



Dynamic changes in murine forebrain miR-211 expression associate with cholinergic imbalances and epileptiform activity

Uriya Bekenstein^a, Nibha Mishra^{a,b}, Dan Z. Milikovsky^{c,d}, Geula Hanin^a, Daniel Zelig^{c,d}, Liron Sheintuch^{c,d}, Amit Berson^a, David S. Greenberg^a, Alon Friedman^{c,d,e}, and Hermona Soreq^{a,b,1}

^aThe Alexander Silberman Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem 9190401, Israel; ^bThe Edmond and Lily Safra Center for Brain Sciences, The Hebrew University of Jerusalem, Jerusalem 9190401, Israel; ^cDepartment of Physiology and Cell Biology, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, 8410501 Israel; ^dDepartment of Cognitive and Brain Sciences, Zlotowski Center for Neuroscience, Ben-Gurion University of the Negev, Beer-Sheva, 8410501 Israel; and ^eDepartment of Medical Neuroscience, Dalhousie University, Halifax, NS B3H 4R2, Canada

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Epilepsy is a common neurological disease, manifested in unprovoked recurrent seizures. Epileptogenesis may develop due to genetic or pharmacological origins or following injury, but it remains unclear how the unaffected brain escapes this susceptibility to seizures. Here, we report that dynamic changes in forebrain microRNA (miR)-211 in the mouse brain shift the threshold for spontaneous and pharmacologically induced seizures alongside changes in the cholinergic pathway genes, implicating this miR in the avoidance of seizures. We identified miR-211 as a putative attenuator of cholinergic-mediated seizures by intersecting forebrain miR profiles that were Argonaute precipitated, synaptic vesicle target enriched, or differentially expressed under pilocarpine-induced seizures, and validated TGFBR2 and the nicotinic antiinflammatory acetylcholine receptor nAChRα7 as murine and human miR-211 targets, respectively. To explore the link between miR-211 and epilepsy, we engineered dTg-211 mice with doxycycline-suppressible forebrain overexpression of miR-211. These mice reacted to doxycycline exposure by spontaneous electrocorticography-documented nonconvulsive seizures, accompanied by forebrain accumulation of the convulsive seizures mediating miR-134. RNA sequencing demonstrated in doxycycline-treated dTg-211 cortices overrepresentation of synaptic activity, Ca²⁺ transmembrane transport, TGFBR2 signaling, and cholinergic synapse pathways. Additionally, a cholinergic dysregulated mouse model overexpressing a miR refractory acetylcholinesterase-R splice variant showed a parallel propensity for convulsions, miR-211 decreases, and miR-134 elevation. Our findings demonstrate that in mice, dynamic miR-211 decreases induce hypersynchronization and nonconvulsive and convulsive seizures, accompanied by expression changes in cholinergic and TGFBR2 pathways as well as in miR-134. Realizing the importance of miR-211 dynamics opens new venues for translational diagnosis of and interference with epilepsy.

acetylcholinesterase | cholinergic | EEG | epilepsy | microRNA

Epileptic seizures are generated when excitation/inhibition imbalances or abnormal afferent signals to a brain region lead to excessive or parallel nonspecific neuronal activation (1). Epilepsy has a wide range of manifestations, from brief loss of consciousness to overt convulsions. All mammalian brains, including healthy human brains, are prone to the generation of seizures, and diverse brain insults (ischemic, traumatic, infectious) reduce the threshold to seizures (2, 3). However, why some brains undergo epileptogenesis and develop recurrent unprovoked seizures following insults while others escape this pathophysiology remains unknown (4). Specifically, genes whose modulation mitigates these processes, and the neurotransmission and/or growth factor pathways involved, remain largely unexplored.

Recent reports point at microRNAs (miRs) as functional regulators of epileptogenesis. MiRs are small noncoding RNA molecules that regulate the expression levels of most protein coding

genes in mammals and may orchestrate whole transcriptional pathways (5). Recent studies on status epilepticus (SE) animal models of epilepsy identified seizure-characteristic miR changes (6). Also, different miRs, including miR-132 (7), miR-34a (8), and miR-146a (9) emerged as actively involved in the pathogenesis of epilepsy. Of growing interest is miR-134, whose silencing exerts protective effects both in reducing the in vivo severity of SE (10, 11) and in in vitro-cultured hippocampal neurons (12), accompanied by numerous links to human epilepsy (13).

Disrupted ACh signaling and malfunctioning of the TGF-β pathway may both lead to excessive neuronal activation and seizures, and underlie some cases of epilepsy (14, 15). However, the molecular mechanisms linking these two pathways with epileptogenesis are only partially known. Disrupted ACh signaling leads to seizures due to the modulatory function of ACh over mammalian brain neurotransmitters such as glutamate or GABA, and affects synaptic transmission in large cortical networks. Cholinergic receptors include muscarinic metabotropic receptors, which function via G proteins, and nicotinic cation-channel receptors, which are exclusively excitatory (17); modulating

Significance

Acute traumatic stress increases the sensitivity to develop epileptic seizures in certain people. It is therefore important to discover physiological mechanisms that avoid epilepsy. To test if rapidly inducible microRNAs (miRs) could mediate such protection, we combined mouse engineering, RNA sequencing, electric recording of brain activity, and learning tests. We discovered that miR-211, originating from an epilepsy-related genomic locus, may be involved, and therefore engineered mice produce a drug-suppressible excess of brain miR-211. In these mice, suppressing miR-211 excess to the original expression levels in normal brains led to electrically recorded epilepsy and hypersensitivity to epilepsy-inducing compounds; it also modified acetylcholine receptor composition. The functional impact of miR-211 dynamics on seizure threshold may enable future development of miR-211-directed therapeutics.

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¹To whom correspondence should be addressed. Email: hermona.soreq@mail.huji.ac.il.

