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Synaptic Structure and Transcriptomic Profiling of Reward and Sensory Brain Areas in Male Mice of Fentanyl Addiction

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Background: Opioid-based medications are powerful analgesics commonly prescribed for pain management, but they are also highly addictive. The over-prescription of opioids analgesics has triggered current opioid crisis, which now has expanded to heroin and illicit synthetic opioids like fentanyl and its analogues. The side effects of fentanyl abuse have been well recognized, yet the underlying molecular adaptations across brain regions upon fentanyl exposure remain elusive.

Methods: The transmission electron microscopy (TEM) and next-generation RNA-sequencing (RNA-seq) were used to investigate the ultrastructure synaptic alterations and transcriptional profiling changes of reward and sensory brain regions in mice after fentanyl exposure.

Results: The naloxone-precipitated acute withdrawal symptoms were observed in mice exposed to fentanyl. Results of TEM showed an increase in the number of synapses, widening of synaptic gaps, and thickening of postsynaptic density in the NAc of the fentanyl addiction mice, accompanied by obvious mitochondrial swelling. RNA-seq identified differentially expressed genes (DEGs) in prefrontal cortex of mice brains after fentanyl exposure, and the expression of some addiction-related genes such as *Calm4, Cdh1, Drd1/2/3/4, F2rl2, Gabra6, Ht2cr, Oprk1* and *Rxfp3* showed the most striking changes among experimental groups. KEGG enrichment analysis indicated that these DEGs were related to the development of addiction behavior, dopaminergic/GABAergic/serotonergic synapse, synapse assembly/synaptic plasticity/synaptic vesicle cycle, cAMP/MAPK signaling pathway, neuroactive ligand-receptor interactions. These transcriptomic changes may be correlated with the structural and behavioral changes observed in fentanyl-exposed mice.

Discussion: The findings of this study contribute to a better understanding of the molecular mechanism of addiction behavior, which is essential for the development of optimized therapy strategies for addicts.

Keywords: fentanyl, addiction, synaptic plasticity, gene expression, transcriptional profiles

Introduction

Opioid-based medications are commonly used but often abused analgesics. Due to their strong addictive properties, synthetic opioids like fentanyl and its analogues have become the primary cause of the deadliest opioid crisis in the United States and elsewhere, and has become an important medical and social issue worldwide. However, the neural mechanisms behind the effects caused by fentanyl and its analogs are somewhat unknown, and there are few ways to prevent and treat the damaging effects of fentanyl abuse. Addiction is a chronic relapsing psychiatric disorder caused by the interaction of drug abuse and the brain reward system. The main manifestations of addiction are compulsive and reckless drug seeking behaviors, loss of ability to limit drug intake, and withdrawal symptoms after cessation of drug use.[1,2] Abuse of addictive drugs can cause damage to

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multiple body systems, especially the nervous system. Actually, converging evidence from many studies reveals that addictive drugs including fentanyl modify synaptic transmission in the mesocorticolimbic dopamine system by regulating synaptic plasticity and dendritic spine stability.[3,4] Moreover, the synaptic and neural circuit changes caused by a drug experience usually persist,[3–5] which lay the foundation upon which further drug-induced adaptations (withdrawal symptoms and relapse behavior) occur.

The prefrontal cortex (PFC) plays a very important role in addiction. It is the final projection area of mesocortical pathway and nucleus accumbens (NAc) related addiction circuitry in the mesolimbic dopaminergic system, which is an integral part of the brain reward system.[6,7] Studies have shown that long-term morphine consumption can cause morphological changes of NAc and PFC neuronal axons in addicted animals, and the changes in neuronal plasticity in NAc and PFC regions are strongly related to drug addiction. For example, transmission electron microscopy (TEM) study of the NAc region in heroin-addicted rats demonstrated a significant increase in synapse number, which was accompanied by mitochondrial swelling and blurring of the synaptic gaps when compared to the control group.[8] Heidari et al also reported an increase in synaptic density and postsynaptic density thicknesses in hippocampal CA1 area of morphine addicted rats.[9]

It is believed that the structural and morphological changes of synapses inevitably lead to functional changes, and are the results of changes in gene expression in relevant brain circuits. According to the literature, several important signal pathways that control neuronal excitability, synaptic plasticity, neurotransmitter transmission and neuroprotection are closely related to addictive behaviors.[10] Moreover, it has been reported that fentanyl-abusing patients are less sensitive to reversal by naloxone (the opioid antagonist). Therefore, restoring drug-induced changes in excitatory synaptic transmission and/or synaptic plasticity in PFC may provide new directions for clinical treatment of fentanyl addiction, and give guidance for selecting effective strategies for addiction intervention and fentanyl withdrawal.[11–13]

To investigate the molecular mechanisms implicated in fentanyl addiction behaviors, and to identify key genes or molecules involved in addiction and synaptic remodeling of neurons, the transcriptional changes in PFC of fentanyl-addicted mice were examined. Meanwhile, the naloxone-induced withdrawal symptoms were recorded and the ultra-structure changes of synapses in the NAc region were assessed. The identified differentially expressed genes (DEGs) in reward and sensory brain regions suggest that transcriptome adaptations may underlie the ultrastructural synaptic changes and withdrawal symptoms observed in fentanyl-exposed mice.

Materials and Methods

Experimental Animals

Thirty-two 8-week-old wild-type adult male pathogen-free C57BL/6 mice (18–25 g) were provided by the Laboratory Animal Center of Zhejiang Chinese Medical University. Four animals were raised in each cage with a 12 h light/dark cycle and a room temperature of 23 ± 1°C, and all animals had free access to water and food. After a 2-week acclimation period, the mice were randomly assigned into either the fentanyl-addicted group (FA) or saline control group (SC), with 16 mice in each group. Animal husbandry and experimental procedures were approved by the Ethics Committee on Animal Experimentation of Zhejiang Chinese Medical University (approval number: IACUC-20230327-05), and carried out according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Reagents

Fentanyl citrate was obtained from the Key Laboratory of Drug Control and Monitoring, National Anti-Drug Laboratory Zhejiang Regional Center. Naloxone was purchased from Sigma-Aldrich (Shanghai, China). These drugs were diluted in sterile 0.9% saline and injected subcutaneously in total volumes of 0.01mL/g mouse (approximately 0.2mL for each mouse). The detailed concentrations were indicated below, and the drug doses were expressed as concentration of free base.

