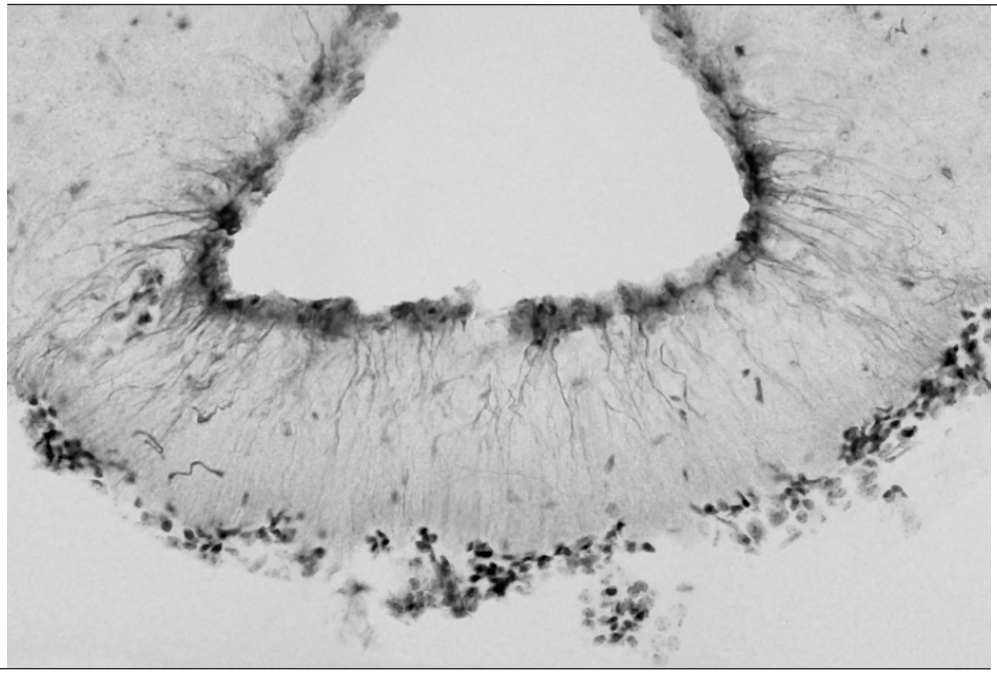
Chapter 5

Doublecortin-like knockdown in hypothalamic tanycytes induce subtle effects on bodyweight and Deiodinase 2 activity.



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Abstract

Doublecortin-like (DCL) is a microtubule-associated protein that is highly homologous to doublecortin and is crucially involved in adult neurogenesis in the hippocampus and forebrain. Previously, we showed high DCL expression in the hypothalamic tanycytes, a cell population involved in the production of thyroid hormones. Therefore, we address the question whether or not DCL is involved in thyroid hormone signalling. To this end, we measured bodyweight, serum T3 and T4 concentrations and D2 activity in hypothalamic tissue of DCL-knockdown (KD) mice and their littermate controls. Furthermore, we measured mRNA expression of TRH, NPY, D2 and D3 in hypothalamic punches containing the ARC-ME or the PVN. We observed a strong reduction in DCL expression in hypothalamic tanycytes, which was associated with reduced body weight growth and a significant increase in D2 activity, the enzyme metabolizing inactive T4 into active T3. However, serum levels of T4 and T3 did not differ between wildtype and DCL-KD animals and also the expression of TRH, NPY, D2 and D3 mRNA in the hypothalamus was not affected by DCL knockdown. Together, our data indicate a role for DCL in the regulation of D2 activity in hypothalamic tanycytes and a possible subtle role in thyroid signalling.

Introduction

The doublecortin-like kinase (DCLK) gene encodes multiple splice variants. One of these is Doublecortin-like (DCL), a microtubule associated protein (MAP) that shares a high amino acid sequence identity with doublecortin over its entire length (Burgess and Reiner, 2002;Sossey-Alaoui and Srivastava, 1999;Vreugdenhil et al., 2001). During embryonic development, DCL is widely expressed in mitotic cells, radial glial cells (RGs) and radial processes. DCL functions as a microtubule stabilizing protein of mitotic spindles in vitro and in vivo (Boekhoorn et al., 2008;Vreugdenhil et al., 2007). In addition, DCL knockdown by RNA-interference technology induces spindle collapse in vitro. DCL knockdown in vivo, by in utero electroporation, leads to a significantly reduced cell number in the inner proliferative zones and a dramatic disruption of most radial processes (Vreugdenhil et al., 2007). Furthermore, DCL is involved in suppression of spine maturation (Shin et al., 2013) and, in the postnatal brain, DCL regulates the transport of the glucocorticoid receptor (GR) in neuronal progenitor cells (NPC's) (Fitzsimons et al., 2008).