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the action of both receptor types results in seizure activity (18). In autosomal dominant nocturnal frontal lobe epilepsy, missense mutations in either the nAChR α 4 or β 2 nicotinic receptor subunits interrupt cholinergic regulation, leading to seizures (19). Also, focal hypercholinergic activity suffices as an initial event in generating network hyperexcitability and seizures (20). Additionally, hippocampal induction of hyperexcitability by cholinesterase inhibitors induces epileptogenesis in rodent models of epilepsy, and injecting the cholinergic agonist pilocarpine initiates epileptic events, seizures, or full SE (21). Nevertheless, mechanisms underlying the control of such modulatory changes and avoidance of epileptogenesis in the healthy brain are still sought after.

Cholinergic-mediated control of excitability may also target nonneuronal components of the neurovascular unit. ACh modulates inflammatory signaling in both the periphery and the brain, which together with blood–brain barrier integrity may critically control network excitability and seizure threshold. For example, brain insults such as trauma may lead to increased permeability of the microvasculature to serum albumin, which then binds TGF β R2 (TGFBR2) receptors on astrocytes, inducing a chain of epileptogenic events. Such events include activation and phosphorylation of Smad2 and downstream transcriptional effects, entailing neuroinflammation, excitatory synaptogenesis, and reduced neuronal inhibitory cues, all leading to chronic epilepsy (22). Concordantly, local or systemic application of losartan, a potential TGF- β -signaling blocker, can reduce albumin-derived TGF- β -signaling and thus block epileptogenesis (23).

Numerous genetic epileptic syndromes are hallmarked by cholinergic dysfunctions that control neuronal excitability and seizure threshold (18, 20). Therefore, we predicted that miR modulators of cholinergic activities may avoid neuronal hypersynchronization by performing coordinated surveillance over signaling networks that keep synchronized firing in check, and sought miRs that may play functional role(s) in inducing and/or suppressing seizure-related cholinergic signaling by affecting synaptic targets. For this purpose, we generated and tested transgenic mouse models, used continuous electrocorticography recordings to detect changes in neocortical activity, and used RNA sequencing (RNA-seq), bioinformatics analyses, and corresponding validations, all of which identified miR-211 as an attenuator of coregulated cholinergic and TGFBR2-associated seizures.

Results

Neuronal miR-211 Controls Cholinergic Synapse Transcripts in the Epileptogenic Brain. To perform a nonbiased search for neuronal miRs regulating synaptic processes and responding to cholinergic seizure-related cues, we intersected publicly available transcriptional profiles of miRs regulating synaptic vesicle transcripts (24), overrepresented in healthy forebrain immune precipitates of neuronal Argonaute 2 (25) and differentially expressed following pilocarpine injection, which induces cholinergic-mediated seizures (26). Of the three miRs that emerged in all three profiles (miR-211-5p, -218-5p, and -27a-3p; Fig. 1A), we experimentally observed hippocampal miR-211 to be down-regulated in acute pilocarpine-injected model mice, compared with saline-injected controls (48 h following injection; Fig. 1B).

Notably, miR-211 is a conserved intragenic miR located within the TRPM1 calcium channel gene (5), itself within the 15q13.3 locus where heterozygote microdeletions [Online Mendelian Inheritance in Man (OMIM) no. 612001] associate with mental retardation and recurrent epileptic seizures (27, 28), and homozygous deletions associate with severe neurodevelopmental problems, including epileptic encephalopathy (29). In proximity to the TRPM1 gene and within the 15q13.3 locus is the nicotinic receptor nAChR α 7 (30) (Fig. 1C), a gain-of-function mutation that results in nicotine-induced seizures (31). In silico miR–target interaction analysis (32, 33) predicted that *Homo sapiens* (hsa)-miR-211 targets nAChR α 7 via

