Age-dependent regulation of synaptic connections by dopamine D2 receptors

Jie-Min Jia, Jun Zhao, Zhonghua Hu, Daniel Lindberg & Zheng Li

Dopamine D2 receptors (D2R) are G protein–coupled receptors that modulate synaptic transmission and are important for various brain functions, including learning and working memory. Abnormal D2R signaling has been implicated in psychiatric disorders such as schizophrenia. Here we report a new function of D2R in dendritic spine morphogenesis. Activation of D2R reduced spine number via GluN2B- and cAMP-dependent mechanisms in mice. Notably, this regulation occurred only during adolescence. During this period, D2R overactivation caused by mutations in the schizophrenia risk gene Dtnbp1 led to spine deficiency, dysconnectivity in the entorhinal-hippocampal circuit and impairment of spatial working memory. Notably, these defects could be ameliorated by D2R blockers administered during adolescence. Our findings suggest an age-dependent function of D2R in spine development, provide evidence that D2R dysfunction during adolescence impairs neuronal circuits and working memory, and indicate that adolescent interventions to prevent aberrant D2R activity protect against cognitive impairment.

D2R belongs to the D2-like (D2, D3 and D4 type) subfamily of dopamine receptors. D2R dysfunction has long been recognized and targeted for therapy in schizophrenia, a debilitating mental disorder. An increase in D2R density is consistently found in schizophrenic brains, and all antipsychotics antagonize D2R. Genetic studies have shown that some genes associated with increased risks of schizophrenia encode proteins that regulate D2R, including dysbindin (Dtnbp1), which controls trafficking of D2R to the cell surface.

Although effective for psychosis, treatment with D2R antagonist has little effect on cognitive impairment, a core symptom of schizophrenia and a major determinant of disability. Proper synaptic connections are essential for cognition. In schizophrenic brains, however, interneuronal connections are impaired. For example, in both the prefrontal cortex and hippocampus of patients with schizophrenia, there is a reduction in the number of dendritic spines, small dendritic protrusions accommodating most excitatory synapses in the brain. In additions, neurons derived from induced pluripotent stem (iPS) cells from patients with schizophrenia exhibit severe impairments in their interneuronal connections. The pathogenic mechanisms underlying synaptic dysconnectivity, however, are still largely unknown.

D2R activation is coupled to a number of signaling pathways. By coupling with G proteins, activated D2R negatively regulates the cAMP-PKA pathway. Activated D2R also induces the formation of the β-arrestin 2–Akt–protein phosphatase 2A signaling complex. In hippocampal, cortical and striatal neurons, brief activation of D2R inhibits currents mediated by NMDA receptors (NMDAR). Alteration of synaptic transmission is often accompanied by structural modification of synapses, such as formation, elimination and morphological changes of dendritic spines. Whether D2R regulates the structure of synapses, however, has not been experimentally tested.

We found that D2R modulated the morphogenesis of dendritic spines in hippocampal neurons via GluN2B- and cAMP-dependent mechanisms. Notably, D2R regulated spines only during postnatal weeks 3–6, and adolescent D2R hyperactivity resulted in a reduction in spine number in mice with deficient expression of the schizophrenia risk gene dysbindin. Even transient suppression of spine development during adolescence by hyperactive D2R adversely affected entorhinal cortex–hippocampal connectivity and working memory in adulthood. These findings suggest a previously unknown function of D2R in spine development and identify a critical period during which dendritic spines are regulated by D2R.

RESULTS

D2R regulates the morphogenesis of dendritic spines

To determine whether D2R regulates the development of neuronal connections, we intraperitoneally injected male C57BL/6 mice (postnatal day 21 (P21), during a period of active spine growth and synaptogenesis) with either vehicle, the D2R agonist quinpirole or bromocriptine, or the D2R antagonist eticlopride. Hippocampal slices were prepared 24 h after injection for diolistic labeling of neurons. Notably, in mice injected with D2R agonists, spine density of hippocampal CA1 neurons was reduced. The spine density of CA1 neurons was unaffected by a single injection of eticlopride (data not shown), but was substantially increased by five daily injections of eticlopride. Thus, D2R activation inhibits spine development, whereas prolonged blockade of D2R activity promotes it.

To test whether the D1-like subfamily of dopamine receptors (D1R-like) also regulate spine development, we injected either vehicle, the D1R-like agonist SKF38393 or the D1R-like antagonist SCH23390 into P21 male C57BL/6 mice and prepared hippocampal slices for diolistic labeling 24 h after injection. The spine density of CA1 neurons...
neurons was comparable in all three groups of mice (Fig. 1a,c). Thus D1R-like receptors do not regulate spine development in hippocampal neurons.

To corroborate the results of our pharmacological experiments, we overexpressed or knocked down D2R in vivo. The CA1 region of male C57BL/6 mice (P21) was injected with lentivirus expressing enhanced green fluorescent protein (EGFP) along with siRNA specific for either Drd2 (D2R, Drd2 siRNA-1) or Drd1 (D1R; Supplementary Fig. 1a–c), or co-injected with the EGFP virus and lentivirus overexpressing either D1R or D2R. At 7 d after injection, brain sections were prepared from injected mice. In CA1 pyramidal neurons transduced with the D2R virus, spine density was reduced, whereas, in those transduced with Drd2 siRNA virus, it was increased (Fig. 1b,d). Transduction of virus expressing D1R or Drd1 siRNA, however, left the numbers of spines intact (Fig. 1b,d). These results confirm the findings of our pharmacological experiments.

The change in spine number may affect synaptic transmission. To test this possibility, we measured miniature excitatory postsynaptic currents (mEPSCs) in mice injected with either the D2R or Drd2 siRNA lentivirus. Although mEPSC amplitude was not changed by overexpressing or knocking down D2R, mEPSC frequency (which positively correlates with synapse number) was reduced in neurons transduced with the D2R virus and increased in neurons transduced with the Drd2 siRNA virus (Fig. 1e,f and Supplementary Fig. 1f). The change in mEPSC frequency was consistent with that in spine number in virus-injected mice. Taken together, these results indicate that D2R activation inhibits spine development.

**D2R regulates maturation and growth dynamics of spines**

Dendritic spines are generally categorized into three groups: mushroom spines with a large head and a constricted neck, thin spines with a small head and a long neck, and stubby spines without constriction between the tip and the neck. Mushroom and thin spines are the primary types of spines in adult brains, whereas stubby spines are primarily found in immature neurons. To determine the effect of D2R activation on spine morphology, we conducted a detailed spine analysis in primary hippocampal neurons.

To visualize spines, we transfected neurons at 14 d in vitro (DIV14) with a construct expressing Venus (a mutant yellow fluorescent protein). At 3 d after transfection, neurons were treated with either the D2R agonists quinpirole or bromocriptine, the D2R antagonist eticlopride, the D1R-like agonist SKF38393, or the D1R-like antagonist SCH23390 for 24 h. Consistent with our in vivo results, the density of total spines (including mushroom, thin and stubby spines) was not changed by treatment with SKF38393 or SCH23390 (Supplementary Fig. 2a,b), but was decreased in both quinpirole- and bromocriptine-treated cells (Supplementary Fig. 2c). Unlike our observations in vivo, treatment with eticlopride did not affect spine density in vitro (Supplementary Fig. 2c), likely because of the lack of dopamine neurons, and thus endogenous D2R activity, in cultured hippocampal neurons.

