Behavioral/Systems/Cognitive

Transient Overexpression of Striatal D_2 Receptors Impairs Operant Motivation and Interval Timing

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The striatum receives prominent dopaminergic innervation that is integral to appetitive learning, performance, and motivation. Signaling through the dopamine D_2 receptor is critical for all of these processes. For instance, drugs with high affinity for the D_2 receptor potently alter timing of operant responses and modulate motivation. Recently, in an attempt to model a genetic abnormality encountered in schizophrenia, mice were generated that reversibly overexpress D_2 receptors specifically in the striatum (Kellendonk et al., 2006). These mice have impairments in working memory and behavioral flexibility, components of the cognitive symptoms of schizophrenia, that are not rescued when D_2 overexpression is reversed in the adult. Here we report that overexpression of striatal D_2 receptors also profoundly affects operant performance, a potential index of negative symptoms. Mice overexpressing D_2 exhibited impairments in the ability to time food rewards in an operant interval timing task and reduced motivation to lever press for food reward in both the operant timing task and a progressive ratio schedule of reinforcement. The motivational deficit, but not the timing deficit, was rescued in adult mice by reversing D_2 overexpression with doxycycline. These results suggest that early D_2 overexpression alters the organization of interval timing circuits and confirms that striatal D_2 signaling in the adult regulates motivational process. Moreover, overexpression of D_2 under pathological conditions such as schizophrenia and Parkinson's disease could give rise to motivational and timing deficits.

Key words: **learning; schizophrenia; Parkinson's disease; dopamine; basal ganglia; conditioning**

Introduction

Plasticity in the striatum is critical for three key aspects of operant behavior: learning, motivation, and timing. Dopamine D_2 receptors, which are highly enriched in striatum, appear to modulate this plasticity. In slice preparations, removal of the inhibition constraint of D_2 signaling changes the direction of plasticity at corticostriatal synapses from long-term depression to long-term potentiation (Calabresi et al., 1997; Centonze et al., 2004). Similarly, pharmacological blockade of D_2 receptors during the training of an appetitive associative task enhances performance in a subsequent drug-free testing session (Eyny and Horvitz, 2003). In contrast, when animals are subject to operant testing while under the direct influence of D_2 antagonists, the drug suppresses operant behavior because of a decrease in aspects of motivation (Wise, 2004; Salamone et al., 2005). Finally, D_2 signaling modulates the timing of operant responding. Both D_2 agonists and antagonists influence potently the ability to time intervals (Drew et al., 2003; Matell et al., 2006; Taylor et al., 2007), leading to the hypothesis that D_2 receptor signaling modulates the speed of an internal clock (Meck, 1986). In line with this, lesions of mesolimbic and nigrostriatal dopamine projections severely impair the ability of rats to time operant fixed intervals (FIs) (Meck, 2006). Importantly, interval timing deficits are also observed in two human conditions, schizophrenia (Johnson and Petzel, 1971; Densen, 1977; Tysk, 1983, 1990; Elvevag et al., 2003, 2004) and Parkinson's disease (Malapani et al., 1998, 2002), diseases in which abnormal dopamine tone is thought to be a key pathology.

Recently, we generated transgenic mice that selectively overexpress D_2 receptors in the striatum (Kellendonk et al., 2006). Patients with schizophrenia show increased striatal D_2 receptor density (Wong et al., 1986; Laruelle, 1998), and the mice were generated to study the behavioral and physiological consequences of this specific molecular alteration in mice. D_2 transgenic mice show increased striatal D_2 receptor density and reduced adenylate cyclase activity in the striatum, consistent with increased D_2 signaling. Behaviorally, these mice show impairments in prefrontal-dependent processes, such as working memory and behavioral flexibility, but are not impaired in tests of spatial learning and anxiety-like behavior (Kellendonk et al., 2006) (E. H. Simpson, C. Kellendonk, and E. R. Kandel, unpublished results). Moreover, developmental overexpression expression of the D_2 receptor is sufficient to induce the deficit in the working memory task because, when transgenic overexpression

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of D_2 receptors is switched off in the adult animal, the deficit is not reversed.

Given the central role of dopamine, and specifically D_2 signaling, in the control of motivational and timing processes, processes thought to be related to the negative and cognitive symptoms of schizophrenia, we sought to determine whether the excess D_2 receptors in the striatum affect these behavioral processes. We show that D_2 transgenic mice have impairments in both the motivation to work for food rewards and interval timing. Whereas the motivational deficits can be ameliorated by shutting off D_2 overexpression in the adult, the timing deficits are not fully rescuable. The results confirm that D_2 receptors are integral for motivational and timing processes and suggest that dysregulation of D_2 signaling may contribute to the motivational and timing impairments observed in human conditions such as schizophrenia and Parkinson's disease.

Materials and Methods

Mice

Transgenic mice were generated as described previously (Kellendonk et al., 2006). Briefly, mice expressing the human D_2 receptor under control of the tet-operator (tet-O) were generated on a C57BL/6-CBA(J) F2 background. tet-O mice were backcrossed for eight generations to the C57BL/6(J) background and then crossed for behavioral studies with mice expressing the tetracycline transactivator (tTA) transgene under the calcium/calmodulin-dependent kinase IIα (CaMKIIα) promoter (Mayford et al., 1996). CaMKII–tTA mice were on 129SveV(Tac)N17 background. Only F1s between backcrossed animals were used for behavioral analysis. Offspring were genotyped by independent Southern blots for tTA and tet-O. To regulate tet-O-driven gene expression, mice were fed doxycycline-supplemented chow (40 mg/kg; Mutual Pharmaceutical, Philadelphia, PA) beginning at 12 weeks of age. Behavioral experiments were commenced after 2 weeks of doxycycline feeding. The following groups of female mice were used in the operant experiments: D_2 receptor overexpressers (double transgenics) off doxycycline (D_2OE) ($n = 10$) and on doxycycline (D_2OE-Dox) ($n = 10$). Because the single transgenes did not have any effect on the behavior, control animals consisted of single-transgenic or wild-type littermates off doxycycline (control) ($n = 10$) and on doxycycline (control-Dox) ($n = 9$). Naive groups of D_2OE ($n = 6$) and control ($n = 6$) female mice were used in the sucrose preference test.

