Mitofusin 2 is necessary for striatal axonal projections of midbrain dopamine neurons

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Mitochondrial dysfunction is implicated in aging and degenerative disorders such as Parkinson’s disease (PD). Continuous fission and fusion of mitochondria shapes their morphology and is essential to maintain oxidative phosphorylation. Loss-of-function mutations in PTEN-induced kinase 1 (PINK1) or Parkin cause a recessive form of PD and have been linked to altered regulation of mitochondrial dynamics. More specifically, the E3 ubiquitin ligase Parkin has been shown to directly regulate the levels of mitofusin 1 (Mfn1) and Mfn2, two homologous outer membrane large GTPases that govern mitochondrial fusion, but it is not known whether this is of relevance for disease pathophysiology. Here, we address the importance of Mfn1 and Mfn2 in midbrain dopamine (DA) neurons in vivo by characterizing mice with DA neuron-specific knockout of Mfn1 or Mfn2. We find that Mfn1 is dispensable for DA neuron survival and motor function. In contrast, Mfn2 DA neuron-specific knockouts develop a fatal phenotype with reduced weight, locomotor disturbances and death by 7 weeks of age. Mfn2 knockout DA neurons have spherical and enlarged mitochondria with abnormal cristae and impaired respiratory chain function. Parkin does not translocate to these defective mitochondria. Surprisingly, Mfn2 DA neuron-specific knockout mice have normal numbers of midbrain DA neurons, whereas there is a severe loss of DA nerve terminals in the striatum, accompanied by depletion of striatal DA levels. These results show that Mfn2, but not Mfn1, is required for axonal projections of DA neurons in vivo.

INTRODUCTION

Parkinson’s disease (PD) is a common neurodegenerative condition characterized by loss of dopamine (DA)-producing neurons in the substantia nigra pars compacta (SNc). Although other populations of neurons are also affected, the hallmark motor symptoms of PD are caused by the resulting DA deficiency in the striatum, the area to which these DA neurons project. The pathophysiological events that lead to the degeneration of DA neurons are unclear, but may involve mitochondrial dysfunction (1). A distinct form of PD, autosomal recessive juvenile parkinsonism (AR-JP), is caused by mutations in the genes PTEN-induced kinase 1 (PINK1), Parkin and DJ-1. Several genes mutated in AR-JP have been linked to the regulation of mitochondrial function. The E3 ubiquitin ligase Parkin and the mitochondrial kinase PINK1 act in a common pathway suggested to be involved in mitochondrial quality control. In cell lines, depolarization of the membrane potential across the inner mitochondrial membrane induces a PINK1-dependent recruitment of cytosolic Parkin to the mitochondrial outer membrane (2). The precise role of Parkin on the outer mitochondrial surface remains to be established and the link to the degeneration of DA neurons is not well understood (3).

We have previously addressed the consequences of mitochondrial dysfunction in DA neurons by creating MitoPark mice that have DA-specific disruption of mitochondrial transcription factor A (4), a dual function protein required for mitochondrial transcription initiation and for packaging of mtDNA into nucleoids (5,6). These mice develop severe motor symptoms due to progressive loss of DA neurons in live mice.
SNc. The degenerating DA neurons in MitoPark mice have abnormal mitochondrial ultrastructure and a fragmented mitochondrial network. Furthermore, the DA neurons in MitoPark mice develop large, dense and membranous intracellular bodies derived from abnormal mitochondria, and the supply of mitochondria to their distal axons is impaired (4,7).

Mitochondria form a functionally interconnected network in the cell by continuous fission and fusion (8,9). Mitochondrial dynamics is essential for embryonic development and studies in differentiated tissues have shown that continuous fission and fusion of mitochondria is necessary for maintaining mtDNA and oxidative phosphorylation capacity (10–12). Mutations in genes regulating mitochondrial fusion and fission cause human neurodegenerative diseases. For instance, mutations in \(\text{OPA1}\) cause atrophy of the optic nerve (13) and mutations in \(\text{MFN2}\) cause a form of hereditary motor and sensory neuropathy (14). It is believed that mitochondrial fusion contributes to maintaining function by allowing an exchange of mtDNA and other matrix components (8). Fission events fragment the mitochondrial network into smaller units to allow mitochondrial transport to different subcellular localizations, such as nerve terminals. Fission has also been reported to have a role in mitochondrial quality control as dysfunctional mitochondria are less prone to fuse with the main mitochondrial network (15) and instead may be degraded by autophagocytosis (16,17).

