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ORIGINAL ARTICLE

Sex-dependent effects of an *Hnrnph1* mutation on fentanyl addiction-relevant behaviors but not antinociception in mice

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Abstract

Opioid Use Disorder (OUD) and opioid-related deaths remain a major public health concern in the United States. Both environmental and genetic factors influence risk for OUD. We previously identified *Hnrnph1* as a quantitative trait gene underlying the stimulant, rewarding, and reinforcing properties of methamphetamine. Prior work shows that hnRNP H1, the RNA-binding protein encoded by *Hnrnph1*, post-transcriptionally regulates *Oprm1* (mu opioid receptor gene)—the primary molecular target for the therapeutic and addictive properties of opioids. Because genetic variants can exert pleiotropic effects on behaviors induced by multiple drugs of abuse, in the current study, we tested the hypothesis that *Hnrnph1* mutants would show reduced behavioral sensitivity to the mu opioid receptor agonist fentanyl. *Hnrnph1* mutants showed reduced sensitivity to fentanyl-induced locomotor activity, along with a female-specific reduction in, and a male-specific induction of, locomotor sensitization following three, daily injections (0.2 mg/kg, i.p.). *Hnrnph1* mutants also required a higher dose of fentanyl to exhibit opioid reward as measured via conditioned place preference (CPP). Male *Hnrnph1* mutants showed reduced fentanyl reinforcement. *Hnrnph1* mutants also showed reduced sucrose motivation, suggesting a reward deficit. No genotypic differences were observed in baseline thermal nociception, fentanyl-induced antinociception, physical or negative affective signs of opioid dependence, or in sensorimotor gating. In the context of our prior work, these findings suggest that *Hnrnph1* dysfunction exerts a selective role in reducing the addiction liability to drugs of abuse (opioids and psychostimulants), which could provide new biological pathways to improve their therapeutic profiles.

KEYWORDS

addictive, analgesia, opiate, pain, psychostimulant

1 | INTRODUCTION

The United States is in the midst of a major opioid addiction epidemic, with over 2 million people estimated to be suffering from Opioid Use Disorder (OUD) and over 67,000 deaths in 2018 (https://www.cdc.gov). A major source of the current opioid epidemic was the over-prescribing of opioid analgesics that began in the late 1990s, based on the premise that pain is the fifth vital sign and that opioids are a safe

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and effective option with low risk for misuse if properly prescribed to pain patients. This philosophy led to a loosening of governmental regulations and opioid over-dispensing.[1] The recognition of a prescription opioid epidemic resulted in the advent of abuse-deterrent formulations of prescription opioids, such as Oxycontin® (containing the mu opioid receptor agonist, oxycodone), which decreased the street value of heroin and made it more marketable.[2,3] Recent laws limit the amount and frequency of opioid that can be dispensed within a prescription (e.g., Prescription Drug Monitoring Programs)[4] and have been associated with a massive shift to illicit heroin use over the past decade.

More recently, synthetic opioids, namely derivatives of the mu opioid receptor agonist fentanyl, have entered the market as they are synthesized more easily and affordably than heroin and can be more readily transported illicitly across borders, because of their increased potency. Thus, fentanyl-derivative drugs are frequently a major component of illicitly sold heroin. Fentanyl-derivative drugs can exhibit potencies ranging from 50 to 10,000 times greater than heroin or morphine, which makes a substantial contribution to the number of opioid-related deaths in the U.S.[5]

While the increased availability of illicit prescription and nonprescription opioids has historically fueled the current opioid epidemic, other environmental risk factors (e.g., early life stress)[6] and genetic factors[7] also contribute to risk for OUD. Twin and family studies estimate the heritability of OUD at greater than 50%.[8,9] However, the genetic basis of OUD remains largely unknown, with only a handful of genome-wide association studies (GWAS) reporting genome-wide significant loci associated with addiction, including *KCNC1* and *KCNG2* (voltage-dependent potassium channel and channel modifier, respectively), *CNIH3* (AMPA receptor axillary protein 3) and most recently *RGMA* (RGM domain family, member 3).[7,10-12] Interestingly, a GWAS identified a noncoding single nucleotide polymorphism (SNP) upstream of *OPRM1* (opioid receptor, mu 1) that was associated with the therapeutic dose of methadone for treating OUD.[13] Larger sample sizes, including an increase in the number of opioid-exposed unaffected controls, will yield several new genome-wide discoveries in the coming years.[10]

