

Loss of the serum response factor in the dopamine system leads to hyperactivity

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ABSTRACT The serum response factor (SRF) is a key regulator of neural development and cellular plasticity, which enables it to act as a regulator of long-term adaptations in neurons. Here we performed a comprehensive analysis of SRF function in the murine dopamine system. We found that loss of SRF in dopaminoceptive, but not dopaminergic, neurons is responsible for the development of a hyperactivity syndrome, characterized by reduced body weight into adulthood, enhanced motor activity, and deficits in habituation processes. Most important, the hyperactivity also develops when the ablation of SRF is induced in adult animals. On the molecular level, the loss of SRF in dopaminoceptive cells is associated with altered expression of neuronal plasticity-related genes, in particular transcripts involved in calcium ion binding, formation of the cytoskeleton, and transcripts encoding neuropeptide precursors. Furthermore, abrogation of SRF causes specific deficits in activity-dependent transcription, especially a complete lack of psychostimulant-induced expression of the *Egr* genes. We inferred that alterations in SRF-dependent gene expression underlie the observed hyperactive behavior. Thus, SRF depletion in dopaminoceptive neurons might trigger molecular mechanisms responsible for development of psychopathological conditions involving hyperactivity.—Parkitna, J. R., Bilbao, A., Rieker, C., Engblom, D., Piechota, M., Nordheim, A., Spanagel, R., Schütz, G. Loss of the serum response factor in the dopamine system leads to hyperactivity. *FASEB J.* 24, 2427–2435 (2010). www.fasebj.org

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NEURONAL PLASTICITY CONFERS to organisms the ability to learn and adapt. Incoming environmental stimuli activate neuronal responses by initiating a chain of events that eventually leads to adaptations in synaptic connections and changes in the pattern of behavior. In this report, we analyze the role of serum response factor (SRF), a key transcription factor for activity-dependent transcription and expression of genes involved in neuronal plasticity

(1). SRF belongs to the group of MADS box transcription factors, which bind to gene promoters containing CA₂G elements. Extracellular stimuli activate SRF through the Ras signaling cascade, which then relays the signal by mitogen-activated kinases to ternary complex factors (*i.e.*, Elk-1, Sap-1, and Net1) and/or SRF itself (2, 3). SRF interacts with activated ternary complex factors on gene promoters of immediate-early genes containing adjacent CA₂G and Ets binding site (EBS) motifs and activates their transcription, as observed in the case of *Fos* (4–6). An alternate mechanism of SRF activation involves the Rho/Rac-induced rearrangements in the cytoskeleton, which enable the interaction of SRF with myocardin-related factors (*i.e.*, MRTF-A/MAL and MRTF-B) (7–10).

The role of SRF in regulating gene expression in response to external signals, coupled with its high expression in neurons, makes it a prime candidate for mediating long-term adaptations in neurons and thus behavioral plasticity (11). This notion is supported by studies on genetic models. Global ablation of SRF in mice leads to impaired mesodermal differentiation and early embryonic lethality (12). Conditional deletion using the Cre/loxP system in differentiating primary neurons causes aberrant neuronal migration in the rostral migratory stream and incorrect neuronal circuit assembly in the hippocampus (13, 14), or, when restricted to mature forebrain neurons, a general lack of gene induction after natural stimuli or electroconvulsive shocks (15). Furthermore, SRF loss in the adult forebrain is associated with the inability to habituate to a novel context and impaired formation of the long-term depression in hippocampal neurons (16).

A particularly high abundance of SRF in the brain is observed in the striatum and nucleus accumbens (11, 17), suggesting that by regulating transcription in the dopamine system, SRF influences the development of striatum-

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dependent dopamine-driven behaviors. Therefore, we studied the role of SRF in the murine dopamine system, its influence on transcription, and the subsequent behavioral consequences by using highly specific and inducible-knockout models.

MATERIALS AND METHODS

Animals

Mice carrying a conditional *Srf* deletion in neurons were generated by crossing the strain carrying the loxP flanked *Srf* (*Srf^{flx1}*) (18) with mice expressing the Cre recombinase in dopaminergic neurons (D1Cre and D1CreERT2 strains) (19) or dopaminergic neurons (DATCre strain) (20). In D1Cre mice trace amounts of Cre are first detected at embryonic day (E) 16 in the striosomal neurons of the striatum, and the recombinase is expressed in the matrix neurons around postnatal day 7 (19). In the D1CreERT2 strain, the recombinase remains inactive until its translocation to the nucleus is activated by tamoxifen. Tamoxifen (1 mg) was dissolved in 100 μ l of sunflower oil with 10% v/v ethanol and injected i.p. 2 \times /d for 5 d. Animals were allowed to recuperate for \geq 2 wk before testing. There was no recombination observed before tamoxifen injection as assayed by crossing with R26lacZ reporter mice (21). The DATCre line used harbors two copies of the Cre transgene and recombinase activity is first observed around E17; efficient deletion of the target is detected perinatally (20).

Transgenic animals from all parental strains had been backcrossed into the C57BL6/N background for \geq 5 generations, and the majority of the experimental mice had \geq 7 generations of backcross. Behavioral experiments were performed on 10- to 15-wk-old transgenic animals using Cre-negative, *Srf^{loxP/loxP}* or *Srf^{loxP/wt}* littermates as controls ($n=7-13$ /genotype/experiment). Mice were housed individually and were provided with water and food *ad libitum*. Light was provided from 6 AM to 6 PM, with constant room temperature and humidity. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe) and performed in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Immunoblotting, immunohistochemistry, and *in situ* hybridization

For immunohistochemistry and *in situ* hybridization, dissected brains were fixed for 48 h in 4% paraformaldehyde and then cut with a Vibratome (Leica, Wetzlar, Germany) at 50 μ m. Free-floating sections were processed for *in situ* hybridization as described previously (20, 22). The following antibodies were used: tyrosine hydroxylase (TH) (1:2000; Upstate Biotechnology, Lake Placid, NY, USA), dopamine receptor D1 (D1) (1:3000; Sigma-Aldrich Corp., St. Louis, MO, USA), NeuN (1:3000; Millipore Corporation, Billerica, MA, USA), cleaved caspase-3 (1:1000; Cell Signaling Technology Inc., Danvers, MA, USA), phospho-anti-cAMP response element-binding protein (CREB) (23) (1:5000), Cre-recombinase (20) (1:2000), and SRF (1:2000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). β -Galactosidase whole-mount stainings were prepared as described previously (20). The following cDNA clones have been used for the generation of riboprobes: *Egr2*, RZPDp981B04110D, RZPD; *Fos*, IMAGp966N2451Q, RZPD; and *Tac1*, RZPDp981E11155D, RZPD.