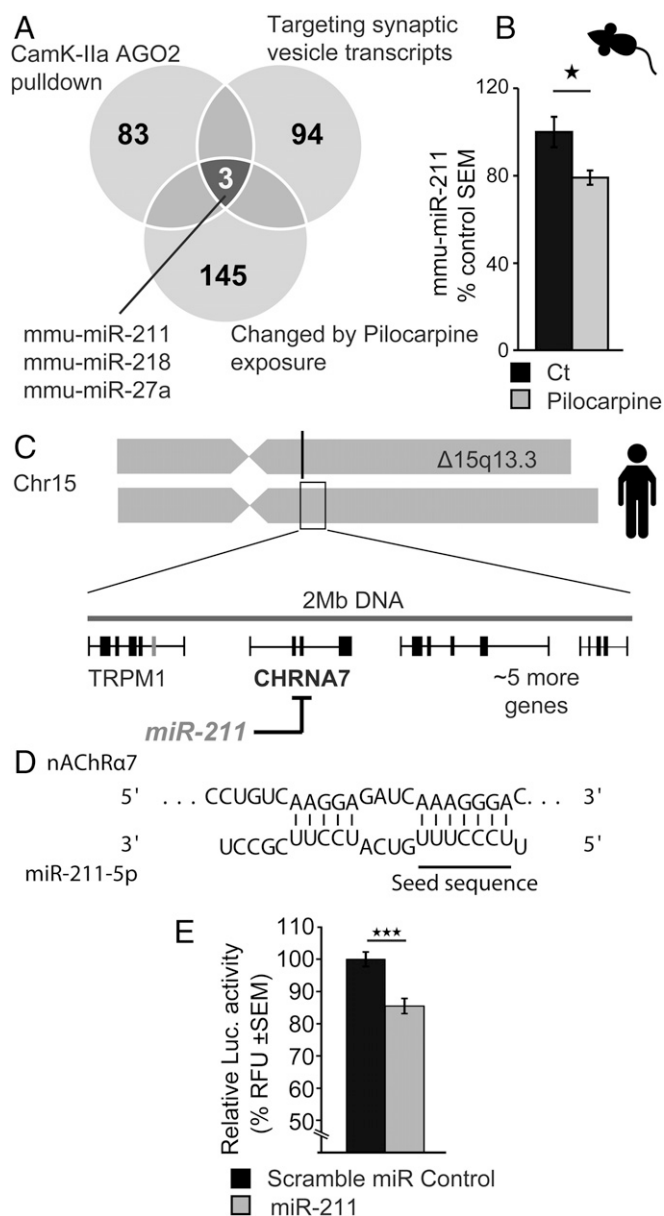


Fig. 1. Identifying MiR-211 as a synaptic candidate associated with cholinergic signaling-induced seizures. (A) Three candidate miRs (miR-211, -218, and -27a) emerged by intersecting rodent miRs whose levels modify following exposure to the cholinergic facilitator pilocarpine (145 miRs) (6); interact with the RNA-induced silencing complex (RISC) protein Argonaute 2 (AGO2) in CamK2a-expressing cells (83 miRs) (25); and target synaptic vesicle transcripts (94 miRs) (75), predicting involvement in cholinergic-related epileptic seizures. (B) qRT-PCR measurements show mmu-miR-211 decline in hippocampal RNA 24 h following exposure to pilocarpine. (C) Human MiR-211, as well as its in silico target, nicotinic nAChR α 7, and five other genes localize to a 15q13.3 chromosomal region where heterozygote deletions entail cognitive impairments with recurrent seizures. (D) The seed domain of hsa-miR-211-5p shows sequence complementarity with the inflammation-regulating nicotinic nAChR α 7. (E) Luciferase assay validated direct targeting by miR-211 of nAChR α 7 in human embryonic kidney cells. Results were considered significant at $*P < 0.05$, $***P < 0.001$, after correction for multiple testing when applicable.

a 7-mer miR response element (Fig. 1D). Validating this prediction, miR-211 expression in cultured HEK293 cells directly down-regulated a luciferase expression construct containing the nAChR α 7 3' UTR (Fig. 1E); taken together, this indicated that miR-211 is both affected by and may control cholinergic transmission in an epilepsy-relevant manner.

C and D). During the subsequent 6 d, ECoG recordings demonstrated spontaneous nonconvulsive seizures in six of eight dTg-211 mice but in none of nine control CamK mice receiving similar Dox treatment (Fig. 2E). Seizures mostly initiated by the third or fourth day after Dox administration (Fig. 2F), parallel to the decline in miR-211 overexpression (Fig. 2C). Identified seizures showed a pattern of low frequency (~5 Hz) and sharp activity (Fig. 2G and H), without observed motor convulsions. Notably, similarly slow hypersynchronous cortical activity is reminiscent of several human syndromes manifested in epilepsy (36), altogether raising the possibility that forebrain miR-211 elevation may be protective against spontaneous nonconvulsive seizures, whereas its reduction induces them.

Forebrain miR-211 Suppression Exacerbates Long-Lasting Pentylene-tetrazole-Induced Seizures and TGF- β Signaling. The spontaneous seizures induced following Dox-suppression in dTg-211 mice suggested that miR-211 decline may also entail a sustained susceptibility to convulsions. To test such an effect, we treated dTg-211 mice with Dox for 6 d, and challenged them and matched controls with the seizure-provoking pentylene-tetrazole (PTZ) agent 5 d after Dox has been removed, when the levels of cortical miR-211 are again elevated (Fig. 3A). Tested dTg-211 mice showed increased susceptibility to convulsive stimuli (Fig. S1A), and ECoG recording of consequent epileptiform spikes in dTg-211 mice showed an increase in spike counts and total numbers (Fig. 3B and C), as well as seizure numbers and latency to first spike and to first seizure compared with CamK control mice (Fig. 3D–F), where miR-211 levels were conspicuously lower (Fig. 3G), all pointing to long-lasting epileptiform impact of Dox in dTg-211 mice.

Given the reported role of TGF β R signaling in epileptogenesis, we next examined if the TGF β R pathway genes that change following status epilepticus were modified in the epilepsy-susceptible dTg-211 mice following Dox. The 3'-UTR of the murine TGF β R2 gene shows seed sequence complementarity with the mature *Mus musculus* (mmu)-miR-211 (Fig. S1B), and an in vitro psiCHECK assay showed direct down-regulation of the murine TGF β R2 3'-UTR reporter by mmu-miR-211, validating this miR–target link (Fig. 3H). Correspondingly, non-Dox-treated frontal dTg-211 cortices showed twofold lower TGF β R2 transcript levels compared with controls (Fig. S2B), alongside ~40% reduced TGF β R2 protein (Fig. 3I; ELISA). Reciprocally, administration of Dox induced a step-wise fourfold increase in TGF β R2 mRNA within 4 d (Fig. 3J). To explore if TGF β R2 pathway genes are globally changed, we turned to unbiased RNA-seq of dTg-211 cortical tissue RNAs (without and with Dox suppression of miR-211 overexpression) compared with matched control tissues. The cDNA libraries showed overall similar sequencing reads distribution across expression level and interrelated tag-wise normalized variance and expression levels (Fig. S2A and B). Nevertheless, comparing dTg-211 brains before and after 5 d of Dox administration showed substantially higher numbers of differentially expressed genes than comparing naive dTg-211 brains to CamK controls (Fig. 3K), suggesting that Dox-induced suppression of miR-211 overexpression may entail an extensive physiological change. Notably, grouped miR-211 target transcripts, as predicted in silico by the TargetScan algorithm (37), showed significant, albeit mild, increases and decreases following Dox administration [empirical cumulative distribution function (ECDF) plots] (Fig. S3). We conclude that the bulk of transcriptome changes induced by miR-211 perturbations occurred in secondarily affected transcripts and that the transcriptional footprint of miR-211 reduction was greater than that of its sustained overexpression.

