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A comparison of striatal-dependent behaviors in wild-type and hemizygous *Drd1a* and *Drd2* BAC transgenic mice

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Abstract

Studies of striatal physiology and motor control have increasingly relied on the use of bacterial artificial chromosome (BAC) transgenic mice expressing fluorophores or other genes under the control of genetic regulatory elements for the dopamine D1 receptor (D1R) or dopamine D2 receptor (D2R). Three recent studies have compared wild-type, D1R and D2R BAC transgenic mice, and found significant differences in physiology and behavior, calling into question the use of these mice in studies of normal circuit function. We repeated the behavioral portions of these studies in wild-type C57BL/6 mice and hemizygous *Drd1a*-tdomato (D1-Tmt), *Drd1a*-eGFP (D1-GFP), and *Drd2*-eGFP (D2-GFP) mice backcrossed into the C57BL/6 background. Our three laboratories independently found that open-field locomotion, acute locomotor responses to cocaine (20 mg/kg), locomotor sensitization to five days of daily injections of cocaine (15 mg/kg) or amphetamine (3 mg/kg), cocaine (20 mg/kg) conditioned place preference, and active avoidance learning to paired light and footshock were indistinguishable in these four mouse lines. These results suggest that while it is crucial to screen new transgenic mouse lines for abnormal behavior and physiology, these BAC transgenic mouse lines remain extremely valuable tools for evaluating the cellular, synaptic, and circuit basis of striatal motor control and associative learning.

Introduction

In recent years, numerous transgenic mouse lines have been generated using bacterial artificial chromosome (BAC) technology (Gong et al., 2003; Gong et al., 2007) in order to express fluorophores or other genes in specific subsets of neurons. This method has allowed neurophysiologists to selectively target subpopulations of neurons in brain slices (Wang et al., 2006; Kreitzer and Malenka, 2007). In particular, basal ganglia neurophysiology has been limited by the fact that striatal principal neurons, medium spiny neurons (MSNs), are divided into two functionally distinct but intermixed populations. MSNs expressing the D1 dopamine receptor form the direct pathway and striatonigral projection, whereas MSNs expressing the D2 dopamine receptor form the indirect pathway and striatopallidal

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projection (Gerfen et al., 1990). These two populations of MSNs are thought to have complementary if not opposing effects on movement (DeLong, 1990) and associative learning (Bach et al., 2008; Lobo et al., 2010; Boschen et al., 2011) so distinguishing them is essential. Historically, these neurons could not be distinguished in physiological recordings without the use of single-cell PCR (Surmeier et al., 1996; Wang et al., 2006), retrograde tracing (Waszczak et al., 1998; Gertler et al., 2008; Planert et al., 2010), or by filling them with a marker followed by post-hoc immunohistochemistry (Bagegta et al., 2011). By permitting prospective identification of cell types and cell-type specific molecular manipulations, BAC transgenic technology has been the foundation for many recent studies elucidating the properties of different populations of striatal neurons, thus enabling a greater understanding of how basal ganglia circuits function in health and disease.

Despite the rapid adoption of these transgenic mouse lines, three recent studies have identified concerning behavioral abnormalities in D1 and D2 BAC transgenic mice (Ade et al., 2011; Bagegta et al., 2011; Kramer et al., 2011). Mice from a D2-GFP mouse line, which express GFP in indirect pathway MSNs, showed changes in dopamine receptor expression, dopamine sensitivity, and open-field behavior as compared to wild-type and D1-GFP mice (Kramer et al., 2011). Abnormal open field behavior was shown in the same mouse line by another laboratory (Ade et al., 2011). A third laboratory found differences in striatal synaptic plasticity, open field locomotion, and active avoidance in D1-GFP mice (Bagegta et al., 2011). These studies appropriately call into question the use of BAC transgenic mouse lines for both striatal physiology and behavior.

We have used similar mouse lines in our three independent laboratories for several years, and therefore sought to determine if these abnormalities were specific to the mouse colonies examined, or were a general feature of these BAC transgenic lines. We report that in assays of open field behavior, acute and chronic responses to psychostimulants, conditioned place preference, and active avoidance learning, hemizygous BAC transgenic D1-Tmt, D1-GFP, and D2-GFP mouse lines are indistinguishable from wild-type C57Bl/6 mice. Thus the previously reported abnormalities in similar BAC transgenic lines cannot be attributed to the presence of the BAC transgenes, and these lines remain a valuable tool for study of striatal circuits in adaptive and pathological behavior.

