MCT8 deficiency in Purkinje cells disrupts embryonic chicken cerebellar development

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Abstract

Inactivating mutations in the human SLC16A2 gene encoding the thyroid hormone transporter monocarboxylate transporter 8 (MCT8) result in the Allan–Herndon–Dudley syndrome accompanied by severe locomotor deficits. The underlying mechanisms of the associated cerebellar maldevelopment were studied using the chicken as a model. Electroporation of an MCT8-RNAi vector into the cerebellar anlage of a 3-day-old embryo allowed knockdown of MCT8 in Purkinje cell precursors. This resulted in the downregulation of the thyroid hormone-responsive gene RORα and the Purkinje cell-specific differentiation marker LHX1/5 at day 6. MCT8 knockdown also results in a smaller and less complex dendritic tree at day 18 suggesting a pivotal role of MCT8 for cell-autonomous Purkinje cell maturation. Early administration of the thyroid hormone analogue 3,5,3’-triiodothyroacetic acid partially rescued early Purkinje cell differentiation. MCT8-deficient Purkinje cells also induced non-autonomous effects as they led to a reduced granule cell precursor proliferation, a thinner external germinal layer and a loss of PAX6 expression. By contrast, at day 18, the external germinal layer thickness was increased, with an increase in presence of Axonin-1-positive post-mitotic granule cells in the initial stage of radial migration. The concomitant accumulation of presumptive migrating granule cells in the molecular layer, suggests that inward radial migration to the internal granular layer is stalled. In conclusion, early MCT8 deficiency in Purkinje cells results in both cell-autonomous and non-autonomous effects on cerebellar development and indicates that MCT8 expression is essential from very early stages of development, providing a novel insight into the ontogenesis of the Allan–Herndon–Dudley syndrome.

Introduction

Thyroid hormones (THs) play an important role in the functional organisation of the cerebellum. Distinct cerebellar cell types depend on adequate TH levels as well as a correct timing of TH access to coordinate key cellular events driving the development and functional maintenance of the cerebellum (Koibuchi 2008, Faustino & Ortiga-Carvalho 2014). As such, induction of hypothyroidism in animal models such as rat and chicken...
results in a number of morphological alterations, including impaired Purkinje cell (PC) dendritic arborisation, delayed granule cell (GC) migration from the external germinal layer (EGL) towards their final cortical position in the internal granular layer (IGL), delayed myelination and changes in synaptogenesis between cerebellar neurons and afferent neuronal fibres (Bouvet et al. 1987, Verhoelst et al. 2004, Anderson 2008, Shimokawa et al. 2014). The role of 3,5,3′-triiodothyronine (T3), the most active TH, in PC dendritogenesis has also been shown in vitro by the addition of T3 to cerebellar cultures resulting in an increase in PC dendritic branching and calibre in a dose- and time-dependent manner (Heuer & Mason 2003). Although these in vitro and in vivo studies clearly demonstrate the impact of TH deficiency on distinct neurodevelopmental processes, they have not addressed the underlying mechanisms of TH action in the developing brain.

As a prerequisite for TH action, intracellular concentrations of THs are tightly regulated by deiodinases and transmembrane transporters (Bianco & Kim 2006, Visser et al. 2008). In recent years, there has been a growing awareness of the requirement for monocarboxylate transporter 8 (MCT8) in neurodevelopment, through its role in the uptake of T3 in central neurons (Heuer et al. 2005, Wirth et al. 2009). Inactivating mutations in the human SLC16A2 gene coding for MCT8 have been identified as the cause of a syndrome of severe psychomotor retardation, known as the Allan–Herndon–Dudley syndrome (AHDS) (Dumitrescu et al. 2004, Friesema et al. 2004, Schwartz & Stevenson 2007). Based on a post-mortem study on a 30th gestational week MCT8-deficient foetus and an 11-year-old AHDS patient, it was suggested that an impaired TH supply to neural cells is the likely cause of the severe insult to the developing brain. Moreover, histological and biochemical abnormalities were compatible with a large number of clinical symptoms related to impaired cerebellar function (Lopez-Espindola et al. 2014).

