



REVIEW

Unique Pharmacology, Brain Dysfunction, and Therapeutic Advancements for Fentanyl Misuse and Abuse

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Received: 17 December 2021 / Accepted: 13 February 2022 / Published online: 15 May 2022
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Abstract Fentanyl is a fully synthetic opioid with analgesic and anesthetic properties. It has become a primary driver of the deadliest opioid crisis in the United States and elsewhere, consequently imposing devastating social, economic, and health burdens worldwide. However, the neural mechanisms that underlie the behavioral effects of fentanyl and its analogs are largely unknown, and approaches to prevent fentanyl abuse and fentanyl-related overdose deaths are scarce. This review presents the abuse potential and unique pharmacology of fentanyl and elucidates its potential mechanisms of action, including neural circuit dysfunction and neuroinflammation. We discuss recent progress in the development of pharmacological interventions, anti-fentanyl vaccines, anti-fentanyl/heroin conjugate vaccines, and monoclonal antibodies to attenuate fentanyl-seeking and prevent fentanyl-induced respiratory depression. However, translational studies and clinical trials are still lacking. Considering the present opioid crisis, the development of effective pharmacological and

immunological strategies to prevent fentanyl abuse and overdose are urgently needed.

Keywords Fentanyl abuse · Neural mechanisms · Pharmacological interventions · Fentanyl vaccines

Introduction

Fentanyl was initially designed and synthesized as a valuable therapeutic for anesthesia and severe pain management. It exerts analgesic and rewarding effects by binding to and activating μ -opioid receptors (MORs). Fentanyl has unique pharmacological properties, including a rapid onset of action, high potency at activating MOR-associated signaling *in vivo* (100-fold more potent than morphine), high lipophilicity, and the induction of muscle rigidity. Fentanyl abuse and overdose are becoming increasingly more common [1, 2]. Moreover, fentanyl is commonly mixed with cocaine, methamphetamine, heroin, and other illicit drugs among addicts. Over 40% of opioid-dependent people who inject drugs report lifetime purposeful fentanyl use, which is independently correlated with younger age, recent daily injection, and moderate/severe depression [3]. In the United States, urine drug test data has shown that positivity rates for non-prescribed fentanyl increased 1850% (from 0.9% in 2013 to 17.6% in 2018) among cocaine-positive samples and increased 798% (from 0.9% in 2013 to 7.9% in 2018) among methamphetamine-positive samples [4]. Positivity rates of co-occurring methamphetamine, cocaine, and heroin have also increased since 2013 among fentanyl-positive urine drug test results [5]. Fentanyl abuse is rarely found in China, and its manufacture, consumption, and storage are strictly controlled [6–9]. All fentanyl-related substances have been

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included in the supplementary list of controlled narcotic drugs and psychotropic substances with non-medical use in China since May 1, 2019 [10] (<http://www.mps.gov.cn/n2254314/n2254487/c6473090/content.html>).

Before and during the COVID-19 pandemic, the misuse and abuse of fentanyl appear to have persistently increased in the United States and elsewhere [11]. Overdose deaths and infections among people who use drugs, including fentanyl, increased during the COVID-19 crisis [12–14]. A study in Ontario, Canada, showed that fentanyl use increased by 108% among patients who were treated with opioid receptor agonists [15]. These patients have a high risk of COVID-19 infection, and their treatment can be problematic under conditions of quarantine and isolation and considering the scarcity of healthcare resources during the pandemic [16]. Prescription fentanyl demand has increased in critically ill COVID-19 patients who require mechanical ventilation, resulting in a shortage of intravenous fentanyl. The combined use of intravenous and transdermal fentanyl may be an option [17, 18]. However, intravenous fentanyl administration can disrupt brainstem respiratory systems, trigger respiratory depression, and impair the microcirculatory compensatory response to hemorrhage in the brainstem and lungs. Therefore, COVID-19 patients who use fentanyl should be closely monitored to avoid the exacerbation of clinical outcomes [19–21].

The present review introduces the receptor pharmacology and behavioral effects of fentanyl and elucidates the potential neural mechanisms that underlie fentanyl abuse from different perspectives, including neural circuit dysfunction and neuroinflammation. We also discuss the progress in the development of pharmacological interventions and active immunopharmacotherapies to attenuate fentanyl abuse and its side-effects.

Abuse Potential of Fentanyl

Despite its widespread use for chronic pain and severe cancer pain in the clinical setting, fentanyl and its analogs have been shown to produce hyperlocomotion and strong rewarding and reinforcing effects and have abuse potential.

Fentanyl Abuse and Withdrawal

Fentanyl induces a strong rewarding and reinforcing effects, which is the main contributor to its abuse and overdose deaths [22, 23]. Similar to morphine, fentanyl and its analogs, including isobutyrylfentanyl, crotonylfentanyl, para-fluorobutyrylfentanyl, β -methylfentanyl, paramethoxyfentanyl, fentanyl carbamate, and 3-furanylfentanyl, have MOR agonist-like hyperlocomotor effects in mice

[24–26]. Self-administration models are commonly used to evaluate the reinforcing effects of drugs. Both male and female rats exhibit stable fentanyl self-administration, drug-primed reinstatement, and yohimbine (stress)- and cue-induced reinstatement [27]. When rats self-administer fentanyl with short access (ShA; 1 h are trained to duration daily) or long access (LgA; 6 h duration daily), both ShA and LgA rats exhibit an increase in craving during fentanyl-, yohimbine-, and cue-induced reinstatement [27], indicating strong fentanyl abuse potential. Monroe and Radke established an aversion-resistant fentanyl self-administration model in mice, and found that when quinine is added to the fentanyl solution, mice still robustly respond and consume the solution in both a home-cage two-bottle choice task and operant response task [28]. In addition, a fentanyl vapor self-administration model has been successfully established in mice. This model recapitulates key features of opioid addiction, including the escalation of fentanyl intake, somatic signs of withdrawal, punishment-resistant fentanyl intake, and relapse [29]. A study that combined fentanyl vapor self-administration and a behavioral economics approach found more inelastic demand for fentanyl and an increase in maximal response output in LgA (12 h) rats compared with ShA (1 h) rats [30]. These studies indicate that fentanyl is a powerful addictive drug with high abuse potential, which partially explain the sharp rise in fentanyl abuse in recent years.

Although chronic high-dose fentanyl administration reduces anxiety-like behavior, lowers sensitivity to painful stimuli, and decreases sensitivity to cocaine, fentanyl withdrawal increases anxiety-like behavior [31]. Spontaneous and naloxone-precipitated withdrawal from 2 weeks of intermittent fentanyl exposure also causes weight loss and increases signs of distress in both mice and rats [32]. States of opioid dependence and withdrawal can profoundly influence fentanyl reinforcement and the efficacy of therapeutic medications. The reinforcing effects of fentanyl increase in morphine-withdrawn rats, reflected by a rightward shift of the fentanyl dose-response curve with an increase in maximal effectiveness, whereas the reinforcing effects of fentanyl decrease in opioid-dependent rats [33]. However, no antinociceptive cross-tolerance occurs with repeated microinjections of morphine and fentanyl into the ventrolateral periaqueductal gray, which may be attributable to the different signaling mechanisms that mediate morphine- and fentanyl-induced antinociception [34, 35].

