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Sex differences in miRNA as therapies for ischemic stroke

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ABSTRACT

MicroRNAs, a subset of non-coding RNAs, are present in virtually all tissues including body fluids and are global regulators of the transcriptome. In view of the expanding number of microRNAs and the large number of gene targets that each microRNA can potentially regulate, they have been compared to hormones in the scope of their effects. MicroRNA have been implicated as biomarkers for several diseases including stroke, as well as chronic conditions that are associated with stroke. Recent research has focused on manipulating miRNA to improve stroke outcomes. Although several miRNAs have been shown to have neuroprotective properties, the overwhelming majority of these studies have employed only male animals. This review will focus on two miRNAs, Let7f and mir363–3p, whose effectiveness as a stroke neuroprotectant is sex-specific.

1. Overview

Stroke is considered a global epidemic, with 15 million projected to suffer a stroke every year and ranked as the second most common cause of mortality worldwide. and the third most common cause of disability-adjusted life years (DALY) (Feigin et al., 2017). The demographics of a stroke-affected individual varies from nation to nation, such that in developing countries stroke occurs among younger populations as compared to developed nations. Almost 20% of first time strokes in India occur in people under 40 years of age (Pandian et al., 2005), while in the US, stroke incidence is most prevalent among older people (65 + years) (Statistics and Research, 2011). Among this elderly demographic, biological sex is a principal intervening variable that contributes to stroke risk. Women are more likely to get a stroke (Petrea et al., 2009), to display more non-classical stroke symptoms, and to have worse stroke outcomes. Women account for 60% of stroke-related deaths (Lloyd-Jones et al., 2010), and while stroke-related death rates have declined over the last 25 years for men, they have not for women (Roger et al., 2011). The 5-year stroke recurrence is also disproportionately higher in females (20%) as compared to males (10%) in the 45–64 age range (Roger et al., 2011). Stroke-related disability is worse in women as evidenced by a 10% discharge of women patients to long-term care as compared to 5% of men, based on a Canadian stroke registry (Kapral et al., 2005). Furthermore, since women live longer than men, it is projected that stroke-related disability and institutionalization will affect women more than men (Lai et al., 2005).

Due to the wide prevalence of stroke worldwide, new stroke therapies are urgently needed to reduce mortality and improve quality of life. A staggering amount of preclinical research has focused on stroke neuroprotectants, including 7000 + publications (NCBI resources, Pubmed, 09/10/2018), but most of these have not successfully translated to the bedside. One explanation that is frequently cited for this failure is the relative lack of validity between the basic science animal models and the clinical stroke population. This discordance formed the basis of the Stroke Therapy Academic Industry Roundtable (STAIR) group, which was convened to make recommendations to improve the quality of preclinical studies of acute stroke therapies. The STAIR recommendations enumerate several ways in which basic science studies could have more translational validity, such as studies including both males and females, young and aging animals, animals with co-morbidities such as type 2 diabetes, hypertension, among others (Fisher et al., 2009). Additionally, STAIR recommendations also propose the use of more than one evaluation for a neuroprotectant (behavior and histology) (Trueman et al., 2017). Thus, although aged women have a higher risk for stroke, worse outcomes, and poorer recovery after ischemia compared to aged men, preclinical studies have largely failed to utilize aging females in these studies. Consequently, clinical trials, based on promising products in preclinical studies, where inclusion of both sexes is required by law have floundered or proven inconclusive.

The search for neuroprotectants is further complicated by evidence that drugs may be effective in one sex but not the other. A historical case in this context is the research and eventual clinical trials of the compound called tirilazad mesylate. A member of a group dubbed

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'Lazaroids' (reviewed in (Cahill and Hall, 2017), due to their remarkable anti-oxidant and neuroprotective properties for TBI and subarachnoid hemorrhage (SAH), tirilazad was tested in several clinical trials but was eventually not approved by the FDA. The first trial found that patients receiving 6 mg/kg/day of tirilazad had reduced mortality and showed better scores on the Glasgow Outcome Scale as compared to the placebo group (Kassell et al., 1996), however it was noted that the beneficial effects of the drug were exclusively restricted to male patients. Subsequent trials with this drug were never designed for a sex-specific analysis, failed to show significant effects and a promising drug was permanently tabled.

