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Gustafsson, Julie Ry; Katsioudi, Georgia; Degn, Matilda; Ejlerskov, Patrick; Issazadeh-Navikas, Shohreh; Kornum, Birgitte Rahbek

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DNMT1 regulates expression of MHC class I in post-mitotic neurons

Julie Ry Gustafsson¹, Georgia Katsioudi¹, Matilda Degn¹, Patrick Ejlerskov², Shohreh Issazadeh-Navikas² and Birgitte Rahbek Kornum¹,³,⁴ *

Abstract
Major Histocompatibility Complex I (MHC-I) molecules present cellularly derived peptides to the adaptive immune system. Generally MHC-I is not expressed on healthy post-mitotic neurons in the central nervous system, but it is known to increase upon immune activation such as viral infections and also during neurodegenerative processes. MHC-I expression is known to be regulated by the DNA methyltransferase DNMT1 in non-neuronal cells. Interestingly DNMT1 expression is high in neurons despite these being non-dividing. This suggests a role for DNMT1 in neurons beyond the classical re-methylation of DNA after cell division. We thus investigated whether DNMT1 regulates MHC-I in post-mitotic neurons. For this we used primary cultures of mouse cerebellar granule neurons (CGNs). Our results showed that knockdown of DNMT1 in CGNs caused upregulation of some, but not all subtypes of MHC-I genes. This effect was synergistically enhanced by subsequent IFNγ treatment. Overall MHC-I protein level was not affected by knockdown of DNMT1 in CGNs. Instead our results show that the relative MHC-I expression levels among the different MHC subtypes is regulated by DNMT1 activity. In conclusion, we show that while the mouse H2-D1/L alleles are suppressed in neurons by DNMT1 activity under normal circumstances, the H2-K1 allele is not. This finding is particularly important in two instances. One: in the context of CNS autoimmunity with epitope presentation by specific MHC-I subtypes where this allele specific regulation might become important; and two: in amyotrophic lateral sclerosis (ALS) where H2-K but not H2-D protects motor neurons from ALS astrocyte-induced toxicity in a mouse model of ALS.

Keywords: MHC class I, Post mitotic neurons, DNMT1, HLA, H2, Autoimmune neurodegeneration

Introduction
Involvement of the immune system is a common trait in several neurodegenerative diseases and autoimmune diseases which affect the central nervous system (CNS) [1, 2], and the etiology of these neurological disorders is often not determined. Many autoimmune diseases are associated with certain Major Histocompatibility Complex (MHC) alleles, including type 1 narcolepsy that has a very strong association with HLA-DQB1*06:02 and are also associated with certain HLA class I alleles [3, 4]. MHC class I signaling has also been suggested to play a role in neurodegenerative disorders such as Parkinson's disease and Amyotrophic Lateral Sclerosis (ALS) [5, 6]. Our goal with the current study was to thus study the regulation of MHC class I (MHC-I) in neurons.

The CNS was originally considered an immune privileged organ [7], however this perception is no longer prevalent. We now know that peripheral T cells can enter the CNS, both when recruited by chemokines upon viral infection of neurons [8] and also as routine monitoring where memory T cells can be reactivated by antigen-presenting dendritic cells in the perivascular and subarachnoid space [9]. A range of autoimmune CNS diseases involving neuronal destruction is likely mediated by CD8+ T cells, as suggested by the presence of CD8+ T cells in affected brain areas in Rasmussen's encephalitis [10], paraneoplastic neurological degeneration [11], and Multiple Sclerosis [12] which is otherwise considered a CD4+ T cell driven disease. CD8+ T cell mediated neuronal destruction depends however on activation by interaction with MHC-I molecules, which are not expressed on healthy post-mitotic neurons under non-pathological conditions [13, 14]. MHC-I is in contrast...
expressed in neurons at early developmental stages but gets downregulated in adulthood [15–17]. During development, MHC-I molecules have been shown to negatively regulate synaptic density [18], and the establishment of cortical connections [19]. In adulthood, the same functions seem to play a role under different pathological conditions. In the middle cerebral artery occlusion (MCAO) model of stroke, MHC-I knock out mice (H2-Kb and H2-Db double knock out) have smaller infarct areas and better behavioral recovery [20]. In contrast, under conditions of axonal injury, MHC class I molecules seem to play a beneficial role and have for instance been found to protect specific synaptic contacts from detachment [21].

MHC-I molecules are readily induced on neurons upon interferon gamma (IFNγ) treatment [13, 14], and is also regulated by neuronal activity during development [16, 22, 23]. Several studies have shown that when MHC-I is induced on neurons, CD8+ T cells can recognize and kill the neurons in culture are post-mitotic [28, 29]. Our specific aim was thus to investigate whether DNMT1 or the other DNA methyltransferases DNMT3a and DNMT3b, in human SK-N-AS cells would affect expression levels of HLA-A and HLA-B. We evaluated the gene expression compared to non-targeting siRNA (baseline, Additional file 1: Figure S1A-F) for each of the genes of interests HLA-A, HLA-B, and β2M, as well as of the targeted genes DNMT1, DNMT3a and DNMT3b, upon treatment for 72 h with either non-targeting siRNA, siRNA against DNMT1, siRNA against DNMT3a, or siRNA against DNMT3b (Additional file 1: Figure S1). Specific downregulation of the targeted DNMT was obtained for all three DNMTs. DNMT1 gene expression was decreased by 43% upon treatment with siRNA against DNMT1 (p = 0.019 versus non-targeting siRNA) (Additional file 1: Figure S1A). This resulted in a significant upregulation of HLA-A expression by 50% (p = 0.0061 versus non-targeting siRNA) (Additional file 1: Figure S1D). We observed a non-significant tendency towards upregulation of HLA-B and β2M as well. DNMT3a levels was decreased by 32% upon treatment with siRNA against DNMT3a (p = 0.017 versus non-targeting siRNA) (Additional file 1: Figure S1B), and DNMT3b levels was decreased by 48% upon treatment with siRNA against DNMT3b (p = 0.029 versus non-targeting siRNA) (Additional file 1: Figure S1C). Neither of these changes resulted in significant effects on HLA-A, HLA-B or β2M, even though we did see tendencies towards upregulation when DNMT3b was knocked down.

