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Overview of microRNA modulation in analgesic research

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Abstract

MicroRNA (miRNA) mediated gene regulation underlies cellular processes and play an important role in the cellular homeostasis and diseases. miRNAs are responsive to pharmacological interventions and miRNA expression is altered by various pharmacological agents. Differences in the endogenous levels of miRNAs can influence drug efficacy and toxicity. Thus, miRNA levels can be potential biomarkers for predicting treatment response, efficacy and toxicity. In addition, elucidating the mechanistic significance of miRNA alterations can aid in the identification of therapeutic targets, patient stratification and personalizing therapy. In this overview, we provide basic information about the properties of miRNA, their modulation and their measurement. We specifically describe examples of miRNA changes induced by different classes of analgesic drugs including opioid, non-opioid and the intermediary ketamine classes and how studying miRNA levels in patients requiring and receiving analgesic therapy can help achieve maximum and safe therapeutic benefit.

Keywords

miRNA; analgesic drugs; pain; drug metabolism; biomarker

Introduction

MicroRNAs (miRNAs) are short non-coding single-stranded RNA molecules that potentiate the epigenetic regulation of a significant proportion of the eukaryotic genome. miRNAs modulate gene expression by binding to the target mRNA predominantly in the 3' untranslated region (3' UTR). Seed sequence complementarity of ~7 base pairs enables miRNA to bind to the target mRNAs resulting in the inhibition of translation or reduction in the stability of the mRNA, both of which can result in decreased expression of the target protein. Numerous studies suggest the involvement of miRNAs in key biological processes including development and cellular homeostasis, and their altered expression is associated with various pathological conditions (Gorski, Vogel, & Doudna, 2017).

The presence of stable miRNAs in bodily fluids and the noninvasive nature of assessing them have resulted in many studies exploring their utility as sensitive and specific

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Conflicts of Interest

The authors declare that they have no competing interests.

biomarkers (De Guire et al., 2013). Drug treatments alter miRNAs in humans and in various animal models. Thus, miRNAs can be predictive biomarkers for therapeutic intervention as well as prognostic markers for treatment response. This overview discusses in brief the basic concepts of the regulatory role of miRNA, the aspects governing their pharmacological modulation during drug responses, and a guide to predicting, assessing and validating molecular interactions. The responses to different classes of analgesic drugs of the opioid, non-opioid and the intermediary ketamine classes are outlined here.

miRNA biogenesis and mechanism of action

miRNAs comprise a group of short (21–23 nucleotides) non-coding single-stranded RNA molecules and play a central role in cellular homeostasis, regulating the expression of more than 60% of the genes at the post transcriptional level. Majority of miRNAs originate from independent loci in the genome but some are processed from within the introns and exons too. The biogenesis of a miRNA is initiated in the nucleus giving rise to a long (2–4 kb) primary transcript known as pri-miRNA. The pri-miRNA is then processed into a precursor miRNA (pre-miRNA) by a microprocessor complex called Drosha. Transnuclear transport proteins like RanGTPase and exportin 5 facilitate the translocation of this pre-miRNA to the cytoplasm where it is cleaved into an imperfectly paired, double stranded miRNA-miRNA* duplex by the RNase III endonuclease, Dicer. One of the complementary strands of the duplex, known as the guide-strand is incorporated into the RNA-induced silencing complex (RISC), while the other strand is degraded. The mature miRNA is loaded into the Argonaute (Ago) protein within the RISC and this enables the presentation of the miRNA seed sequence in a pre-organized A-form helix. The helical form of the 5' seed (nucleotides 2–7) and the conformational change in the Ago protein facilitates the search for the target sequence complementarity. Base-pairing between miRNA and target mRNA can lead to translational repression and when there is high complementarity, to mRNA cleavage (Gorski et al., 2017) (Figure 1). miRNAs are also present in circulation, often within extracellular vesicles or as RNase resistant complexes in association with Ago or lipoproteins. The composition of circulating miRNAs can differ in disease states and has generated tremendous interest both from a biomarker perspective and for their therapeutic potential. Uptake of circulating miRNAs transported by extracellular vesicles can regulate gene expression in recipient cells thereby modulating distant signaling. Thus both cellular and circulating miRNAs play an important role in gene regulation (El Andaloussi, Mager, Breakefield, & Wood, 2013).

miRNA nomenclature, profiling and data analysis

miRNAs are named with the prefix “mir” followed by a dash and a number. Thus in hsa-miR-132, “hsa” represents the species (“hsa” is for Homo sapiens, “mmu” for Mus musculus, and “rno” for Rattus norvegicus). This is followed by “miR” (with capital “R”) for mature miRNA or “mir” (with small “r”) for pri-miRNA, pre-miRNA, or genomic locus. miRNAs with nearly identical sequences are annotated with an additional lower case letter as in hsa-miR-34a and hsa-miR-34b. Though transcribed from different regions in the genome, some pre-miRNAs generate identical mature miRNAs and are indicated with an additional dash-number suffix as in hsa-mir-194-1 and hsa-mir-194-2.

