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Changes in Brain MicroRNAs Contribute to Cholinergic Stress Reactions

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Abstract Mental stress modifies both cholinergic neurotransmission and alternative splicing in the brain, via incompletely understood mechanisms. Here, we report that stress changes brain microRNA (miR) expression and that some of these stress-regulated miRs regulate alternative splicing. Acute and chronic immobilization stress differentially altered the expression of numerous miRs in two stress-responsive regions of the rat brain, the hippocampal CA1 region and the central nucleus of the amygdala. miR-134 and miR-183 levels both increased in the amygdala following acute stress, compared to unstressed controls. Chronic stress decreased miR-134 levels, whereas miR-183 remained unchanged in both the amygdala and CA1. Importantly, miR-134 and miR-183 share a common predicted mRNA target, encoding the splicing factor

SC35. Stress was previously shown to upregulate SC35, which promotes the alternative splicing of acetylcholinesterase (AChE) from the synapse-associated isoform AChE-S to the, normally rare, soluble AChE-R protein. Knockdown of miR-183 expression increased SC35 protein levels in vitro, whereas overexpression of miR-183 reduced SC35 protein levels, suggesting a physiological role for miR-183 regulation under stress. We show stress-induced changes in miR-183 and miR-134 and suggest that, by regulating splicing factors and their targets, these changes modify both alternative splicing and cholinergic neurotransmission in the stressed brain.

Keywords Stress · microRNA · miR-183 · miR-134 · SC35 · Cholinergic

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Introduction

Mammalian psychological stress is known to induce prominent changes in neuronal activity and gene regulation across multiple brain regions (McEwen 2007). Acute and chronic stress both lead to the remodeling of dendrites in the hippocampus (McEwen 1999), which controls learning functions via establishing spatial, episodic, and contextual memory formation (Kenney and Gould 2008; Otto and Eichenbaum 1992). Specifically, in the CA1 region of the hippocampus, both neurons and glia are affected by mental stress (Espinosa-Oliva et al. 2009; Hirata et al. 2009). In the amygdala, stress reactions impact emotion, addiction, fear, and anxiety (Vyas et al. 2002; Walker and Davis 2008). Chronic stress also increases aggression, likely to reflect hyperactivity of the amygdala (Wood et al. 2003). At the physiological level, cholinergic neurotransmission is altered under stress (Kaufer et al. 1998; Soreq and Seidman 2001). These stress-induced changes are mostly attributed to the combinatorial regulation of many genes' altered transcription levels (Anguelova et al. 2000). However, the contribution of posttranscriptional regulation mechanisms to these stress-associated responses is increasingly acknowledged (Battaglia and Ogliari 2005; Gattoni et al. 1996; Meshorer et al. 2005). One such mechanism is the production of multiple proteins with divergent or even opposite functions from a single transcript via alternative splicing, which accounts for much of the proteome's flexibility (Stamm et al. 2005). A case in point is the stress-induced alternative splicing of the primary ACh-hydrolyzing enzyme acetylcholinesterase (AChE), which modifies cholinergic neurotransmission (Meshorer and Soreq 2006) and affects neuronal processes in a pathway involving the splicing factors SC35 and ASF/SF2 (Meshorer et al. 2005). However, the molecular mechanism(s) underlying the apparent relationship between stress, alternative splicing, and cholinergic neurotransmission remain incompletely understood.

An important posttranscriptional mechanism for gene regulation involves microRNAs (miRs). miRs are 20–28-nucleotide noncoding RNAs encoded in the genomes of plants and animals, exerting translational repression and/or degradation of target mRNAs via complementary binding to the 3' untranslated region (UTR; Maniataki and Mourelatos 2005). miR levels vary across cell type, tissue, and developmental stages (Baek et al. 2008; Liang et al. 2007; Plasterk 2006; Sood et al. 2006). Each miR targets multiple mRNAs (Krek et al. 2005; Lewis et al. 2005), which in some cases code for proteins participating in the same signaling pathway (Li et al. 2007). Thus, both the overall profile of miR expression and the expression levels of particular key miRs impact diverse biological processes (Chen et al. 2004; He et al. 2005; Kluiver et al. 2007; Laneve et al. 2007; Li et al. 2007; Thai et al. 2007).

