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Differential regulation of mature and precursor microRNA expression by NMDA and metabotropic glutamate receptor activation during LTP in the adult dentate gyrus *in vivo*

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Abstract

Regulation of microRNA (miRNA) expression and function in the context of activity-dependent synaptic plasticity in the adult brain is little understood. Here, we examined miRNA expression during long-term potentiation (LTP) in the dentate gyrus of adult anesthetized rats. Microarray expression profiling identified a subpopulation of regulated mature miRNAs 2 h after the induction of LTP by high-frequency stimulation (HFS) of the medial perforant pathway. Real-time polymerase chain reaction analysis confirmed modest upregulation of miR-132 and miR-212, and downregulation of miR-219, while no changes occurred at 10 min post-HFS. Surprisingly, pharmacological blockade of *N*-methyl-D-aspartate receptor (NMDAR)-dependent LTP enhanced expression of these mature miRNAs. This HFS-evoked expression was abolished by local infusion of the group 1 metabotropic glutamate receptor (mGluR) antagonist, (RS)-1-aminoadipic acid (AIDA). AIDA had no effect on LTP induction or maintenance, but blocked activity-dependent depotentiation of LTP. Turning to the analysis of miRNA precursors, we show that HFS elicits 50-fold elevations of primary (pri) and precursor (pre) miR-132/212 that is transcription dependent and mGluR dependent, but insensitive to NMDAR blockade. Primary miR-219 expression was unchanged during LTP. *In situ* hybridization showed upregulation of the pri-miR-132/212 cluster restricted to dentate granule cell somata. Thus, HFS induces transcription of miR-132/212 that is mGluR dependent and functionally correlated with depotentiation rather than LTP. In contrast, NMDAR activation selectively downregulates mature miR-132, -212 and -219 levels, indicating accelerated decay of these mature miRNAs. This study demonstrates differential regulation of primary and mature miRNA expression by mGluR and NMDAR signaling following LTP induction, the function of which remains to be defined.

Keywords

depotentiation; gene expression; hippocampus; long-term potentiation; synaptic plasticity

Introduction

Excitatory synapses of the mammalian brain display diverse forms of activity-dependent synaptic plasticity (Bliss et al., 2007; Nelson & Turrigiano, 2008). Bursts of synaptic activity can induce short-term changes in synaptic strength, but more stable modifications

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typically require modulation of gene expression at the transcriptional and post-transcriptional levels. Through post-transcriptional regulation, synaptic activity may dictate the time and place of neuronal protein synthesis (Ashraf & Kunes, 2006; Sutton & Schuman, 2006; Bramham & Wells, 2007; Bramham et al., 2010). Recently, microRNAs (miRNAs) have entered the fray as major regulators of post-transcriptional gene expression. miRNAs are short (19–24 nucleotides) non-coding RNAs that most commonly inhibit protein synthesis by sequence-specific binding to the 3' untranslated region (3' UTR) of target mRNAs and recruitment of an RNA-induced silencing complex (RISC), resulting in reduced translation or mRNA degradation (Standart & Jackson, 2007; Filipowicz et al., 2008). Several hundred miRNAs have been identified in different metazoan organisms. As miRNAs generally bind to numerous target mRNAs, and many mRNAs are regulated by multiple miRNA species, the possibilities for fine orchestration of translation are enormous.

Primary miRNA transcripts are processed in the nucleus by the RNAase III endonuclease Droscha to generate short-hairpin precursors of ~70–100 nucleotides, which are exported from the nucleus and further processed by another RNAase family enzyme, Dicer, to produce a mature miRNA of ~22 nucleotides in length. The activity of miRNAs may therefore be modulated at multiple steps in the biogenesis pathway as well as through regulation of the miRNA-bound RISC (Ashraf et al., 2006; Kosik, 2006; Presutti et al., 2006; Kye et al., 2007; Winter et al., 2009).

miRNAs play coordinating roles in a variety of cellular processes, including cell specification and apoptosis (Bartel, 2004; Chang et al., 2007). In neurons, recent studies have established roles for specific miRNAs in neurogenesis and dendritic spine morphogenesis (Vo et al., 2005; Krichevsky et al., 2006; Schratt et al., 2006; Cao et al., 2007; Fiore et al., 2009; Siegel et al., 2009). In the marine snail *Aplysia*, expression of miR-124 is linked to synapse-specific long-term sensitization (Rajasethupathy et al., 2009). In flies, degradation of the protein Armitage, a component of the miRNA-RISC, promotes synaptic protein synthesis during long-term memory.

Despite the advances in understanding neuronal miRNAs, little is known about miRNA regulation during activity-dependent synaptic plasticity in the adult mammalian brain. We therefore examined miRNA expression following induction of long-term potentiation (LTP) by high-frequency stimulation (HFS) of the perforant path input to the dentate gyrus of anesthetized rats. Using miRNA expression profiling and quantitative reverse transcription polymerase chain reaction (RT-PCR), we identified mature miRNAs with significantly increased (miR-132, miR-212) or decreased (miR-219) expression during LTP. Analysis of the primary and precursor transcripts demonstrated massive metabotropic glutamate receptor (mGluR)-dependent transcription of miR-132 and -212 in dentate granule cells that is functionally correlated with depotentiation rather than LTP. In contrast, activation of *N*-methyl-D-aspartate receptors (NMDAR) during LTP induction selectively downregulated mature miR-132, -212 and -219 levels, indicating stimulation of mature miRNA turnover.

