miR-124 dosage regulates prefrontal cortex function by dopaminergic modulation

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MicroRNA-124 (miR-124) is evolutionarily highly conserved among species and one of the most abundantly expressed miRNAs in the developing and mature central nervous system (CNS). Previous studies reported that miR-124 plays a role in CNS development, such as neuronal differentiation, maturation, and survival. However, the role of miR-124 in normal brain function has not yet been revealed. Here, we subjected miR-124-1/+ mice, to a comprehensive behavioral battery. We found that miR-124-1/+ mice showed impaired prepulse inhibition (PPI), methamphetamine-induced hyperactivity, and social deficits. Whole cell recordings using prefrontal cortex (PFC) slices showed enhanced synaptic transmission in layer 5 pyramidal cells in the miR-124-1/+ PFC. Based on the results of behavioral and electrophysiological analysis, we focused on genes involved in the dopaminergic system and identified a significant increase of Drd2 expression level in the miR-124-1/+ PFC. Overexpression or knockdown of Drd2 in the control or miR-124-1/+ PFC demonstrates that aberrant Drd2 signaling leads to impaired PPI. Furthermore, we identified that expression of glucocorticoid receptor gene Nr3c1, which enhances Drd2 expression, increased in the miR-124-1/+ PFC. Taken together, the current study suggests that miR-124 dosage modulates PFC function through repressing the Drd2 pathway, suggesting a critical role of miR-124 in normal PFC function.

MicroRNAs (miRNAs) are small non-coding RNA molecules regulating gene expression of a great variety of biological processes in plants and animals. A large number of diverse miRNAs are expressed in the vertebrate CNS. MicroRNA-124 (miR-124) is one of the most abundantly expressed miRNAs in mouse and human brains. The nucleotide sequence of miR-124 and its nervous system-specific expression pattern are highly evolutionarily conserved from C. elegans through Drosophila melanogaster, and all vertebrates studied through to humans. Both in human and mouse genomes, miR-124s are encoded on three loci: miR-124-1, -2, and -3. Among the three primary miR-124s (pri-miR-124s), pri-miR-124-1 is predominantly expressed. In mice, retinal non-coding RNA3 (RNR3) functions as a pri-miR-124-1 precursor. We previously identified RNR3-deficient mice (RNR3−/−) by deleting the entire 4.5 kb region harboring RNR3. In the RNR3−/− brain, the level of mature miR-124 was reduced by 60–80% compared with that in the wild-type (WT) brain. RNR3−/− mice showed abnormalities in the CNS such as over-extension of dentate gyrus granule neuron axons and apoptosis of cone photoreceptor cells in the retina. Since in our previous study we confirmed...
that a loss of miR-124 is responsible for the RNCR3−/− abnormalities by in vivo rescue experiment, we will refer to RNCR3−/− mice as miR-124−/− mice in the current study.

In *C. elegans* miR-124 mutants, neurons are normally generated and no overt phenotype was observed. *Drosophila* miR-124 mutants exhibit abnormalities in neuroblast proliferation and neuronal maturation. In vertebrates, consistent with its expression pattern in the developing CNS, miR-124 has been reported to be essential for neuronal differentiation, maturation, synaptic plasticity and progenitor proliferation, however, it should be noted that conflicting results on miR-124 function have been reported. It was reported that neither inhibition nor overexpression of miR-124 affected neuronal differentiation or progenitor proliferation in the chick neural tube. On the other hand, other studies reported that miR-124 promotes both embryonic and adult neurogenesis. In the chick spinal cord, miR-124 was implicated in the stimulation of neuronal differentiation through suppressing the anti-neuronal REST/SCP1 pathway. In addition, miR-124 induces neurogenesis in P19 mouse embryonic cells. In the adult mouse subventricular zone, miR-124 was shown to induce adult neurogenesis through regulation of Sox9.

Human miR-124-1 is located in the chromosome 8p23.1 region. Heterozygous deletions on human 8p23.1 have been reported to be associated with psychiatric disorders, including schizophrenia, autism, and social impairment. Due to its location on 8p23.1 and because of its specific expression to the CNS, human miR-124-1 is implicated to be associated with mental diseases.

Despite of many studies, the role of miR-124 in normal brain function remains unclarified. While miR-124−/− mice show aberrant growth of dentate granule cell axons in the hippocampus, miR-124−/− mice exhibited no substantial morphological defects in the brain development as far as we analyzed. In the current study, we investigated the role and mechanism of miR-124 in normal brain functions using miR-124−/− mice.

Results

A comprehensive behavioral test battery for miR-124−/− mice. To address whether miR-124−/− haploinsufficiency affects normal brain functions, we subjected miR-124−/− mice to a comprehensive behavioral test battery. In the rotarod test, miR-124−/− mice exhibited normal motor function (Fig. 1a; two-way repeated-measures ANOVA: Genotype: *F*(1, 12) = 0.001206, *p* = 0.9729; Time: *F*(8, 96) = 19.94, *p* < 0.0001; Interaction: *F*(8, 96) = 0.1703, *p* = 0.9943). We monitored locomotor activity of the miR-124−/− mice in the open field test, and observed no significant difference in total distance traveled compared with WT control mice (Fig. 1b; two-way repeated-measures ANOVA: Genotype: *F*(1, 12) = 0.02964, *p* = 0.8662; Time: *F*(8, 96) = 18.44, *p* = 0.0001; Interaction: *F*(11, 132) = 0.9734, *p* = 0.4738). To assess anxiety-like behavior, we monitored time spent in the central part of the open field. We also measured the number of entries to the open arm and cumulative time spent in the open arm of the elevated plus maze. There were no significant differences in these parameters between miR-124−/− and WT mice (Fig. 1c–g; unpaired *t* test: *t*(12) = 1.029, *p* = 0.3236; unpaired *t* test: *t*(12) = 1.752, *p* = 0.0959; unpaired *t* test: *t*(12) = 1.676, *p* = 0.1101; unpaired *t* test: *t*(12) = 1.219, *p* = 0.2377; unpaired *t* test: *t*(12) = 0.9325, *p* = 0.3628).

