not show any fluctuations after several years of L-dopa therapy, since such fluctuations are almost invariable in PD patients. Many patients also start to experience some deleterious complications such as hallucinations and compulsive behaviors due to dopaminergic stimulation of the mesolimbic system (Aarsland *et al.*, 2009; Evans *et al.*, 2009; Voon *et al.*, 2009). Patients eventually become disabled. Novel therapeutic interventions in place of oral L-dopa administration are therefore required.

Preclinical Studies with Adeno-Associated Virus (AAV) Vectors

PD is a suitable candidate for gene therapy (Muramatsu *et al.*, 2005). PD is primarily confined to the well-defined nigrostriatal dopaminergic system and it is not necessary to deliver therapeutic genes to the entire brain, but only to a portion of the basal ganglia. Stereotactic surgical techniques to approach basal ganglia are established in clinical practice. In addition, well-characterized rodent and primate PD models are available for testing novel therapeutic interventions.

Viral vectors, in particular vectors derived from adeno-associated virus (AAV), are suitable for the transduction of neurons in the mammalian brain without significant toxicity. Recombinant AAV vectors have been applied in clinical trials for numerous disorders including hemophilia, cystic fibrosis and retinal degeneration (Daya and Berns, 2008). No adverse effects due to the administration of the vectors themselves have so far been found.

Researchers have been developing gene therapy method to restore local dopamine production in the striatum using AAV vectors. Gene transfer of TH, GCH and AADC, or AADC alone into the putamen has lead to behavioral recovery in primate models of PD (Fan *et al.*, 1998; Bankiewicz *et al.*, 2000; Shen *et al.*, 2000; Sanchez-Pernaute *et al.*, 2001; Muramatsu *et al.*, 2002; Bankiewicz *et al.*, 2006; Forsayeth *et al.*, 2006; Li *et al.*, 2006). In our study, cynomolgus monkeys (*Macaca fascicularis*) received the intravenous injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a selective toxin of dopaminergic neurons, once a week until a stable parkinsonian syndrome was achieved (Muramatsu *et al.*, 2002). Then mixtures of three AAV vectors that express TH, AADC, and GCH, respectively were stereotaxically injected into the unilateral putamen. Coexpression of the enzymes in the unilateral putamen resulted in remarkable improvement in manual dexterity on the contralateral to the vectors-injected side. Transduced cells were mainly medium spiny neurons and present in a large region of the putamen (>90% of the putamen). Microdialysis demonstrated that concentrations of dopamine in the vectors-injected putamen were increased in comparison to the control side. Moreover the level of dopamine was remarkably elevated after systemic administration of L-dopa with peripheral decarboxylase inhibitor. Monkeys showed no complications related to the AAV vector injection including dyskinesia.

Clinical Studies of AADC Gene Therapy

Two phase I clinical trials were conducted at the University of California San Francisco (UCSF) (Christine *et al.*, 2009) and Jichi Medical University (JMU)

(Muramatsu *et al.*, 2010) to evaluate the safety and potential efficacy of AAV vector-mediated gene delivery of AADC to the bilateral putamen in combination with the oral administration of L-dopa. Both trials confirmed the safety of the AAV vectors for clinical use in the human brain and the motor symptoms associated with PD were alleviated.

A low dose cohort of five patients received 1×10^{11} vector genome (vg) and a high dose cohort of five patients received 3×10^{11} vg of AAV vector expressing AADC (AAV-AADC) in the UCSF study. The mean improvements of the ten patients in the total score of unified Parkinson's disease rating scale (UPDRS) were 31% (10.5 points) in the "off" medication state and 32% (10 points) in the "on" medication state. The mean improvements on the motor score of UPDRS were 36% (12 points) in the "off" state and 28% (4.3 points) in the "on" state. Positron emission tomography (PET) using [^{18}F]fluoro-L-m-tyrosine (FMT), a tracer for AADC, revealed a 30 and 75% increase in Ki^c values in the putamen of the low-dose and high-dose cohort, respectively, at 6 months after the gene delivery.

Six patients received 3×10^{11} vg of AAV-AADC in the JMU study. The motor function in the "off" state improved to a mean of 46% (11.6 points) based on the motor score of UPDRS at six months after surgery. PET revealed a 56% increase in FMT activity, which persisted for up to 96 weeks (Fig. 2).

It is worth noting that motor function in the "off" state improved in both studies. The anti-parkinsonian effects observed after L-dopa administration have generally been recognized as short- and long-duration responses. The short-duration response roughly parallels the plasma L-dopa concentrations, while the long-duration response builds up over weeks and improves trough (worst) motor performance in the "off" state (Nutt, 2008). The pattern of the short-duration response to L-dopa did not change after gene therapy in the JMU study and the beneficial effect on the "off" state appears to be attributed to augmentation of the long-term response to L-dopa. Although the mechanism underlying the long-duration response is not sufficiently understood, improving the trough or "off" state motor function by augmenting the long-term response would likely reduce motor fluctuation.

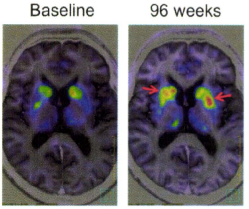

Fig. 2. FMT-PET images. Axial images at the level of the putamen are shown before and 96 weeks after gene therapy. The uptake of FMT was observed to increase after the AAV vector-mediated gene delivery of AADC (red arrows). FMT, 6-[^{18}F]fluoro-L-*m*-tyrosine, a tracer for AADC; PET, positron emission tomography.

