Chapter 19
Cerebellar Epigenetics: Transcription of MicroRNAs in Purkinje Cells

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Abstract Climbing fiber activity is often associated with cerebellar plasticity. However, seemingly simple and short-lasting examples of neuronal plasticity such as Long-Term Potentiation (LTP) are associated with the translation and translocation of as many as 100 proteins. One means of controlling protein translation is through the interaction of microRNAs with mRNAs. MicroRNAs are small non-coding nucleotides that repress translation of mRNAs with complementary nucleotide sequences. A single microRNA may share nucleotide complementarity with as many as 30 different mRNAs, enhancing the scope of its regulatory impact. While microRNAs play an important role in cellular development, apoptosis and microbial defense, the role of microRNAs in the regulation of neuronal activity in adult nervous systems has been unexplored. Possibly, translation of many proteins necessary for neuronal plasticity is regulated by the repressive action of only a few microRNAs. Here we consider how the transcription of microRNAs in cerebellar Purkinje cells is influenced by activation of its climbing fiber synapses. We use horizontal optokinetic stimulation (HOKS) to chronically regulate climbing fiber excitation of floccular Purkinje cells in mice for 0–30 h. We investigate how this activity influences the transcription of microRNAs in Purkinje cells with enhanced climbing fiber signals compared to Purkinje cells with merely spontaneous climbing fiber inputs. HOKS evokes increases in 12 microRNA transcripts in floccular Purkinje cells. One of these microRNAs, miR335, increases 18-fold after 24 h of HOKS. After HOKS is stopped, miR335 transcripts decay with a time constant of ~2.5 h. HOKS evokes a 28-fold increase in pri-miR335 transcripts. These data indicate that the evoked increase in mature miR335 transcripts can be attributed to increased transcription of microRNAs.

Keywords Flocculus • Plasticity • Climbing fiber

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Climbing fiber activity is often associated with cerebellar changes in synaptic efficacy of Purkinje cells. Long-term depression (LTD) in Purkinje cells provides an example of decreased synaptic efficacy of a set of pre-synaptic afferent, parallel fibers, following their conjunctive pairing with a climbing fiber (Ito et al. 1982; Ekerot and Kano 1985; Linden and Connor 1993). The changes in synaptic efficacy observed during LTD last seconds to tens of minutes. These seemingly simple and short-lasting examples of neuronal plasticity are associated with the translation or translocation of as many as 100 proteins (Sanes and Lichtman 1999). Longer-term changes in synaptic efficacy, lasting tens of hours, may involve not only redistribution and targeting of proteins, but changes in gene transcription as well. Here we consider how Purkinje cell activity evoked by natural stimulation of cerebellar climbing fibers causes increased transcription of microRNA.

Conceptually the problem of controlling gene transcription, translation and targeting of multiple proteins could be simplified if the proteins were regulated by common precursors such as microRNAs; small, non-coding RNAs derived from "junk" DNA. A single microRNAs can target the 3′-untranslated regions of as many as 5–30 mRNAs and limit their translation by complementary repression and degradation (Cullen 2004; Robins and Press 2005; Landgraf et al. 2007; Hobert 2008; Eulalio et al. 2009; Guo et al. 2010). While the transcription of microRNAs has been linked to cellular development, apoptosis (Ambros 2004; Reinhart et al. 2000; Harfe 2005; Cullen 2004; Kosik 2006; Schratt et al. 2006; Bushati and Cohen 2007) and microbial defense (Cullen 2004; Bartel 2004; Zeng et al. 2005). microRNAs also regulate functions of adult neurons (Smalheiser and Lugli 2009; Schratt 2009; Konopka et al. 2011; Tognini et al. 2011; Mellios et al. 2011; Barmack et al. 2010).