Previously we described DCL expression in the adult mouse brain (Saaltink et al., 2012). In the hippocampus and subventricular zone (SVZ) of the adult brain, DCL is expressed in a cell population which is also positive for the neurogenesis marker doublecortin (DCX; Saaltink et al., 2012) suggesting a role for DCL in neuronal progenitor cells (NPCs) (Couillard-Despres et al., 2005;Plumpe et al., 2006;Rao and Shetty, 2004). Indeed, conditional knockdown of DCL in adult mice inhibits newborn neurons in the hippocampal dentate gyrus (DG) to mature (chapter 3). DCL is also expressed in other brain areas like suprachiasmatic nucleus (SCN), islands of Calleja (ICj) and hypothalamic tanycytes, but these areas traditionally do not show adult neurogenesis. Since most DCL-related studies showed involvement in neurogenesis, the question comes up what the function of DCL is in these apparent non-neurogenic brain areas.

Although there are several studies suggesting neurogenesis within the hypothalamus (Haan et al., 2013;Lee et al., 2012;Xu et al., 2005), DCL-expressing hypothalamic tanycytes do probably not reflect a population of newborn neurons since DCL is in particular expressed in nearly all β-tanycytes (Saaltink et al., 2012). These β-tanycytes reside in the ventricle wall along the median eminence, which may be considered as a peri- or circumventricular organ (CVO). Tanycytes have a radial glia-like phenotype and express beside DCL, several markers of neural stem cells like nestin (Baroncini et al., 2007;Barrett et al., 2006;Chouaf-Lakhdar et al., 2003;Xu et al., 2005), vimentin (Baroncini et al., 2007;Bolborea et al., 2011;Chauvet et al., 1998;Kameda et al., 2003;Sidibe et al., 2010;Xu et al., 2005) and sox2 (Lee et al., 2012;Li et al., 2012). Beside the blood-brain barrier (BBB), CVO's form an alternative but semi-permeable barrier between the peripheral blood stream and the cerebrospinal fluid

(CSF) (Rodriguez et al., 2005;Rodriguez et al., 2010). Tanycytes play an important role as gatekeeper (Rodriguez et al., 2005) and are equipped with transport machinery to transfer substances from blood to CSF and vice versa (Rodriguez et al., 2010). Hypothalamic tanycytes are also thought to be part of the feedback mechanism that controls the set-point of the hypothalamic-pituitary-thyroid axis (HPT-axis; Coppola et al., 2007). Under influence of thyrotropin-releasing-hormone (TRH) produced in the neurons of the paraventricular nucleus of the hypothalamus (PVN) and released in the median eminence (ME) the thyrotrophes in the pituitary release thyroid-stimulating-hormone (TSH) into the systemic circulation. Circulating TSH stimulates the thyroid gland to produce the thyroid hormones (TH) thyroxine (T4) and tri-iodothyronine (T3). THs induces genomic and non-genomic effects in many tissues in the body (Bassett et al., 2003). T4 is the inactive hormone while T3 is the active form. T4 is converted locally into T3 by deiodinase enzymes. There are several types of deiodinase enzyme of which type 1 and 2 (D1 & D2) convert T4 into T3. D1 is mainly active in the liver and kidney whereas D2 is expressed in the central nervous system, the anterior pituitary, brown adipose tissue and to a lesser extent in skeletal muscle. Within the hypothalamus, D2 is mainly expressed in the arcuate nucleus (ARC) and hypothalamic tanycytes (Guadano-Ferraz et al., 1997;Kalsbeek et al., 2005;Tu et al., 1997). (Crantz et al., 1982;Fekete and Lechan, 2007). Since DCL is highly expressed in tanycytes and has transporting capacities (Fitzsimons et al., 2008), it's tempting to speculate about a role for DCL in tanycyte functioning within the HPT-axis.

In this study we address the question whether conditional DCL knockdown in mice affects tanycytes functioning in thyroid hormone signalling. To this end, we measured bodyweight, serum T3 and T4 concentrations and D2 activity in hypothalamic tissue of both mice with normal DCL expression and in mice with DCL knockdown. Furthermore we measured mRNA expression of TRH, NPY, D2 and D3 in hypothalamic punches containing the ARC-ME or the PVN.

