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Effect of (S)-3,5-DHPG on MicroRNA Expression in Mouse Brain

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Abstract

MicroRNAs are small non-coding RNAs that regulate post-transcriptional gene expression. In the short time since the discovery of microRNAs, the literature has burgeoned with studies focused on the biosynthesis of microRNAs, target prediction and binding, and mechanisms of translational repression by microRNAs. Given the prominent role of microRNAs in all areas of cell biology, it is not surprising that microRNAs are also linked to human diseases, including those of the nervous system. One of the least-studied areas of microRNA research is how their expression is regulated outside of development and cancer. Thus, we examined a role for regulation of microRNAs by neurotransmitter receptor activation in mouse brain. We focused on the group I metabotropic glutamate receptors by using intracerebroventricular injection of the selective agonist, (S)-3,5-dihydroxyphenylglycine (DHPG) in mouse brain. We then examined the expression of microRNAs in the cerebral cortex by Ambion and Invitrogen microarrays, and the expression of mature microRNA sequences by SABiosciences qPCR arrays, at 4, 8 and 24 hours after DHPG injection. These studies revealed that the largest number of significantly regulated microRNAs was detected 8 hours after DHPG injection in the microarrays and qPCR arrays. We then used RNA blots to quantify microRNA expression, and *in situ* hybridization to examine cellular distribution of the microRNAs regulated by DHPG. Bioinformatic analysis of the microRNAs regulated 8 hours after DHPG in all three arrays revealed KEGG pathways that are known to correlate with group I mGluR effects, as well as recently described and novel pathways. These studies are the first to show that DHPG regulates the expression of microRNAs in mouse cerebral cortex, and support the hypothesis that group I mGluRs may regulate microRNA expression in mouse brain.

INTRODUCTION

MicroRNAs are a class of small, non-coding RNAs that function to regulate post-transcriptional gene expression. MicroRNAs bind to complementary sequences in the 3'-untranslated region (3'UTR) of target messenger RNA (mRNA) transcripts, resulting in translational repression and/or accelerated mRNA destabilization (Guo, et al., 2010). The miRBase Sequence Database Release 17 contains 16,772 entries representing hairpin precursor miRNAs, expressing 19,724 mature miRNA products, in 153 species (<http://www.mirbase.org/>) (Griffiths-Jones, et al., 2008). Approximately one-half of microRNA genes are contained within introns of protein-coding transcripts, and they can be differentially processed from the sense and antisense strands of the same hairpin RNA or

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transcripts from the same locus (Amaral, et al., 2008). MicroRNAs are transcribed into primary microRNA transcripts, cleaved to 60–70 nucleotides in the nucleus, and the resulting precursor microRNAs are actively transported to the cytoplasm. There they are cleaved by endonucleases such as *Dicer* to produce mature microRNAs which bind to ribonucleoproteins to form RNA-induced silencing complexes (RISCs). MicroRNAs in RISCs target ~60% of mammalian genes (Friedman, et al., 2009).

There has been intense focus on determining the mechanisms of microRNA-regulated post-transcriptional gene expression, and their roles in development, brain function, and brain disorders (Potkin, et al., 2010, Provost, 2010, Rachidi and Lopes, 2010, Satoh, 2010, Saugstad, 2010, Sonntag, 2010, Yelamanchili and Fox, 2010). However, less attention has focused on the mechanisms which regulate microRNA expression. A recent study revealed that long-term potentiation induced by high-frequency stimulation of the medial perforant pathway and activation of mGluRs and NMDARs resulted in differential regulation of primary and mature microRNAs (Wibbrand, et al., 2010). Thus, we examined the effect of intracerebroventricular (ICV) injection of the group I mGluR-selective agonist, (*S*)-3,5-dihydroxyphenylglycine (DHPG), on microRNA expression in mouse brain. We then used Ambion and Invitrogen microRNA microarrays, as well as the SABiosciences quantitative PCR (qPCR) arrays to profile microRNA expression in total RNA isolated from the cerebral cortex. Further, we used *in situ* hybridization to examine the anatomical distribution, and RNA blot analysis to quantify expression of, select microRNAs in DHPG-treated mouse brain. In addition, we used KEGG analysis to examine pathways that are potentially regulated by the microRNAs significantly altered by DHPG. These studies are the first to show that DHPG regulates the expression of microRNAs in mouse cerebral cortex, and support a potential role for group I mGluRs in the regulation of microRNA expression in mouse brain.

MATERIALS AND METHODS

Materials

DHPG was purchased from Tocris Bioscience (Ellisville, MO). The *mirVana*[™] miRNA Isolation Kits were purchased from Applied Biosystems/Ambion (Austin, TX). The Mouse MicroRNA Genome V2.0 PCR Arrays (MAM-200C) were purchased from SABiosciences (Frederick, MD). The 5′ Digoxigenin (DIG)-labeled Locked Nucleic Acid (LNA) detection probes (scrambled: #99004, hsa-miR-132: #38031, mmu-miR-463: #39591, mmu-miR-709: #39324, has-miR-200c: #38536, hsa-miR-19a: #18091; U6: #99002) were purchased Exiqon Inc. (Woburn, MA).

Experimental Design

We used microarrays, qPCR arrays, *in situ* hybridizations, and RNA blots to analyze the expression of microRNAs in mouse brain after ICV injection of DHPG. A summary of the experimental design for all studies is listed in Table 1. Sample groups included five group of mice: (i) naïve control, (ii) saline injected - 8 hours, (iii) DHPG injected - 4 hours, (iv) DHPG injected - 8 hours, and (v) DHPG injected - 24 hours, prior to sacrifice.

Intracerebroventricular Injection of DHPG

Adult male C57BL/6J mice (25–30 g; Charles River Laboratories, Wilmington, MA) were housed and cared for in the Animal Care Facility of Legacy Research, and all procedures were performed in an AAALAC accredited facility in accordance with approved Institutional Animal Care and Use Committee (IACUC) protocols and principles outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. For ICV injection, the mice were anesthetized with 4% isoflurane (Butler, Dublin, OH),

ventilated with 1.5% isoflurane (70% N₂O, 30% O₂), and rectal and temporalis muscle temperature was maintained at 37°C±0.2°C. A 24-gauge, borosilicate glass Hamilton Syringe (7105KH) was stereotactically placed (Kopf, David Kopf Instruments, Tujunga, CA) in the right and left lateral ventricle of the brain. The stereotaxic coordinates used were: -0.22 mm anterior-posterior (AP) to the Bregma, +/- 1.00 mm medial to lateral (ML), and -2.5 mm dorsal to ventral (DV) (Paxinos and Franklin, 2007). 1 µL of physiological saline (Baxter, Deerfield, IL) or 1 µL of 250 µM DHPG (Tocris) was injected into each lateral ventricle, then the mice were placed in a holding cage for up to 24 hours after DHPG injection and monitored for signs of seizure or other distress. For biochemical studies, the mice were overdosed with isoflurane, the cerebral cortices dissected out and frozen in -20°C methylbutane then the tissues were stored at -80°C until further use. For *in situ* hybridization studies, the mice were deeply anesthetized using isoflurane and transcardially perfused with fresh filter-sterilized 4% paraformaldehyde (PFA) (Sigma-Aldrich, St. Louis, MO) in 1X Tris-buffered saline (TBS, pH 7.0). The whole brain was isolated and incubated at 4°C overnight in fresh 4% PFA in 1X TBS, cryoprotected by incubation in sterile 30% sucrose in 1X TBS for 72 hours, then the tissues were frozen on dry ice and stored at -80°C until further use.