To validate whether a similar regulation of MHC-I genes also existed in mouse cells, we evaluated the expression of H2 genes in mouse N2a neuroblastoma cell lines upon 72 h of treatment with the inhibitor of DNMT1 and DNA methylation 5-aza-2-deoxycytidine (5-aza). The N2a cell line is derived from strain A mice and thus carries the HLA-I subtypes H2-Kk, H2-Dd, and H2-Ld. We used 5-aza rather than PCA in the N2a cell lines, as 5-aza has been used regularly in N2a...
HLA-I subtypes

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expression was also regulated by DNMT1 in

We next aimed at addressing whether MHC-I gene expression of H2-Kd (d), and H2-D1/L (e) in mouse neuroblastoma cell line N2a. 72 h treatment with IFNγ (0.2 ng/μL) in SK-N-AS and 0.5 ng/μL in N2a significantly increased gene expression of HLA-A (a), HLA-B (b), H2-K1 (c) and H2-D1 (d). H2-D1/L gene expression was increased, but not significantly (p = 0.08) (e). Bars represent mean +/- S.E.M., N = 3. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. untreated in a One-Way ANOVA with Dunnett’s post hoc test.

Knockdown of DNMT1 increase MHC-I gene expression in post-mitotic mouse CGNs

We next aimed at addressing whether MHC-I gene expression was also regulated by DNMT1 in post-mitotic neurons. We used CGNs for this purpose. We derived the CGNs from Balb/C mice that carry the HLA-I subtypes H2-Kd, H2-Dd, and H2-Ld. All known small molecule inhibitors of DNA methyltransferases are developed for dividing cells [35], and as expected PCA and 5-aza did not have any significant effect on H2 genes in CGNs (data not shown). Thus we decided to investigate regulation of H2 genes in CGNs by knocking down DNMT1, DNMT3a and DNMT3b, one at a time.

We evaluated the gene expression compared to untreated CGNs (baseline 1, Fig. 2a-f) for each of the genes of interests β2M, H2-K1, H2-D1 and H2-D1/L, as well as of the targeted genes DNMT1, DNMT3a and DNMT3b, upon treatment for 72 h with either non-targeting siRNA, siRNA against DNMT1, siRNA against DNMT3a, or siRNA against DNMT3b (Fig. 2a-e, bars from left to right on X-axis). In baseline conditions the relative expression levels of the different genes differed markedly in CGNs. When compared to the endogenous expression of ACTB and GAPDH, expression levels were: β2M = 19.0%, H2-K1 = 15.3%, H2-D1 = 8.3%, H2-D1/L = 29.5%. IFNγ was used as a positive control for HLA/H2 induction in all experiments, and induced all HLA and H2 genes significantly, except for H2-D1/L which was induced 3.06 fold compared to untreated N2a cells (p = 0.06) (Fig. 1a-e). These results demonstrate that MHC-I gene expression is regulated by DNMT1 in both the human SK-N-AS and the mouse N2a neuroblastoma cell lines.
As observed in the neuroblastoma cell lines, the different genes encoding MHC-I responded differently to the treatments. The level of β2M was unaffected by any of the treatments (Fig. 2d). There was no signal from H2-K1 in any of the samples, suggesting that this gene is not expressed in post-mitotic CGNs, and that it cannot be induced by changes in DNMT activity. Signal from the H2-D1 probe was heavily influenced by non-targeting siRNA (Fig. 2e). Although treatment with DNMT1 siRNA did cause a 2-fold increase in H2-D1 gene expression compared to untreated CGNs, this effect was not significantly different from the H2-D1 expression levels upon treatment with non-targeting siRNA (Fig. 2e). Finally, treatment with siRNA against DNMT1 caused a 1.74 fold increase in H2-D1/L compared to untreated CGNs (Fig. 2f), which was also significantly different from the effect of non-targeting siRNA on H2-D1/L (p < 0.0001) (Fig. 2f). Knockdown of DNMT3a or DNMT3b did not affect the expression levels of H2-D1/L showing that the effect is specific for DNMT1.

Even though Purkinje cells are notoriously difficult to culture, CGN cultures can contain a small number of Purkinje cells. We wanted to test the possibility that the observed changes was caused by large changes in the small fraction of Purkinje cells. We therefore measured the level of calbindin, a well known marker of Purkinje neurons, in our cultures. Calbindin was only borderline detectable by qPCR, and we did not observe any significant differences between the different treatments (data not shown).

**Knockdown of DNMT1 decrease markers of synaptic function in post-mitotic mouse CGNs**

Since MHC-I plays a role in synaptic plasticity during development [18, 19], and further has been suggested to also aid in synaptic pruning in the adult brain [21], we speculated whether the mechanism for upregulation of H2-D1/L following DNMT1 knock down, could be linked to changes in synaptic function in the cultures. To address this we measured two markers of synaptic function: synaptophysin and the vesicular glutamate transporter 1 (VGlut1). Synaptophysin is important for efficient synaptic vesicle trafficking [36] and for activity-dependent synapse formation [37], while VGlut1 is involved in glutamate vesicular release in mature CGNs [38]. In the experiment, DNMT1 gene expression was decreased by 76% upon treatment with siRNA against DNMT1 compared to non-targeting siRNA (n = 5, p = 0.0011) (Fig. 3a), while H2-D1/L was increased 1.76 fold compared to non-targeting siRNA treatment of the CGNs (p = 0.049) (Fig. 3b). In the same experiment, DNMT1 siRNA treatment caused a 89% downregulation of synaptophysin (p = 0.0022) and VGlut1 was decreased by 92% (p = 0.0018) (Fig. 3c).
and d). These results show that knock down of DNMT1 significantly affects expression of synaptic markers, while knock-down of DNMT3a or DNMT3b has no effect.