Methods

Animals

Mice were bred and tested in three different laboratories, located at the Gladstone Institute, Stanford University and the University of Geneva. Hemizygous mice of either sex were used in all experiments. D2-GFP mice were originally obtained from X. William Yang at the University of California, Los Angeles (Kreitzer and Malenka laboratories) or Paul Greengard and Jean-Antoine Girault at The Rockefeller University and University of Pierre and Marie Curie in Paris, respectively (Luscher laboratory), and were back-crossed to C57BL/6 mice for >10 generations. D1-GFP mice were back-crossed to C57Bl/6 mice for 4–5 generations. D1-Tmt line 5 mice were obtained from Nicole Calakos (Shuen et al., 2008). Mice from all three lines were bred against C57Bl/6 mice (Jackson Labs) to obtain hemizygous mice for behavioral experiments. Nontransgenic littermates or wild-type C57Bl/6 mice (Jackson labs) were used as controls. For open-field behavior, acute responses to cocaine, psychostimulant sensitization, and active avoidance learning, wild-type C57Bl/6 (Jackson labs) were used as controls. For acute locomotor responses to cocaine, cocaine sensitization, and cocaine conditioned place preference, nontransgenic littermates were used as an additional control group. All mice were 6–10 weeks of age at the time of behavioral measurements. Mice were housed on a 7 AM – 7 PM light cycle, and testing was performed during daytime hours.

Drugs

Amphetamine and cocaine (Sigma-Aldrich) were dissolved in normal saline and administered intraperitoneally. Amphetamine (0.6 mg/mL) was administered at a dose of 3 mg/kg. Cocaine (2 mg/mL) was administered at a dose of 20 mg/kg for acute locomotor responses and cocaine CPP, and 15 mg/kg (1.5 mg/mL) for assessment of sensitization. Equivalent volumes of saline were injected in control animals.

Behavior

Open field behavior—Naive animals were placed in square polycarbonate cages with opaque white walls measuring 41 cm on a side for a period of 10 minutes. Open field locomotion was recorded and analyzed using video-tracking software (Noldus). Analysis was subdivided into time spent and distance travelled in peripheral areas (2.5 cm or less from the walls of the cage) versus the center, following the method of Bagetta et al., 2011.

Acute locomotor effects of cocaine—Naïve animals were placed in a behavioral chamber (a circular corridor with total diameter 19 cm, width 4 cm) after being injected with either normal saline (0.9%) or cocaine (20 mg/kg). Open field locomotion was monitored using video-tracking software (Any-maze, Stoelting) for a total of one hour after the injection, and distance travelled was divided into 5 minute bins.

Amphetamine sensitization—Animals were habituated to square polycarbonate cages for one hour daily for two days prior to behavioral testing. Open-field locomotion was recorded and analyzed using video-tracking software (Noldus) for one hour before and one hour after administration of amphetamine (3 mg/kg dissolved in normal saline, intraperitoneal injection) or normal saline (intraperitoneal injection). Injections were given and behavioral responses recorded for 5 consecutive days.

Cocaine sensitization—Animals were placed into a polycarbonate cage fitted with a beam-break movement-detection system (MedAssociates), then injected with either cocaine (15 mg/kg) or normal saline (intraperitoneal injection), and returned to the polycarbonate cage for 15 minutes. This procedure was repeated for 5 consecutive days.

Active Avoidance learning—Animals were placed in a behavioral apparatus with two chambers and an open intervening door fitted with electrified steel floor beams (Gemini Avoidance System, San Diego Instruments). For five daily sessions (60 trials per session), after a 30 second dark acclimation period, each trial began with illumination of the chamber in which the mouse was located. After 4 seconds of light, a 0.2 mA shock was applied to the floor of that chamber until the mouse left the chamber, for a maximum of 25 seconds. When the mouse left the chamber, light and shock were co-terminated. If the mouse exited less than 4 seconds after the light began, no shock was delivered. A randomly generated intertrial interval of 5 to 25 seconds separated trials. Conditioned responses were defined as trials during which the mouse left the chamber after the light came on, but prior to the shock being delivered ($\leq 4s$).

