Long-term climbing fibre activity induces transcription of microRNAs in cerebellar Purkinje cells

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Synaptic activation of central neurons is often evoked by electrical stimulation leading to post-tetanic potentiation, long-term potentiation or long-term depression. Even a brief electrical tetanus can induce changes in as many as 100 proteins. Since climbing fibre activity is often associated with cerebellar behavioural plasticity, we used horizontal optokinetic stimulation (HOKS) to naturally increase synaptic input to floccular Purkinje cells in mice for hours, not minutes, and investigated how this activity influenced the transcription of microRNAs, small non-coding nucleotides that reduce transcripts of multiple, complementary mRNAs. A single microRNA can reduce the translation of as many as 30 proteins. HOKS evoked increases in 12 microRNA transcripts in floccular Purkinje cells. One of these microRNAs, miR335, increased 18-fold after 24 h of HOKS. After HOKS stopped, miR335 transcripts decayed with a time constant of approximately 2.5 h. HOKS evoked a 28-fold increase in pri-miR335 transcripts compared with an 18-fold increase in mature miR335 transcripts, confirming that climbing fibre-evoked increases in miR335 could be attributed to increases in transcription. We used three screens to identify potential mRNA targets for miR335 transcripts: (i) nucleotide complementarity, (ii) detection of increased mRNAs following microinjection of miR335 inhibitors into the cerebellum, and (iii) detection of decreased mRNAs following HOKS. Two genes, calbindin and 14-3-3-\(\mu\), passed these screens. Transfection of N2a cells with miR335 inhibitors or precursors inversely regulated 14-3-3-\(\mu\) transcripts. Immunoprecipitation of 14-3-3-\(\mu\) co-immunoprecipitated PKC-\(\gamma\) and GABA\(_{\alpha}\) receptors, horizontal optokinetic stimulation.

1. Introduction

Functional plasticity in the discharge of neurons in the central nervous system (CNS) can be characterized by a variety of physiological and molecular techniques. ‘Habituation’, a decrement in post-synaptic response evoked by repetitive electrical stimulation of an afferent pathway, represents neuronal plasticity in its simplest form [1]. Cerebellar ‘long-term depression’ in Purkinje cells provides an example of decreased synaptic efficacy of a set of electrically stimulated pre-synaptic afferent, parallel fibres, following their conjunctive pairing with another afferent, a climbing fibre [2–4]. The changes in synaptic efficacy observed during ‘long-term depression’ or its excitatory counterpart, ‘long-term potentiation’, have durations of 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 [5]. Longer term changes in synaptic efficacy, lasting tens of hours, may involve not only redistribution and targeting of proteins, but also changes in gene transcription.
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. MicroRNAs target the 3′-untranslated regions of as many as 5–30 mRNAs and limit their translation by complementary repression and degradation [6–11].

While earlier experiments have linked the transcription of microRNAs to cellular development, apoptosis [6,12–17] and microbial defence [6,18,19], it has become increasingly accepted that microRNAs could regulate functions of adult neurons [20–24]. Functionally, the idea that microRNAs could provide a common regulatory element for activity-induced changes in synaptic function is not as far fetched as it might first seem. Approximately one-third of human protein-coding genes are regulated by microRNAs [25]. Only recently has it become possible to investigate how long-term neuronal activity might functionally influence the transcription of specific microRNAs. Here we examine how long-term activation of visual climbing fibres influences transcription of microRNAs transcription of microRNAs in cerebellar Purkinje cells. We show that a specific microRNA, miR335, complements mRNAs of two proteins, 14-3-3- and calbindin. One of these proteins, 14-3-3-6, influences the insertion of GABA_A receptors into the Purkinje cell membrane.