RNA Isolation and Quality Assessment

Total RNA was isolated from the right and left mouse cortex using the mirVana™ kit (Ambion, Austin, TX) according to the manufacturer's instructions and as previously described (Lusardi, et al., 2010). For quantification of microRNA expression, we used the non-radioactive RNA blot protocol as described by Kim et al., (Kim, et al., 2010). The RNA sample concentrations were measured at 260 nm on a NanoDrop2000 spectrophotometer (Thermo Scientific, Wilmington, DE). The quality of each sample was assessed by visualization of RNA distinct 18S and 28S, rRNA and tRNA bands separated on a 1.0% agarose gel in 1X Tris-borate-EDTA (TBE) and detected using the UV transilluminator on a Kodak RT2000 Image station coupled to Kodak 1D 3.6 Network software (Kodak, New York, NY).

Ambion and Invitrogen MicroRNA Microarray

All samples were profiled on the same miRNA-AI (Ambion and Invitrogen) array platform comprised of the Ambion mirVana Probe Set V2 (Ambion/Applied Biosystems) and the Invitrogen NCode Multispecies miRNA Probe Set V2 (Invitrogen Corporation, Carlsbad, CA) at the NIH Microarray Consortium facility at Duke University (Durham, NC). This array combined probes from Ambion mirVana Set2 and Invitrogen NCode multispecies miRNA probe set V2 which contains 1,809 probes including 795 human, 487 mouse, and 282 rat miRNAs, respectively. RNA labeling and hybridization were completed according to the manufacturer's instructions. Briefly, RNA from each of the 21 experimental samples (Table 1) was labeled with Cy5 using miRCURY LNA miRNA Array labeling kit (Exiqon, Woburn, MA). In addition, a pool of the 5 control samples was labeled with Cy3 as a reference for each of the 21 experimental samples. After being labeled, the samples were loaded onto the microarray slides and incubated 16–18 h at 60°C. After hybridization, the slides were washed, dried by centrifugation, and scanned using the Axon GenePix 4000B Scanner (Axon, Sunnyvale, CA). Intensity for the Cy5 and Cy3 channels were set so that the total signal in each channel would be equal.

MicroRNA Microarray Analysis

The miRNA microarray data were analyzed using a Web based miRNA microarray analysis program created by Rob Lusardi (Slowdog Software, Portland, OR, USA)(Lusardi, et al., 2010). The ratio of the median intensities for each signal was calculated: 1 indicated equal quantities of target miRNA in the experimental and pooled control samples, < 1 reduced

quantities of target miRNA in the experimental sample, and > 1 increased quantities of target miRNA in the experimental sample, relative to the pooled controls. Each ratio was $\log_2$ -transformed to produce a normally distributed data set amenable to standard statistical analysis: average log ratio (ALR) of miRNA expression = $\log_2(\text{Cy5/Cy3})$, where Cy5 and Cy3 were the probe intensities of a single miRNA in the individual experimental and pooled control samples, respectively. Preliminary analysis confirmed consistent results among each of the replicates within an experimental group. Student's t-Test was used to identify microRNAs that were significantly changed 4 hours, 8 hours, or 24 hours after bilateral DHPG injection and 8 hours after bilateral saline injection as compared to control mice.

The Ambion multispecies probe set was designed based on mirBase Version 6, and the Invitrogen multispecies set on Version 7. For each probe, we reannotated the probe identifier based on sequences in mirBase Version 16. Only probes with a full length match to a mature mirBase V16 mouse microRNA sequence (MIMAT) were considered for further analysis. We present both the reannotated mirBase ID (MIMAT ID) and mature microRNA (mmu-miR) identifiers in Table 2, as well as the original vendor code for reference. In Supplemental Files S1 and S2, we also include vendor species identification. Based on the sequences provided by the vendors for each probe, some probes (with unique vendor codes) mapped to the same mature microRNA. In addition, many microRNAs were represented in both the Ambion and Invitrogen probe sets, resulting in further overlap. For this analysis, we considered each probe to be individual. For example, mir-124 matched to multiple probes within both the Ambion and Invitrogen sets; each probe is presented individually.

SABiosciences qPCR MicroRNA Array

Mouse MicroRNA Genome V2.0 PCR Arrays (MAM-200C; SABiosciences) were used to quantitatively assay mature microRNA expression in mouse brain. The arrays consisted of the 528 most abundantly expressed and best characterized microRNA sequences in the mouse genome, as annotated by the Sanger miRBase Release 14. For qPCR array analysis we used pooled total RNA samples ($n=4$ each group, Table 1) representing control, 4 hours DHPG, 8 hours DHPG, and 24 hours DHPG treatment groups. The RT2 miRNA First Strand Kit (SABiosciences) was used for Reverse Transcription (RT) of the RNAs, as per the manufacturer's instructions. Total RNA from four mice were pooled for each group (control, 4 hour, 8 hour and 24 hour DHPG). Then 2 μg of pooled total RNA was incubated in RT buffers at 37°C for 2 hours, followed by 95°C for 5 minutes to degrade the RNA and to inactivate the reverse transcriptase. The first-strand cDNA samples were chilled on ice then diluted with RNase-free water. The RT2 SYBR green master mix (PA-012) was used for the qPCR reactions, as per the manufacturer's instructions (SABiosciences). Briefly, the diluted first-strand cDNA was combined with master mix, then 25 μL was aliquoted into each well of six 96 well plates. The six plates were briefly centrifuged then stored at -20°C . For amplification, individual plates were removed from -20°C , defrosted for 5 minutes at RT, briefly centrifuged and placed into a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Parameters were set to: (i) one cycle at 95°C for 10 minutes, (ii) 40 cycles at 95°C for 15 seconds, 60°C for 40 seconds, and 72°C for 30 seconds. A dissociation step set to: (iii) 95°C for 15 seconds; 60°C for 1 minute and 95°C for 15 seconds was performed to ensure that all PCRs generated a single product.