Each Purkinje cell receives synaptic input from only one climbing fiber that makes ~500 glutamatergic synaptic contacts as it envelopes the dendritic tree (Cajal 1911; Granit and Phillips 1956; Konnerth et al. 1990; Harvey and Napper 1991). The climbing fiber evokes the largest EPSP of any known central synapse (Eccles et al. 1967). Activation of climbing fiber synapses on Purkinje cells offers a powerful model system for examining how naturally-evoked neuronal activity influences the transcription of microRNAs and mRNAs. Controlled modulation of climbing fiber activity can be achieved using horizontal optokinetic stimulation (HOKS) to modulate the activity of floccular Purkinje cells. To achieve this objective unanesthetized mice are restrained at the center of a rotating optokinetic sphere (Fig. 19.1b). Rotation of the optokinetic sphere about its vertical axis in the CCW direction excites direction-selective "on" ganglion cells in the right eye while disfacilitating these cells in the left eye (Oyster et al. 1980). Ganglion cell axons from the right eye project to the left nucleus of the optic tract (NOT) in the dorsal midbrain. The axons of neurons in the left NOT descend to the inferior olive where they excite neurons in the left dorsal cap (Maekawa and Simpson 1973; Alley et al. 1975; Barmack and Hess 1980; Simpson et al. 1988). Neurons in the left dorsal cap project as climbing fibers to the right flocculus where they excite Purkinje cells (Fig. 19.1c) (Leonard et al. 1988; Schonewille et al. 2006). Since the visual projections to the flocculus are lateralized HOKS causes increased climbing fiber excitation in one flocculus while reducing it in the other.

