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Interruption of Continuous Opioid Exposure Exacerbates Drug-Evoked Adaptations in Brain Function and Behavior

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ABSTRACT

Background: Drug-evoked adaptations in the mesolimbic dopamine system drive opioid abuse and addiction, but these adaptations vary in magnitude and direction following different patterns of opioid exposure. Despite fundamental links between the pattern of drug exposure and abuse liability, few studies have systematically manipulated the pattern of opioid administration while measuring neurobiological and behavioral impact.

Methods: In male and female mice, we first compared the behavioral consequences of chronic morphine exposure for one week, across multiple doses and with administration patterns that were either intermittent (daily injections) or continuous (osmotic minipump infusion). We then interrupted continuous morphine exposure with twice-daily naloxone injections, and used next-generation RNA sequencing to perform genome-wide transcriptional profiling in the nucleus accumbens and dorsal striatum.

Results: Continuous morphine exposure caused tolerance to both the antinociceptive and psychomotor-activating effects of morphine. In contrast, both intermittent and interrupted morphine exposure caused long-lasting psychomotor sensitization. The interruption of continuous morphine exposure exacerbated drug-evoked transcriptional changes in the nucleus accumbens and dorsal striatum, dramatically increasing the number of differentially expressed transcripts and engaging unique signaling pathways.

Conclusions: These experiments indicate that opioid-evoked adaptations in brain function and behavior are critically dependent on the pattern of drug administration, and exacerbated by interruption of continuous exposure. Maintaining continuity of chronic opioid administration may therefore represent a strategy to minimize detrimental effects on brain reward circuits. This may in turn reduce the risk of progression from opioid use to abuse, promoting safer use of opioid-based treatments for clinical indications.

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INTRODUCTION

Chronic opioid exposure can cause neurobiological adaptations that produce detrimental consequences. The clinical utility of opioids as analgesics is due to activation of opioid receptors in the brain and spinal cord (1), but chronic exposure tends to diminish these effects and produce analgesic tolerance. Conversely, opioid abuse liability is related to activation of receptors in portions of the mesolimbic dopamine system, including the ventral tegmental area and nucleus accumbens (2). Activation of these receptors is positively reinforcing (3, 4) and causes dopamine release in the nucleus accumbens of humans (5) and rodents (6). Chronic opioid exposure produces transcriptional and epigenetic changes in the mesolimbic dopamine system, causing structural and functional remodeling of this circuit that contributes to the development of drug addiction (7, 8)

The effects of chronic opioid exposure on reward-related behavior and mesolimbic dopamine function are surprisingly diverse, ranging from increases in opioid sensitivity (i.e., sensitization) to decreases in opioid sensitivity (i.e., tolerance) (9-11). Variability in these outcomes is correlated with differences in the pattern of drug administration (12, 13). Reward tolerance is typically reported after relatively continuous patterns of opioid administration, whereas reward sensitization commonly observed after more intermittent patterns of opioid exposure (14-20). Divergent effects of continuous and intermittent opioid exposure have also been reported for mesolimbic dopamine release (21-23) and psychomotor activation (24-27). However, few attempts have been made to directly compare different patterns of opioid exposure, while controlling key variables like cumulative dose and peak drug level that can confound comparisons between patterns. Such an analysis may provide novel insight regarding opioid-evoked adaptations in brain function that mediate specific behavioral outcomes.

To examine these issues, we manipulated the pattern of morphine administration to mice in several ways. We first replicated prior reports that intermittent morphine injections produce psychomotor sensitization, whereas continuous morphine infusion produces psychomotor tolerance. Because this comparison was confounded by large differences in peak drug level, we also used daily injections of an opioid receptor antagonist (naloxone) to interrupt continuous morphine administration. This manipulation had no effect on either cumulative dose or peak drug level, but caused a striking switch in behavioral outcome: from transient psychomotor tolerance to durable psychomotor sensitization. We then used next-generation RNA sequencing to perform genome-wide transcriptional profiling in the nucleus accumbens and dorsal striatum after these patterns of morphine exposure.

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Interrupted morphine administration exacerbated transcriptional changes observed in the nucleus accumbens, engaging unique signaling pathways that were unaffected by continuous morphine exposure. Together, our data suggest minimizing the interruption of opioid receptor activation can increase the safety of chronic opioid treatment, by reducing detrimental adaptations in brain reward circuits that promote abuse and addiction.

METHODS AND MATERIALS

Subjects

Male and female C57BL/6J mice were obtained from The Jackson Laboratory (stock #000664) or bred in-house. Constitutive mu opioid receptor (*Oprm1*) knockout mice (28) were obtained from The Jackson Laboratory (stock #007559). All mice were at least 5 weeks old at the beginning of each experiment and housed in groups of 2-5 per cage, on a 12 hour light cycle (0600h – 1800h) at ~23°C with food and water provided ad libitum. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Minnesota.

Drug Exposure

Morphine hydrochloride (Mallinckrodt, St. Louis, MO) was dissolved in sterile saline (0.9%), and delivered subcutaneously by bolus injection (5 mL/kg) or continuous infusion using osmotic minipumps (Alzet Model 2001). After adjusting morphine concentration for body weight, minipumps were filled with 300 µL of solution and primed overnight at 40°C. The following day, mice weighing 15-25 g were anesthetized with 5% isoflurane/95% oxygen. The primed minipump was implanted through a small incision on the rump, which was then closed with wound clips. Carprofen (5 mg/kg, s.c.) was given as an analgesic before surgery and for 3 days following pump implantation or removal. To interrupt continuous morphine exposure, we injected mice with saline or naloxone (0.1-10 mg/kg, s.c.) twice per day, with injections separated by a period of two hours (29). Behavioral assessments were performed prior to naloxone injection on the first day of morphine exposure, and then 24 hours after the final naloxone injection on the last day of morphine exposure.

Behavioral and Pharmacokinetic Assessments

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As previously described (30), we tested open-field locomotor activity in a clear plexiglass arena (ENV-510, Med Associates) housed within a sound-attenuating chamber. The location of the mouse within the arena was tracked in two dimensions by arrays of infrared beams, connected to a computer running Activity Monitor software (Med Associates) that automatically calculates distance travelled. Mice were habituated to the chamber for one hour the day before initiating drug treatment, and then tested on the first and last days of chronic morphine treatment (30 minutes after morphine injections). For morphine challenges, the session length varied as a function of morphine dose: 60 mins (Saline and 2 mg/kg), 90 mins (6.32 mg/kg), or 120 mins (20 mg/kg)

Thermal antinociception was tested on a 55°C hot plate (IITC Life Scientific, Woodland Hills, CA). The day before initiating drug treatment, mice were habituated to the instrument for 60 seconds at room temperature. Immediately before the first drug exposure, we established baseline latency to either jump or lift and lick a hind paw at 55°C. Mice were then tested on the first and last days of chronic morphine exposure (30 minutes after morphine injections), with a maximal cutoff of 30 seconds to prevent tissue damage. The percent maximum possible effect was calculated as (test latency – baseline latency) / (30 sec – baseline latency) x 100.

In a subset of mice, we also collected facial vein blood samples on the first and last days of morphine exposure (5 minutes after injections), to determine serum morphine concentration. These samples were collected in heparinized tubes and centrifuged at 7500 RPM for 3 minutes at 14°C. Serum was transferred to a 5 ml vial and stored at -20°C until analysis. Morphine concentrations were measured by gas chromatography coupled with mass spectrometry as previously described (31, 32).

Gene Expression and RNA Sequencing

RNA sequencing was performed using male mice, while equal numbers of both sexes were used in all other experiments, including targeted gene expression analysis with qRT-PCR. Following six days of chronic treatment, we rapidly removed brains under isoflurane anesthesia, and dissected bilateral nucleus accumbens (core and shell) and dorsal striatum (caudate-putamen) on ice. For qRT-PCR, tissue was snap frozen on dry ice and stored at -80°C. RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. All RNA samples had A260/A280 purity ratio ≥ 2. Reverse transcription was performed using Superscript III (Invitrogen). For each sample, duplicate cDNA preparations were set up. Mouse β-actin mRNA

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was used as the endogenous control. qPCR using SYBR green (BioRad, Hercules, CA) was carried out with a Lightcycler 480 II (Roche) system with the following cycle parameters: 1 x (30 sec @ 95°C), 35 x (5 sec @ 95°C followed by 30 sec @ 60°C). Data were analyzed by comparing the C(t) values of the treatments tested using the ΔΔC(t) method. Expression values of target genes were first normalized to the expression value of β-actin. The median of each cDNA replicate reactions was used to quantify the relative target gene expression. Primers were designed in Primer3 and validated in BLAST and are listed in Supplemental Table S1.

