

# MicroRNA-219 modulates NMDA receptor-mediated neurobehavioral dysfunction

Jannet Kocerha<sup>a</sup>, Mohammad Ali Faghihi<sup>a</sup>, Miguel A. Lopez-Toledano<sup>a</sup>, Jia Huang<sup>a,b</sup>, Amy J. Ramsey<sup>c</sup>, Marc G. Caron<sup>c</sup>, Nicole Sales<sup>d</sup>, David Willoughby<sup>e</sup>, Joacim Elmenf, Henrik F. Hansen<sup>f</sup>, Henrik Orum<sup>f</sup>, Sakari Kauppinen<sup>f,g</sup>, Paul J. Kenny<sup>h</sup>, and Claes Wahlestedt<sup>a,1</sup>

Departments of <sup>a</sup>Molecular and Integrative Neurosciences, <sup>d</sup>Infectology, and <sup>h</sup>Molecular Therapeutics, The Scripps Research Institute, 130 Scripps Way, Jupiter, FL 33458; <sup>b</sup>Departments of Cell Biology and Neurobiology, Duke University Medical Center, Durham, NC 27710; <sup>c</sup>Ocean Ridge Biosciences, 10475 Riverside Drive, Suite 1, Palm Beach Gardens, FL 33410; <sup>e</sup>Santaris Pharma, Boege Alle 3, Horsholm, DK-2970, Denmark; <sup>f</sup>Department of Cellular and Molecular Medicine, Wilhelm Johannsen Centre for Functional Genome Research, University of Copenhagen, Blegdamsvej 3, DK-2200 N, Denmark; and <sup>g</sup>Miami Institute for Human Genomics, University of Miami, South Campus, Building C, 12500 SW 152nd Street, Miami, FL 33177

Edited by Floyd E. Bloom, The Scripps Research Institute, La Jolla, CA, and approved December 12, 2008 (received for review June 17, 2008)

**N-methyl-D-aspartate (NMDA) glutamate receptors are regulators of fast neurotransmission and synaptic plasticity in the brain. Disruption of NMDA-mediated glutamate signaling has been linked to behavioral deficits displayed in psychiatric disorders such as schizophrenia. Recently, noncoding RNA molecules such as microRNAs (miRNAs) have emerged as critical regulators of neuronal functions. Here we show that pharmacological (dizocilpine) or genetic (NR1 hypomorphism) disruption of NMDA receptor signaling reduces levels of a brain-specific miRNA, miR-219, in the prefrontal cortex (PFC) of mice. Consistent with a role for miR-219 in NMDA receptor signaling, we identify calcium/calmodulin-dependent protein kinase II  $\gamma$  subunit (CaMKII $\gamma$ ), a component of the NMDA receptor signaling cascade, as a target of miR-219. In vivo inhibition of miR-219 by specific anti-miR in the murine brain significantly modulated behavioral responses associated with disrupted NMDA receptor transmission. Furthermore, pretreatment with the antipsychotic drugs haloperidol and clozapine prevented dizocilpine-induced effects on miR-219. Taken together, these data support an integral role for miR-219 in the expression of behavioral aberrations associated with NMDA receptor hypofunction.**

cerebral cortex | glutamatergic signaling | regulatory RNA

**N**MDA receptors (NMDA-R) control many executive brain functions, such as working memory, and their dysfunction is implicated in a host of brain disorders (1–4). Notably, hypofunctional NMDA-R signaling, particularly in the prefrontal cortex (PFC), has been implicated in the cognitive and behavioral disturbances characteristic of schizophrenia (5), autism (6, 7), attention deficit hyperactivity disorder (ADHD) (8, 9), mood disorders (10), and other psychiatric illnesses. The cellular mechanisms by which disrupted NMDA-R transmission drives behavioral pathology are still unclear, although several of the major proteins involved in this pathway, such as calcium/calmodulin-dependent protein kinase II (CaMKII) (11), have been identified. In this study, we examine whether neurobehavioral abnormalities associated with NMDA-R hypofunction can be attributed to a novel class of regulatory RNA molecules, microRNAs (miRNAs).

miRNAs have attracted much attention as regulators of neuronal development and synaptic plasticity (12–15). Furthermore, psychiatric disorders such as schizophrenia, autism, and Tourette's syndrome are associated with dysregulated levels of miRNAs (16–20). miRNAs are small ( $\approx 22$  nt) noncoding transcripts that can control expression of protein-coding mRNAs at the posttranscriptional level (21). Pleiotropic miRNAs can control gene expression by binding to complementary sequences in the 3' untranslated region (3' UTR) of target mRNA transcripts to facilitate their degradation and/or inhibit their translation (15, 22, 23). Understanding this layer of gene regulation therefore promises to enrich our knowledge of brain function and pathology.

## Results

### miRNA Regulation by Dizocilpine-Mediated NMDA-R Hypofunction.

Dizocilpine is a highly selective phencyclidine-like NMDA-R antagonist that can rapidly produce schizophrenia-like behavioral deficits in humans and rodents (24). We examined whether a psychotomimetic dose of dizocilpine (0.5 mg/kg, i.p.) altered miRNA expression in brain regions of C57BL/6 mice, by using miRNA microarray profiling as an initial screening approach. Our analysis was focused on the PFC because of the considerable evidence linking this brain region with behavioral pathology in psychiatric illnesses (19). We extracted the small RNAs from the PFC of the mice 15 min after administration of a single dose of dizocilpine, i.e., a time-point at which dizocilpine-induced behavioral disturbances such as hyperlocomotion and stereotypy are readily observed (25). Of note, there was a robust reduction of miR-219 out of 182 miRNAs detectable by microarray in PFC tissues (Table S1). miR-219 is a conserved miRNA expressed in both rodent and human brains, but not in other tissues (26, 27). These data demonstrate that concentrations of a brain-specific miRNA, which may play a role in regulating NMDA-R function, are altered during states of NMDA-R hypofunction.

