Functional integration of complex miRNA networks in central and peripheral lesion and axonal regeneration

Review article

Functional integration of complex miRNA networks in central and peripheral lesion and axonal regeneration

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Abstract

New players are emerging in the game of peripheral and central nervous system injury since their physiopathological mechanisms remain partially elusive. These mechanisms are characterized by several molecules whose activation and/or modification following a trauma is often controlled at transcriptional level. In this scenario, microRNAs (miRNAs/miRs) have been identified as main actors in coordinating important molecular pathways in nerve or spinal cord injury (SCI). miRNAs are small non-coding RNAs whose functionality at network level is now emerging as a new level of complexity. Indeed they can act as an organized network to provide a precise control of several biological processes. Here we describe the functional synergy of some miRNAs in case of SCI and peripheral damage. In particular we show how several small RNAs can cooperate in influencing simultaneously the molecular pathways orchestrating axon regeneration, inflammation, apoptosis and remyelination. We report about the networks for which miRNA-target bindings have been experimentally demonstrated or inferred based on target prediction data: in both cases, the connection between one miRNA and its downstream pathway is derived from a validated observation or is predicted from the literature. Hence, we discuss the importance of miRNAs in some pathological processes focusing on their functional structure as participating in a cooperative and/or convergence network.

Abbreviations: AAV(s), adeno-associated virus(es); ADAM, a disintegrin and metalloprotease; AGO(2), argonaut (2); AKT, protein kinase B; APC, adenomatous polyposis coli; ASO(s), antisense oligonucleotide(s); ATF3, activating transcription factor 3; BBB, blood brain barrier; BCAA, branched chain amino acids; BCAT2, branched-chain aminotransferase; Bcl-2, B-cell lymphoma 2; BDNF, brain-derived neurotrophic factor; C11Orf9, myelin regulatory factor; CAND1, cullin-associated and neddylation-dissociated 1; CREB, cAMP response element binding protein; DGCR8, DiGeorge Syndrome critical region 8; DHA, docosahexaenoic acid; Dkk-1, Dickkopf-related protein 1; DLK, dual leucine zipper kinase; DRG, dorsal root ganglion; Egr2, early growth response 2; FADD, Fas-Associated protein with Death Domain; FAP-1, FAS associated phosphatase-1; FAS, associated protein with death domain; FAS-L, associated protein with death domain ligand; FGF2, fibroblast growth factor 2; FGF9, fibroblast growth factor 9; FGFR2, fibroblast growth factor receptor 2; FoxJ3, forkhead box J3; FOXO, Forkhead box O; Foxp(12), Forkhead box O p(1,2); GAP-43, growth associated protein 43; GFAP, glial brillary acidic protein; GSK-3(β), glycogen synthase kinase 3(β); Hes5, hes family bHLH transcription factor 5; HspB1, heat shock protein B1; IkBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha; IKK, inhibitor of kappa kinase complex; IL-1, interleukin 1; IL-1α, interleukin 1α; IL-1β, interleukin 1β; IL-6, interleukin 6; IRAK1, interleukin 1 receptor associated kinase 1; JNK, c-Jun N-terminal kinase; KLF(6), Krueppel-like factor (6); LIF, leukemia inhibitory factor; LNA, locked nucleic acid; LV, lentiviral vectors; MAG, myelin-associated glycoprotein; MAP1B, microtubule associated protein 1B; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; MCAo, middle cerebral artery occlusion; MecP2, methyl CpG binding protein 2; miRNA(s) or miR(s), microRNA(s); MRF, myelin regulatory factor; mTOR, mechanistic target of rapamycin; NCAM, neural cell adhesion molecule; NeuroD1, neuronal differentiation D1; Neurog2, Neurogenin 2; NF-kB, nuclear factor kappa B; NGF, nerve growth factor; NPC, neuronal progenitor cell; NSCs, neural stem cells; OLs, oligodendrocytes; OPCs, oligodendrocyte progenitor cells; p250GAP, Rho GTPase activating protein 32; p53, tumor protein p53; p73, tumor protein p73; PAK, p21-activated kinases; PDK1/2, phosphatidylinositol-dependent kinase 1/2; PDZ-RhoGEF, Rho guanine nucleotide exchange factor 11; PI3K, phosphoinositide 3-kinases; PMP-22, peripheral myelin protein 22; PRKAG3, protein kinase AMP-activated non-catalytic subunit gamma 3; PTEN, phosphatase and tensine homolog proteins; PUMA, p53 upregulated modulator of apoptosis; rAAV, recombinant AAV; Rac1, Ras-related C3 botulinum toxin substrate 1; REST, RE1-Silencing Transcription factor; RhoA, Ras homolog gene family member A; RISC, RNA-induced silencing complex; ROCK1, rho-associated coiled-coil-containing protein kinase 1; RVG, rabies virus glycoprotein; SARM-1, sterile alpha- and armadillo-motif-containing protein-1; SC(s), Schwann cell(s); SCI, spinal cord injury; SCZ, schizophrenia; siRNA, small interfering RNA; SMA, spinal muscular atrophy; SMAC, second mitochondria-derived activator of caspases; SMAD2, mothers against decapentaplegic homolog 2; SMAD3, mothers against decapentaplegic homolog 3; SOD1, superoxide dismutase 1; Sox(24,6,9,10,11), SRY-box (2,4,6,9,10,11); Sox2, SRY-box 2; Sox4, SRY-box 4; Sox6, SRY-box 6; SPCs, Schwann progenitor cells; STAT3, signal transducer and activator of transcription 3; TDP-43, TAR DNA-binding protein 43; TF, transcription factor; TGF-β, transforming growth factor-β; TGF-β, transforming growth factor-β; TIAM1, T-cell lymphoma invasion and metastasis 2; TLX, tailess; TNF-α, tumor necrosis factor alpha;

TRAF6, TNF receptor associated factor 6; TRBP, trans-activation response RNA-binding protein; wnt, wingless-related integration site; XIAP, x-linked inhibitor of apoptosis protein; ZFP238, zinc finger protein 238

Keywords: Spinal cord injury; Axotomy; Neuroinflammation; Apoptosis; Cooperation; Convergence

1 P~~ERIPHERAL AND CENTRAL AXON DEGENERATION AFTER INJURY~~eripheral and central axon degeneration after injury

Peripheral and central injury (due to motor vehicle accident, falls or sporting injuries) may severely damage axons and/or neurons, leading to a partial or complete motor, sensory and/or autonomic dysfunction, and considerably compromising the patient quality of life. Depending on the injury severity, neural death, inflammation and retrograde degeneration occur at different extent as consequence of specific molecular degenerative mechanisms. We summarize the key events and pathways taking place upon PNS/CNS damage and leading to degeneration.

As concerns PNS, during the first phases after injury, axon and myelin damage occurs both at the proximal and distal segments of the nerve in a process called Wallerian degeneration. In the first hours after the injury the end of the axon swells and in two days the increase of intracellular Ca^{2+} induces axon degeneration through calcium dependent proteolytic enzymes such as calpain (George et al., 1995). The increased Ca^{2+} influx leads to kinase activation such as dual leucine zipper kinase, c-Jun N-terminal kinase (JNK), inhibitor of kappa kinase complex (IKK) and glycogen synthase kinase 3 (GSK-3) whose inhibition protect axons in dorsal root ganglion (DRG) neuron culture (Gerdts et al., 2011; Michaelevski et al., 2010). Pathways involving transforming growth factor-β (TGF-β)/mothers against decapentaplegic homolog 2/3(SMAD2/SMAD3) and sterile alpha- and armadillo-motif-containing protein-1 have been identified as negative signals limiting axonal ability to regrowth (Gerdts et al., 2013; Patent number US7524640B2).

The growth pathway involving phosphoinositide 3-kinases-protein kinase B (PI3K-AKT), whose inhibition is associated with poor growth ability, is repressed by phosphatase and tensin homolog (PTEN) after peripheral nerve injury. Indeed its *in vivo* inhibition in a rat sciatic nerve injury model accelerates axon outgrowth (Christie et al., 2010). Despite the molecular signals inhibiting regeneration, the axons of a peripheral nerve show an intrinsic growth capacity. Within hours from injury, genes with regenerative capabilities are activated: JUN, FOS, activating transcription factor 3 (ATF3) and cAMP response element binding protein (CREB). CREB3 knock out (KO) in DRG culture significantly reduces neurite elongation, demonstrating its fundamental role after injury (Ying et al., 2014). Locally released cytokines, such as leukemia inhibitory factor (LIF) and interleukin-6 (IL-6), activate the downstream growth associated protein 43 (GAP-43) after DRG peripheral injury (Cafferty et al., 2004). GAP-43 is the first recombination activating gene originally discovered by Skene and Willard (1981), whose induction after sciatic nerve injury strongly correlates with regeneration (Skene and Willard, 1981). LIF also activates the Janus kinase 2-*signal transducer and activator of transcription 3* (JAK2-STAT3) pathway in DRG neurons: STAT3 is specifically associated to the early phase of axon regeneration in PNS (Bareyre et al., 2011).

While axon degeneration/regeneration occur, T cells, neutrophils and macrophages infiltrate the injury site, increasing the level of early inflammatory cytokines, tumor necrosis factor alpha (TNF-α) and interleukin-1α (IL-1α) (Wagner and Myers, 1996). In the following days the inflammatory reaction is amplified by macrophages, mast cell recruitment and endothelial cell activation. Macrophages remove myelin debris and myelin associated molecules (like myelin-associated glycoprotein, MAG) that inhibit regeneration (Baichwal et al., 1988; da Costa et al., 1997). Resident macrophages (4% of the total number) rapidly respond to the injury and then circulating macrophages (chemokine c-c motif receptor 2) are recruited to the lesion side where they constitute the main cell components of the bridge for the new growing tissue (Siebert et al., 2000). Moreover they are also recruited by chemokine (c-c motif ligand 2) secreted by SCs that is fundamental in regulating the axonal regrowth program via LIF/STAT3 mechanism. Notably, two distinct populations of macrophages are described: M1 with a proinflammatory function and M2 working in the opposite direction (Ydens et al., 2012).

SCs after injury break off from the damaged axons, dedifferentiate, secrete cytokines and promote immune cell infiltration. JUN is the main factor responsible for the initial transition from myelinating to non-myelinating SCs that allow a complete clearing of the tissue for a successful regeneration. This is consistent with the downregulation of myelin genes (MBP, MAG and peripheral myelin protein 22-PMP-22) and the upregulation of immature SC markers such as neural cell adhesion molecule (NCAM), p75 neurotrophin receptor and glial fibrillary acidic protein (GFAP) (Chen et al., 2007; Jessen and Mirsky, 2008). On the other hand, a set of trophic factors inactive in normal or developing SCs [for instance glial cell line-derived neurotrophic factor, artemin, brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) (Boyd and Gordon, 2003)] appear. In addition, the products of early axon degeneration stimulate SCs to remove myelin debris and recruit macrophages by the toll-like receptors nuclear factor kappa-light-chain-enhancer of activated B cells. Due to the separation from the axons, SCs synthetize TNF-α, IL-1α and interleukin β that in turn contribute to monocyte and macrophages attraction and infiltration (Shamash et al., 2002). Additionally, starting from 4 days after trauma, denervated SCs divide both at the proximal and distal stumps of the lesioned axon (Mitchell et al., 1990; Trapp et al., 1988). If regenerating axons are re-contacted by SCs, they can unwrap it by myelin sheet, otherwise they undergo apoptosis.

If the balance between regenerative/degenerative program is high, a successful nerve regeneration can occur. However the regenerative capabilities of the PNS, albeit considerable, are often not sufficient to guarantee a complete reinnervation and functional recovery: if a disorganized axon sprouting occurs, the circuitry refinement maybe inadequate, resulting in sensory deficit and limited motor control (Battiston et al., 2009; Wu and Murashov, 2013).

Contrarily to the PNS, after a CNS lesion, the spinal cord has a very limited capability to restore its connectivity. This poor ability is due both to a sequence of anatomical changes (including neuron cell death, neuroinflammatory reaction, glial scar formation and myelin inhibitory proteins) that create a growth inhibitory environment, and to the adult physiological inactivation of the growth program by oligodendrocytes (OLs) (Kwon et al., 2004). The time course of the post injury modifications is characterized by an initial phase (0–2 h) of neuronal and glial death associated with hemorrhage and ischemia both rostrally and caudally to the lesion, followed by an acute phase (2h-2 weeks) that consists of an intense inflammation process (microglia, astrocyte, neutrophil and T cell recruitment) resulting in the glial scar formation, neurotoxicity and further cell death and demyelination. Then an intermediate phase characterized by glial scar maturation and inefficient attempts of axon sprouting occurs (2 weeks-6 months). In the last chronic phase (>6 months) the lesion stabilizes: glial scar continuously forms, Wallerian degeneration occurs and axon fragmentation leads to final deficits and symptoms.

More in detail neuron and OL death is the first event, occurring earlier by necrosis and later by apoptosis. As for PNS, the Ca^{2+} influx induced by the lesion leads to calpain activation with mitochondria dysfunction and excitotoxicity damage (mediated by N-methyl-D-aspartate receptor). Reactive oxygen and nitrogen species exacerbate the damage causing OL loss that in turn leads to further neural cell death. One of the first apoptotic pathways activated is the JNK cascade, whose specific inhibition prevents caspase 3 cleavage, promotes locomotor recovery and neuroprotection after SCI (Repici et al., 2012). Upstream caspase activation, associated protein with death domain-associated protein with death domain ligand (FAS-FAS-L) mediated cascade plays a critical role in axon degeneration and OL apoptosis: FAS deficient-mice with a SCI compression show improved locomotor recovery, axonal sparing and myelin preservation (Casha et al., 2005; Yu et al., 2009).

