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Genetic mouse models for behavioral analysis through transgenic RNAi technology

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Pharmacological inhibitors and knockout mice have developed into routine tools to analyze the role of specific genes in behavior. Both strategies have limitations like the availability of inhibitors for only a subset of proteins and the large efforts required to construct specific mouse mutants. The recent emergence of RNA interference (RNAi)-mediated gene silencing provides a fast alternative that can be applied to any coding gene. We established an approach for the efficient generation of transgenic knockdown mice by targeted insertion of short hairpin (sh) RNA vectors into a defined genomic locus and studied the efficiency of gene silencing in the adult brain and the utility of such mice for behavioral analysis. We generated shRNA knockdown mice for the corticotropin-releasing hormone receptor type 1 (*Crhr1*), the leucine-rich repeat kinase 2 (*Lrkk2*) and the purinergic receptor P2X ligand-gated ion channel 7 (*P2rx7*) genes and show the ubiquitous expression of shRNA and efficient suppression of the target mRNA and protein in the brain of young and 11-month-old knockdown mice. Knockdown mice for the *Crhr1* gene exhibited decreased anxiety-related behavior, an impaired stress response, and thereby recapitulate the phenotype of CRHR1 knockout mice. Our results show the feasibility of gene silencing in the adult brain and validate knockdown mice as new genetic models suitable for behavioral analysis.

Keywords: Anxiety, behavior, CRHR1, LRRK2, P2RX7, RNAi, shRNA

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The role of specific genes in behavior is often studied by the use of small molecule inhibitors or the analysis of gene knockout mice. Pharmacological inhibitors can be conveniently applied to wild-type mice, but specific compounds are available only for a subset of proteins. In contrast, knockout mice can be generated for any gene but the generation of these mice is a time-consuming and labor-consuming task. RNA interference (RNAi)-mediated gene silencing could provide a new alternative to these methods because it involves only simple technology and yet allows to target any gene mRNA (Hannon & Rossi 2004). Gene silencing can be either transiently induced in mammalian cells by the direct delivery of short inhibitory (si) RNAs or made permanent through the use of short hairpin (sh) RNA expression vectors placed into the genome. In such vectors, a RNA polymerase III-dependent promoter drives the expression of shRNA molecules that are processed into siRNA and cause permanent gene knockdown (Dykxhoorn *et al.* 2003). The direct delivery of siRNA and viral shRNA vectors into the brain has been shown, but cellular uptake, infection rate and local distribution remain critical parameters of these methods (Lasek *et al.* 2007; Thakker *et al.* 2004; Xia *et al.* 2004). In contrast, transgenic mice that harbor a shRNA vector in all cells provide a defined experimental setup, given that the genomic integration site does not interfere with vector expression. To ensure the efficient and reproducible expression of shRNA vectors in transgenic mice, we previously established a method for the targeted integration of single vector copies into the *Rosa26* locus of embryonic stem (ES) cells by recombinase-mediated cassette exchange (RMCE) (Hitz *et al.* 2007).

It has been shown that shRNA transgenic mouse embryos can display an all-over knockdown phenotype comparable to knockout mice (Kunath *et al.* 2003). However, the utility of shRNA transgenic mice for gene silencing in the adult brain and for behavioral analysis is not documented. In particular, the expression pattern of shRNA vectors in the brain, the efficiency and permanence of gene silencing in aging mice and a comparison to the phenotype of knockout mice have not been reported. To clarify these issues, we generated mice transgenic for shRNA vectors directed against the mRNA of the corticotropin-releasing hormone receptor type 1 (*Crhr1*), the leucine-rich repeat kinase 2 (*Lrkk2*) and the purinergic receptor P2X ligand-gated ion channel 7 (*P2rx7*) genes that are linked to mood disorder, Parkinson's disease and depression, respectively (Lucae *et al.* 2006; Muller & Wurst 2004; Paisan-Ruiz *et al.* 2004; Wasserman *et al.* 2008; Zimprich *et al.* 2004). In these mice, we studied the efficiency of gene silencing in the brain of young and 11-month-old mice, the shRNA expression pattern and assessed the behavioral phenotype of *Crhr1* knockdown mice in comparison to *Crhr1*

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knockout mice (Timpl *et al.* 1998). Our results show the feasibility of gene silencing in the brain of shRNA transgenic mice and validate this approach to produce new genetic models suitable for behavioral analysis.

Materials and methods

Crhr1, Lrkk2, P2rx7 and luciferase-specific shRNA vectors

To generate knockdown mice for the *Crhr1*, *Lrrk2* and *P2rx7* genes, we constructed shRNA expression vectors driven by the human U6 promoter. For this purpose, the U6 promoter and cloning region from the pSHAG plasmid (Paddison *et al.* 2004) was transferred into pBluescript (pBS), opened with *Bse*RI/*Bam*HI and ligated with a *Crhr1*-, a *Lrkk2*- or a *P2rx7*-specific shRNA oligonucleotide pair to generate pU6-shCRHR1, pU6-shLRRK2 or pU6-shP2RX7, respectively. The oligonucleotide pairs with efficient knockdown of the targeted gene that were used for further studies are CRHR1-3 (5′-caaacgtcctggagtatatactgagaagcttgtcagtatatactccaggacgtttgcttttttggaaa-3′ and 5′-gatctttccaaaaaagcaaacgtcctggagtatatactgacaagcttctcagtatatactccaggacgtttgcg-3′) targeting the sequence 5′-gcaaacgtcctggagtatatactga-3′ of the *Crhr1* mRNA, LRRK2-2 (5′-gacagctttccttatttgacttaagaagcttgttaagtcaaataaggaaagctgtccttttttggaaa-3′ and 5′-gatctttccaaaaaaggacagctttccttatttgacttaacaagcttcttaagtcaaataaggaaagctgtccg-3′) targeting the sequence 5′-ggacagctttccttatttgacttaa-3′ of the *Lrrk2* mRNA and P2RX7-1 (5′-gaactcattctttgtcatgagaagcttgtcatgacaaagaatgagttccttttttggaaa-3′ and 5′-gatctttccaaaaaaggaactcattctttgtcatgacaagcttctcatgacaaagaatgagttccg-3′) targeting the sequence 5′-ggaactcattctttgtcatga-3′ of the *P2rx7* mRNA. The U6 promoter-driven luciferase-specific shRNA vector, pU6shLuc-lox-stop-lox, targeting the sequence 5′-gtgcgctgctggtgccaac-3′of humanized firefly luciferase, initially contained a loxP-flanked stop cassette within the loop region (Hitz *et al.* 2007) that was deleted from the genome by mating to a Cre recombinase germline deleter strain. The efficiency of gene silencing induced by these vectors was tested by transient transfection into IDG3.2 ES cells. Because *Lrrk2* and *P2rx7* but not the *Crhr1* gene is expressed in ES cells, *Crhr1* shRNA vectors were cotransfected with a *Crhr1* cDNA expression vector. After 2 days, mRNA from the transfected cells was isolated, converted to cDNA and the reduction of the targeted transcripts was determined by quantitative real-time polymerase chain reaction (qRT-PCR) in relation to samples transfected with an empty pBS-U6 vector without shRNA, as control for nonspecific transfection effects.