SABiosciences qPCR MicroRNA Array Normalization

Mature microRNA values were first normalized by the equation $\Delta\text{Ct} = (\text{Ct}_{\text{moi}} - \text{Ct}_{\text{hskp}})$, where moi refers to the microRNA of interest and hskp refers to the housekeeping gene. Changes in microRNA expression with respect to the control sample were calculated by the equation $\Delta\Delta\text{Ct} = (\Delta\text{Ct}_{\text{timepoint}} - \Delta\text{Ct}_{\text{control}})$; thus, microRNA values increased at a time point will be negative, those decreased will be positive. Fold changes were calculated as

$2^{(-\Delta\Delta Ct)}$. We considered several options for the housekeeping value, including: (i) the average of all probes in an experiment, analogous to the microarray normalization method; (ii) the average of probes that do not change across experimental groups, as recommended by SABiosciences; and (iii) small nucleolar RNAs (Snord85, Snord68, and Snord66) and U6 small nuclear RNA (Rnu6) provided on each plate to serve as unchanging normalization factors. We found that the values of $\Delta\Delta Ct$ at 4 hours and 24 hours were normally distributed and centered around zero regardless of the normalization method (Supplemental file S3). At 8 hours, however, the normalization method had a profound effect on the distribution of $\Delta\Delta Ct$, potentially affecting the interpretation of the regulation of microRNA changes. Specifically, using the average of all probes to normalize results in similar numbers of increased and decreased microRNAs, while the non-changing probes and Snord85 results in a negative bias, and Rnu6 as a normalizing factor results in a slight positive bias. We determined significance as any probe with $\Delta\Delta Ct \geq 1.5$ standard deviations from the experimental average. We found that while the absolute magnitude of $\Delta\Delta Ct$ changed with normalization method, the significance and regulation of significant probes was insensitive to normalization methods. Therefore, we selected the Rnu6 probe for our housekeeping gene to match our RNA blot analysis.

MicroRNAs and KEGG Pathway Analysis

We used the mirPath target prediction tool from DIANA (www.diana.cslap.ece.ntua.gr) to predict functional pathways regulated by our significantly regulated microRNAs (Papadopoulos, et al., 2009), accessed on December 9, 2011. At the 8 hour time point, we evaluated 6 independent microRNA lists: significantly increased and decreased probes from the Ambion probe set, Invitrogen probe set, and the SABiosciences qPCR array. For each set, we considered only probes with a perfect match (including 3' and 5' strand, and mouse species). The resulting KEGG pathways for each of the six sets were filtered to include only those with reported $p \leq 0.05$ ($-\ln(p) > 3.0$). The lists were integrated by microRNA regulation (increased and decreased), then further limited to those KEGG pathways significantly regulated by all three probe sets. DIANA reports the cumulative number of microRNA – gene interactions within a given pathway for a set of input microRNAs, though more genes do not always correlate to higher significance. The sum of genes across the three probe sets was the reference used to sort the resulting pathways.

MicroRNA In Situ Hybridization

PFA-fixed mouse brains were cut into 25 μM sections using a Cryostat CM3050S (Leica Microsystems Inc., Bannockburn, IL) and stored at -80°C . For *in situ* hybridization, the brain sections were removed from -80°C and air-dried at room temperature (at least one hour). The sections were fixed by incubation in imidazole buffer (0.13 M 1-methylimidazole, 300 mM NaCl pH 8.0) twice for 10 minutes, followed by incubation in 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (EDC; Thermo Fisher Scientific, Rockford, IL) solution (16 M EDC, 0.13 M 1-methylimidazole, 300 mM NaCl pH 8.0) for 1 hour at 28°C . The slides were washed once in 0.2 % (w/v) glycine in 1X TBS for 5 minutes, then twice in 1X TBS for 5 minutes each wash. The sections were acetylated by incubation in 0.1 M triethanolamine, 0.5 % (v/v) acetic anhydride for 10 minutes, followed by 3 washes in 1X TBS for 5 minutes each wash. The 5'-DIG-labeled LNA microRNA detection probes were diluted 1:133 in hybridization buffer (50% formamide, 0.3 M NaCl, 20 mM Tris HCl pH 8.0, 5 mM EDTA, 10 mM NaPO_4 pH 8.0, 10% dextran sulfate, 1 $\times$ Denhardt's solution, and 0.5 mg/mL yeast tRNA) and heated at 65°C for 5 minutes, then chilled on ice. Each section was incubated in the miR-probe solution covered with Hybri-slips (Sigma-Aldrich Corp., St. Louis, MO) overnight at 54°C in a sealed container and humidified with tissues soaked in 50% formamide in 1X saline-sodium citrate (SSC) buffer.

After hybridization, the slides were immersed in 5X SSC to remove the coverslips for 15 minutes, then washed twice in 50% formamide, 1X SCC-Tween for 25 minutes; once in 0.2X SSC for 15 minutes; once in 1X TBS for 15 minutes. A Super PAP Pen (Cancer Diagnostics, Inc., Birmingham, MI) was used to draw a circle around each section to provide surface tension to hold antibody solution in place. The sections were then incubated in blocking solution (1X Blocking Reagent, Roche) for 1 hour at room temperature in a humidified chamber, followed by incubation in blocking solution containing a 1:1500 dilution of Alkaline Phosphatase (AP)-conjugated anti-DIG Fab fragment (Roche Applied Science, Indianapolis, IN) for 2 hours at room temperature in a humidified chamber. The slides were washed twice in 1X TBS-Tween for 20 minutes, then twice in 1X TBS for 20 minutes, and the sections were incubated in BM Purple AP substrate (Roche) for 1–3 days in the dark. AP substrate reactions were terminated by washing the slides in 1 mM EDTA, 1X TBS for 10 minutes, followed by a 2 minute wash in deionized water. Sections were stained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA) then washed and dehydrated by consecutive washes in increasing concentrations of EtOH (75–95%) and mounted using Eukitt Mounting Medium (Electron Microscope Sciences, Hatfield, PA). Images were collected on a Leica DM1000 microscope equipped with a DFC295 camera, and images processed using the Leica Application Suite software version 3.7.0 (Leica Microsystems Inc., Bannockburn, IL).

MicroRNA Blot Analysis

RNA samples were separated on 15% denaturing PAGE gels using the SequaGel-UreaGel System (National Diagnostics, Atlanta, Georgia). The RNA was then transferred to Roche Positively Charged Nylon Membrane (Roche Applied Science, Indianapolis, IN) in 1X TBE. The RNA was cross-linked to the membrane by incubation in 16 M EDC, 0.13 M 1-methylimidazole, 300 mM NaCl pH 8.0 at 60°C for 2 hours. The membranes were incubated in ULTRAhyb® Ultrasensitive Hybridization Buffer (Applied Biosystems, Foster City, CA) at 37°C for 30 minutes in a hybridization oven. The membranes were incubated with fresh hybridization buffer containing 5'-DIG-labeled LNA microRNA detection probes at a final concentration of 0.5 nM. Membranes were incubated overnight at 37°C with slow rotation. The blots were washed and detected using the DIG Wash and Block Buffer Set (Roche). The membranes were washed twice with Low Stringent Buffer (2XSSC, 0.1% SDS) at 37°C for 15 minutes, twice with High Stringent Buffer (0.1XSSC, 0.1% SDS) at 37°C for 5 minutes, rinsed with Washing Buffer at 37°C for 10 minutes, then incubated in fresh 1X Blocking Buffer in 1X Maleic acid for 3 hours at room temperature. The membranes were incubated in fresh blocking buffer containing a 1:15,000 dilution of Anti-Digoxigenin-AP (DIG), Fab fragments (Roche; #11093274910) at room temperature for 30 minutes. The membranes were then washed four times for 15 minutes in 1X DIG Washing buffer. Membranes were incubated in 1X Detection Buffer for 5 minutes then incubated with 1:100 dilution of CSPD Disodium Phosphate (Roche) in Detection Buffer at room temperature for 5 minutes. The membranes were placed in a heat-sealed bag for 15 minutes at 37°C in the dark. RNA bands were imaged using a Kodak Imaging System and quantified using ImageJ. Membranes were stripped and reprobbed with U6 which served as a loading control.