2. Long-term stimulation of a climbing fibre pathway

Investigation of the effects long-term synaptic stimulation on the transcription genes and translation of mRNAs requires a chronic preparation that can be effectively stimulated for tens of hours without compromising the health of the animal. The stimulation must also modulate the discharge of neurons in a system with well-understood anatomy and physiology. Once stimulation is stopped, it is essential to analyse transcriptional changes in tissue samples containing cells whose activity has been modulated by the manipulated stimulus. Furthermore, the transcriptional changes evoked in the ‘stimulated’ sample must be compared to an identical ‘unstimulated’ tissue sample obtained from the same animal.

(a) Horizontal optokinetic stimulation

Horizontal optokinetic stimulation (HOKS) can be used to control parametrically an afferent signal to the flocculus. HOKS is generated by rotating a sphere or cylinder with striped vertical meridians on its interior walls about a rabbit (figure 1a). Counter clockwise (CCW) rotation of the sphere or drum with respect to the right eye at velocities of 0.1–10.0 deg s^{-1} increases activity of ‘on’ direction-selective ganglion cells [29]. Clockwise (CW) rotation with respect to the right eye decreases activity of ganglion cells. HOKS has an important behavioural function. It evokes reflexive eye movements termed the ‘optokinetic reflex’. This reflex consists of two phases: slow phases during which the eyes nearly match the velocity of the sphere rotation, and fast phases that quickly reset the initial starting positions of the slow phases. Functionally, the ‘optokinetic reflex’ provides one of several sensory signals that maintain postural stability. The direction selectivity of ganglion cells is reflected in the directional gain (eye velocity/drum velocity) of the evoked eye movement. Monocular viewing of CCW rotation of the drum with respect to the right eye evokes eye movements over a greater range of stimulus velocities than does CW rotation (figure 1c).

Long-term HOKS causes adaptation of central neuronal circuitry. The eyes adapt to retinal slips of contrast-rich environments. This adaptation is reflected in the reduced gain of the optokinetic reflex. When long-term HOKS is stopped, re-adaptation to the absence of HOKS is required. The optokinetic system attempts to re-create a steady-state retinal slip that existed prior to the termination of HOKS. This re-adaptation consists of moving the eyes oppositely to previous direction of HOKS. Consequently, rabbits that have received long-term HOKS and are subsequently placed in the dark develop a large counter nystagmus in which the slow phase of the eye movements reverses and attains velocities (20–50 deg s^{-1}) that are well beyond the range of eye velocities attained during HOKS. This reversed nystagmus persists for 12–36 h (figure 1d) [26]. An analogous vestibular adaptation in humans occurs when one adapts to the motion of a boat at sea (sea legs) and re-adapts to the stability of land upon return (land legs).

(b) Central optokinetic projections from the eye to floccular Purkinje cells

The visual systems of mice and rabbits are predominantly lateralized (figure 1b). Axons of direction-selective ganglion cells project to the contralateral nucleus of the optic tract (NOT) [30]. NOT axons innervate a small cluster of neurons in the inferior olive termed the dorsal cap (dc) [27,31,32]. Approximately, 1000 neurons in the caudal dc respond to HOKS. CCW HOKS of the right eye (figure 1a) increases the discharge of neurons in the left caudal dc. CW HOKS with respect to the right eye increases the discharge of these neurons (figure 1e) [27]. Axons from the dc cross the midline and terminate as climbing fibres on Purkinje cells in the right cerebellar flocculus, ipsilateral to the right eye stimulated in the CCW direction (figure 1a) [32–34].

Each Purkinje cell receives synaptic input from only one climbing fibre which makes approximately 500 glutamatergic synaptic contacts as it envelopes the dendritic tree [35–38]. The climbing fibre evokes the largest excitatory postsynaptic potential (EPSP) of any known central synapse [39]. Climbing fibre-evoked EPSPs trigger a sequence of multiple action potentials termed ‘complex spikes’ (CSs) that last 5–10 ms [36,40–42]. CSs are followed by a Ca^2+ activated increased K^+ conductance [43]. In addition to the low frequency (0.1–4.0 imp s^{-1}) discharge of CSs, Purkinje cells also have shorter duration action potentials termed ‘simple spikes’ (SSs) that discharge at higher frequencies (10–50 imp s^{-1}). It is universally observed that when CSs increase, SSs decrease. The histogram in figure 1f (modified from [43]) illustrates this reciprocal association between CSs and SSs. The earliest part of the reciprocity of CSs and SSs is caused by the Ca^2+ activated increased K^+ conductance [43]. However, in vivo the major component of the CS-evoked decrease in SSs can be attributed to climbing fibre-evoked activity of inhibitory stellate cells. While climbing fibres release glutamate directly onto Purkinje cell dendrites, the glutamate also spills over and excites stellate cells that, in turn, feed back onto Purkinje cells. This spillover-induced inhibition lasts approximately 100 ms [44–48].

