MicroRNA-132 loss is associated with tau exon 10 inclusion in progressive supranuclear palsy

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Received April 14, 2011; Revised June 17, 2011; Accepted July 27, 2011

INTRODUCTION

In neurons, the microtubule-associated protein tau exists as six isoforms produced by the alternative splicing of exons 2, 3 and 10 (1). The exclusion or inclusion of exon 10, respectively, produces tau isoforms with either three (3R-tau) or four (4R-tau) microtubule-binding repeats, which are important for tau function. In humans, only 3R-tau is expressed in the fetal stage, while both 3R-tau and 4R-tau are expressed in a ratio of approximately 1:1 in the adult stage (2). Rodent brains on the other hand express only 3R-tau in fetal and neonatal stages and mainly 4R-tau in the adult stage (3).

Neurofibrillary tangles, constituted of abnormally hyper-phosphorylated and aggregated tau, are observed in more than 20 human neurodegenerative disorders known as tauopathies (1). Both clinical and biochemical evidences suggest that changes in tau exon 10 splicing is causally linked to dementia. More than 35 mutations in the MAPT gene (which encodes for tau) were identified in patients suffering from rare forms of familial frontotemporal dementia and parkinsonism linked to chromosome 17, causing aberrant splicing of tau exon 10, leading to an imbalance in the 4R:3R-tau ratio (4,5). In sporadic tauopathies, imbalances in tau ratios are often observed. For instance, tau deposits found in progressive supranuclear palsy (PSP) or corticobasal degeneration contain mostly 4R-tau isoforms (6,7). Thus, the delicate balance between 3R-tau and 4R-tau seems critical for tau (dys)function, and changes in this ratio may contribute to tau-dependent pathology.
MicroRNAs are short (~21 nt) noncoding RNA molecules that are abundantly expressed in the brain and function as negative regulators of gene expression at the posttranscriptional level (8–10). Mechanistically, they repress translation and/or promote mRNA degradation by binding to the 3′ untranslated region (UTR) of target transcripts (or gene transcripts) (10,11). Each microRNA (miRNA) can target up to several hundred target transcripts in vivo, thus potentially regulating multiple biological pathways (12). Upon neuronal differentiation, the brain-specific miR-124 targets poly(A) tract-binding protein 1 (PTBP1/PTB/ hnRNP I), a global repressor of neuron-specific alternative splicing (13). An opposite mode of action has been described in muscle cells (14). Down-regulation of PTBP1 in neuronal cells leads to the accumulation of correctly spliced Ptbp2 mRNA and a dramatic increase in the PTBP2 (nPTB/brPTB) protein (13,15,16). In the adult brain, PTBP1 is mainly expressed in glial cells, while PTBP2 is predominantly localized in post-mitotic neurons (15). Although less studied, it has been demonstrated that PTBP2 also functions as a repressor of exon inclusion (17); however, this role may vary depending on the physiological context (18,19).

We have previously shown that the loss of miRNA function in post-mitotic neurons in vivo causes endogenous tau hyper-phosphorylation, an effect mediated in part by miR-16 family members and ERK1 (20). In this study, we tested the hypothesis that miRNAs could be involved in the physiological regulation of tau exon 10 splicing, ultimately affecting 3R-tau and/or 4R-tau isoform abundance in disease.