For RNA sequencing, tissue was stored in RNAlater (Qiagen) and total RNA was isolated using the RNeasy Mini Kit (Qiagen) as previously described (33). Total eukaryotic RNA isolates were quantified using a fluorimetric RiboGreen assay. Total RNA integrity was assessed using capillary electrophoresis (Agilent BioAnalyzer 2100), generating an RNA Integrity Number (RIN) that was >8 for all samples. Total RNA samples were converted to Illumina sequencing libraries using the Truseq Stranded mRNA Sample Preparation Kit (Illumina, San Diego, CA, USA). Extracted mRNA was then oligo-dT purified using oligo-dT coated magnetic beads, fragmented and then reverse transcribed into cDNA. The cDNA was adenylated and then ligated to dual-indexed (barcoded) adapters. Truseq libraries were then sequenced 50-bp paired-end run on the Illumina HiSeq 2500, generating ~20 million paired-end reads per run.

Raw Illumina reads were cleaned of low quality bases, adapter contamination, and low complexity sequence with Trimmomatic (34). Cleaned reads were aligned to the *Mus musculus* reference genome, version GRCm38 with HISAT2 (35), with a list of putative splice sites derived from the GTF annotation file. Resulting BAM files were sorted by read name, and counts were generated with HTSeq (36) with the unstranded option. A modification to the GTF file from Ensembl was performed to consider only protein coding genes for the counts. The expression counts matrix was filtered to remove genes with less than 150 total counts across all samples prior to differential expression testing. Nucleus accumbens and dorsal striatum samples were handled separately for all analyses.

Normalization and Differential Expression Tests

For differential expression analysis, filtered expression counts were normalized and variance-stabilized with DESeq2 (37). The variance-stabilized counts for all genes passing the expression filter were decomposed

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with principal components analysis (PCA) to identify potential broad patterns in gene expression differences among samples. Stabilized and filtered counts were also centered about 0 for each gene and used as input for hierarchical clustering. One sample from the nucleus accumbens was identified as an outlier based on both PCA and hierarchical clustering, and was removed from further analyses. Normalized and stabilized counts were exported for other analyses.

Differential expression testing was performed with normalized counts in DESeq2. The saline-saline and saline-naloxone treatments were combined ("control") for differential expression testing, because PCA and hierarchical clustering did not support these treatments being separated. Differentially expressed genes were identified among three comparisons: control vs. continuous, control vs. interrupted, and continuous vs. interrupted. A false discovery rate (q) of 0.05 was used for Benjamini-Hochberg multiple testing corrections (38), with a fold change threshold of 15%.

Pathway and Network Analyses

Differentially expressed genes from DESeq were used to identify overrepresented pathways with Ingenuity Pathway Analysis (IPA) core expression analysis (QIAGEN Inc). Enrichment of gene ontology (GO) terms was performed using g.profiler (39), using a target list of differentially expressed genes, and a background list of all protein coding genes that passed expression filtering criteria for the initial analysis. GO terms that crossed an adjusted P-value of 0.05 were considered as significantly enriched.

Filtered, normalized, and stabilized counts from DESeq2 were scaled to mean 0 and unit variance was used as input for a weighted gene coexpression network analysis (WGCNA) (40, 41). Scale-free topology assumptions and soft power thresholds for network edges were checked with routines within the WGCNA package. Coexpression networks were built separately for each brain region, and then region-specific networks were merged to form a consensus network, merging modules with a Pearson correlation of at least 0.3 between their eigengenes. Eigengene values for each sample were exported. Tests for associations between module eigengene expression value and experimental treatment were performed with a one-way ANOVA. An eigengene-treatment association was considered as significant if its Bonferroni-adjusted P-value was less than 0.05, with the number of modules tested taken as the number of independent hypothesis tests.

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Statistical Analyses

Similar numbers of male and female animals were used in all experiments, except for RNA sequencing, which was only conducted in males to minimize variability. Individual data points from males (filled circles) and females (open circles) are distinguished in figures. Sex was included as a variable in factorial ANOVA models analyzed using IBM SPSS Statistics v24, with repeated measures on within-subject factors, but sex effects were not significant unless noted otherwise. We report significant effects that are critical for data interpretation in the text, but comprehensive reporting of all main effects and interactions from ANOVA models can be found in Supplemental Table S2. For main effects or interactions involving repeated measures, the Huynh-Feldt correction was applied to control for potential violations of the sphericity assumption. This correction reduces the degrees of freedom, resulting in non-integer values. Significant interactions were decomposed by analyzing simple effects (i.e., the effect of one variable at each level of the other variable). Significant main effects were analyzed using Dunnett or Tukey post-hoc tests. Significant simple effects within group are indicated by a hash (#) to the right of group data, while significant simple effects or post-hoc tests between groups are indicated by an asterisk (*) above the data. The Type I error rate was set to $\alpha = 0.05$ (two-tailed) for all comparisons. All summary data are displayed as mean +/- SEM.

RESULTS

Comparison between Intermittent Injection and Continuous Infusion of Morphine

In an initial attempt to compare different patterns of morphine exposure, we delivered morphine subcutaneously for one week as either a daily injection (Figure 1A) or continuous infusion (Figure 1E). We selected one common dose (20 mg/kg/day) for both injection and infusion, but expected bolus injection of this dose to generate peak morphine levels much greater than continuous infusion across 24 hours. We therefore included lower injection doses (2 and 6.32 mg/kg/day), as well as a higher infusion dose (63.2 mg/kg/day), in hopes of identifying injection and infusion doses that produce comparable peak morphine levels.

On the first and last day of morphine exposure, mice were placed in an open field chamber to measure locomotor activity. Morphine caused a dose-dependent increase in distance travelled, regardless of whether the

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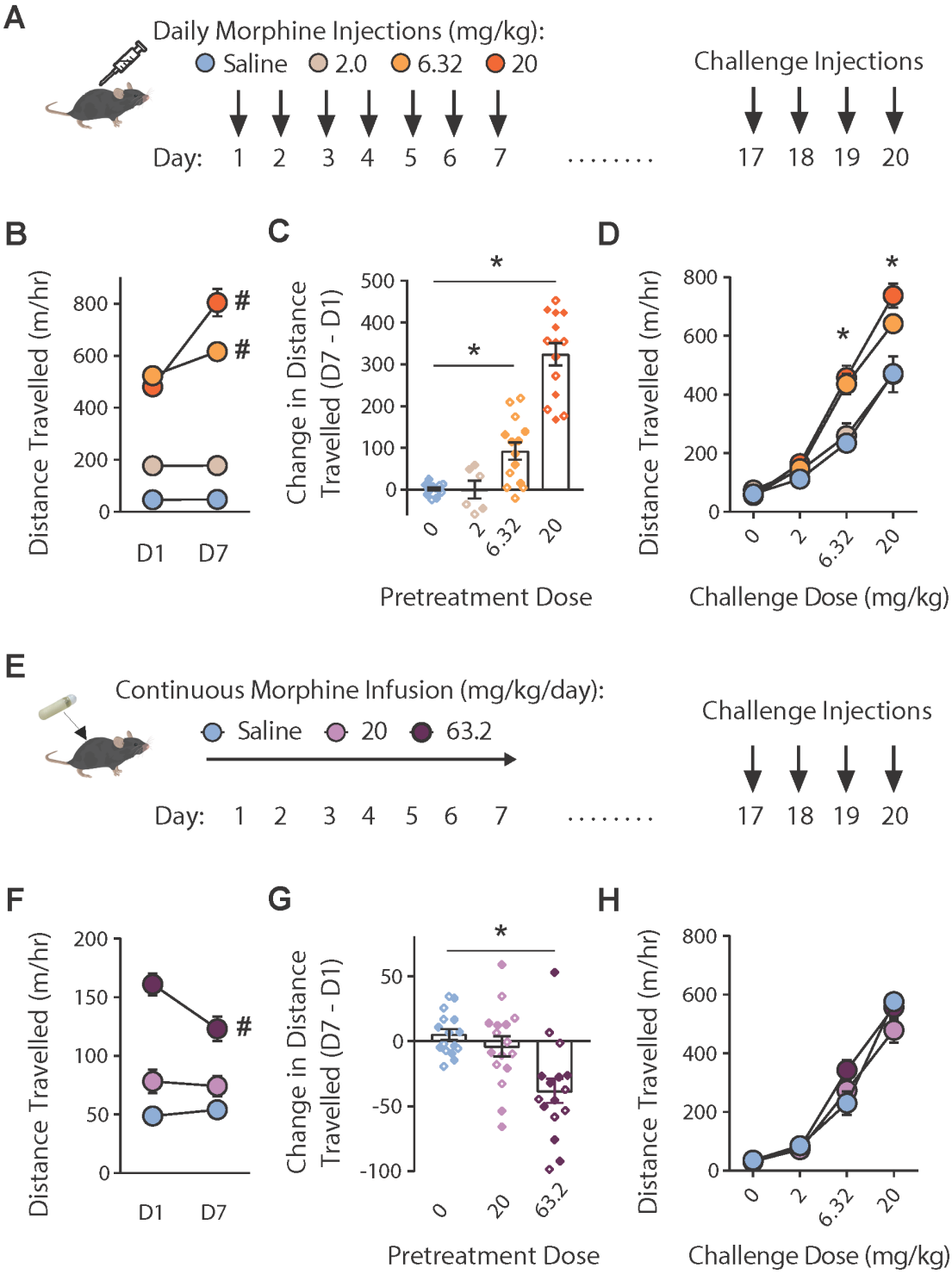