In support of the microarray data, RT-PCR analyses demonstrated that miR-219 levels were significantly reduced by  $\approx 50\%$  (a change from an average cycle threshold value for controls of 28 to 29 after treatment) in the PFC of mice 15 min after injection of dizocilpine (0.5 mg/kg). miR-219 expression levels returned to basal levels by 120 min after treatment (data not shown), a time-point at which dizocilpine-induced behavioral disturbance has greatly dissipated (25, 28). Dizocilpine did not alter miR-219 expression levels in the hippocampus or cerebellum relative to control mice (Fig. 1A), suggesting that the decreased levels of miR-219 may be anatomically restricted during acute treatment. Northern blot analysis of PFC tissue demonstrated that dizocilpine depleted the mature miR-219 transcript, but not its precursor, indicating that the transcription rate of miR-219 was not altered by dizocilpine (data not shown).

The non-NMDA ionotropic glutamate receptor antagonist 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo[*f*]quinoxaline-2,3 dione

Author contributions: J.K., M.A.F., M.A.L.-T., A.J.R., M.G.C., D.W., S.K., P.J.K., and C.W. designed research; J.K., M.A.F., M.A.L.-T., J.H., A.J.R., D.W., P.J.K., and C.W. performed research; A.J.R., M.G.C., D.W., J.E., H.F.H., H.O., S.K., and C.W. contributed new reagents/analytic tools; J.K., M.A.F., M.A.L.-T., J.H., N.S., D.W., P.J.K., and C.W. analyzed data; and J.K., M.A.F., M.A.L.-T., P.J.K., and C.W. wrote the paper.

The authors declare no conflict of interest.

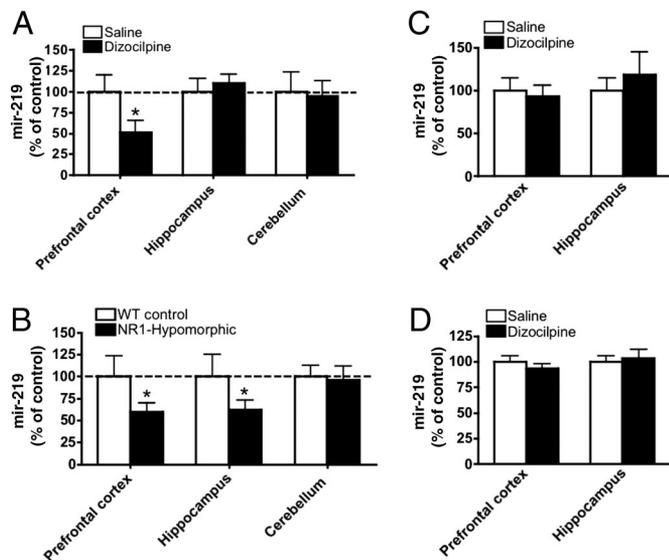
This article is a PNAS Direct Submission.

See Commentary on page 2975.

<sup>1</sup>To whom correspondence should be addressed. E-mail: clawah@scripps.edu.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0805854106/DCSupplemental](http://www.pnas.org/cgi/content/full/0805854106/DCSupplemental).

© 2009 by The National Academy of Sciences of the USA

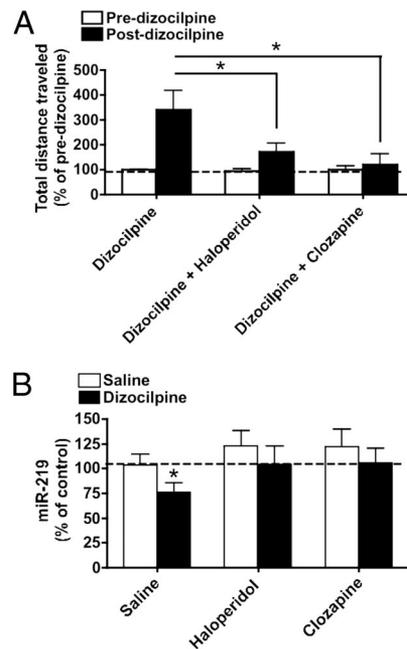


**Fig. 1.** NMDA-R hypofunction is associated with decreased miR-219 expression in prefrontal cortex. (A) miR-219 expression was quantified by RT-PCR from the PFC, hippocampus, and cerebellum of adult male C57BL/6 mice injected with saline ( $n = 9$ ) or dizocilpine (MK-801) (0.5 mg/kg; i.p. injection;  $n = 9$ ) for 15 min. MiR-219 was down-regulated significantly ( $P < 0.05$ ) 15 min after dizocilpine injection in the PFC but not in the hippocampus or cerebellum. (B) Expression of miR-219 was also significantly ( $P < 0.05$ ) down-regulated in the PFC of NR1 hypomorphic mice compared to wild-type (WT) controls ( $n = 5-8$  per group). (C) C57BL/6J mice were chronically treated with dizocilpine (0.2 mg/kg per hour) for 5 days by minipump ( $n = 9$  for wild-type controls and  $n = 10$  for dizocilpine-treated animals). After 5 days of dizocilpine treatment, the brains were removed and the prefrontal cortex and hippocampus were dissected for microRNA analysis. (D) Another group of mice were chronically treated with dizocilpine by i.p. injection (0.5 mg/kg once per day) for 14 days. After 14 days, the PFC and hippocampus were harvested and analyzed for miR-219 expression. (C and D) Chronic treatment with dizocilpine had no significant effect on miR-219 levels with either minipumps (C) or daily injections (D) in any of the brain regions examined.

(NBQX) (1 mg/kg), which does not elicit cognitive nor behavioral deficits reminiscent of psychiatric disorders, did not alter miR-219 expression levels in the PFC (Fig. S1). Thus, NMDA-R hypofunction in particular, and not decreased glutamate-mediated transmission in general, impacts miR-219 concentrations in the PFC.

**miR-219 Responds to Acute Dizocilpine Treatment.** Both acute and chronic dizocilpine treatment are well known to induce changes in locomotor activity and stereotypy. However, there are distinct physiological differences between the two treatments, such as changes in glutamate release (29). Therefore, we chronically treated mice with dizocilpine for 5 days by osmotic minipump (0.2 mg/kg per hour) or by daily i.p. injections (0.5 mg/kg), with animals being killed 15 min after the last injection on the 14th day. In the chronically treated mice (i.p.- and minipump-infused mice), miR-219 levels were not down-regulated in the PFC (Fig. 1 C and D), suggesting that the acute cellular responses to dizocilpine in miR-219 concentrations are subject to desensitization over time.