To test whether miR-124−/− haploinsufficiency contributes to depression-like behavior, we subjected miR-124−/− mice to tail suspension and forced swim tests. miR-124−/− mice exhibited no significant difference in immobility time (Fig. 1h,i; unpaired *t* test: *t*(10) = 0.3745, *p* = 0.7518; unpaired *t* test: *t*(10) = 0.3952, *p* = 0.7010). To test for short-term memory deficit, we conducted a test of spontaneous Y-maze alternations, a spatial working memory task based on the natural tendency of mice to alternate the selection of maze arms. We did not observe differences in spontaneous alternation between miR-124−/− and WT mice (Fig. 1j; unpaired *t* test: *t*(10) = 0.00, *p* = 0.9999).

These results suggest that miR-124−/− haploinsufficiency does not affect normal motor function, anxiety-like behavior, depression-like behavior, or spatial working memory. The previous study shows that miR-124−/− mice exhibit a significantly decreased number of cone photoreceptor cells and an impairment of photopic electroretinogram (ERG) responses. We measured the ERG responses from miR-124−/− mice and observed no substantial ERG abnormalities (Supplementary Material Fig. S1a–c). We also analyzed cone photoreceptor integrity in the miR-124−/− retina by immunofluorescent staining and observed no significant differences between miR-124−/− and control retinas (Supplementary Material Fig. S1d). These results suggest that miR-124−/− mice have no substantial defects in the retina.

Haploinsufficiency of miR-124-1 causes abnormalities in social, psychostimulant induced behaviors, and sensorimotor gating. Since social deficits often appear in psychiatric disorders, we examined the sociability of miR-124−/− mice by social interaction test. Although there was no significant difference in the total duration of contacts between miR-124−/− and WT control mice, the total number of contacts for miR-124−/− mice decreased significantly compared to that of WT mice (Fig. 2a,b; unpaired *t* test: *t*(14) = 0.8695, *p* = 0.3993; unpaired *t* test: *t*(14) = 2.980, *p* = 0.0099). In addition, although the spontaneous locomotor activity in miR-124−/− mice was unchanged in the open field test (Fig. 1b), the total distance traveled in social interaction was significantly decreased (Fig. 2c; unpaired *t* test: *t*(14) = 3.791, *p* = 0.0020). These results suggest that miR-124−/− haploinsufficiency causes social behavior deficits.

A dopaminergic psychostimulant, methamphetamine, raises dopamine levels in the nucleus accum-bens and frontal cortex, and increases locomotor activity. To examine whether miR-124−/− mice show dopamine-associated behavioral deficits, we investigated the response of miR-124−/− mice to methampheta-mine using the open field. We found that the distance traveled by miR-124−/− mice in the open field test increased significantly compared to WT mice after methamphetamine administration (Fig. 2d; two-way repeated-measures ANOVA: Genotype: *F*(1, 13) = 0.714, *p* = 0.0224; Time: *F*(8, 96) = 4.304, *p* < 0.0001; Interaction: *F*(12, 156) = 6.334, *p* < 0.0001). This result suggests that miR-124−/− haploinsufficiency affects dopamine signaling associated with psychostimulant-induced hyperactivity.
Prepulse inhibition (PPI) is a reduction in an acoustic startle response observed when a weak stimulus (pre-pulse) is presented before the startling stimulus. PPI is a measure of sensorimotor gating that is impaired in various neuropsychiatric disorders, including schizophrenia, Huntington’s disease, Tourette’s disease, autism, and panic disorder. To test whether miR-124-1 haploinsufficiency affects sensorimotor gating, we measured PPI in miR-124-1+/− mice. We observed that the amplitudes of acoustic startle response were similar in miR-124-1+/− and WT mice (Fig. 2e; unpaired t test: $t_{(16)} = 2.046$, $p = 0.0576$). In contrast, we found that the percentages of PPI both with 78 dB and 86 dB prepulses decreased significantly in miR-124-1+/− mice (Fig. 2f; unpaired t test: $t_{(16)} = 2.566$, $p = 0.0207$; unpaired t test: $t_{(16)} = 2.745$, $p = 0.0144$), suggesting that miR-124-1 haploinsufficiency results in a deficit of sensorimotor gating in mice.
PFC of WT mice did not affect the amplitudes of the acoustic startle response or PPI (Fig. 2i; two-way ANOVA: Genotype: *p* < 0.01, and total distance traveled during social interaction (c) (n = 8 per genotype; **p** < 0.01). (d) Methamphetamine-induced hyperactivity in miR-124-1/+ mice but not in WT control mice (WT, n = 10; Het, n = 5). (e,f) PPI in miR-124-1/+ mice was impaired, whereas startle response amplitude was not significantly changed compared with that in WT mice (WT, n = 11; Het, n = 7; *p* < 0.05, **p** < 0.01; n.s., not significant). (g) A schematic of the AAV construct expressing miR-124 and Ds-Red (AAV-DsRed-miR-124). AAV-DsRed-miR-124 was injected into the PFC of 2 M WT and miR-124-1/+ mice. Fluorescence indicates the brain section injected with AAV-DsRed-miR-124. L, lateral; M, medial; D, dorsal; V, ventral; PL, prelimbic; IL, infralimbic. (h) Quantification of miR-124 expression level in the PFC of AAV-DsRed-miR-124-injected miR-124-1/+ mice (control AAV-DsRed injected, n = 7; AAV-DsRed-miR-124 injected, n = 4; **p** < 0.01). The dotted line indicates miR-124 expression level in control AAV-DsRed-injected WT mice. (ij) Injection of AAV-DsRed-miR-124 into the PFC rescued the PPI deficit in miR-124-1/+ mice (n = 12-15 per group; *p* < 0.05, **p** < 0.01). Error bars in (a–d) represent ± SD. Box–whisker plots present median (center line), ±1.5 interquartile range (box), minimal and maximal values (whiskers) in (e,f,h–j).