The limited regrowth ability of CNS is probably due to the lack of a sustained upregulation of growth factors. However it is known that when BDNF, neurotrophin 3, 4 and ciliary neurotrophic factor are released at the spinal lesion level, they are able to sustain axon growth after 1–2 months from the injury (Bregman et al., 2002). Similarly, the selective downregulation of axon growth inhibitory molecules, such as Ras homolog gene family member A (RhoA), GSK-3β and PTEN, strongly sustains axon regeneration. Synaptogenesis and neurite outgrowth are reached by a RhoA inhibitory treatment in a rat acute SCI model (Devaux et al., 2017); GSK-3β inhibition significantly reduces secondary damage of SCI and ameliorates motor recovery in injured mice (Cuzzocrea et al., 2006); conditional PTEN depletion enhances the regenerative growth of corticospinal tract in association with motor function restoration in adult mice post-SCI (Danilov and Steward, 2015). Other growth inhibitors belong to 1) chemorepulsive axon guidance molecules, such as the semaphorin family known to be expressed after SCI, or ephrin, netrin and wingless-related integration site (wnt) known to limit axonal elongation in CNS lesion; 2) chondroitin sulfate proteoglycans (like neurocan, brevican, versican), a class of extracellular matrix molecules that are expressed by astrocytes in the glial scar and therefore limit neuronal plasticity. In addition, neurite outgrowth inhibitors, MAG and OLs myelin glycoprotein synergistically restrict axon growth after SCI (Cafferty et al., 2010).

Neural death and axon regeneration proceed in parallel to a demyelinating process that is quite different from the processes activated in the peripheral system. In detail, demyelination is accompanied by oligodendrocytes progenitor cell (OPC) proliferation as demonstrated by the presence of neural/glial antigen 2 positive cells 4 weeks after SCI and increased level of fibroblast growth factor-2 (FGF-2), a mitotic factor that maintains OPC renewal state (McTigue et al., 2001; Mocchetti et al., 1996). However OPC maturation and remyelination is prevented by the Notch1 expression rostral and caudal to the injury (Chen et al., 2005): indeed Notch signals, with “a disintegrin and metalloprotease” (ADAM) molecules as downstream effectors, block myelin maturation. Also NCAM, a factor that prevents myelination, increases in dorsal spinal cord, motor neurons and corticospinal tract after SCI transection (Tzeng et al., 2001).

The apoptosis of neurons and oligodendrocytes (OLs) is also the result of the inflammatory response. Vessels and blood brain barrier breakdown cause a rapid infiltration of neutrophils and T cells, attracted by an initial release of TNF-α and IL-6 (Habgood et al., 2007; Pineau and Lacroix, 2007). The following increased production of cytokines gives rise to the secondary inflammatory response characterized by macrophage and microglia recruitment. The inflammation molecular mediators (as TNF-α) are generally described to have a dual role, neurotoxic or neuroprotective (Kim et al., 2001; Lavine et al., 1998). A similar role is attributed to microglia that after an injury become activated assuming a typical amoeboid structure: microglia depletion can be neuroprotective, but its controlled activation can also be beneficial. This paradox is explained by the presence of two different classes of cells, proinflammatory M1 and anti-inflammatory M2 microglial cells (Kigerl et al., 2009).

Also astrocytes are activated by injury: these cells form a strong inhibitory scar that works as an anatomical barrier for axonal regeneration, in the ineffective attempt to limit the spread of damage and preserve the healthy tissue. Astrocytes are recruited to the injury site by a molecular cascade that includes TGF-β, interleukin-1 (IL-1), interferon-γ and FGF-2, and become hypertrophic with an increased production of GFAP (Cregg et al., 2014).

However, despite the limited axon regrowth potential of CNS, some regeneration associated genes have been described after central lesion. For example, the transcription factor CREB is induced by SCI and is able to promote axon growth and regeneration when overexpressed (Qiao and Vizzard, 2005; Qiu et al., 2002). The transcription factor ATF3 is known to enhances c-Jun mediated sprouting in response to neural axotomy *in vitro* (Pearson et al., 2003). JAK-STAT pathway is described to control the reestablishment of spinal cord continuity in response to a Xenopus Laevis model of spinal cord injury with a differential pathway activation during regenerative and non-regenerative stages (Tapia et al., 2017). GAP-43 can promote axon growth when upregulated after SCI compression (Curtis et al., 1993; Zhang et al., 2005).

Therefore the contribution of several different pathways for PNS-CNS response to injury is quite evident and extremely tangled. However the level of complexity increases considering microRNA (miRNAs/miRs) as new

upstream regulators that, acting as networks, control simultaneously different processes.

2 ORIGIN, BIOGENESIS AND NOMENCLATURE OF MICRORNArigin, biogenesis and nomenclature of micrornas

MiRNAs are small non-coding RNAs about 20–22 nucleotides-long, which negatively regulate gene expression at post-transcriptional level. They were discovered by Lee et al. (1993), who demonstrated that lin-4 “gene” is able to repress the lin-14 gene (Lee et al., 1993). Only in early 2000s, lin-4 and let-7 (the second to be characterized) were described as part of a large group of RNAs present in several organisms (from C. elegans to humans) and involved in different biological processes (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a, 2003b; Pasquinelli et al., 2000). Soon after miRNAs were also found in plants suggesting that they are evolutionary conserved (Dugas and Bartel, 2004; Reinhart, 2002). Since then, research on miRNAs expanded rapidly, demonstrating not only how many different physiological roles they play, but also revealing how their aberrant expression is implicated in several diseases. MiRNAs are highly conserved across species: they appeared first in invertebrates as a defense mechanism against infection, then were conserved through evolution to refine gene expression in more complex organisms (Lee and Ambros, 2001). At present, over 2000 miRNAs have been involved in the regulation of one third of the human genes (Hammond, 2015). These small RNAs are spread in the genome and can be transcribed either as independent units or as located within the intron or an untranslated region of a coding gene (Lim et al., 2003a). Moreover, 50% miRNAs derive from a unique polycistronic unit, sharing the same promoter that gives raise to their mature forms (Lee et al., 2002; Rodriguez et al., 2004). Polycistronic miRNA transcripts, that can be up to ten kb in mammals, are organized in clusters. A cluster is defined as a unit in which miRNAs are closer than 1 kb between each other, allowing their coordinated transcription and function. Similarly, a miRNA family is defined as a group of small RNA deriving from a common ancestor that generally have similar physiological functions but that are not always conserved in primary sequence or secondary structure (Ambros et al., 2003; Griffiths-Jones et al., 2003; Kozomara and Griffiths-Jones, 2014; Wright and Bruford, 2011). The cluster and the family organization underscores the underestimated complexity of the miRNA role. The miRNA maturation process (Fig. 1) can be divided in two steps (Bartel, 2004). The first one consists of miRNA transcription by the RNA polymerase II into the primary miRNA (pri-miRNA) (Lee et al., 2004): this hairpin loop structure undergoes a process of capping (at 5’end), polyadenylation with multiple adenosine (poly-A tail) and splicing. Then, DiGeorge Syndrome critical region 8 (DGCR8)-Drosha association (Filippov et al., 2000; Han et al., 2004) is required for the cleavage of the double-stranded pri-miRNA, thus releasing the hairpin and allowing the formation of the precursor miRNA (pre-miRNA) (Ketting et al., 2001; Knight and Bass, 2001).

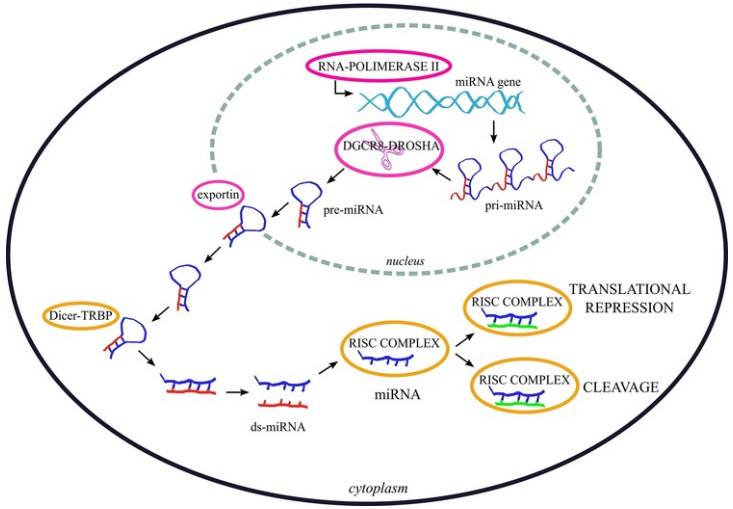

Fig. 1 miRNA maturation process.

alt-text: Fig. 1

Additionally, the nuclear factor TAR DNA-binding protein 43 (TDP-43), involved in neurodegenerative diseases like amyotrophic lateral sclerosis (ALS), selectively affects miRNA synthesis pathway (Buratti et al., 2010) as an accessory component that directly interacts with the Drosha-DGCR8 complex (Kawahara and Mieda-Sato, 2012). Indeed TDP-43 plays a crucial role in the stabilization of Drosha protein during neural differentiation as demonstrated for the first time by a reduced miRNA production in human TDP-43 KO neuroblastoma cell line (Di Carlo et al., 2013). Moreover miRNA-independent gene-regulatory function of miRNA biogenesis enzymes have been demonstrated to control different CNS processes as for instance Drosha control of Neurogenin 2 (Neurog2) and neuronal differentiation 1 (NeuroD1) are essential for neurogenesis maintenance (Knuckles et al., 2012). Indeed TDP-43 activity on Drosha stability is also an indirect way to post-transcriptionally regulate Drosha target, Neurog2, a transcription factor (TF) critical for neural differentiation. TDP-43 exerts both an indirect and direct control on Neurog2 mRNA, that in turn

regulates NeuroD1 expression whose production is again modulated by Drosha. The final effector NeuroD1 induces the expression of genes related to neural differentiation. Independently from Dicer and miRNA biogenesis, Drosha also targets nuclear factor 1 B preventing OL fate acquisition by neural precursors (Rolando et al., 2016). Not only Drosha but also DGCR8 directly affects corticogenesis, as its deletion pushes a premature neural precursor differentiation and an increased number of T-box brain 1 positive neurons (Marinaro et al., 2017).

Both pri- and pre-miRNAs can be modified by RNA-editing, which entails important functional implications related to maturation and target specificity (Blow et al., 2006; Kawahara et al., 2008; Yang et al., 2006). The adenosine deaminases are responsible for this process converting the adenosine of the miRNA stem region into inosine (Yang et al., 2006). Moreover some additional proteins control both pri- and pre-miRNA processing: KH-type splicing regulatory proteins and LIN28 interact with the terminal loop of pri- and pre-miRNAs facilitating and inhibiting respectively DROSHA and Dicer activity (Trabucchi et al., 2009; Viswanathan et al., 2008) Then the enzyme exportin 5 translocates the generated pre-miRNA from the nucleus to the cytoplasm, where the second step occurs (Kim, 2004). Here, the endonuclease Dicer, in association with trans-activation response RNA-binding protein (TRBP), removes the loop from the 5′ and 3′ ends producing a double RNA of 21–22 nucleotides. Usually only one strand becomes the real mature miRNA, while the other is degraded (Khvorova et al., 2003). However, recently next generation sequencing experiments demonstrated that occasionally both strands could be expressed. Therefore i) the standard nomenclature system named “5p” the microRNAs derived from the 5′ arm, and “3p” those derived from the 3′ arm of the hairpin stem loop; ii) they are loaded at the same frequency in the RNA-induced silencing complex (RISC). Additionally, when distinct precursor sequences and genomic loci express identical mature sequences, they are named “1” and “2”, as for example miR-121-1 and miR-121-2. However it is also possible to find “miR-121a” and “miR-121b”, in which letter suffixes denote closely related mature sequences deriving from two different precursors (Kozomara and Griffiths-Jones, 2014). The selected single strand/double strand(s) is/are incorporated in the RISC that, among many other components, contains the Argonaute (AGO) proteins (Vaucheret et al., 2004) essential for the miRNA orientation and interaction with their targets occurring between 6 and 8 nucleotides (seed region) at the 5′ UTR of the miRNA and the mRNA 3′ UTR.

The maturation process described above is the most frequent, but alternative pathways may occur for some miRNA families (Ha and Kim, 2014). Drosha-independent (but still Dicer dependent) pathways have been described for mirtrons (miRNA located in mRNA introns) and miRNAs derived from short hairpin RNAs (Okamura et al., 2007; Xie et al., 2013): in the first case pre-miRNA is generated by splicing, debranching and trimming processes, while in the second the miRNA (for example miR-320) is directly transcribed and transported by exportin 1. Also Dicer-independent biogenesis is possible as in the case of miR-451, that is directly loaded to AGO2 protein to produce the miRNA mature transcript (Cifuentes et al., 2010; Yang et al., 2010). Finally some miRNAs, like let-7 and miR-105, present a short 3′ overhang from Drosha processing that requires terminal uridylyl transferases mono-uridylation in order to be processed by Dicer (Heo et al., 2012).

At the same time, miRNA cleavage/translational repression of the target represents their traditional mechanism of action, but not the only one. Indeed, some miRNAs, during cell quiescence, may directly mediate the upregulation of their targets (Rusk, 2008). The apparently intricated miRNA panorama starts from their biogenesis, reflecting how wide is their role in a great variety of biological processes. The aim of this review is to provide an overview of the complex mechanisms of action of miRNAs into the Nervous System, showing in particular how their expression changes after peripheral nerve and spinal cord injury (SCI).

3 MiRNAs ~~IN PERIPHERAL AND CENTRAL AXON REGENERATION~~in peripheral and central axon regeneration

In the last ten years the increasing interest in miRNAs in the nervous system revealed their specific involvement in many aspects of neuronal function and disease, such as neurite growth and neurodegeneration. To underline the potentially big impact of non-coding RNAs in neurodegenerative diseases, the latter ones are often defined as RNA disorders (Johnson et al., 2012). Moreover, taking into account miRNA intrinsic properties to regulate several biological functions, it is not surprising that they are considered one of the major players in the pathogenesis of CNS/PNS injury.

