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MicroRNA-128 governs neuronal excitability and motor behavior in mice

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Abstract

The control of motor behavior in animals and humans requires constant adaptation of neuronal networks to signals of various types and strengths. We found that microRNA-128 (miR-128), which is expressed in adult neurons, regulates motor behavior by modulating neuronal signaling networks and excitability. miR-128 governs motor activity by suppressing the expression of various ion channels and signaling components of the extracellular signal-regulated kinase ERK2 network that regulate neuronal excitability. In mice, a reduction of miR-128 expression in postnatal neurons causes increased motor activity and fatal epilepsy. Overexpression of miR-128 attenuates neuronal responsiveness, suppresses motor activity and alleviates motor abnormalities associated with Parkinson's–like disease and seizures in mice. These data suggest a therapeutic potential for miR-128 in the treatment of epilepsy and movement disorders.

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miR-128 is one of the most abundant and highest enriched miRNA in the adult mouse and human brain ((1, 2) (Fig. S1A). The expression of miR-128 in the mouse brain increases gradually during postnatal development and peaks in adulthood ((3, 4) (Fig. S1B)). miR-128's expression in diverse brain regions (Fig. S1D) suggests an important role for this miRNA in processes that are common to many neuronal cell-types.

The indication of a potent regulatory role for miR-128 in brain function came from our observation of early-onset fatal epilepsy in mice deficient in miR-128 (Fig. 1A). miR-128 is encoded by two separate genes, *miR-128-1* and *miR-128-2*, on mouse chromosomes 1 and 9 (Fig. S2A, B) or human chromosomes 2 and 3, respectively. In mice, germline *miR-128-2* deficiency results in an 80% reduction of miR-128 expression in the forebrain, whereas ablation of the *miR-128-1* gene eliminates only 20% of miR-128 (Fig. S2A, B). The profound decline in miR-128 expression levels in *miR-128-2*$^{-/-}$ but not *miR-128-1*$^{-/-}$ mice is associated with the development of hyperactivity and increased exploration at 4 weeks of age (Fig. 1A, Fig. S2C, D). The juvenile hyperactivity in *miR-128-2*$^{-/-}$ mice progresses quickly to severe seizures and death at 2–3 months of age (Fig. 1A, B, movie S1). The lethal impact of miR-128 deficiency in mice can be prevented by treatment with the anticonvulsant drug valproic acid (Fig. 1C), thus demonstrating the causal role of seizures in the animals' death.

The hyperactivity and fatal epilepsy in *miR-128-2* deficient mice reflects the ability of miR-128 to control the excitability of postnatal neurons. Selective inactivation of the *miR-128-2* gene in forebrain neurons (*Camk2a-cre; miR-128-2*$^{fl/fl}$) leads to a reduction of miR-128 expression, followed by early onset hyperactivity, seizures, and death, as observed in *miR-128-2*$^{-/-}$ mice (Fig. 1B, D, Fig. S3A). Moreover, correction of miR-128 deficiency by ectopic *miR-128-2* expression in neurons normalizes motor activity and prevents the seizure-induced death (Fig 1E, Fig. S4A, C).

To gain an understanding of the mechanism that mediates miR-128-dependent control of motor activity, and to avoid interference between phenotypes caused by the loss of miR-128 in diverse neuronal cell-types, we restricted the *miR-128-2* deficiency to dopamine responsive neurons that regulate motor behavior in mice and humans. There are two major dopamine responsive Camk2a-expressing neuron types in the mouse forebrain, which have distinct contributions to motor activity (5). While activation of the dopamine 1 receptor expressing neurons (D1-neurons) increases locomotion, activation of dopamine 2 receptor expressing neurons (D2-neurons) reduces locomotion in mice (6). We found that miR-128 deficiency in D1-neurons (*Drd1a-cre;miR-128-2*$^{fl/fl}$), but not in D2-neurons (*A2a-cre;miR-128-2*$^{fl/fl}$), leads to juvenile hyperactivity followed by lethal seizures at around 5 months of age (Fig. 1F, Fig. S3B, C).

