Original Article

MOLECULAR METABOLISM

Adult neural stem cell fate is determined by thyroid hormone activation of mitochondrial metabolism

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ABSTRACT

Objective: In the adult brain, neural stem cells (NSCs) located in the subventricular zone (SVZ) produce both neuronal and glial cells. Thyroid hormones (THs) regulate adult NSC differentiation towards a neuronal phenotype, but also have major roles in mitochondrial metabolism. As NSC metabolism relies mainly on glycolysis, whereas mature cells preferentially use oxidative phosphorylation, we studied how THs and mitochondrial metabolism interact on NSC fate determination.

Methods: We used a mitochondrial membrane potential marker *in vivo* to analyze mitochondrial activity in the different cell types in the SVZ of euthyroid and hypothyroid mice. Using primary adult NSC cultures, we analyzed ROS production, SIRT1 expression, and phosphorylation of DRP1 (a mitochondrial fission mediator) as a function of TH availability.

Results: We observed significantly higher mitochondrial activity in cells adopting a neuronal phenotype *in vivo* in euthyroid mice. However, prolonged hypothyroidism reduced not only neuroblast numbers but also their mitochondrial activity. *In vitro* studies showed that TH availability favored a neuronal phenotype and that blocking mitochondrial respiration abrogated TH-induced neuronal fate determination. DRP1 phosphorylation was preferentially activated in cells within the neuronal lineage and was stimulated by TH availability.

Conclusions: These results indicate that THs favor NSC fate choice towards a neuronal phenotype in the adult mouse SVZ through effects on mitochondrial metabolism.

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Keywords Adult neurogenesis; Mitochondrial metabolism; Thyroid hormone; Neural stem cell fate

1. INTRODUCTION

Neurogenesis persists in the brain of adult mammals in two well-defined niches, the hippocampus and the sub-ventricular zone (SVZ) [1,2]. In both areas, NSCs can differentiate to form neurons or glial cells (oligodendrocytes and astrocytes) [3]. NSCs produce transit amplifying progenitors that predominantly differentiate to neuroblasts which migrate to the olfactory bulb forming interneurons [4]. Progenitors can also acquire an oligodendroglial phenotype, becoming oligodendrocyte precursor cells (OPCs) that mature to oligodendrocytes (OLs) [5]. Diverse internal and external signals modulate NSC differentiation, with thyroid hormones (THs) having a crucial role in promoting neuroblast determination [6]. Understanding homeostatic controls of neuron/glial cell fate decisions is necessary to address how adult neurogenesis is modified in physiological and pathological conditions.

It is well established that NSCs principally rely on aerobic glycolysis before differentiation, contrasting with mature cells, in which metabolism is mainly based on mitochondrial oxidative phosphorylation (OXPHOS) [7,8]. Though generating less ATP, high glycolysis can provide proliferating cells with increased production of nucleotides and lipids [9]. Hypoxia also contributes to maintaining an undifferentiated state, influencing proliferation and cell-fate commitment [10,11]. In turn, changes in metabolic status influence NSCs pluripotency and differentiation [12,13]. According to energy requirements, mitochondria change shape, number and connections, through mitochondrial biogenesis, fission, and fusion. These processes modulate mitochondrial production, growth, interconnections, intracellular dissemination, and destruction of damaged mitochondria [14]. Factors enhancing OXPHOS will involve an increase in these processes. Amongst factors regulating mitochondrial dynamics, DRP1 induces mitochondrial fission. When phosphorylated, DRP1 interacts with FIS1

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Received July 31, 2017 • Revision received August 11, 2017 • Accepted August 14, 2017 • Available online 19 August 2017

http://dx.doi.org/10.1016/j.molmet.2017.08.003

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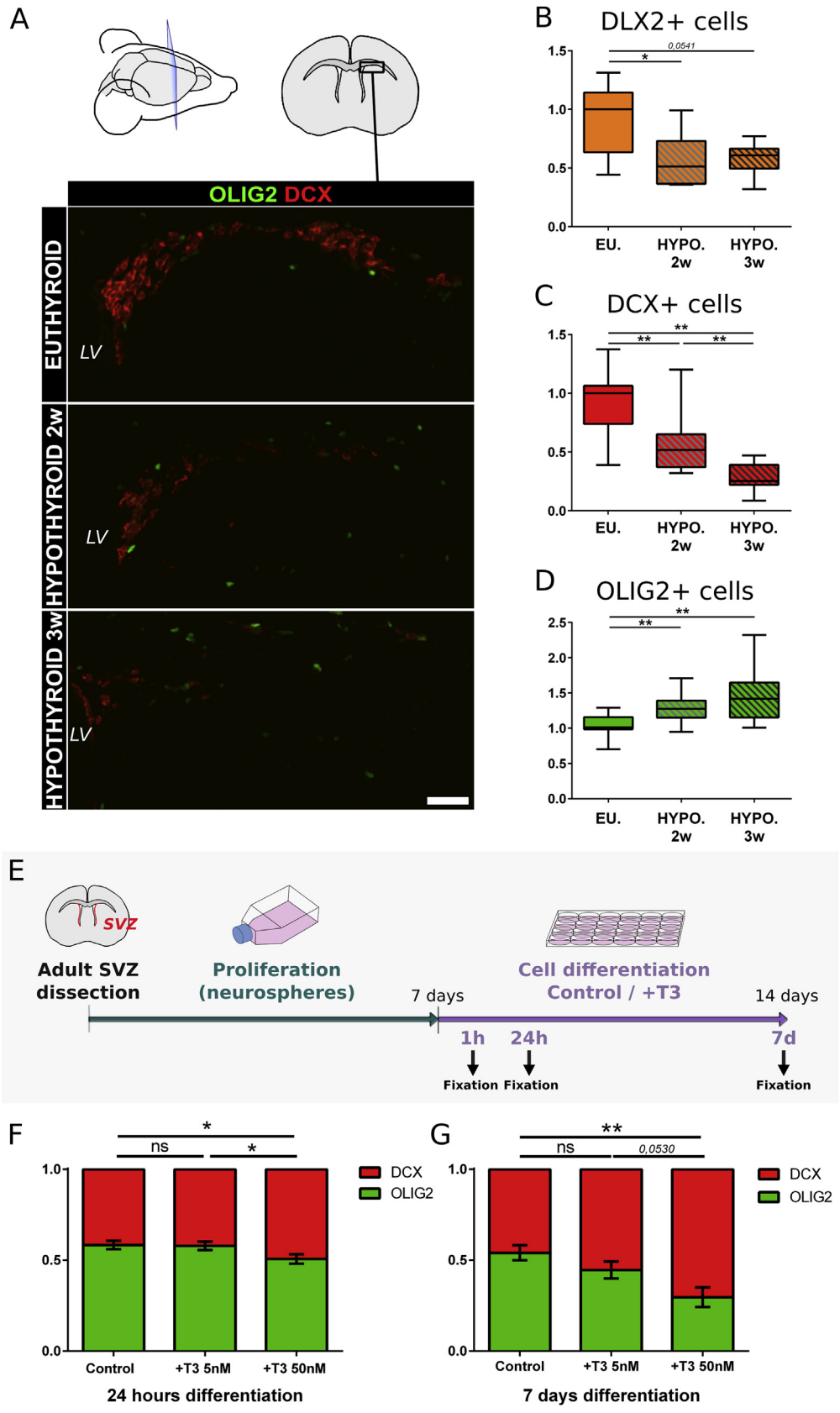

