SUPPLEMENT

Adaptations in Nucleus Accumbens Neuron Subtypes Mediate Negative Affective Behaviors in Fentanyl Abstinence

Supplemental Materials and Methods

Experimental Subject Strain Details

Male and female hemizygous D1-Cre (Line FK150) were used for morphology, electrophysiology, RNAscope, and E2f1 experiments. Male and female hemizygous A2A-Cre (Line KG139) were used for morphology and RNAscope experiments. Homozygous $Rpl22^{HA}$ ("RiboTag") (1) mice expressing Cre-inducible $Rpl22^{HA}$ were crossed to D1-Cre or A2A-Cre mouse lines to generate male and female D1-Cre X $Rpl22^{HA}$ or A2A-Cre X $Rpl22^{HA}$ mice and used for subtype-specific RNAseq and Nanostring. All transgenic mice were bred on C57Bl/6 background.

Fentanyl abstinence

Mice were pair housed across a perforated divider with a sex-matched mouse for the entire experiment (2). Each mouse was provided a single 50 mL conical tube with a rubber stopper and ball-point sipper tube (Ancare) containing 10 µg/mL fentanyl citrate (Cayman #22659) in tap water as their sole liquid source for 5 days. Water control mice received plain tap water in identical tubes. Liquid consumption was determined by weighing the tubes daily, then normalized to individual mouse bodyweight. Following 5 days of fentanyl or water, tubes were replaced with plain tap water for 10 days. On abstinence Day 10, mice were either euthanized for morphology, electrophysiology, or molecular experiments, or transferred to individual housing for behavioral testing unless otherwise noted.

Behavioral Testing

All video-tracking was conducted with TopScan Lite software (Cleversys, Reston, VA, USA). For 3-Chamber social interaction testing, mice were placed in an arena divided into 3 chambers by clear acrylic dividers. The two outer chambers contain wire mesh cups, while the central chamber is empty. The experimental mouse is placed in the central chamber of the arena with two empty cups and allowed to explore for 5 min. Then the experimental mouse is allowed to explore the arena for an additional 5 min with unfamiliar sex-matched adult conspecific in one of the wire mesh cups. The amount of time spent in the chamber containing the cups (empty or novel mouse) is measured with video tracking (2). For elevated plus maze, mice were placed in the center of the maze and their activity in open and closed arms was recorded over 5 min with video tracking (3). For naloxone precipitated withdrawal signs, mice that still had access to fentanyl were injected with 1mg/kg naloxone (4), then placed into an empty glass cylinder. A blinded experimenter scored withdrawal signs for 10 min and determined bodyweight loss after 1hr. Points were assigned as previously (5). (Graded signs: wet dog shakes: 1-2, 2 points; 2+, 4 points; Escape jumps: 2-4, 1 point, 5-9, 2 points, 10+, 3 points; paw tremors: 1-2, 2 points, 3+, 4 points; bodyweight loss in 60 min, 1 point per each 1%; Checked Signs: diarrhea, swallowing movements, teeth chattering, all 2 points each; genital grooming, abnormal posture, 3 points each). All behavioral experiments were conducted during the light cycle. For social stress (3,6) male mice were placed in hamster cages with perforated plexiglass dividers containing a novel, aggressive male CD1-resident. We used only male mice in the social stress paradigm since unmanipulated CD-1s will attempt to mate with female mice. Mice were defeated for 3 min by 3 different residents on a single day, each session separated by 15 min of sensory interaction. 24 hr later, social avoidance was assessed with video-tracking. Experimental mice were placed in an open field containing a perforated box. Time spent around the box (“interaction zone”) was compared between two trials (2.5 min each) during which the box was empty or contained a novel CD-1.

Stereotaxic surgery

At 5-6 weeks of age, D1-Cre mice were anesthetized with isoflurane (4% induction, 1.5% maintenance) and affixed in a stereotaxic frame (Kopf Instruments). An incision was made in the scalp, and holes were drilled to target the NAc core (AP+1.6mm, ML: ± 1.5 mm, DV: -4.4 mm,10º)

(3). 300-400 nL of Cre-inducible, double inverted open (DIO) reading frame adeno-associated viruses (AAVs) were infused bilaterally with Neuro Syringes (Hamilton). The scalp was closed with Vet Bond (3M), and mice were group housed for 3 weeks to allow for recovery and expression.

Immunostatining

Mice were transcardially perfused with 0.1M PBS and 4% paraformaldehyde. Brains were removed and post-fixed for 24 hr, then sectioned to a thickness of 100 µm with a vibratome (Leica, Germany). Slices were washed 3 x 5 min with PBS and blocked for 30 min in in PBS with 3% normal donkey serum (NDS) and 0.3% Triton X-100. Slices were incubated at 4ºC overnight in chicken anti-GFP (1:500; Aves Lab, Tigard, OR, USA; #GFP-1020). Slices were washed 3 x 5 min, then 7 x 60 min, then incubated at 4ºC overnight in Anti-Chicken Alexa 488 (1:500; Jackson Immuno, West Grove, PA, USA; #111-545-144). Slices were washed with PBS as above, mounted with Vectashield, and imaged on a laser-scanning confocal microscope (Leica SP8).

MSN reconstruction and dendrite analysis

D1-Cre or A2A- Cre mice were injected with AAV5-Ef1a-DIO-eYFP (UNC Vector Core, Chapel Hill, NC, USA, diluted to 1.5 $x10^{11}$ VP/mL). Sections containing NAc were sampled from bregma AP:1.5-1.0 mm and Z-stack images were acquired at 0.6 µm increments using a 40x objective. MSNs were 3D reconstructed using Imaris 8.3 software (Bitplane, Oxford Instruments) as previously (3,7). Surfaces were masked to generate a 2D image of a single MSN for Sholl analysis. Concentric ring intersections were determined using the ImageJ Sholl analysis plugin (8) at 10 µm increments from soma.