RESULTS
Identification of brain miRNAs that control tau isoform abundance

We previously showed that tau phosphorylation was enhanced in Dicer conditional KO (cKO) mice (20). In the brains of adult Dicer mutant mice, tau exon 10 inclusion was repressed, leading to the expression of the fetal tau isoform (Supplementary Material, Fig. S1). Because the production of several miRNAs is abolished in this mouse model (20), we hypothesized that miRNAs may contribute to the splicing regulation of tau. In order to identify potential miRNAs that could regulate tau exon 10 splicing, we used the TargetScan logarithm (21) to search for potential miRNA targets sites within the 3′ UTR of splicing factors previously shown to regulate tau exon 10 splicing (Supplementary Material, Table S1). From this list, we chose five brain miRNAs, namely miR-9, miR-124, miR-132, miR-137 and miR-153, predicted to target PTBP1 and PTBP2 as well as six other genes, and expressed these in Neuro2a cells. Forty-eight hours post-transfection, western blot analysis was performed using 3R-tau, 4R-tau and tauC (total tau) antibodies. Surprisingly, all candidate miRNAs significantly affected 3R-tau and 4R-tau expression, albeit at different levels (Fig. 1A and B). While miR-9, miR-124, miR-132, miR-137 and miR-153 decreased the relative 3R-tau levels, miR-9, miR-132 and miR-137 decreased 4R-tau. Notably, the latter three miRNAs as well as miR-124 decreased the overall 4R:3R-tau ratio. We could confirm that exogenous miRNAs were expressed at similar levels when compared with the scrambled control (data not shown). Blocking endogenous miRNA levels with antisense probes reversed the effects of miR-124, miR-132, miR-137 and miR-153 on 4R:3R-tau ratios (Supplementary Material, Fig. S2), indicating that physiologically expressed miRNAs can regulate tau isoform abundance. Interestingly, miR-9 and miR-137 increased, while miR-132 decreased, total tau protein levels (Fig. 1A and B). We used miR-132 and miR-124-transfected cells to confirm at the mRNA level the changes in tau exon 10 splicing (Fig. 1C and D and data not shown). Of mention, a muscle-specific miRNA, miR-206, had no effect on tau protein (data not shown) and mRNA (Fig. 1D). We also performed knockdown experiments using siRNAs directed against murine Mapt, which validated the specificity of our tau antibodies in these cells (Fig. 1E).

Changes in miR-132 and PTBP2 levels in the PSP brain

We next asked whether candidate miRNAs could be affected in sporadic tauopathies where an imbalance in 4R:3R-tau isoform expression has been described. To this end, we used PSP as the disease model. We firstly confirmed the relative increase in 4R:3R-tau ratios in sporadic PSP brain when compared with controls (Supplementary Material, Fig. S3). There, we measured miR-9, miR-124, miR-132, miR-137 and miR-153 expression in control and PSP patients (n = 8 per group) by quantitative reverse transcriptase reaction followed by polymerase chain reaction (qRT–PCR). Mature (functional) miR-132 expression levels were significantly decreased (P = 0.0047, Mann–Whitney t-test) in PSP temporal lobe when compared with non-demented controls (Fig. 2A). Other candidate miRNAs, that is, miR-124, miR-9, miR-137 and miR-153, remained unchanged in these samples (Supplementary Material, Fig. S3). Notably, decreased miR-132 levels were equally observed in PSP prefrontal and temporal lobes displaying abnormal 4R:3R-Tau ratios (Supplementary Material, Fig. S3). These observations prompt us to analyze in more detail miRNA changes in PSP. Global miRNA micro-arrays were performed with groups of four controls and four PSP patients. In these unbiased assays, we could confirm the down-regulation of miR-132 in PSP (Supplementary Material, Table S2). Again, no changes in miR-9, miR-124, miR-137 and miR-153 were observed in these assays. Of interest, other candidate miRNAs listed in Supplementary Material, Table S1 remained unchanged in the microarrays. We thus identified miR-132 to be specifically down-regulated in the PSP brain using two independent methods.

As shown in Supplementary Material, Table S1, miR-132 is predicted to target the splicing factors PTBP2 and ASF/ SRp50a/Sfrs1. Western blot analysis indicated that PTBP2 was significantly up-regulated in PSP brain regions (Fig. 2B and C and Supplementary Material, Fig. S3), while ASF remained unchanged in these samples (data not shown). Similar results were obtained using the neuronal marker NeuN as the normalization control (Supplementary Material, Fig. S3), further strengthening our observations. We also measured PTBP1, which was not affected in disease (Fig. 2B and D and Supplementary Material, Fig. S3). PTBP2 mRNA levels remained quite stable between groups (Fig. 2E), suggesting that part of this misregulation is
posttranscriptional. Based on these results, we focused our investigation on miR-132 and PTBP2.

**PTBP2 is a direct target for miR-132**

The PTBP2 3′ UTR is relatively long (~1300 bp) and highly conserved when compared with its parologue PTBP1 (Fig. 3A). In luciferase assays, miR-132 significantly down-regulated PTBP2, but not PTBP1, expression when compared with a scrambled miRNA control (Fig. 3B and C). Other candidate miRNAs, that is, miR-9, miR-137 and miR-153, did not significantly affect PTBP2 expression using these reporter assays. On the other hand, all miRNAs, except for miR-132, affected PTBP1 expression, which is consistent with the prediction algorithm (Fig. 3A and Supplementary Material, Table S1). As previously reported (13), miR-124 targeted both PTBP2 and PTBP1 luciferase reporter constructs (Fig. 3B and C), therefore serving as a positive control. We further validated the specificity of miR-132 binding on the PTBP2 3′ UTR by mutating the conserved miRNA seed region (see Materials and Methods), which abolished the regulating effect (Fig. 3B, last lane). Endogenous PTBP2, but not PTBP1, was significantly down-regulated in Neuro2a cells treated with pre-miR-132 as assessed by western blot analysis (Fig. 3D and E). The increase in PTBP2 protein levels in the presence of miR-124, miR-9, miR-137 and miR-153 is consistent with the down-regulation of PTBP1 in more physiological conditions. The splicing factor hnRNPA1 did not change in these experiments (Fig. 3D), and therefore served as a normalization control. Taken together, these results demonstrate that miR-132 directly targets PTBP2 to regulate its expression, independently of PTBP1 modulation.