To motivate mice to earn rewards in the operant tasks, they were limited to 1 h daily access to food in the home cage (Isopro RMH 3000 complete mouse diet or doxycycline-supplemented chow; Prolab, Syracuse, NY). Water was available *ad libitum*.

Apparatus

Eight matching experimental chambers (model env-307w; Med-Associates, St. Albans, VT) equipped with liquid dippers were used in the experiment. Each chamber was located in a light- and sound-attenuating cabinet equipped with an exhaust fan, which provided 72 dB background white noise inside the chamber. The internal dimensions of the experimental chamber were 22 × 18 × 13 cm, and the floor consisted of metal rods placed 0.87cm apart. A feeder trough was centered on one wall of the chamber. Inside the trough, an infrared photocell detector (4 mm from trough opening) was used to record head entries into the trough. A reward of one drop of evaporated milk could be provided by raising a dipper located inside the feeder trough. A retractable lever was mounted on the same wall as the feeder trough, 5 cm away. A house light (model 1820; Med Associates) located at the top of the chamber was illuminated throughout all sessions. An audio speaker was positioned 8.5 cm from the floor on the wall opposite the feeder trough. The speaker delivered a brief tone (90 db, 2500 Hz, 250 ms) to signal that the liquid dipper was raised.

Procedure

Histology. After completion of all behavioral experiments, mice were killed by cervical dislocation, and the brains were rapidly removed and frozen. Single-label *in situ* hybridization was performed as described previously (Kellendonk et al., 2006) using antisense oligonucleotides specific to the endogenous D_2 mRNA (AGG CAG GGA GGC GGC AAG CAG CTG CTG TGC AGG CAA GGG GCA GAC) or the transgenic D_2 mRNA (GGA CAG ATT CAG TGG ATC CAT GGT GGC GGC CGA TCC GCT TGG).

For the fluorescent double-label *in situ* hybridization, sections of brain tissue were prepared exactly as described previously (Kellendonk et al., 2006) and then incubated with 3% H_2O_2 in PBS at room temperature for 30 min, washed with PBS, and incubated with 0.1 M triethanolamine-HCl at room temperature for 10 min. After washing, slides were prehybridized at room temperature for 1 h in hybridization solution (50% deionized formamide, 10 mM Tris-HCl, pH 8, 200 μg/ml yeast tRNA, 10% dextran sulfate, 1× Denhardt's solution, 600 mM NaCl, 0.25% SDS, and 1 mM EDTA, pH 8). Slides were then incubated overnight at 68°C in the same solution with both a digoxigenin-labeled cRNA probe against the human growth hormone poly(A) sequence and a fluorescein-labeled probe against a fragment of the acetylcholinesterase gene. After extensive washing, fluorescein was detected using an HRP-conjugated antifluorescein antibody (Roche Diagnostics, Indianapolis, IN) and the tyramide signal amplification–fluorescein detection system (PerkinElmer, Wellesley, MA). Digoxigenin was detected by an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Diagnostics) and a 2-hydroxy-3-naphtoic acid-2-phenylanilide phosphate/Fast Red kit (Roche Diagnostics). Sections were mounted with Vectashield with 4′,6′-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA).

Operant lever press training. Lever press training consisted of two phases. First, mice were trained to consume the liquid reward from the dipper located inside the feeder trough. Mice were placed inside the chambers with the dipper in the raised position, providing access to a drop of evaporated milk. The dipper was retracted 10 s after the first head entry into the feeder trough. A variable intertrial interval ensued, followed by a new trial identical to the first. The session ended after 30 min or 20 dipper presentations. On the following day, mice received another session similar to the first, except that the dipper retraction was response independent. On each trial, the dipper was raised for 8 s and then lowered whether or not mice had made a head entry. Sessions like this continued until a mouse made head entries during at least 20 of 30 dipper presentations in one session. In this and all other segments of the experiment, sessions occurred once per day, 5 d per week.

In the second phase of lever press training, mice were required to press a lever to earn the liquid reward. For the first lever press training session, mice were placed in the chamber for 8 h. At the beginning of the session, the lever was extended into the chamber, and lever presses were reinforced on a continuous reinforcement schedule. In this and all subsequent sessions, the reward consisted of raising the dipper for 5 s. To familiarize mice with the retraction and extension of the lever, after the 20th reinforcement, the lever was retracted. After a variable delay, the lever was extended, and the cycle was repeated. If a mouse did not earn 100 reinforcements in the session, it repeated the procedure the next day. Two days after the first successful lever press training session, mice received a shorter continuous reinforcement training session. The session began with the lever extended. The lever was retracted after every two reinforcements and then reextended after a variable intertrial interval. The session ended when the mouse earned 40 reinforcements or 1 h elapsed. Mice continued receiving sessions like this until they earned 40 rewards in one session. Mice then moved to fixed interval (FI) training.

FI training. In FI training, lever presses were not reinforced until after a fixed interval (timed relative to the lever extension) had elapsed. Mice began on FI-4 s schedule, meaning that the first lever press occurring >4 s after lever extension was reinforced. Each reinforcement was followed by a variable intertrial interval (mean of 30 s; range of 110 s), during which the lever remained retracted, and then a new trial, signaled by the extension of the lever. When a mouse earned at least 40 rewards in one session, the FI duration was extended in the next session. The FI durations were 4, 8, 16, and 24 s. When a mouse reached the criterion of 40 rewards in one session on the FI-24 s, it was moved to peak interval training.