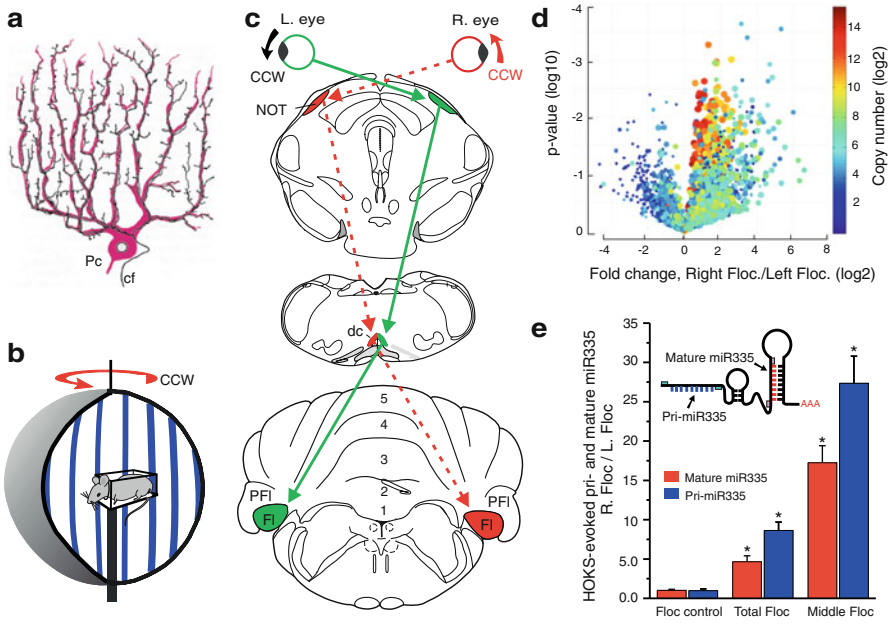

Fig. 19.1 Horizontal optokinetic stimulation (HOKS) evokes climbing fiber induced increases in microRNA transcription in the flocculus. (**a**) Mice are restrained at the center of an optokinetic sphere. Counter clockwise (CCW) rotation of the sphere (HOKS at 6 deg/s) excites direction-selective "on" ganglion cells in the right eye and "disfacilitates" ganglion cells in the left eye. (**b**) A cartoon shows that a single climbing fiber makes extensive synaptic terminals on a Purkinje cell dendritic tree (Redrawn from Cajal 1911). (**c**) A cartoon depicts functional optokinetic pathway from the retina to the ipsilateral flocculus (see text for details). The *dashed lines* indicate the excitatory pathway that originates from the right eye. The *solid lines* indicate the "disfacilitated" pathway from the left eye. (**d**) RNA samples from left and right flocculi are analyzed initially on a microRNA microarray that includes 616 human and 361 mouse mature miRNAs. Captured microRNAs are plotted in a Volcano plot, representing four variables: (1) The fold change for each microRNA 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. (**e**) Specific microRNAs are measured using qPCR. For miR335 both pri-miR335 and mature miR335 are identified using specific primer pairs. Both pri-miR335 and mature miR335 transcripts increase. The location of qPCR primers that flank the targeted nucleotide sequences is indicated for both sets of primers by boxes. Abbreviations: *dc* dorsal cap of the inferior olive, *Fl* flocculus, *NOT* nucleus of the optic tract, *PFl* paraflocculus

Climbing fiber activity increases the transcription of microRNAs in Purkinje cells. HOKS can be maintained for 0–30 h. When it is stopped mice are anesthetized, the left and right flocculi are removed and total RNA is extracted from each flocculus. Changes in microRNA transcripts in "stimulated" and "non-stimulated" flocculi are measured using a microarray (GeneChip® microRNA 2.0, Affymetrix Co).

Three criteria are used to discriminate levels of microRNA transcripts; (1) Fold changes between the left and right flocculus must exceed twofold. (2) The *P*-value of a *t*-test for significance must be <0.005. (3) The average copy number must exceed 256. These three criteria identify 12 microRNAs (miR133, miR7a, miR199a-5p, let71, miR100, miR15a, miR21, miR335-5p, miR361, miR379, miR22, miR126-3p) with increased transcripts in the right flocculus following 24 h of CCW HOKS (Barmack et al. 2010). Three of these microRNAs (miR126, miR335 and miR379) have p-values <0.001 (Fig. 19.1d).

The microRNAs identified by microarrays can be measured with greater accuracy and at reduced cost using quantitative Polymerase Chain Reaction (qPCR). RNA is extracted from the flocculi. cDNAs are synthesized and amplified using primer pairs that identify and amplify specific target sequences. Using qPCR, the transcripts of miR335 increase 18-fold in the "stimulated" vs "unstimulated" flocculus after 24 h of HOKS (Fig. 19.1e).

Proof of climbing fiber-evoked increases in microRNA transcripts is not necessarily proof of increased microRNA transcription. Several enzymatic post-transcriptional factors could contribute to the regulation of microRNAs. However, the transcription of larger pri-microRNAs precedes the action of intra-nuclear enzyme Drosha/Pasha that digests pri-microRNA converting it into pre-microRNA (Lee et al. 2003; Cai et al. 2004). It also precedes the action of cytoplasmic enzymes such as Dicer, thats cut pre-microRNA into a microRNA duplex (Lund and Dahlberg 2006) and Argonaut, that selects the mature microRNA strand from the microRNA duplex (Ghildiyal and Zamore 2009; Johnston and Hutvagner 2011). If climbing fiber-evoked depolarization of Purkinje cells increases mature microRNA transcripts by increasing transcription of pri-microRNAs rather than by post-transcriptional regulatory mechanisms, then it should be possible to measure changes in both pri-microRNAs and mature microRNAs during climbing fiber-evoked Purkinje cell activity. This question can be answered by measuring transcripts of pri-miR335 and mature miR335 in floccular RNA samples extracted from "stimulated" and "non-stimulated" flocculi. Primer binding sites for pri-miR335 are not present on mature miR335 and the primer binding sites on mature miR335 are not accessible until the duplex RNA is cleaved into two strands (Fig. 19.1e). Following 24 h of HOKS, pri-miR335 transcripts from the middle zone of the stimulated (right) flocculus are 28X more than those in the unstimulated (left) left flocculus. Mature miR335 transcripts are 18X more. In samples taken from the whole flocculus pri-miR335 transcripts are 9× more in the right flocculus and mature miR335 transcripts are 5X more. In control samples taken from mice not exposed to HOKS, the ratio of right flocculus/left flocculus for pri-miR335 and mature miR335 is 1 (Barmack et al. 2014). Consequently, we can conclude that climbing fiber excitation increases the transcription of miR335 in Purkinje cells.