**Endogenous PTBP2 regulates tau isoform abundance**

Knockdown of PTBP2, but not PTBP1, in Neuro2a cells caused a significant reduction in relative 4R-tau levels as well as in 4R:3R-tau ratios (Fig. 4A and B). Loss of PTBP2 (and PTBP1) also decreased total tau protein levels, as seen before using pre-miR-132 (Fig. 1A and B). Of mention, double knockdown of PTBP2 and PTBP1 similarly affected 4R:3R-tau ratios. PTBP2 protein levels were strongly
down-regulated in siRNA-treated cells, therefore validating our siRNA conditions (Fig. 4C). As expected, PTBP1 reduction caused an increase in endogenous PTBP2 levels (Fig. 4C). Taken together, these results strongly implicate PTBP2 down-regulation as directly responsible for the effects of miR-132 on tau exon 10 splicing.

Developmental correlation between miR-132, PTBP2 and 4R-tau

Since tau splicing is developmentally regulated, we next sought independent validation for the role of miR-132 and PTBP2 in the regulation of tau splicing. To this end, RT–PCR and western blot analyses were performed using mouse brain homogenates at different developmental stages. As previously documented (2), 4R-tau isoforms increased considerably during post-natal brain development (Fig. 5A). The switch between 3R-tau and 4R-tau isoforms at the mRNA level was most predominant between postnatal day 9 (P9) and 14 (P14), coincident with the lower PTBP2 expression (Fig. 5B). By western blot, fetal tau protein isoform at 52 kDa is seen mainly from P0 to P16, while 4R-tau protein isoform at 55 kDa appears at P9. 4R-tau protein isoform containing exon 2 and 3 sequences starts to be visible at P14. Interestingly, miR-132 increased ~16-fold, while miR-124 and miR-9 remained essentially unchanged, during this time-frame (Fig. 5C). Thus, miR-132 is inversely correlated with PTBP2 and tau exon 10 inclusion in the developing brain.

DISCUSSION

To date, little is known about the molecular mechanisms causing abnormal tau exon 10 alternative splicing in the sporadic tauopathic brain. In this study, we explored whether miRNAs, previously implicated in tauopathies such as Alzheimer’s disease (AD), could indirectly regulate the expression of tau exon 10 trans-acting factors, therefore affecting 4R-tau and 3R-tau isoform abundance. These experiments were encouraged by previous studies showing miRNA-regulated pathways are involved in tau metabolism and toxicity (20,22). In addition, it was shown that PTBP1, a recognized miRNA target gene, affects tau exon 10 splicing in mini-gene systems in vitro (23). Here, we provide evidence that a number of brain-specific/enriched miRNAs, that is, miR-124, miR-9, miR-132, miR-137 and miR-153, could participate in the physiological regulation of tau exon 10 splicing. Analysis of miRNA expression profiles identified miR-132 to be specifically down-regulated in PSP, a major 4R-tau tauopathy. We identified PTBP2 as a direct target for miR-132. Using knockdown studies in neuronal cells, we show that endogenous PTBP2 participates in the regulation of tau isoform abundance. Up-regulation of 4R-tau (corresponding