Tissue culture

The murine hybrid ES cell line IDG3.2, established from a male (C57BL/6J × 129S6/SvEvTac)-F_1 blastocyst (Hitz *et al.* 2007), were used for transient and stable transfections. Cells were grown in Dulbecco's modified Eagle's minimal essential medium (Invitrogen, Paisley, UK) containing 15% fetal calf serum, 20 mM Hepes, 1× non-essential aminoacids, 0.1 mM β-mercaptoethanol and 1.5×10^3 U/ml leukemia inhibiting factor on gelatin-coated culture dishes for transient transfections or on mitomycin-C-treated mouse embryonic fibroblasts for stable transfections and blastocyst injections or tetraploid complementation. Transient transfections with shRNA vectors and *Crhr1* expression vector were performed by electroporation. After transfection, cells were cultivated for 2 days and lysed, and the extracted RNA was analyzed by qRT-PCR.

Quantitative RT-PCR

For qRT-PCR of *in vitro* samples, cells were lysed using 'Invisorb RNA Cell HTS 96-Kit/C' according to manufacturer's protocol (Invitec, Berlin, Germany). Lysates were stored at −80°C and qRT-PCR was performed by Cenix Biosciences (Dresden, Germany). The following primer pairs were used for *Crhr1* CRHR1-F 5′-ccgctacaacaccacaaacaat-3′ and CRHR1-R 5′-agtggcccaggtagttgatgat-3′, for *Lrrk2* LRRK2-F 5′-gttttggatccgtttatcgagc-3′ and LRRK2-R 5′-caactccattaccaacatccga-3′ and for *P2rx7* P2RX7-F 5′-ttgcacatgatcgtcttttcct-3′ and P2RX7-R 5′-tgcagtgtcaaagatgctgtgt-3′. For qRT-PCR of *in vivo* samples, RNA extraction from tissue was performed following RNeasy protocols (Qiagen, Hilden, Germany) and RNA was stored at −80°C. For reverse transcription of RNA, the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Darmstadt, Germany) was used and qRT-PCR with Taqman universal PCR Master Mix (Applied Biosystems) was performed on a Thermo-Cycler (7900 HT Real-Time PCR System; Applied Biosystems). We used the Taqman(R) Gene Expression Assay for specific amplification of P2rx7 products (Assay ID: Mm00440582_m1; Applied Biosystems).

Recombinase-mediated cassette exchange

To insert a single copy of shRNA vectors into the *Rosa26* locus of ES cells by RMCE, we used the ES cell clone IDG26.10-3, derived from the IDG3.2 line, that contains a pair of ϕC31 integrase (C31Int) attP recognitions sites (Hitz *et al.* 2007). For this purpose, shRNA expression units were inserted first into the donor vector pRMCE in-between a pair of C31Int attB recognition sites (Fig. 2b). The *Crhr1* and *Lrrk2* shRNA constructs were cloned as *Not*I/*Eco* RV fragment from pU6-shCRHR1 or pU6-shLRRK2, respectively, into pRMCE, while the pU6shLuc-lox-stop-lox cassette was cloned as *Asi*Si/*Not*I fragment. The *P2rx7* shRNA construct was cloned as Klenow-blunted *Asi*SI/*Fse*I fragment from pU6-shP2rx7 into Klenow-blunted *Sbf*I/*Fse*I-opened pRMCE to generate the shRNA donor vector for RMCE with the shP2RX7 cassette in the opposite direction than the shCRHR1 and shLRRK2 cassettes. Donor vectors were co-electroporated with a C31Int expression vector into mouse acceptor ES cells (clone IDG26.10-3) followed by selection with G418 for 7 days. Resistant colonies were isolated and analyzed for cassette exchange by Southern blotting of *Eco*RV-digested genomic DNA using the Rosa26 5′-probe. Correct cassette exchange was indicated by a 14-kb band in addition to the 11-kb band from the wild-type Rosa26 locus, whereas the original RMCE locus gives rise to a 4.5-kb band. We described previously the generation of the pRMCE and the pU6shRNA vectors, of mouse acceptor ES cells harboring attP sites and of the Rosa26 5′-probe (Hitz *et al.*, 2007).

Generation of mutant mice, breeding and genotyping

Heterozygous mice for the shCRHR1 construct were obtained from RMCE modified IDG26.10-3 ES cells by tetraploid embryo aggregation, as described previously (Nagy *et al.* 1993). Positive ES cells containing the shLRRK2, the shLuc or the shP2RX7 construct, respectively, were injected into C57BL/6N blastocysts. Resulting chimeras were bred to C57BL/6J mice (Charles River, Germany), and offspring were tested for germline transmission. Transgene positive offspring were further backcrossed to C57BL/6J mice and used for phenotyping upon two generations. Genotyping of mice was performed by Southern blot and PCR. For Southern blots, the genomic DNA was digested with *Eco* RV and hybridized with the Rosa26 5′-probe. The Rosa26 5′-probe for Southern blotting is a 448-bp fragment of the *Rosa26*-locus 5′ to the first exon, amplified with the following primer pair: 5′-aaggatactggggcatacg-3′ and 5′-cttctcagctacctttacacacc-3′. The modified *Rosa26* allele, containing the shCRHR1 or the shLRRK2 construct, respectively, is detected by a 14-kb fragment. The modified *Rosa26* allele containing the shP2RX7 construct is detected by a 5.8-kb fragment and the *Rosa26* wild-type locus shows an 11-kb band. The modified *Rosa26* allele containing the shLuc construct including the loxP-flanked stop cassette is detected by a 15-kb fragment and by a 14.2-kb fragment after Cre-mediated deletion of the stop cassette. The stop cassette was deleted from the germ line of shLuc mice by cross to a Cre deleter strain. The Cre transgene was removed by further breeding, and its presence was tested by PCR on tail tip DNA using the primer pair 5′-atgcccaagaagaagaggaaggt-3′ and 5′-gaaatcagtgcgttcgaacgctaga-3′, to amplify a 447-bp fragment. Additionally, the mice were screened by PCR, using the primer pair 5′-gttgtgcccagtcatagccgaatag-3′ and 5′-cacgcttcaaaagcgcacgtctg-3′ that amplify a 280-bp fragment from the pgk-promoter and the neomycin-coding region. Experiments on animals were carried out in accordance with the European Community Council Directive of 24 November 1987 (86/609/EEC). All animal

experiments were approved by the animal welfare and use committee of the local governmental body (Regierung von Oberbayern).