For immunoblotting, striatum was dissected and immedi-

ately homogenized in 1% (w/v) SDS containing 1 mM NaF and 1 mM Na₃VO₄. Proteins (10–20 μ g) were separated by SDS-PAGE and transferred onto a nitrocellulose membrane (S&S BA85, Whatman Schleicher and Schuell, Keene, NH, USA). The following antibodies were used: CREB (1:1000; Cell Signaling Technology Inc.) anti-phospho Ser-133 CREB (1:1000; Cell Signaling Technology Inc.), and anti-GAPDH-MAB374 (1:10000; Millipore Corporation).

Behavioral experiments

Food intake and body weight

Daily measurements of the amount of food intake and body weight were determined during 5 consecutive days in mice at the ages of 6, 10, and 15 wk.

Home cage activity

Locomotor activity in the home cage was monitored by connecting an infrared sensor (Mouse-E-Motion; Infra-E-Motion GmbH, Henstedt-Ulzburg, Germany). A Mouse-E-Motion device was placed above each cage (30 cm from the bottom), so that the mouse could be detected at any position inside the cage. The device was sampling every 4 s whether the mouse moved or not. The sensor could detect body movements of the mouse of \geq 1.5 cm from one sample point to the next. Monitoring of locomotor activity started before the beginning of the experiments and lasted for 4–5 d, and data were collected every 12 h to measure the diurnal pattern of motor activation.

Activity chamber

Animals were placed in activity chambers in which locomotor activity was measured every 10 min for a period of 30 min under novelty or familiarity (24 h later) conditions. Clear Plexiglas boxes of 40 cm in diameter and 40 cm in height were used, and the locomotor activity was measured with a TruScan activity monitoring system (Coulbourn Instruments, Allentown, PA, USA).

Elevated plus maze

The plus maze consisted of 2 open arms and 2 enclosed arms extending from a central platform. The maze was elevated 50 cm above and illuminated from the top at 45 lux. Each mouse was placed at the intersection of the 4 arms of the maze and allowed to explore all 4 arms freely for 5 min.

Cocaine treatment

For the behavioral experiments, cocaine hydrochloride (Sigma-Aldrich Corp.) was dissolved in 0.9% (w/v) sterile saline and administered to the mice i.p. at a dose of 10 mg/kg. For gene expression profiling, mice were injected i.p. with 25 mg/kg cocaine.

Conditioned place preference (CPP)

CPP was induced by 8 alternating injections of cocaine (10 mg/kg i.p.) or saline into the corresponding compartment of the apparatus (spatial place preference boxes; Panlab, Barcelona, Spain). The CPP score represents the difference between the time spent (seconds) in the cocaine or saline-paired compartment during the test day (test duration: 900 s).

Statistics for behavioral experiments

Unless indicated otherwise, statistical analyses were performed with Statistica 6.0 (StatSoft, Tulsa, OK, USA). For comparison of multiple groups statistical significance was determined using ANOVA followed by a Newman-Keuls post-test. For pairwise comparisons, Student's *t* test was used.

Expression profiling

Profiling of gene expression was performed on *Srf*^{D1Cre} animals and controls that were habituated to handling and then injected once with 25 mg/kg cocaine or saline. After 1 h, animals were killed by cervical dislocation; the brains were dissected and fixed overnight in RNAlater solution (Sigma-Aldrich Corp.), and then striatum, including the nucleus accumbens, was microdissected. Total RNA was prepared with the RNeasy Mini Kit (Qiagen, Hilden, Germany), and its quality was assessed on RNA LabChips (Agilent Technologies, Santa Clara, CA, USA). Microarray experiments were performed using GeneChip Mouse Genome 430A 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Labeling of the target RNAs (1–2 µg total RNA), hybridization, and scanning of the microarrays were performed according to the manufacturer's instruction. A total of 12 GeneChips, each representing a single animal, were used for profiling of gene expression after a single cocaine injection, 3 for each treatment/genotype combination.

Analysis of array data was performed as described before (24). In brief, data were normalized, and expression values

were computed using R/Bioconductor (25) and the GCRMA method. Statistical analysis was performed using the LIMMA library; a fold change of measured transcript abundance of ≥ 1.5 and $P < 0.001$ (*t* test) were considered significant. Ontology enrichment analyses were performed using DAVID (26). Visualization of putative transcription factor binding sites in the mouse and human ortholog genes were aligned using LAGAN (27), and bl2seq (28) was performed using the TFBS BioPerl module (29) and matrices from JASPAR (30). Criteria for selecting putative sites were noncoding region with $\geq 80\%$ of sequence conservation between mouse and human located within 2 kb of the transcription start and a matrix score of ≥ 0.9 . All array data were deposited at the Gene Expression Omnibus (GSE10870). Validation of gene expression data was performed with quantitative PCR (qPCR) using fluorescent TaqMan probes (Applied Biosystems, Foster City, CA, USA) according to the accompanying manuals. Abundance of the following transcripts was measured: *Fos*, *Tiparp*, *Nr4a1*, *Gadd45b*, *Egr2*, *Pdyn*, *Siah2*, and *Rgs4*.