To identify miR-128 targets that are responsible for the abnormal motor activity, we analyzed mRNAs associated with the RNA induced silencing complex (RISC) in adult neurons *in vivo*. The RISC-bound mRNAs represent the pool of cellular mRNAs that become a subject of miRNA-mediated suppression (7). We used mice that express the epitope-tagged RISC component Argonaute 2 (Ago2) (8) in Camk2a-neurons (Fig. S5A). Immunoprecipitation of Ago2 (HITS-CLIP, (9)) from the forebrain of these mice yielded the

neuron-specific RISC-associated mRNAs (Fig. S5A, B). The perfect base pairing of at least six nucleotides between the miRNA seed sequence and the 3′untranslated region (3′UTR) of the RISC-associated mRNAs (10) was considered to be the minimal requirement for any potential miRNA-mediated mRNA suppression (Fig. S5B, C). Using these criteria, we found that the miR-128 seed target sequence (ACUGUG) is the most represented hexamer among all RISC-associated mRNAs (Fig. S5C, Table S1)), and identified a total of 1061 potential miR-128 target mRNAs in adult neurons (Table S2).

We investigated these miR-128 target genes by analyzing their expression in neurons deficient for miR-128. We expected that mRNA transcripts that are targeted directly by miR-128 in neurons would show an increased in mRNA expression and subsequent ribosome association in the miR-128 deficient cells. We reasoned that the relative homogeneity of the D1-neuron population might provide the most accurate assessment of miR-128-dependent target genes that are responsible for controlling motor activity. The impact of miR-128 deficiency on mRNA expression was evaluated by D1 cell-type specific Translating Ribosome Affinity Purification (TRAP) in mice (11). The TRAP approach allows a direct comparison between ribosome-associated mRNAs from wild-type and miR-128-deficient D1-neurons *in vivo* (Fig. S6A). Using Sylamer analysis (12), we confirmed the expected enrichment of potential miR-128 binding sites among the most upregulated genes in miR-128 deficient D1-neurons (Fig. S6B). We found that the deficiency of *miR-128* in D1-neurons results in a significant up-regulation of 154 of the predicted RISC-associated miR-128 target genes (Fig. 2A, Table S3). The fact that only ~15% of the potential RISC-associated miR-128 targets display increased expression is likely to reflect the known redundancy among miRNAs. Many mRNAs are regulated by more than one miRNA (13, 14) thus limiting the actual impact of individual miRNA deficiency on the expression of miRNA targets *in vivo*.

Bioinformatic network and pathway analyses of the miR-128 target genes indicates the ability of miR-128 to affect molecular processes that are intrinsically linked to the regulation of neuronal excitability and motor behavior in mice and humans (Fig. 2B). In particular, miR-128 regulates the expression of numerous ion channels and transporters, as well as genes that contribute to neurotransmitter-driven neuronal excitability and motor activity (Fig. 2B, Table S3, S4). Several of these genes are linked to epilepsy in humans, some of which, including the neurotransmitter GABA transporter *Slc6a1*, the high affinity glutamate receptor *Slc1a1*, the voltage gated sodium channels *Scn2b* and *Scn4b*, the voltage-dependent calcium channels *Cacna2d3* and *Cagn2*, as well as the carbonic anhydrase *Car7*, are potential targets of clinically approved anti-seizure drugs (Table S3, S4) (15). The high abundance of extracellular signal regulated kinase (ERK1/2) signaling network components among the miR-128 targets underscores the potential of this miRNA to control signaling processes associated with neuronal excitability (Fig. 2B). Moreover, many of the neuronal signaling proteins and channels that we identified as direct miR-128 target genes are involved in the regulation of upstream signaling events, which can affect ERK activity (Tables S3, S4). While ERK1 and ERK2 are not directly targeted by miR-128, the ERK network appears to be at the center of the miR-128-controlled signaling circuit in neurons. The protein expression levels of potent ERK network regulators, which are directly targeted

by miR-128, such as Pea15a (16), D4Ertd22e/Szrd1 (17), and the TARPP protein that is encoded by the long splice variant of the miR-128-2 host gene *Arpp21* (18, 19), are increased in the striatum of mice with a D1-neuron specific deficiency in miR-128 (Fig. 2C, S7). Furthermore, mice with a D1-neuron specific deficiency of *miR-128-2* display an increase in ERK2 activation as compared to their littermate controls (Fig. 2D). Notably, only ERK2, but not ERK1, displays increased phosphorylation (Fig. 2D). Deficiency of miR-128 in D1-neurons appears to specifically activate ERK2 phosphorylation, without affecting the activation of other MAP kinase pathways components such as the stress-activated protein kinase/Jun-amino-terminal kinase (SAPK/JNK) or protein kinase B (AKT) (Fig. S8). Electrophysiological studies in striatal slices from *Drd1a-cre; miR-128-2*$^{fl/fl}$ mice revealed an increase in D1-neuron excitability. The miR-128 deficient D1-neurons show normal membrane excitability at the soma (Fig. S9A), but display enhanced dendritic excitability (Fig. 3A) as well as a ~20% increase of functional dendritic spines (Fig. 3B, S9B). These findings are consistent with a critical role of the ERK2 network in neuronal excitability and synaptic plasticity (20, 21).