Methods

Animals

Three-month-old DCL KD transgenic (n=12) (chapter 3) and wildtype (n=12) male mice were obtained from our outbred colony (derived from TaconicArtemis, Cologne, Germany). The animals were kept under a 12:12 light-dark cycle (lights on from 7:00 to 19:00 hours), in a temperature-controlled room (23°C). The shRNA system was induced by doxycycline (dox) via dox containing food pellets (Dox Diet Sterile S3888, 200mg/kg, BioServ, New Jersey, USA). Water and food were available ad libitum. After 5 weeks of dox induction the animals were decapitated and blood was collected. Bodyweight was measured regularly. Serum was

stored at -20°C until it was analyzed. The liver, pituitary and hypothalamus (defined rostrally by the optic chiasm, caudally by the mamillary bodies, laterally by the optic tract, and dorsally by the apex of the third ventricle) were isolated and stored immediately in liquid nitrogen. The tissue block containing the hypothalamus (n=6) was used for dissection of the periventricular area (PE) and the arcuate nucleus / median eminence region (ARC-ME). The PE consists of both paraventricular nuclei and the upper part of the ependymal lining of the third ventricle. This area was obtained by punching the hypothalamus with a hollow needle (diameter 1100µm) based on anatomical landmarks (the apex of the third ventricle Franklin K.B.J., 1997). The PE samples may include (part of) the dorsomedial nucleus (DMN) which –like the PVN- contains TRH neurons. The same instrument was used to obtain the ARC-ME samples (see Fig. 1). This experiment was approved by the Local Animal Welfare Committee of the University of Leiden, The Netherlands.

Immunohistochemistry

Wildtype and transgenic animals of 6 weeks old were put on a dox diet (n=6 per group). After 5 weeks the animals were killed and prepared for immunohistochemistry as described previously (Saaltink et al., 2012). In short, animals were deeply anaesthetized by IP injection of sodium pentobarbital (Euthasol 20%, ASTPharma bv, Oudewater, The Netherlands). Thereafter the mice were transcardially perfused with ice-cold 0.1M phosphate buffered saline (PBS) and subsequently with 4% para-formaldehyde in 0.1M PBS (PFA). After perfusion, the mice were decapitated and the heads kept in 4% PFA overnight at 4°C for post fixation. The next day, brains were removed and put in a 15% sucrose solution (0.1M PBS) overnight at 4°C for dehydration. Subsequently, the brains were put in a 30% sucrose solution for another night at 4°C. At the end of the dehydration procedure the brains were removed from the solution and blotted dry before snap-freezing. The brains were kept at -80°C until used for cryosectioning. Serial coronal 30µm-thick sections were obtained using a cryostat (Leica CM 1900, Leica Microsystems, Rijswijk, The Netherlands). All brain sections were collected in 2ml Eppendorfs containing anti-freeze (50%glycerol, 50% 0.2M PB) and stored at -20°C until further use.

Free floating sections were left at room temperature for 15 minutes before being washed in 0.1M phosphate-buffered saline (PBS) and blocked in 2% bovine serum albumin (BSA, sc-2323, Santa Cruz) in PBS for 2 hours. After three washing steps in PBS the primary antibody targeting DCL (Saaltink et al., 2012) was applied to the slides in PBS with 0.3% TX-100 and left at room temperature for 1 hour followed up by overnight incubation at 4°C. Subsequently the slides were washed in PBS and incubated in secondary antibody (Alexa Fluor® 488 donkey anti-rabbit IgG) for 2 hours at room temperature. After washing with PBS the slides were counterstained with Hoechst (1:10000) for 10 minutes and washed again before

they were mounted and covered using Aqua Poly/Mount (Polysciences, Inc.)

RNA isolation and RT-PCR

Punches derived from the PE and ARC-ME were analysed as described earlier (Boelen et al., 2004). mRNA was isolated from hypothalamic brain tissue using the Magna Pure apparatus and the Magna Pure LC mRNA isolation kit II (tissue) (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol. cDNA synthesis was performed with the 1st strand cDNA synthesis kit for RT-PCR (AMV) (Roche Molecular Biochemicals). Real Time PCR was performed using the Lightcycler480 and Lightcycler480SybrGreen I Master mix (Roche Molecular Biochemicals, Mannheim, Germany). Primer pairs for mouse hypoxanthine phosphoribosyl transferase (Hprt), D2 (Dio2), D3 (Dio3) and TRH have been previously described (Boelen et al., 2004). We designed primer pairs for mouse NPY (forward primer 5' -GGGCTGTGTGGACTGACCC-3', reverse primer 5'GGTACCCCTCAGCAGAATG-3'). Annealing temperature in the PCR reaction was 60ºC. Quantification was performed using the LinReg software (Ruijter et al., 2009). The mean of the efficiency was calculated for each assay, samples that had a greater difference than 0.05 of the efficiency mean value, were not taken into account (0-5%). mRNA levels were corrected for housekeeping gene (HPRT) expression.