RESULTS

Regulation of MicroRNAs by DHPG Detected by Ambion and Invitrogen Microarrays

We used the Ambion/Invitrogen (AI) microarray to analyze the relative expression of all microRNA forms expressed in naïve, saline-injected (8 hours), and DHPG-injected (4, 8 and 24 hours) mice. The AI arrays contain the complement to mature microRNA sequence, thus they will detect mature sequences regardless of whether they are part of a mature, precursor, or primary microRNA. Of the 421 microRNA probes on the AI microarray set, a total of 226

were detected in our experiments. Analysis of the microarray data in the Ambion dataset (Supplemental File S1) revealed those microRNAs that are significantly regulated by DHPG at each time point, as depicted by Venn diagram in Figure 1A ($p < 0.05$). Analysis of the microarray data in the Invitrogen dataset (Supplemental File S1) revealed those microRNAs that are significantly regulated by DHPG at each time point, as depicted by Venn diagram in Figure 1B ($p < 0.05$). Together, these two distinct array platforms revealed that *in vivo* activation of group I mGluRs by the selective agonist DHPG leads to significant changes in microRNA expression, with the greatest number of microRNAs altered at 8 hours after DHPG treatment. Table 2 lists the significantly regulated microRNAs detected at 4, 8, or 24 hours after DHPG treatment in the microarrays. Supplemental File 2 lists the significantly regulated microRNAs detected at 8 hours after saline treatment in the Ambion (A) and Invitrogen (B) microarrays that were likely a response to handling and surgery and were thus excluded from further analysis. The results show that the greatest number of altered microRNAs in both the Ambion and Invitrogen platforms is detected 8 hours after DHPG.

Regulation of MicroRNAs by DHPG Detected by SABiosciences qPCR Array

We used the SABiosciences qPCR array to quantify the expression of mature microRNAs in DHPG-injected mice. This array set contained 576 mouse microRNA probes; a total of 528 were detected in our experiments. Heatmaps show the regulation of the microRNAs represented in the qPCR arrays for each of the six 96-well plates in the array sets; green represents decreased expression, and red represents increased expression, of each microRNA relative to control (Supplemental File S3). Analysis of the qPCR array data revealed those microRNAs that are significantly regulated at each time point after DHPG injection (Supplemental File S4). The number and relationship of significantly regulated microRNAs at each time point are depicted by Venn diagram (Figure 1C, left). Corresponding bar graphs illustrate the number of significantly increased and decreased microRNAs at each time point (Figure 1C, right). Table 3 lists the significantly regulated microRNAs detected at 4, 8 and 24 hours after DHPG treatment in the qPCR arrays and their corresponding $\Delta\Delta Ct$ values and fold changes. The normalization methods used for the qPCR data is described in Supplemental File S5. These studies revealed that DHPG induces significant changes in microRNA expression at each time point after DHPG, with the greatest number of changes occurring 8 hours after treatment, similar to the microarray results.

In Situ Hybridization Reveals the Distribution of DHPG-Regulated MicroRNAs

We used *in situ* hybridization to examine the regional distribution of the DHPG-regulated microRNAs. The probes are directed against mature microRNA sequences, thus they will detect mature sequences regardless of whether they are part of a mature, precursor, or primary microRNA. Figure 2 shows mouse brain sections with selected microRNAs (miR-463, 709, 132, 19a, and 200c) as well as scrambled miR probe in control, 4, 8 and 24 hours after DHPG, 8 hours after saline injected, and section with no probe. The results show that miR-463, which had very low abundance in control tissue by the qPCR array ($Ct = 37$), was diffusely expressed, with increased expression at 4 and 24 hours after DHPG, consistent with the qPCR analysis. miR-709 was predicted to increase significantly at 8 hours by qPCR analysis; *in situ* hybridization demonstrates that it was expressed throughout the brain, with enhanced cytosolic expression. Further, miR-709 is increased in all time points, possibly reflecting changes in both the precursor and mature species. In addition, we found a striking increase in the saline injected brain, suggesting that miR-709 may also function in stress response. miR-132 was not predicted to change significantly in any array platform evaluated here; accordingly, the *in situ* hybridization images appear unchanged at each time point. Closer examination does show a slight increase in the hippocampal pyramidal neurons in response to DHPG, which may have been too small to be detected in our cortical tissue

preparations. miR-19a was predicted to decrease at the 8 hour time point; accordingly, the *in situ* hybridization shows lower expression at 8 hours than either the 4 or the 24 hour time point. Again, unanticipated changes in mir-19a expression at 4 and 24 hours may reflect increases in precursor expression not measured by the qPCR array. miR-200c was predicted to have a robust expression by the qPCR array, without significant changes, though the *in situ* hybridization probe did not detect any signal. We included a scrambled probe in our analysis to demonstrate non-specific binding by the *in situ* hybridization protocol, but saw no significant changes in response to DHPG. The saline injection did have a significant response with some probes which may reflect a specific stress response to handling or the surgery. The images show that microRNA expression is altered in the hippocampus and cortex for most probes, and these expression patterns loosely correlate with the qPCR array data.

RNA Blot Analysis of MicroRNA Expression in Mouse Brain

We used RNA blots to quantify microRNA expression in response to DHPG treatment. The probes are directed against mature microRNA sequences, thus they will detect mature sequences regardless of whether they are part of a mature, precursor, or primary microRNA. We focused on miR-709 and miR-132 as the mature miRs were increased and unchanged, respectively, in the qPCR array. Figure 3A shows pre-mir-132 and miR-132 detected on the RNA blot, and their expression is not significantly changed, consistent with the qPCR array. Figure 3B shows 3 forms of pre-mir-709 detected on the RNA blot, however, there is no detectable mature miR-709 band, which increased by 45 fold in the qPCR array. However, our findings are consistent with a study showing that some probes including miR-709 only detect the pre-microRNAs and do not detect the mature microRNA sequences on RNA blots (Tang, et al., 2007). Corresponding graphs show the quantitative analysis of microRNA bands relative to U6.

Pathway Analysis of Predicted Genes Targets of 8 Hour DHPG-Regulated MicroRNAs

We used mirPath, a web-based computational tool, to identify molecular pathways potentially altered by the microRNAs significantly regulated in each of the three probe sets at 8 hours after DHPG injection (Table 4). We first limited the set of KEGG pathways to those with an enrichment p value ≤ 0.05 . We further restricted the pathways of interest to those pathways identified by all three probe sets. For reference, we include the summary number of genes regulated and enrichment p value from each of the three probe sets; the number of genes and enrichment p value for each probe set are included in Supplemental File S6. In addition to pathways well known to be influenced by DHPG in the brain, such as map kinase signaling and long-term potentiation, we found several cancer pathways that have more recently been associated group I mGluR signaling (Shin, et al., 2008), evidence of the specificity of the mirPath KEGG predictions, as discussed further below.