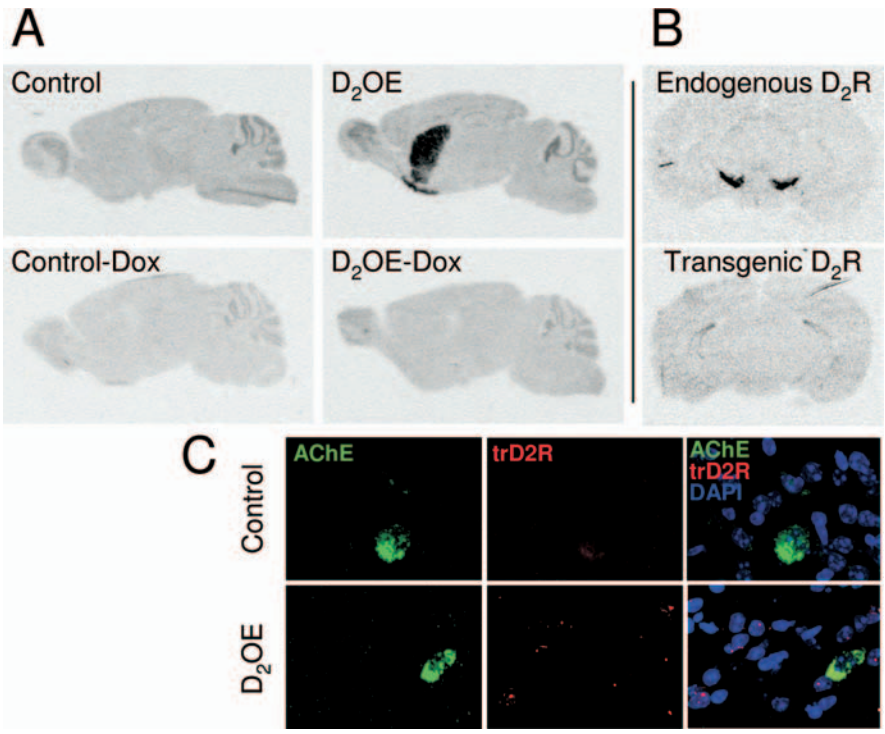

Figure 1. ***A***, *In situ* hybridization against the transgenic D_2 mRNA. Expression was detected in the striatum and olfactory tubercle of D_2OE mice and was completely absent in D_2OE-Dox and control mice. ***B***, *In situ* hybridization of two adjacent 16 μm sections hybridized with an antisense oligonucleotide against the mouse D_2 receptor coding sequence (top) or an antisense oligonucleotide directed against the transgenic D_2 mRNA (bottom). Whereas endogenous D_2 receptors are expressed in dopaminergic midbrain neurons, transgenic D_2 receptors are absent. ***C***, Double *in situ* hybridization using cRNA probes against acetylcholinesterase (AChE; left) and the transgenic D_2 receptor (trD2R; middle). The right panel shows the overlay (with DAPI nuclear stain).

Peak interval training. Peak interval training was used to assess the accuracy with which mice timed the 24 s fixed interval. Sessions consisted of two trial types: FI-24 s trials (as described above) and "peak" trials. Peak trials were probe trials in which the lever was extended for 96 s and lever presses were not reinforced. Peak trials and FI trials were ordered randomly, with the restriction that no more than five peak trials could occur consecutively. The first session of peak interval training consisted of 12 peak trials and 48 FI-24 s trials. If the mice earned at least 40 rewards, then every session thereafter consisted of a maximum of 24 peak and 36 FI-24 s trials. The session duration was capped at 90 min; if mice did not complete all 60 peak and FI trials in this period, the session ended. After 24 sessions of the latter type, mice were moved to the progressive ratio.

Progressive ratio training. Progressive ratio training was used to assess the amount of effort a mouse was willing to expend to obtain a reward. On each trial, the lever was extended, and, after the mouse made a criterion number of lever presses, a reward was delivered. The criterion was set at two lever presses for the first trial and then doubled with each successive trial, such that the second trial required four lever presses, the third trial eight presses, and so forth. The session ended after 2 h or after 3 min had elapsed without a lever press. A mouse's "break point" was defined as the first criterion it was unable to successfully complete.

Sucrose preference test. Mice were housed individually for 4 d in standard mouse cages containing two water bottles: one filled with water and the other with 10% sucrose (w/v) in water. The relative position of the two water bottles was alternated each day. For one 2-d period, mice were food deprived, receiving ~1 h of food access per day. For the other 2 d period, chow was available *ad libitum* in the cage. The order of these periods was counterbalanced between subjects.

Data analysis. The control and control-Dox groups did not differ on any dependent measure (*t* tests, $p > 0.05$), so these groups were collapsed into a single control group. Unless otherwise specified, data were subjected to one-way ANOVA comparing the control (collapsed), D_2OE, and D_2OE-Dox groups. Significant main effects were probed *post hoc* with pairwise Fisher's PLSD tests. ANOVA results are reported in the text, and significant *post hoc* comparisons are indicated in the figures with an asterisk. α was set at 0.05 for all analyses.

Results

D_2 overexpression and regulation by doxycycline

Consistent with previous results (Kellendonk et al., 2006), expression of the transgenic D_2 receptor mRNA was robust and limited to striatum and olfactory tubercle (Fig. 1*A*) (higher-resolution images are shown by Kellendonk et al., 2006). Within the dorsal and ventral striatum, expression of transgenic D_2 appeared to be limited to medium spiny neurons. Double *in situ* hybridization did not detect expression of the transgenic mRNA in acetylcholinesterase-expressing striatal interneurons (Fig. 1*C*). Furthermore, very limited transgene expression was observed in the cortex (Fig. 1*A*,*B*) (for higher-resolution images, see Kellendonk et al., 2006), and no transgene expression was observed in the dopaminergic nuclei of the midbrain (Fig. 1*B*), two areas that provide major inputs to striatum.

Because these experiments required food restriction, we assessed whether 1 h/d of access to doxycycline-supplemented food reverses transgene expression. We determined that transgene expression could be switched off by feeding D_2OE mice for 1 h/d with doxycycline food for 2 weeks (data not shown) (Fig. 1*A* shows animals that were killed after behavioral testing).