Figure 1: Thyroid hormones favor neuronal over glial fate decision *in vivo* and *in vitro*. (A) Coronal sections of dorsal SVZ in euthyroid versus hypothyroid adult mice (two and three weeks, see materials and methods) treated by IHC (OLIG2, in green and DCX, in red). Representative images. Scale bar: 50 μm. LV: Lateral ventricle. (B) Quantification of DLX2+ cell numbers in the adult SVZ of euthyroid and hypothyroid mice. n=4 euthyroid, hypothyroid 2 weeks and hypothyroid 3 weeks. (C–D) Quantification of DCX+ (integrative

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at the mitochondrial surface to induce division [15]. Triiodothyronine (T_3), the active form of THs, promotes DRP1 localization to mitochondria [16], where the protein activates fission. Finally, it is well-documented that THs are major regulators of cell and mitochondrial metabolism [17,18].

Given the twin roles of THs in NSC biology and mitochondrial metabolism, we hypothesized that TH determination of NSC fate could implicate modulation of the glycolysis to OXPHOS metabolism transition. To test this hypothesis, we studied NSC determination both *in vivo* and *in vitro* as a function of TH availability. We provide a number of novel results on the role of TH in activating mitochondria and inducing a neuronal, as opposed to a glial, cell fate. First, we show that TH influences mitochondrial activity and ROS production, being higher in cells adopting a neuronal fate. Second, we find that the mitochondrial fission-inducer DRP1 is activated preferentially in cells differentiating toward a neuroblast phenotype, a process influenced by TH availability. Finally, if mitochondrial activity is blocked by oligomycin, neuronal determination is dramatically reduced. In conclusion, the data show that TH signaling increases mitochondrial dynamics and activates mitochondrial respiration during NSC differentiation, thus directing adult NSC determination to a neuronal fate.

2. MATERIAL AND METHODS

2.1. Animals

C57BL/6 wild-type male mice, 8 weeks old, were purchased from Janvier (Le Genest St. Isle, France). Food and water were available ad libitum. All procedures were conducted according to the principles and procedures in Guidelines for Care and Use of Laboratory Animals and validated by local and national ethical committees.

2.2. *In vivo* studies

2.2.1. Hypothyroid treatments

To induce hypothyroidism, 8 week-old male mice were given iodine-deficient food containing 6-n-propyl-2-thiouracil (PTU) at 0.15% (Harlan Tekland, Madison, WI) for two to three weeks. This method of inducing hypothyroidism is recommended by the American Thyroid Association (ATA) in their rodent guide [19]. It is possible that inducing long-term hypothyroidism or hyperthyroidism affects food intake and body weight. We did not measure the amount of food eaten by hypothyroid mice, but we saw no obvious changes in body weight during the time span of treatment. Hypothyroidism was checked by measuring serum T_4 concentrations at sacrifice, using a T_4 ELISA test (Labor Diagnostika Nord (LDN), Nordhorn, Germany) (Figure S1A) or using RIA (carried out by Academic Medical Center, University of Amsterdam, Netherlands) (Figure S1B).

2.2.2. JC-1 *in vivo* staining

JC-1 dye (2 μL 1 μg/μL) was stereotaxically injected in the lateral ventricle (anterior: 3.65 mm, lateral: 1 mm and depth: 2.1 mm relative to lambda) of 2 month-old euthyroid and hypothyroid mice anesthetized with isoflurane. Mice were sacrificed 4 h after injection and brains fixed in 4% paraformaldehyde (PFA) in PBS (0.1 M, pH 7.4). JC-1 red and green fluorescence were quantified in the cytoplasm of EGFR+, NG2+ and DCX+ cells located in the dorso-lateral part of the SVZ after specific immunostainings. JC-1 red on green fluorescence values were standardized with the global SVZ red on green signal.

2.2.3. Immunohistochemistry (IHC) *in vivo*

Mice were anesthetized with Pentobarbital (130 mg/kg, Centravet) and perfused rapidly through the left heart ventricle with PBS, then with 4% PFA in PBS (0.1 M, pH 7.4). Brains were harvested and post-fixed at 4 °C overnight in the same fixative solution. Brains were cryoprotected in 30% sucrose in PBS at 4 °C, embedded in OCT, frozen, and stored at −80 °C until processed. Brain coronal sections (30 μm thick) were incubated for 1 h in a blocking solution of 10% normal donkey serum (Sigma) and 1% BSA (Sigma) in PBS at room temperature (RT), then incubated with primary antibody diluted in blocking solution at 4 °C overnight. After three 10 min washes in PBS at RT, sections were incubated with fluorescent secondary antibodies (1/500, Invitrogen) in 1% donkey serum and 1% BSA in PBS for 2 h RT. Sections were washed three times 10 min in PBS at RT, incubated with DAPI for 5 min at RT, and mounted onto SuperFrost glass slides (Fisher) Prolong Gold with antifade reagent (Invitrogen). Fluorescence images were acquired using a Leica TCS-SP5 confocal microscope. Images were processed using the FIJI software [20]. All *in vivo* quantifications were done on brain slices located between bregma +0.4 mm and +0.9 mm stereotaxic coordinates.

2.2.4. Characterization of DLX2 as specific marker of adult SVZ neuronal precursors

In the adult SVZ, well-defined markers of oligodendrocyte precursor cells (OPC) are SOX10, NG2, and OLIG2, the latter two being maintained in immature OL [4,21]. If localized in the SVZ, OLIG2+ and NG2+ cells can be considered OPCs as these markers are expressed prior to migration. A well-established marker for differentiating neuroblasts is DCX [22]. DLX2 over-expression acts as a pro-neurogenic factor in the adult SVZ [23], making it a good candidate for being specific to early neuronal commitment in the adult. To ascertain this point, we immuno-assayed adult SVZ for DLX2, OLIG2 and for SOX2, a pluripotency marker expressed in NSC and progenitor cells, the expression of which decreases during differentiation [6]. DLX2 and OLIG2 signals in the SVZ showed sharp mutual exclusion (Figure S2A–C). The only cells expressing both markers also displayed high SOX2 levels (Figure S2C), suggesting that the rare co-expression of OLIG2/DLX2 occurs prior to differentiation. Inversely, nearly all SVZ DCX+ neuroblasts express low levels of DLX2 (Figure S2D). Thus, DLX2 expression decreases with neuronal differentiation enabling DLX2 to be used as a marker of adult SVZ neuronal precursors.