Sex-specific actions of stroke neuroprotectants have been more systematically studied in preclinical models. An example of this is the tetracycline antibiotic, minocycline. Minocycline is clinically well-tolerated and is neuroprotective for ischemic stroke in experimental models and clinical trials (Yrjanheikki et al., 1999; Lampl et al., 2007; Li and McCullough, 2009). However, in C57BL/6 male and female mice subjected to middle cerebral artery occlusion (MCAo), minocycline was effective in reducing infarct volume only in male mice. Furthermore, minocycline was also ineffective in ovariectomized female mice even though male and ovariectomized female mice present similar levels of estrogen (Li and McCullough, 2009). An open-label evaluator-blinded clinical study of minocycline found that oral minocycline, 200 mg daily for 5 days, found a similar effect (Amiri-Nikpour et al., 2015). Male patients had significantly lower NIH stroke scale (NIHSS) scores in the minocycline-treated group compared with controls, while no significant clinical improvement was found between treatment and controls in female patients (Amiri-Nikpour et al., 2015).

This review will focus on sex differences in the effectiveness of microRNAs, a new class of neuroprotective molecules.

2. MicroRNAs

MicroRNAs (miRNAs) are 18–25 nucleotide-length, non-coding RNA molecules that function as master regulatory molecules by controlling large gene networks, either by de-stabilizing mRNA transcripts (Denli and Hannon, 2003), interfering with their translation into proteins (Ambros, 2001), or controlling the methylation state of target genes (Lim et al., 2005). Though relatively few in number, miRNAs are predicted to control a large proportion of the tissue- and cell-specific transcriptome (Krek et al., 2005; Lim et al., 2005) regulating important biological processes including mitosis, tissue-specific cellular differentiation, and cell death (Croce and Calin, 2005), maintaining the pluripotent state of embryonic stem cells (Houbaviy et al., 2003), delaying neuronal maturation (Krichevsky et al., 2003), or promoting neuronal differentiation (Conaco et al., 2006). Montano and Long (2011) have proposed that RNA surveillance by regulatory molecules such as miRNA influence life span and longevity. MiRNAs can occur within exons, introns and polycistronic clusters in the genome, and are usually transcribed as primary transcripts (pri-miRNA) by RNA polymerase II and are processed into a mature form in the cytoplasm. Of relevance to this review is the emerging view that aberrant regulation of miRNAs may play a significant role in diseases such as neurologic disorders (Nelson et al., 2008), inflammation (Rippo et al., 2014) and cancer (Perwez Hussain and Harris, 2007).

MiRNA profiles are altered with stroke in both human (Jickling et al., 2014a, 2014b) and experimental (Jeyaseelan et al., 2008; Dharap et al., 2009, 2011; Sepramaniam et al., 2014) models. Additionally, specific miRNA are associated with pathogenic processes that initiate or exacerbate stroke, such as hyperlipidemia (miR33), hypertension (miR155), atherosclerosis (miR21, miR126), plaque rupture (miR222) (reviewed in (Rink and Khanna, 2011)) and endothelial cell (EC) loss and blood-brain barrier (BBB) breakdown (miR15a) (Yin et al., 2010). MiR17 is reported to be significantly elevated in acute stroke patients as compared to controls (patients with vascular risk but no stroke) (Kim et al., 2015). Plasma levels of miR210 are lowered in ischemic stroke patients as compared to controls, and stroke outcomes are better in patients where miR210 levels are higher (Zeng et al., 2011). A recent study evaluated the association of microRNA polymorphisms with the risk of ischemic stroke in a Chinese population. miR146a/rs2910164 C/G genotypes were significantly associated with increased risk of ischemic stroke, and the association was more pronounced in subjects over 60 years old, females, non-drinkers, and subjects without hypertension or diabetes mellitus (Liu et al., 2014).

