Dopamine D2 Receptor Overexpression Alters Behavior and Physiology in *Drd2-EGFP* Mice

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Bacteria artificial chromosome (BAC) transgenic mice expressing the reporter protein enhanced green fluorescent protein (EGFP) under the control of the D1 and D2 dopamine receptor promoters (*Drd1-EGFP* and *Drd2-EGFP*) have been widely used to study striatal function and have contributed to our understanding of the physiological and pathological functions of the basal ganglia. These tools were produced and promptly made available to address questions in a cell-specific manner that has transformed the way we frame hypotheses in neuroscience. However, these mice have not been fully characterized until now. We found that *Drd2-EGFP* mice display an ~40% increase in membrane expression of the dopamine D2 receptor (D2R) and a twofold increase in D2R mRNA levels in the striatum when compared with wild-type and *Drd1-EGFP* mice. D2R overexpression was accompanied by behavioral hypersensitivity to D2R-like agonists, as well as enhanced electrophysiological responses to D2R activation in midbrain dopaminergic neurons. Dopamine (DA) transients evoked by stimulation in the nucleus accumbens showed slower clearance in *Drd2-EGFP* mice, and cocaine actions on DA clearance were impaired in these mice. Thus, it was not surprising to find that *Drd2-EGFP* mice were hyperactive when exposed to a novel environment and locomotion was suppressed by acute cocaine administration. All together, this study demonstrates that *Drd2-EGFP* mice overexpress D2R and have altered dopaminergic signaling that fundamentally differentiates them from wild-type and *Drd1-EGFP* mice.

Introduction

The use of bacteria artificial chromosome (BAC) transgenic mice has become commonplace in neuroscience research as they are critical for the identification of specific cell types and for the cell-specific expression of Cre recombinase that, when combined with flox genes, can produce targeted gene expression or deletion. BAC transgenic mice carry large DNA clones containing 100,000 to 200,000 bp of genetic code that include complete regulatory sequences for the gene of interest and thus are able to achieve expression patterns that better mimic those of endogenous genes (Heintz, 2001; Gong et al., 2003). In recent years, BAC transgenic mice that express transgenes under the control of the D1 (D1R) and D2 dopamine receptor (D2R) regulatory sequences have become very useful tools for studying striatal function and have improved our understanding of the physiological and pathological function of the basal ganglia circuits (Surmeier et al., 2007, 2008; Kreitzer and Malenka, 2008; Valjent et al., 2009; Tian et al., 2010). Specifically, *Drd1-EGFP* and *Drd2-EGFP* transgenic mice that express enhanced green fluorescent protein (EGFP) in neurons containing D1R or D2R, respectively, have eased the identi-

fication of the two subpopulations of medium spiny neurons (MSNs) in the striatum. MSNs are the main output of the striatum and project to the medial globus pallidus and the pars reticulata region of the substantia nigra (SNr) by way of two distinct and parallel pathways, the indirect and the direct pathways. MSNs of the direct pathway mainly express D1R and send monosynaptic projections directly to the medial globus pallidus and SNr, while those of the indirect pathway mainly express D2R and send projections to the same regions via the lateral globus pallidus and the subthalamic nucleus. Using *Drd1* and *Drd2-EGFP* BAC transgenic mice, several studies have demonstrated cell-specific morphological and cell-membrane properties that distinguish each population of MSNs, as well as cell-specific synaptic plasticity and changes induced by cocaine and other psychostimulants (Lee et al., 2006; Kreitzer and Malenka, 2007; Day et al., 2008; Gertler et al., 2008; Shen et al., 2008).

These BAC transgenic mice were generated by the GENSAT (Gene Expression Nervous System Atlas) project, which is funded by the National Institutes of Health and aims at producing these valuable tools on a large scale for general use within the neuroscience community. Although the benefits of the GENSAT project are vast, one intrinsic limitation is that the BAC transgenic lines were not characterized in depth before becoming available to researchers, who then became responsible for this task. Here, we characterize *Drd2-EGFP* mice and show that they display an elevated D2R expression pattern, and have altered behavioral and physiological responses to D2R-like agonists and cocaine.