**Genetic Disruption of NMDA Signaling Regulates MicroRNA Expression.** Hyperlocomotor activity and increased stereotypic behavior induced by impaired NMDA-R signaling is also exhibited in mice with a hypomorphic mutation in *Grin1* (NMDA-R subunit NR1). This mutation in *Grin1* results in an  $\approx 90\%$  decrease in adult NMDA-R expression levels (25). RT-PCR analyses demonstrated that expression levels of miR-219 were significantly

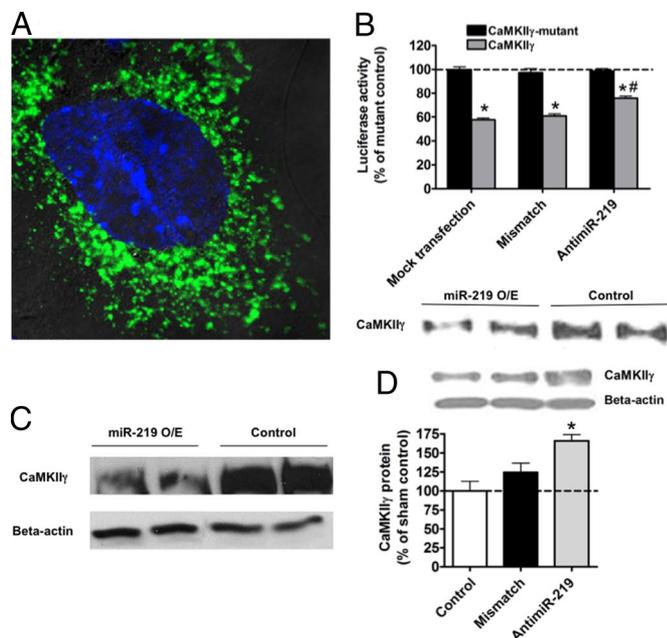


**Fig. 2.** Antipsychotic treatment reverses dizocilpine-mediated miR-219 regulation. (A) Distance traveled over a 2-h time period after pretreatment of mice for 30 min with haloperidol or clozapine (0.1 mg/kg haloperidol, 0.75 mg/kg clozapine; i.p.) (B) Pretreatment of mice for 30 min with haloperidol or clozapine (0.1 mg/kg haloperidol, 0.75 mg/kg clozapine; i.p.;  $n = 5-8$  per group) reversed the effects of dizocilpine administration on miR-219 expression ( $P < 0.05$ ), compared with saline treatment.

lower in the PFC from NR1 mutant mice compared with wild-type controls (Fig. 1B). Additionally, the NR1 hypomorphic mice exhibited a decrease in hippocampal levels of miR-219, another brain region implicated in the presentation of schizophrenia-related symptoms (30).

**Antipsychotic Treatment Modulates miR-219 Expression.** Acute treatment with antipsychotics alleviates the neurobehavioral deficits expressed in humans and rodents with pharmacological (dizocilpine) or genetic (NR1 hypomorphs) NMDA-R dysfunction (31). Therefore, we tested the possibility that pretreatment (30 min) with haloperidol (0.1 mg/kg, i.p.) or clozapine (0.75 mg/kg, i.p.), clinically used typical and atypical antipsychotic drugs, respectively, might impact acute dizocilpine-mediated (0.5 mg/kg, i.p.) regulation of miR-219 levels. Haloperidol and clozapine attenuated dizocilpine-mediated hyperlocomotion (measured as distance traveled) in C57BL/6 mice (Fig. 2A). Consistently, pretreatment with either of the antipsychotic drugs prevented the reduction of miR-219 concentration in the PFC after dizocilpine administration (Fig. 2B).

**CaMKII $\gamma$  Is an mRNA Target of miR-219.** The above data demonstrate that miR-219 expression levels are highly sensitive to disruptions in NMDA-R signaling. Next, we tested the hypothesis that miR-219 may directly modulate NMDA-R signaling by regulating the expression of components in this cascade. To identify protein-coding mRNA transcripts involved in NMDA signaling in the brain that have hybridization sites for miR-219, several bioinformatic programs for prediction analysis were used. We narrowed the number of bioinformatic candidates down to 6 based on their identification by 4 independent programs, including miRanda, TargetScan, PicTar, and RNA22 and a stringent minimum energy cutoff of  $-20$  kcal/mol. CaMKII $\gamma$  was one of the mRNA targets predicted by this list to have strong hybridization sites for miR-219 (Table S2). The



**Fig. 3.** Identification of CaMKII $\gamma$  as an mRNA target of miR-219. (A) miR-219 was subcellularly localized in mouse P19 cells by using an LNA-modified ISH probe. In the P19 cells, miR-219 was concentrated around the nucleus of the neurons, as depicted by an overlay of the green Alexa Fluor 488 signal derived from the LNA-miR-219 probe with the blue nuclear DAPI stain. (B) CaMKII $\gamma$  was identified as an *in vitro* target of miR-219. Wild-type and mutant luciferase reporter constructs containing the miR-219 target site were generated and subsequently transfected ( $n = 6$  for all treatments) into P19 cells. There was significant down-regulation of luciferase signal from the wild-type construct compared to the mutant control, suggesting that endogenous miR-219 indeed represses translation of CaMKII $\gamma$ . Additionally, luciferase signal from the wild-type construct could be partially derepressed in cells, where miR-219 activity was inhibited with an LNA-antimiR molecule complementary to the mature microRNA sequence compared to mismatch (MM) LNA control. One of the primary functions of miRNAs is translational arrest. Hence, protein levels of CaMKII $\gamma$  were subsequently analyzed from P19 cells displaying overexpression (O/E) with miR219 RNA. Western blot analyses revealed a repression of CaMKII $\gamma$  protein levels in miR-219 O/E cells, further validating CaMKII $\gamma$  as a target of this microRNA. (C) To examine the cortical regulation of CaMKII $\gamma$  by miR-219, cortical cells derived from C57BL/6 mice were overexpressed with miR-219 and protein was isolated 48 h after transfection ( $n = 4$  each for miR-219 overexpression and scrambled control). Western blot analysis revealed that overexpression of miR-219 significantly reduced the protein levels of the kinase. (D) CaMKII $\gamma$  protein expression was assessed *in vivo* in LNA-antimiR treated mice. Animals were killed after 7 days of osmotic minipump administration of saline (control), mismatch LNA, or LNA-antimiR-219, and the PFC was subsequently harvested from the brains for protein analysis. Western blot analysis revealed up-regulation of CaMKII $\gamma$  protein in the PFC of LNA-antimiR-219 treated animals compared to mismatch or controls. \*,  $P < 0.05$  for wild-type versus mutant groups; #,  $P < 0.05$  for wild-type construct groups.