Figure 2. Abnormal miR-132 and PTBP2 levels in the PSP brain. (A) qRT–PCR of mature hsa-miR-132 in control ($n = 8$) and PSP ($n = 8$) patients. Here, total RNA extracted from temporal cortex was used. The microRNA miR-16 was used as normalization control (using the average of controls as 1-fold). Similar results were obtained using let-7a as normalization control ($P = 0.0019$, Mann–Whitney $t$-test) (data not shown). (B) Western blot analysis of PTBP2, PTBP1, β-tubulin and NeuN in control ($n = 8$) and PSP ($n = 8$) patients. In these experiments, the radioimmunoprecipitation assay (RIPA)-soluble protein was used from the temporal cortex. (C and D) Quantifications of the PTBP2 protein from temporal lobe. Blots were normalized to β-tubulin. No changes in PTBP1 protein levels were observed in these samples. (E) qRT–PCR of human PTBP2 mRNA levels in control ($n = 8$) and PSP ($n = 8$). Here, GAPDH mRNA was used as normalization control. Statistical significance was determined by a Mann–Whitney $t$-test ($^{*}P < 0.01$). Standard deviation, mean and molecular markers are shown.
to exon 10 inclusion) during post-natal development inversely correlates with PTBP2 expression. During this time frame, miR-132 increased ≏ 16-fold. Taken together, these results provide good evidence that specific miRNAs, via splicing factor regulation, could regulate tau alternative splicing in physiological and possibly pathological conditions.

PTBP1 down-regulation seems sufficient and necessary to induce neuron-specific exon inclusion of neuronal transcripts (13). The decrease in PTBP1 also coincides with the up-regulation of PTBP2, mainly in cultured cells in vitro, which has been implicated in the regulation of alternative splicing of a number of transcripts in Neuro2a cells as well as in other models (24–26). Here, we show that knockdown of PTBP2, but not PTBP1, induced a marked decrease in 4R:3R-tau ratios in Neuro2a cells. These results were accompanied with a small, yet significant, down-regulation of total tau protein (but not mRNA) levels induced by our knockdown experiments. Interestingly, it was shown that PTBP1 can stimulate internal ribosomal entry site (IRES)-mediated translation initiation (reviewed in 25,27). In addition, the human tau mRNA contains an IRES, and IRES-dependent translation plays a role in the synthesis of tau protein (28). Whether PTBP2 (and/or PTBP1) functions in tau translation regulation therefore warrants further investigation.

Targeting of PTBP1 and possibly PTBP2 by miR-124 is one mechanism proposed to operate during early neuronal differentiation. The results obtained herein add to the complexity of this model, suggesting that other miRNAs, such as miR-132, participate in the physiological regulation of PTBP family members. We have previously shown that PTBP1 protein levels are increased in the brains of adult Dicer cKO mice (29). Based on this observation, it is tempting to speculate that the loss of global miRNA function triggers neuronal dedifferentiation. The fact that fetal tau (3R-tau) becomes expressed in the Dicer mutant mice is in line with this hypothesis. Unfortunately, this rather crude mouse model cannot discriminate between the identity and number of miRNAs involved in PTBP and tau exon 10 splicing regulation. Moreover, one can anticipate the activation of several compensatory pathways, including PTBP1 and/or PTBP2 cross-regulation, in the complete absence of miRNAs in vivo. Clearly, specific miRNA gene knockout and/or transgenic mouse models will be required to address this question. Nevertheless, these results provide ‘proof of concept’ that neuronal miRNAs can participate in the splicing regulation of tau exon 10.

Consistent with our hypothesis, we identified specific miRNAs, such as miR-132, which can regulate endogenous 4R:3R-tau ratios in neuronal cells. Obviously, and as discussed above, we cannot exclude a role for additional miRNAs and splicing factors involved in this process. Indeed, several potential miRNA target sites are found in the 3′ UTR of genes previously associated with tau exon 10 inclusion, including PTBP1 and Srs family members (Supplementary Material, Table S1). Moreover, we provide evidence that brain miRNAs miR-124, miR-9, miR-137 and miR-153 could also affect tau exon 10 splicing when expressed in cells. The next goal will be to address the role of these miRNAs (and putative targets) in this process, particularly in the developing brain in vivo.
We and others have shown that 4R-tau becomes expressed during post-natal brain development. Here, we show that PTBP2 is down-regulated during this process, in contrast to what is observed in developing cells in vitro (13,14,15) or during early brain development in vivo (30). The fact that PTBP1 is also repressed during this period (data not shown) suggests a complex regulatory pathway involved in PTBP expression regulation during post-natal brain development, at a time when synaptogenesis occurs. It is likely that PTBP2 loss controls a second splicing transition event where a number of neuronal transcripts, such as tau, are functionally regulated. Alternatively, PTBP2 down-regulation could be important for splicing-independent functions by targeting different RNA transcripts or even miRNAs (24,31). One can also imagine that miRNAs regulate PTBP paralogues in a specific developmental and/or cellular context. For instance, miR-124 could function in early neuronal development, whereas miR-132 acts after birth. Another possibility is that PTBP2 down-regulation is an indirect consequence of its localization in post-mitotic neurons (15). Whether miR-132 and PTBP2 function in tau exons 2 and 3 splicing (32) remains to be determined.