Fentanyl Exposure

For induction of drug dependency, mice in the FA group were injected with freshly dissolved fentanyl solution once a day (at 10 a.m. in the morning) for 14 consecutive days and the fentanyl doses were ascended every five days. The detailed

daily doses were 1.25 mg/kg per mouse from day 1 to day 5, 2.5 mg/kg from day 6 to day 10, and 5.0 mg/kg from day 11 to day 14. At the same time, mice in the SC group received an equal volume of saline injections for 14 days. This pattern of fentanyl administration and doses were selected based in our pre-experiments and previous publications.[14]

Naloxone Precipitated Withdrawal Behavioral Assay

To confirm fentanyl dependency, half of experimental mice in both the FA and SC groups received a subcutaneously injection of naloxone (4 mg/kg) two hours after the final fentanyl or saline injection on day 14 (at 12 a.m.), and these mice were named as FA+NX group. Then, the individual naloxone-precipitated fentanyl withdrawal behaviors were recorded for 60 min immediately following naloxone administration. These withdrawal symptoms were evaluated using criteria reported in previous study with some modification (see Table 1),[15] and totally scored as the sum of the 9 items. In these experiments, no obvious behavioral changes between saline- and saline+naloxone-treated mice were observed, therefore, both the saline- and saline+naloxone-treated mice were considered as control group in the following experiments. The same methodology was employed in the previous study.[16]

TEM

The NAc from brain tissue was cut into 1mm^3 size, and immersed in 2.5% glutaraldehyde fixative (pH 7.2) for 48h. After that, these samples were further fixed with 1% osmium tetroxide, dehydrated with ethanol gradient, embedded with epon812, and cut into ultrathin sections at 50 nm. The prepared samples were double-stained with 1% uranyl acetate and lead citrate, and then photographed by TEM (H-600, Hitachi, Japan), with an accelerating voltage of 80 kV.

Tissue Collection and RNA-Sequencing

Tissue collection from experimental mice was carried out after behavioral tests on day 14. Immediately after decapitation, PFC and NAc regions in the brains were quickly micro dissected using a 2 mm puncher, flash-frozen on dry ice, and stored at −80°C. Each group contained 3 biological repeats. All samples prepared for RNA extraction contained bilateral PFC from one animal. Total RNA was isolated using QIAzol lysis reagent and purified using the RNeasy micro kit (Qiagen, Germantown, MD, USA, Cat#55402828). The concentration and quality of total RNA were assessed using the NanoDropND-1000 (NanoDrop, Wilmington, DE, USA), and then mRNA was purified from 5 mg of total RNA using poly-T oligo magnetic beads. After two rounds of purification, the obtained mRNAs were fragmented with divalent cations, and then reverse transcribed to construct cDNA libraries using the mRNA-Seq Sample Preparation kit. The mRNA sequencing analyses were

Table 1 Scale of Opioid Dependence Withdrawal Symptoms

Symptomatic	Calculation of Points
Abnormal body position	Hair licking, face washing, shrugging, cunnilingus, standing, tail erect (2 points)
Provoke	Contact (1 point)
	Proximity (2 points)
Quiver	Discontinuous (1 point)
	Continuous (2 points)
Kowtow	Discontinuous (0.5 points)
	Continuous (1 point)
Restlessness, irritability, desire to get out of the cage	Light (0.5 points)
	Obvious (1 point)
Shed tears	Appearance of teardrops (4 points)
Have the runs	Soft stools (4 points)
	Thin stools (8 points)
Salivation	Light (1 point)
	Obvious (2 points)
Weight loss (< 1h)	< 2% (0 points); 2–4% (5 points)
	4–6% (10 points); 6–8% (15 points)

performed using the IlluminaNovaseqTM6000 (LC Bio Technology CO, Ltd. Hangzhou, China) platform and three biological repeats were set for each sample. After obtaining the raw sequencing data, the original data were firstly filtered to obtain high-quality clean data, and then these data were aligned to the reference genome of the mouse and analyzed for gene expression quantification, gene differential analysis and enrichment analysis. The criteria to define DEGs was a 50% change in the expression levels (|log 2 Fold change | > 1) and an adjusted P value < 0.05. R statistical software (version 4.0.1) was used for downstream analysis and visualization of the RNA sequencing analysis output, including but not limited to drawing heat maps and Venn diagrams. Pathways (from KEGG) and gene ontology terms (biological process, molecular function and cell compartment) with adjusted P value < 0.05 were selected for further analysis.

Correlation Analysis

The Spearman correlation analysis was performed to determine the connections between top 50 addiction-related DEGs and the synapse index changes using the R statistical programming language (version 4.2.1). After obtaining Spearman's rho values and corresponding P values with purity adjustment, the clustering correlation heatmap with signs was generated using the OmicStudio tools at http://www.omicstudio.cn, and $P < 0.05$ was set as the significance threshold. Circos analysis was carried out to show the correlations of the top 30 highly expressed genes in FA and SC groups. The open-source circos software was obtained from www.circos.ca, and data of top 30 highly expressed genes were reformatted by the R statistical programming language to comply with Circos data file requirements.

Quantitative Real Time-Polymerase Chain Reaction (qPCR)

RNA samples extracted from bilateral PFC regions by TRIzol reagent were also utilized for qRT-PCR analysis, with primer sequences listed in Table S1. The selection of genes for qRT-PCR analysis was based on their relevance to brain function and addiction behavior. A total of 1 μg of RNA was converted into complementary DNA (cDNA) using the PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara, Dalian, China). Subsequently, qPCR was performed using the PowerUp™ SYBR Green Master Mix (PE Applied Biosystems, CA, USA) on ABI Prism 7500 Sequence Detection System, under thermal cycling conditions of an initial denaturation at 95°C for 10s followed by 40 cycles of denaturation at 95°C for 10s and annealing/extension at 58°C for 30s. The *GAPDH* was chosen as the reference gene, and the relative gene expression levels were quantitatively analyzed by the $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out by using SPSS 24.0 software. Comparisons between groups were made using one-way analysis of variance (ANOVA) followed by Student's t test or Tukey's post hoc tests, and differences were considered statistically significant if $P < 0.05$.

Results

Observation of Withdrawal Symptoms

According to the protocol for generating a FA model, 16 mice received increasing amounts of fentanyl exposure for 14 days and 8 of the mice received naloxone injections on day 14. Among these mice, the naloxone precipitated acute withdrawal signs were observed, including jumping, pronounced desire to leave the cage, continuous tremor, face washing, and weight loss. These withdrawal symptoms were not evident in control mice. The withdrawal symptom scores in the SC and FA groups were 2.33 ± 0.94 and 18.33 ± 1.25, respectively, and the differences were highly significant ($P < 0.01$).

TEM Results

TEM was used to observe the ultrastructural changes of NAc in the brain. As shown in Figure 1 and Table 2, FA mice were found to have an increased number of synapses when compared with SC group ($P < 0.05$), and mitochondria in