Influential Factors

The behavioral effects of fentanyl administration are influenced by sex, animal strain, and genetic and environmental factors. Fentanyl potently induces an acute

locomotor response and behavioral sensitization in female rats but more potently induces contextual reward in a conditioned place preference paradigm in male rats [36]. Under a fixed-ratio 5 schedule of reinforcement and multi-day progressive-ratio schedule in a behavioral economics analysis, female rats exhibit greater reinforcing efficacy of fentanyl than male rats. In a fentanyl *versus*. food choice procedure, male rats are more sensitive to fentanyl than females [37]. Remifentanyl self-administration-induced neuroadaptations have been shown to differ between males and females, indicating that sex-specific interventions that target opioid-induced adaptations should be developed [38]. Species differences have been found in the expression of fentanyl withdrawal-associated pain. Spontaneous and ongoing pain is preferentially expressed in rats, whereas withdrawal-associated thermal hyperalgesia is found only in mice [32]. Genetic and environmental factors influence the risk of opioid use disorder. *Hnrnp1* mutant mice exhibit lower behavioral sensitivity to fentanyl, suggesting that the expression of this gene may be a biomarker that reflects addictive properties [39].

Unique Pharmacology of Fentanyl Treatment

Fentanyl is known to be a strong MOR agonist, similar to other opioids, such as morphine. The structural assessment of agonist efficacy at MORs has revealed different patterns between fentanyl and morphine [40, 41]. Principal component analysis and computational functional analysis have identified differences in ligand binding, intracellular signaling events, and receptor conformational changes between fentanyl and morphine, which may contribute to the greater efficacy of fentanyl over morphine [40–42]. Chevalier *et al.* exploited the X-ray structure of the MOR in complex with fentanyl and elucidated detailed fentanyl-MOR interaction profiles, thus providing a structural basis for the binding affinity, ligand recognition, and abuse potential of fentanyl and its analogs [43]. Using molecular dynamics techniques to identify binding events of the MOR with fentanyl and its analogs carfentanil and lofentanil, a previous study found that fentanyl induces the unique rotameric conformation of M153, which is required for inducing β -arrestin coupling [44]. These studies provide structural and molecular insights into the detailed mechanisms by which fentanyl binds to MORs, which is helpful for the further optimization of fentanyl-based analgesics with fewer side-effects and improved safety and efficacy.

Fentanyl and its analogs can have serious side-effects, such as increases in brain and kidney levels of malondialdehyde, neuroadaptive changes in the brain, and acceleration of the onset of acute postoperative pain [45, 46].

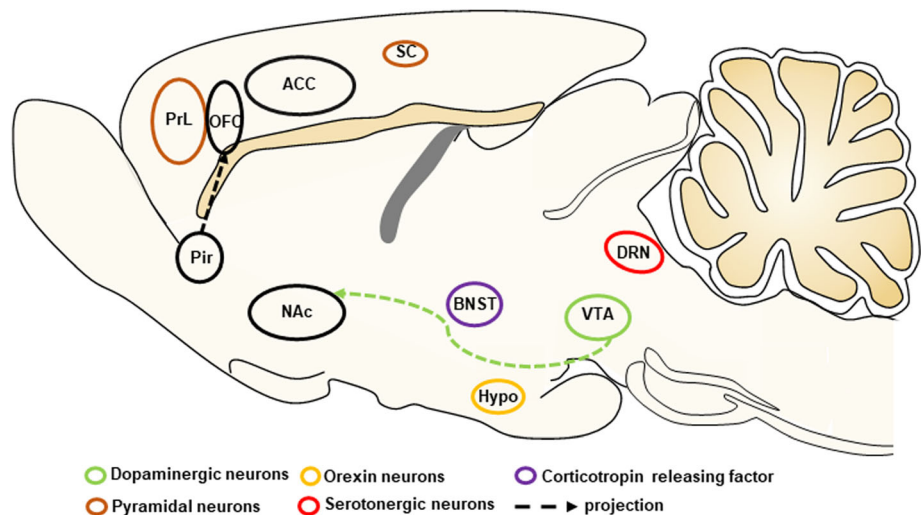
After exposure to fentanyl and its analogs in rats, the metabolite norfentanyl has been detected at high concentrations in urine and remains detectable for 3 days [47]. Fentanyl and its analogs have weak binding affinity for κ -opioid receptors, δ -opioid receptors, and the $\sigma 1$ receptor, which could contribute to the pharmacological actions and side-effects of these drugs [22, 48]. Fentanyl also interacts with non-opioid recombinant human neurotransmitter receptors and transporters, such as the $\alpha 1A$ and $\alpha 1B$ adrenoceptor subtypes and the dopamine D_1 and D_4 receptor subtypes [49]. Human ether-a-go-go-related gene channels have been associated with fentanyl-induced sudden death, and this is exacerbated by hypoxia, hypokalemia, or alkalosis [50, 51]. The complex receptor pharmacology of fentanyl contributes to the different clinical manifestations of fentanyl use, including respiratory and cardiothoracic side-effects and overdose deaths.

Recent studies have reported that fentanyl inhibits the viability and invasion of non-small-cell lung cancer cells by reversing the expression of microRNA-331-3p and histone deacetylase 5 [52]. Fentanyl has been shown to have anti-leukemia activity via suppression of the STAT5 and Ras pathways [53]. Fentanyl inhibits the progression, invasion, and migration of gastric cancer cells by suppressing activity of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/matrix metalloproteinase-9 signaling pathway [54]. Fentanyl also stimulates tumor angiogenesis and promotes the migration of some but not all tested human lung cancer cells; this may be related to the activation of multiple pro-angiogenic signaling pathways, including vascular endothelial growth factor receptor 2/focal adhesion kinase/PI3K/Akt and small guanosine triphosphatases [55]. This study indicates that fentanyl use in cancer patients may have potential adverse effects and should be administered with caution. The β -arrestin 2 pathway plays an important role in recovery from opioid overdose [56], and ketamine may help attenuate some of the adverse physiological consequences of fentanyl use [21]. Li *et al.* found that ketamine combined with fentanyl and dexmedetomidine induces safer and more efficient anesthesia than ketamine alone [57]. These studies provide insights into the molecular basis and signaling pathways that mediate the multiple pharmacological effects of fentanyl and its analogs.

Neural Circuit Dysfunction and Neuroinflammation Underlying Fentanyl Misuse and Abuse

Perinatal fentanyl exposure in newborn male and female mice leads to signs of spontaneous somatic withdrawal-related behavior, an increase in anxiety-like behavior, and impaired sensory function that lasts at least to adolescence

Fig. 1 Schematic of potential neural mechanisms of fentanyl abuse. Specific types of neurons and neural circuits that are involved in fentanyl taking and seeking are shown in a sagittal cross-section of the brain. SC, somatosensory cortex; ACC, anterior cingulate cortex; VTA, ventral tegmental area; NAc, nucleus accumbens; Hypo, hypothalamus; BNST, bed nucleus of the stria terminalis; PrL, prelimbic cortex; OFC, orbitofrontal cortex; Pir, piriform cortex; DRN, dorsal raphe nucleus.



