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Sex Differences in MicroRNA Expression During Developmental in Rat Cortex

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Abstract

There are important sex differences in the risk and outcome of conditions and diseases between males and females. For example, stroke occurs with greater frequency in men than in women across diverse ethnic backgrounds and nationalities. Work from our lab and others have revealed a sex-specific sensitivity to cerebral ischemia whereby males exhibit a larger extent of brain damage following an ischemic event compared to females. Studies suggest that the difference in male and female susceptibility to ischemia may be triggered by innate variations in gene regulation and protein expression between the sexes that are independent of post-natal exposure to sex hormones. We have shown that there are differences in microRNA (miRNA) expression in adult male and female brain following focal cerebral ischemia in mouse cortex. Herein we examine a role for differential expression of miRNAs during development in male and female rat cortex as potential effectors of the phenotype that leads to sex differences to ischemia. Expression studies in male and female cortices isolated from postnatal day 0 (P0), postnatal day 7 (P7), and adult rats using TaqMan Low Density MiRNA arrays and NanoString nCounter analysis revealed differential miRNA levels between males and females at each developmental stage. We focused on the miR-200 family of miRNAs that showed higher levels in females at P0, but higher levels in males at P7 that persisted into adulthood, and validated the expression of miR-200a, miR-200b, and miR-429 by individual qRT-PCR as these are clustered on chromosome 5 and may be transcriptionally co-regulated. Prediction analysis of the miR-200 miRNAs revealed that genes within the Gonadotropin Releasing Hormone Receptor pathway are the most heavily targeted. These studies support that developmental changes in miRNA expression may influence phenotypes in adult brain that underlie sexually dimorphic responses to disease, including ischemia.

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Keywords

microRNA; sex; development; rat; cortex; disease outcome

1. Introduction

There are important sex differences in the risk and outcome of conditions and diseases between males and females. Sex here is defined as being male or female according to reproductive organs and the functions assigned by chromosomal complement (XX for female and XY for male) (Wizemann *et al.*, 2001). Sex differences in physiology and pathophysiology leading to differential conditions or diseases are classified as (1) unique to one sex, (2) those that have greater prevalence in one sex compared with the other, or (3) those with differential age of onset, symptomatology, or response to treatment in one sex compared with the other, as reviewed in (Miller, 2014). Such differential conditions include those related to cardiovascular, respiratory, musculoskeletal, immunological, gastrointestinal, and renal/urological systems, as well as development, and behavioral diseases. We are focused on differential responses to stroke, which occurs more frequently in men than women across diverse ethnic backgrounds and nationalities (Appelros *et al.*, 2009; Bushnell, 2008; Ovbiagele *et al.*, 2013; Persky *et al.*, 2010; Reeves *et al.*, 2008; Saini *et al.*, 2008; Towfighi *et al.*, 2013). We, and others, have shown that the extent of brain damage resulting from cerebral ischemia is sex-specific, with female animals being less sensitive than males (Cheng *et al.*, 2010; Koerner *et al.*, 2007; Lang *et al.*, 2008; Murphy *et al.*, 2004; Siegel *et al.*, 2010; Vagnerova *et al.*, 2008). Studies show that some sex differences are unrelated to sex hormones, suggesting that differential susceptibility to ischemia is triggered by innate variations between the sexes in gene regulation and protein expression between the sexes that are independent of post-natal exposure to sex hormones. In addition, gene profiling shows differential messenger RNA responses in blood between human males and female after ischemic stroke, particularly those for immune, inflammatory, and cell death responses to stroke (Tian *et al.*, 2012).

MiRNAs are short non-coding RNAs that predominantly function as regulators of gene and protein expression, largely by translational repression or mRNA degradation (Guarnieri *et al.*, 2008). MiRNAs have been correlated with several physiological and pathological processes in the brain such as cellular differentiation (Feng *et al.*, 2011), neurological disorders (Saugstad, 2010), ischemic preconditioning (Lusardi *et al.*, 2010) and stroke (Rink *et al.*, 2011; Tan *et al.*, 2011). Recent findings have shown sexual dimorphism of miRNA expression in diverse tissues, implying a critical role of miRNAs in sex differentiation and in sex-specific regulation of tissue development and/or function (Dai *et al.*, 2014).