Minimal HOKS time to detect climbing fiber evoked increases in microRNA transcription is 6 h. It is useful to know the minimal duration of HOKS necessary to evoke a detectable increase in microRNA transcripts. This question is answered in experiments where mice receive HOKS for different durations (0, 3, 6, 12, 24, 30 h).

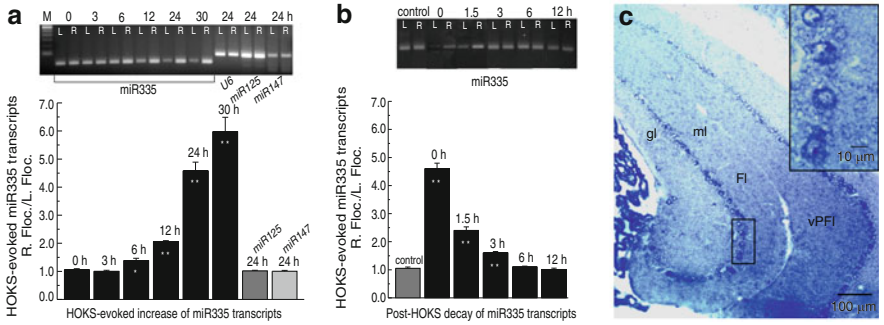

Fig. 19.2 Increased transcription of miR335 by HOKS and its subsequent decay. (**a**) Increasing the duration of HOKS increases the transcription of miR335. Mice receive binocular HOKS for fixed durations of 0–30 h. After HOKS is stopped ward the mice are anesthetized and euthanized. The flocculi are dissected for RNA extraction. cDNAs are synthesized and amplified by PCR. U6 is co-amplified as a loading control. Each reaction is run on a gel and the optical density of the bands is measured photometrically. When the ratio of PCR band density (R. Floc./L. Floc.) is >1 it indicates increased transcription of miR335 in the right flocculus. Two microRNAs, miR125 or miR147 (*gray bars*), are run as controls. Their transcription is not affected by HOKS. HOKS duration is indicated above each gel pair and for each *histogram bar*. (**b**) Transcripts of miR335 in the right flocculus decay rapidly. The transcripts are measured at the indicated times after HOKS is stopped. Three control mice (*gray bar*) receive no HOKS. For both **a** and **b**, each *histogram bar* indicates the mean for three mice. *Error bars* indicate standard error of the mean. *Asterisks* indicate statistical significance using a single factor ANOVA at $P<0.020$ (*) or $P<0.001$ (**). (**c**) A digoxigenin-labeled oligonucleotide complementary to miR335 and immunolabeled with an antibody to digoxigenin hybridizes with the cytoplasmic component of Purkinje cells. The area denoted by the *small box* is shown at higher magnification in the larger insert. Abbreviations: *Fl*, flocculus, *gl* granule cell layer, *ml* molecular layer, *vPFl* ventral paraflocculus (Modified from (Barmack et al. 2010))

Climbing fiber evoked transcription of miR335 can be detected after 6 h of HOKS. The transcription of miR335 increases linearly from 6 to 30 h (Fig. 19.2a).

miR335 transcripts decay following climbing fiber activation with a time constant of 2.5 h. It is important to know for how long the increases in microRNA transcripts persist after HOKS stops. This question can be addressed specifically by exposing mice to a constant duration of HOKS for 24 h. When HOKS stopped, the mice remain within the illuminated sphere for 0.0, 1.5, 3.0, 6.0, or 12 h. Transcripts of miR335 are then measured with qPCR at each of the specified post-stimulus intervals. Using this regimen miR335 transcripts decay to control levels with a time-constant of ~2.5 h (Fig. 19.2b). This rapid decay suggests that while microRNA transcripts cannot account singularly for long-lasting changes in Purkinje excitability observed after HOKS is stopped.