**Blockade of DNMT1 acts in synergy with IFNγ to induce MHC-I gene expression**

IFNγ is a known inducer of MHC-I expression on neurons. It has been reported that IFNγ and the demethylating agent zebularine work in synergy to increase expression of indoleamine 2,3-dioxygenase 1 (IDO1) - another gene known to be induced by IFNγ [39]. We therefore next examined whether the same synergy existed for MHC-I in neurons. To investigate a possible synergistic effect in N2a cells, we lowered the dose of 5-aza to 0.5 μM, and the time of IFNγ treatment to 24 h to avoid ceiling effects that could occur with the 72 h treatment. Due to this short timeframe of IFNγ treatment, we did not observe any induction of H2 genes with IFNγ alone (Fig. 4a-c), as otherwise observed when cells were treated for 72 h (Fig. 1c-e). The low dose of 5-aza alone significantly induced H2-D1/L (p = 0.0007) (Fig. 4c), whereas neither H2-K1 nor H2-D1 were increased upon treatment with 5-aza alone (Fig. 4a-b). When IFNγ and 5-aza were combined, H2-K1 gene expression was increased by 2 fold compared to untreated (p = 0.0007), and this increase was significantly different from both IFNγ alone (p = 0.0003) and 5-aza alone (p = 0.0023) (Fig. 4a). H2-D1 was not induced significantly by the combined treatment with IFNγ and 5-aza (Fig. 4b). Finally, the combined treatment with IFNγ and 5-aza increased H2-D1/L 3.54 fold change compared to untreated (p = 0.001), which was significantly different compared to untreated (p = 0.0007), from the 2.48 fold induction observed upon treatment with 5-aza alone (p = 0.038), as well as from IFNγ treatment alone (p = 0.001) (Fig. 4c). Taken together these results revealed that blockade of

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**Fig. 3** Knockdown of DNMT1 decrease markers of synaptic function in post-mitotic mouse CGNs. Shown is the fold change in gene expression of DNMT1 (a), H2-D1/L (b), synaptophysin (c) and VGlut1 (d) following 72 h siRNA treatment of CGN cultures. Baseline indicate gene expression level in CGNs treated with non-targeting siRNA. Bars represent mean +/- S.E.M, N = 5 from two independent experiments. Stars illustrate effects that are significantly different from non-targeting siRNA group. * p < 0.05, ** p < 0.01, multiplicity corrected p-values in a One-Way ANOVA with Dunnett’s post hoc test.
DNA methyltransfer acts in synergy with IFNγ to induce expression of some but not all H2 alleles in neurons.

We next addressed synergy between IFNγ treatment and knockdown of DNMT1 in CGNs. We chose to focus on DNMT1, as knockdown of DNMT1, rather than DNMT3a or DNMT3b, caused an increase in H2-D1/L. IFNγ treatment was used as positive control for H2 gene induction in CGNs and treatment with 0.005 ng/μl IFNγ for 72 h significantly increased H2-D1 and H2-D1/L gene expression (Fig. 4d-g). Importantly, IFNγ did not increase H2-K1 gene expression to detectable levels. We tested two different treatment combinations and timings. Either IFNγ was added simultaneously with the siRNA against DNMT1 (Fig. 4d-e), or IFNγ was added for 72 h following treatment with siRNA against DNMT1 (Fig. 4f-g). When IFNγ was added simultaneously with the siRNA against DNMT1, H2-D1 (Fig. 4d) and H2-D1/L (Fig. 4e) were induced to the same extent, as when IFNγ was added in combination with non-targeting siRNA, indicating no synergy. This was in contrast to the treatment where IFNγ was added consecutive to siRNA against DNMT1. In this case H2-D1/L was induced 10.78 fold compared to untreated, which was significantly different from the 8.05 fold induction obtained upon treatment with non-targeting siRNA and then IFNγ (p = 0.0067) (Fig. 4g), suggesting synergy between knockdown of DNMT1 and consecutive treatment with IFNγ.

Knockdown of DNMT1 does not increase total MHC-I protein expression in post-mitotic mouse CGNs

We next examined protein expression of MHC-I protein upon knockdown of DNMT1 in CGNs by immunofluorescence (Figs. 5, 6 and 7) and flow cytometry (Fig. 7). As
expected, MHC-I protein was undetectable by immuno-fluorescence in untreated CGNs (Fig. 5a) and in CGNs treated with non-targeting siRNA for 72 h (Fig. 5c), but clearly visible on neurons upon treatment with 0.005 ng/μl IFNγ for 72 h (Fig. 5b). As H2-K1 gene expression was not induced to detectable levels upon IFNγ treatment, the detected MHC-I protein are of the subtypes H2-D1 and/or H2-L. In our experimental group, treatment of CGNs with siRNA against DNMT1 for 72 h did not induce MHC-I protein on the neurons (Fig. 5d).

We then addressed whether the sequential treatment of CGNs with siRNA against for DNMT1 and then IFNγ, caused a synergistically effect on MHC-I protein levels as detected by immuno-fluorescence, as was the case for gene expression. We observed a clear MHC-I protein signal on the neurons treated with IFNγ (Fig. 6a), as well as for CGNs treated with non-targeting siRNA for 72 h plus additional 72 h treatment with IFNγ (Fig. 6b), but the data did not show any additional MHC-I protein upon treatment with siRNA against DNMT1 for 72 h plus additional 72 h treatment with IFNγ (Fig. 6c). These results indicate that the synergistic effect we observed on gene expression level, does not translate into overall MHC-I protein levels, but rather shifts the balance between expressed MHC-I subtypes.