Conditioned Place Preference—Animals were placed in a behavioral apparatus (PanLab) consisting of two compartments distinguished by different floor and wall patterns, separated by a neutral chamber. Video tracking software (Any-maze, Stoelting) was used to determine time spent in each of the chambers. The conditioned place preference (CPP) protocol was as follows: (1) In the preconditioning test (day 1), mice were placed in the neutral area, and the time spent in each compartment was measured for 15 min. (2) For conditioning (days 2–7), after injection of cocaine (20 mg/kg, days 2,4, 6 or 3, 5, and 7) or saline (days 2,4, 6 or 3, 5, and 7), mice were alternatively confined in each compartment for

20 min. Control mice received saline every day. (3) In the postconditioning test (day 8), mice had free access to both compartments, and the time spent in each compartment was measured as in the preconditioning test. Place preference (CPP score) was calculated for each mouse as the difference between postconditioning and preconditioning time spent in drug-paired compartment.

Statistics

Values cited in the text and figures are mean \pm SEM. We performed power calculations to ensure adequate sample size for each behavioral test. Based on a predicted effect size of 1.5 standard deviations (similar to the effect sizes reported in Bagetta et al., 2011 and Kramer et al., 2011 for differences between BAC transgenic and wild-type mice), a sample size of 10 mice per group has a 99% likelihood of detecting a difference between groups using a 2-factor ANOVA with three groups (for example, interaction of genotype and treatment).

Basic locomotor activity measurements and conditioned place preference were compared using the one- or two-factor ANOVA. Acute responses to cocaine, psychostimulant sensitization and active avoidance learning in the three groups were assessed using the repeated-measures ANOVA. Post-hoc comparisons were performed using the Tukey test. Statistical calculations were made using OpenStat software.

Results

Following the same breeding strategy employed for electrophysiology and behavioral studies in our laboratories, we have back-crossed BAC transgenic mice with wild-type C57BL/6 mice in order to generate transgenic mice in a C57BL/6 background. We used hemizygous D2-GFP, D1-GFP or D1-Tmt mice for all experiments. Controls were commercially available C57BL/6 mice from The Jackson Laboratory for all behavior other than conditioned place preference. We first measured open field behavior in naive mice. Representative tracks for the 10 minute session are shown for each of the mouse lines (Figure 1A–C). There was no significant difference between the total distance travelled across groups (Figure 1D, ANOVA: $F_{2,30}=0.12$, $p=0.89$). As two previous studies had shown a difference between locomotion in the peripheral versus center portion of the open field in D1 or D2 BAC transgenic mice (Ade et al., 2011; Bagetta et al., 2011), we subdivided the session into distance travelled and time spent in peripheral versus central areas. We found no significant differences between mouse lines in either distance travelled (Figure 1E, ANOVA: $F_{2,30}=0.43$, $p=0.65$) or time spent in the peripheral sector (Figure 1F, ANOVA: $F_{2,30}=0.60$, $p=0.55$).

We also assessed more complex associative behavior in the BAC transgenic lines by administering a two-way active avoidance test, which has been shown to depend on the striatum (Vecsei and Beal, 1991), as well as on dopamine signaling (Zis et al., 1974; Koob et al., 1984). In this task, animals learn to avoid an aversive stimulus by forming an association between a predictive sensory cue and the aversive stimulus. A previous study showed that BAC *Drd1a*-eGFP reporter mice had deficits in this task (Bagetta et al., 2011). C57BL/6 as well as D1-Tmt and D2-GFP hemizygote mice were trained on the active avoidance task with five daily sessions of 60 trials each. During each trial, a light (conditioned stimulus) signaled to the mouse that a footshock would occur in 4 seconds. Mice learn to leave the chamber prior to the footshock (the conditioned response). Initially, mice display few conditioned responses, instead escaping when the shock is delivered, but over time they form the association and show more conditioned responses (Figure 1G, repeated measures ANOVA: $F_{4,148}=88$, $p<0.0001$ for effect across days). In addition, the delay between onset of the conditioned stimulus (light) and the conditioned response decreased steadily over the training sessions (Figure 1H, repeated measures ANOVA:

$F_{4,148}=40$, $p<0.0001$ for effect across days). Neither of these measures of learning were significantly different between wild-type, D1-Tmt and D2-GFP mice (repeated measures ANOVA: $F_{8,148}=0.838$, $p=0.5711$ and $F_{8,148}=0.585$, $p=0.79$, for interactions between genotype and day).