Any modulation of miRNAs is assessed by observing changes in expression profiles of miRNAs determined by microarray analysis, deep sequencing or real-time quantitative PCR (qPCR). The two commonly used formats of miRNA profiling offered by Applied Biosystems are the Open Array platform, a high-throughput format of a 3072-well microfluidic card with up to 758 miRNAs and controls, and the Taqman low density array (TLDA) microfluidic cards used in two sets of 384-well card medium-throughput format. Additional platforms for determining miRNA levels are available from other vendors including Qiagen (Germantown, MD), Exiqon (Woburn, MA), and nCounter analysis system (Nanostring Technologies, Seattle, WA). Although these approaches provide comprehensive coverage of known miRNAs with high specificity and sensitivity, unlike sequencing, these methods cannot identify novel miRNAs.

Data normalization is crucial to account for variability; the mean or quantile methods in addition to the recommended endogenous control miRNAs are commonly used (Schwarzenbach, da Silva, Calin, & Pantel, 2015). Ct (threshold cycle) is the readout from a qPCR and it is a relative measure of the concentration of target in the PCR reaction. Ct is the number of cycles required for the fluorescent signal to cross the threshold (exceeds background level). Ct values are inversely proportional to the amount of target nucleic acid in the sample, (lower the Ct level the greater the amount of target miRNA in the sample). For calculating the mean normalization, the average (Ct₀) of all miRNAs is subtracted from each Ct value. Quantile normalization uses the ranking of the raw Ct expression values on each plate. Vendors usually recommend endogenous control miRNAs. Alternatively, one can identify endogenous controls appropriate for the current study. Standard deviation of the Ct values across all samples can be used as the stability measure for each miRNA and the most stable miRNAs are then selected as endogenous controls. The delta Ct (Ct - Ct_{reference}) values of the target miRNAs is calculated by subtracting the reference value from the target (Ct target - Ct reference where Ct reference is the endogenous control). Statistical significance of differential expression can be determined for Ct values. Relative expression of target miRNAs is determined using the 2^{-ΔCt} method, followed by hierarchical clustering of the log-transformed fold changes for identifying significantly altered miRNAs compared to control. The critical parameters contributing to the variability in miRNA assessment from bodily fluids have been discussed in depth before (Moldovan et al., 2014).

Prediction and validation of miRNA targets

The human genome is known to currently generate 2588 mature miRNAs (www.mirbase.org). Once the miRNAs of interest are identified, online databases listing bioinformatics prediction of potential target genes (M. M. Akhtar, Micolucci, Islam, Olivieri, & Procopio, 2016) and functional target validation can be pursued (Steinkraus, Toegel, & Fulga, 2016). The biological roles of miRNAs vary between cell types, developmental stages, or in response to external stimuli and disease states. Elucidating the function of miRNAs require combinatorial approaches altering endogenous miRNA levels or activity, and disrupting functional miRNA–target interactions *in vivo*. The seed sequence guides the miRNA to its target mRNA and because it is relatively short, a single miRNA can target many genes and each mRNA can harbor binding sites for multiple miRNAs. Although there are several tools employing distinct parameters and strategies to identify putative

targets, these *in silico* miRNA target predictions require experimental validation. Target validation is initiated by assessing the binding of miRNA to the 3'UTR of the target mRNA and the ability of miRNA to downregulate protein translation or degradation of mRNA or both (See Protocol by Shenoda et al for details). A preliminary assay involves the 3'UTR of the target mRNA cloned downstream of the luciferase gene in a reporter construct expressed in a cell line. A decrease in substrate luminescence thus indicates the binding of miRNA to the 3'UTR leading to lower luciferase protein expression (Shenoda, Ramanathan & Ajit, 2017). After the confirmation of miRNA binding to target mRNA, additional *in vitro* validation studies performed typically involve the transfection of miRNA into cells with endogenous expression of target mRNA and protein. Overexpression of miRNA using mimics or downregulation with antagomirs (miRNA inhibitors), are also commonly used to further validate the miRNA-mRNA interaction. Genetic gain or loss of function studies offer a true physiological *in vivo* validation. Lack of phenotype in loss-of-function studies has been a challenge in the miRNA field and several factors contribute to this. miRNAs are fine tuners of gene regulation; in addition, there are multiple target binding sites within a gene, and often there are several miRNA family members with the same seed sequence. All of these factors can contribute to compensatory mechanisms, easily overriding any functional perturbations resulting from loss or inhibition of an individual miRNA. The influence of miRNAs functioning as a rheostat under a normal or homeostatic state may often be modest but the functions of miRNAs are more pronounced under stress or disease states. Thus focusing investigations on miRNAs altered in disease states provides a strong rationale for further mechanistic studies.