Our recent studies have revealed sex-specific differences in miRNA expression to ischemic insult as well as a universal, ischemia-induced miRNA signature equally present in both male and female brains (Lusardi *et al.*, 2014). These studies suggest that the differential regulation of miRNA responses to ischemia may account for sex differences in ischemic sensitivity in male and female brain. Accordingly, there are differences in the expression of circulating miRNAs between adult and middle aged males and females. Recent studies revealed that the neurotoxic effects of estrogen in older females occurs because of decreased

availability of IGF-1, a neuroprotectant that decreases with advancing age, is down-regulated by estrogen treatment (Selvamani *et al.*, 2010), an effect that is mediated by let7f (Selvamani *et al.*, 2012). Infarct volume and sensory-motor deficits were significantly reduced in adult females compared with middle-aged females, adult males or middle-aged males. Circulating blood miRNA profiling of adult and middle-aged female and male rats subjected to stroke revealed 21 miRNAs differentially regulated due to age, and 78 miRNAs differentially regulated, most by sex, at 2 and 5 days post-stroke. The top targets of these miRNAs were related to growth factor signaling, cell structure and phosphoinositide 3-kinase/Akt and mammalian target of rapamycin signaling (Selvamani *et al.*, 2014). These studies provide evidence that differential miRNA expression in developing and aged brain leads to differential outcomes in stroke between male and female brain.

Given that the female advantage in stroke risk is present in childhood (Golomb *et al.*, 2009), in the present study we examined the developmental expression of miRNAs in male and female P0, P7, and adult rat cortex as potential effectors of differential phenotypes inherent in males and females. We found distinct differences in the expression of miRNAs between male and female cortex, and validated expression of members of the miR-200 family that showed a developmental switch in expression between males and females. These studies suggest that specific miRNAs may be differentially regulated during development in male and female brain, resulting in altered gene and protein expression that may contribute to sex differences-associated disease outcomes, including stroke.

2. Materials and methods

2.1 Experimental Groups

Experiments were carried out on male and female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA, USA). Experiments were carried out in accordance with the National Institutes of Health guidelines for research animal care and approved by the Oregon Health and Science University Animal Care and Use Committee. All rats were maintained on a 12/12 hour light-dark cycles and permitted ad libitum access to food and water. Three developmental stages were analyzed: postnatal day zero (P0), P7, and adult (10 weeks). For the P0 and P7 developmental stage, brains were collected from two entire litters at each development stage to reach n=10 pups/age/sex. Rat pups were sexed using ano-genital distance, with males having a larger genital papilla and longer ano-genital distance than female rodent pups (Liu *et al.*, 2007; Liu *et al.*, 2008). Sex was confirmed by inspecting internal organs and gonads after laparotomy, for example, uterine horns in females and ductus deferens in males. The cortices were dissected from each rat brain, and the tissues were frozen in 2-methyl-2-butane on dry ice then stored at -80°C .

2.2 RNA isolation

Total RNA was isolated from the cortex of 5 individuals within each of 6 groups (male P0, P7, and adult; female P0, P7, and adult). We used the mirVana miRNA Isolation Kit (Life Technologies, Carlsbad, CA, USA), following the recommended protocol for total RNA isolation from frozen tissue. The RNA isolation did not include the “Enrichment Procedure for Small RNA” in the protocol provided with the kit. Total RNA was eluted with 100 μL of

Elution Solution provided with the RNA isolation kit, and the RNA concentrations quantified by spectroscopic measurement of A260. RNA samples were stored at -80°C until further use.

2.3 TaqMan® Low Density Arrays

MiRNA quantification was performed using TaqMan® Low Density Arrays (TLDA) from Applied Biosystems (TaqMan® Array Rodent MicroRNA A+B Cards Set v3.0, catalog #4444909; Life Technologies, Grand Island, NY). The two card sets each contain 384 TaqMan® MicroRNA probes per card based on Sanger miRBase 15, and include 641 rodent miRNAs with 373 unique rat sequences. cDNA was synthesized from 1 µg of total RNA from each individual male and female rat from each developmental stage (P0, P7 and adult) using Megaplex RT primers, as per the manufacturer's instructions. In brief, the protocol consisted of 40 cycles of PCR on a BioRad C1000 thermal cycler (Bio-Rad Laboratories, Hercules, CA) at 16°C for 2 min, 42°C for 1 min, and 50°C for 1 second, followed by 85°C for 5 minutes to degrade the RNA and to inactivate the reverse transcriptase. Equal volumes of individual cDNA reactions were then pooled for each experimental group ($n=5/\text{group}$), and mixed with the TaqMan Universal PCR Master Mix, No AmpErase® UNG for the PCR reactions. The samples were loaded onto TaqMan® Array cards, and run on a QuantStudio Real Time PCR Detection System (Life Technologies). Parameters were set to 1 cycle at 95°C for 10 minutes, then 40 cycles at 97°C for 5 seconds, 60°C for 40 seconds.