Predictably, genes that were down-regulated in dTg-211 forebrains compared with controls were more commonly differentially expressed following Dox administration, in contrast to all genes or all up-regulated genes (Fig. 3L; ECDF, Kolmogorov–Smirnov

test, $P < 0.001$). Also, those genes that were down-regulated in dTg-211 brains showed preferred post-Dox up-regulation (Fig. 3M and N). Numerous TGF- β pathway genes that were modified 12 h following SE (38) were also changed following Dox, either by up- or down-regulation (Fig. 3O), including the Myc proto-oncogene protein (*Myc*), the Chordin (*chrd*), inhibitor of DNA binding 2, HLH protein (*id2*), and SMAD family members 1 and 9 (*smad1*, *smad9*). Together, these findings suggested that the Dox-induced release of TGF β R2 from miR-211-mediated suppression impacted forebrain TGF- β signaling. Murine miR-211 thus emerged as a modulator of epilepsy, whose forebrain suppression induces long-lasting hyperexcitability and epileptiform activity alongside TGF- β pathway alterations.

MiR-211 Decline Induces Transcriptome Changes of Endothelial, Synaptic, and Cholinergic Functions. To explore the global transcriptional changes following miR-211 suppression, and test if they relate to specific brain cell types, we compared dTg-211 brains' transcripts to controls. Specifically, we tested highly expressed genes characteristic of neurons, astrocytes, oligodendrocytes and their progenitor cells (OPCs), microglia, and endothelial cells (39) (Fig. 4A and B). None of the cell type marker groups showed changes in dTg-211 brains compared with controls. However, comparing dTg-211 transcripts in brains with and without Dox (Fig. 4C) demonstrated that 19 of 21 endothelial cell markers, but none of the other cell type markers showed an increase following Dox administration ($P < 0.05$, perturbation analysis; *Methods*). Thus, miR-211 reaction to Dox appeared to potentiate endothelial gene expression, predicting functional relevance for neurovascular unit activities.

Next, we searched for Dox-induced changes in the expression of cholinergic receptor genes. The excitatory muscarinic ACh receptor-5 (30), a positive effector of cholinergic synaptic transmission (31), was elevated by fourfold (Fig. 4D). Likewise, the excitatory nACh α -1 neuronal nicotinic receptor and α -5 nicotinic receptor (40, 41), and the ionotropic α -7 nicotinic receptor, responsible for post- and presynaptic excitation (17) and blocker of inflammation (42), were all elevated. In contrast, the metabotropic muscarinic ACh receptors 4 and 2 (mAChR4, mAChR2) were both twofold reduced following Dox administration (Fig. 4D and scheme in Fig. 4E). mAChR4 is located on both pre- and postsynaptic sites in brain cholinergic synapses, and exerts inhibitory effects on synaptic firing (43) with a role in locomotion. Additionally, we noted increases in butyrylcholinesterase (BChE; Fig. S4B), which hydrolyzes ACh in the brain alongside AChE and is elevated in AD brains (44). In contrast, we noted decrease of ATCA/BNIP-H (Fig. S4C and D), an ataxia-related brain-specific scaffold protein, which was recently found to recruit choline acetyltransferase (ChAT) to neurite terminals, and promote cholinergic signaling (45, 46). Furthermore, within 4 d following Dox administration, dTg-211 mice presented fourfold increases in the forebrain levels of miR-134 (Fig. 4F), known to be causally involved with the induction of convulsive seizures (7, 10, 11, 47).

To examine how extrasynaptic cholinergic imbalance would affect miR-211 expression and the risk of epilepsy, we used transgenic AChE-R (TgR) mice overexpressing the soluble, nonsynaptic stress-induced splice variant of AChE from which the 3'-untranslated region (3'-UTR), which contains the miR regulatory element, had been deleted (Fig. 4G, scheme). TgR mice, which constitutively overexpress AChE-R that catalyzes ACh breakdown in extrasynaptic sites and show chronic stress behaviors, are hypersensitized to nicotine administration (48). Intriguingly, these mice also experienced higher susceptibility to seizures, manifested as larger fraction of mice presenting full SE after pilocarpine injection (Fig. 4H); this was accompanied by shorter latency until SE was observed (Fig. 4I; $P < 0.05$), reduced miR-211 expression in the hippocampus and frontal cortex