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Materials and Methods

Animals. All experiments were performed in accordance with guidelines from the National Institute on Alcohol Abuse and Alcoholism (NIAAA) Animal Care and Use Committee. Homozygote mice of both genders were used in all experiments, unless otherwise stated, and were housed on a 12 h light/dark cycle (0630–1830 light) with food and water *ad libitum*. *Drd1-EGFP* and *Drd2-EGFP* BAC transgenic mice were generated by the GENSAT project (Gong et al., 2003), and wild-type Swiss Webster mice were obtained from Taconic. Two colonies derived from the same founder *Drd2-EGFP* mouse line were used and compared in this study: the original colony established at NIAAA in 2003 from mice received from Dr. Charles Gerfen at the National Institute of Mental Health; and a newer colony (*Drd2-EGFP/M*) established in 2009 from breeder mice obtained from the Mutant Mouse Regional Resource Center (stock No. 000230-UNC). *Drd2-EGFP* heterozygote mice were generated by crossing homozygote *Drd2-EGFP* mice to Swiss Webster wild-type mice and were used only in the F1 generation.

Drugs. Cocaine (Sigma-Aldrich) was dissolved in sterile saline for injections. Quinelorane dihydrochloride (Sigma-Aldrich) stock solution (3 mg/ml) was prepared in 99% EtOH and diluted accordingly in sterile saline before the injections (final EtOH concentration, <0.1%). Tetraotoxin (Ascent), quinpirole (Sigma-Aldrich), sulpiride (Tocris Bioscience), ketanserin (Tocris Bioscience), and [³H]methylspiperone (PerkinElmer) were used for *in vitro* experiments.

Locomotor assessment. Locomotor activity was recorded in cages (10 × 6.5 inches) constructed out of clear polycarbonate walls with stainless steel or polycarbonate floors under dim illumination (~100 lux). Horizontal activity was detected as infrared beam crosses (1 inch spacing, 10 beams per cage) using Opto M3 activity monitors (Columbus Instruments). Before each experiment, mice were assigned to sex- and age-matched groups, and the different genotypes were run in parallel, whenever possible. For assessing acute and chronic responses to cocaine, mice (6–11 weeks old) were weighed and allowed to run freely for 5 min. They were then given an intraperitoneal injection of either vehicle or cocaine (30 mg/kg), and locomotion was measured for the next 20 min. For the cocaine dose–response curve, mice (naive to cocaine) received a vehicle injection for 3 consecutive days and, on the fourth day, were injected with one of three cocaine doses (5, 15, or 30 mg/kg). Experiments for the dose–response curve to D2R agonist were performed following a previous protocol (Ralph and Caine, 2005). Adult mice (2–10 months old) were allowed to run freely in the cage for 1 h, during which time baseline locomotor activity was determined. After this time, mice received an injection of either the vehicle or one of five doses of quinelorane (1, 3, 10, 30, or 300 μg/kg). Mice were then immediately returned to the cage, and horizontal locomotion was measured for 3 more hours. In all cases, locomotor activity is either expressed as the number of infrared beam breaks per minute or normalized to the mean baseline value obtained for each genotype after vehicle injection. Data were fit using the Hill equation: $\text{minimum} + (\text{maximum} - \text{minimum}) / [1 + (\text{EC}_{50}/x)^{\text{rate}}]$.

Membrane radioligand binding assays. Brains were removed from mice (5–12 weeks old) and immediately transferred to cold artificial CSF (ACSF). Striatal samples (20–40 mg) were obtained from thick coronal brain slices (1 mm) by microdissection under a dissection scope and immediately frozen in liquid nitrogen. For each assay, striatum from four mice of each genotype were pooled and stored at –80°C to allow for parallel processing. Tissue was suspended in lysis buffer (5 mM Tris-HCl, 5 mM MgCl₂, pH 7.4), incubated on ice for 10 min, and homogenized using a glass dounce tissue grinder. The suspension was centrifuged at 20,000 × g for 30 min, and the membranes were resuspended in binding buffer (50 mM Tris, pH 7.4) to yield a protein concentration of ~50 μg/ml (Bradford protein assay, Bio-Rad). Membrane preparations were incubated for 90 min at room temperature (RT) with [³H]methylspiperone in a final reaction volume of 1 ml containing the 5HT₂ receptor antagonist ketanserin (50 nM). Nonspecific binding was determined in the presence of (+)-butaclamol (4 μM). Bound ligand was separated by filtration through polyethyleneimine-soaked GF/C filters using a Brandel cell harvester and was analyzed via liquid scintillation spectroscopy (60%

efficiency). The maximum binding concentration (B_{max} ; pmol receptor/mg protein) was estimated as the asymptote value of the isotherm, and the K_d calculated as the concentration of radioligand required to occupy half of the available receptors.