Modulation of SSs in vivo is often attributed to excitatory action of parallel fibre synapses on Purkinje cell dendrites.
Figure 1. Horizontal, optokinetic stimulation (HOKS) modulates the discharge of ‘complex spikes’ (CSs) and ‘simple spikes’ (SSs) in floccular Purkinje cells. (a) Long-term HOKS is evoked by continuously rotating a contour-rich optokinetic drum or sphere about a vertical axis. (b) Counter clockwise (CCW) rotation of the drum increases the discharge of direction-selective retinal ganglion cells in the right eye. These cells terminate in the left nucleus of the optic tract (NOT). NOT neurons excite neurons in the subjacent dorsal cap (dc) of the left inferior olive. Climbing fibres from the dorsal cap cross the midline and terminate on Purkinje cells in the right flocculus (Fl). (c1) Monocular CCW HOKS of the right eyes evokes eye movements in a pattern of slow and fast phases. The velocity of the slow phases nearly matches the velocity of the HOKS over a wide range of velocities (0.10–10 deg s⁻¹). (c2) Monocular CW HOKS of the right eye is less effective in evoking eye movements even at low stimulus velocities. The lower traces in c1 and c2 indicate HOKS velocity. (d) HOKS becomes less effective in evoking eye movements during long-term (12–40 h) stimulation. When long-term HOKS is stopped (indicated by arrow) and the rabbit is placed in the dark, a nystagmus develops in which the direction of the slow phases is opposite to the slow phases evoked by HOKS. This negative optokinetic after-nystagmus, termed OKAN II, is long-lasting (24–36 h). The slow phases of OKAN II attain abnormally high velocities (30–40 deg s⁻¹) that never occur spontaneously or during HOKS (modified from [27]). (e) Multi-neuron recordings from the left caudal dorsal cap of the inferior olive are excited by constant velocity CCW HOKS of the right eye and disfacilitated by constant velocity CW HOKS (modified from [30]). (f) Constant velocity CCW HOKS of the right eye increases and CW HOKS decreases discharge of CSs in a Purkinje cell in the right flocculus. The discharge of SSs decreases during CCW HOKS and increases during CW HOKS (modified from [43]). dc, dorsal cap; Fl, flocculus; PFl, paraflocculus.
This attribution of SS modulation to excitatory parallel fibre signals lacks experimental support. In the vestibular cerebellum, the discharges of CSs and of vestibular primary afferent mossy fibres increase during ipsilateral roll-tilt. The discharges of SSs are oppositely modulated, ruling out the possibility that the discharges of SSs are determined primarily by mossy fibre-evoked parallel fibre discharges [58,59].

The primary role of climbing fibres in modulating the antiphasic response of SSs is underscored by mutation of a gene, Ptf1a::cre;Robo3lox/lox [60]. In such mutants, the normally crossed climbing fibre projection to the contralateral cerebellum is routed ipsilaterally. As we have already observed, CCW optokinetic stimulation of the right eye in normal mice increases the discharge of CSs and decreases the discharge of SSs in the right flocculus. However, in mutants the CCW stimulation of the right eye now decreases the discharge of CSs and increases discharge of SSs in the right flocculus. The directional polarity of the HOKS-evoked CSs and SSs is reversed. Their reciprocity persists. The discharges of SSs would not reverse if they were evoked by mossy fibre projections to granule cell-parallel fibre projections. Mossy fibre projections remain undisturbed in the mutants.