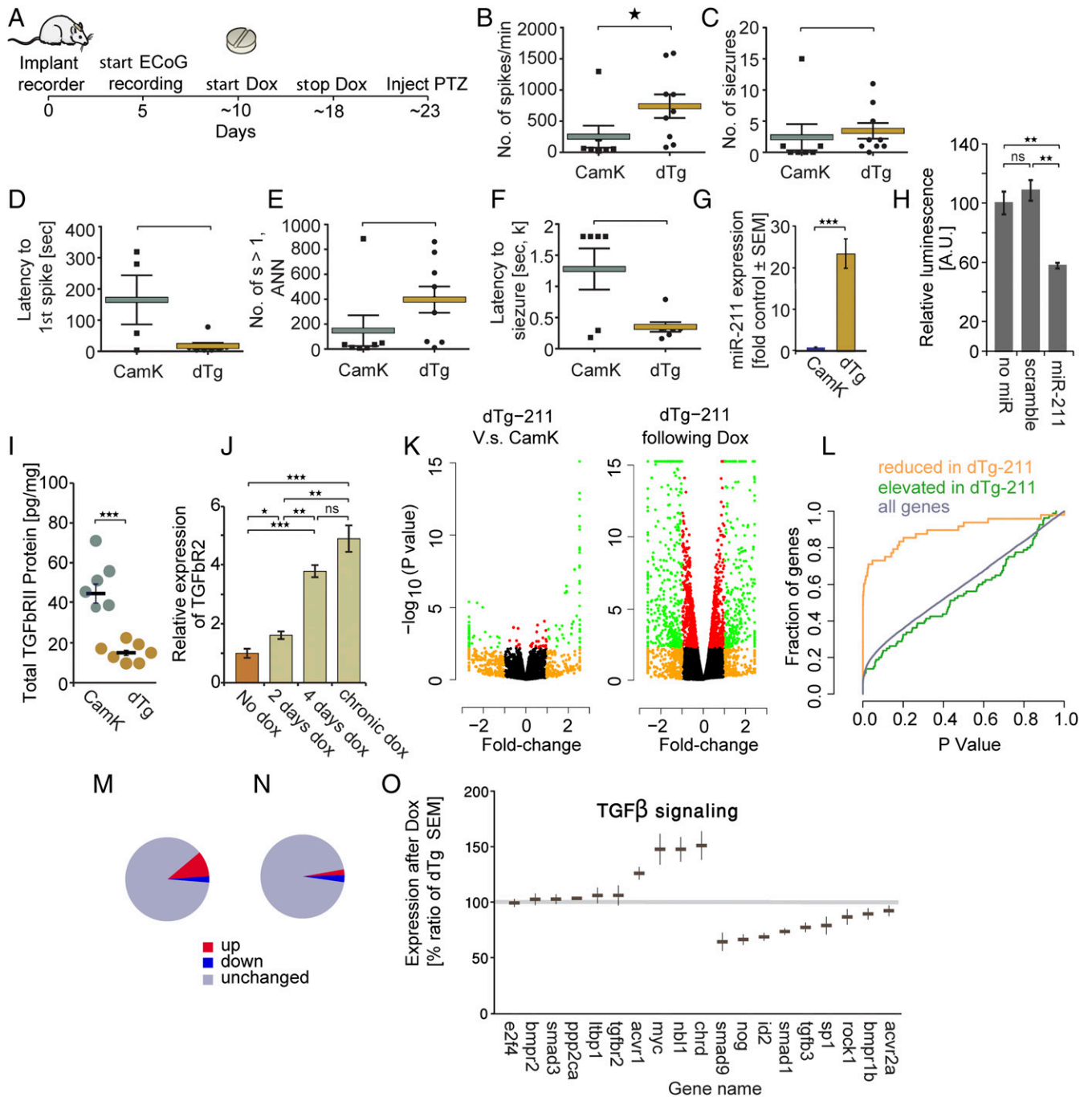


Fig. 3. Dox-treated dTg-211 mice show sustained susceptibility to PTZ-induced convulsions alongside TGFBR-associated gene changes in RNA sequence. (A) Scheme of PTZ injection 4 d after 5-d Dox administration, aimed to examine long-term susceptibility to this convulsant. See Fig. S3 for increased manual convulsions index scores in dTg-211 mice. (B) ECoG recording shows larger spikes per minute counts, reflecting seizure susceptibility in PTZ-exposed dTg-211 mice compared with CamK controls. (C) Number of seizures. (D) Latency to first spike. (E) Number of seizure events by neuronal networks analysis. (F) Latency to first seizure. (G) DTg-211 mice regained miR-211 overexpression after Dox removal, at the time of PTZ test. (H) Luciferase validation tests of miR-211 targeting of the murine TGFBR2 3'-UTR but not a control sequence. (I) Reduced TGFBR2 protein concentration (twofold) in dTg-211 frontal cortex (ELISA, $n = 7 + 7$, $P < 0.001$). (J) Increased TGFBR2 mRNA levels following Dox administration. (K) Fold-change volcano plot differences for dTg-211 with/without Dox (Right) compared with dTg-211/CamK brains (Left). Dots represent genes, with positive or negative twofold change (orange), passing cutoff threshold for significance (red), both (green), or unmodified (black). (L) ECDF plots show differential expression (P values) following Dox of reduced (orange) but not elevated genes (green) in dTg-211 cortices or in all genes (gray). (M) Cortical genes up-regulated in dTg-211 are reduced (red) following Dox compared with (N) all genes. (O) Per-gene fold changes following Dox for TGF β -signaling genes modified 12 h following status epilepticus (38). Results were considered significant at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, after correction for multiple testing when applicable. ns, not significant.

compared with controls (Fig. 4J), and overexpression of miR-134 in the prefrontal cortex and hippocampus (Fig. 4K), possibly in relation to their hypersynchronous state. Thus, modified

cholinergic regulation in TgR mice reciprocally modified fore-brain miR-211 and miR-134 levels in a similar fashion as well as exacerbated susceptibility to epileptic seizures.