D_2 overexpression delays acquisition of lever pressing

All subjects required either two or three dipper training sessions to learn to consume the milk rewards, and there were no between-group differences in the speed (number of sessions) with which this response was acquired (p values >0.20). There were, however, modest differences in the speed with which lever pressing was acquired. The control and D_2OE-Dox groups each required a median of one session to reach the criterion of 40 rewards in one session (the criterion for moving to FI training), but the D_2OE group required a median of two sessions. These values differed significantly ($F_{(2,36)} = 3.93$; $p = 0.028$). Despite this difference, all mice eventually met the continuous reinforcement acquisition criterion and then successfully completed the sequence of four FI schedules. There were no differences between groups (p values >0.10) in the number of sessions required to progress through the sequence of FI schedules.

D_2 overexpression reduces operant responding and impairs interval timing

Peak interval training was used to assess the accuracy and precision with which mice learned the target interval of 24 s. In peak interval training, mice received both FI-24 s trials and interspersed peak trials, on which the lever was extended for 96 s but lever presses were not reinforced. To assess acquisition of the task

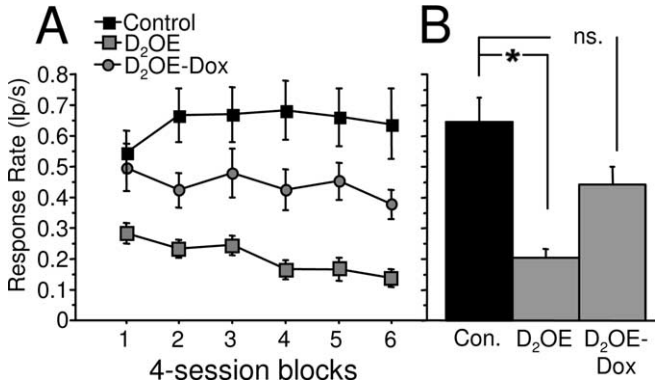

Figure 2. Mean response rate on FI trials as a function of session (***A***) and averaged across all sessions (***B***). Response rate was reduced in D_2OE mice, and this reduction was partially ameliorated in D_2OE-Dox mice. *$p < 0.05$. ns, Not significant.

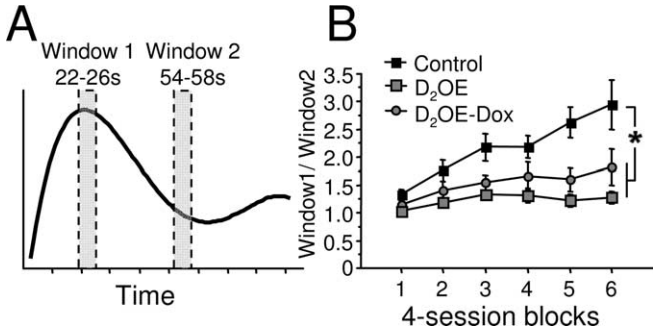

Figure 3. The development of operant response timing as a function of training session. ***A***, To assess timing of operant responding, we compared the response rate during seconds 22–26 (Window 1) with that of seconds 54–58 (Window 2). ***B***, The window 1/window 2 ratio increased as a function of sessions, but the increase was greater in control mice than in D_2OE and D_2OE-Dox mice. *$p < 0.05$.

and motivation to earn rewards, we first examined response rates during the FI-24 s trials as a function of session (Fig. 2). Note that, because mice had already learned to lever press on an FI schedule before starting the peak interval task, average response rates were relatively high in the early sessions of peak interval training and did not increase markedly with more training (Fig. 2*A*). One-way ANOVA revealed that the groups differed in their FI response rates ($F_{(2,36)} = 8.98$; $p < 0.001$). As shown Figure 2*B*, *post hoc* tests indicated that response rate was significantly reduced in D_2OE mice relative to controls, but the intermediate response rate exhibited by D_2OE-Dox mice did not differ significantly from controls or D_2OE mice.

Generally, timing of the 24 s target interval is evidenced on peak trials by an increase in response rate as the 24 s target duration approaches, a high rate of responding on or around the target time, followed by a decrease in response rate after the target interval has passed. Timed responding of this sort usually develops gradually with training, as the height of the peak in responding near the target time increases over sessions (Balsam et al., 2002; Drew et al., 2005) and/or the response rates before and after the target time decrease over sessions (Guilhardi and Church, 2005). To assess the development of timed responding in D_2 overexpressing mice, we examined the change over sessions in response rates during two "windows" of the trial, representing a period near the target time when a high response rate would be expected and from a period slightly more than twice the target time when low response rates would be expected (Fig. 3*A*). Window 1 consisted of seconds 22–26 and window 2 of seconds 54–58 (peak trials only). Good timing would be characterized by a response rate in window 1 ($rate_{w1}$) that exceeds that of window 2 ($rate_{w2}$). The response rate during each of these windows was computed for each session and each mouse. We then computed the ratio of $rate_{w1}$ to $rate_{w2}$ ($rate_{w1}/rate_{w2}$). The value of this ratio increased over sessions, consistent with an improvement in timing over sessions (Fig. 3*B*). However, the magnitude of the increase over sessions was markedly larger in control mice (on and off doxycycline) than in D_2OE mice (on and off doxycycline). These observations were confirmed with a 3 (group) × 6 (session block) ANOVA, which detected a significant session × group interaction ($F_{(10,180)} = 2.65$; $p = 0.005$), as well as significant main effects of group ($F_{(2,36)} = 6.80$; $p = 0.003$) and session ($F_{(5,180)} = 8.30$; $p < 0.001$). The data also show that interval timing was not fully rescued by switching off transgene expression in D_2OE mice (D_2OE-Dox differs significantly from control).