Electrophysiology

For electrophysiological recordings, D1-Cre mice expressing AAV9-Ef1a-DO-TdTomato-DIO-EGFP (9) were deeply anesthetized with isoflurane and perfused with ice cold NMDG-substituted artificial cerebrospinal fluid (ACSF) prior to decapitation. NMDG-ACSF contained (in mM) 92 N-methyl-D-glucamine, 2.5 KCl, 1.25 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 25 glucose, 0.5 $CaCl_2$ and 10 $MgCl_2$. The brain was dissected out and 300 µm thick coronal slices containing nucleus accumbens were cut in ice-cold NMDG-ACSF with a vibratome. Slices were then placed in interface slice

chambers at room temperature and allowed to rest for at least an hour before recording in Na-ACSF containing (in mM) 120 NaCl, 3 KCl, 1.0 NaH_2PO_4, 1.5 $MgSO_4 \cdot 7H_2O$, 2.5 $CaCl_2$, 25 $NaHCO_3$, and 20 glucose. Pipettes with access resistance between 2-6 MΩ were filled with a K-gluconate based internal pipette solution containing (in mM) 105 K-gluconate, 5 KCl, 2 $MgCl_2 \cdot 6H_2O$, 10 HEPES, 4 Mg-ATP, 0.3 Na-GTP, 1 EGTA, and 10 Na_2-Phosphocreatine. Whole-cell recordings of D1 or D2 medium spiny neurons were done in the nucleus accumbens core. Cell type identification was done via fluorescent imaging while patching with D1- and D2-expressing MSNs expressing GFP and TdTomato respectively. Following patching, 5 minutes was allowed for the internal pipette solution to fill the cell before recordings were performed. Recordings were performed using CV203 BU headstage, Axopatch 200B amplifier, Digidata 1440A digitizer and Clampex 10.5 software (Axon Instruments). If the cell had an access resistance above 25 MΩ, it was excluded. Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the absence of TTX while holding the membrane potential at -50 mV and analyzed by event detection in MiniAnalysis (Synaptosoft). All other electrophysiological data were analyzed in Clampfit 11.2. Intrinsic membrane properties were recorded by applying a 500 ms long -5 mV step from a holding potential of -70 mV. Access resistance was determined by the peak of the capacitive current deflection, membrane resistance by the mean current deflection after the cell had reached a steady state, and capacitance by the area of the current deflection. The resting membrane potential was recorded in current-clamp mode, while applying no holding current. Cell excitability was recorded by applying 500 ms long 20 pA current steps and counting the number of evoked action potentials. The threshold potential was measured as the membrane potential where the potential began to deflect to initiate the first recorded action potential. The Rheobase was recorded by applying a 300 pA current ramp and measuring the current required to evoked the first action potential. Current-clamp recordings were performed while using a holding current that kept the membrane potential at -70 mV at rest. We analyzed cells located only in the NAc core and the core/shell boundary. Cells were excluded from the experiment if they failed Grubb's Outlier test (α=0.05) for a given intrinsic measurement.

RNA isolation

Polyribosomes were immunoprecipitated from NAc of D1-Cre-RiboTag and A2A-Cre-RiboTag mice as described previously (3,6,7,10). Briefly, four 14-gauge NAc tissue punches per mouse (4 mice pooled/sample) were collected on Day 10 of fentanyl abstinence. Tissue punches were homogenized by douncing in 1 mL homogenization buffer, and the supernatant was incubated with 5 µl anti-HA antibody (Covance, Princeton, NJ, USA: #MMS-101 R) at 4ºC overnight with constant rotation. Samples were then incubated with 400 uL of protein G magnetic beads (Life Technologies, Carlsbad, CA, USA #100.09D) overnight at 4ºC with constant rotation. Beads were washed in a magnetic rack with high-salt buffer. RNA was extracted with a DNase step (Qiagen, Germantown, MD, USA) using the RNeasy mini kit (Qiagen #74104) by adding lysis buffer supplemented with beta mercaptoethanol and following the manufacturer's instructions. RNA concentration and quality was determined with a Bioanalyzer (Agilent).

RNAseq and WGCNA

For RNA sequencing, only samples with RNA integrity numbers >8 were used. 6 samples/sex/drug/cell-type were submitted for RNA sequencing at the UMSOM Institute for Genome Sciences (IGS) and processed as described previously (7). Libraries were prepared from 10 ng of RNA from each sample using the Smart-Seq v4 kit (Takara). Samples were sequenced on an Illumina HiSeq 4000 with a 75 bp paired-end read. An average of 64–100 million reads were obtained for each sample. Reads were aligned to the mouse genome (Mus_musculus.GRCm38) using TopHat (version 2.0.8; maximum number of mismatches = 2; segment length = 30; maximum multi-hits per read = 25; maximum intron length = 50,000). The number of reads that aligned to the predicted coding regions was determined using HTSeq. We applied two strategies to characterize gene expression changes in these data. First, we sought to identify individual genes with significant gene expression changes following abstinence from fentanyl. We used limma-trend to fit log2-normalized read counts per million to a linear model and tested for significant effects of fentanyl in each cell type, separately in males and in females, as well as treating sex as a covariate. Second, we characterized the effects of fentanyl on gene co-expression networks. As a starting point for this analysis, we used limma-trend to select the set of genes that exhibited a nominally-significant effect (p-value < 0.05) of fentanyl, sex, or cell type. We then used weighted gene co-expression network analysis (WGCNA) to characterize co-expressed modules

among these genes, separately within D1 and D2 MSNs. Module detection was performed with the blockwiseModules() function with power = 10, corType = 'bicor', networkType = 'signed', minModuleSize = 25, reassignThreshold = 0, mergeCutHeight = 0.25, minMEtoStay = 0, and otherwise default parameters. We tested for differential expression of module eigengenes with limma, using posthoc contrasts to estimate effects of fentanyl while adjusting for sex differences, as above. Hub genes for each module were defined by calculating the Pearson correlation between the eigengene and each gene in the module.

