

Figure 1. The lesion, presenting on the left side of the dorsal caudal medulla. Part B is taken from Olszewski J, Baxter D. *Cytoarchitecture of the Human Brain Stem*. 2nd ed. Basel, NY: Karger; 1982.

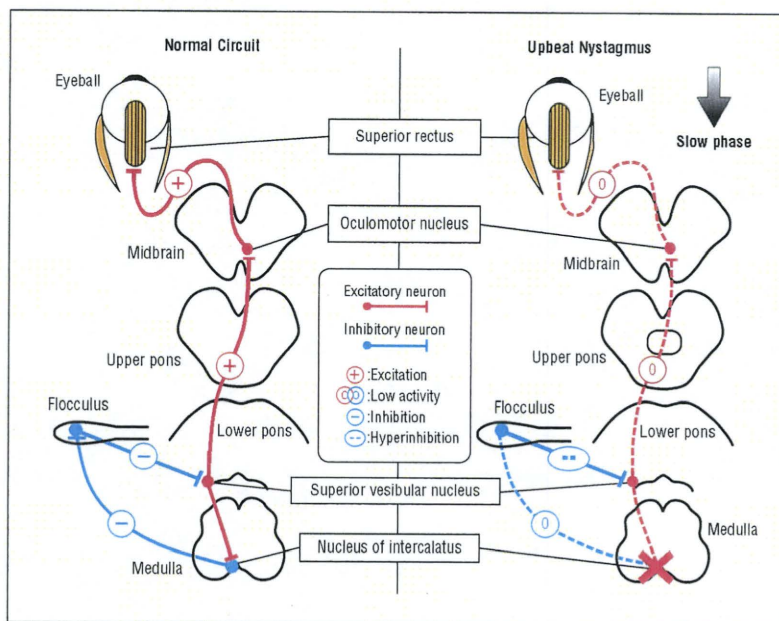


Figure 2. Schematic diagram. Modified with permission from *Brain*.⁴

Anti-N-methyl-D-aspartate Receptor Encephalitis During Pregnancy

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Objective: To report 3 patients who developed anti-N-methyl-D-aspartate receptor encephalitis during pregnancy.

Design: Case reports.

Setting: University hospitals.

Patients: Three young women developed at 14, 8, and 17 weeks of gestation acute change of behavior, prominent psychiatric symptoms, progressive decrease of consciousness, seizures, dyskinesias, and autonomic dysfunction.

Main Outcome Measures: Clinical, radiological, and immunological findings.

Results: The 3 patients had cerebrospinal fluid pleocytosis, normal magnetic resonance imaging, and electroencephalogram showing slow activity. All had higher antibody titers in cerebrospinal fluid than in serum and 2 had ovarian teratomas that were removed. The pregnancy was terminated in 1 patient with recurrent bilateral teratomas. All patients had substantial neurological recoveries, and the 2 newborns were normal. Results of extensive antibody testing in 1 of the babies were negative.

Conclusion: The current study shows that anti-NMDAR encephalitis during pregnancy can have a good outcome for the mother and newborn.

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ANTI-N-METHYL-D-ASPARTATE receptor (NMDAR) encephalitis is a synaptic autoimmune disorder that is likely mediated by antibodies against the NR1 subunit of the receptor.¹ Despite the severity of the disorder, most patients have substantial recoveries. Because the disease frequently affects women of childbearing age and the antibody subtypes (IgG1, IgG3) can cross the placenta, there is concern about the effects of the disorder during pregnancy.² We report 3 patients who developed the disorder during pregnancy.

REPORT OF CASES

Clinical features from the patients are described in this section and summarized in the **Table**. Antibodies to NMDAR were detected as reported elsewhere¹ and the titers were determined by serial dilution (starting at 1:10). The baby of patient 1 had antibody studies in the umbilical cord, serum, cerebrospinal fluid (CSF), and amniotic fluid.

CASE 1

A 19-year-old woman presented at 14 weeks of gestation with 2 weeks of headache and malaise followed by bizarre behavior and paranoid

delusions resulting in hospitalization. Over the course of a week, her mental status worsened until she was minimally responsive. She had a generalized seizure that was treated with fosphenytoin and lorazepam, and she was intubated for airway protection. A bedside electroencephalogram (EEG) revealed generalized slowing but no epileptic activity. On examination, she was minimally responsive to noxious stimuli, had generalized hyperreflexia, and moved all limbs spontaneously. Results of magnetic resonance imaging (MRI) and CSF studies are described in the **Table**. Treatment with acyclovir was started for presumptive viral encephalitis.

On the third day in the intensive care unit, she developed repetitive pursing of the lips and furrowing of her brow without EEG correlates. These movements became more frequent and the dyskinesias spread to her limbs. By day 8, she developed diaphoresis, tachycardia, mydriasis, and hypertension. These symptoms were difficult to control despite treatment with fentanyl, lorazepam, propofol, bromocriptine, and β -blockers.

On day 5, a tracheostomy was performed. Treatment with intravenous immunoglobulin did not result in improvement. On day 23, an MRI of the abdomen and pelvis revealed a 2.5 × 3-cm left ovarian simple cyst. On day 43, NMDAR antibodies were identified in CSF. A second course of intravenous immunoglobulin treatment along with 1 g of methylprednisolone was given daily for 5 days. On day 50,

Table. Clinical Information and Antibody Titers

Patient	First Symptom of Encephalitis During Pregnancy	Other Symptoms	Teratoma	EEG	CSF WBC Count, / μ L ^a	Week of Pregnancy, Antibody Titers in Serum and CSF	Outcome ^b
1	14th wk: Headache, malaise, bizarre behavior	Seizure, orofacial and limb dyskinesias, autonomic instability, minimally responsive	Left immature teratoma	Slow activity	244	14th wk: serum, ND; CSF, 1:80; 21st wk: serum, ND; CSF, 1:10; 32nd week: serum, ND; CSF, ND; baby at birth: serum, CSF, and umbilical cord blood, ND	Cesarean section at week 38; healthy baby; home on day 184; substantial recovery at 2-mo follow-up
2	8th wk: Abnormal, stereotyped behavior	Orofacial dyskinesias, autonomic dysfunction, seizures, minimally responsive, respiratory depression	Bilateral mature teratomas	1-Hz spikes, slow activity predominantly in frontal lobes	57	10th wk: serum, ND; CSF, 1:320; 13th week: serum, ND; CSF, 1:40	Bilateral tumor removal; pregnancy terminated; right ovary left to preserve fertility; home with minimal deficits on day 87
3	17th wk: Affective and behavioral change	Orofacial movements, tonic seizure without EEG correlate, episodes of agitation alternating with minimal response	No tumor or cyst	Generalized high-amplitude slow activity, 2 Hz	11	19th wk: serum, 1:320; CSF, 1:640; 5 mo postdelivery: serum, 1:80; CSF, NA	Spontaneous delivery at 37 wk of pregnancy; healthy baby; home 23 wk after symptom presentation; full recovery at last follow-up

Abbreviations: CSF, cerebrospinal fluid; EEG, electroencephalogram; NA, not applicable; ND, not detectable (<1:10); NMDAR, *N*-methyl-D-aspartate receptor; WBC, white blood cell.

^aNormal, $\leq 4/\mu$ L. All 3 patients had normal CSF protein and glucose concentrations.

^bDays relate to duration of stay in the hospital.

a left oophorectomy was performed, revealing an immature teratoma. On day 52, plasmapheresis was initiated, with a total of 7 treatments over 2 weeks. On day 72, she became more alert, responded to voice, and tracked objects. Throughout the hospitalization, the fetus was monitored weekly by Doppler ultrasonography, showing normal heart tones. Obstetric ultrasonography performed at weeks 20 and 26 revealed normal fetal anatomy and appropriate growth for gestational age.

The patient remained in the intensive care unit because of persistence of sympathetic storms. By day 107, she was following simple commands and the sedation was slowly weaned. On day 127, she began mouthing words. A cesarean section and concomitant surgical staging was performed on day 166 (at 37 weeks of gestation, following amniocentesis confirming fetal lung maturity). The infant weighed 6 lb 3 oz and Apgar scores were 3 at 1 minute and 6 at 5 minutes. All pathological specimens (left adnexa, pelvic lymph nodes, and peritoneal samples) were negative for tumor. Over the next 3 days, the patient was weaned from the ventilator and she made steady gains in physical therapy. By day 184, she was able to ambulate with a walker and her speech was fluent, but she was only oriented to self. Her progress was hampered by impulsivity, short-term memory loss, and physical deconditioning. Two months after discharge, she was functioning independently at home, although she was persistently impulsive and complained of somnolence. The infant has met all developmental milestones to date.

CASE 2

A 20-year-old woman developed change of behavior during the eighth week of pregnancy. She became argumentative, refused to talk and eat, and developed stereotyped behaviors, such as walking endlessly around a room or filling and emptying a glass with water. Two days before hospital admission, semi-

rhythmic movements including blinking, licking, and tongue protrusion were noted. One day before admission, she developed hyperthermia and decreased level of consciousness and had a seizure. She had a history of bilateral ovarian teratomas that were removed when she was 16 years of age.

At admission, she had neck stiffness, without fever, and showed repetitive orofacial movements. She was poorly responsive to verbal and painful stimuli and had generalized hyperreflexia. Ancillary test results are described in the Table. Intravenous acyclovir and methylprednisolone administration were started. On day 3, cardiac pauses up to 5 seconds were noted (Figure, A). Over the next few days, she developed hypersalivation and generalized tonic convulsions. On day 13, status epilepticus and respiratory depression led to intubation and mechanical ventilation. On day 15, a pelvic computed tomographic scan revealed bilateral ovarian tumors (Figure, B, arrows); 2 days later, a left salpingo-oophorectomy and removal of both tumors was performed, and the pregnancy was terminated. Pathological studies confirmed bilateral mature teratomas.

From days 23 to 27, she received intravenous immunoglobulin and sedation with midazolam was discontinued. She gradually started tracking objects and following commands but continued having partial seizures that were treated with carbamazepine and gabapentin. On days 32 to 36, intravenous immunoglobulin administration was repeated, and by day 43, she was able to breathe spontaneously. By day 52, she was able to drink; the last seizure was observed on day 53. On day 64, she was eating regularly, and a few days later, she was able to walk. Her Mini-Mental State Examination score was 27 of 30 on day 85 and she was discharged home with minimal deficits on day 87.

CASE 3

A 19-year-old pregnant woman presented at 17 weeks of gestation with acute-onset behavioral change, including increas-

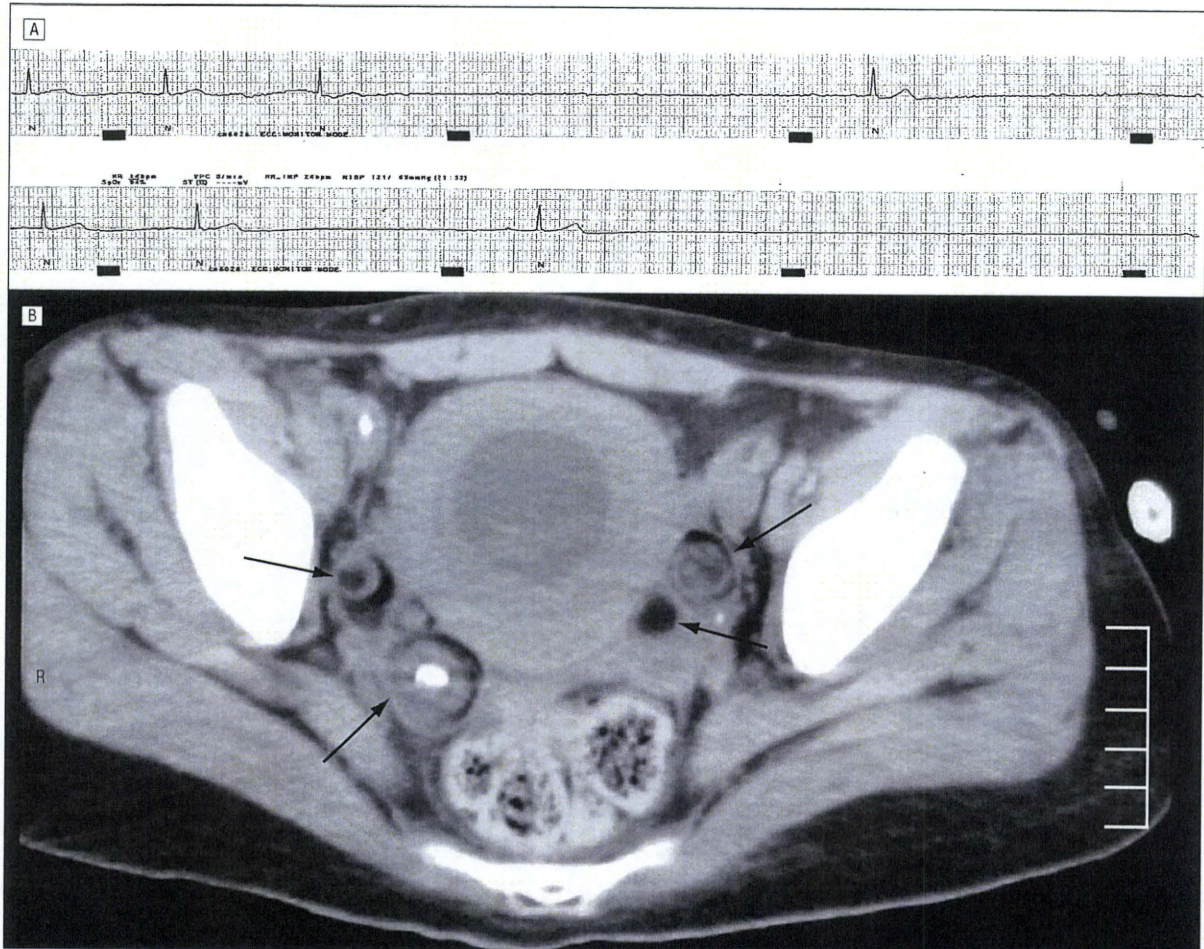


Figure. Cardiac pauses and bilateral teratomas in patient 2. A, Cardiac pauses up to 5 seconds were noted on day 3 of hospital admission. B, A pelvic computed tomographic scan revealed bilateral recurrent teratomas (arrows).