Quantitative polymerase chain reaction. Total RNA was purified from microdissected striatal tissue from two adult mice from each genotype (treated as independent samples) using RNeasy Micro (Qiagen), and cDNA was synthesized using iScript cDNA Synthesis Kit (Bio-Rad). Primers for D2R amplification (forward: 5'-ATC TCT TGC CCA CTG CTC TTT GGA-3'; reverse: 5'-ATA GAC CAG CAG GGT GAC GAT GAA-3') and probes containing a 5' 6-FAM and a 3'-Black Hole Quencher-1 (5'-TG TAT CAT TGC CAA CCC TGC CTT CGT-3') were synthesized by Integrated DNA Technologies. Quantitative PCR (qPCR) runs were performed using TaqMan Fast Polymerase (Applied Biosystems) in a StepOnePlus Real-Time PCR system. Cycling conditions were as follows: initial hold at 95°C for 20 s; 40 cycles of step 1 (95°C for 1 s); and set 2 (60°C for 20 s). Samples were run in quadruplicates, and negative controls were run in parallel. Relative quantification was calculated using the $\Delta\Delta C_t$ method (StepOne System Software, Applied Biosystems).

Fast-scan cyclic voltammetry. Coronal brain slices (400 μm) containing the nucleus accumbens core from adult mice (2–4 months old) were prepared as described previously (Mateo et al., 2005). Slices were kept in oxygenated modified Krebs's buffer as follows (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.2, CaCl₂ 2.4, MgCl₂ 1.2, NaHCO₃ 25, glucose 11, HEPES 20, L-ascorbic acid 0.4; at RT until required. Recordings were made at 34°C in a chamber perfused at a rate of 1 ml/min. Cylindrical carbon-fiber microelectrodes (50–100 μm exposed fiber) were prepared with T650 fibers (6 μm diameter, Goodfellow) and inserted into a glass pipette (Cahill et al., 1996). The carbon-fiber electrode was held at –0.4 V, and the potential was ramped to +1.2 V and back at 400 V/s every 100 ms. Dopamine (DA) release was evoked by a rectangular, electrical pulse stimulation (50–600 μA, 0.6 ms/phase, biphasic) applied every 5 min. Data collection was done using LabVIEW software (National Instruments). Ten cyclic voltammograms of charging currents were recorded as background before stimulation, and the average was subtracted from data collected during and after stimulation. Baseline DA signals were collected for 5 min before drug application. The decay of mean DA transient for each condition was fit using a single exponential, and the decay time constant (τ) was calculated as k^{-1} (k = first-order rate constant).

Sequencing. Clone GENSAT-BX37 was obtained from the Children's Hospital Oakland Research Institute and grown in media with chloramphenicol (Sigma). DNA was extracted using a Large-Construct Kit (Qiagen). Sequencing was performed by Macrogen USA using the forward primer 5'-CAT GGT CCT GCT GGA GTT CGT G-3' and the reverse primer 5'-CGT CGC CGT CCA GCT CGA CCA G-3'.

Electrophysiology. Horizontal slices (250 μm) containing the ventral tegmental area (VTA) were prepared using a vibrating tissue slicer (VT-1200S, Leica) and allowed to recover for 30 min at 33°C in oxygenated standard artificial CSF containing the following (in mM): NaCl 124, NaH₂PO₄ 1, KCl 2.5, MgCl₂ 1.3, CaCl₂ 2.5, glucose 20, and NaHCO₃ 26.2. Interpeduncular fossa and medial terminal nucleus were used as landmarks to locate midbrain DA neurons. Their identity was further confirmed by the characteristic spontaneous activity at 2–5 Hz (in cell-attached) and the presence of I_h current (Zhang et al., 2010). Whole-cell voltage-clamp recordings were performed using patch electrodes (4–6 MΩ) filled with the following (in mM): KMeSO₄ 138, MgCl₂ 2, CaCl₂ 2, HEPES 10, EGTA 10, GTP-Na 0.3, and Na₂ATP 4, pH 7.2–7.4 (295–300 mOsm) at 35°C. Cells were held at –55 mV, and series resistance (6–25 mΩ) and input resistance were monitored throughout the recording. Data were collected using a Multiclamp 700B amplifier (Molecular Devices), and currents were low-pass filtered at 2 kHz and digitized at 5 kHz. The D2-like receptor agonist quinpirole (30, 90, 200, or 1000 nM) was added to the ACSF solution. Data were fit using the Hill equation: $\text{minimum} + (\text{maximum} - \text{minimum}) / [1 + (\text{EC}_{50}/x)^{\text{rate}}]$, where the minimum was set to zero.

Statistics. Data are presented as the mean ± SEM, and differences were determined using a *t* test or an ANOVA followed by a Tukey test for multiple comparisons.

