Published in final edited form as:

Neuroscience. 2010 December 15; 171(3): 655–665. doi:10.1016/j.neuroscience.2010.09.039.

CLIMBING FIBERS INDUCE microRNA TRANSCRIPTION IN CEREbellAR PURKINJE CELLS

Neal H. Barmack, Zuyuan Qian, and Vadim Yakhnitsa

Department of Physiology and Pharmacology, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, USA

Abstract

The coordinated expression of as many as 100 proteins may be required to sustain simple changes in synaptic transmission. While each protein may be regulated separately, the translation of multiple proteins could be regulated by microRNAs. MicroRNAs are short non coding RNAs that translationally repress cognate sequences in targeted mRNAs. If these targeted sequences are shared across several genes, then a single microRNA could, effectively regulate the activity of several genes in parallel. Here we investigate whether microRNA transcription is influenced by naturally evoked synaptic activity at the climbing fiber-Purkinje cell synapse in the mouse cerebellar flocculus. Mice received 24 h of binocular horizontal optokinetic stimulation (HOKS) evoking sustained increases in climbing fiber activity to Purkinje cells in one flocculus and decreases to Purkinje cells in the other. Increased climbing fiber activity increased transcription of twelve microRNAs in the flocculus. The transcription of one of these microRNAs, miR335, was proportional to duration of stimulation, increasing 18-fold after 24 h of HOKS. We localized miR335 transcripts to Purkinje cells using hybridization histochemistry. Transcripts of miR335 decayed to baseline within 3 h after HOKS was stopped. We identified mRNA targets for miR335 using multiple screens: sequence analysis, microinjection of miR335 inhibitors and identification of mRNAs whose transcription decreased during HOKS. Two genes, calbindin and 14-3-3-θ passed these screens. Our data suggest that microRNA transcription could provide an important synaptic or homeostatic mechanism for the regulation of proteins that contribute to Purkinje cell plasticity.

Keywords

microRNA; climbing fibers; inferior olive; cerebellum; optokinetic; plasticity

INTRODUCTION

RNA transcription and translation are key steps in the expression of proteins essential for establishing intermediate and long term memories (Chamberlain et al., 1963; Costa-Mattioli et al., 2009; Hydén and Egyházi, 1962; Mercer et al., 2008). Multiple transcriptional and translational events occur over a time course of several hours in response to synaptically-evoked neuronal activity. No fewer than 100 proteins have been implicated in the cellular

© 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Correspondence: Neal H. Barmack, Ph.D., Department of Physiology and Pharmacology, Oregon Health & Science University, 3181 SW Sam Jackson Park Road, Portland, Oregon 97239, USA, **TEL:** 503-418-2560, barmackn@ohsu.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

changes associated with long-term potentiation and depression (Sanes and Lichtman, 1999). Possibly each transcriptional event associated with cellular plasticity can be attributed to a gene-specific promoter. Alternatively, one or few microRNAs (miRNAs) could target multiple mRNAs, repress their translation and account for a variety of transcriptional events that contribute to long term memory formation (Mercer et al., 2008) or homeostatic regulation (Turrigiano, 2008).

microRNAs are strings of 19–22 nucleotides derived from non-coding DNA and regulate gene translation by complementary repression or degradation of mRNAs (Cullen, 2004; Hobert, 2008). A single miRNA targets as many as 5–30 mRNAs (Robins and Press, 2005) potentially influencing translation of as many proteins (Landgraf et al., 2007). miRNAs also regulate protein translation by targeting transcription factors (Kosik, 2009). miRNA transcription has been associated with cellular differentiation and development (Ambros, 2004; Cullen, 2004; Kosik, 2006; Muddashetty and Bassell, 2009; Pena et al., 2009; Schratt et al., 2006), microbial defense (Bartel, 2004; Cullen, 2004; Zeng et al., 2005), oncogenesis (Tavazoie et al., 2008; Yuan et al., 2008) as well as the occurrence of diseases such as macular degeneration (Castanotto and Rossi, 2009) and spinocerebellar ataxia (Lee et al., 2008).

While miRNAs are expressed in fully mature neurons, such as cerebellar Purkinje cells, there are only hints that miRNAs have important non-developmental functions. Conditional knock-out of the cytoplasmic miRNA processing enzyme, Dicer, in Purkinje cells, induces cell death and ataxia (Schaefer et al., 2007). Cultured hippocampal neurons depolarized by bath-applied increases in KCl, evince increased transcription of a cluster of miRNAs under the control of a transcription factor known to influence dendritic spine morphogenesis (Fiore et al., 2009; Nudelman et al., 2010; Wayman et al., 2008). Changes in miRNA transcription evoked by physiological stimulation *in vivo* could indicate a role for miRNA in translational control of multiple proteins involved in neuronal plasticity.

If physiological stimulation modulates miRNA transcription in mature neurons, these transcriptional changes would occur over several hours or even tens of hours. This requires the application of natural stimulation to a neural system with well understood anatomy and physiology. Once stimulation is finished it is essential to analyze transcriptional changes in stimulated tissue samples relative to transcriptional changes in nearly identical control samples from the same animal.

We used prolonged horizontal optokinetic stimulation (HOKS) of the visual climbing fiber pathway to the cerebellar flocculus to control climbing fiber synaptic activity in a restricted region of the flocculus. In essence, we investigated the effects of sustained activity of climbing fiber synapses on floccular Purkinje cells.

HOKS is induced by placing an animal at the center of a sphere that slowly rotates about a vertical axis. Horizontal rotation of the contrast-rich interior wall of the sphere evokes increased activity of “on” direction-selective ganglion cells, with receptive fields of tens of degrees in the eye stimulated in the posterior→anterior (P→A) direction and decreased activity of ganglion cells in the eye stimulated in the A→P direction (Fig. 1A) (Oyster et al., 1980). Direction-selective ganglion cells project to the contralateral nucleus of the optic tract (NOT) (Simpson et al., 1988). The axons of NOT neurons descend to the dorsal cap of Kooy (dc) in the ipsilateral inferior olive (Maekawa and Simpson, 1973) (Fig. 1B). Climbing fibers emerge from the dc and cross the midline to terminate on Purkinje cells in the contralateral cerebellar flocculus (Alley et al., 1975; Schonewille et al., 2006). Because of the lateralized projections of the mouse visual system, binocular HOKS increases climbing fiber activity in the flocculus ipsilateral to the P→A stimulated eye and reduces it in the

flocculus ipsilateral to the A→P stimulated eye. This allows us to sample two oppositely stimulated flocculi and to compare transcriptional differences for miRNAs from the same animal.