Two homologous large GTPases, mitofusin 1 (Mfn1) and Mfn2, govern fusion of the mitochondrial outer membrane in mammals (9,18). Studies have identified Parkin as a possible regulator of mitofusin levels by direct ubiquitination to facilitate subsequent proteasome-mediated degradation (19,20). Consistent with this idea, the \(Drosophila\) Mfn homolog Marf accumulates in \(Drosophila\) PINK1 and Parkin mutants (21), and genetic manipulations that promote fission or decrease fusion can partially suppress \(Drosophila\) mutant phenotypes (22,23).

We have addressed the roles of Mfn1 and Mfn2 in vivo and report here that loss of Mfn1 in DA neurons has no apparent effect. In contrast, loss of Mfn2 in DA neurons causes severe mitochondrial fragmentation and enlargement accompanied by respiratory chain deficiency. Interestingly, Mfn2 is critical for DA innervation of the striatum, as young adult knockout mice have normal numbers of DA neurons in SNc, whereas the DA nerve terminals in the striatum are almost completely absent. Mfn2 is thus essential for DA neuron function and critical for axonal arborization.

RESULTS

Generation of conditional knockout mice with disruption of Mfn1 or Mfn2 in DA neurons

We assessed the expression of Mfn1 and Mfn2 mRNA in the mouse brain by \textit{in situ} hybridization and found that transcripts of both genes were ubiquitously expressed in the mouse brain, including in midbrain DA neurons (Supplementary Material, Fig. S1, outlined regions). Next, we obtained conditional knockout alleles for Mfn1 and Mfn2 by gene targeting in the mouse (Supplementary Material, Fig. S2A–D). We tested
these knockout alleles by disrupting both genes in the heart by crossing $Mfn1^{loxP}$ and $Mfn2^{loxP}$ mice with $Ckmm$-cre mice (24). Disruption of $Mfn1$ (genotype $Mfn1^{loxP}/Mfn1^{loxP}; +/Ckmm$-cre) or $Mfn2$ (genotype $Mfn2^{loxP}/Mfn2^{loxP}; +/Ckmm$-cre) in the heart resulted in a very profound decrease in $Mfn1$ and $Mfn2$ mRNA (Supplementary Material, Fig. S2E and F) and protein, respectively (Supplementary Material, Fig. S2G). These results demonstrate that breeding of $Mfn1^{loxP}$ and $Mfn2^{loxP}$ mice to cre-expressing transgenic mice can efficiently ablish the expression of $Mfn1$ and $Mfn2$ in a selected tissue. With this information at hand, we proceeded to study the role of $Mfn1$ and $Mfn2$ in DA neurons by breeding $Mfn1^{loxP}$ and $Mfn2^{loxP}$ mice to DAT-cre mice. The mice with disruption of $Mfn1$ in DA neurons (genotype $Mfn1^{loxP}/Mfn1^{loxP}; +/DAT-cre$, hereafter denoted $Mfn1^{DA}$ mice) were clinically unaffected, when followed until 1 year of age. In contrast, mice with disruption of $Mfn2$ in DA neurons (genotype $Mfn2^{loxP}/Mfn2^{loxP}; +/DAT-cre$, hereafter denoted $Mfn2^{DA}$ mice) had a drastically shortened life span and died before the age of 7 weeks (median survival, 5.8 weeks; Fig. 1A). The $Mfn1^{DA}$ mice had normal body weight (Supplementary Material, Fig. S3A), whereas the $Mfn2^{DA}$ mice had reduced body weight at the age of 5 weeks (Fig. 1B). We found no differences in spontaneous locomotion or rearing in $Mfn1^{DA}$ mice in comparison with controls (Supplementary Material, Fig. S3B and C). In contrast, the $Mfn2^{DA}$ mice had significantly decreased rearing activity (Fig. 1C), whereas locomotion, assessed as horizontal beam breaks, was unchanged (Fig. 1C and D). The total distance traveled was significantly increased in $Mfn2^{DA}$ mice (Fig. 1C). Increased total distance with similar amounts of horizontal beam breaks can result from increased diagonal locomotion across the cage floor, possibly reflecting increased stress. As the decreased rearing activity of $Mfn2^{DA}$ mice may prevent them from reaching food on the cage top, a cohort of mice were provided moist food on the cage floor. Despite this, $Mfn2^{DA}$ mice continued to lose weight, became severely hypokinetic and had to be sacrificed at 11–12 weeks of age due to poor general condition (data not shown).