The flocculus has the distinct advantage of being physically accessible. After HOKS is stopped, a mouse is anaesthetized, euthanized and both flocculi are identified and removed. In the mouse, the flocculus is spindle shaped. It is approximately 1.1 mm in axial length, approximately 400 μm at its peak diameter and weighs approximately 400 μg. The climbing fibres that are activated by HOKS project to the middle third of the flocculus, making it possible to obtain a more concentrated sample of activated cells [61]. Experimental samples of RNA are obtained from Purkinje cells in the middle segment of the right flocculus that are excited by monocular CCW HOKS of the right eye. RNA samples can also be obtained from unstimulated Purkinje cells in the left flocculus, thereby providing ‘control’ samples of microRNAs with which stimulated samples of microRNAs are compared.

3. microRNA pathway

Regulation of microRNA consists of a cascade of enzymatically controlled pathways as illustrated in figure 2. In the nucleus RNA polymerase II, Pol II, transcribes a microRNA gene into an unstructured primary-microRNA, pri-microRNA, several hundreds of nucleotides in length and containing one or more microRNA stem–loops [63,64]. Class 2 RNase III enzymes, Drosha/Pasha, cleave pri-microRNA into a characteristic stem–loop structure approximately 70 base pairs long [65]. Drosha caps the pre-microRNA with a polyadenylated...

Figure 2. Regulatory pathways for microRNA transcription. See text for abbreviations and discussion (adapted from [62]). (Online version in colour.)
nucleotide [66]. Pre-microRNA is transferred to the cytoplasm by Exportin-5, a member of the karyopherin family that recognizes the two-nucleotide overhang left by Drosha at the 3’ end of the pre-microRNA hairpin [67,68]. Once in the cytoplasm, the pre-microRNA is cleaved by an endonuclease in the RNase III family, Dicer, into short duplex fragments, dsRNA, that are approximately 22 nucleotides long. These fragments have a two-base overhang on the 3’ end [69]. Although either strand of the duplex may potentially act as a functional microRNA, one strand, mature microRNA, is recognized by an Argonaute protein. It cleaves the microRNA duplex and selects the strand that becomes the mature microRNA [70,71]. Mature microRNA is incorporated into the RNA-induced silencing complex (RISC) where it de-adenylates its target mRNA. This promotes de-capping and degradation of the mRNA rather than translational repression [10,11].

microRNA is associated with structural plasticity [6,12,15,16,72–75], microbial defence [6,18,19] and oncogenesis [76,77]. More recently, microRNAs have been implicated in modifying the discharge of neurons in the CNS [78–81]. In neurons, an activity-dependent microRNA could repress the translation of multiple mRNAs. Conversely, several activity-dependent microRNAs could target a single mRNA, thereby allowing independent regulation of protein expression by multiple transcriptional pathways [9,20,21,79,82].

4. Climbing fibre discharge increases transcripts of microRNAs in the flocculus

Investigations of microRNAs most often begin with a screen for microRNA transcripts associated with a developmental stage, a disease or an experimental treatment. Here, we begin with a microarray screen for transcripts associated with the synaptic events triggered in a cerebellar Purkinje cell, by a single climbing fibre synaptic input. CCW HOKS with respect to the right eye increases the discharge of climbing fibres that project to right flocculus. When HOKS is stopped 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 were used to discriminate levels of microRNA transcripts: (i) fold changes between the left and right flocculus must exceed twofold; (ii) the p-value of a t-test of significance must be less than 0.005; (iii) the average copy number must exceed 256. These three criteria identified 12 microRNAs whose transcription is differentially increased in the right flocculus following 24 h of HOKS [62]. These microRNAs include miR133, miR7a, miR199a-5p, let7i, miR100, miR15a, miR21, miR335-5p, miR361, miR379, miR22 and miR126-3p. Three of these microRNAs (miR126, miR335 and miR379) have p-values of less than 0.001.