Each Purkinje cell receives a projection from a single climbing fiber that makes ~500 synaptic contacts on the Purkinje cell dendritic tree (Fig. 1C) (Harvey and Napper, 1991). Climbing fiber discharge evokes an iconic EPSP-action potential sequence in Purkinje cells termed the “complex spike” (Granit and Phillips, 1956). Consequently, olivo-cerebellar circuitry, driven by HOKS, allows study of sustained excitatory synaptic signaling at the climbing fiber-Purkinje cell synapse.

Prolonged HOKS evokes profound changes in posture and balance. In rabbits, HOKS evokes a pattern of eye movements, termed optokinetic after-nystagmus, that outlasts the duration of the HOKS by 24–48 h (Barmack and Nelson, 1987). In mice, prolonged HOKS induces an ataxia in which mice are initially unable to stand on their rear legs without losing their balance as they twist in a direction opposite to the former HOKS (unpublished observations). At a cellular level, prolonged HOKS (6 – 144 h) evokes a 12-fold increase in transcription of corticotropin-releasing factor mRNA in dorsal cap neurons (Barmack and Young, 1990). HOKS also modulates Purkinje cell expression of proteins, such as calbindin and 14-3-3- θ known to be involved in signal transduction (Barmack and Qian, 2002).

The cerebellum also receives inputs mediated by mossy fibers and distributed to Purkinje cells via granule cell parallel fibers. However, the receptive field structure, spatial distribution and velocity sensitivity of mossy fibers remains to be worked out. Currently, there is no evidence that indicates that optokinetically driven mossy fibers have any influence whatsoever on floccular Purkinje cells that respond to HOKS.

Optokinetically-evoked climbing fiber activity increased transcripts of twelve miRNAs. We localized the transcription of one of these miRNAs, miR335, to Purkinje cells. We demonstrate that miRNAs interact with proteins that contribute to the adaptive response of mature neurons. This is the first instance in which miRNA transcription is modulated by neuronal activity at a defined synapse *in vivo* in the mammalian brain using natural stimulation.

EXPERIMENTAL PROCEDURES

Horizontal optokinetic stimulation

All animal procedures were approved in advance by the Institutional animal care and use committee of Oregon Health & Science University. C57bl6 mice were partially restrained in plastic tubes near the center of an optokinetic sphere with a diameter of 30 cm (Fig. 1A). The sphere rotated at a constant velocity, 6 deg/s, in the P→A direction with respect to the right eye and A→P direction with respect to the left eye. The inside wall of the sphere was striped vertically. Mice were removed from the sphere every 8 h, weighed, and given food and water for 20 min. The maximum duration of HOKS was 30 h. During monocular HOKS a stationary white opaque barrier obscured vision of the left eye of the moving interior wall of the optokinetic sphere by 190 deg horizontally and 160 deg vertically.

Anesthesia and surgical removal of the flocculus

Mice were euthanized with an intramuscular injection of ketamine hydrochloride (100 mg/kg), xylazine (12 mg/kg) and acepromazine maleate (2.4 mg/kg). The mouse was decapitated. The brain was rapidly removed and placed in ice-cooled saline. The flocculi were removed under an X25 operating microscope. We used a forceps with fine tips to separate the flocculus from the ventral paraflocculus by dividing the two structures along the

posterolateral fissure. We used a fine dissection needle to isolate the flocculus from the dorsal cochlear nucleus and to detach the flocculus from the inferior and middle cerebellar peduncles. The spindle shaped mouse flocculus is ~1.1 mm in axial length, ~400 μ m at its peak diameter and weighs ~400 μ g.

miRNA microarray analysis

miRNA expression profiling of mouse flocculus was performed using a two-color microarray platform (Asuragen Technical Services, Austin, TX). Two sample pairs of 100 ng of total RNA each were labeled at 3' ends with biotin (Amersham Biosciences, Piscataway, NJ). These samples were used for hybridization on an Ambion custom-manufactured Affymetrix GeneChip™, containing >13,000 miRNA probes derived from the Sanger microRNABase (Griffiths-Jones et al., 2006) and other published reports (Bentwich et al., 2005; Berezikov et al., 2005; Cummins et al., 2006). The microRNABase includes 616 human and 361 mouse mature miRNAs. Antigenomic probe sequences were used for estimating background signal (Affymetrix, Palo Alto, CA). Other non-miRNA control probes in the microarray lacked sequence homology to the human genome and were used for spike-in external reference controls. For each hybridization, samples of RNA from the left and right flocculi of three mice were combined. Hybridizations were performed in three separate experiments, using a total of nine mice. Data from these hybridizations were combined and plotted for the population.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and real time PCR

Total RNA from each flocculus was extracted with a Mini-RNA isolation kit (Zymo Research, Orange, CA). Aliquots of total RNA (5 – 20 ng) were subjected to cDNA synthesis using miRNA-specific primers (miR335, miR125, miR147) and primers specific for U6 snRNA. All reactions were performed using the miRCURY LNA miRNA PCR System (Exiqon, Vedbaek, Denmark). After a 10 min incubation at 95°C, 40 cycles of PCR cycling was performed for 10 s at 95°C and 20 s at 60°C. All measurements of miRNA transcription were normalized against U6 snRNA levels and analyzed using Kodak 1D image analysis software.

In some experiments, miR335 was analyzed using real time PCR on a Bio-Rad CFX96 real time system (Biorad, Hercules, CA), using the following protocol: 10 min at 95°C, 40 cycles of 10 s at 95°C and 20 s at 60°C. Fluorescent signals were generated using SYBR Green “master mix” (Exiqon).

Hybridization histochemistry

We hybridized 10–15 μ m sections of the flocculus with short (~20 nt) “locked nucleic acid-modified oligonucleotide probes” (LNA-DNA probes) that target mature miRNAs (Obernosterer et al., 2007). Mice were anesthetized and perfused with sterile saline chilled to 4°C, followed by 4% paraformaldehyde containing 0.15% picric acid in 0.1 M PBS (pH 7.2). The brain was removed and dehydrated in graded sucrose (10–20%) in 0.1M PBS (pH 7.4). Brains were frozen and cut on a cryostat. Coronal sections were thaw-mounted onto glass slides. Each probe was labeled at the 3' and 5' ends with digoxigenin (Exiqon, Vedbaek, Denmark). The incubation temperature for probes was fixed at their melting point (50–65°C). Hybridized probes were detected using an anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche, Mannheim, Germany) with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) used as the substrate.

Microinjection of miRNA inhibitors into cerebellum and mRNA analysis

The efficacy of two miR335 inhibitors (miR-5' inhibitor: UCAAGAGCAAUAACGAAAAAUGU and miR-3' inhibitor: UUUUUCAUUAUUGCUCUGACC (Invitrogen, Carlsbad, CA) was confirmed using PC 12 cells. These miR335 inhibitors were mixed equally (0.8 µg/µl) in a transfection solution (PolyPlus Corp, Illkirch, France). We used a glass micropipette to pressure inject 200–600 nl of this solution into folia 7–8 of the left posterior vermis. An equal volume of transfection solution without the miRNA inhibitors was injected into the right folia. The microinjections were made 0.7 mm on either side of the midline.