MicroRNAs as stroke neuroprotectants: Manipulating levels of microRNAs using either mimic sequences or inhibitory sequences (antagomirs) have been shown to mediate neuroprotection in stroke models. A Pubmed database search using the following terms: 'microRNA and stroke', 'microRNA and MCAo', 'microRNA and ischemia and infarct' yielded 241 articles. Publications that focused on microRNA profiling were excluded. Only those publications which reported a direct effect on stroke outcomes after treatment with a miRNA, either by injection of oligonucleotides, or transfections, were included. Thus, we also excluded 'association' type studies where a drug treatment was shown to regulate a specific miRNA while also improving (or worsening) stroke outcomes, unless treatment with that miRNA was also shown to alter stroke outcomes. Additionally, only those studies were included where the miRNA treatment was conducted in vivo. These criteria resulted in a curated list of 97 publications (Supplementary Table 1), which was manually checked to determine if the study used males, females or both. The vast majority (93/97) used males only; the remaining 4 studies used both sexes. Of these 4, one study used both sexes, but the data was not analyzed separately for each sex (Piao et al., 2018) and will not be discussed here. Another group examined the effect of miR181b inhibition on stroke outcomes in females (Stary et al., 2017), which built on their previous study where they reported the effects of this miRNA in males (Xu et al., 2015). Inhibition of this microRNA was neuroprotective in both sexes. Moreover, there was an interaction of miR-181b inhibition and estradiol treatment in females which resulted in greater neuroprotection than either treatment separately (Stary et al., 2017). This study has been discussed in an excellent recent review (Kaidonis et al., 2018). The remaining 2 publications tested Let7f and mir363–3p in both males and females and showed sex-specific effects in each case (Selvamani et al., 2012; Selvamani and Sohrabji, 2017), and are the primary focus of this review.

2.1. Mir Let7f

Selection of Let7f: Let7f is one of several miRNAs that target the peptide hormone insulin-like growth factor (IGF)-1. IGF-1 is implicated as a risk factor in both stroke and CVD. Lower IGF-1 levels significantly increase the risk of stroke and higher IGF-1 levels are associated with improved stroke outcomes, suggesting that circulating IGF-1 levels may have predictive value for ischemic stroke outcome (De Smedt et al., 2011; Dong et al., 2014; Tang et al., 2014). IGF-1 levels decrease with age in all species and this loss of IGF-1 may underlie the increased risk of stroke observed in humans, and the increased severity of stroke in older animals as compared to the young animals (reviewed in (Sohrabji and Williams, 2013).

IGF-1 is also a well-known neuroprotectant, and has been shown to reduce cell loss in several acute neural injuries as well as ischemic stroke (reviewed in (Sohrabji, 2015)). The availability of IGF-1, along with its binding protein IGFBP3, is decreased with age (Rosario, 2010) and the ratio IGF-1 to IGFBP3 declines faster in males than females (Waters et al., 2003). Studies using male animal models support the hypothesis that IGF-1 plays a pivotal role in maintaining brain functions in acute ischemic stroke through various mechanisms including neuronal survival, anti-inflammation, and/or anti-thrombotic effects (Li et al., 2010; Jin et al., 2013; Patel et al., 2013). Exogenous IGF-1 treatment intranasally, intravenously, or intracerebroventricularly has been shown to decrease ischemic stroke injury (Liu et al., 2004; Rizk

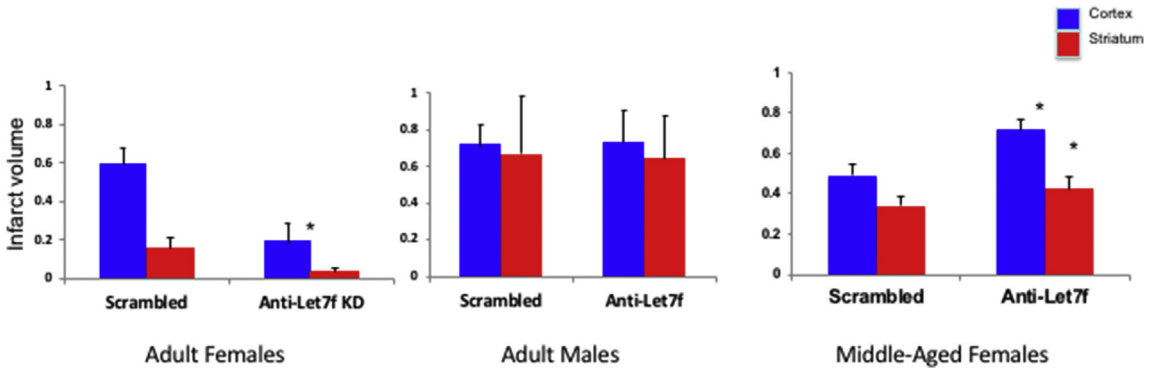

Fig. 1. Effect of anti-Let7f treatment on infarct volume: Adult (6 month old) males and females and middle-aged (10–12 month old) acyclic females were subject to ischemic stroke and treated with anti-Let7f 4 h after the onset of ischemia. Infarct volume in the cortex and striatum was reduced by anti-Let7f treatment in adult females (A), had no effect on adult males (B), and increased infarct volume in the cortex of middle-aged females (C). (Panels A and B reproduced from Selvamani et al., 2012). N = 6–8 per group; *: $p < 0.05$.