Materials and methods

Electrophysiology and intrahippocampal infusion

Animal experiments were carried out in accordance with the European Community Council Directive of 24 November 1986 (86/609/EEC) and approved by the Norwegian Committee for Animal Research. Experiments were performed on 45 adult male Sprague–Dawley rats. The electrophysiological procedures have been detailed elsewhere (Messsaoudi et al., 2002; Panja et al., 2009). Briefly, rats were anesthetized with urethane and electrodes were inserted for selective stimulation of the medial perforant pathway and recording of evoked potentials in the hilar region of the dentate gyrus. Recordings were done with borosilicate

glass micropipettes (tip size 1–5 μm) filled with 1 M NaCl (input impedance 1–1.5 M Ω). Drugs were infused with a second micropipette (tip size 10–15 μm) connected via a polyethylene (PE50) tube to a 5- μL Hamilton syringe (Reno, NV, USA) and infusion pump. The two micropipettes were clamped together on a micromanipulator with a vertical tip separation of 700 μm . The tip of the infusion cannula was located in deep stratum lacunosum-moleculare of field cornu ammonis (CA) 1, approximately 300 μm from the nearest medial perforant path–granule synapses in the upper blade of the dorsal dentate gyrus. Test pulses were applied at 0.033 Hz throughout the experiment, except during the period of HFS. The HFS paradigm for LTP induction consisted of eight pulses at 400 Hz, repeated four times, at 10-s intervals. Three sessions of HFS were given, with 5 min between each HFS. A low-frequency stimulation (LFS) group received test pulses (one pulse every 30 s) but not HFS. Depotentiation was elicited by applying 5 Hz stimulation for 2 min (600 pulses) starting 2 min post-HFS.

Drugs

CPP [(R,S)-3-22-carboxypiperazin-4-yl-propyl-1-phosphonic acid; Tocris Cookson, UK] was dissolved in saline and injected i.p. at a dose of 10 mg/kg, 90 min prior to HFS. AIDA [(R,S)-1-aminoindan-1,5-dicarboxylic acid; Tocris] was dissolved in 1 mM sodium hydroxide and further diluted with 0.9% sodium chloride to a final concentration of 50 mM and pH adjusted to 7.4. Actinomycin D (ACD; 5 mg/mL in saline; Sigma, St Louis, MO, USA) was infused 2 h before HFS.

RNA purification

Urethane-anaesthetised rats were killed by decapitation and the dentate gyrus was rapidly microdissected on ice and homogenized as previously described (Wibrand et al., 2006). Total RNA containing short RNAs was extracted from homogenate samples using the *mirVana*TM PARIS miRNA Isolation kit (Ambion, Austin, TX, USA). The RNA was eluted in 100 μL of nuclease-free water, and RNA quality and quantity was determined spectrophotometrically.

miRNA expression profiling

mirVana-purified RNA (20 μg) was sent to LC Sciences (Houston, TX, USA) for microarray expression profiling (<http://www.lcsciences.com>). RNA samples were size fractionated using a YM-100 Microcon centrifugal filter (from Millipore), and the isolated small RNAs (< 300 nt) were 3'-extended with a poly(A) tail using poly(A) polymerase. An oligonucleotide tag was then ligated to the poly(A) tail for later fluorescent dye staining. Hybridization was performed using $\mu\text{ParaFlo}$ microfluidic chips (LC Sciences). Each detection probe consisted of a chemically modified nucleotide coding segment (21–35 nucleotides) complementary to mature target miRNA (miRBase <http://microrna.sanger.ac.uk/sequences/>) and a spacer segment of polyethylene glycol to extend the coding segment away from the substrate. Hybridization images were collected using a laser scanner (GenePix 4000B; Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analysed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression).

cDNA preparation and real-time PCR

For the analysis of *zif268*, 500 ng of total RNA was used as an input to generate cDNA using the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The cDNA was diluted 10-fold, and 5 μL was used as template for semiquantitative real-time RT-PCR together with the iQSYBR Green Supermix (Bio Rad, Hercules, CA, USA).

Samples were assayed in triplicate and normalized to polyubiquitine (Alme et al., 2007). Primers used to detect *zif268* and polyubiquitine: forward and reverse *zif268* (5' - AACAAACCCTACGAGCACCTG-3' and 5' - AGGCCACTGACTAGGCTGAA-3'), and forward and reverse polyubiquitine (5' - GGCAAGACCATCACCTAGA-3' and 5' - GCAGGGTTGACTCTTTCTGG-3').

Changes in mature miRNA levels were determined using the TaqMan® microRNA Reverse Transcription kit and TaqMan® microRNA Assays (Applied Biosystems, Foster City, CA, USA) according to manufacturer's protocol. cDNA (15 µL) was generated from 30 ng of total RNA, and 5 µL of a threefold dilution was used for real-time PCR reactions. Three small RNAs (y1, snoRNA, Rnu6B) and one miRNA were considered for normalization in an initial set of samples. In the end, Y1 and miR-16 were selected for normalization based on their sufficiently high and stable levels of expression among samples. The TaqMan assays used are listed in Table S1.