Once an initial inquiry has been narrowed using microarrays, one can use qPCR to measure specific transcripts from total RNA isolated from stimulated and unstimulated flocculi. We initially focused on one of the microRNAs, miR335, that had the greatest fold change of any of the candidate microRNAs. The duration of HOKS was varied (figure 3c). An increase in miR335 transcripts could be detected after HOKS of only 6 h. After 30 h of HOKS, miR335 transcripts in the right flocculus increased by a factor of 6 relative to transcripts in the left flocculus.

5. miR335 transcripts decay rapidly after horizontal optokinetic stimulation is stopped

If increased climbing fibre activity is associated with an increase in one or more microRNA transcripts, then it is important to know for how long the increases in microRNA transcripts persist when the increased climbing fibre activity stops. This question has been addressed specifically for HOKS-induced transcripts of miR335 by stimulating mice in the CCW direction with respect to the right eye for 24 h. When HOKS stopped, the mice remained within the illuminated sphere for 0.0, 1.5, 3.0, 6.0 or 12 h. Transcripts of miR335 were measured with qPCR at each of the specified post-stimulus intervals. miR335 transcripts decayed to control levels with a time-constant of approximately 2.5 h (figure 3e). This rapid decay suggests that microRNA transcripts cannot account singularly for long-lasting changes in Purkinje excitability observed after HOKS is stopped.

6. Probes for six microRNAs hybridize with Purkinje cells

While HOKS increases microRNA transcripts in the flocculus, it was by no means certain that the transcripts were localized exclusively to Purkinje cells. Consequently, we used six ‘locked nucleic acid-modified oligonucleotide probes’ to examine whether they hybridize with mature microRNAs (miR15, miR21, miR335, miR361 and miR379) in Purkinje cells as well as cerebellar interneurons. The probe for miR335 hybridized with Purkinje cell soma (figure 3c), but not with nuclei. This confirms that the probe targets the mature miR335 and not the unedited longer pri-miR335. Probes for miR15, miR21 and miR361 also hybridized with Purkinje cells and weakly with stellate cells (not shown). A scrambled probe, having the same GC content as the probe for microR335, failed to hybridize with either Purkinje cells or other cerebellar neurons (not shown). These data confirm that the microRNAs detected in RNA samples are derived principally from Purkinje cells.

7. Climbing fibres induce transcription of pri-miR335 in Purkinje cells

Proof of increased microRNA transcripts is not necessarily proof of increased transcription. Several post-transcriptional factors could possibly contribute to the regulation of microRNAs. While a promoter initiates transcription of a pri-microRNA, this pri-microRNA could, in principle, be regulated enzymatically by Drosha/Pasha as it is reduced to a pre-microRNA (figure 2). Similarly, the pre-microRNA could be regulated by Exportin as it passes from the nucleus into the cytoplasm. In the cytoplasm, Dicer might provide a rate limiting step in the formation of the microRNA duplex. A similar role has been proposed for Argonaute whose expression could also be a rate limiting post-transcriptional step in the biogenesis of mature microRNA transcripts [71]. RISC could also regulate mature microRNA transcripts by shielding them from enzymatic decay [83]. While these possible regulatory functions make sense developmentally as a means of controlling post-transcriptional processing of multiple microRNAs...
qPCR primers that provided independent measurements of pri
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simultaneously, they might corrupt the specificity of
microRNA signalling.

If climbing fibre-evoked depolarization of Purkinje cells
increases mature microRNA transcripts by increasing transcrip-
tion 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 fibre-evoked Purkinje cell activity. We designed
qPCR primers that provided independent measurements of pri-
miR335 and mature miR335. The 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 (figure 4). We exposed
mice to 24 h of HOKS, increasing the climbing fibre input to
Purkinje cells. We also increased the sensitivity of our measure-
ments by restricting Purkinje cell samples to the middle zone of
the flocculus. This is the zone to which climbing fibres that are
responsive to HOKS project [34]. Following 24 h of HOKS,