We further analyzed the effect of D2R agonists on different types of spines and on filopodia, thin and pointy dendritic protrusions that may be spine precursors. As a result of confocal microscopy’s limited spatial resolution, we classified all spines with a narrow neck...
Figure 2 D2R regulates the morphogenesis and growth dynamics of dendritic spines in cultured hippocampal neurons. Cultured hippocampal neurons were transfected with the Venus or indicated constructs at DIV14 and imaged at DIV17. (a) Representative images of transfected neurons (top, scale bar represents 20 µm) and dendrites at a higher magnification (bottom, scale bar represents 5 µm). (b,c) Quantification of (a). (d) Representative images of cultured hippocampal neurons. Scale bars represent 20 µm (top set of images) and 5 µm (bottom set of images). (e,f) Representative time-lapse images of neurons (top, scale bar represents 20 µm) and dendrites at a higher magnification (bottom, scale bar represents 5 µm) before (0 min) and 60 min after quinpirole treatment. (h) Quantification of the conversion between different types of dendritic protrusions during the 60-min imaging period. M, mushroom spines; T, thin spines; F, filopodia; S, stubby spines. The results were replicated in three independent experiments. Histograms show one of the three replicates (n = 15 neurons for each condition). Data are presented as mean ± s.e.m. Two-tailed Mann-Whitney test was used to calculate P values for comparison with vehicle-treated cells in b, c, h and i, and between vehicle- and quinpirole-treated cells transfected with the same construct in e and f.

and a head into a combined ‘mushroom/thin’ spine group. Notably, quinpirole and bromocriptine treatment reduced the density of mushroom/thin spines and increased filopodium density, but did not affect stubby spine density (Fig. 2a,b). Moreover, the neck of mushroom/thin spines in neurons treated with quinpirole or bromocriptine was elongated (Fig. 2a,c).

To confirm that the effects of quinpirole and bromocriptine on spines are mediated by D2R, we transfected neurons (DIV14) with a construct expressing Drd2 siRNA along with the Venus construct. At 3 d after transfection, we treated neurons with quinpirole for 24 h and analyzed dendritic protrusions in transfected neurons. Only protrusions that did not contact axons of transfected neurons were analyzed. Although transfection with the Drd2 siRNA construct did not influence spine density, spine dimension, filopodium density or quinpirole-induced elevation of filopodium density, it did abolish quinpirole’s effects on both the density and length of mushroom/thin spines (Fig. 2d–f).

To determine whether the effects of siRNAs are a result of Drd2 knockdown, we co-transfected neurons with the Drd2 siRNA construct and a construct expressing Drd2 with silent mutations in the siRNA-binding region, rendering it resistant to Drd2 siRNA (Drd2-M; Supplementary Fig. 1a,b). The effect of quinpirole on spines was restored in co-transfected cells (Fig. 2d–f), confirming that quinpirole reduces spine density by activating D2R. Given that
only D2R was only knocked down in postsynaptic neurons, these results indicate that postsynaptic D2R mediates quinpirole’s effects on mushroom/thin spines (but not on filopodium).

D2R overexpression alone enhanced quinpirole’s effects on spine density (Fig. 2d–f), suggesting that the level of D2R positively correlates with the size of quinpirole’s effect. Spine density in neurons overexpressing D1R, however, was not affected by the D1R agonist SKF38393 (Supplementary Fig. 2a,b). Thus, the ineffectiveness of SKF38393 on spines is not a result of inadequate D1R on hippocampal neurons.

Given that long spines and filopodia are usually found in immature neurons19, their increase by D2R agonist suggests that D2R activation inhibits spine maturation. To test this possibility, we imaged the same neurons (DIV17) before and 1 h after quinpirole treatment. Quinpirole treatment increased the rate of both spine addition and retraction and the conversion of mushroom/thin spines to filopodia, but reduced the conversion of filopodia to mushroom/thin spines (Fig. 2g–i). Thus, D2R activation enhances the dynamics of spine growth, inhibits the conversion of dendritic protrusions from immature to mature appearance and destabilizes mature spines. Taken together, our results from both in vivo and in vitro experiments indicate that D2R activation restricts spine number during spine development by inhibiting spine maturation.

D2R regulates spines via GluN2B and cAMP

To explore the mechanisms by which D2R regulates spines, we first tested whether D2R activation alters synaptic protein expression. Primary hippocampal neurons (DIV17) were treated with quinpirole for 24 h and then stained for the AMPA receptor subunits GluA1 and GluA2 and the presynaptic proteins Bassoon and Synaptophysin. Expression of these proteins was not affected by quinpirole (Supplementary Fig. 3a,b).

We then tested whether NMDAR is involved in the regulation of spines by D2R, as NMDAR is required for spine maturation in hippocampal neurons11,23. We treated primary hippocampal neurons with quinpirole and the NMDAR antagonist (2R)-amino-5-phosphonovaleric acid (AP5). Although AP5 did not affect either spine density or size, it did abolish quinpirole-induced changes (Fig. 3a–c). Thus, NMDAR activity is required for D2R to inhibit spine morphogenesis.

**Figure 3** GluN2B is required for D2R-mediated regulation of dendritic spines. (a–f) Cultured hippocampal neurons transfected with the Venus construct were treated with vehicle or chemicals as indicated. Representative images of transfected neurons (top, scale bars represent 20 µm) and dendrites at a higher magnification (bottom, scale bars represent 5 µm) are shown in a and d. Quantification of a is shown in b and c. Quantification of d is shown in e and f; the results were replicated in three independent experiments and histograms show one of the three replicates (n = 15 neurons for each condition). (g,h) Hippocampal slices were prepared from C57BL/6 mice (P21) that were intraperitoneally injected with chemicals as indicated and diolastically labeled. Representative images are shown in g and quantification is presented in h; n = 15 neurons from 3 slices from 3 mice for each condition. Scale bar represents 5 µm. (i,j) Cultured cortical neurons (DIV17) were treated with quinpirole and then harvested for immunoblotting. Representative cropped immunoblots for GluN1, GluN2A and GluN2B are shown in i; the full-length images are shown in Supplementary Figure 7a,b. Data are quantified in j; n = 3 experiments for each condition. Scale bar, 20 µm for images of neurons and 5 µm for images of dendrites. Data are presented as mean ± s.e.m. Two-tailed Mann-Whitney test was used to calculate P values for comparison between cells treated with indicated chemicals and those treated with the same chemical and quinpirole. Quinpirole, 1 µM; AP5, 50 µM; ifenprodil, 3 µM; Ro256981, 1 µM; TCN201, 10 µM. *P < 0.05, **P < 0.01, ***P < 0.001.
modulates NMDAR-mediated synaptic transmission, as brief D2R activation does. We analyzed evoked NMDAR-mediated excitatory postsynaptic currents (EPSCs\textsubscript{NMDA}) in hippocampal slices taken from 3-week-old mice that were intraperitoneally injected with quinpirole, bromocriptine or vehicle. EPSCs were recorded from CA1 neurons by stimulating the Schaffer collateral pathway. The input-output relationship of EPSCs\textsubscript{NMDA} was indistinguishable between vehicle- and D2R agonist–injected mice (Supplementary Fig. 3c). Thus, prolonged D2R activation does not alter NMDAR-mediated currents.