To amplify precursor sequences, the forward and reverse primers were designed to bind within the stem portion of the precursor miRNA. To amplify the primary transcript specifically, at least one primer was placed outside the precursor in the 5' or 3' flanking sequence. The precursor primers will amplify both precursor and longer transcripts, whereas primers annealing in the primary transcript will amplify the long primary sequence only. Precursor and primary sequences were obtained from the miRNA registry and UCSC Genome Browser, respectively (Griffiths-Jones, 2004; Kuhn et al., 2007). Primers were designed using Primer3 (Rozen & Skaletsky, 2000). Oligonucleotide sequences used for priming and PCR are listed in Tables S2 and S3.

Semiquantitative real-time RT-PCR of precursors and primary transcripts was carried out according to Jiang et al. (2005) and Schmittgen et al. (2008), with minor modifications. Briefly, total RNA was treated with RNase-free DNase (Ambion), and 500 ng of RNA was reverse-transcribed using a mix of gene-specific reverse miR primers (final concentration 10 µM) and the SuperScript III First Strand Synthesis System (Invitrogen). An initial step of 80°C for 1 min was added to the SuperScript III protocol to denature the hairpin structures. cDNA was diluted 20-fold, and 5 µL was used in each PCR reaction using the iQ SYBR Green Supermix (Bio Rad). PCR was performed for 20 s at 95°C and 1 min at 60°C for 40 cycles, followed by a thermal denaturation protocol to ensure amplification of a single product. For some primer combinations it was necessary to increase the annealing temperature to 62°C to get a more specific product (pri-132). The mature miR-219 is generated from two genes, miR-219-1 and miR-219-2. We were unable to amplify the precursor of miR-219-1, possibly due to its extremely low abundance, and therefore focused our analysis on miR-219-2.

Changes in relative concentration were calculated as the difference in threshold cycles (CT) between the left dentate gyrus (experimental) and right dentate gyrus (control). CT was calculated by subtracting the CT of the housekeeping gene from the CT of the gene of interest. Fold change was generated using the equation 2^{-CT} . Student's *t*-test was used dentate gyrus for statistical analysis.

miRNA *in situ* hybridization

At the end of LTP recordings rats were intracardially perfused with 4% paraformaldehyde (PFA). The brain was removed and submerged sequentially in 4% PFA for 24 h at 4°C and 30% sucrose for 48 h at 4°C. On the following day the brains were frozen in CO₂ gas, and 30-µm-thick coronal sections were cut on a Leica CM3050S cryostat using Richard-Allan Sec5e blades. Sections were immediately stored in phosphate buffer containing 0.1% azide at 4°C.

For primary miRNA *in situ* hybridization, riboprobes were prepared from genomic rat DNA using the following PCR primers; fw-212-cluster 5 gaggggacctgagaagcag3 and bw-212-cluster 5 gctctgtatctgcccacacc3, and cloned into the pCR[®]II-TOPO[®] vector (Invitrogen). The Arc RNA probe was prepared from a cDNA insert matching the first 2975 nucleotides of the Arc mRNA (GenBank accession number NM-019361) and cloned into the pCR[®]II-TOPO[®] vector. Antisense and sense probes were transcribed from linearized plasmids using T7 and SP6 polymerase in the presence of digoxigenin (DIG) labeling mix (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. *In situ* hybridization was performed on 30- μ m-thick floating sections, as described previously (Wibrand et al., 2006). Visualization was done either with the chromogenic substrates nitro blue-tetrazolium-chloride and 5-bromo-4-chloro-indolyl-phosphate (Roche) or with a fluorescent alkaline substrate (Fast Red Tablets; Roche).

In situ hybridization of mature miRNA was performed using locked nucleic acid (LNA) probes, as previously described (Pena et al., 2009). In tissues fixated with PFA only, significant amounts of mature miRNAs are released and diffuse out of the tissue during the *in situ* hybridization procedure. This is avoided by adding a fixation step with 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC). Unlike formaldehyde, EDC reacts with the 5 phosphate end of the miRNA, condensing it with the protein matrix to form stable linkages. Short oligo probes with LNA modifications are commonly used for the detection of mature miRNAs in Northern blots and during *in situ* hybridization (Kloosterman et al., 2006; Obernosterer et al., 2007). The spiking of LNA nucleotides in the probes increases the sensitivity and specificity of binding to short RNAs. The method of Pena and colleagues was applied with minor modifications for use on floating sections (modifications listed in supporting Appendix S1). Sections were rinsed in Tris-buffered saline (TBS) and incubated with proteinase K for 5 min at 37°C, washed twice in TBS, then post-fixed for 5 min in 4% PFA. After washing once in 0.2% glycine/TBS and twice in TBS, sections were incubated in freshly prepared 1-methylimidazole solution, and then immersed in EDC fixative for 60 min at room temperature. Sections were washed again, followed by acetylation with triethanolamine and acetic anhydride, to inactivate endogenous alkaline phosphates and peroxidases. After 10 min of prehybridization, sections were incubated overnight in 4 pmol of LNA probe diluted in 200 μ L hybridization buffer. A hybridization temperature of 20°C below the T_m of the experimentally determined miRNA–LNA probe duplex was used. The LNA probes were synthesized and melting temperatures were experimentally determined in the Tuschl laboratory (Pena et al., 2009). After post-hybridization washes, the sections were treated with 3% hydrogen peroxide and washed, before being blocked and incubated with anti-DIG-AP for 1 h at room temperature (Roche). LNA probes were visualized with either the NBT/BCIP chromogen system or the Cy3 fluorescent system. The NBT/BCIP chromogen system produces a purple reaction product in the presence of alkaline phosphatase (Roche). The TSA Plus Cy3 System (PerkinElmer Life Sciences) were used for observing dendritic staining and gives an orange-red fluorescent staining. Slides for fluorescent staining were mounted with Prolong[®] Gold antifade reagent with DAPI (Invitrogen).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

