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Ablation of the pro-inflammatory master regulator miR-155 does not mitigate neuroinflammation or neurodegeneration in a vertebrate model of Gaucher's disease

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ABSTRACT

Bi-allelic mutations in the glucocerebrosidase gene (*GBA1)* cause Gaucher's disease, the most common human lysosomal storage disease. We previously reported a marked increase in miR-155 transcript levels and early microglial activation in a zebrafish model of Gaucher's disease (*gba1*$^{-/-}$). miR-155 is a master regulator of inflammation and has been implicated in a wide range of different neurodegenerative disorders. The observed miR-155 upregulation preceded the subsequent development of widespread pathology with marked neuroinflammation, closely resembling human Gaucher's disease pathology. We now report similar increases of miR-155 expression in mammalian models of GD, confirming that miR-155 upregulation is a shared feature in glucocerebrosidase (GCase) deficiency across different species. Using CRISPR/Cas9 mutagenesis we then generated a miR-155 mutant zebrafish line (miR-155$^{-/-}$) with completely abolished miR-155 expression. Unexpectedly, loss of miR-155 did not mitigate either the reduced lifespan or the robust inflammatory phenotypes of *gba1*$^{-/-}$ mutant zebrafish. Our data demonstrate that neither neuroinflammation nor disease progression in GCase deficiency are dependent on miR-155 and suggest that miR-155 inhibition would not be a promising therapeutic target in Gaucher's disease.

1. Introduction

miR-155 is a typical multifunctional miRNA which is involved in a wide range of different immune and inflammatory processes (Faraoni et al., 2009). Inflammation is increasingly recognised as a key mechanism leading to neurodegenerative diseases with an observed up-regulation of miR-155 in Parkinson's disease (PD), multiple sclerosis (MS), motor neuron disease (MND), Alzheimer's disease (AD) and other dementias (Butovsky et al., 2015; Caggiu et al. 2018; Chen et al., 2018; Tili et al. 2018). In MND, miR-155 is strongly upregulated in the spinal cord of both familial and sporadic MND with dysregulation of previously validated miR-155 downstream targets in the lumbar ventral horn (Butovsky et al., 2015). Genetic ablation of miR-155 ameliorated disease severity and prolonged survival in SOD1^{G93A} mice, a widely

used rodent model of MND. Furthermore, treating SOD1^{G93A} mice with anti-miR-155 at the beginning of disease onset also improved the disease phenotype and prolonged the lifespan, suggesting that miR-155 could be a therapeutic target for human MND (Butovsky et al., 2015).

In post-mortem studies of PD patient brains, activated microglia and increased concentrations of pro-inflammatory cytokines have been identified within the substantia nigra (Boka et al., 1994; McGeer et al., 1988). An increase in pro-inflammatory cytokines in both PD patient serum and cerebrospinal fluid (CSF) has been reported (Dobbs et al., 1999; Mogi et al., 1994). Distinct cytokine profiles may be associated with more aggressive progression of the disease (Williams-Gray et al., 2016). Genetic studies of risk factors for PD and epidemiological studies also support the role of inflammation in PD (Kaur et al. 2017). miR-155 is significantly upregulated in the substantia nigra pars compacta
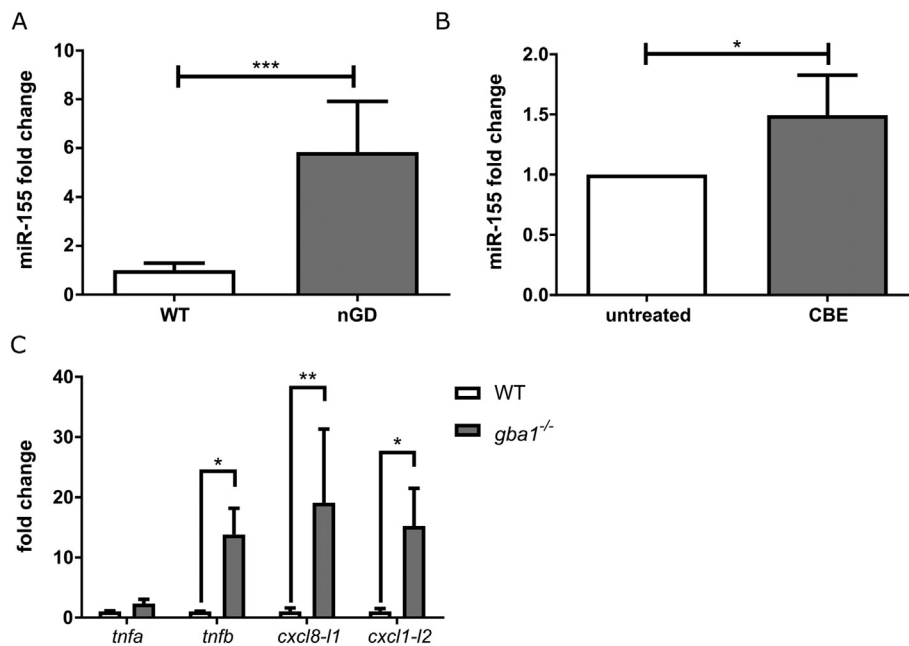