Deiodinase type 2 activity

The hypothalamic block was homogenized on ice in 10 volumes of PED50 (0.1M sodium phosphate, 2 mM EDTA and 50 mM DTT pH 7.2) using a Polytron (Kinematica, Luzern, Switzerland). Homogenates were immediately processed for D2 measurement as previously described (Kwakkel et al., 2009). Protein concentration was measured with the Bio-Rad protein assay using bovine serum albumin (BSA) as the standard following the manufacturer's instructions (Bio-Rad Laboratories, Veenendaal, The Netherlands). D2 activity was measured in duplicate using 75 µl of homogenate. Samples were incubated for 4 hours, at 37 °C, in a final volume of 0,15 ml with 1 or 500 nM T4 (blank as high concentration of substrate (T4) saturates D2), 500 nM PTU (to block D1) and 2×105 cpm [3'5'- (125)I]T4. The reaction was stopped by cooling the samples on ice and adding 0.15 ml of ice-cold ethanol. After centrifugation, 0.125 ml of the supernatant was added to 0.125 ml 0.02 M ammonium acetate (pH 4), and 0.1 ml of the mixture was applied to 4.6 x 250 mm Symmetry C18 column connected to a Waters HPLC system (Model 600E pump, Model 717 WISP autosampler, Waters, Etten-Leur, The Netherlands).

Mobile phase A: 0.02 M ammonium acetate (pH 4.0), mobile phase B: acetonitril. The column was eluted with a linear gradient (28–42% B in 15 min) at a flow of 1.2 ml/min. The activity of T4, and T3 in the eluate was measured online using a FSA flow detector (150TR) van Perkin Elmer (Perkin Elmer, Groningen, The Netherlands). D2 activity was expressed