CaMKII family of kinases are considered core regulators of NMDA signaling that can modulate NMDA-R trafficking and activity state, and can be locally translated in dendrites to promote rapid plasticity (32, 33). Therefore, we focused on this target for further *in vitro* and *in vivo* analysis.

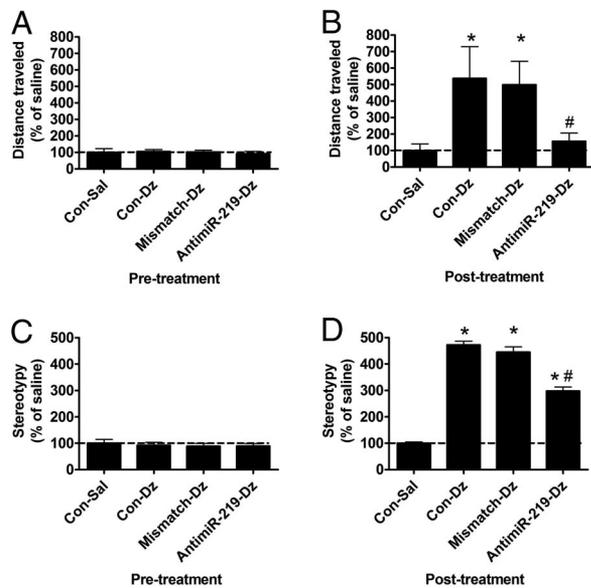
To establish an *in vitro* cell system for further examination of CaMKII $\gamma$  regulation by miR-219, we differentiated mouse P19 cells into neurons. Subcellular analysis of miR-219 expression in the P19 cells with a locked nucleic acid (LNA)-modified probe (LNA-FISH) revealed a cytoplasmic signal for the mature miR-219 transcript (Fig. 3A). In addition, signal for miR-219 was also detected along neurite outgrowths (Fig. 3A), a pattern of expression similar to other miRNAs shown to play a role in

synaptic plasticity (34). The dendritic expression pattern of miR-219 noted here is consistent with a recent study demonstrating high concentrations of miR-219 in cell soma and dendritic regions of cultured primary rat hippocampal neurons (34).

By using the P19 cells as a model system, we investigated whether CaMKII $\gamma$  is a target for miR-219 regulation. A portion of the CaMKII $\gamma$  3'UTR encompassing the putative miR-219 target site, or a mutated sequence as a control, was engineered downstream of a luciferase gene in a vector. Luciferase activity from cells transfected with the wild-type CaMKII 3'UTR construct was repressed by  $>40\%$  ( $P < 0.05$ ) compared with cells transfected with the mutant control. To assess whether the CaMKII $\gamma$  luciferase levels could be restored with inhibition of miR-219 expression, we transfected cells with an LNA-modified antisense inhibitor of miR-219 (LNA-antimiR-219). LNA is a bicyclic RNA analog in which the ribofuranose ring in the sugar-phosphate backbone is structurally constrained by a methylene bridge between the 2'-oxygen and the 4'-carbon atoms resulting in a locked 3'-endo conformation characteristic of RNA. This modification results in reduced conformational flexibility and increased local organization of the phosphate backbone. When used in antisense constructs, LNA substantially increases potency both *in vitro* and *in vivo* (35). The repression of luciferase activity was significantly released when the P19 cells were cotransfected with LNA-antimiR-219, suggesting that endogenous miR-219 hybridizes to the CaMKII 3'UTR and thereby inhibits translation of the encoded gene (Fig. 3B). Expression levels of miR-9, another brain-enriched miRNA, were unaltered in LNA-antimiR-219-treated P19 cells (Fig. S2), suggesting that the LNA antimiR acted by selectively targeting miR-219 and not by inducing a generalized decrease in miRNA expression. Furthermore, protein levels of CaMKII $\gamma$  were reduced in P19 cells upon overexpression of miR-219 (Fig. 3B), whereas CaMKII $\gamma$  mRNA levels were unchanged (Fig. S3).

**miR-219 Represses CaMKII $\gamma$  *In Vitro* in Cortical Cells.** To address whether the results we obtained with the P19 cells could be extrapolated to primary neuronal cells, we evaluated the regulation of CaMKII $\gamma$  by miR-219 *in vitro* in cortical cells derived from C57BL/6 mice. Cortical cells in which miR-219 was overexpressed showed a robust reduction in CaMKII $\gamma$  protein levels (Fig. 3C). Additionally, treatment of cortical cells for 18 h with dizocilpine increased the protein levels of CaMKII $\gamma$ , consistent with up-regulated levels of CaMKII $\gamma$  in the PFC of dizocilpine-treated mice (Fig. S4). Collectively, these data support the bioinformatic predictions that CaMKII $\gamma$  mRNA is indeed a target of miR-219 and are consistent with a primary translational repression mechanism for miR-219 regulation of CaMKII $\gamma$  expression.

***In Vivo* Inhibition of miR-219 Alters CaMKII $\gamma$  Expression.** Next, we examined the role of miR-219 in regulating CaMKII $\gamma$  expression levels *in vivo* in the brains of mice. To date, very few putative targets for microRNA regulation have been verified *in vivo*. Mice were implanted s.c. with osmotic minipumps that delivered continuous infusions (0.5  $\mu\text{L}/\text{h}$ ) of LNA-modified oligonucleotides or saline. Tubing was tunneled from the osmotic minipump to a chronic indwelling intracerebroventricular cannula, such that the oligonucleotides were delivered continuously into the third ventricle of the forebrain (36, 37). LNA-modified oligonucleotides were previously shown to be nontoxic in the rodent brain (35), and as antimiRs (i.e., designed to hybridize with miRNAs) they have been shown to potentially antagonize the biological activity of miRNAs in rodents (38) and nonhuman primates (39). Mice were treated in the above manner with LNA-antimiR-219 (antimiR-219), a stringently mismatched LNA control having only 2 mismatches in the miR-219 recognition sequence and which does not target any known transcript