To characterize the timing deficits in more detail, we next examined the full distributions of lever press responses during the peak trials. We limited our analysis to the final four sessions of peak interval training, which was the period in which control mice exhibited asymptotic performance in terms of both response rate and timing precision. We plotted the number of responses per second during peak trials, averaging across all peak trials in the last four sessions of training (Fig. 4*A*). Consistent with impaired timing precision, the curves of D_2OE and D_2OE-Dox mice are flatter than those of control mice. In addition, the D_2OE mice have a reduced peak height, which is consistent with decreased motivation, but this deficit was partially rescued in D_2OE-Dox mice. In Figure 4*B*, the same data are normalized (response rates are expressed as a proportion of the maximum response rate). In the normalized functions, it is apparent that the timing deficit is partially corrected in the D_2OE-Dox mice, which show a maximum at 24 s target time, whereas the maximum is shifted rightward in the D_2OE mice off doxycycline.

We then explored the timing deficit in quantitative terms. Because the distribution of responding on peak trials in normal subjects generally assumes an approximately normal or Gaussian distribution, it is possible to characterize response timing quantitatively by fitting a Gaussian probability density function to each subject's peak trial data (Buhusi and Meck, 2000). The Gaussian includes parameters representing peak height (a), peak width (b), and peak location (x_o) (Fig. 5*A*). Using a curve-fitting software (SigmaPlot; Systat Software, San Jose, CA), we fit Gaussians to each subject's peak trial data (1 s bins, averaged across the final four sessions of training, as above) and then examined whether the obtained best-fit parameter values differed by group. The software conducted an iterative search to find the parameters that produced the best fit of the data. The mean best-fit parameters for each group are shown in Figure 5.

ANOVA revealed significant main effects of group on peak height ($F_{(2,36)} = 7.06$; $p = 0.003$), width ($F_{(2,36)} = 3.53$; $p = 0.040$), and location ($F_{(2,36)} = 7.82$; $p = 0.002$), as well as on variance accounted for by the Gaussian model ($F_{(2,36)} = 4.40$; $p = 0.020$). Peak height (a) (Fig. 5*B*) was significantly reduced in D_2OE and D_2OE-Dox mice. As in our other analyses, this reduction was partially rescued in D_2OE-Dox mice, but the rescue did not reach statistical significance (D_2OE vs D_2OE-Dox, $p = 0.239$). Peak width (b) (Fig. 5*C*) was significantly greater in D_2OE-Dox than in control mice. Although peak width also appeared to be increased in D_2OE mice, this group did not differ significantly from controls ($p = 0.112$) or from the D_2OE-Dox

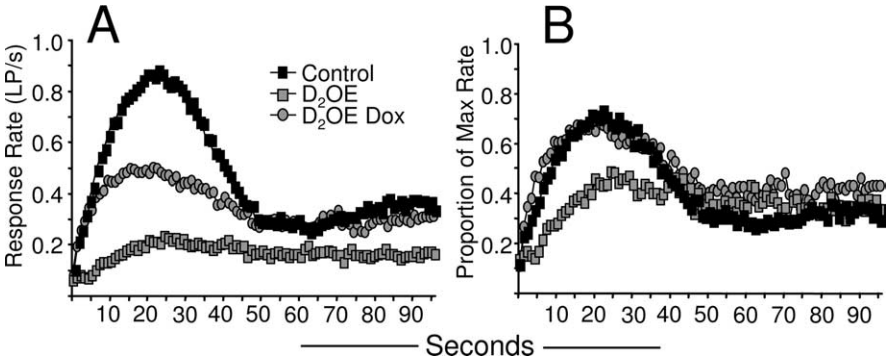

Figure 4. Timing of operant responses during the final four sessions of the peak interval procedure. ***A***, Mean response rate during peak trials as a function of time in the trial. In ***B***, response rates are expressed as a proportion of the per-trial maximum response rate. Both D_2OE and D_2OE-Dox mice have broader distributions of responding, consistent with reduced timing precision. Response rate was reduced in D_2OE mice, and this deficit was partially rescued in D_2OE-Dox mice.

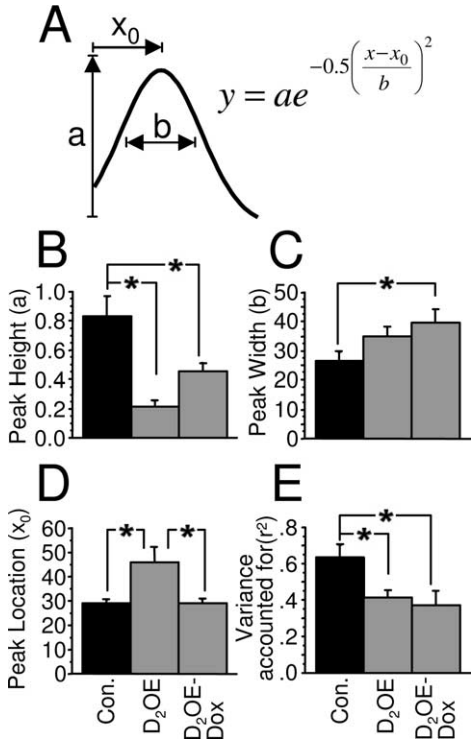

Figure 5. Mathematical modeling of peak interval performance. Peak trial data were fit to a Gaussian probability density function, illustrated in ***A***. ***B–E*** show the mean best-fit parameter values from the Gaussian fits. ***B***, Peak height was reduced in D_2OE mice but rescued in D_2OE-Dox mice. ***C***, Peak width was significantly increased in D_2OE-Dox mice; the D_2OE group did not differ significantly from either of the other groups. ***D***, Peak location was significantly increased (right-shifted) in D_2OE mice, and this increase was rescued in D_2OE-Dox mice. ***E***, The Gaussian function provided a better fit for the control data than for data from either D_2OE or D_2OE-Dox mice. *$p <$ 0.05.

group ($p = 0.437$). Peak location (x_0) (Fig. 5*D*) was significantly increased in D_2OE mice relative to controls, but this increase was ameliorated in D_2OE-Dox mice. Finally, the Gaussian model provided a better fit (r^2) (Fig. 5*E*) to the control data than to either the D_2OE or the D_2OE-Dox data. Overall, the modeling indicates that timing precision was impaired in both the D_2OE and D_2OE-Dox mice, although the normal peak location in the D_2OE-Dox group suggests at least a partial rescue of the timing deficit after reversing D_2 receptor levels to normal levels.