**Loss of Mfn2 in DA neurons causes severe respiratory chain deficiency without recruitment of Parkin to the defective mitochondria**

Next, we investigated the number of midbrain DA neurons by immunolabeling to detect tyrosine hydroxylase (TH) expression (Fig. 2A). We quantified SNc DA neurons by stereology and found normal numbers of neurons in both $Mfn1^{DA}$ (Fig. 2B) and $Mfn2^{DA}$ mice (Fig. 2C) at 5 weeks of age.

To assess mitochondrial morphology in DA neurons, we used the $lox$-Stop-lox-mito-YFP reporter mice in conjunction with the DAT-cre allele, as described previously (7). The mitochondrial morphology was normal in $Mfn1^{DA}$ mice at the age of 6 and 18 weeks (Fig. 3A). In contrast, the mitochondria in $Mfn2^{DA}$ mice were spherical and enlarged (Fig. 3B). These abnormal mitochondria weremunoreactive with antibodies against the mitochondrial matrix protein superoxide dismutase 2 (SOD2) and the outer mitochondrial membrane protein TOM20 (Fig. 3C), demonstrating at least some preservation of function. We also performed electron microscopy of DA neurons in $Mfn2^{DA}$ mice and found abnormal, rounded mitochondria with disorganized cristae (Fig. 3D), having a size corresponding to that of the enlarged mitochondria visualized by light microscopy (Fig. 3B). We observed no neurofibrillar tangles by electron microscopy of $Mfn2^{DA}$ neurons.

The profound mitochondrial morphological abnormalities motivated us to use an enzyme histochemical approach to simultaneously assess the activities of cytochrome c oxidase (COX) and succinate dehydrogenase (SDH). This COX/SDH assay showed a marked reduction in COX activity and increased SDH activity in midbrain DA neurons of $Mfn2^{DA}$ mice at 5 weeks of age. Open bars, control ($n = 3$); filled bars, knockout ($n = 3$) mice. Error bars indicate ± SD. (C) Relative number of TH-positive neurons in the midbrain of $Mfn1^{DA}$ mice at 5 weeks of age. Open bars, control ($n = 3$); filled bars, knockout ($n = 3$) mice. Error bars indicate ± SD.

Figure 2. No loss of DA neurons in $Mfn1^{DA}$ or $Mfn2^{DA}$ mice. (A) TH immunoreactive DA neurons in SNc of control and $Mfn2^{DA}$ mice (scale bar: 200 μm). (B) Relative number of TH-positive neurons in the midbrain of $Mfn1^{DA}$ mice at 5 weeks of age. Open bars, control ($n = 3$); filled bars, knockout ($n = 3$) mice. Error bars indicate ± SD. (C) Relative number of TH-positive neurons in the midbrain of $Mfn2^{DA}$ mice at 5 weeks of age. Open bars, control ($n = 3$); filled bars, knockout ($n = 3$) mice. Error bars indicate ± SD.
Loss of targeting of DA axons to the striatum in the absence of Mfn2

Mfn1<sup>DA</sup> mice had no cell loss in SNc (Fig. 2B) and maintained a normal DA innervation of the striatum (Fig. 5A and B). Likewise, there was no decrease in the number of TH-positive cell bodies in SNc in 5-week-old Mfn2<sup>DA</sup> mice (Fig. 2C), although the somata displayed pathological alterations, such as irregular margins and elongated nuclei (Fig. 3B and C). Strikingly, the Mfn2<sup>DA</sup> mice showed a profound lack of DA innervation (88% reduction) in the striatum (Fig. 5C and D). We...
measured levels of DA and its major metabolites in different brain regions by high-performance liquid chromatography (HPLC) and found no significant differences in Mfn1DA mice at 50 weeks of age (Fig. 6A–C). In contrast, we found a severe reduction in DA in the striatum of Mfn2DA mice at 5 weeks of age (Fig. 6D). We also found increased 3,4-dihydroxyphenylacetic acid (DOPAC)/DA and of homovanillic acid (HVA)/DA ratios at 5 weeks of age (Fig. 6D). We also found increased COX activity and increased SDH activity. (B) Stereotaxic delivery of AAV encoding mCherry-Parkin to midbrain DA neurons expressing mito-YFP in Mfn2DA mice at 4 weeks of age (n = 3). Cells were analyzed 7 days after injections (scale bar: 10 μm).