Continuous Synthesis of Dopamine

Gene transfer of AADC alone in combination with oral L-dopa administration would be a safer strategy for initial clinical trials because excess production of dopamine could be avoided by reducing the dose of L-dopa. However, TH and GCH gene transfer into striatal cells may appear offer a more efficient method of supplying L-dopa continuously in comparison to the administration of exogenous L-dopa and avoid motor fluctuations associated with intermittent administration of L-dopa.

A phase I/II trial involving the triple gene transfer of TH, GCH and AADC into the bilateral putamen using a vector derived from the equine infectious anemia virus (EIAV) has been initiated at the Henri Mondor Hospital in France. Two dose levels have been evaluated in cohorts of three patients per dose (Oxford BioMedica, 2010). The first cohort of patients, treated at the lowest initial dose level, has now completed two-year assessments. The mean improvement from baseline in the motor score of UPDRS was 20%, in two of the three patients sustained effects of 30% improvement were observed without any increase in L-dopa dose. The third patient remained at levels of improvement similar to baseline. The maximum improvement in motor function at one year was 56% in the higher dose group and the mean was 28% for both doses relative to the patients' pre-treatment motor function.

Although it is difficult to package multiple genes in a single AAV vector because of the limited capacity on the size of the DNA (<5 Kb), one cell could be simultaneously transduced with different AAV vectors (Shen *et al.*, 2000; Muramatsu *et al.*, 2002). AAV vectors would therefore be the next choice for clinical trials for triple gene transfer of the TH, AADC, and GCH.

Other Strategies and Future Prospects

Gene therapy for PD has been tested in clinical trials due to the development of efficient viral vectors (Kaplitt, 2010). Two other strategies are developed in addition to dopamine replacement. One is designed to protect nigrostriatal projection by producing neurturin, a trophic factor for dopaminergic neurons, in the putamen. The other strategy is to modulate the neural activity along the output pathway of the basal ganglia by transducing the subthalamic nucleus with vectors expressing glutamic acid decarboxylase (GAD-65, GAD-67), a key enzyme for synthesis of the inhibitory transmitter γ-aminobutyric acid (GABA). Initial results of phase I studies indicate that these two strategies are also promising (Kaplitt *et al.*, 2007; Marks *et al.*, 2008). However, preliminary results of a phase II double-blind study of the neurturin strategy failed to show significant beneficial effects over the sham operation group, thus earlier intervention may be required for success of this kind of neuro-protective approach in future trials. Another phase I/II study is ongoing in which the patients receive AAV vectors expressing neurturin both in the putamen and the SNc.

There are several PD symptoms that do not respond well to L-dopa, such as cognitive dysfunction, depressive state, frozen gait, posture reflex disturbance and

sleep disturbance (Sethi, 2008). Effective therapeutic genes must be identified for the treatment of these symptoms, and then to be delivered into the appropriate areas of the brain. The development of vector constructs that avoid over-expression is also required for increasing safety (Li *et al.*, 2006). Gene therapy is therefore expected to become the therapy of choice for PD in the near future.

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The current status of gene therapy for Parkinson's disease

Shin-ichi Muramatsu, M.D, Ph.D

Division of Neurology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, JAPAN

ABSTRACT

The recent development of viral vectors, especially vectors derived from adeno-associated virus (AAV), has translated gene therapy for Parkinson's disease (PD) from animal experiments into clinical trials. The current gene therapy protocols used are based on three major strategies. The first protocol involves local production of dopamine via the introduction of dopamine-synthesizing enzyme genes into the putamen. The aromatic L-amino acid decarboxylase (AADC) gene has been transferred in this manner with the aim of efficiently converting orally administered L-dopa. The delivery of triple genes including tyrosine hydroxylase (TH), guanosine triphosphate cyclohydrolase I (GCH) and AADC is also being undertaken, and is aimed at continuously supplying dopamine into the putamen. The second protocol involves the protection of nigrostriatal projections via the production of neurturin, a trophic factor for dopaminergic neurons in the putamen. The final method includes the modulation of neural activity along the output pathway of the basal ganglia by transducing the subthalamic nucleus with vectors expressing glutamic acid decarboxylase (GAD-65, GAD-67), a key enzyme required for the synthesis of the inhibitory transmitter γ-aminobutyric acid (GABA). The initial results of phase 1 studies using AAV vectors have not only confirmed the safety of these vectors, but have also revealed the alleviation of motor symptoms associated with PD.

KEY WORDS : Adeno-associated virus, Aromatic L-amino acid decarboxylase, Neurturin, Glutamic acid decarboxylase, Positron emission tomography
Corresponding Author : Shin-ichi Muramatsu, M.D, Ph.D, E-mail : muramats@jichi.ac.jp; Tel: +81-285-58-7352, Fax: +81-285-44-5118

doi : 10.5214/ans.0972-7531.1017209

Introduction

Almost two decades have passed since the first gene therapy clinical trial was conducted for the treatment of adenosine deaminase deficiency at the National Institute of Health in the United States in 1990. Although gene therapy appeared to be a ground breaking form of medical treatment at the time, it has not proven to be as successful in treating disease as initially anticipated. A patient with ornithine transcarbamylase deficiency died of systemic inflammatory response syndrome (SIRS) following the administration of a large quantity of adenoviral vector into the hepatic artery.[1] In addition, some children with X-linked severe combined immunodeficiency (X-SCID) developed leukemia due to the activation of an oncogene after gene therapy using a retroviral vector.[2,3] These reports describing the severe, adverse effects of gene therapy dampened the excitement underlying the success of gene therapy treatment to some extent. However, encouraging results have been obtained more recently with clinical studies for Parkinson's disease (PD).[4-7]

Table. Gene therapy clinical trials for Parkinson's disease.