RESULTS

Targeted ablation of *Srf* in either dopaminoceptive or dopaminergic neurons

To characterize the role of SRF in the murine dopamine system, we used the Cre/loxP system for selective

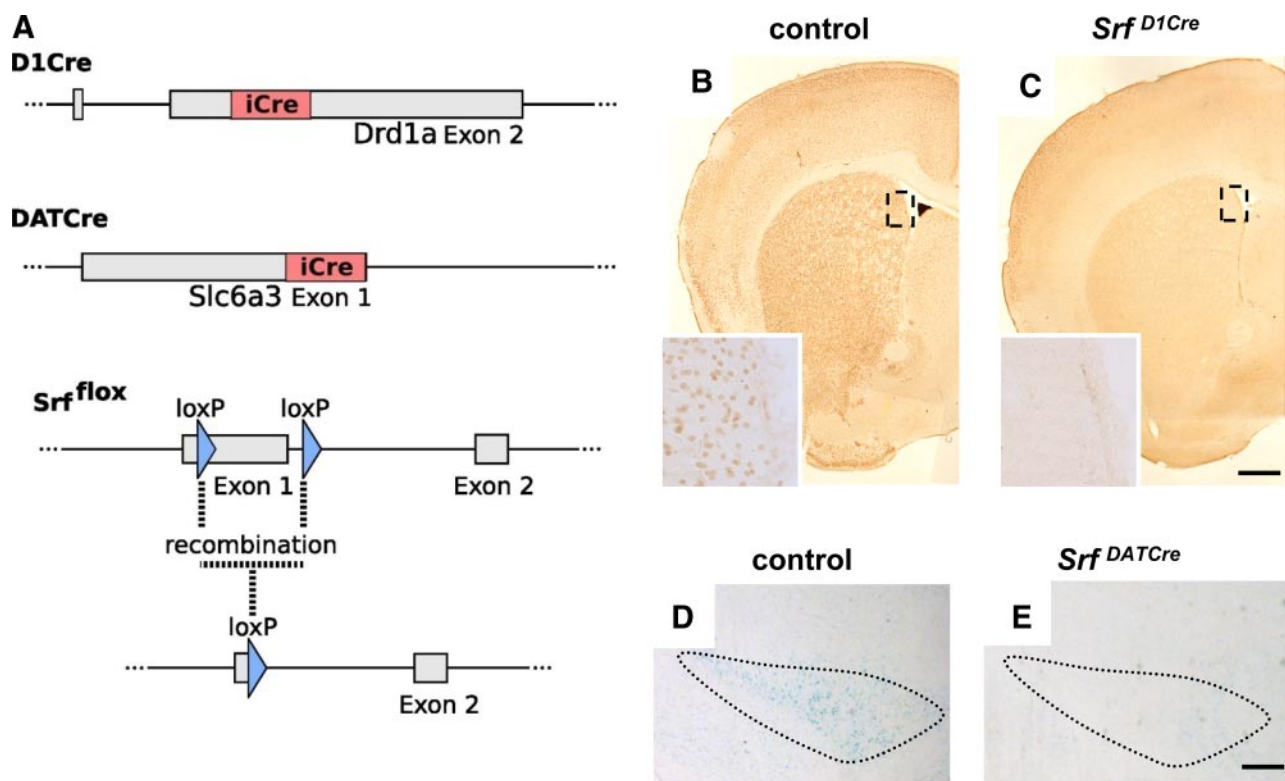


Figure 1. Cre/loxP-mediated deletion of *Srf*. **A**) Schematic representation of Cre/loxP-mediated deletion of the first exon in the floxed *Srf* gene (*Srf*^{loxP}) in dopaminoceptive (D1Cre) and dopaminergic (DATCre) neurons. **B**, **C**) Immunostaining confirms ablation of SRF in *Srf*^{D1Cre} mice. Coronal brain sections were incubated with anti-SRF antibodies and a peroxidase-conjugated secondary antibody and stained with 3,3'-diaminobenzidine (brown). Representative sections containing the striatum from control (**B**) and *Srf*^{D1Cre} animals (**C**). Insets: higher-magnification images of the mediodorsal striatum, adjacent to the lateral ventricle (dashed squares). **D**, **E**) Ablation of SRF in dopaminergic neurons in the ventral midbrain of *Srf*^{DATCre} mice. Sections from control (**D**) and *Srf*^{DATCre} mice (**E**) were prepared as described above and then stained with the HistoGreen substrate (blue-green). Ventral tegmental area/substantia nigra area is encircled by a dotted line. Scale bars = 200 µm (**B**, **C**); 100 µm (**D**, **E**).

neuronal-type specific gene deletion. Transgenic animals harboring a variant of the *Srf* gene (*Srf^{flex1}*), in which part of the first exon is flanked by loxP sites, were crossed with mice expressing the Cre-recombinase under the transcriptional control elements of either the dopamine receptor D1 (*D1*, *Drd1a*) or the dopamine transporter (DAT, *Slc6a3*) (18–20) (Fig. 1A). In *Srf^{D1Cre}* mice, loss of SRF was observed in the striatum, nucleus accumbens, olfactory tubercle, CA1 and CA2 regions of the hippocampus, and, in addition, the lower layers of the cortex (Fig. 1B, C and Supplemental Fig. S1). The deletion in *Srf^{DATCre}* mice was restricted to dopaminergic neurons, in particular in the ventral midbrain (Fig. 1D, E). The general brain anatomy of both *Srf^{D1Cre}* and *Srf^{DATCre}* mice appeared unaltered, as assessed by Nissl, TH, and D1 receptor staining (Supplemental Fig. S1). We did not observe signs of neuroinflammation or cell loss. *Srf^{D1Cre}* and *Srf^{DATCre}* mice were born at the expected Mendelian ratios, but for *Srf^{D1Cre}* animals increased mortality during early life was observed, with ~25% of mutant mice dying before weaning. No alter-

ations in phenotype were observed in heterozygous mice.

Constitutive *Srf* ablation in dopaminoceptive but not dopaminergic neurons induces hyperactivity

We observed that *Srf^{D1Cre}* animals were smaller than their control littermates and weighed on average 25% less at the age of 8 wk. However, they continuously gained weight, and at 15 wk of age no differences were observed in body weight of *Srf^{D1Cre}* mice compared with controls (Supplemental Fig. S2A; 2-way ANOVA for repeated measures: genotype $F_{1,15}=7.8$, $P<0.01$; time \times genotype $F_{2,30}=6.9$, $P<0.005$), which was associated with significantly increased food intake in mutant mice (Supplemental Fig. S2B; $t(14)=-4.9$, $P<0.01$).