Nanostring

40 ng of RNA per sample was processed with the nCounter Master Kit (Nanostring Technologies) by UMSOM IGS on a custom-made gene expression Code set (Sequences available in Supplemental File 4). Data were analyzed using cell-type, treatment, and sex as factors with nSolver Analysis software (Nanostring Technologies, RRID:SCR_021712) using Aars, Pum1, and Hsp90ab1 as housekeeping genes.

Gene Ontology and transcription factor analysis

We performed Gene Ontology (GO) Analysis using the BINGO plugin for Cytoscape (11) using the mouse annotation from geneontology.org in August 2019. We queried over-represented GO terms in each WGCNA module under the "molecular function," and "cellular-component" ontologies using the whole annotation as a reference set, and a Benjamini & Hochberg False Discovery Rate (FDR) correction of 0.05. We identified common transcriptional regulators of the Green module using the iRegulon plug-in in Cytoscape (12) using program default settings with the exception of a more stringent enrichment score threshold of 3.5.

Cell-type specific E2f1 manipulation

Mouse E2f1-myc-Flag ORF clone was obtained from Origene (#MR206856). We PCR amplified the ORF using Phusion DNA polymerase (New England Biolabs #E0553), then cloned into the NheI and AscI restriction site of pAAV-Ef1a-DIO-eYFP (Addgene #27056). Vector sequences were confirmed by digestion and sequencing. AAV9-Ef1a-DIO-E2f1-myc-Flag was packaged at the University of Maryland Virus Vector Core Facility.

In Vitro Experiments

Neuro2A cell cultures were maintained in DMEM supplemented with 10% FBS and Penicillin/streptomycin (all from Thermo Fisher Scientific). Cell cultures of passage number between 5-10 were used for the entire experiment. 24 hours prior to transfection, cells were seeded at the density of 1.5 X 10^5 cells per well in 24 well plates. Transfection was performed using Invitrogen LF3000 reagents following the manufacturer's standard protocol. Cells were harvested 48 hours post transfection for either RNA or protein sample preparation.

For western blotting, cell lysates were prepared from 12 wells (2 pooled/condition for N=3 samples each) with RIPA buffer (Sigma-Aldrich R0278) supplemented with Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich P5726), Phosphatase Inhibitor Cocktail 3 (Sigma-Aldrich P0044), Complete Protease Inhibitor Cocktail (Roche 11873580001). Electrophoresis was performed using Mini-Protean TGX Gels (Biorad 4569033) followed by the transfer onto a nitrocellulose membrane (Biorad 1620094). After blocking in 5% skim milk/Tris-buffered saline supplemented with 0.1% Tween 20 (TBST), membranes were incubated with primary antibody overnight at 4^{o}C followed by secondary antibody incubation for 1 hour at room temperature. Blot images were captured using Biorad ChemiDoc MP Imager, and the band intensities were quantified relative to GAPDH band intensities using ImageJ (NIH). The following primary antibodies were used at 1:1000 dilution: E2F1 (Abcam ab137415), PRKCE (Proteintech 20877-I-AP), GRAMD1B (Proteintech 24905-I-AP), NLGN2 (ProSci 7969), GAPDH (Cell Signaling 2118s).

For qPCR, RNA extraction samples were prepared from 12 wells (6 samples/condition) using the Qiagen RNeasy micro plus kit (Qiagen 74034). Neuro2a cells were harvested directly from the wells with application of Buffer RLT and physical scraping using a cell scraper. Purified RNA was quantified with a Nanodrop (Thermo), then 500ng of complementary DNA (cDNA) was synthesized using the reverse transcriptase iScript complementary DNA synthesis kit (Bio-Rad, Hercules, CA; # 1708891), then diluted to a concentration of 2.5 ng/µL. Relative mRNA expression changes were measured by quantitative PCR using Perfecta SYBR Green FastMix (Quantabio, Beverly, MA; #95072) with a Bio-Rad CFX384 qPCR system. Primer sets are available in the Key Resources Table. Fold change expression was calculated using the 2^{-ddCt} method with *Gapdh* as the reference gene.

Cut & Run qPCR

Nucleus Accumbens tissue punches were collected on abstinence Day 10, flash frozen on dry ice, and stored at -80°C. Cut & Run was performed using Cell Signaling Cut & Run Kit (#86652) following the manufacturer's standard protocol. Tissue punches were homogenized by douncing in buffer supplemented with spermidine and protease inhibitor cocktail according to the manufacturer's recipe. Enriched chromatins were purified using Cell Signaling ChIP and Cut & Run DNA Purification Kit (14209) following the manufacturer's standard protocol. The following antibodies were used at 2ul per sample for negative control and 5ul per sample for all remaining antibodies: Normal Rabbit IgG (Cell Signaling #66362, negative control), H3K4me3 (Cell Signaling #9751, positive control), E2F1 (Invitrogen #32-1400). Expression changes were measured using qRT-PCR with PerfectaSYBR Green FastMix (Quanta, Beverly, MA, USA). Samples were normalized to negative control and compared with IgG. Samples were excluded if the positive control did not show enrichment relative to negative control. Primer sequences can be found in the Key Resources Table.

Data and code availability

Data : RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table. Differential expression and WGCNA data have been deposited at Mendeley and are publicly available as of the date of publication. The DOI is listed in the key resources table. All other data reported in this paper will be shared upon request.