Transfection efficiency is not always complete in post-mitotic cells and can vary between the individual cells within the culture, and we therefore next aimed at investigating MHC-I protein expression by immunofluorescence on neurons that had taken up high amount of the siRNAs against DNMT1. We therefore transfected CGNs with a mix of the previously used SMARTpool against DNMT1 and a Cy3-labelled single siRNA against DNMT1, and performed immunofluorescence for MHC-I and β-III-tubulin in conjunction with detection of the Cy3 signal from the siRNA mixture (Fig. 7). Only 3:7 siRNA molecules were Cy3-labelled to ensure that only cells that had taken up a significant amount of siRNA were visibly labelled. This approach also allowed us to use the original SMART pool in combination with the Cy3-labelled siRNA to secure consistency. Using this
procedure, the Cy3-siRNA was indeed detectable on neurons (arrow), but the neurons positive for Cy3-siRNA did not display MHC-I protein (Fig. 7c), confirming our results. Untreated CGNs expressed no MHC-I protein (Fig. 7a), whereas 72 h treatment with 0.005 ng/μl IFNγ induced MHC-I protein on the neurons (Fig. 7b). We supplemented the immunofluorescence with flow cytometry studies, to be able to examine all neurons in the culture in a systematic way (Fig. 8). We gated the neurons by size (Fig. 8a), and viability (Fig. 8b), and then analyzed the β-III-tubulin-positive population (Fig. 7c) for extracellular MHC-I protein expression (Fig. 8d). Histogram of MHC-I signal shows overlay of untreated CGNs (purple), CGNs treated with non-targeting siRNA (green), CGNs treated with siRNA against DNMT1 (red) and CGNs treated with siRNA against DNMT1 and IFNγ for 72 h (blue) (Fig. 8d). IFNγ treatment induced a MHC-I + population of neurons (49%) with a median fluorescence intensity of 360, whereas treatment of CGNs with siRNA against DNMT1 did not induce extracellular MHC-I on the neurons (Fig. 8d) confirming our immunofluorescence data. We next addressed whether synergy between knockdown of DNMT1 and IFNγ existed on the MHC-I protein level. Histograms of MHC-I signal in Fig. 8e-f show overlay of CGNs treated with non-targeting siRNA and IFNγ (light green), and CGNs treated with siRNA against DNMT1 and IFNγ (pink), and Fig. 8e shows the results from the cells receiving siRNA and IFNγ simultaneously (like Fig. 4d-e), whereas Fig. 8f shows the results from the cells receiving IFNγ after the siRNA (like Fig. 4f-g and Fig. 6). The percentage of neurons displaying MHC-I signal, as well as the median fluorescence intensity of the PE-MHC-I signal, were in both instances comparable between the cells receiving non-targeting siRNA and IFNγ, and the cells receiving siRNA against DNMT1 and IFNγ, indicating no synergy with regard to total MHC-I protein level.

**Discussion**

In contrast to other nucleated cells, most post-mitotic neurons do not express MHC-I under non-pathological
conditions [13, 14]. However, during several pathological conditions MHC-I expression can be induced in neurons where they are believed to play an active role in the disease process. This is the case for both autoimmune diseases, where neurons can be killed by CD8+ T cells [24–26], and for neurodegenerative disorders [5, 6]. Knowledge on the molecular mechanisms regulating neuronal MHC-I might thus be of relevance in a range of CNS diseases. We chose to address whether MHC-I expression on neurons is regulated by DNMT1. DNMT1 is traditionally thought of as the maintenance DNA methyltransferase during cell division, but today we know that DNMT1 mRNA and protein is expressed in post-mitotic neurons [30, 31], suggesting a more complex role. It was also previously believed that methylation patterns were stable in post-mitotic neurons, but it is now clear that active demethylation is an ongoing process that in neurons is tightly regulated by multiple enzymes including the ten eleven translocation (TET) enzyme TET1 [40–42], and Gadd45b (growth arrest and DNA-damage-inducible 45b) which is induced upon neuronal stimulation [43].

MHC-I expression is controlled by methylation in tumour cell lines and human PBMCs [44, 45]. We here show that this is also the case in neuron-like cell cultures, by showing that two inhibitors of DNA methylation (PCA and 5-aza) increased the expression of genes encoding MHC-I in human and mouse neuroblastoma cell lines. This effect was observed upon 72 h of incubation with the compounds, and, as we assume that the cells have divided in this timespan, this effect most likely is dependent on the passive demethylation process of dividing cells.

Importantly, we next examined non-dividing post-mitotic CGNs. Here we show that a decrease in DNMT1 specifically causes an increase in H2-D1/L gene expression, while H2-K1 gene expression was still undetectable after DNMT1 knockdown. Importantly, knockdown of DNMT3a and DNMT3b did not change H2 levels. This suggests that DNMT1 does indeed have a more complex role in post-mitotic neurons than merely being the maintenance DNA methyltransferase.

The increase in MHC-I mRNA expression we observed was not sufficient to cause an upregulation of MHC-I protein on the surface. For MHC-I protein to be expressed on the surface, the MHC-I chain needs to form a stable complex with β2M [46]. β2M baseline expression was high in CGNs and did not change with DNMT1 knockdown, we therefore suspect that β2M is not the rate limiting step for MHC-I surface expression. Instead additional stimulation is likely needed for MHC-I to be expressed on the surface. It has for example been shown that TAP1 and TAP2 expression is needed for surface expression of MHC-I [47, 48].
relevance of additional factors has been suggested before in a study of cultured rat hippocampal neurons [49]. The authors showed that 40% of untreated neurons expressed MHC-I transcripts, but only 10% of the neurons expressed β2M mRNA, and none expressed TAP1 and TAP2 mRNA [49]. Interestingly in the same culture system, MHC-I was upregulated not only by IFNγ but also in neurons that had been electrically silenced by treatment with tetrodotoxin (TTX) [14]. Upregulation of the entire machinery for MHC-I expression, has also been shown to occur with aging [50].