Despite the generation and extensive study of different MCT8-deficient animal models (Dumitrescu et al. 2006, Heuer & Visser 2013, de Vrieze et al. 2014, Zada et al. 2014), the exact pathway by which MCT8 regulates cerebellar circuit organisation remains unclear. Mct8-deficient zebrafish display an underdeveloped cerebellum and exhibit clear locomotor deficits (de Vrieze et al. 2014, Zada et al. 2014). In mice, however, apart from phenocopying the altered serum TH profile, Mct8 knockout (KO) results in an apparently unaffected cerebellar structure (Trajkovic et al. 2007, Koibuchi 2009, Heuer & Visser 2013), coupled with normal performance in locomotor behavioural tests (Wirth et al. 2009). The differences between those animal models are likely to be explained by the presence of additional TH transporters, such as organic anion transporting polypeptide 1C1 (OATP1C1) (Mayerl et al. 2014) and large type amino acid transporter 2 (LAT2) (Wirth et al. 2009, Heuer & Visser 2013, Nunez et al. 2014) that can compensate for the reduced TH uptake at the rodent blood–brain barrier and neuronal membrane, respectively. Correspondingly, Mct8/Oatp1c1 double KO mice display neurological and locomotor deficits, which can be attributed to impaired TH transport across the blood–brain barrier (Fu et al. 2013, Mayerl et al. 2014). However, this model is less appropriate to assess the in vivo role of MCT8 at the level of the neurons themselves.

Recently, potential therapeutic agents have been identified that act as TH analogues, mimic TH transcriptional action and bypass MCT8 deficiency via (an) unidentified transporter(s) (Visser & Visser 2012). Although some of them were shown to have beneficial effects in Mct8 KO zebrafish larvae (Zada et al. 2014) and in Mct8/Oatp1c1 double KO mice (Horn et al. 2013, Kersseboom et al. 2014), a recent clinical trial in patients treated in the first year of infancy failed to improve mental status, probably because neurological damage was already irreversible at the onset of treatment (Verge et al. 2012). A better understanding of the function of MCT8 at the neuronal level during embryonic development is therefore an important objective. We turned to the chicken embryonic cerebellum, which provides an excellent in vivo model system for analysis of gene function on account of its accessibility, large embryo size and ease of delivery of highly efficient RNA interference (RNAi) tools (Rao et al. 2004, Stern 2005, Das et al. 2006). Moreover, chicken PCs abundantly express MCT8 mRNA during the first two-thirds of embryonic development (Delbaere et al. 2015). In this study, we used an in vivo knockdown strategy to silence early MCT8 expression in developing PCs and investigated the resulting cerebellar phenotype at different developmental stages.

**Methods**

**Animals**

All in vivo experiments were performed on chicken embryos (Gallus gallus, White Leghorn strain). Fertilised eggs were obtained from a commercial hatchery (Wyverkens, Halle, Belgium) and incubated in a forced draft incubator (38°C, 50% relative humidity, tilting at
a 45° angle every hour) until the desired developmental stage. Chicken embryonic development takes 21 days from the start of incubation (E0) till hatching at E20. All experimental protocols were approved by the Ethical Committee for Animal Experiments of the KU Leuven (P108/2012 and P005/2016) and were carried out in strict accordance with the European Communities Council Directive (2010/63 EU).

**In ovo electroporation**

Fertilised chicken eggs were incubated for three days prior to cutting a small window in the eggshell. Using a pressure micro-injector (Femtojet, Eppendorf), the fourth ventricle was completely filled with approximately 2 µg/µL plasmid vector (100–200 nL), solely or in combination: pRFPRNAiA (empty control red fluorescent protein (RFP) vector), MCT8-RNAi vector and pCaβ-eGFP-m5 (Yaneza et al. 2002). Injection solutions were supplemented with 0.1% Fast Green (Sigma-Aldrich) to allow visualisation. The RNAi vector for optimal gene silencing in chicken cells was kindly supplied by Prof. S. Wilson (University of Sheffield, UK). To develop the gene-specific RNAi vectors for knockdown of MCT8, the standard pRFPRNAiA vector was modified by cloning synthetic miRNA hairpins into the first hairpin site within the miRNA operon expression cassette, according to the protocol from Das et al. (2006). The sequence targeted was TTGCAGGATACTTCAACGATT, which the first nucleotide was changed to a guanine in antisense riboprobes as previously described (Das et al. 2006). This sequence was used to target MCT8 mRNA expression was first tested and confirmed in *in vitro* Co-expression of RFP by the vector allowed accurate in *in vivo* monitoring of transfection efficiency in target cells/tissues. Co-injection of the pCaβ-eGFP-m5 plasmid allowed high resolution imaging of the PC dendritic tree at E18. For electroporation, a small tungsten needle cathode (CUY614, 200 µm, Sonidel) and a larger plate anode (CUY661, 3 × 7 mm, Sonidel) were positioned on both sides of the cerebellar primordium. Six 50 ms pulses of 12 V with pulse intervals of 200 ms were applied using an ECM 830 Electro Square Porator (BTX, Harvard Apparatus). Eggs were subsequently resealed and incubated until the desired stage.