Partial rescue of the PPI deficit in miR-124-1−/− mice. We observed multiple types of behavior deficits in miR-124-1−/− mice: social deficits, methamphetamine-induced hyperactivity, and impairment of PPI. Since all of these phenotypes are associated with the impairment of prefrontal cortex (PFC) functioning,26–28, we focused on the role of miR-124 in the PFC. First, we examined expression of miR-124 in the PFC from miR-124-1−/− on the role of miR-124 in the PFC. We prepared an adeno-associated virus (AAV) expressing DsRed and miR-124 (AAV-DsRed-miR-124) (Fig. 2g). We injected AAV-DsRed-miR-124 or control AAV-DsRed into the PFC of 2 M WT and miR-124-1−/− mice, and measured PPI at 2 weeks (wks) after AAV injection. We confirmed that injection of AAV-DsRed-miR-124 significantly increases the expression level of miR-124 in the PFC of miR-124-1−/− mice (Fig. 2h; Mann–Whitney U test: *p* = 0.0061). Injection of AAV-DsRed-miR-124 or control AAV-DsRed into the PFC of WT mice did not affect the amplitudes of the acoustic startle response or PPI (Fig. 2i; two-way ANOVA: Genotype: *F*1,49 = 2.301, *p* = 0.1357; Virus: *F*1,49 = 7.740, *p* = 0.0076; Interaction: *F*1,49 = 0.7856, *p* = 0.3798). In contrast, the impaired PPI with an 86 dB prepulse in miR-124-1−/− mice recovered with the injection of AAV-DsRed-miR-124 (Fig. 2j; two-way ANOVA: Genotype: *F*1,49 = 1.161, *p* = 0.2865; Virus: *F*1,49 = 0.4301,
Figure 3. The effect of miR-124-1 haploinsufficiency on synaptic transmissions in layer 5 pyramidal cells in the PFC. (a) A schematic representing the recording region (red square) in PFC slices. PL, prelimbic; IL, infralimbic. (b) IR-DIC image of the PL region and the location of stimulating and recording electrodes. (c) Representative images showing biocytin-filled layer 5 pyramidal cells in WT control (left) and miR-124-1+/− mice (right). (d) Example average (n = 20) traces of EPSCs (left) and IPSCs (right) evoked by paired-pulse electrical stimulation at three stimulus intensities in WT (upper, black) and miR-124-1+/− mice (lower, red). (e) The average peak amplitude of evoked EPSCs (left) and IPSCs (right) was plotted against the stimulus intensity. Vertical and horizontal error bars indicate the SEM of the amplitude of evoked responses and the intensity of stimulation, respectively (n = 11 per genotype; *p < 0.05). (f) Paired-pulse ratio (PPR) of EPSCs (left) and IPSCs (right), respectively (WT, n = 9; Het = 11). (g) Example average (n = 20) traces of EPSCs before (pre, lighter color) and during (darker color) quinpirole application in WT (upper, black) and miR-124-1+/− (lower, red) mice. (h) Plots of the peak amplitude of EPSCs before (pre) and during quinpirole application...
in WT (black, left) and miR-124-1/+ (red, right) mice (n = 10 per genotype; *p < 0.05, ***p < 0.001). (i) The ratio of EPSC amplitude before and during quinpirole application in WT (black, left) and miR-124-1/+ (red, right) mice. (j) Example average (n = 20) traces of IPSCs before (pre, lighter color) and during (darker color) quinpirole application in WT (upper, black) and miR-124-1/+ (lower, red) mice. (k) Plots of the peak amplitude of IPSCs before (pre) and during quinpirole in WT (black, left) and miR-124-1/+ (red, right) mice (n = 11 per genotype; **p < 0.0001; n.s., not significant). (l) The ratio of IPSC amplitude before and during quinpirole application in WT (black, left) and miR-124-1/+ (red, right) mice (*p < 0.05).

\[ p = 0.5150; \text{Interaction: } F(1,40) = 2.737, p = 0.0632; \text{two-way ANOVA: Genotype: } F(1,49) = 7.184, p = 0.0100; \text{Virus: } F(1,41) = 2.768, p = 0.1025; \text{Interaction: } F(1,40) = 5.393, p = 0.0244. \] These results suggest that the expression level of miR-124 in the PFC is critical for normal sensorimotor gating in mice.

**miR-124-1 haploinsufficiency enhances synaptic transmission in layer 5 pyramidal cells in the PFC.** To investigate the effect of miR-124-1 haploinsufficiency on synaptic transmission in the PFC, we conducted whole-cell recordings from layer 5 pyramidal neurons in PFC slices prepared from miR-124-1/+ mice (Fig. 3a–c). To make a comparison of the strength of excitatory and inhibitory synaptic inputs to the pyramidal cells between the WT control and miR-124-1/+ mice, we recorded excitatory postsynaptic currents (EPSCs) at −70 mV and inhibitory postsynaptic currents (IPSCs) at 0 mV in response to electrical stimulation of presynaptic fibers at three different intensities (Fig. 3d). The slopes of the stimulus-response curves for EPSCs and IPSCs were both significantly steeper in miR-124-1/+ compared with WT mice (Fig. 3e; unpaired t test: \( t_{(18)} = 2.301, p = 0.0329 \); unpaired t test: \( t_{(20)} = 2.611, p = 0.0167 \)). There were no significant differences in the paired-pulse ratio of EPSCs or IPSCs between WT and miR-124-1/+ mice (Fig. 3f; unpaired t test: \( t_{(13)} = 0.08295, p = 0.9348 \); unpaired t test: \( t_{(20)} = 0.7042, p = 0.4894 \)). These results suggest that both excitatory and inhibitory synaptic transmissions in layer 5 pyramidal neurons are enhanced by miR-124-1 haploinsufficiency.