miR-18 and miR-124 were recently reported to regulate glucocorticoid receptors, suggesting involvement in a variety of systemic stress responses (Vreugdenhil et al. 2009). However, the mechanistic involvement of miRs at large in psychological stress remains unknown. We predicted that miR-mediated regulation contributes to the yet incompletely understood link(s) between the molecular and the physiological reactions of particular brain regions to psychological stress and more specifically to the regulation of alternative splicing. To test for miR involvement in governing the region-specific stress-induced changes in alternative splicing, we studied the expression profiles of miRs in the hippocampal CA1 and the central amygdala brain regions in stressed and control rats and challenged the relevance of observed changes by manipulating miR-183 levels in cultured cells.

Materials and Methods

Immobilization Stress Adult male rats (Charles River; 200–225 g) were subjected to a single 4-h session of immobilization stress (acute stress group), 4 h of complete immobilization stress per day for 14 days (chronic stress group), or brief daily handling (no-stress group). Twenty-four hours after the last stress or handling session, the animals were sacrificed, and tissues were carefully dissected, flash-frozen, and stored at -80°C . RNA was then purified using the mirVana kit (Ambion), which preserves short RNAs, from the dissected central amygdala and hippocampus CA1 region. For each brain region in each of the three groups, pooled samples were generated from the RNA of three to four rats.

Spotted Array Methods Spotted array methods were adapted from Ben-Ari et al. (2006). The mirVana oligo set (Ambion, Austin, TX, USA; Cat. Num. 1564V1) was used to construct an in-house array with >200 spotted probes complementary to known human and mouse miRs. Dye-swapping tests served to exclude dye-specific labeling differences (Dombkowski et al. 2004). Labeling used the CyDye reactive dye pack (Amersham, NSW, Australia), as instructed 2 dye-swapped arrays were used for every comparison, each with at least 6 replicate spots per miRNA probe. Hybridization was performed in chambers (Corning, NY, USA) for 15 h at 64°C . Scanning used an Affymetrix 428 Array Scanner at 532 and 650 nm, controlled by the “Jaguar” software (Affymetrix, CA, USA), and results were exported to the “Imagene” program (BioDiscovery Inc., CA, USA) for quantification. Data normalization, exclusion of unreliable spots, and combination of the information from all slides were performed using the Normalize Suite (Beheshti et al. 2003). Significantly altered transcripts (with a p value of the sign-test smaller than 0.05), which were not

disqualified due to any quality parameter, were identified by setting an arbitrary threshold.

Cell Culture Chinese hamster ovary (CHO) cells were grown in 5-ml flasks in Dulbecco’s modified Eagle’s medium (Biological Industries, Beit Haemek, Israel) supplemented with 10% fetal bovine serum and 2 mL-glutamine (Biological Industries). Transfection of pre-miR-183 and anti-miR-183 oligonucleotides (Ambion) was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at 80–90% confluence. For each transfection sample, 3 µg of oligonucleotide or plasmid and the lipofectamine reagent were both diluted in 0.25–1 ml of unsupplemented growth medium. After 5 min, the diluted oligonucleotide and lipofectamine were combined, incubated for 20 min, and added to the cells. Medium was replaced at 6 h, and cells were harvested 24 h posttransfection. A scrambled sequence oligonucleotide and lipofectamine treatment alone served as negative controls; a green fluorescent protein (GFP) expression vector served to assay transfection efficiency.

Quantitative RT-PCR Total RNA including transcripts that are 200 bases and smaller was extracted using the mirVana (Ambion, Austin, TX, USA) and the miRNeasy (QIAGEN) isolation kits from cultured cell samples. Contaminating DNA was removed with DNA-free (Ambion). RNA concentration was determined using the NanoDrop ND-1000 instrument (NanoDrop Technologies, Wilming-

ton, DE, USA). Reverse transcription (RT) of miRs was performed using SuperScript III First-Strand Synthesis Systems kit reagents for RT-polymerase chain reaction (PCR; Invitrogen) as detailed elsewhere (Raymond et al. 2005). Primer extension quantitative PCR (QPCR) was conducted as previously described (Raymond et al. 2005) using Power SYBR Green PCR Master Mix (Applied Biosystems), LNA-modified forward primer, and a universal reverse primer and amplified using the ABI-7900HT instrument (Applied Biosystems) equipped with dedicated software (ver. 2). Triplicate values of each treatment were normalized to β-actin mRNA, 5S rRNA, or the U6 small RNA. Absolute quantification of miR levels was performed using standards containing known dilutions of a commercial pre-miR-183 (Ambion). mRNA RT and QPCR were performed as previously described (Gilboa-Geffen et al. 2007). Triplicate values of each sample were normalized to β-actin or GAPDH mRNA.