We suggest, that by knocking down DNMT1 we create a situation where H2-D1/L loci become more accessible for the translational machinery. This will increase the baseline transcription of the gene slightly, as we see in our data. The effect is however small and confined to the mRNA level. For MHC class I protein to be expressed more factors such as β2M, TAP1 and TAP2 are needed. Changes in DNMT1 levels thus only affects which H2-genes are expressed but it does not affect the total rate of protein expression.

If what we suggest is true, and lowering DNMT1 levels indeed causes the H2-D1/L loci to become more accessible, then we should be able to predict that any stimulus that induces MHC class I protein expression would induce even more H2-D1/L expression in cells with lowered DNMT1. This was indeed the case. We first confirmed that MHC-I protein on the surface of neurons can be induced by IFNγ alone as previously reported [13, 14], and in addition we showed that in CGNs, IFNγ treatment only induce H2-D1 and H2-D1/L gene expression and not H2-K1 gene expression. When stimulating the CGNs with IFNγ after DNMT1 knockdown, H2-D1/L expression was increased by 10.78 fold compared to untreated CGNs, in contrast to 8.05 fold in CGNs treated with non-targeting siRNA. The same synergistic effect was observed in N2a neuroblastoma cell line, where combined treatment with IFNγ and 5-aza led to higher gene induction of H2-D1/L gene expression than the treatment with either one individually. In contrast to CGNs, H2-K1 was also induced to a higher extent by IFNγ in N2a cells following 5-aza treatment. Synergy
between changes in methylation and IFNγ signaling has been shown before, in a previous study showing that IFNγ and the demethylating agent zebularine acted synergistically to increase the gene expression of IDO1 [39].

Non-targeting siRNA across several experiments consistently induced H2-D1 gene expression or interfered with its detection for unknown reasons, and it was thus not possible to distinguish if the H2-D1 induction upon knockdown of DNMT1 was due to lower levels of DNMT1, or just the mere presence of a siRNA. However, the same effect was not observed upon introduction of siRNA against DNMT3a or DNMT3b, suggesting that H2-D1 does indeed increase upon DNMT1 knockdown, and that the signal with non-targeting siRNA was due to a technical problem with the probes. The probe detecting both H2-D1 and H2-L (H2-D1/L collectively) was not influenced by non-targeting siRNA supporting this idea. With current available probes we cannot distinguish the signals from H2-D1 and H2-L.

Our results consistently show that knockdown of DNMT1 does not cause an increase of total MHC-I protein in neurons, not even in the neurons displaying the highest uptake of siRNA against DNMT1. Two different antibodies and techniques were used, strengthening the credibility of the result. For flow cytometry studies we investigated surface MHC-I, and the possibility that intracellular MHC-I protein was increased without being translocated to the cell surface thus exists, however in that case we would have expected to observe an intracellular MHC-I signal in immunofluorescence. Another issue is that the antibodies used for immunofluorescence and flow cytometry are not subtype-specific antibodies, but rather were chosen based on their previously reported performance in immunofluorescence and flow cytometry [23, 51].

It would be interesting to investigate MHC-I protein using subtype-specific antibodies, but we were not able to obtain a sufficient signal-to-noise ration with the currently available subtype-specific MHC-I antibodies.

Our data suggest that DNMT1, when present in neurons, can inhibit expression of the H2-D1/L loci. Whether this is through a direct effect on methylation status of the H2-D1 or H2-L loci, or through some other mechanism, is still unknown. The mechanism could also be indirect through changes in the transcriptional machinery induced by the lack of DNMT1 activity. Interestingly, we observe that this effect of DNMT1 does not affect all H2 loci to an equal extend. Since we at the same time did not observe any changes in total surface expression of MHC-I protein, this suggests that it might instead shift the balance of available MHC-I protein from one subtype to another. This could be highly important in the context of autoimmunity, where epitope presentation to T-cells depend on the MHC-I subtype. The phenomenon of differential response of the different MHC-I subtypes have been reported before in peripheral blood leukocytes, colon mucosa, and larynx mucosa, with some MHC-I subtypes having a lower constitutive expression and being more inducible [52, 53]. This could also play an important role in the development of neurodegenerative diseases such as ALS. It has been shown in a mouse model of ALS, that increasing MHC class I expression on motor neurons protects the neurons against astrocyte toxicity [6]. The result was that the mice survived longer and had a better motor performance. Interestingly, this effect was only seen with H2-K and not H2-D. This could perhaps be attributed to the fact that H2-D1 is inhibited by the presence of DNMT1 in mature motor neurons while H2-K is not.