DISCUSSION

Little is known about the response of microRNAs as a result of specific receptor signaling. Microarray studies have shown regulated expression of hippocampal microRNAs in response to the induction of mGluR-dependent LTD (Park and Tang, 2008) or LTP (Wibrand, et al., 2010). Thus, we examined the direct effects of the group I mGluR-specific agonist, DHPG on microRNA expression by ICV injection in adult mouse brain, a well-established model for examining *in vivo* responses. We profiled microRNA expression using Ambion and Invitrogen microarrays, as well as a SABiosciences qPCR array which detects mature mouse microRNA sequences. Our studies show that DHPG injection leads to the regulation of microRNA expression in mouse cerebral cortex at 4, 8 and 24 hours after treatment, with the greatest number of microRNA changes detected at 8 hours after DHPG

in all array platforms. The mechanisms for regulation of microRNA expression by DHPG are not yet known. However, potential mechanisms might include changes in gene transcription or post-translation modification of microRNA processing proteins or RNA-induced silencing complexes, or a combination of both mechanisms mediated by group I mGluR activation.

Our understanding of microRNAs, their sequences, and physiological roles is continuously expanding, as evidenced by the rapid changes in mirBase version numbers (www.mirBase.org). Changes include newly identified microRNAs, nomenclature changes, 3' and 5' strand distinctions, and deletions of sequences. To ensure that our analysis reflects the most recent understanding of microRNAs in mouse brain, we used the probe sequences provided by Ambion and Invitrogen to reannotate the microarray identifiers. We used the most stringent condition, 100% sequence match with no deletions or gaps in mouse specific mature sequences, to ensure only high confidence annotations were used. In this process, we found good correspondence among reannotated genes (Supplemental File S1). The growing repositories of microarray data provide a wealth of knowledge; however, to fully exploit this resource, it will be necessary to reannotate archived data to the most current curated version of mirBase (Git, et al., 2010).

The analysis of qPCR array data relies on a “housekeeping” gene that does not change across experimental conditions to normalize data sets. As with other quantifiable methods, the identification of a reliable unchanging reference is a continuing challenge. We examined several methods for normalization including normalizing to the average of all probes, analogous to the microarray method, by unchanging targets as recommended by the vendor, and by small nucleolar and nuclear RNAs present on all plates in the qPCR array. The housekeeping genes provided by the array manufacturer (Snord85, Snord66, Snord68, and Rnu6) did not change uniformly across experimental groups. Regardless of the normalization method selected, at 4 and 24 hours after DHPG, the ΔCt values followed a Gaussian distribution, and the $\Delta\Delta\text{Ct}$ values were centered around zero, suggesting that relatively few of the microRNAs changed significantly. However, at 8 hours, while the ΔCt distribution was still Gaussian (Supplemental File S5), the $\Delta\Delta\text{Ct}$ distribution was skewed significantly depending on the method employed, making the use of a $\Delta\Delta\text{Ct}$ threshold for significant increase or decrease difficult to establish. Therefore, we chose to determine significance based on the difference from the average $\Delta\Delta\text{Ct}$ for each time point, which was also insensitive to normalization method at each time point examined. These results highlight that, while qPCR is a quantifiable method, the selection of housekeeping genes can have a profound effect on the interpretation of “up” or “down” regulation of a specific microRNA.

We found little overlap in the microRNAs identified by microarray and qPCR array methods. This likely reflects technology differences such as the distinct probe designs used by each vendor and the evolution of mirBase. This finding is in accord with a recent rigorous study which revealed that such outcomes represent inherent problems within and between the different assays (Git, et al., 2010). The study examined identical RNA samples on 6 distinct microRNA microarrays obtained from different vendors, and found that there was little correlation between the datasets. Only 1 of 6 microarray vendors (Agilent) used probes specifically targeted to the mature microRNA sequence, while the others used probes that can detect the mature microRNA sequence but can also detect microRNA sequences in the primary and precursor transcripts. Our samples were analyzed by an array comprised of both Ambion and Invitrogen probe sets, which showed high levels of false positives and negatives in the Git study.

Perhaps the most significant difference between the two platforms is that qPCR selectively amplifies mature microRNAs and the microarrays are qualitative indicators of primary, precursor, and mature microRNA transcripts. The selection of either method must be based on specific experimental needs. For example, the cost of array processing makes the use of experimental replicates feasible in most cases. Multiple replicates makes the data amenable to familiar statistical techniques, and can reveal relatively small magnitude changes from control. The qPCR is a more precise, quantifiable method, but significantly increases the cost of for experimental or technical replicates, and requires the use of a threshold to establish significance; for difficult-to-obtain tissue, this may be a rational trade off. MicroRNA microarray and qPCR array technologies will continue to evolve, as they have for mRNA analysis. Git and colleagues also found inconsistencies between data obtained from microarrays and qPCR arrays, as well as from NextGen sequencing (Git, et al., 2010). However, even with discrepancies between the Ambion, Invitrogen, and SABiosciences qPCR array datasets, the most compelling results from these studies was that DHPG consistently induced significant changes in microRNA expression at each time point examined, with the greatest changes seen 8 hours after DHPG injection. In addition, the KEGG pathway analysis (discussed below) identified specific validated group I mGluR-associated pathways, giving credence to the specificity of the reported microRNA response to DHPG.

We examined the anatomic distribution of select microRNAs using *in situ* hybridization, and quantified microRNA expression by RNA Blot. As with the microarrays, the probes for these experiments can recognize primary, precursor, and mature forms of the microRNA. The tissue we used for both the microarrays and qPCR array consisted of the cerebral cortex, which includes both the cortex and hippocampus. The *in situ* hybridization revealed regional differences in the effects of DHPG. For example, while miR-132 was not predicted to change in the microarray platforms, there is a slight increase in the pyramidal neurons of the hippocampus, which was likely too small to be detected in the cerebral cortex preparations used in these experiments. This was further confirmed by the RNA blots, which demonstrate no significant change in the mature miR-132 bands. The diffuse distribution of the mir-19a probe follows the pattern of reduced expression at 8 hours relative to the 4 and 24 hour time point, and may reflect a dendritic localization, though additional experiments would be necessary to confirm this. While miR-709 was predicted to increase significantly by the qPCR array, and the *in situ* hybridization confirms this increased expression, there is also an increase in the saline injected brain suggesting that while miR-709 may have a DHPG specific response, it is also likely to have a role in the endogenous stress response from either the handling or the surgery.

Previous studies used deep sequencing to identify novel microRNAs in mouse embryo, including the mature miR-709 sequence (Mineno, et al., 2006). Target validation studies using luciferase reporter assays were used to examine the effect of the precursor form (mir-709), not the mature sequence, on a predicted target (Tamminga, et al., 2008). These studies suggest that a mature miR-709 might exist, or that a unidentified mature microRNA resides within the precursor mir-709 sequence. However, the RNA blot studies from our lab and others (Tang, et al., 2007) show abundant expression of precursor mir-709, but not mature miR-709, suggesting that a mature miR-709 as currently annotated in MirBase Version 16 does not exist. It is interesting to note that the annotated mature miR-709 sequence is located within 1 nucleotide of the 3' end of the precursor sequence, which might have enabled erroneous detection of a 'mature' sequence by the qPCR array primers. Thus, further studies will be necessary to validate that a mature microRNA sequence is located within the precursor mir-709 sequence in adult mouse brain, and/or to examine the possibility that precursor microRNAs such as mir-709 can serve a functional role without further processing into a mature form.