At the end of electrophysiological recording rats were decapitated, and the dentate gyrus was rapidly dissected on ice and homogenized. Samples were boiled in sample buffer (Bio-Rad) and resolved on 10% or 8% SDS–PAGE minigels. Proteins were transferred to polyvinylidene difluoride membranes (Amersham Biosciences), which were then blocked, probed with antibodies and developed using chemiluminescence reagents (ECL, Amersham Biosciences). The blots were scanned using Gel DOC EQ (Bio-Rad), and band intensities

were quantified using analytical software (Quantity one 1D analysis software; Bio-Rad). Proteins were normalized to α -tubulin. Significant differences between the treated and non-treated dentate gyrus were determined using Student's *t*-test for dependent samples. The *P*-value for significance was 0.05. Antibodies used for Western blotting were as follows: anti-anti-methyl CpG-binding protein (MeCP2; 1 : 1000; Millipore Temecula, CA, USA), p250 GTPase-activating protein (p250GAP; 1 : 1000; gift of Takanobu Nakazawa, U. Tokyo, Japan), anti-Arc (C7) (1 : 500; Santa Cruz Biotechnology) and anti- α -tubulin (1 : 1000; Sigma).

Results

Modulation of mature miRNA expression during NMDAR-dependent LTP

We sought to identify miRNAs associated with NMDAR-dependent LTP induction at medial perforant-granule cell synapses in the dentate gyrus. A spaced HFS paradigm was used to induce non-decremental protein synthesis-dependent LTP in urethane-anesthetized rats (Messaoudi et al., 2002, 2007). As shown in Fig. 1, HFS resulted in a robust and stable increase in the slope of field excitatory postsynaptic potential (fEPSP) and amplitude of the population spike (Fig. 1A–C). A second group of rats received HFS following systemic (i.p.) injection of the competitive NMDAR antagonist, CPP. As previously shown (Williams et al., 1995; Messaoudi et al., 2002), LTP of the fEPSP and population spike was inhibited in CPP-treated rats. No changes in synaptic efficacy were observed in a third group of rats receiving LFS only. As a positive control for NMDAR-dependent gene regulation, we examined expression of immediate-early gene *zif268* (also known as *Egr1*) in homogenate samples from microdissected dentate gyrus (Cole et al., 1989; Havik et al., 2003). At 2 h post-HFS, *zif286* mRNA levels in the HFS-treated dentate gyrus were significantly elevated 2.8-fold above the contralateral, control dentate gyrus (Fig. 1D). This increase was abolished in the CPP group and absent in the LFS group. These results confirmed generation of stable NMDAR-dependent LTP associated with robust changes in gene expression.

Microarray expression profiling was performed to screen for LTP-regulated miRNAs 2 h post-HFS. MirVana-purified RNA from the HFS-treated and contralateral control dentate gyrus from two animals was differentially hybridized to rat miRNA chips (MiRat_8.0_060307) representing all miRNA transcripts listed in Sanger miRBase Release 8.0. Figure 2A shows miRNAs exhibiting mean changes of at least 20%. By this arbitrary criterion 10 miRNAs showed increased expression (rno-miRNA-28, -103, -107, -125a, -132, -151*, -212, -320, -485, -543) and 11 miRNAs showed decreased expression (rno-miRNA-17, -19b, -21, -23a, -23b, -138, -181b, -219, -247, -338, -494), of a total of 237 probes on the chip.

Real-time RT-PCR analysis was used for independent validation and further study of three candidate regulated miRNAs (Fig. 2B). In agreement with the array data, miR-132 and miR-212 levels were significantly elevated, while miR-219 levels were significantly decreased at 2 h post-HFS in treated dentate gyrus relative to untreated control dentate gyrus. This regulation was HFS dependent, as no changes in miRNA expression were observed in rats receiving LFS only. We anticipated that blockade of LTP by CPP would eliminate or reduce the changes in miRNA expression. Instead, each of the three miRNAs exhibited enhanced expression when HFS was applied in the presence of CPP. Thus, miR-132 levels were elevated from 1.38-fold in the HFS group to 1.83-fold in the HFS + CPP group, miR-212 levels increased from 1.26- to 1.59-fold, and miR-219 levels flipped from a decrease of 0.68-fold in the HFS group to an increase of 1.27-fold in the HFS + CPP group (Fig. 2C). The effects of CPP can be attributed to block of HFS-evoked NMDAR activation rather than block of basal NMDAR activity, as the HFS-treated and control dentate gyrus both received CPP via the systemic injection.

