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## The emerging roles of microRNAs in CNS injuries

**Oneil G. Bhalala, Maya Srikanth, and John A. Kessler**

Department of Neurology, Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, IL 60611, USA.

### Abstract

The consequences of injuries to the CNS are profound and persistent, resulting in substantial burden to both the individual patient and society. Existing treatments for CNS injuries such as stroke, traumatic brain injury and spinal cord injury have proved inadequate, partly owing to an incomplete understanding of post-injury cellular and molecular changes. MicroRNAs (miRNAs) are RNA molecules composed of 20–24 nucleotides that function to inhibit mRNA translation and have key roles in normal CNS development and function, as well as in disease. However, a role for miRNAs as effectors of CNS injury has recently emerged. Use of bioinformatics to assess the mRNA targets of miRNAs enables high-order analysis of interconnected networks, and can reveal affected pathways that may not be identifiable with the use of traditional techniques such as gene knock-in or knockout approaches, or mRNA microarrays. In this Review, we discuss the findings of miRNA microarray studies of spinal cord injury, traumatic brain injury and stroke, as well as the use of gene ontological algorithms to discern global patterns of molecular and cellular changes following such injuries. Furthermore, we examine the current state of miRNA-based therapies and their potential to improve functional outcomes in patients with CNS injuries.

### Introduction

CNS injuries such as spinal cord injury (SCI), traumatic brain injury (TBI) and stroke are common pathologies, collectively affecting over 13.5 million people in the USA alone.<sup>1–3</sup> These injuries constitute a major cause of morbidity and mortality, as beneficial treatments for these complex pathologies are lacking, and obstacles remain with regard to development of effective therapies. Successful treatments for CNS injury must minimize both short-term and long-term cellular damage. Furthermore, identification of factors that lead to irreversible damage requires analyses at both the cellular and molecular level, from disruptions in tissue to alterations in signalling pathways. Microarray technology has been used to uncover some of these factors, and has revealed transcriptome changes in tissue from patients with—as well as in animal models of—CNS injuries.<sup>4</sup> Through microarray studies, a role for protein-coding mRNAs in CNS injury has been revealed; however, the family of noncoding RNAs (ncRNAs) has been increasingly implicated in many diseases of the CNS.<sup>5,6</sup>

microRNAs (miRNAs)—short ncRNAs that modulate protein expression levels by antagonizing mRNA translation—are emerging as powerful regulators of cellular function.<sup>7</sup> As a single miRNA can target and block translation of numerous mRNAs, these molecules

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Correspondence to: O. G. Bhalala oneil-bhalala@fsm.northwestern.edu.

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are capable of altering cellular function at a network level and are, therefore, a particularly potent means of governing cell biology and behaviour. In light of the central roles of miRNAs in CNS development and disease, microarray studies of these molecules in CNS injuries have recently been performed.<sup>8,9</sup> These studies provide the highest-order view to date of the molecular and cellular changes that occur after CNS injury. From this view, global patterns of change in the affected tissue as well as the circulating blood and cerebrospinal fluid (CSF) can be determined, providing insight into pathways that can be therapeutically targeted.

In this Review, we provide an overview of miRNA biogenesis and its role in CNS development, as well as summarizing data from recent miRNA microarray studies of SCI, TBI and stroke. Furthermore, common themes of miRNA biology and function that have arisen from these studies are discussed, and conserved pathways in the CNS injury response are identified. Finally, the potential of miRNA-based therapies as a novel approach to ameliorate the morbidity and mortality associated with CNS injury is highlighted.

## miRNA structure and function

miRNAs are single-stranded ncRNA molecules composed of 20–24 nucleotides that regulate protein expression at the epigenetic level.<sup>10</sup> They are initially transcribed from genomic DNA, largely by RNA polymerase II, in the form of primary miRNA transcripts (pri-miRNA; Figure 1).<sup>11</sup> Pri-miRNAs, which can be thousands of base pairs in length, form functional secondary structures that contain stem-loops consisting of inverted repeats.<sup>11,12</sup> These loops are recognized and cleaved by the endonuclease Droscha (also known as ribonuclease III), which leads to liberation of a precursor miRNA (pre-miRNA) from the pri-miRNA.<sup>13,14</sup> Through the function of exportin-5 (a protein that is involved in nuclear export of RNA), pre-miRNA is transported from the nucleus into the cytoplasm, where Dicer and TAR RNA-binding protein process it to form a duplex of the mature miRNA strand and a complementary strand of similar length (designated miRNA\*).<sup>15–17</sup> The mature miRNA strand is generally biologically active, whereas the miRNA\* strand is not. Consequently, the latter strand is degraded, although exceptions to this rule have been noted.<sup>18,19</sup>

The RNA-induced silencing complex (RISC), which contains the Argonaute family of proteins that have a key role in mediation of RNA interference, facilitates targeting and binding of the miRNA to mRNA.<sup>20–22</sup> The resultant effect of the miRNA–mRNA complex is decreased protein production, as translation and stabilization of the mRNA is inhibited.<sup>23</sup> Quantitative proteomics studies have found that under physiological conditions, a single miRNA can downregulate expression of hundreds of proteins, but the magnitude of change is small (less than fourfold).<sup>24</sup> Moreover, reduction of protein levels is predominately due to mRNA destabilization rather than inhibition of translation.<sup>25,26</sup> These findings suggest that although a given miRNA may have broad effects, multiple miRNAs targeting genes of similar pathways are needed to cause functionally relevant changes in the cell.

miRNAs target mRNAs containing regions that are complementary to the miRNA ‘seed sequence’, a region encompassing nucleotides 2–7 at the 5′ end of the miRNA that has a critical role in miRNA–mRNA binding.<sup>27</sup> The miRNA binding sites on mRNA are typically conserved between species, and are found in the 3′ untranslated region (UTR), although other binding locations have been described.<sup>10</sup> A given miRNA can have multiple binding sites in the same mRNA, thereby enhancing its overall effect. Not all mRNAs are targeted by miRNA, however: it is estimated that only one-third of mRNAs contain miRNA binding sites.<sup>28</sup> Some mRNA transcripts that lack miRNA binding sites were found to contain a short 3′ UTR and, therefore, lacked an miRNA binding domain. Such transcripts escape

miRNA-mediated repression, and many of these ‘short’ mRNAs were found to regulate key cell functions such as DNA repair, protein synthesis and proliferation.<sup>29,30</sup>