2.4 MiRNA expression data analysis - TaqMan® Low Density Arrays

TLDA probe sequences were reannotated to rat-specific sequences in miRBase 20; only probes with exact matches to mature rat sequences (353) were included for further analysis. TLDA miRNA values were normalized to the average of 6 replicates of U6 snRNA standard on each TLDA card set. For each miRNA on a card, delta Ct (ΔCt) was calculated as ($\text{Ct}_{\text{U6}} - \text{Ct}_{\text{miRNA}}$). At each time point, we calculated the differential expression for each miRNA between male and female as $\Delta\text{Ct} = (\text{Ct}_{\text{male}} - \text{Ct}_{\text{female}})$; thus, miRNA with higher expression in males than in females have a positive ΔCt value, while miRNA with lower expression in males than in females will have a negative ΔCt value. ΔCt values greater than 1.0 were considered enriched in males, and values less than -1.0 were considered enriched in females.

2.5 NanoString nCounter® analysis

MiRNA expression was examined using total RNA isolated from 2 male rat cortices and 2 female rat cortices from each developmental stage. The RNA was analyzed using NanoString nCounter® Rat miRNA Expression Arrays (catalog #GXA-RMIR-12; NanoString Technologies, Seattle, WA). The NanoString miRNA panel contains 423 probes for rat miRNAs from miRBase 20. Total RNA (100 ng) was used as input for nCounter miRNA sample preparation reactions and the reactions were performed, as per the manufacturer's instructions (NanoString Technologies). Small RNA sample preparation involves the ligation of a specific DNA tag onto the 3' end of each mature miRNA. These tags normalize the melting temperatures of the miRNAs and provide a unique identification for each miRNA species in the sample. Excess tags were then removed, and the resulting material was hybridized with a panel of miRNA: tag-specific nCounter capture and

barcoded reporter probes. Hybridized probes were then purified and immobilized on a streptavidin-coated cartridge using the nCounter Prep Station (NanoString Technologies). Data collection was carried out on the nCounter Digital Analyzer (NanoString Technologies) following manufacturer's instructions to count individual fluorescent barcodes and quantify target RNA molecules present in each sample. For each assay, a high-density scan (600 fields of view) was performed.

2.6 MiRNA expression data analysis - NanoString

All miRNA sequences on the NanoString platform matched miRBase 20 sequences exactly. Raw intensity values of the NanoString nCounter Rat miRNA Expression Arrays for 12 samples normalized according to the "top-100 miR normalized counts" by the vendor. Linear calibrations were performed for each sample according to the standards included in each sample, converting counts into femtomolar (fM) concentrations. Counts below 100 (~0.5 fM) could not be distinguished from negative control values and should be considered "not present." The average of the two experimental replicates was calculated and used for comparison with the pooled TLDA data. At each time point, the relative expression between male and female was calculated as $\text{Log}_2(\text{Male/Female})$, resulting in positive values for miRNA enriched in the male population, and negative values for miRNA enriched in the female population.

2.7 Real-time qRT-PCR assays

We validated the expression of three miRNAs identified in the TLDA and NanoString studies using total RNA from individual male and female rats that was pooled for the TLDA arrays (n=5/age/sex). Detection of miRNAs was completed with a 2-step qRT-PCR assay, using the miScript PCR System (Qiagen, Valencia, CA). One μg of total RNA was converted to cDNA, for RNA from each individual sample, with the miScript II RT Kit, following the standard protocol with the miScript HiSpec Buffer. Negative control samples included a reverse transcription reaction with no template RNA (RT-NTC). The resulting 10 μL of RT products (or No RT controls) were diluted to a total volume of 100 μL with the addition of 80 μL RNase/DNase Free water, and stored at -80°C before use in PCR assays. 2 μL of cDNA was assayed in each PCR reaction well. miScript PCR Primer Assays (Qiagen) were used for detection of mature miRNA, using the miScript SYBR Green PCR Kit for qRT-PCR assays. The following primer sets were used:

Rn_miR-200a	5' UAACACUGUCUGGUAACGAUGU	(#MS00000581)
Rn_miR-200b	5' UAAUACUGCCUGGUAUGAUGAC	(#MS00000588)
Rn_miR-429	5' UAAUACUGUCUGGUAUGCCGU	(#MS00001071)

Assays were performed with the standard recommended reaction mix (25 μL volume per reaction) in 96-well reaction plates, using a ViiA 7 Real Time PCR Detection System (Life Technologies).