When tested in an unfamiliar activity chamber, *Srf^{D1Cre}* mice exhibited continuously increased horizontal locomotor activity over time, compared with controls, indicating an impairment in habituation to novelty (Fig. 2A;

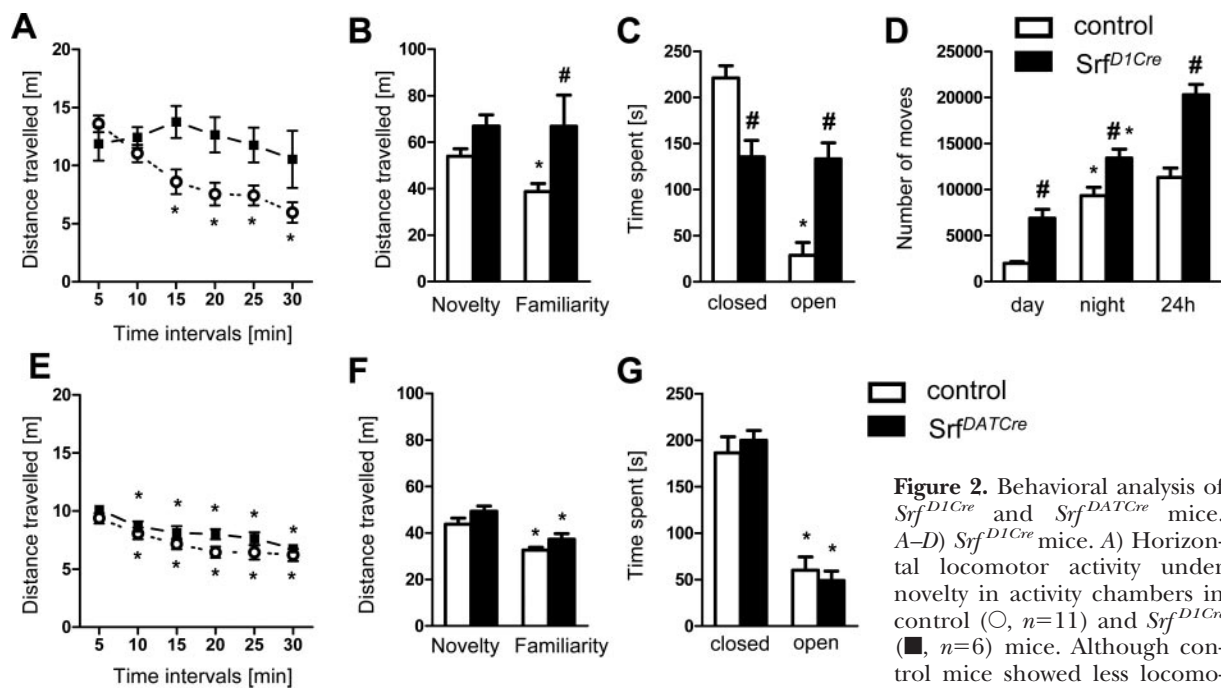


Figure 2. Behavioral analysis of *Srf^{D1Cre}* and *Srf^{DATCre}* mice. A–D) *Srf^{D1Cre}* mice. A) Horizontal locomotor activity under novelty in activity chambers in control (○, $n=11$) and *Srf^{D1Cre}* (■, $n=6$) mice. Although control mice showed less locomotor activity over time (ANOVA

for repeated measures: time effect $F_{5,75}=5.1$, $P<0.001$), *Srf^{D1Cre}* mice showed no decrease in locomotor activity over time, indicating an impairment in habituation. $*P<0.01$ vs. first time interval. B) When tested again in the activity chambers 24 h later, control mice displayed a significant reduction in locomotor activity compared with the first exposure. In contrast, *Srf^{D1Cre}* mice showed the same degree of activity during the second exposure, with a significant genotype effect. $*P<0.01$ vs. novelty; $\#P<0.01$ vs. control. C) *Srf^{D1Cre}* mice (■) spent equal times in open and closed arms of the elevated plus maze, whereas control mice (□) spent significantly more time in the closed arm (2-way ANOVA indicated a genotype effect $F_{1,15}=15.3$, $P=0.001$, and an arm effect $F_{1,15}=21.1$, $P<0.001$, as well as an arm \times genotype effect $F_{1,15}=20$, $P<0.001$). $*P<0.001$ vs. closed; $\#P<0.001$ vs. control. D) Day and night locomotor activity in home cage in control and *Srf^{D1Cre}* mice. Both genotypes displayed higher activity during the active phase of the cycle (2-way ANOVA indicated a phase effect $F_{1,15}=27.8$, $P<0.001$). However, *Srf^{D1Cre}* mice, in comparison with control animals, showed highly increased locomotor activity during both phases (2-way ANOVA revealed a genotype effect $F_{1,15}=12.7$, $P<0.01$). $*P<0.001$ vs. day; $\#P<0.01$ vs. control. E–G) *Srf^{DATCre}* mice. E) Horizontal locomotor activity under novelty in activity chambers in control (○, $n=13$) and *Srf^{DATCre}* (■, $n=13$) mice. Habituation to novelty is normal in *Srf^{DATCre}* mice, as indicated by a similar decrease in horizontal locomotor activity over time in control and mutant mice. $*P<0.01$ vs. first time interval. F) *Srf^{DATCre}* mice also displayed a reduction in locomotor activity after a second exposure 24 h later. $*P<0.001$ vs. novelty. G) There were no differences in the elevated plus-maze test between *Srf^{DATCre}* and control mice (genotype effect $F_{1,24}=0.02$, $P=0.9$), and both genotypes spent significantly more time in the closed arm than in the open arm (arm effect $F_{1,24}=62.6$, $P<0.001$). $*P<0.001$ vs. closed.

2-way ANOVA for repeated measures: time \times genotype $F_{5,75}=3.8$, $P<0.01$). In fact, this effect was still present when the mice were retested after 24 h under the same conditions (Fig. 2B; habituation \times genotype $F_{1,15}=2.1$, $P<0.05$). In addition, the response to a novel anxiogenic environment was altered in these mice. In the elevated plus maze, Srf^{D1Cre} mice spent equal times in both the closed-protected and open-unprotected arms (Fig. 2C) with an increased percentage of visits into the open arms compared with control littermates (Supplemental Fig. S3A; $t(15)=-5.7$, $P<0.001$).