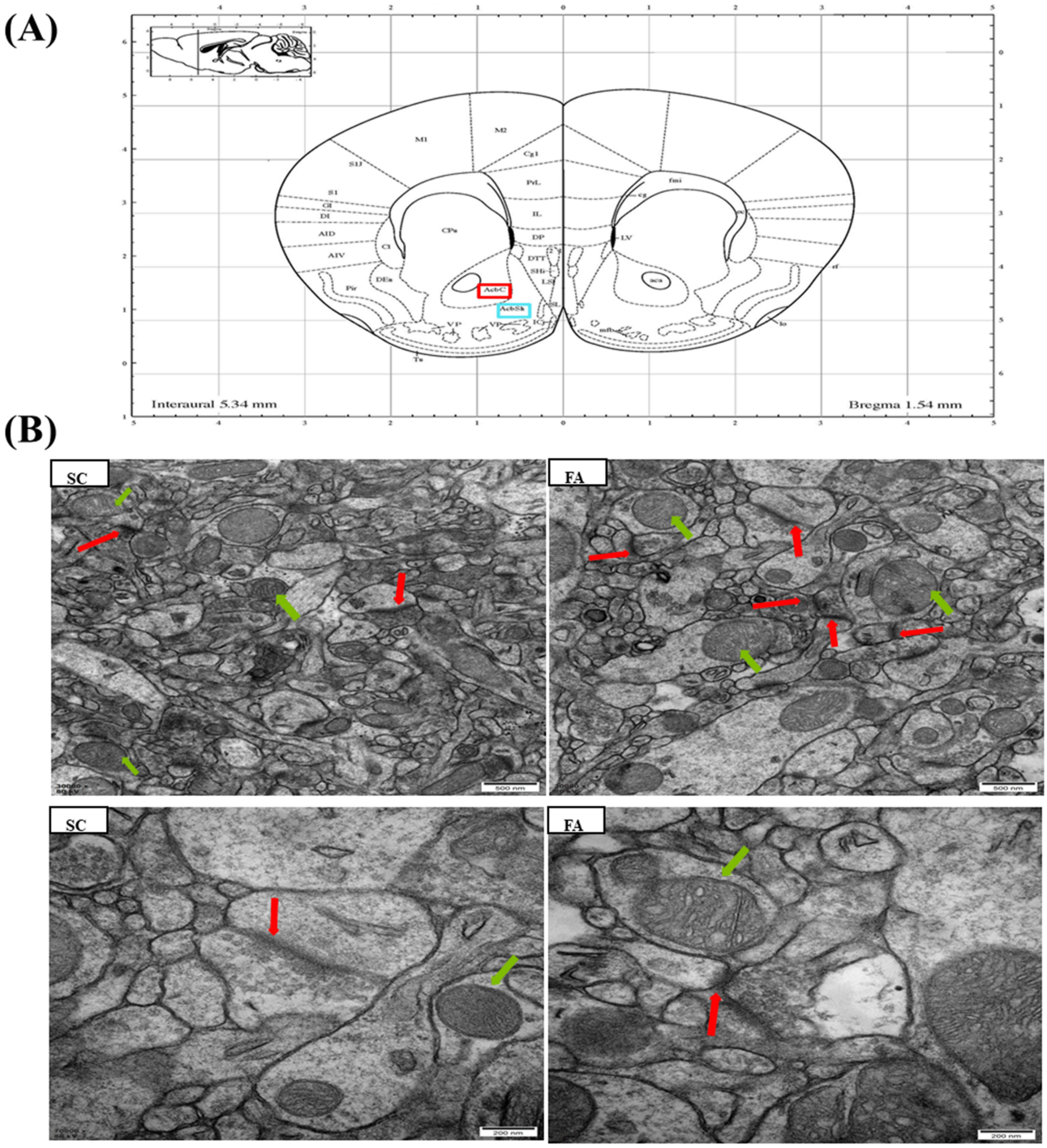

Figure 1 Synaptic ultrastructure of the nucleus accumbens of mice in SC and FA groups. (**A**) Schematic of areas within the nucleus accumbens core (AcbC; red squares) and shell (AcbSh; blue squares) analyzed at approximately Bregma 1.54 mm. (**B**) Representative TEM images of nucleus accumbens of SC and FA groups. Left, SC groups (×30,000, scale bars = 500 nm) and SC groups (×80,000, scale bars = 200 nm); Right, FA groups (×30,000, scale bars = 500 nm) and SC groups (×80,000, scale bars = 200 nm). The red arrows indicate synapses, while the green arrows indicate mitochondria.

neurons were swollen. In addition, the FA group had ruptured mitochondrial membranes (Figure 1D). Synaptic structural index showed that the synaptic gap in the FA group was wider than that in the SC group ($P < 0.05$), and the postsynaptic density was also thicker than that in the SC group ($P < 0.05$) (Table 2).

Table 2 Number of Synapses and Synaptic Structural Indices in the NAc Region of Mice

Group	Number of Synapses	Thickness of Synaptic Cleft (nm)	Thickness of Postsynaptic Density (nm)
SC(n=8)	6.00 ± 0.83*	15.97 ± 0.79*	43.65 ± 2.11*
FA(n=8)	8.33 ± 0.47*	18.50 ± 0.97*	50.41 ± 2.13*
P	<0.05	<0.05	<0.05

Note: *denotes significant alterations in synaptic indices between FA and SC groups.

Overview of Transcriptome Analysis

After filtering out the nonsensical sequences (including adaptors, poly-N, reads shorter than 150 nt and undetermined bases), the expression levels of 36,503 transcripts were obtained according to the Illumina sequencing data. Using the fold changes cutoff ratio of ≥ 2 and $P < 0.05$, 631 DEGs were identified when the FA group was compared with SC group, of which 465 genes were down-regulated and 166 genes were up-regulated (Figure 2A). And the comparison of FA+NX vs SC group yielded 493 DEGs, including 197 up-regulated transcripts and 296 down-regulated transcripts (Figure 2B).

GO and KEGG Enrichment Analysis of Differentially Expressed Genes

The identified DEGs were further assigned to GO and KEGG enrichment analysis. As shown in Figure 2C, the most significantly enriched GO terms in the comparison of FA vs SC group ($P < 0.05$) were "Integral component of membrane", "extracellular region", "dopamine neurotransmitter receptor activity", "synaptic transmission, dopaminergic" and "inflammatory response". KEGG enrichment analysis of these DEGs showed the most profoundly enriched pathways ($P < 0.05$) were "steroid biosynthesis", "neuroactive ligand-receptor interaction", "inflammatory mediator regulation of TRP channels", "ECM-receptor interaction", and "serotonergic synapse" (Figure 2D). On the other hand, the comparison of the FA+NX vs SC group revealed a significant enrichment of GO terms ($P < 0.05$) including "Integral component of membrane", "extracellular region", "neuropeptide signaling pathway", "inflammatory response", and "calcium ion binding" (Figure 2E). And the KEGG terms "neuroactive ligand-receptor interaction", "calcium signaling pathway", "focal adhesion", "ECM-receptor interaction", "PI3K-Akt signaling pathway", and "cytokine-cytokine receptor interaction" were found to be significantly enriched ($P < 0.05$) in the comparison of FA+NX vs SC (Figure 2F).

Following, the pathways closely related to synaptic plasticity and substance addition were analyzed. As summarized in Table 3, *Adora2a, Drd1/2/3, Gabra6* and *Oprk1* involved in morphine addiction and dopaminergic synapse pathways, as well as *Ntrk1* and *Ptpn7* in cAMP/MAPK signaling pathway were strikingly down-regulated in FA and FA+NX groups when compared to controls. On the other hand, the expression levels of *Drd4/5* and *Ptgs2* involved in dopaminergic/serotonergic synapse, and *F2rl2* and *Rxfp3* in neuroactive ligand-receptor interaction pathway were up-regulated in FA and FA+NX groups. For the comparison of FA vs FA+NX groups, the alteration of *Calm4, Mapk13, Slc6a3* and *Slc18a1* transcripts were most pronounced.