Diffusion of a microinjected miRNA inhibitor across the midline into the site of the other microinjected inhibitor would tend to minimize expected differences. We tested the extent of diffusion of microinjections by injecting 800 nl of a GFP labeled vector into the posterior cerebellum. The extent of diffusion was contained within a 1 mm sphere of tissue.

Three days after the microinjection of miR335 inhibitors, mice were anesthetized, decapitated and the injected folia were removed. We extracted total RNA from each sample and analyzed it with a Mouse Genome 430 2.0 GeneChip™ array (Affymetrix, Palo Alto, CA). The genome array image scan measured the average intensity values for each array probe.

Statistical analysis

Statistical analyses for both miRNA and mRNA genome array image scans were performed using a MAS 5.0 algorithm (Hubbell et al., 2002; Liu et al., 2002) to produce gene-level data for each probe set on the array. Array data were normalized using global scaling to an average target intensity of 350. Detection calls were based on the detection p-value for each probe set determined with a Wilcoxon rank-sum test of probe signals. Pair-wise comparisons of samples were based on a Wilcoxon signed-rank test performed using the MAS 5.0 algorithm. The magnitude and direction of gene transcript changes were determined using a one-step Tukey's bi-weight method to calculate signal log ratio estimates.

RESULTS

HOKS evokes increased transcription of miRNAs

Prolonged binocular HOKS evoked increased transcription of several miRNAs. We exposed three groups of three mice to HOKS for 24 h at 6 deg/s in the P→A direction with respect to the right eye and A→P direction with respect to the left eye. After the binocular HOKS was stopped, the mice were anesthetized and euthanized. The left and right flocculi were removed. Total RNA was extracted from each flocculus and combined within each group to obtain sufficient amounts to analyze on a miRNA microarray. We used the miRNA microarray to compare the miRNAs in the RNA samples extracted from the right flocculi (increased climbing fiber activity) with the samples extracted from the left flocculi (decreased climbing fiber activity). Global changes in miRNA transcription are illustrated in an asymmetric "volcano plot" (Fig. 2A) that includes data from the transcription of 361 miRNAs. We screened these transcripts using three criteria: 1) Changes between the left and right flocculus exceed 2.2 fold. 2) The p-value of a t-test of significance is <0.005. 3) The average copy number for each microRNA exceeds 256. These three criteria identified twelve miRNAs whose transcription was differentially increased in the right flocculus. Three of these miRNAs (miR126, miR335 and miR361) had p-values <0.001 (Fig. 2B). We chose one of these, miR335, for detailed analysis because its fold change was the highest of any of the candidate miRNAs. The mature nucleotide sequence of miR335 is: 16 - UCA GAG

CAA CGA AAA AUG U - 38 (www.mirbase.org, Accession MIMAT0000766, mmu-miR-335-5P).

HOKS-evoked transcription of specific miRNAs

We used RT-PCR to measure the transcription of miR335 after different durations of HOKS. We ran RT-PCR reactions using two pairs of primers. One pair amplified miR335 and the second pair amplified the spliceosomal RNA, U6, used as a loading control. Amplified RNAs were run on gels and the density of each band was measured photometrically.

If HOKS caused no change in miR335 transcription, then the PCR-amplified miR335 transcripts from the right and left flocculi would be equal and their ratio (right flocculus/left flocculus) would equal one. Binocular HOKS for 6 h was sufficient to increase the transcription of miR335 in the right flocculus (Fig. 3A). The transcription of miR335 increased with increasing duration of HOKS. After 30 h of HOKS, miR335 transcripts in the right flocculus exceeded those in the left by 6-fold. We ran two miRNAs, miR125 and miR147, in addition to U6, as controls. miR125 was highly expressed in the cerebellum, but its transcription was not influenced by HOKS. miR147 was weakly transcribed in the cerebellum and also not influenced by HOKS.

HOKS evokes increased miR335 transcripts due to excitatory action of the climbing fiber signal in the ipsilateral flocculus

The use of binocular HOKS raised the logical possibility that the differential transcription of miR335 was induced not by increased climbing fiber activity in the right flocculus, but by the reduced climbing fiber activity in the left flocculus. We tested this possibility by exposing three mice to monocular HOKS of the right eye in the P→A direction for 24 h. The field of view of the left eye was obscured by a gray cardboard, depriving the left eye of HOKS in the A→P direction, while maintaining a comparable light flux. Monocular HOKS increased the transcription of miR335 in the right flocculus relative to the non-stimulated left flocculus by a factor of 4.7 (Fig. 3B). Together the miRNA microarray and the RT-PCR data establish that enhanced climbing fiber activity evoked by HOKS increases the transcription of miR335 in the flocculus.

When HOKS is stopped miR335 transcripts rapidly decay

The possible role of miRNAs in synaptic plasticity is, in part, determined by how long an increased level of transcripts remain after the stimulus that induced transcription is stopped. The time course of miRNA decay determines the dynamics of its repressive action on protein translation. We addressed this question specifically for HOKS-induced transcription of miR335 by exposing mice to monocular HOKS in the P→A direction with respect to the right eye for 24 h. When the rotation of the drum was stopped, mice remained in the illuminated and stationary drum for variable durations before being anesthetized and euthanized. We had demonstrated previously that the residual effects of HOKS were prolonged when rabbits were maintained in an illuminated environment rather than in darkness (Barmack and Nelson, 1987). HOKS-induced miR335 transcripts decayed with a time-constant of ~2.5 h (Fig. 3C). Consequently miR335 repression of its target mRNAs abates rapidly when HOKS is stopped.

Peak numbers of miR335 transcripts occur in floccular horizontal climbing fiber zone

Measurements of miRNAs understate the effects of HOKS on transcription within the flocculus for two reasons. First, climbing fibers that respond to HOKS terminate in a physiologically-defined floccular zone located in the middle of the flocculus (Schonewille et

al., 2006). This horizontal zone occupies ~400 μm of the ~1.1 mm axial length of the flocculus. It is flanked by zones in which Purkinje cells respond to vertical OKS (Fig. 4A, horizontal zone is indicated in red). By including the entire flocculus in our sample, we diluted RNA obtained from neurons in the horizontal zone with RNA obtained from neurons in zones not directly influenced by HOKS. Second, our floccular samples included several neuronal and glial cell types. Specific miRNAs are not expressed equally in all cell types. We addressed the first of these two limitations by dissecting three approximately equal segments along the folial axis of the right flocculus after mice were exposed to 24 h of monocular HOKS, P→A with respect to the right eye. We used real time RT-PCR to measure transcription of miR335 in each segment with respect to transcription of miR335 in the undivided right flocculus. We also measured the transcription of U6, using it to correct for unequal loading of RNA samples. miR335 transcripts were maximal in the middle segment, encompassing the horizontal climbing fiber zone. This segment had a transcription ratio of 18.0 ($p < 0.001$) (Fig. 4B). Given the variability of free hand dissection of the flocculus, it is surprising that the rostral and caudal segments of the right flocculus were as devoid of miR335 transcripts as observed.