Southern blotting

DNA was extracted from tail tissue with the Wizard genomic DNA extraction kit (Promega, Mannheim, Germany). About 10 μg of genomic DNA were digested with *Bam*HI, run on a 0.8% agarose gel and blotted on a nylon membrane (Hybond N^+; Amersham). Hybridization was performed overnight at 65°C with the Rosa26 5′-probe, labeled with ^{32}P (5′-[α-^{32}P]dCTP; Amersham, Piscataway, NJ, USA). After washing, the membrane was exposed to a BioMax MS1 film (Kodak, Stuttgart, Germany).

Northern blotting

Whole brains or brains parts (with separated cerebellum, for *Crhr1*) were used for RNA extraction with TriReagent (Ambion, Warrington, UK). Northern blots for mRNA were performed with the NorthernMax-Gly system (Ambion). Membranes were hybridized overnight at 65°C (42°C for β-actin) with a ^{32}P-labeled DNA probe against the *Crhr1*, *Lrrk2* or *β-actin* mRNA, respectively. After washing, the membrane was exposed to a BioMax MS1 film (Kodak). Quantification of band intensities was performed with an imaging plate and the FLA-8000 instrument (Fujifilm, Düsseldorf, Germany). The *Crhr1*-specific probe was the 700-bp *Pst*I/*Bsp*HI fragment of the *Crhr1* cDNA. For detection of the *Lrrk2* transcript, the ^{32}P-labeled 1.9 kB *Bam*HI/*Hin*dIII fragment of the *Lrrk2* cDNA was used. The *β-actin* transcript was detected with a probe detecting nucleotide 762-1837 of the mouse cytoplasmic *β-actin*, purchased from Ambion.

In situ *hybridization*

In situ hybridization was carried out on 20-μm thick coronal sections with ^{35}S radiolabeled LNA oligoprobes (Thomsen *et al.* 2005). Mice were anesthetized and perfused with 4% paraformaldehyde (PFA). The brains were removed, postfixed in 4% PFA overnight at 4°C and stored at 4°C in 25% sucrose. Brains were cut on Cryostat (MICROM, Heidelberg, Germany) in 20-μm thick sections, mounted on Super-Frost® Plus slides (Menzel-Glaeser, Braunschweig, Germany) and stored at −20°C. Upon lipid removal and dehydration (70% ethanol, 1 min; 80% ethanol, 1 min; 95% ethanol, 2 min; 100% ethanol, 1 min; chloroform, 4 min; 100% ethanol, 1 min; 95% ethanol 2 min), slides were air dried for 30 min at RT. Afterward, the slides were prehybridized (100 μl HybMix per slide, 1 h at 45°C), incubated with radiolabeled LNA oligoprobes (for one slide: 1×10^6 c.p.m. in 100 μl HybMix) in a humid chamber at 45°C overnight, washed [1× saline sodium citrate (SSC) with 0.05% Tween20 and 2 μM dithiothreitol (DTT), 4 × 15 min at 55°C; 0.2× SSC with 0.05% Tween20 and 2 μM DTT, 2 × 15 min at 55°C; 0.2× SSC with 0.05% Tween20 and 2 μM DTT, 55°C cooling down to RT for 30°C; 0.1 × SSC, 2 × 5 min], dehydrated (70% ethanol, 95% ethanol, 100% ethanol, each 2 min) and air dried. The hybridized slides were dipped in autoradiographic emulsion (Kodak NTB2), developed after 2 weeks and counterstained with cresyl violet according to standard protocols. HybMix for hybridization contains50% deionized formamide, 4× SSC, 0.5× Denhardt's solution, 1% Sarcosyl (N-lauroylsarcosine), 20 mM Na-phosphate buffer and 10% Dextran sulfate. LNA oligoprobes for CRHR1 (5′ gcaaacgtcctggagtatatactga 3′) and LRRK2 (5′ gacagctttcmcttatttgamcttaa 3′) were obtained from Exiqon, Denmark. About 3 pmol LNA oligonucleotides were labeled with ^{35}S by terminal transferase according to manufacturer's protocol (Roche, Mannheim, Germany), purified with mini Quick Spin Oligo Columns according to manufacturer's instructions and measured in a scintillation counter.

Western blotting

Total protein was extracted from brain tissue. Tissue was homogenized in radioimmuno precipitation assay (RIPA) buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 0.25% sodiumdesoxycholat, 150 mM NaCl, 1 mM EDTA, protease inhibitor), sonificated and centrifuged. About 50 μg protein of each sample were run on a 10% Tris–HCl gel (Bio-Rad, Munich, Germany) and blotted on a PVDF membrane (Pall, Bad Kreuznach, Germany). After blocking with 4% skim milk, the membrane was incubated with the primary antibody for 1 h, washed with Tris-buffered saline with Tween 20 (0.05%) (TBST), incubated with the secondary antibody for 1 h and washed with TBST. The detection reaction was initiated with enhanced chemiluminescense (ECL) detection reagents (Amersham), and the membrane was exposed to Hyperfilm (Amersham) for 5 seconds to several minutes. For quantification of band intensities, ECL plus was used instead of ECL detection reagent and chemifluorescent bands were detected with the FLA-8000 fluorescent image analyzer (Fujifilm). Antibodies used were the rat monoclonal anti-LRRK2 antibody 1E11 (E. Kremmer, Helmholtz Zentrum München) and horseradish-peroxidase-conjugated goat anti-rat (Jackson Immuno Research, Newmarket, UK).

Behavior analysis

For the light/dark box test of shCRHR1 mice, 29 male (14 mutants and 15 controls) and 22 female mice (7 mutants and 15 controls) at the age of 4 months and for the test of shLuc mice 15 male (5 mutants and 10 controls) and 24 female mice (11 mutants and 13 controls) at the age of 7 months were analyzed. Construction of the arena and further details were described previously (Timpl *et al.* 1998). Each mouse was placed in the center of the black compartment and allowed to freely explore the apparatus for 5 min. Behaviors were observed by a trained observer. Data were analyzed with respect to time spent in both the compartments and the tunnel. An entry into a compartment was defined as placement of all four paws into the compartment. The box was cleaned before each trial. Data were expressed as mean + SEM and statistically analyzed using SPSS software (SPSS Inc., Chicago, IL, USA). Data were analyzed by two-factorial analysis of variance (ANOVA, factors sex and genotype), with an accepted level of significance of $P < 0.05$. Because the ANOVA did not show any significant sex and genotype interactions, data of both sexes were pooled.