Gene	AADC		TH/GCH/AADC	Neurturin		GAD	
Function	Convert L-dopa to dopamine		Synthesis of dopamine from tyrosine	Neurotrophic factor for dopaminergic neurons		Synthesis of inhibitory neurotransmitter GABA	
Vector	AAV		EIAV	AAV		AAV	
Phase	I		I	I	II	I	II
Institute	UCSF[7]	JMU	Henri Mondor Hospital[22]	UCF[8]	Multi-center[28]	NYP Hospital[5]	Multi-center
Dose	$9x10^{10}$ $3x10^{11}$	$3x10^{11}$	1x 2x	$1.3x10^{11}$ $5.4x10^{11}$	$5.4x10^{11}$ Sham	$3.5x10^{9}$ $1x10^{10}$ $3.5x10^{10}$	$1x10^{11}$ Sham
Number of subjects	10	6	6	12	54	12	40
Target	Putamen (Bilateral)		Putament (Bilateral)	Putamen (Bilateral)		STN (Unilateral)	STN (Bilateral)
PET tracer	[^{18}F] fluro-m-tyrosine			[^{18}F] fluro-DOPA		[^{18}F] fluro-deoxyglucase	

AADC, aromatic L-amino acid decarboxylase; AAV, adeno-associated virus; EIAV, equine infectious anemia virus; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GCH, guanosine triphosphate cyclohydrolase I; TH, tyrosine hydroxylase; STN, subthalamic nucleus. Dose of AAV vectors are represented as vector genome/patient.

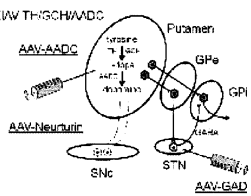

Figure. Current gene therapy strategies for the treatment of Parkinson's disease

Three gene therapy strategies are currently available for the treatment of (PD. 1) production of dopamine. AAV vectors expressing AADC or EIAV vector expressing TH, GCH and AADC are infused into the putamen. 2) Protection of the nigrostriatal pathway. The neurotrophic factor neurturin is produced continuously in the putamen. 3) Modification of neuronal activity in the STN by introducing the GAD gene.

AADC, aromatic L-amino acid decarboxylase; AAV, adeno-associated virus; EIAV, equine infectious anemia virus; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GCH, guanosine triphosphate cyclohydrolase I; GPe, globus pallidus external segment; GPi, globus pallidus internal segment; TH, tyrosine hydroxylase; SNc, substantia nigra pars compacta; STN, subthalamic nucleus.

Adeno-associated virus vectors

Technology that efficiently introduces a therapeutic gene into target cells is essential for successful gene therapy. Viral vectors, in particular vectors derived from adeno-associated virus (AAV), have been shown to be suitable for the transduction of neurons in the mammalian brain using stereotaxic surgery.[8] AAVs are small (25 nm), single stranded DNA viruses that belong to Parvoviridae.[9] No clear pathogenicity in humans has been reported, and many adults synthesize antibodies against AAVs following latent infection in childhood.[10,11] The AAV genome exists in episomes in the nucleus and is rarely incorporated in the chromosomes. To date, more than one hundred genotypes have been defined for primate AAVs. The vast majority of vectors used in clinical applications are derived from serotype 2 (AAV2).

AAV2 has a 4.7-kb genome, both ends of which contain hairpin structures called inverted terminal repeats (ITR). A region encoding the non-structural protein Rep and capsid protein VP are also present. These regions for Rep and VP can be substituted with a therapeutic gene when constructing vectors. AAV vectors express an exogenous gene in the brain for long term use (more than seven years) and do not produce any significant inflammatory or immuno-logical reactions.[12] Although the size of the genes that can be inserted into the AAV2 genome is limited to less than 4.5 kb, most of the therapeutic genes fit into this range. It has also been shown that several AAV vectors are able to infect one cell at the same time and can express plural genes.[13] AAV vectors have been applied in clinical trials for numerous disorders including hemophilia,[14] cystic fibrosis[15] and retinal degeneration.[16] To date, adverse effects due to the administration of the vectors themselves have not been found. In addition to gene therapy, AAV vectors have also been recently used as a genetic tool in the neurosciences.[17,18]

Gene transfer of dopamine-synthesizing enzymes

Dopamine is synthesized in the brain from diet-derived L-tyrosine. Three enzymes are essential in this production process.[19] Tyrosine hydroxylase (TH) is the rate-limiting enzyme that converts L-tyrosine to L-3, 4-dihydroxy-phenylalanine (L-dopa). Guanosine triphosphate cyclohydrolase I (GCH) is the rate-limiting enzyme that synthesizes the essential TH co-factor tetrahydrobiopterine (BH_4), while aromatic L-amino acid decarboxylase (AADC) converts L-dopa to dopamine. These three enzymes are transported from the substantia nigra in an anterograde manner to the striatum. A severe loss of the nerve terminals in advanced Parkinson's disease (PD) is associated with an 80-95% depletion of striatal enzyme activity. Gene transfer of TH, GCH and AADC,[13,20] or AADC alone,[12,21] into the striatal neurons has led to behavioral recovery in primate models of PD.