HOKS-evoked miR335 transcription occurs in Purkinje cells

Having demonstrated that the HOKS-evoked miR335 transcription was maximal in the floccular horizontal climbing fiber zone ipsilateral to the eye stimulated in the P→A direction, we next used hybridization histochemistry to identify the cell(s) in which transcription of miR335 occurred (see **EXPERIMENTAL PROCEDURES**). We made a hybridization probe for miR335 and hybridized the probe with transverse sections through the cerebellum. The miR335 probe hybridized with its complement in cerebellar Purkinje cells (Fig. 5A,B,F). Purkinje cell somas, but not nuclei, were densely labeled, confirming that the probe targeted the mature miR335, not the pri-miR335 --- the unedited, longer, primary transcript.

Purkinje cells were not the only labeled neurons. Large interneurons (10–15 μm) in the granule cell layer were also labeled, although less intensely (Fig. 5F). These were likely a subset of Golgi cells, but since cell processes were not labeled, this identification is tentative. As a negative control we incubated floccular sections with a scrambled probe having the same GC content as the probe for microR335. The scrambled probe failed to label cells (Fig. 5E). As a positive control we incubated sections with a probe for the ubiquitously expressed U6. This probe clearly labeled both the soma and nuclei of Purkinje cells as well as cells in the molecular and granule cell layers (Fig. 5C,D).

Microinjection of miR335 inhibitors confirms target mRNAs

Identification of miR335 in floccular Purkinje cells as one of the miRNAs whose transcription was influenced by climbing fiber activity raised yet another challenge. How could we identify the target mRNAs for miR335? While nucleotide sequences offer clues to finding complementary sequences of target mRNAs, the set of mRNA targets that have complementarity, even under the most stringent conditions, is unacceptably large. Based on sequence information and using stringent complementary nucleotide matching requirements, more than 149 target mRNAs have sufficient complementarity to hybridize with miR335.

We used miRNA inhibitors to establish a reduced set of target mRNAs empirically. By microinjecting a mixture of two miRNA inhibitors of miR335 directly into the left posterior vermis (folia 7 and 8), we identified target mRNAs whose transcripts were increased. We injected an equal volume of transfection solution without the inhibitors into the right posterior vermis. Three days after the injections were made we extracted RNA from the inhibitor-injected and control-injected folia. Using a mRNA microarray we measured the

effects of the miR335 inhibitors on mRNA transcripts. The microarray detected 28 increased mRNA transcripts in the folia injected with miR335 inhibitors relative to the folia receiving control injections (see Table 1).

Enhanced climbing fiber input decreases mRNA transcripts in flocculus

Having identified mRNAs whose transcripts were increased following injections of miR335 inhibitors, we manipulated miR335 in the opposite sense by obtaining samples of floccular tissue that received 24 h of HOKS-induced increases in miR335. These samples were analyzed using an mRNA microarray. We looked for a subset of mRNAs whose transcripts were reduced after 24 h of binocular HOKS. Microarray measurements of mRNAs in mice were combined with “Differential Display” studies of the effects of HOKS on mRNA transcription in rabbits (Barmack and Qian, 2002). We identified 42 mRNAs whose transcription was reduced during HOKS-evoked increases in climbing fiber discharge. Three of these mRNA transcripts (calbindin, 14-3-3- θ and zinc finger protein) were complementary to miR335. Two of these three transcripts, calbindin and 14-3-3- θ , following microinjections of miR335 inhibitors.

DISCUSSION

Stimulus-induced increases in miRNA transcription

Optokinetically-evoked climbing fiber activity evoked increased transcription of twelve miRNAs in the flocculus. The increased transcription of one of these miRNAs, miR335, was large (18-fold) and restricted to Purkinje cells and a subset of Golgi cells. These are the only two cerebellar neurons onto which climbing fibers are known to make functional synapses (Hámori and Szentágothai, 1980; Sugihara et al., 1999). The optokinetically-evoked increase in miR335 probably underestimates the actual change in transcription that occurs in Purkinje cells, since our micro-dissected floccular samples undoubtedly included Purkinje cells not driven by HOKS-evoked climbing fiber activity.

Transcripts of miR335 increased with increasing duration of HOKS up to the 30 h limit used in this experiment. This finding contrasts with a previous report showing that transcripts of miR132 peaked in hippocampal neurons 45 min after the onset of pilocarpine induced seizures (Nudelman et al., 2010). The subsequent decay of miR132 transcripts was analogized to the action of “immediate early genes.” miR335 transcription does not share this profile. The rapid decay of miR335 transcripts following the cessation of HOKS argues for time-limited effects of miRNA transcription and suggests that miRNAs play a short term role in altered function.

miR335 reduces transcripts of calbindin and 14-3-3- θ

Using four separate screens we showed that two genes, calbindin and 14-3-3- θ , interacted with miR335. Prolonged HOKS in rabbits reduced transcription and expression of calbindin in the flocculus that was ipsilateral to the eye stimulated in the P→A direction (Barmack and Qian, 2002) consistent with the present finding that transcription of miR335 increased in the flocculus that received increased climbing fiber synaptic input during HOKS.

Calbindin buffers intracellular calcium and prevents damage of intracellular organelles, such as mitochondria, from large calcium transients associated with cellular depolarization (Baimbridge et al., 1992; Rintoul et al., 2001). Climbing fiber synaptic excitation evokes an amalgam of sodium and calcium spikes that raises intracellular concentrations of calcium in Purkinje cells (Lasser-Ross and Ross, 1992; Llinás and Sugimori, 1980). Larger intracellular calcium concentrations induced by miR335 repression of calbindin translation, could either be thought of as increasing the likelihood of cytotoxicity or, alternatively, as an intracellular

signal that reflects changes in synaptic activity --- or both, depending on the levels of evoked changes in calcium concentration. In many excitable cells, calcium concentration is detected by calcium sensors, potassium channel-interacting proteins that interact with Kv4 low voltage-activated A-type potassium channels and regulate spike timing and dendritic excitability (Anderson et al., 2010). It is conceivable that miR335 participates in such a homeostatic function in Purkinje cells by regulating the efficacy of parallel fiber synapses following long-term climbing fiber activation.