With bioinformatics algorithms such as PicTar, miRanda and TargetScan, the mRNA targets of miRNAs can be predicted. As described above, one miRNA can bind to numerous different mRNAs, and a given mRNA can be bound by different miRNAs. Consequently, a given miRNA can modulate many members of a family of genes that are functionally related, thereby enhancing the overall effect of miRNA-mediated inhibition. Conversely, a single mRNA can be strongly repressed through concerted upregulation of the multiple miRNAs that target that mRNA. The various modes through which miRNAs can regulate gene networks highlight the potency of these molecules.

## miRNAs in CNS development

Numerous sequencing studies in organisms ranging from *Caenorhabditis elegans* to humans have revealed that expression of miRNAs is ubiquitous.<sup>15,31–34</sup> In mice, global depletion of miRNAs, which is achieved through deletion of the gene that encodes Dicer, leads to arrested development at embryonic day 7.5,<sup>35</sup> highlighting the vital role of these molecules in general development. miRNAs are also indispensable for development of the CNS, as targeted ablation of the Dicer gene in nervous system tissue during development yields a smaller cortex,<sup>36,37</sup> and deletion of Argonaute proteins (and, therefore, miRNA function) prevents neural tube closure.<sup>38</sup>

Numerous miRNAs are found in the CNS, and many are specific to a given lineage or cell type.<sup>32,39–43</sup> For example, miRNA-9 (miR-9) is expressed in neurogenic regions of the brain where it regulates neural stem cell numbers and functions by binding the 3′ UTR of *TLX*, which encodes a nuclear receptor that maintains proliferation of neural stem cells while inhibiting their differentiation.<sup>44,45</sup> Neuronal miRNAs include miR-124, expression of which increases on neuronal differentiation and reaches maximal levels in mature neurons.<sup>33,46</sup> Targets of miR-124 include protein jagged-1 (Jag-1, also known as Notch ligand receptor), SOX-9 (an adult neurogenesis transcription factor), and DLX2 (a transcription factor involved in neuronal subtype specification).<sup>46,47</sup> Oligodendrocyte-specific miRNAs include miR-219, miR-338 and miR-17.<sup>48</sup> miR-219 and miR-338 promote differentiation of oligodendrocyte precursor cells (OPCs) by repressing expression of proteins that promote OPC proliferation, such as platelet-derived growth factor receptor  $\alpha$ , and the transcription factors SOX-6 and HES-5.<sup>49,50</sup> By contrast, miR-17 promotes OPC proliferation by targeting inhibitors of the AKT pathway.<sup>51</sup> Astrocytic miRNAs have also been identified and include miR-23, miR-26 and miR-29.<sup>52,53</sup> Deletion of Dicer from the astrocytic lineage results in aberrant astrogliogenesis and astrocytic function;<sup>43,54,55</sup> however, the specific mRNA targets of these miRNAs in astrocytes are less well-delineated than in other neural cell types.

## miRNA modulation following CNS injury

CNS injury causes profound cellular changes that occur as a result of dysregulation of signalling pathways and structural proteins. As miRNAs have emerged as important regulators of molecular networks, insight into their modulation following tissue damage could provide a global perspective of the injury response. In the sections that follow, recent miRNA microarray studies of SCI, TBI and stroke are reviewed, and how our knowledge of injury-associated miRNAs can inform therapeutic strategies for these injuries is also discussed.

## Spinal cord injury

SCI has an estimated worldwide annual incidence of 11–60 cases per million people,<sup>56</sup> resulting in an estimated financial burden of more than US\$1 million per patient over an average lifetime.<sup>57</sup> miRNA microarray studies can inform strategies for development of more-effective treatments for SCI, as they provide a broad perspective of the complex molecular and cellular changes that occur after injury.

One microarray study of a contusion model of SCI in rats found that, compared with baseline, over 35% of the miRNAs expressed in the spinal cord were significantly affected within the first 7 days following injury.<sup>58</sup> The affected miRNAs were differentially regulated, with some demonstrating a sustained increase in expression after injury and others a sustained decrease. According to the target prediction algorithm miRanda, upregulated miRNAs (such as miR-1 and miR-206) targeted anti-inflammatory and antioxidant mRNAs. Conversely, downregulated miRNAs, including miR-34a and miR-181a, were predicted to target proinflammatory mRNAs such as tumour necrosis factor and IL-1 $\beta$ . Furthermore, antiapoptotic and proapoptotic mRNAs were identified as targets of miRNAs that showed increased and decreased expression, respectively. Similar observations were made in another microarray study of a contusion SCI model in rats,<sup>59</sup> which found 343 miRNAs to be modulated following injury, most being downregulated by day 7 post-injury compared with baseline. Interestingly, as the injury response progressed, the number of miRNAs that were downregulated gradually increased, whereas the number of upregulated miRNAs remained constant. These data, together with a separate analysis that assessed changes in mRNAs in similar model of SCI,<sup>60</sup> suggest that a negative correlation exists between miRNA and mRNA expression patterns in SCI.

In another SCI model, the number of miRNAs noted to be downregulated compared with baseline was higher at day 14 than at day 4 post-injury.<sup>61</sup> Moreover, this study found a direct correlation between the number of miRNAs affected and injury severity. *In situ* hybridization analyses have confirmed the heterogeneity of temporal expression of miRNAs following SCI, but also revealed spatial heterogeneity of miRNAs after injury. Decreased expression of miR-129-1/2 (a member of the miR-129 family of miRNAs, which prevent post-mitotic cells from re-entering the cell cycle<sup>62</sup>) was initially confined to perilesion white matter tracts up to day 4 post-injury, but had spread towards the rostrum by day 14.<sup>61</sup> Expression of miR-1 (a regulator of transcription, differentiation and cell motility), however, was downregulated at the injury site at both day 4 and day 14 post-injury. Reduction of miR-1 and miR-129 levels could cause cells such as neurons to become aberrantly mitotic, and could explain the increase in apoptosis that is observed at the injury site after SCI.