In hippocampal neurons, NMDAR is composed of GluN1, GluN2A and GluN2B subunits. To determine whether a specific NMDAR subunit mediates D2R's effects on spines, we treated primary hippocampal neurons (DIV17) with quinpirole along with the GluN2A antagonist TCN201 or the GluN2B antagonists ifenprodil or Ro25-6891. Although ifenprodil and Ro25-6891 obliterated quinpirole-induced changes in dendritic protrusions, TCN201 did not (Fig. 3d–f). Likewise, quinpirole-induced spine reduction was abolished in mice (male, C57BL/6, P21) that were intraperitoneally injected with ifenprodil or Ro25-6891, but not TCN201 (Fig. 3g,h). Thus, GluN2B, but not GluN2A, cooperates with D2R to control spine number.

To determine how GluN2B assist D2R to regulate spines, we first tested whether chronic D2R activation, similar to acute D2R activation, induces GluN2B dephosphorylation at Ser1303. The level of phosphorylated GluN2B was comparable to quinpirole-treated and control cells (Supplementary Figs. 3d,e and 7c). Similarly, after prolonged D2R activation, phosphorylation of GluA1 at Ser845, which is also reduced by acute D2R activation\textsuperscript{26}, remained unchanged (Supplementary Fig. 3d,e).

We next examined whether D2R activation influences GluN2B expression. Total GluN2B expression and that of GluN1 and GluN2A was indistinguishable between quinpirole-treated and control cells (Fig. 3i,j). A surface biotinylation assay, however, revealed that quinpirole treatment greatly reduced surface expression of GluN2B, but not that of GluN1 or GluN2A (Fig. 3i,j and Supplementary Fig. 7a,b).

To confirm the result of the biotinylation assay, we transfected cultured hippocampal neurons (DIV17) with GFP-tagged GluN2B and GluN2A, treated them with quinpirole for 24 h, and stained surface-expressed, GFP-tagged NMDAR with an antibody to GFP. On the surface of quinpirole-treated cells, we detected normal levels of GluN2A and reduced levels of GluN2B (Supplementary Fig. 3f–i). This change was likely a result of excessive GluN2B endocytosis, as it was obliterated by treatment with dynasore, a dynamin inhibitor that blocks GluN2B endocytosis\textsuperscript{17} (Supplementary Fig. 3h,i). The GluN2B antagonists ifenprodil and Ro25-6891 also blocked the quinpirole-induced decrease in surface GluN2B expression (Supplementary Fig. 3h,i). To determine whether the change in surface GluN2B is required for the effects of quinpirole on spines, we treated hippocampal neurons (DIV17, transfected with the Venus construct for 3 d) with quinpirole and dynasore. The effect of quinpirole on spines was abolished by dynasore treatment (Supplementary Fig. 3j,k). Thus, GluN2B internalization is required for D2R to regulate spine development.

Given that D2R activation reduces cAMP formation and Akt activity\textsuperscript{13}, we also tested whether these molecular changes contribute to the effects of D2R activation on spines. We treated primary hippocampal neurons (DIV17, 3 d after transfection with the Venus construct) with quinpirole and either the adenyl cyclase activator forskolin or the PI3K activator 740 Y-P. Although forskolin blocked quinpirole-induced changes in dendritic protrusions, 740 Y-P did not (Fig. 4). Treatment with either forskolin or 740 Y-P alone did not affect dendritic protrusions (Fig. 4). Thus, D2R regulates spine development through the cAMP, but not Akt, cascade. Taken together, these results indicate that both GluN2B internalization and cAMP downregulation are required to affect spine development.

**D2R-dependent spine deficiency in dysbindin mutant mice**

Disturbances of the density and functions of D2R are consistently found in the brains of schizophrenics\textsuperscript{1}. To determine whether D2R pathology contributes to spine abnormalities, we examined dendritic spines in sandy mice, which harbor a spontaneous deletion in dysbindin, express no dysbindin protein and have increased expression of D2R on the cell surface\textsuperscript{3,4}. We analyzed spines in both cultured hippocampal neurons and slices from sandy mice. Although the density of mushroom/thin spines was decreased in primary hippocampal neurons from sandy mice (DIV17, 3 d after transfection with the Venus construct), that of filopodia was increased, and mushroom/thin spines were elongated (Fig. 5a–c). Likewise, in the CA1 region of sandy mice injected with lentivirus expressing EGFP (P28, 1 week after injection), spine density was less than in their wild-type littermates (Fig. 5d,e).

To test whether elevated D2R activity in sandy mice contributes to the alteration in spines, we injected lentivirus expressing Drd2 siRNA...
into the CA1 region of sandy mice. Spine density was increased in transduced cells (Fig. 5d,e). Thus, the reduction in spine density in sandy mice stems from overactive D2R.

Given that mEPSCs are affected by D2R activation in wild-type mice (Fig. 1e,f), we asked whether it was also changed in sandy mice. mEPSCs were recorded in the CA1 region of hippocampal slices. In sandy mice, mEPSC frequency was reduced, whereas mEPSC amplitude was comparable with that in wild-type mice (Fig. 5f,g and Supplementary Fig. 4). These results are consistent with previous findings in sandy mice27. The change in mEPSC frequency in sandy mice was abolished by injecting lentivirus expressing Drd2 siRNA (Fig. 5f,g), indicating that it is caused by D2R overactivation. Moreover, D2R knockdown in sandy mice resulted in an increase in mEPSC amplitude (Supplementary Fig. 4). This effect is likely a result of the interaction of D2R with other molecules or signaling pathways altered in sandy mice, as D2R knockdown did not alter mEPSC amplitude in wild-type mice (Supplementary Fig. 1f).

Consistent with elevated surface expression of D2R in sandy mice4, the levels of cAMP in the hippocampus of sandy mice were reduced by 30.0 ± 10.6% (P = 0.045). Moreover, enhancing cAMP production by injecting forskolin rescued the spine deficiency in sandy mice (Fig. 5h,i). Forskolin injection did not later spine density in wild-type mice (Fig. 5h,i). These findings suggest that AMP reduction in sandy mice contributes to spine defects. Taken together, these results suggest that overactivation of D2R as a result of mutations in the dysbindin gene impairs spine development.