14-3-3- θ binds to constitutively-expressed PKC- γ in Purkinje cells. Knock-down of 14-3-3- θ , reduces PMA-induced PKC phosphorylation of proteins (Qian and Barmack, 2006). Consequently, miR335 could influence how PKC is mobilized for protein phosphorylation. Phosphorylation of GABA_A receptors at multiple PKC phosphorylation sites influences GABA_A receptor clustering (Brandon et al., 2000; Herring et al., 2005; Krishek et al., 1994; Meier and Grantyn, 2004; Moss et al., 1992; Terunuma et al., 2008). Again, it is conceivable that miR335, as well as other miRNAs influence Purkinje cell excitability through the regulation of GABA_A receptor clustering.

miRNA transcription and behavioral plasticity

While we have used HOKS to reveal the effects of prolonged climbing fiber activity on Purkinje cell discharge, the reader may wonder whether HOKS effectively models Purkinje cell activity generated under more nearly physiological conditions. In fact, prolonged HOKS is a common occurrence. In the laboratory we create the stimulus condition in which the world slowly spins about the animal. In nature, this never occurs. However, it is mimicked by posturally induced movements of the animal with respect to a static world. Postural perturbations induced by visual, proprioceptive or vestibular stimulation during development and during movement commonly provoke HOKS. Floccular climbing fiber activity evoked by HOKS corrects these postural imbalances. No experimental or theoretical observations suggest that this correction occurs with a time course of a few minutes.

Multiple targeting mRNAs by miRNAs

Another means of assessing the importance of miRNA targeting of specific mRNAs is to establish whether there is multiple targeting. *Zinc finger proteins* have nucleotide sequences that are complementary to 10/12 miRNAs whose transcription was increased by HOKS. *Zinc finger proteins* have DNA-binding domains that determine DNA structure. They participate in the replication, repair and modification of DNA (Wolfe et al., 2000). *RNA binding motif protein* has nucleotide sequences that are complementary to 9/12 miRNAs. *RNA binding motif proteins* associate with both single and double stranded RNAs through an RNA recognition motif and participate in multiple processes of gene expression, chromatin remodeling, transcription, RNA splicing, mRNA stabilizing and translation (Lunde et al., 2007). *Transmembrane proteins* have nucleotide sequences complementary to 8/12 miRNAs. They are constituents of transporters, ion channels, receptors, enzymes and structural membrane-anchoring domains (Ponta et al., 2003).

We tested only one of the twelve miRNAs whose transcription in the flocculus was enhanced by HOKS-evoked climbing fiber activity. Additional testing of other miRNAs will be required to obtain a complete picture of how Purkinje cells respond to a maintained climbing fiber activity. Ultimately these findings may generalize to other synaptic circuitry.

Cellular analysis of microR335 function

The advantage of using HOKS to induce changes in floccular Purkinje cells is that the optokinetic system of mammals allows controlled natural stimulation of a defined set of spatially compact floccular Purkinje cells. The ability to exercise control of a natural

stimulus for tens of hours and to obtain both experimental and control tissue samples that allow measurement of RNA transcripts is perhaps unique to the flocculus. This has led to the discovery that the transcription of twelve miRNAs in the flocculus is influenced by climbing fiber activity and that miR335 translationally represses two proteins; calcium binding protein and 14-3-3-θ. Undoubtedly we have underestimated the proteins whose expression is influenced by climbing fiber activity, due to the stringency of the criteria of the present experiment. We have also not yet examined the interaction of other modulated miRNAs on protein expression.

Future prospects of miRNAs in neural systems

We initially posed the question of whether a few miRNAs could simplify the regulation of more than 100 proteins necessary for the changes that occur during learning or homeostatic regulation of neuronal activity. The answer to this question is not definitive, but it seems likely that miRNAs provide one means of simplifying this control problem.

The value of this experimental approach lies not with discovery of every miRNA whose transcription is related to climbing fiber activity. Rather this approach will establish clusters of miRNAs that co-regulate protein translation during activity of multiple neural systems that respond to glutamatergic transmission. This may lead to a better understanding of the subcellular changes that account for neuronal plasticity. It may also lead to prospective pharmacological targets to suppress the consequences of abnormal glutamatergic activity.

Further analysis of the cellular consequences of this miRNA regulation of protein translation might best be accomplished using *in vitro* preparations in which the consequences of miRNA transcription on target mRNAs can be studied directly. Ultimately it will be possible to return to the intact system to study the behavioral effects of manipulating various combinations of miRNAs on behavior.

Acknowledgments

This research was supported by NEI-EY018561 and by NIDCD DC006668.

REFERENCES

- Alley K, Baker R, Simpson JJ. Afferents to the vestibulo-cerebellum and the origin of the visual climbing fibers in the rabbit. *Brain Res.* 1975; 98:582–589. [PubMed: 1182539]
- Ambros V. The functions of animal microRNAs. *Nature.* 2004; 431:350–355. [PubMed: 15372042]
- Anderson D, Mehaffey WH, Iftinca M, Rehak R, Engbers JD, Hameed S, Zamponi GW, Turner RW. Regulation of neuronal activity by Cav3-Kv4 channel signaling complexes. *Nat Neurosci.* 2010; 13:333–337. [PubMed: 20154682]
- Baimbridge KG, Celio MR, Rogers JH. Calcium-binding proteins in the nervous system. *TINS.* 1992; 15:303–309. [PubMed: 1384200]
- Barmack NH, Nelson BJ. Influence of long-term optokinetic stimulation on eye movements of the rabbit. *Brain Res.* 1987; 437:111–120. [PubMed: 3427473]
- Barmack NH, Qian Z. Activity-dependent expression of calbindin in rabbit floccular Purkinje cells modulated by optokinetic stimulation. *Neuroscience.* 2002; 113:235–250. [PubMed: 12123701]
- Barmack NH, Young WSI. Optokinetic stimulation increases corticotropin-releasing factor mRNA in inferior olivary neurons of rabbits. *J Neurosci.* 1990; 10:631–640. [PubMed: 2303865]
- Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell.* 2004; 116:281–297. [PubMed: 14744438]
- Bentwich I, Avniel A, Karov Y, Aharonov R, Gilad S, Barad O, Barzilai A, Einat P, Einav U, Meiri E, Sharon E, Spector Y, Bentwich Z. Identification of hundreds of conserved and nonconserved human microRNAs. *Nat Genet.* 2005; 37:766–770. [PubMed: 15965474]