As well as global miRNA changes, the roles of individual miRNAs in SCI have been elucidated through the use of animal models; some examples are described below.

**miR-20a**—A role for miR-20a in SCI was implicated following the observation that this miRNA was upregulated in a murine transection model of SCI.<sup>63</sup> Infusion of miR-20a into the uninjured spinal cord induced inflammation and neuronal cell death similar to the pathology observed after trauma-induced SCI in patients. Inhibition of miR-20a activity *in vivo* by use of an miRNA inhibitor ameliorated the effects of SCI, improving hindlimb motor recovery and decreasing neuronal death. Mechanistically, miR-20a was found to target the 3' UTR of the mRNA encoding the basic helix–loop–helix protein neurogenin-1 (NGN-1, or Neurog1), a transcription factor that is involved in neuronal differentiation and specification.<sup>64</sup> Importantly, NGN-1 infusion into the spinal cord increased expression of myelin and neuronal markers after transection-induced injury in mice, and markedly improved functional recovery.<sup>64</sup> These findings validate the functional relevance of the interaction between miR-20a and NGN-1, but miR-20a has also been shown to target

STAT3 (a key mediator in the SCI response),<sup>65,66</sup> suggesting that miR-20a can affect the response to SCI via multiple pathways.

**miR-486**—miR-486 is another miRNA that has a profound role in regulating SCI pathology. Similar to miR-20a, miR-486 expression was increased at day 7 post-injury in a murine contusion model of SCI, and was detected in motor neurons by *in situ* hybridization.<sup>67</sup> Infusion of miR-486 into the spinal cords of uninjured mice produced SCI-like effects, with decreased motor function and increased neuronal death.<sup>67</sup> One of the validated targets of miR-486 is NeuroD6, a protein that is important for neuronal differentiation and oxidative stress response.<sup>68</sup> As predicted, NeuroD6 levels were suppressed after SCI in the mouse model.<sup>67</sup> Infusion of a duplexed small interfering RNA (siRNA) that targeted miR-486 caused an increase in NeuroD6 protein levels, which, in turn, increased expression of the reactive oxygen species (ROS) scavenger proteins glutathione peroxidase 3, selenoprotein N and thioredoxin. Such downstream effects caused a decrease in the magnitude of neuronal death and led to a significant improvement in motor recovery. These data suggest that miR-486 is an attractive therapeutic target for SCI, as inhibition of this miRNA leads to a reduction of ROS-mediated cell death, thereby increasing neuronal survival and functional recovery.

**miR-21**—Studies published in 2011 and 2012 revealed that miR-21 is involved in regulation of the response to SCI,<sup>61,69,70</sup> but this findings was somewhat unexpected as miR-21 was first identified as an ‘oncomiR’ (an miRNA related to cancer pathogenesis) owing to its high expression in many cancers including glioblastoma multiforme.<sup>71–73</sup> A role for miR-21 has now been identified in other pathologies such as hypertrophic cardiomyopathy.<sup>74</sup> In a murine contusion model of SCI, miR-21 levels increased modestly around the lesion site within the first 2 weeks post-injury. In the chronic stages following injury, however, a greater upregulation of miR-21, particularly colocalized with astrocytes, was observed.<sup>70</sup>

The function of miR-21 in SCI was further elucidated using transgenic mice in which miR-21 was either overexpressed or inhibited specifically in astrocytes.<sup>70</sup> Astrocytes normally respond to SCI by becoming hypertrophic and increasing their expression of intermediate filament protein<sup>75,76</sup>—changes that are thought to be beneficial by facilitating repair of the blood–brain barrier (BBB) and limiting infiltration of inflammatory cells into the CNS.<sup>77</sup> Interestingly, the beneficial hypertrophic response to SCI was attenuated in transgenic mice that overexpressed miR-21, whereas inhibition of miR-21 augmented the astrocytic response, even in the chronic stages of SCI recovery when astrocytic hypertrophy has normally diminished.<sup>70</sup> Inhibition of miR-21 in astrocytes also had a non-cell-autonomous effect, as the number of axons within the glial scar also increased.

miR-21 is regulated by signalling pathways, such as the bone morphogenetic protein (BMP) pathway, that are pivotal to both the detrimental and recovery responses following SCI.<sup>78</sup> Signalling through BMP receptor type 1a (BMPRIa) reduced miR-21 expression in astrocytes, whereas signalling through BMPRIb had the opposite effect.<sup>53</sup> Of note, the effects of BMPRIa knockout were similar to those observed in mice when miR-21 was overexpressed, whereas knockout of BMPRIb caused a response similar to that observed following miR-21 inhibition. Collectively, these results suggest that BMP-mediated effects in SCI occur via regulation of miR-21.

**Exercise-induced miRNAs**—Exercise following SCI has shown promise as a means to improve functional recovery,<sup>79</sup> and research suggests that miRNAs can mediate this recovery process. For instance, in rats that were made to exercise after SCI, levels of the proapoptotic miRNA miR-15b were significantly decreased, whereas miR-21 (which targets

and blocks the proapoptotic protein programmed cell death protein 4) was upregulated.<sup>80</sup> miR-21 also targets PTEN, a negative regulator of the AKT–mTOR (mammalian target of rapamycin) pathway, which governs proliferation and growth. Conditional knockout of PTEN in corticospinal tract neurons enhanced regeneration of axons through the injured tissue.<sup>81</sup> An mTOR-targeting miRNA, miR-199a-3p, was also found to be downregulated by exercise.<sup>69</sup> These data suggest that the beneficial aspects of exercise after SCI are, at least in part, attributable to changes in miRNAs that have the net effect of promoting cellular plasticity while inhibiting neuronal apoptosis. Interestingly, the findings on miR-21 suggest that the effects of this miRNA on proliferation and growth vary with cell type, with an inhibitory role in astrocytes but a positive effect in neurons. To maximize the therapeutic potential of miRNAs, cell-specific modulation of these molecules may, therefore, be necessary.

