks from our data and evaluate the activity of these gene regulatory

networks in cells from different cell-type clusters, using the SCENIC R package (version 1.3.1).

Briefly, this analysis was performed as follows: with the gene expression matrix as input, gene

sets that were co-expressed with potential regulators or transcription factors (TFs) were

identified using the GENIE3 algorithm (implemented using the GENIE3 R package (version

1.16.0) (Huynh-Thu et al., 2010)). Using the RcisTarget R package (version 1.14.0) (Aibar et

al., 2017), *cis*-regulatory motif analysis was performed to further exclude false positives and

indirect targets of TFs and get direct-binding targets. With this step, only those co-expression

modules that showed significant motif enrichment of a potential regulator (TF) were selected,

and these final modules of TFs and their putative downstream target genes were referred to as

'regulons'. Finally, the AUCell algorithm (Aibar et al., 2017) was used to quantify the activity of

these regulons in cells by assigning an enrichment or activity score to each regulon. This was

calculated as an 'area under the recovery curve' (AUC) across the ranking (based on

expression values) of all genes expressed in a cell. The top 5 regulons specifically active in a

selected cluster (compared to other clusters) were identified using the calcRSS() and

plotRSS_oneSet() functions in the AUCell R package (version 1.16.0). The differentially active

regulons across exercise and control cells within a cluster were determined using the

FindMarkers() function in Seurat by setting the argument assay = 'AUC', where the 'AUC'

assay slot in the Seurat object contained the regulon AUC score matrix generated by AUCell.

For functional annotation and enrichment analysis of selected differentially expressed genes or

differentially active regulons, an overrepresentation analysis was carried out using one of the

following methods: clusterProfiler R package (version 4.0.0) (Yu et al., 2012), WebGestalt R

package (version 0.4.4) (Liao et al., 2019) and the ShinyGO web application (Ge et al., 2020).

Gene regulatory relationship networks were constructed using the output of the SCENIC

workflow and the Cytoscape software (version 3.9.1) (Shannon et al., 2003).

RNA velocity analysis and PAGA velocity graph generation

To understand cellular dynamics at a deeper level, we used the scVelo Python toolkit (version

0.2.4) (Bergen et al., 2020) with default parameters on our dataset, which utilizes splicing

kinetics to calculate RNA velocity estimates for single cells. Using these velocity estimates, we

used the PAGA graph abstraction method (Wolf et al., 2019) within the scVelo workflow to infer

connectivities and directed transitions between selected cell-type clusters in our data. A PAGA

graph was then visualized by associating a node with each cluster of interest and connecting

these nodes based on the statistical measure of connectivity or transition between them.

Figure Legends

Fig. 1: Single-nucleus RNA sequencing analysis of the whole hippocampus in exercising vs. sedentary mice reveals changes in abundance of specific cell-types. A. Experimental design: The cohort of WT mice belonging to the exercising experimental group, or 'runners' ($n = 4$), had free access to running wheels in their cages for a duration of 4 weeks. Mice belonging to the control or 'sedentary' group ($n = 4$) were similarly housed but the running wheels were blocked. The 4 week-long voluntary exercise paradigm was followed by isolation of hippocampal nuclei for single-nucleus RNA sequencing. **B.** UMAP plots showing clusters of nuclei or 'cells', colored by experimental group **(left panel)** and cell-type **(right panel)**. *(ExN1-13: excitatory neurons; InN1-4: inhibitory neurons; ODC: oligodendrocytes; OPC: oligodendrocyte precursor cells; AST: astrocytes; MGL: microglia)* **C.** Violin plots showing average module score (expression) of marker genes specific to the different cell types/hippocampal regions, after cell-type specific annotation of clusters *(ExN: excitatory neurons; InN: inhibitory neurons; ODC: oligodendrocytes; OPC: oligodendrocyte precursor cells; AST: astrocytes; MGL: microglia; CA: Cornu Ammonis; DG: Dentate Gyrus).* **D.** Bar graph indicating the proportions of broad cell-types observed in the dataset *(ExN: excitatory neurons; InN: inhibitory neurons; ODC: oligodendrocytes; OPC: oligodendrocyte precursor cells; AST: astrocytes; MGL: microglia)* **E.** UMAP plot with cell clusters colored by average module score (expression) of marker genes specific to the dentate gyrus (DG), highlighting the two DG excitatory neuron clusters. **F.** Analysis of differences in cell-type proportions between cells from exercise and control samples, using permutation testing. The x-axis denotes the fold difference in cell-type proportions (log2 scale). Points marked in red indicate clusters with significantly different proportions of cells between the two groups. Horizontal lines around the points indicate the confidence interval for the magnitude of difference for a specific cluster, calculated via bootstrapping. **G.** UMAP plot with cells colored by Augur cell-type prioritization upon perturbation resulting from exercise, measured using area under the curve (AUC) scores.

Fig. 2: Selective loss of InN2 inhibitory neuron cluster upon exercise indicates a role of the transcription factor Prdm16. A. UMAP plot with cells colored by experimental group, showing the loss of InN2 inhibitory neurons upon exercise. **B.** Heatmap plots of functional annotation for specific genes among the top 50 markers of the InN2 cluster that had significantly enriched GO (biological process, molecular function and cellular component) terms. **C.** Cell-type specific regulons for InN2 cluster identified using the SCENIC workflow. The y-axis denotes the regulon specificity score (RSS) (with high RSS values indicating high cell-type/cluster specificity, and vice versa). The x-axis denotes the rank of each regulon within the selected cluster, based on the RSS. The top 5 ranked regulons for InN2 are labeled on the plot, with the number of genes comprising each regulon indicated within parentheses. *(Regulons ending with "extended" also include motifs linked to the transcription factor by lower*

confidence annotations). **D.** Dotplot showing significant GO biological process terms enriched among the
collective list of genes and transcription factors (TFs) making up the top 5 regulons, as indicated in the
RSS-Rank plot in (C). **E.** Violin plots depicting the normalized expression of Prdm16 **(top panel)**, and
the average module score (expression) for the genes included in the Prdm16 regulon **(bottom panel)** in
all clusters. **F.** Violin plot showing the expression of Prdm16 in InN2 cluster, split between exercise and
control cells. **G.** Network plot showing the 17 genes comprising the Prdm16 regulon. **H.** Dotplot showing
significant GO biological process terms enriched among the list of 17 Prdm16 regulon genes.