Fig. 1. miR-155 and associated inflammatory markers are upregulated in different models of GCase deficiency.

(A) miR-155 expression is upregulated in the cortex of the Gba$^{flox/flox}$; Nestin-Cre neuropathic Gaucher disease mouse model ($n = 6$, $p = .0004$, one-tailed t-test) and (B) in cultured murine microglia treated with the GCase inhibitor CBE ($n = 3$, $p = .031$, one-tailed t-test). (C) Expression of miR-155 downstream targets *tnfa*, *tnfb*, *cxcl8-l1* and *cxcl8-l2* (zebrafish orthologues of human TNFa and IL-8) in 12 wpf zebrafish brain ($n = 3$, two-way ANOVA). * $p < .05$, ** $p < .01$, *** $p < .001$. Error bars = SD.

(SNpc) of mice with adeno-associated-virus-mediated expression of alpha-synuclein. This rodent model of PD demonstrated microgliosis and a 30% loss of dopaminergic neurons. In contrast, $miR-155^{-/-}$ mice transduced with alpha-synuclein showed attenuated inflammatory responses and near complete neuroprotection (Thome et al., 2016).

Heterozygous mutations in *GBA1* are the most common risk factor for PD (Gegg and Schapira, 2018) whilst bi-allelic *GBA1* mutations cause Gaucher's disease (GD), the most common lysosomal storage disorder (Davidson et al., 2018). A key pathological feature of GD is the infiltration and accumulation of Gaucher cells in many different organs, particularly the haemopoietic system (Baris et al., 2014). Gaucher cells are infiltrating glycolipid-engorged macrophages and the source of increased inflammatory markers observed in GD patients (Panicker et al., 2014). Neuroinflammation is thought to be a major contributing feature to neurodegeneration in GD, as demonstrated by the upregulation of inflammatory markers, astrogliosis, and microglial activation correlating with neuronal loss in two murine models of GD (Farfel-Becker et al. 2011; Massaro et al., 2018; Vitner et al., 2012). Microglial activation is therefore a common feature in both PD and GD brains (Ginns et al., 2014). Of note, plasma levels of monocyte-associated inflammatory markers (including IL8, MCP-1, MIP1α, SCF, and PARC) are increased in $GBA1^{+/-}$ PD patients and increased IL8 levels were also associated with poorer cognition (Chahine et al., 2013). This suggests that inflammation may be a particularly important aspect of pathogenic mechanism in GBA-linked PD.

We recently described a *gba1* mutant zebrafish line ($gba^{-/-}$) generated by TALEN mutagenesis which faithfully modelled key aspects of human GD, including Gaucher cell accumulation, neurodegeneration and marked elevation of GD biomarkers. We observed early activation of immune/inflammatory mechanisms with microglial activation and increased *miR-155* transcription levels at 4 or 5 days post fertilisation (dpf) respectively. Of note, both the early microglial activation and the accompanying miR-155 activation dramatically preceded the onset of a change in phenotype which only occurred at around 8–10 weeks with resulting lethality around 12 weeks of age. Compared to the larval stages, we observed a striking inflammatory phenotype at 12 weeks, with a further increase of miR-155 levels and marked microglial accumulation (Keatinge et al., 2015).

The aim of this study was therefore to investigate whether miR-155 upregulation may be a universal feature of GCase deficiency in mammalian models systems of GD and to determine whether miR-155

ablation may have a neuroprotective effect in *gba1* deficiency.

