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review

MicroRNA regulators of cholinergic signaling link neuromuscular, cardiac and metabolic systems

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ABSTRACT

The neurotransmitter acetylcholine (ACh) notably regulates many brain and bodily functions, including metabolic as well as heart and muscle activities. Concordantly, acquired changes in ACh signaling lead to significant and widespread systemic effects. Those can be observed both when ACh signaling fails, as in the motor neuron disease amyotrophic lateral sclerosis (ALS) and when it over-reacts, as in the hyper-cholinergic excitation following organophosphate poisoning. To explore the corresponding molecular mechanisms, we focused on regulation of ACh signaling by microRNAs (miRs). Current research implicates miRs as post-transcriptional modulators of gene expression, playing pivotal, rapid and interactive roles across various systems. To interrogate the systemic role of ACh-regulating miRs (CholinomiRs), we sought evidence for CholinomiRs with dual or triple roles in neuromuscular junctions (NMJ), heart development and functioning, and/or metabolic systems. Here, we report key links between CholinomiRs with known cardiac and metabolic roles, including the nicotinic acetylcholine receptor-targeted miR-1 and the acetylcholinesterase-targeted miR-132, and NMJ-related metabolic regulating miRs such as the histone deacetylase 4-targeted miR-206. Taken together, this information indicates a bridging role for CholinomiRs that may be relevant both for NMJ degeneration and the metabolic changes observed in ALS patients, and for the cardiac irregularities and NMJ degeneration reported following organophosphate poisoning. Uncovering the potentially causal involvement of CholinomiRs in balancing neuromuscular, cardiac and metabolic functions might improve our understanding of the inter-tissue communication and the processes of reaching homeostatic states which are essential for balancing between seemingly separate body systems, allowing a more encompassing look on disorders involving impaired cholinergic signaling.

CHOLINERGIC SIGNALING IMPAIRMENTS SPAN MULTIPLE TISSUES

The neurotransmitter acetylcholine (ACh) has been the very first chemical neurotransmitter to be identified *(1,2)*. ACh notably regulates numerous brain and bodily functions, including metabolic *(3,4,5)*, cardiac *(6)* and muscle development and activities *(7,8,9)*. Compatible with the crucial regulatory role of this neurotransmitter in many multi-tissue functions, acquired changes in ACh signaling lead to significant and widespread systemic effects *(10,11,12)*. Those can be observed both when ACh signaling fails, as in the motor neuron disease amyotrophic lateral sclerosis (ALS) *(13,14,15)* and when it over-reacts, as in the hyper-cholinergic excitation following organophosphate poisoning *(16,17)*. Based on this compelling evidence, we predicted the

existence of a rapid and efficient surveillance mechanism(s) which can send messages between different tissues when ACh signaling is imbalanced, and adjust its levels to the new needs for reaching homeostasis. In the following, we argue that miRs targeted to cholinergic genes and their upstream regulators may fit this description.

MIRS AS A REGULATORY SYSTEM

MiRs are small non-coding RNA molecules that are involved in post-transcriptional regulation of mRNA translation and stability. By partially binding to sequences primarily located on the 3'-untranslated region (3'-UTR) of mRNA molecules, miRs can lead to simultaneous de-adenylation, translational repression and cleavage of those mRNA molecules that carry such complementary motifs *(18)*. Functional studies indicate miRs involvement in a broad range of cellular and developmental processes such as the cell cycle *(19)*, learning and memory formation and maintenance *(20)*, energy metabolism *(21)* and many others. The mRNA targets of miRs include a sequence of at least 7 nucleotides which is complementary to the 'seed' region of the miR, and recent evidence suggests that other characteristics add to the specificity of that pairing *(22)*. While some miRs are tissue-specific, a significant amount of them is present in different tissues. One miR can thus simultaneously influence multiple pathways, and regulate different, yet specific targets. Furthermore, miRs may be transported between tissues, for example by being packaged in exosomes *(23,24,25)*, adding inter-tissue communication to their surveillance power. Altogether, miRs provide a rapid and economically efficient regulatory step over gene expression as well as inter-tissue coordination of multi-organ functioning, precisely those features that one would predict for ACh controlling entities.