Correlation Analysis of Transcriptional Data with Synaptic Changes

In order to gain a deeper understanding of the relationship between DEGs and the synaptic changes, the correlation analysis was performed between number of synapses, thickness of synaptic cleft, thickness of postsynaptic density, and top 50 DEGs. As shown in Figure 3, that the expression of *Nod2* was strongly positively correlated with thickness of synaptic cleft (rho = 0.961, $P = 0.003$), and *Drd4* was strongly positively correlated with number of synapses (rho = 0.986, $P = 0.003$). On the other hand, the expression level of *Sema5b* was strongly negatively correlated with thickness of postsynaptic density (rho = −0.967, $P = 0.002$), and the expression levels of *Cysltr2, Nlrp3, Tacr1, Aplnr, Gbp2* and *Col6a3* were strongly negatively correlated with number of synapses (rho = −0.968, $P = 0.002$; rho = −0.955, $P = 0.003$; rho = −0.986, $P = 0.003$; rho = −0.921, $P = 0.008$; rho = −0.928, $P = 0.008$; rho = −0.917, $P = 0.009$; respectively).

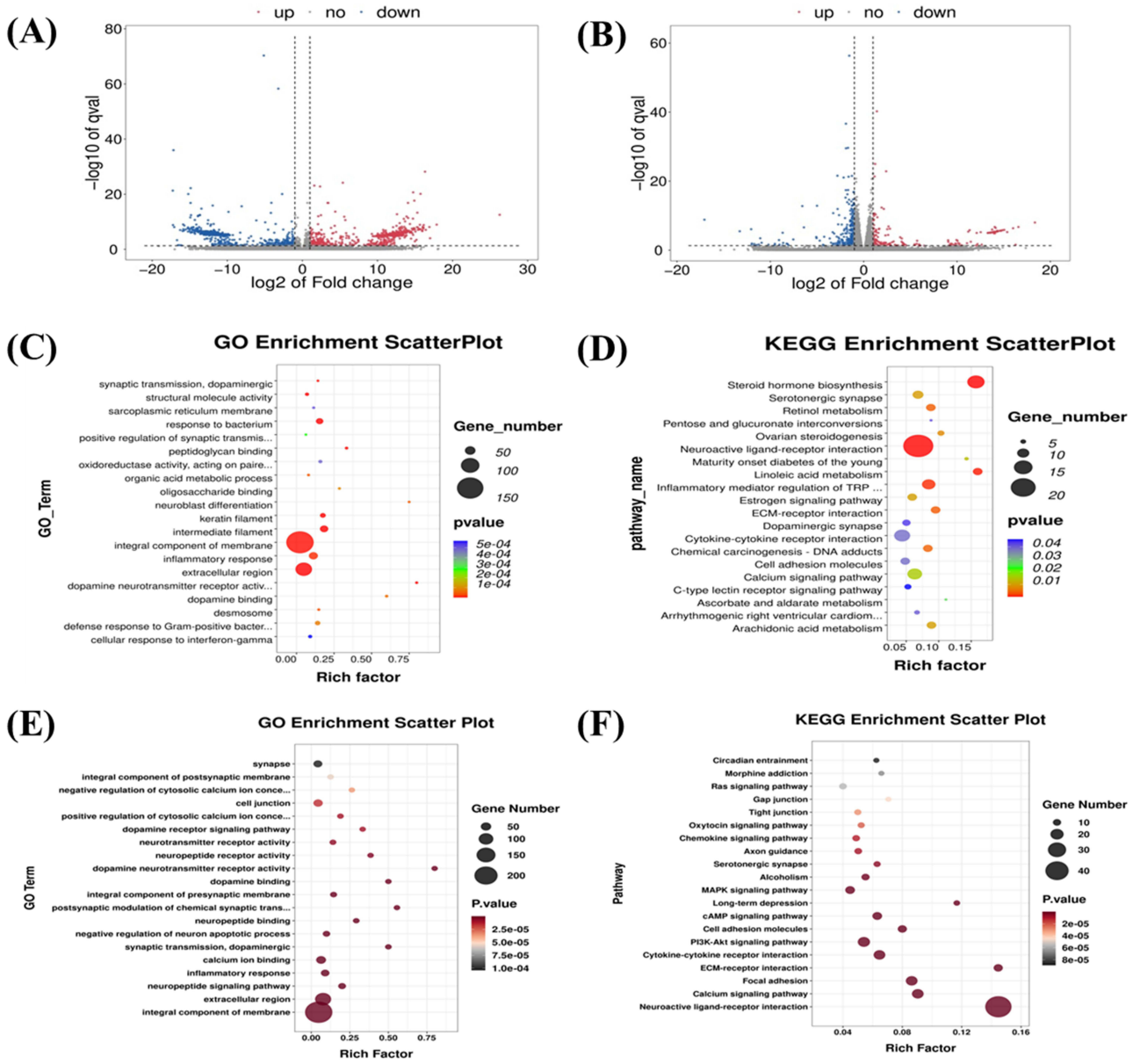

Figure 2 Changes of the transcriptomic profiles in prefrontal cortex regions of mice in SC, FA and FA+NX groups. (**A**) the volcano plot of significant DEGs in the comparison of FA group vs SC group (fold change ≥ 2, $P < 0.05$); (**B**) the volcano plot of significant DEGs in the comparison of FA+NX group vs SC group (fold change ≥ 2, $P < 0.05$); (**C**) Bubble diagram of top 20 enriched GO terms for DEGs in the comparison of FA group vs SC group ($P < 0.05$); (**D**) Bubble diagram of top 20 enriched GO terms for DEGs in the comparison of FA+NX group vs SC group ($P < 0.05$); (**E**) Bubble diagram of top 20 enriched KEGG pathways for DEGs in the comparison of FA group vs.SC group ($P < 0.05$); (**F**) Bubble diagram of top 20 enriched KEGG pathways for DEGs in the comparison of FA+NX group vs SC group ($P < 0.05$).

The qRT-PCR Confirmation of RNA-Seq Differentially Expressed Genes

To validate the RNA-Seq results, 9 genes potentially associated with fentanyl addiction behavior were chosen to perform the qRT-PCR. As shown in Figure 4, most of the tested genes exhibited significant differential expression consistent with the trends observed in the transcriptome data. Among them, *Drd1, Drd2, Htr2c, Oprm1* and *Syt4* did not show significant differential expression between FA and FA+NX groups. Therefore, these data basically confirmed the reliability and accuracy of RNA-seq analysis.