Supplemental References

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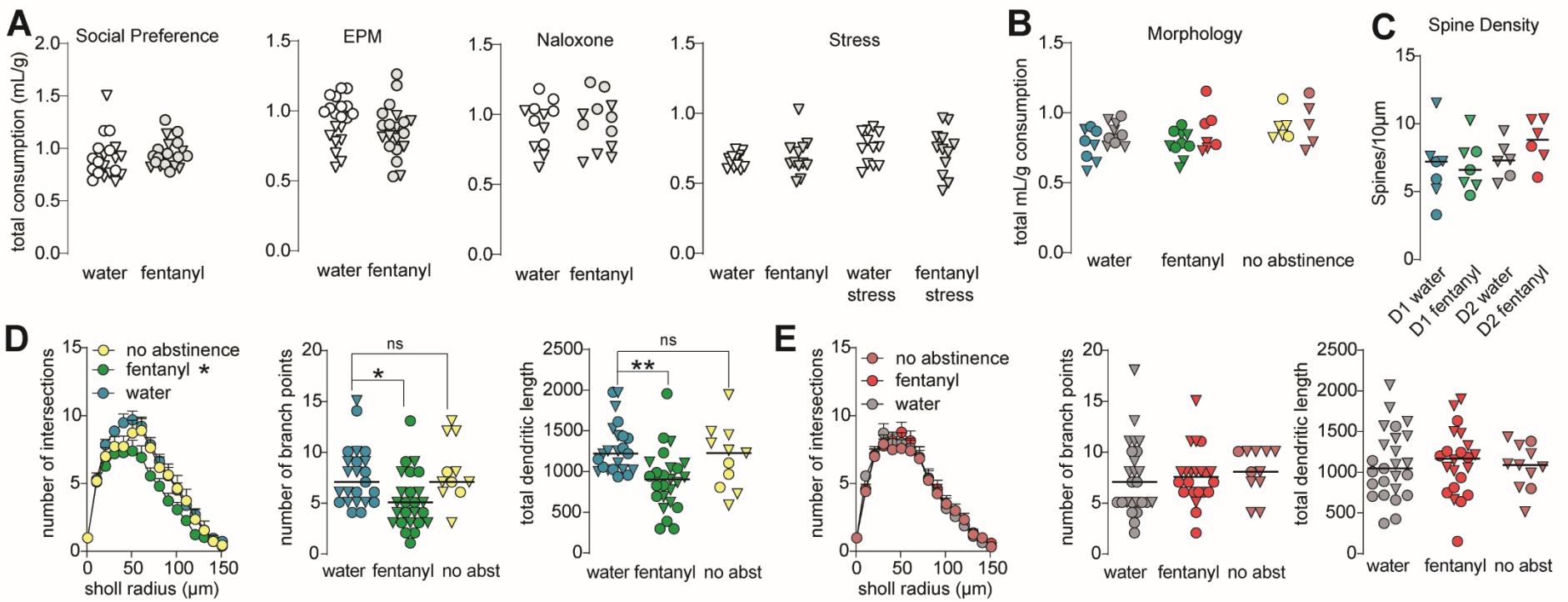

Figure S1. **Related to Figure 1.**
(**A**) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in male (triangles) and female (circles) C57BL/6 mice used in behavioral experiments reported in Figure 1B,C,E,F. (**B**) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in D1- and A2A-Cre mice used in morphology experiments reported in Figure 1I-L, and S1D below. Symbols are colored according to genotype and treatment. Circles represent females, triangles represent males. (**C**) Spine density from a subset of water and fentanyl abstinent D1- and D2-MSNs. Each triangle or circle represents average spine density in a male or female mouse, respectively. (**D**) Comparison of D1-MSN dendritic complexity between D1-Cre mice exposed to 5 days of fentanyl (no abstinence), and the water or fentanyl abstinent D1-Cre mice from Fig 1I-J, reproduced here to aid visual comparison. (Dunnet's post-hoc: No abstinence vs water p's>0.05 for all measures, Fentanyl abstinence vs water p's<0.05, n=59 cells from 24 total mice). (**E**) Comparison of D2-MSN dendritic complexity between A2A-Cre mice exposed to 5 days of fentanyl (no abstinence), and the water or fentanyl abstinent A2A-Cre mice from Fig 1K-L reproduced here to aid visual comparison (All p's >0.05, n=58 cells from 22 total mice). Detailed Statistics in Supplemental File 1. Note: water and fentanyl abstinence morphology data in panels D and E are the same as in Fig 1I-L to avoid unnecessary repetitive experiments.