The whole PNS regenerative process is driven by a sequence of cellular and molecular mechanisms and involves also miRNAs, as demonstrated by recent deep sequencing studies revealing an abnormal miRNA expression after peripheral nerve injury *in vivo* (Li et al., 2012; Yu et al., 2011a). Aiming at reestablishing their normal expression, a series of studies focused on the modification of a single or entire miRNA family. For instance, the inhibition of let-7 miRNA both *in vitro* (co-culture of SCs and DRG neurons) and *in vivo* (nerve transection in adult male rats) increases NGF secretion and enhances axonal outgrowth within a regenerative microenvironment (Li et al., 2015). Acting on a different pathway, miR-26a supports mammalian axon regeneration through the inhibition of GSK-3β activity in a model of adult mouse DRGs and sciatic nerve crush (Jiang et al., 2015). The same small RNA also promotes neurite outgrowth *in vitro* via the suppression of PTEN expression, one of the best characterized pathways for axonal growth (Cui et al., 2015; Li and Sun, 2013). Another important player in regulating axonal growth is miR-21, which is induced after DRG *in vivo* axotomy and can promote regeneration under its overexpression (Strickland et al., 2011b). In a similar model (DRG neuronal culture study) both miR-431 gain of function and knockdown of its downstream effector Kremen 1 (wnt-βcatenin canonical pathway) significantly enhance axon outgrowth (Wu and Murashov, 2013). Therefore, by acting on the same and/or different pathways, there is evidence of the influence of miRNAs in the field of peripheral nerve regeneration. On the other hand in the CNS context in which sprouting is limited and axon regeneration almost absent, miRNAs represent major players in the maladaptive changes occurring after SCI. As for the PNS, several studies analyzed the global profiling of miRNA expression after SCI obtaining differential expression for a great number of small RNAs (Liu et al., 2009). Indeed they can play either a protective or a detrimental role (Nieto-Diaz et al., 2014; Ning et al., 2014). Some miRNAs acting on different pathways promote functional recovery. For instance, an increased axon density within the lesion site was shown after miR-21 inhibition in astrocytes in a SCI mouse model (Bhalala et al., 2012). Even better, miR-133b is an important determinant for axon regrowth and functional recovery by reducing RhoA protein in an adult zebrafish SCI model (Yu et al., 2011b). Considering that some

miRNAs are evolutionarily conserved, the ability of let-7 to turn back the clock on regeneration in adult C. elegans neurons suggests that its potential could also be applied for mammalian axon regrowth (Nix and Bastiani, 2013; Zou et al., 2013). Moreover further evidence for the regenerative potential of some miRNAs comes from *in vitro* studies, such as the role exerted on embryonic cortical neurons when the miR-17-92 cluster is overexpressed: indeed this cluster acts on PTEN pathway as many other small RNAs, included the miR-29 family (Zhang et al., 2013; Zou et al., 2015a). It is noteworthy that miR-17-92 and STAT3 are linked in a positive feedback loop in which STAT3 activation is related to an overexpression of this miRNA cluster in retinoblastoma cells *in vitro* (Jo et al., 2014): although such connection has been only confirmed in cancer pathways so far, it could affect also the axon growth pathways, since STAT3 is a crucial TF in neurite outgrowth (Jo et al., 2014). Somewhere in between peripheral and central nervous damage, miR-142-3p seems to be a potential target to enhance central regeneration of primary sensory neurons by inhibiting the adenylyl cyclase 9 molecule (Wang et al., 2015a).

The list of miRNAs with regrowth potential is long in the literature and, despite the encouraging results achieved, we are still far from a full understanding of their mechanisms. The main reason lies in the difficulty to connect every single element to reconstruct a broken system. This is particularly true for miRNAs, since each of them is only a piece of a complex and perfectly functioning puzzle. For this reason it is necessary to re-think of miRNAs not as acting singularly, but as coordinated members of different groups. Therefore in the following paragraphs, we will show some examples of miRNA actions, demonstrating their synergistic role in well-known molecular pathways related to axonal growth, inflammation, apoptosis and remyelination. Even though CNS and PNS differ for many aspects, we try to describe miRNAs of the two systems in the same pathway, since we consider that some possible common cascade and/or functions maybe reasonably integrated.

4 MiRNAs ~~AND TRANSCRIPTION FACTORS AS REGULATORS OF AXON GROWTH AND REGENERATION~~and transcription factors as regulators of axon growth and regeneration

As previously mentioned, immediately after injury there is a large increase in the expression of regeneration associated genes, a phenomenon controlled by the early activation of a specific TF program. TFs are DNA-binding proteins able to amplify the number of genes to switch on/off and the subsequent cascades of cellular signaling. Both TFs and miRNAs exert an extensive effect on gene expression, since the former control almost all genes, whereas the latter roughly 20% of animal transcripts. It is well known that TFs [such as tumor protein p53 (p53), c-Jun, CREB, RE1-Silencing Transcription factor (REST), STAT3, Forkhead box O (FOXO), Krueppel-like factor (KLFs), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kb) and SRY-box containing gene (Sox)] are connected to axon growth and regeneration abilities as well as miRNAs emerged as important players for the survival and differentiation of neurons during development and after injury (Fineberg et al., 2009; Lagos-Quintana et al., 2001). Therefore establishing a direct link between TFs and miRNAs can help to better understand the complexity of the axon growth/regeneration process.

One of the most studied TF in the nervous system is STAT3, a component of the 7 mammalian STATs broadly expressed in cortex, striatum, forebrain and hippocampus both at embryonic and adult stages (De-Fraja et al., 1998). In adults, STAT3 is expressed at very low basal level but for instance it is reactivated from 6 h~~ours~~ to 1 month after a sciatic nerve injury without a correspondent re-expression after a central dorsal column injury (Qiu et al., 2005). Indeed STAT3 reactivation seems to be a specific paradigm to promote regrowth in the PNS. However elevated STAT3 levels are present after optic nerve crush and STAT3 *in vitro* KO in hippocampal neurons leads to a decreased BDNF-induced neurite growth (Fischer et al., 2000; Ng et al., 2006). Such STAT3 role can be regulated by miRNA activity. STAT3 inhibition by two different miRNAs (miR-125b and miR-124) has been demonstrated both *in vitro* and *in vivo*. miR-125b upregulation during SH-SY5Y cell differentiation was experimentally correlated to the downregulation of 10 different genes that in turn repress pathways involved in neuronal differentiation: for instance, by inhibiting the activity of GRB2-associated-binding protein 2, miR-125b activates STAT3 and its PI3K-AKT signaling cascade, known to control axon growth (Le et al., 2009) (Fig. 2). Acting on the same pathway, miR-124 is significantly downregulated *in vivo* after an hypoglossal motor neuron injury, suggesting that its repression has a crucial role for an appropriate nerve regeneration by the consequent STAT3 upregulation. The positive effect due to miR-124 downregulation can be also enhanced by the simultaneously upregulation of KLF6, another growth promoting TF (Nagata et al., 2014) (Fig. 2). The importance of miR-124 effect on STAT3 is also demonstrated in cultured hippocampal neurons: when induced by a specific small interfering RNA (siRNA) in a methyl donor deficiency mouse model, miR-124 upregulation inhibits STAT3 signaling cascade and enhances neurite outgrowth (Kerek et al., 2013). miR-124 is also known to regulate Ras-related C3 botulinum toxin substrate 1(Rac1) through Ras homolog gene family, member A (RhoA-GTP) (Fig. 3) that is described as a regulator of STAT3 (Raptis et al., 2011).

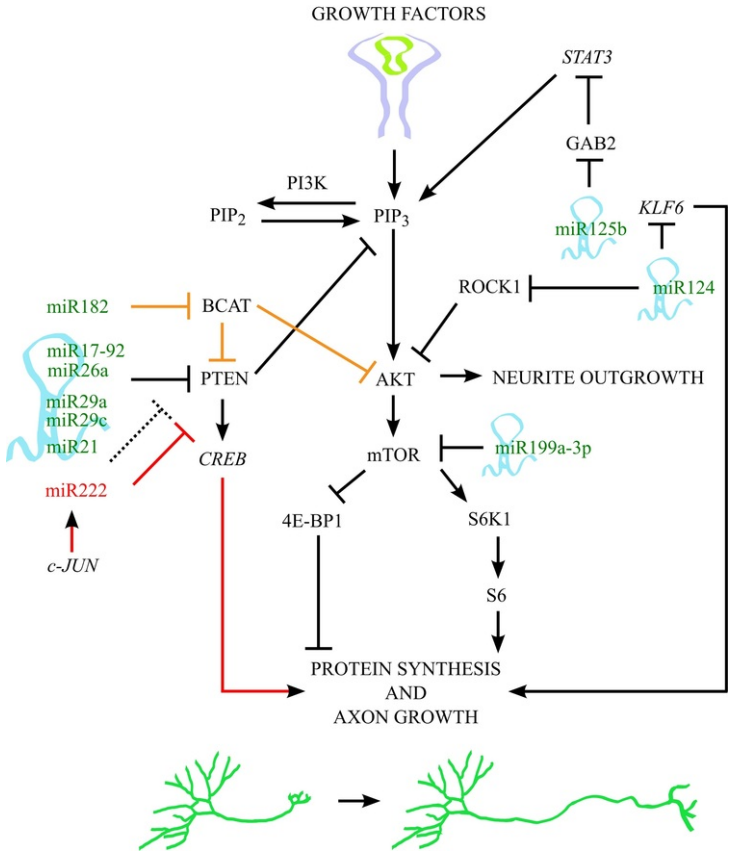

Fig. 2 miRNAs in axonal growth network-1, focused on PTEN-AKT-mTOR; continuous lines indicate validated interactions and dotted line identifies an hypothesized interaction; miRNAs in green, red and black colors refer respectively to CNS, PNS and CNS-PNS. TFs are written in italic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 2

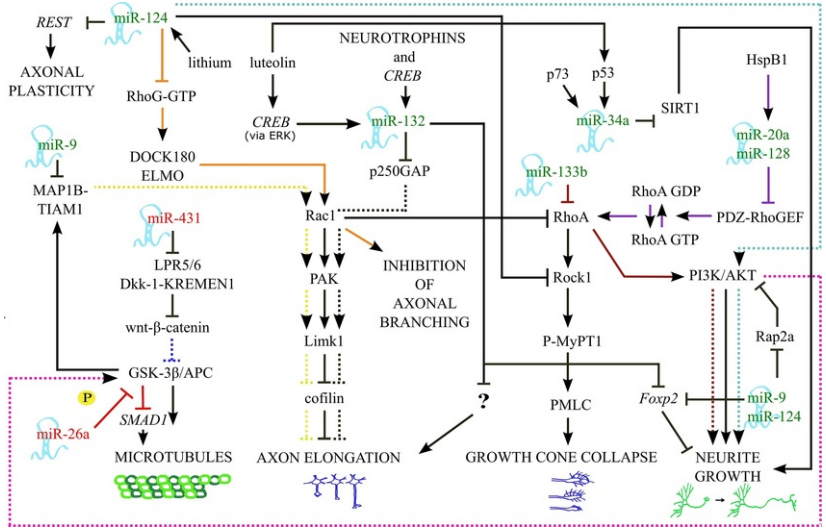

Fig. 3 miRNAs in axonal growth network-2 (2.1-2.2), whose core molecules are represented by GSK-3β, Rac1, RhoA and PI3k/AKT; miRNAs in green and red colors refer respectively to CNS and PNS. Continuous lines indicate validated interactions, whereas dotted line hypothesized interactions. TFs are written in italic. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 3

Another important TF, involved in different biological processes, is p53, a member of the tumor suppressors family. In the nervous system p53 is known as pro-apoptotic factor active in case of trauma and neurodegenerative diseases (Floriddia et al., 2012; Qi et al., 2016). Moreover p53 is also required for neurite outgrowth and regeneration, acting on Coronin 1b and Ras related protein Rab13 (cytoskeleton molecules) as demonstrated both *in vitro* in different cell lines (human epithelial 293, H1299, pheocromocytoma PC-12) and rat primary cortical neurons and *in vivo* (murine model of facial nerve injury) (Di Giovanni et al., 2006): in the last case, p53 KO mice showed a lower number of regenerative fibers compared to WT mice.

The link between this TF and miRNAs is well demonstrated in the following *in vitro* experiment: p53 upregulation induced by luteolin (a flavonoid present in vegetables) increases the expression of miR-34a, that targeting sirtuin 1 (SIRT1) promotes neurite outgrowth of PC12 cells (Chen et al., 2015). Similarly, the conditional miR-7 ablation in the murine cortex revealed that its expression is essential for a complete transition from radial glial cells to intermediate progenitors acting on adenylate kinase 1 and tumor protein p21 factors, two molecules implicated in the p53 pathways. Only the presence of miR-7 and its subsequent action on p53 pathway delete the microcephalic phenotype of an abnormal brain development (Pollock et al., 2014). Moreover miR-7 has been described to reduce the neurite outgrowth of neuroblastoma cell line *in vitro* likely acting on the same TF through a not specified molecular cascade (Chen et al., 2010).

Another important factor is c-Jun that is highly expressed during neural embryonic and postnatal development in CNS, and then decreased in adulthood (Mellström et al., 1991; Wilkinson et al., 1989). However its expression increases again after the peripheral (but not the central) degeneration segment of DRG neurons *in vivo* (Broude et al., 1997). C-Jun, by a specific miRNA interaction, can be indirectly correlated to a second TF, CREB, a known target of PTEN (Gu et al., 2011). As shown in Fig. 2 (red lines) after an *in vivo* sciatic nerve transection the increased level of c-Jun induces miR-222 upregulation that in turn decreases PTEN level, allowing the maintenance of CREB phosphorylation. So that CREB transcriptional control is the final step promoting neurite outgrowth (Zhou et al., 2012). miRNA-CREB interaction can be also considered in the opposite direction, since CREB can induce miR-132 that in turn promotes neurite outgrowth. This connection has been proved in an *in vitro* model of PC12 cells where luteolin induces miR-132 expression through CREB-extracellular signal–regulated kinase (ERK) mediated phosphorylation (Lin et al., 2012), without identifying the target responsible of this effect. Vo et al. (2005) demonstrated the same miRNA function on PC12 cells, providing the GTPase-activating protein Rho GTPase activating protein 32 (p250GAP) as miR-132 target and suggesting that in both works miR-132 could act in the same pathway (Vo et al., 2005) (see miR-132 in axonal growth network Fig. 3). miR-132 and miR-212 are located in the same genome locus and are controlled by two TFs, CREB and REST. Moreover REST is the target of miR-9, one of the most expressed miRNA in brain already mentioned to control axon length *in vitro* and *in vivo* (Fig. 3). Moreover miR-9 and REST seem to work in a feedback regulatory loop: during SK-N-BE (human neuroblastoma cell line) proliferation, REST represses miR-9 expression but upon differentiating stimulus, REST dismission and CREB activation induce miR-9 expression that in turn inhibits REST in order to maintain the differentiation program (Laneve et al., 2010). As depicted in Fig. 3, REST is also targeted by miR-124 as recently demonstrated by ~~Doeppner and coworkers (2017)~~Doeppner et al. (2017): in this study the authors revealed that the intraperitoneal injection of lithium after an *in vivo* ischemic stroke (mouse model) increased the level of miR-124 that in turns represses REST thus promoting angioneurogenesis and axonal plasticity (Fig. 3)

(Doeppner et al., 2017). Since miR-9 is widely expressed in neurons and involved in several biological processes, is not surprising it was found to be involved in a feedback regulatory loop with tailess (TLX), a forebrain-restricted TF that regulates neurogenesis maintaining neural stem cells (NSCs) in an undifferentiated proliferative state (Roy et al., 2004; Shi et al., 2004). Indeed miR-9 negatively regulates NSC and retinal progenitor cell proliferation and accelerates differentiation directly inhibiting TLX that in turn is able to repress miR-9 in a regulatory loop necessary to have a proper balance between NSC proliferation and differentiation (Hu et al., 2014; Zhao et al., 2009). Producing the same effect of miR-9 on NSCs, also other miRNAs participate in this regulatory loop such as let-7b (also acting on protein phosphatase 2 catalytic subunit alpha), let-7a and let-7d that inhibits TLX that in turn upregulates miR-9 level (Ni et al., 2014; Song et al., 2015; Zhao et al., 2010a~~; Zhao et al.~~, 2013). On the contrary, TLX inhibits two miRNAs (miR-219 and miR-137) that negatively regulate NSC proliferation (Murai et al., 2016; Sun et al., 2011).