Table 3 List of Significant DEGs Related to Addiction and Their Fold Changes in PFC of Mice Among Experimental Groups

Gene Name	Description	FA/SC	FA+NX/SC
	Amphetamine/Alcoholism/Cocaine/Morphine addiction		
Adora2a[c,d]	Adenosine A2a receptor	0.23	0.15
Arc	Activity regulated cytoskeletal-associated protein	1.05	0.47
Calm4[a,c]	Calmodulin 4	0.00	11.35
Drd1[a,c,d]	Dopamine receptor D1	0.39	0.15
Drd2[a,c,d]	Dopamine receptor D2	0.11	0.05
Gabra6[a,d]	Gamma-aminobutyric acid (GABA) A receptor, subunit alpha 6	0.00	0.20
Gabrq[a,d]	Gamma-aminobutyric acid (GABA) A receptor, subunit theta	0.35	0.16
Gnb4[a]	Guanine nucleotide binding protein (G protein), beta 4	0.61	0.40
Kcnj5[a]	Potassium inwardly-rectifying channel, subfamily J, member 5	0.32	0.69
Oprk1[d]	Opioid receptor, kappa 1	0.28	0.05
Oprm1[d]	Opioid receptor, mu 1	0.52	0.31
Rgs9	Regulator of G-protein signaling 9	0.68	0.26
Slc6a3[a]	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	1.78	0.01
Slc18a1[a]	Solute carrier family 18 (vesicular monoamine), member 1	1.46	0.18
	Dopaminergic/GABAergic/Serotonergic synapse[a]		
Drd3[d]	Dopamine receptor D3	0.01	0.01
Drd4[d]	Dopamine receptor D4	6.53	5.41
Drd5[c,d]	Dopamine receptor D4	2.76	0.32
Htr1d[c,d]	5-Hydroxytryptamine (serotonin) receptor 1D	0.30	0.35
Htr2c[d]	5-Hydroxytryptamine (serotonin) receptor 2C	0.41	0.13
Ptgs2	Prostaglandin-endoperoxide synthase 2	2.02	0.84
	Synapse assembly/Synaptic plasticity/Synaptic vesicle cycle[b]		
Cdh1	Cadherin 1	0.61	2.54
Cplx4	Complexin 4	0.00	0.38
Egr2	Early growth response 2	0.82	0.44
Syt4	Synaptotagmin IV	0.23	0.69
	cAMP/MAPK signaling pathway[c]		
Adcyap1[d]	Adenylate cyclase activating polypeptide 1	2.02	1.86
Mapk13[a]	Mitogen-activated protein kinase 13	0.16	2.56
Ntrk1	Neurotrophic tyrosine kinase, receptor, type 1	0.02	0.00
Ptpn7	Protein tyrosine phosphatase, non-receptor type 7	0.10	0.14
	Neuroactive ligand-receptor interaction[d]		
F2rl2	Coagulation factor II (thrombin) receptor-like 2	3.04	1.80
Rxfp3	Relaxin family peptide receptor 3	2.58	1.65

Note: The genes also involved in dopaminergic/GABAergic/serotonergic synapse were marked with superscript of the letter a; Genes also involved in synapse assembly/synaptic plasticity/synaptic vesicle cycle were marked with superscript of the letter b; Genes also involved in cAMP/MAPK signaling pathway were marked with superscript of the letter c; Genes also involved in neuroactive ligand-receptor interaction pathway were marked with superscript of the letter d.

Discussion

Both the PFC and NAc are considered to be the primary regions involved in the generation of natural and drug reward effects.[9] They also play an important role in behavioral changes related to addiction and are targeted by many addictive drugs. In this study, we present comprehensive synapse morphological alterations in the NAc region and transcriptional changes in PFC region of mice exposed to fentanyl. Results show that mice exposed to fentanyl exhibited prominent naloxone precipitated acute withdrawal symptoms, and caused 631 DEGs in reward and sensory brain regions.

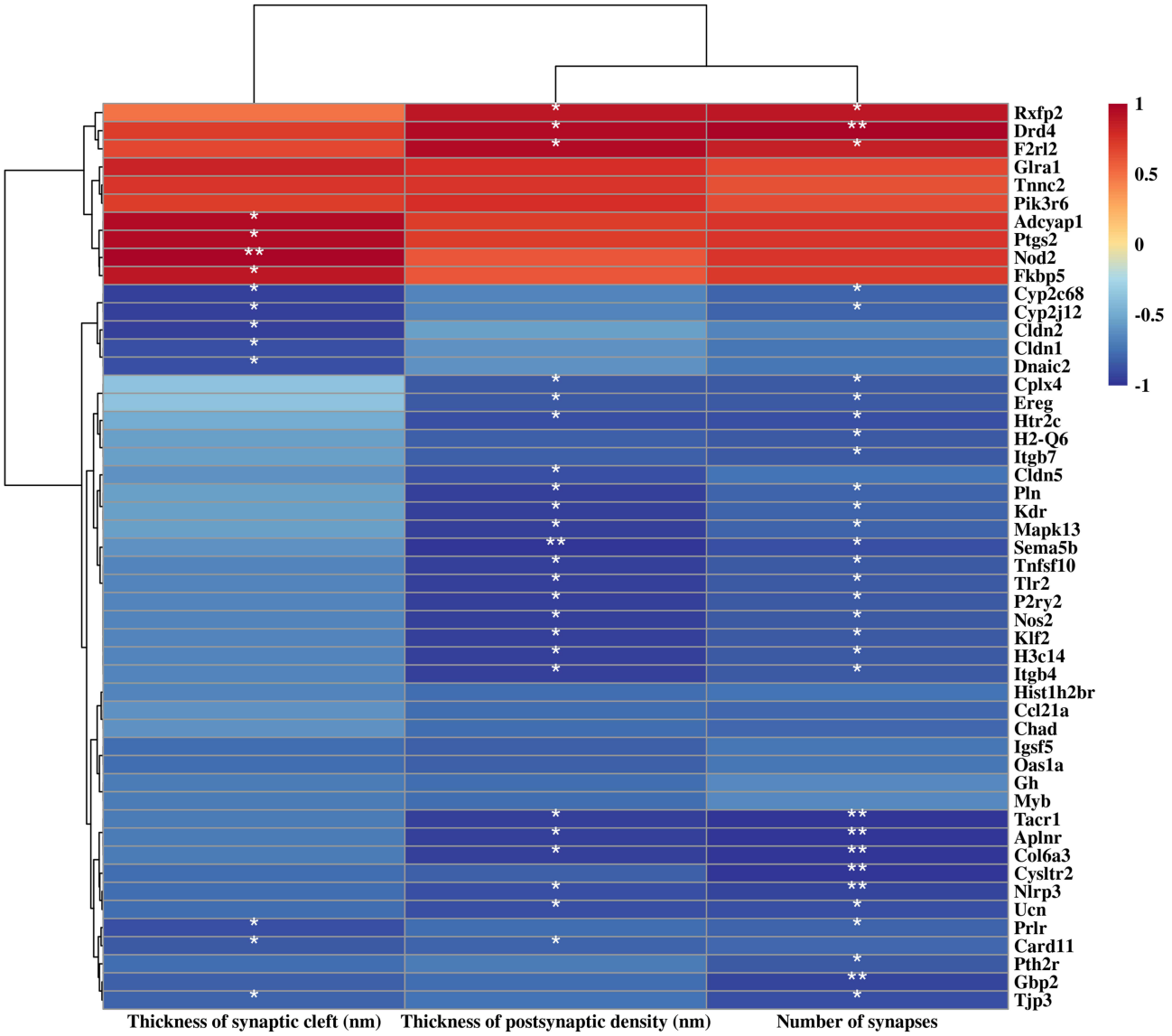

Figure 3 Heatmap showing the significant correlations between the top 50 DEGs and synaptic changes. Spearman's rho values are signed and color-coded according to their respective status: Red blocks indicate the positive correlation and the blue blocks indicate the negative correlation. The intensity of the color reflects the strength of the correlation, with darker shades indicating stronger associations. Significant correlation are denoted by $*P < 0.05$ or $**P < 0.01$.