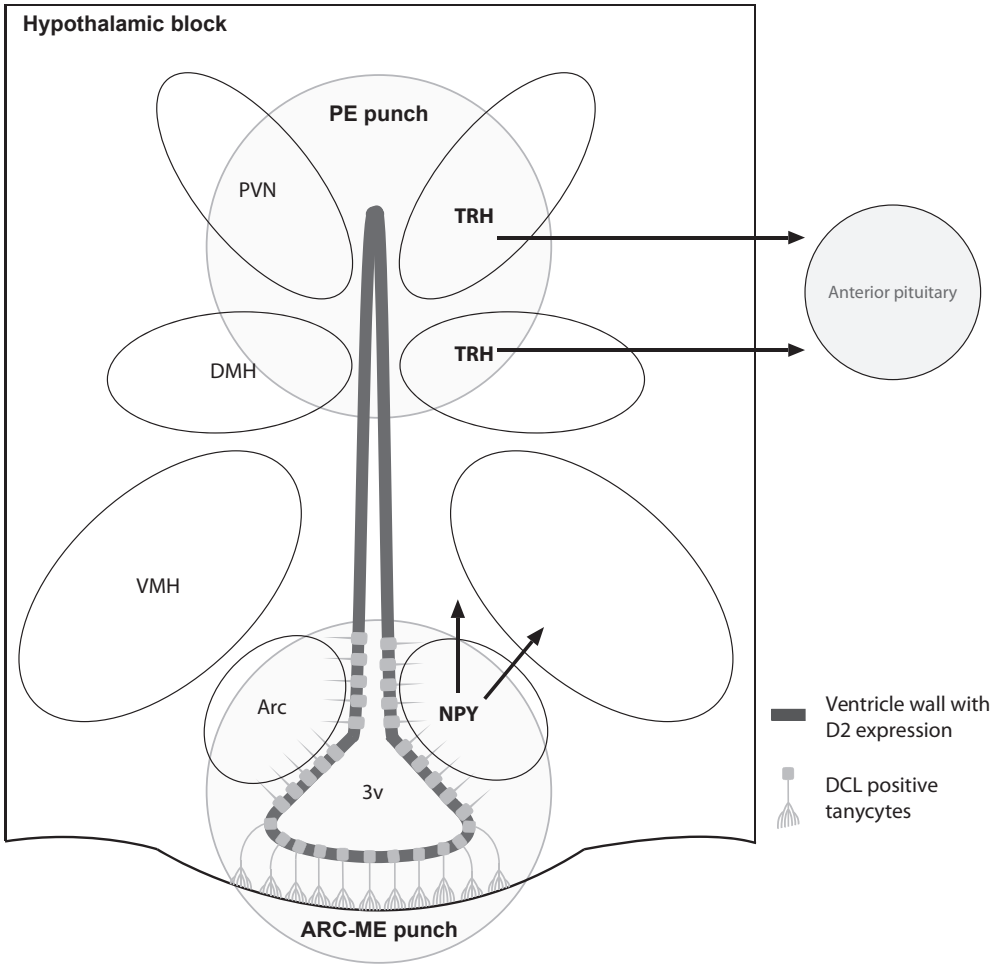

Figure 1: Schematic overview of the hypothalamic block from which the punches are derived. The periventricular area (PE) punch contains nuclei around the upper part of the wall of the third ventricle (3v) including the paraventricular nucleus (PVN) and parts of the dorsomedial hypothalamus (DMH). Both nuclei contain TRH producing neurons which stimulate the anterior pituitary to release TSH. The ARC-ME punch contains tissue from the arcuate nucleus (ARC) and the median eminence (ME) including the lower part of the ventricle wall. This part of the ventricle wall also contains DCL positive tanycytes. The ARC is characterized by the expression of NPY. The ventral medial hypothalamus (VMH) is not punched. D2 is expressed along the whole ventricle wall (red).

as fmol of generated T3 per minute per mg of tissue. The amount of generated T3 was calculated using the values of the 1nM T4 incubations minus the mean of the 500 nM T4 incubations.

Thyroid hormone levels

Serum T3 and T4 were measured with in-house radio immunoassays (RIAs) (Wiersinga and Chopra, 1982) as described before (Boelen et al., 2004). All samples of one experiment were measured within the same run (intra-assay variability T3: 3.6% and T4: 6.6%).

Statistics

Results are expressed as mean ±S.E.M. and unless stated otherwise a Student's t-test was performed using Prism 4.00 (GraphPad Software Inc., San Diego, CA). qPCR data is tested with a General Linear Model (GLM) for univariate analysis of variance in SPSS statistical software version 20 (IBM, SPSS Inc. Chicago,IL). P values less than 0.05 were considered as statistically significant.

Results

To knockdown DCL protein expression in hypothalamic tanycytes we put wildtype and DCL-KD animals on a dox diet. Five weeks later, mice were sacrificed and prepared for immunohistochemical analysis. DCL protein expression was mainly found in the lower part of the third ventricle wall close to the median eminence. In line with our previous findings (Saaltink et al, 2012) wildtype animals showed a clear DCL signal in both dendrites and cell bodies of hypothalamic tanycytes (Fig. 2 A&C). In contrast, DCL expression was strongly reduced in tanycyte dendrites and only a weak signal was left in the ventricle wall in DCL-KD littermates (Fig. 2 B&D).

Thyroid hormone signalling also affects energy balance and basal energy metabolism and therefore might affect bodyweight. We measured the bodyweight of the experimental animals before and after dox induction. At the start of the experiment there was a small, but significant, difference (1.23 ± 0.49 g, p=0.016) in bodyweight between wildtype (22.29 ± 0.29 g, N=24) and DCL-KD (21.05 ± 0.40 g, N=24) animals (Fig. 3A). This difference was increased after 5 weeks of dox induction (Fig. 3B). Wildtype animals (25.36 ± 0.44 g, N=24) were still significantly heavier than DCL-KD animals (23.04 ± 0.48g , N=24), but the difference was more pronounced (2.32 ± 0.65 g, p<0.001). Wildtype animals gained relative more bodyweight (13.8% ± 1.4, N=24) than their DCL-KD littermates (9.4% ± 0.9, N=24) (Fig.3C).

As tanycytes are involved in thyroid hormone signalling and express D2, we measured the activity of this enzyme. We found a marked increase in hypothalamic D2 activity in the hypothalamic block (Fig. 4C) of DCL-KD mice (0.074 fmol/mg/min ± 0.005, N=5) compared to wildtype littermates (0.030 fmol/mg/min ± 0.003, N=6). Despite the significant increase