**TA3 treatment**

Injection and electroporation of the control RFP vector or the MCT8-RNAi vector was performed at E3 as described above. At E4 and E5, 25 µL of a 0.01 mM 3,5,3′-triiodothyroacetic acid (TA3) solution (Sigma-Aldrich; 5 ng/g egg weight diluted in 0.9% NaCl with 0.1% DMSO) or vehicle was administered by dripping on the area vasculosa, and eggs were further incubated until sampling at E6. At least 5 animals per condition were included. To define the treatment dose, we performed preliminary experiments on non-electroporated embryos administering different doses of TA3 or vehicle and selected the highest dose where no apparent adverse effects on early embryonic development were detected.

**Tissue sampling**

The cerebellum was collected at different stages: E6, E10, E14 and E18. Tissue samples were fixed overnight in 4% paraformaldehyde (PFA) in PBS (4°C), and then incubated overnight in 20% sucrose in PBS (4°C) for cryoprotection. Samples were finally embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands) and stored at −80°C until cryosectioning. 30 µm sections were made for analysis at E18, whereas 12 µm sections were made for all other analyses.

**In situ hybridisation**

*In situ* hybridisation (ISH) was performed on 12 µm cryosections using digoxigenin (DIG)-UTP-labelled antisense riboprobes as previously described (Hidalgo-Sanchez et al. 2005, Geysens et al. 2012). Antisense and sense probes for chicken MCT8 and RORα were synthesised following the manufacturer’s recommendations (Roche Diagnostics S.L., Applied Science). Probes were detected using an anti-DIG antibody (Roche Diagnostics) conjugated with alkaline phosphatase (AP). A combination of Nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Promega) was used as colorimetric substrate for the final AP reaction. Brightfield images were obtained with a Zeiss Imager Z1 microscope using the digital image processing software Zen 2012 (Carl Zeiss). For every gene, ISH was performed on at least 3 animals to confirm the observed level of expression.

**EdU pulse-labelling assay**

Injection and electroporation of the control RFP vector or the MCT8-RNAi vector was performed at E3 as described previously. Four hours prior to sampling at E10, 400 µL of a 500 µM 5-ethyl-2′-deoxyuridine (EdU) solution was administered by dripping on top of the embryo.
GC precursor proliferation was visualised on coronal sections using the commercial Click-IT EdU Alexa Fluor 488 Imaging Kit (Life Technologies). Fluorescent labelling was then visualised using a confocal microscope and analysed using the image processing software ImageJ (NIH). Per animal, the counting of EdU-positive cells was performed in a 100×200 µm² region selected in the dorsolateral cerebellum, above a widespread transfected area in the cerebral anlage. At least 5 animals per condition were included. The corresponding region in the untransfected side was used as an internal control.

**Immunohistochemistry**

Immunohistochemical stainings were performed using mouse-anti-LHX1/5 (1:200; 4F2, Developmental Studies Hybridoma Bank (DSHB), Iowa University, USA), mouse-anti-Paired Box protein 6 (PAX6) (1:200; DSHB) and mouse-anti-Axonin-1 (1:30; DSHB). Signal detection was achieved via Alexa488-conjugated secondary antibodies (Invitrogen) or the TSA FT amplification system (Perkin Elmer). Images were taken with the Zeiss Imager Z1 or confocal microscope, followed by analysis with ImageJ. The fraction of LHX1/S-positive cells within the RFP-expressing population was counted in an area of 200×200 µm² on two representative coronal sections for each animal. At least 30 transfected cells per cerebellum were included. The thickness of the Axonin-1-positive layer and the number of Axonin-1-expressing cells, defined as the number of DAPI-positive cells within the Axonin-1-positive layer, were analysed on at least 3 representative sections per animal, in two selected areas of 300×300 µm² per section. All stainings were analysed on at least 5 animals per condition.

**Morphological analysis of the PC dendritic tree**

To investigate the impact of early MCT8 deficiency on the late PC dendritic tree (E18), the control RFP vector or the MCT8-RNAi vector was electroporated in combination with the pCAβ-eGFP-m5 plasmid at E3. Digital Z-stack images of individual PCs expressing both plasmids and located in similar areas of 30 µm sagittal sections within folium V to VII were obtained with a confocal microscope. The total area covered by the dendritic tree as visualised by GFP expression for 5–6 individual PCs per animal was determined using ImageJ, on at least 5 animals per condition. The height of the PC was measured as the distance starting from the root of the primary dendrite up to the end of the longest dendrite. Additionally, PC soma area and the length of the primary dendrite were obtained. Sholl analysis was performed to determine the number of branching points, as well as the total amount of intersections per PC (Kutzing et al. 2010). The centre of the PC soma was chosen as the mid-point, and the number of branching points was measured every 10 µm, starting at 10 µm (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

**Analysis of EGL thickness and number of DAPI-positive cells in the ML**

EGL thickness was measured at E10 in the region described in 'EdU pulse-labelling assay’ section. Again, the corresponding region in the untransfected side was included as an internal control. To discriminate between a transient or persistent effect on EGL thickness, measurements were performed at E18 at a location featured by strong transfection in the inner region of folium V to VII. Per animal, two 100×100 µm² windows were drawn above a well-transfected area to count the number of DAPI-positive cells in the molecular layer (ML). At least 5 animals were included per condition.