Meanwhile, NAc ultrastructural analysis revealed an obvious increase in synapse number, widening of synaptic gaps, thickening of postsynaptic density, and ruptured mitochondrial membranes in FA-treated mice compared to mice in SC group. KEGG enrichment analysis indicated some of the DEGs were involved in neurotransmitter and synaptic signal transmission, thus might contribute to the development of fentanyl addiction. These results suggest that these transcriptional alterations may underlie drug-seeking behavior and psychological dependence. Therefore, precisely interventions at the genetic level could potentially prevent or reduce these neuro-molecular changes, as well as abstinence symptoms.

According to previous reports, the persistent synaptic changes within the mesolimbic reward areas in the abstinence period have a profound influence on behaviors, and they are believed to be the reasons for the development of substance addiction.[17] Among transcripts directly involved in addiction behaviors, the down-regulation of *Calm4, Gabra6, Oprk1* and *Oprm1* in FA-treated mice was most noticeable. *Calm4* is involved in the Ca^{2+}/CaMKII/CREB signaling pathway, which is important in the formation of long-term-potentiation (LTP) and thus a central regulator of synaptic plasticity, learning and drug addiction.[18,19] The dysfunction of *Gabra6* is associated with anxiety-like traits and is closely linked to increased psychological stress response and decreased GABA transmission.[20,21] The *Oprk1* is a candidate underlying

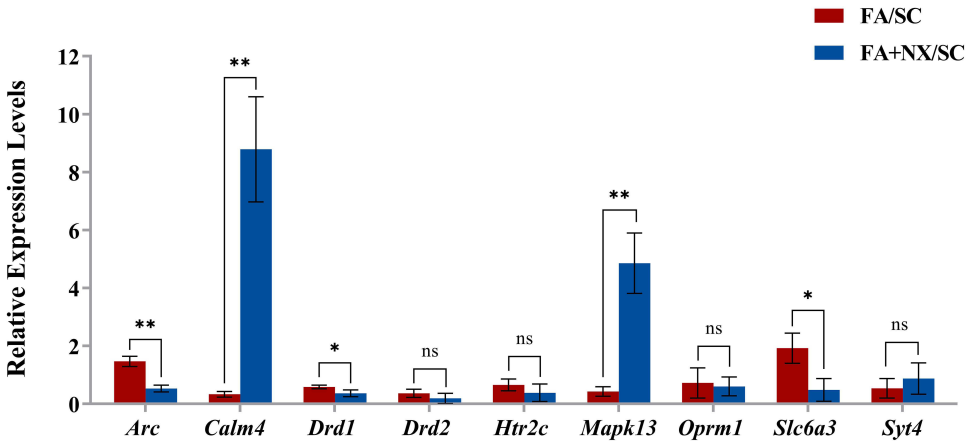

Figure 4 Comparison of the relative expression levels of genes potentially associated with fentanyl addiction behavior in SC, FA and FA+NX groups by qRT-PCR. Genes were randomly selected, and their expression levels in SC group were set as 1, while their expression abundance in FA and FA+NX groups were relatively quantified to the SC group. *GAPDH* gene was used as the internal reference gene, and the results were mean ± standard deviation (SD) (n=3). Significant fold changes in expression levels between different groups were denoted by $^{**}P < 0.01$ and $^{*}P < 0.05$, whereas no significant changes were represent as ns.

opioid addiction. A negative correlation between *Oprk1* expression and fentanyl exposure was observed in frontal-projecting claustrum neurons in a recent study, and genetic variants of *Oprk1* are closely associated with substance dependence risk.[22–24] O*prm1* encodes the mu opioid receptor, which is the primary site of action for morphine and fentanyl. The decreased O*prm1* levels in FA-treated mice were consistent with the reduced O*prm1* mRNA transcription observed in the postmortem brains of opioid users and infant's cord blood or saliva with neonatal abstinence syndrome reported in previous studies.[25,26] And *Oprm1* suppression following acute and chronic exposure to opiates might be associated with hypermethylation of its promoter sequence.[27]

The analysis of transcripts related to dopaminergic, GABAergic, and serotonergic synapses revealed significant alterations in dopamine receptors and 5-hydroxytryptamine receptors among the experimental groups. This suggests the potential impacts on neurotransmission within these pathways. *Ht2cr* is the most extensively studied serotonin receptor, it is also the key modulator of dopamine output that controls the neurochemical and behavioral effects of psychostimulants, particularly the in vivo effects of cocaine.[28,29] Research has indicated that decreased *5-Htr2c* may increase the urge for addiction, while agonists of *5-Ht2cr* are effective in reducing craving and preventing relapse.[30] The *Drd1, 2, 3, 4* and *5* encode dopamine receptors, which play distinct regulatory roles in various neuropsychiatric disorders with different distribution characteristics in brain regions.[31] Among them, *Drd4* is predominantly expressed in PFC, specifically positioned on inhibitory GABAergic interneurons and excitatory glutamatergic pyramidal neurons. As such, it acts as a critical regulator in morphine addiction.[32,33] According to previous reports, *Drd4* activation in PFC strengthens the rewarding effects of opioids, while blocking *Drd4* with antagonist PNU-101387G prevents amphetamine-induced behavioral sensitization.[31] The role of *Drd1* was opposite to *Drd4* in blocking the recall of fear memories and preventing the enhancement of opioid reward effects.[34] Additionally, lower levels of *Drd2* have been well recognized in substance use disorders, which may reflect a homeostatic downregulation of *Drd2* after excessive drug use.[35] For transcripts related to synapse assembly, synaptic plasticity and synaptic vesicle cycle, *Cplx4* was most significantly down-regulated in FA-treated mice. *Cplx4* encodes a member of the SNARE complex regulator, which controls the rate and Ca^{2+} sensitivity of SNARE-mediated synaptic vesicle fusion. It is also involved in regulating the synaptic vesicles released by photoreceptor ribbon synapses. Therefore, the decreased *Cplx4* levels in FA-treated mice may be related to the blurry vision reported by individuals of opioid overdose.[36] These transcriptional changes among experimental groups are in line with behavior alterations such as the emergence of withdrawal symptoms, indicating the correlation between differential gene expression, modified synaptic plasticity alterations and fentanyl addiction.

Previous studies have suggested that MAPK/cAMP signaling pathways and neuroactive ligand-receptor interaction regulate neurotransmitter transduction and synaptic plasticity in neurons, which lead to long-lasting changes in memory function and addictive properties.[37–40] These pathways were found to be significantly enriched after fentanyl treatment in

this study, supporting the neuropathological status of these mice. Table 3 presents that most of the transcripts involved in MAPK/cAMP pathways are down-regulated except *Adcyap1*. *Adcyap1* encodes the neuropeptide also known as pituitary adenylate cyclase-activating polypeptide. Consistent with the findings in this study, it has been reported that *Adcyap1* levels are positively correlated with anxiety-like behaviors in mice.[41] As a neurodevelopment-related pathway, neuroactive ligand-receptor interaction regulates the release of neurotransmitters such as dopamine and serotonin. Among transcripts involved in this pathway, the expression changes of *F2rl2* and *Rxfp3* are particularly noticeable. Both the activation of *F2rl2* and *Rxfp3* are highly responsive to stimuli, especially neurogenic stressors, and they regulate behavioral responses as well as key neural processes including hippocampal theta rhythm and related psychiatric conditions.[42] Thus, the expression trends of these genes after fentanyl exposure were consistent with their reported function in neuropsychiatric disorder.