Figure 3. HOKS increases miR335 transcripts in flocculus. (a) Mice received binocular HOKS for 0–30 h. After HOKS, the mice were anaesthetized and euthanized.
The flocculi were dissected for RNA extraction, cDNAs were synthesized and amplified by PCR. U6 was co-amplified as a loading control. Each reaction was run on a
gel and the optical density of the bands was determined photometrically. The ratio of PCR band density (R. Flocc./L. Flocc.) indicates increased transcripts of miR335 in
the right flocculus. No increase was observed for transcripts of miR125 or miR147. Increasing the duration of binocular HOKS increased the transcripts of miR335. The
duration of HOKS is indicated above each gel pair. 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 *

Transcripts of miR335 in the right and left flocculi were measured at the indicated times after HOKS was stopped. Each histogram bar indicates the mean for
three mice. Three control mice (left bar) received no HOKS. Asterisks indicate statistical significance using a single factor ANOVA (p < 0.001). (b) Transcripts of miR335 decayed rapidly following 24 h of HOKS.
Transcripts of miR335 in the right and left flocculi were measured at the indicated times after HOKS was stopped. Each histogram bar indicates the mean for
three mice. Three control mice (left bar) received no HOKS. Asterisks indicate statistical significance using a single factor ANOVA (p < 0.001). (c) miR335 is tran-
scribed in cerebellar Purkinje cells. Coronal sections through the cerebellar flocculus and paraflocculus were hybridized to a digoxigenin-labelled oligonucleotide
complementary to miR335 and immunolabelled with an antibody to digoxigenin. The area denoted by a boxed outline is shown at higher magnification in
(d). The arrowheads indicate Purkinje cells with cytoplasmic, but not nuclear, immunolabelling. Fl, flocculus; gl, granule cell layer; ml, molecular layer; vPFl, ventral
paraflocculus (adapted from [62]). (Online version in colour.)
Figure 4. HOKS-evoked climbing fibre discharge increases transcription of pri-miR335 as well as miR335 in Purkinje cells. The relative number of transcripts of both pri-miR335 and mature miR335 increased after 24 h of HOKS (P > 0.001 with respect to the right eye). qPCR primers were constructed to sample both pri-miR335 (concentrated in the nucleus) and mature miR335 (exported to the cytoplasm). Binocular HOKS increased transcripts of pri-miR335 and miR335 in six mice. The ratio of qPCR (R. Floc./L. Floc.) increased for both pri-miR335 and mature miR335 for samples from the whole flocculi (total Floc). An even larger increase was obtained when floccular tissue samples were restricted to 400 μm segment that corresponds to the middle floccular region (middle Floc.). Transcripts were also measured from floccular tissue obtained from three unstimulated control mice (control). Error bars indicate standard error of the mean. Asterisks indicate statistical significance using a single factor ANOVA (p < 0.001). (Online version in colour.)

Figure 5. miR335 inversely controls transcripts of 14-3-3-ζ in vitro. N2a cells were transfected with either a miR335 inhibitor or a miR335 precursor. After 24 h total RNA was prepared from the cells. Transcripts of miR335, U6, 14-3-3-ζ and actin were measured by qPCR. Transcripts of miR335 were normalized with respect to U6 transcripts. Transcripts of 14-3-3-ζ and 14-3-3-ε were normalized with respect to actin transcripts. Transcripts of miR335, 14-3-3-0 and 14-3-3-ε from transfected cells were plotted relative to the transcripts obtained from untransfected cells. Treatment of N2a cells with the miR335 inhibitor decreased transcripts of miR335 and increased transcripts of 14-3-3-0, but had no effect on transcripts of 14-3-3-ε. Treatment with the miR335 precursor increased miR335 and decreased 14-3-3-0, but had no effect on 14-3-3-ε. Error bars indicate standard error of the mean. Asterisks indicate statistical significance using a single factor ANOVA (p < 0.001).
for U6 served as a loading control for miR335. Transcripts for actin served as a loading control for calbindin, 14-3-3-α and 14-3-3-ε. Following transfection of N2a cells with the miR335 inhibitor, transcripts of miR335 decreased, while transcripts of 14-3-3-α increased. Conversely, transfection of N2a cells with the miR335 precursor increased transcripts of miR335 and decreased transcripts of 14-3-3-α. The selectivity of these interactions is supported by the absence of changes in transcripts of the control isoform, 14-3-3-ε. Although the equivalent experiment using calbindin transcripts as targets has not yet been completed, these data show that modulation of transcripts for 14-3-3-α can be induced directly by modulation of miR335.