Figure 1. Comparing intermittent injection and continuous infusion of morphine. ***(A)*** *Intermittent exposure consisted of seven daily injections of morphine, followed 10 days later by challenge with injection of escalating morphine doses (n=6-14/group).* ***(B)*** *Locomotor activity after the first (D1) and last (D7) day of exposure.* ***(C)*** *Change in locomotor activity on D7 versus D1, depicted for individual mice at each dose.* ***(D)*** *Locomotor activity following challenge injections with ascending doses of morphine.* ***(E)*** *Continuous infusion of morphine via osmotic minipump for seven days, followed 10 days later by challenge with injection of escalating morphine doses (n=8/group).* ***(F)*** *Locomotor activity on the first (D1) and last (D7) day of exposure.* ***(G)*** *Change in locomotor activity on D7 versus D1, depicted for individual mice at each dose.* ***(H)*** *Locomotor activity following challenge injections with ascending doses of morphine. *p<0.05 between groups, Dunnett post-hoc test. #p<0.05 for simple effect within group.*

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drug was delivered by intermittent injection (Figure 1B; main effect of Dose: $F_{3,40}$ = 105.26, p < .001) or continuous infusion (Figure 1F; main effect of Dose: $F_{2,42}$ = 40.83, p < .001). As expected, the magnitude of psychomotor activation on day 1 was greater after bolus morphine injection: distance travelled after 20 mg/kg morphine was >6 times higher after injection (524 ± 15 m/hr) versus infusion (78 ± 10 m/hr). The same pattern was observed for serum morphine levels (Supplemental Figure S1A). For both acute psychomotor activation and serum morphine levels, the largest infusion dose (63.2 mg/kg/day) produced an effect approaching but still lower than the smallest injection dose (2 mg/kg).

After one week of chronic morphine exposure, intermittent injection and continuous infusion caused opposite forms of psychomotor adaptation. Daily injection caused psychomotor sensitization, which was most robust at higher doses (Figure 1C; Dose x Day interaction: $F_{3,40}$ = 53.85, p < .001). Conversely, continuous infusion caused psychomotor tolerance, which was also most robust at the highest dose (Figure 1G; Dose x Day interaction: $F_{2,42}$ = 8.94, p = .001). To examine the persistence of psychomotor tolerance and sensitization, we waited 10 days after the end of chronic treatment, and then challenged all groups with injection of ascending morphine doses. Psychomotor sensitization caused by intermittent injection persisted during this challenge test (Figure 1D; Pretreatment Dose x Challenge Dose interaction: $F_{7.49,99.89}$ = 9.19, p < .001), whereas the psychomotor tolerance caused by continuous infusion did not persist (Figure 1H; Pretreatment Dose x Challenge Dose interaction: $F_{5.75,51.73}$ = 2.00, p = .085).

We also tested thermal antinociception on the first and last day of chronic morphine exposure (Supplemental Figure S1B). Both intermittent injection and continuous infusion caused dose-dependent antinociception on the first day of morphine exposure, and the magnitude of this effect diminished over days, indicating the development of antinociceptive tolerance. These results show that different patterns of chronic morphine exposure cause similar adaptations in some behavioral responses (e.g., antinociception), but divergent adaptations in other behaviors (e.g., psychomotor activation), because these behaviors are mediated by adaptations in distinct neural circuits. Our data also highlight the difficulty in simultaneously controlling peak drug level and total dose when comparing injections and infusions, motivating the development of a new model to facilitate rigorous analysis of morphine exposure pattern.

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Comparison between Continuous and Interrupted Infusion of Morphine

Our next strategy to compare different patterns of morphine exposure was inspired by a classic study of neonatal opioid exposure pattern in rats (29), which used daily injections of a competitive opioid receptor antagonist to interrupt the action of a long-acting opioid receptor agonist. We implemented this approach to interrupt the continuous activation of opioid receptors produced by subcutaneous morphine infusion (63.2 mg/kg/day), to gain control of total morphine dose as well as peak morphine level. We administered two injections of naloxone each day (Figure 2A), separated by an interval of two hours (29). We selected a high dose of naloxone (10 mg/kg) to fully interrupt activation of opioid receptors by morphine, as preliminary experiments showed this naloxone dose had more robust effects than lower doses, including weight loss following each pair of daily injections (Supplemental Figure S2).

In open field tests of locomotor activity (Figure 2B), we observed psychomotor tolerance in control mice receiving continuous morphine exposure (i.e., daily injections of saline). However, in mice receiving interrupted morphine exposure (i.e., daily injections of naloxone), the direction of behavioral plasticity was reversed and we instead observed psychomotor sensitization (Morphine x Naloxone x Day interaction: $F_{1,86} = 10.32$, $p = .002$). When we calculated the change in distance travelled on Day 7 versus Day 1 (Figure 2C), we noted that the degree of sensitization after interrupted morphine was numerically larger in males (71.64 $\pm$ 29.11 m/hr) than in females (17.12 $\pm$ 12.25 m/hr). However, there were no significant main effects or interactions involving sex (Supplemental Table S2). Serum morphine levels were equivalent between groups on the last day of chronic morphine exposure (Figure 2D), and the development of antinociceptive tolerance was not affected by daily injections of naloxone (Figure 2E), making the divergent psychomotor plasticity particularly striking. We also confirmed that psychomotor sensitization and other behavioral effects of interrupted morphine were absent in mu opioid receptor knockout mice (Supplemental Figure S3), as previously reported for intermittent morphine injections (28, 42). Psychomotor sensitization is thus a common behavioral consequence of both interrupted and intermittent morphine exposure that depends upon mu opioid receptor signaling.