2.8 MicroRNA target prediction

We used the microRNA.org rat Target Sites Prediction database (Betel *et al.*, 2008), and downloaded the ‘Good mirSVR score, Conserved miRNA’ from <http://www.microrna.org/> (August 2010 Release) (Betel *et al.*, 2008). We first queried microRNA.org to identify the predicted targets of the individual miRNAs validated by qRT-PCR (miR-200, miR-200b and miR-429). We further queried the predicted targets of all miR-200 family members (miR-200a, miR-200b and miR-429 that are clustered on chromosome 5, and miR-200c and miR-141 encoded on chromosome 4) using three target prediction databases: rat microRNA.org (Betel *et al.*, 2008), rat TargetScan Release 6.2 (Friedman *et al.*, 2009; Garcia *et al.*, 2011; Grimson *et al.*, 2007; Lewis *et al.*, 2005), and mouse PicTar (there is no rat data in PicTar) (Krek *et al.*, 2005). We then used the protein targets identified in the prediction programs to query the PANTHER 9.0 program (<http://www.pantherdb.org/>), which identifies pathways of the predicted targets, as well as gene ontology (Mi *et al.*, 2013; Mi *et al.*, 2009).

3. Results

3.1 MicroRNA Platforms

We used two platforms to analyze the global expression of miRNAs in male and female cortex: (1) the TaqMan® Array Rodent MicroRNA arrays (Life Technologies) as a Taqman qRT-PCR approach, and (2) the NanoString nCounter® analysis (NanoString) which uses molecular “barcodes” and single-molecule imaging to detect and count RNAs without PCR amplification. The TLDA arrays contain probes for 641 and 373 unique miRNAs for mouse and rat, respectively. For initial studies we used a very conservative and restricted analysis of the data, and included only those 353 miRNAs detected in the samples that showed a 100% match to mature rat miRNA sequences documented in miRBase Version 20. We then identified 280 miRNAs detected in common between the 353 rat-specific miRNAs in the TLDA arrays and the 423 rat miRNAs on the NanoString platform, as shown in the Venn diagram (Figure 1). We focused on these high confidence miRNAs as this allowed an initial analysis of only those rat specific miRNAs probed in both platforms.

3.2 MicroRNAs differentially regulated during development in male and female rat cortex

We examined the expression of RNA samples isolated from P0, P7, and adult male and female cortices. The results from the TLDA arrays show that there are distinct differences in miRNA expression between males and females at each developmental time point (n=5 pooled samples/sex/age). The results from the NanoString studies support that there are distinct differences in miRNA expression between males and females at each developmental time point (n=2 individual samples/age). The miRNAs showing differential expression in both the TLDA and NanoString platforms are shown in Table 1. These data show that there are several miRNAs that are expressed uniquely at P0, P7, or adult in male or female brain. The data also show that members of the miR-200 family of miRNAs (miR-141-3p, miR200a-3P, miR200b-3P, and miR-429) showed a sex-specific switch in expression: females had higher levels of these miRNAs than males at P0, but males had higher levels than females at P7 and adults. Similarly, miR-875 expression was higher in females at P0 and P7 but higher in males in adults, while miR-935 expression was higher in males at all

developmental stages. We also identified miRNAs with changes in expression during development unique to either the TLDA array or NanoString platform (raw data for all miRNAs provided in Supplemental Table 1). Specifically, the sex- and age-dependent expression profiles measured for miR-200a, miR-200b, and miR-429 is demonstrated as assessed by TLDA arrays (Figure 2A. TLDA) and by the NanoString platform (Figure 2B. NS). Together, these results show that there are miRNAs unique to each developmental stage in both male and female brain, as well as distinct changes in miRNA expression between males and females during development.

3.3 Validation of miR-200 family of microRNAs

We focused further individual qRT-PCR validation studies on the miR-200 family members miR-200a, miR-200b, and miR-429 encoded as a gene cluster on rat chromosome 5 as developmental changes in their expression between males and females may result from co-transcriptional regulation. We did not include miR-141 and miR-200c for validation studies as these genes are encoded on chromosome 4, and miR-200c was excluded from this restricted, high confidence analysis because it did not meet the criteria for a 100% match with the rat sequence (Supplemental Table 1), which leaves the primer efficiency for qRT-PCR assay questionable. Each miRNA qRT-PCR assay was performed in triplicate for individual samples. The Ct values were calculated using automated cycle threshold and baseline for all reactions, and technical replicates averaged. Prism was used to calculate a two-way ANOVA for each miRNA. In each case, the main effect was due to age ($p < 0.0001$), and in each case, there was an interaction between age and sex ($p < 0.05$). The validation assays of miR-200a, miR-200b, and miR-429 in individual male and female cortices at P0, P7, and adult ($n = 5/\text{sex/age}$) supported the TLDA and NanoString data showing a developmental switch in expression (Figure 2C. qRT-PCR). The qRT-PCR values are graphed as Ct values in order to directly compare them to the NS data, which is reported as Femtomolar concentration, and not as a fold change, thus allowing miRNA changes in expression (increased/decreased) from the three distinct platforms to be compared. The Ct values for all miRNAs in each group are listed in Supplemental Table 1. These studies validated that the expression of the miR-200 cluster encoded on rat chromosome 5 (miR-200a, miR-200b, and miR-429) is higher in females at P0, but that the expression is switched to higher in males at P7 and this expression profile is maintained in male adults, consistent the TLDA and NanoString data.