Results

Mice carrying the BAC GENSAT-BX37, *Drd2*-EGFP mice bred normally and were indistinguishable in appearance from wild-type Swiss Webster (weight at 7–10 weeks old for *Drd2*-EGFP females = 28.1 ± 0.5 g; *Drd2*-EGFP males = 33.9 ± 0.7 g; wild-type females = 27.5 ± 0.6 g; wild-type males = 35.9 ± 0.7 g). The genetic background of *Drd2*-EGFP mice is mainly Swiss Webster, an outbred strain, with some contribution from the FVB/N strain because the transgene was injected in FVB/N fertilized oocytes and backcrossed to Swiss Webster mice. *Drd1*-EGFP mice were generated in the same fashion and share the same mixed background as *Drd2*-EGFP mice. Thus, *Drd1*-EGFP mice are a useful control for issues concerning the strain genetic background, and they have been frequently compared with *Drd2*-EGFP mice (Lee et al., 2006; Surmeier et al., 2007; Day et al., 2008; Gertler et al., 2008). Throughout this study, wild-type Swiss Websters and *Drd1*-EGFP mice were used and compared with *Drd2*-EGFP mice to help determine differences among transgenic lines and deviations from the unmodified background strain.

Increased D2R membrane expression and mRNA levels in *Drd2*-EGFP mice

Membrane expression of D2R was determined using radioligand binding assay on membrane extracts from the striatum of *Drd2*-EGFP and wild-type mice. The saturation binding isotherm for these genotypes showed no significant changes in receptor affinity (K_d) ($F_{(3,11)} = 0.21$, $p = 0.88$) but did show a significant increase in B_{max} , which was confirmed by a one-way ANOVA ($F_{(3,13)} = 6.7$, $p < 0.01$). Specifically, *Drd2*-EGFP mice displayed an increase in B_{max} that was $\sim 40\%$ higher than wild-type ($B_{max} = 1.32 \pm 0.06$ pmol/mg for wild-type and 1.80 ± 0.04 pmol/mg for *Drd2*-EGFP; $q = 4.9$; $p < 0.05$, Tukey test) with no corresponding change in affinity ($K_d = 0.21 \pm 0.03$ nM for wild-type mice and 0.20 ± 0.02 nM for *Drd2*-EGFP mice; $p = 0.99$, Tukey test) (Fig. 1A–C). To address whether this effect was a unique property of this particular mouse colony housed at the NIAAA, an independent colony of *Drd2*-EGFP mice was set up with outside breeders and named *Drd2*-EGFP/M (also derived from the only founder line). These mice also showed an increase in B_{max} compared with wild-type mice that was equivalent to that seen in our original colony, with no change in affinity ($B_{max} = 1.75 \pm 0.13$ pmol/mg; $q = 4.7$; $p < 0.05$; $K_d = 0.22$ nM; $p = 0.97$, Tukey test) (Fig. 1A–C). To determine whether EGFP expression in the striatum was responsible for this change, *Drd1*-EGFP mice were tested and no difference was de-

Figure 1. Overexpression of dopamine D2R subunit in the striatum of *Drd2*-EGFP mice. **A**, Saturation binding isotherm for D2R using [³H]-methylspiperone performed on striatal membrane extracts ($n = 12$ –14 mice/strain). **B**, B_{max} values obtained from the radioligand binding assay. **C**, K_d values for D2R binding. **D**, **E**, Representative plots of qPCR assays ($n = 8$ /genotype) using primer sets for the endogenous control β -actin gene “*actb*” (**D**) and *drd2* (**E**). **F**, *Drd2* mRNA levels expressed relative to wild-type mice. **G**, Linear representation of the clone RP23–161H15, which contains *Drd2* (red), *Ankk1* (black), and *Ttc12* (blue). Exons and introns are represented by solid and open boxes, respectively. **H**, Inferred schematic of GENSAT-BX37 containing EGFP downstream of the *Drd2* start codon. **I**, Enlarged diagram of upstream and downstream sequences around EGFP (green) deduced from the sequencing results. Left, Upstream sequence aligns with intron 1 of *Drd2* (red). Right, Downstream sequence aligns with the C terminus of EGFP (green), a part of the insertion cassette (polyA, in gray), and intergenic sequence between *Ankk1* and *Ttc12* (red). * $p < 0.05$ compared with wild type.

tected in B_{max} or K_d compared with wild-type mice ($B_{max} = 1.36 \pm 0.10$ pmol/mg; $q = 0.4$; $p = 0.99$; $K_d = 0.22$ nM; $p = 0.93$, Tukey test) (Fig. 1A–C).