We used the bioinformatics tool mirPath by DIANA (Papadopoulos, et al., 2009) to perform *in silico* validation of the microRNA predictions for each of the probe sets used in these experiments, separating out the microRNAs that increased from those that decreased. Here, we present KEGG pathways predicted by all three of the probe sets (Ambion, Invitrogen, and SABiosciences qPCR) at 8 hours after DHPG injection. In addition to the pathways familiar to neuroscientists, there were several cancer and pancreas/insulin/diabetes related pathways. A PubMed search revealed that mGluRs have been identified both in cancers within the brain (de Groot and Sontheimer, 2011) and unassociated with nervous tissue (Li, et al., 2005, Namkoong, et al., 2007, Stepulak, et al., 2009). Also unexpected was the role of mGluRs in the regulation of insulin secretion in the pancreas (Brice, et al., 2002, Storto, et al., 2006). These results illustrate the growing potential of using *in silico* methods to validate the specificity of global assays. While this study is not translational, these results further illustrate the role of bioinformatics tools to anticipate potential unexpected drug interactions in the development of clinical therapeutics.

In conclusion, these studies show that DHPG regulates microRNAs in mouse brain, and support a potential role for mGluRs in the regulation of microRNA expression. These results warrant further studies to identify the pathways and mechanisms regulating changes in microRNA expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Temporal Regulation of MicroRNAs by DHPG

Venn diagrams (left) show the number and relationships of microRNAs significantly regulated at 4, 8 and 24 hours after DHPG detected by the Ambion (A) and Invitrogen (B) microarrays, and the SABiosciences qPCR array (C). The corresponding bar graphs (right) show the number of microRNAs that increased and decreased at each time point after DHPG treatment. Microarray significance was determined by t-test with a significance of $p \leq 0.05$; qPCR array significance was determined by a threshold greater than 1.5 standard deviations from the mean.

Figure 2. Cellular Distribution of MicroRNAs

Representative images of *in situ* hybridization for miR-463, 709, 132, 19a, 200c and scrambled miR probes (n=3 experimental replicates). Expression was evaluated in control (untreated) mouse brain and at 4, 8 and 24 hours after DHPG, and 8 hours after saline injection. Sections with no probe are included for reference.

Figure 3. RNA Blot Analysis of MicroRNA Expression

Representative RNA blots show the expression of miR-709 (A) and miR-132 (B) in naïve control (C), 8 hours (D8) and 24 hours (D24) after DHPG. The corresponding graphs show the quantification of each RNA band relative to U6.

Table 1

Experimental Design

Experimental Group	Microarray Reference	MicroRNA Microarray	Mouse Genome PCR Array	In Situ	MicroRNA Blots
Naïve Control	n=5 C1-C5 Pooled	n=5 C1-C5	n=4 C1, 2, 4 & 5 Pooled	n=3 C6-C8	n=3 C9-C11
Saline 8 hours		n=5 S1-S5		n=3 S6-S8	
DHPG 4 hours		n=4 D4,1-D4,4	n=4 D4,1-D4,4 Pooled	n=3 D4,5-D4,7	
DHPG 8 hours		n=4 D8,1-D8,4	n=4 D8,1-D8,4 Pooled	n=3 D8,6-D8,8	n=3 D8,9-D8,11
DHPG 24 hours		n=3 D24,1-D24,3	n=4 D24,1-D24,3 Pooled	n=3 D24,6-D24,8	n=3 D24,9-D24,11

Table 2A

MicroRNAs Significantly Regulated 4 Hours After DHPG

MirBase			Ambion			Invitrogen		
MIMAT ID	mmu-mir	SM Code	Sig	ΔALR	Code	Sig	ΔALR	
0000134	mir-124	10245	0.05	0.27				
0000134	mir-124	10691	0.025	0.28				
0000139	mir-127	10400	0.05	0.16				
0004324	mir-181d	11118	0.025	-0.28				
0000664	mir-219-5p	10664	0.0125	1.24				
0003151	mir-378	11360	0.05	0.44				
0000747	mir-382	10924	0.05	-0.13				
0000748	mir-383	10308	0.05	0.36				
0001090	mir-409-3p	10163	0.025	-0.46				
0003127	mir-484	10379	0.05	0.63				
0003182	mir-494	10710	0.025	-0.70				
0000523	let-7c				1268	0.05	-0.15	
0000663	mir-218				1143	0.025	-0.73	
0000743	mir-379				2276	0.05	-0.27	

Table 2B

MicroRNAs Significantly Regulated 8 Hours After DHPG

MirBase			Ambion			Invitrogen		
MIMAT ID	mmu-mir	SM Code	Sig	ΔALR	Code	Sig	ΔALR	
0000521	let-7a	10050	0.05	-0.21	1030	0.025	-0.23	
0000522	let-7b	11050	0.025	-0.26	1431	0.0125	-0.30	
0000523	let-7c	10436	0.025	-0.26	1268	0.0125	-0.22	
0000128	mir-30a	11062	0.05	-0.32	1460	0.05	-0.26	
0000704	mir-361	10085	0.0125	-0.41	2296	0.05	-0.28	
0003185	mir-369-5p	10963	0.005	-0.56	1867	0.025	-0.54	
0000740	mir-376a	10504	0.025	-0.29	1945	0.025	-0.27	
0000743	mir-379	10316	0.0025	-0.65	2276	0.0025	-0.74	
0000218	mir-24-1*	10073	0.025	0.70				
0000534	mir-26b	11070	0.05	-0.84				
0000515	mir-30d	10756	0.05	-0.25				
0000545	mir-98	10426	0.05	0.94				
0000132	mir-99b	11021	0.005	-0.34				
0000133	mir-101a	10537	0.0125	0.58				
0000546	mir-103	10632	0.025	-0.23				
0000647	mir-107	10056	0.025	-0.22				
0000134	mir-124	10691	0.0125	0.26				
0000134	mir-124	10245	0.05	0.23				
0000136	mir-125b-5p	10148	0.0125	-0.23				
0000140	mir-128	10639	0.025	-0.10				
0000140	mir-128	10159	0.0125	-0.19				
0000152	mir-140*	11041	0.025	0.77				
0004537	mir-154*	10731	0.05	0.31				
0000210	mir-181a	10421	0.005	-0.39				
0004324	mir-181d	11118	0.0025	-0.43				
0000517	mir-192	10456	0.0125	-0.49				
0000664	mir-219-5p	10664	0.0005	2.56				