3.4 MicroRNA target prediction

We first queried microRNA.org to identify the predicted targets of the individual miRNAs validated by qRT-PCR (miR-200, miR-200b and miR-429), and used PANTHER pathway analysis to identify protein pathways targeted by these three miRNAs. The results revealed that the top pathways targeted by the miR-200 family are the Gonadotropin releasing hormone receptor pathway, EGF receptor signaling pathway, Huntington disease, and the Wnt signaling pathway, (Table 2). We then expanded the query to include all miR-200 family members (miR-200a, miR-200b and miR-429, and 141 and miR-200c) using three target prediction databases: microRNA.org, TargetScan 6.2, and PicTar, and used PANTHER pathway analysis to identify protein pathways targeted by these five miRNAs. The results revealed that consistent with the initial target analysis, the top pathways targeted

by the miR-200 family in all three prediction programs are the Gonadotropin releasing hormone receptor pathway, EGF receptor signaling pathway, Huntington disease, and the Wnt signaling pathway (Supplementary Table 2). Proteins in these pathways include the cAMP-response element binding protein (CBP/CREBBP) and mitogen-activated protein kinases (MAPKs) (Table 3, Supplementary Table 2), which function as transcriptional regulators associated with hormonal responses and sex differences to injury.

4. Discussion

MiRNAs have been linked to many physiological and pathological processes, and recent studies have focused on how sex might influence miRNA regulation of gene expression (Dai *et al.*, 2014; Morgan *et al.*, 2012; Sharma *et al.*, 2014). We recently reported that there are distinct miRNA responses to ischemia in male and female brain (Lusardi *et al.*, 2014). Other studies have demonstrated that miRNA levels can be altered in a sex-specific manner in normal and injured brain (Koturbash *et al.*, 2011; Pak *et al.*, 2013; Parsons *et al.*, 2008; Siegel *et al.*, 2011). Sex differences in miRNA expression have been shown in developing human brain (Ziats *et al.*, 2013a) and such differences in males and females have implications for autism spectrum disorder (Ziats *et al.*, 2013b). Studies documenting changes in miRNAs during postnatal maturation and after manipulations to disturb puberty revealed the potential involvement of developmental changes in Lin28/let-7 expression in rat hypothalamus as effectors in the mechanisms permitting/leading to puberty onset (Sangiao-Alvarellos *et al.*, 2013). In addition, divergent patterns of cellular distribution and mRNA expression of Lin28 and Lin28b, RNA-binding proteins involved in the control of miRNA synthesis, especially of the let-7 family, suggested distinct functional roles of these two related miRNA-binding proteins in male gonadal development (Gaytan *et al.*, 2013). The studies herein support that there are sex differences in miRNA expression in rat cortex at different developmental stages, suggesting that a developmental switch in miRNA expression may lead to phenotypic changes in males and females that result in distinct differences in disease progression and outcomes for each sex.

The finding that miR-200a, miR-200b, and miR-429 are differentially regulated during development in male and female cortex is intriguing due to their clustered gene location on rat chromosome 5, and potential for co-transcriptional regulation. Previous studies showed selective upregulation of the miR-200 family by three hours following ischemic preconditioning in adult male mice (Lee *et al.*, 2010). Our recent studies revealed significant upregulation of miR-200a and miR-200b by 8 hours following focal ischemia in both male and female mouse cortex (Lusardi *et al.*, 2014). It is also interesting to note that the miR-200a and miR-141 sequences contain a single different seed sequence (AACACUG) compared with the other miR-200 family members (AAUACUG), and may bind to substantially different target genes.

Bioinformatic analysis of the miR-200 miRNAs revealed target genes that function as transcriptional regulators associated with hormonal responses and sex differences to injury such as the CBP/CREBBP, MAPKs, and STAT proteins. CBP protein is widely expressed in brain, and is found in high levels in the hypothalamus, preoptic area, thalamus, amygdala, hippocampus, cortex, and cerebellum (Stromberg *et al.*, 1999). CBP functions as a nuclear

receptor coactivator that acts in concert with the steroid receptor coactivator-1 (SRC-1) to enhance estrogen receptor and progesterone receptor transcriptional activity *in vitro* (Smith *et al.*, 1996) and *in vivo* (Molenda *et al.*, 2002). Both neuron-specific RNA interference and neurons derived from CBP heterozygous knockout mice showed increased damage after oxygen-glucose deprivation (OGD) *in vitro* (Yildirim *et al.*, 2014). Studies also show that MAPK/ERK signaling is critical for estradiol neuroprotection in focal and global ischemia (Lebesgue *et al.*, 2009).