**Figure 4.** Dysfunctional mitochondria in Mfn2-deficient DA neurons do not recruit Parkin. (A) COX and SDH activity in control and Mfn2-deficient DA neurons at 5 weeks of age. Increased blue staining indicates decreased COX activity and increased SDH activity. (B) Stereotaxic delivery of AAV encoding mCherry-Parkin to midbrain DA neurons expressing mito-YFP in Mfn2DA mice at 4 weeks of age (n = 3). Cells were analyzed 7 days after injections (scale bar: 10 μm).

**DISCUSSION**

High energy demands and the requirement to maintain cellular homeostasis at sites distant to the cell body likely make neurons particularly vulnerable to mitochondrial dysfunction. There is emerging evidence that degeneration of axons may occur independently and by mechanisms distinct from those of the cell body and that loss of dopaminergic axons is an early event in the progression of PD (28). This phenomenon is illustrated in Mfn2DA mice, where we find that loss of Mfn2 has a profound effect on DA innervation of the striatum at a time point when the DA nerve cells in SNc are respiratory chain deficient, but still viable. A progressive loss of nigro-striatal projections, which precedes that of DA cell bodies, is also seen in a similar model with disruption of Mfn2 in its DA neurons (29). Consistent with these observations, it has been reported that the overexpression of mutant forms of the Mfn2 protein, which cause hereditary motor and sensory neuropathy, leads to axonal degeneration and impaired mitochondrial movement without loss of cell bodies in cultured neurons (30). Axon terminals are diminished already in very young (~3.5 weeks old) Mfn2DA mice, which suggest the occurrence of axonal degeneration in early postnatal life or impaired formation during development. The DA-containing nigrostriatal system appears around embryonic day E13 in rats and mice (31,32), but the formation of a terminal DA-containing dense network in the striatum is largely a postnatal event that takes place during the first 4 weeks of life in rodents (33,34). It is possible that loss of Mfn2 impairs axon development during this period by preventing axonal transport of mitochondria. We have previously shown that mitochondrial dysfunction impairs anterograde supply of mitochondria in DA neurons (7) and Mfn2 has also been reported to play a direct role in axonal transport of mitochondria (35), in addition to its role in fusion that is required to maintain respiratory chain function.

Although the number of SNc DA neurons is intact in Mfn2DA mice, mitochondria in the soma appear as separate, respiratory chain-deficient organelles. Such a condition should promote Parkin-mediated mitochondrial degradation (17). However, we were unable to detect any in vivo translocation of Parkin to these aberrant mitochondria. A possible explanation is that the mitochondrial membrane potential is maintained above the threshold required for Parkin translocation, as previously discussed in the context of the MitoPark mice, that also display no in vivo recruitment of Parkin to the enlarged and severely respiratory chain-deficient mitochondria (7).

It remains unclear why two homologous mitofusins have evolved in mammals. Mfn1 and Mfn2 can functionally replace each other to maintain mitochondrial fusion in vitro, but both genes are critical for embryonic development (10).
Despite similar expression levels, we find a striking discrepancy between loss of \( \text{Mfn1} \) and \( \text{Mfn2} \) in DA neurons. Whereas loss of \( \text{Mfn2} \) causes abnormal mitochondrial morphology, respiratory chain dysfunction and severe lack of DA terminals in the striatum, loss of \( \text{Mfn1} \) does not significantly affect DA neuron survival or function. However, preliminary results indicate that DA-specific \( \text{Mfn1} \) and \( \text{Mfn2} \) double-knockout mice have an even shorter lifespan than the \( \text{Mfn2}^{\text{DA}} \) mice, suggesting a partial suppression of the \( \text{Mfn2} \) knockout phenotype by \( \text{Mfn1} \). These findings are similar to reports of the disruption of \( \text{Mfn1} \) and \( \text{Mfn2} \) in cerebellar Purkinje cells. Also here, the neurons are largely unaffected by the loss of \( \text{Mfn1} \), whereas loss of \( \text{Mfn2} \) causes a profound neurodegeneration (11). It thus seems likely that \( \text{Mfn2} \) has an additional role in neurons besides promoting mitochondrial fusion. For example, \( \text{Mfn2} \) may be of particular importance for axonal transport of mitochondria (35) and has also been reported to mediate the interaction between mitochondria and the endoplasmatic reticulum (36).