ing nervousness, irritability, and anxiety. She stopped walking and communicating but would say the same word repetitively and was transferred to a psychiatric facility. Her bizarre behavior continued; for example, she was frequently hitting the walls, taking cold baths, and accusing the physicians of “being murderers.” She had fluctuating periods of decreased level of consciousness and agitation. She had a partial tonic seizure involving the left arm without EEG correlate; this showed generalized high-amplitude slow activity (2 Hz). Because of progressive deterioration and the presumptive diagnosis of viral encephalitis, she was transferred 1 week later to a neurology unit. On physical examination, her temperature was 37.1°C, and she had no neck stiffness. She developed hyperhidrosis and repetitive semirhythmic oral movements, like automatisms. Brain computed tomography and MRI were normal. The CSF showed lymphocytic pleocytosis (white blood cell count, 11/ μ L) with normal protein and glucose concentrations. Results of extensive viral studies and autoimmune and paraneoplastic markers were negative. Anti-NMDAR antibodies were identified in her serum and CSF. A second EEG showed no changes compared with the previous study. Magnetic resonance imaging of the abdomen and pelvis and abdominal ultrasonography were normal.

From day 19, the patient was treated with intravenous methylprednisolone (500 mg/d for 5 days) without clinical

improvement, and a similar course of methylprednisolone treatment was started on day 35. After this second treatment, the orofacial dyskinesias subsided, but she continued with a decreased level of consciousness. On day 54, she had a generalized tonic-clonic seizure and treatment with phenobarbital was started. One month later, her level of consciousness started to progressively improve. Throughout the hospitalization, she did not develop hypoventilation. The fetus was monitored regularly by Doppler ultrasonography, showing normal heart tones. At 37 weeks of pregnancy, she spontaneously delivered a healthy 2892-g baby with Apgar scores of 8 at 1 minute and 9 at 5 minutes. Her Mini-Mental State Examination score was 24 of 30, and she was discharged 3 weeks later. At the last follow-up, she was fully functional and had returned to work. The child remains healthy with no obvious adverse effects.

DETECTION OF NMDAR ANTIBODIES

All 3 patients had higher NMDAR antibody titers in CSF than serum (Table). No antibodies were identified in the amniotic fluid, umbilical cord blood, serum, or CSF from the baby of patient 1. The baby of the other patient was not examined for antibodies.

To our knowledge, these are the first reported patients with anti-NMDAR encephalitis diagnosed during pregnancy. The 3 patients had substantial neurological recoveries, although in 1 case the pregnancy was terminated because of the severity of neurological symptoms, presence of recurrent bilateral teratomas, and early stage of pregnancy. The newborns of the other 2 patients were healthy and their physical and cognitive milestones are being closely followed up. Concern for the fetus and newborns is warranted in this disorder as studies indicate that NR1 antibodies from patients decrease NMDAR clusters in vitro and in animal models.^{1,3} Moreover, the antibodies are IgG1 and IgG3, which are the subtypes involved in autoimmune newborn illnesses, such as congenital lupus.⁴

The good outcome of the 2 neonates of our study is likely due to several factors, including the variable effects of autoimmune disorders on the fetus. For example, despite experimental models showing that Ro/SSA antibodies cause congenital heart block, only 2% to 5% of neonates from patients with these antibodies have congenital heart block.⁵ Two additional factors relate to the levels of serum maternal antibodies and the timing of transplacental transfer of IgG. IgG1 and IgG3 cross the placenta by binding to an Fc neonatal receptor present in syncytiotrophoblasts.⁶ This mechanism of placental transfer develops around weeks 14 to 16, resulting initially in very low levels of fetal blood IgG that gradually increase until the time of delivery.⁴ Additionally, the fetal blood-brain barrier becomes functional by the end of the second trimester. Our patients developed symptoms between 8 and 17 weeks of pregnancy when the IgG placental transfer is absent or limited, and assuming the immune response was triggered systemically, the levels of serum NMDAR antibodies decreased rapidly. In fact, 2 patients had negative serum but positive CSF antibody titers (both tested at initial dilution 1:10) by the time they were diagnosed with anti-NMDAR encephalitis, explaining the absence of NMDAR antibodies in the baby who was tested.

With a sharp increase in the number of cases with anti-NMDAR encephalitis, more patients will be identified during pregnancy. This study suggests that these patients and the newborns can do well. The concern should be the search (and removal) of a teratoma along with supportive care of the mother and fetus. Treatment with corticosteroids, intravenous immunoglobulin, and plasmapheresis was well tolerated but the effects could not be assessed because of the close temporal association with tumor removal in 2 patients. The third patient only received corticosteroids, with questionable improvement of the dyskinesias. The recovery seemed to accelerate af-

ter giving birth; this and the predominance of the disorder in young women bring into consideration a possible role of hormonal factors that needs further study.

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Retinoid X Receptor Gamma Control of Affective Behaviors Involves Dopaminergic Signaling in Mice

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SUMMARY

Abnormal signaling by retinoids or n-3 polyunsaturated fatty acids has been implicated in clinical depression. The converging point in activities of these two classes of molecules is transcriptional activation of retinoid X receptors (Rxr). We show here that ablation of *Rxr γ* in mice leads to depressive-like behaviors including increased despair and anhedonia, which were accompanied by reduced expression of dopamine D2 receptor in the shell of nucleus accumbens (NAc) and altered serotonin signaling. While abnormal serotonin signaling is not sufficient to generate the depressive behaviors, increasing D2r expression by chronic fluoxetine (Prozac) treatment or adenoassociated virus type2 (AAV2) mediated expression of *Rxr γ* or D2r in the NAc of *Rxr γ ^{-/-}* mice normalizes depressive-like behaviors in *Rxr γ ^{-/-}* animals. Conversely, NAc infusion of raclopride, a D2r antagonist prevents AAV2-*Rxr γ* -mediated rescue of despair behaviors in *Rxr γ ^{-/-}* mice. Combined, our data argue that control of NAc D2r expression is critical for *Rxr γ* -mediated modulation of affective behaviors.

INTRODUCTION

Dopaminergic signaling and in particular its mesolimbic pathway plays an important, reinforcing role in regulation of motivated behaviors. Abnormally low dopaminergic signaling has been suggested to be involved in clinical depression (Millan, 2006; Nestler and Carlezon, 2006). Classical antidepressant treatments increase dopaminergic tone and its signaling via different dopamine receptor subtypes, which suggests a direct implication of dopamine in the efficiency of such treatments (Renard et al., 2001; Valentini et al., 2004; Willner et al., 2005). The dopaminergic reuptake inhibitor, Bupropion, has been found effective as an antidepressant treatment (Foley et al., 2006), although its

actions also implicate noradrenergic transmission. Furthermore, some of the dopaminergic receptor ligands, such as bromocriptine and pergolide or pramipexole, are effective in the treatment of depression either as a monotherapy or as adjuvants (Corrigan et al., 2000; Mattes, 1997; Theohar et al., 1982). Although the dopamine receptor specificity of these agents is variable, all of them act as agonists of dopamine D2 receptor (D2r), which suggests that this receptor plays a particular role in the regulation of affective behaviors. This possibility is further supported by preclinical studies in rodent models used for research on depression. Thus, chronic mild stress leads to a reduction of D2r expression in the nucleus accumbens (Willner, 1997), and activation of D2 receptors has an anti-depressant action in animal models of despair (Brocco et al., 2006; Siuciak and Fujiwara, 2004). Moreover, chronic antidepressant treatments including selective serotonin reuptake inhibitors (SSRIs), which primarily modulate serotonergic transmission, can also increase D2r expression in humans and rodents (Ainsworth et al., 1998; Dziedzicka-Wasylewska et al., 1997; Larisch et al., 1997). In line with such findings, an inhibition of D2r in human and animal models prevents antidepressant activities of fluoxetine (Prozac) and/or other antidepressants (Willner et al., 2005, and references therein).

The expression of D2r is modulated at the transcriptional level by retinoic acid (RA), an active form of vitamin A (Krezel et al., 1998; Samad et al., 1997). Such control implicates activities of retinoic acid receptors (*Rar α* , *Rar β* , *Rar γ*) and retinoid X receptors (*Rxr α* , *Rxr β* , *Rxr γ*), which in the form of heterodimers act as transcription factors and mediate RA signaling in vivo (Kastner et al., 1997). *Rar β* and *Rxr γ* are the predominant retinoid receptors expressed in the striatum, including the nucleus accumbens (Krezel et al., 1999; Zetterström et al., 1999). Concomitant ablation of these receptors in *Rar β /*Rxr γ** double-knockout mice leads to strong reduction of D2r expression in the dorsal and ventral striatum and marked locomotor deficits (Krezel et al., 1998). The involvement of murine retinoid receptors in the control of dopaminergic signaling in the striatum might suggest a potential role of the retinoid pathway in modulation of affective behaviors. Such modulation is further suggested by clinical data on depression associated with altered retinoid signaling in *acne vulgaris* patients treated with isotretinoin (Bremner and McCaffery,

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2008). Rxr's were also proposed to mediate genomic actions of n-3 polyunsaturated fatty acids (n-3 PUFAs) (de Urquiza et al., 2000; Lengqvist et al., 2004). Such functions of Rxrs could be directly relevant for the pathology of affective disorders, as decreased n-3 PUFA signaling has been suggested to be associated with depression and use of n-3 PUFAs such as docosahexaenoic acid or eicosapentaenoic acid were reported beneficial in clinical conditions (Logan, 2004; Peet and Stokes, 2005) and in animal models used in research on depression (Carlezon et al., 2005; Naliwaiko et al., 2004). We have combined pharmacological and genetic approaches to address the role of retinoid receptors in control of affective behaviors and implication of dopaminergic signaling in such control.

RESULTS

Increased Despair Behaviors in *Rxr γ* Null Mice Can Be Normalized by Antidepressant Treatment

To address the contribution of retinoid receptors in control of despair behaviors we have studied the effects of the loss of function of *Rar β* and/or *Rxr γ* in mice on performance in the forced swim test. Concomitant ablation of *Rar β* and *Rxr γ* in *Rar β ^{-/-}Rxr γ ^{-/-}* double null mutant mice led to a marked increase of the immobility time, which attained 129 ± 4.3 s and was significantly longer ($p < 0.001$) than in wild-type (WT) control mice, which remained immobile for 71 ± 6.2 s (Figure 1A). The increased immobility in the double-mutant mice was principally due to the loss of function of *Rxr γ* , since single *Rxr γ ^{-/-}* mutants displayed similar high immobility time of 117 ± 4 s, whereas inactivation of *Rar β* did not affect immobility time in this task (64.9 ± 6.8 s; $p > 0.05$). An abnormal locomotor behavior is unlikely to account for the increased immobility time of *Rxr γ ^{-/-}* mice in the forced swim test, since *Rxr γ ^{-/-}* mice did not differ from their WT littermates with respect to spontaneous locomotion in actimetric cages, novelty-induced locomotion in the open field test, or locomotor coordination in the rotarod task (Krezel et al., 1998; see Figure S1 available online).

Since despair behaviors belong to the core symptoms of depression, we have explored whether antidepressant treatment could improve the performance of *Rxr γ ^{-/-}* mice. In agreement with previous reports of SSRI activities in C57BL6J and 129SV mouse strains (Dulawa et al., 2004), a 21 day chronic treatment with fluoxetine at the dose of 20 mg/kg/24hr did not affect performance of WT mice in the forced swim test, but such treatment reversed the despair behavior in *Rxr γ ^{-/-}* mice (Figure 1B), as illustrated by a significant *genotype* \times *fluoxetine* treatment interaction ($F[1,35] = 23.2$, $p < 0.001$). Thus, high immobility of vehicle treated *Rxr γ ^{-/-}* mice was reduced in fluoxetine treated *Rxr γ ^{-/-}* animals, which behaved comparably to vehicle treated WT mice (Figure 1B).