[58, 59]. Moreover, this exposure impairs synaptic transmission in the somatosensory cortex (SC) and anterior cingulate cortex (ACC), reduces ketamine-evoked cortical oscillations, and reduces the morphology of basal dendrites of pyramidal neurons in the SC [59]. Fentanyl binds to MORs in molecular-layer interneurons to reduce γ -aminobutyric acid (GABA)ergic neurotransmission to Purkinje cells, thus inhibiting sensory information processing [60]. In addition, fentanyl and morphine exposure during therapeutic hypothermia does not impair neurodevelopment and does not significantly affect cognition, language, or motor function in infants, but the long-term effects need further investigation [61]. Here, we describe the potential neural mechanisms that are involved in the pathophysiology of fentanyl abuse and fentanyl-related side-effects (Fig. 1).

Reward and Stress Circuits

Drug addiction is viewed as a disorder of dopamine neurotransmission and the reward system in the brain, especially dopamine-releasing neurons in the ventral tegmental area (VTA) and its extensive projections [62, 63]. Previous studies have shown that fentanyl vapor self-administration causes long-lasting neuroadaptations of GABA_B receptor-mediated currents in VTA dopamine neurons [29]. Both subcutaneous and intravenous fentanyl administration significantly increase dopamine release in the nucleus accumbens (NAc) and have reinforcing effects [64]. RNA sequencing has revealed that repeated fentanyl withdrawal elicits distinct transcriptional activity in the VTA and NAc in male and female rats [65]. In addition, fentanyl exhibits an affinity for, and effects on, recombinant human neurotransmitter transporters, including dopamine receptor subtypes [49]. These studies indicate that the

dopamine system mediates the rewarding and reinforcing effects of fentanyl abuse.

The orexin system is involved in the pathophysiology of opioid addiction, and orexin receptor antagonism attenuates drug-seeking behavior and relapse [66, 67]. Intermittent fentanyl self-administration induces a multifaceted addiction-like state and increases the number of orexin neurons in both the dorsomedial/perifornical hypothalamus and lateral hypothalamus [68]. The orexin receptor 1 antagonist SB-334867 decreases fentanyl intake, decreases motivation for fentanyl, and attenuates the cue-induced reinstatement of fentanyl-seeking [68, 69]. Intravenous fentanyl also decreases glutamate release and increases GABA release in the anterior hypothalamus, measured by the push-pull superfusion technique, and this is reversed by intrahypothalamic administration of the opioid receptor antagonist naloxone [70]. In addition, corticotropin-releasing factor (CRF) in the bed nucleus of the stria terminalis (BNST) is a critical driver of stress-induced relapse to drug-seeking. Intra-BNST microinjections of the CRF₁ receptor antagonist R121919 decreases cue-induced fentanyl-seeking and fentanyl-associated conditioned reinforcement in rats [71].

The SC and dorsal raphe nucleus (DRN) are critical regions involved in nociception and may also play a critical role in the process of fentanyl misuse. Fentanyl administration alters the firing activity of neurons and serotonin (5-hydroxytryptamine, 5-HT) efflux in the DRN, which may involve both opioid and 5-HT_{1A} receptors [72, 73]. Autoradiographic mapping has shown that sufentanil tolerance is associated with the large-scale downregulation of MOR binding sites in various brain regions, including the DRN and SC [74]. Fentanyl decreases synaptic excitation and inhibits nociceptive stimulus-induced cortical activity in both the ACC and SC [59, 75]. Synchronization between the right SC and right hippocampus is

also impaired postoperatively after anesthesia with fentanyl and droperidol [76].

Chronic drug use is often accompanied by the loss of cognitive control, which is closely linked to the dysfunction of frontal cortex regions. Remifentanyl self-administration causes a long-lasting hypoactive basal state of pyramidal neurons in the prelimbic cortex (PrL) in females, and this is driven by a reduction of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor-mediated excitatory synaptic transmission [38]. In addition, remifentanyl self-administration has bidirectional effects on PrL layer 5/6 pyramidal neuron GABA_B-G protein-coupled inwardly rectifying K⁺ channel signaling in males [38]. Moreover, the chemogenetic activation of PrL pyramidal neurons reverses the deficits in cognitive flexibility, the basal hypoactive state, and remifentanyl-induced plasticity [38]. Reiner *et al.* developed a rat model of relapse to drug-seeking after food choice-induced voluntary abstinence and found that relapse to fentanyl-seeking is associated with an increase in activation in the orbitofrontal cortex (OFC) and its projection from the piriform cortex (Pir) but not in projections from the basolateral amygdala or thalamus. Using pharmacological inactivation and the anatomical disconnection of projections between the Pir and OFC, previous studies found that Pir–OFC projections play a critical role in relapse to fentanyl-seeking after voluntary abstinence [77, 78]. Future studies that use neural circuit-defined imaging and manipulation techniques may shed further light on the neurobiology of fentanyl relapse.

Neuroinflammation

Opioid misuse, tolerance, and withdrawal have been reported to induce innate and adaptive neuroimmune dysfunction, affect neuronal function, and modulate the activation and phenotyping of microglial cells, thus producing proinflammatory effects in the brain [79]. Fentanyl self-administration profoundly alters innate immune proteins in the NAc and hippocampus. Fentanyl administration increases the expression of cytokines (e.g., tumor necrosis factor α [TNF α], interleukin-1 β [IL-1 β], and IL5), chemokines (e.g., C-C motif chemokine 20), and interferon (IFN) proteins (e.g., IFN β and IFN γ) in the NAc, decreases several interleukins (e.g., IL-4 and IL-7), chemokines (e.g., granulocyte-macrophage colony-stimulating factor, monocyte chemoattractant protein 1, and chemokine ligand 5), and interferons (e.g., IFN β and IFN γ), and stimulates IFN gene protein expression in the hippocampus, which may initiate fentanyl misuse and perhaps the development of opioid use disorder [80, 81]. Fentanyl administration increases mechanical and thermal hyperalgesia, increases the expression of proinflammatory

cytokines (e.g., IL-1 β , IL-6, and TNF- α) in the spinal cord and dorsal root ganglia, and activates microglia in the spinal cord in rats [82]. Repeated fentanyl administration activates the NLRP3 inflammasome in astrocytes and serotonergic neurons and promotes NLRP3-dependent pyroptosis in the DRN. Furthermore, the antiinflammatory agent minocycline and the NLRP3 inhibitor MCC950 prevent fentanyl-induced antinociception and hyperalgesia [83]. Fentanyl has also been shown to reverse the lipopolysaccharide-induced release of TNF- α , IL-1 β , and IL-10 by inhibiting Toll-like receptor-4 (TLR4) expression and glycogen synthase kinase-3 β activation [84]. These studies indicate that neuroinflammation plays an important role in opioid-induced analgesia and fentanyl-induced hyperalgesia.