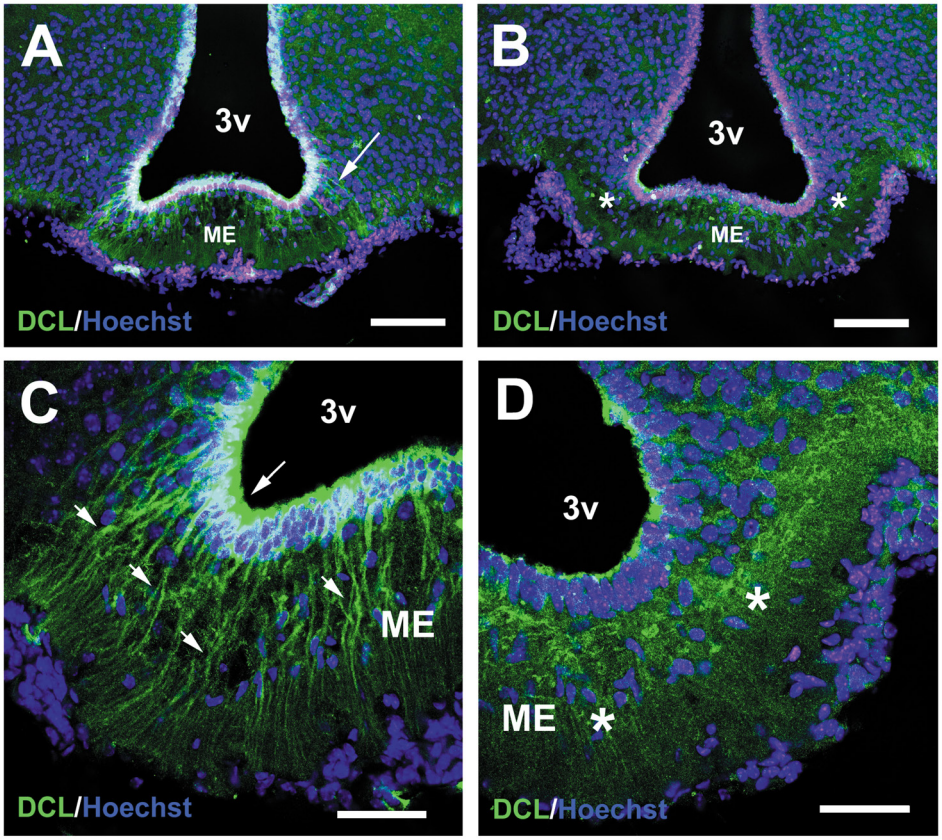

Figure 2: DCL-KD in hypothalamic tanycytes. A) Strong DCL expression in the wall of the third ventricle (3v). DCL positive projections in median eminence (ME) are clearly visible (arrows). B) DCL expression in the 3v wall is strongly reduced (asterix). C) Higher magnification of tanycytes close to the ME with DCL positive basal processes extending into the ME (arrows). D) Although some DCL signal is left, strong reduction of DCL expression is visible after siRNA mediated DCL knockdown. Scale bars measure in A and B 40µm, in C and D 100µm.

in D2 activity, DCL knockdown did not affect D2 mRNA expression in either the PE or the ARC-ME (Fig. 5A). Similar results were found for D3 mRNA; DCL knockdown did not affect D3 mRNA expression in PE and ARC-ME punches (Fig. 5B). Since hypothalamic D2 converts peripheral T4 into T3 and is thought to be involved in the set-point of the HPT-axis, we also measured serum T3 and T4 levels. DCL knockdown did not affect serum levels of either T3 or T4 (see Fig. 4A & B). In addition, DCL knockdown did not affect mRNA expression of several HPT-axis related genes in either the PE or ARC-ME punch (Fig. 5). However, both TRH and NPY mRNA levels did exhibit the expected area specificity with significant higher levels of TRH in the PE punch ($F(1)=60.53$, $p<0.001$, Fig. 5C) and a significantly higher NPY mRNA expression in the ARC-ME punch ($F(1)=43.50$, $p<0.001$).

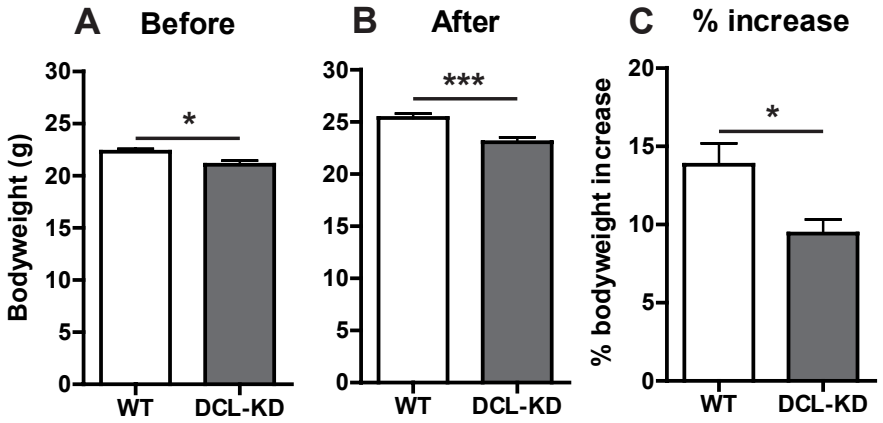

Figure 3: Bodyweight before and after doxycycline induction. A) Wildtype (WT) (22.29 ± 0.2887 g, N=24) and DCL-KD (21.05 ± 0.3996 g, N=24) littermates differed in bodyweight at the start of the experiment (1.233 ± 0.4930 g, p=0.016). B) After 5 weeks of dox induction, WT animals (25.36 ± 0.4381 g, N=24) were still significantly heavier than DCL-KD animals (23.04 ± 0.4774g , N=24), but the difference had increased (2.321 ± 0.6479 g, p<0.001). C) WT animals gained relative more bodyweight (13.82% ± 1.370, N=24) than their DCL-KD littermates (9.418 %± 0.9036, N=24).