Modulation of miRNAs in pain and analgesia

miRNAs regulate a complex network of molecular processes and many of these processes are involved in mediating, regulating or attenuating drug effects (M.-P. Li et al., 2016; Rupaimoole & Slack, 2017). miRNA can act as an intermediate between the drug and the physiological systems. For example, drug modulation of miRNA may regulate downstream effectors responsible for the therapeutic effects of the drug. Several studies have examined alterations in miRNA expression in tissue samples collected from rodent models of inflammatory and neuropathic pain models and suggest a role for miRNAs in mediating pain (McDonald & Ajit, 2015). It has been found that miRNAs target key regulators of pain processing such as mu opioid receptors (Hwang, Wagley, Law, Wei, & Loh, 2012), γ -aminobutyric acid- α 1 (GABA $_{\alpha 1}$) (Sengupta et al., 2013), cyclooxygenase 2 (N. Akhtar & Haqqi, 2012), TRPV1 (X. Li et al., 2011) and multiple Na⁺ and Ca²⁺ channels (Favereaux et al., 2011; von Schack et al., 2011). Moreover, modulating several miRNAs are useful in attenuating or preventing pain (Huang et al., 2016; McDonald & Ajit, 2015). miRNA expression profiles are altered in several pain related disorders including complex regional pain syndrome (CRPS) (Orlova et al., 2011), fibromyalgia (Bjersing, Lundborg, Bokarewa, & Mannerkorpi, 2013), migraine (Andersen, Duroux, & Gazerani, 2016; Tafuri et al., 2015), osteoarthritis (Beyer et al., 2015) and rheumatoid arthritis (Pauley et al., 2008). Circulating miRNA levels are altered by pharmacological treatments (Cheng et al., 2013) including analgesic therapies (Douglas et al., 2015) indicating a potential role for miRNA in mediating drug effects.

Role of miRNAs in regulating pharmacodynamics of analgesic drugs

Opioids—Opioids, one of the most potent classes of analgesics, have been used for decades to treat chronic pain, while exhibiting the highest substance abuse liability. Opioids exert their action by binding to opioid receptors of the mu-(MOR), delta-(DOR) and kappa-(KOR) classes. The relationship between opioids and miRNA is rather complex (Hwang et al., 2012). An altered expression of several miRNAs was observed in central nervous system (CNS) regions where opioid activity occurs (Dave & Khalili, 2010; He, Yang, Kirkmire, & Wang, 2010). Many of the altered miRNAs are involved in opioid receptor regulation (Qifang Wu et al., 2013). MOR expression is regulated by the miRNA let-7 through binding to its 3' UTR. The let-7 (let-7) gene was first discovered in the nematode as a key developmental regulator and is highly conserved across species. Morphine upregulates let-7 expression in SH-SY5Y cells and in a mouse model of opioid tolerance, thereby mediating opioid tolerance via downregulation of MOR. Inhibition of let-7 attenuated opioid tolerance in mice (He et al., 2010).

miR-23b is another *trans*-acting factor that represses MOR translation efficiency. Long-term morphine treatment increased miR-23b expression in a dose- and time-dependent manner (Wu, Zhang, Law, Wei, & Loh, 2009). Another miRNA that targets MOR is miR-339-3p. Treatment of mice with opioid agonists (fentanyl or morphine) increased the levels of miR-339-3p, which was shown to bind to the 3' UTR of the MOR transcript, thereby regulating protein levels. This specific effect was blocked by the opioid antagonist naloxone suggesting that miR-339-3p may serve as a negative feedback modulator of MOR signals by regulating intracellular MOR biosynthesis (Qifang Wu et al., 2013). Thus, altering the levels of specific miRNAs in certain pain conditions can potentially affect opioid activity by targeting MOR.