MIRS IN THE NEUROMUSCULAR JUNCTION

The great majority of miR studies primarily focus on their impact on tumor biology and/or the nervous system. Nevertheless, an increasing amount of research provides evidence for miRs involvement in muscle development and maintenance. We predicted that CholinomiRs, those miRs which regulate ACh-related functions *(1)* in one or more systems would also be involved in ACh roles in other tissues. A prominent example is that of miR-1, which was initially discovered in nematodes as a regulator of two subunits of the nicotinic ACh receptor nAChR (UNC-29 and UNC-63) *(26)* and has more recently been shown to be important for post-mitotic growth of larval muscle in a fly model *(27)*. In this experiment *Drosophila* miR-1 (DmiR-1) knock-out mutant lethality was rescued when a DmiR-1 transgene was expressed in the mesoderm and muscle. It was further suggested by the researchers that miR-1 essentially maintained muscle cell identity and survival by inactivating the expression of non-muscle genes. This is corroborated by evidence showing that miR-1 operates as a down-regulator of the histone deacetylase HDAC4, which is known to inhibit muscle cell differentiation *(28,29)*. Furthermore, miR-1 was shown, alongside miR-206 which is also targeted to HDAC4, to promote the differentiation of skeletal muscle satellite cells in favor of proliferation, allowing growth and repair of postnatal skeletal muscle *(30,31)*. Via HDAC4-targeting, miR-206 promotes muscle regeneration and re-innervation following injury *(32,33)*. Also, increases in the levels of miR-132, a known regulator of Acetylcholinesterase (AChE), potentiate ACh signaling in brain and body alike *(34,35)*. MiR-132 has further been proposed as a biomarker for the moto-neuron disease spinal muscular atrophy (SMA), showing significantly higher levels in patients as opposed to controls *(36)*. Thus, several known miRs play significant roles in various events along the life span of motor neurons, NMJs and muscles across multiple organisms, such as development, differentiation, functional maintenance, and regeneration after injury.

MIRS IN HEART DEVELOPMENT AND FUNCTION

Multiple miRs have been identified in cardiac tissue at all stages of development. Cardiac-specific knockout of Dicer, a gene encoding an RNase III endonuclease that is essential for miR processing, leads to rapid heart failure and postnatal lethality, showing the importance of cardiac miRs *(37)*. For the purpose of this review, we will focus mainly on miR-1 and miR-132 in cardiac functioning. Importantly, miR-1 promotes pluripotent progenitor cells or stem cells to adopt cardiac characteristics during cardiogenesis *(38)*. Furthermore, miR-1 is causally involved in the electrophysiological functioning of the heart, via regulating levels of the GJA1 and KCNJ2 channels that are believed to contribute to the arrhytmogenic potential *(39)*. In mouse models, miR-1 over-expression impairs cardiac contractile function, most likely by targeting cMLCK and CaM, as well as by inducing anterior-ventricular block *(40, 41)*. CaM is an upstream activator of CaMKII *(42)*. A decrease in CAMKII activity has been reported to initiate changes in myofibril thick filament structure, resulting in decreased interaction of myosin heads with actin thin filaments *(43)*. The cMLCK kinase phosphorylates regulatory light chains in the heart, and a decrease in RLC phosphorylation has been shown to promote myocyte hypertrophy in vivo *(44)*. MiR-1 is also dysregulated in mouse ventricles during development of severe hypertrophic cardiomyopathy and heart failure *(45)*. Thus, accurate miR-1 levels are important for correct cardiac functioning.

MiR-1 is not the only miR involved with cardiac activities. Rather, miR-132, apart from its neural and immune role, is also involved in heart functioning, along

Table 1. *miR-1, miR-132 and miR-206 presence and influence in cardiac, metabolic and muscular system*