Inactivation of *Rxr γ* Leads to Anhedonia, Which Can Be Normalized by Antidepressant Treatment

To better evaluate the involvement of retinoid receptors in the control of affective behaviors, we investigated hedonic behaviors in the sucrose preference test in *Rar β ^{-/-}* and *Rxr γ ^{-/-}* single and compound mutant mice. During the active, night phase of the circadian cycle WT mice displayed clear preference for

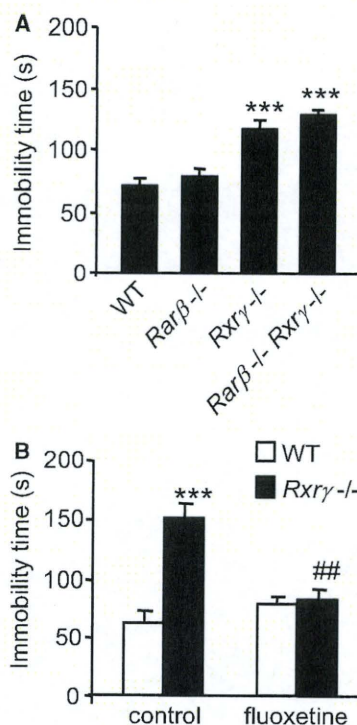


Figure 1. Increased Despair Behavior in *Rxr γ* Null Mutant Mice Is Reversible by Chronic Antidepressant Treatment

The time of immobility in the forced swim test was measured in naive $n_{WT} = 6$ and $n_{Rar\beta^{-/-}} = 8$, $n_{Rxr\gamma^{-/-}} = 8$ single- and $n_{Rar\beta^{-/-}Rxr\gamma^{-/-}} = 8$ double-mutant littermates (A). Chronic, 21-day long treatment with fluoxetine (20 mg/kg/24 hr) reduced the immobility time in $n_{Rxr\gamma^{-/-}} = 10$ mice as compared to $n_{Rxr\gamma^{-/-}} = 10$ mice fed with control diet but not in $n_{WT} = 8$ mice as compared to $n_{WT} = 11$ mice fed with control diet (B). Data are presented as mean values \pm SEM. *** $p < 0.001$ with respect to nontreated (A) or vehicle-treated (B) WT mice; ## $p < 0.01$ as compared with vehicle-treated *Rxr γ ^{-/-}* mice. See also Figure S1.

a 1% sucrose solution as compared to plain water, since sucrose represented $91.3\% \pm 2.7\%$ of total liquid consumption. In compound *Rar β ^{-/-}Rxr γ ^{-/-}* mutant mice, such a preference was absent as sucrose consumption reached $58.1\% \pm 10.6\%$ of total liquid intake (Figure 2A), which was significantly less than preference in WT mice ($p < 0.05$) and not different from a chance level of 50% ($t = -0.1$, ns, one-group t test). Such anhedonic behavior was due to ablation of *Rxr γ* , since *Rxr γ* single null mutants displayed similar loss of sucrose preference and consumed $57.8\% \pm 10.8\%$ of sucrose solution, whereas *Rar β ^{-/-}* mice consumed sucrose solution at $92\% \pm 2.1\%$ and were indistinguishable from their WT controls. The total liquid intake during the sucrose preference test was not different between WT and mutant mice (4.6 ± 0.2 g for WT, 4.9 ± 0.3 for *Rar β ^{-/-}*, 4.9 ± 0.2 for *Rxr γ ^{-/-}* and 5.2 ± 0.3 for *Rar β ^{-/-}Rxr γ ^{-/-}* mutants). The absence of sucrose preference in *Rar β ^{-/-}Rxr γ ^{-/-}* and *Rxr γ ^{-/-}* mice is unlikely to result from gustative deficits since all groups preferred water to 1% sucrose on the first presentation of sucrose drink. Thus, during 3 hr of the first

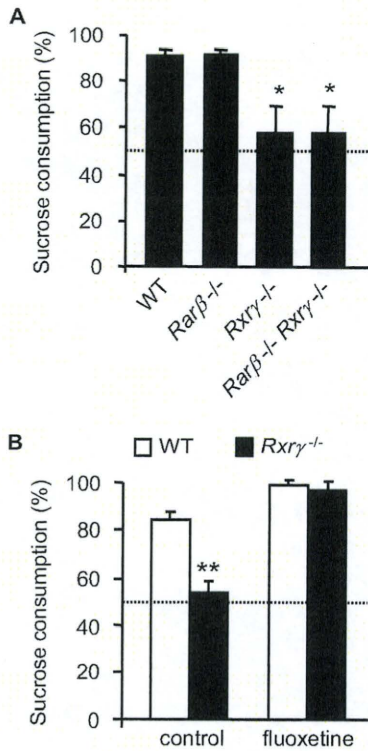


Figure 2. Loss of Sucrose Preference in *Rxrγ*^{-/-} Mice Is Reversible by Chronic Antidepressant Treatment

Sucrose preference was measured as percent of sucrose solution consumption with respect to total amount of liquid consumed during the night phase in $n_{WT} = 11$ and $n_{Rar\beta^{-/-}} = 11$, $n_{Rxr\gamma^{-/-}} = 7$, and $n_{Rar\beta^{-/-}Rxr\gamma^{-/-}} = 7$ null mutant mice (A). Chronic, 19-day long treatment with fluoxetine (20 mg/kg/24 hr) normalized sucrose preference in $n_{Rxr\gamma^{-/-}} = 18$ mice as compared to $n_{Rxr\gamma^{-/-}} = 22$ mice fed with control diet but not in $n_{WT} = 13$ mice as compared to $n_{WT} = 15$ mice fed with control diet (B). Data are presented as mean values \pm SEM. * $p < 0.05$ significantly different from WT group; ** $p < 0.01$ different from vehicle treated WT mice.

testing session, in sucrose-naive WT mice sucrose solution constituted $43\% \pm 2.5\%$ of total liquid consumption, as compared to $44\% \pm 2.1\%$ for *Rarβ*^{-/-}, $37\% \pm 5\%$ for *Rxrγ*^{-/-}, and $32.4\% \pm 7.3\%$ for *Rarβ*^{-/-}*Rxrγ*^{-/-} mice, which for all groups was significantly less than the chance level of 50% ($t > 3.5$ for any of the comparisons, $p < 0.05$, one-group t test). Such avoidance of sucrose solution by sucrose-naive mice results from a natural tendency for reserved consumption of novel food/drink and provides evidence for recognition of 1% sucrose taste. A chronic, 19 day antidepressant treatment with 20 mg/kg/24hr of fluoxetine, reversed the sucrose preference deficits in *Rxrγ*^{-/-} mice (Figure 2B), which is reflected by significant *genotype* \times *treatment* interaction ($F[1,64] = 7.9$, $p < 0.01$). Thus, fluoxetine-treated *Rxrγ*^{-/-} mice preferred sucrose solution to water similarly to WT control mice, and the percentage of sucrose solution consumed by fluoxetine-treated *Rxrγ*^{-/-} mice was significantly higher than in vehicle-treated mutant animals ($p < 0.001$).

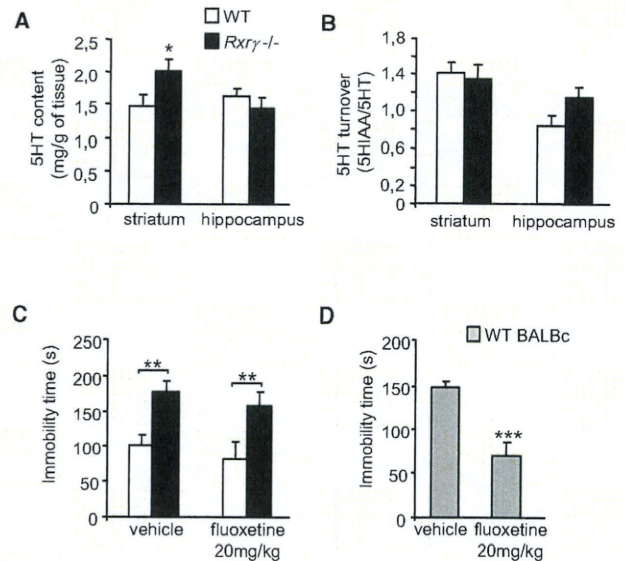


Figure 3. Abnormal Serotonergic Signaling Is Not Sufficient to Generate Despair Behavior in *Rxrγ*^{-/-} Mice

Total tissue levels of 5HT were standardized by tissue weight and compared for the hippocampus and striatum in $n_{WT} = 6$ and $n_{Rxr\gamma^{-/-}} = 6$ mice (A). For the same animals and structures the serotonin turnover was calculated as ratio of standardized measures of 5HIAA and 5HT (B). Acute treatment with fluoxetine (20 mg/kg) did not alter immobility time in the forced swim test neither in $n_{Rxr\gamma^{-/-}} = 8$ mice as compared to $n_{Rxr\gamma^{-/-}} = 8$ vehicle-treated mice nor in $n_{WT} = 7$ mice as compared to $n_{WT} = 9$ vehicle-treated mice (C), whereas the same dose significantly reduced immobility time in control strain of $n_{BALBc} = 6$ mice as compared to $n_{BALBc} = 6$ vehicle-treated mice (D). Data are presented as mean values \pm SEM. * $p < 0.05$ or ** $p < 0.01$ for selected comparisons and *** $p < 0.001$ in comparison with vehicle treated BALBc mice.

Abnormal Serotonergic Signaling Is Not Sufficient to Generate Depressive Behaviors in *Rxrγ*^{-/-} Mice

Efficiency of fluoxetine to reverse affective abnormalities could suggest that altered serotonergic signaling is at the origin of depressive-like behaviors in *Rxrγ*^{-/-} mice. To address this issue, we carried out global evaluation of serotonergic signaling focusing on hippocampus and striatum (including NAc), the two regions differentially innervated by dorsal and median raphe 5HT inputs suggested to play a role in control of affect (Lechin et al., 2006). HPLC measurements of 5HT and its metabolite 5HIAA in tissue homogenates revealed a significant increase of 5HT levels in the striatum of *Rxrγ*^{-/-} mice (2.01 ± 0.18 ng/g for *Rxrγ*^{-/-} and 1.46 ± 0.17 for WT mice; $t = 2.2$, $p = 0.05$), which was not accompanied by altered metabolism of serotonin (Figure 3A). Although there was no significant difference in 5HT levels in the hippocampus, *Rxrγ*^{-/-} displayed strong tendency ($t = 1.92$, $p = 0.08$) for increased metabolism of 5HT in this region (Figure 3B). Such abnormalities in the distribution of 5HT did not correlate with abnormal expression of 5HT1a receptor, prominently involved in control of 5HT tone and proposed to play a role in control of affective behaviors and in actions of SSRI antidepressants (Blier et al., 1998; Blier and Ward, 2003). Indeed, the relative 5HT1a mRNA levels (standardized with respect to

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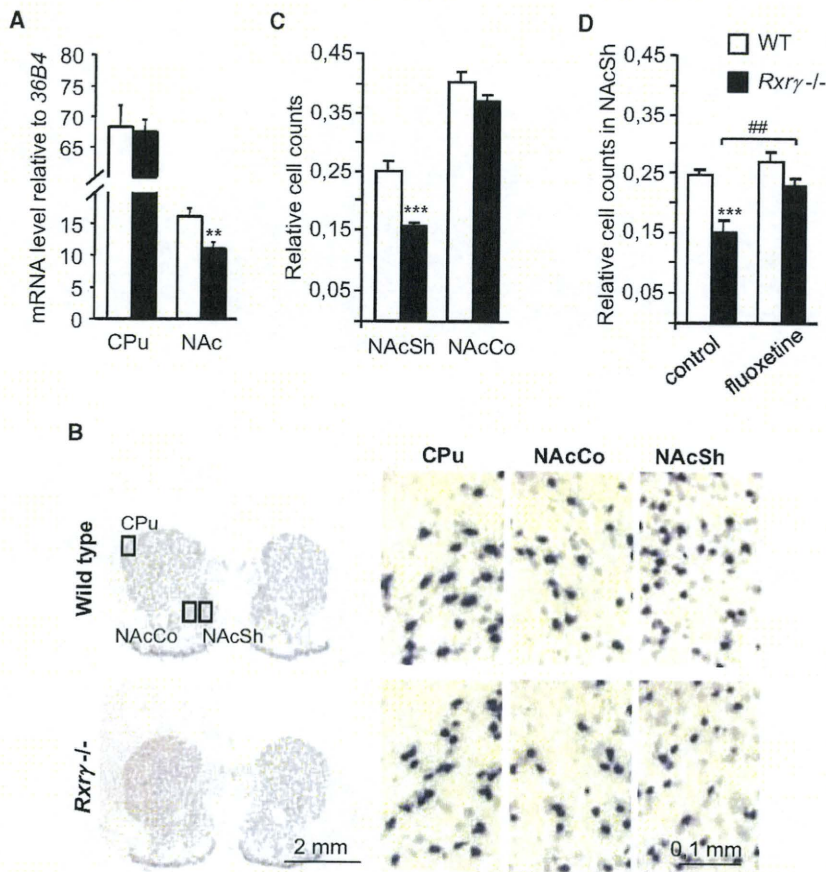


Figure 4. Decreased Expression of Dopamine D2r Receptor mRNA in the Nucleus Accumbens of *Rxrγ*^{-/-} Mice Is Reversed by Chronic Fluoxetine Treatment

D2r mRNA levels were measured by quantitative real-time RT-PCR and are presented as relative to the expression of the housekeeping gene 36B4, $n_{WT} = 9$, $n_{Rxr\gamma^{-/-}} = 10$ (A). In situ hybridization detection of D2r mRNA is shown in the whole striatum (left), and at high magnification in selected regions (boxed) of the caudate putamen (CPu), nucleus accumbens shell (NAcSh), and core (NAcCo) in WT and *Rxrγ*^{-/-} mice (B). The numbers of D2r-positive cells in the shell and core of the NAc are presented as relative to D2r cell counts in the adjacent dorsal part of the CPu on the same section, for $n_{WT} = 6$ and $n_{Rxr\gamma^{-/-}} = 6$ mice (C). The effects of 19 days of chronic fluoxetine treatment on the relative numbers of D2r positive cells in NAcSh in $n_{WT} = 3$ and $n_{Rxr\gamma^{-/-}} = 5$ mice were compared with $n_{WT} = 4$ and $n_{Rxr\gamma^{-/-}} = 4$ mice fed control diet (D). Data are presented as mean values \pm SEM. ** $p < 0.01$; *** $p < 0.001$ different from corresponding WT group or ##, $p < 0.01$ for selected comparison. See also Figure S2.