D2R regulates spine number in an age-dependent manner

To determine whether D2R also regulates spine numbers in mature hippocampal neurons, we injected vehicle or quinpirole into 2–12-week-old male C57BL/6 mice and prepared hippocampal slices for diostolic labeling at 24 h after injection. In vehicle-injected mice, spine density of CA1 neurons showed an upward trajectory from 2–8 weeks of age, then dropped slightly at 12 weeks (Fig. 6a,b). Notably, quinpirole injection caused a reduction of spine density only in mice aged 3–6 weeks, but not in those aged 2, 8 or 12 weeks (Fig. 6a,b). To confirm the effect of quinpirole in adult mice, we injected 8-week-old C57BL/6 mice with lentivirus expressing EGFP alone or with Drd2, and analyzed spines in hippocampal slices prepared from injected mice 1 week after injection. Spine density was unaffected by overexpressing Drd2 in adult mice (Supplementary Fig. 5a,b). These results indicate that D2R regulates spine number in an age-dependent manner.

Next, we examined whether spine deficiency in sandy mice is also age dependent. Although the spine density of CA1 neurons was lower in 3–6-week-old sandy mice than in their wild-type littermates, spine density in the two groups was comparable at 2, 8 and 12 weeks of age (Fig. 6c,d). To determine whether spines in adult sandy mice are regulated by D2R, as in adolescent sandy mice (Fig. 5d,e), we injected lentivirus expressing Drd2 siRNA and EGFP into the CA1 region of 8-week-old sandy mice and analyzed spines in hippocampal slices 1 week after injection. Spine density was comparable in virus-injected and control mice (Supplementary Fig. 5a,b). Thus, the effect of genetic injection of D2R on spines is also age dependent.
As all antipsychotics are D2R blockers\textsuperscript{29}, we tested whether antipsychotic treatment can ameliorate the spine deficiency in sandy mice. We injected the typical antipsychotic loxapine (a potent D2R blocker)\textsuperscript{29,30} and the atypical antipsychotic clozapine (a potent serotonin receptor antagonist and a weak D2R blocker)\textsuperscript{30,31} into sandy mice and wild-type littermates at P21. Hippocampal slices were prepared 24 h after injection for diolistic labeling of neurons. Notably, loxapine injection, which had no effect on spine density in wild-type mice, increased spine density of CA1 neurons in sandy mice to a level comparable to that in their wild-type littermates (Fig. 6e,f). Clozapine injection, however, had no effect on spine density in either sandy or wild-type mice (Fig. 6e,f). These results indicate that typical antipsychotics acting on D2R, but not atypical antipsychotics with less affinity for D2R, can rescue spine defects in sandy mice if applied during the period in which spines are sensitive to D2R activity. Taken together, these findings suggest that the effect of D2R activation on dendritic spines is age dependent and that adolescence is a critical period in which D2R hyperactivity reduces spine number.

GluN2B determines the age dependency of D2R function

During brain development, NMDARs in the forebrain switch from predominantly containing GluN2B to GluN2A\textsuperscript{25}. Given that we found that GluN2B is required for D2R-mediated spine regulation, we hypothesized that the developmental change in NMDAR composition might account for the fact that D2R no longer affects spine numbers in mature brains. To test this possibility, we first analyzed the temporal pattern of GluN2B expression in the mouse hippocampus. Immunoblotting of hippocampal lysates revealed that GluN2B expression was decreased and GluN2A expression was increased in adulthood (Supplementary Figs. 5c,d and 7d).

To determine whether the changes in GluN2A and GluN2B expression influence the effects of D2R activation on spines, we transfected young hippocampal neurons (DIV18, expressing more GluN2B and less GluN2A) with GluN2A and mature hippocampal neurons (DIV56, expressing less GluN2B and more GluN2A) with GluN2B. Although mature neurons no longer altered spine density following quinpirole treatment, overexpression of GluN2B restored their ability to respond to quinpirole (Fig. 7a–c). In contrast, deviating GluN2A expression from its normal developmental trajectory, either by overexpressing GluN2A in young neurons or by blocking GluN2A activity with TCN201 in mature neurons, left quinpirole’s effects on spines unchanged (Fig. 7). These results indicate that the decrease in GluN2B rather than the increase in GluN2A expression that prevents mature neurons from altering their spine density following D2R activation.

Adolescent D2R hyperactivity impairs neural connectivity

Although spine density returns to normal in adult sandy mice that experienced spine loss during adolescence as a result of D2R hyperactivity, perturbation of dendritic spines during this period may permanently alter these circuits, given that adolescence is a period in which neuronal circuits mature and are refined\textsuperscript{32,33}. To test this possibility, we examined the entorhinal cortex–CA1 connection, as neurons in the layer III of the entorhinal cortex send mono-synaptic projections to CA1 neurons\textsuperscript{34,35}.

The retrograde tracer cholera toxin subunit B (CTB) was injected into the CA1 region of the right hippocampus. At 24 h after injection, horizontal brain sections containing the entorhinal cortex were prepared. In wild-type mice, neurons retrogradely labeled by CTB were found in both the medial entorhinal cortex (MEC) and lateral entorhinal cortex (LEC) (Fig. 8a). In contrast, the number of labeled neurons was markedly reduced in the MEC, but increased in the LEC, of sandy mice, and the ratio of labeled MEC-to-LEC neurons was therefore decreased (Fig. 8a–c). To test whether these changes are a result of D2R hyperactivity during adolescence, we intraperitoneally injected sandy mice with eticlopride from P21 to P35 (once daily) and injected them with CTB at 8 weeks of age. Notably, eticlopride treatment restored the ratio of labeled MEC-to-LEC neurons (Fig. 8a,b). We also tested the effect of feeding sandy mice water supplemented with eticlopride (ad libitum) from P21 to 35. Given that feeding and injecting eticlopride had comparable effects on the ratio of labeled MEC-to-LEC neurons (Supplementary Fig. 6a), we merged the data from the injected and fed groups (Fig. 8b,c).

To determine whether the effect of eticlopride on neuronal connectivity in sandy mice is age dependent, we fed adult sandy mice eticlopride from P56 to P70 and injected them with CTB at 13 weeks of age. The ratio of labeled MEC-to-LEC neurons was comparable in treated and untreated sandy mice (Fig. 8a–c). Thus, eticlopride treatment in adolescence, but not in adulthood, can correct connectivity in the entorhinal cortex–hippocampal circuit in sandy mice.
These findings indicate that adolescent D2R hyperactivity causes perturbations of adult neural circuits.

To corroborate the results obtained with sandy mice, we examined the effect of adolescent D2R hyperactivity on the entorhinal cortex–hippocampal connection in wild-type mice. Wild-type mice were fed quinpirole from P21 to P28 and injected with CTB at 8 weeks of age. Quinpirole treatment resulted in a decrease in the number of retrogradely labeled cells in the MEC and in the ratio of labeled MEC-to-LEC neurons (Fig. 8a–c and Supplementary Table 1). In contrast, quinpirole treatment in adult mice (from P56 to P63 and injected with CTB at 12 weeks of age) did not change the ratio of labeled MEC-to-LEC neurons (Supplementary Fig. 6b). These results confirm that adolescent D2R hyperactivity adversely affects the entorhinal cortex–hippocampal connectivity. Taken together, our findings indicate that adolescent D2R hyperactivity disturbs the establishment of adult patterns of neural connectivity and that treatments need to be administered during adolescence to alleviate this effect.