Enhanced ERK2 activation is linked to increased motor activity and seizures in mice (22–24). The hyper-activation of ERK2 and concomitant increase in D1-neuron sensitivity to dopamine occurs also during Parkinson-like disease in mice caused by chemically induced depletion of dopamine in the mouse striatum (25–27). The reduced levels of dopamine and concurrent increase of D1-neuron sensitivity result in hyper-responsiveness to the motor activity-inducing effects of dopamine (26–28). In humans, the D1-neuron hyper-responsiveness is one of the major causes of dyskinesia, a side effect of L-Dopa treatment in Parkinson's disease (25–27).

We found that miR-128 deficiency in striatal D1-neurons mimics the hypersensitivity of D1-neurons in mice suffering from Parkinson's-like syndrome. The deficiency of *miR-128* in D1-neurons enhances motor activity in response to Drd1-specific agonist treatment in mice (Fig. 3C). The D1-neuron hyper-responsiveness to the Drd1-agonist is also associated with an increase in ERK2 phosphorylation in the striatum of *Drd1a*-cre; *miR-128-2*$^{fl/fl}$ mice (Fig. 3D). The increase in dopamine sensitivity and enhanced ERK2 activation in mice with Parkinson's-like disease are accompanied by increased expression of dopamine-induced immediate early genes (IEG) in D1-neurons (25–27). Similarly, Drd1-agonist treatment enhances IEG expression in *miR-128* deficient D1-neurons as compared to the D1-neurons of control mice (Fig. 3E). The increased locomotor activity characteristic of *Drd1a*-cre; *miR128-2*$^{fl/fl}$ mice was normalized by pharmacological inhibition of the mitogen-activated protein kinase kinase MEK1, a major activator of ERK2 in neurons. *In vivo* administered MEK1-specific inhibitor SL327 does not affect motor activity in wild type mice (22) but does normalize ERK2 phosphorylation and motor activity in the mutant mice (Fig. 4A). In turn, overexpression of miR-128 in Camk2a-neurons is associated with reduced ERK2 activation (Fig. S10A) and decreased motor activity (Fig. S4B) in mice. The effect of increased miR-128 expression in adult neurons protects mice against abnormal motor activities associated with chemically-induced Parkinson's disease (Fig. 4B, S10B) and seizures (Fig. 4C).

In summary, we have identified miR-128 as a modulator of signaling pathways that control neuronal excitability and motor activity in mice. The human *miR-128-2* gene on chromosome 3p lies within a region that has been linked to idiopathic generalized epilepsy (29, 30). It is tempting to speculate that changes in miR-128 or miR-128 target gene expression could be a potential cause of increased neuronal excitability and epilepsy in humans. Our understanding of miR-128's role in neuronal signaling could prove advantageous in the design of novel therapeutics for epilepsy and motor disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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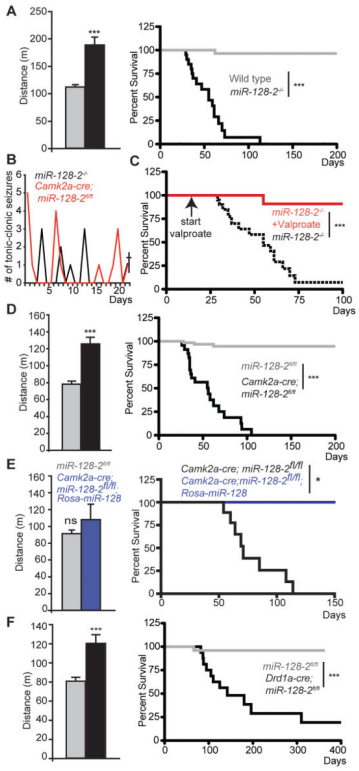