- Berezikov E, Guryev V, van de Belt J, Wienholds E, Plasterk RH, Cuppen E. Phylogenetic shadowing and computational identification of human microRNA genes. *Cell*. 2005; 120:21–24. [PubMed: 15652478]
- Brandon NJ, Delmas P, Kittler JT, McDonald BJ, Sieghart W, Brown DA, Smart TG, Moss SJ. GABAA receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. *J Biol Chem*. 2000; 275:38856–38862. [PubMed: 10978327]
- Castanotto D, Rossi JJ. The promises and pitfalls of RNA-interference-based therapeutics. *Nature*. 2009; 457:426–433. [PubMed: 19158789]
- Chamberlain TJ, Halick P, Gerard RW. Fixation of experience in the rat spinal cord. *J Neurophysiol*. 1963; 26:662–673. [PubMed: 14019944]
- Costa-Mattioli M, Sossin WS, Klann E, Sonenberg N. Translational control of long-lasting synaptic plasticity and memory. *Neuron*. 2009; 61:10–26. [PubMed: 19146809]
- Cullen BR. Transcription and processing of human microRNA precursors. *Mol Cell*. 2004; 16:861–865. [PubMed: 15610730]
- Cummins JM, He Y, Leary RJ, Pagliarini R, Diaz LA Jr, Sjoblom T, Barad O, Bentwich Z, Szafranska AE, Labourier E, Raymond CK, Roberts BS, Juhl H, Kinzler KW, Vogelstein B, Velculescu VE. The colorectal microRNAome. *Proc Natl Acad Sci U S A*. 2006; 103:3687–3692. [PubMed: 16505370]
- Fiore R, Khudayberdiev S, Christensen M, Siegel G, Flavell SW, Kim TK, Greenberg ME, Schratt G. Mef2-mediated transcription of the miR379–410 cluster regulates activity-dependent dendritogenesis by fine-tuning Pumilio2 protein levels. *EMBO J*. 2009; 28:697–710. [PubMed: 19197241]
- Granit R, Phillips CG. Excitatory and inhibitory processes acting upon individual Purkinje cells of the cerebellum in cats. *J Physiol (Lond)*. 1956; 133:520–547. [PubMed: 13368102]
- Griffiths-Jones S, Grocock RJ, van DS, Bateman A, Enright AJ. miRBase: microRNA sequences, targets and gene nomenclature. *Nucleic Acids Res*. 2006; 34:D140–D144. [PubMed: 16381832]
- Hámori J, Szentágothai J. Lack of evidence of synaptic contacts by climbing fibre collaterals to basket and stellate cells in developing rat cerebellar cortex. *Brain Res*. 1980; 186:454–457. [PubMed: 7357462]
- Harvey RJ, Napper RM. Quantitative studies on the mammalian cerebellum. *Prog Neurobiol*. 1991; 36:437–463. [PubMed: 1947173]
- Herring D, Huang R, Singh M, Dillon GH, Leidenheimer NJ. PKC modulation of GABAA receptor endocytosis and function is inhibited by mutation of a dileucine motif within the receptor beta 2 subunit. *Neuropharmacology*. 2005; 48:181–194. [PubMed: 15695157]
- Hobert O. Gene regulation by transcription factors and microRNAs. *Science*. 2008; 319:1785–1786. [PubMed: 18369135]
- Hubbell E, Liu WM, Mei R. Robust estimators for expression analysis. *Bioinformatics*. 2002; 18:1585–1592. [PubMed: 12490442]
- Hydén H, Egyházi E. Nuclear RNA changes of nerve cells during a learning experiment in rats. *Proc Natl Acad Sci U S A*. 1962; 48:1366–1373. [PubMed: 14450327]
- Kosik KS. The neuronal microRNA system. *Nat Rev Neurosci*. 2006; 7:911–920. [PubMed: 17115073]
- Kosik KS. MicroRNAs tell an evo-devo story. *Nat Rev Neurosci*. 2009; 10:754–759. [PubMed: 19738624]
- Krishek BJ, Xie X, Blackstone C, Haganir RL, Moss SJ, Smart TG. Regulation of GABAA receptor function by protein kinase C phosphorylation. *Neuron*. 1994; 12:1081–1095. [PubMed: 8185945]
- Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO, Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, Novosel A, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De VG, Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di LR, Wernet P, Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, Brownstein MJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T. A mammalian microRNA expression atlas based on small RNA library sequencing. *Cell*. 2007; 129:1401–1414. [PubMed: 17604727]

- Lasser-Ross N, Ross WN. Imaging voltage and synaptically activated sodium transients in cerebellar Purkinje cells. *Proc R Soc Lond [Biol]*. 1992; 247:35–39.
- Lee Y, Samaco RC, Gatchel JR, Thaller C, Orr HT, Zoghbi HY. miR-19, miR-101 and miR-130 co-regulate ATXN1 levels to potentially modulate SCA1 pathogenesis. *Nat Neurosci*. 2008; 11:1137–1139. [PubMed: 18758459]
- Liu W, Mei R, Di X, Ryder TB, Hubbell E, Dee S, Webster TA, Harrington CA, Ho M, Baid J, Smeekens SP. Analysis of high density expression microarrays with signed-rank call algorithms. *Bioinformatics*. 2002; 18:1593–1599. [PubMed: 12490443]
- Llinás R, Sugimori M. Electrophysiological properties of in vitro Purkinje cell somata in mammalian cerebellar slices. *J Physiol (Lond)*. 1980; 305:171–195. [PubMed: 7441552]
- Lunde BM, Moore C, Varani G. RNA-binding proteins: modular design for efficient function. *Nat Rev Mol Cell Biol*. 2007; 8:479–490. [PubMed: 17473849]
- Maekawa K, Simpson JI. Climbing fiber responses evoked in vestibulocerebellum of rabbit from visual system. *J Neurophysiol*. 1973; 36:649–666. [PubMed: 4541327]
- Meier J, Grantyn R. Preferential accumulation of GABAA receptor gamma 2L, not gamma 2S, cytoplasmic loops at rat spinal cord inhibitory synapses. *J Physiol*. 2004; 559:355–365. [PubMed: 15235089]
- Mercer TR, Dinger ME, Mariani J, Kosik KS, Mehler MF, Mattick JS. Noncoding RNAs in Long-Term Memory Formation. *Neuroscientist*. 2008; 14:434–445. [PubMed: 18997122]
- Moss SJ, Doherty CA, Haganir RL. Identification of the cAMP-dependent protein kinase and protein kinase C phosphorylation sites within the major intracellular domains of the beta 1, gamma 2S, and gamma 2L subunits of the gamma-aminobutyric acid type A receptor. *J Biol Chem*. 1992; 267:14470–14476. [PubMed: 1321150]
- Muddashetty R, Bassell GJ. A boost in microRNAs shapes up the neuron. *EMBO J*. 2009; 28:617–618. [PubMed: 19295499]
- Nudelman AS, DiRocco DP, Lambert TJ, Garelick MG, Le J, Nathanson NM, Storm DR. Neuronal activity rapidly induces transcription of the CREB-regulated microRNA-132, in vivo. *Hippocampus*. 2010; 20:492–498. [PubMed: 19557767]
- Obernosterer G, Martinez J, Alenius M. Locked nucleic acid-based in situ detection of microRNAs in mouse tissue sections. *Nat Protoc*. 2007; 2:1508–1514. [PubMed: 17571058]
- Oyster CW, Simpson JI, Takahashi ES, Soodak RE. Retinal ganglion cells projecting to the rabbit accessory optic system. *J Comp Neurol*. 1980; 190:49–61. [PubMed: 7381054]
- Pena JT, Sohn-Lee C, Rouhanifard SH, Ludwig J, Hafner M, Mihailovic A, Lim C, Holoch D, Berninger P, Zavolan M, Tuschl T. miRNA in situ hybridization in formaldehyde and EDC-fixed tissues. *Nat Methods*. 2009; 6:139–141. [PubMed: 19137005]
- Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol*. 2003; 4:33–45. [PubMed: 12511867]
- Qian Z-Y, Barmack NH. Serine phosphorylation influences interaction of GABA-A receptor trafficking and clustering proteins. *Society for Neuroscience*. 2006; 38
- Rintoul GL, Raymond LA, Baimbridge KG. Calcium buffering and protection from excitotoxic cell death by exogenous calbindin-D28k in HEK 293 cells. *Cell Calcium*. 2001; 29:277–287. [PubMed: 11243935]
- Robins H, Press WH. Human microRNAs target a functionally distinct population of genes with AT-rich 3' UTRs. *Proc Natl Acad Sci U S A*. 2005; 102:15557–15562. [PubMed: 16230613]
- Sanes JR, Lichtman JW. Can molecules explain long-term potentiation? *Nat Neurosci*. 1999; 2:597–604. [PubMed: 10404178]
- Schaefer A, O'Carroll D, Tan CL, Hillman D, Sugimori M, Llinas R, Greengard P. Cerebellar neurodegeneration in the absence of microRNAs. *J Exp Med*. 2007; 204:1553–1558. [PubMed: 17606634]
- Schonewille M, Luo C, Ruigrok TJ, Voogd J, Schmolesky MT, Rutteman M, Hoebeek FE, De Jeu MT, De Zeeuw CI. Zonal organization of the mouse flocculus: physiology, input, and output. *J Comp Neurol*. 2006; 497:670–682. [PubMed: 16739198]

- Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, Greenberg ME. A brain-specific microRNA regulates dendritic spine development. *Nature*. 2006; 439:283–289. [PubMed: 16421561]
- Simpson JJ, Leonard CS, Soodak RE. The Accessory Optic-System - Analyzer of Self-Motion. *Ann NY Acad Sci*. 1988; 545:170–179. [PubMed: 3239883]
- Sugihara I, Wu H, Shinoda Y. Morphology of single olivocerebellar axons labeled with biotinylated dextran amine in the rat. *J Comp Neurol*. 1999; 414:131–148. [PubMed: 10516588]
- Tavazoie SF, Alarcon C, Oskarsson T, Padua D, Wang Q, Bos PD, Gerald WL, Massague J. Endogenous human microRNAs that suppress breast cancer metastasis. *Nature*. 2008; 451:147–152. [PubMed: 18185580]
- Terunuma M, Xu J, Vithlani M, Sieghart W, Kittler J, Pangalos M, Haydon PG, Coulter DA, Moss SJ. Deficits in phosphorylation of GABA(A) receptors by intimately associated protein kinase C activity underlie compromised synaptic inhibition during status epilepticus. *J Neurosci*. 2008; 28:376–384. [PubMed: 18184780]
- Turrigiano GG. The self-tuning neuron: synaptic scaling of excitatory synapses. *Cell*. 2008; 135:422–435. [PubMed: 18984155]
- Wayman GA, Davare M, Ando H, Fortin D, Varlamova O, Cheng HY, Marks D, Obrietan K, Soderling TR, Goodman RH, Impey S. An activity-regulated microRNA controls dendritic plasticity by down-regulating p250GAP. *Proc Natl Acad Sci U S A*. 2008; 105:9093–9098. [PubMed: 18577589]
- Wolfe SA, Nekludova L, Pabo CO. DNA recognition by Cys2His2 zinc finger proteins. *Annu Rev Biophys Biomol Struct*. 2000; 29:183–212. [PubMed: 10940247]
- Yuan W, Sun W, Yang S, Du J, Zhai CL, Wang ZQ, Zhang J, Zhu TH. Downregulation of microRNA-142 by proto-oncogene LMO2 and its co-factors. *Leukemia*. 2008; 22:1067–1071. [PubMed: 17972953]
- Zeng Y, Cai X, Cullen BR. Use of RNA polymerase II to transcribe artificial microRNAs. *Methods Enzymol*. 2005; 392:371–380. [PubMed: 15644193]

Figure 1. Horizontal optokinetic stimulation (HOKS) and its effects on neuronal circuitry

A. Mice were restrained at the center of an optokinetic drum. They received either binocular or monocular HOKS at 6 deg/s for 3–30 h in the P→A direction with respect to the right eye. **B.** Horizontal direction-selective ganglion cells in the retina project to the contralateral nucleus of the optic tract (NOT). NOT neurons project to neurons in the ipsilateral caudal dorsal cap (dc) of the inferior olive. Dorsal cap neurons project as climbing fibers to the contralateral flocculus (FI). **C.** Each floccular Purkinje cell (Pc) is multiply innervated by a single climbing fiber (cf) (Redrawn from Cajal, 1911). **Abbreviations:** MTN, medial terminal nucleus; PFI, paraflocculus; 1–5, folia of the anterior vermis.

Figure 2. Differential expression of miRNA in mouse flocculus evoked by HOKS

Three groups of three mice received binocular HOKS for 24 h in the P→A direction with respect to the right eye. Total RNA was extracted from the left and right flocculi of each mouse and combined for each group. The RNA from each group was analyzed on a miRNA microarray, and subsequently combined for analysis. **A.** Each data point in the Volcano plot represents four variables: 1) The fold change for each miRNA is represented on abscissa (right floc./left floc), 2) Statistical probability transcription difference (p-value) of a miRNA between the left and right flocculi is represented on left ordinate, 3) Average copy number (color coded) is represented on right ordinate and 4) Frequency of occurrence a miRNA relative to all other sampled miRNAs is represented as data point diameter. **B.** Twelve

miRNAs were screened using three criteria: 1) A fold change >2 , 2) A p-value <0.005 , and 3) An average copy number >256 . Error bars indicate standard error of the mean. Histogram color indicates copy number according to the right ordinate in **A**.