Gut microbiota and gut-brain communication also play a critical role in opioid use and opioid tolerance [85, 86]. Chronic opioid use alters fecal microbial diversity and composition and induces gut microbial dysbiosis in both rodents and humans, accompanied by increases in peripheral and central inflammation [87–89]. Moreover, microbial metabolites, such as short-chain fatty acids, are reduced, and intestinal and blood-brain barrier integrity is disrupted after opioid use, which could modulate the development, maturation, and function of microglia and exacerbate the neuroimmune responses [87, 89, 90]. Neuroinflammation that is induced by alterations of the microbiota can ultimately contribute to behaviors that are associated with opioid dependence [91, 92]. However, the effects of fentanyl use on the gut microbiota remain unknown. Future studies are needed to investigate the influence of alterations of the microbiota on brain immunity and function.

Pharmacological Interventions to Prevent Fentanyl Abuse and Side-effects

Pharmacotherapies that target brain opioid receptors, including agonists (e.g., methadone and buprenorphine) and antagonists (e.g., naltrexone and naloxone), have been approved by the Food and Drug Administration (FDA) for the treatment of opioid use disorder. A meta-analysis has shown that these treatments can reduce mortality among opioid users [93]. A long-acting injection of extended-release buprenorphine has been shown to be feasible for users of heroin-containing fentanyl in an open-label trial [94]. However, these pharmacotherapies are limited by high relapse rates, a high risk of precipitating withdrawal in individuals who are exposed to fentanyl, and weak efficacy in preventing fentanyl overdose [95]. Thus, new approaches and novel medications are still needed for the treatment of opioid use disorder.

Pharmacological Interventions to Prevent Fentanyl-Seeking

Here, we summarize the currently available pharmacological interventions to attenuate fentanyl taking and seeking in rodents and nonhuman primates (Table 1). These interventions mainly target opioid receptors and neurotransmitter and neuropeptide systems. Intravenous and subcutaneous administration of the long-lasting MOR antagonist methocinnamox reverses and prevents fentanyl-induced antinociception and respiratory depression in rats [96]. Methocinnamox attenuates heroin self-administration in rhesus monkeys, which continues to display efficacy even at very low plasma levels [97]. Contingent administration of the κ -opioid receptor agonists U50488 and nalfurafine decrease fentanyl choice, which is blocked by the selective κ -opioid receptor antagonist norbinaltorphimine [98]. Levo-tetrahydropalmatine is an antagonist of dopamine receptors that suppresses the rewarding effects of fentanyl in the conditioned place preference paradigm by inhibiting the phosphorylation of extracellular signal-regulated kinase (ERK) and cyclic adenosine monophosphate response element binding protein (CREB) in the hippocampus, NAc, and prefrontal cortex in mice [99]. The acute stimulation of 5-HT_{2A} receptors with the psychedelic 2,5-dimethoxy-4-iodoamphetamine decreases the economic demand for fentanyl and food after intermittent and continuous-access self-administration [100]. In a rat model of relapse, the glucagon-like peptide-1 receptor (GLP-1R) agonist exendin-4 reduces fentanyl self-administration in rats but causes malaise-like behavior. A novel dual agonist of GLP-1Rs and neuropeptide Y₂ receptors, GEP44, reduces fentanyl self-administration with fewer adverse effects compared with exendin-4 [101, 102]. The growth hormone secretagogue receptor antagonist JMV2959 reduces fentanyl-induced conditioned place preference and fentanyl self-administration [64]. The glucocorticoid receptor antagonist PT150 reduces footshock-induced fentanyl-seeking [103]. Intra-BNST injections of the CRF₁ receptor antagonist R121919 decreases cue-induced responding to fentanyl in both opioid-dependent and nondependent rats [71]. Compared with ShA and LgA to fentanyl, intermittent access increases the motivation for fentanyl and total intake, which is reversed by the orexin receptor-1 antagonist SB334867 [68].

Pharmacological Interventions to Attenuate Fentanyl-Induced Respiratory Depression

Fentanyl overdose fatalities and the opioid crisis are mainly attributable to drug-induced respiratory depression. Fentanyl overdose induces hippocampal ischemia, accompanied by delayed leukoencephalopathy [104]. Fentanyl but not morphine alters mitochondrial morphology, depending

on the dosage and cell type, and this may be associated with neuronal apoptosis [105, 106]. Fentanyl-induced respiratory depression is associated with electrocortical changes, underscoring the importance of considering the activity of cortical and subcortical areas when evaluating fentanyl-induced respiratory depression [107]. Fentanyl and its analogs can rapidly produce airway closure and rigidity in the upper airway and diaphragm chest wall, which may involve α -adrenergic and cholinergic receptor-mediated mechanical failure of the respiratory and cardiovascular systems [49, 108].

We summarize currently available pharmacological interventions to prevent fentanyl-induced respiratory depression in Table 2. D-amphetamine hastens the recovery of arterial pH and partial pressure of CO₂, O₂, and sO₂ in rats, promotes the alleviation of respiratory depression, and escalates the return of consciousness following high-dose fentanyl [109]. The G protein-biased cannabinoid CB₂ receptor agonist LY2828360 decreases fentanyl-induced respiratory depression in wildtype mice but not in G protein-biased CB₂ receptor knockout mice [110]. The acyclic cucurbit[n]uril molecular container calabadiol 1 reverses fentanyl-induced respiratory depression in rats [111]. The selective α 4 β 2 nicotinic receptor agonist A85380 confers robust and rapid reversal of fentanyl-induced respiratory depression, without any obvious side effects. A85380 also significantly decreases respiratory depression and apnea when co-administered with fentanyl and remifentanyl [112]. Brackley *et al.* found that the peptide oxytocin receptor agonist oxytocin acetate and the non-peptide oxytocin receptor agonist WAY-267464 without vasopressin-1A receptor cross-activation rescues fentanyl-induced respiratory depression [113]. The clinically approved superoxide dismutase mimetic 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl (Tempol) has also been reported to attenuate the cardiorespiratory depressant effects of fentanyl without affecting its analgesic effects [114].

Altogether, mounting preclinical data provide proof-of-concept of the efficacy of potential pharmacological interventions to attenuate fentanyl-seeking and prevent fentanyl-induced respiratory depression, demonstrating their potential for the treatment of fentanyl abuse and overdose. However, translational studies and clinical trials still need to be conducted.

Progress in the Development of Anti-Fentanyl Conjugate Vaccines as Candidate Medications

The management and prevention of fentanyl-related overdoses are notable challenges. Anti-opioid vaccines may be a promising therapeutic approach to prevent opioid overdose and reduce opioid-induced behavior, including drug

Table 1 Summary of recent findings on pharmacological interventions to attenuate fentanyl taking and seeking.