Reduced Dopamine D2 Receptor Signaling in the Nucleus Accumbens of *Rxrγ*^{-/-} Mice Is Reversible by Fluoxetine Treatment

The despair behavior and anhedonia and their reversal by chronic antidepressant treatment in *Rxrγ*^{-/-} mice, indicates that

expression of the house keeping gene 36B4) were comparable between *Rxrγ*^{-/-} and their WT controls in the NAc (2.2 ± 0.4 for *Rxrγ*^{-/-} and 2.7 ± 0.3 for WT; $t = -1$, ns) and hippocampus (1.4 ± 0.2 for *Rxrγ*^{-/-} and 1.9 ± 0.3 for WT mice; $t = -1.5$, ns).

To investigate functional relevance of abnormal serotonergic signaling for depressive-like behaviors in *Rxrγ*^{-/-} mice we have tested whether acute fluoxetine treatment can reverse increased despair in the forced swim test. In contrast to chronic treatment, acute administration of fluoxetine at 20 mg/kg (IP, 30min prior to the forced swim test) did not alter increased immobility of *Rxrγ*^{-/-} mice as there was no significant interaction for *genotype* \times *fluoxetine* treatment ($F[1,28] = 3E-4$, ns) and the main effect of *genotype* ($F[1,28] = 19.4$, $p < 0.001$) remained significant despite of the treatment (Figure 3C). To control for the efficiency of acute fluoxetine treatment we used wild-type BALBcByJ (BALBc) mice, the strain susceptible to reveal antidepressant activities of fluoxetine (Lucki et al., 2001), and we found that 20 mg/kg of fluoxetine was sufficient to significantly reduce despair behaviors in this strain (Figure 3D). We concluded that inefficiency of acute fluoxetine treatment to modulate despair behaviors suggests that abnormal serotonergic signal is not sufficient to generate depressive behaviors and adaptive changes associated with chronic fluoxetine treatments might be at the origin of affective abnormalities in *Rxrγ*^{-/-} mice.

null mutation of *Rxrγ* leads to deficits resembling some of the core symptoms of depression. We hypothesized that abnormal function of the nucleus accumbens (NAc), the key structure implicated in the control of motivated behaviors and one of the primary sites of *Rxrγ* expression (Krezel et al., 1999) might be at the origin of the behavioral abnormalities in *Rxrγ*^{-/-} mice. As dopaminergic signaling in the NAc is critically involved in the modulation of motivated behaviors and in the mechanisms of antidepressant activities including fluoxetine, we examined the expression of dopaminergic D1 and D2 receptors in *Rxrγ*^{-/-} mice. Using real-time quantitative RT-PCR, we found a significant 32% reduction of *D2r* expression in the NAc of *Rxrγ*^{-/-} mice ($t = -3.16$, $p < 0.01$; Figure 4A). Interestingly, no such reduction ($t = -0.22$, ns) was observed in the dorsal caudate putamen (CPu). The inactivation of *Rxrγ* did not affect expression of D1r as *D1r* RNA levels were not significantly different between WT and *Rxrγ*^{-/-} mice in the NAc (11.7 ± 1.0 versus 10.6 ± 1.0 units; $t = -0.76$, ns) and dorsal striatum (37.2 ± 2.2 versus 39.9 ± 0.8 units; $t = 1.35$, ns), as measured by RT-PCR and calculated relative to expression of a reference housekeeping gene (Figure S2).

In order to further investigate the regionalization and the origin of reduced levels of D2r mRNA in the NAc we carried out in situ hybridization (ISH) studies (Figure 4B). Comparisons of *Rxrγ*^{-/-} mice and their WT controls revealed that the number of neurons

expressing D2r was significantly reduced in the shell of the NAc (152 ± 4 versus 206 ± 6 ; $t = -8$, $p < 0.001$) and the core of the NAc (294 ± 6 versus 338 ± 16 ; $t = -2.44$; $p < 0.05$), but not in the dorsal striatum (798 ± 17 versus 841 ± 34 ; $t = -1.1$, ns). To minimize cell counting errors related to differences in the signal intensity between different sections, which may account for more discrete changes, we took advantage of expression of D2r in the dorsal striatum (CPU), which was not affected by ablation of *Rxr γ* ^{-/-} and we used this region as our internal (intra-section) control to calculate relative changes in D2r cell numbers. To this end we divided cell counts in the NAcSh or NAcCo by those obtained for the adjacent region of CPU on the same section. We found a strong (36%) reduction in relative cell number only in the NAcSh (0.16 ± 0.01 for *Rxr γ* ^{-/-} as compared to 0.25 ± 0.02 for WT; $t = -4.7$, $p = 0.001$), but not in the NAcCo (0.37 ± 0.01 for *Rxr γ* ^{-/-} versus 0.40 ± 0.02 for WT; $t = -1.5$, ns), of *Rxr γ* ^{-/-} mice (Figure 4C). The difference in the magnitude of changes in D2r expression might be related to much weaker expression of *Rxr γ* in the NAcCo as compared to shell region (Krezel et al., 1999). Reduction of D2r in the NAcSh might be functionally relevant as chronic fluoxetine treatment, in addition to reversing depressive-like behaviors, increased also the relative number of D2r positive cells in the NAcSh of behaviorally naive *Rxr γ* ^{-/-} mutants (0.15 ± 0.02 for nontreated *Rxr γ* ^{-/-} versus 0.23 ± 0.01 for fluoxetine treated *Rxr γ* ^{-/-} mice; $t = -3.4$, $p < 0.01$), but not WT mice (0.24 ± 0.01 for nontreated WT versus 0.27 ± 0.02 for fluoxetine treated mice; $t = -1.2$, ns; Figure 4D).

A decrease of D2r-positive cell number in the NAcSh of *Rxr γ* ^{-/-} mice is most probably related to reduced transcription of D2r, rather than to the loss of a subpopulation of D2r expressing neurons. Supporting this hypothesis, the number of cells expressing mRNA coding for enkephaline, a neuropeptide found predominantly in D2r expressing neurons, was not significantly reduced (124.3 ± 2.8 enkephaline-positive cells in the NAc shell of *Rxr γ* ^{-/-} mice, as compared to 133.6 ± 3 cells in WT animals; $t = -2.34$, ns). Thus, the reduced number of D2r-expressing neurons could reflect a general decrease of D2r transcription in the NAc shell, with a reduction below the detection threshold level in neurons expressing low levels of D2r. Alternatively, it might be related to reduced transcriptional control of D2r restricted to a selected neuronal population. To address this issue, we quantified the intensity of D2r expression in the ISH experiments, using the ImageJ software (see Experimental Procedures). We found that the mean intensity of the D2r signal in the NAc shell was not different between WT and *Rxr γ* ^{-/-} mice when comparing absolute mean values (122.9 ± 2.2 for WT and 125.1 ± 2 for *Rxr γ* ^{-/-} mice; $t = 0.7$, ns) or when such measures were normalized with respect to the intensity of D2r expression in the dorsal striatum within the same brain section, where D2r expression was not affected by ablation of *Rxr γ* (1.05 ± 0.01 for WT and 1.08 ± 0.02 for *Rxr γ* ^{-/-} mice; $t = 1$, ns). These data suggest that *Rxr γ* might control expression of D2r in a selected subpopulation of D2r neurons.

Finally, to assess whether cell- and regional-specific reduction of D2r mRNA expression leads to abnormal D2r activities, we investigated neuronal activation in *Rxr γ* ^{-/-} mice in response to haloperidol, a D2 preferential antagonist. To this end, we studied

the induction of *c-fos* protein expression, a molecular marker related to neuronal activity and plasticity. An acute treatment with haloperidol (1 mg/kg) increased the number of *c-fos* positive cells in various regions of the striatum, including the shell and core of the NAc and CPU in all tested mice (Figures 5A and 5B). However, in the shell of the NAc the magnitude of this increase was significantly lower in the *Rxr γ* ^{-/-} than in WT mice as reflected by the significant interaction between *genotype* and *treatment* ($F[1,14] = 5.6$, $p < 0.05$) and PLSD Fischer post hoc analysis ($p < 0.01$). Such difference was not observed in the core of the NAc or in the dorsal CPU in the same sections (compare NAc-Sh with NAc-Co and CPU for WT-Hal and KO-Hal in Figure 5C). To study whether such a decrease reflects the action of haloperidol or is related to the stress inflicted during drug injection, we also evaluated the numbers of *c-fos* positive cells in saline-injected mice and found that these numbers were not significantly different between WT and *Rxr γ* ^{-/-} mice (compare WT-veh and KO-veh in Figure 5C). Thus, the haloperidol-specific induction of *c-fos* in the shell of the NAc, calculated as the ratio of *c-fos* positive cells in the haloperidol-treated mice with respect to saline-treated mice, was lower by 48% in *Rxr γ* ^{-/-} mice as compared to their WT controls (Figure 5D). To validate these findings functionally we tested the locomotor effects of low, non-cataleptic doses of haloperidol, which have been proposed to involve post-synaptic dopamine D2 receptors in the nucleus accumbens (Messier et al., 1992; Millan et al., 2004; Pijnenburg et al., 1976). All mice treated with haloperidol displayed reduction of locomotor activity in the novel environment of the open field, although such reduction was different depending on the genotype (significant *genotype* \times *treatment* interaction, $F[4,68] = 2.7$; $p < 0.05$). Post hoc analysis revealed that the decrease of locomotion was significantly lower in *Rxr γ* ^{-/-} mice as compared to WT controls for haloperidol doses of 0.1 and 0.2 mg/kg ($p < 0.05$; Figure 6A). These effects of haloperidol cannot be attributed to an altered susceptibility of *Rxr γ* ^{-/-} mice to develop catalepsy, since a 0.2 mg/kg dose did not induce catalepsy in WT and *Rxr γ* ^{-/-} mice, whereas a high dose of haloperidol (2 mg/kg) induced comparable degrees of catalepsy in both genotypes (Figure 6B).