miR-124a and miR-134 were used as negative controls as these miRNAs are expressed in granule cells of the adult dentate gyrus but were not regulated on the microarray. miR-124a is implicated in the regulation of adult neurogenesis (Cheng et al., 2009), while miR-134 functions in activity-dependent plasticity of dendritic spines during development (Schratt et al., 2006). RT-PCR analysis showed that miR-124a and -134 expression are not significantly affected by HFS in the presence or absence of NMDAR block (Fig. 2C). Next we examined expression of all miRNAs (miR-124a, 132, -134, -212, -219) at 10 min post-HFS, considering that changes in miRNA expression might peak shortly after LTP induction. However, at this early time point RT-PCR analysis showed no significant effects of HFS on miRNA expression in the presence or absence of CPP (Fig. 2B). Thus, LTP is associated with NMDAR-dependent downregulation of select mature miRNAs on a time course that is delayed relative to LTP induction.

AIDA blocks HFS-evoked modulation of mature miRNA levels and inhibits depotentiation

If NMDAR signaling downregulates miRNA expression, what is responsible for the increase in expression observed during LTP and following blockade of LTP with CPP? There must be an opposing system that upregulates miRNA expression. We considered group 1 mGluRs as intriguing candidates for miRNA regulation. While mGluRs are not required for LTP, these receptors are activated by HFS of the medial perforant pathway and play critical roles in depotentiation and metaplasticity (Martin & Morris, 1997; Wu et al., 2004; Kulla & Manahan-Vaughan, 2007; Abraham, 2008).

mGluR function in LTP and depotentiation was assessed using the Group 1 mGluR specific antagonist, AIDA. AIDA (1 μ L, 50 mM, 16 min) or vehicle control was infused 45 min prior to HFS through a glass pipette located in stratum lacunosum-moleculare of CA1, some 300 μ m from the nearest medial perforant path synapses in the upper blade of the dorsal dentate gyrus. As shown in Fig. 3A, AIDA had no effect on baseline fEPSP responses or the magnitude or stability of LTP as monitored for up to 4 h post-HFS. AIDA also had no effect on low-frequency test responses during 2 h of recording. Depotentiation was evoked by applying 5 Hz stimulation for 2 min starting 2 min after HFS (Martin & Morris, 1997). In both AIDA and vehicle-infused controls, 5 Hz stimulation resulted in a rapid decrease of fEPSP slope values to baseline followed by a partial recovery of potentiation by 30 min post-HFS. In vehicle controls the level of LTP remained strongly reduced for the duration of recording (mean fEPSP increase of $21.21 \pm 3.4\%$ at 2 h post-HFS; Fig. 3B). In AIDA-infused rats, however, the LTP continued to slowly recover, and by 2 h post-HFS was indistinguishable from controls that received HFS and AIDA without 5 Hz stimulation (mean fEPSP increase at 2 h post-HFS was $41.6 \pm 3.7\%$ in HFS + AIDA + 5 Hz group and $49.9 \pm 4.6\%$ in the HFS + AIDA group). Stimulation (5 Hz) alone had no effect on baseline responses (data not shown). Thus, in agreement with early reports, mGluR activation contributes to activity-dependent destabilization of LTP.

The results from the RT-PCR analysis are shown in Fig. 3C. AIDA treatment blocked the changes in miRNA expression observed following application of HFS alone or in combination with CPP, but had no effect on basal levels of expression in a control group receiving LFS only.

HFS-evoked expression of the primary miR-132 and miR-212 is mGluR dependent

The analysis so far has revealed opposing modulation of mature miRNA levels by mGluR and NMDAR signaling during LTP. Synaptic activity-evoked changes in mature miRNA levels could reflect a number of processes, including alterations in mature miRNA turnover, processing of miRNA precursors, as well as miRNA transcription. Focusing on transcriptional regulation, we examined expression of the primary (pri) miRNA transcripts

at 10 min and 2 h post-HFS (Fig. 4A). Massively enhanced expression of pri-miR-132 and pri-miR-212 expression was observed. These changes were less than 10-fold at 10 min post-HFS and increased to more than 50-fold at 2 h post-HFS, whereas pri-miR-219 and pri-miR-134 expression were unchanged at both time points. No changes in the expression of pri-miRNA transcripts were observed in the control LFS group. Remarkably, infusion of AIDA prior to HFS completely abolished the upregulation of pri-miR-132 and -212. In contrast, both pri-miRNAs were strongly induced by HFS in the presence of CPP, and this increase was also abolished by AIDA. The same pattern of results was obtained by RT-PCR analysis of precursor (pre) miRNA (Fig. 4B), the immediate product of pri-miRNA cleavage by Drosha. Thus, HFS of the perforant path induces massive mGluR-dependent expression of primary and precursor miR-132 and miR-212.