Two phase I clinical trials were conducted at the University of California San Francisco (UCSF) and Jichi Medical University (JMU) to evaluate the safety and potential efficacy of AAV vector-mediated gene delivery of AADC to the bilateral putamen. Alleviation of motor symptoms associated with PD was observed in both trials. In the UCSF study,[7] a low dose cohort of five patients received 110^{11} vector genome (vg) and a high dose cohort of five patients received 310^{11} vg of AAV-AADC. The mean improvements of the ten patients in the total score of unified Parkinson's disease rating scale (UPDRS) were 31% (10.5 points) in the off-state and 32% (10 points) in the on-state. The mean improvements in the motor score of UPDRS were 36% (12 points) in the off-state and 28% (4.3 points) in the on-state. Positron emission tomography (PET) using [^{18}F]fluoro-m-tyrosine (FMT), a tracer for AADC, revealed a 30 and 75% increase in K^c values in the putamen of the low-dose and high-dose cohort, respectively, at 6 months after the gene delivery. In the JMU study, six patients received 310^{11} vg of AAV-AADC. Motor function in the off-state improved to a mean of 46% (11.6 points) based on the UPDRS scores at six months after surgery, without any apparent changes in the short-duration response to levodopa. PET revealed a 56% increase in FMT activity, which persisted for up to 96 weeks (manuscript in preparation). Phase 2 trials of AAV-AADC are currently in the planning stages.

Using a vector derived from the equine infectious anemia virus (EIAV), a type of lentivirus, a phase I/II trial involving the triple gene transfer of TH, GCH and AADC into the bilateral putamen has been initiated at the Henri Mondor Hospital in France.[22] Two dose levels have been evaluated in cohorts of three patients per dose. All patients treated at the second dose level have completed their six-month assessments and have shown improvement in motor function in the off-state when evaluated on the motor score of UPDRS. The mean improvement was 34% relative to the patients' pre-

treatment motor function.[22] Studies into the high dose (5 times the lower dose) cohort are planned. Using this triple gene transfer, dopamine will be continuously produced in the putamen and may reduce "wearing-off" effects by avoiding pulsatile stimulation of dopaminergic receptors.

Gene transfer of neurturin

An alternative approach to gene therapy for PD is the protection of the nigrostriatal pathways from progressive degeneration by providing genes encoding for neurotrophic factors. Neurturin is a natural analog of glial cell line-derived neurotrophic factor (GDNF).[23] GDNF is a small glycoprotein that provides strong trophic support for the dopaminergic neurons. However, GDNF protein has limited usefulness as a therapeutic agent due to its short duration of activity and poor ability to cross the blood-brain barrier. In animal models of neurotoxin induced PD, viral vector-mediated gene delivery halts ongoing degeneration of the nigrostriatal pathway, resulting in functional recovery, even after substantial numbers of dopaminergic cells have been depleted.[24,25]

A phase I gene therapy trial that introduced the neurturin gene into the bilateral putamen was conducted at the UCSF[6]. The first six patients entered into the study receiving a dose of 310^{11} vg, while the next six patients received a dose of 5.410^{11} vg of AAV vector. A mean improvement of 36% (14 points) in the UPDRS motor score in the off-state and a mean increase of 25% (2.3 h) in on time without troublesome dyskinesia were observed one year after surgery. Subsequently, a phase II double-blind control study was undertaken at nine academic institutions in the United States, and in which one-third of a total of 54 patients received sham surgery (partial burr hole without infusion of vectors). Significant differences were not obtained in terms of the degree of motor performance between the gene transfer group and the control group at 12 months, although some treatment effects have since been observed in 30 subjects followed for 18 months under blind conditions.[26] Analysis of post-mortem brain tissue from two patients treated with AAV vectors demonstrated that neurturin was expressed in the putamen, but not in the substantia nigra. Earlier intervention may be required for success in future trials of this kind of neuro-protective approach, as most of the nigrostriatal fibers have already been lost when the PD symptoms appear.

Gene transfer of glutamic acid decarboxylase

In PD, depletion of dopamine in the striatum leads to an increase in the activity of the subthalamic nucleus (STN).[27] The increased excitatory drive of the STN to the internal portion of the globus pallidus (GPi) and to the substantia nigra pars reticulata (SNr) then exerts inhibitory effects on the thalamo-cortical projection and brainstem nucleus, resulting in motor symptoms such as bradykinesia and rigidity. During the last two decades, deep brain stimulation of the STN, which is thought to modify STN output by high-frequency stimulation, has become routine treatment for advanced PD patients and has resulted in the improvement of motor function. Gene transfer of glutamic acid decarboxylase (GAD-65 and GAD-67), a rate-limiting enzyme required for the synthesis of inhibitory transmitter γ-aminobutyric acid (GABA), into the STN is aimed at converting excitatory output to inhibitory output, thus, obtaining a similar effect to electrical stimulation.[28]

An open-label phase I clinical trial has been conducted at the New York Presbyterian Hospital.[5] A total of 12 patients in three dose-escalation cohorts received AAV-GAD into the unilateral STN contra-lateral to more severe motor symptoms. At 12 months after the vector infusion, the mean improvement on motor score of UPDRS was 27% in the off-state and 24% in the on-state.[5] A PET scan using [^{18}F]fluorodeoxyglucose (FDG) as a tracer revealed a decrease in uptake into the ventrolateral nucleus (VL) and dorsomedial nucleus (MD) of the thalamus on the operated side, and an increased uptake in the ipsilateral premotor and motor cortical regions.[29]

The underlining physiological changes in PD include, in addition to increases in the firing rate of the STN, the tendency of pallidal neurons to fire in more irregular patterns, as well as abnormal oscillatory synchronization in the basal ganglia.[30] Thus, mechanisms underlying how DBS and AAV-GAD gene therapy is effective remain to be defined.[31] A double-blinded phase II study of AAV-GAD infusion into the bilateral STN is currently underway for 40 subjects, including 20 subjects that received sham surgery.