Rxr γ in the Nucleus Accumbens Is Critical for Control of Despair and Hedonic Behaviors and Modulation of D2r Expression

To investigate whether *Rxr γ* expression in the nucleus accumbens shell plays a role in the control of depressive-like behaviors and expression of dopamine D2r, we carried out functional rescue experiments using stereotaxic injection of adenoassociated virus (AAV2) expressing *Rxr γ* , in the NAc of *Rxr γ* null mutants. Using immunohistochemical analysis, we could clearly detect the expression of *Rxr γ* protein in the WT noninjected mice (top panels in Figure 7A) or in the NAcSh of *Rxr γ* ^{-/-} mice infected with AAV2-*Rxr γ* vector (bottom panels in Figure 7A), but not in *Rxr γ* ^{-/-} animals infected with AAV2-*Gfp* virus (middle panels in Figure 7A). The virus-mediated expression of *Rxr γ* in *Rxr γ* ^{-/-} mice was detectable bilaterally at bregma 1.1 and 1.4 and specifically in the NAcSh in 5 (out of 10) mice injected with AAV2-*Rxr γ* whereas for AAV2-*Gfp* infected mice, such pattern of *Gfp* expression was identified in 7 (out of 10) animals. In the

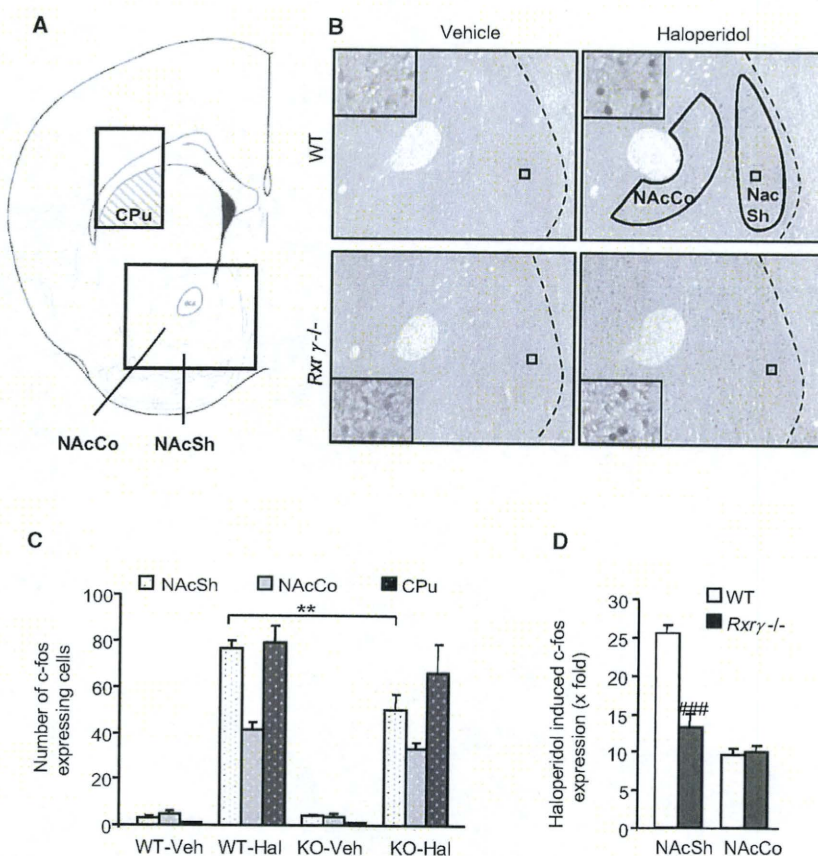


Figure 5. Haloperidol Induction of *c-fos* Expression Is Impaired in the NAc Shell of *Rxrγ*^{-/-} Mutants

The brain regions used for *c-fos* counts are schematized (A). *c-fos* positive cells were scored in selected regions of the dorsal striatum (striped area; CPu) (A), the nucleus accumbens shell (NAcSh) and core (NAcCo) (B). *c-fos*-positive cells were counted for each structure and are presented as means \pm SEM (C) or as the ratio of haloperidol/vehicle induced *c-fos* cells for each genotype (D). Each experimental group consisted of $n = 6$ animals. Data are presented as mean values \pm SEM. ** $p < 0.01$, ### $p < 0.001$.

remaining animals ($n = 5$ for AAV2-*Rxrγ* and $n = 3$ for AAV2-*Gfp*) viral infection was unilateral or not restricted to the NAc (e.g., spreading into ventral septum) and these mice were excluded from the analysis of behavioral data. The infection of *Rxrγ* null mutant mice with the AAV2-*Rxrγ* expressing vector led to a significant decrease of despair behaviors ($t = 2.8$, $p < 0.05$) and anhedonia ($t = -2.7$, $p < 0.05$) as compared to *Rxrγ*^{-/-} mice infected with the *Gfp*-expressing virus (Figures 7B and 7C). Such behavioral effects of *Rxrγ* expression in the NAc were not confounded by altered locomotor activity, as spontaneous locomotion in the actimetric cages or novelty induced locomotion in the open field test were comparable between the groups (Figure S3). During the sucrose preference test the total amount of liquid consumed during the testing session was not different among the groups and was on average 4.7 ± 0.4 mg/night.

In addition to reversal of behavioral deficits, re-expression of *Rxrγ* in the nucleus accumbens of *Rxrγ*^{-/-} mice led to an increase of the number of D2r expressing neurons. In the NAc shell of *Rxrγ*^{-/-} infected with AAV2-*Rxrγ* we identified 190.2 ± 11.1 D2r-positive neurons, which was significantly more than 153.2 ± 11.2 neurons in AAV2-*Gfp* infected mutant mice ($t = -2.34$, $p < 0.05$), which was also reflected by relative measures of D2v positive cell numbers with respect to adjacent CPu region (0.23 ± 0.01 for AAV2-*Rxrγ* as compared to 0.18 ± 0.01 in AAV2-*Gfp* infected *Rxrγ*^{-/-} mice; $t = -3.1$, $p < 0.05$). To address

whether such an increase of D2v expression in the NAcSh is relevant to depressive-like behaviors in *Rxrγ*^{-/-} mice we have blocked D2r signaling in the NAcSh by bilateral infusion of the D2r antagonist raclopride (5 μ g/side) in AAV2-*Rxrγ* rescued *Rxrγ*^{-/-} mice. We found that blocking D2r signaling compromised antidepressant effects of AAV2-mediated re-expression of *Rxrγ* in *Rxrγ*^{-/-} mice since such animals remained immobile in the forced swim test for 130.6 ± 13.4 s, which was significantly longer ($t = 4.1$, $p < 0.01$) than *Rxrγ*^{-/-} mice which were infected with AAV2-*Rxrγ* and infused with ACSF vehicle (57.9 ± 9.7 s; Figure 7D). Although raclopride infusion into the NAc led to a slight tendency to

reduce general locomotor activity as measured in the open field (Figure 7E), such reduction was not significant ($t = -0.87$, $p = 0.4$) and cannot account for increased immobility in the forced swim test.

AAV2-Mediated Expression of D2r in the Nucleus Accumbens Reverses Depressive-like Behaviors of *Rxrγ*^{-/-} Mice

In order to further address the role of a reduction of D2r expression in the control of depressive-like behaviors in *Rxrγ*^{-/-} mice, we increased D2r signaling in the nucleus accumbens by AAV2 mediated expression of D2r. Seven out of nine injected *Rxrγ*^{-/-} mice were retained for statistical analysis as they displayed bilateral D2r expression revealed by increased number of D2r positive neurons in the NAc (210.7 ± 10.5 in AAV2-D2r mice as compared to 153.2 ± 11.2 in AAV2-*Gfp* infected *Rxrγ*^{-/-} mice; $t = -4.3$, $p < 0.01$). The increase of D2r-positive neurons was specific to the shell of NAc and was not present in the adjacent, dorsal part of the striatum on the same sections (Figure 8A). Such expression was functionally relevant as it increased locomotor activity in the open field (Figure 8B), which attained 122.3 ± 10 m for AAV2-D2r mice as compared to 96.9 ± 3.7 m of distance covered by AAV2-*Gfp* mice ($t = 2.35$, $p < 0.05$). Increased activity resulted from abnormal reactivity to a novel environment and could be further demonstrated by increased

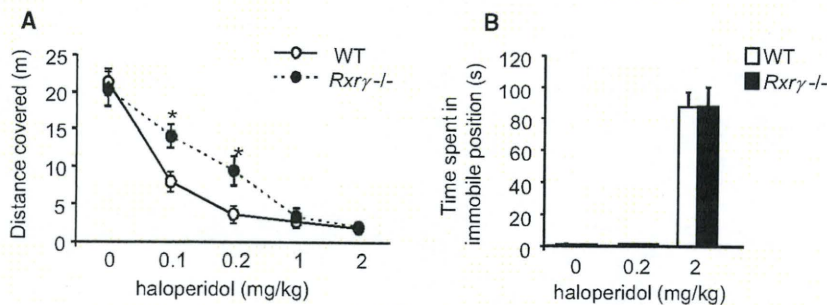


Figure 6. Behavioral Responses to Haloperidol in *Rxrγ*^{-/-} Mice

Twenty minutes after treatment with saline (0) or haloperidol (0.1, 0.2, 1, or 2 mg/kg), locomotor activity was measured in WT and *Rxrγ*^{-/-} mice in the open field test during 5 min. The number of animals tested in each genotype/treatment group was: $n_{WT/0} = 7$, $n_{Rxr\gamma^{-/-}/0} = 7$, $n_{WT/0.1} = 8$, $n_{Rxr\gamma^{-/-}/0.1} = 10$, $n_{WT/0.2} = 8$, $n_{Rxr\gamma^{-/-}/0.2} = 7$, $n_{WT/1} = 7$, $n_{Rxr\gamma^{-/-}/1} = 7$, $n_{WT/2} = 7$, $n_{Rxr\gamma^{-/-}/2} = 10$ (A). Catalepsy was measured in the bar test 30 min after vehicle (0) or haloperidol (0.2 or 2mg/kg) injection (B). The number of animals tested in each genotype/treatment group was: $n_{WT/0} = 8$, $n_{Rxr\gamma^{-/-}/0} = 8$, $n_{WT/0.2} = 10$, $n_{Rxr\gamma^{-/-}/0.2} = 7$, $n_{WT/2} = 8$, $n_{Rxr\gamma^{-/-}/2} = 8$. Data are presented as mean values \pm SEM. * $p < 0.05$ with respect to WT haloperidol-treated group.

locomotion in the actimetric cages, which was evident during the first hr of the 32 hr test (Figure S3), thus reflecting enhanced D2r signaling (Ouagazzal and Creese, 2000; Zhang et al., 1996). In the forced swim test *Rxrγ*^{-/-} mice infected with AAV2-D2r remained immobile for 58 ± 11.1 s, which was significantly less ($t = -3.5$, $p < 0.01$) than 127.9 ± 16.7 s for AAV2-Gfp mice (Figure 8C). AAV2-mediated D2r expression also normalized anhedonia of *Rxrγ*^{-/-} mice. Indeed, *Rxrγ*^{-/-} mice infected with AAV2-D2r preferred sucrose to water and consumed $73.8\% \pm 6.9\%$ of sucrose as opposed to significantly lower ($t = 2.62$, $p < 0.05$), $53.3\% \pm 4.3\%$ for AAV2-Gfp control mice (Figure 8D).

DISCUSSION

We provide here evidence that a specific retinoid receptor is implicated in the control of affective behaviors in mice. We show that null mutation of *Rxrγ* leads to increased despair behavior in the forced swim test and anhedonia, the key symptom of depression as measured in the sucrose preference paradigm. Our studies of single and compound *Rxrγ*^{-/-} and *Rarβ*^{-/-} mutant mice provide also evidence that *Rarβ* might not be the heterodimerization partner of *Rxrγ* in control of affective behaviors. Although we cannot exclude some functional redundancy between *Rar*'s in their interactions with *Rxrγ*, it is unlikely that *Rarα* or *Rarγ*, the two other *Rar* isoforms, may functionally compensate for the loss of *Rarβ*, since these receptors display very limited coexpression with *Rxrγ* (Krezel et al., 1999). Considering that in addition to *Rar*'s, the *Rxr*'s interact with other members of the nuclear receptor superfamily, the nature of the heterodimerization partner of *Rxrγ* remains to be determined.

The behavioral abnormalities displayed by *Rxrγ*^{-/-} mice are of particular relevance for research on depression, as they resemble some of the core symptoms specific to depressive disorders and they could be reversed by chronic fluoxetine treatment. Such functions of *Rxrγ* are specific to central control of affective behaviors, since *Rxrγ* null mutant mice do not present dysfunction of the peripheral nervous system or muscles and with the exception of compromised working memory (Wietrzyk et al., 2005), they do not display any other apparent abnormalities (Krezel et al., 1996, 1998). Thus, considering efficiency of

chronic treatment with fluoxetine to reverse anhedonia and despair we speculated that abnormal serotonin signal might be at the origin of depressive-like phenotype in *Rxrγ*^{-/-} mice. Although such hypothesis could be further supported by increased 5HT tissue levels in the striatum and a strong tendency ($p = 0.08$) for increased 5HT turnover in the hippocampus of knockout mice, we found that acute modulation of serotonergic signaling by single treatment with fluoxetine did not affect performance of *Rxrγ*^{-/-} mice in the forced swim test, suggesting strongly that altered serotonin signaling is not sufficient to generate depressive-like behaviors in these mice. In consequence, we considered that long term, adaptive changes associated with chronic fluoxetine treatment might be relevant for beneficial effects of this antidepressant treatment and for the mechanisms of depressive-like behaviors in *Rxrγ*^{-/-} mice. We investigated, therefore, dopaminergic signaling, known to be modulated by chronic fluoxetine treatment and the key neurotransmission pathway involved in the control of motivated behaviors. The dopamine D2 receptor has been suggested to be particularly relevant to such regulations, and its potential implication in depressive disorders and role in antidepressant therapies have been investigated (Dailly et al., 2004; Millan, 2006; Nestler and Carlezon, 2006). In addition, D2r is known to be a direct transcriptional target of retinoid receptors (Krezel et al., 1998; Samad et al., 1997). We found that the inactivation of *Rxrγ* led to a significant reduction in *D2r* mRNA expression specifically within the nucleus accumbens, whereas the expression of *D1r* was not affected in any part of the striatum. Interestingly, our in situ hybridization analysis suggested that reduced *D2r* expression may concern only a subpopulation of neurons in the shell of NAc, since in this region (1) the number of enkephalin positive neurons, a distinct marker of D2r neurons was not altered in *Rxrγ*^{-/-} mice, (2) the intensity of *D2r* signal was not reduced in *D2r* positive neurons of *Rxrγ*^{-/-} mice indicating that reduced *D2r* expression is not generalized, whereas (3) chronic fluoxetine treatment increased the number of *D2r* positive neurons in *Rxrγ*^{-/-} mice, but not in WT mice.