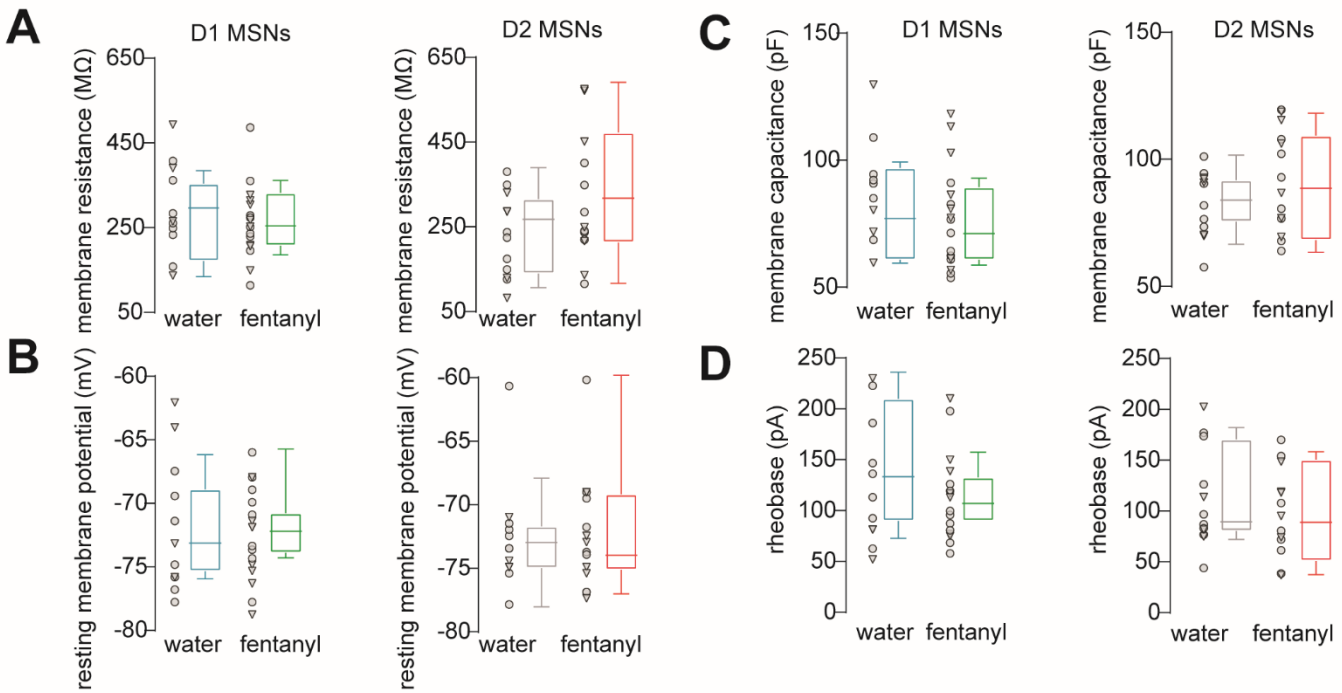

Figure S2. Related to Figure 2

(A) Membrane resistance, (**B**) resting membrane potential, (**C**) membrane capacitance, and (**D**) rheobase in D1- and D2-MSNs from water and fentanyl abstinent mice. Individual data points represent individual neurons; triangles denote neurons from males. Box and whiskers plots show the median and range for data nested by mouse. Detailed Statistics in Supplemental File 1.

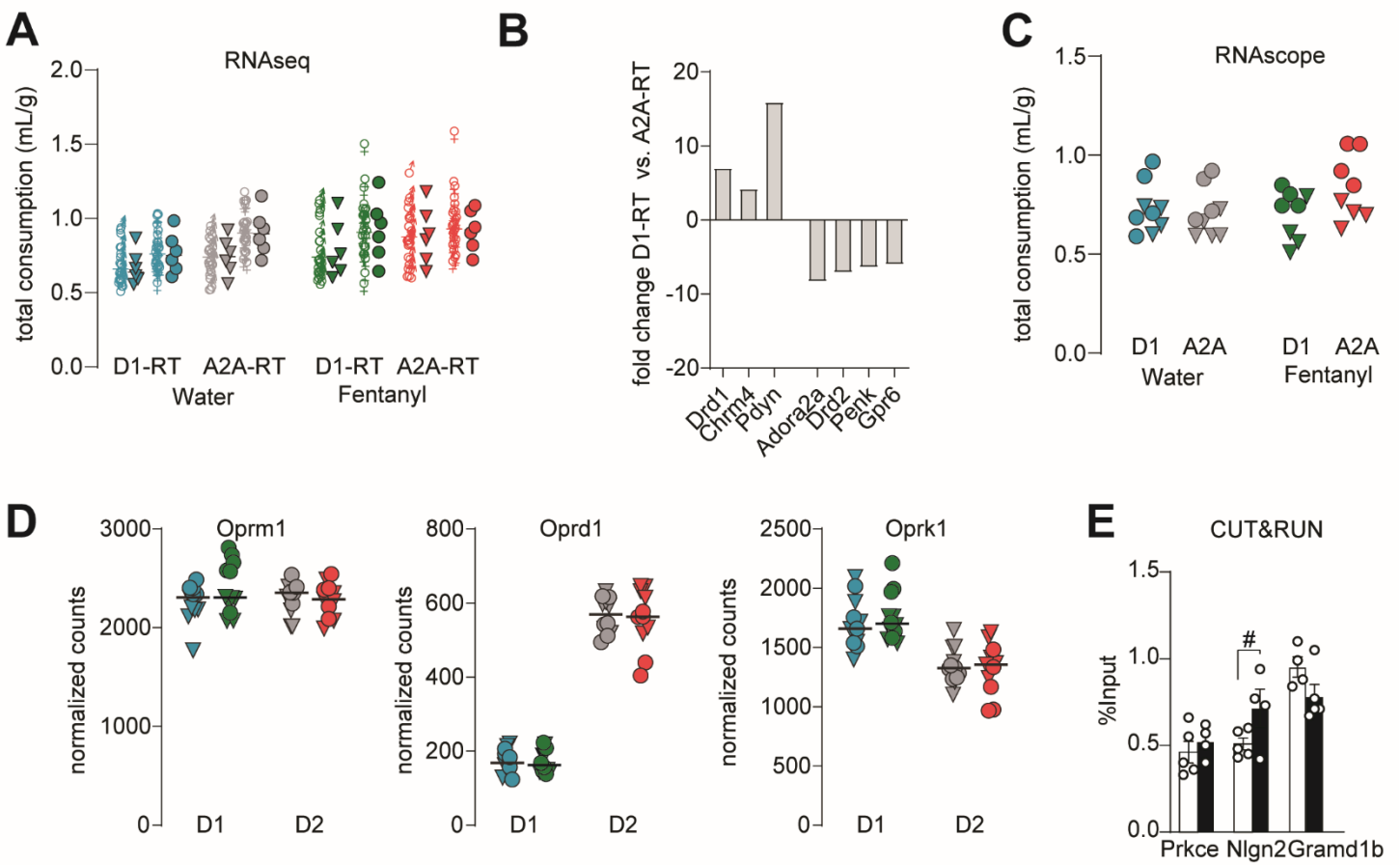

Figure S3. Related to Figures 3 and 4.