Adolescent D2R hyperactivity impairs working memory

The entorhinal cortex–hippocampal circuit is essential for spatial working memory. To determine whether D2R hyperactivity affects spatial working memory, we conducted behavioral tests with sandy mice that were fed eticlopride and wild-type mice that were fed quinpirole during adolescence. At 8 weeks of age, we conducted the spontaneous Y maze test to analyze spontaneous spatial working memory and the open field test to measure locomotor activity. Wild-type and sandy mice given untreated water were used as controls. In the Y maze test, sandy mice had a lower alternation score, corresponding to poorer spatial working memory, than wild-type control mice (Fig. 8d). The performance of wild-type mice fed quinpirole in the Y maze was also reduced (Fig. 8d), suggesting that overactivation of D2R impairs working memory. Notably, the alternation score of sandy mice was improved by eticlopride treatment during adolescence (Fig. 8d).

In the open field test, sandy mice traveled longer distances than wild-type mice (Fig. 8e), consistent with their reported hyperactivity. Eticlopride treatment during adolescence, however, did not change sandy mice's locomotor activity (Fig. 8e). For wild-type mice, locomotion tested at 4 weeks after feeding with quinpirole during adolescence was comparable with that in untreated mice (Fig. 8e and Supplementary Table 2), suggesting that hyperactivity might be caused by acute, but not long-lasting, effects of D2R activation on brains.

To determine whether eticlopride treatment in adult sandy mice can also improve working memory, we fed 8-week-old sandy mice eticlopride–supplemented water for 2 weeks and conducted behavioral tests at 13 weeks of age. In contrast with the improvement seen when eticlopride was applied in adolescence, working memory performance was not changed (Fig. 8d). Hyperactivity in sandy mice, however, was ameliorated by eticlopride treatment in adulthood (Fig. 8e), excluding the possibility that eticlopride treatment in adulthood has no effect on behavior.

Taken together, these findings indicate that overactivation of D2R during adolescence causes impairments of spatial working memory in adulthood. Blocking D2R during this critical period in sandy mice, moreover, improves their spatial working memory thereafter.
D2R hyperactivity during adolescence impairs the entorhinal-hippocampal circuit and spatial working memory. Sandy mice were treated with eticlopride (5 µg ml⁻¹) during either adolescence (P21–35) or adulthood (P56–70). Wild-type mice were treated with quinpirole (2.5 µg ml⁻¹) from P21 to P28. At 3–4 weeks after treatment, mice were used for the retrograde tracing experiment and behavioral tests. (a) Representative images of retrogradely labeled neurons in the entorhinal cortex. Scale bar represents 100 µm. (b) The ratio of retrogradely labeled MEC to LEC neurons. (c) The number of retrogradely labeled neurons in each section (150 µm in length along the MEC-LEC axis) of the entorhinal cortex normalized to the total number of labeled neurons in the entorhinal cortex. O in the x axis indicates the border between the MEC and LEC. n = 5 mice for each condition. (d) Alternation scores from the Y maze test. (e) Total distance traveled during 30 min in the open field test. Pooled data from two independent experiments are shown in d and e. Data are presented as mean ± s.e.m. Mann-Whitney test was used for statistical analysis. P values less than 0.05 for comparison between untreated and eticlopride-fed sandy mice (blue), between sandy and wild-type mice (yellow), and between untreated and quinpirole-fed wild-type mice (orange) are shown in c; P values less than 0.05 for comparison between sandy and wild-type mice are shown in e; the complete list of P values for c and e is provided in Supplementary Table 1. *P < 0.05, **P < 0.01.

**DISCUSSION**

Our results describe an age-dependent function of D2R in controlling spine development. We found that D2R hyperactivity during the critical period when spines are subject to regulation by D2R impairs the establishment of the entorhinal-hippocampal circuit and working memory in adult mice. Our results consistently confirmed the theory that overactivation of D2R during adolescence impairs spine development, neural circuits and working memory. We excluded Drd2 and Drd1 knockout mice from our study because we feared that their retarded growth, reduced body weight, smaller brains and shorter dendrites in cortical neurons indicated global developmental defects.

The effect of intraperitoneal injection of D2R agonist on spine density of hippocampal neurons could be caused by activation of D2R on postsynaptic neurons, on presynaptic neurons or in extra-hippocampal areas. The results of our transfection of Drd2 siRNA into postsynaptic hippocampal neurons in vitro and D2R overexpression or knockdown in vivo indicate that the spine phenotype is a result of D2R’s actions on postsynaptic neurons. Filopodia, however, might be regulated by presynaptic D2R, as postsynaptic D2R knockdown did not change D2R agonist–induced filopodium overgrowth.

Our results indicate that NMDAR, particularly receptors containing the GluN2B subunit, is required for D2R to induce spine changes. Unlike brief D2R activation, however, chronic D2R activation did not affect NMDAR-mediated currents or GluN2B phosphorylation. It was D2R activation–induced GluN2B endocytosis that was required for the effect of D2R on spines. In addition to GluN2B, we found that forskolin blocked the effect of D2R activation on spines, consistent with the known role of cAMP in spine morphogenesis.

In both wild-type and sandy mice, we found that D2R regulated spines only during postnatal week 3–6, the period of active spine growth, synaptogenesis and adolescent development in mice. Moreover, we found that it was the developmental decrease in GluN2B expression that prevented spines in mature brains from changing in response to D2R activation. Regulation of spines by D2R during adolescence had robust effects on the shaping of neuronal circuits. These effects were retained and manifested in adulthood. Adolescence precedes early adulthood, the usual age of onset for schizophrenia in humans. By extrapolation, D2R-dependent spine morphogenesis may be important for the pathophysiology of schizophrenia.

Our finding that D2 blockers administered during adolescence in mice can prevent D2R hyperactivity–induced impairments in spine development and working memory suggests that young human subjects with D2R hyperactivity, perhaps from mutations in the *dysbindin* gene, could benefit from such D2R antagonism. Further studies examining the effects of D2R activation in human prefrontal cortex and in animal models of schizophrenia will help elucidate the role of D2R in the pathophysiology of schizophrenia.
gene, might benefit from prodromal medication that targets D2R to protect spine development and cognition. Further studies in humans will be needed to test this possibility.

METHODS
Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS
We thank H. Arnheiter (NINDS/NIH) for critical discussion of the manuscript and E.J. Sherman for editing the manuscript. This work was supported by the Intramural Research Program of the National Institute of Mental Health (ZIA MH002381 to Z.L.).