### Traumatic brain injury

TBI is a leading cause of mortality in the general population in the USA, accounting for over 50,000 deaths annually.<sup>1</sup> Within the armed forces, however, post-deployment surveys indicate that up to 20% of soldiers who have returned from Iraq have experienced at least one TBI.<sup>82</sup> Similar to their role in SCI, miRNAs may be important mediators of the profound molecular and cellular changes that occur after TBI in both the short and the long term. Microarray analyses in rat models of TBI have revealed dynamic temporal regulation of miRNA expression within the cortex, with the numbers of downregulated and upregulated miRNAs peaking at 24 h and 72 h post-injury, respectively.<sup>83</sup>

**miRNA changes in the hippocampus**—Structural changes within the hippocampus are among the most frequent sequelae of TBI; in one MRI study, hippocampal atrophy was observed in 59% of patients with TBI.<sup>84</sup> Furthermore, hippocampal pathology is a major predictor of the cognitive and memory deficits in many individuals who have experienced a TBI.<sup>85</sup> Microarray studies in animal models of TBI have revealed significant changes in miRNA expression within the hippocampus. Of the 444 verified rodent miRNAs, 35 were found to be upregulated and 50 downregulated after a TBI.<sup>86</sup> According to gene-ontology bioinformatics analyses, the predicted targets of these miRNAs are involved in signal transduction, transcription, proliferation and differentiation.

In another study of TBI in rodent models, deep sequencing identified eight upregulated and 13 downregulated miRNAs at 24 h post-injury, whereas three were upregulated and 13 downregulated at day 7 post-injury.<sup>87</sup> In this study, the miRNAs that showed changes in expression during the acute post-injury phase were predicted to target genes involved in apoptosis, protein folding, and aerobic respiration—processes that are associated with pathology and stress management in the cell. By contrast, miRNAs that were dysregulated in chronic stages of injury were predicted to regulate genes involved in cytoskeletal organization and intracellular trafficking—processes linked to brain repair mechanisms. As observed in other CNS injuries, miR-21 was upregulated in the TBI-affected hippocampus, particularly in the dentate gyrus and CA3 region.<sup>88</sup> Expression of protein cell death protein 4, a target of miR-21, was decreased in these regions, identifying a potential miR-21-regulated survival mechanism in cells that are affected by TBI.

**miRNAs as biomarkers**—The role of miRNAs in TBI may not be purely mechanistic. When treating a patient with suspected TBI, timely diagnosis is difficult, as cognitive effects of the injury may not manifest for days or weeks, by which point the therapeutic window has narrowed. miRNA-based bio-markers of brain injury in the serum and CSF could serve as tools to quickly identify affected patients after suspected TBI. Studies of CSF from TBI-affected rats found a significant increase in levels of one miRNA, miR-let-7i, as early as 3 h

post-injury.<sup>89</sup> Prediction analysis revealed that miR-let-7i targets TBI-related proteins such as S100B and UCH-L1, suggesting a possible role for miR-let-7i in regulating TBI pathology.<sup>90–92</sup> Studies in patients with TBI have identified three other miRNAs—miR-16, miR-92a, and miR-765—that can serve as diagnostic biomarkers for severe brain injury. Notably, levels of miR-16 and miR-92a are also increased in the plasma of patients with mild TBI.<sup>93</sup> Collectively, these studies highlight miRNAs as promising diagnostic tools in TBI.

**miRNA changes following treatment**—One of the most promising treatments for TBI is hypothermia, a therapy that reduces prolonged neurological damage and improves functional outcomes.<sup>94–96</sup> Interestingly, some miRNAs that show altered expression after TBI are also temperature-sensitive. In a rat fluid-percussion model of TBI, the levels of many miRNAs that were upregulated after TBI were reduced under hypothermic conditions.<sup>97</sup> One such miRNA, miR-9, is predicted to reduce expression of proteins known to interact with actin-binding proteins and basal plasma membranes, suggesting that an increase in miR-9 levels after TBI would perturb cytoskeleton and cell-adhesion properties, thereby increasing cell death. Reduction in the levels of miR-9, therefore, could contribute to the enhanced cell survival that is observed with hypothermia treatment. Further experiments in animal models of TBI are needed to determine whether modulation of miR-9, either alone or in combination with other miRNAs, can lead to improvements following TBI.

## Stroke

One in six people worldwide will have at least one stroke in their lifetime.<sup>98</sup> Stroke can result in extensive tissue damage as well as long-term cognitive and motor deficits, which are potentiated by cerebral oedema and neuronal death among other phenomena. Identification of the miRNAs that are involved in regulation of stroke-related cellular and molecular networks could provide insight into new therapeutic avenues. As with TBI, dysregulation of miRNAs following stroke can be assessed in peripheral blood samples. One analysis of whole blood from patients who had experienced a stroke revealed that of the 836 miRNAs tested on the array chip, 157 were differentially regulated: 138 upregulated and 19 down regulated.<sup>99</sup> Ontological analyses predicted that the targets of the dysregulated miRNAs were involved in angiogenesis, hypoxia, endothelial cell regulation, and the immune response. Surprisingly, the miRNA profile of large-artery strokes was distinct from that of small-artery strokes, suggesting that the vessel affected by stroke can be identified through analysis of whole-blood miRNA levels.<sup>99</sup>

**miRNA-based differentiation of stroke subtypes**—miRNA expression profiles can be used to differentiate between ischaemic and haemorrhagic stroke.<sup>100</sup> Analysis of rat blood at day 1 post-injury revealed that 10 and 20 miRNAs were upregulated in ischaemic and haemorrhagic stroke models, respectively. Of these miRNAs, only eight were common to both models. 65 and 21 miRNAs were downregulated in ischaemic and haemorrhagic strokes, respectively, with 14 miRNAs being shared between the stroke subtypes. Analysis of miRNA levels in brain tissue from the animals led to a similar conclusion: miRNA changes in ischaemic or haemorrhagic stroke are largely distinct, but also show some overlap. These studies suggest that a similar set of cellular functions, such as cell cycle and cell death, are affected in both types of stroke; however, as distinct subsets of miRNAs are dysregulated in each stroke subtype, some pathways could be differentially affected depending on stroke aetiology. Further investigation is needed to discern which pathways are unique to each stroke subtype and how they can be exploited to maximize detection and treatment of stroke.