DNMT1 have been studied in post-mitotic neurons by others. DNMT1 has for example been found to colocalize with GAD67-positive GABAergic neurons in many parts of the brain [54]. Curiously, Fan et al. found no effect of postnatal brain-specific knockdown of DNMT1, neither on global methylation levels nor on neuronal long-term survival [31]. The simultaneous gene deletion of DNMT1 and DNMT3a however, affected synaptic function, decreased learning and memory formation, and caused upregulation of MHC-I and STAT1 gene expression in adult mouse hippocampus [55]. It might seem contradictory that post-mitotic cells would depend on continuous methylation, however with the discovery of an oxidized variant of 5mC, the 5-hydroxymethylcytosine (5hmC), which was discovered simultaneously in mouse ESCs [42] and in adult mouse cerebellar Purkinje cells [56], the concept of active demethylation arose. Methylated DNA can be demethylated without cell division, when 5hmC is converted to 5C through a series of events involving the TET enzymes and base excision repair pathways [40, 42]. Active demethylation is stimulated by neuronal activity acting through TET1 and Gadd45b [40–43]. For active demethylation to occur, and thus for the neurons to depend on re-methylation by DNMT1, it might be a prerequisite that the neurons have formed an active network. CGNs are glutamatergic but receive mostly inhibitory inputs from GABA and glycine containing synapses. This cannot be reproduced in vitro, and this might be an important limitation in our study. It is also possible that such inherent properties of the neuronal cultures vary from one preparation of CGNs to another. In light of this, it would be interesting to study knockdown of DNMT1 in other in vitro models, such as organotypic slice cultures, or in combination with induction of active demethylation. We do detect synaptic markers in our CGN cultures, and interestingly observe a strong effect of DNMT1 downregulation on these synaptic markers. This is particularly interesting as MHC-I are known to be involved in synaptic plasticity. It is tempting to
speculate that lack of DNMT1 causes an upregulation of MHC-I, which in turn causes elimination of synapses in the CGN culture. The MHC-I surface protein would be removed in this process explaining why we do not see it. However, from our present data we can not say anything about the direction of the correlation between increased MHC-I expression and lower expression of synaptic markers. It could also be that DNMT1 plays an important role in controlling the transcriptional processes related to synapse maintenance, and that the upregulation of MHC-I is secondary to this effect. DNA methylation status has indeed been shown to control transcription-dependent regulation of glutamatergic synaptic homeostasis [57].

In conclusion we have shown that MHC-I gene expression is regulated by DNMT1 in human and mouse neuroblastoma cell lines, and that the gene expression of some but not all H2 subtypes is upregulated in post-mitotic CGNs upon knockdown of DNMT1. We moreover show that IFNγ can act in synergy with these treatments to further increase H2-D1/L gene expression. Importantly, this effect differs between H2 subtypes.

Methods
SK-N-AS cell culturing and addition of procarainamide and IFNγ
The human neuroblastoma cell line SK-N-AS was obtained from ATCC. SK-N-AS cells were cultured in a 50/50 mixture of Ham's F-12 media (Gibco, Life Sciences), and Iscove's Modified Dulbecco's Medium (IMDM) (Biowest, Nuaillé, France), supplemented with 10% Fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), and 0.5% penicillin-streptomycin (P/S) (Lonza Ltd., Basel, Switzerland). SK-N-AS cells were seeded at a density of 3 × 10^4 cells/ml at day 1 in Ham's F-12/IMDM containing 10% FCS and 0.5% P/S, then on day 2 media was changed to Ham's F-12/IMDM containing 2% FCS and procarainamide (PCA) in the dose range 0.1–2 mM or 0.02 ng/μl IFNγ was added to the cells. At day 5 the cells were harvested for RNA extraction.

siRNA treatment of SK-N-AS cells
SK-N-AS cells were seeded at a density of 75 × 10^4 cells/ml at day 1 in Ham's F-12/IMDM containing 10% FCS and 0.5% P/S. On day 2 media was changed to Ham's F-12/IMDM without FCS and P/S containing instead 15 nM siRNA and Lipofectamine 2000 (Life Technologies). The siRNAs used were Ambion™ Select siRNA against human DNMT1 (s4215), DNMT3a (s200426) and DNMT3b (s4221). After 5 h of transfection, the media was changed to Ham's F-12/IMDM containing 10% FCS and 0.5% P/S and the cells were incubated for 72 h at 37 °C before harvest and RNA extraction.

N2a cell culturing and addition of 5-aza-2-deoxycytidine and IFNγ
The murine neuroblastoma cell line N2a was obtained from ATCC. Undifferentiated N2a cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) high glucose (Biowest, Nuaillé, France), supplemented with 10% Fetal calf serum (FCS) (Sigma-Aldrich, St. Louis, MO, USA), and 0.5% penicillin-streptomycin (Lonzia Ltd., Basel, Switzerland). N2a cells were seeded at a density of 4.5 × 10^4 cells/ml at day 1 and 0.5 ng/μl IFNγ was added on day 2. 5-aza-2-deoxycytidine (5-aza) was added on day 2, 3, and 4 in the doses 5 μM and 80 μM, and at day 5 the cells were harvested for RNA purification. The 5-aza treatment was renewed every 24 h, due to the short half-life of this compound. When addressing possible synergy between 5-aza treatment and IFNγ treatment, the same protocol for 5-aza treatment was followed, but with the addition of 0.05 ng/μl IFNγ at day 4 and with a lower concentration of 5-aza (0.5 μM).

Preparation and culturing of primary cultures of cerebellar granular neurons
Primary CGN cultures were prepared from 7 to 9 BALB/cJ BomTac (Taconic, Denmark) pups at postnatal day six, essentially as described by Schousbo et al. [58]. Cerebellum was isolated from a maximum of two pups at a time, the meninges removed on ice in a dissection buffer containing Krebs-Ringer buffer (Substrate-department, Faculty of Health Science, University of Copenhagen), Bovine Serum Albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), MgSO₄ (RegionH Apoteket), and HEPES buffer (Gibco, Life Sciences), and the remaining tissue was mechanically and enzymatically homogenized by chopping and trypsinization. Cells were then washed in a buffer containing trypsin inhibitor and DNase I (Sigma-Aldrich, St. Louis, MO, USA), centrifuged briefly at 100 rpm to stratify cells, and the uppermost layers were transferred to Krebs-Ringer buffer supplemented with BSA (Sigma-Aldrich, St. Louis, MO, USA), MgSO₄ (RegionH Apoteket), HEPES (Gibco, Life Sciences), CaCl₂ (RegionH Apoteket). Upon centrifugation for 10 min. at 700 rpm, the pelletted cells were resuspended in NBM-A (Gibco, Life Sciences) supplemented with B27 (Gibco, Life Sciences), BSA (Sigma-Aldrich, St. Louis, MO, USA), Glutamax (Gibco, Life Sciences), and HEPES (Gibco, Life Sciences). Cells were seeded at a density of 4 × 10^5 cells/ml in 24-well plates coated with poly-D-lysinse (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C, 5% CO₂. 24 h after seeding of cells, cytosine β-D-arabinofuranoside hydrochloride (Ara-C) (Sigma-Aldrich, St. Louis, MO, USA) was added to the media to a final concentration of 10 μM to inhibit the growth of glial cells.
siRNA for primary cultures of cerebellar granular neurons