One limitation of these studies is the lack of potentially new miRNAs not included in the current TLDA arrays. While the current version of the TLDA array contains probes based on miRBase 15, there are 728 mature rat miRNA sequences annotated in miRBase 20 (June 2013 Release). In this study, reannotation of the miRNA probes from the TLDA arrays to identify rat-specific probes present in the current miRBase 20 resulted in a total of 373 probes that are 100% identical to miRBase 20. The curators of miRBase have commented on the need for “high confidence” confirmation of newly annotated miRNAs. MiRBase is a community resource with an inclusive policy that accepts data from manuscripts describing novel miRNAs as the primary requirement for deposition of sequences in the database. Since 2007, the overwhelming majority of miRNAs deposited in miRBase have been predicted from small RNA deep sequencing experiments, which can predict hundreds of novel miRNAs. However, a small number of poorly performed analyses can swamp the *bona fide* miRNA gene set with dubious annotations, as claimed in (Brown *et al.*, 2013; Hansen *et al.*, 2011; Meng *et al.*, 2012; Wang *et al.*, 2011). Given that the most common reason that a sequence misses out on being called “high confidence” is insufficient reads (<10) mapping to both arms of the hairpin precursor, however, the miRBase curators have developed a new method to automatically categorize a subset of miRNAs in miRBase as “high confidence” using the aggregated read data (Kozomara *et al.*, 2014).

In conclusion, our studies support that there is differential expression of miRNAs during development in male and female rat cortex. We are currently validating expression of additional miRNAs identified in these studies, and identifying the targets of and performing functional assays for these miRNAs to determine their role in sex-specific outcomes to ischemia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- MicroRNAs are differentially expressed during development in male and female rats.
- There is a developmental switch in expression of the miR-200 family.
- At P0, expression of the miR-200 family is higher in females than males.
- At P7 and adult, expression of the miR-200 family is higher in males than females.
- The miR-200 targets include the Gonadotropin Releasing Hormone Receptor pathway.

Figure 1. Venn diagram of rat miRNA probes in the TLDA and NanoString platforms
The diagram shows the number of rat miRNA probes in the TLDA (353) and NanoString (423) platforms, and the number of miRNA probes in common to both platforms (280).

Figure 2. Expression of the miR-200 family of miRNAs in male and female cortex during development

A. Expression of miR-200a, miR-200b, and miR-429 in male (black) and female (gray) presented as Ct values from the TLDA rodent arrays (n=5 pooled cortices/sex/stage). B. Expression of miR-200a, miR-200b, and miR-429 from individual male (black) and female (gray) (n=2 individual cortices/sex/stage) presented as femtomolar (fm) concentration values from the rat NanoString platform. C. Expression of miR-200a, miR-200b, and miR-429 in male (black) and female (gray) presented as mean Ct $\pm$ SE from individual qRT-PCR assays (n=5 individual cortices/sex/stage).

Table 1
MicroRNAs differentially expressed in male and female cortex during development

The table lists miRNAs identified in rat cortex with expression greater in males versus females, and for these with expression greater in females versus males, at developmental stages P0, P7, and adult rat. MiRNAs are annotated from miRBase 20, and the Accessions are the unique MIMAT numbers for each miRNA. The list includes miRNAs unique to male or female at each developmental stage, those miRNAs that show a developmental switch in expression (the miR-200 family of miRNAs (indicated by bold font) and miR-875 (indicated by italic font), and miR-935 that shows higher expression in males at all developmental stages (indicated by *).