2.3. *In vitro* studies

2.3.1. Primary adult NSC neurosphere culture

Five male mice were sacrificed per neurosphere culture. Brains were removed and lateral SVZ dissected under a binocular dissection microscope in DMEM-F12-glutamax 1/50 Glc 45% and incubated with papain for 30 min at 37 °C with pipette dissociation every 10 min to obtain a single-cell suspension. Cells were collected by centrifugation

density) and OLIG2+ cell numbers in the adult SVZ of euthyroid and hypothyroid mice. n = 12 euthyroid and hypothyroid 2 weeks, n = 7 hypothyroid 3 weeks. (E) Schematic timeline illustrating the experimental design for *neurosphere assay* derived from adult mouse SVZ. Dissected cells are grown with proliferating factors (bFGF and EGF) for 7 days to form neurospheres. After plating, dissociated cells from neurospheres differentiate in multi-well plates for 1 h to 7 days without proliferation factors, in control medium or with T_3 (5 and 50 nM). (F–G) Relative proportion of OLIG2+ and DCX+ cells after 24 h and 7 days of differentiation at each T_3 dose. Pool of three experiments. p-value: *<0.05, **<0.01, ns: non-significant. Kruskal–Wallis followed by Mann–Whitney tests.

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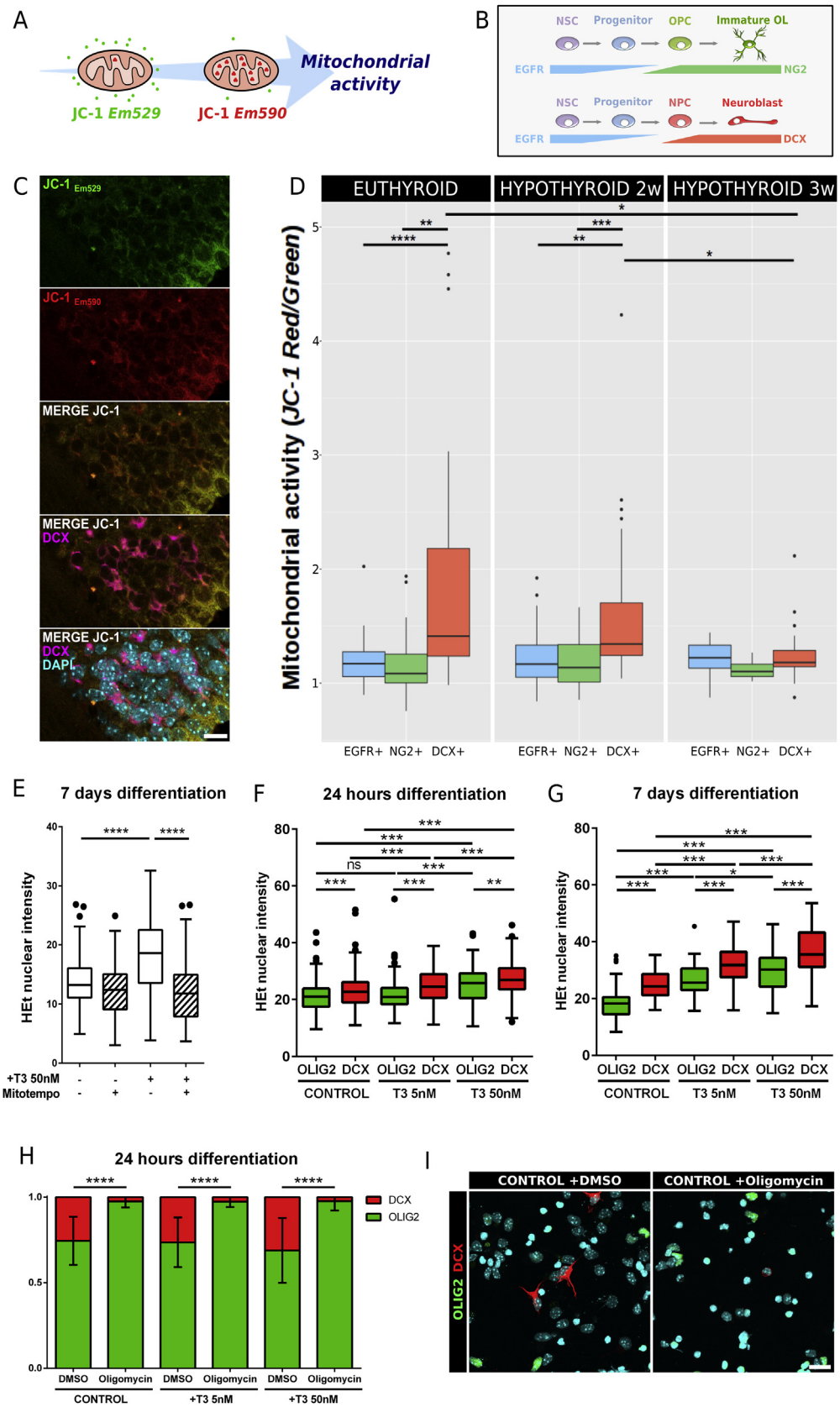

Figure 2: Mitochondrial activity and ROS production are higher in cells adopting a neuronal fate and are influenced by thyroid hormones. (A–D) Mitochondrial activity is higher in cells adopting a neuronal fate and is decreased by hypothyroidism specifically in neuroblasts *in vivo*. (A) Schematic representation of JC-1 dye mechanism of action. Excited at a wavelength of 488 nm, JC-1 monomers outside of mitochondria exhibit green fluorescence, whereas JC-1 aggregates inside mitochondria exhibit a red fluorescence

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(1000 rcf, 5 min). After resuspension, cells were cultured in complete culture medium (DMEM-F12 [Gibco], 40 μg/ml insulin [Sigma], 1/200 B-27 supplement [Gibco], 1/100 N-2 supplement [Gibco], 0.3% glucose, 5 nM Hepes, 100 U/ml penicillin/streptomycin) containing 20 ng/ml of EGF and 20 ng/ml of FGF2 (Peprotech), in a 5% CO_2 environment at 37 °C for 7 days to obtain primary neurospheres. To analyze cell differentiation, neurospheres were dissociated into single cells and 50 000 cells were plated per well on Poly-D-Lysine-coated glass coverslips in 24-well plates in complete culture medium without EGF/FGF2 for 7 days maximum.

To test the effect of T_3 on cell differentiation, T_3 was added to the differentiation medium (5 nM or 50 nM). Medium was renewed every two to three days during differentiation. Cells were then fixed with 4% paraformaldehyde for 10 min RT at different differentiation time points and used for immunocytochemistry (see below).