Future prospects

Owing to the development of efficient viral vectors, gene therapy for PD has been tested in clinical trials, with the initial results of phase I studies proving encouraging. In contrast to cell transplantation, immunosuppressive drugs are not necessary for the current gene therapy strategies. If the primary purpose of treatment is the supplementation of dopamine into the striatum for improving motor performance, then gene therapy appears to be simpler than cell transplants. Cell therapy may prove useful in treating Parkinsonism including cerebral ischemia, striato-nigral degeneration and cortico-basal degeneration, where neurons in the striatum are damaged. However, several PD symptoms that L-dopa is not effective at rescuing including cognitive dysfunction, depressive state, frozen gait, posture reflex disturbance and sleep disturbance have also been reported.[32] For the treatment of these symptoms, effective therapeutic genes must be identified and delivered into the appropriate areas of the brain. Development of vector constructs that avoid over-expression is also required for increasing safety.[33] Although AAV and EIAV are reported to be non-pathogenic for humans, the long-term safety must be confirmed. It is expected that in the near future gene therapy will become the general choice for the treatment of PD.

Competing interests – None
Received Date : 13 February 2010
Revised Date : 7 June 2010
Accepted Date : 7 July 2010

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

Agnieszka Krzyżosiak,[1,2,3,6] Monika Szyszka-Niagolov,[1,2,6] Marta Wietrzych,[1,2] Serge Gobaille,[4] Shin-ichi Muramatsu,[5] and Wojciech Krężel[1,2,*]
[1]IGBMC (Institut de Génétique et de Biologie Moléculaire et Cellulaire), BP 10142, Inserm, U 596, CNRS, UMR 7104 Illkirch F-67400, France
[2]Université de Strasbourg, Strasbourg F-67000, France
[3]Wrocław University of Technology, Faculty of Chemistry, Division of Biochemistry, Wybrzeże Wyspiańskiego 27, 50-370 Wrocław, Poland
[4]IFR37 de Neurosciences, Faculté de Médecine, 11 rue Humann, 67085 Strasbourg, France
[5]Division of Neurology, Department of Medicine, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
[6]These authors contributed equally to this work
*Correspondence: krezel@igbmc.u-strasbg.fr
DOI 10.1016/j.neuron.2010.05.004

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 *Rxrγ* 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 Rxrγ or D2r in the NAc of $Rxr\gamma^{-/-}$ mice normalizes depressive-like behaviors in $Rxr\gamma^{-/-}$ animals. Conversely, NAc infusion of raclopride, a D2r antagonist prevents AAV2-*Rxrγ*-mediated rescue of despair behaviors in $Rxr\gamma^{-/-}$ mice. Combined, our data argue that control of NAc D2r expression is critical for Rxrγ-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 (Rarα, Rarβ, Rarγ) and retinoid X receptors (Rxrα, Rxrβ, Rxrγ), which in the form of heterodimers act as transcription factors and mediate RA signaling in vivo (Kastner et al., 1997). Rarβ and Rxrγ 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 *Rarβ/Rxrγ* 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,

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 *Rxrγ* 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 Rarβ and/or Rxrγ in mice on performance in the forced swim test. Concomitant ablation of *Rarβ* and *Rxrγ* in *Rarβ*$^{-/-}$ *Rxrγ*$^{-/-}$ 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 Rxrγ, since single *Rxrγ*$^{-/-}$ mutants displayed similar high immobility time of 117 ± 4 s, whereas inactivation of Rarβ 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 *Rxrγ*$^{-/-}$ mice in the forced swim test, since *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice (Figure 1B), as illustrated by a significant *genotype* × *fluoxetine* treatment interaction ($F[1,35] = 23.2$, $p < 0.001$). Thus, high immobility of vehicle treated *Rxrγ*$^{-/-}$ mice was reduced in fluoxetine treated *Rxrγ*$^{-/-}$ animals, which behaved comparably to vehicle treated WT mice (Figure 1B).

Inactivation of Rxrγ 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 *Rarβ*$^{-/-}$ and *Rxrγ*$^{-/-}$ single and compound mutant mice. During the active, night phase of the circadian cycle WT mice displayed clear preference for a 1% sucrose solution as compared to plain water, since sucrose represented 91.3% ± 2.7% of total liquid consumption. In compound *Rarβ*$^{-/-}$*Rxrγ*$^{-/-}$ mutant mice, such a preference was absent as sucrose consumption reached 58.1% ± 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 *Rxrγ*, since *Rxrγ* single null mutants displayed similar loss of sucrose preference and consumed 57.8% ± 10.8% of sucrose solution, whereas *Rarβ*$^{-/-}$ mice consumed sucrose solution at 92% ± 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 *Rarβ*$^{-/-}$, 4.9 ± 0.2 for *Rxrγ*$^{-/-}$ and 5.2 ± 0.3 for *Rarβ*$^{-/-}$*Rxrγ*$^{-/-}$ mutants). The absence of sucrose preference in *Rarβ*$^{-/-}$ *Rxrγ*$^{-/-}$ and *Rxrγ*$^{-/-}$ 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