2. Methods

2.1. Zebrafish husbandry

All larval and adult zebrafish were housed at the University of Sheffield; experimental procedures being in accordance UK Home Office Animals (Scientific Procedures) Act 1986 (Project licence PPL 70/8437, held by Professor Oliver Bandmann). Adult zebrafish were housed at a density of 40 per tank, on a cycle of 14 h of light, 10 h of dark. Adults and embryos were kept at constant temperature of 28 °C.

The $gba1^{-/-}$ and $pink1^{-/-}$ mutant lines were previously described in (Keatinge et al., 2015) and (Flinn et al., 2013) respectively. The *miR-155* mutant line was generated by the CRISPR/Cas9 method, using a gRNA targeting a *Hyp188III* site within the miR-155 coding region (ultramer sequence 5'-AAAGCACCGACTCGGTGCCACTTTTTCAAGTTG ATAACGGACTAGCCTTATTTTAACTTGCTATTTCTAGCTCTAAAACATC ACGATTAGCATTAAACCTATAGTGAGTCGTATTACGC-3'). Briefly, guide RNAs (gRNAs) were generated from a DNA ultramer template (IDT) and transcribed using a T7 MEGAshortscript™ kit (Invitrogen). gRNAs were then co-injected into one-cell stage zebrafish embryos with Cas9 protein (NEB). F0 injected embryos were raised to 3 months then crossed to wild type (WT) to identify founders. The progeny of selected F0 founders were then outcrossed to generate the F1 generation where appropriate mutant alleles were identified and selected for further study. The line was genotyped using primers F 5'-TATGACGTCCACAT GCATGC and R 5'-GATGTTCAGACGGTTCGCTC.

$miR-155^{+/-}$ were crossed to $gba1^{+/-}$ to generate double heterozygous individuals. These were subsequently in-crossed to generate double mutants. At each in-cross larvae were genotyped at 3dpf by larval tail biopsy as previously described (Wilkinson et al., 2013) and each genotype raised in genotype-specific tanks at a density of 10–15 fish per tank. All individuals were re-genotyped at 10 weeks post-fertilisation.