To inhibit mitochondrial superoxide production, MitoTEMPO (Sigma) 20 μM was added to culture medium during 7 days of cell differentiation. To measure cellular ROS production, cells were exposed to Hydroethidin (HEt) (ThermoFisher) 40 μM 0.4% DMSO in culture medium during 30 min before fixation. HEt transformation to ethidium bromide in presence of superoxide ions has been quantified in cells nuclei. To inhibit ATP synthase and OXPHOS, oligomycin (Sigma) 1 μM was added to culture medium during cell differentiation.

2.3.2. Immunostaining *in vitro*

Cells fixed on glass coverslips were blocked with 10% donkey serum and 1% BSA in PBS for 30 min at RT. Incubation with primary antibody was performed in blocking solution overnight at 4 °C or for 2 h at RT. After three 5 min washes in PBS at RT, cells were incubated with fluorescent secondary antibodies in 1% donkey serum and 1% BSA in PBS for 1 h at RT. Cells were washed three times in PBS, incubated with DAPI for 5 min at RT and mounted on SuperFrost glass slides with Prolong Gold antifade reagent. Fluorescence images were acquired using a Leica TCS-SP5 confocal microscope and processed using the FIJI software [20]. All *in vitro* quantifications were done on Max Intensity Z projection of 4 μm stack images. At least 10 40× images were quantified per slide per experiment.

2.4. Statistical analysis

Statistical analyses were carried out with R and GraphPad Prism. Statistical differences were analyzed with appropriate tests, indicated in figure legends, to compare control and treated groups.

2.5. Antibodies and reagents

A complete list of antibodies and reagents used is presented in supplemental experimental procedures.

3. RESULTS

3.1. Thyroid hormone availability differentially modulates mitochondrial activity and ROS production in neuronal and glial lineages

To understand the implication of mitochondrial metabolism in thyroid hormone (TH) control of NSC fate, we analyzed mitochondrial activity *in vivo* and *in vitro* in neuronal and glial lineages. A first step was to verify that THs favor neuronal over glial fate decision in a dose and time dependent manner *in vivo* and *in vitro*. This objective required an unambiguous marker of neuronal precursor, shown in Figure S2 (see details in Experimental procedures). We quantified cell populations in mice fed control or hypothyroid diet for two (2w) or three weeks (3w). Hypothyroidism time-dependently decreased both DLX2+ neuronal precursor cell (NPC) and DCX+ neuroblast numbers, whilst increasing OLIG2+ OPCs in the latero-dorsal SVZ (Figure 1A–D). Thus, low TH availability *in vivo* favors adult NSCs determination to an oligodendrocyte fate, at the expense of DLX2+ NPCs and DCX+ neuroblasts. To test the consequences of TH excess on NSC fate, we used an *in vitro* assay, in which adult SVZ-derived proliferating NSCs and progenitors form neurospheres in the presence of growth factors. After 7 d, neurospheres were dissociated and differentiation induced without growth factors (Figure 1E). We quantified DCX+ and OLIG2+ cells after 24 h and 7 d in differentiating conditions $\pm T_3$, (5 or 50 nM). After 24 h, T_3 (50 nM) decreased the proportion of OLIG2+ cells ($p < 0.05$). This decrease in OLIG2+ cells was highly significant ($p < 0.01$) at 7 d differentiation with T_3 (50 nM), accompanied by increased proportions of DCX+ neuroblasts (Figure 1F and G). Thus, *in vivo* and *in vitro* T_3 availability controls neuronal *vs* glial fate in the adult SVZ.

To quantify mitochondrial activity in cells committed towards a glial or a neuronal fate in the SVZ, we used JC-1. This dye enters mitochondria as a function of their membrane potential. In active mitochondria, the respiratory chain transfers protons into the inter-membrane space, increasing membrane potential and favoring JC-1 entry. JC-1 particles emit a green fluorescence in the cytoplasm, and aggregate to emit a red fluorescence inside mitochondria (Figure 2A). Following intra-cerebro-ventricular injection (ICV), we determined JC-1 ratios (red/green) as a function of cell type using cytoplasmic immuno-staining for proliferating cells (EGFR), for NPCs and neuroblasts (DCX) and for OPCs (NG2) (Figure 2B and C). Figure 2C shows a representative example of JC-1 red/green signals in DCX+ SVZ cells in the niche. To determine how TH availability affected JC-1 ratios in different cell types, we compared euthyroid to hypothyroid mice (2 or 3 weeks) (Figure 2D). In euthyroid mice, quantifying JC-1 ratio showed that mitochondrial activity was significantly higher in DCX+ neuroblasts than in EGFR+ proliferating NSCs and progenitors ($p < 0.001$) or in NG2+ OPCs

that is proportional to mitochondrial membrane potential. (B) Membrane and cytoplasmic markers were used to quantify red and green JC-1 signals in the SVZ in respectively, neural stem cells and progenitors (EGFR), neuronal precursor cells and neuroblasts (DCX), oligodendrocyte precursor cells and immature oligodendrocytes (NG2). (C) Representative image of JC-1 red and green fluorescence after ICV injections of the dye into the lateral ventricle of a euthyroid mice. Coronal sections were treated for immunohistochemistry using antibodies against DCX (purple). Scale bar: 10 μm (D) JC-1 red on green fluorescence values for EGFR+, NG2+ and DCX+ cells located in the latero-dorsal part of the SVZ of euthyroid and hypothyroid (2 or 3 wks) adult mice. EUTHYROID: n = 16, HYPOTHYROID 2 wks: n = 14, HYPOTHYROID 3 wks: n = 9. p-Value: *<0.05, **<0.01, ***<0.001, ****<0.0001. Kruskal–Wallis test followed by a Dunn's multiple comparison post-hoc test. (E–I) ROS production is increased by T3 *in vitro* and is higher in DCX+ cells. (E) HEt nuclear intensity after 7 d differentiation of adult NSC in different conditions of T_3 availability and in the presence or absence of MitoTEMPO (20 μM). Administration of MitoTEMPO counteracts T3-induced increase in ROS production. (F) HEt nuclear intensity after 24 h of adult NSC differentiation in control medium or with T3 (5 or 50 nM) in DCX+ and OLIG2+ cells. (G) HEt nuclear intensity after 7 d of adult NSC differentiation in control medium or with addition of T3 (5 or 50 nM) in DCX+ and OLIG2+ cells. (H) Relative proportion of OLIG2+ and DCX+ cells after 24 h differentiation for each dose of T3, in presence or absence of oligomycin (1 μM). (I) Representative immunocytochemistry on cells differentiated for 24 h in control conditions (DMSO) or oligomycin (1 μM) (OLIG2: green, and DCX: red). p-Value: *<0.05, **<0.01, ***<0.001, ****<0.0001. ns: non-significant; Kruskal–Wallis followed by Mann–Whitney tests.