et al., 2007; Lioutas et al., 2015; Sohrabji, 2015). Studies of middle-aged, acyclic female rats have demonstrated that post-stroke IGF-1 replacement, alone or in combination with estradiol, exerts neuroprotective effects (Selvamani and Sohrabji, 2010a; c; Selvamani and Sohrabji, 2010b; Bake et al., 2014). IGF-1 adminstered to estrogen deficient (but not ovariectomized) middle-aged females also reduces stroke-induced damage and motor impairment in the aging brain and reduces blood brain barrier disruption and neuroinflammation (Bake et al., 2014). Since IGF-1 is reduced in the aging animal (and human), identifying miRNAs that target this peptide hormone could be engineered to improve IGF-1 levels. Our studies show that 2 miRNAs that target IGF-1, Let7f and mir1 are elevated in middle-aged females as compared to young females, consistent with the fact that IGF-1 is decreased in this older group (Selvamani et al., 2012).

Effect of Let7f on stroke outcomes: In view of the evidence that Let7f is increased and IGF-1 decreased in the forebrain of middle-aged females, this group was treated with antagomirs (inhibitors of Let7f) by intracerebroventricular (ICV) injection 4 h after stroke. This treatment was effective in reducing infarct volume only in young females but not in males or ovariectomized females (Selvamani et al., 2012) (Fig. 1). Moreover, Let7f antagomirs to middle-aged females, paradoxically, increased infarct volume (Fig. 1), indicating that miRNA treatment is likely to be influenced by the hormonal milieu of the organism. In view of the fact that miRNAs have multiple target genes, some of which are benign and others malignant, the same miRNA may elicit different responses in 'estrogen-sufficient' young females and 'estrogen-deficient' males, ovariectomized young females and middle-aged females. Paradoxically, Let7f targets not only IGF-1 but also caspase-3, which is a cell death effector. Thus, suppressing Let7f in middle-aged females may have created a more toxic environment by increasing caspase-3. As described later (under section '*Caspase*'), caspase-3 appears to be an important sex and age-specific target for modifying stroke outcomes.

2.2. Mir-363-3p

Selection of miR363-3p: MiR363–3p was identified as a potential stroke neuroprotectant from a profiling study, specifically, by comparing circulating miRNAs from young and middle-aged male and female rats. As described in several studies, young females have smaller infarct volumes and less sensory-motor impairment as compared to age-matched males (Alkayed et al., 1998; Park et al., 2006). With age, recovery from stroke is much worse, and surprisingly, aged females display larger infarct volumes and worse stroke outcomes as compared to age-matched males (Liu et al., 2009a). Furthermore, epidemiological data shows that stroke risk and severity, which is low in females prior to menopause, is similar or worse than males, after menopause (Reeves et al., 2008). To capture this midlife transition, our studies included reproductive senescent (middle-aged) female rats. Reproductive senescent female rats have typically had 5-6 pregnancies, are currently acyclic (constant diestrus) with a hormonal profile reminiscent of menopause (low estrogen, elevated FSH) (Jezierski and Sohrabji, 2001; Selvamani and Sohrabji, 2010b). Reproductive senescent females have worse stroke outcomes as compared to young (normally cycling) females, and show no advantage compared to age-matched males. By exploiting naturally occurring differences in stroke outcomes between adult and middle-aged males and females, we could potentially identify miRNAs associated with 'good' and 'bad' stroke outcomes.

An early study of miRNA profiles in the ischemic and non-ischemic cortex performed 8 h after stroke, found specific cohort of miRNAs that were similarly regulated (increased or decreased) in males and females. A smaller subset, which included miR509–3p and miR883b-3p were regulated in an opposite manner in males and females (1–3 days) (Lusardi et al., 2014), suggesting that miRNA profiles may reflect sex differences in stroke severity. With the current recognition that stroke is multi-organ disease, we profiled miRNA in both brain tissue and in circulation. Originally thought to be exclusively intracellular, miRNAs are also found in many body fluids, including blood (Weber et al., 2010). Since the response to stroke is modified by the immune and endocrine system, miRNAs in circulation potentially represent a composite response of these tissues to stroke.