Table 3 shows that *Calm4, Mapk13, Slc6a3* and *Slc18a1* were the most significantly altered transcripts in the comparison of FA vs FA+NX. Previous studies have clearly demonstrated the association between opioid dependence and the alteration of Ca^{++} homeostasis, and the striking up-regulation of *Calm4* in FA+NX group might contribute to naloxone-induced fentanyl withdrawal syndrome observed in these mice.[43] *Slc6a3* encodes dopamine transporter 1, an integral membrane protein responsible for recycling dopamine from the synaptic cleft back into the presynaptic neuron.[44] *Slc18a1* encodes vesicular monoamine transporter 1, which is responsible for presynaptic transport and packaging of dopamine, norepinephrine, serotonin, and histamine.[45] The decreased expression of *Slc6a3* and *Slc18a1* in FA+NX group may reflect dysregulation of dopamine transmission in NAC regions. Taken together, these data indicate that the activated MAPK pathway, increased Ca^{++} influx and dysregulated dopamine transmission play central roles in the development of fentanyl withdrawal symptoms.

Conclusion

The alterations of synapse and transcriptional profiles in reward and sensory brain areas after fentanyl exposure were investigated in this study. Most of these DEGs were reportedly correlated to various neuropsychiatric disorders, including remodeling ECM, modulating synapse assembly and synaptic plasticity, regulating neurotransmitter transduction, and altering the formation of LTP. These data provide valuable insights into the molecular mechanism underlying addiction behavior, and it is expected that precise intervention strategies may be developed to prevent or reduce these neuromolecular changes and possibly mitigate behavioral adaptations in illicit fentanyl-abusing population.

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Disclosure

The authors declare no conflict of interest in this work.

References

1. Fattore L, Diana M. Drug addiction: an affective-cognitive disorder in need of a cure. *Neurosci Biobehav Rev*. 2016;65:341–361. doi:10.1016/j.neubiorev.2016.04.006
2. Hyman SE, Malenka RC, Nestler EJ. Neural mechanisms of addiction: the role of reward-related learning and memory. *Annu Rev Neurosci*. 2006;29:565–598. doi:10.1146/annurev.neuro.29.051605.113009
3. Thomas MJ, Kalivas PW, Shaham Y. Neuroplasticity in the mesolimbic dopamine system and cocaine addiction. *Br J Pharmacol*. 2008;154(2):327–342. doi:10.1038/bjp.2008.77
4. Dacher M, Nugent FS. Opiates and plasticity. *Neuropharmacology*. 2011;61(7):1088–1096. doi:10.1016/j.neuropharm.2011.01.028
5. Lüscher C, Malenka RC. Drug-evoked synaptic plasticity in addiction: from molecular changes to circuit remodeling. *Neuron*. 2011;69(4):650–663. doi:10.1016/j.neuron.2011.01.017
6. Ikemoto S. Dopamine reward circuitry: two projection systems from the ventral midbrain to the nucleus accumbens-olfactory tubercle complex. *Brain Res Rev*. 2007;56(1):27–78. doi:10.1016/j.brainresrev.2007.05.004