To examine the persistence of psychomotor sensitization after interrupted morphine exposure, we removed pumps on the final day of chronic exposure and tested locomotor activity 24 hours later. Distance travelled after saline challenge was decreased in both morphine groups, reflecting a state of spontaneous

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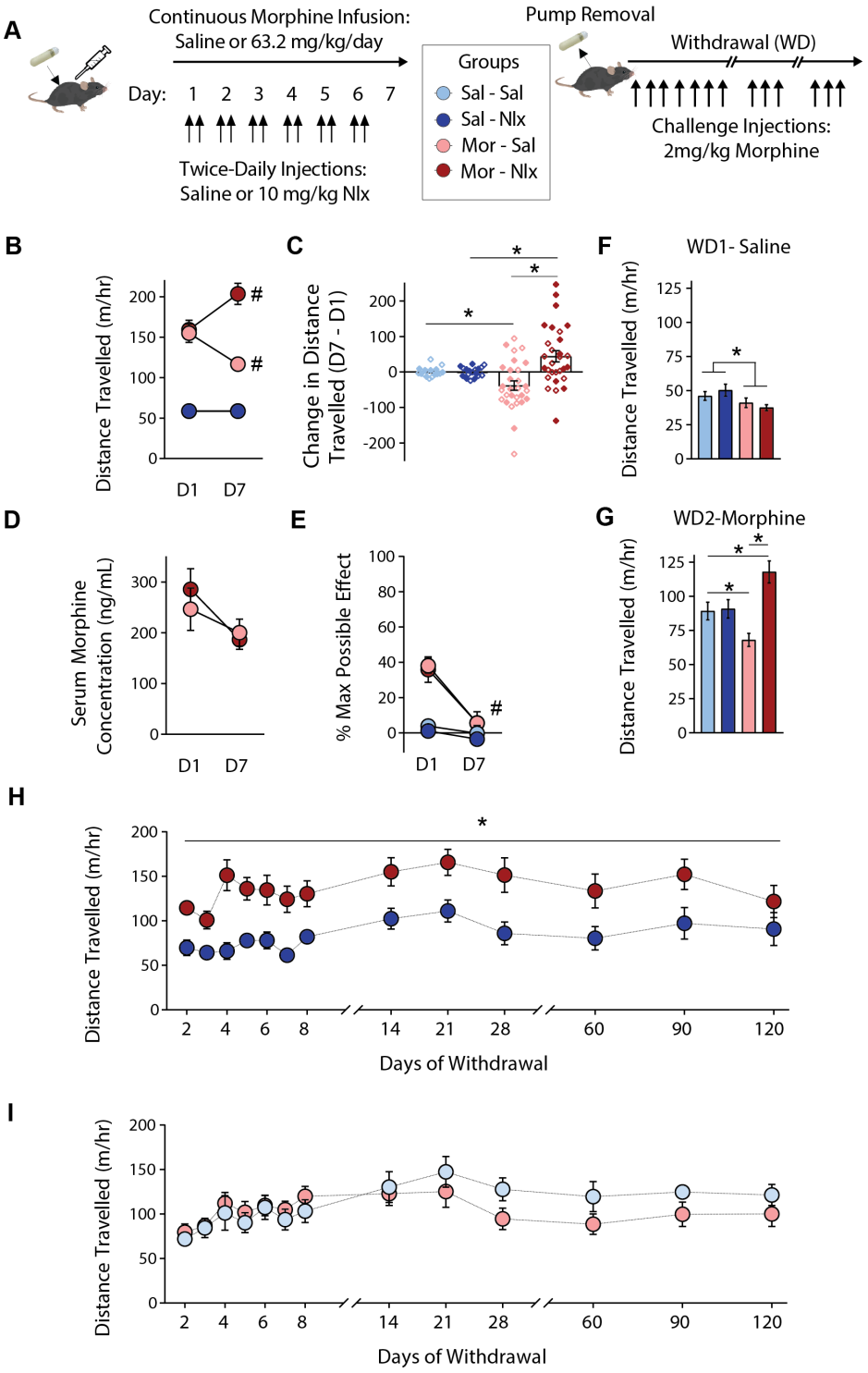

Figure 2. Interruption continuous morphine exposure with daily naloxone injections. ***(A)*** *Continuous infusion of morphine or saline for one week, interrupted by twice-daily injections (separated by 2 hours) of saline or naloxone. followed by challenge injections of morphine (n=18-29/group).* ***(B)*** *Locomotor activity on the first (D1) and last (D7) day of exposure; note that data points from Sal-Sal and Sal-Nlx groups are overlaid.* ***(C)*** *Change in locomotor activity on D7 versus D1, depicted for individual mice at each dose. *p<0.05 between groups, Tukey post-hoc test.* ***(D)*** *Serum morphine concentrations taken on D1 and D7 (n=5/group).* ***(E)*** *Thermal antinociception on the hot plate, measured on D1 and D7. #p<0.05 for simple effect within group.* ***(F)*** *Open field locomotor activity 24 hours following pump removal. *p<0.05, main effect of Morphine.* ***(G)*** *Locomotor response to morphine challenge 48 hours after pump removal. *p<0.05 between groups, Tukey post-hoc test.* ***(H-I)*** *Locomotor response to morphine challenges during extended withdrawal in groups that previously received daily injections of naloxone (**H**) or saline (**I**) (n=12/group). * p<0.05, main effect of Morphine.*

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withdrawal (Figure 2F; main effect of Morphine: $F_{1,40} = 12.16$, $p = .001$). The next day, all mice were challenged with injection of 2 mg/kg morphine (Figure 2G). Psychomotor activation was blunted by previous exposure to continuous morphine, while sensitization persisted after interrupted morphine (Morphine x Naloxone interaction: $F_{1,64} = 13.37$, $p = .001$), suggesting both effects last for 48 hours after chronic exposure. To map the time course of this effect, a subset of mice continued receiving 2 mg/kg morphine challenges, as repeated exposure to this dose causes negligible psychomotor plasticity (Figure 1C). We again observed a significant Morphine x Naloxone interaction ($F_{1,39} = 13.97$, $p < .001$), and thus separately analyzed the simple effect of morphine in each injection group. In mice that received naloxone injections, the psychomotor sensitization produced by interrupted morphine exposure was evident in response to daily and weekly challenges, but then gradually diminished over subsequent months (Figure 2H; main effect of Morphine: $F_{1,17} = 14.21$, $p = .002$). In mice that received saline injections, the psychomotor tolerance produced by continuous morphine dissipated almost immediately (Figure 2I; main effect of Morphine: $F_{1,18} = 1.23$, $p = .28$). The durability of psychomotor sensitization after interrupted morphine exposure thus mirrors the effect of daily morphine injections (43, 44), whereas psychomotor tolerance is more transient (45).

Striatal Gene Expression after Continuous and Interrupted Morphine Exposure

The nucleus accumbens plays a key role in expression of psychomotor sensitization to morphine (46, 47) and other drugs of abuse (48), so we next compared the impact of continuous and interrupted morphine exposure on gene expression in this brain region. To minimize variability related to sex differences, we used only male mice in this experiment, since interrupted morphine caused more robust sensitization in males. At the end of chronic treatment, we dissected the nucleus accumbens as well as the dorsal striatum (Figure 3A), and used next-generation RNA sequencing to perform genome-wide transcriptional profiling. We defined differential gene expression with a fold change threshold of 15%, while controlling false discovery rate at $q < 0.05$. With these criteria, we did not detect any differential gene expression between the two control groups with saline pumps (plus or minus naloxone injections), so these groups were combined to form a single control group for all comparisons with chronic morphine exposure (Table S3).