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We observed hypersensitivity of locomotor activity induced by methamphetamine administration and impaired PPI in miR-124-1/+ mice (Fig. 2d,f). Previous studies have reported that absence of Dopamine D2 receptor (Drd2) attenuates the disruption of PPI induced by amphetamine29, and methamphetamine-induced hyperactivity30. To investigate whether dopamine regulation of local circuit processing in the PFC is affected by dopamine, we examined the effects of Drd2 activation on synaptic transmission in miR-124-1/+ mice. We observed a significant reduction in EPSC amplitude after the application of 5 μM quinpirole, a Drd2 agonist, in both miR-124-1/+ mice and WT mice, but no significant difference in the level of reduction between them (Fig. 3g–i; paired t test: \( t_{(39)} = 3.165, p = 0.0115 \); paired t test: \( t_{(54)} = 5.436, p = 0.0004 \); unpaired t test: \( t_{(18)} = 0.6279, p = 0.5379 \)). On the other hand, the amplitude of IPSCs significantly decreased by quinpirole application in miR-124-1/+ mice but not in WT mice (Fig. 3j–l; paired t test: \( t_{(10)} = 2.054, p = 0.0670 \); paired t test: \( t_{(10)} = 6.770, p < 0.0001 \); unpaired t test: \( t_{(20)} = 2.213, p = 0.0387 \)). These results suggest that Drd2-mediated dopaminergic modulatory effects on inhibitory transmissions increased in the pyramidal cells in the PFC of miR-124-1/+ mice. We speculated that PPI deficit and methamphetamine-induced hyperactivity observed in miR-124-1/+ mice are likely due to modulation of the dopaminergic system by miR-124 reduction in the PFC.

**Drd2 is up-regulated in the miR-124-1/+ PFC.** To explore the molecular mechanism of behavioral deficits observed in miR-124-1/+ mice, we focused on genes involved in the dopaminergic system. We searched for miR-124 target sites in 3′UTR of dopamine-related genes, including dopamine receptor genes (Drd1–5), a dopamine transporter gene (DAT), a dopamine synthesis gene (TD), and a dopamine metabolism gene (MAOB), and found that only the 3′UTR of Drd2 possesses an evolutionally conserved miR-124 target sequence (Fig. 4a). We observed that total Drd2 (containing both long and short variants) increased in 2 M miR-124-1/+ PFC (Fig. 4b; unpaired t test: \( t_{(13)} = 2.749, p = 0.0189 \)).

To examine the targeting effect of miR-124 on the Drd2-3′UTR, we performed a luciferase reporter assay. We prepared reporter plasmids containing native Drd2-3′UTR (Drd2-3′UTR) and Drd2-3′UTR with mutations in the miR-124 target site (Drd2-3′UTRmut) (Fig. 4c). We observed that luciferase activity shows no obvious change between the cells transfected with Drd2-3′UTR and those transfected with Drd2-3′UTRmut (one-way ANOVA: \( F(3,8) = 4.156, p = 0.0476 \)). This result suggests that Drd2 is not a direct target of miR-124, but is indirectly regulated by miR-124 in the PFC.

**Expression level of Drd2 in the PFC affects PPI.** To test whether Drd2 suppression in the miR-124-1/+ PFC rescues the PPI, we used AAVs expressing shDrd2 to knockdown Drd2 (AAV-shDrd2) or a control shRNA (AAV-shControl) (Fig. 4d). We injected AAV-shDrd2 into the miR-124-1/+ PFC at 2 M and measured PPI at 2 wks after injection. Acoustic startle amplitude was unchanged between AAV-shControl- or AAV-shDrd2-injected mice (Fig. 4e; Mann–Whitney U test: \( p = 0.9336 \)). No significant change of PPI with a 78 dB prepulse was observed in these mice, in contrast, a significant recovery of the PPI deficit was observed by knockdown of Drd2 in the PFC with an 86 dB prepulse (Fig. 4f; Mann–Whitney U test: \( p = 0.1650 \); Mann–Whitney U test: \( p = 0.0274 \)). These results suggest that the impaired PPI in miR-124-1/+ mice is at least partially caused by Drd2 increase in the PFC.

To investigate whether Drd2 overexpression in the PFC affects PPI, we prepared AAVs expressing Drd2 (AAV-Drd2) or EGF (AAV-EGF), and co-injected them into the PFC of WT mice at 2 M (Fig. 4g). We observed no significant change of startle amplitude between control AAV-EGF- and AAV-Drd2-injected mice (Fig. 4h; Mann–Whitney U test: \( p = 0.2890 \)). No significant change of PPI with a 78 dB prepulse was observed in these mice, however, we observed a significant PPI decrease with an 86 dB prepulse in mice with Drd2 overexpression.
by AAV-Drd2 injection into the PFC (Fig. 4i; Mann–Whitney U test: p = 0.1375; Mann–Whitney U test: p = 0.0350). These results suggest that Drd2 level change is at least partially responsible for the sensorimotor gating abnormality in the PFC of miR-124-1+/− mice.

In the reporter assay, we did not observe a significant change in luciferase activity between native and mutated Drd2-3′UTR constructs (Fig. 4c). How was Drd2 expression enhanced in the miR-124-1+/− PFC? To address this question, we further screened miR-124 target genes in the PFC. To identify miR-124 target genes in the PFC, we performed high-throughput mRNA sequencing (RNA-seq) analysis using RNAs isolated from miR-124-1+/− PFC or control PFC at 2 M (Fig. 5a). We identified 3,480 upregulated genes (more than 1.1-fold increase) in the miR-124-1+/− PFC. We next searched for putative target genes of miR-124 in the context of PFC development. We
mice)35. The reduction of expression in the hippocampus (approximately 80% of non-stressed control miR-124 expression was significantly down-regulated by cotransfection with the -3′ Nr3c1 genes in the analysis (Supplementary Material Fig. S2d; unpaired t test: t(4) = 0.0051). These results suggest that Nr3c1 is directly regulated by miR-124 in the PFC. Drd2 is likely to be regulated indirectly in the PFC by miR-124 through regulation of Nr3c1 expression change.