The siRNAs against mouse DNMT1, DNMT3a and DNMT3b were bought as a pool of four siRNAs targeting the same gene in different sites, called SMARTpool, from the Accell range by Dharmacon™, part of GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom (See Additional file 1: Table S1). Negative control siRNA, “Accell Non-targeting #1” was designed, modified and microarray-confirmed by Dharmacon™ to have minimal targeting of known genes in mouse cells. The siRNAs were diluted in the supplied 5XsiRNA buffer diluted in RNase-free water, according to the manufacturer's instructions, aliquoted and stored at −20°C.

Addition of IFNγ and siRNA to primary cultures of cerebellar granular neurons

On the day of siRNA transfection the normal growth media of CGNs was exchanged with preheated and CO₂-equilibrated NBM-A media supplemented with B27, Glutamax and HEPES containing 10 μM Ara-C and 1 μM siRNA, but excluding BSA. Transfection efficiency was assessed using a Cy3-labelled non-targeting siRNA (Additional file 1: Figure S1). For studies of the effect of siRNA on H2 and DNMT genes (Figs. 3 and 4), the effect of siRNA on MHC-I protein by immunofluorescence (Figs. 5 and 7), and the effect of siRNA on H2 protein by flow cytometry (Fig. 8), CGNs were treated with 1 μM siRNA SMARTpool or non-targeting siRNA control for 72 h prior to harvesting or fixation for further processing. For detection of the neurons receiving the SMARTpool against DNMT1 by immunofluorescence (Fig. 7), the SMARTpool against DNMT1 was mixed 7:3 with a custom-made Cy3-labelled siRNA against DNMT1, and the mix was then added to cultures in equimolar amount with regards to the SMARTpool against DNMT1 as was used in previous experiments. In these experiments, treatment with 0.005 ng/μl IFNγ for the same 72 h prior to processing was used as a positive control for H2 induction. When testing synergy between DNMT1 knockdown and IFNγ treatment, two different protocols were tested. Either 0.005 ng/μl IFNγ was added on the same day as the siRNAs, followed by RNA extraction 72 h later (Fig. 4d-e). Alternatively cells were first subjected to 72 h of siRNA treatment, then media was exchanged to normal growth media including 10 μM Ara-C and BSA, and 0.05 ng/μl IFNγ was added to the cells, which were then incubated for another 72 h before harvest for RNA extraction (Fig. 4f-g) or fixation for immunofluorescence (Fig. 6).

Quantification of gene expression

Cells were harvested in RLT buffer on ice, and RNA was purified using RNasey® Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction for harvesting animal cells. The quality and concentration of the purified RNA was determined by measuring optical density (OD, 260/280) on a NanoDrop 2000c Spectrophotometer (ThermoFischer Scientific, Waltham, MA USA). The purified RNA was used to generate cDNA using QuantiTect® Reverse Transcription Kit, which includes an enzymatic removal of all genomic DNA (Qiagen, Hilden, Germany). Real-time quantitative PCR was performed in an Applied Biosystems Quant Studio™ 7 Flex station (Life Technologies™, Applied Biosystems, Foster City, CA, United States). The PCR primers and FAM-conjugated TaqMan® Gene Expression Assays probes (Additional file 1: Table S2) were from LifeTechnologies (Applied Biosystems, Foster City, CA, United States). The real-time PCR was carried out with TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster City, CA, United States), which contained AmpliTaq® Gold DNA Polymerase, AmpErase, UNG, dNTPs with dUTP, and optimized buffer components. The thermal cycle conditions were: part A: 50 °C for 2 min to activate the polymerase, part B: 95 °C for 10 min, part C: 95 °C for 15 s for denaturation, and part D: 60 °C for 1 min for annealing and extension, repeating part C and D for 45 cycles. We assessed the degree and specificity of the knockdown of targeted genes; DNMT1, DNMT3a and DNMT3b using real time quantitative PCR (Figs. 2 and 3). Evaluation of an actual functional effect of the knockdown was performed by measuring if the gene expression of positive control genes NNAT and S100A10 were upregulated. The two genes NNAT and S100A10 have previously been reported to be upregulated upon knockdown of DNMT1 in a neuronal cell line [59]. For details, see Additional file 1: Figure S2. MHC-I gene expression was evaluated using probes against HLA-A, HLA-B and HLA-C in human SK-N-AS neuroblastoma cell line, and for mouse cells using probes against β2-microglobulin (β2M), H2-D1, H2-K1 and a probe detecting H2-D1 and H2-L (H2-D1/L) as it is not possible to choose TaqMan probes specifically for H2-L. Endogenous expression of ACTB and GAPDH was measured and used to calculate relative expression levels between samples. The signal obtained from HLA-C in SK-N-AS cells, and from H2-K1 in CGNs was undetectable (Ct (HLA-C) > 35, Ct (H2-K1) > 40), and was not further analyzed.