**Modulation of cerebral oedema**—Cerebral oedema is a common consequence of stroke that contributes to tissue and cellular damage following ischaemia. Levels of aquaporin proteins, which function as cellular water conduits, are known to increase after cerebral ischaemia,<sup>101</sup> although their exact role in this process is unclear. Some reports based on rodent models suggest that reduction of aquaporin-4 levels after stroke leads to a reduction in oedema,<sup>102</sup> whereas others suggest that upregulation of aquaporin-4 is required to facilitate oedema clearance.<sup>103</sup> In a rodent middle cerebral artery occlusion (MCAO) model, levels of miRNAs that are predicted to target aquaporins (such as miR-30a-3p and miR-383) were decreased and aquaporin-4 levels were increased after ischaemia.<sup>104</sup> In addition, miR-320a, which is also downregulated in MCAO, has validated binding sites in the 3' UTR of the aquaporin-1 and aquaporin-4 mRNAs.<sup>105</sup> Collectively, these studies suggest that modulation of miRNAs can be used to regulate aquaporins and, therefore, affect oedema following cerebral ischaemia.

**miRNAs and neuronal cell death**—Neuronal cell death is another prominent feature observed after stroke. In accordance with this phenomenon, levels of miRNAs that regulate apoptosis are altered following cerebral ischaemia. For example, miR-497, which targets the antiapoptotic genes *Bcl-2* and *Bcl-w*, was upregulated in infarcted regions in a mouse model of MCAO.<sup>106</sup> Importantly, inhibition of miR-497 in mice reduced infarct size while enhancing neurological function. *Bcl-W* is an identified target of miR-29b, an miRNA that was found to be upregulated in neurons after MCAO.<sup>107</sup> miR-21 is also thought to increase neuronal survival after stroke by targeting and blocking translation of the gene that encodes Fas ligand (a receptor that signals to induce apoptosis).<sup>108</sup> Such a mechanism could explain preservation of neurons within the ischaemic boundary zone—a region in which miR-21 levels are increased following ischaemia.

ROS are also a key trigger of neuronal apoptosis. Scavengers of ROS, such as superoxide dismutase 2 (SOD2), help to protect neurons from such stress.<sup>109</sup> Interestingly, SOD2 is targeted by miR-145, another miRNA that is upregulated after MCAO. In a mouse model of MCAO, infusion of an miR-145 antagonist (antagomiR-145) caused an increase in neuronal SOD2 expression and a reduction in infarct size.<sup>110</sup> This study suggests the existence of a class of miRNAs that can be modulated to improve neuronal survival after ischaemic events.

**miRNAs in ischaemic preconditioning**—A theoretical strategy for neuroprotection in stroke is ischaemic preconditioning (IPC), which involves brief exposure to nonlethal ischaemia to induce protective mechanisms in the tissue during phases of prolonged ischaemia.<sup>111</sup> Microarray studies in animal models have shown that levels of numerous miRNAs are rapidly altered following IPC. Such alterations include upregulation of miRNAs that target members of the mitogen-activated protein kinase (MAPK) and mTOR signalling pathways.<sup>112</sup> Conversely, miRNAs that were down regulated seemed to target signalling via Wnt. miRNA-based modulation of these three pathways (MAPK, mTOR and Wnt) can increase cytoprotection and cellular regeneration in the affected tissue. The miR-200 family was also upregulated within 3 h of IPC, with the neuroprotective effect driven by downregulation of prolyl-hydroxylase 2, a negative regulator of the pro-survival protein hypoxia-inducible factor-1 $\alpha$ .<sup>113</sup> Another study identified methyl CpG binding protein 2 (MeCP2)—a global transcriptional regulator and putative mediator of the beneficial effects of IPC—as a target of many of the downregulated miRNAs.<sup>114</sup> This observation provided an explanation for the increase in MeCP2 protein expression that was observed after IPC despite the fact that the mRNA levels of MeCP2 remained unchanged.<sup>115</sup> These studies serve as guidance for the translation of neuroprotective mechanisms of IPC from bench to bedside.



## Common themes of dysregulated miRNAs

Many of the studies reviewed here rely on miRNA microarray data to develop an understanding of the CNS injury response. Although this method of analysis provides a global view of the changes that occur during injury, microarrays have restrictions, such as a limited miRNA expression profile of the microarray chip and need for validation of affected miRNAs. Importantly, many of the predicted mRNA targets of identified miRNAs remain undefined and require further investigation. Analysis of the collective results from microarray studies can, however, provide insights into the characteristics of the CNS injury response. A meta-analysis of micro array studies unmasks the miRNA response kinetics after injury, revealing that many miRNAs are altered as early as day 1 post-injury (Table 1). This observation is consistent with reports from studies that used *in vitro* models of CNS injuries or synthetic inducible miRNA reporter systems.<sup>116,117</sup> The finding of early post-injury modulation of miRNAs suggests that molecules involved in regulation of miRNA expression are at least partially present before injury, and that they do not wholly rely on protein production following activation of signalling components. Furthermore, spatial expression profiles indicate that the affected miRNAs lie within and adjacent to the injury site, suggesting that miRNA modulation involves both autocrine and paracrine signalling. However, as changes in blood miRNAs were also detected, systemic signalling is also likely to be involved in the modulation of miRNA levels following injury.