Several studies reported that miR-124 plays roles in pathogenesis of psychiatric disorders including Alzheimer’s disease and frontotemporal dementia (FTD)28,34. In their studies, human brains with Alzheimer’s disease exhibited a slight decrease of miR-124 expression, and the FTD mouse model with an impairment of age-dependent sociability also exhibited miR-124 down-regulation in the PFC. The impaired sociability of the FTD mouse model was shown to be accompanied with up-regulation of AMPA receptor subunits (Gria2, 3 and 4) following miR-124 down-regulation. We examined expression of Gria2, 3 and 4 in the 2 M miR-124-1–/– PFC by qRT-PCR. However, the expression of these genes was not significantly altered (Supplementary Material Fig. S2a; unpaired t test: t(11) = 1.461, p = 0.1720; unpaired t test: t(11) = 0.9990, p = 0.3393; unpaired t test: t(11) = 0.5248, p = 0.6101). A recent study reported that mice exposed to chronic ultra-mild stress exhibited depression-like change of Gsk3b in the 2 M miR-124-1–/– hippocampus by qRT-PCR. We observed a decrease of miR-124 (approximately 73% of WT control mice, Supplementary Material Fig. S2b; unpaired t test: t(4) = 3.805, p = 0.0190) but no significant change of Gsk3b in the miR-124-1–/– hippocampus was detected (Supplementary Material Fig. S2c; unpaired t test: t(4) = 0.8932, p = 0.4222). Another study showed that small GTPase regulatory gene EPAC-deficient mice, which exhibit an impairment of learning and social interaction, displayed an elevation of miR-124 expression in the cortex and hippocampus, leading to down-regulation of miR-124 target gene Zif268 (Egr1)36. We did not observe a significant expression change of Zif268 in the 2 M miR-124-1–/– PFC and hippocampus by qRT-PCR analysis (Supplementary Material Fig. S2d; unpaired t test: t(4) = 1.631, p = 0.1312; unpaired t test: t(4) = 0.5992,
Thus, the expression levels of the previously reported miR-124 target genes, Gria2, Gria3, Gria4, Gsk3b, and Zif268, in the miR-124-1+/− PFC were not significantly affected compared to those in the control PFC. Since the experimental design of these studies is different from our study35,36,38, future studies are needed to elucidate the cause of varied outcomes.

Discussion
In the current study, several lines of evidence suggest that impaired sensorimotor gating in miR-124-1+/− mice is likely due to abnormal dopaminergic system by miR-124 reduction in the PFC. First, our whole-cell recordings from layer 5 pyramidal cells in the miR-124-1+/− PFC revealed that the amplitude of IPSCs significantly decreased by Drd2 agonist quinpirole application. Second, we found that Drd2 expression is upregulated in the miR-124-1+/− PFC. Third, injection of AAV-shDrd2 into the PFC of miR-124-1+/− mice partially rescued PPI impairment. Fourth, overexpression of Drd2 in the WT PFC by injection of AAV-Drd2 caused a PPI abnormality. In addition, a previous study reported that injection of dopamine receptor agonist apomorphine into the mouse PFC significantly compromises PPI37. Accordingly, our results suggest the aberrant Drd2 pathway in the PFC is a major cause of sensorimotor gating deficit observed in miR-124-1+/− mice.

Several previous studies reported in vivo microRNA functions in normal brain using mouse mutants. In the striatum, miR-128 regulates motor behavior by modulating the ERK signaling network and neuronal excitability38. miR-137, a schizophrenia risk gene product, controls synaptic plasticity and memory in the hippocampus39. miR-338-3p in the auditory thalamus regulates auditory thalamus development and acoustic-startle response40. In the current study, we showed that miR-124 is necessary for the normal brain function in the PFC. Whole-cell recordings from layer 5 pyramidal neurons in miR-124-1+/− PFC slices revealed that synaptic transmission into pyramidal neurons in the PFC is enhanced by miR-124-1 heterozygous deficiency. Previous studies showed the link between function of pyramidal neurons in the PFC and animal behavior. Loss of Dysbindin-1, whose genetic variation is associated with risk for schizophrenia, shows impairments in working memory, PPI, and higher locomotor activity. In the Dysbindin-1 mutant PFC, enhanced excitability of pyramidal neurons was observed38. DISC1 knockdown in the mouse PFC leads to deficits in PPI and methamphetamine-induced hyperactivity27. In DISC1 knockdown mice, attenuated electrophysiological responsiveness of pyramidal neurons in the PFC was observed. The mice lacking GluN2C, an NMDA receptor subunit, exhibited abnormalities in cognition and PPI as well as activity alteration of layer 5 pyramidal neurons in the PFC42. Since none of Dysbindin-1, DISC1 and GluN2C transcripts contain the miR-124 target sequence, we speculate that these genes function in distinct pathways from that of miR-124 in normal brain function. We cannot rule out the possibility that the abnormal electrophysiological properties of pyramidal neurons in the miR-124-1+/− PFC are due to indirect effects from other cells. Future analysis using PFC pyramidal neuron specific-miR-124 conditional knock-out mice may clarify this point.

It was previously shown that Nr3c1 3′ UTR contains a miR-124 target sequence and that luciferase reporter expression was downregulated by miR-124 in a cultured cell system45. In the current study, we observed upregulation of Nr3c1 by reduction of miR-124 in vivo, suggesting that Nr3c1 is an endogenous target of miR-124 in the prefrontal cortex. Blocking of Nr3c1 function decreases the Drd2 expression level, and affects the dopaminergic pathway in the frontal cortex of one mouse model of adolescent stress33. Conditional deletion of Nr3c1 in dopaminergic neurons affects the motivation of mice to self-administer cocaine45. These observations as well as our luciferase reporter assay suggest that Drd2 is regulated indirectly in the PFC by miR-124 through modulation of Nr3c1 expression change. To examine this point in vivo, we performed injection of an Nr3c1 antagonist into the miR-124-1+/− PFC, however, we did not observe a statistically significant change of PPI or an expression change of the Drd2 mRNA by RT-qPCR analysis using the whole PFC. It is possible that the Drd2 expression in the PFC is redundantly regulated by Nr3c1 and other molecules. There might be another possibility that only a subset of PFC neuronal cells is regulated by Nr3c1 and the expression change of Drd2 might be difficult to be detected in the whole PFC by RT-qPCR analysis.

Heterozygous deletions on human 8p23.1 containing miR-124-1 locus are associated with psychiatric disorders15–18. Interestingly, a recent genome-wide association study reported that the inversion polymorphism in the 8p23.1 region containing the human miR-124-1 locus is associated with neuroticism45. Further investigation of miR-124 mutations and/or expression levels in human neuropsychiatric disorder patients, including schizophrenia, Alzheimer’s disease, FTD, autism, and social impairment, may advance our understanding on the pathogenesis and/or exacerbation of these diseases.