Next we examined the acute locomotor response to cocaine. Cocaine blocks the reuptake of dopamine at dopaminergic axon terminals, thus amplifying any existing dopamine signaling, which typically results in increased locomotion. A prior study showed a near-absent response to acute cocaine administration in homozygous D2-GFP mice (Kramer et al., 2011). In contrast, we found that wild-type C57Bl/6, hemizygous D1-GFP and D2-GFP mice all responded to cocaine with a brisk increase in locomotion, (Figure 2A–C; repeated measures ANOVA: $F_{10,750}=94$; $p<0.0001$), which was significantly greater than in saline-injected mice. The responses to cocaine in all three groups were statistically indistinguishable (Figure 2A–C, repeated measures ANOVA: $F_{20,750}=0.65$, $p=0.52$). These findings demonstrate that BAC transgenic animals show an acute response to cocaine comparable to that seen in wild-type animals.

Psychostimulant sensitization and conditioned place preference are commonly used as a model for certain features of reward learning and addiction (Robinson et al., 1998; Tzschentke, 1998; Vezina and Leyton, 2009). Both phenomena are thought to involve modulation of midbrain dopamine circuitry and its striatal targets (Anderson and Pierce, 2005; Hikida et al., 2010; Wolf and Ferrario, 2010). In cocaine sensitization, the locomotor response to a fixed dose of psychostimulant is enhanced on each subsequent day of exposure, until a plateau is reached. This behavior was impaired in a prior study of D2-GFP homozygotes (Kramer et al., 2011). We performed cocaine sensitization by giving one group of mice daily intraperitoneal injections of saline, the other daily injections of cocaine (15 mg/kg), and measuring the locomotor response in each. Wild-type animals injected with cocaine showed both an acute increase in locomotion and subsequent robust increases in locomotion over the course of five days (Figure 3A, F), while saline-injected animals moved a similar distance each day. D1-Tmt, D2-GFP, and wild-type mice showed strong locomotor sensitization (repeated measures ANOVA: $F_{4,104}=23.8$, $p<0.0001$ for effect across days). To confirm this behavior using a different dopaminergic drug, we examined amphetamine locomotor sensitization as well. Wild-type, D1-Tmt, and D2-GFP mice all showed robust levels of locomotor sensitization in response to the daily amphetamine treatments (Figure 3E,F,G; repeated measures ANOVA: $F_{4,96}=94.4$, $p<0.0001$ for effect across days, summarized in Figure 3H). The lack of differences between wild-type, D1-Tmt and D2-GFP mice in either cocaine (repeated measures ANOVA: $F_{8,104}=0.671$, $p=0.72$ for interaction between genotype and day) or amphetamine locomotor sensitization (repeated measures ANOVA: $F_{8,96}=1.44$, $p=0.19$ for interaction between genotype and day) demonstrate that this extensively studied form of behavioral plasticity is normal in these BAC transgenic lines.

Finally, to further examine whether dopamine-dependent learning was normal in these mouse lines, we evaluated D1-GFP and D2-GFP hemizygotes and wild-type littermates for their ability to develop cocaine-induced conditioned place preference (CPP). In this task, which was developed as an assay of drug-seeking behavior (Tzschentke, 1998), animals were exposed to cocaine in one behavioral context and to saline in another, over several learning sessions, then assayed for their preference for the cocaine-paired chamber (CPP score). Experiments were performed in four groups of mice: D1-GFP hemizygotes and their nontransgenic littermates, and D2-GFP hemizygotes and their nontransgenic littermates. All groups showed a significant difference between saline and cocaine CPP scores (Figure 4; ANOVA: $F_{1,32}=55$, $p<0.0001$ for saline vs cocaine), which was indistinguishable across groups (ANOVA: $F_{2,32}=1.33$, $p=0.28$ for interaction between genotype and drug treatment),

demonstrating that both BAC transgenic lines tested have intact and normal cocaine CPP. These results showing intact cocaine CPP further support the hypothesis that BAC transgenic mouse lines have normal striatal- and dopamine-dependent behavior.

Discussion

Using mice from three separate colonies and employing different behavioral measurement techniques, we found that open field behavior, acute response to cocaine, and three types of striatal-dependent behaviors are indistinguishable in wild-type and hemizygous D1- and D2-BAC transgenic mice. These findings contradict recent reports that D1 and D2 BAC transgenic mice display abnormal behavior and strongly suggest that the BAC transgene insertion does not account for the observed behavioral changes. An important feature of our studies was the use of commercially available wild-type controls, which increase the ability to detect the effects of genetic background and/or inbreeding of mice, in addition to the effects of transgenic material. Using both wild-type and littermate controls in our cohorts of mice, we found no differences in behavior across mouse lines.