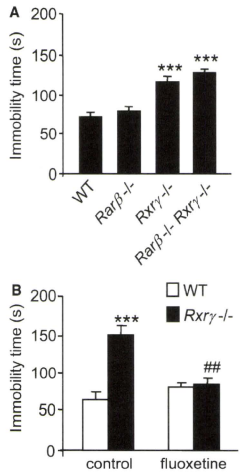

Figure 1. Increased Despair Behavior in *Rxrγ*$^{-/-}$ Null Mutant Mice Is Reversible by Chronic Antidepressant Treatment

The time of immobility in the forced swim test was measured in naive $n_{WT} = 6$ and $n_{Rarβ-/-} = 8$, $n_{Rxrγ-/-} = 8$ single- and $n_{Rarβ-/-Rxrγ-/-} = 8$ double-mutant littermates (A). Chronic, 21-day long treatment with fluoxetine (20 mg/kg/24 hr) reduced the immobility time in $n_{Rxrγ-/-} = 10$ mice as compared to $n_{Rxrγ-/-} = 10$ 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 ± SEM. ***$p < 0.001$ with respect to nontreated (A) or vehicle-treated (B) WT mice; ##$p < 0.01$ as compared with vehicle-treated *Rxrγ*$^{-/-}$ mice. See also Figure S1.

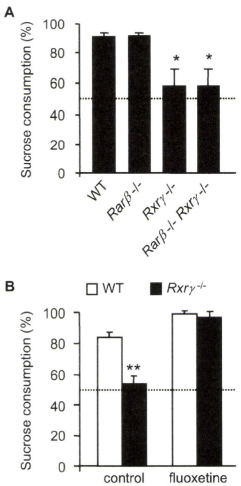

Figure 2. Loss of Sucrose Preference in *Rxrγ*$^{-/-}$ Mice Is Reversible by Chronic Antidepressant Treatment

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, $n_{Rx\gamma-/-}$ = 7, and $n_{Rar\beta-/-Rx\gamma-/-}$ = 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 ± SEM. *$p < 0.05$ significantly different from WT group; **$p < 0.01$ different from vehicle treated WT mice.

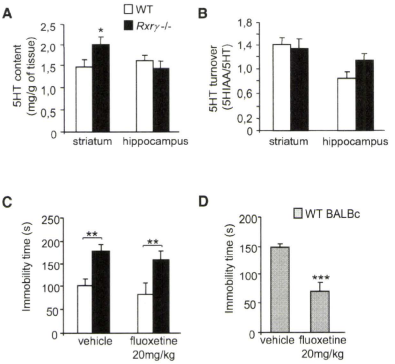

Figure 3. Abnormal Serotonergic Signaling Is Not Sufficient to Generate Despair Behavior in *Rxrγ*$^{-/-}$ Mice

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 ± SEM. *$p < 0.05$ or **$p < 0.01$ for selected comparisons and ***$p < 0.001$ in comparison with vehicle treated BALBc mice.

testing session, in sucrose-naive WT mice sucrose solution constituted 43% ± 2.5% of total liquid consumption, as compared to 44% ± 2.1% for *Rarβ*$^{-/-}$, 37% ± 5% for *Rxrγ*$^{-/-}$, and 32.4% ± 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* × *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 ($p < 0.001$).

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

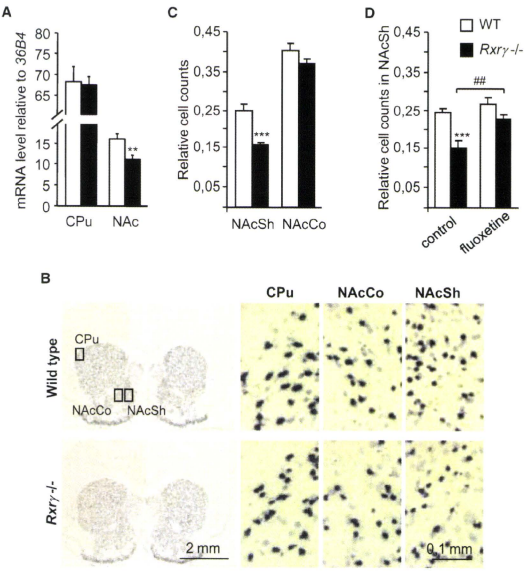

Figure 4. Decreased Expression of Dopamine D2r Receptor mRNA in the Nucleus Accumbens of *Rxrγ*$^{-/-}$ Mice Is Reversed by Chronic Fluoxetine Treatment

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 ± SEM. $^{**}p < 0.01$; $^{***}p < 0.001$ different from corresponding WT group or ##, $p < 0.01$ for selected comparison. See also Figure S2.

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* × *fluoxetine* treatment ($F[1,28] = 3E\text{-}4$, 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.