**Fig. 4.** Antagonism of miR-219 in vivo by LNA-antimiR alters dizocilpine-induced behavior. The effects of in vivo antagonism of miR-219 in the brain on dizocilpine induced behavior were examined in mice. Adult male C57BL/6 mice were administered saline ( $n = 5$ ), a 2-nt mismatch LNA control ( $n = 5$ ; 0.24 mg per day), or LNA-antimiR complementary to miR-219 ( $n = 5$ ; 0.24 mg per day) for 5 days by osmotic minipump. After 5 days of saline (surgical controls) or oligonucleotide administration, mice were habituated in an Open-Field box before acute administration of dizocilpine. Data were recorded for total distance traveled and stereotypy during the 2-h pretreatment habituation and for 90 min after dizocilpine (Dz) or saline (Sal) administration (the 30- to 90-min interval is shown here for the posttreatment data). Hyperlocomotion (measured as distance traveled) (A and B), as well as stereotypy (C and D), were all significantly attenuated after Dz challenge in the LNA-antimiR-219 treated mice when compared to the mismatch or Dz-treated controls. \*,  $P < 0.05$  compared with Sal treated controls; #,  $P < 0.05$  for Dz controls compared with Dz-treated groups.

(mismatch), or saline (control). The dose of LNA-antimiR-219 was chosen based on a previous study that successfully used a similar antimiR approach to reduce brain expression levels of miR-132 in vivo (26). After 7 days of continuous LNA-antimiR infusion, expression levels of CaMKII $\gamma$  protein were assessed in the PFC. Western blot analysis demonstrated that CaMKII $\gamma$  protein concentrations in the PFC were significantly increased in vivo in the LNA-antimiR-219-treated animals compared with mismatch- or saline-treated groups (Fig. 3D). This observation supports a tonic inhibitory role for endogenous miR-219 on local CaMKII $\gamma$  protein levels. Taken together, these data demonstrate that CaMKII $\gamma$  expression levels in the PFC are regulated by miR-219. Further, these data support the hypothesis that miR-219 represents an integral component of NMDA-R signaling.

**In Vivo Inhibition of miR-219 Modulates NMDA-R-Mediated Neurobehavioral Dysfunction.** Next, we tested the functional relevance of altered miR-219 expression levels in vivo during states of NMDA-R hypofunction. Specifically, we examined whether inhibition of miR-219, by using LNA-antimiR-219 delivered into the brain according to the same chronic infusion procedure described above, modified the degree of behavioral disruption induced by dizocilpine. In mice, hyperlocomotion and stereotypy are readily assayed behavioral features of dizocilpine-induced NMDA-R hypofunction. In vivo inhibition of miR-219 did not alter locomotor activity (distance traveled) or the expression of stereotyped behaviors under baseline conditions (Fig. 4 A and C). However, mice treated with LNA-antimiR-219 displayed markedly altered hyperlocomotion and stereotypy 30 min after

dizocilpine administration (an average of 850 cm distance traveled and 6,240 stereotypy counts for 1 h) when compared with mice receiving the LNA mismatch or saline (an average of 3,200 cm distance traveled and 9,800 stereotypy counts for 1 h) via osmotic minipump. Furthermore, these effects persisted for 1 h (Fig. 4 B and D).

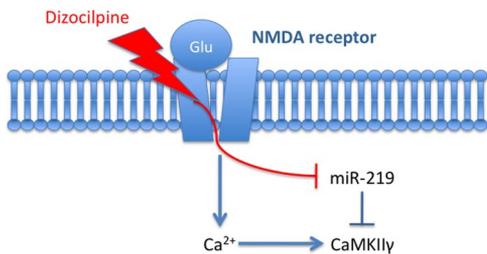
## Discussion

MicroRNAs have recently been implicated in neuronal functions both in vitro and in vivo. Our data support the existence of a previously uncharacterized microRNA modulator of NMDA-R signaling cascades and associated neurobehavioral functions. In the present study, we observed a robust change of miR-219 in the PFC of mice with hypofunctional NMDA-R activity. The down-regulation of miR-219 appears to be linked with acute pharmacological suppression or developmentally down-regulated NMDA-R function. Indeed, both the LNA-antimiR-219-treated and NR1 hypomorphic mice (25) exhibit similar locomotor responses to acute administration of dizocilpine.

There are various animal paradigms which model the hypothesis that a state of NMDA-R hypofunction parallels the diverse range of symptoms in schizophrenic patients. Acute treatment of rodents with an NMDA-R antagonist (dizocilpine) has been reported to resemble the metabolic changes in patients experiencing an acute state of psychosis (40). We show that the acute impact of dizocilpine on miR-219 coincides with the expression of locomotor deficits, indicating a rapidly on-setting regulatory role of the microRNA in neurobehavioral dysfunction. Additionally, it is well known that there are compensatory physiological changes in animals chronically treated with dizocilpine, which shifts the balance of the neurotransmitter system and the corresponding neurological impairment, including significant changes in cognitive tests and the induction of locomotor activity with exposure to stress (40). The adaptive cellular changes in chronically treated mice are consistent with our finding that miR-219 is unaltered following chronic dizocilpine administration. It could be expected that the NR1 hypomorphic mice would have a similar cellular response as chronically treated wild-type mice, however, they show maintained decreased expression of miR-219. It has been shown that administration of NMDA antagonists such as dizocilpine in infant mice can alter the development of the cerebral cortex (41), a brain region strongly implicated in the pathogenesis of schizophrenia (42). This point could help explain the slightly different results obtained with hypomorphic mice, whose NMDA-mediated glutamate signaling is impaired throughout development. In fact, when we compared other regions of the brain, we also observed lower levels of miR-219 in the hippocampus, a region which showed no change of the microRNA in genetically unmodified mice.

The behavioral deficits prompted by pharmacological or genetic knockdown of NMDA-R function can be alleviated with currently prescribed therapeutics (25). Acute treatment with antipsychotics, such as clozapine, blocks the hyperlocomotor symptoms induced by NMDA-R hypofunction in dizocilpine-treated animals or NR1 hypomorphs (25, 43). In our studies, a possible mechanism by which these clinically effective antipsychotic agents attenuated the acute inhibitory effects of dizocilpine on miR-219 concentrations is by decreasing the neurochemical and behavioral deficits associated with NMDA receptor hypofunction. Consistent with this hypothesis, it has been shown that haloperidol and clozapine can facilitate NMDA receptor function (44) and counter the inhibitory effects of dizocilpine on NMDA receptor-mediated transmission (45).