Stress response

The male mice previously tested in light/dark box (14 mutants and 15 controls) were analyzed 1 week later for stress response. For evaluation of the endocrine response to stress, we collected blood samples immediately after 10-min restraint stress, for which animals were placed in a 50-ml conical tube with the bottom removed. Stress experiments were performed in the afternoon (1600–1700 h). Blood was collected through orbital sinus puncture in 1.5-ml EDTA-coated microcentrifuge tubes (Kabe Labortechnik, Nümbrecht-Elsenroth, Germany). All blood samples were kept on ice and later centrifuged for 15 min at 6000 rpm at 5°C. Plasma was transferred to 1.5-ml microcentrifuge tubes and stored frozen at −20°C until the determination of corticosterone. Plasma corticosterone concentrations were measured by a commercially available RIA kit (MP Biomedicals Inc., Illkirch, France). Data were analyzed by two-factorial ANOVA (factors *genotype* and *treatment*), with an accepted level of significance of $P < 0.05$.

Results

Generation of shRNA vector transgenic mice

To generate knockdown mice for the *Crhr1*, *Lrrk2* and *P2rx7* genes, we constructed several U6 promoter-driven shRNA expression vectors for each gene and tested their silencing efficiency by transient transfection into ES cells. Because *Crhr1* is not expressed in ES cells, *Crhr1* shRNA vectors were cotransfected with a *Crhr1* cDNA expression vector. After 2 days, the reduction of the targeted transcripts was determined by qRT-PCR using cDNA from transfected cells in relation to a sample receiving control vector. The most active *Crhr1*-specific vector showed a knockdown level of 78%, while the best *Lrrk2* and *P2rx7* vectors reached an overall silencing efficiency of 80% and 68%, respectively (Fig. 1). In control experiments, we determined transfection efficiency in

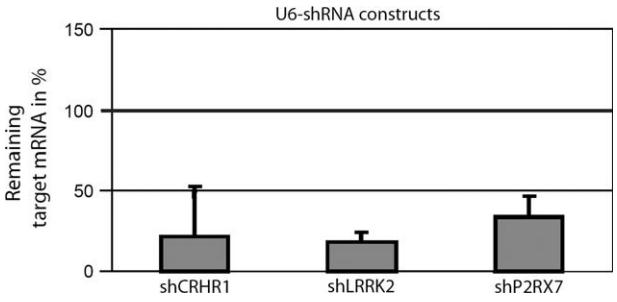

Figure 1: Gene silencing efficiency of *Crhr1-, Lrrk2-* and *P2rx7*-specific shRNA vectors *in vitro*. The shRNA vectors shCRHR1-3, shLRRK2-2 and shP2RX7-1 were transfected into ES cells, and the knockdown efficiency of the Crhr1, Lrrk2 and P2rx7 mRNAs was determined after 2 days by qRT-PCR on cDNA in relation to a sample receiving a control vector. The *Crhr1* shRNA vector was cotransfected with a *Crhr1* cDNA expression vector.

this system of 90% against a background of 10% non-transfected cells (data not shown). We therefore estimate that the absolute efficiency of the *Lrrk2* and *P2rx7* shRNA vectors lies in the range of 90% and 75%, respectively. These three most potent shRNA vectors were further selected for the production of shRNA transgenic mice. In addition, we used an established shRNA vector with specificity for firefly luciferase (Hitz *et al.* 2007), that has no predicted target sequence in the mouse genome, to control for potential nonspecific effects of *in vivo* shRNA expression. To generate transgenic mice, we inserted single shRNA vector copies into the *Rosa26* locus of ES cells by RMCE using an ES cell line that contains a pair of C31Int attP recognitions sites (Fig. 2a) (Hitz *et al.* 2007). The shRNA expression units were inserted into the RMCE donor vector pRMCE in-between a pair of C31Int attB recognition sites (Fig. 2b). Upon cotransfection of the shRNA donor vectors and a C31Int expression plasmid, ES cell colonies that underwent RMCE were isolated by selection for the neomycin resistance marker (Fig. 2c) and RMCE events were confirmed by Southern blot analysis (Fig. 2d). ES cell clones harboring correct RMCE events were further used for the production of germline-transmitting chimaeras through standard blastocyst injection or by aggregation with tetraploid morulas (Nagy *et al.* 1993). Chimaeric males were mated to normal C57BL/6J females; shRNA transgenic offspring identified by PCR and Southern blot analysis (Fig. 2d) were used for further breeding. All shRNA transgenes were efficiently transmitted through the germ line, and the shRNA transgenic mice were healthy, fertile and exhibited the same body weight as nontransgenic littermate controls.

shRNA expression in the brain of shCRHR1 and shLRRK2 mice

Using the shCRHR1 and shLRRK2 transgenic mouse lines, we asked whether shRNAs are ubiquitously expressed in the brain and their presence is compatible with normal brain development. We analyzed the brain of shCRHR1, shLRRK2 and nontransgenic control mice by Nissl staining and did not observe any obvious morphological alterations between these groups (Fig. 3). Thus, shRNA expression from the U6shRNA/*Rosa26* configuration does not interfere with normal brain development. Because the *in vivo* activity pattern of