At the moment, it is not clear why reduction of *D2r* expression was not observed in the dorsal striatum of *Rxrγ*^{-/-} mice, which together with the shell of NAc are the brain regions with the most prominent expression of *Rxrγ* (Krezel et al., 1999). One

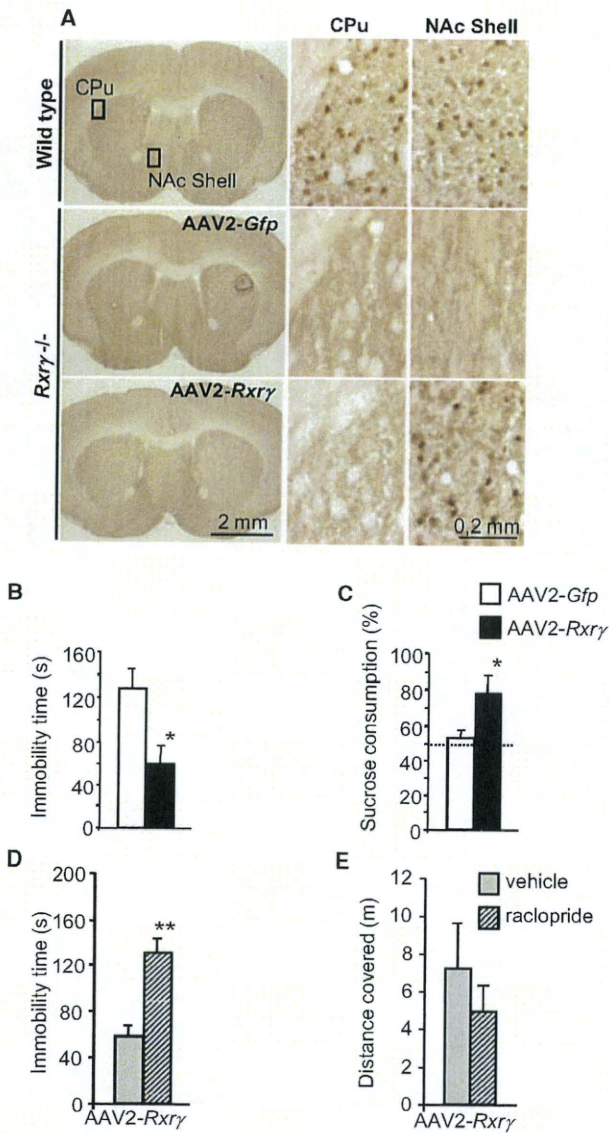


Figure 7. Re-expression of *Rxrg* in NAc Shell Reverses Depressive-like Behaviors in *Rxrg*^{-/-} Mice through D2r-Dependent Mechanism
Immunohistochemical detection of *Rxrg* in the dorsal caudate putamen (CPu) and nucleus accumbens (NAc) shell of WT noninfected mice (A, top row) or *Rxrg*^{-/-} mice after infection with AAV2 vector expressing Gfp (A, middle row) or *Rxrg* (A, bottom row). Re-expression of *Rxrg* in the NAc shell reduced immobility in the forced swim test (B) and restored sucrose preference (C) in *Rxrg*^{-/-} mice as compared with *Rxrg*^{-/-} mice infected with AAV2-Gfp. Acute inhibition of D2r signaling in the NAc shell after bilateral infusion of raclopride (5 μg/side) prevented AAV2-*Rxrg* rescue of depressive-like behaviors in *Rxrg*^{-/-} mice as measured in the forced swim test for *n*_{raclopride} = 5 and *n*_{ACSF} = 7 infused mice (D). Locomotor activity in the open field test was studied in the same mice 2 days after forced swim test (E). Data are presented as mean values ± SEM. **p* < 0.05 in comparison with *Rxrg*^{-/-} mice infected with AAV2-Gfp; ***p* < 0.01 in comparison with ACSF infused control group. See also Figure S3.

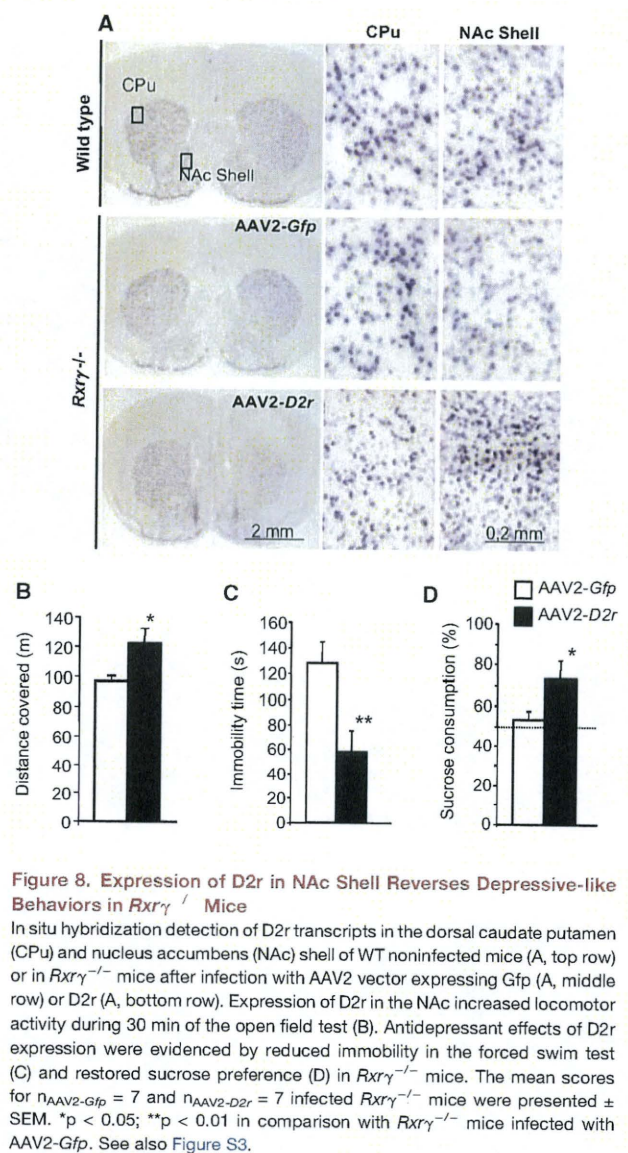


Figure 8. Expression of D2r in NAc Shell Reverses Depressive-like Behaviors in *Rxrg*^{-/-} Mice
In situ hybridization detection of D2r transcripts in the dorsal caudate putamen (CPu) and nucleus accumbens (NAc) shell of WT noninfected mice (A, top row) or in *Rxrg*^{-/-} mice after infection with AAV2 vector expressing Gfp (A, middle row) or D2r (A, bottom row). Expression of D2r in the NAc increased locomotor activity during 30 min of the open field test (B). Antidepressant effects of D2r expression were evidenced by reduced immobility in the forced swim test (C) and restored sucrose preference (D) in *Rxrg*^{-/-} mice. The mean scores for *n*_{AAV2-Gfp} = 7 and *n*_{AAV2-D2r} = 7 infected *Rxrg*^{-/-} mice were presented ± SEM. **p* < 0.05; ***p* < 0.01 in comparison with *Rxrg*^{-/-} mice infected with AAV2-Gfp. See also Figure S3.

possible explanation is that transcriptional control of D2r expression in the dorsal striatum is subject to marked functional redundancy between *Rxrg* and *Rxrα* and/or *Rxrβ*. Such hypothesis is supported by an overall reduction of striatal D2r expression and severe locomotor deficits displayed by *Rxrβ/Rxrg* double null mutants, these defects being absent in the corresponding single null mutants (Krezel et al., 1998).

The reduction of D2r mRNA expression in the NAc of *Rxrg*^{-/-} mice is relevant for D2r functions, as we observed that *c-fos* induction by haloperidol treatment, a D2r antagonist was blunted in mutant mice. In concordance with the topography of deficits in D2r expression, reduced activation of *c-fos* expression was observed in the shell of the NAc, indicating that the reduction of D2r functions might be restricted to this region of the ventral striatum. Such observations are further supported by a blunted

locomotor response of *Rxr γ ^{-/-}* mice to low, noncataleptic doses of haloperidol. Thus, *Rxr γ* null mutant mice were less prone to reduction of locomotor activity in response to haloperidol treatment, suggesting compromised D2r responsiveness in the ventral striatum, the region strongly implicated in the control of horizontal locomotion (Amalric and Koob, 1993; Messier et al., 1992; Pijnenburg et al., 1976; Zhang et al., 1996).

Reduced D2r signaling in *Rxr γ ^{-/-}* mice might be directly related to depressive-like deficits displayed by these mice. In line with this hypothesis, we found that chronic fluoxetine reversal of depressive-like behaviors was accompanied by an increase of D2r expression in the NAcSh of *Rxr γ ^{-/-}* mice. To further explore the behavioral relevance of compromised D2r signaling in *Rxr γ ^{-/-}* mice and the implication of *Rxr γ* in such control, we carried out rescue experiments by virus-mediated re-expression of *Rxr γ* in *Rxr γ ^{-/-}* mice. We found that re-expression of *Rxr γ* in the shell of NAc is critical for modulation of D2r expression and affective behaviors. This was illustrated by an increase of the number of D2r expressing neurons in the NAc and a reversal of behavioral deficits in the forced swim and sucrose preference tests following AAV2 mediated re-expression of *Rxr γ* in the NAc of *Rxr γ ^{-/-}* mice. An increase of D2r expression in NAc appeared to play a critical role in the antidepressant-like activities of *Rxr γ* , since infusion of raclopride, a D2r/D3r antagonist, prevented the rescue of despair behaviors in *Rxr γ ^{-/-}* mice infected with AAV2-*Rxr γ* . Furthermore, we showed that a long-lasting increase of D2r signaling by AAV2 mediated expression of D2r in the NAcSh of *Rxr γ ^{-/-}* mice reversed both despair behaviors in the forced swim and anhedonia in the sucrose preference test. The functionality of viral expression of D2r was confirmed by an increased number of D2r neurons, but also by an increase in novelty induced locomotion as tested in the open field or actimetric cages, which is in agreement with stimulating locomotor effects D2r activation in NAc (Ouagazzal and Creese, 2000; Zhang et al., 1996). Interestingly, such increased locomotion was not observed following re-expression of *Rxr γ* in *Rxr γ ^{-/-}* mice even though it also increased expression of D2r. Such difference might be related to quantitative and qualitative differences in D2r expression, which might have been stronger and display distinct, cell type-specific activities after infection with AAV2-*D2r* as compared to mice infected with AAV2-*Rxr γ* . Although such increased activity may confound results of the forced swim test, we suggest that reduced immobility, induced by AAV2-mediated expression of D2r in *Rxr γ ^{-/-}* mice reflects antidepressant activities since (1) inhibition of D2r signaling by raclopride, which prevented AAV2-*Rxr γ* rescue of despair behaviors in *Rxr γ ^{-/-}* mice, was devoid of nonspecific behavioral effects on locomotion as measured in the open field test; (2) viral expression of D2r also normalized anhedonia in *Rxr γ ^{-/-}* mice, a distinct measure of depressive-like behaviors, not affected by locomotor side effects of AAV2-*D2r* infection. Finally, considering that AAV2 infections lead to low levels of retrograde transduction (Patema et al., 2004), our data on antidepressant effects of AAV2-*D2r* infection of *Rxr γ ^{-/-}* mice suggest the role of post-synaptic D2r in NAc in control of affective behaviors.

In conclusion, this study provides the first evidence that the loss of *Rxr γ* signaling leads to depressive-like behaviors in mice

and indicates that decreased dopamine D2r signaling in the shell of the NAc plays a critical role in *Rxr γ* control of affective behaviors. Considering that retinoids or n-3 PUFAs (de Urquiza et al., 2000; Lengqvist et al., 2004; M.W. et al., unpublished data) can modulate *Rxr* activities in vitro and in vivo, the present data might be of direct relevance for antidepressant activities of n-3 PUFAs reported in clinical conditions (Logan, 2004; Peet and Stokes, 2005) or depression associated with isotretinoin treatment (Bremner and McCaffery, 2008; Kontaxakis et al., 2009). In addition, mnemonic deficits specific to working memory, which were described in *Rxr γ ^{-/-}* mice (Wietrych et al., 2005) might be relevant to cognitive deficits associated with depression. Such deficits, although not considered as the core symptoms of depression, are found in most forms of clinical depression. Consequently, our data suggest that *Rxr γ* is a potential novel target for antidepressant treatments. Unlike conventional neuropharmacology, treatments targeting retinoid receptor(s) could modulate availability of specific neurotransmitter receptors by fine, transcriptional control of their expression. Thus, *Rxr* ligands such as bexarotene (Targretin), used so far in cancer treatment, might be potentially interesting for clinical trials in treatment of depressive disorders.