Other TFs targeted by miR-9 belong to the FOXO family known to maintain the proliferative potential of neural stem cells. Indeed a coordinated action of miR-9 on FOXO1 contributes to neural stem/progenitor differentiation (Kim et al., 2015). FOXO1 as well as Forkhead box P2 (Foxp2) (another member of the same family) are both targets of miR-9 and miR-132, and the second one might contribute to the progression of Alzheimer's disease through downregulation of tau protein networks (Lau et al., 2013). Both these miRNAs and Foxp2 are known to be involved in neurite outgrowth during development; however a precise control of neurite extension during cortical development seems to be achieved by the convergent action of miR-9 and miR-132 to regulate the expression of Foxp2 (Fig. 3) (Clovis et al., 2012). miR-9 also contributes to modify motor neuron columnar organization and axonal projection by acting on Foxp1 during development of spinal cord chicken embryos (Otaegi et al., 2011).

Finally, another TF known to play a crucial role in spinal cord injury is Sox11, a member of the 20 Sox family factors expressed in neural differentiation both in CNS and PNS (Hargrave et al., 1997; Uwanogho et al., 1995). Higher level of Sox11 are detectable both after optic nerve injury in regenerating zebrafish model and in DRG neurons when the regenerative program starts (Tanabe et al., 2003; Veldman et al., 2007). The corticospinal tract after SCI fails to express Sox11, while its expression induced by viral vectors promotes sprouting and axon growth at both acute and chronic phase (Wang et al., 2015b). This effect can be explained by the ability of Sox11 to increase the expression of BDNF and induce the neurogenic differentiation of endogenous neural stem cells after SCI (Guo et al., 2014). Even if Sox11-miRNA connection has not been experimentally proved, it is possible to hypothesize a link between miR-145 and Sox2, another TF of the same family involved in neural differentiation after SCI. Indeed several works demonstrated the requirement of Sox2-positive cells for spinal cord regeneration in xenopus tadpoles and xenopus laevis SCI model (Gaete et al., 2012; Muñoz et al., 2015). In murine SCI model Sox2 mediated reprogramming by virus injection was able to induce the generation of new neurons, a mechanism poorly activated in mammals after SCI (Wang et al., 2016b). This phenomenon can be related to the upregulation of miR-145 after *in vivo* SCI contusion: this miRNA directly recognizes Sox2 in neural stem cells, while the anti-miR-145 reverts the effect on the target (Morgado et al., 2016).

Therefore miRNAs and transcription factors are strictly intertwined and act as a powerful regulatory network in controlling axon growth and regeneration.

5 MiRNAs ~~IN AXONAL GROWTH NETWORK: PTEN PATHWAY~~in axonal growth network: PTEN pathway

miRNAs participate in one of the main molecular cascades regulating axon growth, i.e. the PTEN pathway. Indeed, acting as an inner regulatory system, they represent a promising tool still quite mostly unexplored. As shown in Fig. 2, axonal growth is under the control of different growth factors, such as nerve growth factor, that stimulate the conversion of lipid second messenger phosphatidylinositol (4,5) bisphosphate (PIP_2) into phosphatidylinositol (3,4,5) trisphosphate (PIP_3) by PI3K. Then PIP_3 activates AKT through phosphatidylinositol-dependent kinase 1/2 (PDK1/2) recruitment. AKT in turn switches on a large spectrum of downstream effectors. Among them, the mechanistic target of rapamycin (mTOR) synthetizes raw material for axon extension and GSK-3β promotes cytoskeleton reorganization (as shown further in Fig. 3). Unfortunately, after an injury, this pathway is inhibited by PTEN that antagonizes PI3K activity, thus resulting in the failure of axon regeneration (Chadborn et al., 2006; Park et al., 2010; Rodgers and Theibert, 2002). To promote neuronal regeneration, several strategies (molecular deletion, shRNA, antagonists or inhibitors) have been applied at different levels of this cascade. However, until now, none of them can be completely restored the pre-injury condition, due to the complex network of cooperating molecules that orchestrate the different steps of the process. Based on the literature (Li and Sun, 2013; Zhang et al., 2013; Zou et al., 2015a~~, Zou et al.~~, 2015b), we identified a miRNA network acting on the PTEN pathway (Fig. 2). miR-17-92 cluster and miR-26a directly repress PTEN expression in primary cortical neurons during development, whereas miR-29a and miR-29c have the same effect on PC12 neural differentiating cell lines. When these miRNAs are overexpressed, PTEN protein levels decrease to 41% (miR-17-92 cluster), 38.3% (miR-26a) 44% (miR-29a), and 54% (miR-29c), validating this molecule as one of their main targets. Further evidence of the effect of these miRNAs on PTEN is the upregulation of some downstream effectors, such as mTOR and AKT: the downregulation of PTEN is thus associated with the enhancement of axonal outgrowth for miR-17-92 and of neurite outgrowth for miR-29a miR-29c and miR-26a. Indeed the inhibition of miR-17-92 cluster revealed a suppressed axonal growth by 50% during 60 min of *in vitro* observation. miR-21 is involved in decreased expression of PTEN as well. It contributes to explain the mechanism underlying the benefits of docosahexaenoic acid (DHA), a fatty acid promoting functional recovery after SCI. In fact, DHA *in vivo* administration significantly upregulates miR-21 that in turn downregulates PTEN in corticospinal neurons. The decreased PTEN levels are accompanied by an increase in neurite outgrowth of primary cortical neurons *in vitro*, supporting a key role for miR-21 in the enhancement of neuroplasticity after SCI. Similarly *in vivo* a single acute (1-2 days), but not delayed, administration of DHA induces a significant sensorymotor and functional recovery after rat cervical hemisection (Liu et al., 2015c). miR-26a and miR-222 have been described to repress PTEN respectively in bupivacaine DRG induced nerve injury, and in adult DRG after sciatic nerve transection models, thus promoting nerve regeneration (Cui et al., 2015; Zhou et al., 2012). Some small RNAs indirectly influence mTOR or AKT in order to reach the same goal. For example, miR-222 has been studied both *in vitro* (PC12 cell line) and *in vivo* (laryngeal nerve injury in rabbit): when activated by BDNF administration, miR-222 acts as a promoter of mTOR upregulation (probably by

inhibiting PTEN, dotted line in Fig. 2), significantly inducing neurite outgrowth, increasing the number of regenerating fibers, decreasing fibrous connective tissue and restoring nerve conduction velocity. Similarly, when SCI rats undergo cycling exercise to stimulate neuron regenerative potential, the expression of miR-199-3p is reduced and mTOR expression results increased: the raised regenerative neuronal potential observed should be attributed to the synergistic effects of miR-199-3p and miR-21, that are responsible for PTEN mRNA decrease (Liu et al., 2012; Xie et al., 2015). Moreover also miR-124 indirectly targets AKT through the repression of rho-associated coiled-coil-containing protein kinase 1 (ROCK1), a protein serine/threonine kinase and the major downstream effector of RhoA GTPase. Gu et al. determined that the PI3 K/AKT signalling acts downstream ROCK1 whose repression by miR-124 can activate AKT. The activated AKT is then able to induce neurite outgrowth and elongation during neural development of neuroblastoma cell line [BE (2) M17] and mouse P19 cells (Gu et al., 2014).

Recently another miRNA emerged as a new regulator of PTEN pathway: indeed the overexpression of miR-182 in cultured murine cortical neurons increases the complexity of the dendritic branches and promote axon outgrowth by inhibiting PTEN and activating AKT signal through a decreased expression of branched-chain aminotransferase (BCAT2) (Wang et al., 2017) (orange line Fig. 2).

6 MiRNAs ~~IN AXONAL GROWTH NETWORK: GSK-3β PATHWAY~~in axonal growth network: GSK-3β pathway

During development, axon growth is mediated by several pathways that can be reactivated and/or inhibited in the mature nervous system as a consequence of a lesion. Moreover, one single molecule can participate in different pathways and/or be the target of different miRNAs. A paradigmatic example of these intersections is represented by miR-124, previously involved in neurite elongation of M17 cells (neuroblastoma cell line) *in vitro* through ROCK1-PI3K/AKT regulation (Gu et al., 2014). As shown in Fig. 3, PI3K/AKT signaling is also linked to axonal microtubule assembly. The inhibition of GSK-3β leads to the dephosphorylation of GSK-3β substrates that regulate the dynamics and stability of axon microtubules (Zhou and Snider, 2006). PI3K-GSK-3β signal represents a key step in the axon specification and growth of hippocampal neurons (Jiang et al., 2005; Yoshimura et al., 2005), making possible to establish a connection between miR-124 and GSK-3β through PI3K-AKT signal (blue dotted line). Recently, it has been shown that miR-124 operates synergistically with miR-9 to promote neuronal differentiation and dendritic branching of neural stem cells: these miRNAs, repressing their common target (Rap2a protein), elicit the inhibition of Rap2a protein on AKT. AKT in its active form phosphorylates and suppresses GSK-3β thus leading to neuronal development and maturation (Hur and Zhou, 2010). GSK-3β could be also a downstream effector of the wnt-β-catenin cascade, which is activated after SCI and promotes several biological functions including axon remodeling. In the classical pathway, wnt binds to its receptors (Low-density lipoprotein receptor-related protein 5 and frizzled) to signal to the GSK-3β/adenomatous polyposis coli (APC) complex and finally to β-catenin that translocates to the nucleus to elicit a change in the cytoskeleton components. However, wnt inhibitors (like Dickkopf-related protein 1 (Dkk-1) and Kremen 1) are overexpressed early after a SCI or nerve crush, thus inhibiting the cellular processes activated by β-catenin (Fernández-Martos et al., 2011). As illustrated in Fig. 2, GSK-3β could be the converging point of two molecular cascades (PI3K-AKT- GSK-3β/APC and wnt-β-catenin-GSK-3β) that probably reach the same effect, i.e. the reorganization of microtubules. Moreover as reported in subsection 4, miR-9 and TLX are involved in a feedback regulatory loop that control neurogenesis: also GSK-3β downregulates TLX together with high IL-1β upregulation thus inhibiting hippocampal neurogenesis. Indeed both GSK-3β, TLX and IL-1β are dysregulated in neurodegenerative and psychiatric disorders suggesting that a complex interaction among all these factors could result in a complete regulation of neurogenesis and axon growth (Green and Nolan, 2012).

Another miRNA can be inserted in the wnt-β-catenin-GSK-3β molecular cascade as a regulatory molecule of the cytoskeleton dynamics. Indeed miR-431 is induced after sciatic nerve crush and is able to stimulate the regeneration of cultured DRG axons silencing Kremen-1, an antagonist of wnt-β-catenin signaling (Wu and Murashov, 2013): Kremen 1 was identified as one of the main targets of miR-431; moreover, the *in vivo* mRNA and protein expression in DRG neurons is reduced in presence of miR-431. The effect of this miRNA consists in a significant increase in axon length (about 30%) after its overexpression, whereas cells treated with a miR-431 inhibitor display a reduction in axon branching by 35%. Similarly, evidence of miRNA action on GSK-3β has been demonstrated by the administration of miR-26a inhibitor after a nerve crush that can impair *in vivo* axon regeneration. The endogenous regulatory effect of miR-26a is mediated again by GSK-3β as demonstrated by *in vitro* and *in vivo* experiments in DRG neurons. However, in this case, GSK-3β has a direct effect on gene expression controlling the TF SMAD1, a TF known to promote axon outgrowth after axotomy (Saijilafu et al., 2013; Zou et al., 2009). GSK-3β regulation of axon growth is mediated both via controlling microtubules dynamics, at the growth cone level, or via controlling gene expression at the soma level (Jiang et al., 2015). We can finally conclude that a low level of GSK-3β needs to be endogenously maintained by miRNAs in order to support an efficient regeneration process.

7 MiRNAs ~~IN AXONAL GROWTH NETWORK: MAP1B-Rac1 PATHWAY~~in axonal growth network: MAP1B-Rac1 pathway

The mitogen-activated protein kinase (MAPK) system is found upstream GSK-3β activation and makes it phosphorylate MAP1B stimulating axon growth (Goold and Gordon-Weeks, 2005): this link has been proved in a context in which NGF partially contributes to axon elongation through MAPK- GSK-3β-MAP1B cascade (Fig. 3). MAP1B is also the target of miR-9, an highly conserved miRNA detected in the axon of primary cortical neurons. By acting locally in the axon, this miRNA is able to control axon length both *in vitro* (E17 primary cortical neurons) and *in vivo* (E 14.5 embryos brain) during development. In particular, the axon extension is allowed by preventing the interaction between miR-9 and MAP1B (Dajas-Bailador et al., 2012): the authors attributed this effect to a short stimulation with BDNF and the consequent increase of MAP1B protein level. MAP1B is a fundamental stabilizer of axonal microtubules, and it is highly expressed at the distal tip of the growing axons. Indeed MAP1B deficient mice present a delay in axon outgrowth and a reduced rate of its elongation. Montenegro-Venegas et al. (2010) demonstrated the role of this protein in the cross-talk between microtubules and actin filament: when MAP1B is present, T-cell lymphoma invasion and metastasis 2 (TIAM1, a guanine exchange factor) binds to microtubules, activates Rac1 that in turn inhibits cofilin, thus allowing actin polymerization and axon growth (the pathway is briefly illustrated in Fig. 3). Considering the miR-9 effect on primary cortical neurons through MAP1B silencing, we can suppose that miR-9 suppresses the axon

elongation process acting on this pathway. Moreover one of its first elements (Rac1 GTP, the activated form of Rac1) is a “crossroad” directed to different molecules and signal cascades, and possibly regulated by several miRNAs, like miR-9, miR-124 and miR-132 (Fig. 3). Indeed the last one (whose expression is highly induced by neurotrophins and CREB) promotes neurite outgrowth by p250GAP, a Rho family GTPase-activating protein, inhibition during cortical neuron morphogenesis *in vitro* (Vo et al., 2005): its downstream molecules can be pinpointed in Rac1 pathway, involved in actin polymerization (Fig. 3, dotted yellow lines). Indeed, the link miR-132-p250GAP-Rac1- p21-activated kinases (PAK) is involved in the remodeling and maintenance of spine growth and probably in the axon growth (Lai and Ip, 2013).