2.2. Gene expression analyses

RNA was extracted from zebrafish adult brains (12wpf), cortex of 21 day old Gba$^{flox/flox}$; nestin-Cre and Gba$^{flox/WT}$; nestin-Cre mice, or BV2 microglial cells at specific time points using TRIzol® (Life
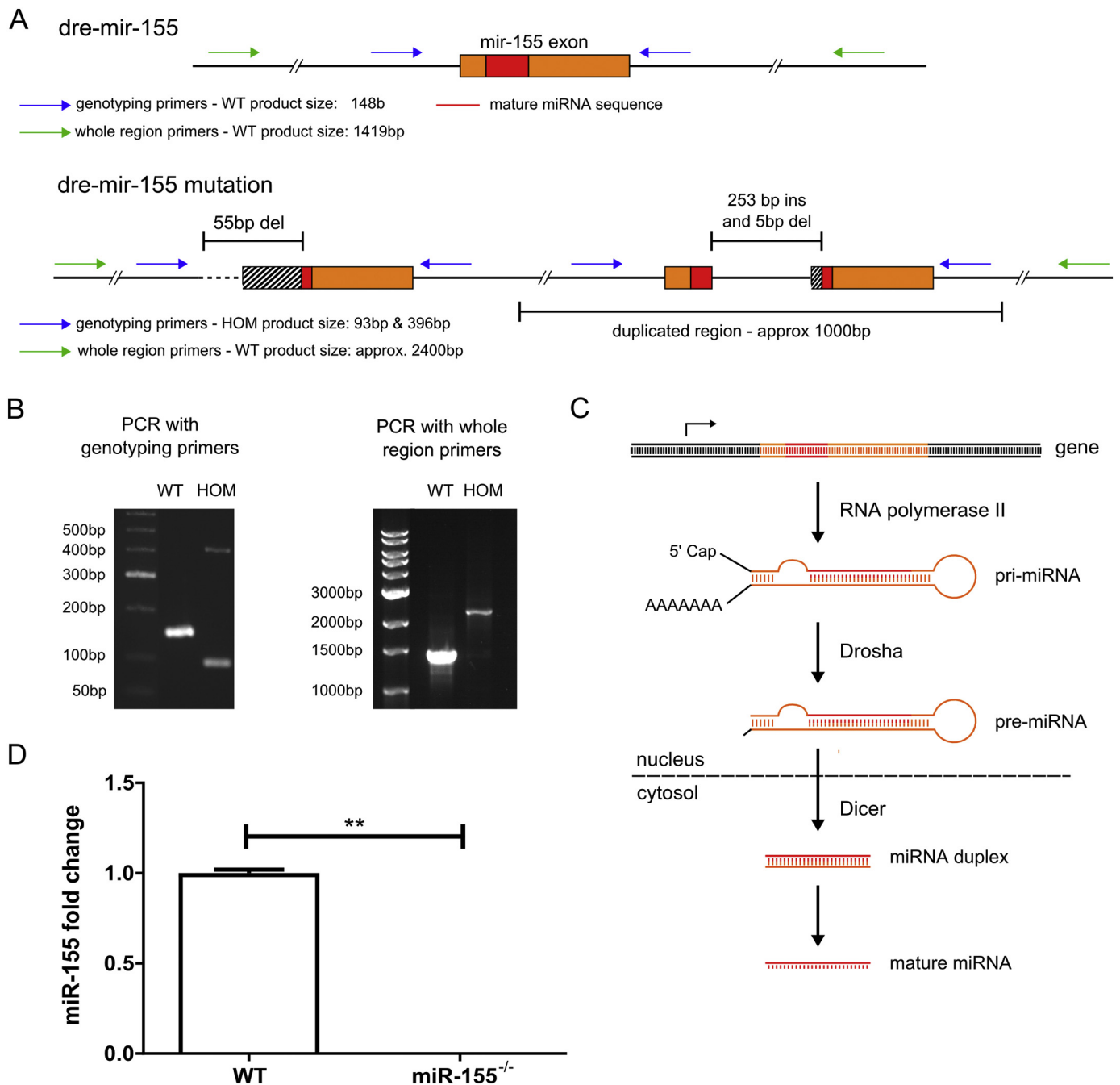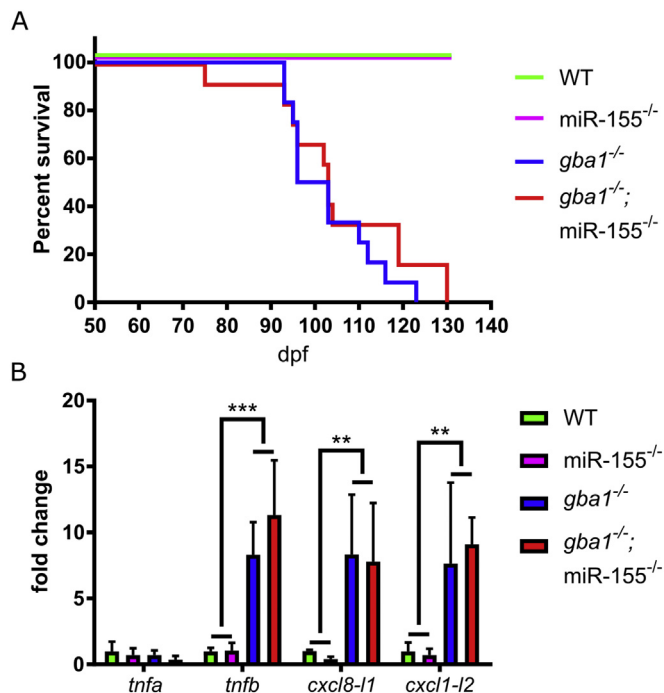
Fig. 2. Generation and validation of a miR-155 knockout line.
CRISPR/Cas9 technology was used to generate a *miR-155* knockout line. (A) This schematic shows the miR-155 gene of wild type (WT) *mir-155* and the mutation generated by CRISPR/Cas9 mutagenesis. Briefly, in the mutant the whole *mir-155* exon is duplicated (1000 bp duplicated region); in the first copy of *mir-155* there is a 55 bp deletion, and in the second copy there is a 253 bp insertion and 5 bp deletion. Both mutations disrupt the mature miR-155 sequence. (B) PCRs with genotyping and whole region primers confirm this complex mutation. In WT, only one amplicon of 148 bp, corresponding to the *mir-155* exon, is generated using the genotyping primers (blue). In homozygous mutants (HOM), two amplicons are generated, one for each *mir-155* exon. The amplicon spanning first exon generates a 93 bp product reflecting the 55 bp deletion. The amplicon spanning the second exon generates a 396 bp product reflecting the 253 bp insertion and 5 bp deletion. The whole region primers (green) generate an amplicon of 1419 bp in WT and an amplicon of approximately 2400 bp in the HOMs reflecting the 1000 bp insertion. The location of the PCR primers used to generate these products are shown in (A). (C) Schematic miRNA processing pathway, illustrating how the miR-155 mutation is predicted to result in abolished production of mature miR-155. (D) The predicted complete loss of mature miR-155 was validated by qPCR for *miR-155* transcripts with miR-155 being undetectable in $miR\text{-}155^{-/-}$ (n = 3, $p = .0044$, one-tailed t-test). ** $p < .01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Technologies™). cDNA was synthesised using a Verso™ reverse transcription kit (Thermo Fisher Scientific). Gene expression was determined using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies) on a Stratagene MxPro 3000P (Stratagene) qPCR machine. For all experiments, *ef1α* was used as a reference gene.