Stage	Male > Female		Female > Male	
	rno-miR	rno Accessions	rno-miR	rno Accessions
P0	miR-129-5p	MIMAT0000600	miR-107-3p	MIMAT0000826
	miR-148b-5p	MIMAT0004645	miR-141-3p	MIMAT0000846
	miR-22-3p	MIMAT0000791	miR-143-3p	MIMAT0000849
	miR-299a-5p	MIMAT0000901	miR-182	MIMAT0005300
	miR-32-5p	MIMAT0000811	miR-183-5p	MIMAT0000860
	miR-326-3p	MIMAT0000560	miR-193-3p	MIMAT0000868
	miR-329-3p	MIMAT0000566	miR-196a-5p	MIMAT0000871
	miR-330-5p	MIMAT0004641	miR-200a-3p	MIMAT0000874
	miR-338-3p	MIMAT0000581	miR-200b-3p	MIMAT0000875
	miR-339-5p	MIMAT0000583	miR-327	MIMAT0000561
	miR-499-5p	MIMAT0003381	miR-336-5p	MIMAT0000576
	miR-582-5p	MIMAT0012833	miR-429	MIMAT0001538
	miR-935*	MIMAT0012845	<i>miR-875</i>	MIMAT0012842
P7	miR-10a-5p	MIMAT0000782		
	miR-125a-3p	MIMAT0004729		
	miR-141-3p	MIMAT0000846	miR-1188-5p	MIMAT0017854
	miR-142-5p	MIMAT0000847	miR-421-5p	MIMAT0001320
	miR-182	MIMAT0005300	miR-673-5p	MIMAT0005328
	miR-183-5p	MIMAT0000860	miR-743b-3p	MIMAT0005280
	miR-196a-5p	MIMAT0000871	<i>miR-875</i>	MIMAT0012842
	miR-200a-3p	MIMAT0000874		
	miR-200b-3p	MIMAT0000875		
	miR-20b-3p	MIMAT0003212		
	miR-211-5p	MIMAT0000882		
	miR-219a-2-3p	MIMAT0005446		
	miR-32-5p	MIMAT0000811		
	miR-347	MIMAT0000598		
	miR-361-5p	MIMAT0003117		
	miR-429	MIMAT0001538		
	miR-490-3p	MIMAT0012823		

	Male > Female		Female > Male	
Stage	rno-miR	rno Accessions	rno-miR	rno Accessions
	miR-743a-3p	MIMAT0005334		
	miR-874-3p	MIMAT0005284		
	miR-879-5p	MIMAT0005287		
	miR-935*	MIMAT0012845		
Adult	miR-141-3p	MIMAT0000846		
	miR-152-3p	MIMAT0000854		
	miR-200a-3p	MIMAT0000874	miR-125b-1-3p	MIMAT0004730
	miR-200b-3p	MIMAT0000875	miR-154-5p	MIMAT0000856
	miR-327	MIMAT0000561	miR-183-5p	MIMAT0000860
	miR-342-5p	MIMAT0004652	miR-204-5p	MIMAT0000877
	miR-350	MIMAT0000604	miR-300-3p	MIMAT0000902
	miR-351-5p	MIMAT0000608	miR-345-5p	MIMAT0000594
	miR-429	MIMAT0001538	miR-485-5p	MIMAT0003203
	miR-668	MIMAT0012839	miR-493-3p	MIMAT0003191
	miR-743a-3p	MIMAT0005334	miR-500-3p	MIMAT0005321
	miR-873-5p	MIMAT0005339	miR-598-5p	MIMAT0005324
	miR-875	MIMAT0012842		
	miR-935*	MIMAT0012845		

Table 2
Predicted targets of miR-200a, miR-200b, and miR-429

The miRNA target prediction from microRNA.org revealed 259 predicted gene targets of the miR-200a, miR-200b, and miR-429. PANTHER pathway analysis identified the categories most targeted by these miRNAs as proteins in the Gonadotropin releasing hormone receptor pathway, the EGF receptor signaling pathway, Huntington disease, and the Wnt signaling pathways. Parenthesis indicates the accession number for each protein category.

Protein Category (Accession)	# Genes	% of Gene hit vs. total # Genes	% of Gene hit vs. total # Protein Class hits
Gonadotropin releasing hormone receptor pathway (P06664)	34	2.80%	5.70%
EGF receptor signaling pathway (P00018)	22	1.80%	3.70%
Huntington disease (P00029)	21	1.70%	3.50%
Wnt signaling pathway (P00057)	19	1.50%	3.20%
Integrin signaling pathway (P00034)	15	1.20%	2.50%
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	15	1.20%	2.50%
FGF signaling pathway (P00021)	15	1.20%	2.50%
p53 pathway (P00059)	14	1.10%	2.30%
Parkinson disease (P00049)	14	1.10%	2.30%
Interleukin signaling pathway (P00036)	14	1.10%	2.30%
Apoptosis signaling pathway (P00006)	12	1.00%	2.00%
Angiogenesis (P00005)	12	1.00%	2.00%
TGF-beta signaling pathway (P00052)	12	1.00%	2.00%
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	12	1.00%	2.00%
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	12	1.00%	2.00%
PDGF signaling pathway (P00047)	11	0.90%	1.80%
Dopamine receptor mediated signaling pathway (P05912)	11	0.90%	1.80%
Nicotinic acetylcholine receptor signaling pathway (P00044)	10	0.80%	1.70%
Metabotropic glutamate receptor group III pathway (P00039)	9	0.70%	1.50%
p53 pathway feedback loops 2 (P04398)	9	0.70%	1.50%
Alzheimer disease-presenilin pathway (P00004)	8	0.60%	1.30%
Alzheimer disease-amyloid secretase pathway (P00003)	8	0.60%	1.30%
VEGF signaling pathway (P00056)	8	0.60%	1.30%
T cell activation (P00053)	8	0.60%	1.30%
Ionotropic glutamate receptor pathway (P00037)	8	0.60%	1.30%
Nicotine pharmacodynamics pathway (P06587)	8	0.60%	1.30%
Cytoskeletal regulation by Rho GTPase (P00016)	8	0.60%	1.30%
Adrenaline and noradrenaline biosynthesis (P00001)	7	0.60%	1.20%
Ras Pathway (P04393)	7	0.60%	1.20%
Blood coagulation (P00011)	7	0.60%	1.20%