Enhanced expression of mature and primary miR-132 in dentate granule cell somata

miRNA *in situ* hybridization for mature miR-132 was performed on coronal brain sections from dorsal hippocampus collected 2 h post-HFS, using LNA probes for which optimal melting temperatures for hybridization were determined (Pena et al., 2009). In agreement with the RT-PCR analysis, miR-132 staining was elevated in the HFS-treated dentate gyrus relative to contralateral control (Fig. 5A, top panel). Sections incubated with no probe (Fig. 5A; lower panel) exhibited only low levels of background staining. HFS had no effect on the staining of two non-regulated miRNAs, miR-124a (Fig. 5A, middle panel) and miR-378 (not shown). Upregulation of mature miR-132 was restricted to the granule cell body layer with no changes in staining in the granule cell dendritic field, although staining within the proximal dendrites of granule cells and pyramidal cells was clearly seen by fluorescence using the tyramide signal amplification system (Fig. 5A and B).

The precursors of miR-132 and miR-212 are known to be transcribed from a common locus as one long primary transcript (Vo et al., 2005). Cloning primers were designed to amplify a 490-nucleotide-long sequence covering both precursors (see Materials and methods for primer sequences). Using riboprobes covering the common primary transcript, we observed a marked enhancement of pri-miR-132/-212 expression following LTP induction (Fig. 5C, upper panel). This upregulation is transcription dependent as it was completely abolished by prior infusion of the RNA synthesis inhibitor ACD (Fig. 5C, lower panel). *In situ* hybridization using either colorimetric or fluorescence detection localized the changes in primary and mature miR-132 expression to granule cell somata of the upper and lower blades of the dentate gyrus, with no detectable changes in the dentate molecular layer (Fig. 5C and D). Thus, *in situ* hybridization confirmed the RT-PCR analysis, and localized the enhancement in primary and mature miR-132 expression to granule cell somata.

Previous *in vitro* studies in primary hippocampal neuronal cultures have identified two common targets of miR-132 and -212: the Rac/Rho-family p250GAP and MeCP2 (Vo et al., 2005; Klein et al., 2007; Wayman et al., 2008). We performed Western blots for these proteins in homogenate samples from microdissected dentate gyrus collected 2 h post-HFS. There were no differences in expression between HFS-treated and contralateral control dentate gyrus for p250GAP ($1.8 \pm 3.7\%$) or MeCP2 ($1.4 \pm 4.2\%$), whereas expression of activity-regulated cytoskeleton-associated protein (Arc) was strongly elevated ($208 \pm 20\%$).

Discussion

This study has uncovered novel features of miRNA regulation during LTP in the dentate gyrus of intact adult rats. Based on real-time PCR analysis of selected candidate miRNAs from a microarray screen, we demonstrated upregulation of miR-132 and -212, and downregulation of miR-219 expression during LTP. It was anticipated that inhibition of LTP with an NMDAR antagonist would attenuate or eliminate these changes in mature miRNA

levels. Although LTP was blocked, miR-132 and miR-219 both exhibited enhanced expression when HFS was applied in the presence of CPP, while the sign of miR-219 expression switched from negative to positive. These results couple LTP to NMDAR-dependent downregulation of mature miR-132, -212 and -219. The regulation appears to be coordinate and specific insofar as expression of miR-124a and miR-134, both of which are expressed in granule cells, was unaffected by HFS in the presence or absence of NMDAR blockade. Furthermore, the regulation by NMDAR signaling appears to be specific to metabolism of these mature miRNAs, as NMDAR blockade had no effect on the expression of their primary and precursor transcripts.

Seeking to explain the synaptic activity-dependent enhancement in miRNA expression, we turned to examine a possible role for mGluR signaling. We found that primary and precursor miRNA transcripts for miR-132 and -212 (but not -219) are transcriptionally upregulated more than 50-fold by a mechanism that is completely blocked by the group 1 mGluR antagonist, AIDA. Parallel increases in pri- and pre-miRNA levels at 10 min post-HFS attest to the rapid transcription and processing of primary transcripts. Changes in mature miRNA expression were detected at 2 h only, indicating a slower processing of hairpin precursors to mature miRNA. Changes in mature miRNA expression were also much smaller (less than twofold). This difference suggests limited precursor processing to mature miRNA. However, the relative differences may also reflect high levels of basal (pre-existing) mature miRNA expression compared with the primary transcripts. *In situ* hybridization analysis showed no expression of primary miR-132/212 in non-stimulated tissue, whereas mature miR-132 was clearly expressed.

Functionally, mGluR activation in the dentate gyrus has been implicated in depotentiation, metaplasticity and long-term depression, rather than LTP (Wu et al., 2004; Kulla & Manahan-Vaughan, 2007; Naie et al., 2007). In agreement with these studies we find that AIDA has no effect on LTP maintenance, but blocks the ability for depotentiation. Thus, LTP is associated with rapid mGluR-dependent transcription miR-132 and miR-212. This miRNA transcription is not required for LTP maintenance under standard conditions, but could serve to modulate LTP stability through regulation of depotentiation or other mGluR-dependent mechanisms. Taken together, the present results indicate that HFS of the perforant pathway activates two parallel processes: (i) NMDAR-dependent regulation of mature miRNA metabolism; and (ii) mGluR-dependent activation of miR-132 and -212 transcription.