AUTHOR CONTRIBUTIONS
J.-M.J. conducted the experiments and data analysis. J.Z. analyzed mEPSCs. D.L. contributed to the CTB experiment. Z.L. and J.-M.J. designed the experiments and wrote the manuscript. Z.H. generated the constructs and lentivirus expressing Drd1, Drd2 and the siRNAs against them.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Howes, O.D. & Kapur, S. The dopamine hypothesis of schizophrenia: version III—the final common pathway. Schizophr. Bull. 35, 549–562 (2009).
2. Miyamoto, S., Duncan, G.E., Marx, C.E. & Lieberman, J.A. Treatments for schizophrenia: a critical review of pharmacology and mechanisms of action of antipsychotic drugs. Mol. Psychiatry 10, 79–104 (2005).
3. Iizuka, Y., Sai, Y., Weinberger, D.R. & Straub, R.E. Evidence that the BLOC-1 protein dysbindin modulates dopamine D2 receptor internalization and signaling but not D1 internalization. J. Neurosci. 27, 12390–12395 (2007).
4. Ji, Y. et al. Role of dysbindin in dopamine receptor trafficking and cortical GABA function. Proc. Natl. Acad. Sci. USA 106, 19593–19598 (2009).
5. Green, M.F. Stimulating the development of drug treatments to improve cognition in schizophrenia. Annu. Rev. Clin. Psychol. 3, 159–180 (2007).
6. Stephan, K.E., Balderweg, T. & Friston, K.J. Synaptic plasticity and disconnection in schizophrenia. Biol. Psychiatry 59, 929–939 (2006).
7. Garey, L.J. et al. Reduced dendritic spine density on cerebral cortical pyramidal neurons in schizophrenia. J. Neurosci. 25, 446–453 (1998).
8. Glantz, L.A. & Lewis, D.A. Decreased dendritic spine density on prefrontal cortical pyramidal neurons in schizophrenia. Arch. Gen. Psychiatry 57, 65–73 (2000).
9. Kolomeets, N.S., Orlovskaya, D.D., Rachmanova, V.I. & Uranova, N.A. Ultrastructural alterations in hippocampal mossy fiber synapses in schizophrenia: a postmortem morphometric study. Synapse 57, 47–55 (2009).
10. Kolluri, N., Sun, Z., Sampson, A.R. & Lewis, D.A. Lamina–specific reductions in dendritic spine density in the prefrontal cortex of subjects with schizophrenia. Am. J. Psychiatry 162, 1200–1202 (2005).
11. Li, Z. & Sheng, M. Some assembly required: the development of neuronal synapses. Nat. Rev. Mol. Cell Biol. 4, 833–841 (2003).
12. Bremond, K.J. et al. Modelling schizophrenia using human induced pluripotent stem cells. Nature 473, 221–225 (2011).
13. Bonci, A. & Hapf, F.W. The dopamine D2 receptor: new surprises from an old friend. Neuron 47, 335–338 (2005).
14. Beazely, M.A. et al. D2-class dopamine receptor inhibition of NMDA currents in prefrontal cortical neurons is platelet-derived growth factor receptor–dependent. J. Neurochem. 98, 1657–1663 (2006).
15. Kotecha, S.A. et al. A D2 class dopamine receptor transactivates a receptor tyrosine kinase to inhibit NMDA receptor transmission. Neuron 35, 1111–1122 (2002).
16. Liu, X.Y. et al. Modulation of D2R-NR2B interactions in response to cocaine. Neuron 52, 897–909 (2006).
17. Li, Y.C., Xi, D., Roman, J., Huang, Y.Q. & Gao, W.J. Activation of glycogen synthase kinase-3 beta is required for hypodopamine and D2 receptor-mediated inhibition of synaptic NMDA receptor function in the rat prefrontal cortex. J. Neurosci. 29, 15551–15563 (2009).
18. Boyer, C., Schikorski, T. & Stevens, C.F. Comparison of hippocampal dendritic spines in culture and in brain. J. Neurosci. 18, 5294–5300 (1998).
19. Peters, A. & Kaiserman–Abramoff, I.R. The small pyramidal neuron of the rat cerebral cortex. The perikaryon, dendrites and spines. Am. J. Anat. 127, 321–355 (1970).
20. Harris, K.M. Structure, development, and plasticity of dendritic spines. Curr. Opin. Neurobiol. 9, 343–348 (1999).
21. Nagai, T. et al. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. Nat. Biotechnol. 20, 87–90 (2002).
22. Yuste, R. & Bonhoeffer, T. Genesis of dendritic spines: insights from ultrastructural and imaging studies. Nat. Rev. Neurosci. 5, 24–34 (2004).
23. Bourne, J.N. & Harris, K.M. Balancing structure and function at hippocampal dendritic spines. Annu. Rev. Neurosci. 31, 47–67 (2008).
24. Tseng, K.Y. & O’Donnell, P. Dopamine-glutamate interactions controlling prefrontal cortical pyramidal cell excitability involve multiple signaling mechanisms. J. Neurosci. 24, 5131–5139 (2004).
25. Traynelis, S.F. et al. Glutamate receptor ion channels: structure, regulation and function. Pharmacol. Rev. 62, 405–496 (2010).
26. Håkansson, K. et al. Regulation of phosphorylation of the GluR1 AMPA receptor by dopamine D2 receptors. J. Neurochem. 96, 482–488 (2006).
27. Chen, X.W. et al. DnTNP1, a schizophrenia susceptibility gene, affects kinetics of transmitter release. J. Cell Biol. 181, 791–801 (2008).
28. Arranz, M.J. & de Leon, J. Pharmacogenetics and pharmacogenomics of schizophrenia: a review of last decade of research. Mol. Psychiatry 12, 707–747 (2007).
29. Glazer, W.M. Does loxapine have “atypical” properties? Clinical evidence. J. Clin. Psychiatry 60 (suppl. 10), 42–46 (1999).
30. Giovanni, G.D., Matteo, V.D. & Esposito, E. Serotonin-Dopamine Interaction: Experimental Evidence and Therapeutic Relevance (Elsevier Science, 2008).
31. Meltzer, H.Y. & Massey, B.W. The role of serotonin receptors in the action of atypical antipsychotic drugs. Curr. Opin. Pharmacol. 11, 59–67 (2011).
32. Tau, G.Z. & Petersen, B.S. Normal development of brain circuits. Neuropsychopharmacology 35, 147–168 (2010).
33. Jaaro-Peled, H. et al. Neurodevelopmental mechanisms of schizophrenia: understanding disturbed postnatal brain maturation through neuregulin-1–Erbb4 and DISC1. Trends Neurosci. 32, 485–495 (2009).
34. Suh, J., Rivest, A.J., Nakashiba, T., Tominaga, T. & Tonegawa, S. Entorhinal cortex layer III input to the hippocampus is crucial for temporal association memory. Science 334, 1415–1420 (2011).
35. Andersen, P., Morris, R., Amaral, D., Bliss, T. & O’Keefe, J. The Hippocampus Book (Oxford University Press, 2007).
36. Schultz, H., Sommer, T. & Peters, J. Direct evidence for domain-sensitive functional subregions in human entorhinal cortex. J. Neurosci. 32, 4716–4723 (2012).
37. Hasselmo, M.E. & Stern, C.E. Mechanisms underlying working memory for novel information. Trends Cogn. Sci. 10, 487–493 (2006).
38. Cho, Y.H. & Jaffard, R. Spatial location learning in mice with ibotenic lesions of the entorhinal cortex or subiculum. Neurobiol. Learn. Mem. 64, 285–290 (1995).
39. Papaleo, F. et al. Dysbindin-1 modulates prefrontal cortical activity and schizophrenia-like behaviors via dopamine/D2 pathways. Mol. Psychiatry 17, 85–98 (2012).
40. Talbot, K. The sandy (sdy) mouse: a dysbindin-1 mutant relevant to schizophrenia research. Prog. Brain Res. 179, 87–94 (2009).
41. Bakl, J.H. et al. Parkinson-like locomotor impairment in mice lacking dopamine D2 receptors. Nature 377, 424–428 (1995).
42. Drago, J. et al. Altered striatal function in a mutant mouse lacking D1A dopamine receptors. Proc. Natl. Acad. Sci. USA 91, 12564–12568 (1994).
43. Wang, H.D., Stanwood, G.D., Grandy, D.K. & Deutch, A.Y. Dysdopaminergic dendrites in prefrontal cortical pyramidal cells of dopamine D1 and D2, but not D4, receptor knockout mice. Brain Res. 1300, 58–64 (2009).
44. Xi, M. et al. Dopamine D1 receptor mutant mice are deficient in striatal expression of dynorphin D2 receptors. J. Neurochem. 96, 729–742 (1994).
45. Ji, Y., Pang, P.T., Feng, L. & Lu, B. Cyclic AMP controls BDNF-induced TrkB phosphorylation and dendritic spine formation in mature hippocampal neurons. Nat. Neurosci. 8, 164–172 (2005).
46. Fox, J.G. et al. The Mouse in Biomedical Research (Academic Press, 2007).
47. Lewis, D.A. & Levitt, P. Schizophrenia as a disorder of neurodevelopment. Annu. Rev. Neurosci. 25, 409–432 (2002).
ONLINE METHODS