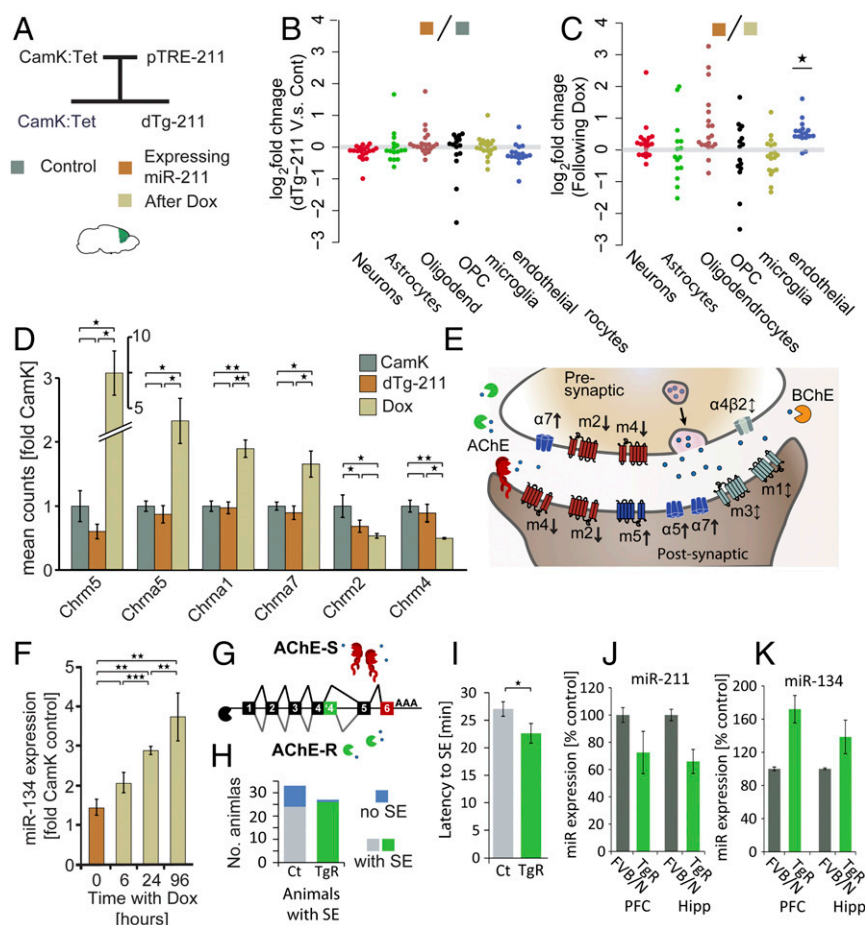


Fig. 4. MiR suppression in dTg-211 mice alters cell type marker genes and cholinergic receptors, and a cholinergic mouse model shows concordant miR changes alongside increased seizure susceptibility. (A) Experimental setup: CamK:TtA mice bred with Tg-pTRE-211 mice generated dTg-211 mice and littermate CamK controls. Illumina-compatible libraries from frontal cortex RNA of mice before or under doxycycline (color-coded squares) were sequenced. (B) Sustained cell-type marker (39) in dTg brains. (C) Elevated endothelial marker genes following Dox. (D) Modified muscarinic and nicotinic cholinergic receptors in Dox-treated dTg-211 brains. (E) Scheme of cholinergic receptors and regulators (shown in D) in brain cholinergic synapses. Note Dox-induced down-regulation of cholinergic receptors suppressing synaptic transmission: CHR2 and CHR4 (m2 and m4); and up-regulation of facilitators CHRM5 (m5), CHRNA5, and CHRNA7 ($\alpha 5$ and $\alpha 7$). (F) MiR-134 up-regulation in dTg-211 mice following Dox administration parallels the time frame of seizure induction in this model. (G) Scheme of the synaptic and nonsynaptic AChE transcript variants and corresponding protein forms. (H) Mice overexpressing the nonsynaptic cholinergic enzyme AChE-R (TgR) (76) show higher propensity with (I) shorter latency for status epilepticus event following pilocarpine injection, alongside (J) miR-211 reduction and (K) miR-134 elevation in prefrontal cortex (PFC) and hippocampus (Hipp) of TgR mice. Results were considered significant at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, after correction for multiple testing when applicable.

Dox-Induced Decline of Forebrain miR-211 Impairs Numerous Signaling Pathways.

To test for phenotypic outputs that may contribute to the synergic effects of transcript changes in functionally related proteins, we established protein–protein interaction (PPI) networks (49). We used genes that were both highly (top 3,000 genes) and differentially expressed [false discovery rate (FDR) < 0.01] following Dox to generate a network of PPI-annotated partners defined by 61 core proteins, and that included first neighbor-interacting proteins of 515 edges and 434 nodes (Fig. 5A). Enriched up-regulated and down-regulated genes and pathways within this network included numerous synaptic vesicle cycle genes (e.g., reduced Vamp2, Snap25; Fig. 5B). Intriguingly, 19 of the 61 core proteins within this network related to the cholinergic synapse KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway 04725 ($P < 10^{-8}$; Fig. 5C). Also, numerous neuronal-related pathways were either up- or down-regulated (Fig. 5D and E; see Table S2 for full list of pathways), including calcium-signaling (KEGG 04020) and synaptic vesicle cycle (KEGG 04721) pathways.

A possible drawback for examining transcriptional effects using Tet-Off mice is that Dox administration may itself impact the

transcriptional profile. To test for this possibility, we examined if cholinergic synapse and TGF- β pathway genes change in publicly available transcriptional data of other Dox administration models (Fig. S5). The outcome profile was unique and nonoverlapping, indicating that miR-211 decline underlines diverse, rapid changes in forebrain cholinergic synapses of dTg-211 mice that are unrelated to the effect of Dox itself. To conclude, miR-211 suppression led to selective transcriptional changes in specific functional groups that control cholinergic synaptic transmission and/or affecting hyperexcitability.

MiR-211 Excess Associates with Impaired Learning Strategy in dTg-211 Mice.

Cholinergic transmission in the mammalian brain has important roles in learning and memory through numerous circuits feeding into the cortex (17). Concordantly, enrichment analysis using the RNA sequencing data identified functional Dox-induced outputs in the dTg-211 cortical datasets. Gene Ontology (GO) (24) showed 166 genes with log fold change less than 0.5 or greater than 2, FDR-corrected P value < 0.01 , and absolute expression levels greater than log cpm of 6.2 (Dataset S1). GO annotation enrich-

these trials was also affected, with slower strategy progression toward directed platform search over days (Fig. 6C). In a probe trial examining reference memory with the platform removed, CamK controls showed clear quadrant preference but dTg-211 mice lost the capacity to locate the platform quadrant (Fig. 6D), possibly indicating short memory duration even once the task had been learned. In contrast, dTg-211 mice performed similarly to CamK controls in locomotion-based tests for anxiety and exploratory behavior, such as the elevated plus maze (Fig. S6 A and B) and the open field test (Fig. S6 C and D). Thus, dTg-211 mice presented a specific functional phenotype of impaired allocentric navigation, a disability attributed to functional impairments in the hippocampus, entorhinal cortex, and some surrounding structures, the functioning of which is strongly modulated by the cholinergic system (51, 52).