The NMDAR-dependent decrease in mature miRNA levels could reflect inhibition of precursor processing or degradation of mature miRNA. As pre-miRNA levels were not detectably altered by NMDAR blockade, the most likely explanation is net degradation (decay) of mature miRNA. At present, little is known about decay mechanisms for miRNAs once they are bound to their mRNA targets. A better understanding of the relationship between cytoplasmic processing (P) bodies (putative sites of mRNA storage and degradation) and translational repression by miRNAs is likely to be important. While target-bound miRNAs are generally stable, subpopulations of miRNAs may undergo rapid degradation in the context of activity-dependent relief from miRNA inhibition (translational derepression; Parker & Sheth, 2007; Cougot et al., 2008; Franks & Lykke-Andersen, 2008; Tang et al., 2008; Zeitelhofer et al., 2008). This scenario fits with the role of NMDARs in post-transcriptional activation of protein synthesis during LTP. Furthermore, studies in hippocampal neuronal cultures show that NMDAR signaling dynamically alters the localization and composition of dendritically localized P-bodies, as reflected by rapid exchange of the decapping enzyme Dcp1a and the depletion of Argonaute 2, a key protein of the miRNA-RISC (Cougot et al., 2008).

Smalheiser and colleagues have recently provided evidence for activity-dependent local processing of pre-miRNA at synapses (Lugli et al., 2005, 2008). Mature miRNA present in synaptoneurosome fractions of adult brain tissue derives at least in part from processing of local precursors (Lugli et al., 2008). Evidence suggests that NMDAR activation results in the proteolytic liberation of Dicer from the postsynaptic density and subsequent activation of its RNAase III activity (Lugli et al., 2005). Mature miRNAs regulated by this mechanism should be rapidly elevated at synaptic sites following NMDAR activation and LTP induction, while the corresponding precursor levels should decrease. Such a pattern of regulation was not observed in the present study, but our measurements of miRNA expression in whole dentate gyrus homogenates (rather than synaptic fractions) cannot be used to address the question of local precursor processing in LTP. Taken together, however, these studies have revealed an unexpected regulation of the mature miRNA expression by NMDAR-dependent and transcription-independent mechanisms.

Coordinate action of many miRNAs is crucial for biological processes, such as cell fate determination and apoptosis. Co-transcriptional regulation is one way by which such coordination is thought to be achieved (Cheng et al., 2007; He et al., 2007). Evidence suggests that miR-132 and -212 are co-transcriptionally regulated from a stable intron of a cryptic non-coding RNA (Vo et al., 2005). An important common target of miR-132 and -212 is the Rac/Rho-family p250GAP. In cultured hippocampal neurons, activity-dependent expression of miR-132 regulates dendritic morphogenesis by decreasing synthesis of p250GAP (Vo et al., 2005; Wayman et al., 2008). The transcription of miR-132 in this context is CREB dependent, and stimulated by NMDAR and brain-derived neurotrophic factor (BDNF)-activated signaling pathways. *In vitro* studies also indicate a key role for miR-132 transcription in the homeostatic regulation of MeCP2 during neural development (Klein et al., 2007). The present work on LTP in the adult gyrus shows that transcription of pri-miR-132 and -212 is strongly dependent on mGluR rather than NMDAR activation. Changes in mature miR-132 and miR-212 during LTP reflected the opposing effects of mGluR and NMDAR signaling. Interestingly, while net levels of mature miRNA were significantly increased, no changes in the expression of p250GAP or MeCP2 protein were detected. Taken together, the results suggest that transcriptional regulation of miR-132/212 and its impact on target protein expression differs substantially between the developmental setting of embryonic neurons and LTP in the adult brain.

The present study gives the first insights into regulation of miRNA expression during LTP in the adult mammalian brain. It will be important in future work to determine the full range of miRNAs regulated by NMDAR and mGluR signaling, and to identify possible roles for activity-dependent miRNA expression and turnover in sculpting protein synthesis-dependent forms of synaptic plasticity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ACD	actinomycin D
AIDA	(RS)-1-aminoindan-1,5-dicarboxylic acid

CA	cornu ammonis
CPP	(R,S)-3-22-carboxypiperazin-4-yl-propyl-1-phosphonic acid
DIG	digoxigenin
EDC	1-ethyl-3-(3-dimethyl-aminonpropyl) carbodiimide
fEPSP	field excitatory postsynaptic potential
HFS	high-frequency stimulation
LFS	low-frequency stimulation
LNA	locked nucleic acid
LTP	long-term potentiation
MeCP2	methyl CpG-binding protein
mGluR	metabotropic glutamate receptor
miRNA	microRNA
NMDAR	<i>N</i> -methyl-D-aspartate receptor
p250GAP	p250 GTPase-activating protein
PFA	paraformaldehyde
RISC	RNA-induced silencing complex
RT-PCR	reverse transcription polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TBS	Tris-buffered saline

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Figure 1.