**Statistics**

For all analyses, normal distribution of data was first tested by the Kolmogorov–Smirnov test. The fraction of LHX1/5-positive cells in the RFP-expressing population, the thickness of the Axonin-1-positive layer, the number of Axonin-1-expressing cells, EGL thickness at E18 as well as the number of DAPI-positive cells in the ML were all analysed by unpaired two-tailed Student’s t-test. As an internal control could be included, EGL thickness at E10, number of EdU-positive cells and results from the Sholl analysis were analysed by two-way repeated measures analysis of variance (ANOVA) followed by Bonferroni post hoc test to detect the significant differences between the means. Analysis for morphometric measurements of the dendritic tree area was performed by Mann–Whitney U test. All graphs are shown as scatterplots with mean± standard deviation (s.d.), and n representing the number of biological replicates. All statistical tests were performed in GraphPad Prism for Windows, version 5.00 (GraphPad Software), and differences were considered significant when P<0.05.
Results

Targeted electroporation of MCT8-RNAi at E3 knocks down MCT8 expression in PCs

For in vivo transfection of the chicken cerebellar primordium, the plasmid vector was electroporated at E3 into the cerebellar anlage, which at that stage, contains a large proportion of PC precursors (Luo & Redies 2004). The unidirectional electric field created by positioning the two electrodes on both sides of the cerebellum, allowed transfection of the left cerebellar lobe as confirmed at E6 (Fig. 1A). Analysis of the RFP signal on transverse sections showed successful transfection of cells throughout the ventricular zone and mantle layer, which contains early post-mitotic PCs (Fig. 1B and C). As a reliable chicken MCT8-antibody is still lacking, knockdown efficiency was evaluated using ISH. To rule out non-specific effects of electroporation, MCT8 expression was examined after electroporation with the control RFP vector (Fig. 1D). This revealed a pronounced MCT8 expression (Fig. 1E)

Figure 1
In vivo monitoring of transfection and validation of MCT8 knockdown. (A) Co-expression of RFP allows accurate in vivo monitoring of the transfection efficiency in the left cerebellar lobe, as checked at E6. (B) Scheme of the dissected hindbrain depicting the two cerebellar lobes, which are unfolded (white arrows in C) and coronally sectioned (dotted line). (C) The transfected side (TRS) is marked by a diffuse RFP signal, whereas the opposing side is untransfected (UTS). Scale bar: 200 µm. (D, E and F) The majority of transfected cells migrate from the ventricular zone towards the cerebellar mantle region (delineated by white dotted line). ISH analysis after electroporation with the control RFP vector reveals strong MCT8 mRNA expression (black arrow) at both the TRS (white arrow) and UTS and excludes the possible influence of the electroporation procedure. Scale bar: 50 µm (G, H and I) Transfection with the MCT8-RNAi vector results in a clear reduction of MCT8 mRNA expression (black dotted arrow) as seen at the TRS (white dotted arrow). Scale bar: 50 µm. (J, K and L) Pronounced RFP signal is still found in the more mature cerebellum at E18 in the soma as well as the dendritic tree of PCs, the latter being visualised by co-electroporation of the pCaj-eGFP-m5 plasmid. Scale bar: 20 µm. C, caudal; Cb, cerebellum; D, dorsal; F, frontal; H, hindbrain; IV, fourth ventricle; TRS, transfected side; UTS, untransfected side; V, ventral.
in RFP-expressing transfected mantle layer cells, which was identical to that of the untransfected lobe (Fig. 1F). By contrast, in embryos electroporated with the MCT8-RNAi vector (Fig. 1G), there was a clear reduction in MCT8 expression in differentiating PCs at the transfected side (Fig. 1H) compared to the untransfected side (Fig. 1I).