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($p < 0.01$) (Figure 2D, left panel). The significantly higher level of JC-1 ratios in DCX+ neuroblasts *versus* EGFR+ NSC/progenitors or NG2+ OPCs was lost in hypothyroid mice (3w, Figure 2D right panel), due to a strong decrease in mitochondrial activity in the DCX+ cells that was more marked as a function of duration of hypothyroidism. Three weeks hypothyroidism significantly reduced JC-1 ratios in DCX+ cells compared to 2 w ($p > 0.5$) (Figure 2D).

Increased mitochondrial activity drives reactive oxygen species (ROS) production. We used hydroethyrine (HEt) to measure ROS production within different cell types derived from adult NSCs *in vitro*. In the presence of the superoxide anion (O_2^-), the main species of ROS, HEt, is oxidized to form ethidium bromide (EB), which intercalates with DNA, staining nuclei red [24]. To confirm that HEt signal relates to mitochondrial activity, we quantified nuclear HEt in cells differentiated in the presence of MitoTEMPO, a mitochondria specific ROS-scavenger, $\pm T_3$ (50 nM). ROS scavenging by MitoTEMPO did not significantly reduce HEt nuclear intensity in control cells but significantly abrogated the T_3-dependent increase in ROS production (Figure 2E). Thus, T_3-dependent mitochondrial activity increases ROS production in neurospheres that have been dissociated and allowed to differentiate. We used HEt to quantify ROS production in OLIG2+ and DCX+ cells after 24 h and 7 d *in vitro* differentiation. DCX+ neuroblasts produced more ROS than OLIG2+ OPC and immature OL at both differentiation times in control conditions (Figure 2F and G), confirming results obtained with JC-1 *in vivo* (Figure 2D). Adding T_3 to the differentiation medium increased ROS production in both lineages, at each time point, but DCX+ cells always displayed significantly higher ROS production (Figure 2F and G). Additionally, neuroblasts were more T_3 sensitive: DCX+ cells responding significantly to 5 nM T_3 at 24 h whereas OLIG2+ OPCs did not (Figure 2F). To investigate whether blocking mitochondrial activity affected cell fate choice, we used oligomycin [25]. Figure 2H and I shows that oligomycin significantly increased the proportion of OLIG2+ cells at the expense of DCX+ neuroblasts in all conditions. Thus, 50 nM T_3 is not sufficient to counteract the effect of blocking mitochondrial activity with oligomycin.

Overall, these results show T_3 availability to induce mitochondrial activity and ROS production, steps required for adult NSC determination to a neuroblast phenotype. In contrast, OLIG2+ cells are less sensitive to T_3, only responding to high concentrations (24 h, 50 nm T_3). Even after 7 d OLIG2+ cells still displayed lower mitochondrial activity than DCX+ neuroblasts.

3.2. The mitochondrial activity regulator SIRT1 is expressed in a fate-specific manner

We next analyzed interactions between TH and another regulator of cell metabolism and mitochondrial biogenesis, the protein-deacetylase SIRT1. SIRT1 maintains the undifferentiated state of adult NSCs/progenitors and its expression decreases as a function of neuronal and oligodendrocyte differentiation [26,27]. As SIRT1 is a coactivator of TRβ and Trα [28] it could influence TH effects on metabolic changes during NSC fate decisions.

SIRT1 was expressed in a large subset of SOX2+ cells *in vivo*, but virtually excluded from SOX10+ OPCs (Figure 3A, upper panels). In contrast, we found a positive correlation between SIRT1 and DLX2 and SIRT1 expression in most, but not all, SVZ DCX+ neuroblasts (Figure 3A lower panels). We then tested how TH availability modulated SIRT1+ cell numbers in the dorsal SVZ. Hypothyroidism (3w) significantly decreased SIRT1+ numbers (Figure 3B). To investigate this further, we carried out *in vitro* experiments analyzing SIRT1 expression as a function of lineage. Figure 3C shows that after 1 h, 24 h or 7 d differentiation, SIRT1 was expressed in most high SOX2-expressing OLIG2+ OPCs (proliferating OPCs), but was largely excluded from OLIG2+SOX2− cells maturing toward an oligodendrocyte phenotype. Notably, DCX+ neuroblasts predominantly expressed SIRT1, while less than 10% OLIG2+SOX2− cells expressed it (Figure 3D). All DLX2+ NPCs expressed SIRT1 at 1 h, 24 h and 7 d differentiation with or without T_3 (Figure S3A and B). These results confirm the *in vivo* observations of preferential maintenance of SIRT1 in cells undergoing determination toward a neuronal rather than an oligodendrocyte phenotype. Interestingly, TH availability did not appear to affect SIRT1 expression within each lineage (Figure 3E).

3.3. T_3 preferentially activates the fission-inducing factor DRP1 in cells acquiring a neuronal fate

We next tested whether T_3 availability modulates mitochondrial fission through DRP1 activation (phosphorylation) during neurogenesis and if this process occurs in a fate-specific manner during the glycolysis to OXPHOS metabolic switch. We first analyzed which cell types express the Ser616 phosphorylated form of DRP1 ($pDRP1^{S616}$), usually associated with fission. $pDRP1^{S616}$ was mainly found in a sub-population of $SOX2_{low}$ committed progenitors and in neuronal lineage cells (DLX2+ and DCX+), but not in SOX10+ or OLIG2+ OPCs (Figure 4A). Further, there was an overall decrease in the $pDRP1^{S616}$ signal in hypothyroid mice (Figure 4B), revealing a global reduction of $pDRP^{S616}$ cells, consistent with decreased neuroblast numbers (Figure 1B and C).

CDK1 mediates Ser616 activating DRP1 phosphorylation [29,30] while CDK5-mediated Ser616 phosphorylation of DRP1 inhibits mitochondrial fission during neuronal maturation [31]. To assess the activating or inhibiting nature of $pDRP1^{S616}$ in the adult SVZ cells *in vivo*, we analyzed CDK5 and CDK1 expression. While CDK1 was found in DCX+ neuroblasts, CDK5 was not, confirming the fission-activating nature of $pDRP1^{S616}$ in differentiating neuroblasts (Figure S4).

To characterize more precisely phosphorylation-dependent DRP1 activation in each cell lineage, we used *in vitro* studies to quantify total DRP1 and $pDRP1^{S616}$ in differentiating cells. After 24 h, $pDRP1^{S616}$ was low in SOX2+ progenitors and increased significantly as a function of differentiation in both lineages (Figure 4C). Although significant, the increase was only 20% during differentiation from SOX2+/OLIG2-to SOX10+, and from to OLIG2+ OPCs and immature OLs. In contrast, during SOX2+ cell determination to DLX2+ NPCs and DCX+ neuroblasts it was >300% (Figure 4C–E and Figure S5A–C). Quantifying total DRP1 in different cell types showed that DRP1 protein levels were opposite to changes in $pDRP1^{S616}$ signal, underlining that increased DRP1 phosphorylation correlates with differentiation (Figure 4F and Figure S5D–F). After 7 d differentiation, $pDRP1^{S616}$ levels were reduced in DCX+ neuroblasts, showing no difference with those in OLIG2+ cells (Figure 4H). Thus, DRP1 activation peaks during neuronal determination, and decreases with maturation.