BAC transgenic mouse lines are increasingly critical tools for study of neural circuits, particularly those involving the striatum. Given that behavioral abnormalities were reported in several of these lines, we performed several well-established behavioral assays which reflect dopamine signaling and striatal function. These tests assessed motor and limbic aspects of basal ganglia function, as well as experience-dependent behavioral plasticity (Redgrave et al., 2010). Normal behavioral results in our cohorts strongly suggest that neural circuits mediating these behaviors are also normal. There are several possible explanations for the discrepancy between our results and those of the other groups using D1 and D2 BAC transgenic lines: (1) the use of hemizygous vs. homozygous animals, (2) differences in breeding strategy, and/or (3) differences in genetic background. Our use of hemizygous mice which have been outbred over many generations to C57BL/6 mice can reduce the impact of within-colony breeding. In contrast, generating homozygous mice involves some degree of inbreeding, which can increase the likelihood of maintaining inbred copy number variations of the BAC transgenes and may also promote the expression of additional genetic polymorphisms that affect behavior. The more subtle differences in behavior seen in D1-GFP and D2-GFP hemizygotes as compared to D2-GFP homozygotes (Ade et al., 2011; Bagetta et al., 2011) support this hypothesis. Furthermore, genetic background may interact with BAC transgenes to attenuate or enhance behavioral abnormalities (Chan et al, co-submitted manuscript).

Our results suggest that BAC transgenic mouse lines used to study striatal function remain valuable tools and, in contrast to previous reports, do not necessarily display abnormal behaviors in common assays of striatal function. However, together with these previous reports, our results suggest that there are likely to be additional factors beyond the presence of the transgene(s) that determine behavior. One particularly important factor may be the breeding strategy that is used to generate the animals. Moreover, using C57Bl/6 mice as strain controls should enhance the sensitivity to any abnormal behavior. The continued use of these experimentally powerful BAC transgenic mouse lines will have to be monitored carefully to avoid contamination of results by the additional effects that transgenic manipulation may cause and conclusions will always need to be supported by the judicious use of control subjects.

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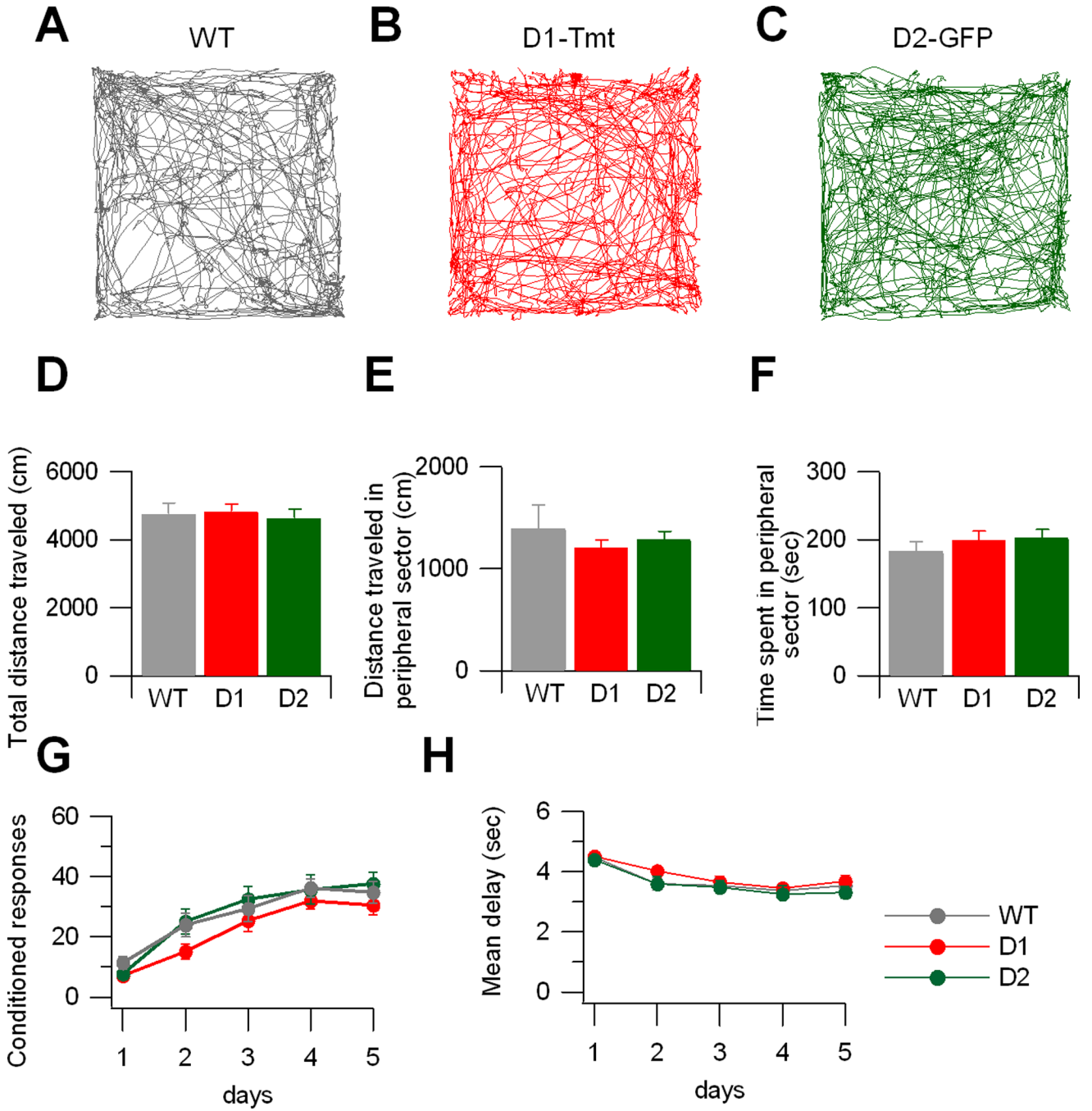