To determine whether altered D2R gene expression could account for the increased surface expression, quantitative PCR was performed to measure D2R mRNA levels in all genotypes. Primers were designed to amplify mRNA for both short and long isoforms of D2R from striatal total RNA. The assay revealed that mice from *Drd2*-EGFP and *Drd2*-EGFP/M had significantly higher levels of D2R mRNA compared with wild-type mice (Fig. 1D–F). D2R mRNA levels were normalized to the levels of β -actin mRNA in each sample as an endogenous control. *Drd2*-

EGFP mice showed two times higher levels of D2R mRNA, and *Drd2*-EGFP/M showed 1.5 times higher levels, than those of wild-type mice ($F_{(3,24)} = 102$; $p < 0.01$ ANOVA and Tukey test; $q = 7.75$; $p < 0.01$).

The results suggest that higher D2R mRNA levels could account for the increased membrane expression in both mouse colonies of *Drd2*-EGFP, yet the molecular mechanism remains unclear. One possibility considered was the presence of additional copies of the *Drd2* gene in the transgene. The original BAC RP23–P161H15 contains a segment (217,489 bp) of mouse chromosome 9 (49,082,781–49,300,270), which includes the following three genes: *Drd2* (dopamine receptor D2); *Ankk1* (ankyrin repeat and kinase domain-containing 1); and *Ttc12* (tetratricopeptide repeat domain 12) (Fig. 1G). This BAC was modified to express EGFP downstream of the D2R start codon and was named GENSAT-BX37 (Fig. 1H) (note: only A-box 3' primer sequence is reported at <http://www.gensat.org/bacreport.jsp>). We sequenced the flanking regions around EGFP in GENSAT-BX37 using primers anchored in EGFP and proceeding upstream and downstream (supplemental material, available at www.jneurosci.org as supplemental material). The sequencing results using the upstream-directed primer confirmed that EGFP was inserted directly after intron 1 of the *Drd2* gene (the start codon is located in exon 2) (Fig. 1H,I), which is in agreement with the reported 3' primer used for EGFP insertion. The sequence obtained for the downstream flanking region corresponded to the intergenic sequence between the *Ankk1* and *Ttc12* genes, suggesting that the *Drd2* and *Ankk1* genes were removed when EGFP was inserted. These results rule out the possibility of additional functional copies of the *Drd2* present in the BAC, but also suggest that the *Ttc12* gene was left behind in GENSAT-BX37.

Enhanced potency of D2R agonist at the cellular and behavioral level

We next sought to investigate the functional consequences of D2R overexpression. With this purpose, whole-cell voltage-clamp recordings were performed from midbrain dopaminergic neurons in acute brain slices prepared from wild-type, *Drd2*-EGFP, and *Drd1*-EGFP transgenic mice. Midbrain dopaminergic neurons express somatic D2R that couple to G-protein-gated inwardly rectifying K^+ channels and, when activated, produce an outward current (Beckstead et al., 2004) that can be used to assess the functional consequences of D2R overexpression. DA neurons were identified by their characteristic I_h current and low firing frequency, and in *Drd2*-EGFP mice were also identified by their green fluorescence (Fig. 2A). The D2R-like agonist quinpirole (200 nM) evoked an outward current that was approximately twofold larger in *Drd2*-EGFP mice (wild-type = 54.3 pA and *Drd1*-EGFP = 83.8 pA, while *Drd2*-EGFP = 161 pA; $F_{(2,18)} = 4.78$; $p < 0.05$, Tukey Test, *Drd2*-EGFP vs wild-type; $q = 4.18$; $p < 0.05$) (Fig. 2D). A dose–response curve was built to compare the currents evoked by multiple concentrations of quinpirole between the genotypes. Quinpirole-evoked currents had a lower EC_{50} in *Drd2*-EGFP mice than in wild-type and *Drd1*-EGFP mice, indicating an enhanced cellular potency for D2R agonist in the *Drd2*-EGFP genotype (EC_{50} : wild-type mice = 392 nM; *Drd1*-EGFP mice = 260 nM; *Drd2*-EGFP mice = 60 nM) (Fig. 2B).