Protein Category (Accession)	# Genes	% of Gene hit vs. total # Genes	% of Gene hit vs. total # Protein Class hits
5HT2 type receptor mediated signaling pathway (P04374)	7	0.60%	1.20%
PI3 kinase pathway (P00048)	6	0.50%	1.00%
Oxidative stress response (P00046)	6	0.50%	1.00%
Synaptic vesicle trafficking (P05734)	6	0.50%	1.00%
p53 pathway by glucose deprivation (P04397)	6	0.50%	1.00%

Table 3**Proteins in the GNRH Pathway**

The table includes proteins in the Gonadotropin releasing hormone receptor pathway (GNRH) that are potential targets of the miR-200 family of miRNAs. The list includes the Gene symbol, the Gene Name, and the Gene Ontology (GO) Molecular Function.

Gene	Gene Name	GO Molecular Function
Adcyap1	Pituitary adenylate cyclase-activating polypeptide	neuropeptide hormone activity
Adipor1	Adiponectin receptor 1, isoform CRA_a	receptor activity
Anxa5	Annexin A5	
Ascl1	Achaete-scute homolog 1	sequence-specific DNA binding transcription factor activity
Atf3	Cyclic AMP-dependent transcription factor ATF-3	sequence-specific DNA binding transcription factor activity
Cacna1c	Voltage-dependent L-type calcium channel subunit alpha-1C	voltage-gated calcium channel activity
Crebbp	CREB-binding protein	transcription cofactor activity
Drd2	D(2) dopamine receptor	G-protein coupled receptor activity
Dusp1	Dual specificity protein phosphatase 1	phosphoprotein phosphatase activity
Gata2	Endothelial transcription factor GATA-2	sequence-specific DNA binding transcription factor activity
Gata4	Transcription factor GATA-4	sequence-specific DNA binding transcription factor activity
Gnai3	Guanine nucleotide-binding protein G(k) subunit alpha	pyrophosphatase activity; adenylate cyclase activity
Gnb1	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	GTPase activity
Id2	DNA-binding protein inhibitor ID-2	sequence-specific DNA binding transcription factor activity
Irs1	Insulin receptor substrate 1	
Itpr1	Inositol 1,4,5-trisphosphate receptor type 1	receptor activity
Jun	Transcription factor AP-1	sequence-specific DNA binding transcription factor activity
Map2k6	Mitogen-activated protein kinase kinase 6	
Map3k8	Mitogen-activated protein kinase kinase kinase 8	
Mapk14	Mitogen-activated protein kinase 14	protein kinase activity
Mapk9	Mitogen-activated protein kinase 9	protein kinase activity
Nab1	NGFI-A-binding protein 1	
Nfya	Nuclear transcription factor Y subunit alpha	sequence-specific DNA binding transcription factor activity
Nr3c1	Glucocorticoid receptor	ligand-activated sequence-specific DNA binding RNA polymerase II transcription factor activity
Pcaf	Protein Pcaf (Fragment)	acetyltransferase activity
Pla2g4a	Cytosolic phospholipase A2	phospholipase activity
Prkab1	5'-AMP-activated protein kinase subunit beta-1	kinase activity
Ptk2b	Protein-tyrosine kinase 2-beta	non-membrane spanning protein tyrosine kinase activity
Rap1b	Ras-related protein Rap-1b	GTPase activity
Scg2	Secretogranin-2	neuropeptide hormone activity
Smad2	Mothers against decapentaplegic homolog 2	sequence-specific DNA binding transcription factor activity
Smad5	Mothers against decapentaplegic homolog 5	sequence-specific DNA binding transcription factor activity
Tgfb2	Transforming growth factor beta-2	growth factor activity