Timing impairments in D_2OE mice were not caused by a failure to earn rewards

We next explored whether the timing impairments in D_2OE and D_2OE-Dox mice might be secondary to their impairment in lever press response rate. Mice responding at a low rate might receive less accurate information about the target time because they may receive fewer rewards and because the rewards earned may come later in the trial (i.e., more distant from the target time). Therefore, we analyzed the number of rewards earned and the latency to earn rewards during the final four sessions of training (Fig. 6). As shown in Figure 6*A*, D_2OE mice showed a very modest but statistically significant decrease in the number of rewards earned ($F_{(2,36)} = 13.03$; $p < 0.001$). *Post hoc* tests confirmed that the D_2OE group differed from both the other groups, but the D_2OE-Dox and control groups did not differ from each other. The latency to earn rewards was defined as the median latency on FI trials for the mouse to earn a reward after the 24 s FI had elapsed (Fig. 6*B*). This measure, too, differed by group ($F_{(2,36)} = 7.04$; $p = 0.003$). *Post hoc* tests confirmed that D_2OE mice had an increased latency to earn rewards relative to the other groups. Importantly, the D_2OE-Dox and control groups did not differ from each other.

These data indicate that, because of their low FI response rate, D_2OE mice likely received less accurate information about the target time. However, D_2OE-Dox and control mice earned the same number of rewards and showed no differences in the latency to earn the rewards. This suggests that the timing deficits in D_2OE-Dox mice did not arise from their receiving less information about the target time.

D_2 overexpression produces a reversible impairment in operant motivation

D_2OE mice exhibited a reduced operant response rate in the peak procedure, and this reduction in response rate was at least partially rescued in D_2OE mice treated with doxycycline. Is this decrease in motivation the underlying cause for the decrease in the response rate? To address this question, we used the progressive ratio task, which assesses the amount of effort a mouse will expend to obtain a reward, to directly assess motivation in these mice. Mice received five sessions of progressive ratio training, and data were aggregated over all sessions. We first examined how long mice responded on the progressive ratio schedule before reaching the criterion of 3 min without a response, which terminated the session. Survival functions were generated by plotting as a function of session time the percentage of cases (a case was one subject's performance in one session) in which the subject was still responding on the progressive ratio schedule (Fig. 7*A*). Cases in which the mouse continued responding until the session timed-out at 2 h were included in the analysis as censored cases. The survival functions were analyzed using the Mantel–Cox log-rank test, which detected an overall effect of group ($\chi^2 = 28.1$; $p < 0.001$). The overall effect was followed up with pairwise comparisons using the same test. The D_2OE group differed from both the D_2OE-Dox group ($\chi^2 = 10.7$; $p = 0.001$) and the control group ($\chi^2 = 26.5$; $p < 0.001$), but the D_2OE-Dox and control groups did not differ from each other ($\chi^2 = 1.8$; $p =$ 0.179). We also examined the number of rewards earned per session (Fig. 7*B*), which differed by group ($F_{(2,36)} = 13.08$; $p <$

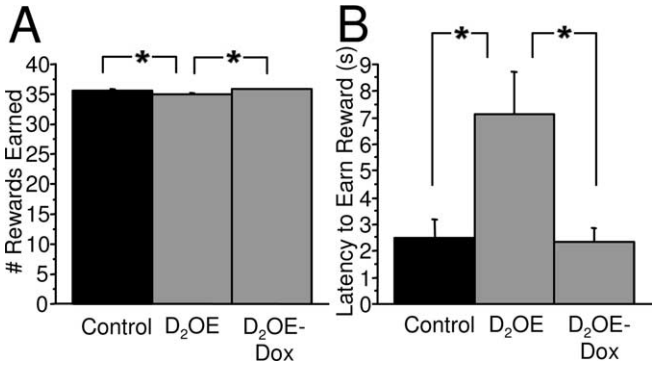

Figure 6. On FI trials, mean number of rewards earned per session (***A***) and mean latency to earn the reward after the FI had elapsed (***B***). D_2OE mice earned slightly but significantly fewer rewards and took longer to earn rewards than the other groups. Both of these deficits were rescued in the D_2OE-Dox mice. *$p < 0.05$.

0.001). *Post hoc* tests confirmed that D_2OE-Dox earned fewer rewards than both control and D_2OE-Dox mice. The groups also differed in the number of lever presses made per session (Fig. 7*C*) ($F_{(2,36)} = 9.68$; $p < 0.001$). Both the D_2OE and D_2OE-Dox groups differed from controls on this measure, indicating that rescue in the response rate of the D_2OE-Dox group was incomplete. Finally, to confirm that mice were interested in the reward, we examined the number of head entry responses into the food source during periods when reward was available (i.e., when the dipper was up). As shown in Figure 7*D*, the groups did not differ on this measure ($F_{(2,36)} = 1.31$; $p = 0.282$), indicating that all groups were pursuing the rewards when they were available.

D_2 overexpression did not alter sucrose preference

The decrease in motivation could be attributable to a reduction in the reinforcing effects of the reward. Mice were rewarded with sweet evaporated milk in the progressive ratio task. To address whether D_2OE mice value sweetness as a reinforcer, we tested D_2OE and control mice for sucrose preference using the two-bottle choice test. Sucrose consumption as a proportion of total liquid consumed did not differ between D_2OE and control mice, regardless of whether the test was conducted under food deprivation (Fig. 8*A*) or not (Fig. 8*B*). The data were subjected to 2 (group) × 2 (deprivation state) ANOVA. The effects of group ($F_{(1,10)} = 0.11$), deprivation state ($F_{(1,10)} = 3.14$; $p = 0.11$), and the interaction ($F_{(1,10)} = 0.18$) all failed to reach significance.