For miRNA qPCR analysis, RNA was extracted as above. For zebrafish and mouse brain RNA the RNA concentration was accurately quantified using the QuantiFluor™ RNA system (Promega) and the Qubit® fluorometer (Life Technologies). 100 ng of total RNA was reverse-transcribed and subsequently qPCR was performed using TaqMan

Fig. 4. miR-155 ablation does not reduce microglial accumulation in $gba1^{-/-}$ zebrafish.
(A-B) Brains from WT, $gba1^{-/-}$, miR-155$^{-/-}$ and $gba1^{-/-}$;miR-155$^{-/-}$ zebrafish were labelled using a microglial antibody marker 7.4.C4. Sections from comparable parts of the (A) optic tectum and (B) cerebellum are shown for each genotype, oriented rostral to left; scale bar for all images is shown in bottom left panel. (C-D) Microglial abundance was analysed for each genotype (2 sections each from 4 brains for each genotype). Immunoreactive microglia were quantified within each brain region of interest (C) optic tectum, (D) cerebellum and expressed as the density of microglia per square mm of brain region imaged ($n = 8$, two-way ANOVA). ** $p < .01$, **** $p < .0001$. Error bars = SD.

of miR-155$^{-/-}$ zebrafish (Fig. 2D, $n = 3$, $p = .0044$, one-tailed *t*-test).

3.3. Genetic ablation of miR-155 does not increase survival or alter inflammatory phenotypes of $gba1^{-/-}$ zebrafish

To assess the effects of loss of miR-155 in $gba1^{-/-}$ zebrafish, *miR-155*$^{+/-}$ animals were crossed with $gba1^{+/-}$ to generate $gba1^{+/-}$; *miR-155*$^{+/-}$ zebrafish; these were subsequently in-crossed to generate double homozygous mutants ($gba1^{-/-}$;miR-155$^{-/-}$). Until 12 weeks post fertilisation, $gba1^{-/-}$ zebrafish are visibly indistinguishable from their wild-type siblings. Around this age they develop a rapidly progressive movement phenotype and become emaciated. This is caused by extensive neurodegeneration accompanied by a strong

neuroinflammatory phenotype (Keatinge et al., 2015). Unexpectedly, genetic ablation of *miR-155* did not modulate the lifespan (Fig. 3A) of $gba^{-/-}$ zebrafish (median survival 99.5dpf in $gba1^{-/-}$ and 103dpf in $gba1^{-/-}$;miR-155$^{-/-}$, $n = 13$) or the emergence of abnormal motor function (data not shown).

We next determined whether this absence of rescue in this model was attributable to lack of involvement of neuroinflammation in the phenotype versus miR-155 being unnecessary for neuroinflammation in this model. We had previously demonstrated marked microglial accumulation in the optic tectum and cerebellum in end stage $gba1^{-/-}$ zebrafish (Keatinge et al., 2015). Genetic ablation of miR-155 did not ameliorate upregulation of *tnfb* or *cxcl8-l1/l2* (Fig. 3B), or CNS microglial accumulation in $gba1^{-/-}$ brains (Fig. 4). Unexpectedly, in the

optic tectum, $gba1^{-/-}$;$miR\text{-}155^{-/-}$ zebrafish had a further 40% increase in microglial abundance compared to $gba1^{-/-}$ (Fig. 4C; 218 microglia/mm^2 to 150 microglia/mm^2, $n = 8$, $p < .01$, two-way ANOVA with Tukey's post-tests).