Figure 1. Open field locomotor activity and active avoidance learning are similar in wild-type, D1-Tmt, and D2-GFP mouse lines. A, B, C. Example traces of locomotor activity recorded in a novel cage for 10 minutes in each mouse line. D, E, F. Summary of locomotor activity in each session (n=10 wild-type mice, n=10 D1-Tmt mice, and n=12 D2-GFP mice). D. Total distance travelled. E. Total distance travelled. F. Time spent in the peripheral sector. G, H. Active avoidance behavioral training (n=15 wild-type, n=10 D1-Tmt, and n=12 D2-GFP mouse lines). G. Number of conditioned responses per 60-trial session on each of 5 consecutive training days. H. Mean delay time between the onset of the light cue and the

mouse leaving the chamber on each training day. In all panels, wild-type is shown with grey symbols, D1-Tmt with red symbols, and D2-GFP with green symbols.

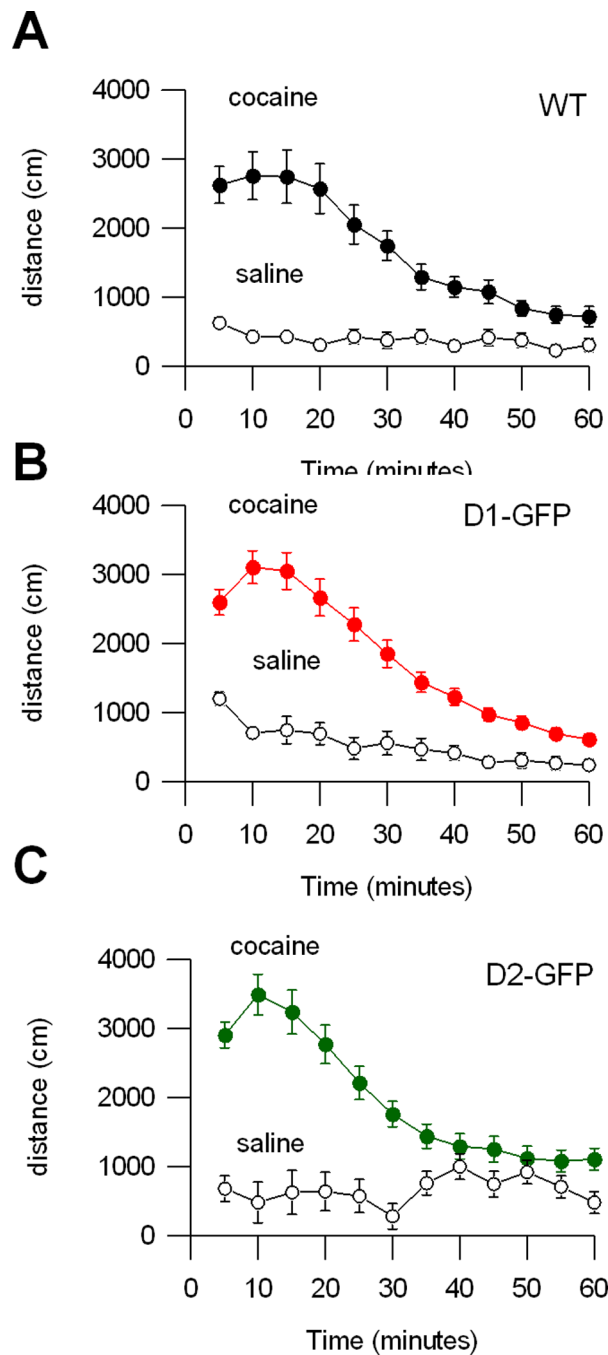


Figure 2.

Acute locomotor responses to cocaine are similar in wild-type, D1-GFP, and D2-GFP mouse lines. A, B, C. Total distance travelled following intraperitoneal injection of saline (open symbols) or cocaine (filled symbols) in wild-type C57Bl/6 (n=12 saline and 12 cocaine), D1-GFP +/- mice (n=4 saline and n=23 cocaine), D2-GFP +/- mice (n=6 saline and n=22 cocaine).