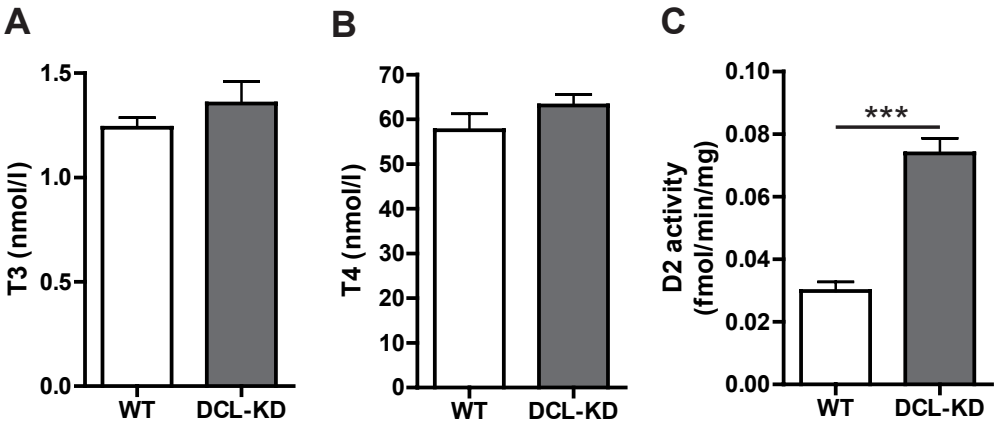

Figure 4: TH levels in blood plasma. A) T3 levels in peripheral blood serum did not differ between WT (1.238 ± 0.04816 N=13) and DCL-KD (1.355 ± 0.1047 N=11) animals. B) Even so did T4 levels not differ between WT (57.54 ± 3.681 N=13) and DCL-KD (63.09 ± 2.470 N=11) animals. C) DCL-KD animals have a more than two times higher D2 activity (0.074 fmol/mg/min ± 0.005, N=5) compared to wildtype littermates (0.030 fmol/mg/min ± 0.003, N=6).