(A) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in male and female D1-RiboTag (RT) and A2A-RT mice used in RNAseq and Nanostring experiments in Figures 3 and 4. Tissue from mice that consumed similar amounts were pooled to generate 6 samples/sex/condition/genotype, resulting in an average consumption value per sample (filled circles next to individual points). Triangles denote male samples; samples are colored based on treatment and genotype. (**B**) Nanostring validation showing expression of D1-MSN specific genes *Drd1*, *Chrm4*, and *Pdyn* are enriched, and D2-MSN genes *Adora2a, Drd2, Penk*, and *Gpr6* expression are impoverished in D1-RT samples. (**C**) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in D1 and A2A-Cre mice used for RNAscope experiments in Figure 4F. Triangles denote male mice. Symbols are colored according to genotype and treatment. (**D**) Nanostring counts of mu, delta, and kappa opioid receptors in samples from water and fentanyl abstinent D1- and A2A-RTmice. Triangles denote male samples; lines denote median, symbols are colored according to genotype and treatment. (**E**) Cut&Run chromatin profiling in total NAc from individual water (white) and fentanyl abstinent (black) mice. Regions containing predicted E2f1 binding sites for Prkce, Nlgn2, and Gramd1b were amplified with qPCR. Data are presented as (mean ± sem) expression in E2f1-immunoprecipitated samples relative to input. (#, p=0.08 water vs fentanyl abstinence for Nlgn2). Detailed Statistics in Supplemental File 1.

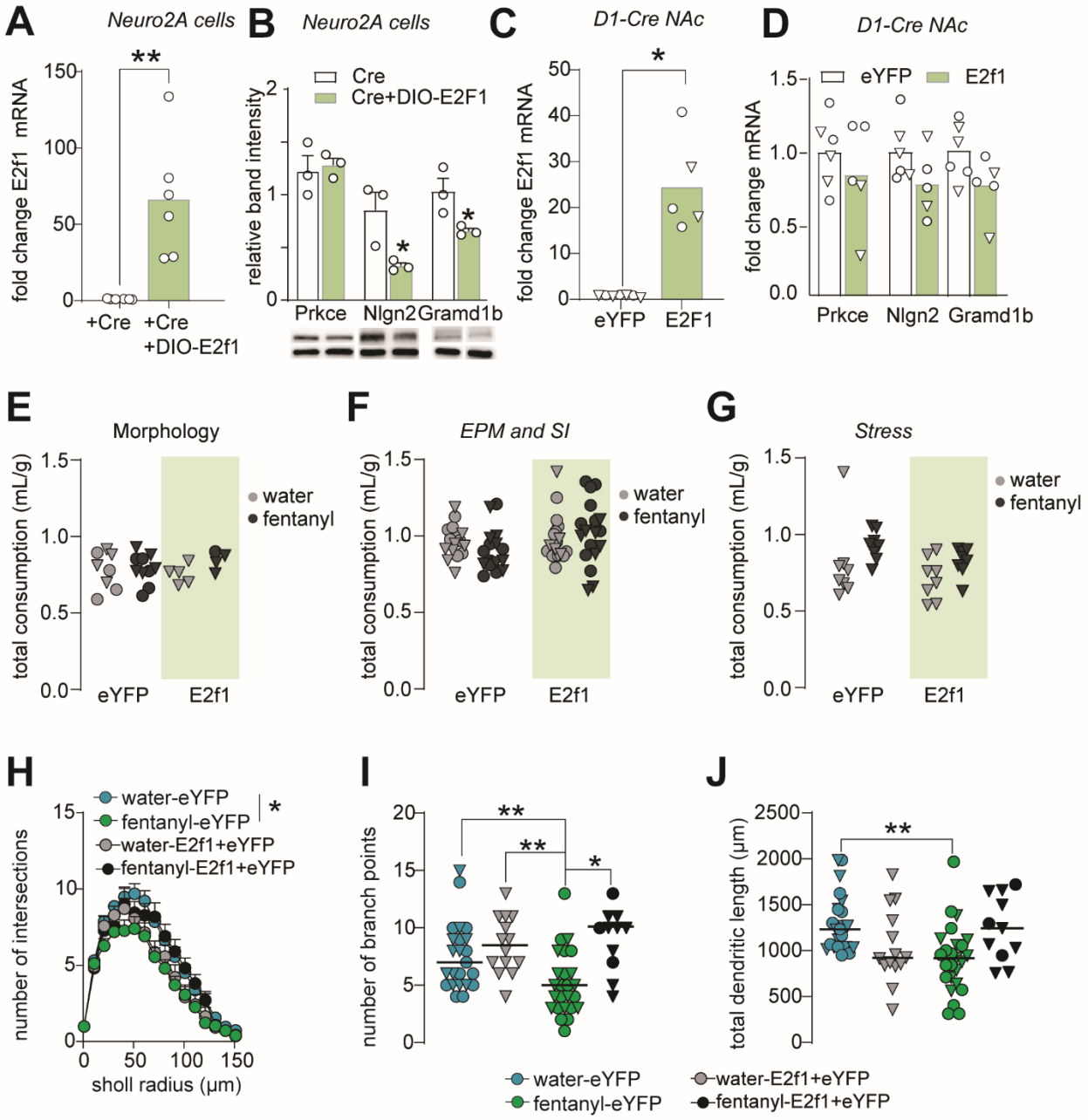

Figure S4. Related to Figure 5.

(**A**) Fold E2f1 expression in Neuro2A cells transfected with Cre only (white), or Cre and DIO-E2f1 (green). Bars represent mean. (**, p=0.010, t-test). (**B**) Western blot showing downregulation of E2f1 targets Nlgn2 and Gramd1b at protein level in Neuro2A cells. Top bands are the protein of interest, and bottom are Gapdh used to normalize expression. (n=6 cultures/condition pooled 2 cultures/sample; *,p<0.05, t-tests). (**C**) Fold E2f1 expression in total NAc of D1-Cre mice expressing DIO-eYFP (white) or DIO-E2f1 (green). Bars represent mean (**, p= 0.007, t-test); triangles denote males. (**D**) Fold expression of E2f1 target genes in total NAc of D1-Cre mice expressing DIO-eYFP (white) or DIO-E2f1 (green). Bars represent mean; triangles denote males. (Prkce, p=0.4, Nlgn2, p= 0.1, Gramd1b, p=0.08, t-tests). (**E**) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in D1-Cre mice used in morphology experiments in Fig 5D-G. Triangles denote males. The eYFP group is the same as the D1-Cre in Fig S1B, reproduced here to aid visual comparison. (**F**) Total liquid consumption across 5 days of water or fentanyl normalized to individual mouse bodyweight in D1-Cre mice used in Fig5I-J, and (**G**) Fig 5K. (**H**) Comparison of D1-MSN Sholl intersections between fentanyl and water abstinent D1-Cre mice with or without Intra-NAc DIO-E2f1 (mean± sem; *, p=0.014, Fentanyl-eYFP vs Water-eYFP; Sidak's post-hoc after Greenhouse Geiser-corrected ANOVA) (**I**) Comparison of D1-MSN branch points and (**J**) total dendritic length in fentanyl and water abstinent D1-Cre mice with or without Intra-NAc DIO-E2f1 (Branch points: **, p<0.002, *, p=0.022; Dendritic length: **, p=0.002; Sidak's post-hoc after corrected ANOVA). Triangles denote males. Detailed Statistics in Supplemental File 1. Note: water and fentanyl eYFP data in panel S4H-J are the same as in Fig 1I-J to avoid unnecessary repetitive experiments.