Analysis of predicted targets of miRNAs that are modulated following SCI, TBI and stroke indicate that some of the targeted pathways—such as apoptosis, inflammation, cell proliferation and cell differentiation—are shared between these injuries (Table 1). However, close inspection reveals that a given cellular function is often regulated by a different set of miRNAs in each injury. Regulation of neuronal differentiation, for example, is affected in the various CNS injuries, but different miRNAs and gene targets have been implicated in each. As detailed above, miR-20a is upregulated after SCI and targets the proneural gene *Ngn1*.<sup>63</sup> In stroke, however, miR-124, which promotes neuronal differentiation of neural progenitor cells by targeting *Jag1* mRNA, is downregulated.<sup>47</sup> Apoptosis is regulated by miR-15b and miR-486 after SCI,<sup>67,80</sup> but is regulated by miR-29b, miR-145 and miR-497 following stroke.<sup>106,107,110</sup> Despite such injury-specific miRNAs, certain miRNAs are modulated in all injury types: miR-21 is the most frequently reported of such miRNAs, and may serve as a common cell-death regulator in SCI, TBI and stroke.

The findings of miRNA studies build on our knowledge gained from mRNA-based microarray analysis of CNS injuries. A common theme that has arisen from both mRNA and miRNA studies is the complexity of pathway regulation, as well as recognition of miRNAs as a means to regulate similar pathways through different gene targets. Therapeutic strategies can be designed to target miRNAs that affect common pathways, thereby enabling modulation of mRNA levels to increase cell survival, reduce inflammation, and promote cell proliferation and differentiation in the various injuries. Moreover, as unique sets of miRNAs are altered in each injury type, miRNAs can potentially serve as diagnostic biomarkers for the different types and subtypes of CNS injuries.

## miRNA-based therapies

As described above, miRNA levels are altered after CNS injuries, and such changes have profound effects on the downstream targets of these molecules. Restoration or inhibition of miRNA activity, therefore, represents a therapeutic opportunity to modulate cellular process and improve outcomes in patients with CNS injuries. The current state of miRNA-based therapies—including miRNA amplifiers and inhibitors, and artificial miRNAs—are discussed below.

## miRNA amplifiers

Few methods that aim to exogenously increase levels of miRNAs after CNS injury have been developed. One such method involves miRNA mimics: modified double-stranded RNA molecules with one strand containing the same sequence as the mature miRNA. Notably, naturally occurring RNA molecules contain uridine bases and uridine–guanine nucleotide pairs that induce a nonspecific immune response by activating Toll-like receptors.<sup>118</sup> As the miRNA mimic sequence cannot be changed without its efficacy or specificity being compromised, chemical modifications of these nucleotide bases are necessary to minimize the immune reaction. For example, a 2′ ribose modification to replace guanine with cyclopentyl-guanine in a miRNA mimic can markedly reduce immune activation while maintaining miRNA activity (Figure 1).<sup>119</sup> Modifications to other regions of the molecule, such as the 3′ overhang, have also been shown to increase miRNA-mimic stability by reducing the extent of nuclease degradation of the mimic.<sup>120</sup> *In vivo* studies of miRNA mimics have been performed in other fields such as oncology.<sup>121</sup> Studies of miRNA mimics in the context of patients with stroke, TBI or SCI are lacking, but these molecules could also be an effective therapy for CNS injuries if they can be deployed in an appropriate manner.

Another strategy for increasing miRNA levels *in vivo* is to introduce the pri-miRNA or pre-miRNA sequence into the target tissue with the use of a DNA-based plasmid (Figure 1). Successful application of this technology has been demonstrated in a stroke model in which a plasmid encoding pri-miR-181 was introduced into the brain using intraventricular infusion.<sup>122</sup> This process led to production of functional miR-181 molecules that successfully targeted heat shock protein 70 family members, and to a reduction in infarct size. One benefit of plasmid-based expression of miRNAs is that the amount of mature miRNA can be amplified by increasing transcription of the plasmid. This method, however, relies on the action of endogenous enzymes such as Drosha and Dicer, the levels and function of which may be reduced in the damaged tissue.<sup>123</sup>

## miRNA inhibitors

Owing to concerted research efforts to block the function of oncomiRs, marked progress has been made in the development of miRNA inhibitors: antisense sequences that bind mature miRNA molecules and block their function.

**AntagomiRs**—AntagomiRs are first-generation miRNA inhibitors that contain 2′-*O*-methyl-modified nucleotides, terminal phosphorothioates and 3′-end-conjugated cholesterol groups—features that increase *in vivo* stability and bio-availability of the inhibitor (Figure 1).<sup>124</sup> AntagomiRs have been effectively delivered to many tissues, including the CNS,<sup>125</sup> but as high doses of these inhibitors are likely to be needed for *in vivo* efficacy, more-potent strategies may be required.<sup>126</sup>

**Locked nucleic acids**—Locked nucleic acids (LNAs) offer one strategy for high-potency miRNA inhibition (Figure 1). LNAs are modified oligonucleotides that contain locked ribose rings. The modifications to these molecules are designed to minimize endonuclease degradation while markedly enhancing their capacity to bind to target miRNA.<sup>127,128</sup> In mice and nonhuman primates, LNA molecules have demonstrated low toxicity and enhanced *in vivo* function compared with antagomiRs.<sup>129,130</sup> In chimpanzees that had been infected with hepatitis C virus, intravenous administration of an LNA that targeted miR-122 (an miRNA that is essential for replication of hepatitis C virus) reduced not only the levels of this miRNA in the liver but also the number of circulating hepatitis C viral particles.<sup>131</sup> Following such promising results, a phase II clinical trial of the miR-122-targeting LNA was initiated, and has recently been completed, in patients with chronic hepatitis C.<sup>132</sup>

**miRNA sponges**—Although antagomiRs and LNAs are tools that enable knockdown of miRNA functions, their effects are likely to be transient, as the half-life for these molecules in serum is just a few days.<sup>133,134</sup> To overcome this problem and obtain persistent inhibition, miRNA-sponge constructs have been developed. An miRNA sponge is an artificial RNA transcript containing multiple repeats that are complementary to the target miRNA (Figure 1).<sup>135</sup> Consequently, the sponge serves as a competitive inhibitor, sequestering the miRNA from its endogenous targets. Retroviruses and lentiviruses have been used to integrate plasmid DNA encoding the sponge transcript into the brains of mice, and this approach led to stable *in vivo* inhibition of the target miRNA.<sup>136,137</sup> Sponge transcripts are promising tools for treatment of CNS injuries, as inhibition of the miRNAs that are upregulated during injury is likely to improve patient recovery.