Based on the results reported in this study, we propose that miR-219 negatively regulates the function of NMDA receptors. Indeed, a recent study reported that overexpression of miR-219 in cortical cells inhibits Ca<sup>2+</sup> influx through NMDA-R signaling transduction upon receptor activation in vitro. We show that



**Fig. 5.** Schematic of miR-219 regulation in NMDA signaling. Outlined in the schematic, we propose that miR-219 negatively regulates the function of NMDA receptors. The silencing of miR-219 releases the translational repression of the microRNA on the 3'UTR of the CaMKII $\gamma$  mRNA, thus providing a compensatory mechanism to maintain NMDA-R function during acute antagonism of the receptor.

CaMKII $\gamma$ , an integral downstream responder to NMDA-mediated Ca<sup>2+</sup> signaling, is a target of miR-219 *in vitro* and *in vivo*. The silencing of miR-219 in the murine brain releases the translational repression of the microRNA on the 3'UTR of the CaMKII $\gamma$  mRNA, thus providing a compensatory mechanism to maintain NMDA-R function during acute antagonism of the receptor and to attenuate associated behavioral manifestations (Fig. 5)

Recent clinical trials in schizophrenia (31) have indicated that glutamate receptors may be viable targets for novel therapeutics. We propose that miR-219 may play a role in the locomotor deficits exhibited in acute schizophrenia through the regulation of glutamatergic NMDA-R signaling cascades. It will be important in future work to test the effects of antimiR-219 on other behavioral deficits induced by NMDA receptor ligands like dizocilpine, especially those not dependent on locomotor activity. For instance, it will be interesting to test whether inhibition of miR-219 signaling can alter schizophrenia-like deficits in sensorimotor gating or the deficits in brain reward function induced by dizocilpine.

In summary, we report that miR-219 appears to be an integral component of the NMDA-R signaling cascade. This microRNA responds rapidly to alterations in NMDA-R signaling, exerts translational control of CaMKII $\gamma$  expression, and contributes to altered behavioral manifestations.

## Materials and Methods

**RNA Isolation.** Total RNA was isolated from all samples by using phenol- and chloroform-based extractions. Up to 30 mg of tissue was homogenized in 1,000  $\mu$ L of RNeasy Lysis Buffer (Qiagen), followed by addition of 250  $\mu$ L of chloroform and vortexing for 1 min. The RNA was precipitated with 2 $\times$  volumes of isopropanol overnight at  $-20^{\circ}\text{C}$  before being pelleted with a 30-min centrifugation at 12,000  $\times$  g. The RNA pellets were resuspended in RNeasy Lysis Buffer (Qiagen). Up to 10  $\mu$ g of RNA for each sample was treated with Turbo DNase (Ambion) for 1 h at  $37^{\circ}\text{C}$  to remove any residual genomic DNA.

**microRNA Array Profiling.** Samples of small RNA (0–200 nt) were obtained by size-fractionation on YM-100 ultrafiltration columns (Millipore) and were 3'-end-labeled with Oyster-550 fluorescent dye by using the Flash-Tag kit (Genisphere). The labeled RNA was hybridized overnight to epoxide glass slides double-spotted with the NCode version 2.0 oligonucleotide probe set (Invitrogen). Microarrays were scanned on an Axon GenePix 4000B scanner (Molecular Devices), and data were extracted from images by using GenePix V4.1 software (Axon Laboratories).

**miRNA-Specific RT-PCR.** All miRNA RT-PCR experiments were performed using the microRNA assays from Applied Biosystems. For all reactions, 10 ng of total RNA was reverse-transcribed with miRNA-specific primers (Applied Biosystems) and a mouse primer to U6 for an endogenous control (Applied Biosystems). The cDNA was amplified by RT-PCR using universal Taqman mix and microRNA-specific (Applied Biosystems) according to the manufacturer's protocol. All reactions were analyzed by using the  $\Delta\Delta\text{Ct}$  calculation procedure (Applied Biosystems).

**Luciferase Reporter Constructs.** Luciferase constructs were generated by using the pGL3 control vector and ligation using T4 DNA ligase of annealed oligos containing  $\approx$ 50 base-pairs of sequence surrounding the putative miRNA target site. All ligated products were transformed into *Escherichia coli* DH5 $\alpha$  cells and verified by sequencing.

**Cell Culture and Transfections.** Mouse P19 embryonic carcinoma cells (American Type Culture Collection) were differentiated into neuronal-like cells according to a protocol adapted from Jones-Villeneuve (46). Differentiated cells were transfected overnight with 0.15% Lipofectamine2000 (Invitrogen) and 100 ng of luciferase vector or 20 nM of LNA oligonucleotide, accordingly. Additionally, all luciferase experiments were cotransfected with 5 ng of a control *Renilla* pRL-TK (Promega) vector for normalization. Luminescence for luciferase-based assays was read on the Analyst Multimode Reader (Molecular Devices).

Cortical cells derived from C57 mice were purchased from Lonza-Walkersville Corporation. A total of 400,000 cells were plated into each well of a poly-D-lysine coated plate and transfected 5 days later with 0.1% Lipofectamine and 30 nM of synthetic miR-219 RNA. For dizocilpine exposures (50  $\mu$ M), cells were allowed to mature for 14 days before treatment.

**Western Blot.** Protein was isolated by sonication of cells or tissue in a standard solubilization buffer of 20 mM Hepes, 100 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton-X, and a protease-inhibitor mixture. Protein levels were quantified by standard Bradford assay before gel electrophoresis and subsequent transfer of proteins to a nylon membrane. The membrane was blocked with 5% nonfat dry milk before incubation with the primary antibody (Millipore). Antibody-bound proteins were visualized by chemiluminescence and autoradiography.

**In Situ Hybridization (ISH).** miRNAs were detected in fixed P19 cells by using Digoxigenin (Dig)-labeled LNA probes from Exiqon. The cells were prehybridized with a buffer containing 50% formamide, 5 $\times$  SSC, 0.1% Tween, 9.2 mM citric acid, 50  $\mu$ g/mL heparin, and 500  $\mu$ g/mL yeast RNA at  $22$ – $25^{\circ}\text{C}$  below the  $T_m$  of the LNA probe. The prehybridized samples were then incubated overnight in the hybridization containing 20 nM of the probe. Hybridization signal was detected by using an Alexa Fluor 488–streptavidin tyramide amplification kit (Invitrogen) before confocal microscopy.