In contrast, parallel analysis of Srf^{DATCre} mutants did not reveal alterations in any of the behaviors tested; *i.e.*, motor activity and anxiety-like behavior did not differ between mutant and control mice when placed in the activity chamber (Fig. 2E, F) and the elevated plus maze, respectively (Fig. 2G and Supplemental Fig. S3B). Therefore, alterations in motor activity and emotionality appeared to be associated with loss of SRF in dopaminergic neurons. To study further the observed phenotype in Srf^{D1Cre} mutants, we measured home cage activity by means of the E-Motion system. Control and Srf^{D1Cre} mice displayed a typical diurnal pattern of activity, with increased activity during the dark phase (Fig. 2D; 2-way ANOVA indicated a phase effect $F_{1,15}=27.8$, $P<0.001$). However, diurnal activity analyzed in 12-h intervals was altered in Srf^{D1Cre} animals because they displayed a significant increase in motor

activity during both the active and resting phases, compared with controls, respectively (genotype effect $F_{1,15}=12.7$, $P<0.01$). Consequently, the overall activity of Srf^{D1Cre} mice during a complete 24-h day was also increased (Fig. 2D; $t(15)=-3.6$, $P<0.01$).

Behavioral responses to psychostimulants in Srf^{D1Cre} or Srf^{DATCre} mice were not altered. They displayed normal locomotor activation after i.p. injection of 10 mg/kg cocaine and developed similar conditioned place preference as controls (Supplemental Fig. S4). Therefore, we conclude that the mutations do not impair psychostimulant-induced dopamine-dependent behaviors.

Inducible loss of SRF in D1 neurons in adult mice also leads to hyperactivity

The Cre/loxP system restricts the mutation spatially to specific groups of neurons. However, it does not provide temporal control to the onset of the deletion; hence, the observed phenotype may result, at least in part, from a variety of compensatory effects, *e.g.*, abnormal development of the mesocorticolimbic system. To circumvent those compensatory mechanisms, we have generated the D1CreERT2 transgenic mouse, in which activation of the Cre recombinase and subsequent induction of mutagenesis require the presence of the synthetic steroid tamoxifen (Fig. 3A–D) (22, 31), and

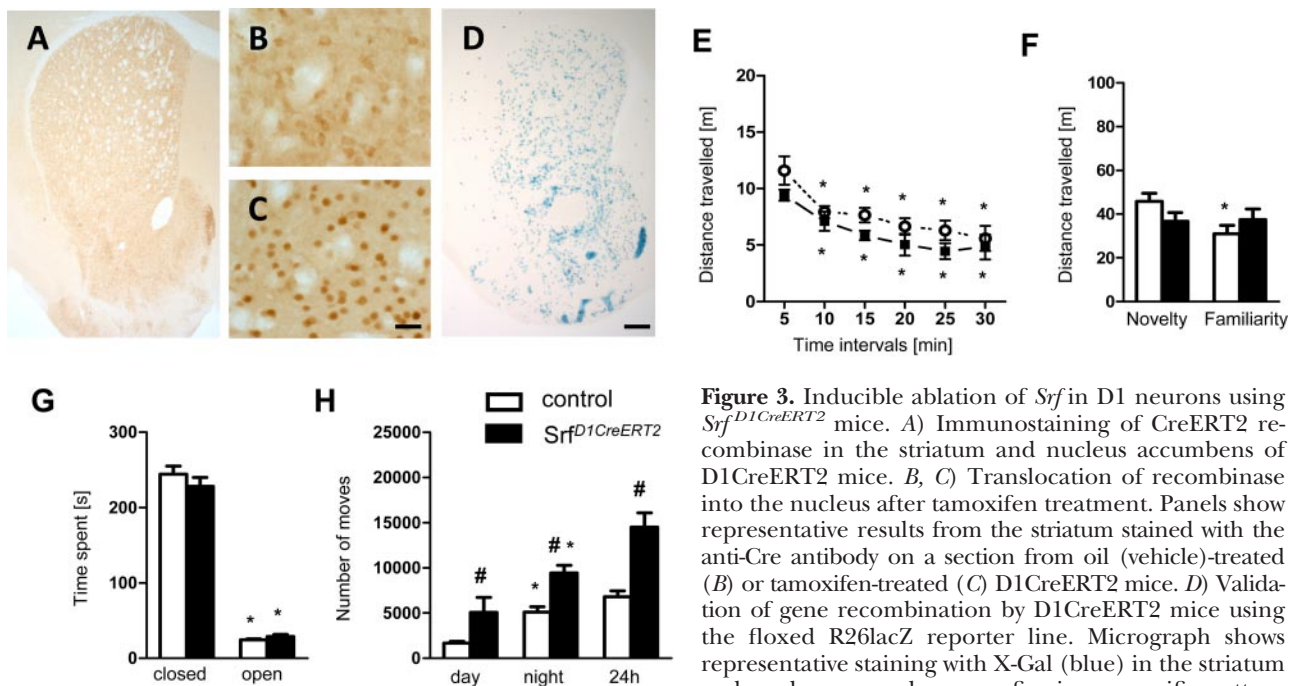


Figure 3. Inducible ablation of *Srf* in D1 neurons using $Srf^{D1CreERT2}$ mice. A) Immunostaining of CreERT2 recombinase in the striatum and nucleus accumbens of D1CreERT2 mice. B, C) Translocation of recombinase into the nucleus after tamoxifen treatment. Panels show representative results from the striatum stained with the anti-Cre antibody on a section from oil (vehicle)-treated (B) or tamoxifen-treated (C) D1CreERT2 mice. D) Validation of gene recombination by D1CreERT2 mice using the floxed R26lacZ reporter line. Micrograph shows representative staining with X-Gal (blue) in the striatum and nucleus accumbens, confirming a specific pattern

of recombination. E) Habituation to novelty in activity chambers was not impaired in $Srf^{D1CreERT2}$ mice, because their horizontal locomotor exploratory activity decreased over time to an extent comparable to that seen with control mice $*P<0.05$ vs. first time interval. F) When tested 24 h later, $Srf^{D1CreERT2}$ mice showed no habituation to their locomotor response (ANOVA for repeated measures: habituation \times genotype $F_{1,12}=9.9$, $P<0.01$), whereas control mice displayed habituation. $*P<0.01$ vs. novelty. G) In the elevated plus maze test, no differences were found between control and inducible-knockout genotypes (genotype effect $F_{1,12}=0.9$, $P=0.3$), as all mice spent significantly more time in the closed arm than in the open arm (arm effect $F_{1,12}=585.8$, $P<0.001$). $*P<0.001$ vs. closed. H) Day/night and 24-h measurements of locomotor activity in home cage in control (\square , $n=7$) and $Srf^{D1CreERT2}$ animals (\blacksquare , $n=7$). Both genotypes displayed higher activity during active night phase of cycle (phase effect $F_{1,12}=15.1$, $P<0.01$). Similar to Srf^{D1Cre} mice, inducible $Srf^{D1CreERT2}$ mice showed highly increased locomotor activity in both phases (genotype effect $F_{1,12}=23$, $P<0.001$). $*P<0.01$ vs. day; $\#P<0.001$ vs. control.

crossed these mice with the floxed *Srf* mice. The resulting *Srf*^{D1CreERT2} animals were injected with tamoxifen at an age of 8 wk. Loss of SRF was subsequently observed in ~56% of neurons of the nucleus accumbens and a smaller proportion of dorsal striatum cells, as well as scattered neurons in lower layers of the cortex and other areas known to express D1 dopamine receptors (Supplemental Fig. S5).