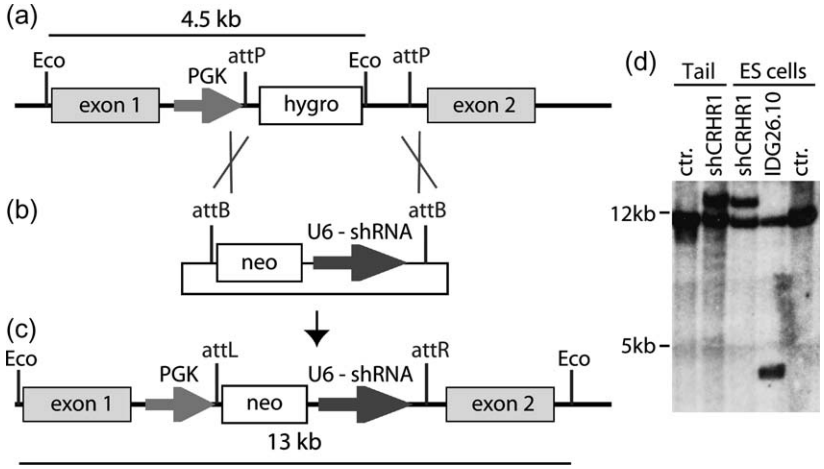

Figure 2: Strategy for the generation of shRNA transgenic mice. (a) Acceptor ES cells harbor a *Rosa26* locus modified by a pair of attP sites and a pgk-promoter-driven hygromycin resistance gene. Upon C31 integrase-mediated recombination between the modified Rosa26 locus and the RMCE donor vector (b), containing a pair of C31 integrase attB sites, the hygromycin-coding region becomes replaced by a neomycin resistance coding region and the U6 promoter-driven shRNA unit (c). *Eco* RV recognition site (Eco) and genomic DNA fragments for Southern blotting (recognized by Rosa 5′probe) are indicated in a and b. (d) Southern blot analysis of RMCE ES cell clones and mice containing the shCRHR1 shRNA vector. Genomic DNA was digested with *Eco* RV. The wild-type *Rosa26* locus appears as 11-kb band and acceptor ES cells harboring the hygromycin resistance gene (IDG26.10) show an additional 4.5-kb band. Upon RMCE, the correct clone, containing a neomycin resistance gene and the U6-shRNA construct (shCRHR1), is identified by a 14-kb band. The ES cell clone shCRHR1 was used to generate transgenic mice. Tail DNA from these mice was used for genotyping. In transgenic mice both, the 11 kb wild-type band and the 14 kb transgene band were present.

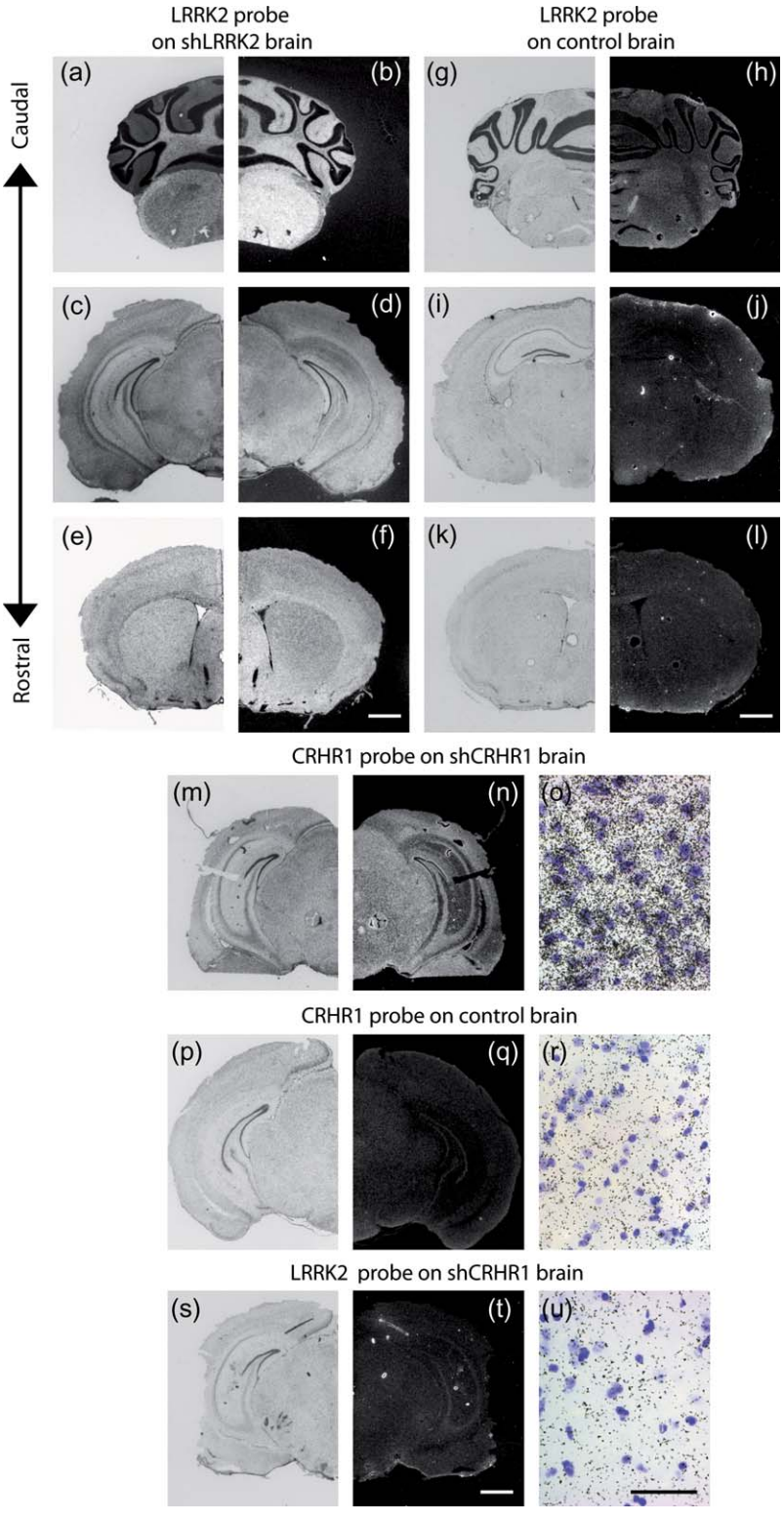

Figure 3: shRNA expression in the brain of *Crhr1* and *Lrrk2* shRNA mice. Coronal sections from brains of shLRRK2 mice (a, b, c, d, e and f), shCRHR1 mice (m, n, o, s, t and u), and wild-type controls (g, h, l, j, k, l, p, q and r) were hybridized with specific LNA probes against shLRRK2 (a–l; s–u) or shCRHR1 (m–r). Hybridization signal appears white in dark field (b, d, f, h, j, l, n, q and t) and black in bright field (o, r and u). Strong hybridization signals are seen in all brain regions of shLRRK2 mice using the shLRRK2 specific LNA probe (b, d and f), while only background signals are visible in control sections (h, j and l) or in sections from shCRHR1 mice (t and u). *In situ* hybridization using a shCRHR1-specific LNA probe resulted in strong signals in sections from shCRHR1 mice (n) but not in controls (q). The specificity of the shCRHR1 and shLRRK2 probes, respectively, is shown in higher magnification images in o, r and u. a, c, e, g, i, k, m, o, p, r, s, u, bright field images; b, d, f, h, j, l, n, q, t, dark field images; Scale bars: 1 mm in f, l and t; 50 μm in u.

the U6 promoter is unknown, we further analyzed the expression of shRNAs in adult brain. Coronal brain sections from adult shCRHR1 and shLRRK2 mice were hybridized with ^{35}S-dATP labeled locked nucleic acid (LNA) oligonucleotides that recognize the antisense strand of the shRNAs. In addition, Nissl staining was performed to highlight the nuclei

and endoplasmatic reticulum of all cells, whereas glial fibers, dendrites or axons are not stained. As shown in Fig. 3, the sections from adult shCRHR1 and shLRRK2 mice exhibited strong hybridization signals throughout the brain, while non-transgenic controls showed essentially no signals except background. No obvious cross-reaction between the *Lrrk2*-specific probe and the *Crhr1* shRNA was found, indicating high specificity of the used LNA oligonucleotides (Fig. 3t). We conclude that the human U6 promoter embedded within the *Rosa26* locus leads to ubiquitous shRNA expression in the adult brain and does not disturb brain development.