Discussion

Overexpression of D_2 receptors in the striatum caused three impairments in the peak interval timing procedure: a reduction in operant response rate, suggesting reduced motivation, a broadening of the distribution of operant responses in time, consistent with an impairment in timing precision, and an increase in the latency of the peak in response rate, consistent with an impairment in timing accuracy. Both the decreased response rate and the timing accuracy deficit were improved by switching off D_2 overexpression. Subsequent testing in the progressive ratio operant task confirmed that D_2 overexpressing mice exhibited reduced operant motivation and that this impairment was ameliorated by switching off D_2 overexpression.

Striatal D_2 overexpression impairs interval timing

As expected, control mice showed a defined peak in responding at the target time in the peak interval procedure. D_2OE mice, however, exhibited broader distributions of responding on peak trials, with little or no peak at the target time. Quantitative analysis of the peak trial data confirmed that the distributions of D_2OE mice were broader, less Gaussian, and less accurate than those of control mice. Only the timing accuracy impairment was rescued in D_2OE mice treated with doxycycline.

A reduction in response rate was also apparent in both the D_2OE and D_2OE-Dox mice, although the reduction was more modest in the latter group. There is the possibility that this response rate reduction produced the timing impairments we observed. Indeed, D_2OE mice earned fewer rewards and had a significantly greater latency to earn rewards in the peak interval task than controls, which means the D_2OE mice received less accurate information about the target interval. Both of these deficits were corrected in D_2OE-Dox mice, however. Because the timing accuracy deficit was corrected in D_2OE-Dox mice but the timing precision deficit was not, we suspect that only the accuracy deficit had a motivational origin. The timing precision deficit appears to have a different underlying mechanism, such as an inherent deficit in the ability to time or to modulate behavior based on temporal information.

D_2 overexpression reduces operant motivation

To directly assess operant motivation, we used the progressive ratio task, which assesses the amount of effort a subject will expend to earn a reward. D_2OE mice ceased responding significantly earlier in the progressive ratio task, earned fewer rewards, and made fewer responses. These deficits were ameliorated in D_2OE-Dox mice, indicating that the progressive ratio impairment in D_2OE mice results from concurrent overexpression of the D_2 receptor.

Theoretically, impaired performance in the progressive ratio is open to two general classes of explanation: a decrease in an internal drive state or an increase in the subjective cost of earning the rewards. Changes in drive states could result from a reduction in the hedonic value of rewards ("liking") or incentive motivation ("wanting") (Berridge, 2004). Changes in subjective cost could include the presence of a motor impairment that makes lever pressing more onerous. As the literature on neuroleptic effects on operant behavior attests, it can be challenging to discriminate among these explanations (Wise, 2004).

However, we have reason to believe that the progressive ratio impairment is attributable to a deficit in wanting or incentive motivation and not to a motor impairment or a blunting of hedonic impact. First, D_2OE mice showed normal activity and velocity in the open field and perform normally in the motorically demanding Morris water maze, in which swim speed is not affected by D_2 overexpression (Kellendonk et al., 2006), suggesting that general motor ability is intact. Second, D_2OE mice responded normally to reward presentation, suggesting that rewards maintained their reinforcement value. In the progressive ratio task, when rewards were presented, D_2OE mice approached them as frequently (Fig. 7*D*) as controls. Furthermore, D_2OE mice exhibited a normal preference for sucrose when sucrose solution and water were simultaneously available in the preference test.

The motivational phenotype of D_2OE mice resembles that produced by administration of D_2 antagonist drugs or striatal dopamine depletion, manipulations that suppress operant lever pressing but do not reduce consumption of free rewards (Salamone et al., 1994, 2005) and do not affect hedonic responses to food rewards (Berridge et al., 1989; Pecina et al., 1997). Thus, dopamine depletion is thought to specifically impair secondary rein-

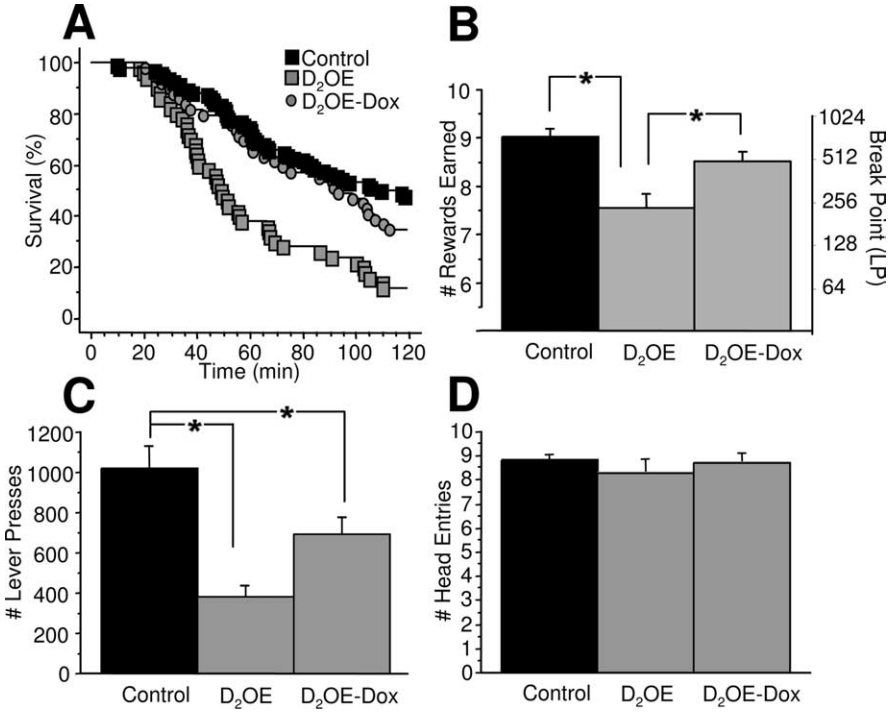

Figure 7. Performance in the progressive ratio task. ***A***, Kapan–Meier survival function plotting the percentage of subjects continuing to respond on the progressive ratio schedule as a function of session time. D_2OE mice ceased responding significantly sooner than the other groups. ***B***, Mean number of rewards earned per session and break point (last ratio completed). D_2OE mice earned fewer rewards and had a lower break point than the other groups. ***C***, Mean number of lever presses per session. D_2OE and D_2OE-Dox mice made fewer responses than controls. The difference between D_2OE and D_2OE-Dox approached significance ($p = 0.08$). ***D***, Mean number of head entries to the food compartment while food rewards were available (i.e., the dipper was up). The groups did not differ on this measure, indicating that all groups approached rewards when they were available. $^*p < 0.05$.