In summary, our results demonstrate that mammalian DA neurons in vivo critically depend on \( \text{Mfn2} \), but not \( \text{Mfn1} \), to maintain mitochondrial morphology, axon integrity and DA-mediated motor control. A picture is now emerging from experimental mouse work that argues that mitochondrial dysfunction preferentially affects the DA innervation of the striatum by impairing axonal targeting, as demonstrated in this paper, mitochondrial axonal transport (4,7) or release of DA from nerve terminals in the striatum (37). These experimental findings shed important new light on the possible role of mitochondrial dysfunction in PD and are consistent with observations that the DA neuron pathology in human PD patients starts peripherally with the degeneration of the striatal DA nerve terminals (28).

MATERIALS AND METHODS

Breeding of mice

Mice with loxP-flanked \( \text{Mfn1} \) or \( \text{Mfn2} \) alleles were obtained from Taconic and crossed with \( \text{DAT-cre} \) mice (4). The resulting double heterozygous offspring were, in turn, crossed with homozygous \( \text{Mfn1}^{\text{loxP/loxP}} \) or \( \text{Mfn2}^{\text{loxP/loxP}} \) mice to generate DA-specific knockouts for \( \text{Mfn1} \) (\( \text{Mfn1}^{\text{loxP/loxP}; +/\text{DAT-cre}} \)) or \( \text{Mfn2} \) (\( \text{Mfn2}^{\text{loxP/loxP}; +/\text{DAT-cre}} \)), respectively. In a subset of matings, the \( \text{Gt(Rosa26)Sor conditional YFP} \) allele was introduced by an additional cross in the parental generation.

Mice were fed ad libitum (R70 Standard Diet; Lactamin), had free access to water and were kept on a 12:12 h light:dark cycle at 24°C. Experiments were approved by the Animal Ethics Committee of the North Stockholm region and conducted in accordance with international standards on animal welfare and Swedish law.

RNA expression analysis

Total RNA was extracted from heart samples using TRIzol (Invitrogen). Reverse transcription was conducted on 2 \( \mu \text{g} \) of
total RNA by using the high-capacity cDNA reverse transcription kit (Applied Biosystems). Semi-quantitative real-time polymerase chain reaction was subsequently performed using specific TaqMan probes for the Mfn1 and Mfn2 transcripts (Mm01289372_m1 and Mm00500120_m1 gene expression assays, Applied Biosystems). The transcript levels were normalized to β-2 microglobuline RNA (Mm00437762_m1 gene expression assay, Applied Biosystems).

**Behavior analysis**

Mice were analyzed at 5 weeks of age (n = 17) using an animal activity monitoring system (VersaMax, AccuScan Instruments). Following habituation to the dimly lit, low-noise and ventilated experimental room for at least 30 min, mice were placed individually in activity cages (40 × 40 and 30 cm high). A grid of infrared light beams at floor level and 7.5 cm above recorded spontaneous horizontal and vertical activities, respectively, over a period of 1.5 h during the light phase (between 4 and 6 p.m.).

**In situ hybridization**

To detect the expression of gene transcripts, the following oligonucleotide probes were used: TH, 5′-GGT GTG CAG CTC ATC CTG GAC CCC CTC CAA GGA GCG CT-3′; Mfn1, 5′-AAC GCT CTC TCT TTC GCA CGT TCT GCC AGC TCA TAA AGG TA-3′ and Mfn2, 5′-ACT GTC CCA GCA AAA AGG GTA TGA GGG GCG AGT GAG CAC AAG-3′. Probes were labeled with 33P, as described (38).