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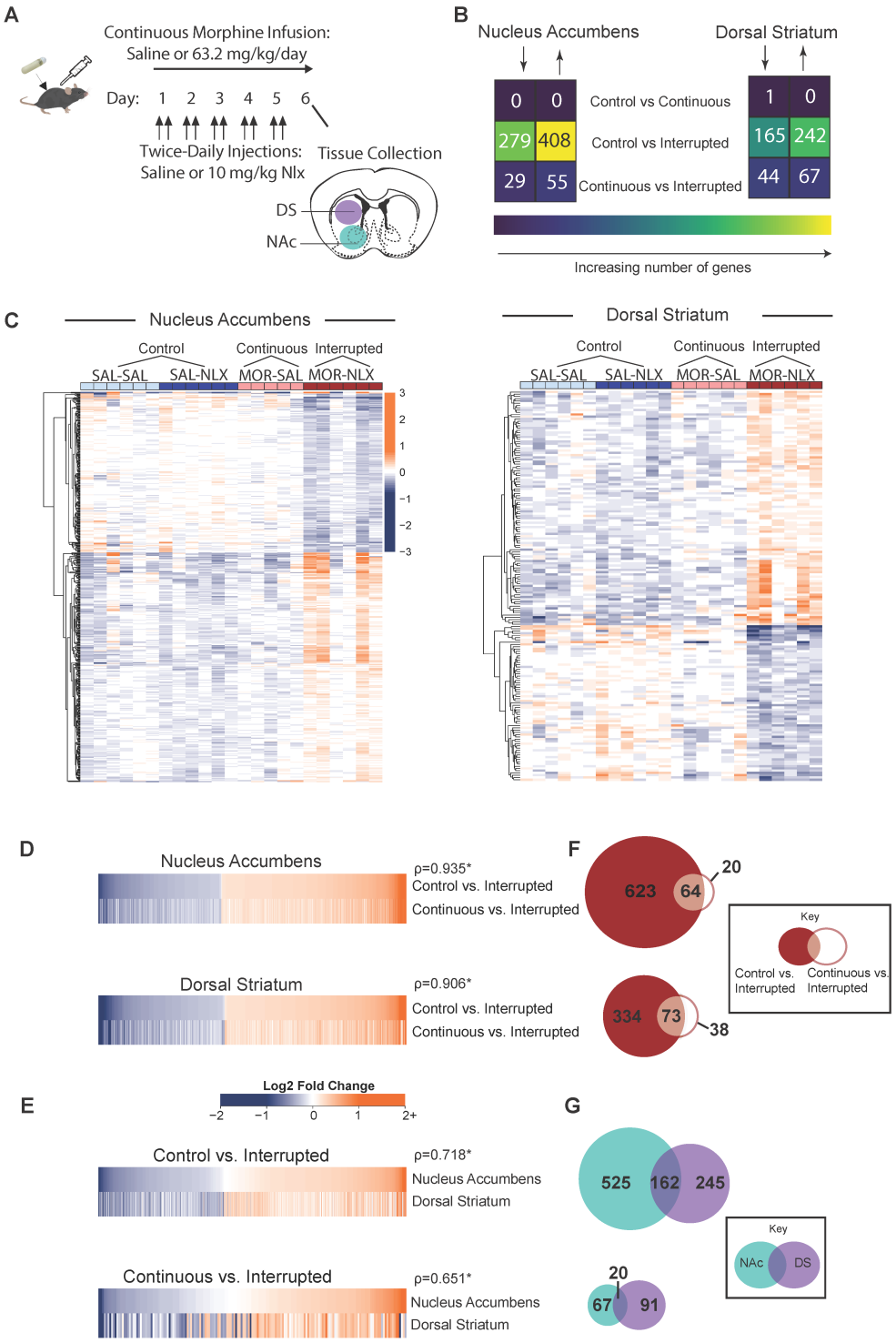

Figure 3. Differential gene expression after continuous or interrupted morphine. ***(A)*** *Microdissection of brain tissue for RNA sequencing from the nucleus accumbens (NAc) or dorsal striatum (DS), after six days of continuous or interrupted morphine exposure (n=5-6/group).* ***(B)*** *The number of differentially expressed genes (DEGs) that were significantly up- or down-regulated in each brain region; note that mice implanted with saline pumps have been combined to form a single control group.* ***(C)*** *Heatmaps showing normalized level of DEGs for each individual sample.* ***(D-E)*** *Union heatmaps showing aligned expression of genes in each brain region that were differentially expressed in comparisons between either Control vs. Interrupted or Continuous vs. Interrupted (**D**), or genes for each comparison that were differentially expressed in nucleus accumbens or dorsal striatum (**E**), aligned by fold change in the first comparison.* ***(F-G)*** *Venn diagrams showing the number of shared and unique DEGs for Control vs. Interrupted and Continuous vs. Interrupted comparisons in each brain region (**F**), or for nucleus accumbens and dorsal striatum in each comparison **(G)**.*

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Compared to the control group, interrupted morphine significantly regulated 687 transcripts in the nucleus accumbens and 407 transcripts in the dorsal striatum (Figures 3B-C). These changes are not due to residual presence of naloxone, as the last naloxone injection occurred the day before tissue collection. Surprisingly, continuous morphine significantly regulated only one gene (*Sst*) in the dorsal striatum. To further assess the impact of continuous morphine exposure, we performed a less stringent analysis using a statistical threshold of $p < 0.05$, while maintaining a fold change threshold of 15% (Supplemental Figure S4). With these criteria, continuous morphine significantly regulated 112 genes in the nucleus accumbens and 294 genes in the dorsal striatum, comparable to previous studies of continuous morphine exposure (49). Interrupted morphine still had substantially greater impact, significantly regulating 1,389 and 1,382 genes in the nucleus accumbens and dorsal striatum, respectively. Using our original statistical criteria ($q < 0.05$), direct comparison of continuous and interrupted morphine (Figure 3B) also revealed differential gene expression in both the nucleus accumbens (84 transcripts) and dorsal striatum (111 transcripts). Both of these groups were exposed to identical total doses and peak levels of morphine, making these differences particularly striking.

We conducted additional analyses of the gene sets regulated by interrupted morphine in comparison to either continuous morphine or control. First, we used a union heat map to align transcripts that were differentially expressed in either comparison, and sorted by fold change after interrupted morphine (Figure 3D). To quantify the degree of similarity in these patterns, we calculated Spearman's rank correlation coefficient, which was very high in both the nucleus accumbens ($\rho = 0.935$, $p < .001$) and dorsal striatum ($\rho = 0.906$, $p < .001$). We also used union heat maps to visualize the differentially expressed genes between the nucleus accumbens and dorsal striatum (Figure 3E), and found significant positive correlations when comparing interrupted morphine to control ($\rho = 0.718$, $p < .001$), as well as interrupted versus continuous morphine ($\rho = 0.651$, $p < .001$).

Second, we generated Venn diagrams of differentially expressed transcripts, which showed that the majority of genes regulated by interrupted versus continuous morphine were also regulated in comparison to control (Figure 3F). Outside of the intersection between these comparisons, a number of genes were significantly regulated by interrupted morphine in comparison to control, but not continuous morphine. Visual inspection of the union heat maps show that all of these genes tended to change in the same direction when comparing interrupted versus continuous morphine (Figure 3D), even if this tendency was not statistically significant. There

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was less overlap in differentially expressed genes between brain regions than between treatment comparisons (Figure 3G), consistent with a lower degree of correlation in union heat maps. Together, these results indicate interrupted morphine exacerbates some of the effects of continuous morphine, while also causing unique changes not produced by continuous morphine, with both effects more robust in the nucleus accumbens.

Genes and Pathways Regulated by Interrupted Morphine Exposure

We visualized individual up- and down-regulated genes using volcano plots, with fold change as the abscissa and statistical reliability (q-value) as the ordinate. Note that interactive version of these volcano plots have been included as electronic supplemental material. In both the nucleus accumbens (Figure 4A) and dorsal striatum (Figure 4B), two of the most dramatic effects of interrupted morphine versus control were downregulation of Rbm3 and Cirbp. Continuous morphine exposure was previously reported to downregulate both of these genes in the nucleus accumbens (49). The degree of Rbm3 and Cirbp downregulation was significantly greater when the morphine exposure was interrupted versus continuous, in both nucleus accumbens (Figure 4E) and dorsal striatum (Figure 4F). Changes in Rbm3 expression were validated using quantitative RT-PCR on tissue from an independent cohort of male and female mice (Supplemental Figure S5A), highlighting a molecular consequence of continuous morphine that is exacerbated when exposure is interrupted.

In the nucleus accumbens, Gabra2 was significantly upregulated by interrupted morphine, in comparison to either control (Figure 4A) or continuous morphine (Figure 4E). Gabra2 was also significantly upregulated by interrupted morphine in the dorsal striatum, in comparison to either control (Figure 4B) or continuous morphine (Figure 4F). In the nucleus accumbens, additional GABA and glycine receptor subunits were upregulated by interrupted morphine in comparison to control (Gabrb2 and Glra2) or continuous morphine (Glra3). We also noted that several glutamate receptor subunits (Gria4, Grin2d, Grin3a) were upregulated by interrupted morphine, but only in comparison to control in the nucleus accumbens. These changes are consistent with plasticity of excitatory and inhibitory synaptic transmission caused by intermittent morphine exposure (50-52).