Many of the miRNAs dysregulated in pain can potentially alter the opioid system. For example, OPRM1 gene which encodes for MOR is a predicted (not experimentally validated) target of miR-34a and miR-34c, two miRNAs belonging to miR-34 family. miR-34a was downregulated in CRPS patients resistant to ketamine therapy (Douglas et al., 2015). In investigating the mechanistic significance of this change, it was shown that miR-34a is a negative regulator of corticotrophin releasing hormone receptor 1 (CRHR1). The biological effects of Corticotropin-releasing hormone (CRH) are mediated through its receptor CRHR1 which is a key regulator of pro-opiomelanocortin (POMC). POMC is a precursor polypeptide that is cleaved posttranslationally to generate a number of bioactive peptides including β -endorphin. Authors conclude that miR-34a, by targeting and reducing the expression of CRHR1, can impair the CRH/CRHR1/POMC axis (Shenoda, Alexander, & Ajit, 2016).

Non-opioid analgesics—Celecoxib is a selective cyclooxygenase 2 (COX2) inhibitor used to treat inflammatory pain. Induction of COX2 largely accounts for the high prostanoid production at the site of inflammation (Chen, Yang, & Grosser, 2012). Celecoxib administration for 7 days in a complete Freund's adjuvant (CFA) model of inflammatory pain showed that several miRNAs dysregulated by CFA were restored to baseline levels after drug administration (Qureshi et al., 2015). Celecoxib was able to reverse the CFA-induced

downregulation of miR-211, miR-342-5p, and miR-411 suggesting a role for these miRNAs in regulating inflammation (Qureshi et al., 2015). These studies suggest that reversal of miRNA expression to physiological levels may be important for the therapeutic effect of celecoxib.

Ketamine, a commonly used anesthetic, is an N-methyl D-aspartate (NMDA) receptor antagonist and has recently been employed in treatment of depression. Though a noncompetitive antagonist of the NMDA receptor, ketamine has affinities for other receptors and transporters. A recent study showed that the rapid antidepressant actions of ketamine are independent of NMDAR inhibition but involve early and sustained activation of AMPARs (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors) (Zanos et al., 2016). Subanesthetic doses of ketamine administered intravenously produce analgesia, an effect largely attributed to NMDA receptor antagonism. Although debated for its potential abuse and side effects, intravenous administration of ketamine over several days with antipsychotics and antihallucinogenics, has been shown to provide relief to CRPS sufferers, especially in patients for whom all other treatments have failed. Although ketamine can reduce pain, approximately a third of the patients do not respond to ketamine therapy (Niesters, Martini, & Dahan, 2014).

In a recent proof-of principle study, intravenous ketamine treatment induced circulating miRNA alterations in blood from patients with CRPS and demonstrated differential expression of 14 miRNAs in good and poor responders before and after ketamine treatment, indicating miRNA profile can be predictive of treatment response (Douglas et al., 2015). The 14 miRNAs were different in the two groups. This indicates that ketamine elicited different responses in the two groups suggesting that the commonality in disease symptoms does not necessarily indicate dysregulation of the same molecular pathways. Investigating the genes targeted by these differentially regulated miRNAs can be beneficial in obtaining insights into previously unexplored mediators in CRPS.

Downregulation of miR-206 in the hippocampus of ketamine-treated rats was hypothesized to be involved in ketamine mediated anti-depressant effect by targeting BDNF (X. Yang et al., 2014). High doses of ketamine negatively impact learning and memory functions via neurodegeneration, effects thought to be mediated via downregulation of miR-214 with subsequent upregulation of PTEN protein leading to neuronal apoptosis (J. J. Wang et al., 2014). Similar observations were reported in mice where elevated levels of mir-34a and mir-124 correlated with anesthesia-induced neurotoxicity through reduction of their targets fibroblast growth factor 1 receptor (FGF1R) and AMPA receptor respectively, leading to apoptosis of hippocampal neurons (Xu et al., 2015). Downregulation of these miRNAs improved survival, learning and behavioral performance in mice. Ketamine is also used as an anesthetic; miRNA expression changes after exposure to volatile anesthetics including propofol and ketamine was reviewed recently (Neudecker, Brodsky, Kreth, Ginde, & Eltzschig, 2016).