Figure 1. miR-128 controls motor behavior in mice

(A) Deficiency in *miR-128-2* causes hyperactivity and premature death in mice. (Left panel) Motor activity was determined by measuring total horizontal distance in a 60 min open field assay (n=23 and 12). (Right panel) The lifespans of *miR-128-2*$^{-/-}$ mice and littermate controls are shown (n= 20 and 46). **(B, C) miR-128 deficiency causes fatal seizures that can be prevented by anti-convulsant treatment (B)** Representative display of spontaneous tonic-clonic seizure episodes in *miR-128-2*$^{-/-}$ (black) or *Camk2a-cre; miR-128-2*$^{fl/fl}$ mice (red) during a 22-day observation period. **(C)** The lifespans of control *miR-128-2*$^{-/-}$ (dotted line, as shown in **A**) or sodium valproate-treated (red, n=11) *miR-128-2*$^{-/-}$ mice are shown **(D) Deficiency in miR-128 in postnatal neurons causes hyperactivity and fatal epilepsy.** Motor activity and survival rates of *Camk2a-cre; miR-128-2*$^{fl/fl}$ mice (n=21 and 25) and littermates (n=8 and 47) are shown. **(E) Ectopic expression of miR-128 normalizes hyper-locomotion and prevents death of *Camk2a-cre; miR-128-2*$^{fl/fl}$ mice.** Motor activity in *Camk2a-cre; miR-128-2*$^{fl/fl}$; *Rosa-miR-128* (n=4, blue) and wild-type mice (n=10, gray) are shown. The lifespans of *Camk2a-cre; miR-128-2*$^{fl/fl}$ mice in the presence (n=4, blue) or absence (n=9, black) of ectopic miR-128 expression are shown. **(F) miR-128 deficiency in D1-neurons causes hyperactivity and fatal epilepsy.** Motor activity (n=26 and 42) and lifespans (n= 16 and 28) of mice with a D1-neuron specific miR-128 deficiency or control mice are shown. Error bars show s.e.m., Welch's t-test, non-significant (ns), * p≤0.05, ** p≤0.01, *** p≤0.001. Kaplan-Meier graph shows survival curves of mutant and littermate control mice, *** p≤0.001, log rank tests.

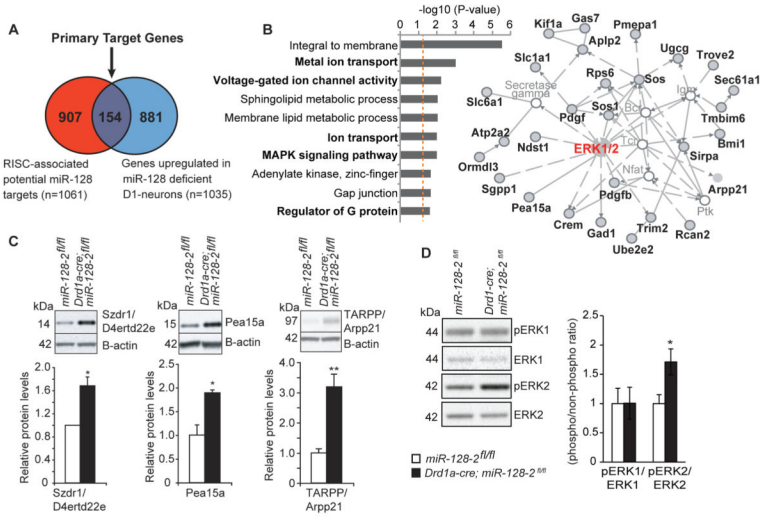

Figure 2. miR-128 controls signaling protein expression and activation of the ERK signaling network in neurons

(A) Venn diagram shows the RISC-associated mRNA targets of miR-128 (red) and mRNAs that are up-regulated in miR-128 deficient D1-neurons (blue). The overlapping 154 mRNAs are considered as direct miR-128 targets. **(B) (Left)** Gene ontology annotations of the 154 miR-128 target genes are shown with pathway enrichment presented as −log10 (p-value). The dotted orange line indicates p= 0.05. **(Right)** The components of the ERK1/2 network ($p=10^{-46}$, right-tailed Fisher's exact test) that are directly targeted by miR-128 are indicated in solid grey. **(C)** Expression levels of miR-128-targeted ERK regulators in the striatum of *Drd1a-cre; miR-128-2*$^{fl/fl}$ and littermate controls were analyzed by Western blotting (n=4 each). **(D) Increased ERK2 phosphorylation in the striatum of mice with D1-neuron-specific miR-128 deficiency.** Representative Western blot analysis of ERK1/2 phosphorylation in the striatum of control and *Drd1a-cre; miR-128-2*$^{fl/fl}$ mice is shown; bar graphs display phospho-ERK/ERK protein ratios (n=4). Error bars show s.e.m., Welch's t-test, * p≤0.05, ** p≤0.01.