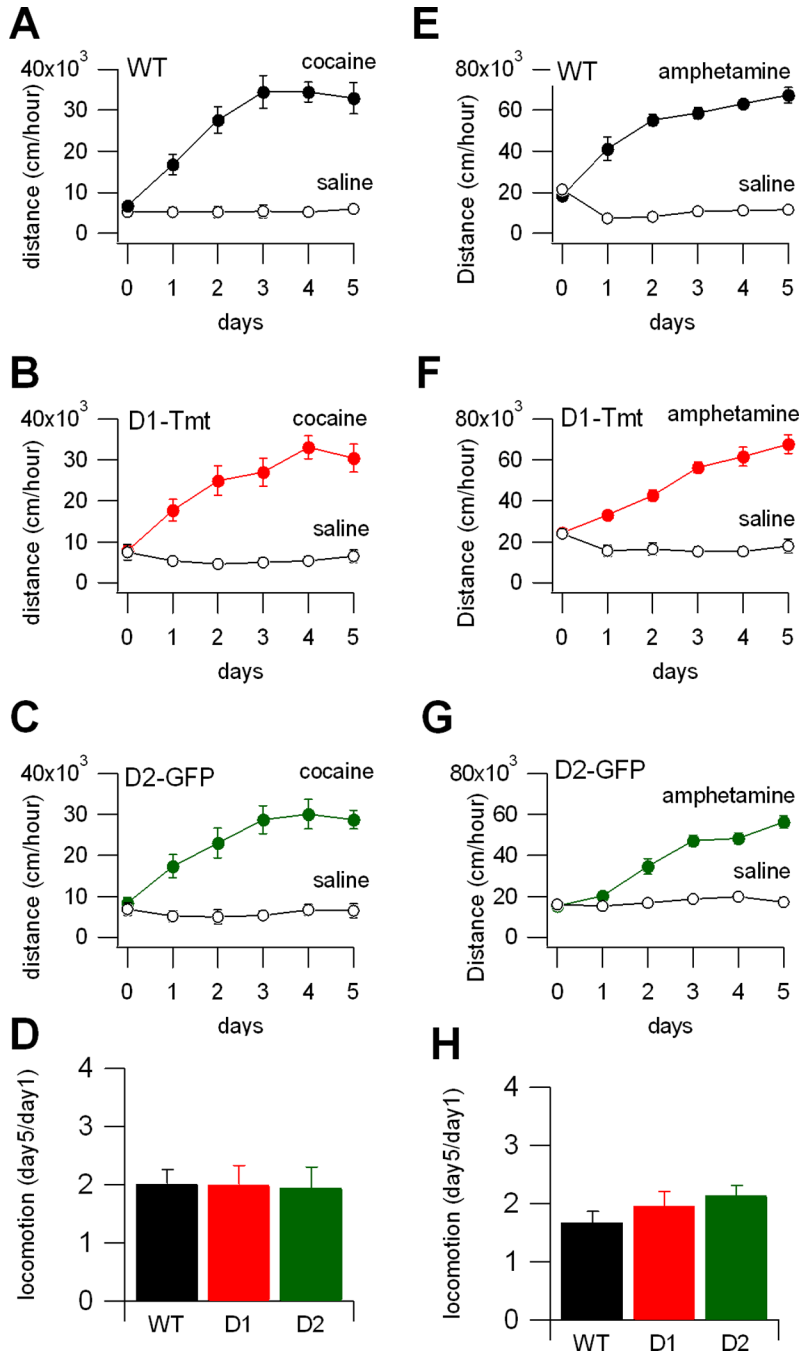


Figure 3. Psychostimulant locomotor sensitization is similar in wild-type, D1-Tmt, and D2-GFP mouse lines. A, B, C, Total distance travelled following intraperitoneal injection of saline (open symbols) or cocaine (filled symbols, 15 mg/kg) in wild-type C57Bl/6 (n=7 saline and n=10 cocaine), D1-Tmt (n=6 saline and n=8 cocaine), and D2-GFP (n=6 saline and n=8 cocaine) mice. D. Summary of ratio of locomotion on day 5 to day 1 of cocaine for each of the mouse lines. E, F, G. Total distance travelled following intraperitoneal injection of saline (open symbols) vs