**Animals.** Adult male C57BL/6J mice (Jackson Labs) were used for all studies. Mice weighing 20–25 g at the beginning of the experiments were housed in groups of 4 per cage. Mice were housed in a humidity- and temperature-controlled ( $22^{\circ}\text{C}$ ) vivarium on a regular light cycle (lights on at 8:00 a.m.), with food and water available ad libitum. NR1 hypomorphic mice were described previously (31) except that the mice used in this study were backcrossed on a C57BL/6J background for at least 10 generations. NR1 mice and their wild-type littermates were killed, and the brains were rapidly removed and frozen on dry ice until used. All procedures were conducted in strict accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Chronic Dizocilpine Treatments.** Microosmotic pumps (model 1002; Alzet) were prepared in an aseptic tissue culture hood with a 100- $\mu$ L volume of 2-mg dizocilpine (Sigma). This dose was calculated to deliver 0.2 mg/kg dizocilpine every hour. Twelve-week-old wild-type mice weighing 25 g were anesthetized i.p. with 10 mg/kg xylazine/100 mg/kg ketamine and implanted s.c. with microosmotic pumps. Five days after implantation of the minipumps, mice were killed by cervical dislocation, and the brains were rapidly removed and frozen in isopentane over dry ice. For chronic treatment by i.p. injection, 12-week-old wild-type mice were administered 0.5 mg/kg dizocilpine once daily for 14 days. Fifteen minutes after the last dose on the 14th day, animals were killed and the PFC and hippocampus were rapidly dissected from the brain for microRNA analysis.

**Oligonucleotides.** The LNA-antimiR molecules were synthesized as unconjugated oligonucleotides with a phosphodiester backbone. The perfectly matching LNA-antimiR oligonucleotide: 5'-Tg<sup>m</sup>CgtTTggACAaT<sup>m</sup>C-3' (capital letters, LNA; <sup>m</sup>C, LNA methylcytosine; lowercase letters, DNA) was complementary to nucleotides 2–16 in the mature miR-219 sequence. The mismatch LNA control oligonucleotide was synthesized with the following sequence: 5'-Tg<sup>m</sup>CgtTAGgAcTaT<sup>m</sup>C-3'.

**Intracranial Surgery.** Mice weighing 20–25 g were anesthetized by inhalation of vapor isoflurane/oxygen mixture (1–3%) and positioned in a stereotaxic frame (Kopf Instruments) before cannulation. The incisor bar was adjusted to

the “flat-skull” position, and a 22-gauge stainless steel cannula (6 mm in length; Plastics One) was implanted into the third ventricle (anteroposterior, –1.94 mm from bregma; medialateral,  $\pm 0$  mm from midline; dorsoventral, –1.4 mm ventral to skull surface). Four indentations were made in the skull to accommodate screws that, together with the application of dental acrylic, held the cannulae in place.

**Osmotic Minipump Implantation.** After successful implantation of intraventricular cannulae, mice were prepared with s.c. osmotic minipumps [model 2002 (14 day)] delivering a constant flow of 0.5  $\mu$ L of solution per h (Alzet Osmotic Pumps). Sterile tygon tubing was connected to the output of each minipump at one end, and the other end tunneled under the animals' skin to exit at the base of the skull. This tubing was then connected to the indwelling intraventricular cannula, which was implanted as described above. The surgical wound resulting from minipump implantation was closed with 9-mm stainless steel wound clips (Becton Dickinson Primary Care Diagnostics). After the relevant time-point of exposure to LNA-antimiR, LNA-antimiR mismatched control

oligonucleotide, or saline delivered directly into the third ventricle, mice were killed and their brains were harvested for biochemical analyses.

**Locomotor Activity and Stereotyped Behaviors.** Horizontal (ambulation) and vertical locomotor behaviors and stereotyped behaviors of mice were automatically recorded in transparent 25-  $\times$  42-  $\times$  19-cm plastic cages equipped with computer-controlled photocells automatically monitoring the movements (Photobeam Activity System; San Diego Instruments). The cages were arranged on the shelves and illuminated by artificial lights with an average intensity of 290 lux. Locomotion and stereotypy was recorded in 30-s periods.

**Statistics.** Results are given as mean  $\pm$  SEM. Where appropriate, statistical analysis was performed with ANOVA tests for comparisons between groups. The null hypothesis was rejected at the  $P < 0.05$  level.

**ACKNOWLEDGMENTS.** This work was supported by National Institute of Mental Health Grant 1R01MH083733-01 and in part by the Scripps Florida Funding Corporation.