### Delivery mechanisms

Development of miRNA mimics and inhibitors has a promising future, but targeted delivery of these molecules, in particular into the CNS, remains a major obstacle. Direct administration of miRNA therapeutics, for example into the ventricles or parenchyma, is one potential delivery method, but the resulting tissue damage and immune-system activation present some drawbacks. Delivery methods that afford passage across the BBB have, therefore, been explored. One such method involves exosomes—endogenous lipid-membrane vesicles that are secreted by cells and can serve as carriers for RNA molecules.<sup>138,139</sup> On their entry into the systemic circulation, exosomes can cross the BBB and deliver siRNA molecules for gene silencing.<sup>140</sup> Although the exosome delivery method has not yet been applied to miRNAs, its use for CNS-targeted miRNA-based therapies is likely to be a natural extension (Figure 1).

### Artificial miRNAs

An emerging idea in the field of RNA interference therapy is the use of miRNA-mediated silencing to control gene expression in an artificial manner. After injury to the CNS, many deleterious mRNAs—such as those encoding proteins involved in apoptosis and inflammation—are upregulated.<sup>141,142</sup> Artificial miRNAs that target groups of mRNAs involved in such deleterious processes could be a useful therapeutic strategy. Indeed, with the aid of computer algorithms, artificial miRNAs have been developed that can be used to identify miRNA seed sequences in genes of interest.<sup>143,144</sup> By mining the vast amount of available mRNA microarray data for transcriptome changes seen after CNS injuries, seed sequences that are common to different mRNAs can be identified and exploited to generate artificial miRNAs. Such an approach could enable precise gene expression control with minimal off-target mRNA binding, as artificial miRNA sequences can be adjusted to bind only to the intended transcript.

### Conclusions and future directions

The fact that the cellular and molecular changes that occur after CNS injuries are diverse provides a substantial challenge for single-target therapies. Identification of the miRNAs that are altered following CNS injury provides a means to elucidate global patterns within these changes. An analysis of recent miRNA microarray studies indicates that pathways such as apoptosis, inflammation and cell proliferation are among the most commonly affected pathways in CNS injuries, and that changes in these pathways are conserved across CNS injuries from SCI to stroke. Consequently, therapies aimed at modifying these pathways could be broadly applicable to a variety of CNS injuries, but multifaceted therapies that target many components in multiple pathways are also likely to be required. Exploitation of miRNA-based therapeutics is an appealing means of accomplishing this goal.

Promising advances in synthetic miRNA amplifiers and inhibitors have been made with regard to improved bioavailability, function, and adverse-effect profiles. The success of studies in which known miRNAs were targeted, such as the clinical trial of miR-122 in hepatitis C,<sup>132</sup> provide inspiration for development of novel miRNA-based therapies. Furthermore, the power of bioinformatics and target-prediction algorithms can be harnessed for the design of artificial miRNAs that target a diverse set of mRNA transcripts involved in the common pathways of CNS injuries. Full realization of the potential of miRNAs, however, will require validation of findings from animal models and in human tissue; only then can microarray data and rapid miRNA pharmaceutical development be combined to help improve outcomes after CNS injuries.

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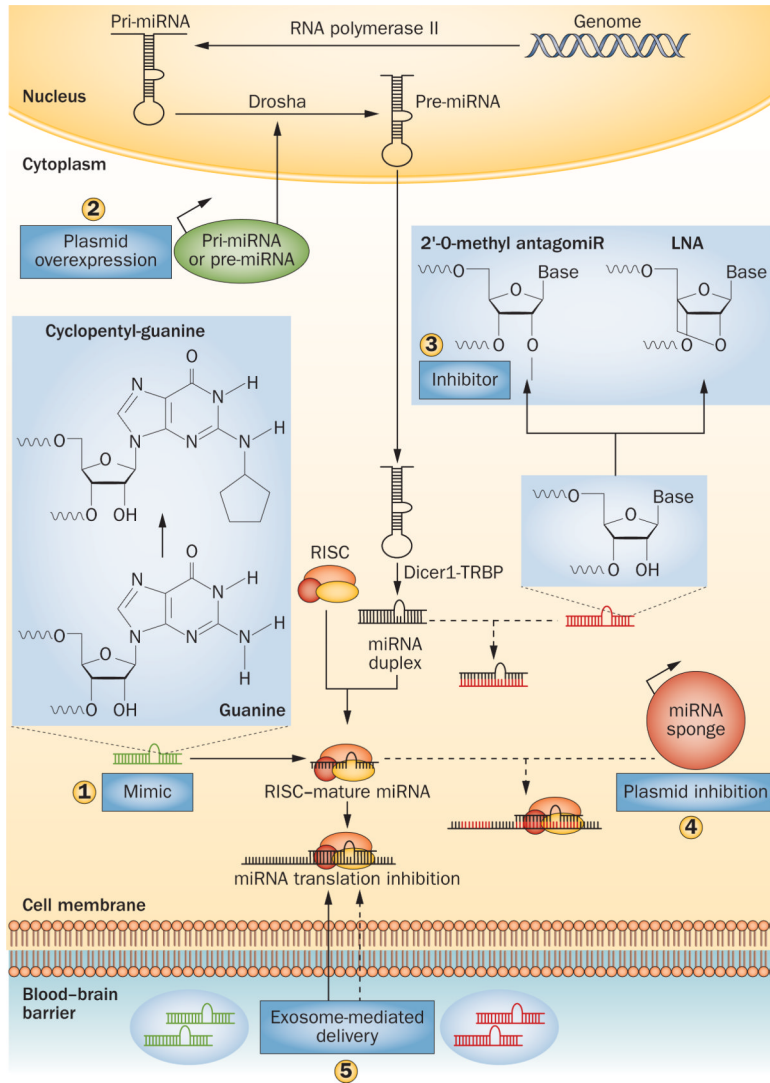
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### Key points