10. 14-3-3-α Interacts with PKC-γ to serine phosphorylate GABA<sub>Α</sub>γ<sub>2</sub>

While climbing fibre-evoked increases in transcription of miR335 in Purkinje cells inversely regulate transcripts of 14-3-3-α, the significance of this regulation for cerebellar synaptic transmission remains to be demonstrated. In Purkinje cells, 14-3-3-α interacts exclusively with PKC-γ, one of several constitutively expressed isoforms of protein kinase C. PKC-γ is critically involved in the phosphorylation of cerebellar proteins. When 14-3-3-α was immunoprecipitated, it co-immunoprecipitated PKC-γ. It also co-immunoprecipitated GABA<sub>Α</sub>γ<sub>2</sub>, a subunit of the pentameric GABA<sub>Α</sub> receptor implicated in its membrane clustering [85–91].

Using N2a cells, we knocked down 14-3-3-α and PKC-γ to test how they contribute to the serine phosphorylation of GABA<sub>Α</sub>γ<sub>2</sub>. N2a cells were transfected with siRNAs or miRNAs designed specifically to knock down either 14-3-3-α or PKC-γ. N2a cells were divided into groups. One group was treated with 200 nM of phorbol 12-myristate-13-acetate (PMA), to increase native PKC activity. The other group did not receive PMA treatment. Knockdown of either 14-3-3-α or PKC-γ reduced serine phosphorylation of GABA<sub>Α</sub>γ<sub>2</sub> only in N2a cells that also were activated by PMA (figure 6a) [85].

We examined the possibility that knockdown of either 14-3-3-α or PKC-γ reduced the cell surface expression of GABA<sub>Α</sub>γ<sub>2</sub> using an assay that preferentially biotinylates cell surface proteins [85]. Again we compared the efficacy of the knockdowns in N2a cells that were treated with PMA in contrast to those that were untreated (figure 6b). In PMA-treated N2a cells, knockdown of either 14-3-3-α or PKC-γ reduced cell surface expression of GABA<sub>Α</sub>γ<sub>2</sub>.

11. Functional effects of miR335 repression of 14-3-3-α mRNA

This review has provided evidence that long-term synaptic activation of Purkinje cells by climbing fibres increases transcription of several microRNAs. Specifically, we have shown HOKS increases climbing fibre discharge and that this increased discharge increases transcription of miR335 in floccular Purkinje cells 18-fold [62]. Undoubtedly, we have underestimated the magnitude of the climbing fibre-induced increase in transcription of miR335. Floccular tissue samples always include Purkinje and other cell types that are unresponsive to HOKS or did not express miR335. Increased transcription of miR335 in Purkinje cells can be detected after 6 h of HOKS.

Figure 6. Knockdown of 14-3-3-α and PKC-γ in N2a cells reduces serine phosphorylation of GABA<sub>Α</sub>γ<sub>2</sub>, and reduces its cell surface expression. 14-3-3-α and PKC-γ were knocked down independently in N2a cells by siRNA treatment or miRNA transfection. The N2a cells were also treated with 200 nM of phorbol 12-myristate-13-acetate (PMA), a PKC activator, or received no PMA treatment. (a) PMA treatment increased serine phosphorylation of GABA<sub>Α</sub>γ<sub>2</sub> in N2a cells (p < 0.005). Knockdown (KD) of 14-3-3-α or PKC-γ decreased serine phosphorylation only in cells treated with PMA (p < 0.050, indicated by asterisk). (b) KD of 14-3-3-α or PKC-γ decreased cell surface expression of GABA<sub>Α</sub>γ<sub>2</sub> in N2a cells treated with PMA (p < 0.050, indicated by asterisk). Cell surface expression of GABA<sub>Α</sub>γ<sub>2</sub> was measured by selective biotinylation of membrane proteins. Measurements were normalized with respect to control cells treated with vehicle alone. The error bars indicate one standard error of the mean (adapted from [91]).