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 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ versus 0.40 ± 0.02 for WT; t = −1.5, ns), of *Rxrγ*$^{-/-}$ mice (Figure 4C). The difference in the magnitude of changes in D2r expression might be related to much weaker expression of Rxrγ 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 *Rxrγ*$^{-/-}$ mutants (0.15 ± 0.02 for nontreated *Rxrγ*$^{-/-}$ versus 0.23 ± 0.01 for fluoxetine treated *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice when comparing absolute mean values (122.9 ± 2.2 for WT and 125.1 ± 2 for *Rxrγ*$^{-/-}$ 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 Rxrγ (1.05 ± 0.01 for WT and 1.08 ± 0.02 for *Rxrγ*$^{-/-}$ mice; t = 1, ns). These data suggest that Rxrγ 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 *Rxrγ*$^{-/-}$ 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 in *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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* × *treatment* interaction, F[4,68] = 2.7; p < 0.05). Post hoc analysis revealed that the decrease of locomotion was significantly lower in *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice to develop catalepsy, since a 0.2 mg/kg dose did not induce catalepsy in WT and *Rxrγ*$^{-/-}$ mice, whereas a high dose of haloperidol (2 mg/kg) induced comparable degrees of catalepsy in both genotypes (Figure 6B).

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

To investigate whether Rxrγ 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 Rxrγ, in the NAc of *Rxrγ* null mutants. Using immunohistochemical analysis, we could clearly detect the expression of Rxrγ protein in the WT noninjected mice (top panels in Figure 7A) or in the NAcSh of *Rxrγ*$^{-/-}$ mice infected with AAV2-*Rxrγ* vector (bottom panels in Figure 7A), but not in *Rxrγ*$^{-/-}$ animals infected with AAV2-*Gfp* virus (middle panels in Figure 7A). The virus-mediated expression of Rxrγ in *Rxrγ*$^{-/-}$ 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-*Rxrγ* whereas for AAV2-*Gfp* infected mice, such pattern of Gfp expression was identified in 7 (out of 10) animals. In the

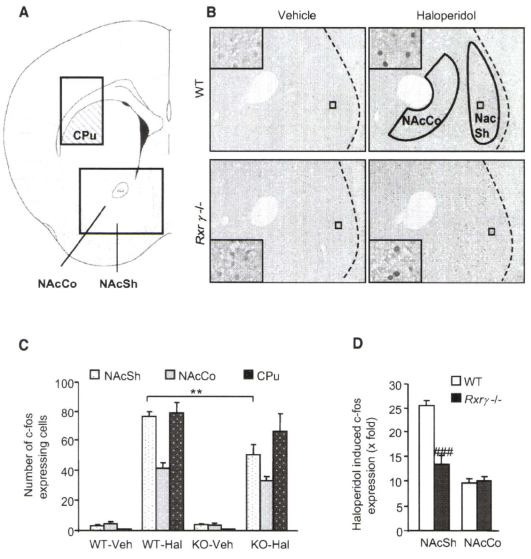

Figure 5. Haloperidol Induction of c-*fos* Expression Is Impaired in the NAc Shell of *Rxrγ*$^{-/-}$ Mutants

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 ± 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 ± 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 μg/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

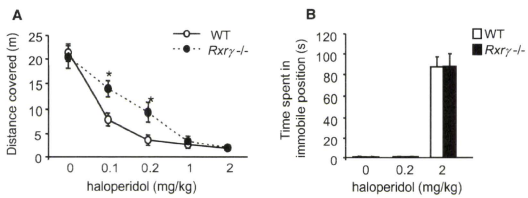

Figure 6. Behavioral Responses to Haloperidol in *Rxrγ*$^{-/-}$ Mice

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 ± 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% ± 6.9% of sucrose as opposed to significantly lower ($t = 2.62$, $p < 0.05$), 53.3% ± 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 isotypes, 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 (Wietrzych 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 enkephaline 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

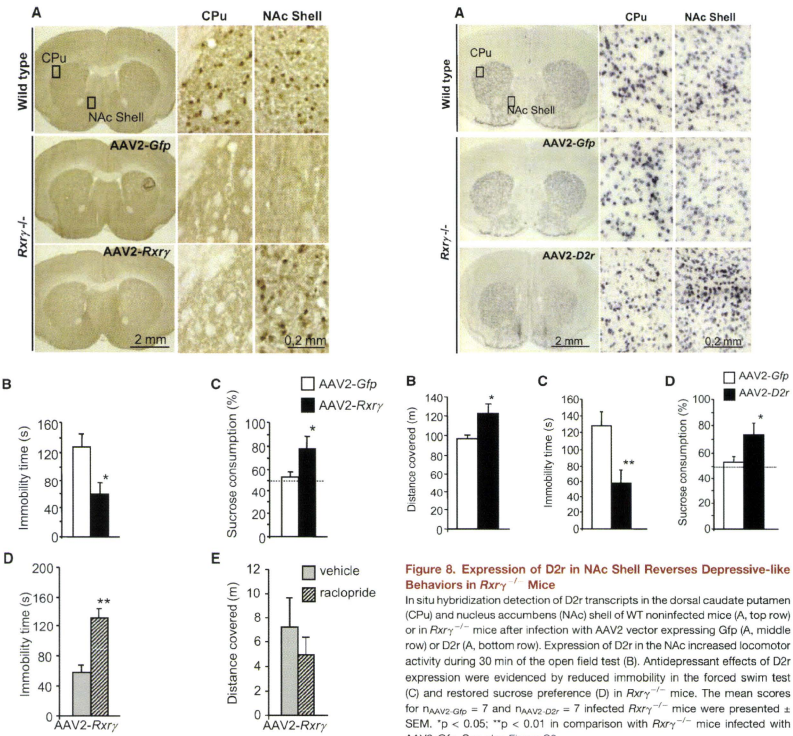

Figure 8. Expression of D2r in NAc Shell Reverses Depressive-like Behaviors in *Rxrγ*$^{-/-}$ Mice

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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice. The mean scores for $n_{AAV2\text{-}Gfp} = 7$ and $n_{AAV2\text{-}D2r} = 7$ infected *Rxrγ*$^{-/-}$ mice were presented ± SEM. $^{*}p < 0.05$; $^{**}p < 0.01$ in comparison with *Rxrγ*$^{-/-}$ mice infected with AAV2-*Gfp*. See also Figure S3.