Immunoblots These were performed as previously described (Berson et al. 2008) using mouse monoclonal antibodies (Zymed 339400) against arginine–serine-rich (SR) proteins and mouse monoclonal antibodies (SC-32293) targeted to α-tubulin.

Statistics Student's *T* test, the Kolmogorov–Smirnov two-sample test, and the nonparametric Wilcoxon test were employed for defining the significance of differences between analyzed groups. *p* values <0.05 were considered

Table 1 miRs with a mean LR change >0.25 in absolute value (*p*<0.05) 24 h after acute or chronic immobilization stress in rats, sorted by brain region and stress regimen (left to right)

	AMY-acute	AMY-chronic	HIP-acute	HIP-chronic		AMY-acute	AMY-chronic	HIP-acute	HIP-chronic	
up	miR106b	miR1-2	miR1-2	miR132	down	let7a-1	let7a-1	let7f-2	let7c	
	miR134	miR15a	miR376b	miR17-5p		miR202	let7c	miR124a-1	let7f-1	let7f-1
	miR183	miR190	miR182*	miR208		miR361	let7f-1	miR138-1	miR100	miR100
	miR382	miR193	miR424	miR23a		miR376b	let7f-2	miR15b	miR134	miR148a
		miR208	miR190	miR369		miR381	miR103-1	miR202	miR148a	miR148a
		miR22	miR19a	miR376b		miR9-1	miR134	miR422a	miR16-1	miR16-1
		miR322	miR208	miR410			miR138-1	miR9-1	miR182*	miR182*
		miR361	miR216				miR182		miR219-1	miR219-1
		miR369	miR32				miR216		miR22	miR22
		miR376a					miR222		miR221	miR221
		miR376b					miR298		miR30a-3p	miR30a-3p
		miR381					miR323		miR330	miR330
							miR34a		miR376a	miR376a
							miR368		miR9-1	miR9-1
					miR9-1		miR96	miR96		
Total	4	12	9	7	Total	6	16	7	15	

miRs with established brain or stress-related functions are shown in italics. miR-1 targets the nAChR (Simon et al. 2008); miR-134 regulates dendritic spine development (Schratt et al. 2006); miR-132 and miR-182* regulate AChE levels (Shaked et al., submitted). miR-17-5p (Hebert et al. 2009) and miR-124 (Makeyev et al. 2007) control neuronal development and differentiation

significant. Hierarchical clustering of array data was performed using MeV rev.4 software (Saeed et al. 2003) for Mac OS X, with Euclidian metrics and default parameters.

Results

Amygdala and Hippocampus Display Distinct Stress-Induced Changes in miR Profiles Following Acute or Chronic Stress

To test if stress-responsive brain regions display altered miR profiles following psychological stress, miR-specific spotted arrays were hybridized with short RNAs from the central amygdala or the CA1 region of the hippocampus of rats subjected to acute or chronic stress or from the corresponding regions of nonstressed controls. Several miRs appeared to be differentially regulated in the stress-responsive brain regions by acute and chronic stress (Table 1). In both regions, we observed, more decreases than increases in miR levels under chronic stress (Table 1), possibly indicating a tendency of upregulated expression of the target mRNAs of these miRs under stress. Additionally, chronic stress resulted in numerically larger changes than acute stress in both brain regions. In the central amygdala, ten different miRs were modified (upregulated or downregulated) under acute stress, as compared to 28 following chronic stress, whereas the hippocampal CA1 region showed 16 and 22 modified miRs after acute and chronic stress, respectively. Moreover, the expression profiles of stress-responsive miRs were different in the analyzed regions, so that there was very limited overlap between miRs modified in each of these regions under acute and chronic stress. For example, in the central amygdala, miR Let-7a-1 was the only miR affected by both acute and chronic stress, whereas, in the hippocampal CA1 region, miR-376b and miR-208 both increased under either acute or chronic stress whereas miR-9-1 decreased in both these conditions. All of the other changes were unique to either acute or chronic stress and particular to the analyzed brain regions.