Our studies compared adult and middle-aged males and females, and confirmed that young females have the best stroke outcomes, as estimated by small infarct volumes and low sensory motor impairment, as compared to adult males and middle-aged males and females (Selvamani et al., 2014). MiRNA profiling in plasma samples showed that one miRNA, miR-363–3p was elevated in young animals (irrespective of sex) at 2d post stroke and then increased further at 5d after stroke only in young females (Selvamani et al., 2014). At 5d post stroke, this miRNA was significantly inversely correlated with infarct volume (Selvamani and Sohrabji, 2017). To directly assess if mir-363–3p was mechanistically linked to stroke outcomes, two complementary approaches were used. Young females were injected with an antagomir to miR363–3p 4 h after the onset of ischemia, which significantly increased infarct volume. Conversely, middle-aged females were injected with miR363–3p mimics, 4 h after the onset of ischemia, which significantly decreased infarct volume and reduced motor impairment. Remarkably, young and middle-aged males injected with this mimic had no improvement in their stroke recovery (Selvamani and Sohrabji, 2017).

MiR363 is identified as a tumor suppressor in various cancers, including hepatocellular carcinoma, breast cancer, head and neck cancer, gastric cancer, colorectal cancer and neuroblastoma (Floyd et al., 2014; Song et al., 2015; Wang et al., 2016). MiR363–3p is predicted to regulate caspase-3, the cell death effector (Floyd et al., 2014). In females,

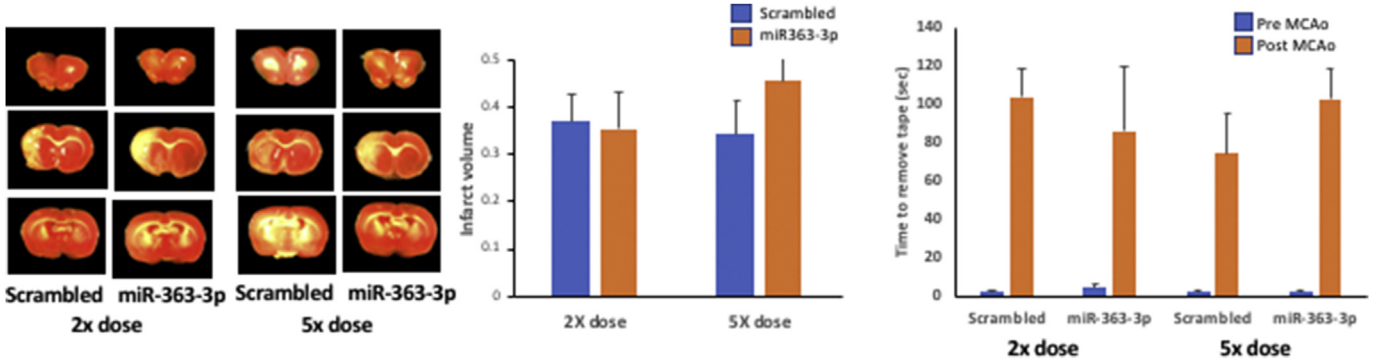

Fig. 2. Effect of miR363–3p doses on infarct volume in males: Adult (6 month old) males were subject to ischemic stroke and treated with miR-363–3p mimics 4 h after the onset of ischemia. Two doses of miR363–3p were used (14 μg/kg, 35 μg/kg), which was 2X and 5X respectively of the dose used in Selvamani and Sohrabji (2017). Infarct volume estimated from TTC stained sections showed no significant difference between miR363–3p treated groups as compared to controls injected with a scrambled miRNA. Similarly, no improvement was seen in the adhesive removal test with either dose. Histogram depict mean ± SEM., N = 9 per group for scrambled miRNAs and 2X miR363–3p and N = 14 for 5X miR363–3p mimic treated group.

mir363–3p regulated both caspase-3 expression and activity, while it had no effect on either measure in males. One explanation for this sex-specific effect is that the 'dose' of mir363–3p was sufficient for middle-aged females, but insufficient for males. Subsequently, increasing the dose of mir363–3p by 2-fold or 5-fold also failed to improve infarct volume or behavior (Fig. 2).

An alternate hypothesis is that mir363–3p is ineffective in males because cell death in males is not ameliorated by caspases and may depend on alternate cell death pathways. Some support for this hypothesis comes from studies showing that cell death pathways in ischemic stroke are sexually dimorphic (Reeves et al., 2008; Yuan et al., 2009; Liu et al., 2011; Gibson, 2013). Mitochondria play a central role in regulating apoptotic cell death. Oxidative stress caused by ischemia can stimulate the release of apoptogenic signaling molecules, such as caspases, cytochrome c, apoptosis-inducing factor (AIF), and endonuclease G, resulting in caspase-dependent or caspase-independent cell death pathways. Caspase-induced cell death results from activation of endonucleases leading to numerous small cleaved DNA fragments. This process is initiated by the release of mitochondrial cytochrome c and formation of the apoptosome. Apoptotic cell death can also be mediated by Poly ADP ribose polymerase (PARP)-1, also known as caspase-independent cell death. Poly-ADP-ribose (PAR) polymers, formed by PARP-1 activation, stimulate the release of mitochondrial apoptosis inducing factor (AIF) (Fan et al., 2017).