Involvement of miRNAs in analgesic toxicity—Overdose of acetaminophen, one of the most widely used analgesic, results in liver injury (Amar & Schiff, 2007). Sulfation and glucuronidation pathways involved in metabolizing acetaminophen become saturated during

acute overdose, and excess drug is then processed via cytochrome P450 enzymes to the toxic metabolite, N-acetyl-p-benzoquinone imine (NAPQI). Although NAPQI can be neutralized by intracellular glutathione (GSH), during overdose cellular levels of GSH is insufficient to eliminate the toxic metabolite, resulting in hepatotoxicity. Although administration of N-acetylcysteine following early detection within 6–8 h of overdose is crucial, hepatotoxic effects are seen 24 h later, and necessitate early diagnostic biomarker detection. Another study showed 44 altered miRNAs in the plasma of a mice subjected to acetaminophen overdose (K. Wang et al., 2009). Higher levels of mir-122 and mir-192 were present in the plasma of acetaminophen overdosed mice, while these were decreased in liver tissue. On the other hand, mir-710 and mir-711 were elevated in liver tissues but were lower in plasma samples. Similar acetaminophen toxicity studies carried out in patients showed alterations in 11 miRNAs including mir-122, that differed between plasma of acetaminophen overdosed patients and a similar but an unrelated condition of ischemic hepatitis (Ward et al., 2014). mir-122, among eight other miRNAs elevated in serum of children with acetaminophen toxicity (Xi Yang et al., 2015) was the most characteristic miRNA biomarker, in all the above studies, and is coincidentally a liver specific miRNA accounting for 70% of liver miRNAs. Additionally, in all the studies, changes in miRNA expression were detected as early as 3 h following drug administration and was much more robust in the diagnosis of hepatotoxicity compared to the standard protein biomarker, ALT (alanine transferase) enzyme detection.

Modulation of analgesic efficacy and toxicity by miRNAs—miRNAs regulate the expression of drug-metabolizing enzymes, transporters, and nuclear receptors, all of which can contribute to inter-individual differences in drug response (He, Chevillet, Liu, Kim, & Wang, 2015). Variations in miRNA expression can influence miRNA-mRNA interactions. Elucidating how these miRNAs can affect drug metabolism and efficacy can provide new insight on differences in treatment response. This approach was used to determine the mechanistic basis of two differentially expressed circulating miRNAs, miR-548d (Douglas et al., 2015) and miR-34a in CRPS (Shenoda et al., 2016). Ketamine metabolism is a complex process in which multiple active metabolites are generated and proposed to contribute, at least partially, to ketamine activity. Most ketamine metabolites are inactivated and eliminated through glucuronidation, specifically by UDP glucuronosyl transferases (UGT). miR-548d negatively regulates UGT1A1 *in vitro* in hepatic cells. Based on this finding, the downregulation of miR-548d-5p in poor responders to ketamine was postulated to influence the expression of its target mRNA UGT1A1. Thus, a higher UGT1A1 activity can lead to higher levels of inactive glucuronide conjugates and thereby can minimize the therapeutic efficacy of ketamine in poor responders (Douglas et al., 2015). In another study with an overlapping set of patients, lower pre-treatment levels of miR-34a was linked to a poor ketamine response (Shenoda et al., 2016). Thus, biomarker discovery can be extended towards mechanistic studies to provide molecular insights distinguishing responders from non-responders prior to treatment. It is crucial that these findings be confirmed in larger patient cohorts and future studies can be focused on the most relevant miRNAs identified.

Conclusions

The pharmacology of analgesics has been studied for decades and the relationship between the drugs and their metabolic pathways are well established. Although advances in miRNA biology have been more recent, the importance of miRNAs is unprecedented owing to their critical biomarker utility, which was unattainable by traditional diagnostic markers. While drugs that produce active metabolites structurally identical to other opioids can complicate efforts to monitor patients for abuse, others, like acetaminophen that can lead to overdose toxicity, pose diagnostic biomarker detection challenges for early therapeutic intervention. These can be circumvented by relying on circulating miRNAs that may be characteristic of pathological conditions, which regulate key enzymes of the drug metabolic pathways or receptor expression. In addition, a deeper understanding of the miRNA pharmacology could also assist in determining treatment. A patient's miRNA signature may differ even prior to treatment initiation and changes in signature may correlate with treatment outcome. Identifying the potential target genes for individual miRNAs could provide insights into the molecular underpinnings of different pathologies and may help identify novel targets. Thus, assessing miRNA changes before and after treatment will provide insight into miRNA signatures and its alteration in good responders, non-treatment (placebo) responders and poor responders. Samples from both unsuccessful and successful clinical trials can provide meaningful insights and guidance for future and ongoing trials.

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Figure 1.
Schematic representation of miRNA biogenesis.