8 MiRNAs I~~N AXONAL GROWTH NETWORK: RhoA-PI3K-AKT PATHWAY~~n axonal growth network: rhoA-PI3K-AKT pathway

Another molecule controlled by miRNAs in the axonal growth network is MAP1B. According to the role attributed to MAP1B during axon development (Del Rio et al., 2004; DiTella et al., 1996; Montenegro-Venegas et al., 2010), it activates Rac1 pathway that inhibits RhoA, thus avoiding the growth cone collapse (Fig. 3). Similarly, miR-133b is a suppressor of RhoA, contributing *in vitro* to neurite outgrowth both in PC12 cell lines and primary cortical neurons (E18; (Lu et al., 2015)). Extracellular signal-regulated kinases and PI3K/AKT are the main effectors of the signaling pathway downstream miR-133-RhoA. As already described above, PI3K/AKT is one of the best known pathway involved in axon growth and is probably linked to GSK-3β, another key player of such process. The role of miR-133 emerges even more significant if we consider the work of Xin et al. (2012): neurite outgrowth of primary cultured neurons isolated from post-middle cerebral artery occlusion brain was stimulated by miR-133b-enriched exosomes derived from mesenchymal stromal cells, through RhoA repression. Therefore miR-133 seems to be responsible for axon growth not only during development, but also during brain repair (Xin et al., 2012). In addition, Yu et al. (2011a,b) described the essential role of miR-133 after SCI in adult zebrafish. miR-133 is endogenously overexpressed six hours after the lesion, but its inhibition blocks the regeneration process increasing RhoA protein level, thus likely activating the growth cone collapse (Fig. 3, brown dotted lines). Indeed the injured adult zebrafish, following miR-133 inhibitor administration, showed a reduced locomotor recovery and a low number of neurons with regenerating axons (Yu et al., 2011b). Its beneficial effect has been recently demonstrated also in mammals by the lentiviral injection of miR-133b in an adult injured spinal cord: RhoA has been validated as its direct target, whose donwnregulation was associated to mice locomotor recovery already at 4 weeks after the lesion (Theis et al., 2016). Finally, the indirect connection among other two miRNAs with RhoA pathway was demonstrated by Sun et al. (2013), that analyzed the mechanism of action of the heat shock protein B1 (HspB1). HspB1 is able to repress RhoA (thus promoting neurite extension), silencing the Rho GTPase, Rho guanine nucleotide exchange factor 11 (PDZ-RhoGEF), an exchanging factor that promotes the switch between RhoA GDP (inactive) and GTP active. PDZ-RhoGEF repression is driven by miR-20a and miR-128, whose expression is specifically enhanced by HspB1 ((Sun et al., 2013) violet dotted line, Fig. 3). In fact, inhibitors of miR-20a and miR-128 block the neurite growth of E17 cortical neurons promoted by HspB1, thus confirming the molecular cascade analyzed. Even though the downstream effectors of RhoA are not described here, it can be reasonably supposed a connection with the PI3-K/AKT pathway that directly promotes neurite growth (brown dotted line Fig. 3). In conclusion, this molecular cascade could be a pivotal pathway not only during development, but also after an injury. Indeed all the molecules described have been found in neurites and growth cones, and are produced in the axonal protein synthesis occurring after injury.

9 MiRNAs ~~IN NEUROINFLAMMATION FOLLOWING~~in neuroinflammation following SCI

The pathophysiology of SCI is also characterized by the activation of the inflammatory response to the lesion. miRNAs are one of the main regulators of inflammation in several neurodegenerative diseases [multiple sclerosis, ALS and Alzheimer’s disease, for example] as well as in SCI. Here we describe one of the most common inflammatory pathways, analyzing the contribution of some miRNAs altered after SCI. As shown in Fig. 4, the nuclear factor kappa B (NF-kB) canonical pathway, a transcription factor for cytokines, adhesion molecules and anti-apoptotic proteins, can be activated by different extracellular signals such as TNF-α and interleukin 1 (IL-1). Through their receptors, they activate IKK, constituted by IKK-α IKK-β and IKK-γ, that induces the phosphorylation of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IkBα), an inhibitory protein bound to NF-kB. The IkBα phosphorylation results in its release by NF-kB that is translocated to the nucleus by importin (Baeuerle and Baltimore, 1988; Baldwin, 1996). Here it can bind the DNA, thus inducing the production of pro-inflammatory cytokines. The immune mediator TNF-α is the first target of four different miRNAs. This pro-inflammatory and pro-apoptotic factor increases after injury, but its expression can be modulated by miR-181a, miR-125b, miR-411 and miR-99a. In normal conditions, miR-181a is enriched in astrocytes both in culture and in mouse cerebral cortex *in vivo*; however, its *in vitro* (primary astrocytes culture) knocking down enhances the production of cytokines such as TNF-α or IL-6 (Hutchison et al., 2013). Whether these molecules are the direct targets of miR-181a is not fully understood: indeed Hutchinson et al. (2013) have conferred a neuroinflammatory role to this miRNA, validating methyl CpG binding protein 2 (MecP2) and x-linked inhibitor of apoptosis protein (XIAP) as its direct targets. These two molecules are generally involved in apoptosis, but are likely also connected in astrocytes responses. Moreover, miR-181a is downregulated in a contusive SCI model 7 days post injury (Liu et al., 2009) with a good correlation between the microarray data and the RT-PCR results. The role of miR-181, analyzed by miRanda database, revealed TNF-α as one of its potential targets. In a similar experiment with a model of moderate contusive SCI, microarray data showed a decreased level of miR-181 after injury, and the pathway analysis of the networks affected by miRNAs put this small RNA as a controller of the inflammatory process (Yunta et al., 2012). Conversely, miR-125b directly targets the 3′ untranslated region of TNF-α transcript, thus controlling its production in the endotoxin shock (Tili et al., 2007). Even though here is not related to CNS, we hypothesize that the NF-kB pathway is conserved in different systems. Liu et al. (2009) described also a downregulation of miR-125, hypothesizing that changes in its expression could be related to infiltration and activation of astrocytes and other cells types. Despite they did not describe a clear role for this miRNA, there is a consistent literature conferring an inflammatory role to miR-125 (Pogue et al., 2010; Tili et al., 2007; Xia

et al., 2009). Also miR-411 and miR-99a have been related to TNF-α by in silico prediction. The connection with this cytokine is even stronger considering their *in vivo* downregulation after SCI, that should lead to increased inflammation (Liu et al., 2009; Yunta et al., 2012). Proceeding through the pathway, miR-9, miR-199 and miR-146a are negative regulators of NF-kB, IKK-β, and NF-kB respectively. Based on prediction data, the *in vivo* downregulation of miR-9 and miR-199 after SCI leads to higher level of NF-kB and IKK-β (Chen et al., 2008; Wang et al., 2011). The link between these miRNAs and their targets is based on some demonstrations derived by inflammation and cancer studies, that make this connection likely valid also for the traumatic conditions. IKK-β has been identified for the first time as a target of miR-199 in a study relative to the inflammatory properties of ovarian cancer cells (Chen et al., 2008). Moreover after an acute SCI miR-199b levels in activated microglia decrease with a consequent activation of IKK-β-NF-kB signaling pathway. *In vivo* overexpression of this miRNA reverses the upregulation of its target (IKK-β) resulting in the suppression of inflammatory response and improving animal performance (grip strength and rotarod tests) (Zhou et al., 2016). On the other hand, Bazzoni et al. (2009) showed that miR-9 (human polymorphonuclear neutrophils and monocytes) acts on NF-kB with a feedback control loop in which NF-kB increases miR-9 that in turn tunes its levels (Bazzoni et al., 2009). The same auto-regulatory loop has been demonstrated both for miR-9 by Wang et al. (2011) in hepatoma cells (Wang et al., 2011), and for miR-146a whose increased expression 7 days after injury negatively represses NF-kB (Bethea et al., 1998; Taganov et al., 2006). miR-146a is expressed in reactive cultured human astrocytes and its overexpression has an anti-inflammatory effect, reducing the levels of several cytokines as IL-6 (Iyer et al., 2012; Liu et al., 2009; Yunta et al., 2012). Indeed its expression has been correlated with a positive functional score after SCI, revealing its concrete anti-inflammatory role (Strickland et al., 2011a). Moreover miR-146a has been recently involved in a feedback mechanism (Fig. 4): its upregulation in SCI is driven by proinflammatory cytokines, whose expression increases *in vivo* during the firsts day after SCI. Indeed the induced miR-146a overexpression in acute phase is a consequence of the initial inflammation which is then blocked by interleukin 1 receptor associated kinase 1- TNF receptor associated factor 6 (IRAK1-TRAF6-MAPK) inactivation by miR-146a (Wei et al., 2016). Therefore, the authors indicated miR-146a as a potential therapeutic anti-inflammatory target in SCI. Finally, let-7 results downregulated at 1, 3 and 7 days post injury (Nieto-Diaz et al., 2014). This miRNA directly inhibits IL-6 during the inflammatory response related to cancer cell transformation (Iliopoulos et al., 2009).

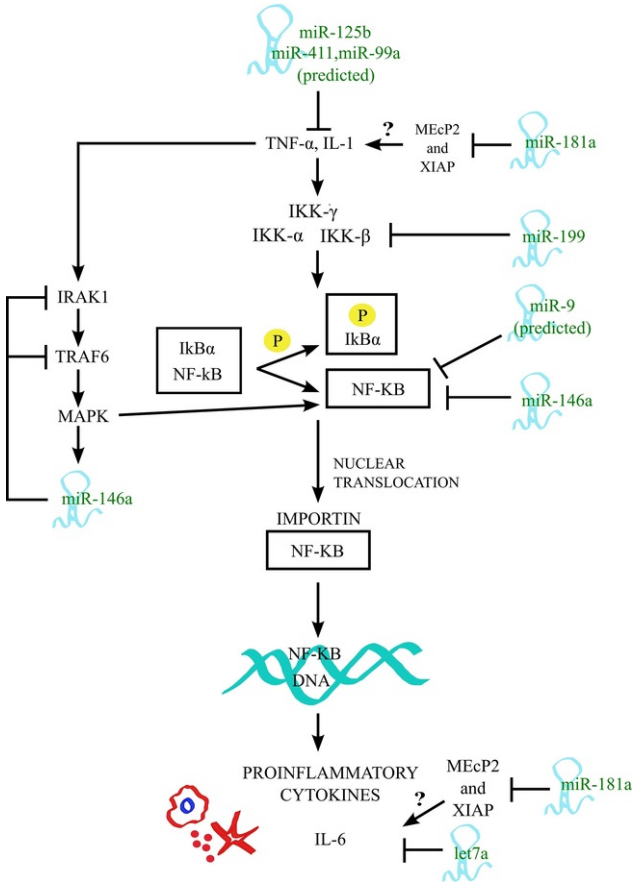

Fig. 4 miRNAs in inflammation, with particular emphasis on cytokines (TNF-α, IL-1, IL-6) and NF-kB role. P in the yellow circle stands for phosphorylation. Question marks indicate an unproved interaction between the molecules indicated; miRNAs in green

color refer to CNS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 4

10 MiRNAs ~~IN APOPTOSIS FOLLOWING~~in apoptosis following SCI

The role of miRNAs appears pivotal also in orchestrating the mechanisms of apoptosis, equally important during nervous system development and in case of trauma.

miRNA biogenesis is essential for the correct CNS development as demonstrated by Dicer and microprocessor complex deletion/downregulation. *In vivo* Dicer deletion in midbrain dopamine neuron, Purkinje cells, cortical and hippocampal neurons, striatal neurons and motor neuron progenitors causes apoptosis, brain abnormalities and decreases neural differentiation (Chen and Wichterle, 2012; Cuellar et al., 2008; Davis et al., 2008; Kim et al., 2007; Schaefer et al., 2007). More in detail *in vivo* conditional Dicer ablation in dorsal telencephalon at embryonical stage revealed that differentiation and survival of newborn neurons is much more affected by miRNA absence than the maintenance of progenitor expansion (De Pietri Tonelli et al., 2008). Also the conditional deletion of DGCR8 in post-mitotic neurons (hippocampus and cortex) partially causes brain weight reduction, microcephaly and apoptosis (Babiarz et al., 2011; Shiohama et al., 2003). Moreover the AGO1-4 ES cell line deficiency undergoes a deficit in miRNA-mediated translation repression and apoptosis as a result of Bim upregulation (Su et al., 2009).