Analysis of gene silencing in shCRHR1, shLRRK2 and shP2RX7 mice

To determine the efficiency of gene silencing in the brain of shCRHR1 mice, we first analyzed the level of *Crhr1* transcripts by Northern blot hybridization. RNA was isolated from the brain of three 2-month-old shCRHR1 and littermate control mice, excluding the cerebellum that was analyzed separately and hybridized with a *Crhr1* cDNA probe or a *β-actin* probe as loading control. As shown in Fig. 4 (a), the samples from the shCRHR1 brains showed strongly diminished levels of *Crhr1* transcripts. By quantitative analysis and normalization to the actin signal of each sample, we determined a knockdown efficiency of 80% compared with the littermate controls. The cerebellum of nontransgenic control mice shows approximately eightfold higher levels of *Crhr1* transcripts compared with other brain regions, as judged from the exposure time required to produce comparable signal strengths (6 h for cerebellum and 72 h for brain). Despite higher levels of target transcripts in the cerebellum the knockdown of the *Crhr1* mRNA was comparable to that seen in brain (excluding cerebellum) and was quantified as a value of 85%. Thus, the gene silencing efficiency in the adult brain was comparable to the 78% knockdown determined for the *Crhr1* shRNA vector in transiently transfected ES cells (Fig. 1) and an increased transcript level did not diminish this value.

We were further interested whether the efficiency of gene silencing observed in young adult mice is stable over time and also valid for 11-month-old mice. Therefore, we repeated the *Crhr1* Northern blot analysis with RNA from the brain of 11-month-old shCRHR1 and littermate control mice. As shown in Fig. 4 (a), 11-month-old shCRHR1 mice show essentially the same reduction of *Crhr1* transcripts in brain and cerebellum as seen in younger mice. Upon quantification and normalization to the actin hybridization signals, the extent of gene silencing was determined to be 76% in brain and 82% in cerebellum. Thus, shRNA expression from our U6shRNA/*Rosa26* configuration is stable over time, and RNAi-mediated gene suppression occurs undisturbed over many months in the adult brain. However, because of the lack of suitable antibodies we could not analyze the knockdown of the CRHR1 protein in shCRHR1 mice.

To determine the extent of gene silencing in the shLRRK2 mouse strain, we performed a Northern blot analysis using brain RNA at the age of 6 weeks. The *Lrrk2* transcript is well detectable in wild-type brain but below the limit of detection in the samples from a shLRRK2 mouse (Fig. 4b). The level of gene silencing was quantified to reach a level of at least 82%.

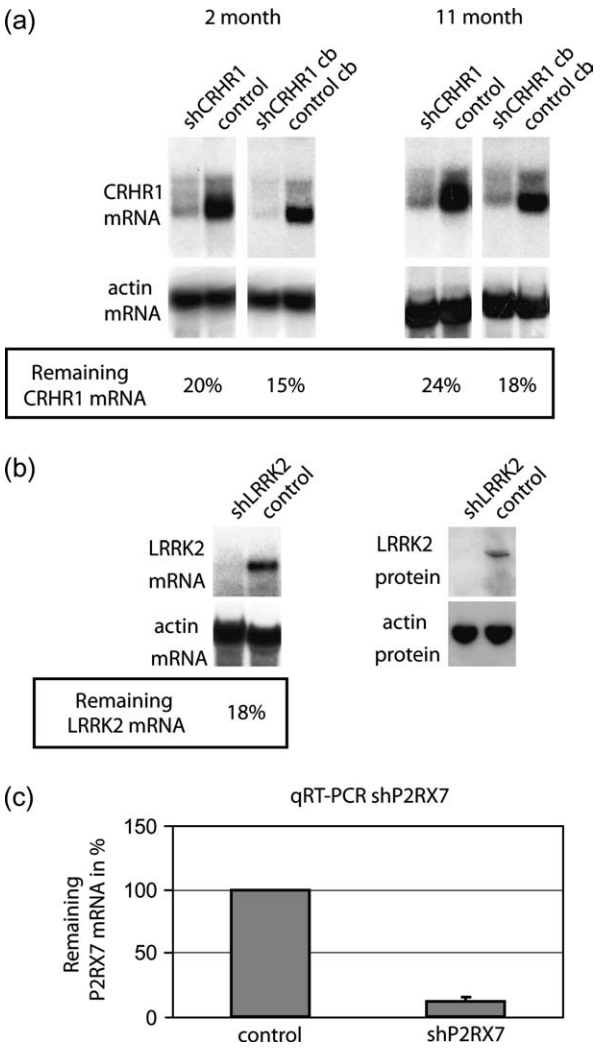

Figure 4: Efficiency of gene silencing in the brain of *Crhr1, Lrrk2* and *P2rx7* shRNA mice. (a) Northern blot analysis of *Crhr1* mRNA in the brain, and separately in the cerebellum, of 2- and 11-month-old shCRHR1 and control mice. (b) Brains from 6-week-old shLRRK2 and control mice were used to analyze the *Lrrk2* gene knockdown at the mRNA and protein level. Quantification results for Northern blots in a and b are indicated. (c) Knockdown of *P2rx7* gene expression in the brain of shP2RX7 mice, determined by qRT-PCR using brain cDNA from five shP2RX7 and five littermate control mice. The mean value of gene expression in controls was set as 100%; the bars represent triplicate measurements with standard deviation for shP2RX7.

Protein from the same tissue sample was analyzed for the LRRK2 protein by Western blot using a LRRK2 specific antibody. As shown in Fig. 4b, the LRRK2 protein was well detectable in the wild-type control but fell below the detection limit in the shLRRK2 brain, indicating a corresponding suppression of the LRRK2 mRNA and protein. The Northern and Western blot analyses were repeated with three shLRRK2

and control mice at an age of 5 months and showed essentially the same results (data not shown). The knockdown efficiency in shP2RX7 mice was analyzed by qRT-PCR. Total RNA was extracted from brains of five shP2RX7 mice and five littermate control mice at an age of 6–8 weeks. The mean knockdown efficiency was determined as 88% (Figure 4, c).

In conclusion, shRNA-mediated mRNA silencing occurs in the adult brain as efficient as found *in vitro* in ES cells; it remains stable over time in aging mice and leads to the corresponding silencing of a target protein.