Organism	Function affected	Body system	miRNA
Drosophila, Mouse, Rats	Mediates cell cycle arrest and differentiation of cardiomyocyte during chamber development *(75)* Promotes electrical and contractile heart irregularities *(76,39,40,41)*	Heart	miR-1
Xenopus laevis, Human (*in vitro*), *C. elegans*	Promotes myogenesis during development *(28)* Downregulates muscle sensitivity to and pre-synaptic release of ACh *(26)*	Muscle	
Human (*in vitro*)	Downregulation PPP-dependent NADPH production and ribose synthesis *(77)*	Metabolism	
Mouse	Upregulation in cardiomyocytes promoting hypertrophy and heart failure *(48)*	Heart	miR-132
Mouse	Downregulation AChE levels *(58)*	Muscle	
Human	Promotes inflammation in visceral adipose tissue *(78,79,80,81)*	Metabolism	
Drosophila	Promotes cardiomyocyte hypertrophy *(49)*	Heart	miR-206
Mouse	Promotes muscle cell re-innervation *(32,33)*	Muscle	
Human (*in vitro*)	Downregulation PPP-dependent NADPH production and ribose synthesis, Downregulates gluco-kinase activity *(15,73)*	Metabolism	

with its tandem miR-212. Mir-132 is highly expressed in rat hearts and aortic wall following hypertension and cardiac hypertrophy *(46)*. In addition, miR-132 and miR-212 levels are upregulated by hypertrophic stimuli in mice and are both necessary and sufficient to drive the hypertrophic growth of cardiomyocytes *(47)*. Consequently, miR-212/132^{KO} mice that lack these two important miRs are protected from pressure-overload-induced heart failure, whereas miR-212/132 overexpression in cardiomyocytes leads to pathological cardiac hypertrophy and severe heart failure and death in mice *(47)*. In *Drosophila* as well, miR-206 is involved in cardiac hypertrophy through its YAP target *(48)*, although little is known concerning this miR's role in the fly's cardiac function. To conclude, miR-1, miR-132 and miR-206 are all important for correct heart development and their dysregulated levels may cause pathological cardiac conditions.

CHOLINOMIRS AT THE NMJ: ALS AND ORGANOPHOSPHATE POISONING AS MODELS

The NMJ is a specialized synapse, enabling communication between the nervous system and skeletal muscles; and it makes use of the chemical transmitter ACh for relaying electrical impulses. Notably, ACh signaling in the NMJ can be regulated by CholinomiRs at multiple stages *(49)*. In *C. elegans*, miR-1 regulates the expression of the UNC-29 and UNC-63 nAChR subunits as well as the muscle transcription factor MEF-2, such that its levels regulate presynaptic ACh secretion *(26)*. In mammals, miR-1 provides a surveillance over epigenetic processes in the NMJ by targeting the histone deacetylase HDAC4 *(29,30)*. Intriguingly, HDAC4 also operates as a mediator of long-lasting stress-inducible changes in AChE's promoter choices in the hippocampus *(50)*. This histone modifier also regulates nAChR expression following skeletal muscle denervation *(51)*, making miR-1 a global upstream regulator of ACh signaling. HDAC4 is also elevated in the skeletal muscle of patients with the motor-neuron disease ALS *(52)*. Predictably, miR-206, having an identical seed sequence to miR-1, also regulates HDAC4 levels *(33)*. Multiple studies have provided evidence for the important role of miR-206 in muscle regeneration and protection from muscular atrophy *(53)*. In a mouse model for ALS, miR-206^{KO} mutants showed faster progression of the disease, most likely due to delayed muscle re-innervation compared to mice with the functioning miR-206 allele *(33)*. At a later stage of ACh signaling, where ACh degradation terminates such signals, miR-132 emerged as direct regulator of AChE levels *(34)*.

Organophosphate poisoning, most commonly caused by exposure to insecticides or nerve agents, interrupts ACh signaling at all of the above systems and more. Such poisoning causes drastic hyper-cholinergic stimuli by instantaneously arresting ACh degradation by AChE, and one outcome of such poisoning involves rapid changes in AChE gene expression *(54)*. In civil terms, agricultural use of organophosphate insecticides is very common, with an estimate of a hundred thousand fatalities annually and many severe symptoms, including cognitive and cardiac ones among survivors *(55,56)*. The organophosphates inactivate AChE by phosphorylating the serine hydroxyl residue on the enzyme *(57)*. This leads to ACh accumulation at the NMJ causing hyper-cholinergic stimulation. Moreover, mice exposed to miR-132 antisense molecules prior to organophosphate poisoning sustain higher AChE levels and show a higher survival and recovery rate compared to naïve animals *(58)*. Collectively, this evidence suggests causal involvement of CholinomiRs in both normal neuromuscular development as well as pathological states such as ALS and following organophosphate poisoning.