Similar to *Srf*^{D1Cre} animals, *Srf*^{D1CreERT2} mice showed a lean phenotype and increased food intake compared with control tamoxifen-injected animals (Supplemental Fig. S6; body weight $t(12)=2.2$, $P<0.05$, and food intake $t(12)=-4.8$, $P<0.001$). Although the first reaction to a novel environment (activity chamber) is not impaired in *Srf*^{D1CreERT2} mice, *i.e.*, they show a similar decrease of locomotor activity compared with control animals over a 30-min test interval (Fig. 3E; 2-way ANOVA, time effect $F_{5,60}=25.3$, $P<0.001$ but no genotype effect), they show no reduction in locomotor activity when tested again 24 h later (Fig. 3F), indicating an impairment to habituate to a familiar environment (habituation \times genotype $F_{1,12}=9.9$, $P<0.01$). Unlike in *Srf*^{D1Cre} animals, reactivity to the exposure of an elevated plus maze is normal in *Srf*^{D1CreERT2} mice (Fig. 3G; genotype effect $F_{1,12}=0.9$, $P=0.3$). A pronounced phenotype, however, is seen again in the home cage. As in *Srf*^{D1Cre} animals, *Srf*^{D1CreERT2} mice show a strong hyperactivity in the home cage during day and night phases (Fig. 3H; 2-way ANOVA revealed a genotype effect $F_{1,12}=23$, $P<0.001$), which is also significant when analyzed for the complete 24-h period [$t(12)=-4.8$, $P<0.001$].

Loss of SRF alters basal expression of plasticity-related genes and causes specific deficits in activity-dependent transcription

To identify possible molecular mechanisms underlying the observed behavioral alterations, we analyzed gene expression profiles under basal conditions in *Srf*^{D1Cre} and control mice. A total of 125 gene transcripts displayed significantly different abundance in *Srf*^{D1Cre} mice compared with controls (Fig. 4 and Supplemental Tables S1 and S2). The three most common Gene Ontology (GO) terms associated with 71 transcripts with decreased abundance in transgenic animals were calcium ion binding (*i.e.*, *Prkcc*, *Prkcm*, and *Cdh9*), organ development (*i.e.*, *Srf*), and cytoskeleton activity, particularly actin microfilament structure (*i.e.*, *Actb*, *Actg1*, *Tpm1*, and *Flna*) (Fig. 4A). This observation is in good agreement with the reported roles of SRF in transcription of several key genes involved in neuronal development and plasticity (13, 14, 32). Furthermore, loss of SRF led to increased abundance of 54 transcripts in the striatum of *Srf*^{D1Cre} animals. Among the most prominent changes was a strong, >4-fold, increase in the abundance of the *Igh-6* and *Pdyn* (prodynorphin) transcripts (Fig. 4A). In addition, *Tac1* (tachykinin-1, substance P) transcript abundance was also up-regulated, as indicated by *in situ* hybridization, showing an increase in the striosomes of the dorsal striatum (Supplemental Fig. S7). Thus, markers of the striatonigral

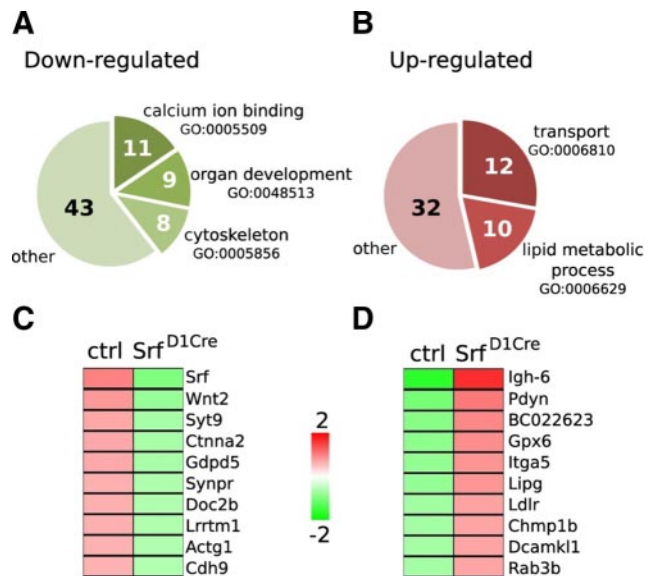


Figure 4. *Srf*^{D1Cre} mice display altered basal gene expression levels in the striatum and nucleus accumbens. *A, B*) Numbers of significantly (fold change >1.5, t test $P<0.001$) down-regulated (*A*) or up-regulated (*B*) transcripts, with most commonly occurring level 3 or 4 ontology terms indicated. If a gene has been assigned more than one GO term (*e.g.*, *Ldlr*), it is counted under one category only. *C, D*) Heat maps show 10 transcripts with the greatest decrease (*C*) or increase (*D*) in abundance in mutant mice. Each column corresponds to one genotype; rows represent transcripts as indicated at right (full names are listed in Supplemental Table S1). Colors indicate normalized expression values, as shown by scale in the middle.

system (*Pdyn* and *Tac1*) were up-regulated, but no changes were observed in transcripts associated with striatopallidal neurons (*Penk*, *A2a*, and *Drd2*). Finally, we found that several gene transcripts involved in sterol transport and metabolism, including *Lipg*, *Ldlr*, *Sc4mol*, *Cyp51*, and *Dhcr7*, were up-regulated in *Srf*^{D1Cre} mice (Fig. 4A and Supplemental Table S2).