Anxiety and stress response of shCRHR1 mice

To compare behavioral phenotypes of knockdown and knockout mice, we solely analyzed shCRHR1 mice because for *Lrrk2* and *P2rx7* results from knockout mice have not been reported. Knockout mice for the *Crhr1* gene exhibit a phenotype characterized by reduced anxiety-related behavior and an impaired stress response (Timpl *et al.* 1998). To assess whether the 80% knockdown of the *Crhr1* mRNA leads to a similar phenotype, we compared the anxiety-related behavior of shCRHR1 and littermate controls in the light/dark box and the plasma levels of corticosterone upon restraint stress. We observed no significant interaction of sex and genotype in time spent in dark compartment ($F_{1,47} = 0.18$; NS) or lit compartment ($F_{1,47} = 0.03$; NS), respectively. However, shCRHR1 knockdown mice spent significantly more time in the aversive lit compartment than nontransgenic littermate controls (factor genotype: $F_{1,1} = 4.09$, $P < 0.05$; Fig. 5, a). shCRHR1 mice spent the same time in the dark compartment as control animals (factor genotype: $F_{1,1} = 1.49$, NS). These data suggest that, as expected, shCRHR1 mice are less anxious. To control for potential nonspecific effects of *in vivo* shRNA expression, we also compared groups of shLuc transgenic mice and nontransgenic littermates in the light/dark box. shLuc mice express shRNA specific for the luciferase reporter gene that has no predicted target sequence in the mouse genome. Both groups showed a higher preference for the lit compartment than shCRHR1 or control mice but did not significantly differ in the time spent in the lit compartment (factor genotype: $F_{1,1} = 1.84$, NS) or the dark compartment (factor genotype: $F_{1,1} = 0.83$, NS). Thus, shRNA expression in shLuc mice does not alter anxiety-related behavior in the light/dark box, suggesting that the reduced anxiety of shCRHR1 mice is caused by the specific knockdown of the *Crhr1* mRNA. Furthermore, the corticosterone levels in the plasma of shCRHR1 males upon 10 min restraint stress were significantly lower (interaction stress × genotype: $F_{1,25} = 11.7$, $P < 0.01$; factor stress: $F_{1,1} = 88.9$, $P < 0.0001$; factor genotype $F_{1,1} = 3.99$, NS) than in a group of nontransgenic littermate controls (Fig. 5b). An impaired stress response resulting in lower corticosterone levels in the plasma is also known from *Crhr1* knockout mice. Thus, shCRHR1 mice show the same key phenotypic characteristics as *Crhr1* knockout mice. We conclude that an 80% reduction in the expression of the *Crhr1* gene is sufficient to induce this phenotype. The behavioral characterization of shLRRK2 and shP2RX7 mice is the subject of further, ongoing studies.

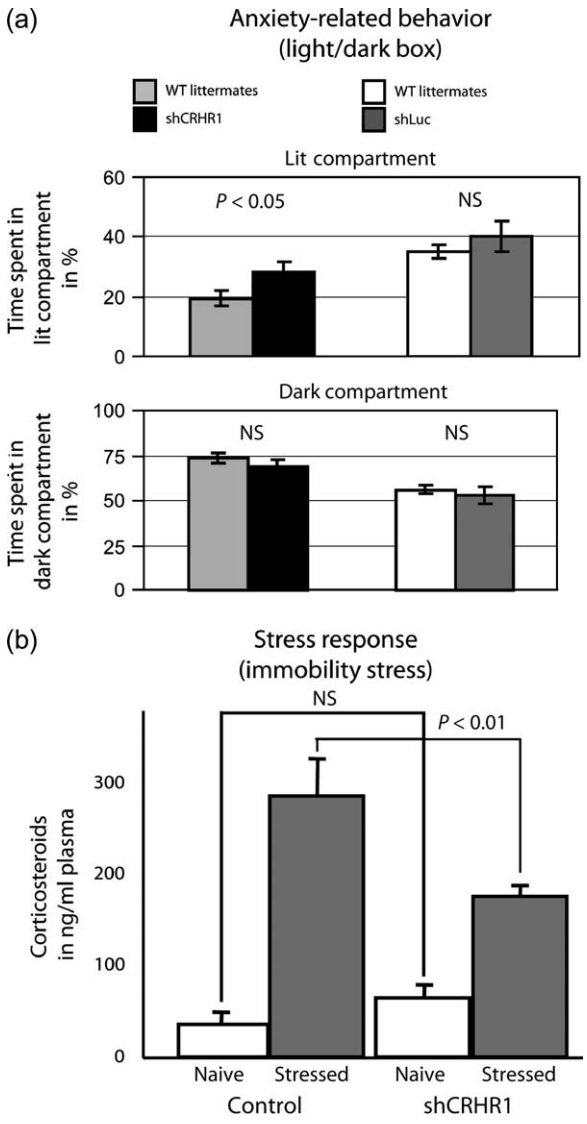

Figure 5: Phenotypic characterization of *Crhr1* shRNA mice. (a) Anxiety-related behavior of 29 shCRHR1 and 22 wild-type (WT) littermate control mice in the light/dark box test. The shCRHR1 mice spent significantly ($P < 0.05$) more time in the lit compartment indicating decreased anxiety-related behavior in shCRHR1 mice. The time spent in the dark compartment did not significantly differ between both shCRHR1 mice and their WT littermates. Separately tested mice expressing shRNA against Luciferase (shLuc) were used as control (16 shLuc mice and 23 WT littermates). No significant alterations were observed between shLuc mice and their WT littermates. Results are expressed as mean value + SEM. (b) The response to immobility stress was analyzed by determining plasma corticosterone levels in naive and stressed shCRHR1 and control mice ($n = 6$–9 males per group). Data were analyzed by two-factorial analysis of variance (ANOVA, factors genotype and treatment), with an accepted level of significance of $P < 0.05$. *Post hoc* comparisons are indicated. Results are expressed as mean value ± SEM.

Discussion

In this study, we show that shRNA vector transgenic mice are a suitable approach for the silencing of target genes in the adult mouse brain. To show the robustness and reproducibility of our RNAi strategy, we generated three shRNA expressing mouse lines with specificity for the *Crhr1*, *Lrrk2* and the *P2rx7* gene. We found that the U6 promoter enables ubiquitous shRNA expression in the brain that gene silencing is constant over time with equal efficiency in young, adult or old mice and that the suppression of a target gene mRNA and protein are corresponding Furthermore, we provide the first validation that shRNA mice are suitable for behavioral analysis because *Crhr1* knockdown mice recapitulate the key phenotypes of *Crhr1* knockout mice, decreased anxiety-related behavior and stress responsiveness. Taken together, these results are the first demonstration that shRNA transgenic mice provide a well working tool for research on gene function in the whole brain.