To confirm and visualise the persistent expression of the RNAi vector in PCs at E18, the vector was electroporated together with the pCAβ-eGFP-m5 plasmid carrying an eGFP coding sequence downstream of a β-actin promoter. At that stage, PCs are organised into a distinct monolayer with characteristic dendritic trees projecting into the

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Figure 2

Decreased RORα and LHX1/5 expression in MCT8-RNAi-transfected PCs at E6. (A and B) Post-mitotic PCs at the TRS of the E6 cerebellum show markedly reduced RORα mRNA expression (black arrow) in contrast to expression at the UTS, indicating a negative influence of MCT8 knockdown on early PC dendritic differentiation. Scale bar: 50 μm. (C, D and E) Immunolabelling of the PC-specific differentiation marker LHX1/5 (green) reveals strong overlap of LHX1/5- and RFP-expressing cells (visible as yellow cells) in the control condition (n = 6), as seen on detailed pictures of the TRS and UTS (black dotted boxes in C). (F, G and H) The fraction of LHX1/5-positive cells in the MCT8-RNAi-transfected population (n = 5) is significantly decreased as compared to that in the control RFP-transfected population (**P < 0.001, two-tailed Student's t-test). High magnification views (white boxes) show yellow cells as a result of strong overlap of LHX1/5 and RFP signal (white arrows). This overlap is greatly reduced in the MCT8-RNAi condition compared to the control condition. Scale bar: 20 μm. L, left; R, right; TRS, transfected side; UTS, untransfected side.
ML. After electroporation at E3, the vast majority of transfected PCs expressed both RFP and GFP (Fig. 1J, K and L), indicating the efficient uptake and expression of both plasmids at least until E18, well beyond E14 when MCT8 expression in PCs is downregulated (Delbaere et al. 2015). Importantly for our experimental design, plasmid expression in the cerebellar cortex was exclusively restricted to PCs with no fluorescence visible in either Bergmann glia or GCs. In addition, no RFP signal was found in the choroid plexus, ruling out the silencing of MCT8 at the blood–cerebrospinal fluid barrier as a cause of any observed phenotype (Supplementary Fig. 2).

MCT8-RNAi transfection suppresses PC markers

To assess the effects of MCT8 knockdown on early PC maturation, we examined the mRNA expression of the TH-responsive nuclear transcription factor retinoic acid receptor-related orphan receptor alpha (RORA), which is expressed in PCs undergoing early dendritogenesis (Boukhtouche et al. 2010). Transfection of the MCT8-RNAi construct strongly reduced RORA mRNA expression at E6 (Fig. 2A) when compared to expression within the presumptive PC layer of the untransfected side in the same embryo (Fig. 2B). Using immunolabelling, we then examined the LIM homeobox domain transcription factor 1/5 (LHX1/5), which in E6 cerebellum is specifically expressed in early PCs (Zhao et al. 2007). We consistently selected a corresponding region in the transfected and opposing untransfected side (Fig. 2C). Electroporation with the control RFP vector revealed that the majority of transfected cells were LHX1/5 positive (yellow cells in Fig. 2D). The untransfected side contained a similar number of LHX1/5-expressing cells (Fig. 2E), indicating normal early PC differentiation in the cerebellar anlage in both transfected and untransfected sides. By contrast, transfection with the MCT8-RNAi vector resulted in the unilateral loss of LHX1/5 expression (Fig. 2F and G). Intriguingly, right next to the RFP-expressing population, clear LHX1/5 expression was noticed in probably later born untransfected PCs, indicating that the effect is restricted to MCT8-RNAi-transfected cells. The effect of KD was quantified by comparing the fraction of LHX1/5-positive cells within the RFP-expressing population of the transfected sides (Fig. 2H). Expression of the MCT8-RNAi vector led to a significant reduction in presumptive PCs (P < 0.001).

Figure 3
TA3 administration at E4 and E5 partially rescues LHX1/5 expression in MCT8-RNAi-transfected cells at E6. (A, B and C) Administration of TA3 at E4 and E5 after electroporation of the control RFP vector at E3 did not increase the number of transfected LHX1/5-expressing cells at E6 (n = 7) as compared to the vehicle solution only (n = 5, P = 0.68, two-tailed Student’s t-test). (D, E and F) In contrast, administration of TA3 partially restored the fraction of LHX1/5-expressing cells within the MCT8-RNAi-transfected population (n = 8) as compared to vehicle (n = 10, ***P < 0.001, two-tailed Student’s t-test). Scale bar: 20 µm.
We then tested if supplementation with the TH analogue TA3, which can enter cells independently from MCT8, could rescue LHX1/5 expression in MCT8-RNAi-transfected cells. We first administered either vehicle or TA3 at E4 and E5 after electroporation of the control RFP vector at E3 (Fig. 3A and B). The fraction of LHX1/5-expressing cells within the RFP-expressing population at E6 was similar in both conditions (Fig. 3C: P=0.68). As expected, after vehicle administration, MCT8 knockdown by MCT8-RNAi transfection resulted in a reduction in LHX1/5-positive cells. However, this effect was significantly reduced after administration of TA3 (Fig. 3D and F: P<0.001), although there were still fewer LHX1/5-expressing cells than in control-transfected animals (compare Fig. 3C with F).