T_3 effects on $DRP1^{S616}$ in DCX+ neuronal versus OLIG2+ glial cells at 24 h and 7 d differentiation were assessed next. At 24 h differentiation, T_3 (5 nM) significantly increased $pDRP1^{S616}$ in DCX+ cells, but not in OLIG2+ cells (Figure 4G). After 7 d differentiation, the only increase in $pDRP1^{S616}$ intensity was seen in the neuronal lineage, again in response to T_3 (5 nM) (Figure 4H). Differences in $pDRP1^{S616}$ signal were not accounted for by increased DRP1 expression as there were no major differences in DRP1 levels in OLIG2+ and DCX+ cells. Similarly, smaller responses to T_3 (5 nM) were limited to neuroblasts (Figure S5H and I). Overall, these results demonstrate that T_3 specifically induces DRP1 phosphorylation at early stages of NSC/progenitor cell determination toward a neuronal fate.

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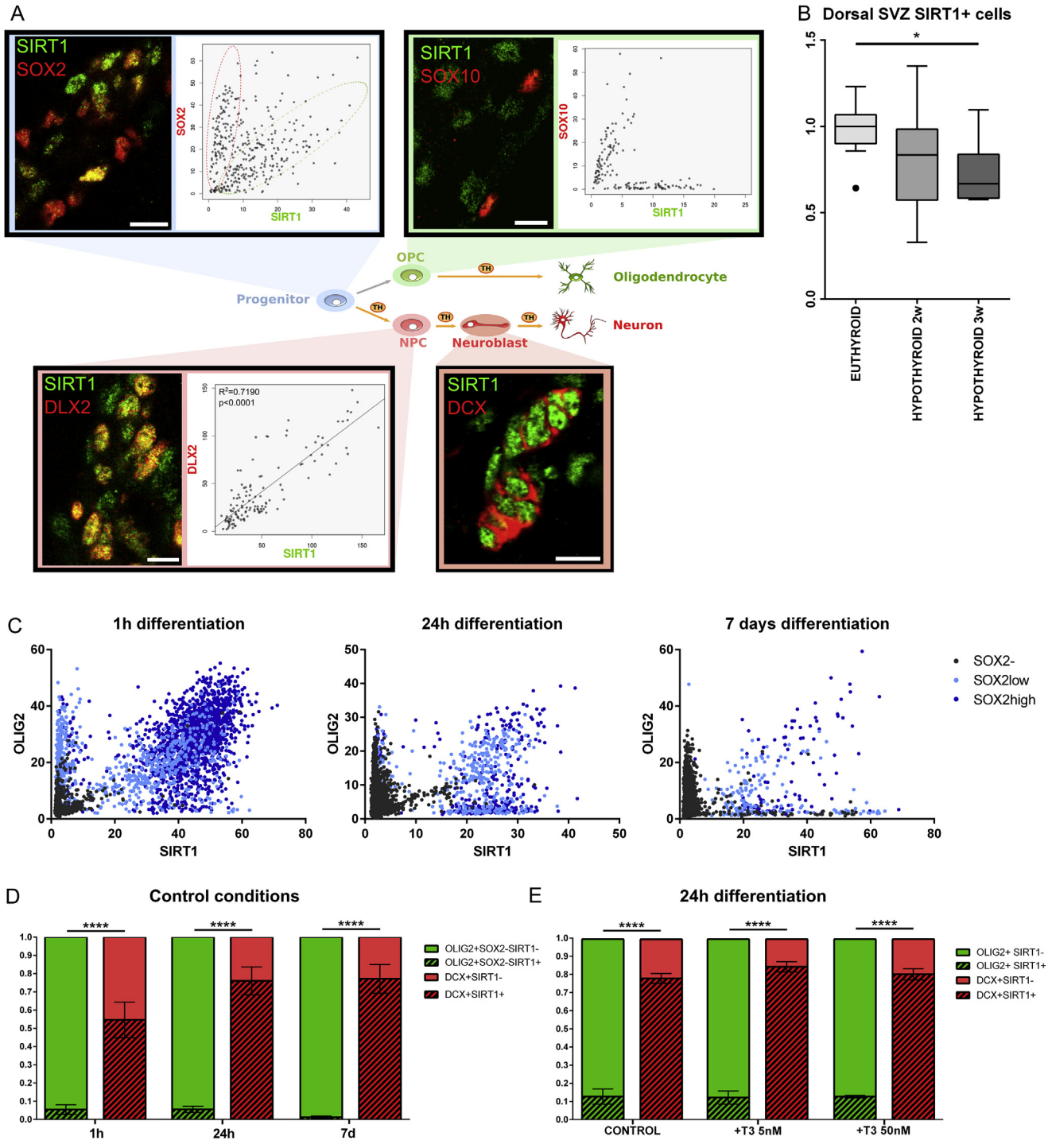

Figure 3: SIRT1 expression is maintained at early stages of neuroblast determination. (A) *In vivo* co-immuno-stainings performed on wild-type adult mice SVZ. SIRT1 (green) expression is detected in a sub-population of SOX2+ progenitors and is mainly excluded from SOX10+ OPCs. SIRT1 is positively correlated with DLX2 expression in neuronal precursor cells (R2 = 0.719, $p < 0.0001$), and expression is maintained in most DCX+ neuroblasts. (B) Quantification of the number of SIRT1+ cells in the dorsal SVZ of euthyroid and hypothyroid (2 and 3 wks) adult mice. Four experiments were pooled, n = 12 euthyroid, n = 10 hypothyroid 2 wks, n = 6 hypothyroid 3 wks (C) SIRT1/OLIG2 cell by cell nuclear relative fluorescence after 1 h, 24 h or 7 d of differentiation *in vitro*. SOX2 positive cells are represented by blue dots. (D) Proportion of DCX+ and OLIG2+ cells positive for SIRT1 after 1 h, 24 h and 7 d differentiation in control conditions. (E) Proportion of DCX+ and OLIG2+ cells positive for SIRT1 after 24 h differentiation in control medium or with T_3 (5 and 50 nM). p-Value: *<0.05, ****<0.0001. Kruskal–Wallis followed by Mann–Whitney tests.