4. Discussion and conclusions

There is growing interest in the role of epigenetic mechanisms in neurodegeneration but observations are frequently limited to model systems of one species and can be of uncertain functional relevance.

The mature sequence of *miR-155* is 100% conserved between zebrafish and humans, similarly, the predicted binding sites in the 3'UTRs of many of the known targets of miR-155 are also conserved between zebrafish and humans. It is thought that miR-155 exerts its pro-inflammatory effects through targets such as SOCS1 which have key function as negative regulators on cytokines (Wang et al. 2018; Yao et al., 2012). Suppression of SOCS1, and other similar targets, by miR-155 leads to an elevation of cytokine levels and pro-inflammatory effects (Testa et al., 2017). The miRNA target prediction software TargetScan predicts a miR-155 binding site in position 15–21 in the 3'UTR of zebrafish *socs1*.

We showed that miR-155 was upregulated in three vertebrate models of GCase deficiency: $gba1^{-/-}$ zebrafish, Gba$^{flox/flox}$; Nestin-Cre mice, and mouse BV2 microglia treated with CBE, a GCase inhibitor. We also showed that the orthologues of *TNFα* and *IL-8*, inflammatory markers which are regulated by direct targets of miR-155 (Lippai et al. 2013; Min et al., 2014; Pathak et al. 2015), are also highly upregulated in the brains of *gba1* deficient zebrafish. IL-8 was shown to be upregulated in PD patients with *GBA* mutations (Chahine et al., 2013) and TNFα is upregulated in GD patient serum and in the brain of Gba$^{flox/flox}$; Nestin-Cre mice (Barak et al., 1999; Vitner et al., 2012). These findings demonstrate that the pathogenic processes leading to miR-155 upregulation and inflammation is present in both mouse and zebrafish models of GCase deficiency.

We used CRISPR/Cas9 to generate a *miR-155* mutant line and demonstrated with the Taqman™ miRNA assay that mature miR-155 is not produced in this *miR-155* mutant line. In *miR-155* mutants, the inflammatory marker *cxcl8-l1*, a classical *miR-155* downstream target, was reduced 50% compared to WT zebrafish, however this did not reach significance. Unexpectedly, loss of miR-155 did not have an effect on the survival or inflammatory phenotypes of *gba1* mutant zebrafish. This suggests that, at least in zebrafish, miR-155 does not play a causative role for neuroinflammation in GCase deficiency and suggests redundancy of inflammatory pathways. Loss of miR-155 had no effect on the upregulation of *tnfb* or *cxcx8-l1/l2* in $gba1^{-/-}$ zebrafish. This may be because of alternate pathways, which are not dependant on miR-155 targets, leading to their upregulation and the loss of repression of miR-155 targets may not be strong enough to overcome this effect. We cannot comment whether miR-155 is able to regulate *socs1* in zebrafish; luciferase assays or western blots are required to determine the effect of miRNAs on their direct targets as they exert their effects post-transcriptionally. It is possible that although the mature miR-155 sequence is conserved in zebrafish the binding sites in key anti-inflammatory targets may not be as well conserved and therefore miR-155 may exert a weaker, or have no effect on these targets.

Although zebrafish are a powerful model for genetic studies and 70% of human genes have at least one clear orthologue, many genes have two orthologues due to a large genome duplication event in the zebrafish genome (Howe et al., 2013). In many cases it is not clear which gene copy is functional or, where both are, which is most closely related to its human counterpart. Our study provides evidence that the two zebrafish orthologues of *TNFα*, *tnfa* and *tnfb*, function differently. We showed that in brains of 3-month-old *gba1* mutant zebrafish *tnfb* was upregulated 8-fold while *tnfa* was unchanged, this highlights the importance of investigating both orthologues where they are present.

Our observation of marked pro-inflammatory changes in $gba1^{-/}$ $^{-}$;$miR\text{-}155^{-/-}$ zebrafish suggests redundancy of pro-inflammatory pathways in GCase deficiency, at least in zebrafish this contrasts with a possible role of miR-155 in other forms of neurodegeneration. The observed rescue effect of miR-155 inactivation in mouse model systems of MND, PD and EAE may either reflect a more specific involvement of miR-155 in these conditions or reduced redundancy in mammalian model systems.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2019.04.008.

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