MCT8-RNAi transfection of PCs results in a reduction in GC precursor proliferation

The nuclear transcription factor RORα has been shown to regulate the expression of sonic hedgehog (SHH) in PCs, thereby promoting proliferation of GC precursors (Gold et al. 2007, Jetten 2009). As we found that MCT8 knockdown decreased RORα expression in early PCs, we also examined the expanding GC precursor population in the EGL, which in the chicken embryo can be detected from E8. The cerebellum was electroporated at E3 and examined in coronal sections at E10 using incorporation of the proliferation marker EdU (4-h pulse), which identifies the superficial EGL. Because the EGL is generated from the rhombic lip after E6, RFP is excluded from the proliferative EGL after electroporation with our RFP-expressing vectors.

Figure 4
Reduced GC precursor proliferation and EGL thickness in the E10 cerebellum after MCT8-RNAi transfection. (A) EdU-positive cells (green) identify the EGL in the dorsolateral cerebellum (white arrows), whereas PCs expressing the control RFP vector or the MCT8-RNAi vector (red) reside exclusively in the underlying cerebellar anlage. Scale bar: 200 µm. (B, C, D and E) High magnification views of TRS and UTS (white dotted boxes in A) clearly show the EdU-positive cells in the EGL (region enclosed by white dotted lines in B, C, D and E) in both conditions. Scale bars: 20 µm. (F and G) EGL thickness as well as the number of EdU-positive cells in the EGL is similar in the UTS of both conditions and in the TRS of the cerebellum transfected with the control RFP vector, but is significantly lower after transfection with the MCT8-RNAi vector (n=5–7, **P<0.01, two-way repeated measures ANOVA, followed by a Bonferroni post hoc test). (H and I) PAX6 expression (green) is less prominent at the TRS in the MCT8-RNAi condition. Scale bar: 50 µm. Cb, cerebellum; IV, fourth ventricle; TRS, transfected side; UTS, untransfected side.
at E3 (Fig. 4A). In cerebellar lobes transfected with the control RFP vector (Fig. 4B), there was no reduction in the number of EdU-positive cells or the EGL thickness compared to the untransfected side (Fig. 4C). However, when transfected with the MCT8-RNAi vector, both the thickness of the EGL and the number of proliferating cells were clearly reduced at the transfected compared to the untransfected side (Fig. 4D and E). The overall density of DAPI-positive cells in the EGL was also reduced (data not shown). Quantification of the number of EdU-positive cells (Fig. 4F) and the EGL thickness (Fig. 4G) supports a significant reduction in cell division within the EGL ($P<0.01$). In addition, immunolabelling of PAX6, a marker for both GC precursors and post-mitotic GCs (Chung et al. 2010) was reduced after MCT8-RNAi electroporation (Fig. 4H and I). Altogether, this reflects a decreased GC precursor pool expansion and a stalled maturation of GCs in the EGL.

**MCT8-RNAi transfection of PCs results in a retardation of GC maturation**

To investigate whether MCT8 knockdown delays GC maturation, we examined the time course of depletion of the EGL as GC precursors undergo terminal differentiation and migrate to the IGL. In the normal E18 chicken cerebellum, the EGL has decreased significantly in thickness (Verhoest et al. 2004). After electroporation at E3, transfected PCs are found in a monolayer in the cerebellar folia and sagittal sections show a clearly defined PCL, ML and EGL. In contrast to a decreased thickness at E10, the EGL overlying PCs transfected with MCT8-RNAi vector was thicker than that in controls (Fig. 5A and D). Within the ML, a scattering of DAPI-labelled cells correspond to ML interneurons and migrating post-mitotic GCs. The number of DAPI-positive cells in the ML at E18 was larger (Fig. 5E) compared to the control condition (Fig. 5B). Quantification of EGL thickness (Fig. 5G) and number of neurons in the ML (Fig. 5H) revealed significant increases ($P<0.05$ and $P<0.01$, respectively). As post-mitotic GCs start their migration towards the IGL, they also express Axonin-1 (Fig. 5C) (Baeriswyl & Stoeckli 2008). The Axonin-1-positive layer was thicker (Fig. 5F and I; $P<0.01$) and the number of Axonin-1-expressing cells was higher after MCT8 knockdown (Fig. 5F and J; $P<0.01$). This combination of features is consistent with a stalled maturation of GCs and a perturbed inward migration to form the IGL.