4. DISCUSSION

Adult NSC-driven neurogenesis generates both neurons and glial cells [4], processes that require precise control of NSC fate decisions. THs commit NSCs to a neuronal phenotype [6]. Here we show that TH signaling favors neuronal fate in the adult SVZ through induction of mitochondrial respiration. Studying how TH signaling and mitochondrial metabolism affect NSC decisions requires specific markers of

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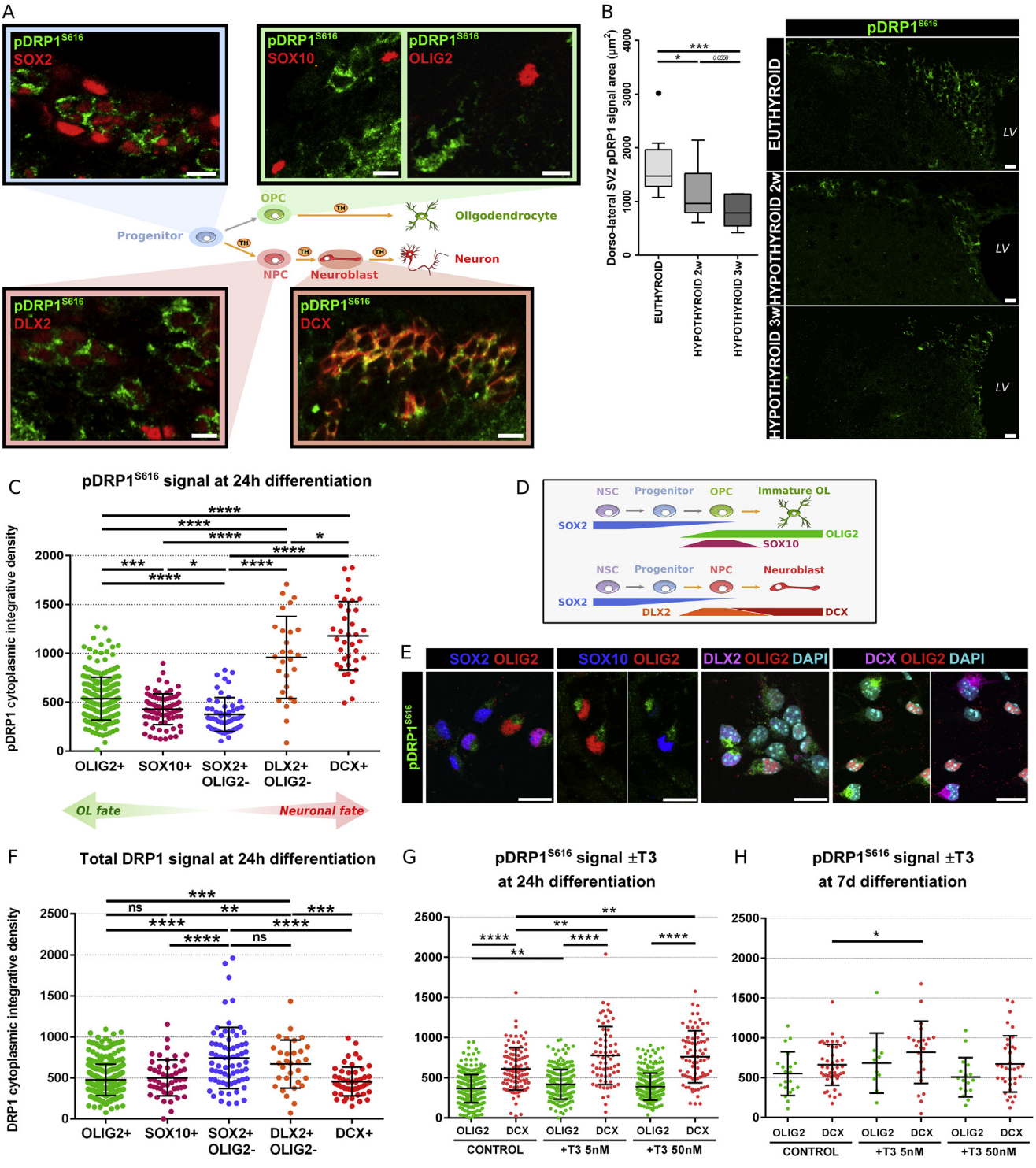

Figure 4: Thyroid hormones activate DRP1 phosphorylation in cells adopting a neuronal fate. (A–B) pDRP1^{S616} is mainly expressed in cells adopting a neuronal fate *in vivo* and decreased following a short-term hypothyroidism. (A) Immunohistochemistry stainings performed on wild-type adult mice SVZ using antibodies against pDRP1^{S616} (green), DLX2, DCX, SOX2, SOX10, and OLIG2 (red). pDRP1^{S616} is expressed in SOX2^{low} more committed progenitor cells, DLX2+ neuronal precursor cells (NPC) and DCX+ neuroblasts. pDRP1 is not detectable in SOX2^{high} early progenitors nor in SOX10+ and OLIG2+ oligodendrocyte precursor cells (OPC). (B) Immunohistochemistry showing pDRP1^{S616} expression in dorsal SVZ and quantification of pDRP1^{S616} signal in dorsal SVZ of euthyroid and hypothyroid (2 and 3 wks) adult mice. n = 12 (EUTHYROID and HYPOTHYROID 2 wks) or n = 7 (HYPOTHYROID 3 wks). Scale bar: 10 µm (C–H) DRP1 Ser616 phosphorylation is higher in the neuronal lineage and T_3 activate DRP1 phosphorylation. (C) Quantification of pDRP1^{S616} cytoplasmic signal of SOX2+, SOX10+, OLIG2+, DLX2+, and DCX+ cells after 24 h of NSC differentiation *in vitro*. Co-immunostainings were done with antibodies directed against pDRP1^{S616}, OLIG2, and a third marker (SOX2, SOX10, DLX2, DCX). (D) Markers used for specific cell type detection. (E) Representative images of pDRP1^{S616} immunostainings at 24 h differentiation. Scale bar: 10 µm. (F) Quantification of total DRP1 cytoplasmic signal in SOX2+, SOX10+, OLIG2+, DLX2+ and DCX+ cells after 24 h differentiation. (G–H) Quantification of the cytoplasmic signal of pDRP1^{S616} in OLIG2+ and DCX+ cells after 24 h and 7 d differentiation in control conditions or with T_3 (5 nM or 50 nM). p-Value: *<0.05, **<0.01, ***<0.001, ****<0.0001. Kruskal–Wallis followed by Mann–Whitney tests.

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early neuroblast determination. We demonstrate that DLX2 expression is specific to NPCs in the adult mouse SVZ, allowing the use of this marker to distinguish between NPC and OPC.

NSC rely primarily on glycolytic metabolism, whereas most mature neural cells use mainly OXPHOS-based energy [7]. Furthermore, oxygen availability impacts NSC differentiation capacity and whether the NSC becomes a neuron, an astrocyte, or an oligodendrocyte [13,32]. We show first, that the transition toward an OXPHOS-based metabolism occurs in the neurogenic lineage during early cell-fate commitment and, second, that it is a TH-dependent process. Further, our results show that this metabolic switch occurs simultaneously with activation of mitochondrial fission through phosphorylation of DRP1, also induced by THs. Lastly, expression of the metabolic regulator SIRT1 is preferentially maintained in cells differentiating to neuroblasts. These experiments thus underline the importance of TH-dependent activation of mitochondrial OXPHOS during adult NSC determination to a neuronal phenotype. In the glial lineage, there is also a switch to OXPHOS-based metabolism, but this occurs later in the differentiation process.