7. Morales M, Pickel VM. Insights to drug addiction derived from ultrastructural views of the mesocorticolimbic system. *Ann N Y Acad Sci*. 2012;1248:71–88. doi:10.1111/j.1749-6632.2011.06299.x
8. Xia B, Li Y, Li R, et al. Effect of sirtuin-1 on synaptic plasticity in nucleus accumbens in a rat model of heroin addiction. *Med Sci Monit*. 2018;24:3789–3803. doi:10.12659/msm.910550
9. Heidari MH, Amini A, Bahrami Z, Shahriari A, Movafag A, Heidari R. Effect of chronic morphine consumption on synaptic plasticity of rat's hippocampus: a transmission electron microscopy study. *Neurol Res Int*. 2013;2013:290414. doi:10.1155/2013/290414
10. Yang HY, Wu ZY, Wood M, Whiteman M, Bian JS. Hydrogen sulfide attenuates opioid dependence by suppression of adenylate cyclase/cAMP pathway. *Antioxid Redox Signaling*. 2014;20(1):31–41. doi:10.1089/ars.2012.5119
11. Coffin PO, Maya S, Kahn JG. Modeling of overdose and naloxone distribution in the setting of fentanyl compared to heroin. *Drug Alcohol Depend*. 2022;236:109478. doi:10.1016/j.drugalcdep.2022.109478
12. Kelly E, Sutcliffe K, Cavallo D, Ramos-Gonzalez N, Alhosan N, Henderson G. The anomalous pharmacology of fentanyl. *Br J Pharmacol*. 2023;180(7):797–812. doi:10.1111/bph.15573
13. Slavova S, Costich JF, Bunn TL, et al. Heroin and fentanyl overdoses in Kentucky: epidemiology and surveillance. *Int J Drug Policy*. 2017;46:120–129. doi:10.1016/j.drugpo.2017.05.051
14. Uddin O, Jenne C, Fox ME, Arakawa K, Keller A, Cramer N. Divergent profiles of fentanyl withdrawal and associated pain in mice and rats. *Pharmacol Biochem Behav*. 2021;200:173077. doi:10.1016/j.pbb.2020.173077
15. Maldonado R, Negus S, Koob GF. Precipitation of morphine withdrawal syndrome in rats by administration of mu-, delta- and kappa-selective opioid antagonists. *Neuropharmacology*. 1992;31(12):1231–1241. doi:10.1016/0028-3908(92)90051-p
16. Listos J, Listos P, Baranowska-Bosiacka I, et al. Linagliptin, a selective dipeptidyl peptidase-4 inhibitor, reduces physical and behavioral effects of morphine withdrawal. *Molecules*. 2022;27(8):2478. doi:10.3390/molecules27082478
17. Olusakin J, Kumar G, Basu M, et al. Transcriptomic profiling of reward and sensory brain areas in perinatal fentanyl exposed juvenile mice. *Neuropsychopharmacol*. 2023;48(12):1724–1734. doi:10.1038/s41386-023-01639-8
18. Pilar Garcia-Pardo M, Roger-Sanchez C, Rodriguez-Arias M, Minarro J, Asuncion Aguilar M. Pharmacological modulation of protein kinases as a new approach to treat addiction to cocaine and opiates. *Eur J Pharmacol*. 2016;781:10–24. doi:10.1016/j.ejphar.2016.03.065
19. Song X, Cui Z, He J, Yang T, Sun X. κ-opioid receptor agonist, U50488H, inhibits pyroptosis through NLRP3 via the Ca2+/CaMKII/CREB signaling pathway and improves synaptic plasticity in APP/PS1 mice. *Mol Med Rep*. 2021;24(1):529. doi:10.3892/mmr.2021.12168
20. Gonda X, Sarginson J, Eszlari N, et al. A new stress sensor and risk factor for suicide: the T allele of the functional genetic variant in the GABRA6 gene. *Sci Rep*. 2017;7:12887. doi:10.1038/s41598-017-12776-8
21. Trucco EM, Hicks BM, Villafuerte S, Nigg JT, Burmeister M, Zucker RA. Temperament and externalizing behavior as mediators of genetic risk on adolescent substance use. *J Abnorm Soc Psychol*. 2016;125(4):565–575. doi:10.1037/abn0000143
22. Terem A, Fatal Y, Peretz-Rivlin N, et al. Claustral neurons projecting to frontal cortex restrict opioid consumption. *Curr Biol*. 2023;33(13):2761–2773. doi:10.1016/j.cub.2023.05.065
23. Xu K, Seo D, Hodgkinson C, Hu Y, Goldman D, Sinha R. A variant on the kappa opioid receptor gene (OPRK1) is associated with stress response and related drug craving, limbic brain activation and cocaine relapse risk. *Transl Psychiatry*. 2013;3:e292. doi:10.1038/tp.2013.62
24. Zhang H, Kranzler HR, Yang BZ, Luo X, Gelernter J. The OPRD1 and OPRK1 loci in alcohol or drug dependence: OPRD1 variation modulates substance dependence risk. *Mol Psychiatry*. 2008;13(5):531–543. doi:10.1038/sj.mp.4002035
25. Wachman EM, Hayes MJ, Lester BM, et al. Epigenetic variation in the mu-opioid receptor gene in infants with neonatal abstinence syndrome. *J Pediatr*. 2014;165(3):472–478. doi:10.1016/j.jpeds.2014.05.040
26. Sun N, Yu LA, Gao YB, et al. MeCP2 epigenetic silencing of *Oprm1* gene in primary sensory neurons under neuropathic pain conditions. *Front Neurosci*. 2021;15:743207. doi:10.3389/fnins.2021.743207
27. Reid KZ, Lemezis BM, Hou TC, Chen R. Epigenetic modulation of opioid receptors by drugs of abuse. *Int J Mol Sci*. 2022;23(19):11804. doi:10.3390/ijms231911804
28. Bubar MJ, Cunningham KA. Serotonin 5-HT2A and 5-HT2C receptors as potential targets for modulation of psychostimulant use and dependence. *Curr Top Med Chem*. 2006;6(18):1971–1985. doi:10.2174/156802606778522131
29. Dhonnchadha BAN, Cunningham KA. Serotonergic mechanisms in addiction-related memories. *Behav Brain Res*. 2008;195(1):39–53. doi:10.1016/j.bbr.2008.06.026
30. Fletcher PJ, Grottick AJ, Higgins GA. Differential effects of the 5-HT(2A) receptor antagonist M100907 and the 5-HT(2C) receptor antagonist SB242084 on cocaine-induced locomotor activity, cocaine self-administration and cocaine-induced reinstatement of responding. *Neuropsychopharmacol*. 2002;27(4):576–586. doi:10.1016/S0893-133X(02)00342-1
31. Botticelli L, Micioni Di Bonaventura E, Del Bello F, et al. Underlying susceptibility to eating disorders and drug abuse: genetic and pharmacological aspects of dopamine D4 receptors. *Nutrients*. 2020;12(8):2288. doi:10.3390/nu12082288
32. Di Ciano P, Grandy DK, Le Foll B. Dopamine D4 receptors in psychostimulant addiction. *Adv Pharmacol*. 2014;69:301–321. doi:10.1016/b978-0-12-420118-7.00008-1
33. Rivera A, Suarez-Boomgaard D, Miguelez C, et al. Dopamine D4 receptor is a regulator of morphine-induced plasticity in the rat dorsal striatum. *Cells*. 2022;11(1):31. doi:10.3390/cells11010031
34. Li JJ, Szkudlarek H, Renard J, Hudson R, Rushlow W, Laviolette SR. Fear memory recall potentiates opiate reward sensitivity through dissociable dopamine D1 versus D4 receptor-dependent memory mechanisms in the prefrontal cortex. *J Neurosci*. 2018;38(19):4543–4555. doi:10.1523/jneurosci.3113-17.2018
35. Burns JA, Kroll DS, Feldman DE, et al. Molecular imaging of opioid and dopamine systems: insights into the pharmacogenetics of opioid use disorders. *Front Psychiatry*. 2019;10:626. doi:10.3389/fpsyt.2019.00626
36. Babai N, Sendelbeck A, Regus-Leidig H, et al. Functional roles of complexin3 and complexin4 at mouse photoreceptor ribbon synapses. *J Neurosci*. 2016;36(25):6651–6667. doi:10.1523/jneurosci.4335-15.2016
37. Bingor A, Azriel M, Amiad L, Yaka R. Potentiated response of ERK/MAPK signaling is associated with prolonged withdrawal from cocaine behavioral sensitization. *J Mol Neurosci*. 2021;71(11):2229–2236. doi:10.1007/s12031-021-01799-6
38. Hu Y, Fang Z, Yang Y, Rohlsen-Neal D, Cheng F, Wang J. Analyzing the genes related to nicotine addiction or schizophrenia via a pathway and network based approach. *Sci Rep*. 2018;8:2894. doi:10.1038/s41598-018-21297-x

39. Todd D, Clapp M, Dains P, Karacay B, Bonthius DJ. Purkinje cell-specific deletion of CREB worsens alcohol-induced cerebellar neuronal losses and motor deficits. *Alcohol*. 2022;101:27–35. doi:10.1016/j.alcohol.2022.02.005
40. Torres OV, Jayanthi S, McCoy MT, Cadet JL. Selective activation of striatal NGF-TrkA/p75NTR/MAPK intracellular signaling in rats that show suppression of methamphetamine intake 30 days following drug abstinence. *Int J Neuropsychopharmacol*. 2018;21(3):281–290. doi:10.1093/ijnp/pyx105
41. Shintani Y, Hayata-Takano A, Yamano Y, et al. Small-molecule non-peptide antagonists of the PACAP receptor attenuate acute restraint stress-induced anxiety-like behaviors in. *Biochem Biophys Res Commun*. 2022;631:146–151. doi:10.1016/j.bbrc.2022.09.079
42. Olucha-Bordonau FE, Albert-Gasco H, Ros-Bernal F, et al. Modulation of forebrain function by nucleus incertus and relaxin-3/RXFP3 signaling. *CNS Neurosci Ther*. 2018;24(8):694–702. doi:10.1111/cns.12862
43. Britch SC, Walsh SL. Treatment of opioid overdose, current approaches and recent advances. *Psychopharmacology*. 2022;239(7):2063–2081. doi:10.1007/s00213-022-06125-5
44. Meaden CW, Mozeika A, Asri R, Santos CD. A review of the existing literature on buprenorphine pharmacogenomics. *Pharmacogenomics J*. 2021;21(2):128–139. doi:10.1038/s41397-020-00198-1
45. Lohoff FW, Dahl JP, Ferraro TN, et al. Variations in the vesicular monoamine transporter 1 gene (VMAT1/SLC18A1) are associated with bipolar disorder. *Neuropsychopharmacol*. 2006;31(12):2739–2747. doi:10.1038/sj.npp.1301196