For the generation of knockdown mice, we inserted shRNA vectors into the *Rosa26* locus of ES cells by site-specific recombination. This approach allows to establish knockdown mice more rapidly and with less effort as compared to the generation of knockout mice by homologous recombination. Furthermore, the insertion of shRNA vectors into the *Rosa26* locus guarantees for reproducible expression of any shRNA vector and excludes negative influences of chromosomal integration sites known for randomly integrated transgenes.

The gene silencing efficiency achieved in the brain of adult shRNA transgenic mice was comparable to the knockdown determined *in vitro* upon transient shRNA vector transfection (compare Fig. 1 with Fig. 4). The described knockdown mouse lines argue for the reproducibility of this method and further offer the capability to predict the efficiency of gene silencing before generating mouse lines. Therefore, this strategy allows generating shRNA knockdown mice that exhibit a preselected level of silencing of a targeted gene.

Because it is known that shRNAs can silence mRNAs with less than 100% sequence homology (Anderson *et al.* 2008; Svoboda 2007), phenotypes observed in a single knockdown mouse strain potentially result from such off-target effects. Therefore, it will be usually necessary to confirm new findings with a second mouse strain expressing a different shRNA directed against the same target gene, although in exceptional cases, results from a single shRNA transgenic mouse strain were also acceptable (Kissler *et al.* 2006). By the use of the *Rosa26*/RMCE approach, the additional work to generate side-by-side two shRNA strains is limited. However, in this study, the shCRHR1 mice recapitulated the *Crhr1* knockout phenotype suggesting specific *Crhr1* silencing.

Besides off-target effects, shRNA expression from the modified Rosa26 locus may elicit other nonspecific side-actions. To control for such effects, we generated mice expressing shRNA against luciferase that has no target in the mouse genome. These mice appear healthy with normal body weight and did not show significant differences to their littermates in the light/dark box, indicating that brain shRNA expression as such does not alter anxiety-related behavior. Both shLuc and control mice, however, exhibited increased preference for the lit compartment compared with shCRHR1 and control mice. A possible reason for this is the age difference of the strains at the time of phenotyping (4 vs. 7 months) or unrecognized variations of the experimental settings or environment over time because these strains were phenotyped at an interval of several months.

By comparison of heterozygous and homozygous knockout mice, it is well known that a 50% reduction of the expression of a targeted gene in heterozygote mutants rarely results in a detectable phenotype. However, little is known about phenotypes resulting from a 70% to 80% reduction of target gene expression. This intermediate range may represent the variation accounting for human disease alleles caused by point mutations or reduced expression levels of genes like the fibroblast growth factor receptors (Fgfr) in major depressive disorder (MDD). In MDD patients, reduced expression of the Fgfr2 and Fgfr3 genes have been described in certain brain areas (Evans *et al.*, 2004). Through RNAi technology, it would be possible to mimic the diminished expression of Fgfr2 and Fgfr3 by generation of a conditional Fgfr2/Fgfr3 double-knockdown mouse strain. Although efficient silencing of genes can lead to a reduction of about 95% of gene expression residual protein is still remaining. Interestingly many mutations associated to human diseases are probably also not null mutations (Chen *et al.* 2006). Therefore, the incomplete suppression of gene function in shRNA transgenic mice could mimic the genetic configuration in such patients and may provide a naturalistic way to study the involvement of partial loss-of-function alleles in human diseases. In this context the knockdown mice for the *Lrrk2* and *P2rx7* genes presented in this work could be readily used as human disease models. Several studies suggest a role of the *Lrrk2* gene in Parkinson's disease (PD) and of *P2rx7* in MDD (Lucae *et al.* 2006; Paisan-Ruiz *et al.* 2004; Zimprich *et al.* 2004) making the respective shRNA mice useful for further studies.

The development of genetic mouse models that mimic human disease conditions to elucidate pathogenic mechanisms is the most direct application of the presented RNAi *in vivo* technology. Another interesting application of this technology is drug target validation studies to estimate the therapeutic potential of small molecule protein inhibitors. Although such compounds are not available for most protein classes knockdown mice could be used to explore the therapeutic potential of all genes of the mammalian genome. Using therapeutic siRNA molecules RNAi-based drugs are already subject of numerous clinical studies and may considerably expand in future the space of the 'druggable' genome (Gonzalez-Alegre & Paulson 2007). A first step toward the noninvasive delivery of siRNA molecules into the brain has been recently reported but further improvements of siRNA delivery systems are required (Kumar *et al.*, 2007). Increased siRNA stability by chemical modifications or the use of nanoparticles or pegylated immunoliposomes are promising developments that could allow therapeutic application of siRNAs (Elmen *et al.* 2005; Panyam *et al.* 2002; Pardridge 2007).

The downregulation of gene expression by continuously delivered siRNAs through osmotic pumps or by stereotactic injection of viral shRNA vectors were the only successful applications of RNAi to analyze gene function in behavior

(Lasek *et al.* 2007; Senechal *et al.* 2007; Thakker *et al.* 2004). One disadvantage of this method is the intense consumption of expensive siRNAs. Furthermore, it is uncontrollable which cells will be affected by the infused siRNAs. Generating knockdown mice with the here described strategy allows bodywide expression of shRNAs in virtually all cells and tissues of the mouse. Although we present in this study constitutive gene knockdown mice modifications of this strategy are possible. The possibility of generating mice with simultaneous knockdown of several genes with one shRNA vector can accelerate the generation of double mutant mice (Steuber-Buchberger *et al.* 2008). Additionally the use of Cre recombinase dependent conditional shRNA expression vectors offers the possibility to knockdown genes in a time and tissue specific manner while Tet-inducible shRNA expression allows reversible gene knockdown (Hitz *et al.* 2007; Seibler *et al.* 2007). These modifications and variations of RNAi technology enable a detailed analysis of gene function in neurological and psychiatric mouse disease models. There is, however, no definitive rule to decide whether, for a particular experiment, bodywide or conditional gene silencing is more appropriate because this depends on the specific biological question and the gene of interest. For the use of RNAi, we recommend the same decision as usually taken for the construction of knockout mice: unless a conditional mutant, that increases the work and time load, is not mandatory, the most straightforward approach is to work with the germline/bodywide mutant. The specific advantage of these mutants is that gene function can be tested within the whole brain in a single type of mutant.

Taken together, our approach to generate knockdown mice represents a new fast and reproducible tool to generate mouse models for behavioral analysis. Furthermore, knockdown mice will be helpful to validate future siRNA based gene therapies.

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