We next tested whether enhanced cellular potency of a D2R-like agonist was also reflected in enhanced behavioral potency. While rats typically show a biphasic locomotor response to D2R-like agonists, mice display a dose-dependent decrease in locomotor activity (Halberda et al., 1997; Ralph and Caine, 2005). Mice

Figure 2. Increased behavioral and cellular sensitivity to D2R agonist in *Drd2*-EGFP mice. **A**, EGFP-positive dopaminergic neurons in the VTA. **B**, Dose–response curve of outward current evoked by different quinpirole concentrations from DA neurons ($n = 3–6$ and $3–10$, respectively, for wild-type and *Drd2*-EGFP mice). **C**, Representative traces of quinpirole (200 nM) elicited outward current. **D**, Mean quinpirole (200 nM) evoked outward current between genotypes ($n = 6, 5$, and 10 , respectively, for wild-type, D1, and D2 mice). **E**, **F**, Dose–response curves showing normalized locomotor activity versus quinlorane dose. Values represent infrared beam breaks over 3 h postinjection time normalized to the mean value obtained for each genotype after saline injection ($n = 4–10$). y-axis label for **E** also refers to the y-axis label for **F**.

were allowed to habituate to the activity cage for 1 h, they then received intraperitoneal injections of saline or different doses of quinlorane, and locomotion was measured during the next 3 h (postsaline locomotor activity: wild-type mice = 1174 ± 222 counts/3 h; *Drd1*-EGFP mice = 714 ± 104 counts/3 h; *Drd2*-EGFP mice = 5939 ± 873 counts/3 h; *Drd2*-EGFP/M mice = 2354 ± 595 counts/3 h). *Drd2*-EGFP and *Drd2*-EGFP/M mice showed potentiated responses to low doses of D2R-like agonist (at $3 \mu\text{g/kg}$: $F_{(3,19)} = 4.67$; $p < 0.05$, Tukey test wild-type vs *Drd2*-EGFP and vs *Drd2*-EGFP/M; $q = 4.5$ and 4.4 , respectively; and $p < 0.05$). These responses were reflected in a leftward shift of the dose–response curve and lower EC_{50} compared with wild-type and *Drd1*-EGFP mice (EC_{50} : *Drd2*-EGFP mice = $1.5 \mu\text{g/kg}$; *Drd2*-EGFP/M mice = $1.2 \mu\text{g/kg}$; *Drd1*-EGFP mice = $6 \mu\text{g/kg}$; and wild-type mice = $65 \mu\text{g/kg}$) (Fig. 2E,F). Note that *Drd2*-EGFP mice exhibited hyperactivity, which is shown in more detail later in Figure 4A,B. As a result, locomotor activity after saline injection was lower in control mice than in *Drd2*-EGFP mice, but all control mice still displayed sufficiently high counts to allow for the detection of the inhibitory effects of D2R-like agonist on locomotion.

Altered dopaminergic signaling and impaired cocaine effect on DA uptake

Fast-scan cyclic voltammetry was used to investigate the levels and kinetics of DA release and uptake evoked by electrical stimulation in the nucleus accumbens core (Fig. 3). DA transients for wild-type and *Drd1*-EGFP mice did not differ in any parameters, and hence data from these two genotypes were pooled together as controls for statistical purposes. This singular value was then compared against *Drd2*-EGFP mice in all voltammetry analyses. The peaks of the evoked DA transients were similar among genotypes (mean control = $1.16 \pm 0.08 \mu\text{M}$; *Drd2*-EGFP mice = $1.08 \pm 0.1 \mu\text{M}$; $n = 13$ –10). However, the descending phase of the transient was much slower in *Drd2*-EGFP mice (Fig. 3*B,C*). Quantification of the mean decay time constant (τ) confirmed a significant increase in the τ in *Drd2*-EGFP mice ($526 \pm 47 \text{ ms}$) compared with control mice ($375 \pm 23 \text{ ms}$; $t_{(12)} = 2.8$; $p < 0.05$) (Fig. 3*C*). As a consequence of the severe slowdown of the DA clearance in *Drd2*-EGFP mice, the area of the DA transient was increased by ~ 2.5 times in these mice (area controls = 0.67 ± 0.08 ; *Drd2*-EGFP mice = 1.2 ± 0.16 ; $n = 10$ –13; $t_{(23)} = 3.0$; $p < 0.01$) (Fig. 3*D*).

The kinetics of the descending portion are dependent on the rate of DA uptake (Wightman et al., 1988). Cocaine blocks the DA transporter and slows down the clearance of DA in control mice, so we asked whether cocaine actions were affected in *Drd2*-EGFP mice. Figure 3*E,F* compares the effect of cocaine ($10 \mu\text{M}$) on the evoked DA transients of control and *Drd2*-EGFP mice, and displays the τ value obtained after cocaine application normalized to their own baseline τ obtained before drug application. The results showed that the cocaine effects on τ are greatly reduced in *Drd2*-EGFP mice, indicating that cocaine, at this concentration, did not slow down DA clearance noticeably in *Drd2*-EGFP mice (norm $\tau = 6.7 \pm 0.93$ and 2.22 ± 0.70 for controls and *Drd2*-EGFP mice, respectively; $t_{(6)} = 3.9$; $p < 0.01$).