Next we analyzed the consequences of SRF loss on the regulation of activity-dependent transcription in the striatum of *Srf*^{D1Cre} animals after treatment with cocaine, a powerful activator of dopamine signaling (33). Using microarray profiling, we found that loss of SRF caused a specific and complete lack of induction of *Egr1*, *Egr2*, and *Egr4* transcripts (Fig. 5A and Supplemental Table S3). However, there was no general impairment in the induction of activity-regulated gene expression after cocaine stimulation. Several genes displayed enhanced induction, resulting in 100 transcripts that were significantly increased after cocaine treatment. For example, the expression of *Fos*, *Junb*, and *Arc* was induced to similar or greater degrees as in control animals. Validation of the expression profiling by qPCR confirmed array results in all cases tested (Fig. 5B and Supplemental Fig. S8).

To explain the selective impact of SRF loss on the inducibility of the *Egr* family of genes, we performed an *in silico* comparative analysis of mouse and human activity-dependent gene promoters. A strongly evolutionary conserved arrangement of CARG elements with Elk-1/Sap-1 binding sequences within ~20 bp in the 3'

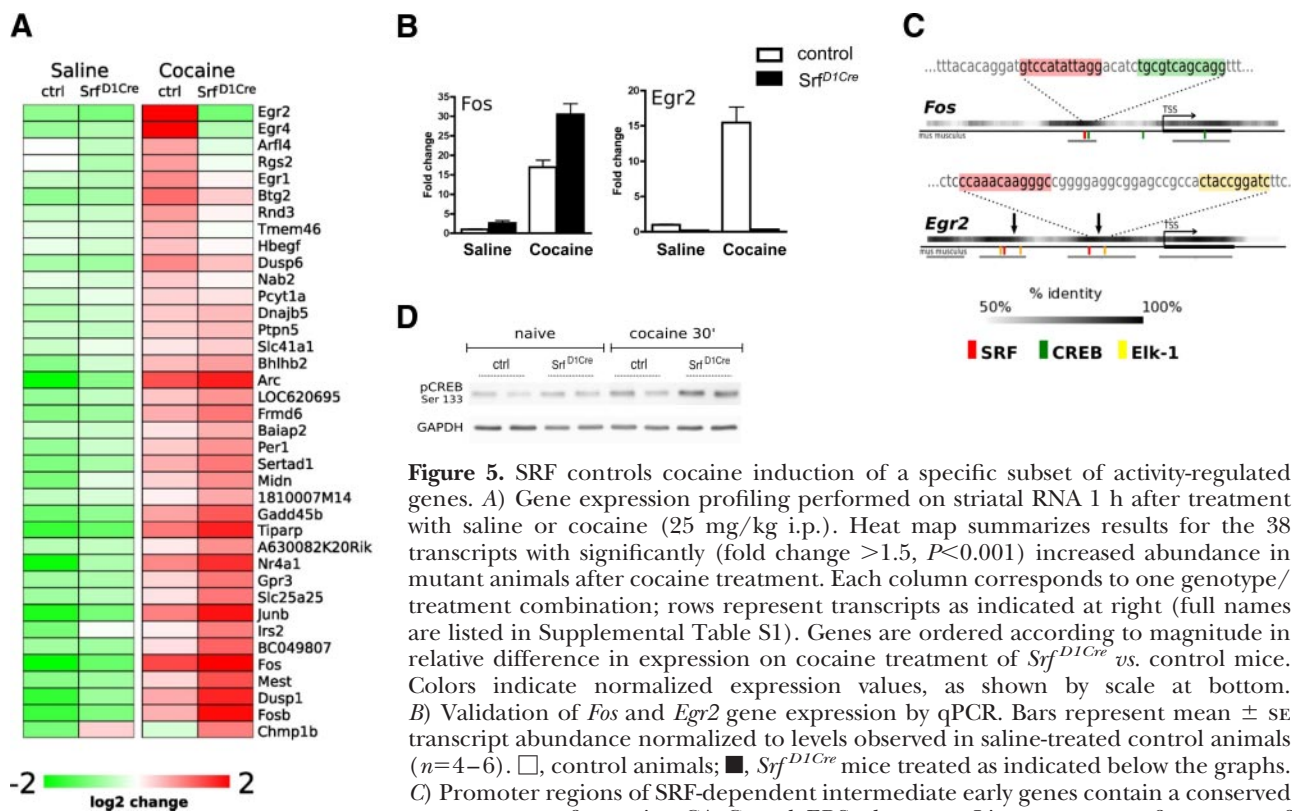


Figure 5. SRF controls cocaine induction of a specific subset of activity-regulated genes. **A)** Gene expression profiling performed on striatal RNA 1 h after treatment with saline or cocaine (25 mg/kg i.p.). Heat map summarizes results for the 38 transcripts with significantly (fold change >1.5, $P < 0.001$) increased abundance in mutant animals after cocaine treatment. Each column corresponds to one genotype/treatment combination; rows represent transcripts as indicated at right (full names are listed in Supplemental Table S1). Genes are ordered according to magnitude in relative difference in expression on cocaine treatment of *Srf^{D1Cre}* vs. control mice. Colors indicate normalized expression values, as shown by scale at bottom. **B)** Validation of *Fos* and *Egr2* gene expression by qPCR. Bars represent mean \pm SE transcript abundance normalized to levels observed in saline-treated control animals ($n = 4-6$). □, control animals; ■, *Srf^{D1Cre}* mice treated as indicated below the graphs. **C)** Promoter regions of SRF-dependent intermediate early genes contain a conserved arrangement of putative CArG and EBS elements. Lines represent fragments of mouse genomic DNA, with identity to human sequence shown as different shades of gray. High-homology fragments are underscored with gray bars. TSS indicates transcription start site; predicted binding sites based on JASPAR matrices are highlighted for SRF (red), CREB (green), and the Ets protein Elk-1 (yellow). Arrangements of CArG and EBS sites within ~20 bp are indicated by arrows. **D)** Western blot analysis shows an increase in phospho-CREB abundance in the striatum of *Srf^{D1Cre}* mice 30 min after cocaine injection (25 mg/kg i.p.).

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direction is detected in the *Egr2*, *Egr1*, and *Egr4* genes (Fig. 5C and Supplemental Fig. S9). The promoter of *Fos* contains CArG sequences in proximity to the transcription start, but the presence of an adjacent putative EBS just 5' to the CArG element is scored only when less stringent criteria are applied, and no conserved CArG and EBS motif arrangement is detected in other activity-dependent gene promoters (e.g., *Fosb*, Supplemental Fig. S9).