It is becoming increasingly acknowledged that changes in miRNA expression patterns could contribute to neurodegenerative disease (9,33,34). This is well documented in AD, for instance, where abnormal miRNA levels could contribute to increased Aβ production (35,36). Here we report that miR-132 is reduced in the PSP brain (Supplementary Material, Table S2), further strengthening our observations. The function of miR-132 in neurons is still under investigation, but has been linked to neuronal differentiation, neurogenesis, neuronal outgrowth and sprouting, as well as spine density (37–39). In future studies, it will be important to elucidate the impact of miR-132 down-regulation on tau pathology and neuronal function in vivo. Obviously, our post-mortem brain studies cannot elucidate the cause-consequence relationship between miR-132 loss and disease progression. Animal studies are currently underway to address this issue.

In conclusion, our study proposes a novel molecular pathway where changes in miRNA expression patterns could contribute to the abnormal splicing of tau in disease. Whether this phenomenon occurs in neurons in vivo is likely but remains to be determined experimentally. Of course, this study does not address the role of tau isoform imbalance in PSP pathology and neurodegeneration per se. Nevertheless, the observed down-regulation of miR-132, resulting in changes in PTBP2 as well as other genes involved in neuronal function, supports the miRNA ‘multiple-hit’ hypothesis (9) in PSP and likely other neurodegenerative disorders displaying tau pathology.

MATERIALS AND METHODS

Patient information

The non-dementia controls (n = 8) and PSP dementia (n = 8) patient brains were obtained from the Douglas Hospital.
Research Centre brain bank in Montreal, Canada, in accordance with the national ethical committee protocols and in agreement with the local Centre de recherche du CHUQ ethical committee. Blocks from the temporal, parietal and prefrontal lobes taken from the same individuals were dissected from each case and snap frozen in liquid nitrogen. Total RNA from human brain was extracted on ice using the miRVana PARIS kit (Ambion) according to the manufacturer’s instructions. The mean age average of patients was: controls 70.8 ± 8.2 years and PSP 73.3 ± 11.7 years. The mean age average of post-mortem interval (PMI) values was: controls 19.2 ± 8.9 h and PSP 23.9 ± 10.9 h. No correlations were observed between PMI and miRNA/mRNA/protein quantifications (data not shown). Both control and PSP patient groups consisted of four males and four females.

Cell line and transfections

Mouse neuroblastoma Neuro2A cells were cultured in DMEM medium supplemented with 10% fetal bovine serum as described previously (40). Transfections were performed as before (41) using 50 nm of pre-miRs (Pre-miR miRNA Precursor Molecules, Ambion) or 50 nm (final concentration) siRNAs against murine Ptbpl, Ptbp2 and/or Tau (Mapt) (ON-TARGETplus pool, Dharmacon). Control-scrambled miRNA or siRNA sequences (Ambion, Dharmacon) were used in respective experiments. Forty-eight hours post-transfection, cells were processed for immunoblot analysis.

RNA extraction, PCR and quantitative RT–PCR

Total RNA was extracted from cells and brain using Trizol (Invitrogen) according to the manufacturer’s instructions. PCR procedures were carried out as described previously (40). Primer sequences are found in Supplementary Material, Table S3. For miRNA quantifications, probe-specific TaqMan miRNA assays (Applied Biosystems) were used according to the manufacturer’s instructions. Relative expression was calculated by using the comparative CT method. RNU19 (for mouse) and miR-16 (for human) (Applied Biosystems) were used as a normalization control, as before (29,35).

Microarrays

Information can be found in Supplementary data.
Statistical methodologies

Statistical significance was determined using a Student’s unpaired t-test or Mann–Whitney t-test as indicated in the text. Calculations were made using GraphPad Prism 5 software.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

ACKNOWLEDGEMENTS

We would like to thank Dr Douglas P. Black (Howard Hughes Medical Institute, UCLA, CA, USA) for generously providing us with the PTBP2 monoclonal antibody. We also thank Claudia Goupil (Quebec), Joanie Baillargeon (Quebec) and Sebastien Carlier (Lille) for technical expertise.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the French National Research Agency, the Canadian NSERC Individual Discovery 092850 grant, the Alzheimer Society of Canada (grant to C.D.), the Scottish Rite Charitable Foundation of Canada, and the Research on Alzheimer’s Disease and Related Disorders Quebec-France-Canada Collaboration grant (TAUMIRNA).

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