Regarding CNS/PNS trauma, as previously mentioned, the sequence of anatomical changes occurring after an injury is also characterized by neuron cell death through the mechanism of apoptosis: during the secondary damage of SCI, it is modulated by a variety of factors, including several miRNAs. At least 7 known miRNAs participate in the control of the main steps of the apoptotic molecular cascade. As shown in Fig. 5, the main component of this pathway consists of B-cell lymphoma 2 (Bcl-2, anti-apoptotic factor) that inhibits the release of cytochrome c by mitochondria and the consequent activation of caspases, thus leading to the final repression of the apoptotic process. Moreover other molecular cascades [those of FAS-~~L~~caspase 8, PTEN-AKT and second mitochondria-derived activator of caspases (SMAC)-XIAP], again regulated by miRNAs, can contribute to the apoptotic mechanisms (Jovanovic and Hengartner, 2006). miR-29b is involved in cell death as a regulator of some pro-apoptotic genes like PUMA, both in a model of forebrain ischemia (Ouyang et al., 2013) and SCI (Liu et al., 2015b). Liu et al. (2015b) demonstrated a decreased expression of miR-29b and an increase in some pro-apoptotic genes after a contusive SCI: more in detail, they demonstrated that transfection of miR-29 mimic *in vitro* (Neuro-2A) decreased the expression of p53 upregulated modulator of apoptosis (PUMA), Bcl-2-associated death promoter, Bcl-2-like protein 11 and phorbol-12-myristate-13-acetate-induced protein 1. Similarly, miR-29 inhibitor reduces Neuro-2A cell viability as well as the miR-29 mimic abolishes the expression of active caspase-3 *in vivo*. Therefore, as schematized in Fig. 5, an overexpression of miR-29b could repress PUMA that generally promotes the apoptotic process by Bcl-2 inhibition. Furthermore, in the same paper the authors conclude that the neural cell death in SCI is the result of a cooperative contribution of miR-29b and miR-20a: the expression of miR-20a increases after SCI thus directly downregulating its target Mcl-1, an anti-apoptotic factor. However only the coinjection of miR-20a inhibitor and miR-29b mimic into the lesion spinal cord completely abolishes caspase 3 activation.

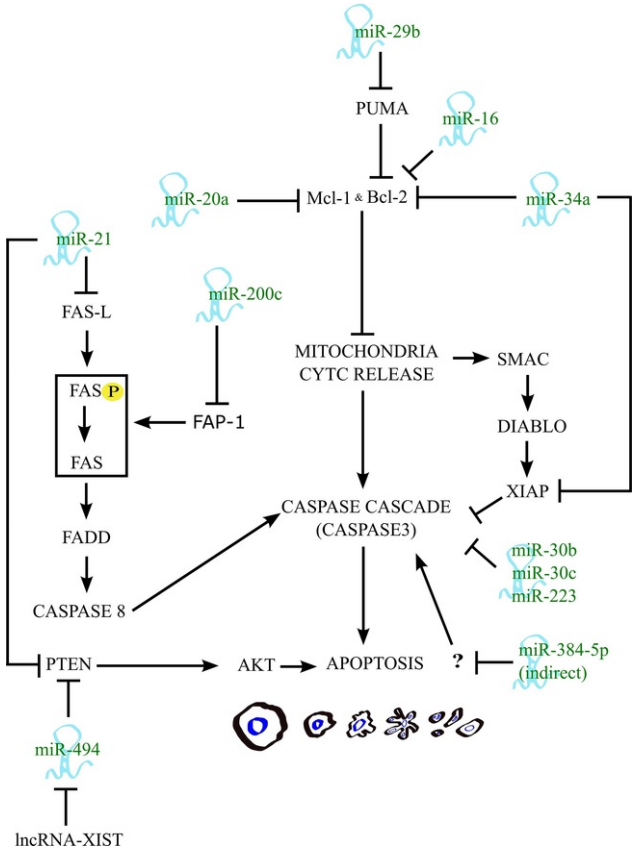

Fig. 5 miRNAs in the apoptotic pathway in which Bcl-2 and caspases are the main players; miRNAs in green color refer to CNS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alt-text: Fig. 5

Bcl-2, together with XIAP, can also be target of miR-34a and miR-16. The direct link between miR-34a and the two anti-apoptotic proteins is described in an experimentally induced traumatic brain injury model in which miR-34 overexpression (*in vitro* and *in vivo*) causes the increase of caspase 11 (Truettner et al., 2013). On the contrary, miR-34 downregulation 4 h after SCI is probably an attempt to contain cell death (Ning et al., 2014). Thence it is likely that miR-34 exerts its pro-apototic role directly targeting Bcl-2 and XIAP and thus increasing caspase levels (Fig. 5). miR-16 can be considered a proapoptotic miRNA as demonstrated by its *in vivo* increased expression 10 days after SCI with a correspondent downregulation of its target, Bcl-2 (Liu et al., 2010).

On the contrary, miR-21, whose expression is increased after contusion SCI in rats (Hu et al., 2013), plays a different role. Knocking down miR-21 with antagomir-21 attenuates the recovery of hindlimb motor function with a decreased spinal tissue sparing and increased lesion volume. Moreover the anatagomir-21 group causes increased apoptosis following SCI, with FAS-L and PTEN validated as direct targets or miR-21. Indeed, *in vivo* treatment with anatagomir-21 results in increased FAS-L and PTEN level, both apoptotic promoters through Fas cell surface death receptor −associated protein with Death Domain-CASPASE 8 (Fas-FADD-CASPASE 8) and AKT cascade (Fig. 5). Moreover through Fas-FADD-CASPASE 8 pathway miR-200c seems to be involved in the FAS-FAS-L interaction by directly targeting FAS associated phosphatase-1 (FAP-1). miR-200c is significantly increased after SCI and its upregulation in BV-2 cells dramatically promotes apoptosis, downregulating FAP-1 and thereby activating FAS signaling cascade (Yu et al., 2014).

Furthermore PTEN is also the target of miR-494, a miRNA downregulated after SCI contusion in rats. Restoring the normal expression of this miRNA inhibits apoptosis and promotes functional recovery after SCI in mice (Zhu et al., 2017). In this case it has been recently described that miR-494 activity is regulated by another small RNA: lncRNA-XIST, a long non coding RNA whose upregulation after SCI reduces the beneficial activity of miR-494 (Gu et al., 2017). Finally, also caspase activation is regulated by some miRNAs. As previously described by a microarray analysis, miR-30b and miR-30c are both downregulated after a spinal cord contusion (Liu et al., 2009). Caspase 3 is a main

target of these two miRNAs, that are described as negative regulators of cell death induced by cell detachment (anoikis) in tumor cell lines model. miR-30b and miR-30c seem able to block anoikis, by inhibiting caspase3 (Moreno-Mateos et al., 2013). Therefore, miR-30 downregulation after SCI may contribute to the exacerbation of secondary SCI.

Also miR-223 and miR-384-5p can act on caspase 3: miR-223 is expressed at high levels at different time points (1, 3, 7 and 14 days) after SCI and its inhibition significantly improved rat motor recovery with a decrease in cell apoptosis. Similarly, the cleaved caspase3 is indirectly upregulated by miR-384-5p in a hypoxic cell line model (Bao et al., 2013): considering the inhibitory general role of miRNAs, in both cases, they probably act on an unknown factor/molecule (question mark in Fig. 4) that in turn activates caspase3. The involvement of these two miRNAs in the apoptotic pathway is straightforward but need to be clarified in order to be exploited for therapeutic purposes.

11 M~~YELINATION-RELATED MIRNAs FOLLOWING CNS and PNS LESION~~yelination-related MIRNAs following CNS and PNS lesion

Myelination is a finely-tuned process essential for both the normal development of CNS/PNS and repear after an injury: indeed some of the mechanisms described during development (see below) can be recapitulated in response to a trauma (McTigue and Tripathi, 2008) as schematized in Table 1. According to a well-defined spatiotemporal differentiation cascade, undifferentiated OPCs and Schwann progenitor cells (SPCs), in CNS and PNS respectively) can give raise to mature OLs and SCs, responsible for axonal myelination. The balance between promoting and inhibiting factors precisely orchestrates such differentiation. miRNAs play a critical role in regulating the production and differentiation of both myelinating cells and in maintaining their identity (He et al., 2012), as demonstrated by the selective depletion of Dicer in OLs and SCs: indeed postnatal OL-specific Dicer-floxed mice present an increased demyelination combined with inflammatory astrocytosis and microgliosis, oxidative damage and neuronal degeneration (Shin et al., 2009). Similarly, Dicer deletion from mouse SCs prevents the formation of a mature myelin sheath (Pereira et al., 2010; Yun et al., 2010). Moreover, miRNAs can induce the switch from neurogenesis to gliogenesis during spinal cord development, as OL transcription factor 1-Dicer KO mice completely lack oligodendrogenesis and astrogliogenesis in the ventral neuroepithelial cells (Zheng et al., 2010). In literature the list of myelination-related miRNAs is quite long (Bremer et al., 2011; Dugas and Notterpek, 2011; He et al., 2012; Lau et al., 2008; Yun et al., 2010) although only some of them appear to be crucial to complete the myelination process. Furthermore, despite the distinct CNS and PNS pathways (Svaren, 2014), some of these main myelination-related miRNAs (miR-9, miR-138 and miR-338) can be detected in both systems. For example, miR-9 is involved both in OPC and SPC maturation acting on the same target, i.e. PMP-22 predominantly expressed in SCs. However, while in the CNS PMP-22 expression is inhibited by high miR-9 levels *in vitro*, in the PNS PMP-22 expression is not affected by low miR-9 levels (Lau et al., 2008; Verrier et al., 2009). Similarly, miR-219, miR-138 and miR-338 can regulate OL or SC maturation respectively in the CNS and the PNS. In the CNS, these miRNAs have been identified by qRT-PCR as the three main small RNAs in differentiating OLs with an increase fold change of 100, 30 and 30 respectively. Even though miR-219 alone is necessary and sufficient to promote OL differentiation, all three miRNAs are involved in different stages of this process. miR-219 directly inhibits OPC proliferation factors, such as platelet-derived growth factor α, Sox6, hes family bHLH transcription factor 5 (Hes5), zinc finger protein 238 (ZFP238), forkhead box J3 (FoxJ3) and platelet derived growth factor receptor α (PDGFRα) and its overexpression is also able to induce OPC differentiation by inhibiting the same targets (Dugas et al., 2010; Zhao et al., 2010b). Sox6, Hes5 and ZFP238 are also targeted by miR-338 that, in addition, is predicted to inhibit fibroblast growth factor receptor 2 (FGFR2, an OPC mitogen receptor). Some pro-neurogenic factors, such as NeuroD1, Isl1 and Otx2, are presumably inhibited by miR-338 and miR-219, thus conferring to these miRNAs multiple roles in OL differentiation (Dugas et al., 2010; Zhao et al., 2010b). miR-138 is described to extend the early stages of OL differentiation (so that the cells can produce mature myelin sheath), likely acting on Sox4, which, in combination with Sox6, inhibits the process. As previously stated, these three miRNAs are also key players during SC differentiation albeit targeting different molecules. miR-138 represses three genes usually expressed in undifferentiated SCs, cyclinD1, Sox2 and Jun (Yun et al., 2010). On the other hand miR-338 expression is under the control of Sox10, a transcription factor that control OL and SC differentiation, indicating that this miRNA may play a role in peripheral myelination even though its targets are not clearly identified (Gokey et al., 2012; Yun et al., 2010). Therefore, these miRNAs are shared between CNS and PNS, but they maintain different mechanisms of action. Considering the pivotal role of miRNAs during myelin formation/maintenance in CNS and PNS, one can foresee their involvement in disease and injury. Indeed miRNAs can act in demyelinating diseases such as multiple sclerosis, leukodystrophy, ischemic stroke, peripheral nerve injury and SCI. After SCI, OLs undergo acute necrosis and apoptosis with a progressive reduction in cell number until 3 weeks after injury (Crowe et al., 1997). The demyelination occurring after the trauma is partially counteracted by a spontaneous remyelination generally starting 2 weeks post injury (Harrison and McDonald, 1977). OPCs are responsible for this: the reactivation of developmental factors induce their proliferation and migration into the lesion area (Almad et al., 2011). On the contrary, SCs react more robustly to a peripheral nerve injury compared to OLs in the CNS: as the wallerian degeneration (i.e. the degeneration of the distal axon) takes place, distally to the lesion SCs start to dedifferentiate, remove the myelin debris and recruit macrophages for further clearing the damaged area. Several molecular pathways are activated during this process, finally leading to a regained contact between axons and dedifferentiated SCs that can now differentiate into mature myelinating cells (Glenn and Talbot, 2013). How miRNAs contribute to myelin destruction/formation in CNS/PNS is not completely understood. It has been demonstrated that the same miRNAs implicated in OL and SC maturation during development can switch their expression after SCI and/or peripheral nerve injury, although their role remain debated in these conditions: at least two of the main miRNAs previously described (miR-9 and miR-219, together with miR-384-5p) seem to be promising circulating biomarkers for evaluating the severity of SCI, since their concentration in the murine serum is strongly upregulated 12 h after the injury (3 h and 24 h in the case of miR-9) (Hachisuka et al., 2014). Moreover miR-219 (and in particular its form miR-219-2-3p) and miR-338 are downregulated after contusive SCI (Liu et al., 2009; Zhao et al., 2010b). Although a specific role for many of these miRNAs after SCI has not been identified, it is likely they are needed at different time points after the lesion to reestablish the OL population both by increasing OPC proliferation and by promoting OL differentiation. As a consequence of their redundant function, some miRNAs may concurrently influence different biological processes. For example miR-9, previously described as involved in the axon growth (Fig. 3) and inflammatory networks (Fig. 4), could be also a