Animals, plasmids and reagents. All animal procedures followed the US National Institutes of Health guidelines Using Animals in Intramural Research and were approved by the National Institutes of Mental Health Animal Care and Use Committee. Four mice were housed in one cage in a room with a 12-h light/dark cycle. Sandy mice that had been backcrossed with the C57BL/6 mice for more than ten generations were purchased from the Jackson Laboratory. Mice were intraaperitoneally injected with various drugs at a volume of 10 mg kg⁻¹. Annulled oligos containing siRNA sequences targeted to rat Drd2 (Drd2 siRNA-1: 1138′–CTCGTGCTGTTCTCAGTCT–1156′, a region conserved between rats and mice; Drd2 siRNA-2: 1269′–CCCCCATCATCCTACACC–1′; Drd2 siRNA-3: 530′–CAGCAGCATGATGTGATG–548′) and mouse Drd1 (1075′–GAGACTGTAAGCATCAACA–1095′), and the cDNAs of myc-tagged Drd1 and HA-tagged were inserted into the pcSuper and the pPRlRin lentiviral vectors. cDNAs for Drd2, Beclin1, ATG5 and siRNA-resistant Drd2 (C1140G, G1146T, C1149T, C1157T, generated by mutagenesis using the KOD kit, Novagen) were cloned into the pcGW vector behind the HA tag. The N-terminal-tagged GluN2A and GluN2B constructs were kind gifts from K. Roche (National Institute of Neurological Disorders and Stroke). The Drd1 cDNA was obtained by PCR from a construct purchased from Origene and cloned into the pcGW vector behind the myc tag. The following reagents were obtained commercially: quinpirole, eticlopride,loxapine, temprodil, Ro25-6981, dynasore and antibody to actin (1:1000, A2044). Anti-PRKCA, anti-p44/42 MAPK, anti-p38 MAPK, anti-GAPDH, anti-SPCP3, anti-GluN1 from BD Pharmingen (1:500, 556308); antibody to GluN2B from Upstate (1:1000, 06-600); antibody to Bassoon from Assay designs (1:1000, VAM-PS003), antibody to GluA2 from Chemicon (1:1000, MAB397). DiI was purchased from Sigma (D282).

Acute hippocampal slices. Mice were decapitated after anesthetization with an overdose of isoflurane. The brain was placed in ice-cold artificial cerebrospinal fluid (ACSF; pH 7.4, bubbled with 95% O₂, 5% CO₂), which consisted of 124 mM NaCl, 26 mM NaHCO₃, 1.25 mM NaH₂PO₄, 2.5 mM CaCl₂, 1.3 mM MgSO₄, and 10 mM d-glucose. Transverse hippocampal slices were sectioned in ice-chilled, oxygenated ACSF with a Leica VT1000S vibratome. Cells were cultured in the CA2 area, and the recording electrode was placed on the cell body of CA1 neurons. Stimuli were delivered to the electrode at an interval of 20 s. Bicuculline (10 µM) was added to the ACSF to block GABA_A receptors. The patch pipette (3–5 MΩ) solution was composed of 130 mM cesium methanesulfonate, 8 mM NaCl, 4 mM Mg-ATP, 0.3 mM Na-GTP, 0.5 mM EGTA, 10 mM HEPES and 5 mM QX-314 at pH 7.3. EPCs_MDA was recorded at a holding potential of +40 mV. EPCs_MDA was measured as the peak amplitude of EPCs in the presence of the AMPA receptor blocker NBQX (20 µM), or measured at ~50–70 ms after the peak of EPCs_MPA without NBQX. Given that EPCs_MDA measured with the NBQX and the kinetics methods were comparable, we pooled data from both methods for statistical analysis. For mEPSC analysis, action potentials were blocked by adding 1 µM tetrodotoxin to ACSF. The series resistance and input resistance were monitored on-line and analyzed with the Clampex program off-line. Only cells with a series resistance of <25 MΩ and a <10% drift in both series resistance and input resistance during the recording period were included.

Primary hippocampal neuron culture, transfection and immunocytochemistry. Hippocampal and cortical neuron cultures were prepared from embryonic day 18–19 rat embryos as previously described. Hippocampal neurons were seeded on coverslips coated with 30 µg ml⁻¹ of poly-d-lysine and 5 µg ml⁻¹ of laminin at a density of 330 cells per mm². Cortical neurons were seeded on plates coated with 30 µg ml⁻¹ poly-d-lysine at a density of 650 cells per mm². Neurons were transfected with Lipofectamine 2000 (Invitrogen). For immunocytochemistry of synaptic proteins, neurons were fixed with 4% formaldehyde in PBS containing 4% sucrose, and incubated with primary antibodies in blocking buffer containing 0.3% Triton X-100 (vol/vol) overnight at 4 °C, followed by staining with Alex 488- or Alex 555-conjugated secondary antibodies. For surface staining of GFP-GluN2A/GluN2B, neurons were incubated with an antibody to GFP in blocking buffer without Triton X-100 overnight at 4 °C, then incubated with an Alex 488-conjugated secondary antibody to label surface GFP-GluN2A/GluN2B. After rinse, cells were permeabilized and incubated with the antibody to GFP again at 25 °C for 2 h, then with an Alex 555-conjugated secondary antibody to label intracellular GFP-GluN2.

cAMP assay. Hippocampi were removed from the mouse brain and homogenized with a motor-driven homogenizer for 20 strokes in the homogenization buffer provided by the cAMP assay kit (R&D Systems, KGE002B). The homogenates were sonicated with a Virstec cell dismuter (Model 16-850) and measured for protein concentration. 100 µg proteins were used for each cAMP assay according to the manufacturer's manual.