For unbiased identification of other signaling pathways regulated by interrupted morphine exposure, we used Ingenuity Pathway Analysis (Qiagen, Frederick, MD). Several canonical pathways (unfolded protein response, endoplasmic reticulum stress, aldosterone signaling in epithelial cells) were significantly regulated in

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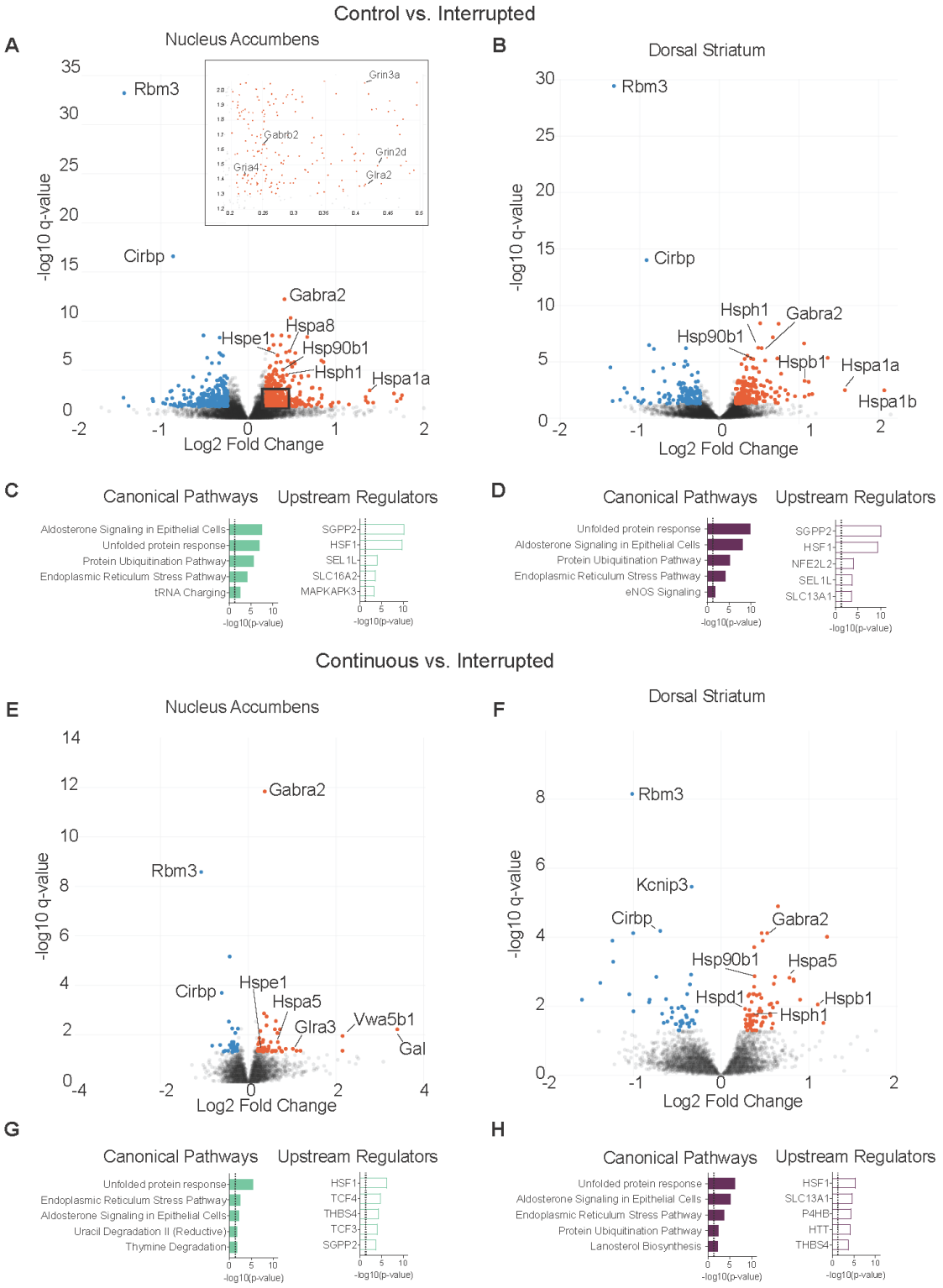

Figure 4. Characterization of differentially expressed genes. ***(A-B)*** *Volcano plots of significantly up- and down-regulated genes in the comparison between Control vs. Interrupted morphine, from either nucleus accumbens* ***(A)*** *or dorsal striatum* ***(B)****; inset shows magnification of highlighted region.* ***(C-D)*** *Bar graphs showing the top five canonical pathways (left) and upstream regulators (right) identified by Ingenuity Pathway Analysis for nucleus accumbens* ***(C)*** *or dorsal striatum* ***(D)****.* ***(E-F)*** *Volcano plots of significantly up- and down-regulated genes in the comparison between Continuous vs. Interrupted morphine, from either nucleus accumbens* ***(E)*** *or dorsal striatum* ***(F)****.* ***(G-H)*** *Bar graphs showing the top five canonical pathways (left) and upstream regulators (right) identified by Ingenuity Pathway Analysis for nucleus accumbens* ***(G)*** *or dorsal striatum* ***(H)****. Dotted line on bar graphs indicates p=0.05.*

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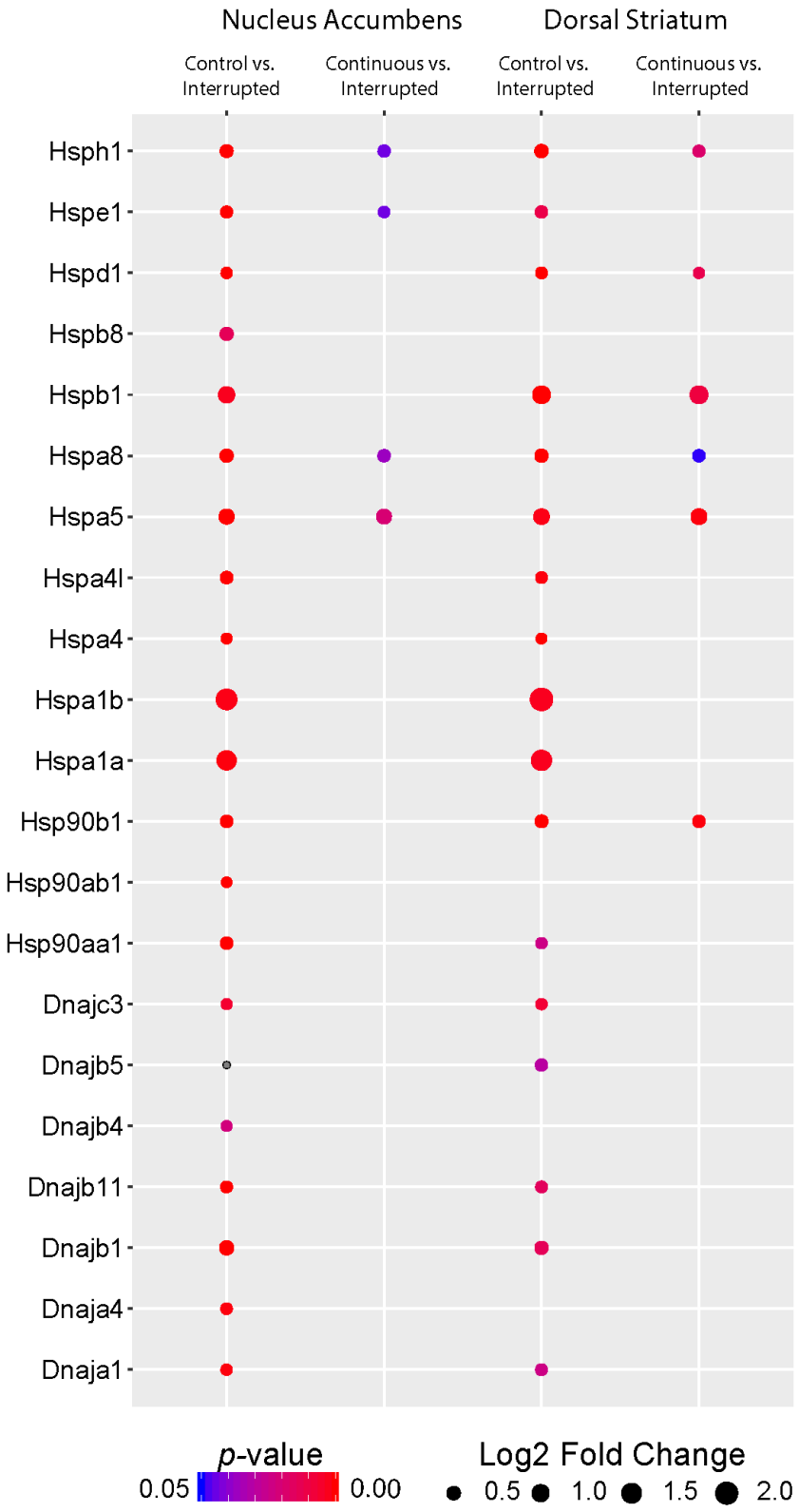