regulator of myelination: the study of Zhou S and coll. described miR-9 downregulation after rat sciatic nerve transection as an important mechanism to allow SC migration in a regenerative attempt (Zhou et al., 2014). Also miR-138 (a key regulator of OL development) has been associated to SCI: however in this case it has been only described to promote neuroplasticity through vimentin repression *in vitro* (Fitzpatrick et al., 2015; Qian et al., 2015). The connection among SCI, miRNAs and myelin regulation remains unclear as demonstrated by several examples. In addition to miR-138, other eight miRNAs (miR-146a, miR-181a, miR-145, miR-17, miR-148b-3p, miR-1, miR-221 and miR-222) have been related to OL or SC maturation, but they are also differentially involved after SCI with distinct and additional functions. For example, miR-146a, in addition to its anti-inflammatory capabilities (Fig. 4) (Yunta et al., 2012), also plays a role in the regulation of OPCs/OLs after a lesion, as it has been proposed as a possible oligodendrogenesis inducing factor after middle cerebral artery occlusion (MCAo). Moreover, *in vitro* it is able to promote neuronal progenitor cell (NPC) differentiation into O4-positive OPCs (targeting IRAK1 and thus increasing the expression of myelin proteins) (Liu et al., 2016). A role for miR-146a in myelination can be also hypothesized, since it can push OLs to differentiate when activated by thymosin β4 acting on IRAK1 and TRAF6 (Santra et al., 2014). Another interesting example comes from miR-181a: in a contusive SCI model, it participates to the inflammation process, as previously described (Fig. 4), being downregulated 7 days post injury (Liu et al., 2009). This miRNA is generally classified as a tumor suppressor in gliomas, but it can also stop OPC proliferation and start OL differentiation *in vitro* (Dugas et al., 2010). Similarly, miR-145 (together with miR-199a-5p) is a regulator of myelin regulatory factor (C11Orf9), the human analog of myelin regulatory factor (MRF), one of the mouse protein necessary for OL maturation (Letzen et al., 2010). As for miR-146a, also its expression is altered after SCI, but miR-145 acts on different pathways: indeed the contusion SCI can upregulate miR-145, whose potential targets are anti-apoptotic genes, such as the Bcl-2 (Liu et al., 2009). Another miRNA upregulated after a spinal contusive lesion and involved in the control of OL number is miR-17 (Liu et al., 2009). This small RNA is a member of the miR-17-92 cluster, previously described in PTEN pathway (Fig. 2): their lack causes the reduction in myelinating cell number in vivo, while their expression promotes OPC proliferation by AKT signal (Budde et al., 2010). Other interesting examples consist in miR-148b-3p and miR-1 (Liu et al., 2009; Yunta et al., 2012). The first is downregulated after SCI with an anti-apoptotic function, while the second is upregulated after SCI with a pro-inflammatory and apoptotic function. Moreover, they are both described as miRNAs regulating *in vitro* proliferation and migration of SCs acting directly on cullin-associated and neddylation-dissociated 1 (CAND1-miR-148b-3p) and BDNF (miR-1) (Qian et al., 2016; Yi et al., 2016). More in detail, miR-1 is a negative regulator of BDNF that results downregulated after peripheral nerve injury thus reflecting the high BDNF level detected. Differently, miR-221 (upregulated after SCI and enriched in sympathetic neurons) and miR-222 can promote SC proliferation and migration *in vitro* as demonstrated by LASS2 repression after sciatic nerve injury (Liu et al., 2009; Natera-Naranjo et al., 2010; Yu et al., 2012b). Another set of miRNAs (miR-145, miR-338 and miR-138) have been specifically related to SC development and play a major role after PNS injury. miR-145, also described in CNS, has been correlated to the defined “injury response gene cluster” by Chang et al. (2013) but it was previously related to SC postnatal development (Verrier et al., 2010). miR-338 (myelination-related miRNA during development) is one of 22 miRNAs potentially linked to nerve regeneration, as suggested by a recent miRNA sequencing profile study after sciatic nerve injury: this miRNA targets ADAM17, a protein which seems essential for CNS myelination (Palazuelos et al., 2014; Yu et al., 2011a). miR-338 role in peripheral injury is also demonstrated *in vivo* by its therapeutic efficacy, when transfected in combination with miR-21, after sciatic nerve injury. When the injured animals are treated with these two miRNAs, 8 weeks after surgery, axon diameter and myelin thickness increase, assuring a significant functional recovery compared to the sham group (Wang et al., 2016a). Concerning miR-138, interestingly during development it represses anti-myelinating factors such as Sox2 and c-Jun to promote myelin formation. However, after a PNS injury its downregulation activates a regenerative response to allow early growth response 2 (Egr2) (one of miR-138 target) exert its role as pro-myelinating factor *in vivo* (Adilakshmi et al., 2012). Finally two miRNAs upregulated after nerve injury, miR-132 and miR-182, play opposite roles on SC proliferation and migration. While the increased expression of miR-132 promotes *in vitro* SC migration targeting protein kinase AMP-activated non-catalytic subunit gamma 3 (PRKAG3) gene and thus facilitating the regeneration process, miR-182 inhibits the same processes acting on fibroblast growth factor 9 (FGF9) and neurotrimin (Yao et al., 2016; Yu et al., 2012a). In conclusion, a complex crosslink involving myelination-related miRNAs during development and after an injury is evident both for CNS and PNS. Although the intertwined connections between the two systems are poorly understood, investigating the role of miRNAs related to myelination will help to fill the gaps in an overall framework.

Table 1 Myelination-related miRNAs in CNS and PNS in physiological and pathological conditions. In the columns CNS and PNS injury UP and DOWN indicate miRNA expression changes.

alt-text: Table 1

	Cell type/ animal model	CNS	PNS	CNS target(S))	PNS target(S))	Function(S) in CNS	Function(S) in PNS	CNS injury	PNS injury	Other implications
miR-9	OL, SC primary culture	✓	✓	PMP-22	PMP-22	inhibition of PNS specific molecules	compact myelin formation	UP biomarker for SCI severity	DOWN SC migration	axon growth (Fig. 3) Inflammation (Fig. 4)
miR-219	OPC primary culture	✓	X	PDGFRα, Sox6, Hes5, ZFP238, FoxJ3, NeuroD1, Isl1, Otx2	X	OL differentiation	X	DOWN Biomarker for SCI severity	X	X
miR-338	OPC primary culture	✓	✓	PDGFRα, Sox6, Hes5, ZFP238, NeuroD1, Isl1, Otx2,FGFR2	ADAM17	OL differentiation	likely peripheral myelination	DOWN undetermined	UP increased axon and myelin size; recovery	X
miR-138	OPC primary culture,	✓	X	Sox4, vimentin, Egr2	CYCLIN	OL differentiation	SC	UP neuroplasticity	DOWN regenerative	X

	SC Dicer1 KO mice				D1, Sox2, Jun		differentiation		response	
miR-146a	OPC primary culture	✓	X	IRAK1, TRAF6	X	from NPC to O4-OPCs; OL differentiation	X	UP OL genesis after MCAo	X	Inflammation (Fig. 4)
miR-181a	Induced OLs	✓	✓	undetermined	X	tumor suppressor in glioma cells	OL differentiation	DOWN undetermined	X	Inflammation (Fig. 4)
miR-145	Induced OLs, SC Dicer1 KO	✓	X	MRF	X	OL maturation	SC maturation	UP undetermined	X	X
miR-17~~-92OL miR-17-~~–92	OL miR-17–92 KO mice	✓	X	AKT signal	X	control of OL number	X	UP undetermined	X	axon growth (Fig. 2)
miR-132	SC primary culture	✓	✓	X	PRKAG3	X	undetermined	X	UP SC migration	X
miR-182	SC primary culture	✓	✓	X	FGF9, neurotrimin	X	undetermined	X	UP Inhibition of SC migration	X
miR-148b-3p	SC primary culture	✓	✓	undetermined	CAND1	undetermined	SC proliferation and migration	DOWN anti-apoptotic in SCI	undetermined	X
miR-1	SC primary culture	✓	✓	undetermined	BDNF	undetermined	SC proliferation and migration	UP pro-inflammatory and apoptotic	DOWN BDNF increase	X
miR-221	SC primary culture	✓	✓	undetermined	LASS2	undetermined	undetermined	UP undetermined	UP SC proliferation and migration	X
miR-222	SC primary culture	✓	✓	undetermined	LASS2	undetermined	undetermined	X	UP SC proliferation and migration	axon growth (Fig. 2)

12 H~~OW TO MANIPULATE~~ow to manipulate miRNAs

The pathways above mentioned demonstrate the central role of miRNAs in case of trauma in modulating axonal growth, inflammation, apoptosis and remyelination: therefore the manipulation of their expression in neurons can be pivotal i) to understand their role in neural diseases and neuropathological conditions as SCI; ii) to exploit their therapeutic potential.

The best way to study the physiological functions of miRNAs both *in vitro* and *in vivo* is to overexpress/downregulate them either in the whole organism or in a particular cell type. Expression in neurons can be modified by transfection, transduction of miRNA plasmid or miRNA mimics/miRNA antagonists. However, the insertion of artificial miRNAs in primary culture neurons is generally toxic and poorly efficient, while *in vivo* their delivery is affected by their inefficient uptake, stability and regulation of expression. miRNA overexpression may elucidate the effect of the inhibition of a specific protein or overcome a miRNA downregulation. It is obtained by administrating plasmids containing a specific miRNA gene or artificial miRNA mimics delivered by viral or not viral vectors. miRNA mimics are synthetized as double-stranded RNA oligonucleotides with different types of chemical modification that make them suitable miRNA substitutes both *in vitro* and *in vivo*. These formulations protect miRNA oligonucleotides from enzyme degradation making the cellular uptake efficient. The efficiency of plasmid/miRNA mimics depends on the transfection technique.

12.1 T~~RANSFECTION METHODS~~ransfection methods

The most common approach to deliver a miRNA plasmid/miRNA mimic into the cells is lipofection: it consists in embedding the miRNA gene plasmid into liposomes, exploiting the capability of these vesicles to fuse with the cell membrane. Thus lipofection conjugates the anionic charge of the miRNA with a mix of lipids with cationic head groups. Lipofection has been for example used to study miR-485 overexpression role in controlling the dendritic spine number and synapse formation in hippocampal neurons culture (Cohen et al., 2011). Moreover an *in vivo* approach, by tail injection of a glycol-modified liposome, was equally efficient in delivering miR-126 (promoting angiogenesis through vascular endothelial growth factor inhibition) to the thigh muscles of an ischemic mouse model (Endo-Takahashi et al., 2015). Alternatively, a polymeric delivery system, the cationic polymers polyethylene imine, again creates a good cationic shielding. miRNA plasmids/mimics can also be encapsulated and delivered by exosome nanoparticles. Exosome-mediated transfer of miRNAs is a mechanism of information exchange occurring spontaneously among cells

(Valadi et al., 2007). For example, modified exosomes loaded with exogenous siRNA have been introduced in a murine brain, leading to BACE 1 knockout specifically in neurons (Alvarez-Erviti et al., 2011). Moreover, the tail injection of miR-124 rabies virus glycoprotein (RVG)-modified exosome in a mouse model of ischemic stroke limited the injury spread by promoting cortical neurogenesis (Yang et al., 2017). The efficiency achieved by the packaging and the cationic charge and exosome encapsulation lead in both cases to an efficient miRNA delivery, suggesting liposomes and cationic polymers polyethyleneimine as promising miRNA-based therapeutic strategies.

Although no clinical trial based on miRNA transfection is today available for the treatment of CNS/PNS disorders, some of the above mentioned transfection methods (by liposomes, nanoparticle or polyethyleneimine) have already found their application in cancer therapy: for example, miR-RX34 intravenous liposome injection and miR-16 mimic (called TargomiR) have both successfully completed a phase I clinical trial for primary liver cancer and malignant pleural mesothelioma, suggesting their viability (www.clinicaltrials.gov). This probably means that the use of miRNAs as therapeutic drugs could be clinically employed also in the neurological field in the near future.

Another highly efficient technique in neurons is magnetofection that consists in the association of nucleic acid with cationic magnetic nanoparticles that are then transported into the cells by a magnetic field. A good neuron viability and a high transfection rate can also be achieved by electroporation and nucleofection, a method that transfers directly the substrate into the cell nucleus and the cytoplasm (Zeitelhofer et al., 2007). However magnetofection, electroporation and nucleofection are valid techniques for *in vitro* miRNA studies before cell plating, but they cannot be used for *in vivo* application. All these delivery systems are considered stable, non-immunogenic and suitable for specific modifications needed to reach a good efficiency.

12.2 V~~IRUSES~~iruses

Viral vectors, such as adeno-associated viruses (AAVs), lentiviruses and retroviruses, can reach a high efficiency expression rate in neurons conferring *in vitro* and *in vivo* other type of properties. AAVs are able to transduce both dividing and not dividing cells with the main advantage to produce a long term expression of the transgene located in its genome. Tissue-specific tropism, high transduction efficiency, relatively non pathogenic and safety make these viruses ideal as miRNA mimics and antagonist transductors. The choice among about 12 AAV serotypes with different ways of administration makes possible miRNA manipulation *in vivo*. For example, the AAV9 encoding an artificial miRNA against superoxide dismutase 1 (SOD1) protein was efficiently injected in the cerebral ventricles of a mouse model of ALS showing its therapeutic effect on both upper and lower motor neurons (Stoica et al., 2016). Long lasting gene expression and low toxicity can also be achieved by recombinant AAV (rAAV): they are less dangerous and more workable compared to other viruses, as demonstrated by cerebrospinal fluid injection of an rAAV-artificial miRNA targeting SOD1 in an ALS murine model leading to KO of SOD1 protein and slowing disease progression (Wang et al., 2014). Moreover rAAV vectors are able to easily cross the BBB and efficiently transduce both neurons and astrocytes: for instance rAAV9 has been tested *in vivo* for the treatment of spinal bulbar muscular atrophy with good results in terms of mouse behaviour, body weight and survival (Miyazaki et al., 2012).

Also lentiviral (LV) vectors can be employed, as demonstrated *in vitro* by lentiviral-mediated miR-92 overexpression in cerebellar granule neurons (Barbato et al., 2010). Their higher efficiency rate makes them suitable vectors also *in vivo*: for example, they have been successfully used in an ischemic mouse brain to increase the progenitor number and the microvessel density (miR-210-LV); (Zeng et al., 2016) and in a mouse model of brain injury to exert a neuroprotective role (miR27a-LV; Sun et al., 2017). However LV employment needs to be carefully considered because of *ex vivo* manipulation and the risk of mutagenesis.

The specificity in infecting dividing cells makes retroviruses useful to study the role of a specific miRNA during neurogenesis. This is the case of miR-137, whose overexpression by retroviruses in dentate gyrus postnatal neurons allowed to define the miRNA roles in the inhibition of dendritic morphogenesis, maturation and spine development both in cultured neurons and *in vivo* (Smrt et al., 2010). Nowadays several clinical trials using AAVs, rAAVs and LVs are ongoing for gene therapy of cancer, Parkinson's disease, Alzheimer's disease and Spinal muscular atrophy (SMA), demonstrating these methods as safe and tolerable also for future miRNA approaches. However although viruses can mediate an high miRNA-efficiency delivery, their therapeutic use in CNS needs to be carefully considered in terms of immunogenicity and oncogenesis. Indeed innate immune response in mouse brain as consequence of AAV administration has been already reported (Lowenstein et al., 2007). Moreover the cost for an high-quality and −quantity production of viral vectors further limits their application.

On the contrary, miRNA loss of function is used to reveal the role of a specific miRNA/miRNA cluster with several possible strategies. One tool consists in total or conditional (tissue-specific deletion) knockout murine lines which allow to investigate the specific role of a single miRNA in the CNS. Even though CNS application of this technique can be found in the literature, the knockout of a single miRNA is only partially efficient due to functional redundancy and miRNA cooperation. Moreover in case of miRNA cluster (notably, miRNAs derive from the same gene locus), the complete deletion of the locus results in the loss of function of different miRNAs thus making difficult to distinguish which is the miRNA responsible for the knockout phenotype (Ruberti et al., 2012).