**MCT8-RNAi transfection results in smaller and less complex PC dendrites at E18**

To examine if our treatment led to persistent defects in the mature PC phenotype, we performed a morphometric

![Figure 5](image-url)
MCT8 deficiency in humans results in a strongly disrupted cerebellar development, accompanied by impaired locomotor function. As Mct8 KO mice do not display any cerebellar abnormalities, and Mct8/Oatp1c1 double KO mice cannot be used to assess the function of MCT8 directly at the neuronal level, new models are required to further unravel the mechanisms underlying MCT8-deficient brain development (Wirth et al. 2014). Here, we used the chicken cerebellum as a model to investigate the effects of early MCT8 deficiency on PC differentiation in more detail and find out how this affects cerebellar development.

PC dendritic maturation is disrupted by early knockdown of MCT8

Dendritogenesis is one of the processes most severely affected by early MCT8 deficiency in PCs. The stunting of dendritic arborisation in our model was less pronounced than that in studies in TH-deficient rodent and chicken brain (Bouvet et al. 1987, Anderson 2008), where TH availability was globally disrupted. Despite reduction in dendritic complexity, the depth of the ML was not noticeably altered in our model (compare Fig. 5A and D). This suggests a specific failure of dendritic trees to undergo a normal programme of developmental branch formation rather than an overall stunting of PC growth. As MCT8 is highly expressed in PCs during the first two weeks of chicken embryonic development (Delbaere et al. 2015), our hypothesis is that the consequences of TH transporter disruption are PC-autonomous and MCT8 is crucial during early stages of development. A similar analysis of dendritic arborisation in PCs transfected at E3 with the control RFP vector or the MCT8-RNAi vector (Fig. 6A and B). MCT8-RNAi transfection resulted in a visibly reduced elaboration of the primary dendrite, reflected in a significantly smaller dendritic tree area (Fig. 6C: \(P<0.01\)). This was accompanied by a reduction in the length of the longest dendrite (Fig. 6D: \(P<0.05\)). MCT8-RNAi-transfected PCs showed a reduced total amount of intersections as determined by Sholl analysis (Fig. 6E: \(P<0.05\)). Further analysis confirmed this was due to a decrease in number of branching points distal to the soma (70, 80 and 90\(\mu m\)) in MCT8-RNAi-transfected PCs (Fig. 6E: \(P<0.001, P<0.001\) and \(P<0.05\), respectively). The length of the primary dendrite and size of the cell soma were not affected (data not shown).

Discussion

MCT8 deficiency in humans results in a strongly disrupted cerebellar development, accompanied by impaired locomotor function. As Mct8 KO mice do not display any cerebellar abnormalities, and Mct8/Oatp1c1 double KO mice cannot be used to assess the function of MCT8 directly at the neuronal level, new models are required to further unravel the mechanisms underlying
dendritic phenotype was observed in mice expressing a dominant-negative TH receptor mutation solely in PCs, supporting a role for intrinsic TH signalling in dendrite elaboration (Yu et al. 2015). As in human (Wirth et al. 2009, Bernal et al. 2015), MCT8 deficiency in chicken PCs most likely cannot be compensated by the presence of additional TH transporters as confirmed by ISH (Delbaere et al. 2015). This lack of redundancy is therefore a reasonable explanation for the severe consequences of MCT8 mutations/knockdown for cerebellar development in contrast to the situation in rodents.

One likely mediator of TH-dependent PC dendritic differentiation is RORα (Boukhtouche et al. 2010). In staggerer mice, carrying a mutation in RORα, there is a reduction in cerebellar size, loss of PCs and GCs and retention of immature properties of PCs late into development, including stunted dendritic arbours lacking distal spiny branchlets (Gold et al. 2007, Jetten 2009). This ligand-dependent nuclear transcription factor is highly expressed in early post-mitotic PCs (Jetten 2009, Boukhtouche et al. 2010, Delbaere et al. 2015), and we have shown here that its expression in the chicken embryo is MCT8 dependent. Additionally, a concomitant loss of LHX1/5-positive expression suggests fundamental changes in PC specification. Zhao and coworkers showed that early post-mitotic PC differentiation is compromised in mutant mice lacking Lhx1/5, leading to a substantial loss in PCs (Zhao et al. 2007), raising the possibility of some degree of PC loss after MCT8 disruption in our model. Using the bioinformatical tool PROMO to identify putative transcription factor binding sites (Messeguer et al. 2002, Farre et al. 2003), a candidate TH receptor α1 response element was identified at 220 and 330 bp of the chicken LHX1 and LHX5 coding sequence, respectively. Correspondingly, the TH receptor α gene (THRA) is strongly expressed in chicken embryos at these early stages of PC development (Vancamp P, Delbaere J & Darras VM, unpublished observations), suggesting that impaired T3-mediated transcriptional action could underlie the MCT8-deficient phenotype. The fact that the effects of targeted MCT8 deficiency in the chicken embryo are similar to those seen in mice expressing a dominant-negative TH receptor in PCs (Fauquier et al. 2014, Yu et al. 2015) clearly stresses the importance of MCT8 at the neuronal level independent of its role in TH transport across brain barriers.