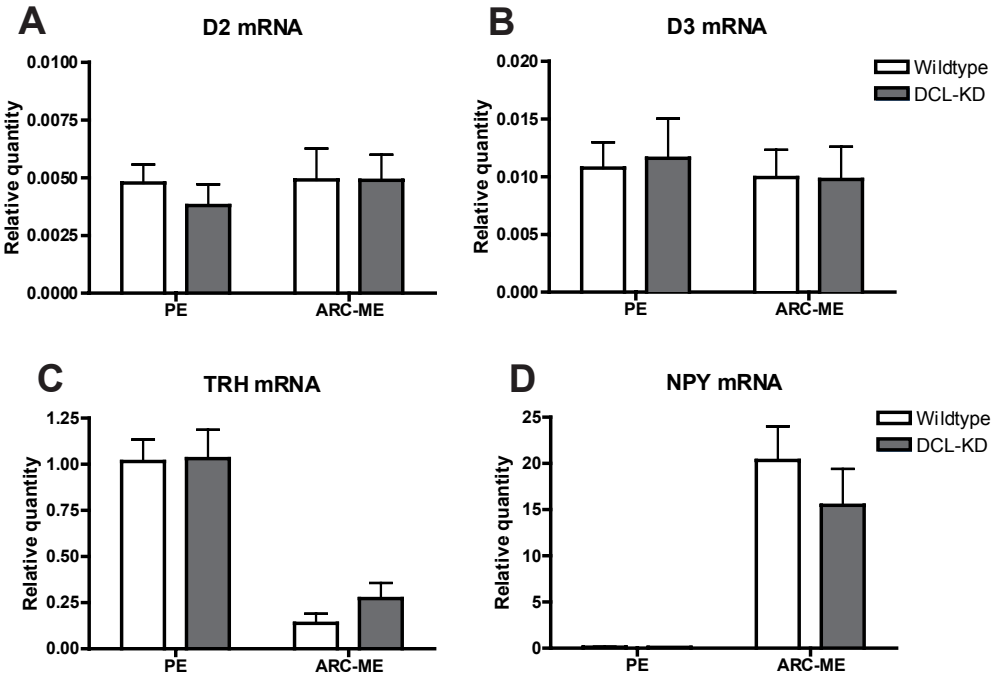

Figure 5: mRNA levels of 4 genes involved in the HPT-axis. A) Despite the significant increase in D2 activity, DCL knockdown did not affect D2 mRNA expression in all hypothalamic tissue ($F(1)=0.191$, $p>0.05$). Furthermore, levels of D2 mRNA did not differ between both PVN and ARC/median eminence ($F(1)=0.294$, $p>0.05$). B) Similar results were found for D3 mRNA; DCL knockdown did not affect D3 mRNA expression between wildtype and DCL-KD animals ($F(1)=0.0.013$, $p>0.05$) and between both PVN and ARC/median eminence (($F(1)=0.201$, $p>0.05$). C) DCL knockdown did not affect TRH mRNA expression levels ($F(1)=0.502$, $p>0.05$) within the hypothalamus. However, a difference between both punches show area specificity since PVN and ARC/median eminence differ in TRH mRNA levels ($F(1)=60.53$, $p<0.001$). D) NPY mRNA expression is characteristic for the arcuate nucleus. A strong significant difference was found between both area's in favour of ARC in all animals ($F(1)=43.50$, $p<0.001$). DCL knockdown did not affect NPY mRNA expression ($F(1)=0.816$, $p>0.05$).

Discussion

Here, we have studied the possible role of DCL in hypothalamic tanycytes in thyroid hormone metabolism by using genetically modified mice with a knockdown of DCL after dox administration in food pellets. We observed a strong reduction in DCL expression in hypothalamic tanycytes which was associated with reduced body weight growth and a significant increase in D2 activity, the enzyme metabolizing inactive T4 into active T3. However, serum levels of T4 and T3 did not differ between wildtype and DCL-KD animals and also the expression of TRH, NPY, D2 and D3 mRNA in the hypothalamus was not affected by DCL knockdown. Toge-

ther, our data suggest a subtle role for DCL in the regulation of D2 in hypothalamic tanycytes which is however not affecting the set-point of the HPT-axis.

We observed a small, but significant difference in bodyweight between wildtype and DCL-KD littermates. After the dox-induced knockdown of DCL, the transgenic mice showed a smaller increase of bodyweight compared to the wild types. The observed difference in hypothalamic D2 activity between DCL-KD and wildtype mice cannot explain the reduced increase in bodyweight in DCL-KD animals since serumT4 and T3 levels were unaffected. A possible explanation for the observed differences in body weight might be leakage of the siRNA system. Leakage of the tet repression function has been reported and might lead to reduced DCL expression which is further decreased by dox administration. However, whether or not leakage affected early bodyweight development in our mice is presently unknown. Another possible explanation for reduced bodyweight increase might be aberrant glucose sensing. Glucose sensing is also an important function of tanycytes and ATP plays an important role in tanycytes mediated glucose sensing within the hypothalamus (Frayling et al., 2011). Interestingly, DCL has recently been shown to reside in mitochondria where DCL knockdown results in decreased cytochrome C activity and ATP production (Verissimo et al., 2013) suggesting that DCL knockdown might affect glucose sensing by reducing ATP production. However, if glucose sensing is affected and if impaired glucose sensing affects bodyweight remains to be elucidated.

D2 in the hypothalamic tanycytes converts inactive T4 into active T3. DCL knockdown animals displayed higher hypothalamic D2 activity than WT littermates. Although D2 activity is increased after DCL knockdown, D2 mRNA expression is not affected in both hypothalamic PE and ARC-ME punches suggesting post-transcriptional regulation. D2 activity is amongst others regulated by ubiquitination via an ATP-dependent process (Gereben et al., 2000;Steinsapir et al., 2000). As DCL knockdown leads to reduction of ATP production (Verissimo et al., 2013), increased D2 activity in DCL knockdown mice might be the result of a blockade of ubiquitination-mediated D2 degradation.
Higher amounts of D2 activity would be expected to result in an increased production of local T3 and thereby lower hypothalamic TRH expression. This is however not the case, despite higher amounts of D2 activity, TRH mRNA expression in the PE area is similar in WT and DCL-KD mice.

Serum thyroid hormone levels were not affected by DCL knockdown either. Determination of hypothalamic T3 levels might be necessary in order to establish the function of the tanycytes with respect to local T3 production by D2. Since tanycytes in the ME create a private milieu within the hypothalamus by functioning as a barrier between peripheral blood and hypothalamus, T3 levels can be different between peripheral blood and hypothalamic

tissue (Rodriguez et al., 2010). Also measurement of TSH hormone levels might provide more useful information about the consequences of increased D2 activity on the HPT-axis function.

All together, DCL knockdown affects tanycytes functioning in thyroid hormone signalling. However, this effect seems to be limited to hypothalamic D2 activity. Although D2 activity is increased, THR mRNA expression and serum thyroid hormone concentrations are not affected by DCL knockdown. Whether increased D2 activity is a direct or indirect result of DCL knockdown remains to be elucidated.

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