12.3 A~~NTISENSE OLIGONUCLEOTIDES~~ntisense oligonucleotides (ASOs)

Due to these limitations, other strategies have been developed, such as ASOs that target the seed region of the mature sequence of a specific miRNA by silencing or degrading it. Compared to a complete knockout, ASOs allow to perform an inhibition limited in time and dependent on ASO pharmacokinetics. Different types of ASOs can be classified depending on the chemical modifications: unmodified RNAs, 2′-O-Methyl RNAs, 2′O-methoxyethyl RNAs, locked nucleic acid (LNA) and phosphorothioate RNAs. The first miRNA inhibitors used in mammals were antagomirs (Krützfeldt et al., 2005), a member of 2′-OMe RNAs, that have a good inhibition effect in brain when delivered locally but not

systemically (Krützfeldt et al., 2007). For example, high miR-223 levels after a rat spinal cord compression were suppressed by the intrathecal injection of the antagomir-223, inducing functional recovery and angiogenesis, and limiting apoptosis *in vivo* (Liu et al., 2015a). However, antagomirs must be delivered in high doses to be effective and they are generally used to downregulate specific mRNAs to study their function. Alternatively, 2′O-methoxyethyl RNA and LNA have been developed as more resistant to many endonucleases with respect to antagomirs. For example, miR-210-LNA has been used in a rat model of ischemic brain injury, providing a neuroprotective effect even 4 h after its intracerebroventricular (ICV) injection (Ma et al., 2016).

12.4 miRNA-~~MASKING ASOs/SPONGES~~masking ASOs/sponges

Alternatively miRNA function may be inhibited by interfering with the miRNA-mRNA interaction using the "miRNA-masking" ASOs. Such modified oligonucleotides, first developed by Xiao et al. (2007), bind to the 3′ UTR of the miRNA target instead of linking directly to the miRNA seed region (Xiao et al., 2007). Similarly, competitive inhibitors called "miRNA sponges" have been developed as RNA transcripts containing multiple binding sites for miRNA seed regions (Ebert et al., 2007). They can be delivered by vectors transfected into the cells, thus inhibiting the miRNA function (Brown and Naldini, 2009). As they are synthetized with several binding sites, they can be used to silence even an entire family of miRNAs. Moreover, sponges with the RNA polymerase II as promoter linked to a fluorescent reporter have been constructed in order to detect and quantify sponge-treated cells. The potential of this strategy is demonstrated by its application in several cell culture and *in vivo* experiments. As an example, the plasmid GFP sponges for miR-9 family have been electroporated in mouse embryonic stem cells *in vitro* to generate a GFP-miR-9-sponge mouse line, allowing the conditional inactivation of the entire miRNA family in a spatiotemporal-controlled way: thus showing that miR-9 family is essential in the control of dendritic growth during development (Giusti et al., 2014). Even if the design of a miRNA sponge is not difficult and quite fast, the efficacy in inhibiting miRNA is not easy, due to the requirement of a reporter assay to validate the target gene. Moreover it is difficult to establish the degree of inhibition of an entire miRNA family.

A variant of the "miRNA sponge" tool are tough decoy RNAs, which obtained a long term suppression over 1 month in mammalian cells. These molecules are stem loop structures, acting again as competitive inhibitors, with specific miRNA binding sequences located in the middle of the molecule (Carè et al., 2007; Staton et al., 2011).

miRNA target protectors can also be used to study the role of miRNA in regulating a specific gene; they are designed to bind the miRNA target site on the gene of interest, consequently blocking miRNA action on its target as it was firstly demonstrated *in vivo* for miR-430 family and TGF-β (Choi et al., 2007). For instance they have been used to first demonstrate that miR-101 binds at the specific 242–248 position of the amyloid precursor protein 3′ UTR regulating the target level in HELA cell culture (Long and Lahiri, 2011).

In conclusion, different strategies are now available for miRNA overexpression or inhibition, although all the approaches display both advantages and limitations, that need to be carefully considered before their application. As a valid alternative to miRNA use, currently the most common therapeutic strategy to inhibit a specific mRNA is RNA interference technique by siRNA. siRNA are 21-nucleotides double stranded RNA molecules discovered to be potent gene expression inhibitors (Fire et al., 1998). Due to their specificity, siRNA technology is widely employed in the study of nervous system diseases. However siRNAs also show some limitations, due to their instability, poor cellular uptake, inability to cross the BBB, and immunogenicity (Gomes et al., 2015). To overcome these limitations several strategies have been proposed: the best results in terms of stability and permeability are reached by chemical conjugation and stabilization with functional molecules, while their cellular specificity is improved by nanoparticles (NPs) based system (such as RVG peptides, chitosan or lipid NPs). The potential of siRNA strategy in CNS-PNS is demonstrated by the numerous *in vivo* studies: for example, a specific PUMA siRNA treatment has been delivered by ICV injection in subarachnoid haemorrhage rat model reducing mortality rate, cerebral oedema and neural deficits (Yan et al., 2011). Another successful example of functional siRNA modification is provided by Al-Jamal et al. (2011): in this study a carbon-nanotube Caspase-3 siRNA has been injected directly into the cerebral cortex of ischemic mice reducing neurodegeneration and promoting functional motor recovery (Al-Jamal et al., 2011). An efficient siRNA delivery is also reached by systemic administration as demonstrated by a polyethyleneimine modified claudin-5 siRNA, able to reduce oedema and cognitive impairment of traumatic brain injury mice (Campbell et al., 2012). Moreover another valid application is described by Alvarez-Erviti et al. (2011) that, by intravenously injecting RVG-β secretase1 exosome siRNA, could reduce protein expression not only in neurons but also in microglia and OLs. Finally, recently the intranasal administration of microglia cells transfected with polyethyleneimine modified Beclin1 siRNA has been found in the cytoplasm of neurons and glial cells in the prefrontal cortex of C57BL6/J mice 4 h and 24 h post-delivery, suggesting this method as a not invasive technique for therapy (Rodriguez et al., 2017).

Thanks to the encouraging preclinical results, there are several siRNA-based protocols in clinical trials: most of them are mainly related to the treatment of cancer (Chakraborty et al., 2017) and inflammatory disorders (for example the siRNA against syk kinase gene produced by Exccelair company and used for the treatment of asthma) (Chakraborty et al., 2017). As concerns the neurological field, it is noteworthy that 13 siRNAs for ocular diseases are now in clinical trials: in particular, safety and preliminary efficacy tests of siRNA-027 and Cand5 have been successfully completed for the treatment of macular degeneration by intravitreal injections (www.clinicaltrials.gov). Moreover intrathecal infusion of two antisense oligonucleotides (ISIS 333611 and Nusinersen) completed phase I clinical trial for the treatment of ALS and SMA respectively (Chiriboga et al., 2016; Miller et al., 2013).

In conclusion, considering the advances and promising results obtained in the above mentioned examples, we can speculate that the use of miRNAs and/or siRNAs in the clinical application for the therapy of CNS/PNS traumatic disorders could be not so far.

13 C~~ONCLUSION REMARKS: miRNA COOPERATION AND CONVERGENCE~~onclusion remarks: miRNA cooperation and convergence

SCI is a complex sequence of events that interact among each other producing the devastating conditions that affect millions of people: indeed, after a trauma, several biological processes (e.g., axonal degeneration/regeneration, inflammation, cell death, myelin loss), apparently independent, are actually intertwined. In this scenario, miRNAs work as a perfectly coordinated network in which every element is just a part of the whole. Here the complexity of the system has been illustrated in some molecular pathways (Figs. 2–5), where every single miRNA can act on different mRNAs as well as a single molecule can be targeted by several miRNAs. As inner regulatory system, miRNAs achieve a precise control of different nervous processes coordinating their actions to orchestrate a complex gene expression network. Such a complicated overview of the miRNA system comes from the demonstration that the repression exerted by one single miRNA is not sufficient to influence an entire biological pathway, as already showed in neocortical development studies (Baek et al., 2008; Barca-Mayo and De Pietri Tonelli, 2014; Selbach et al., 2008). Two main theories have been proposed to explain how miRNAs can manage simultaneously a precise control of several processes. The first one is based on the "cooperation hypothesis": according to Schouten et al. (2013), the cooperativity theory refers to a potentiation of the translational repression achieved by the interaction of different miRNAs on multiple seed regions on the same mRNA 3′ UTR (Schouten et al., 2013). The pathways proposed in the present review well fit with this concept and explain why the manipulation of one single miRNA may result in no effect due to the compensation exerted by the other miRNAs binding the same target. During the last years, another theory emerged referring to the "convergent miRNA action". miRNA convergence is defined as "the synergic action of one or more individual miRNAs that by acting on different seed regions in one or more target genes result in a regulatory effect" (Barca-Mayo and De Pietri Tonelli, 2014). More in detail, the authors recognize three kinds of convergence: "on target", when two or more miRNAs act on the same molecule; "on pathway", when miRNA(s) can target different molecules belonging to the same pathway; "on function", when one or more miRNAs act on different elements of different pathways that share the same biological function. All these types of convergence can be also found in the pathways described above, even though generally every miRNA presented here has been studied individually for its specific effect in PNS/CNS. Therefore the "convergent theory" integrates the "cooperation concept", contributing to render miRNA functioning even more complicated. Some examples of convergent miRNA actions in the CNS have been already described in cortical development studies, including i) the control of proliferation and commitment of neural stem cells/NPCs; ii) the balance between survival and differentiation of cortical progenitors; iii) the specification of cortical cell subtypes and the control of neurite outgrowth; iv) the migration of the glutamatergic cortical neurons (Barca-Mayo and De Pietri Tonelli, 2014). Based on the literature, in the present review we reported some examples of miRNA convergence, gathering different data (experimentally demonstrated or simply hypothesized) and showing how the miRNA synergistic effect can result in a functional outcome. Actually some reports tried to define and exploit the possible synergic effect of several miRNAs acting on the same pathway: for istance, miR-20a and miR-128 are an example of "convergence on target", since, repressing PDZ-RhoGEF, they can promote the neurite growth of E17 cortical neurons *in vitro* ((Sun et al., 2013) Fig. 2). Even more representative, is the "convergence on pathway" of miR-21 and miR-199a-3p in SCI as mediators of synaptic plasticity: in this case the elevated expression of miR-21 and the decreased expression of miR-199a-3p (induced by animal exercise after injury) well correlate with PTEN repression and mTOR enhancement respectively ((Liu et al., 2012) Fig. 1). A synergistic effect is also described for miR-20a and miR-29b in controlling the apoptotic process in SCI: *in vitro* their combination contributes to the repression of the anti-apoptotic myeloid cell leukemia sequence-1 (miR-20a upregulation) and increases the levels of some apoptosis promoters like Bad, Bim, Noxa and Puma (miR-29b downregulation). The *in vitro* data are supported by the proof that *in vivo* miR-20a inhibitor and miR-29b mimic injections decrease the level of caspase-3 in a cooperative way (Liu et al., 2015b). Another recent example of cooperative action on the same function comes from an *in vitro* study on NSCs: three miRNAs (miR-124, miR-138 and miR-137) cooperatively drive differentiation acting on a set of genes connected in a network of TFs with Sp1 as the major node (Santos et al., 2016).

The convergent/combinatorial concept can also explain the synergistic effect of two or more miRNAs compared to their individual efficacy. As previously reported, miR-9 and miR-124 demonstrated to have a stronger effect in promoting dendritic branching of differentiated neurons (*in vitro* NSC) when acting together instead of exerting their role alone (Xue et al., 2016). Also miR-23a and miR-125b are individually able to decrease musashi 1 expression level (a protein involved in neuronal progenitor proliferation) in neuronal stem/progenitor cells, but their synergistic activity produces a stronger effect (Gioia et al., 2014). Very recently a group of 11 miRNAs has been identified to synergistically control the fate of adult hippocampal neural stem cells in *in vitro* (adult NSCs) and *in vivo* Dicer KO model (Pons-Espinal et al., 2017). Another example comes from PNS injury. The transfection of miR-338 and miR-21 8 weeks after a sciatic nerve lesion promotes a functional recovery in terms of sciatic nerve injury, electrophysiological analysis, axon diameter, myelin sheath thickness, G-ratio and ratio of wet weight of the gastrocnemious muscle (Wang et al., 2016a). Due to the still little number of miRNA synergy examples in the nervous system, we cite some references coming from other domains, where the concept of "synergy" has already emerged. For example, the treatment of non-small cell lung cancer with the combination of let-7b and miR-34a overcomes the anti-proliferative effect obtained by individual miRNA administration (Stahlhut and Slack, 2015). Similarly, only the combination of miR-499 and miR133 is able to significantly increase the expression of two cardiac specific genes, revealing their synergistic effect on cardiac differentiation *in vitro* (Pisano et al., 2015). Even if combinatorial approaches are now emerging as new way to study miRNA function, the full description of miRNA networks remains a big challenge. The reason for this complexity derives both from the miRNA redundant structure and from our lack of knowledge: indeed, although about 2000 mature miRNAs have been sequenced in humans, we are probably still far from the total number. Every year new miRNAs are annotated in the main database (miRBase), some associated with validated targets, some just connected with predicted targets and some completely unknown. The synergistic strategy adopted by miRNA is also an important concept for several diseases, as they generally involve several different miRNAs rather than one. Indeed computational studies demonstrated that miRNAs in complex diseases cooperate and act on genes with similar functions, thus exploiting a synergism in function even more significant than in normal cases (Na and Kim, 2013; Xu et al., 2011). In this sense the manipulation of one single miRNA cannot be the most effective therapeutic strategy for SCI or CNS/PNS pathologies, but nevertheless allows researchers to better understand miRNA mechanisms of action and

add new elements to the miRNA complex networks. In conclusion, a global view is necessary to understand the complexity of a system in which every single miRNA operates only in convergence with other members. This is still a new and underestimated concept, but fundamental to understand how miRNAs achieve such a precise coordinated regulation.

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Highlights

- miRNAs are major players in the pathogenesis of CNS/PNS injury.
- miRNAs synergically control axon regrowth, inflammation, apoptosis and myelination.
- miRNA mechanism of action is proposed as a cooperative and/or convergent network.

Queries and Answers

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