PARP: Poly (ADP-ribose) polymerase-1 (PARP-1) activation plays a key role in the pathogenesis of cardiovascular and inflammatory diseases (Beneke, 2008; Peng et al., 2012; Song et al., 2013; Sun et al., 2015). Several studies suggest that pharmacological inhibitors of PARP-1 differentially affect males and females in response to ischemic stroke (Mabley et al., 2005; McCullough et al., 2005), although the PARP-1 inhibitor, MP-124, can provide stroke neuroprotection in both sexes in monkeys (Matsuura et al., 2011). In males, the classical (3-aminobenzamide) or selective (PJ34) PARP inhibitors reduce infarct volume and enhance long-term stroke recovery by mechanisms including suppression of the post-stroke neuroinflammatory response (Takahashi et al., 1999; Couturier et al., 2003; Hamby et al., 2007), or by reducing NAD depletion and PAR formation in male Sprague-Dawley rats (Fujio et al., 2009). Li et al. (Li and McCullough, 2009) found that minocycline which is effective in reducing stroke severity only in male mice, is ineffective in PARP-1 null male mice. Furthermore, downstream pathways of PARP, including apoptosis inducing factor (AIF) and poly (ADP-ribose) polymerase (PAR) polymers also mediate cell death after ischemic insult only in males (Yuan et al., 2009). This suggests that PARP-1 inhibition may be a male-specific pathway, and may underlie the sex dimorphism in PARP treatment studies (Hagberg et al., 2004; Mabley et al., 2005; McCullough et al., 2005; Lang and McCullough, 2008; Li and McCullough, 2009; Liu et al., 2011).

Thus it is possible that miRNAs that target PARP-1 may be more effective for stroke neuroprotection in males, than those targeting caspases. In silico analysis indicates that miR222–3p and miR335 may target PARP1. A cocktail of miR222–3p and miR335 mimics administered intravenously to male rats 4 h after stroke however failed to reduce infarct volume or improve motor behavior in this group (Fig. 3).

Caspases: Under ischemic conditions, caspase pathways are activated in both sexes after stroke, however regulating this pathway promotes neuroprotection in females but not males. The selective pan-

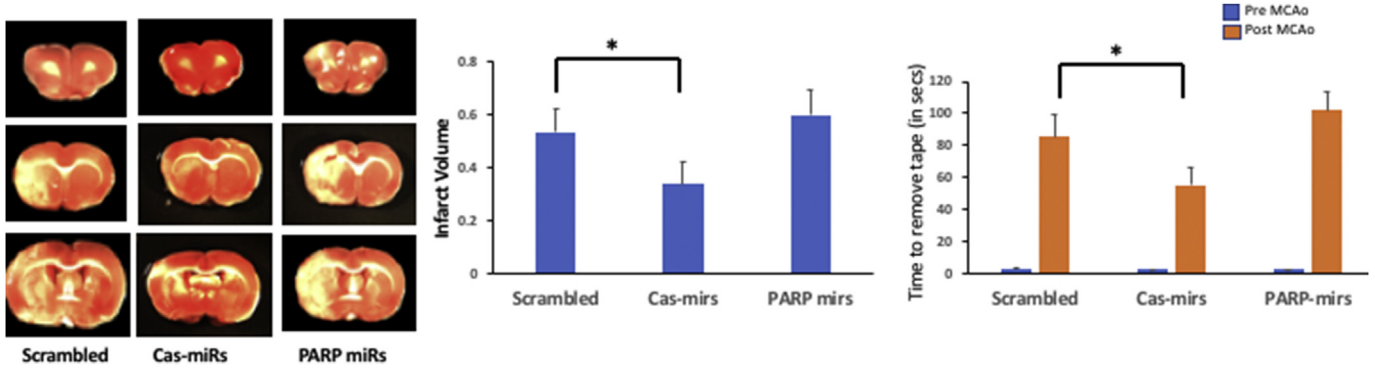

Fig. 3. Effect of miRNA cocktail on infarct volume in males: Adult (6 month old) males were subject to ischemic stroke and treated with a mixture of miRs (miR222–3p and miR335–5p) that target PARP and miR363–3p, let-7f and miR98 that target caspase were injected by tail vein to male rats 4 h after stroke. PARP-miRs did not affect infarct volume, while Cas-miRs significantly reduced infarct volume. Similarly, Cas-miRs reduced adhesive removal time, indicating better sensory motor recovery in this group. N = 8 for Scrambled miRs and Cas-miRs and N = 5 in the PARP miRs group. *: $p < 0.05$.