In a step-by-step approach, we have identified the miRNAs with which miR335 interacts and how the proteins expressed by these miRNAs functionally contribute to the regulation of synaptic functions of Purkinje cells. This approach begins with increased climbing fibre discharge and ends with increased membrane clustering GABA<sub>Α</sub> receptors (figure 7): (a)
Transcription of miR335 is increased by climbing fibre discharge. Increased transcription of miR335 complements 14-3-3-μmRNA and decreases expression of 14-3-3-μ protein. (a) Increased climbing fibre discharge reduces transcripts of GABARAP (the mechanism of this reduction is not now known). (b) 14-3-3-μ associates with both PKC-γ and GABA_A_γ2. This association causes serine phosphorylation of GABA_A_γ2. (c,d) GABA_A receptor activated protein (GABARAP) and serine-phosphorylated GABA_A_γ2 form a complex with N-ethylmaleimide-sensitive factor (NSF). (e) The trafficking complex forms a vesicle. (f) Once assembled, the vesicle can be inserted into the plasma membrane. (g) The diffusion of GABA_A Rs within the plasma membrane is limited by interaction with gephyrin, forming clusters of GABA_A receptors.

Although we have shown that miR335 interacts with calbindin, we have not yet shown how calbindin might also contribute to the alteration of synaptic activity. Decreased expression of calbindin may have multiple regulatory functions. It buffers intracellular calcium and prevents damage of intracellular organelles, such as mitochondria, from large calcium transients associated with cellular depolarization [92,93]. Climbing fibre-evoked depolarization of Purkinje cells consists of sodium and calcium spikes that raise the intracellular concentration of calcium [94,95]. Larger intracellular calcium concentrations induced by miR335 repression of calbindin translation could increase the likelihood of cytotoxicity or, alternatively, could act as an intracellular signal that reflects changes in synaptic activity. In many excitable cells, calcium concentration is detected by calcium sensors that interact with Kv4 low voltage-activated A-type potassium channels. These sensors may regulate spike timing and dendritic excitability [96]. So it is possible that miR335 is a key component in a pathway that regulates the excitability of parallel fibre synapses on Purkinje cells.

12. Normal and abnormal functions of microRNAs in Purkinje neurons

Although this review has focused on the functions of a single microRNA, it is not the only microRNA that targets calbindin or 14-3-3-μ mRNA. Certainly, higher forms of learning will not be explained by reference to a single microRNA. Rather it seems likely that multiple microRNAs participate in different forms of neuronal plasticity. The interactions revealed by these experiments may generalize to non-cerebellar synaptic systems that use either glutamate or GABA as neuronal transmitters.

Climbing fibre activity increased transcripts of 12 microRNAs in Purkinje cells. Five of these transcripts have nucleotide sequences that are complementary to 14-3-3-μ mRNA. This raises the possibility that multiple microRNAs act in parallel to control of a common target mRNA. While analysis of such parallel pathways introduces a daunting level of complexity, these pathways offer a variety of options...
for comparing the conditions during which one or several of the pathways are activated.

Regulation of multiple microRNAs that target common mRNAs might also prove useful in treating cerebellar disorders. Already microRNA dysfunction has been linked to neurological diseases such as cerebellar ataxia [78,97], spinal muscular atrophy [98] and polyglutamine-induced neurodegeneration [99]. Pharmacological treatment that targets specific microRNAs or that targets specific proteins which themselves are influenced by multiple microRNAs may provide novel therapeutic approaches for regulating aberrant neuronal excitability.

References