Clustering analysis of rat central amygdala and hippocampus CA1 array data (Fig. 1a) confirmed that the majority of miRs represented on the array are differentially regulated by acute and chronic stress. The stress-induced changes in miR profiles in the two brain regions were also dissimilar in the clustered profiles. Thus, the changes observed varied both for the different brain regions and for the different stress paradigms employed. Furthermore, several of the miRs showed distinct patterns of regulation, suggesting functional relevance. Thus, miR-17-5p is upregulated in the hippocampus CA1 region under

chronic stress and was recently shown to suppress the amyloid precursor protein (APP) and to undergo down-regulation in the course of neuronal development and differentiation (Hebert et al. 2009). Additionally, several let-7 family members were downregulated in both the

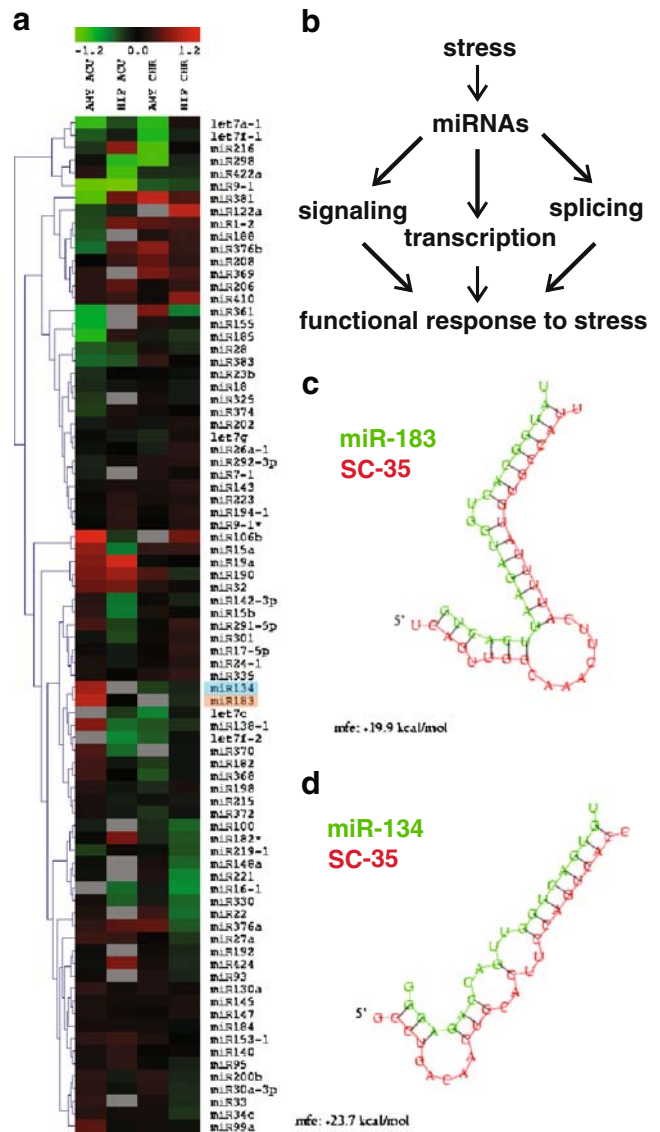


Figure 1 Stress-responsive miR-134 and miR-183 are predicted to target the splicing factor SC35. **a** Hierarchical clustering of signal ratios of miRNAs that passed the quality control in at least three out of four comparisons, from left to right: amygdala (central nucleus) acute/control, hippocampus (CA1) acute/control, amygdala chronic/control, CA1 chronic/control. SC35-targeting miR-134 and miR-183 are emphasized in blue and orange, respectively. Gray fields denote signals that did not pass the more stringent quality control due to low expression levels or high variability between spots. **b** Predicted targets of stress-regulated miRNAs seem to affect all the major stages of gene regulation. **c, d** PicTar-predicted (pictar.bio.nyu.edu) duplexes for miR-183 (**b**) and miR-134 (**c**; in green) with 3' UTR of SC35 mRNA (in red)

central amygdala and the hippocampal CA1 under both acute and chronic stress and were recently reported to similarly suppress APP in the course of development in *Caenorhabditis elegans* (Niwa et al. 2008).