DA release is modulated by presynaptic D2R, and, given our findings of D2R overexpression and enhanced potency, it became important to address the effect of D2R antagonist on evoked DA release. In agreement with previous data (Kennedy et al., 1992; Phillips et al., 2002), sulpiride ($2 \mu\text{M}$) had minimal or no effect on the peak of the evoked DA transient in control mice. However, sulpiride increased the peak of evoked DA release in *Drd2* mice by $\sim 60\%$ (Fig. 3*E*) ($t_{(7)} = 5.5$, $p < 0.01$), revealing some degree of tonic inhibition mediated by D2R in these mice.

Hyperlocomotion and paradoxical response to cocaine in *Drd2*-EGFP mice

When locomotor behavior was measured in naive animals placed in a novel environment, *Drd2*-EGFP and *Drd2*-EGFP/M mice

Figure 3. Altered DA release and uptake in the nucleus accumbens core. **A**, Representative DA traces (with cyclic voltammograms in insets) and color plots. In color plots, the voltammetric current is encoded in color in z-axis and plotted against the applied potential (y-axis) and time (x-axis). Arrows mark the time of electrical stimulation, and the white dashed line marks the faradaic currents at the oxidation potential for DA. **B–D**, Peak DA concentration (**B**), decay time constant (τ) (**C**), and area under the curve (**D**) for the DA transients evoked by electrical stimulation in brain slices of wild-type (black, $n = 3$), *Drd1*-EGFP (white, $n = 10$), and *Drd2*-EGFP (red, $n = 10$) mice. **E**, Representative DA transients evoked by stimulation before (open circle) and after cocaine application ($10 \mu\text{M}$, solid circles) in wild-type (black) and *Drd2*-EGFP (red) mice. **F**, Decay time constant (τ) of the DA transients in the presence of cocaine normalized to the mean baseline τ before cocaine for each genotype. **G**, Peak DA concentration in the presences of sulpiride ($2 \mu\text{M}$) normalized to mean peak before sulpiride for each genotype. * $p < 0.05$ compared with controls.

showed increased activity compared to both control genotypes (Fig. 4*A,B*) (beam breaks/h: *Drd1*-EGFP mice = 1479 ± 113 ; wild-type mice = 1660 ± 157 ; *Drd2*-EGFP/M mice = 2533 ± 181 ; *Drd2*-EGFP mice = 4126 ± 248 ; $n = 16$ –24; $F_{(3,137)} = 41$; $p < 0.01$, ANOVA; * $p < 0.01$ and NS; $p = 0.89$, Tukey test). Additionally, *Drd2*-EGFP mice showed no increase and even a slight dose-dependent decrease in locomotion after acute cocaine administration (Fig. 4*C*). In control mice, chronic cocaine administration caused locomotor sensitization, which was characterized by a further increase in locomotor response over the acute effect of approximately fivefold, relative to saline, with repeated cocaine injections (*Drd1*-EGFP mice: day 1 = 73.4 ± 11.4 ; day 10 = 161 ± 36.9 ; $q = 8.8$; $p < 0.01$; $n = 17$, Tukey test) (Fig. 4*D,E*). However, *Drd2*-EGFP and *Drd2*-EGFP/M mice failed to show a sensitized response to cocaine (*Drd2*-EGFP mice: day 1 = 117 ± 24.1 ; day 10 = 67.0 ± 12.9 ; $n = 12$; *Drd2*-EGFP/M mice: day 1 = 59.8 ± 12.9 ; day 10 = 83.0 ± 16.7 ; $n = 12$; all $p > 0.05$,

Figure 4. Hyperactivity and deficient acute and chronic response to cocaine in *Drd2*-EGFP mice. **A**, Locomotor activity recorded in novel cage (for 1 h in 2 min bins) $n = 18$ –25. **B**, Total locomotor activity in novel cage for each genotype. **C**, Dose–response curve for cocaine intraperitoneal administration, $n = 8$ –10. **D**, Locomotor response to the first and the last day of cocaine treatment for each genotype, $n = 3$ –15. **E** ($n = 15$), **F** ($n = 8$), **G** ($n = 12$), **H** ($n = 9$), Daily locomotor response to repeated cocaine injections. * $p < 0.05$ compared with wild-type.

Tukey test) (Fig. 4*F*, *G*). Furthermore, heterozygote mice for the BAC transgene also failed to show locomotor sensitization to cocaine ($F_{(9,84)} = 10.4$; $p < 0.01$; *Drd2*-EGFP^{+/-} mice: day 1 = 48.4 ± 8.8 ; day 10 = 36.6 ± 21.7 ; $n = 3$; *Drd2*-EGFP/M^{+/-} mice: day 1 = 61.6 ± 11.9 ; day 10 = 68.8 ± 12.3 ; $n = 9$; all $p > 0.05$, Tukey test) (Fig. 4*H*).