Notably, both memory loss and perturbed cholinergic function are reminiscent of Alzheimer's disease (53). Also, miR-211 levels are elevated in epileptic human brain tissues (47), and epileptic events are arguably common in the AD brain (54). Therefore, the possibility of a miR-211 involvement in this condition should be explored. Indeed, postmortem entorhinal cortices from AD patients showed twofold increases in miR-211 levels compared with matched controls (Fig. 6E; $P < 0.05$). Of note, in temporal lobe samples of a discrete cohort of AD patients, no significant change in miR-211 levels was noted (Fig. S6B). Together, these findings may indicate multileveled roles of miR-211 dynamics in cholinergic-mediated neuronal functions.

Discussion

Our study implicated miR-211 as functionally involved in cholinergic surveillance that may limit the risk of neuronal hypersynchronization in the mammalian brain. We combined forebrain RNA-seq analyses with electrocorticography tests of engineered mice presenting conditional miR-211 excess, or overexpressing a miR refractory AChE-R. This exploration linked forebrain miR-211 decreases with spontaneous ECoG-detected nonconvulsive seizures, exacerbated susceptibility to PTZ-induced seizures and increased miR-134 levels, accompanied by comodified synaptic, cholinergic, and TGFBR2 pathways, with a selective impact on endothelial brain cells, indicating involvement of the neurovascular unit (55). The changes associated with miR-211 decline may all add to the risks of convulsive seizures in a long-lasting manner, together attributing several hallmarks of the dynamics and efficacy of cholinergic-mediated susceptibility for seizures in the adult mammalian brain to miR-211 suppression.

ACh-related mechanisms are widely involved in, and sufficient for, generating epileptic seizures (56), with cholinergic imbalances hallmarking several epileptic syndromes (57) and cholinergic agonists inducing limbic seizures (58). Recent independent reports implicated miRs in both initiating and changing the response to seizure-inducing agents (59), but did not explore the putative regulatory role of miRs controlling neuronal cholinergic transmission (57) in avoiding such seizures and affecting the susceptibility to epilepsy. To address these questions, we intersected datasets for miRs targeting synaptic functions (24), experimentally found in neurons (25) and modified following pilocarpine injection (26). This search identified miRs that are directly or consequentially affected by epileptic perturbations, and that potentially function in the synapse. Of these miRs, we focused on miR-211, which was significantly reduced in our hands, either directly or consequentially by pilocarpine injection, and is one of those few genes that are lost in the 15q13.3 deletion syndrome (27, 60) (OMIM no. 612001), which is commonly manifested in epilepsy (27, 28). We also demonstrated miR-211 regulation over the human nAChR α 7 nicotinic receptor, mapped within this genomic locus (61), and whose gain-of-function mutation associates with seizure induction in patients (29). Additionally, miR-211 targets the angiotensin-1 (*ANGPT1*) gene, where a single nucleotide variation disrupting its binding site associates with elevated risk of ischemic stroke (62), a condition with diverse

links to epileptic seizures (4). The miR-211 candidate thus emerged as putatively linked to epilepsy in several ways.

Notably, our work differs from many other miR-focused reports, which depict functional regulation by miRs by assessing their steady-state levels. Rather, we observed limited transcriptional changes in dTg-211 forebrains overexpressing the miR compared with controls, yet found dramatic transcriptional and electrophysiological changes in the brain following the dynamic reduction of miR-211 to its basal levels. That the decline of miR-211 has a more extensive effect than its steady-state level suggests that the function of this miR can be only partially assessed by single time-point measurements. Thus, Dox-induced suppression of the miR-211 transgene in dTg-211 mice induced both nonconvulsive seizures and entailed increased susceptibility to convulsive seizures while elevating the TGFBR2 transcript and protein levels. Because these ECoG-documented changes were absent in control mice, they unlikely reflect the mere impact of Dox, but rather point to a sustained epileptiform effect of miR-211 down-regulation, with possible relevance to human epileptogenic conditions. Additionally, RNA-seq showed a selective elevation in 19 of 21 epithelial marker genes, indicating cellular composition changes of the microvasculature (63), reported to hallmark and contribute to epileptogenesis, with possible gross changes in the epithelial transcriptional landscape.

Intriguingly, the observed seizures were characterized by sharp 5-Hz activity oscillations, with similarity to spike and wave seizures reminiscent of those of absence epilepsy patients. These human syndromes and their corresponding mouse models are manifested in epilepsy, which affects GABAergic modulatory neurotransmission but lacks observed motor convulsions (36, 64). Also, we noted expression changes concordant with systematic facilitation of cholinergic synaptic activation following Dox; specifically, this involved simultaneous elevation in the excitatory nicotinic receptors 5 and 7 and muscarinic receptor 5, and down-regulation of the inhibitory muscarinic receptors 2 and 4. We hypothesized that the muscarinic receptor 1 (mAChR1) will be concordantly modified; supporting this notion, qRT-PCR assays showed significant mAChR1 reduction in dTg-211 mice, and elevation following Dox suppression (Fig. S4E). That we did not observe a parallel change in the sequencing experiment may reflect mRNA editing of this gene's transcripts (15), and for which alignment of sequencing reads is sensitive. We also observed elevation of the ataxia-related scaffold protein ATCAY/BNIP-H gene, which affects shuttling of the ACh synthesizing enzyme ChAT to neurite terminals (45, 46), and mutations in which associate with variable psychomotor retardation phenotypes and cerebellar dysfunction, together indicating multileveled exacerbation of cholinergic signaling.

After Dox has been removed and forebrain miR-211 levels were reelevated, we observed increased susceptibility of dTg-211 mice to PTZ-induced convulsions, suggesting an apparent link between miR-211 expression dynamics to convulsions. Alternative interpretations may, however, exist. For example, abrupt miR-211 reduction may function as a priming event for broad neurological changes, including susceptibility for convulsions, such as those implied by the selective elevation of epithelial marker genes. Alternatively, or in addition, sustained miR-211 elevation may have a supportive or even a merely ancillary role in the complex genetic makeup protecting the neuronal network from hyperexcitability, whereas its perturbation is functionally potent enough to disrupt that balance. Further work would be required to explore these possibilities.