NMDAR-dependent LTP induction and *zif268* mRNA expression in the dentate gyrus *in vivo*. (A) Time course plots show changes in the medial perforant path-evoked fEPSP slope expressed as percentage of baseline. Groups of rats received HFS in the presence of the NMDA receptor antagonist CPP, or LFS only. CPP was injected i.p. at a dose of 10 mg/kg, 90 min prior to HFS. Values are means $\pm$ SEM, $n = 5$ in all groups. (B) Samples of averaged field potentials traces (four sweeps) collected at the end of baseline recording (baseline) and 2 h post-HFS. Scale bars: 5 mV and 2 ms. (C) Mean changes in fEPSP slope and population spike amplitude. *Significantly different from baseline $P < 0.05$. (D) Changes in *zif268* mRNA levels in the dentate gyrus (treated/contralateral control). PCR reactions were

performed in triplicate and normalized to polyubiquitine. *Significantly different from contralateral control ($P < 0.05$). HFS, $n = 8$; HFS + CPP, $n = 5$; LFS, $n = 5$.

Figure 2.

Modulation of mature miRNA expression following NMDAR-dependent LTP induction. (A) Microarray expression profiling was performed using total RNA purified from dentate gyrus homogenate samples collected 2 h post-HFS. miRNA levels are expressed as mean fold change (based on 12 spots of each probe on the chip) in the HFS-treated dentate gyrus relative to the contralateral dentate gyrus. Measurements were obtained in two rats, and only miRNAs exhibiting a mean change of 20% or more are shown. (B and C) Real-time RT-PCR analysis of selected miRNAs. Groups of rats received: HFS, HFS following i.p. injection of the NMDAR antagonist, CPP, or LFS only. Tissue was collected 10 min and 2 h post-HFS (electrophysiology data shown in Fig. 1). PCR reactions were performed in

triplicate, and normalized to miR-16 and Y1. Values are means (+ SEM) changes in miRNA levels. The RT-PCR analysis confirmed upregulation of miR-132 and miR-212, and downregulation of miR-219 during LTP. Note that NMDAR block enhances HFS-induced expression of these miRNAs. Negative controls from the microarray analysis (miR-124a and miR-134) were not regulated in any of the experimental groups. *Significantly different from contralateral control, $P < 0.05$. HFS 10 min, $n = 5$; HFS + CPP 10 min, $n = 5$; HFS, 2 h $n = 8$; CPP + HFS, 2 h $n = 5$; LFS 2 h, $n = 5$.

Figure 3.

The mGluR antagonist AIDA blocks HFS-evoked modulation of mature miRNA levels and inhibits depotentiation. (A) Time course plot of fEPSP changes in rats receiving intrahippocampal infusion of AIDA (1 μ L, 16 min, 50 μ M, black bar) and i.p. injection of CPP (10 mg/kg) 90 min prior to HFS, HFS and AIDA, or LFS alone. $N=5$ in all groups. Stable LTP is evoked in the presence of AIDA. (B) Depotentiation was evoked by applying 5-Hz stimulation for 2 min starting 2 min after HFS. In AIDA-infused rats, depotentiation recovered to the original level of LTP by approximately 2 h. (C) Real-time RT-PCR was performed on tissue obtained from treatment groups in (A). HFS treatment group from Fig. 2 is added for comparison. Tissue was collected 2 h post-HFS. PCR reactions were

performed in triplicate and normalized to miR-16 and Y1. Values are means (+ SEM) changes in miRNA levels. $n = 5$ in all groups.

Figure 4.

HFS-evoked transcription of miR-132 and -212 is mGluR dependent. Fold changes in primary (A) and precursor (B) miRNA transcripts were determined by real-time PCR. PCR reactions were performed in triplicate and normalized to miR-16. Values are means (+ SEM) relative to contralateral control. *Significantly different from control, $P < 0.05$. Tissue was collected 10 min post-HFS in the HFS 10 min group. All other tissue was collected 2 h post-HFS or after 2 h of LFS only. HFS 10 min, $n = 4$; HFS, $n = 6$; HFS + CPP, $n = 5$; HFS + AIDA + CPP, $n = 5$; LFS, $n = 5$.

Figure 5. HFS-evoked expression of miR-132 is transcription dependent and localized to dentate granule cell somata. *In situ* hybridization was used to visualize the expression and localization of mature miR-132 and the primary transcript encoding both miR-132 and -212 (pri-miR-132/212 cluster). Staining was performed on coronal sections from dorsal

hippocampus of PFA-fixed brains collected 2 h post-HFS. (A) Mature miR-132 expression was elevated in the ipsilateral (HFS-treated) dentate gyrus relative to the contralateral (CONTRA) dentate gyrus. Enhanced expression was restricted to the granule cell layer (arrow). No changes in miR-124a expression were observed. Fluorescence *in situ* hybridization revealed staining of mature miR-132 and miR-124a in the proximal dendrites of control non-stimulated granule cells and CA1 pyramidal cells. The staining was strongest in the proximal dendrites of CA1 pyramidal cells (panel B). LTP induction had no detectable effect of the pattern of dendritic miRNA expression. (C) Expression of pri-miR-132/212 cluster was robustly induced in granule cell somata. This expression was abolished by treatment with the transcription inhibitor ACD prior to HFS. (D) Fluorescently labelled probes confirmed 132/212-cluster expression in granule cell somata. Arc mRNA was used a positive control for upregulated expression in granule cell dendrites.