We also show mitochondrial activity and ROS production to be higher in cells differentiating to a neuronal than to a glial lineage in the adult brain. This result corroborates the observation that, in contrast to neurons, mature glial cells maintain high rates of glycolysis, allowing a better adaptability to metabolic stress [33,34]. Further, the JC-1 data showed that the decreased numbers of cells differentiating to neuroblasts in hypothyroid conditions was accompanied by a diminished cellular respiration in this cell type, confirming the crucial role of TH signaling in modulating mitochondrial respiration during cell determination. Another result showing that mitochondrial activity is essential for TH-induction of NSC fate determination came from use of oligomycin, an inhibitor of mitochondrial ATP synthase. Applying this drug overrode TH induction of neuroblast determination. Thus, TH induction of neuroblast cell fate requires mitochondrial OXPHOS activity.

Differential expression of TR coregulators could be another important modulator of TH influence on NSC fate choice. The NAD^+-dependent deacetylase SIRT1 can act as a coactivator for TRβ and TRα [28,35]. SIRT1 is known to be involved in regulating several metabolic pathways, especially mitochondrial function through PGC1α and AMPK [36–38]. SIRT1 also has an important role in stemness maintenance by directly regulating SOX2 [39], OCT4 [40] and NANOG [41], three major transcription factors required for pluripotency and inhibition of differentiation. As expected, our results show that SIRT1 is largely expressed in adult NSC-derived SOX2+ progenitors, but also preferentially maintained in early differentiation stages of the neuronal lineage. In contrast, virtually no SOX10+/OLIG2+ engaged OPCs still expressed this deacetylase. Although a decrease in the total number of SIRT1+ cells was observed *in vivo* in the SVZ of hypothyroid mice, we saw no significant change in SIRT1 expression in either OLIG2+ or DCX+ cells when modifying T_3 availability *in vitro*. The decrease seen in hypothyroid mice *in vivo* correlates with the observed decrease in DLX2+ and DCX+ cells, confirming SIRT1 expression maintenance in the neuronal lineage. It can be hypothesized that while SIRT1 is repressed in pluripotency of SOX2+ progenitors when differentiation starts, its roles as TR coactivator and mitochondrial metabolism modulator are boosted during early neuronal but not early OL determination. Which factors induce SIRT1 in the neuronal lineage will be an interesting question to address in future studies.

Besides SIRT1, TH signaling impacts other modulators of mitochondrial metabolism, either through TH nuclear receptors or through non-genomic processes. T_3 promotes DRP1 localization on mitochondria [16], where the protein can induce fission. We showed that T_3 induces DRP1 activation through phosphorylation of its Ser616 residue, an event that enables DRP1 translocation to the mitochondrial membrane. Though our results showed a slight decrease in DRP1 expression during NSC differentiation, we observe a clear increase in the amount of $pDRP1^{S616}$, that is much larger in DLX2+ NPCs than in SOX10+ OPCs. It is worth noting that repression of DRP1 *in vitro* is known to lead to a reduction in oxygen consumption [42]. Thus, cell fate-dependent induction of mitochondrial activity by THs occurs through changes in mitochondrial dynamics, and particularly modulation of mitochondrial fission. This result adds to previous published findings showing that DRP1 is required for reduction of *Sox2*, *Oct4*, and *Nanog* pluripotency genes during embryonic neurogenesis [43]. Furthermore, it is consistent with the increased mitochondrial dissemination observed during neuronal differentiation and maturation [44]. Fission allows mitochondria to disperse inside the cell, a process likely linked to axonal and dendritic development.

These results lead to the conclusion that local control of TH signaling affects mitochondrial activity and cell fate determination. THs could impact mitochondrial metabolism of differentiating NSCs through different modes of action. Through nuclear TR, T_3 can activate expression of both PGC1a and TFAM, two factors inducing mitochondrial protein expression and biogenesis, processes, which in turn increase mitochondrial dynamics [45–47]. But THs have also been shown to increase mitochondrial respiration efficiency through non-nuclear early effects [48,49]. What is more, two truncated variants of TRα, p28 and p43, are found specifically in mitochondria [50,51]. T_3 induces p28 import into mitochondria inner membrane, where the respiratory chain is located, but the precise function of p28 is not yet known [52]. However, it is known that p43 activates mitochondrial protein synthesis and mitochondrial biogenesis in presence of T_3 [53–56]. Thus, these processes could be important for early influence of THs on mitochondrial metabolism and further accompany nuclear receptor-mediated T3 effects on mitochondrial biogenesis during NSC determination.

5. CONCLUSIONS

Our results show that THs favor adult NSC fate choice toward a neuronal phenotype in the adult sub-ventricular zone (SVZ) through induction of mitochondrial respiration. Mitochondrial activity and ROS production are higher in cells differentiating to a neuronal than to a glial lineage in the adult brain and are influenced by THs. SIRT1 is preferentially maintained in cells engaged in a neuronal differentiation, but does not appear to be directly impacted by TH availability. We also show that THs promote DRP1 activating phosphorylation preferentially in cells acquiring a neuronal fate, in line with the idea of an increase in mitochondrial dynamics and dissemination during cell determination. Further research on how metabolic controls contribute to NSC fate decision processes in the adult brain will be critical in the context of neurodegenerative diseases, both to understand mechanisms underlying disease susceptibility and progression and to develop strategies to direct neurogenesis to neuronal or glial cell fates.

AUTHOR CONTRIBUTIONS

Conceptualization, J.-D.G., B.A.D. and S.R.; Methodology, J.-D.G., S.R. and A.S.; *In vivo* experiments, J.-D.G., S.R., M.L., M.P.-J.; *In vitro* experiments, A.S., J.-D.G., C.L., C.N.V, K.L.B.; Image acquisition, J.-D.G., A.S., M.L., C.N.V., M.P.-J.; Analyses, J.-D.G., A.S., M.L., C.N.V.; Writing, J.-D.G., B.A.D. and S.R.

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ACKNOWLEDGMENTS

This work was supported by the Centre National de la Recherche Scientifique (CNRS), the Museum National d'Histoire Naturelle (MNHN), the EU FP7 contract Thyrage (Grant no. 666869), ANR grants Thrast (ANR-11-BSV2-019-02) and OLGA (ANR-14-CE13-0022). J.-D.G. received PhD grants from région Île-de-France DIM Cerveau&Pensée and fondation pour la recherche médicale (FRM) (FDT20160435031). We thank M.-S. Aigrot for advice on experimental techniques, M. Gèze for technical assistance, S. Sosinsky and F. Uridat for the excellent animal care.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2017.08.003.

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