Figure 5. Upregulated transcription of heat shock proteins after interrupted morphine exposure. *Columns represent individual comparisons between interrupted morphine and either control or continuous morphine for each brain region. Rows represent individual transcripts, with dot size representing fold change and dot color indicating statistical significance.*

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all comparisons of interest across both striatal subregions (Figure 4). Changes in similar pathways (e.g., protein folding, protein processing in the endoplasmic reticulum) were found using gene ontology analysis (Supplemental Figures S6-8). The majority of individual molecules causing significant changes in these pathways were heat shock proteins (Table S4). In comparison to control, interrupted morphine upregulated transcripts encoding numerous individual heat shock proteins in the nucleus accumbens and dorsal striatum, with several also upregulated in the comparison between interrupted and continuous morphine (Figure 5). We validated changes in several of these transcripts using quantitative RT-PCR on tissue from an independent cohort of male and female mice (Supplemental Figure S5B). Significant changes in two top upstream regulators, heat shock transcription factor 1 (HSF1) and sphingosine-1-phosphate phosphatase 2 (SGPP2), were also driven primarily by heat shock proteins (Table S5). Increased transcription of heat shock proteins therefore represents a unique consequence of interrupted morphine exposure, as previously reported after daily injections of morphine (53), but not after continuous morphine exposure (49).

Weighted Gene Coexpression Network Analysis

As a complementary method to identify patterns of transcriptional regulation caused by interrupted morphine exposure, we analyzed RNA sequencing data from the nucleus accumbens and dorsal striatum using weighted gene co-expression network analysis (54). This computational method identifies groups of genes or “modules” that exhibit correlated patterns of expression across samples and treatment conditions. Importantly, these modules are identified using gene expression data from the entire transcriptome, without a need to set binary thresholds for differential gene expression. This analysis generated 18 consensus modules from the nucleus accumbens and dorsal striatum data sets (Figure 6A), which were given arbitrary color names.

The magnitude of gene regulation within each module can be summarized by an eigengene value calculated for each individual sample. We performed one-way analysis of variance on these eigengene values, comparing the control group with continuous and interrupted morphine treatment. Following a Bonferroni correction for multiple comparisons across all 18 modules, the magenta module showed significant regulation in both nucleus accumbens and dorsal striatum, while the turquoise and lime modules were only significantly regulated in the nucleus accumbens (Figure 6A). In all three modules, large eigengene values were only

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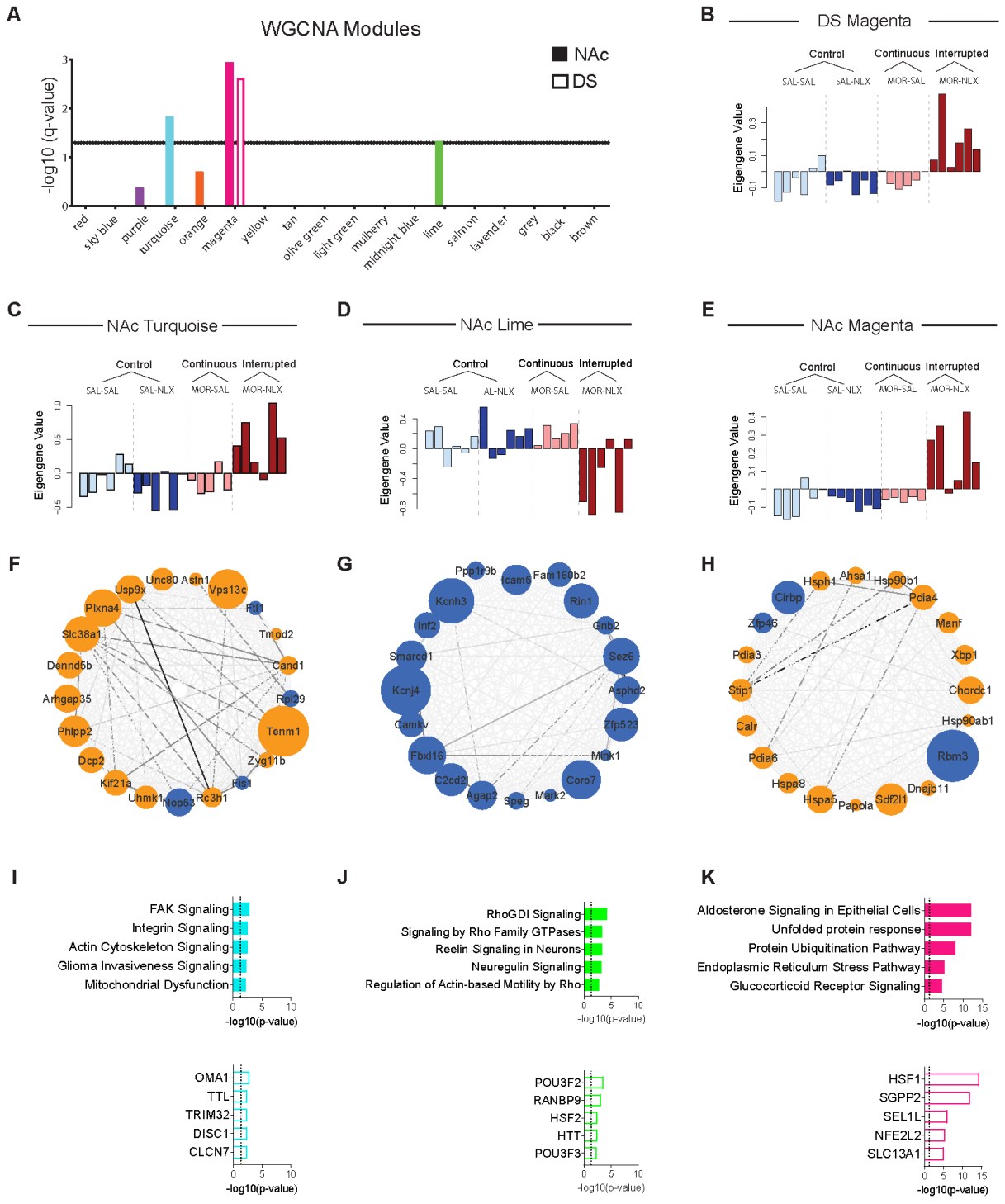

Figure 6. Weighted gene co-expression network analysis. (A-E) *Consensus modules identified across the nucleus accumbens and dorsal striatum, with bars depicting Bonferroni-corrected p-value from a one-way ANOVA on eigengene values across treatment conditions* ***(A)****. Note that colors used to identify each module are arbitrary. Eigengene values for individual samples from the dorsal striatum* ***(B)*** *and nucleus accumbens* ***(C-E)****, are shown for the magenta module* ***(B & E)****, turquoise module* ***(C)****, and lime module* ***(D)****.* ***(F-G)*** *Top 20 connected genes in the turquoise module* ***(F)****, lime module* ***(G)****, and magenta module* ***(H)****, with node size scaled by fold change in the Control vs. Interrupted Morphine comparison, and node color indicating upregulation (orange) or downregulation (blue). The weight of edges connecting nodes is scaled such that strongest connections are darkest in color.* ***(I-K)*** *Ingenuity Pathway Analysis of canonical pathways (top) and upstream regulators (bottom) for genes in the turquoise module* ***(I)****, lime module* ***(J)****, and magenta module* ***(K)****.*

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observed after interrupted morphine, while eigengene values after continuous morphine group were similar to control values (Figure 6B-E). The fact that more modules are significantly regulated in the nucleus accumbens versus the dorsal striatum corroborates our analysis of differential gene expression (Figure 3B).