Several of the stress-regulated miRNAs have validated target mRNA transcripts and functional significance. Importantly, the functions of several of these targets are largely relevant to brain stress responses in general and to cholinergic stress reactions in particular. For example, miR-1 targets and regulates two different subunits of the nicotinic ACh receptor (nAChR) and alters presynaptic ACh secretion (Simon et al. 2008). We have recently shown that miR-132 and miR-182* regulate AChE levels downstream of the transcriptional regulator nuclear factor kappa B and AP1 signaling (Shaked et al., submitted). Other miRNAs appear to control neuronal development and differentiation, both of which are modified under stress. These include miR-17-5p (Hebert et al. 2009) and miR-124, which promotes neuronal differentiation by targeting PTBP1, a repressor of alternative splicing in nonneuronal cells (Makeyev et al. 2007).

Stress-Regulated miR-134 and miR-183 Are Both Predicted to Target Stress-Responsive Transcripts

miR-134 and miR-183 were both upregulated by acute stress in the central amygdala; miR-134 additionally showed a trend of downregulation following chronic stress in both the central amygdala and the hippocampus CA1 region (Table 1, Fig. 1a). These two miRNAs share a number of putative targets as predicted by the PicTar algorithm (Krek et al. 2005; Table 2), suggesting common regulatory roles. Specifically, these predicted targets included two different transcription factors (ZFPM2, CBFA2T1), one of which was reported to be causally involved with controlling oxidative stress and inflammation (Martinez et al. 2004), and several signaling molecules (integrin- β , casein kinase 1G3, OGT), one of which (integrin- β) is involved in regulating shear stress (Chen et al. 1999) and another (OGT) functioning as a chaperone to control heat shock reactions (Sohn et al. 2004). Finally, three of these predicted targets are known regulators of alternative splicing (MBNL1, CUGBP2, SFRS2), with CUGBP2 also

Table 2 PicTar-predicted targets common to miR-134 and miR-183

Human RefSeq	miR-134 score	miR-183 score	Annotation
NM_012082	6.49	3.37	Zinc finger protein, multitype 2 (ZFPM2)
NM_002211	5.61	5.93	Integrin, beta 1 (ITGB1), transcript variant 1A
NM_019063	4.56	2.15	Echinoderm microtubule-associated protein-like 4 (EML4)
NM_016279	4.12	3.51	ZFPM2
NM_004349	3.97	2.27	Core-binding factor, runt domain, alpha subunit 2 (CBFA2T1), transcript variant 1
NM_175634	3.97	2.27	CBFA2T1 transcript variant 2
NM_175635	3.97	2.27	CBFA2T1 transcript variant 3
NM_175636	3.97	2.27	CBFA2T1 transcript variant 4
NM_003016	3.9	2.22	Splicing factor, arginine/serine-rich 2 (SFRS2, SC35)
NM_004384	3.21	2.91	Casein kinase 1, gamma 3 (CSNK1G3)
NM_173683	2.99	2.48	Chromosome 8 open reading frame 21 (C8orf21)
NM_182485	2.38	1.46	Cytoplasmic polyadenylation element binding protein 2 (CPEB2), transcript variant B
NM_182646	2.38	1.46	CPEB2 transcript variant A
NM_003605	2.36	2.97	O-linked N-acetylglucosamine (GlcNAc) transferase (OGT), transcript variant 3
NM_006561	2.32	1.34	CUG triplet repeat, RNA binding protein 2 (CUGBP2)
NM_021038	2.22	1.55	Muscle blind-like (<i>Drosophila</i> ; MBNL1), transcript variant 1
NM_207292	2.22	1.55	MBNL1 transcript variant 2
NM_207293	2.22	1.55	MBNL1 transcript variant 3
NM_207294	2.22	1.55	MBNL1 transcript variant 4
NM_207295	2.22	1.55	MBNL1 transcript variant 5
NM_207296	2.22	1.55	MBNL1 transcript variant 6
NM_207297	2.21	1.54	MBNL1 transcript variant 7