Drug	Route of administration	Pharmacological target	Subjects	Paradigm (fentanyl dose, model)	Main findings	References
SB-334867	IP	Orexin-1 receptor antagonist	Male Sprague-Dawley rats	Self-administration and behavioral economics procedure	SB-334867 decreases motivation for fentanyl without affecting drug consumption at null cost	Fragale <i>et al.</i> [69]
R121919	Intra-BNST injections	Corticotropin-releasing factor-1 receptor antagonist	Male Sprague-Dawley rats	2.5 mg/kg, infusion, self-administration	These patterns of responding with R121919 treatment result in less fentanyl-associated conditioned reinforcement during test	Gyawali <i>et al.</i> [71]
JMV2959	IP	Growth hormone secretagogue receptor antagonist	Male adult Wistar rats	20 or 30 µg/kg, SC; 10 µg/kg, IV	Pretreatment with JMV2959 significantly reduces the number of active lever presses and reduces fentanyl seeking/relapse-like behavior in rats on day 12 of forced abstinence	Sustkova-Fiserova <i>et al.</i> [64]
Methocinnamox	SC	µ-Opioid receptor antagonist	Male and female rhesus monkeys	0.00032 mg/kg, infusion, self-administration	Methocinnamox selectively reduces opioid self-administration and remains effective at times when its plasma levels are very low	Maguire <i>et al.</i> [97]
U50488 and nalfurafine	IV	κ-Opioid receptor agonists	Male and female Sprague-Dawley rats	0, 0.32–10 µg/kg, infusion, self-administration	Both U50488 and nalfurafine decrease fentanyl choice when administered contingently	Townsend [98]
PT150	IP	Glucocorticoid receptor antagonist	Male and female Sprague-Dawley rats	2.5 µg/kg, infusion, self-administration	Both footshock and yohimbine reinstate fentanyl-seeking; only footshock-induced reinstatement is decreased by PT150 (50 and 100 mg/kg)	Hammerslag <i>et al.</i> [103]
Levo-tetrahydropalmatine	IV	Dopamine receptor antagonist	Male C57BL/6 mice	0.05 mg/kg, conditioned place preference	Levo-tetrahydropalmatine suppresses the rewarding properties of fentanyl-induced conditioned place preference; the inhibitory effect may be related to the suppression of ERK and CREB phosphorylation in the hippocampus, nucleus accumbens, and prefrontal cortex in mice	Du <i>et al.</i> [99]
GEP44	SC	Novel dual agonist of glucagon-like peptide-1 receptors and neuropeptide Y Y ₂ receptors	Male Sprague-Dawley rats	2.5 µg/kg, self-administration	GEP44 attenuates opioid taking and seeking at a dose that does not suppress food intake or produce adverse malaise-like effects in fentanyl-experienced rats	Zhang <i>et al.</i> [101]
2,5-Dimethoxy-4-iodoamphetamine (DOI)	IP	Psychedelic 5-HT _{2A} receptor agonist	Male Sprague-Dawley rats	2.5 µg/kg, injection, self-administration, intermittent and continuous schedules	DOI acts through 5-HT _{2A} receptors to alter economic demand for fentanyl; in economic food demand experiments, DOI (0.4 mg/kg) increases demand elasticity and reduces food consumption	Martin <i>et al.</i> [100]

Table 1 continued

Drug	Route of administration	Pharmacological target	Subjects	Paradigm (fentanyl dose, model)	Main findings	References
SB334867	IP	Orexin-1 receptor antagonist	Male Sprague-Dawley male rats	Intermittent self-administration	Addiction-like behaviors induced by intermittent access to fentanyl are reversed by SB-334867	Fragale <i>et al.</i> [68]

IP, intraperitoneal; SC, subcutaneous; IV, intravenous; BNST, bed nucleus of the stria terminalis.

Table 2 Summary of recent findings on pharmacological interventions to prevent fentanyl-induced respiratory depression.

Drug	Route of administration	Pharmacological target	Subjects	Paradigm (fentanyl dose, model)	Main findings	References
A85380	SC	$\alpha 4\beta 2$ nicotinic acetylcholine receptor	Male and female Sprague-Dawley rats	20 $\mu\text{g}/\text{kg}$, IV	Co-administration of A85380 (0.06 mg/kg) and fentanyl or remifentanyl markedly reduces respiratory depression and apnea and enhances fentanyl-induced analgesia	Ren <i>et al.</i> [112]
D-amphetamine	IV	Dopamine D ₁ receptor	Male and female Sprague-Dawley rats	55 $\mu\text{g}/\text{kg}$, IV	D-amphetamine attenuates respiratory acidosis, increases arterial oxygenation, and accelerates the return of consciousness in the setting of fentanyl intoxication	Moody <i>et al.</i> [109]
LY2828360	IP	G protein-biased cannabinoid CB ₂ receptor	Wildtype and CB ₂ knockout mice	0.2 mg/kg, IP	Combination of CB ₂ agonist and fentanyl may represent a safer adjunctive therapeutic strategy compared with a narcotic analgesic alone by attenuating the development of opioid-induced respiratory depression	Zavala <i>et al.</i> [110]
Calabadiol 1	IV	Acyclic cucurbit[n]uril molecular container	Male Sprague-Dawley rats	12.5 or 25 $\mu\text{g}/\text{kg}$, IV	Calabadiol 1 selectively and dose-dependently reverses the respiratory and central nervous system side-effects of fentanyl	Thevathasan <i>et al.</i> [111]
Oxytocin and WAY-267464	IP	Oxytocin receptor	Male Sprague-Dawley rats	60 nmol/kg, IV	Without vasopressin 1A receptor cross-activation, peptide and non-peptide agonist activation of oxytocin receptors (oxytocin and WAY-267464) rescue fentanyl-induced respiratory depression	Brackley <i>et al.</i> [113]
Methocinnamox	IV and SC	μ -Opioid receptor	Male Sprague-Dawley rats	0.0032–0.178 mg/kg, IV	Methocinnamox reverses and prevents fentanyl-induced antinociception and respiratory depression	Jimenez <i>et al.</i> [96]

IP, intraperitoneal; IV, intravenous; SC, subcutaneous.

self-administration in mice, rats, and nonhuman primates [14, 115]. Vaccines produce fentanyl-specific antibodies and sequester large amounts of these drugs in blood, thus reducing biodistribution in the brain and preventing binding to targeted receptors in the brain [116, 117]. Compared with FDA-approved small-molecule pharmacotherapies (e.g., methadone, buprenorphine, and naltrexone), fentanyl vaccines offer several potential clinical benefits, including prolonged protection because of the

relatively long serum half-life, fewer side-effects by directly binding and sequestering drugs in the periphery, high selectivity, and no abuse liability. Vaccines that target opioids (e.g., heroin, morphine, oxycodone, and hydrocodone) and psychostimulants (e.g., cocaine and methamphetamine) have shown promising results in animal models and are currently in various stages of preclinical development [118–121]. Anti-fentanyl vaccines are still in the early stages of development. Currently, several fentanyl