MirBase			Ambion			Invitrogen		
MIMAT ID	mmu-mir	SM Code	Sig	ΔALR	Code	Sig	ΔALR	
0000669	mir-221	10337	0.005	-0.32				
0000567	mir-329	10406	0.05	0.49				
0000586	mir-340-3p	10993	0.05	0.36				
0000741	mir-377	10524	0.025	-1.06				
0000747	mir-382	10924	0.05	-0.17				
0001420	mir-433	10774	0.05	-0.29				
0003127	mir-484	10379	0.05	2.94				
0003486	mir-491	10185	0.025	0.37				
0003182	mir-494	10710	0.025	-0.94				
0000524	let-7e				1421	0.05	-0.16	
0000125	mir-23b				1930	0.025	-0.30	
0000125	mir-23b				1113	0.025	-0.27	
0000537	mir-27a				1485	0.0125	0.21	
0000126	mir-27b				2303	0.05	-0.21	
0000126	mir-27b				2304	0.05	-0.18	
0000130	mir-30b				2280	0.0125	-0.23	
0000248	mir-30e				2282	0.025	-0.37	
0000249	mir-30e*				2052	0.05	1.31	
0000386	mir-106b				1282	0.05	-0.29	
0000141	mir-130a				2005	0.0125	-0.44	
0000224	mir-194				1416	0.05	-0.32	
0000225	mir-195				1311	0.05	-0.24	
0000747	mir-382				2057	0.05	-0.21	
0001422	mir-434-3p				2338	0.0125	-0.17	
0001533	mir-448-3p				2324	0.00125	1.70	
0003184	mir-487b				1817	0.05	-0.27	
0003469	mir-690				1763	0.05	-0.61	
0003496	mir-706				1862	0.05	1.46	

Table 2C

MicroRNAs Significantly Regulated 24 Hours After DHPG

MirBase			Ambion			Invitrogen		
MIMAT ID	mmu-mir	SM Code	Sig	Δ ALR	Code	Sig	Δ ALR	
0000525	let-7f	10902	0.05	0.17				
0000527	mir-16	10339	0.025	0.27				
0000219	mir-24	10737	0.0125	0.34				
0004632	mir-29c*	11335	0.05	0.37				
0000540	mir-93	10951	0.05	0.65				
0000545	mir-98	10426	0.05	0.83				
0000133	mir-101a	10537	0.0125	0.91				
0000134	mir-124	10245	0.0125	0.40				
0000134	mir-124	10691	0.0025	0.44				
0000140	mir-128	10639	0.05	0.11				
0000146	mir-134	10341	0.025	0.90				
0000674	mir-181c	10181	0.05	0.55				
0000225	mir-195	10827	0.05	0.28				
0000664	mir-219-5p	10664	0.0125	1.89				
0001095	mir-370	10482	0.0125	0.57				
0000739	mir-375	10327	0.05	-2.74				
0000745	mir-380-3p	10619	0.00125	1.89				
0001420	mir-433	10774	0.05	-0.19				
0001419	mir-433*	10797	0.05	0.55				
0001632	mir-451	10789	0.005	0.79				
0003485	mir-455*	10529	0.0125	2.89				
0003486	mir-491	10185	0.025	0.29				
0003453	mir-497	11293	0.025	0.27				
0000521	let-7a				1428	0.025	0.10	
0000526	mir-15a				1312	0.05	0.52	
0000531	mir-22				1022	0.025	0.24	
0000537	mir-27a				1485	0.05	0.21	

MirBase			Ambion			Invitrogen		
MIMAT ID	mmu-mir	SM Code	Sig	Δ ALR	Code	Sig	Δ ALR	
0000536	mir-29c				1310	0.05	0.27	
0000746	mir-381				2171	0.05	1.17	

Table 3A

MicroRNAs Significantly Regulated 4 Hours After DHPG

MIMAT ID	Mature mmu-miR	ΔAct	Fold Change	MIMAT ID	Mature mmu-miR	ΔAct	Fold Change
0004621	let-7b*	-1.80	10.45	0004896	miR-590-3p	-3.50	7.70
0004660	miR-19a*	-2.64	5.96	0004837	miR-615-5p	-2.90	11.20
0004628	miR-21*	-3.29	8.91	0003726	miR-675-3p	-3.71	21.93
0004856	miR-105	-5.74	73.26	0003457	miR-680	-2.88	12.86
0004533	miR-141*	-2.17	6.52	0003459	miR-682	-3.09	11.51
0004826	miR-146b*	-2.01	9.09	0004189	miR-693-3p	-2.31	8.25
0005443	miR-181a-2*	-5.85	45.10	0003472	miR-693-5p	-2.37	14.67
0000518	miR-196a	-5.42	9.29	0003487	miR-697	-3.35	10.89
0004663	miR-200c*	-0.20	0.71	0004236	miR-741	-2.52	6.48
0004572	miR-290-3p	-4.20	7.29	0004838	miR-742*	-2.32	6.21
0003190	miR-291b-3p	-3.70	9.61	0004841	miR-871	-3.32	5.80
0000369	miR-292-5p	-1.82	5.72	0004855	miR-876-3p	-2.20	7.59
0000371	miR-293	-2.79	14.77	0004933	miR-878-3p	-3.49	15.62
0000372	miR-294	-3.03	11.43	0004845	miR-881*	1.10	0.56
0004574	miR-294*	-4.67	12.34	0005836	miR-1186	-3.27	17.81
0004579	miR-302a*	-4.30	11.92	0005837	miR-1187	-3.20	13.59
0003375	miR-302c*	-5.00	22.86	0005847	miR-1190	-3.17	11.67
0004643	miR-331-5p	-4.51	10.97	0005850	miR-1192	-3.31	14.98
0004656	miR-345-3p	-4.06	11.35	0005460	miR-1224	-2.33	6.75
0000739	miR-375	1.35	0.42	0007864	miR-1897-5p	-2.79	11.75
0004758	miR-463	-3.83	30.59	0007875	miR-1898	-2.92	11.92
0002104	miR-463*	-3.57	23.34	0007869	miR-1899	-2.86	7.09
0002110	miR-469	-2.47	7.70	0007870	miR-1900	-3.49	18.06
0002111	miR-470	-6.72	115.76	0009391	miR-1928	-4.62	35.38
0004888	miR-493	-3.89	16.39				