Materials and Methods
Animal care. All recombinant mouse experiment procedures were approved by the Institutional Safety Committee on Recombinant DNA Experiments (approval ID 04220), Animal Experimental Committees of the Institute for Protein Research at Osaka University (approval ID 29-01-2), Committee on Animal Research at Kyoto University Graduate School of Medicine (approval ID MedKyo17071), and Experimental Animal Care Committee at National Institute for Physiological Sciences (approval ID 17A18). These procedures were performed in compliance with the institutional guidelines. Mice were housed in a temperature-controlled room at 22 °C with a 12 h light/dark cycle. Fresh water and rodent diet were available at all times.

Generation of miR-124-1+/− C57BL/6 N strain mice. We backcrossed miR-124-1+/− 129S6/SvEvTac mice46 with C57BL/6 N strain mice, using the speed congenic method (Central Institute for Experimental Animals, Japan)47, and generated miR-124-1+/− C57BL/6 N mice. We confirmed that over 99% of microsatellite DNA markers are from the C57BL/6 N genetic background.
Plasmid construction. The Drd2 3′ UTR or Nr3c1 3′ UTR fragment was amplified by PCR using KOD Plus (Toyobo), and cloned into pcDNA-Blunt vector (Invitrogen). The primer sequences are as follows: for Drd2 3′ UTR, forward, 5′-TGAGCTCGAGCGGAGTTGAGACGTAAGTT-3′ and reverse, 5′-CAGGGCTAGGACTCCCTG-3′; for Nr3c1 3′ UTR, forward, 5′-CTGCTGAGCCCTGAGCTCTCTG-3′ and reverse, 5′-GAATTCGCCACCATGGATCCACTGAACCTGTCCTGGTAC-3′. Mutations in the seed match region were introduced by PCR. The primer sequences are as follows: Drd2 3′ UTRmut, 5′-GCAAAGTGGAGGGCTGAGTCATGC-3′ and 5′-GAGGCAGCTGCAGGAGAGAAGACT-3′; Nr3c1 3′ UTRmut, 5′-CTTGCTGAGCCCTGAGCTCTCTG-3′ and reverse, 5′-GAATTCGCCACCATGGATCCACTGAACCTGTCCTGGTAC-3′. The fragments of native and mutated Drd2 3′ UTR or Nr3c1 3′ UTR were ligated with pmirGLO vector (Promega) to generate pmirGLO-Drd2 3′ UTR, pmirGLO-Nr3c1 3′ UTRmut. To generate AAV-DsRed or AAV-DsRed-miR-124 construct, the fragment of CAG-DsRed or CAG-DsRed-miR-124-2 derived from RIP and RIP-miR-124-2 vectors (a gift from Tom Maniatis, Harvard University) was inserted into pAAV-IRES-hrGFP vector (Agilent technologies). For the production of AAV-shDrd2 and AAV-shControl, a target sequence for Drd2 (shDrd2) or shControl was subcloned into pcDNA-mU6 vector (Takara). The mU6-shControl or mU6-shDrd2 fragment was inserted into the pAAV-CAG-AcGFP vector. For the production of AAV-Drd2, full-length cDNA fragment of mouse Drd2 (NCBI #NM_010077) was amplified by PCR using mouse brain cDNA. The primer sequences are 5′-GAATTCGCCACCATGGATCCACTGAACCTGTCCTGGTAC-3′ and 5′-GAGGCACTGGCAGAGAAGACT-3′. The fragment was ligated with pAAV-CBh vector, in which the CBh promoter fragment was inserted into pAAV-CAG-mCherry. To generate pAAV-EGFP, the fragment of native and mutated Drd2 3′ UTR or Nr3c1 3′ UTR were ligated with pmirGLO vector (Promega) to generate pmirGLO-Drd2 3′ UTR, pmirGLO-Nr3c1 3′ UTRmut. To generate AAV-DsRed or AAV-DsRed-miR-124 construct, the fragment of CAG-DsRed or CAG-DsRed-miR-124-2 derived from RIP and RIP-miR-124-2 vectors (a gift from Tom Maniatis, Harvard University) was inserted into pAAV-IRES-hrGFP vector (Agilent technologies).

Immunofluorescent staining of brain sections. For immunohistochemistry, 30-μm brain sections were washed twice in PBS, permeabilized with 0.1% Triton X-100 in PBS, and then incubated with PBS containing 4% donkey serum for 1 h for blocking. The samples were incubated with a primary antibody at 4 °C overnight. After PBS-washing, these samples were incubated with fluorescent-labeled secondary antibodies at room temperature for 2 h. The specimens were observed under a laser confocal microscope (LSM700, Carl Zeiss). Hoechst (Sigma) was used for nuclear staining. Primary antibodies are as follows: rat anti-GFAP (Nacalai, 40404-26, 1:1000) and rabbit anti-DSRed (Clontech, 632496, 1:1000) antibodies. We used Cy3-conjugated (Jackson ImmunoResearch Laboratories, 1:500) and Alexa Fluor 488-conjugated (Sigma, 1:500) secondary antibodies.

High-throughput mRNA sequencing (RNA-seq) analysis. Total RNAs from the mouse PFC were extracted using Trizol (Invitrogen). Library preparation was performed using 1 μg of total RNA and NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs). RNA-seq was done with an Illumina HiSeq 1500 for 51 bp single-end. Approximately 40 M reads were obtained in each sample. The reads were mapped against mouse reference sequence. TIGAR2 was run with default settings. The expression level of each gene was quantified as FPKM (fragments per kilobase of exon per million mapped fragments).

qRT-PCR. Total RNA was extracted using Trizol reagent (Invitrogen), and reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) with random hexamers and Oligo dT (Invitrogen). Quantitative PCR was performed using a SYBR GreenER qPCR SuperMix Universal (Invitrogen) and Thermal Cycler Dice Real Time System Single MRQ TP870 (Takara) according to the manufacturer's instructions. Quantification was carried out by Thermal Cycler Dice Real Time System software version 2.0 (Takara). Nucleotide sequences of primers are as follows: for Nr3c1, forward, 5′-CATTTGGCCCTGGTGGAGATCA-3′ and reverse, 5′-GTTAGCCAGGTAAGACTCTCTGG-3′; Rpl4, forward, 5′-GATATGCACTGGTTGCCTGC-3′ and reverse, 5′-GTTAGCCAGGTAAGACTCTCTGG-3′. To quantify Drd2 expression level, we used the TaqMan probe (Applied Biosystems) and StepOnePlus Real-Time PCR System (Applied Biosystems). To detect mature miR-124, total RNA isolated using the Trizol reagent was reverse transcribed using the TaqMan reverse transcription reagent kit (Applied Biosystems) according to the manufacturer's protocol. qRT-PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) and specific TaqMan mature-miRNA Assays for miR-124 (Applied Biosystems).