Discussion

This study provides an extensive characterization of the BAC transgenic *Drd2*-EGFP and *Drd1*-EGFP mice and reveals that *Drd2*-EGFP mice are not comparable to controls with regard to their dopaminergic system. *Drd2*-GFP transgenic mice, a common tool used to study the physiological and morphological properties of medium spiny neurons in the striatum and nucleus accumbens, have altered D2R expression and signaling. High levels of D2R mRNA in the dorsal striatum are likely to explain the receptor overexpression seen at striatal membranes as well as the hypersensitivity to D2R-like agonists observed in these mice. The data also suggest that the receptor overexpression extends beyond the striatum to midbrain dopaminergic neurons that also express D2R. Additionally, neurochemical analysis of DA terminal function showed slower clearance of extracellular DA in *Drd2*-EGFP mice that led to prolonged DA transients and could

account for the increased locomotor activity observed in these mice.

Drd2-EGFP mice are hyperactive, and acute cocaine administration decreases the locomotor activity. A similar paradoxical response to acute psychostimulants has been described in a mouse model of attention deficit/hyperactivity disorder in which 6-OHDA lesions were performed at neonatal age, resulting in hyperactivity at puberty and an inhibitory locomotor response to methylphenidate and amphetamine (Avale et al., 2004). Furthermore, *Drd2*-EGFP mice failed to display locomotor sensitization, which has been observed, to different degrees, in all reported mouse strains.

It is important to note that this characterization has mainly been performed on homozygous BAC transgenic mice, and thus these results and conclusions are limited to *Drd2*-EGFP homozygote mice. While most previous studies have used homozygote *Drd2*-EGFP mice, a recent study used heterozygote mice (Lerner et al., 2010). Heterozygote mice might display a milder phenotype (however, see Fig. 4*H*), and their use, in combination with appropriate controls, could be recommended over the use of homozygote mice. Nevertheless, further characterization of heterozygote *Drd2*-EGFP mice would be needed if used.

Two different mouse colonies carrying the GENSAT-BX37 clone showed similar alterations in the behavioral, neurochemical, and physiological responses to DA and D2R agonist. At the same time, mice with a similar genetic background but carrying a different BAC clone that produces EGFP expression in a separate subset of medium spiny neurons in the striatum (*Drd1*-EGFP mice) did not show this phenotype. This result rules out a potential contribution of the mixed genetic background of these BAC transgenic mice. However, the results cannot rule out that EGFP expression selectively in D2R-expressing striatal neurons is responsible for the altered gene expression.

The results of this study seem to indicate that the BAC clone itself is responsible for the altered gene expression either because of a positional effect caused by the clone insertion event or the presence of the transgene in one or more copies. Multiple mechanisms can account for the altered gene expression, including positional effects related to the random process of the transgene insertion and the random number of copies that are integrated in each pedigree. The random insertion process may lead to the interruption of a gene or regulatory sequence, or it might position the transgene under the control of a transcriptional enhancer which, in turn, would modify the expression levels of the reporter gene or its expression pattern. For these reasons, the scientific community has always recommended the generation of at least three to four founder lines with independent insertion sites of the same transgene. Unfortunately, there is only one founder line for *Drd2*-GFP mice, and this precludes further investigation of the plausible positional effects.

Sequencing analysis of the BAC clone GENSAT-BX37 seemed to confirm that indeed the coding sequence for D2R was removed and replaced by EGFP. However, the Ttc12 gene might still be present, and this could account for the altered gene expression. Indeed, a previous study reported that mice carrying a modified version of the GENSAT-BX37 showed a 1200-fold higher mRNA level for *Ttc12* in D2R-expressing neurons of the striatum compared with other neurons in wild-type mice or *Drd1*-GFP mice (Heiman et al., 2008). The function of this gene product is unknown, but allelic variations in this region of the genome have been linked to alcohol dependence (Yang et al., 2007) and could possibly be involved in the regulation of the dopaminergic system in adults and/or during development. Other transgenic mice gen-

erated from a modified RP23–161H15 clone or GENSAT-BX37, such as the *Drd2-Cre* transgenic mice, could express similar alterations. Thus, it is important to perform similar analyses and to quantify D2R expression levels if using them for experiments. The conclusions from this study highlight the importance of thorough characterization of the transgenic mice used in neuroscience research and call the community to re-examine the results obtained using *Drd2-EGFP* mice.

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