Immunofluorescence

CGNs were seeded on Nunc™ LabTek™ chamber slides (ThermoFischer Scientific, Waltham, MA, USA), coated with poly-D-lysine (Sigma-Aldrich, St. Louis, MO, USA). Cells were either left untreated, or treated with 0.005 ng/μl IFNγ, or transfected with (A) non-targeting siRNA, (B) SMARTpool against DNMT1 or (C) mix of SMARTpool against DNMT1 and Cy3-labelled siRNA against DNMT1,
in NBM-A media as described above, and after 72 h of incubation fixated in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, United States). Cells were incubated 20 min at room temperature in blocking buffer (PBS containing 2% BSA (Sigma-Aldrich, St. Louis, MO, USA), 0.3% TritonX-100 (AppliChem, Darmstadt, Germany), and 5% Normal Goat Serum (Dako, Glostrup, Denmark)). Primary antibodies diluted in blocking buffer were added after blocking, and incubated overnight at room temperature. Primary antibodies were: rat monoclonal IgG2a anti-mouse MHC class I antibody (1:200, clone ErHr52, BMA Biomedicals, Augst, Switzerland), mouse monoclonal IgG1 anti-β-III-tubulin antibody (1:50, clone TU-1, Santa Cruz, Texas, USA), and rabbit polyclonal anti-DNMT1 antibody (NB100–264, Novus Biologicals, CO, USA). After three washing steps in PBS, fluorophore-conjugated secondary antibodies diluted in blocking buffer were added, and incubated for 1 h at room temperature. Secondary antibodies were: Alexa Fluor® 488 goat anti-mouse (1:2000), Alexa Fluor® 555 goat anti-rat (1:2000) and Alexa Fluor® 488 goat anti-rabbit (1:1000) (all Life Technologies, OR, USA). 4′,6-Diamidino-2-Phenyindole, Dilactate (DAPI) (Life Technologies, OR, USA) were added to the cells immediately upon incubation with secondary antibody, and incubated 8 min at room temperature. Following three washing steps in PBS, and one quick rinse in milliQ water, the slides were mounted with ProLong® Antifade Gold mounting media (Life Technologies, OR, USA). Immunofluorescence images were taken with a Zeiss LSM510 confocal scanning microscope, and analyzed using the Carl Zeiss imaging software Zen Black.

Flow cytometry
CGNs were at day 5 in vitro (DIV5) left untreated, transfected with non-targeting siRNA or with DNMT1 SMARTpool, or treated with 0.005 ng/μl IFNy. At DIV8 cells were harvested by brief trypsinization (2 min, 37 °C) in NBM-A containing BSA, transferred to 5 ml Falcon tubes for antibody staining, and pelleted at 400 RPM, 5 min, 4 °C. Staining was performed as follows with washes in in 3 ml PBS (Biowest, Nuaillé, France) between each step. First, cells were stained for live/dead cells for 30 min at 4 °C using the Fixable Viability Dye eFluor 506 (eBioscience, Aarhus, Denmark) diluted in PBS. Blocking of unspecific signal was done by incubation for 20 min on ice in 10% FCS (Merck Millipore, Berlin, Germany) diluted in PBS. Staining was performed for 20 min on ice with PE-conjugated rat IgG2aκ anti-mouse MHC-I antibody (clone M1/42, 125,505, BioLegend, Copenhagen, Denmark) or PE-conjugated rat IgG2aκ isotype control (clone RTK2758, 400,507, BioLegend, Copenhagen, Denmark), both diluted to 1 μg/ml PBS containing 2% FCS. If needed, cells were fixated in PBS containing 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, United States) for 15 min at room temperature on a shaker and permeabilized in PBS containing 0.7% Tween20 (Bio-Rad, CA, USA) for 15 min at room temperature on a shaker. Intracellular staining was performed over 30 min at room temperature on a shaker with Alexa Fluor 647-conjugated mouse IgG2aκ anti-β-III-tubulin antibody (clone AA10, 657,405, BioLegend, Copenhagen, Denmark) or Alexa Fluor 647-conjugated mouse IgG2aκ isotype control (clone MOPC-173, 400,239, BioLegend, Copenhagen, Denmark), both diluted to 0.5 μg/ml in PBS containing 0.5% Tween20, 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 10% Donkey Normal Serum (DAKO, Glostrup, Denmark). Finally, cells were resuspended in either PBS containing 2% FCS (extracellular staining only, cells not fixated) or in PBS (intracellular staining, cells fixated) and run on a FACSVersus flow cytometer (BD Biosciences, NJ, USA) equipped with FACSuite software for data analysis.

Statistics
The expression of the targeted gene was calculated using the ΔΔCt method, relative to expression levels of the housekeeping genes ACTB and GAPDH. Data are presented as mean fold change with upper and lower intervals calculated as fold change +/- standard error of the mean (S.E.M.). Statistical significance was tested using One-Way or Two-Way ANOVA based on the ΔΔCt values with a Dunnett’s multiple comparison test. The p values reported are corrected for multiple comparisons. For testing the effect of treatments, a One-Way ANOVA with a Dunnett’s multiple comparison test against untreated or non-targeting siRNA was used. For testing synergy between two treatments, a Two-Way ANOVA with a Dunnett’s multiple comparison test against the combined treatment, was used.

Additional file

Additional file 1: Figure S1. Transfection efficiency of Cy3-labelled siRNA in CGNs. CGNs were transfected with Cy3-labelled non-targeting siRNA as described in Materials and Methods. After 72 h incubation the cells were fixated and mounted using mounting media containing DAPI. Confocal images were taken with a Zeiss LSM510 confocal scanning microscope, and analyzed using the Carl Zeiss imaging software Zen Black.

Abbreviations
ADCA-DN: Autosomal dominant cerebellar ataxia, deafness and narcolepsy; CGNs: Cerebellar granule neurons; DNMT1: DNA methyltransferase 1; MHC: Major Histocompatibility Complex; MHC-I: MHC class I.
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Availability of data and materials
The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
JG, GK and MD performed the experiments. MD, PD, SN and BRK supervised the performance of experiments. PD and SN assisted with experimental design and data analysis. JG and BRK conceived the study, planned all experiments, analyzed data and wrote the manuscript. All authors assisted in writing the manuscript and approved the final version.

Ethics approval and consent to participate
All experiments involving research animals were conducted adhering to the guidelines and regulations given by the Danish Research Animal Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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