The main organophosphate-induced damage is done at the NMJ, where AChE inactivation causes ACh accumulation and overstimulation followed by blocking of ACh signaling *(59)*. Cardiac irregularities, along with a wide range of other symptoms, occur frequently following organophosphate poisoning; and arrhythmias can often be the cause of death *(60,61,62)*. Both miR-132 and miR-1 overexpression contribute to cardiac irregularities *(39,40,41,46,47)* and have roles in neuromuscular desensitization *(26,58)*. Therefore, miR-1 and miR-132 may modulate both the cardiac and the neuromuscular symptoms observed following organophosphate poisoning. Such a mechanism might provide a possible explanation for the very rapid and widespread symptoms following exposure to organophosphates, concordant with the rapid and efficient miR biogenesis. That miR-1 regulates retrograde signaling by targeting MEF-2, decreasing presynaptic ACh release is compatible with that prediction. It is tempting to speculate that miR-1 levels rise, which decreases presynaptic ACh release following organophosphate poisoning, in an attempt to compensate for the synaptic over-activation following AChE inhibition. Given that miR-132 downregulation increases survival and recuperation after exposure compared to naïve animals *(58)*, it might be interesting to see if miR-1 downregulation provides the same results and if such treatment exerts any changes at the cardiac level.

MIR-206 AS A BRIDGE BETWEEN ALS-INDUCED NMJ DEGENERATION AND METABOLIC CHANGES

Despite technological challenges, miRs are rapidly rising as possible biomarkers and therapeutic targets for numerous pathological states *(56, 61)*. In a study aiming to find biomarkers for ALS, researchers compared miR alterations from skeletal muscle and plasma in the ALS mouse model to the levels of the affected miRs in the serum from human ALS patients *(64)*. ALS is notably characterized by cycles of denervation and subsequent re-innervation. Correspondingly, elevated levels of miR-206, involved in muscle re-innervation, emerged as a promising biomarker for ALS. It is thus possible that the initial denervation in NMJ's induces increases in miR-206 levels which promotes re-innervation.

Aside from motor neuron degeneration, ALS is associated with several defects in energy metabolism *(65,66, 67,68)*, and a better metabolism correlates with longer survival of ALS patients *(69,70,71)*. Alongside other metabolic features, augmenting glucose intolerance is considered a sign of deteriorating and dysregulated metabolic homeostasis progressing along with the disease *(66,72)*. Interestingly, recent research has identified miR-206's involvement in the downregulation of enzymes involved in metabolism. Thus, miR-206^{KO} mutant mice show increased glucose tolerance and potentiated transcription of glucose metabolism-related enzymes *(73)*. This suggests that over-expression of miR-206, as can be observed in ALS patients and the corresponding mouse models, exerts detrimental effects on glucose tolerance. It is tempting to suggest that the attempt for NMJ re-innervation involves increasing miR-206 levels, but damages metabolic function, in turn leading to more NMJ degeneration. According to this prediction, miR-206 might operate both as a slowing and as an accelerating factor for ALS symptoms.

CONCLUDING REMARKS

The apparent and predicted involvement of miRs in modulating the effects of ALS and the NMJ, as well as cardiac and metabolic symptoms following organophosphate poisoning might provide new ways to detect and treat such pathological states. The involvement of miRs might also explain the differences between people in the severity and progression of the pathologies, as single nucleotide polymorphisms (SNPs) in the miR target genes might lead to differential downregulation of the miR targets. This is corroborated by the elevated AChE levels in carriers of a miR-608 disrupting SNP in the AChE gene *(74)* as well as by the differences in miR-206 and HDAC4 expression between patients with rapidly decaying and slowly progressing ALS *(33)*. Although there was a significant difference between ALS patients and control subjects in miR-206 expression, no difference was measured in miR-206 levels between patients with rapidly progressing ALS and long surviving ALS patients. The levels of the miR-206-targeted HDAC4, on the other hand, were significantly higher in rapidly progressive ALS, suggesting impaired binding of miR-206 to its target, possibly due to a yet undefined SNP or other, further removed molecular differences. In conclusion, further research concerning CholinomiRs involvement in these pathological states, as well as in normal development is required and could provide us with better targeted therapeutics.

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