**Immunohistochemistry and microscopy**

Mice were perfused with Ca2+/Mg2+-free Tyrode’s solution followed by 4% paraformaldehyde with 0.4% picric acid in 0.16 M phosphate buffer. The brains were dissected out, post-fixed for 2 h and equilibrated with 10% sucrose buffer containing 0.1% sodium azide. The brains were frozen on CO2 ice and cryosectioned to obtain 14 or 20 μm sections. Rehydrated sections were immunolabeled overnight with primary antibodies against TH (1:500; Pel-Freez), Tom20 (1:400; Santa Cruz) and SOD2 (1:200; Upstate). For non-fluorescent labeling, biotinylated secondary antibodies (1:400; Vector Laboratories) were used and detected using a peroxidase substrate (Vector SG, Vector Laboratories). For fluorescence labeling, Cy3-conjugated secondary antibodies (1:400; Jackson Biolabs) were used. Confocal images were acquired by sequential scanning using an LSM510 Meta microscope (Zeiss). YFP was excited with the 514 nm argon laser and emission was detected at 520–542 nm. Cy3 and mCherry were excited with a 543 nm helium-neon laser and detected at 553–649 and 584–638 nm, respectively.
Coronary midbrain cryosections (20 μm thickness) were thawed and every sixth section was immunolabeled for TH. SNc was outlined as described (39). A microscope with a motorized stage (Nikon E600) controlled by stereology software (Stereologer, SRC; version 2001) was used for design-based stereology. SNc was outlined using a 4× objective and cells counted using a 60×/1.4NA oil objective. Counting frames were spaced 200 μm apart. A coefficient of error of <0.1 was accepted.

Image analysis and quantifications

ImageJ was used for the measurement of TH density in the striatum. For the quantification of striatal mitochondria, images were obtained using a 40× objective and cells counted using a 60×/1.4NA oil objective. Counting frames were spaced 200 μm apart. A coefficient of error of <0.1 was accepted.

Measurements of monoamines

The brains (n = 7 per genotype) were rapidly dissected, chilled in ice-cold saline and bilateral pieces of the striatum, midbrain and frontal cortex dissected and frozen on dry ice. Tissue pieces were weighed and homogenized by sonication in 0.1 M perchloric acid containing 0.4 mM sodium bisulfite followed by centrifugation and filtration of the supernatant. Noradrenalin, DA, DOPAC, HVA and 5-HT in the supernatants were separated by HPLC on a reverse-phase column (Reprosil-Pur, C18-AQ 150 × 4 mm, 5 μm particle diameter; Dr Maisch HPLC GmbH) with a mobile phase of a 0.05 M sodium phosphate/0.03 M citric acid buffer containing 0.1 mM ethylenediaminetetraacetic acid, with various amounts of methanol and sodium-l-octane sulfonic acid. Monoamine levels were measured by electrochemical detection as previously described (37) and expressed as ng/g wet weight of tissue.

Electron microscopy

Mice were perfused with 2% glutaraldehyde in 440 mM Milloig's buffer (pH 7.4). The brains were removed and a 1-mm-thick coronal section containing the SNc dissected. The SNc of each side was trimmed from the surrounding tissue and cut to generate three pieces of 1–2 mm³ that were further post-fixed in 2% glutaraldehyde solution. The specimens were further trimmed, osmicated, dehydrated and embedded in Durcupan (Fluka, Buchs, Switzerland). Ultrathin sections from SN were collected on formvar-coated copper grids, contrasted with uranyl acetate and lead citrate and examined by electron microscopy (CM12; Philips, Eindhoven, the Netherlands).

Stereotactic injections

Mice anesthetized with isoflurane and buprenorphine (0.075 mg/kg s.c.) were put in a stereotactic frame (Stoelting). A midline skin incision was made and a burr hole was made over each side of the midbrain. Viral constructs (1 × 10⁹ viral genomes in 2 μl) were injected just above SNc (2.9 mm caudal to bregma, 1.3 mm lateral to the midline and 4.2 mm ventral to dura mater) using a 10 μl Hamilton syringe with a custom-made 33G needle (45° type 4 tip). A microsyringe pump controller (World Precision Instruments) was used to deliver the volume at a speed of 300 nl/min. The needle was left in situ another 4 min prior to retraction. The scalp was closed using a 6-0 Ethicon suture.

Statistical analysis

The error bars on figures represent the mean ± SD of all determinations. Two-tailed unpaired t-tests were used to assess statistical significance in figures with *P < 0.05, **P < 0.01 and ***P < 0.001, respectively. Statistics were performed using appropriate software (GraphPad Prism version 5.0; GraphPad Software Inc.).
Conflict of Interest statement. None declared.

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