We generated connectivity diagrams to visualize “hub” genes with the highest degree of connectivity within each module (Figure 6F-H and Table S4). In the magenta module, heat shock proteins were again represented among these hub genes, including the two hub genes with greatest connectivity (Hspa5 and Hspa8). Cirbp and Rbm3 were also among the 20 most connected hub genes in the magenta module (Figure 6H). Ingenuity Pathway Analysis of magenta module genes (Figure 6K) identified many of the same canonical pathways (unfolded protein response, endoplasmic reticulum stress, aldosterone signaling in epithelial cells) and upstream regulators (HSF1, SGPP2) detected in our analysis of differentially expressed genes (Figure 4). Genes in the turquoise and lime modules (Figure 6I-J) implicated additional signaling pathways in the nucleus accumbens, including upregulation of genes involved in FAK and actin cytoskeleton signaling (turquoise module), as well as downregulation of genes involved in signaling by Rho family GTPases (lime module). These results support the conclusion that interruption of continuous morphine exposure exacerbates drug-evoked adaptations in the nucleus accumbens, and provide convergent evidence that heat shock signaling may contribute to the consequences of intermittent opioid exposure.

DISCUSSION

The neurobehavioral impact of chronic opioid exposure has been investigated using a wide variety of drug administration regimens. Our data provide clear evidence that the pattern of opioid administration can fundamentally alter drug-evoked adaptations in brain and behavior. Using psychomotor activity as a proxy indicator of adaptations in the mesolimbic dopamine system (55), we first replicated prior work showing robust psychomotor sensitization after intermittent morphine injections (24-27), while continuous morphine infusion caused psychomotor tolerance. The interruption of continuous opioid receptor stimulation with daily antagonist injections caused a striking behavioral switch, from transient psychomotor tolerance to durable psychomotor sensitization. This behavioral switch was associated with dramatic changes in gene expression in the nucleus accumbens and dorsal striatum, measured using next-generation RNA sequencing.

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Our results are consistent with classic studies comparing intermittent injection and continuous infusion of cocaine or amphetamine at a fixed daily dose. Psychostimulants also cause sensitization of dopamine-related behaviors after intermittent but not continuous exposure (56-60), with corresponding changes in dopamine release (61, 62) and receptor function (63-66). However, our data clearly illustrate this comparison is confounded by large differences in peak drug level, which is much higher following a bolus injection than after continuous infusion of the same dose. Multiple daily morphine injections at escalating doses also produce different effects than single daily injections at a fixed dose (24-26, 67, 68), but these treatment regimens differ with respect to cumulative drug exposure. It is thus inherently challenging to manipulate the pattern of drug exposure while controlling pharmacokinetic factors like peak drug level and cumulative dose. This challenge extends to studies of psychostimulant self-administration, where accumulating evidence indicates intermittent access sensitizes the mesolimbic dopamine system and enhances drug seeking/taking (69-77), but it remains impossible to precisely control peak drug level and total intake when drug delivery is not controlled by the experimenter.

To overcome these challenges, we developed a new strategy to interrupt the continuity of opioid receptor stimulation during morphine infusion, through daily administration of a competitive antagonist (naloxone). This manipulation produced psychomotor sensitization, whereas uninterrupted morphine exposure caused psychomotor tolerance, confirming that the relative continuity or intermittency of chronic morphine exposure dictates the form of psychomotor adaptation. Importantly, naloxone treatment had no effect on the development of antinociceptive tolerance or serum morphine level, confirming tight control of peak drug level as well as cumulative dose. The psychomotor sensitization that developed during interrupted morphine exposure also persisted for weeks to months following pump removal, and this durability contrasted with dissipation of psychomotor tolerance in a matter of days. The transient nature of psychomotor tolerance suggests it involves neurobiological adaptations that are not primarily responsible for persistent features of addiction, such as vulnerability to relapse.

The divergent behavioral effects of continuous and interrupted morphine exposure were mirrored at the level of gene expression in the nucleus accumbens and dorsal striatum. We collected tissue for RNA sequencing on the last day of chronic morphine exposure, when the continuous and interrupted morphine groups exhibited opposite changes in psychomotor response, despite identical serum morphine levels and cumulative dosing. In

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both the nucleus accumbens and dorsal striatum, the number of differentially expressed transcripts was substantially greater after interrupted versus continuous morphine. In fact, after controlling for false discovery rate, no genes in the nucleus accumbens and only one gene in the dorsal striatum reached statistical criteria for differential expression after continuous morphine. The impact of continuous morphine on striatal gene expression is thus less severe than interrupted morphine, as previously reported for chronic morphine treatment and morphine withdrawal in other brain regions (78).

A number genes that were differentially expressed after interrupted morphine encode synaptic receptors for glutamate (Gria4, Grin2d, Grin3a), GABA (Gabra2, Gabrb2), and glycine (Glra2, Glra3). This is consistent with well-established effects of chronic drug exposure on synaptic transmission in the nucleus accumbens (8), including opioid-evoked plasticity at excitatory and inhibitory synapses (50-52). The WCGNA turquoise and lime modules, which were only significantly regulated in the nucleus accumbens, include signaling molecules related to the actin cytoskeleton as well as FAK, integrin, and Rho signaling, These signaling pathways are linked to addiction (79, 80) and could contribute to structural remodeling of synaptic connections caused by morphine (51, 81). The robust upregulation of Gabra2 by interrupted morphine exposure is notable because polymorphisms in this gene are associated with heroin dependence in humans (82). This upregulation of Gabra2 we observe following interrupted morphine contrasts with the downregulation previously reported in the nucleus accumbens after continuous morphine (52), further highlighting the differential impact of these two patterns of exposure.

Interrupted morphine exposure also upregulated a number of genes encoding heat shock proteins, while robustly downregulating transcription of two cold shock proteins, Rbm3 and Cirbp. Genes encoding heat and cold shock proteins were all contained within the WCGNA magenta module, indicating correlated expression across samples and treatments. Changes in expression of both heat and cold shock proteins have previously been reported in striatal tissue following opioid treatment (49, 83-87). These gene expression changes could be related to opioid-induced temperature fluctuations in the nucleus accumbens (88), though they may also indicate a general response to cellular stress that is independent of temperature. Striatal expression of heat shock proteins is tied to psychomotor sensitization, withdrawal, and other behavioral responses to opioids (85, 89-94), supporting functional relevance of the transcriptional changes we observe.

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Ingenuity Pathway Analysis identified similar canonical pathways, including unfolded protein response and endoplasmic reticulum stress, using either differentially expressed gene lists or the WCGNA magenta module. These pathways were enriched in heat shock proteins that also predicted activation of heat shock transcription factor 1 (HSF1) as a top upstream regulator. HSF1 is a DNA-binding protein activated by high temperature and other forms of cellular stress, and regulates expression of heat shock proteins and other molecular chaperones (95). HSF1 has long been implicated in cancer, and has more recently been linked to degeneration of striatal medium spiny projection neurons in Huntington's disease (96). HSF1 has not previously been investigated in the context of addiction, but could be targeted using therapeutic interventions currently under development for cancer and neurodegenerative disease (97, 98). We are currently conducting experiments to determine whether genetic manipulation of HSF1 in mice alters responsiveness to opioid administration.

A key question arising from our data is whether the effects of interrupted opioid exposure are relevant to abuse and addiction. A large body of research has demonstrated that periodic interruption of alcohol exposure exacerbates detrimental effects (99), leading to widespread implementation of chronic intermittent exposure models. Similarly, we propose that human opioid abuse is a fundamentally intermittent activity, routinely interrupted by periods of sleep or limited drug supply (100). Patients taking prescription opioids purported to provide continuous pain relief also frequently report gaps in drug action (101). This latter scenario may be most analogous to our model of interrupted exposure, in that opioid use is not initially motivated by positive reinforcement, but may sensitize brain reward systems and leave individuals vulnerable to subsequent drug abuse and addiction. Our data show that interruption of continuous opioid administration exacerbates transcriptional changes in brain reward circuits, producing an increased behavioral sensitivity to opioids that persist for months. Maintaining continuity of chronic opioid administration may therefore represent a strategy to minimize detrimental effects on brain reward circuits, consistent with the low abuse liability of methadone and therapeutic benefits of maintenance therapy with long-lasting agonists (102). This may in turn reduce the risk of progression from opioid use to abuse, promoting safer use of opioids for clinical indications and helping to combat the opioid epidemic.

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DISCLOSURES

The authors declare no competing financial interests.

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