- Lau CG, Zukin RS (2007) NMDA receptor trafficking in synaptic plasticity and neuropsychiatric disorders. *Nat Rev Neurosci* 8:413–426.
- Coyle JT (1996) The glutamatergic dysfunction hypothesis for schizophrenia. *Harvard Rev Psychiatry* 3:241–253.
- Purcell AE, Jeon OH, Zimmerman AW, Blue ME, Pevsner J (2001) Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* 57:1618–1628.
- Dorval KM, et al. (2007) Association of the glutamate receptor subunit gene GRIN2B with attention-deficit/hyperactivity disorder. *Genes Brain Behav* 6:444–452.
- Javitt DC (2007) Glutamate and Schizophrenia: Phencyclidine, N-Methyl-D-Aspartate Receptors, and Dopamine-Glutamate Interactions. *Int Rev Neurobiol* 78:69–108.
- Carlsson ML (1998) Hypothesis: Is infantile autism a hypoglutamatergic disorder? Relevance of glutamate-serotonin interactions for pharmacotherapy. *J Neural Transm* 105:525–535.
- Moy SS, et al. (2008) Development of a mouse test for repetitive, restricted behaviors: Relevance to autism. *Behav Brain Res* 188:178–194.
- Lehohla M, Kellaway L, Russell VA (2004) NMDA receptor function in the prefrontal cortex of a rat model for attention-deficit hyperactivity disorder. *Metab Brain Dis* 19:35–42.
- Adams J, et al. (2004) Glutamate receptor, ionotropic, N-methyl D-aspartate 2A (GRIN2A) gene as a positional candidate for attention-deficit/hyperactivity disorder in the 16p13 region. *Mol Psychiatry* 9:494–499.
- Beneyto M, Meador-Woodruff JH (2007) Lamina-specific abnormalities of NMDA receptor-associated postsynaptic protein transcripts in the prefrontal cortex in schizophrenia and bipolar disorder. *Neuropsychopharmacology* 9:2175–2186.
- Cammarota M, et al. (2002) Participation of CaMKII in neuronal plasticity and memory formation. *Cell Mol Neurobiol* 22:259–267.
- Schratt GM, et al. (2006) A brain-specific microRNA regulates dendritic spine development. *Nature* 439:283–289.
- Miska EA, et al. (2004) Microarray analysis of microRNA expression in the developing mammalian brain. *Genome Biol* 5:R68.
- Sempere LF, et al. (2004) Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biol* 5:R13.
- Klein ME, Impy S, Goodman RH (2005) Role reversal: The regulation of neuronal gene expression by microRNAs. *Curr Opin Neurobiol* 15:507–513.
- Abelson JF, et al. (2005) Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science* 310:317–320.
- Hansen T, et al. (2007) Brain expressed microRNAs implicated in schizophrenia etiology. *PLoS ONE* 2:e873.
- Burmistrova OA, et al. (2007) MicroRNA in schizophrenia: Genetic and expression analysis of miR-130b (22q11). *Biochemistry* 72:578–582.
- Perkins DO, et al. (2007) microRNA expression in the prefrontal cortex of individuals with schizophrenia and schizoaffective disorder. *Genome Biol* 8:R27.
- Nomura T, et al. (2008) MeCP2-dependent repression of an imprinted miR-184 released by depolarization. *Hum Mol Genet* 17(8):1192–1199.
- Kosik KS (2006) The neuronal microRNA system. *Nat Rev Neurosci* 7:911–920.
- Kosik KS, Krichevsky AM (2005) The elegance of the microRNAs: A neuronal perspective. *Neuron* 47:779–782.
- Miska EA (2005) How microRNAs control cell division, differentiation and death. *Curr Opin Genet Dev* 15:563–568.
- Heresco-Levy U, Javitt DC (1998) The role of N-methyl-D-aspartate (NMDA) receptor-mediated neurotransmission in the pathophysiology and therapeutics of psychiatric syndromes. *Eur Neuropsychopharmacol* 8:141–152.
- Mohn AR, Gainetdinov RR, Caron MG, Koller BH (1999) Mice with reduced NMDA receptor expression display behaviors related to schizophrenia. *Cell* 98:427–436.
- Cheng HY, et al. (2007) microRNA modulation of circadian-clock period and entrainment. *Neuron* 54:813–829.
- Lukiw WJ (2007) Micro-RNA speciation in fetal, adult and Alzheimer's disease hippocampus. *NeuroReport* 18:297–300.
- Druhan JP, Rajabi H, Stewart J (1996) MK-801 increases locomotor activity without elevating extracellular dopamine levels in the nucleus accumbens. *Synapse* 24:135–146.
- Zuo DY, et al. (2006) Effect of acute and chronic MK-801 administration on extracellular glutamate and ascorbic acid release in the prefrontal cortex of freely moving mice on line with open-field behavior. *Life Sci* 78:2172–2178.
- Gasabella B, Cunningham MG, Bolshakov VY, Benes FM (2008) Amygdala-dependent regulation of electrical properties of hippocampal interneurons in a model of schizophrenia. *Biol Psychiatry*, in press.
- Patil ST, et al. (2007) Activation of mGlu2/3 receptors as a new approach to treat schizophrenia: A randomized Phase 2 clinical trial. *Nat Med* 13:1102–1107.
- Suen PC, et al. (1998) NMDA receptor subunits in the postsynaptic density of rat brain: Expression and phosphorylation by endogenous protein kinases. *Brain Res Mol Brain Res* 59:215–228.
- Miller S, et al. (2002) Disruption of dendritic translation of CaMKIIalpha impairs stabilization of synaptic plasticity and memory consolidation. *Neuron* 36:507–519.
- Kye MJ, et al. (2007) Somatodendritic microRNAs identified by laser capture and multiplex RT-PCR. *RNA* 13:1224–1234.
- Wahlestedt C, et al. (2000) Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *Proc Natl Acad Sci USA* 97:5633–5638.
- Thakker DR, et al. (2004) Neurochemical and behavioral consequences of widespread gene knockdown in the adult mouse brain by using nonviral RNA interference. *Proc Natl Acad Sci USA* 101:17270–17275.
- Thakker DR, et al. (2005) siRNA-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol Psychiatry* 10:782–789.
- Elmen J, et al. (2007) Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. *Nucleic Acids Res* 36:1153–1162.
- Elmen J, et al. (2008) LNA-mediated microRNA silencing in non-human primates. *Nature* 452:896–899.
- Bubenikova-Valesova V, Horacek J, Vrajova M, Hoschl C (2008) Models of schizophrenia in humans and animals based on inhibition of NMDA receptors. *Neurosci Biobehav Rev* 32:1014–1023.
- Kaindl AM, et al. (2008) Brief alteration of NMDA or GABAA receptor mediated neurotransmission has long-term effects on the developing cerebral cortex. *Mol Cell Proteomics* 7:2293–2310.
- Di Pietro NC, Seamans JK (2007) Dopamine and serotonin interactions in the prefrontal cortex: Insights on antipsychotic drugs and their mechanism of action. *Pharmacopsychiatry* 40(Suppl 1):S27–S33.
- Corbett R, et al. (1995) Antipsychotic agents antagonize non-competitive N-methyl-D-aspartate antagonist-induced behaviors. *Psychopharmacology* 120:67–74.
- Ninan I, Jardemark KE, Liang X, Wang RY (2003) Calcium/calmodulin-dependent kinase II is involved in the facilitating effect of clozapine on NMDA- and electrically evoked responses in the medial prefrontal cortical pyramidal cells. *Synapse* 47:285–294.
- White IM, et al. (1995) Phencyclidine-induced increases in striatal neuron firing in behaving rats: reversal by haloperidol and clozapine. *J Neural Transm Gen Sect* 102:99–112.
- Jones-Villeneuve EM, McBurney MW, Rogers KA, Kalnins VI (1982) Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J Cell Biol* 94:253–262.