Figure 7. Re-expression of *Rxrγ* in NAc Shell Reverses Depressive-like Behaviors in *Rxrγ*$^{-/-}$ Mice through D2r-Dependent Mechanism

Immunohistochemical detection of Rxrγ in the dorsal caudate putamen (CPu) and nucleus accumbens (NAc) shell of WT noninfected mice (A, top row) or *Rxrγ*$^{-/-}$ mice after infection with AAV2 vector expressing Gfp (A, middle row) or *Rxrγ* (A, bottom row). Re-expression of *Rxrγ* in the NAc shell reduced immobility in the forced swim test (B) and restored sucrose preference (C) in $n_{Rxr\gamma-/-} = 7$ mice as compared with $n_{Rxr\gamma-/-} = 5$ mice infected with AAV2-*Gfp*. Acute inhibition of D2r signaling in the NAc shell after bilateral infusion of raclopride (5 μg/side) prevented AAV2-*Rxrγ* rescue of depressive-like behaviors in *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice infected with AAV2-*Gfp*; $^{**}p < 0.01$ in comparison with ACSF infused control group. See also Figure S3.

possible explanation is that transcriptional control of D2r expression in the dorsal striatum is subject to marked functional redundancy between Rxrγ and Rxrα and/or Rxrβ. Such hypothesis is supported by an overall reduction of striatal D2r expression and severe locomotor deficits displayed by *Rxrβ*/*Rxrγ* 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice to low, noncataleptic doses of haloperidol. Thus, *Rxrγ* 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 *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ mice. To further explore the behavioral relevance of compromised D2r signaling in *Rxrγ*$^{-/-}$ mice and the implication of Rxrγ in such control, we carried out rescue experiments by virus-mediated re-expression of Rxrγ in *Rxrγ*$^{-/-}$ mice. We found that re-expression of Rxrγ 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 Rxrγ in the NAc of *Rxrγ*$^{-/-}$ mice. An increase of D2r expression in NAc appeared to play a critical role in the antidepressant-like activities of Rxrγ, since infusion of raclopride, a D2r/D3r antagonist, prevented the rescue of despair behaviors in *Rxrγ*$^{-/-}$ mice infected with AAV2-*Rxrγ*. Furthermore, we showed that a long-lasting increase of D2r signaling by AAV2 mediated expression of D2r in the NAcSh of *Rxrγ*$^{-/-}$ 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 Rxrγ in *Rxrγ*$^{-/-}$ 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-*Rxrγ*. 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 *Rxrγ*$^{-/-}$ mice reflects antidepressant activities since (1) inhibition of D2r signaling by raclopride, which prevented AAV2-*Rxrγ* rescue of despair behaviors in *Rxrγ*$^{-/-}$ 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 *Rxrγ*$^{-/-}$ 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 (Paterna et al., 2004), our data on antidepressant effects of AAV2-*D2r* infection of *Rxrγ*$^{-/-}$ mice suggest the role of postsynaptic D2r in NAc in control of affective behaviors.

In conclusion, this study provides the first evidence that the loss of Rxrγ 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 Rxrγ 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 *Rxrγ*$^{-/-}$ mice (Wietrzych 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 Rxrγ 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 bexaroten (Targretin), used so far in cancer treatment, might be potentially interesting for clinical trials in treatment of depressive disorders.

EXPERIMENTAL PROCEDURES

Animals

Rarβ$^{-/-}$ and *Rxrγ*$^{-/-}$ single mutant, and *Rarβ*$^{-/-}$*Rxrγ*$^{-/-}$ 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-naive 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

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 *Rxrγ* (AAV2-*Rxrγ*), *D2r* (AAV2-*D2r*), and *EGfp* (AAV2-*Gfp*) contained the entire cDNA sequences of *Rxrγ* (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 iodoxale 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 *Rxrγ*$^{-/-}$ male mice (n = 29) at the age of 8 months. Each animal was anaesthetized using ketamine (100 mg/kg)/xylasine (10 mg/kg) solution and 0.7 μl of AAV2-*Rxrγ*, 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

Rxrγ$^{-/-}$ mice (n = 15) aged between 4 and 5 months were infected with AAV2-*Rxrγ* 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 $CaCl_2$, 1 mM $MgCl_2$, 0.27 mM NaH_2PO_4, 1.2 mM Na_2HPO_4 [pH 7.4]) and infusions of 0.25μl of raclopride (5 μg/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-*Rxrγ* 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 *Rxrγ*$^{-/-}$ 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 μm thick cryosections. Similarly, dorsolateral striatum (CPu) was collected using 0.8 mm punch from four subsequent frozen sections of 300 μm. 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 20min in a total volume of 20 μl 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 μM) following the manufacturer's instructions. All of the reactions were performed in triplicate with the following cycling