Given that we observed increased transcription of CREB-dependent transcripts, we considered whether abrogation of SRF indeed results in increased activity of CREB. Therefore, we analyzed the levels of phospho-CREB (active form of CREB phosphorylated at Ser-133 in the striatum 30 min after cocaine injection (25 mg/kg i.p.) and found that phospho-CREB abundance is increased in *Srf^{D1Cre}* animals compared with controls (Fig. 5D). As independent validation of the observed alterations in CREB activity and selective deficit in activity-dependent transcription we extended our analysis to dopaminergic neurons of *Srf^{DATCre}* animals. The abundance of phospho-CREB was assessed *via* immunohistochemistry (Supplemental Fig. S10A, B). Quantification reveals that loss of SRF leads to a significant increase of phospho-CREB in TH-positive neurons (Supplemental Fig. S10C).

Taken together, the gene expression studies and *in silico* analyses indicate that although SRF is essential for cocaine-induced stimulation of *Egr1*, *Egr2*, and *Egr4*

expression, it might not be a critical factor for transcription of other activity-regulated genes in neurons of the basal ganglia or ventral midbrain.

DISCUSSION

We find that *Srf* ablation restricted to dopaminergic neurons results in increased food intake, motor hyperactivity, and deficits in habituation processes. The behavioral phenotype is accompanied by altered expression of genes related to calcium ion binding, cellular cytoskeleton activity, vesicle trafficking, and neuropeptide signaling. Furthermore, loss of SRF abolishes induction of *Egr* genes after cocaine treatment, whereas expression of most other activity-dependent genes, including *Fos*, is still strongly activated. In summary, our data indicate a specific and restricted role in regulation of transcription by SRF in dopaminergic neurons, and its impairment results in hyperactivity.

The behavioral data obtained in the activity chamber indicate that both constitutive and inducible *Srf* ablation in dopaminergic (but not dopaminergic) neurons results in a deficit in habituation to a novel environment. This phenotype was found to be more pronounced in mice with constitutive (*Srf^{D1Cre}*) SRF ablation compared with inducible (*Srf^{D1CreERT2}*) SRF loss. Reduced or even a lack of habituation could be explained by the deletion of *Srf* in the CA1

region of the hippocampus and thereby resulting inability to form an immediate memory of a novel context (16, 34). In the elevated plus maze, the mutant mice did not show unconditioned responses associated with aversive environments, suggesting an impaired reaction to aversive environments and a deficit in behavioral inhibition as well. Alternatively, these data could indicate an attention deficit derived from the inability to process the emotional significance of the environmental stimuli.

Independently of the ability to process selectively relevant contextual stimuli, SRF seems to be involved in the control of motor activity. Activity measurements in the home cage, a nonstimulating environment, revealed that both *Srf*^{D1Cre} and *Srf*^{D1CreERT2} mice displayed a strong motor hyperactivity. This phenotype bears a resemblance to the human pathological condition, as children suffering from attention deficit hyperactivity disorder (ADHD) show more pronounced hyperactivity in nonstimulating environments than in novel situations (35). Because of higher energy consumption, hyperactivity in general is accompanied by reduced weight gain and increased food intake, phenotypic features that we also observe in *Srf*^{D1Cre} and *Srf*^{D1CreERT2} mice.

In line with these findings, we found at a molecular level changes in gene transcription caused by SRF loss that are parallel to human pathological changes. Genetic association studies in patients with ADHD have identified significant correlations with polymorphisms occurring within genes involved in dopamine signaling (e.g., *DRD3*, *DRD1*, *DAT*, or *DDC*) or synaptic functions (e.g., *ADRA2C*, *CHRNA4*, *SYT1*, *SYP*, or *SNAP25*) (36–39). Orthologs of those genes also have altered expression in *Srf*^{D1Cre} mice (*Syp* and *Adra2c*; in addition, *t* test $P < 0.1$ for *Ddc*, *Snap25*, and *Chrna4*). Therefore, there is significant over-representation of ADHD-associated genes among transcripts with changed abundance after SRF loss [χ^2 $P < 0.01$ for genes reported by Guan *et al.* (36)].

Interestingly, we find that only a selective group of activity-dependent genes is affected by SRF ablation after cocaine induction, most prominently, the *Egr* genes, which is in agreement with a previous report (15). Accordingly, *in silico* promoter analysis revealed a strong evolutionary-conserved arrangement of CArG and EBS elements in *Egr1*, *Egr2*, and *Egr4* genes. Although the *Egr3* probe set on the array seemed to be nonfunctional, our preliminary *in situ* hybridization data (not shown) as well as reports from other model systems confirm that it is also regulated by SRF (40). Conversely, SRF is not essential for cocaine-stimulated transcriptional induction of *Fos* and some other activity-regulated genes in the dopamine system. This finding is particularly surprising considering the role attributed to SRF based on cell-line studies (11) or neuronal knockout (15). However, in the latter report the transcriptional *in vivo* activity of SRF was studied in cortical and hippocampal neurons after electroconvulsive shock or exposure to a novel environment.

From this and other studies (15, 24, 41), it seems likely that CREB and SRF play complementary roles in the activity-dependent gene regulation. Thus, most SRF-dependent genes are generally less dependent on CREB and *vice versa* (compare this study and refs. 15, 32, 41). In addition to its function as a traditional transcription factor, the ability of SRF to act as a functional repressor of

transcription (32) and as a transcriptional activator of specific microRNAs (42) may contribute to the observed behavioral defects in the mutant mice as well as to the increase in activation of several activity-dependent genes (i.e., *Fosb* or *Junb*). In the latter case, the increased levels of CREB phosphorylation in the mutant mice may also be relevant.

Despite hyperactivity being a frequent problem in several human neuronal disorders, notably ADHD, its underlying cause is poorly understood. We provide evidence that SRF is involved in the etiology of motor hyperactivity, by triggering alterations in the regulation of basal ganglia motor circuits. We clearly demonstrate that specific ablation of SRF in dopaminergic neurons causes molecular alterations, which result in a behavioral hyperactivity phenotype. Thus, *Srf*^{D1Cre} and *Srf*^{D1CreERT2} mice represent useful models to study interactions between genetic and environmental factors underlying hyperactivity. **[F]**

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