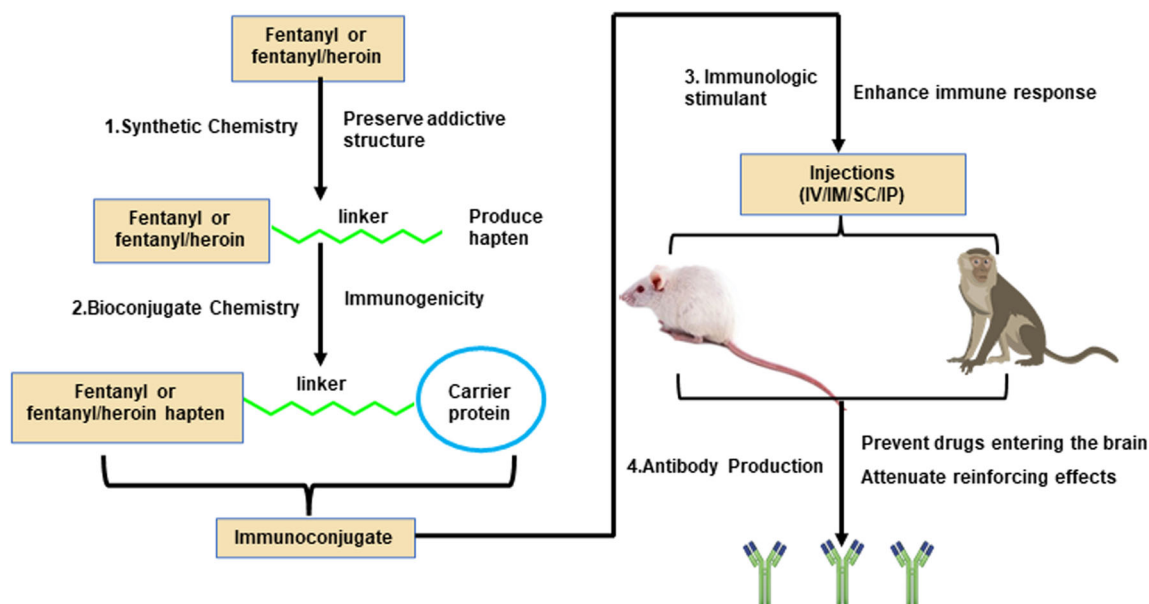


Fig. 2 Design and production of fentanyl and fentanyl/heroin conjugate vaccines. Using synthetic chemistry, a specific linker is added to the fentanyl or fentanyl/heroin without affecting biological functionality to create a hapten. Linkage of the fentanyl or fentanyl/heroin hapten to the carrier protein occurs through bioconjugate chemistry, and subsequent adjuvating of the immunologic stimulant generates complete vaccines. These vaccines have been tested in

and fentanyl/heroin vaccines have been developed with different designs (Fig. 2), including the optimization of fentanyl-based haptens and linker chemistry, the choice of carrier proteins and adjuvants, delivery strategies, and the development of novel carriers and delivery platforms (Table 3).

Fentanyl Vaccines in Mice and Rats

Considering the devastating opioid crisis worldwide, different carrier proteins, adjuvants, delivery strategies, and fentanyl-based haptens are being developed to improve fentanyl vaccine efficacy, shelf life, and safety. A vaccine that consists of a fentanyl-based hapten that is conjugated to the keyhole limpet hemocyanin (KLH) carrier protein or GMP-grade subunit KLH reduces fentanyl distribution in the brain and reduces fentanyl-induced antinociception and respiratory depression in mice and rats [122]. Fentanyl-tetanus toxoid (TT) conjugate vaccines elicit high anti-fentanyl antibody titers and ablate lethal doses of fentanyl and its analogs, including 3-methylfentanyl and α -methylfentanyl [123]. A fentanyl-TT conjugate vaccine has also been reported to decrease fentanyl reinforcement, increase food reinforcement in a fentanyl *versus*. food choice procedure, and prevent the expression of opioid withdrawal-associated increases in fentanyl choice in male

mice, rats, and nonhuman primates, where they have been shown to enhance innate and adaptive immunity, produce fentanyl-specific antibodies, sequester opioid drugs in the periphery, and prevent them from entering the brain, thus attenuating the reinforcing effects of fentanyl or fentanyl-contaminated heroin. IV, intravenous; IM, intramuscular; SC, subcutaneous; IP, intraperitoneal.

and female rats [124, 125]. Carfentanil is ~100-fold more potent than fentanyl and can be lethal at extremely low doses. Pretreatment with the opioid receptor antagonist naltrexone decreases the seeking of fentanyl and heroin but not carfentanil [126]. Recently, two synthetic vaccines, Carfen-*ester*-TT and Carfen-*p-phenyl*-TT, have been shown to reduce carfentanil biodistribution in the brain and blunt carfentanil-induced antinociception and respiratory depression [127].

A recent study found that a fentanyl vaccine that is adjuvanted with a heat-labile toxin of *E. coli*, including dmLT and LTA1, elicits higher levels of anti-fentanyl antibodies than the traditional adjuvant alum. Vaccination with sublingual dmLT or intranasal LTA1 confers the robust blockade of fentanyl-induced analgesia and strong central nervous system penetration of anti-fentanyl antibodies [128]. This study also found that dmLT and LTA1 adjuvants and mucosal delivery enhance the immunogenicity and efficacy of fentanyl conjugate vaccines. A novel fentanyl vaccine, composed of a novel fentanyl hapten (para-AmFenHap), a safe and immunogenic carrier protein TT, and a potent liposomal adjuvant (ALF43A) that contains monophosphoryl lipid A, has also been synthesized and shown to prevent the side-effects of fentanyl [129]. The stimulation of TLR5 may also be an option to improve antibody production and enhance innate and adaptive immunity during active vaccination against

Table 3 Summary of active immunopharmacotherapies developed for fentanyl abuse.

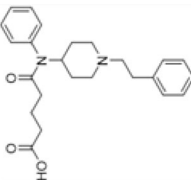
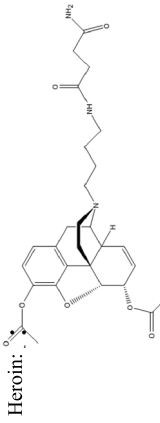
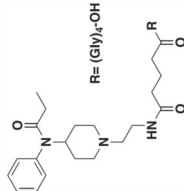
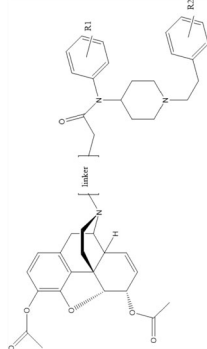
Vaccine	Hapten structure	Carrier protein	Adjuvant	Route of administration	Vaccinations/overall time	Subjects	Main findings	References
1		TT	Alum and CpG	SC	3 injections (0, 2, 4 weeks)	Male Swiss-Webster mice	Single conjugate vaccine elicits high levels of antibodies with cross-reactivity for a wide panel of fentanyl analogs and protects against lethal fentanyl doses	Bremer <i>et al.</i> [123]
2	Heroin:  Fentanyl: Same structure as vaccine 1 Same structure as vaccine 1	TT or KLH	Alum and CpG	SC	3 injections (0, 2, 4 weeks)	Female BALB/c mice	Admixture vaccines sequester opioid drugs in blood and reduce fentanyl-induced antinociception	Hwang <i>et al.</i> [142, 143]
3	Same structure as vaccine 1	TT	CpG and alhydrogel	IV	3 injections (0, 3, 17 weeks)	Male and female Sprague-Dawley rats	Vaccination is very effective in preventing the binding of fentanyl to μ -opioid receptors in the central nervous system and reduces fentanyl-induced antinociception and its reinforcing effects	Townsend <i>et al.</i> [124]
4	Same structure as vaccine 1	TT	Alhydrogel	IM	6 injections (0, 2, 4, 9, 19, 44 weeks)	Adult male rhesus monkeys	The vaccine increases plasma fentanyl levels and reduces fentanyl-induced antinociception	Tenney <i>et al.</i> [133]
5		KLH	Alum	SC	3 injections (0, 2, 4 weeks)	Male BALB/c mice and Holtzman rats	The vaccine reduces fentanyl-induced antinociception in hot-plate test, respiratory depression, and bradycardia over a range of cumulative subcutaneous fentanyl doses	Raleigh <i>et al.</i> [122]
6		KLH	Alum and CpG ODN 1826	IP	4 injections (0, 2, 4, 7 weeks)	Female BALB/c mice	New vaccines developed from chemically contiguous haptens composed of both heroin- and fentanyl-like domains; they confer robust protection against heroin but attenuate protection against fentanyl	Natori <i>et al.</i> [141]