Surface biotinylation assay. Primary cortical neurons were cultured on 10-cm plates and treated with quinpirole at DIV17. At 24 h after treatment, neurons were chilled on ice, incubated with 0.25 mg ml⁻¹ EZ-link sulfo-NHS- LC-LC-Biotin for 45 min on ice. After rinse with ice-cold PBS (containing 0.1 mM Ca²⁺, 4 mM Zn²⁺), extra EZ-link sulfo-NHS-LC-LC-Biotin was quenched with 10 mM glycine. Neurons were washed with cold PBS, lysed in RIPA buffer and analyzed for protein concentration. Cell lysates containing the same amount of proteins were incubated with streptavidin for 2 h at 4 °C and centrifuged. Precipitates were washed three times with RIPA buffer, resuspended in 2× SDS-PAGE gel-loading buffer, and boiled at 95 °C for 5 min for immunoblotting. Image was used for quantification of immunoblots.

Image acquisition and dendritic spine analysis. Confocal images were acquired by using a Zeiss LSM510 confocal microscope with a 63× (NA 1.4) objective for cultured neurons and an Olympus FV1000 confocal microscope with a 60× (NA 1.35) objective for hippocampal slices and time-lapse imaging. Images were collapsed into two-dimensional projections with LSM 5 browser and Olympus fluoview version 2.1c viewer and analyzed with Metamorph software (Molecular Devices) for spine density, spine dimension and integrated fluorescence intensity of stained protein. Images were acquired at 1.024 × 1.024 p.p.i. resolution. Two or three secondary or tertiary dendrites (50–200 µm long, 20–100 µm from the soma) from each neuron were analyzed for spine analysis. Dendrites, the width of spine head (the diameter of spine heads’ largest section that is perpendicular to spine necks) and the length of spine neck (from the bottom of spine heads to the junction between spines and dendritic shafts) were traced manually and measured by Metamorph. The number of dendritic protrusions were counted manually. Images were taken and analyzed blind to the treatment.

Reverse transcription PCR (RT-PCR). Cultured cortical neurons were lysed in Trizol. The mRNA was extracted and transcribed into cDNAs with the oligo(dt) 20 primer. The following primers (Drd1, 5′ primer: CATTCTGAAACTCTCGGTGA, 3′ primer: GTGTGCTACCTCGTGTTCT; Drd2, 5′ primer: CATTGTCTGGTCCTTGTCCT and 3′ primer: GCCAGCAGATGAGGATGA; Actin: 5′ GCTCCTCGTGAGGGCAAGTACTC, 3′ CTCATGCTACTCTCGTGTGCTG) were used for RT-PCR.

Brain injection and sectioning. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg per kg of body weight) and xylazine (10 mg per kg), and mounted in a stereotaxic apparatus. 1 µl lentivirus or 200 nl CTB–Alexa 555 (1 mg ml⁻¹, Invitrogen) was injected into the CA1 area of the right hippocampus using a Hamilton syringe (1.8 mm caudal to bregma, 1.5 mm lateral to the midline, 2.1 mm below the surface of the cerebral cortex). After 7 d (for lentivirus injection) or 24 h (for CTB injection) after injection, mice were perfused with...
4% paraformaldehyde in PBS and cut coronally (for spine analysis) or horizontally (for CTB tracing), using a cryostat, into 50-µm sections. Brain sections were mounted onto slides in mounting media with DAPI (for CTB injection) or without DAPI (for lentivirus injection), and imaged with a Zeiss confocal microscope (63× for lentivirus injection and 10× for CTB injection).

**Behavioral tests.** Mice for behavior tests were housed under a reversed 12-h light/dark cycle and tested during the dark phase. Mice were transferred to the test room at 1 h before the behavioral test for acclimatization. The behavioral apparatus was cleaned with 70% ethanol (vol/vol) and dried with paper towels after each trial. For the Y maze test, mice were placed at the end of one of the three arms of a Y maze with three identical arms that were 120° apart, and allowed to explore the arena freely for 8 min. The number and sequence of arm entries were recorded manually. A consecutive entry into three different arms was counted as an alternation. The alternation score was computed with the formula: [(total number of alternations)/(total number of arm entries) – 2] × 100. Mice staying in one arm for longer than 2 consecutive minutes or with less than 12 arm entries were excluded from the statistical analysis. The open field test was conducted 1 d after the Y maze test. Mice were placed in the center of the test chamber (49 × 49 × 40 cm) illuminated at 20 lx, and allowed to explore the chamber for 30 min. Mice were videotaped during the test. The total travel distance was analyzed with the TopScan software (Clever System). Mice were randomly assigned to the various experimental groups. Behavioral tests were video-recorded and analyzed blind to the treatment.

**Statistical analysis.** No statistical methods were used to predetermine, but our sample sizes are similar to those reported in previous publications. The data were analyzed with the Kolmogorov-Smirnov and χ² test for distribution. As all our data were not normally distributed, the two-tailed Mann-Whitney test was used to calculate P values. The results of statistical tests were not corrected for multiple comparisons.

48. Tabuchi, K. et al. A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* **318**, 71–76 (2007).
49. Fiorentino, H. et al. GABA(B) receptor activation triggers BDNF release and promotes the maturation of GABAergic synapses. *J. Neurosci.* **29**, 11650–11661 (2009).
50. Cai, Y.Q. et al. Central amygdala GluA1 facilitates associative learning of opioid reward. *J. Neurosci.* **33**, 1577–1588 (2013).
51. Fukata, Y. et al. Local palmitoylation cycles define activity-regulated postsynaptic subdomains. *J. Cell Biol.* **202**, 145–161 (2013).
52. Wikkie, M.P. et al. The relationship between NMDA receptor function and the high ammonia tolerance of anoxia-tolerant goldfish. *J. Exp. Biol.* **214**, 4107–4120 (2011).
53. Li, Z. et al. Caspase-3 activation via mitochondria is required for long-term depression and AMPA receptor internalization. *Cell* **141**, 859–871 (2010).
54. O’Brien, J.A. & Lummis, S.C. Diolistic labeling of neuronal cultures and intact tissue using a hand-held gene gun. *Nat. Protoc.* **1**, 1517–1521 (2006).
55. Chan, L., Terashima, T., Urabe, H., Lin, F. & Kojima, H. Pathogenesis of diabetic neuropathy: bad to the bone. *Ann. NY Acad. Sci.* **1240**, 70–76 (2011).
56. Jiao, S. & Li, Z. Nonapoptotic function of BAD and BAX in long-term depression of synaptic transmission. *Neuron* **70**, 758–772 (2011).
57. Belforte, J.E. et al. Postnatal NMDA receptor ablation in corticolimbic interneurons confers schizophrenia-like phenotypes. *Nat. Neurosci.* **13**, 76–83 (2010).