Fig. 3: Increased abundance of neurons in ExN11 cluster suggests increased neurogenesis upon
exercise. A. (left panel) UMAP plots highlighting the ExN11 cluster, split by the experimental conditions
(exercise and control). **B.** Dotplot showing significant GO biological process terms enriched among the
top 50 genes differentially expressed between ExN11 and ExN1 clusters, and among the top 50 genes
differentially expressed between the ExN11 cluster and the ODC and OPC clusters. **C.** Partition-based
graph abstraction (PAGA) graph for InN2, ExN11 and ExN1 clusters, with velocity directed edges
constructed from RNA velocity measurements. Edges denote either connectivities (dashed) or
transitions (solid/arrows). **D.** Box-plots showing a significant decrease in the proportion of developing
cells (ExN11/InN2) and an increase in the proportion of mature granule cells (ExN1) upon exercise.
Each dot indicates one sample (n=4/4 for exercise and control samples). **E**. Violin plots showing
normalized expression of selected marker genes for radial glia/immature neurons/exercise-mediated
neurogenesis. **F.** Cell-type specific regulons for ExN11 cluster identified using the SCENIC workflow.
The y-axis denotes the regulon specificity score (RSS) (with high RSS values indicating high cell-
type/cluster specificity, and vice versa). The x-axis denotes the rank of each regulon within the selected
cluster, based on the RSS. The top 5 ranked regulons for ExN11 are labeled on the plot, with the
number of genes comprising each regulon indicated within parentheses. **G.** Dotplot showing significant
GO biological process terms enriched among the collective list of genes and transcription factors (TFs)
making up the top 5 regulons, as indicated in the RSS-Rank plot above. **H.** Combined gene-regulatory
network plot with TFs specific to InN2 and ExN11 clusters, and their respective regulon genes (Larger
nodes represent genes/TFs that connect two or more regulon networks).

Fig. 4: Differentially expressed genes in excitatory neurons suggest regulation of synaptic
plasticity and neuron differentiation to promote increased neurogenesis upon exercise. A. (top
panel) and **B. (top panel)** UpSet plots depicting (A) commonly upregulated and (B) commonly
downregulated genes upon exercise, between different clusters. Each row corresponds to a cluster, and
bar charts on the right show the size of the set of genes up-/downregulated upon exercise in that cluster.
Each column corresponds to a possible intersection (commonly up-/downregulated gene(s)): the filled-in
cells show which cluster set is part of an intersection. Gene names are labeled for intersections with < 2
common genes among clusters. **A. (bottom panel)** and **B. (bottom panel)** Dotplots showing GO
biological process terms enriched among (A) genes significantly upregulated and (B) genes significantly

downregulated upon exercise, in clusters with significantly enriched terms. **C.** Heatmap depicting the
average regulon activity scores (RAS) for regulons with significant differential activity between cells from
exercise and control conditions, in specific clusters. The number of genes comprising each regulon is
indicated within parentheses. Regulons within red boxes indicate those upregulated upon exercise, and
the ones within blue boxes indicate the ones downregulated upon exercise. **D.** Cell-type specific
regulons for ExN6 cluster identified using the SCENIC workflow. The y-axis denotes the regulon
specificity score (RSS) (with high RSS values indicating high cell-type/cluster specificity, and vice versa).
The x-axis denotes the rank of each regulon within the selected cluster, based on the RSS. The top 5
ranked regulons for ExN6 are labeled on the plot, with the number of genes comprising each regulon
indicated within parentheses. **E.** Violin plots showing the average module score (expression) of the 3
regulons specific to ExN6 that also show upregulated activity upon exercise (*Hes1*, *Klf8*, *Nr2f2*). **F.**
Dotplot showing significant GO biological process terms enriched among the collective list of genes and
transcription factors (TFs) making up the top 5 regulons, as indicated in the RSS-Rank plot in (C). **G.**
Network plot with TFs and their respective regulon genes, specific to or showing differential activity in
ExN6 upon exercise, indicating gene regulatory interactions in ExN6 cluster (Genes/TFs that are
significantly deregulated upon exercise or are specifically overexpressed in ExN6 are represented bigger
in size, while selected genes belonging to these regulons which are deregulated in the same direction as
the regulon TF are colored in red or blue).
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Data availability

snRNA-seq data is available via GEO accession number GSE234278.

Conflict of interest

The authors declare no conflict of interest.

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Contributions

A.M., M.R.I. and A.F. conceptualized and planned the project and analysis. M.R.I. and D.L. organized and performed the behavioral experiments. M.R.I and M.S.S. prepared the samples for snRNA-seq and M.S.S. performed the nuclei isolation and sorting. L.K. and S.B. prepared the sequencing libraries and performed the snRNA sequencing. D.M.K. provided bioinformatics support and performed demultiplexing, alignment and count matrix-generation from the raw snRNA-seq data. A.M. analyzed the snRNA-seq data, visualized and interpreted results. A.M. and A.F wrote the manuscript, with input from other co-authors.

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