Table 3 continued

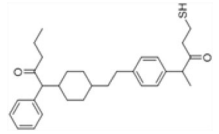
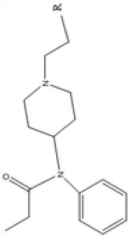
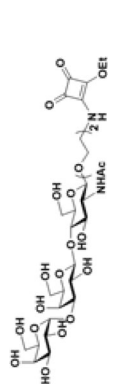
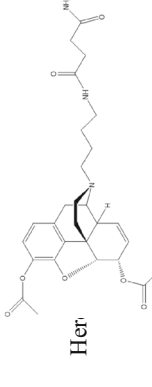
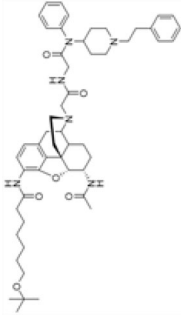
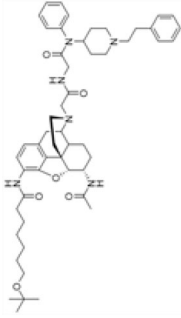
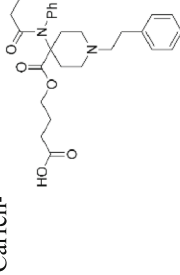
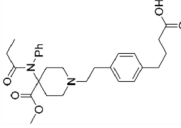
Vaccine	Hapten structure	Carrier protein	Adjuvant	Route of administration	Vaccinations/overall time	Subjects	Main findings	References
7	Same structure as vaccine 5	KLH	Alum	IP	3 injections (0, 3, 8 weeks)	Female A/J mice	Monoclonal antibodies generated in hybridomas from mice vaccinated with a fentanyl conjugate vaccine reverse fentanyl/carfentanil-induced antinociception	Smith <i>et al.</i> [135]
8		TT	ALF43A	IM	4 injections (0, 3, 6, 14 weeks)	Female BALB/c mice	The vaccine induces high-affinity antibodies against fentanyl and its highly potent analogs and protects mice against fentanyl-induced antinociceptive effects	Barrientos <i>et al.</i> [129]
9		KLH or CRM	Alum	IM	4 injections (0, 14, 28, 29 weeks)	Male BALB/c mice and male and female Sprague-Dawley rats	Prophylactic vaccination reduces fentanyl- and sufentanil-induced respiratory depression, antinociception, and bradycardia; therapeutic vaccination reduces intravenous fentanyl self-administration	Robinson <i>et al.</i> [131]
10		OVA	Alum	IP	3 injections (2 weeks)	α1,3GalT knockout mice	The vaccine reduces psychoactive effects of fentanyl without addition of the immunostimulant CpG oligodeoxynucleotide	Wang <i>et al.</i> [132]
11	 Her	CRM or TT	Alhydrogel	IV	4 injections (0, 14, 42, 70 days)	Male and female Sprague-Dawley rats	The vaccine attenuates thermal antinociceptive effects of fentanyl/heroin but has no effects on fentanyl/heroin mixture self-administration	Townsend <i>et al.</i> [139]
12	Fentanyl: Same structure as vaccine 1 Same structure as vaccine 5	KLH	Alum	IP	3 injections (0, 2, 4 weeks)	Male and female BALB/c mice and male Sprague-Dawley rats	α-Fentanyl monoclonal antibodies generated from hybridomas <i>via</i> magnetic enrichment; passive immunization reduces fentanyl-induced antinociception, respiratory depression, and bradycardia	Baehr <i>et al.</i> [136]

Table 3 continued

Vaccine	Hapten structure	Carrier protein	Adjuvant	Route of administration	Vaccinations/overall time	Subjects	Main findings	References
13	Same structure as vaccine 1 	CRM	δ -Inulin	SC	3 injections (2 weeks) in mice 4 injections (0, 1, 2, 4 months) in monkeys	Male BALB/c mice and cynomolgus monkeys	A novel vaccine against heroin and fentanyl developed through the optimization of adjuvants and enhancing stability <i>via</i> lyophilization; it produces high-affinity antibodies and reduces fentanyl/heroin-induced antinociception	Blake <i>et al.</i> [137]
14		CRM	Alum	IP	4 injections (0, 2, 4, 7 weeks)	Female BALB/c mice	The vaccine with an optimized dual hapten produces antibodies with nanomolar affinities and blocks the analgesic effects of fentanyl-contaminated heroin	Park <i>et al.</i> [138]
15	Same structure as vaccine 1	TT	Liposomal	IM	4 injections (0, 3, 6, 14 weeks)	Female BALB/c mice	Combining TT-6-AmHap and TT-para-AmFen-Hap yielded an effective bivalent vaccine that ablates the effects of heroin and fentanyl	Barrientos <i>et al.</i> [140]
16	Same structure as vaccine 7	CRM	dmLT or LTAI	IM	2 injections (0, 4 weeks)	Female BALB/c mice	dmLT or LTAI adjuvants and mucosal delivery may be attractive strategies for improving the efficacy of vaccines against fentanyl use disorder	Stone <i>et al.</i> [128]
17	Same structure as vaccine 1	CRM	Alum	IM	4 injections (0, 3, 8, 15 weeks)	Rhesus monkeys	Fentanyl vaccine effectively reduces fentanyl vs food choice in rhesus monkeys	Townsend <i>et al.</i> [133]
18	Carfen-  Carfen-p-phenyl: 	TT	alum and CpG	IP	4 injections (0, 2, 4, 9 weeks)	Female Swiss-Webster mice	The two synthetic vaccines reduce carfenamil-induced antinociception and respiratory depression	Eubanks <i>et al.</i> [127]

TT, tetanus toxoid; CpG, cytosine-phosphodiester-guanine oligodeoxynucleotide 1826; KLH, keyhole limpet hemocyanin; CRM, diphtheria toxin; OVA, ovalbumin; IV, intravenous; IM, intramuscular; SC, subcutaneous; IP, intraperitoneal.

fentanyl and its analogs [130]. Robinson *et al.* developed prophylactic vaccination and therapeutic vaccination that consists of novel fentanyl-based haptens that are conjugated to carrier proteins. Prophylactic vaccination reduces fentanyl- and sufentanil-induced respiratory depression, antinociception, and bradycardia in mice and rats, and therapeutic vaccination reduces fentanyl self-administration in rats [131]. Wang *et al.* developed a new fentanyl conjugate vaccine that is conjugated to the Gal α 1-3Gal epitope, which is based on preformed antibody-assisted antigen presentation, and this vaccine reduces fentanyl-associated side-effects [132].