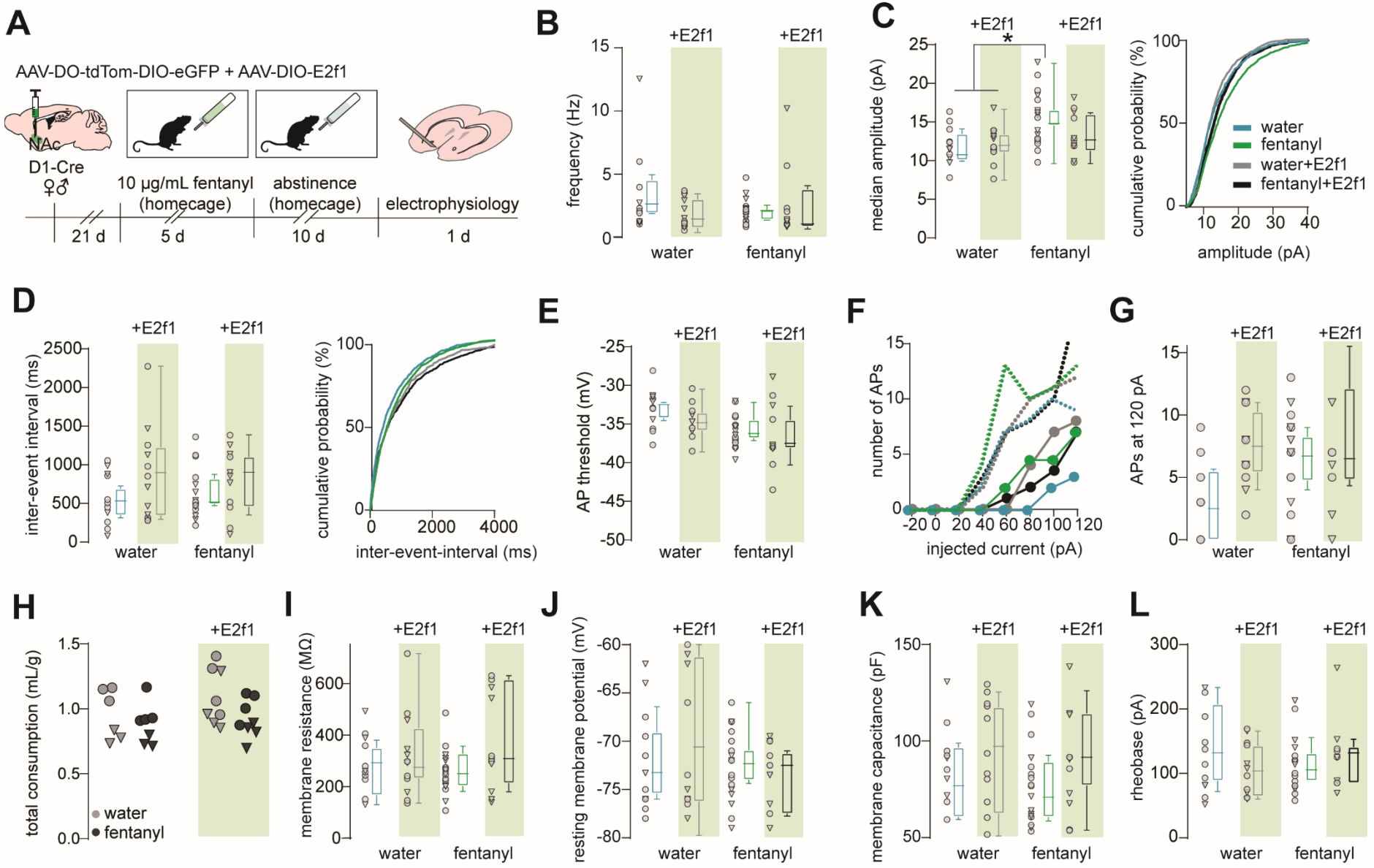

Figure S5, related to Figure 5

(**A**) Timeline for E2f1 electrophysiology experiments. Male and female D1-Cre mice received intra-NAc AAV-DO-TdTomato-DIO-eGFP to label MSN subtype and AAV-DIO-E2f1 for E2f1 overexpression in D1-MSNs. Following homecage water or fentanyl abstinence, slices containing the NAc were collected for patch-clamp recording. (**B**) Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded while holding the membrane potential at -50 mV and analyzed by template-based event detection. Comparison of median sEPSC frequency between water and fentanyl abstinent mice