Animal behavior assays. Locomotor activity. Spontaneous locomotor activity was measured as described previously. Briefly, each mouse was placed at the center of open field box (W × D × H = 40 × 40 × 27 cm), and the total distance traveled and the time spent in the center area (W × D = 20 × 20 cm) were scored for 60 min using EthoVision software (Noldus). To examine methamphetamine-induced hyperactivity, mice received one intraperitoneal injection of methamphetamine (1 mg/kg) after 60 min of habituation in open field box.

Rotarod. Rotarod was performed as described previously. Male mice were tested on an accelerating rotarod (4–50 rpm in 5 min per test), 3 tests a day (30-min break in-between), around the same time of the day over 3 consecutive days, and the latency to fall was scored.

Elevated plus maze. Elevated plus maze was performed as described previously. The plus maze consisted of a plus-shaped apparatus with two open and two closed arms, each with an open roof, elevated 40 cm from the floor. Male mice were put into center of the plus maze and its free movement was recorded for 10 min using EthoVision software.
PPI. The startle response and PPI were measured using a startle reflex measurement system (SR-LAB) as previously described with minor modifications. The test session began by placing a male mouse in a plastic cylinder and leaving it undisturbed for 30 min. The background white noise level in the chamber was 70 dB. A prepulse-pulse trial started with a 50-ms null period, followed by a 20-ms prepulse white noise (74, 78, 82, 86, or 90 dB). After a 100-ms delay, the startle stimulus (40-ms, 120 dB white noise) was presented, followed by a 290-ms recording time. The total duration of each trial was 500 ms. A test session consisted of six trial types (pulse-only trial, and five types of prepulse-pulse trial). Six blocks of the six trial types were presented in a pseudorandomized order such that each trial type was presented once within a block. The formula $\text{100} - \left( \frac{\text{Response on acoustic prepulse-pulse stimulus trials}}{\text{Startle response on pulse-only trials}} \right) \times 100$ was used to calculate %PPI.

**Forced swim test.** Each mouse was placed in a transparent glass cylinder (8 cm in diameter × 20 cm high), containing water at 22–23 °C to a depth of 15 cm, and forced to swim for 10 min. The duration of immobility was measured using digital counters with infrared sensors.

**Tail suspension test.** Mice were suspended upside-down by the tail for 10 min. The session was recorded by a video camera and the duration of immobility was measured.

**Social interaction.** Social interaction was performed as described previously. Two mice of identical genotypes previously housed in different cages, were placed into an open field together and allowed to explore freely for 10 min. The behavior was recorded by a CCD camera and automatically analyzed by EthoVision software. The total duration of contacts, the number of contacts, and total distance traveled were measured. Contacts were defined as follows: if the nose of either of the two mice got close to the nose, body or hip of another mouse within 2–3, 5.5–6.5, or 2-3 cm, respectively, the behavior was considered contact.

**Y-maze.** Y-maze was performed as described previously. Each mouse was placed in the center of the symmetrical Y maze and was allowed to explore freely for 10 min. The sequence and total number of arms entered was recorded, using EthoVision software. The sequence triads, in which all three arms were represented (ABC, ACB, BAC, BCA, CAB, and CBA), were calculated as successful alternations to evaluate the normal cognition and working memory of the last arm entered. The percentage of spontaneous alternation is as follows. A number of triads containing entries into all three arms/maximum possible alternations (the total number of arms entered – 2) × 100.

**Slice preparation for electrophysiology.** miR-124-1 heterozygote mice and their littermate control mice of either sex at postnatal 32–35 days were deeply anesthetized with isoflurane and sodium pentobarbital (50 mg/kg, i.p.), and perfused transcardially with ice-cold normal artificial cerebrospinal fluid (ACSF) containing the following: 126 mM NaCl, 3 mM KCl, 1.3 mM MgSO4, 2.4 mM CaCl2, 1.2 mM NaH2PO4, 26 mM NaHCO3, and 10 mM glucose, saturated with 95% O2 and 5% CO2. The brains were removed from the mice, and coronal slices (300–μm thick) were prepared from the PFC using a vibrating microslicer (VT1200S; Leica) and recovered in an interface chamber at 33 °C for 1 hour. The slices were then maintained in a submerged chamber at room temperature.