Table 3B

MicroRNAs Significantly Regulated 8 Hours After DHPG

MIMAT ID	Mature mmu-miR	ΔAct	Fold Change	MIMAT ID	Mature mmu-miR	ΔAct	Fold Change
0004858	miR-18b	3.82	0.07	0001095	miR-370	-3.79	13.83
0000651	miR-19a	3.70	0.08	0000739	miR-375	3.78	0.07
0004660	miR-19a*	-1.93	3.81	0003511	miR-450b-5p	4.04	0.06
0000513	miR-19b	3.80	0.07	0003742	miR-455	-2.04	4.11
0004628	miR-21*	-3.61	12.21	0002106	miR-465a-5p	-1.96	3.89
0004856	miR-105	-3.00	8.00	0004882	miR-466f-3p	-3.33	10.06
0004669	miR-125b-3p	-2.34	5.06	0002111	miR-470	-5.75	53.82
0004531	miR-135a*	-2.79	6.92	0002112	miR-471	-1.92	3.78
0000151	miR-140	3.62	0.08	0004888	miR-493	-1.59	3.01
0005443	miR-181a-2*	-3.52	11.47	0004837	miR-615-5p	-3.55	11.71
0004541	miR-188-3p	-4.36	20.53	0004943	miR-653	4.36	0.05
0000223	miR-193	-1.63	3.10	0004897	miR-654-5p	-1.87	3.66
0000518	miR-196a	-3.15	8.88	0003479	miR-669c	-3.14	8.82
0000235	miR-202-3p	-1.76	3.39	0004821	miR-671-3p	-1.69	3.23
0004546	miR-202-5p	3.40	0.09	0003731	miR-671-5p	-1.66	3.16
0000520	miR-208a	-3.50	11.31	0003726	miR-675-3p	-2.87	7.31
0004939	miR-208b	-3.37	10.34	0003464	miR-686	-2.03	4.08
0004572	miR-290-3p	-2.30	4.92	0003499	miR-709	-5.51	45.57
0000368	miR-291a-3p	-1.72	3.29	0004237	miR-742	-2.53	5.78
0000370	miR-292-3p	-4.36	20.53	0004840	miR-743b-3p	4.59	0.04
0000369	miR-292-5p	-1.60	3.03	0004841	miR-871	-2.67	6.36
0004573	miR-293*	3.53	0.09	0004861	miR-877	-1.87	3.66
0004574	miR-294*	-2.62	6.15	0004933	miR-878-3p	-2.58	5.98
0000376	miR-298	-1.60	3.03	0005836	miR-1186	-2.13	4.38
0004579	miR-302a*	-1.73	3.32	0005837	miR-1187	-2.57	5.94
0003374	miR-302b	3.60	0.08	0005860	miR-1199	-1.92	3.78
0003375	miR-302c*	-3.13	8.75	0005460	miR-1224	-1.85	3.61
0004867	miR-327	-1.93	3.81	0007869	miR-1899	-1.81	3.51
0004656	miR-345-3p	-2.48	5.58	0007870	miR-1900	-7.31	158.68

Table 3C

MicroRNAs Significantly Regulated 24 Hours After DHPG

MIMAT ID	Mature mmu-miR	ΔAct	Fold Change	MIMAT ID	Mature mmu-miR	ΔAct	Fold Change
0000208	miR-10b	-2.99	7.94	0003478	miR-467b*	0.66	0.63
0004538	miR-10b*	0.43	0.74	0002111	miR-470	-6.72	105.30
0004660	miR-19a*	-2.64	6.23	0002112	miR-471	-2.86	7.25
0004628	miR-21*	-3.29	9.77	0004888	miR-493	-3.89	14.81
0000541	miR-96	-3.08	8.45	0004896	miR-590-3p	-3.50	11.30
0004856	miR-105	-5.74	53.38	0004837	miR-615-5p	-2.90	7.46
0005443	miR-181a-2*	-5.85	57.61	0003479	miR-669c	-3.13	8.74
0000211	miR-182	-3.76	13.53	0004821	miR-671-3p	-3.67	12.71
0000212	miR-183	-3.65	12.54	0003726	miR-675-3p	-3.71	13.07
0000518	miR-196a	-5.42	42.76	0003452	miR-678	0.48	0.72
0001081	miR-196b	-2.88	7.35	0003457	miR-680	-2.88	7.35
0004860	miR-197	0.42	0.75	0003459	miR-682	-3.09	8.51
0000519	miR-200a	-2.60	6.06	0003467	miR-688	0.45	0.73
0004863	miR-220	1.20	0.43	0003487	miR-697	-3.35	10.18
0004572	miR-290-3p	-4.20	18.36	0004841	miR-871	-3.32	9.98
0003190	miR-291b-3p	-3.70	12.98	0004937	miR-875-5p	0.74	0.60
0000371	miR-293	-2.79	6.91	0004933	miR-878-3p	-3.49	11.22
0000372	miR-294	-3.03	8.16	0004844	miR-880	0.51	0.70
0004574	miR-294*	-4.67	25.43	0004845	miR-881*	1.10	0.47
0004576	miR-296-3p	-2.83	7.10	0004847	miR-882	0.87	0.55
0004579	miR-302a*	-4.30	19.68	0004851	miR-883b-3p	0.48	0.72
0003375	miR-302c*	-5.00	31.96	0005836	miR-1186	-3.27	9.64
0004643	miR-331-5p	-4.51	22.76	0005837	miR-1187	-3.20	9.18
0004656	miR-345-3p	-4.06	16.66	0005847	miR-1190	-3.17	8.99
0000739	miR-375	1.35	0.39	0005850	miR-1192	-3.31	9.91
0001537	miR-429	-2.83	7.10	0007864	miR-1897-5p	-2.79	6.91
0004870	miR-453	0.41	0.75	0007875	miR-1898	-2.92	7.56
0004758	miR-463	-3.83	14.21	0007869	miR-1899	-2.86	7.25
0002104	miR-463*	-3.57	11.86	0007870	miR-1900	-3.49	11.22

MIMAT ID	Mature mmu-miR	$\Delta\Delta Ct$	Fold Change	MIMAT ID	Mature mmu-miR	$\Delta\Delta Ct$	Fold Change
0002106	miR-465a-5p	-2.67	6.36	0009391	miR-1928	-4.62	24.56

Table 4
Kegg Pathways for Predicted Genes Targeted by MicroRNAs Regulated 8 hours after DHPG

KEGG Pathway	Pathway mmu ID	# Genes miRNA Down	Summary In(-p) Down	# Genes miRNA Up	Summary Up In(-p)
MAPK signaling pathway	04010	225	57.75	161	52.49
Regulation of actin cytoskeleton	04810	200	65.15	125	34.5
Focal adhesion	04510	188	65.56	122	41.99
Wnt signaling pathway	04310	144	53.29	90	29.4
Colorectal cancer	05210	100	54.64	69	41.95
TGF-beta signaling pathway	04350	93	39.36	61	25.46
Melanoma	05218	86	50.88	58	36.31
Glioma	05214	86	65.83	60	53.34
Chronic myeloid leukemia	05220	79	32.72	52	21.43
Oxidative phosphorylation	00190	13	25.56	10	14.51
Ubiquitin mediated proteolysis	04120	122	39.71		
Insulin signaling pathway	04910	121	30.57		
Tight junction	04530	101	16.93		
Melanogenesis	04916	99	38.6		
ErbB signaling pathway	04012	97	49.2		
Prostate cancer	05215	93	38.23		
GnRH signaling pathway	04912	87	24.51		
Renal cell carcinoma	05211	86	51.94		
Pancreatic cancer	05212	83	42.74		
Long-term potentiation	04720	78	45.19		
Gap junction	04540	75	16.04		
Adherens junction	04520	72	25.45		
mTOR signaling pathway	04150	70	49.99		
Non-small cell lung cancer	05223	70	50.02		
VEGF signaling pathway	04370	68	23.38		
Phosphatidylinositol signaling system	04070	65	19.53		
Acute myeloid leukemia	05221	64	29.69		
Endometrial cancer	05213	63	36.68		
Type II diabetes mellitus	04930	54	29.09		

KEGG Pathway	Pathway mmu ID	# Genes miRNA Down	Summary ln(-p) Down	# Genes miRNA Up	Summary Up ln(-p)
Pyrimidine metabolism	00240	17	12.99		
Axon guidance	04360			90	38.35
T cell receptor signaling pathway	04660			54	14.51
Circadian rhythm	04710			13	10.94