EXPERIMENTAL PROCEDURES

Animals

Rar β ^{-/-} and *Rxr γ ^{-/-}* single mutant, and *Rar β ^{-/-}Rxr γ ^{-/-}* double mutant male mice as well as their wild-type (WT) control mice were raised on a mixed genetic background (60% C57BL/6J and 40% 129SvEms/J) from heterozygous crosses as described (Krezel et al., 1996), and tested at the age of 4–5 months. All mice were housed in groups of 4–5 mice per cage in a 7 a.m.–7 p.m. light/dark cycle in individually ventilated cages, type "MICE" (Charles River, France). Food and water were freely available. All experiments were carried out in accordance with the European Community Council Directives of 24 November 1986 (86/609/EEC) and in compliance with the guidelines of CNRS and the French Agricultural and Forestry Ministry (decree 87848).

Behavioral Procedures

Forced Swim Test

The forced swim paradigm (Dalvi and Lucki, 1999) was carried out between 1 p.m. and 4 p.m. in a 2-l glass beaker half-filled with water at 22°C–23°C (the water depth was 15 cm). All mice were tested only once in this task. To this end, each mouse was lowered gently into the water and the time of immobility was scored during a 6 min testing period. The mouse was judged immobile when it floated in an upright position and made only small movements to keep its head above the water. After 6 min, the mouse was taken out of the water, left to dry under a red light lamp and returned to its home cage.

Sucrose Preference Test

This task, designed to measure hedonic behaviors in mice (Moreau, 1997; Nestler et al., 2002), is based on the palatable nature of sucrose observed in a number of mouse strains. Mice were first habituated to experimental conditions by an overnight housing in individual cages equipped with one bottle filled with water. On the first day of the test, sucrose-naïve mice were placed at 5 p.m. in the same individual cages with one bottle filled with water and another with 1% sucrose solution. Three hours later (8 p.m.) the bottles were weighed to measure liquid consumption and were replaced in cages until morning to continue habituation to experimental conditions. Over 2 additional days, animals were further habituated to sucrose solution in their home cages. The measures of an overnight consumption were then carried out from 5 p.m. until 8 a.m. to evaluate sucrose preference. Mice were not water deprived at any moment, in order to measure spontaneous sucrose preference and exclude any potential emotional confounds induced by stress of water

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deprivation. The sucrose preference was expressed as the percent of sucrose solution consumed with respect to total liquid consumption.

Actimetry

Spontaneous activity was measured in actimetric cages (Immetronic, Pessac, France) with the help of two arrays of infrared beam photo-cells installed on the side walls in each individual cage. Mice were placed in actimetric cages at 11 a.m. and their activity was recorded over 32 hr including a habituation period between 11 a.m. and 7 p.m. and a complete dark/light cycle until 7 p.m. of the next day.

Open Field

Mice were tested in parallel in five automated open-fields (44.3 × 44.3 × 16.8 cm) made of PVC with transparent walls and a black floor, covered with transparent PVC (Panlab, Barcelona, Spain). The open fields were placed in a room homogeneously illuminated at 150 Lux. Unless otherwise specified each mouse was placed in the periphery of the open field and allowed to explore freely the apparatus for 30 min, with the experimenter out of the animal's sight. Activity parameters including distance traveled over the test session were calculated automatically.

Catalepsy Test

Mice were injected intraperitoneally with 0.2 or 2 mg/kg of haloperidol (Sigma) and after 30 min were placed in the test cage with their forelimbs on the wooden transversal bar fixed at a level of 3 cm above floor level. The latency to move out from the bar was scored and used as index of catalepsy.

Production and Use of Adenoassociated Virus (AAV) Vectors

For generation of AAV vectors we used a vector plasmid containing an expression cassette, in which a human cytomegalovirus immediate-early promoter (CMV promoter) was followed by the first intron of the human growth hormone gene, the cDNA of interest, woodchuck hepatitis virus posttranscriptional regulatory element (WRPE; nucleotides 1093 to 1684, GenBank accession number J04514) and simian virus 40 polyadenylation signal sequence. This expression cassette was inserted between the inverted terminal repeats (ITR) of the AAV-2 genome as described (Li et al., 2006). The viral vectors used for expression of *Rxr γ* (AAV2-*Rxr γ*), *D2r* (AAV2-*D2r*), and *EGfp* (AAV2-*Gfp*) contained the entire cDNA sequences of *Rxr γ* (GenBank accession number NM_009107), *D2r* (long isoform, GenBank accession number NM_010077.2), or *EGfp*, respectively. We used two helper plasmids, pAAV-RC and pHelper, harboring the AAV *rep* and *cap* genes, and the *E2A*, *E4*, *VA1* genes of the adenovirus genome, respectively (Agilent Technologies, Santa Clara, CA). HEK293 cells were cotransfected with pAAV-RC and pHelper plasmids using the calcium phosphate coprecipitation method. AAV particles were then harvested and purified by two sequential continuous iodixole ultracentrifugations. The vector titer was determined by quantitative PCR of DNase-I-treated vector stocks, and were estimated at 10^{10} to 10^{12} vector genome copies (vg).

For rescue experiments and *D2r* expression we used behaviorally naive *Rxr γ ^{-/-}* male mice ($n = 29$) at the age of 8 months. Each animal was anaesthetized using ketamine (100 mg/kg)/xylazine (10 mg/kg) solution and 0.7 μ l of AAV2-*Rxr γ* , AAV2-*D2r*, or AAV2-*Gfp* suspension was injected bilaterally into the nucleus accumbens (bregma = +1.5; lateral = ± 0.7 ; ventral = +4.2, the coordinates identified prior to experiments using dye injections and corresponding to bregma = +1.3; lateral = ± 0.5 ; ventral = +4.0 position in the Mouse Brain Atlas; Paxinos and Franklin, 2001) using a stereotaxic apparatus (Precision Cinematographique, Paris, France). The injection was carried out at 50 nl/min using a Harvard Apparatus PHD 2000 pump (Holliston, USA), and the injectors were withdrawn from the brain 20 min after the end of the injection. After placing stitches each animal was left to awake in the temperature-conditioned cage. Mice were tested 4 weeks later and their brains were removed for post hoc analyses.

Drug Infusion Procedure

Rxr γ ^{-/-} mice ($n = 15$) aged between 4 and 5 months were infected with AAV2-*Rxr γ* as described. Four weeks later, mice were anaesthetized with ketamine/xylazine solution and 8 mm long stainless-steel guide cannulas (0.4 mm external diameter; Cortat, Courrendlin, Switzerland), were positioned bilaterally 1 mm above the NAcSh (bregma = +1.5; lateral = ± 0.7 ; ventral = +3.2) using stereotaxic apparatus (Precision Cinematographique, Paris, France). The

cannulas were fixed to the skull with anchoring screws and dental cement. Stainless steel stylet rods were inserted into the cannulas to prevent occlusion. On the day of the experiment raclopride (a D2/D3 specific antagonist soluble in aqueous solutions; Sigma) was dissolved in fresh artificial cerebrospinal fluid (ACSF, which consisted of 3 mM KCl, 140 mM NaCl, 2 mM glucose, 1.2 mM CaCl₂, 1 mM MgCl₂, 0.27 mM NaH₂PO₄, 1.2 mM Na₂HPO₄ [pH 7.4]) and infusions of 0.25 μ l of raclopride (5 μ g/side) or vehicle (ACSF) were performed at 100 nl/min using Harvard Apparatus PHD 2000 pump and stainless-steel injector needles (0.28 mm external diameter) that protruded from the cannula by 1 mm, into the NAcSh. Three minutes after injection injector needles were removed from the brain, the stylet rods were replaced in cannula guides and mice were transferred to their home cage for 5 min prior to the forced swim test. Forty-eight hours later, mice were semirandomly infused with raclopride or ACSF to test the locomotor effects of such treatments. For this experiment mice were placed in the open field immediately after removing injectors and placement of stylet rods and their activity was scored 5 min later during 5 min. Three out of fifteen mice were excluded from analysis due to unilateral AAV2-*Rxr γ* infection or incorrect guide placement, and two mice could not be infused for the open field test since the stylets remained blocked.

Pharmacological Treatments

Haloperidol (Sigma-Aldrich) was dissolved in acetic acid solution and pH was neutralized with NaOH. For *c-fos* expression studies, mice were injected intraperitoneally (IP) with saline or 1 mg/kg of haloperidol, 90 min prior to sacrifice, whereas for analysis of the open field behavior saline or haloperidol were injected 20 min prior to the test and animals were tested for 10 min. IP injection was also used for acute fluoxetine (Lilly France) treatment 30 min prior to forced swim test. For chronic treatment, fluoxetine was added to the standard chow diet. Accordingly, we supplemented standard chow in powdered form with fluoxetine to attain the dose of 20 mg of fluoxetine per kg of body weight during 24 hr. To calculate such a dose, the food consumption was first estimated experimentally to be 4 g of food pellets per 24 hr per animal. Fluoxetine-supplemented food pellets were immediately lyophilized and stored at -20°C until use. For treatment, standard chow pellets were replaced by fluoxetine-supplemented food pellets and were provided ad libitum in standard home cages throughout treatment period. The consumption of fluoxetine-containing pellets did not differ from the consumption of nonsupplemented food pellets in control cages. WT and *Rxr γ ^{-/-}* mice treated with fluoxetine or fed control diet were all tested for sucrose preference on the nineteenth day of treatment, and in the forced swim test on the twenty-first day of treatment, with the exception of mice used for evaluation of fluoxetine effects on *D2r* expression, which were all behaviorally naive.

Quantitative RT-PCR**Dissection of Brain Areas**

Mice were killed by cervical dislocation. Whole brains were extracted, fresh-frozen in OCT, and kept at -80°C until use. Tissue corresponding to the nucleus accumbens (NAc) was collected with 0.5 mm punch from three subsequent 300 μ m thick cryosections. Similarly, dorsolateral striatum (CPU) was collected using 0.8 mm punch from four subsequent frozen sections of 300 μ m. The accurate location of these brain structures was based on visual inspection of each section using a stereomicroscope (Leica, Wild M715) and its comparison with the stereotaxic atlas of mouse brain; Paxinos and Franklin, 2001). Tissue samples were placed on dry ice and kept at -80°C until use.

Quantitative RT-PCR

Total RNA extraction was carried out using the RNeasy Micro Kit protocol (QIAGEN, France). Total RNA from each tissue sample was transcribed into cDNA using QuantiTect Reverse Transcription Kit according to the manufacturer's recommendation. Briefly, the reaction was carried out at 42°C for 20 min in a total volume of 20 μ l and was inactivated at 95°C . Twenty-times-diluted cDNA was used as a template, and quantitative real-time PCR was run in a LightCycler 480 (Roche, Diagnostics, Mannheim, Germany) using LightCycler SYBR Green kit (Roche, Diagnostics) with cDNA and gene-specific primers (100 μ M) following the manufacturer's instructions. All of the reactions were performed in triplicate with the following cycling

protocol: 10 min of heat activation of the enzyme at 95°C, 45 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 30 s, and extension at 72°C for 20 s. Fluorescence detection was performed at 72°C. Gene-specific primers were designed using Primer3 software (primer3) to amplify fragments of 150–250 bp as follows: for *D2r* (Drd2; NM 010077) forward, TCGCC ATTGTCTGGGTCCTG; reverse, TGCCCTTGAGTGGTGTCTTC; *D1r* (Drd1a; NM 010076) forward, AAGATGCCGAGGATGACAAC; reverse, CCCTC TCCAAAGCTGAGATG. The transcript amounts evaluated for *D1r* and *D2r* were normalized for the quantity and quality of each sample by division by the amount of transcript of the housekeeping gene acidic ribosomal phosphoprotein P0 (Arbp or 36B4; NM 007475) in the same sample and such relative values were presented in Figures 4A and 2S. *36B4* transcript amount was quantified using forward primer ACCCTGAAGTGCCTCGACATC and reverse primer AGGAAGGCCTTGACCTTTTC.

In Situ Hybridization and Analysis of Expression Levels

In situ hybridization (ISH) was performed on 14 μ m thick frozen sections with digoxigenin-labeled riboprobes synthesized from a 1680 bp *D2r* cDNA template and an enkephaline 800 bp cDNA template as described (Krezel et al., 1998). Hybridization conditions were as described previously (Krezel et al., 1998) and are available on the <http://empres.har.mrc.ac.uk/> website. The amount of probe used for hybridization and signal detection conditions was adapted to avoid saturation of the chromogenic labeling (see below). Expression patterns were documented using a macroscope (Leica M420) or microscope (DM4000B), both connected to a Photometrics camera with the CoolSNAP (v. 1.2) software (Roger Scientific, Chicago, IL).