- MicroRNAs (miRNAs) are critical for normal development but also have a role in disease, particularly of the CNS
- miRNA microarrays provide the highest-order view of changes that occur following injuries to the CNS, such as stroke, spinal cord injury or traumatic brain injury
- These CNS injuries affect key cellular pathways such as apoptosis, inflammation and cellular proliferation, all of which are regulated by miRNAs
- miRNA-based therapies that augment or inhibit miRNA activity, and with enhanced bioavailability and function, are currently being developed
- Artificial miRNAs can be designed to bind to and modify groups of mRNA transcripts that are not naturally targeted by endogenous miRNAs

### Review criteria

PubMed was searched for articles, including advance online publications published up to 26<sup>th</sup> January 2013, using the following search terms: “microRNA”, “CNS injury”, “spinal cord injury”, “traumatic brain injury”, “stroke”, “ischaemia”, “microRNA mimics”, “antagomiRs”, “locked nucleic acids”, “microRNA sponge”, and “artificial microRNA”. Full versions of review and original articles were assessed for appropriateness. References were also examined to identify additional articles not captured using the search terms listed. The database at [www.ClinicalTrials.gov](http://www.ClinicalTrials.gov) was searched for miRNA-based therapies.



**Figure 1.** Therapeutic modulation of miRNA activity. miRNA mimics are molecules that can be used to overexpress miRNAs (1). They contain modified cyclopentyl-guanine bases, which enable association with the RISC within the cytoplasm to inhibit mRNA translation. Pri-miRNAs and pre-miRNAs can be expressed within cells by a plasmid, and the transcript is then processed by endogenous enzymes into mature miRNA (2). 2'-O-methyl antagomiRs and LNAs inhibit miRNA function by binding to mature miRNAs, thereby preventing their interaction with target mRNAs (3). Plasmids encoding an miRNA sponge transcript can be used for stable miRNA inhibition (4). The sponge transcript contains repeats of complementary sequences to the target miRNA (red regions of transcript), thereby sequestering miRNAs away from endogenous targets. Exosomes are BBB-crossing lipid vesicles that can deliver either miRNA mimics or inhibitors to modulate miRNA activity (5). Solid arrows indicate pathways that lead to increased miRNA activity; dashed arrows indicate pathways that lead to inhibition of miRNA activity. Abbreviations: BBB, blood-brain barrier; LNA, locked nucleic acid; miRNA, microRNA; pre-miRNA, precursor miRNA; pri-miRNA, primary miRNA; RISC, RNA-induced silencing complex; TRBP, TAR RNA-binding protein.

**Table 1**

Summary of miRNA microarray studies in CNS injury

Study	Tissue analysed	Time points analysed	Pathways predicted to be modulated	Number of miRNAs increased at last time point	Number of miRNAs decreased at last time point
<b>Spinal cord injury</b>					
Liu <i>et al.</i> (2009) <sup>58</sup>	Rat spinal cord	4 HPI, 1 DPI, 7 DPI	Apoptosis Inflammation Antioxidation	30	16
Strickland <i>et al.</i> (2011) <sup>61</sup>	Rat spinal cord	4 DPI, 14 DPI	Apoptosis Inflammation Cell proliferation Cell differentiation Cell cycle	3	32
Yunta <i>et al.</i> (2012) <sup>59</sup>	Rat spinal cord	1 DPI, 3 DPI, 7 DPI	Apoptosis Inflammation Cellular regeneration	11	192
<b>Traumatic brain injury</b>					
Lei <i>et al.</i> (2009) <sup>83</sup>	Rat cortex	6 HPI, 24 HPI, 48 HPI, 72 HPI	Not performed	19	5
Redell <i>et al.</i> (2009) <sup>86</sup>	Rat hippocampus	3 HPI, 24 HPI	Cell proliferation Cell differentiation Cell cycle Protein modification	24	28
Hu <i>et al.</i> (2012) <sup>87</sup>	Rat hippocampus	24 HPI, 7 DPI	Apoptosis Transcription Cytoskeleton arrangement Cell adhesion	3	13
Balakathiresan <i>et al.</i> (2012) <sup>89</sup>	Rat serum and cerebrospinal fluid	3 HPI, 24 HPI	Axon guidance Cytoskeleton arrangement Cell adhesion Intracellular signalling	35	4
Redell <i>et al.</i> (2012) <sup>93</sup>	Human blood plasma	3 DPI	Apoptosis Cell proliferation Cell cycle Angiogenesis	27	33
<b>Stroke</b>					
Jeyaseelan <i>et al.</i> (2008) <sup>104</sup>	Rat brain and blood	24 HPI, 48 HPI	Aquaporins Cellular regeneration Extracellular matrix remodelling	32 (brain) 10 (blood)	49 (brain) 15 (blood)
Dharap <i>et al.</i> (2009) <sup>110</sup>	Rat cortex	3 HPI, 6 HPI, 12 HPI, 1 DPI, 3 DPI	Antioxidization Inflammation Ion channels Neurotransmitter receptors Transcription factors	24	23
Tan <i>et al.</i> (2009) <sup>99</sup>	Human peripheral blood	6–18 months post-injury	Angiogenesis Cell proliferation Hypoxia Inflammation Intracellular signalling	176	23

Abbreviations: DPI, days post-injury; HPI, hours post-injury; miRNA, microRNA.