expressing DO-tdTomato-DIO-eGFP with or without DIO-E2f1. Individual data points represent individual neurons, triangles represent neurons from males. Box and whiskers plots show the median and range for data nested by mouse. (**C**) Comparison of median sEPSC amplitude (*, p's<0.04 Fentanyl-eGFP vs water-eGFP, vs water-E2f1+eGFP, Sidak's after ANOVA), and cumulative probability in D1-MSNs from water and fentanyl abstinent mice that received DO-tdTomato-DIO-eGFP with or without DIO-E2f1. (**D**) Comparison of median sEPSC inter-event-interval and cumulative probability in D1-MSNs from water and fentanyl abstinent mice that received DO-tdTomato-DIO-eGFP with or without DIO-E2f1. (**E**) Comparison of the membrane potential required to elicit an action potential (AP), and (**F**) number of APs elicited by injecting current in 20 pA intervals (median+range, circles and dashed lines, respectively), in D1-MSNs from water or fentanyl abstinent mice that received DO-tdTomato-DIO-eGFP with or without DIO-E2f1. (RMANOVA, current step x virus Interaction, p=0.05) (**G**) Comparison of the number of APs elicited by 120 pA injection in D1-MSNs from water and fentanyl abstinent mice that received DO-tdTomato-DIO-eGFP with or without DIO-E2f1. (ANOVA, main effect of virus, p=0.037). (**H**) Total liquid consumption across 5 days for male and female D1-Cre mice used in all electrophysiology experiments in Figs 2 and 5. Comparison of (**I**) membrane resistance, (**J**) resting membrane potential, (**K**) membrane capacitance, and (**L**) rheobase in D1-MSNs from water and fentanyl abstinent mice that received DO-tdTomato-DIO-eGFP with or without DIO-E2f1. Detailed Statistics in Supplemental File 1. Note: The +E2f1 data in S5C, E, G are reproduced from Fig 5N-P and are provided to aid visual comparison. The data without E2f1 in S5B-G are reproduced from Fig 2C-G, and the data in S5I-L reproduced from D1-MSNs in Fig S2A-D.

Table S1. **Related to Figure 3.** WGCNA modules with significant effect of fentanyl abstinence. Module number and color, number of genes in the module, the top 10 hub genes, log fold change and P value for fentanyl abstinence in D1+D2, log fold change and P value in D2, log fold change and P value in D1

Module	Color	nGenes	Top 10 Hub Genes	logFC fentanyl	P.Value fentanyl	logFC fentanyl in D2	P.Value fentanyl in D2	logFC fentanyl in D1	P.Value fentanyl in D1
D1.AE9	blue	206	Dph1 ,Por, Rarb, Cpne2, Wdr18, Trap1, Mpp1, Ctnnbl1, Mgat2, Psen1	1.275507548	0.022152801	0.065283232	0.864442247	1.210224316	0.002687039
D1.AE3	gray	417	Arl6, Ptpra, Ndfip1, Vapa, Prps1, Psmc4, Serinc1, Nap1l2, Cops4, Ppp2ca	1.058554651	0.011768987	-0.024136538	0.932810796	1.082691189	0.000440705
D1.AE11	yellow	104	Prepl, Gm5499, Mtrex, Rars2, Ythdf2, Rnf180, Tm9sf2, Mmachc, Fbxl5, Fbxo3	0.765040912	0.028942308	-0.179467198	0.457610027	0.944508109	0.000287229
D2.AE14	brown	79	Pdia6, Hspa5, Rexo4, Cys1, Dusp6, Egr4, Nr4a1, Atf3, Rnf32, Prps1l3	0.784637105	0.087862045	-0.092543165	0.772253365	0.87718027	0.008416034
D2.AE11	purple	155	Bag5, Ptcd2, Fbxl5, Pitpnb, Timp2, Recql, Pcyox1, Rarb, Mgat2, Bhlhb9	0.604751646	0.185267545	-0.152888763	0.632753305	0.757640409	0.021515141
D2.AE8	black	194	Tmem158, Hmbs, Asphd2, Angptl6, Xxylt1, Limd2, Rnf166, Ap1s1, Cnot8, Itpa	0.754070936	0.044917604	0.42029745	0.110810545	0.333773485	0.202966648
D2.AE2	magenta	361	Tubb2b ,Dlx1, Plpp3, Slc1a3, Arx, Fabp7, Pla2g7, Ndrg2, Mlc1, Cd9	-0.979626559	0.001505807	-0.817756422	0.00024248	-0.161870138	0.432854847
D2.AE15	cyan	72	Ttc39b, Gria3, Kcna2, Csnk2a2, Bicd1, Ank2, Ppm1k, Cacna1a, Arap2, Map7d2	-0.417589101	0.293221885	0.16685955	0.550809096	-0.58444865	0.041023264
D1.AE10	red	169	Ctxn3, Trdn, Gcnt1, Nacc2, Gng4, Ntn4, Stard8, Kcnab3, Lgr6, Gprc5b	-1.385223155	0.006879069	-0.498026518	0.156246406	-0.887196637	0.013668682
D1.AE4	green	388	Cdk5r1, Syt7, Nlgn2, Bcl7a, Syngap1, Prkce, Nat8l, Rab35, Pura, Gramd1b	-0.971595699	0.026780641	0.023067331	0.939001119	-0.99466303	0.001834251
D1.AE18	orange	41	Atf4, Ssh2, Ank2, Chmp4b, Dcp2, Lars2, Gm15564, Psmg3, Marf1, Pex11a	-1.267043609	0.010388206	-0.027922498	0.933847195	-1.239121111	0.000593379

See separate Excel file for:

Supplemental File 1. Detailed statistical analysis including accounting for sex as a biological variable. Associated figure, comparison groups, and statistical tests used. N's represent number of individual mice, n's represent number of individual cells. The F, T, or K-S D column contains the given source of variation used in ANOVA, or specifies t for t-tests and K-S for Kolmogorov Smirnov Distance. P values and corresponding significance stars are shown for each comparison. If corrections for sphericity/variance were made they are as indicated. Notes specify instances in which only certain comparisons are shown for brevity's sake.

Supplemental File 2. Related to Figure 4. Enriched Gene Ontology terms for 11 selected WGCNA modules

Supplemental File 3. Related to Figure 4. Transcriptional regulation analysis, predicted E2f1 target genes and motif ID's from iRegulon.

Supplemental File 4. Nanostring CodeSet information