Figure 3. Binocular and monocular HOKS increases transcription of miR335

A. Mice received binocular HOKS for 0–30 h. The right eye was stimulated in the P→A direction, the left eye in the A→P direction. After HOKS, the mice were anesthetized and euthanized. The flocculi were dissected for RNA extraction. cDNAs were synthesized and amplified by PCR. U6 was co-amplified and used as a loading control. Each reaction was run on a gel to determine the optical density of the amplified bands. The ratio of PCR band density (R. Floc./L. Floc.) shows increased transcription in the right flocculus of miR335, but not for miR125 or miR147. Increasing the duration of binocular HOKS increased the transcription of miR335. The duration of HOKS is indicated above each gel pair. Each histogram bar in **A**, **B** and **C** indicates the mean for 3 mice. Error bars indicate standard error of the mean. Asterisks indicate statistical significance using a single factor ANOVA at $P < 0.020$ (*) or $P < 0.001$ (**). **B.** Monocular HOKS of the right eye in the P→A direction for 24 h evoked increased transcription of miR335. HOKS of the left eye was prevented by a white cardboard monocular occluder that blocked vision of the contrast-rich interior wall of the rotating sphere. Transcription of control miRNAs, miR125 and miR147, was unaffected. Asterisk indicates statistical significance (t-test, $P < 0.001$). **C.** Monocular HOKS of the right eye in the P→A direction for 24 h was performed in 15 mice. Transcripts of miR335 in the right and left flocculi were measured at the indicated times after HOKS was stopped. The decay in transcripts had a time constant of ~2.5 h. Three control mice (gray bar) received no HOKS. Asterisks indicate statistical significance using a single factor ANOVA ($P < 0.001$).

Figure 4. HOKS-evokes transcription of miR335 in horizontal climbing fiber zone

Three mice received monocular P→A HOKS of the right eye for 24 h. HOKS stimulation of the left eye was obscured by stationary white opaque barrier. After HOKS was terminated, the mice were euthanized. The right flocculus was removed and divided rostro-caudally, *in situ*, into three fractions from which RNA was extracted separately. The left flocculus was removed intact. **A.** Planar map of five climbing fiber floccular zones. Purkinje cells in zones 1 and 3 (green) respond to vertical optokinetic stimulation (VOKS). Cells in zone 2 (red) respond to HOKS. Cells in zone 4 (green with red stripes) respond to VOKS and partially to HOKS. Cells in C2 respond to neither HOKS nor VOKS (see, Schonewille et al, 2006). **B.** Real time PCR was used to measure monocular HOKS-evoked changes in miR335

transcripts in the right flocculus. Each of the right floccular fractions was normalized using U6 transcripts and plotted relative to transcripts from the unstimulated left flocculus. Error bars indicate standard error of the mean. Asterisk indicates statistical significance of increased transcripts of miR335 in the middle fraction of the right flocculus (t-test, $p < 0.001$).

Figure 5. miR335 is transcribed in cerebellar Purkinje cells

Coronal sections through the cerebellar flocculus and paraflocculus were hybridized to a digoxigenin-labeled oligonucleotide and immuno-labeled with an antibody to digoxigenin. **A.** Hybridization of digoxigenin-labeled miR335 labels Purkinje cells. **B.** Higher magnification view of Purkinje cells shows labeling in the cytoplasm, but not in the nuclei. Large neurons in the granule cell layer were also labeled (indicated by arrow heads). **C.** Control hybridization with a digoxigenin-labeled probe for U6, labeled Purkinje cells and cells in the granule and molecular layers. **D.** Higher magnification view shows labeling in the cytoplasm, and nuclei of Purkinje cells as well as other neurons. **E.** Control hybridization with a scrambled oligonucleotide probe failed to label any neurons. **F.** A coronal section through the paraflocculus, hybridized with the miR335, probe shows labeling of Purkinje cells and large cells in the granule cell layer. **Abbreviations:** Fl, flocculus; gl, granule cell layer; dPFI and vPFI, dorsal and ventral paraflocculus;

Table 1
Interactions of twelve miRNAs and forty-two mRNAs isolated following prolonged HOKS

HOKS of the right eye in the P→A direction for 48 h at 6 deg/s decreased transcription of forty-two mRNAs in the right flocculus of rabbits. These are listed in the yellow column. Twelve miRNAs whose transcripts were increased in the right flocculus by P→A stimulation of the right eye at 6 deg/s for 24 h are listed in the blue columns. The black crosses (+) in each column indicate predicted interactions with mRNAs based on primary sequence complementarity. The red column indicates the effects of micro-injections of specific miR335 inhibitors into the left paravermal folia 7 and 8. The red crosses (+) indicate increased transcripts of mRNAs isolated from the left paravermal folia relative to the transcripts isolated from the right.

	miRNAs identified by miR335 inhibition												
	7a	7i	15	21	22	100	126	133	199	335	335	361	379
Optokinetically-evoked mRNAs													
Acyl CoA binding Protein													
Acyl-CoA synthetase II			+										
ATP synthase β subunit	+	+										+	
BC-2 protein													
Calbindin										+			
Cytochrome C oxidase subunit - II													
Cytochrome C oxidase subunit - VIc													
DBP-5 protein													
Elongation factor 1γ													
GABAA receptor associated protein								+					
GTP-binding protein												+	
Heat shock protein			+						+		+	+	+
HnRNP F protein													
Hypothetical protein							+						
Inositol polyphosphate-5-phosphatase	+	+					+				+		
Kinesin related protein			+				+						
Lactate dehydrogenase-B (LDH-B)													
Microtubule-associated protein			+						+			+	
Mitochondrial protein 18kDa													
NADH dehydrogenase												+	
Neurofilament protein												+	

	miRNAs identified by miR335 inhibition												
	Optokinetically-evoked miRNAs												
	7a	7i	15	21	22	100	126	133	199	335	335	361	379
Optokinetically-evoked mRNAs													
Phosphatidylethanolamine binding protein													
Phospholipase D1													
Putative transmembrane protein 47													
Response Genes to Complement (RGC-32)													
Retinoic acid repressible protein -1													
Rabaptin-5 protein													
RNA binding motif protein	+	+	+	+	+		+	+	+			+	
Ribosomal protein L11													
S-100 protein b													
Serine/threonine protein kinase TAO1	+	+	+	+			+				+		
TATA box binding protein			+						+				
TGF- β receptor interacting protein 1													
Thyrotropin releasing hormone receptor								+					
Transmembrane protein 97	+	+	+	+	+		+	+	+			+	
Translationally controlled tumor protein			+										
Tumor associated protein int-6													
Transhyretin precursor													
Tyrosine kinase receptor													
21 kDa polypeptide												+	
14-3-3 protein			+	+	+					+	+		
Zinc finger protein 238	+	+	+	+	+		+	+	+	+		+	+