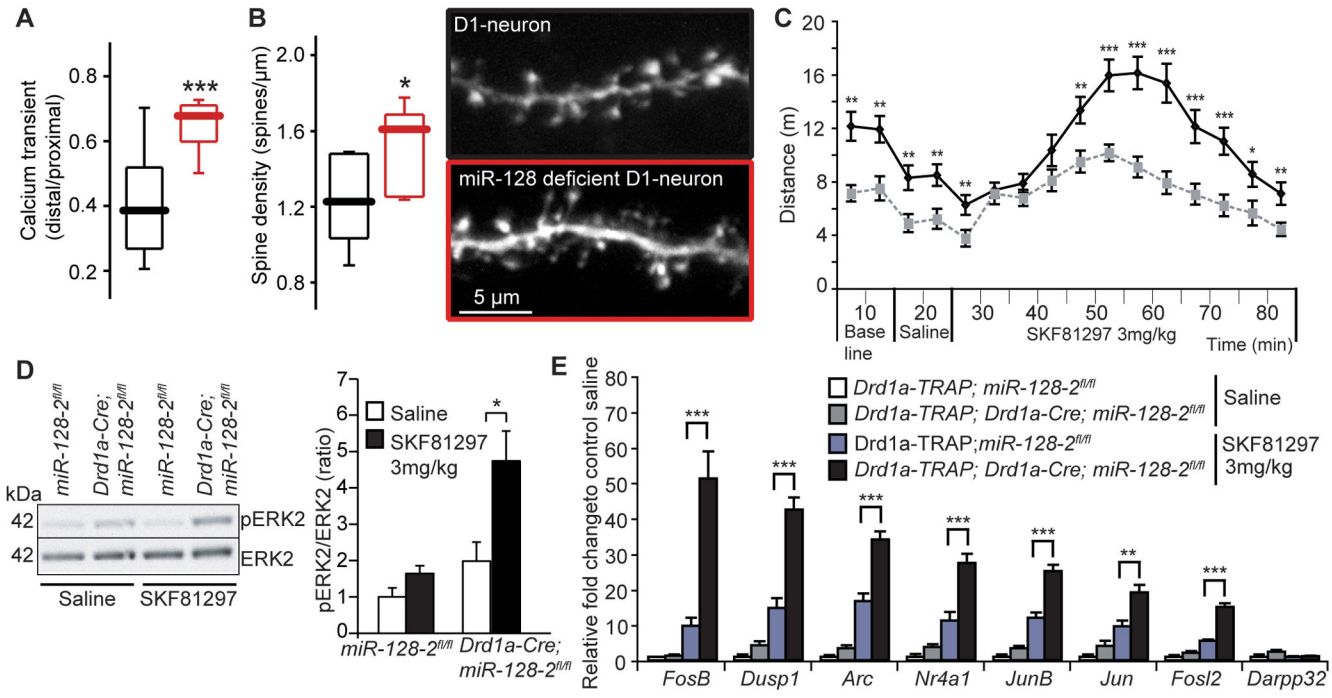

Figure 3. miR-128 controls D1-neuron excitability and responsiveness to dopamine. (A, B) miR-128 regulates D1-neuron dendritic excitability and number of spines

(A) Single action potentials were generated in the soma and action potential invasion was calculated by dividing the distal calcium signal by the maximum proximal calcium signal per cell (n=4 cells, 11–21 shafts per group). Mann-Whitney nonparametric test, *** p≤0.001 (**B)** Representative maximum intensity projection images of distal dendrites in control and mutant D1-neurons are shown. Boxplots display population spine densities (n=10–11 cells per group). Mann-Whitney nonparametric test, error bars show 90th percentile interval, * p≤0.05**. (C–E) miR-128 regulates motor response, ERK2 phosphorylation, and immediate early gene (IEG) induction upon dopamine D1 receptor (Drd1) activation in D1-neurons. (C)** Motor activity of *Drd1a-cre; miR-128-2$^{fl/fl}$* and control mice (n=25 and 30) was evaluated in an open-field chamber. Saline and 3mg/kg Drd1 agonist SKF81297 were injected i.p. at 10 and 20 minute, respectively. (**D)** ERK2 phosphorylation was quantified by Western blotting of striatal lysates derived from *Drd1a-cre; miR-128-2$^{fl/fl}$* and control mice that received saline or D1-agonist SKF81297 injection (n=5 each). Bar graph displays the ratio of phospho-ERK2 to total ERK2 expression. **(E)** IEG and D1-neuron-expressed *Darpp32* gene expression levels were measured by qRT-PCR of D1-neuron specific polyribosome-associated mRNAs purified from saline or SKF81297 treated *Drd1a-TRAP; Drd1a-cre; miR-128-2$^{fl/fl}$* and control mice (n=5 each). Error bars display s.e.m., Welch's t-test, * p≤0.05, ** p≤0.01, ***p p≤0.001.