**Electrophysiology.** Prefrontal cortical slices containing the prelimbic region were transferred into a submerged-type chamber perfused with normal ACSF. An infrared differential interference contrast video microscopy with an X40, 0.8NA water immersion lens (BX-50WI, Olympus) was used to visualize and target layer 5 pyramidal neurons for whole-cell recordings. Patch pipettes (5–7 MΩ) were filled with a solution containing the following: 130 mM Cs-glucuronate, 8 mM CsCl, 1 mM MgCl2, 0.6 mM EGTA, 10 mM HEPES, 3 mM MgATP, 0.5 mM Na3GTP, 10 mM Na-phosphocreatine, and 0.2% biocytin (pH 7.3 adjusted with CsOH). The membrane potential of the recorded cells was held at the reversal potential of inhibitory postsynaptic currents (IPSCs, -70 mV) or excitatory postsynaptic currents (EPSCs, 0 mV) for EPSC and IPSC recordings, respectively. We did not use series resistance compensation and selected cells with a series resistance <25 MΩ for analysis. Electrical paired-pulse stimulation was applied at 0.2 Hz through a glass pipette placed in layer 2 just below layer 1, and whole-cell recordings were obtained using patch pipettes located 65 ± 9.0 μm (mean ± SEM) lateral to the stimulating electrode. The interval of the paired-pulse stimulation was 50 and 100 ms for EPSC and IPSC recordings, respectively. To examine the relationship between stimulus intensity and response amplitude, we used stimulus currents that evoked the minimum (3–7 μA), intermediate, and maximum (10–140 μA) EPSC amplitudes. The current intensities used for evoking EPSCs were the same as those employed for evoking IPSCs in each cell. Quinpirole at 5 μM was added to the bath solution and the effect was assessed 10 min after the application. All of the whole-cell recordings were conducted using a Multiclamp 700B amplifier, and data were analyzed using pClamp9 software (Molecular Devices). After whole-cell recording, the slices were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) overnight. To visualize the recorded neurons, the slices were incubated with streptavidin conjugated to Alexa 488 (1:1000; Life Technologies) in 25 mM PBS containing 0.1% Triton X-100 overnight at room temperature. Images of slices were obtained using an A1R confocal microscope (Nikon) with a 40x objective.

**AAV production.** AAV was produced by triple transfection of an AAV vector plasmid, an adenovirus helper plasmid, and an AAV helper plasmid (pAAV2/9) into AAV-293 cells by the calcium phosphate method. The cells were harvested at 72 h after transfection, and lysed by four freeze-and-thaw cycles. The supernatant was collected by centrifugation, and treated with benzonase nuclease (Novagen) for 10 min at 45 °C to eliminate cellular DNA/RNA and excess plasmid DNAs. The viruses were purified by iodixanol step gradient. The gradient was formed in Ultra-Clear centrifuge tubes (14 × 95 mm, Beckman) by first adding 1.59 ml of 54% iodixanol (Axis-Shield) in

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PBS-MK buffer (1 × PBS, 1 mM MgCl₂, and 25 mM KCl) and then overlaying 1.59 ml of 40% iodixanol in PBS-MK buffer, 2.12 ml of 25% iodixanol in PBS-MK buffer containing phenol red, and 3.18 ml of 15% iodixanol in PBS-MK buffer containing 1 M NaCl. Finally, the lysate was applied on top of the gradient. The tubes centrifuged for 3 h at 40,000 rpm at 18°C in a SW40Ti rotor. The 54–40% fraction containing virus was collected using an 18-gauge needle. The fraction was concentrated using an Amicon Ultra Centrifugal Filter Ultracel-100 K (Millipore). A titrator of each AAV (in vector genomes (VG)/mL) was determined by qPCR using SYBR Green ER Q-PCR Super Mix (Invitrogen) and Thermal Cycler Dice Real Time System Single MRQ TP870 (Takara). The primers used for AAV titrations are as follows: for DsRed, forward, 5′-AACAAGTGAAGTTATCATCGGCCTGA-3′ and reverse, 5′-AGCTTGGCG TCCACGTAGTAGTAC-3′; EGFP, forward, 5′-GAAGGCGATCGACCTAACGAAGGA-3′ and reverse, 5′-CTTGTAGCGGTTCTGTGTCGTC-3′; CbH promoter, forward, 5′-TCCATTGACGTC AATGGTGAGT-3′ and reverse, 5′-CATTGACG TCAATAGGGGCGTAC-3′.

Stereotaxic viral injection. AAVs were injected into the PFC of WT and/or miR-124-1+/- mice (for PFC, +1.69 mm posterior to the bregma, 0.3 mm lateral from the midline, −1.5 mm depth from the dura). The PPI of injected mice were measured 2 wks after the injection. Coronal sections of the PFC were prepared and immunostained with an anti-GFP or anti-DsRed antibody, followed by the Alexa Fluor 488-conjugated or Cy3-conjugated secondary antibody. Immunofluorescence was detected with a laser confocal microscope (LSM700, Carl Zeiss).

Luciferase reporter assay. We transfected 0.1 μg of the luciferase reporter vectors (Drd2-3′UTR, Drd2-3′UTRmut, Nr3c1-3′UTR, or Nr3c1-3′UTRmut) and 1.1 μg of the miR-124 expression vector (pBasi-mU6-shControl or pBasi-mU6-pre-miR-124-1) into HEK293 cells in a 12-well plate using the calcium phosphate method. A β-galactosidase expression vector (β-SV; Promega) was cotransfected for normalization of transfection efficiency. After transfection, the cells were incubated for 48 h and lysed with Reporter Lysis Buffer (Promega). The luminescence signal was detected using a GloMax Multi+ detection system (Promega).

Electroretinogram recordings. The method of recording the electroretinograms (ERGs) was performed as described previously with some modifications. In brief, mice were dark adapted for more than 4 h, and then anesthetized with an intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. ERG responses were measured using the PuREC system with LED LS-100 (Mayo Corporation). After mice were light adapted for 10 min, the photopic ERGs were recorded on a rod-suppressing white background of 1.3 log cd sm⁻². Four levels of stimulus intensities ranging from −0.5 to 1 log cd sm⁻² were used for the photopic ERG recordings. Sixteen responses were averaged for photopic recordings.

Statistical analysis. Statistical analyses were performed using GraphPad Prism version 6.04 (GraphPad Software). Single comparisons were performed using paired or unpaired Student's t test, and multiple comparisons were performed using one-way ANOVA with post hoc Tukey–Kramer test. The statistical significance of experiments involving three or more groups and two or more treatments was assessed by two-way ANOVA with post hoc Tukey–Kramer test. The statistical significance of samples is indicated in the figure legends. Asterisks indicate significance values as follows: *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.

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Author Contributions
T.K., Y. Omori, T.H. and T.F. designed the project. T.K., Y. Omori, S.W., E.T., H.Y., T.C., M.F., M.M., T.S., S.H., Y. Ohkawa, Y.Y., T.H. and T.F. performed experiments and analyzed data. T.K., Y. Omori, Y.Y., T.H. and T.F. wrote the manuscript.

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