Fentanyl Vaccines in Nonhuman Primates and Monoclonal Antibodies against Fentanyl

In non-opioid-dependent rats and rhesus monkeys, fentanyl vaccine administration blunts fentanyl reinforcement and increases food reinforcement for a prolonged period [124, 133]. A fentanyl-CRM₁₉₇ conjugate vaccine produces anti-fentanyl antibodies and attenuates fentanyl *versus* food choice in male and female rhesus monkeys [133]. A fentanyl-TT conjugate vaccine increases plasma fentanyl levels ~6-fold and shifts fentanyl potency at least 10-fold in both schedule-controlled responding and thermal nociception assays in male rhesus monkeys [134]. Monoclonal antibodies (mAbs), especially 6A4, have been generated in hybridomas derived from mice vaccinated with a fentanyl conjugate vaccine. These mAbs have been reported to sequester large amounts of these drugs in blood, reverse fentanyl/carfentanil-induced antinociception, and prevent fentanyl-induced lethality, which could be a promising approach to treat opioid overdose and opioid use disorder [135]. α -Fentanyl mAbs have been generated from mice immunized with a fentanyl-sKLH conjugate vaccine. Pretreatment with these mAbs reduces fentanyl-induced antinociception, respiratory depression, and bradycardia in mice and rats [136]. These studies indicate that mAbs against fentanyl and its analogs have potential applications for the treatment of opioid use disorder and the prevention of overdose and toxicity.

Fentanyl/Heroin Vaccines

Overdose deaths caused by opioids have increased substantially in recent years, attributable to the adulteration of these drugs with fentanyl. Many immunopharmacotherapeutic studies have focused on developing new vaccines that can effectively target dual haptens to produce antibodies that are able to sequester fentanyl and contaminated opioids in the periphery. Blake *et al.* developed a heroin/fentanyl combination vaccine that consists of inulin-based formulations

(Advax) that contain a CpG oligodeoxynucleotide and act as effective adjuvants when combined with a heroin conjugate. This vaccine produces high-opioid-affinity serum antibodies and reduces antinociception in both mice and cynomolgus monkeys [137]. By implementing an optimized dual hapten, a vaccine has been developed to produce antibodies that are able to bind fentanyl-contaminated heroin in the periphery, thus effectively blocking their analgesic effects [138]. A dual fentanyl/heroin vaccine generates high-affinity anti-fentanyl and anti-heroin antibodies and decreases the antinociceptive potency of a fentanyl/heroin mixture but does not attenuate combined fentanyl/heroin self-administration [139]. By combining TT-6-AmHap (a heroin monovalent vaccine) and TT-para-AmFenHap (a fentanyl monovalent vaccine) to formulate a bivalent vaccine that is adjuvanted with liposomes that contain monophosphoryl lipid A adsorbed on aluminum hydroxide, a study found that this bivalent conjugate vaccine induces dual immunogenic responses that ablate the effects of both heroin and fentanyl in mice [140]. A chemically contiguous heroin-fentanyl vaccine that uses a hapten with one epitope that has domains for both fentanyl and heroin has also been developed. This vaccine confers protection against heroin and fentanyl in an antinociception analysis [141]. Admixture vaccination strategies have also been applied to target two different drug species and combat fentanyl-adulterated heroin [142, 143]. These new fentanyl/heroin vaccines appear promising but need further testing for possible translation to clinical use.

Conclusions and Perspectives

In conclusion, fentanyl and its analogs have complex receptor pharmacology and produce multifaceted behavioral effects and clinical characteristics, including analgesia, anesthesia, sedation, and respiratory and cardiothoracic side-effects. Fentanyl transdermal microneedles have a rapid onset of antinociceptive action, providing an effective mode of opioid delivery for immediate pain relief with limited side-effects [144]. Microneedles that are loaded with low doses of fentanyl may reduce the risk of overdose fatalities. Developing novel technologies to reduce the clinical dose of fentanyl may be useful for mitigating the opioid crisis. Mechanistically, the behavioral effects of fentanyl likely result from a combination of its chemistry, receptor pharmacology, receptor signaling, rapid distribution to the central nervous system, neural circuit dysfunction, and neuroinflammation. However, the detailed neurobiological mechanisms that contribute to fentanyl abuse and overdose remain largely unknown. Novel effective pharmacological and psychosocial treatments and vaccines are urgently needed.

Drug self-administration procedures and investigations of the choice between opioids and non-drug reinforcers are recommended to evaluate the effectiveness of candidate interventions in preclinical studies. States of opioid dependence and withdrawal should also be considered because such states can profoundly influence the results. The currently available small-molecule pharmacotherapies that mainly target MORs have limited efficacy to combat fentanyl abuse and overdose. Further elucidation of the molecular and structural bases of biased signaling and interactions between fentanyl and MORs is promising for optimizing fentanyl-based analgesics with fewer side-effects, a better safety profile, and higher efficacy. Future studies are needed to improve the effectiveness of opioid medications, such as G-protein biased MOR agonists, and develop novel non-opioid medications that target other systems, such as neurotransmitter and neuropeptide systems. Traditional Chinese medicine, including herbal therapy and acupuncture, has few side-effects and could also be considered for the treatment of fentanyl addiction [145–147].

Anti-fentanyl vaccines produce antibodies that sequester opioid drugs in the periphery and prevent them from entering the brain and activating reward circuits. They have high selectivity and long-lasting efficacy and do not interfere with endogenous opioids, thus providing safe and cost-effective interventions for the management of fentanyl abuse and overdose. However, although several fentanyl and fentanyl/heroin vaccines have been shown to be effective in rodents and nonhuman primates, these vaccines are still in preclinical stages and need to be translated to the clinical treatment of opioid use disorder and fentanyl overdose. To facilitate preclinical-to-clinical translation and increase the success of drug discovery, it is critical to develop clinically viable formulations and optimize rational vaccine designs to maximize efficacy, understand the immunological mechanisms that underlie drug-motivated behavior and vaccine-evoked immune responses, and identify biomarkers that are predictive of individual variability and support patient stratification.

Acknowledgements This review was supported by the National Key Research and Development Program of China (2019YFC0118604) and the National Natural Science Foundation of China (32071058).

Conflict of Interest The authors declare that they do not have any conflicts of interest.

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