ZFPM2 and CBFA2T1 are transcription factors (CBFA2T1 is involved with controlling oxidative stress and inflammation (Martinez et al. 2004)); integrin- β , casein kinase 1G3, and OGT are signaling molecules (integrin- β is involved in regulating shear stress (Chen et al. 1999)); OGT controls heat shock reactions (Sohn et al. 2004). MBNL1, CUGBP2, and SFRS2 are known regulators of alternative splicing (CUGBP2 is involved in RNA editing (Anant et al. 2001); SC35 accumulates in the PFC following acute stress and regulates the stress-induced alternative splicing of AChE mRNA in brain neurons (Meshorer et al. 2005))

prominently involved in RNA editing (Anant et al. 2001). Together, this agrees with a central functional role of the stress-regulated miRs, impacting all the major tiers of gene regulation (scheme, Fig. 1b). Notably, miR-134 and miR-183 were both predicted by PicTar to bind the 3' UTR of SFRS2 (SC35) mRNA (Fig. 1c, d). The SC35 protein accumulates in the prefrontal cortex (PFC) following acute stress and regulates the stress-induced alternative splicing of AChE mRNA in brain neurons (Meshorer et al. 2005). However, long-term excess of AChE induces a reduction in SC35 (Ben-Ari et al. 2006). Therefore, we selected the miR-183/SC35 regulatory module for a functional validation study.

Manipulating miR-183 Levels Affects SC35 Levels in Cultured Cells

To test the direct effects of miR-183 on SC35 levels, CHO cells were transfected with a pre-miR-183 precursor stem loop, an anti-miR-183 oligo, or a GFP expression vector as negative control. Transfection with pre-miR-183 elevated miR-183 levels by up to four orders of magnitude; the QRT-PCR analysis did not differentiate between the mature and precursor forms (as tested in QRT-PCR calibration; data not shown). Anti-miR-183 did not alter the amount of endogenous miR-183 (Fig. 2a), consistent with a functional interference effect rather than degradation of the endogenous miR. Anti-miR-183 elevated SC35 protein levels by more than twofold (quantification of immunoblot, Fig. 2b). Conversely, pre-miR-183 suppressed SC35 protein levels, although not dramatically; this can be attributed to only partial processing of the exogenous precursor. Thus, tests in cultured cells validated miR-183-mediated suppression of SC35 protein levels.

Discussion

The molecular mechanisms underlying the brain's response to psychological stress are still incompletely understood. Posttranscriptional regulation by miRs can add flexibility and robustness to the control of alternative splicing, in line with the observed complexity of the mammalian stress response at the physiological level (McEwen 2007). In our current study, changes in miR expression profiles were characterized within the stress-responsive amygdala and the CA1 region of the hippocampus of rats subjected to immobilization stress. Microarray analysis and cell culture validation experiments identified specific miRs (e.g., miR-183, miR-134) as regulators of key splicing factors (e.g., SC35 which controls the stress-induced alternative splicing of AChE mRNA; scheme, Fig. 2c). Together, these findings support the notion that stress responses in the mammalian

brain involve miR-mediated control of other posttranscriptional regulators of gene expression, including (but not limited to) proteins regulating alternative splicing.

miR-134, a previously reported regulator of dendritic spine development in the brain (Schratt et al. 2006), is