It has been shown that TA3 can mimic T3 action in the brain and that its uptake in neuronal and fibroblast cell lines of AHDS patients is MCT8 independent (Messier & Langlois 2000, Kersseboom et al. 2014). We found that TA3 administered at E4–E5 is capable of partially rescuing the LHX1/5 phenotype confirming that the effects of MCT8 knockdown can be attributed to the reduced availability of TH. Our results lend support to arguments made elsewhere (e.g. Visser et al. 2016) for both early screening for MCT8 mutations and their treatment through generation of early therapeutic interventions that may rescue neuronal differentiation before irreversible neurological damage occurs.

Figure 7
Theoretical model summarising the importance of MCT8 in PCs for cell-autonomous and non-autonomous development. During cerebellar development, T3 is taken up by MCT8-expressing Purkinje cells (PCs). T3 excess is prevented by deiodinase type 3 (D3)-dependent inactivation, whereas a sufficient amount can bind to the TH receptor α1 (TRα1), ultimately regulating the expression of TH-responsive factors like RORα and LHX1/5, which govern cell-autonomous PC differentiation. In the absence of MCT8, TA3 can enter the PC via a presently unknown transporter and mimic T3 action. The nuclear transcription factor RORα also affects the downstream expression of sonic hedgehog (SHH), a diffusible factor that stimulates granule cell precursor (GCP) proliferation in the external germinal layer (EGL). GCPs and post-mitotic granule cells (GCs) express PAX6, whereas GCs in the initial stage of migration express Axonin-1 (Ax-1) and mature during inward migration to form the internal granular layer.
MCT8-deficient PCs disrupt GC precursor proliferation and post-mitotic GC maturation

Our electroporation strategy targeted a large proportion of cerebellar cells at E3. However, due to the dynamics of GC proliferation, the EGL was not transfected allowing to assess the non-autonomous effects of PC disruption on GC precursor development. In addition, neither Bergmann glia nor choroid plexus, both of which are important mediators of normal cerebellar growth, were transfected in this strategy. GC precursors assemble as transient superficial EGL comprising dividing cells. Post-mitotic GCs then migrate inward to form the IGL (Wingate 2001). PCs regulate the proliferative expansion of GC precursors in the EGL by the production of SHH (Goldowitz & Hamre 1998, Lewis et al. 2004), a downstream target of RORs, and levels of SHH-dependent GC proliferation affect cerebellar size (Butts et al. 2014). Our results indicate that TH-dependent processes in PCs affect non-autonomous aspects such as GC proliferation and differentiation. EGL thickness, proliferation and GC migration were affected downstream of MCT8 knockdown in PCs. Thus, although it is thought that GCs have an autonomous requirement for TH during their late maturation events, as shown by the delay in GC migration in hypothyroid rat and chicken (Verhoelst et al. 2004, Shimokawa et al. 2014), they are also indirectly dependent for normal development on TH signalling in PCs (Fig. 7). Similar non-autonomous effects on GC maturation were also demonstrated in mice expressing a dominant-negative TH receptor in PCs (Fauquier et al. 2014, Yu et al. 2015).

Changes in PC dendritogenesis might also be secondarily affected by both reduced signalling of the EGL (Boukhtouche et al. 2010) and a reduction in parallel fibre inputs (Sotelo & Dusart 2009). Although RORα offers an attractive cell-autonomous explanation for PC dysgenesis, the role of GC–PC signalling cannot be discounted. In future studies, combinatorial electroporation of the cerebellum at different stages (early for PC and late for GC) and the application of cell-specific enhancers to drive gene expression raise the possibility of independently regulating the expression of genes within PC and GC populations to finely dissect these cell–cell interactions.

Conclusion

In the present study, the combination of RNAi and in vivo electroporation in the chicken embryo has proved a valuable model to unravel the functional involvement of the MCT8 transporter in early neural circuit organisation in the cerebellar primordium. Our results suggest that the expression of MCT8 in PCs is essential from the very early stages of development and underline the importance of both cell-autonomous and non-autonomous signalling converging on common pathways for cerebellar development. Restoring early T₃ transcriptional action in PCs in MCT8 deficiency, the cause underlying AHDS, may therefore improve coordinated intercellular cross-talk, thereby contributing to global cerebellar development.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-16-0323.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J D and P V designed the research studies in collaboration with R W, M G and V D, who have expertise in cerebellar development and the techniques used in this study. S V H and N B contributed by designing and validating the RNAi vectors, as well as designing the probes for ISH. J D and P V conducted all further experiments, acquired and analysed all data and were responsible for writing the manuscript. V D was the supervisor of this research study.

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