caspase inhibitor, quinoline-Val-Asp(Ome)-CH2-O-phenoxy (Q-VD-OPh), shows neuroprotective effects in both neonatal and adult female mice but not in males of either age (Renolleau et al., 2007; Liu et al., 2009b). Surprisingly, a caspase 9-specific inhibitor delivered intranasally spares brain damage and improves neurological outcome post stroke in male mice and rats (Akpan et al., 2011), suggesting that specific caspases may mediate cell death in males and females. MiR363–3p targets caspase-3 and appears not to improve stroke in male animals, but it is possible that miRNAs that target other members of the caspase family may be more effective in males. In addition to miR363–3p, Let7f and miR98 have consensus targets in the caspase-3 gene as well as caspase-12, and the caspase recruitment domain (CARD) proteins CARD14 and CARD16. As shown in Fig. 3, a combination of miR363, Let7f, and miR98 administered to male rats 4 h after ischemia significantly reduced infarct volume and improved sensory motor behavior. Thus, it may be the case that the caspase pathway may be a druggable target for both males and females, albeit requiring different dosing or regulating different components of the pathway. More studies are clearly needed to address this gap in the literature. It may be that the cohort of caspase targeting miRNA is low in males as compared to middle-aged females, where only one or few of these microRNA is depleted, such that middle-aged females can be improved with just one type of miRNA treatment but in males a mixture of these caspase-regulating microRNAs are needed. Collectively, these data suggest that factors that control sex-specific miRNA expression need to be carefully investigated.

3. Why do sex differences in miRNA occur?

Broadly speaking, sex differences in miRNA expression may be due to either the gonadal steroid environment or due to sex chromosomes. Some evidence exists for contributions from both sources.

Chromosomal basis for sex differences: The X chromosome is larger and has many more genes compared to the Y chromosome. Many genes on the X chromosome are linked to inflammation and this has been suggested to underlie the increased autoimmune disease seen in women (Chitnis et al., 2000) and the increased susceptibility to infection in men (vom Steeg and Klein, 2016). With respect to miRNA, 118 miRNA are located on the human X chromosome, while only 4 are located on the Y chromosome (Kozomara and Griffiths-Jones, 2014). Furthermore, although X-inactivation ensures that genes from the second X chromosome will be suppressed, about 15% (Carrel and Willard, 2005) to 23% (Tukiainen et al., 2017) of X-linked genes escape X-inactivation. The degree of escape varies between individuals and between tissues, and includes miRNA (Song et al., 2009), especially those located at the periphery of the XY body (Sosa et al., 2015). This suggests the potential for increased gene dosage in females as compared to males.

Several miRNA located on the X-chromosome appear to target cell death pathways. Mir363–3p discussed above is exclusively located on the X-chromosome and Let7f is located on both chromosome 9 and the X chromosome (Kent et al., 2002). In addition, members of the miR106-363 cluster, such as miR18b, miR19b, miR20b, are also located on the X chromosome, and have been shown to play in role in both proliferation and apoptosis. Thus, miRNA on the X chromosome disproportionately target apoptotic genes and may facilitate stroke outcomes through this mechanism. Curiously, however, the aging female has a lower expression of miR363 (Selvamani and Sohrabji, 2017), which suggests that perhaps epigenetic modifications may further affect the expression of miRNAs.

Gonadal steroid regulation of miRNA: The endocrine environment of the organism also influences the type of miRNAs that are likely to be expressed, and, in turn, miRNA regulate hormone receptors and therefore hormone-dependent cell signaling. This reciprocal regulation is well-documented for the estrogen receptor system. A recent study examined estrogen mediated regulation of miRNA in zebra fish, and found that a 12 h treatment of 17b-estradiol up-regulated two miRNA's, miR196b and let-7h and down-regulated miR130c and miR101d (Cohen et al., 2008). Estradiol has been shown to regulate microRNA and pri-microRNAs in breast cancer cells (Paris et al., 2012; Tilghman et al., 2012), the majority of which were suppressed by the hormone (Maillot et al., 2009). Furthermore, forced expression of these miRNAs reduced the effect of estrogen on cell proliferation, suggesting that hormone-dependent downregulation of microRNA is mechanistically linked to its tumorigenic properties.