At the cellular level, miR-211 reacts to endoplasmic reticulum stress by targeting the proximal promoter of the proapoptotic transcription factor chop/gadd153, which attenuates apoptotic processes, allowing the cell to reestablish homeostasis and avoid cell death (65); this may indicate attenuation by miR-211 of stressful cellular signals, inversely proposing that its down-regulation may exacerbate such stress-induced reactions. If indeed the observed

miR-211 elevation in the Alzheimer's entorhinal cortex relates to the disease pathophysiology, it may suggest an explanation to the greater risk for unprovoked seizures in early onset patients and those with more severe disease (66), where miR-211 increases would prove insufficient in avoiding seizures. This expression change, not seen in temporal lobe samples, may relate to the prominent cholinergic innervation of the entorhinal cortex by the medial septum.

Neuronal networks operate over a wide range of activity levels, and their imbalance underlies many neurological diseases, including epilepsy. Our study indicates that miR-211-induced transcription changes may be involved. Concordantly, PPI network analysis showed modified cholinergic synapse pathway genes (KEGG 04725) under miR-211 decline. Additionally, this decline affected protein kinase C gamma and inositol 1,4,5-trisphosphate receptor type 1, which modulate ion metabolism at the cholinergic synapse, and are linked to ataxia (67, 68), indicating globally modified synaptic composition and function under reduced miR-211 expression. Supporting this notion, a *Caenorhabditis elegans* gain-of-function mutation in a neuronal ACh receptor, *acr-2(gf)*, causes an epileptic-like convulsion behavior, and the behavioral and physiological effects of *acr-2(gf)* require the activity of the TRPM1-similar channel *GTL-2* (69). Thus, the functional link among ACh, the miR-211 host gene TRPM1, and epilepsy spans many species.

TgR mice overexpressing the nonsynaptic AChE-R splice variant show both enhanced seizure susceptibility and miR-211 reduction, which agrees with the role of miR-211 as both a regulator of and affected by cholinergic signaling-mediated seizures. Compatible with this notion, we identified a time-dependent induction of miR-134 in both TgR mice and within the 4 d following Dox administration to dTg-211 mice, alongside the susceptibility to seizures. Notably, miR-134 levels increase in temporal lobe samples from temporal lobe epilepsy patients and from mice 24 h after induction of status epilepticus (10). Moreover, antagomir silencing of miR-134 reduces recurrent seizures and development of epileptogenesis, and miR-134 affects long-term depression via the Pumilio-2/Polo-like kinase 2 (*Pum2/Plk2*) pathway, leading to inhibition due to altered subunit composition of plasma membrane-localized AMPA receptors (70), perhaps indicating reversibility of miR-211 decreases.

Pathway analysis performed on all genes that were differentially expressed in dTg-211 cortices following Dox indicated modified cognition, learning, and memory-related GO terms, suggesting that mice expressing miR-211 may have altered memory abilities, possibly due to the role of synaptic cholinergic signaling in memory (71). In the Morris water maze, dTg-211 mice presented delayed progression in search strategy and reduced reference memory abilities in probe trials. The impaired allocentric navigation phenotype may be attributed to impaired hippocampal, entorhinal cortex and some surrounding structures, the functioning of which is strongly modulated by the cholinergic system (51, 52, 72).

In conclusion, we show that dynamic reduction of miR-211 overexpression in forebrain neurons leads to major transcriptional

change, including elevated levels of endothelial marker genes and enriched GO pathways relating to synapse genes, neurotransmitter functioning, higher brain functions, and transporter activity. MiR-211 decreases further induce overall potentiation of cholinergic synapses, and spontaneous ECoG documented nonconvulsive seizures in the brains of tested mice. Our findings present the dynamic changes in miR-211 and miRs at large as possible mediators and attenuators of seizures induced by cholinergic transmission imbalances. The long-lasting functional effects of miR-211 on the process of epileptogenesis and its implications for diagnostic and therapeutic avenues thus require further research.

Methods

Transgenic Mice Generation and Experiments. We generated pTRE-miR-211 mice by cloning the premmu-miR-211 sequence into a pTRE-tight vector, followed by pronuclear injection at the Weizmann Institute of Science Animals Facility. Mice were held in specific pathogen-free conditions at the Hebrew University, an Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited institute. All procedures, including animal tests, were approved by the Institutional Ethics Committee of The Hebrew University of Jerusalem (ethics no. NS-16-14729-4), with concordance to AAALAC International guidelines. tTA-CamKa strain was acquired, backcrossed, and crossed and housed as in *SI Methods*. dTg mice and CamK littermates were housed together and administered Dox in parallel. The cholinergic muscarinic agonist pilocarpine (Sigma; 290–340 mg/kg) was i.p. injected to mice as in ref. 73. Behavioral tests, including Morris water maze, elevated plus maze, and open field tests were performed as described in *SI Methods*.

Human-Derived Samples. Postmortem samples of entorhinal cortex from AD and age-matched controls were obtained from the Netherlands Brain Bank (NBB) at the Netherlands Institute for Neuroscience (73). Samples were collected following written informed consent for a brain autopsy by the NBB.

Statistical Analyses. Differential expression in sequencing experiments was derived from adjusted *P* values in DESeq (74) on R platform, after FDR correction. For specific genes presented, fold change and ratio values are shown as mean \pm SEM. For cholinergic receptors, *t* test was used on normalized count data. For enrichment of cell type marker genes, *P* value was defined based on permutation analysis. Box and whisker plots show second and third quintiles for box, and 1.5 quantile distances from median for whiskers, as by convention. Results were considered significant if $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, after correction for multiple testing when applicable. For ECoG seizure and spike observations, a Mann–Whitney test was used.

Additional methods are described in *SI Methods*.

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