Figure 8. Preference for sucrose solution in the two-bottle choice test. Amount of sucrose solution consumed is expressed as a proportion of total liquid consumed (sucrose plus water). Both control and D_2OE mice exhibited a strong preference for the sucrose solution that was not affected by food deprivation.

forcement or incentive motivation but leave primary motivation and hedonic responses intact.

The similarity among the behavioral effects of D_2 overexpression, neuroleptic administration, and striatal dopamine depletion is surprising. The latter two manipulations, when induced acutely, decrease striatal D_2 activity, whereas D_2 overexpression increases net D_2 receptor activity (Kellendonk et al., 2006). One possible explanation for the paradoxical similarity among the behavioral effects of these manipulations is that the transgenic D_2 overexpression, which is chronic, leads to a compensatory downregulation of D_2-coupled intracellular signaling pathways that are downstream or independent of the cAMP pathway. A second possibility is that D_2 receptor upregulation activates constitutively signaling pathways that are independent of dopamine. A third possibility is that in D_2 transgenic mice the number of cells that express D_2 receptors in the direct pathway is increased compared with wild-type animals attributable to the nature of the CaMKII-α promoter. Finally, it has been postulated recently that D_1 and D_2 receptors may form heterodimers and that these heterodimers signal through G_q, CaMKII, and Ca^{2+} rather than via the traditional cAMP pathway (Rashid et al., 2007). An increased activation of the G_q pathway may therefore contribute to the behavioral deficits in the progressive ratio task.

Relationship to disease

Altered striatal D_2 receptor levels are associated with schizophrenia, drug addiction, and Parkinson's disease. The current mouse model may provide information about how alterations in D_2 receptor levels contribute to these diseases. Because in schizophrenia increased density and occupancy of D_2 receptors has been observed (Wong et al., 1986; Laruelle, 1998; Abi-Dargham et al., 2000), one specific implication of the present data is that the dysregulation of D_2 signaling in the striatum could contribute to the timing (Johnson and Petzel, 1971; Densen, 1977; Tysk, 1983, 1990; Elvevag et al., 2003, 2004) and motivational deficits seen in schizophrenia. Because reversing D_2 overexpression in the adult mouse did not fully rescue the timing deficits, the deficits appear to result from excess D_2 receptor expression during development and adulthood. In patients, treatment with neuroleptic drugs does not completely ameliorate timing and other cognitive impairments (Mishara and Goldberg, 2004), suggesting that these symptoms may be caused by similar underlying mechanisms.

In schizophrenia patients, motivational deficits are also resistant to antipsychotic medication that antagonizes D_2 receptors (Miyamoto et al., 2005). In contrast, the motivational deficits in D_2 overexpressing mice were ameliorated by reversing D_2 overexpression in adulthood. One reason for this apparent dissociation may be that D_2 antagonist drugs, at doses used in schizophrenic patients, block 60–80% of striatal D_2/D_3 receptors (Bigliani et al., 1999; Xiberas et al., 2001; Kessler et al., 2005), whereas in the mouse model, switching off the transgene removes only the additional D_2 receptors (an ~13% reduction). Moreover, D_2 antagonist drugs are known to cause motivational impairments in animal models (Wise, 1982; Ettenberg, 1989). The current data suggest that it may be possible to improve negative symptoms in schizophrenia by reducing D_2 receptor density to normal levels. Alternatively, excess D_2 receptors may affect motivation independently of dopamine signaling, for example by low constitutive activity or by being part of the synaptic scaffolding complex. We expect that the current mouse model will be useful for identifying new classes of compounds that are effective in treating the motivational impairment.

Timing deficits are also seen in Parkinson's disease, in which striatal D_2 receptor overexpression is the result of degeneration of nigrostriatal dopaminergic neurons that occurs progressively

over many years (Sawle et al., 1993; Turjanski et al., 1997; Ryoo et al., 1998; Kaasinen et al., 2000; Thobois et al., 2004). Although the D_2 overexpression is reversed by dopamine replacement therapy (Turjanski et al., 1997; Thobois et al., 2004), both medicated and unmedicated Parkinson's disease patients show poor precision in interval timing tasks (Artieda et al., 1992; Pastor et al., 1992; O'Boyle et al., 1996; Malapani et al., 1998, 2002), suggesting that, as in D_2OE mice, the deficit might be a consequence of long-term compensatory changes rather than acute increases in D_2 levels.

The motivational phenotype of D_2OE mice is interesting with respect to theories of drug addiction. Elevated D_2 receptor levels are thought to be protective against addiction. For instance, among individuals with a family history of alcoholism, those with higher D_2 receptor levels were less likely to be alcoholics (Volkow et al., 2006). It is also known that some drugs of abuse have a smaller hedonic impact in individuals with high D_2 receptor levels (Volkow et al., 1999, 2002). The present results suggest that D_2 receptor levels also modulate responses to natural rewards. However, we found no evidence that elevated D_2 levels reduced the hedonic impact of natural rewards. Instead, D_2 overexpression appeared to specifically reduce incentive motivation, because D_2OE mice showed impaired operant responding but normal responses to rewards when they were presented. These results suggest that elevated D_2 receptor levels may protect against addiction by reducing the incentive properties of drugs rather than by reducing their hedonic impact.

Consistent with pharmacological and lesion studies implicating striatal D_2 receptors in the control of operant motivation and timing, our data indicate that overexpression of D_2 receptors in the striatum impairs these aspects of operant behavior. The data further suggest that excess D_2 in disease states such as schizophrenia could give rise to impairments in these domains, whereas it may be protective against drug addiction.

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