MiRNA also regulate the estrogen receptor. In MCF-7 cells, miR148a knockdown significantly increased ERα protein levels, while overexpression of this microRNA significantly decreased ERα expression and abrogated estradiol-induced cell survival and migration (Ma et al., 2017). MiR206 has two consensus sequences in the 3' UTR region of the ERα gene, and overexpression of this miRNA in MCF7 cells also decreased ERα expression, and conversely, blocking miR206 increased ERα expression (Adams et al., 2007). Regulation of miR206 was, in turn, affected by 17β-estradiol or the ERα agonist, PPT, while the ERβ agonist DPN had no effect (Adams et al., 2007), suggesting a feedback loop between miRNA and estrogen signaling. Similarly, transfection of miR22 mimetics in PxBC-3 pancreatic cells or curcumin-induced miR-22 expression suppressed ERα and SP1 (Sun et al., 2008).

MiRNAs also regulate genes that co-function with estrogen. CYP1B1, a cytochrome p450 that is highly expressed in estrogen target tissues such as the breast, uterus and ovaries, is regulated by the miRNA-27b, such that inhibition of miR-27b increased CYP1B1 expression (Tsuchiya et al., 2006). Mertens-Talcott et al. (2007) found that in estrogen receptor-positive breast cancers miR-27a regulated the transcription factors SP1, SP3 and SP4, which are known to stabilize ERα to estrogen responsive elements (Krishnan et al., 1994). Pertinent to this review is an in depth study of miRNA regulation by estrogen in young (3 month old) and aged (18 month old) female rats (Rao et al., 2013). This study showed that estradiol regulation of miRNA in the forebrain was modified by the age of the animal as well as the specific brain region. Bioinformatics further showed that predicted target genes of estradiol-regulated miRNAs included those related to neuronal function.

Although it is not currently known whether chromosome or gonadal sex is a more important contributor to miRNAs that regulate stroke outcomes, some answers may lie in studies of the four-core genotype (FCG), where the relative contributions of chromosomal and gonadal sex can be evaluated separately. Ablation of the *sry* gene from the Y chromosome results in a chromosomal (XY) male with ovaries rather than testes (Arnold and Chen, 2009). Cross breeding this mouse to one where the *sry* gene is relocated to an autosome results in 4 genotypes: XY male with testes, XY male with ovaries, XX female with testes, XX females with ovaries. This mouse model has been used to study several neurologic diseases with sex-specific outcomes to precisely determine the contribution of sex steroids and sex chromosomes. In experimental autoimmune encephalomyelitis (EAE) and pristane-induced lupus, for example, sex chromosome complement (XX) was a greater indicator of disease burden than sex steroids (Smith-Bouvier et al., 2008) In the case of stroke, the contributions of sex steroids versus sex chromosome revealed a complex interaction with age. Thus, in young FCG mice, genotypes with ovaries, irrespective of chromosomal sex, displayed better stroke outcomes as compared to genotypes with testes. Moreover, ovariectomy of the XX or XY genotypes caused a loss of this neuroprotection (Manwani et al., 2015), indicating that sex hormones were an important determinant of stroke recovery. However, in aged FCG animals, this phenotype was reversed, such that ischemic stroke severity was worse in genotypes with XX chromosomes, irrespective of gonadal sex (McCullough et al., 2016). It would be worthwhile to know the miRNA expression profiles of the young and aged FCG to determine the contribution of X-linked miRNA to stroke recovery. In a study of gonadal fat in FCG mice, unique sets of miRNA were regulated by gonadal steroids and by sex chromosomes (Link et al., 2017). However, this remains to be studied in tissues such as brain, spleen and

circulation, which affect stroke outcomes.

4. Conclusion

MicroRNAs represent a potent new class of drugs that may improve stroke outcomes, possibly, by targeting a large portion of the transcriptome. However, similar to other drugs that are considered stroke neuroprotectants, they need to be tested in a variety of models including both sexes, ages, different types of stroke models and for short- and long-term effects. Sex differences in the effectiveness of miRNAs (reviewed here) underscore the importance of such granular analyses.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2018.10.021.

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