amphetamine (filled symbols, 3 mg/kg) in wild-type C57Bl/6, D1-Tmt +/-, and D2-GFP +/- mice (n=4 saline and n=8 cocaine for each group). H. Summary of ratio of locomotion on day 5 to day 1 of amphetamine for each of the mouse lines.

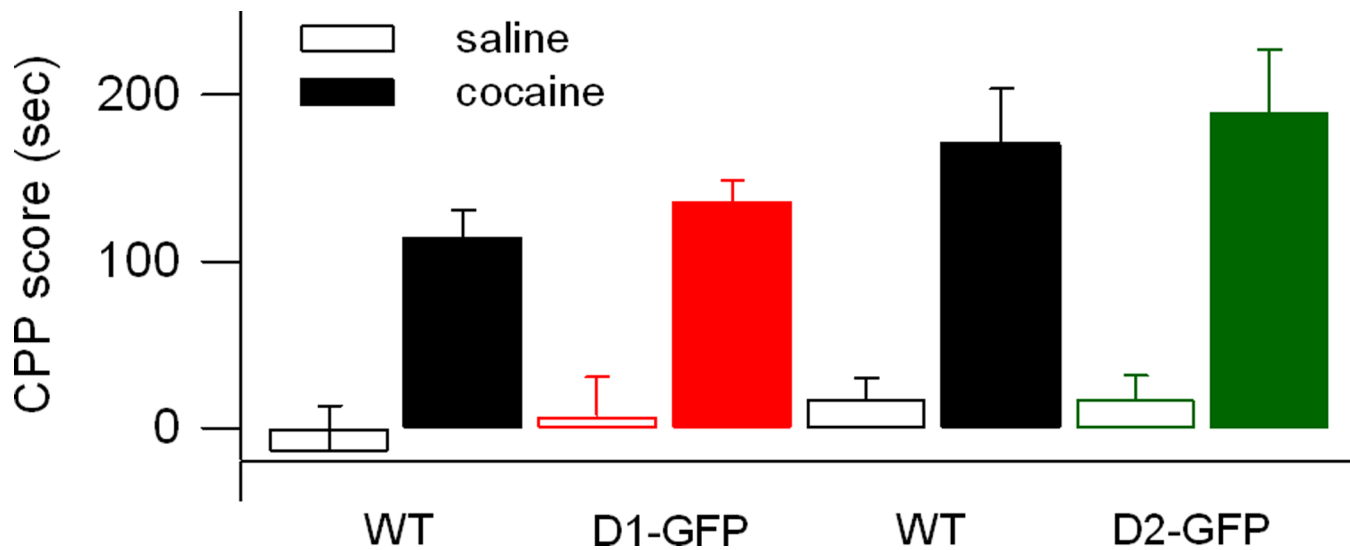


Figure 4. Cocaine conditioned place preference is similar in D1-GFP, and D2-GFP hemizygotes and littermate controls. CPP score in wild-type (D1-GFP $-/-$) littermates (n=4 saline, n=7 cocaine); D1-GFP $+/-$ (n=7 saline, n=10 cocaine), wild-type (D2-GFP $-/-$) littermates (n=4 saline, n=6 cocaine); and D2-GFP $+/-$ (n=5 saline, n=6 cocaine).