Hybridization histochemistry localizes microRNA transcripts to Purkinje cells. While HOKS increases microRNA transcripts in the flocculus, it is by no means certain that the transcripts are localized exclusively to Purkinje cells. This localization can be tested using "locked nucleic acid-modified oligonucleotide probes" to examine whether they hybridize with mature microRNAs. A probe for miR335

hybridizes with Purkinje cell soma, but not with Purkinje cell nuclei (Fig. 19.2c). This confirms the cytoplasmic location of the mature miR335 in Purkinje cells, rather than the nuclear location of the unedited longer pri-miR335. Probes for other microRNA transcripts that increase with HOKS, miR15, miR21 and miR361, also hybridize with Purkinje cells and weakly with stellate cells (not shown). A scrambled probe, having the same GC content as the probe for miR335, fails to hybridize with either Purkinje cells or other cerebellar neurons (not shown). These data confirm that the microRNAs evoked by climbing fiber activity are localized primarily to Purkinje cells.

Screens for discovering mRNAs targeted by microRNAs. Having established that microRNA transcription in Purkinje cells is influenced by climbing fiber activity, it would be useful to identify the proteins whose translation is repressed by miR335. The nucleotide sequence of miR335 offers the first clue to the identity of target mRNAs that have complementary sequences. However, the set of complementary mRNA targets for a particular microRNA is unacceptably large. A stringent screening of two data bases (microRNA Registry and EnsEMBL) reveals more than 149 mRNAs with sequence complementarity to miR335. Second, we can functionally reduce the number of potential mRNAs targeted by miR335 by using an mRNA array (Genome 430 2.0, Affymetrix) in conjunction with same duration HOKS used to identify miRNAs. This functional mRNA screen identifies mRNAs whose transcripts decrease following HOKS. It allows that not all such decreases can be attributed to increased miR335 repression. This screen reveals a total of 42 mRNA transcripts that decrease after HOKS (Barmack and Qian 2002; Barmack et al. 2010). Third, we can microinject specific miR335 inhibitors directly into the cerebellum and identify mRNA transcripts that increase as a consequence of the microinjection, again using an mRNA microarray. This approach generates 28 mRNAs whose transcripts increase following microinjection of miR335 inhibitors. In sum, we can identify 149 mRNA transcripts with nucleotide sequences complementary to that of miR335. We can detect 42 mRNA transcripts that decrease following HOKS and we can identify 28 mRNA transcripts that increase following a microinjection of microR335 inhibitors. This approach yields two mRNAs that satisfy all three screens; 14-3-3-θ and calbindin (Barmack et al. 2010, 2014).

microRNAs and cerebellar function. Cerebellar plasticity will not be explained by reference to a single microRNA. Rather it seems likely that multiple microRNAs act in parallel to control a common target mRNA. Furthermore, a specific mRNA may have a nucleotide sequence that offers complementary targets to several microRNAs. Gaining a functional understanding of the interactions between microRNAs and mRNAs may prove useful in treating cerebellar disorders. Already microRNA dysfunction has been linked to neurological diseases such as cerebellar ataxia (Schaefer et al. 2007; Barnes et al. 2011), spinal muscular atrophy (Haramati et al. 2010) and polyglutamine-induced neurodegeneration (Bilen et al. 2006). Pharmacological treatments that target specific microRNAs or proteins, whose translation is repressed by microRNAs, may provide novel therapeutic approaches for treating the consequences of aberrant neuronal excitability.

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