For the analysis of cell counts and expression levels of *D2r*, the images were transformed into gray scale and analyzed using ImageJ software (Rasband, 1997). The strongest signal observed for any of the neurons in any of the brain sections remained between 67 and 95 units in a 0 to 255 unit gray scale (0 corresponding to black), being thus 25%–30% below full saturation conditions in order to enable quantitative analysis of signal intensity. For each animal, the cell number and intensity of cellular signal was evaluated within selected regions of CP_u, NAcSh and NAcCo on the same sections (for region selection see Figures 5A and 5B) at bregma 1.10 and 1.40 (Paxinos and Franklin, 2001). The mean values of cell counts or intensity for each region were calculated and compared as described in Results.

Immunohistochemistry and *c-fos* Cell Counts

Coronal sections (14 μ m thick) from unfixed frozen brains of 4-month-old *Rxry*^{-/-} mice and their WT littermates were collected on super-frost slides and stored at -80°C until analysis. Sections were postfixed in 4% paraformaldehyde and treated with 1% H₂O₂ to block endogenous peroxides. For detection of Rxry, we used rabbit anti-Rxry polyclonal antibody (SC555, batch A111, Santa Cruz, US), whereas *c-fos* was detected using rabbit anti-*c-fos* polyclonal antibody (1:1000, Chemicon). Both primary antibodies were detected using the ABC system (Vector, USA) according to the manufacturer's manual. For each animal and section, corresponding brain regions were identified according to the mouse brain atlas (Paxinos and Franklin, 2001) and *c-fos*-positive cells were counted from identical surfaces defined by region-corresponding auto-shape figures (Figures 5A and 5B) at two levels of the striatum (bregma 1.10 and 1.40; Mouse Brain Atlas; Paxinos and Franklin, 2001). The mean cell counts for each brain region were calculated and compared as described in Results.

HPLC Measures of Serotonin and Its Metabolites in the Brain Tissue

The brain samples of *n* = 6 WT and *n* = 6 *Rxry*^{-/-} male littermates (4 months old) were weighed immediately after collection and frozen at -80°C until use. Before analysis, samples were thawed, and homogenized in 10 volumes (w/v) of 0.1 M HClO₄ containing the internal standard DHBA (125ng/ml). The homogenates were centrifuged at 12,000 rpm for 20 min at 4°C and supernatant was retained for analysis. Serotonin and its metabolite 5-hydroxyindoleacetic acid (5HIAA) were evaluated using high performance liquid chromatography (HPLC) with electrochemical detection. The chromatographic system consisted of a 25 cm \times 4.6 mm Hypersyl C18 ODS column (particle size 5 μ m, Biochrom, France). The column was kept at a constant temperature of 30°C. The flow rate was 1.2 ml/min with a back pressure of

1500 psi (Waters Instrumentation). The system was linked to a Waters model 460 electrochemical detector with a glassy-carbon electrode. Detector potential was maintained at 0.85 V (reference, Ag/AgCl electrode). The mobile phase consisted of 0.05 M NaH₂PO₄ and 0.1 mM EDTA (pH adjusted to 4.85 with NaOH) in double-distilled water with methanol (6%). The system was calibrated by injecting various amounts (3.4 pg–34 ng) of standard solutions, containing 1.1 ng of internal standard DHBA (3–4 dihydroxybenzylamine 1 mM in HClO₄ 0.1 M). The supernatant of each sample was injected onto the column, and peak identification was performed by comparing retention times with the calibration solution. Results were expressed in ng/g \pm SEM.

Statistical Analysis

The comparisons of behavioral performance in *Rar β* ^{-/-}/*Rxry*^{-/-}, *Rar β* ^{-/-}, and *Rxry*^{-/-} null mutant mice were carried out using the protected least significant difference (PLSD) Fischer test. The pharmacological data for the treatments in WT and *Rxry*^{-/-} mice were analyzed using two-way analysis of variance (ANOVA)—with treatment and genotype as two independent factors and behavioral responses as dependent variables. Comparison of the evolution of locomotor performance in the open field or actimetric cages were evaluated using ANOVA on repeated-measures. Global and post hoc statistical analyses were performed using the PLSD Fischer test and for two-group comparisons using student t test (see t values in the text). Significant differences are indicated in the corresponding figures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at doi:10.1016/j.neuron.2010.05.004.

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A Phase I Study of Aromatic L-Amino Acid Decarboxylase Gene Therapy for Parkinson's Disease

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Gene transfer of dopamine-synthesizing enzymes into the striatal neurons has led to behavioral recovery in animal models of Parkinson's disease (PD). We evaluated the safety, tolerability, and potential efficacy of adeno-associated virus (AAV) vector-mediated gene delivery of aromatic L-amino acid decarboxylase (AADC) into the putamen of PD patients. Six PD patients were evaluated at baseline and at 6 months, using multiple measures, including the Unified Parkinson's Disease Rating Scale (UPDRS), motor state diaries, and positron emission tomography (PET) with 6-[¹⁸F]fluoro-L-m-tyrosine (FMT), a tracer for AADC. The short-duration response to levodopa was measured in three patients. The procedure was well tolerated. Six months after surgery, motor functions in the OFF-medication state improved an average of 46% based on the UPDRS scores, without apparent changes in the short-duration response to levodopa. PET revealed a 56% increase in FMT activity, which persisted up to 96 weeks. Our findings provide class IV evidence regarding the safety and efficacy of AADC gene therapy and warrant further evaluation in a randomized, controlled, phase 2 setting.

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INTRODUCTION

Dopamine replacement has been the standard pharmacotherapy for motor impairment in Parkinson's disease (PD). Although virtually all patients benefit from levodopa at an early stage of the disease, severe loss of nigrostriatal nerve terminals in advanced PD leads to profoundly decreased activities of dopamine-synthesizing enzymes, including aromatic L-amino acid decarboxylase (AADC), an essential enzyme that converts levodopa to dopamine. Failure to respond to levodopa therapy may result from a reduction in AADC activity, decreased dopamine storage capacity in synaptic vesicles, postsynaptic changes in striatal output neurons, and abnormalities

of nondopaminergic neurotransmitter systems.^{1,2} Systemic administration of high-dose levodopa enhances oscillations in motor performance and complications, including hallucinations, due to dopaminergic stimulation of the mesolimbic system.

One potential treatment for advanced PD is gene therapy to restore striatum-selective dopamine production. In addition to AADC, tyrosine hydroxylase, which converts L-tyrosine to levodopa, and guanosine triphosphate cyclohydrolase I, which catalyzes biosynthesis of the essential tyrosine hydroxylase cofactor, tetrahydrobiopterine, are necessary for efficient synthesis of dopamine.² Viral vector-mediated gene transfer of these dopamine-synthesizing enzymes has been shown to achieve behavioral recovery in animal PD models, with efficient transduction of striatal neurons that escape degeneration.³⁻⁶ When tyrosine hydroxylase and guanosine triphosphate cyclohydrolase I are expressed in the striatum, levodopa can be synthesized continuously. This strategy would be useful for reducing motor fluctuations associated with intermittent levodopa intake. Gene transfer of AADC alone in combination with oral levodopa administration would be a safer strategy for initial clinical trials. In the latter approach, the patients still need to take levodopa to control motor symptoms, but excess production of dopamine could be avoided by reducing the dose of levodopa. We assessed the safety, tolerability, and the potential efficacy of intraputamenal infusion of recombinant adeno-associated virus (AAV) serotype 2 vector encoding human AADC (AAV-hAADC-2) in patients with mid- to late-stage PD. We also examined whether the short-duration response to levodopa, the antiparkinsonian response that parallels the plasma levodopa levels, would change after gene therapy.⁷

RESULTS

Patient disposition and baseline characteristics

Six patients (4 men, 2 women), mean age 60 (range, 51–68) years, were enrolled (Table 1). The mean disease duration was 10 (range, 5–18) years, and time on levodopa was 9.3 (range, 5–15) years. The average baseline daily levodopa and levodopa equivalent doses were 642 and 808 mg, respectively.

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Table 1 Patients' baseline characteristics

Subject	Age (years)	Sex	Disease duration (years)	Time on levodopa (years)	Levodopa dose (mg)	Levodopa equivalents (mg)
A-1	51	M	11	9	600	900
A-2	63	M	9	9	450	650
A-3	66	F	7	7	500	700
A-4	58	M	11	11	700	700
A-5	68	F	18	15	1,000	1,100
A-6	56	M	5	5	600	800
Mean (SD)	60 (6.5)	67% M	10 (4.5)	9.3 (3.4)	642 (196)	808 (169)

Abbreviations: F, female; M, male.

Patients are listed in the order in which they received treatment. Levodopa equivalents were estimated as follows: 100 mg of levodopa with a dopa-decarboxylase inhibitor is equivalent to 0.8 mg talipexole, 1 mg pergolide, 1 mg pramipexole, and 1.5 mg cabergoline.

Primary end point

The procedure was well tolerated. All patients completed all protocol-defined visits. One patient (patient A-2) had a venous hemorrhage in the right frontal lobe just below a burr hole that was found on CT scan 3 days after infusion. The patient used his left arm less frequently than his right arm for 3 weeks; this was assumed to reflect mild frontal lobe dysfunction and resolved completely. Mild, transient headache around the burr holes was present for 2 days after surgery in all patients. There were no significant laboratory test abnormalities. All patients had mildly increased titers of anti-AAV2-neutralizing antibodies 6 months after treatment, which tended toward baseline concentrations thereafter (Table 2).

Clinical evaluations

The clinical results are summarized in Table 3. Intraputaminally AAV-hAADC-2 infusion significantly improved both total and motor scores of the unified Parkinson's disease rating scale (UPDRS) in the OFF state. Five of six patients showed substantial improvement in UPDRS motor ratings in the OFF state (Figure 1). Changes in the UPDRS ON state and the percent of ON state hours in a day were not significant. One patient with relatively mild motor symptoms at baseline did not improve on UPDRS (A-3 in Figure 1). However, this patient showed a remarkable increase in mobile time as measured by the diaries (28% at baseline to 58% at 6 months after gene transfer; Figure 2). The daily dose of levodopa was unchanged in two patients (A-2 and A-5) and reduced in three patients (A-1, A-3, and A-5) at 6 months. Patient A-6, who had daytime sleepiness, preferred to reduce pramipexole instead of levodopa after gene therapy.

The last three patients underwent the levodopa test after our institutional review board confirmed the safety of AADC gene transfer in the first three patients. The short-duration response to levodopa did not change significantly after gene therapy in these three patients, though UPDRS motor scores at 6 months showed slight improvement at 30 minutes in patient 5 and at 120 minutes in patient 4 after levodopa intake (Figure 3). Significantly higher peak plasma levodopa concentrations were observed in these two patients after gene therapy.

The mini-mental state examination (MMSE) and geriatric depression scale (GDS) scores did not change significantly.

Table 2 Changes in neutralizing AAV2 antibody titers in sera following gene therapy

Subject	Pre	2 weeks	6 months	1 year
A-1	1:2	1:4	1:4	1:4
A-2	<1	1:32	1:4	1:2
A-3	1:32	1:64	1:64	1:32
A-4	1:32	1:32	1:256	1:64
A-5	1:4	1:32	1:32	1:32
A-6	<1	1:16	1:32	1:32

Abbreviations: AAV, adeno-associated virus.

Titers are determined by *in vitro* assay and represented as "1:" dilutions.

Table 3 Clinical outcomes of six patients

	Baseline	6 months	P value
UPDRS Total OFF	53 (12.4)	38 (10.1)	0.049*
UPDRS Total ON	15 (7.2)	10.7 (2.9)	0.262
UPDRS Part III (Motor) OFF	25.3 (9.4)	13.7 (6.0)	0.024*
UPDRS Part III (Motor) ON	5.2 (4.6)	1.8 (1.5)	0.120
Percent day spent in mobile state	48.8 (12.9)	55.4 (14.8)	0.348
Daily levodopa equivalents dose, mg	808 (169)	707 (233)	0.097

Abbreviations: OFF, off-medication state; ON, on-medication state; UPDRS, Unified Parkinson's Disease Rating Scale.

Data are presented as means (SD). The UPDRS scores in each patient did not change during the 2 months of the screening period.

* $P < 0.05$.

PET analysis

PET imaging revealed increased 6-[¹⁸F]fluoro-L-m-tyrosine (FMT), a tracer for AADC, activity 4 weeks postoperatively, which persisted at 6-month evaluation (Figure 4). The mean increase in FMT uptake from baseline in the combined (right and left) putamen at 24 weeks was 56%. Two patients (A-1 and A-2) who had PET scans 96 weeks after surgery showed persistently increased FMT uptake. In these two patients, motor performance in the OFF state also maintained its improvement at 96 weeks.

DISCUSSION

Extensive preclinical studies on both rodent and nonhuman primate models of PD have shown that AAV vectors can express exogenous genes for a long time in the brain target areas without