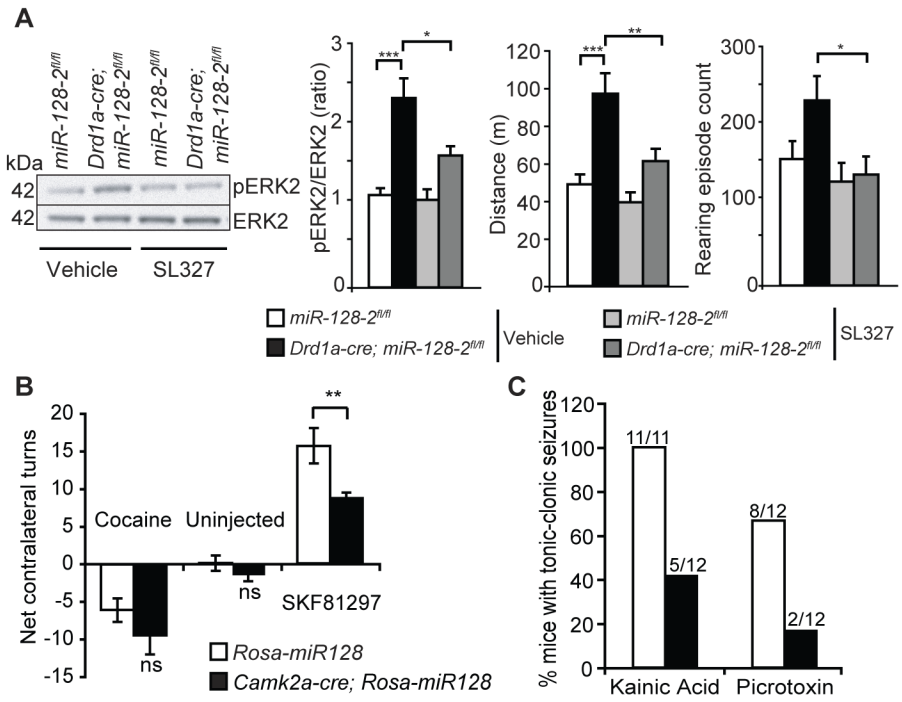

Figure 4. Abnormal motor activity caused by miR-128 deficiency is corrected by pharmacological ERK inhibition or ectopic miR-128 expression

(A) *Drd1a-cre; miR-128-2*$^{fl/fl}$ and littermate control mice were injected i.p. with either vehicle or 12 mg/kg of the MEK1 inhibitor SL327 (n=5/group). Western blot analysis of ERK2 phosphorylation at 30 min after drug injection (left) and motor activity following vehicle or SL327 injection (right) are shown. 2-way ANOVA followed by Bonferroni post-test. Error bars show s.e.m., * p≤0.05, ** p≤0.01, *** p≤0.001. (**B) Overexpression of miR-128 suppresses D1-neuron hyper-responsiveness in the dopamine-depleted striatum.** The number of contralateral rotations at baseline and in response to cocaine (10 mg/kg) or D1-agonist SKF81297 (5 mg/kg) in unilateral 6-OHDA lesioned *Camk2a-cre; Rosa-miR-128* or control mice (n=11/group) are shown. Error bars show s.e.m., Welch's t-test, ** p≤0.01. **(D) miR-128 reduces the susceptibility to chemically-induced seizures in mice.** The numbers of *Camk2a-cre; Rosa-miR-128* or littermate control mice (n=12/group) that exhibit tonic-clonic seizures 60 minutes after i.p. injection of pro-convulsive drugs kainic acid (30mg/kg, p-value=0.005) or picrotoxin (3mg/kg, p-value=0.04) are shown. p-values were calculated by Fisher's exact test.