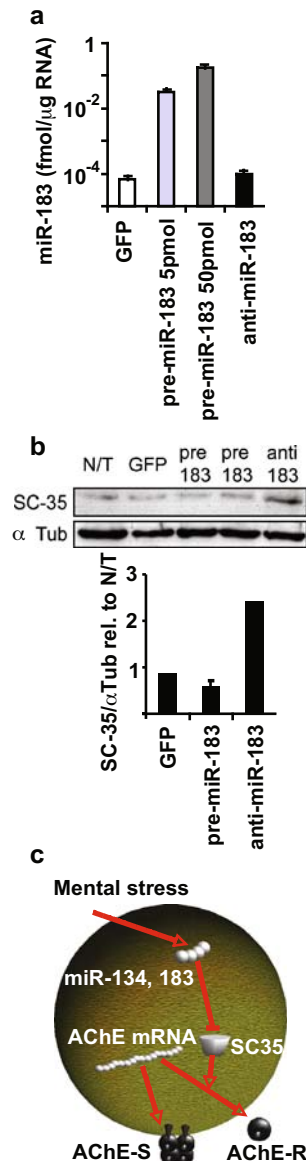


Figure 2 miR-183 regulates the splicing factor SC35. **a** QRT-PCR for miR-183 in CHO cells transfected with pre-miR-183 or anti-miR-183 (Ambion) and GFP-transfected controls. Bars: SD; *t* test $p < 10^{-5}$ for both concentrations of pre-miR-183 compared to GFP control. **b** Scan and quantification of immunoblot for SC35 in cells in **a** with α -tubulin used for normalization. **c** Suggested interplay of alternative splicing and miRs (exemplified by miR-134, miR-183, and their target, the splicing factor SC35) contributes to the complexity of stress responses in the brain, including regulation of the alternative splicing choice between the major “synaptic” AChE transcript AChE-S and the normally minor stress-induced variant AChE-R

elevated in both the hippocampus CA1 and the central amygdala under acute stress and downregulated in both these brain regions under chronic stress, while miR-183, also upregulated in both regions under acute stress, maintains unchanged levels in the chronically stressed hippocampus CA1 and central amygdala. This tentatively suggests the relevance of the observed miR-183 and miR-134 changes to both the acute and chronic stress-induced alterations in SC35. Apart from targeting the splicing factors SC35, MBNL1, and CUGBP2, both miR-134 and miR-183 are predicted to target other splicing factors (e.g., SRP46 and SFRS11, respectively), potentially expanding the impact of these miRs on the pre-mRNA splicing machinery. Both SRP46 and SFRS11 are SR proteins that participate in composing the multicomponent splicing complex and play pivotal roles in both regular and alternative splicing. SRP46 is an SC35 homolog (Soret et al. 1998), while SFRS11 regulates the alternative splicing of Tau, the missplicing of which causes frontotemporal dementia with Parkinsonism linked to chromosome 17, an autosomal dominant neurodegenerative disorder (Wu et al. 2006). Additionally, miR-183 is predicted to bind two sites on the 3' UTR of profilin 2 (PFN2) mRNA. PFN2 regulates actin polymerization, determining dendritic spine morphology in neurons; its knockout severely affects neurotransmitter homeostasis and behavior in mice (Witke 2004). Elevation of PFN2 mRNA was observed in nucleated blood cells from bipolar-disorder-diagnosed twins in five monozygotic twin pairs, compared with their healthy twins (Matigian et al. 2007). Thus, miR-134 and miR-183 both appear to regulate known mediators of neuronal stress reactions, compatible with our current findings and relevant to stress-associated diseases.

There is compelling evidence that bipolar disorder, as well as schizophrenia and some neurosensory pathologies, involves hypercholinergic neurotransmission, and nAChRs were suggested as targets for treatment (McEvoy and Allen 2002; Shytle et al. 2002). The local and temporal regulations of cholinergic neurotransmission via the alternative splicing of AChE are therefore of high relevance to the understanding and treatment of mental stress and disease. We previously demonstrated a shift in the splicing pattern of AChE following acute stress (Kaufer et al. 1998). Furthermore, alternative splicing of the AChE mRNA transcript modifies the effect of the resultant protein product with regards to fibril formation from the APP C-terminal peptides, from an upregulation by the primary “synaptic” AChE-S protein (Inestrosa et al. 1996) to suppression by the stress-induced AChE-R variant (Berson et al. 2008). Our current findings suggest that SC35 mediated alternative splicing of AChE, and, downstream to it, the amyloid fibril formation outcome in neurodegeneration processes is subject to regulation by stress-responding miRs.

In conclusion, our data point at miR functions in regulating alternative splicing under mental stress. The micromanagement of alternative splicing thus merits special attention.

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