RNA binding proteins (RBPs) are a diverse class of molecules that bind to RNA and regulate all aspects of RNA metabolism, including post-transcriptional processing, transport, cellular localization and local translation. RBPs, including heterogeneous nuclear ribonuclear proteins (hnRNPs), can translocate to the cytoplasm following exposure to various extracellular stimuli (e.g., stressors and neuronal activation) and regulate local translation underlying activity-dependent synaptic plasticity.[14] Acute and repeated exposure to drugs of abuse induces activity-dependent synaptic plasticity within the limbic system, including the mesocorticolimbic circuit that underlies behavioral manifestation of the addictions.[15] Because RBPs are positioned to locally and rapidly regulate synaptic protein translation following drug-induced modulation of cellular activity and to regulate long-term drug-induced changes in nuclear gene transcription, there is a growing appreciation that RBPs likely play a pivotal role in synaptic plasticity underlying addiction.[16]

We used an unbiased, forward genetic and fine mapping approach to positionally clone and validate the RBP heterogeneous nuclear ribonuclear protein H1 (*Hnrnph1*) as a quantitative trait gene underlying the locomotor stimulant response to methamphetamine.[17] Differential exon usage analysis identified a set of four quantitative trait variants within the 5′ UTR of *Hnrnph1* that decrease 5′ UTR usage, decrease hnRNP H protein expression, and functionally decrease luciferase reporter expression.[18] *Hnrnph1* mutants harboring a frameshift mutation in the first coding exon show decreased methamphetamine-induced reward, reinforcement, and dopamine release.[19] Furthermore, D1 dopamine receptor activation caused an increase in nuclear hnRNP H immunofluorescence in primary rat cortical neurons that was blocked with a D1 dopamine receptor antagonist,[20] suggesting postsynaptic modulation of hnRNP H in response to D1 receptor signaling.

With regard to opioids, *HNRNPH1* contributes to post-transcriptional processing of *Oprm1*, the gene encoding the mu opioid receptor, including 5′ UTR-mediated translational repression[21] and splicing.[22] The mu opioid receptor is the primary molecular target underlying the addictive and therapeutic properties of opioid drugs.[23] In support of a potential role for *Hnrnph1* in OUD, an intronic variant in *OPRM1* that affects hnRNP H1 binding was associated with the severity of heroin dependence and alternative splicing of *OPRM1* in humans.[22]

Genetic loci and genetically engineered mutations can exert pleiotropic behavioral effects on multiple classes of drugs of abuse,[24] including psychostimulants and opioids.[25-28] Thus, in the present study, we examined OUD-related traits in *Hnrnph1* mutant mice that display deficits in methamphetamine-induced locomotor activity, reward, reinforcement and dopamine release.[19] We employed fentanyl as our mu opioid receptor agonist of choice, given its high degree of selectivity for the mu opioid receptor,[29] its rapid achievement of peak plasma and brain concentration and antinociceptive action following systemic administration,[30] and the global prevalence of abuse and deaths associated from its illicit use.[5] Because both psychostimulant- and opioid-induced locomotor activity depend on dopamine release in the striatum,[31-33] we hypothesized that *Hnrnph1* mutants would display reduced sensitivity to fentanyl-induced locomotor activity and perhaps other behaviors related to drug reward and reinforcement.

Herein, we examined a large battery of behavioral traits that model various aspects of OUD, including acute sensitivity and sensitization to fentanyl-induced locomotor stimulation, conditioned and state-dependent reward as measured via CPP, reinforcement as measured via oral self-administration under operant-conditioning procedures, acute antinociception and tolerance, as well as affective and physiological signs of opioid dependence during fentanyl withdrawal. Importantly, we included sex as a biological variable to examine potential Genotype × Sex interactions in mediating the behavioral effects of *Hnrnph1* deletion on OUD-relevant behaviors. These results identify select, sometimes sex-dependent, changes in fentanyl-induced behavioral phenotypes in *Hnrnph1* mutant mice.

2 | MATERIALS AND METHODS

A portion of the Methods can be found in Supporting Information that describes the protocols used for assessing fentanyl tolerance, acoustic startle, light-dark shuttle box, novel object, marble burying, force swim test (and reversibility with buspirone) and fentanyl dependence.

2.1 | Mice

Hnrnph1 mutant mice (*Hn1+/–*) were generated using TALENs targeting the first coding exon of *Hnrnph1* (exon 4, UCSC Genome Browser; https://genome.ucsc.edu/), resulting in a frameshift mutation and a premature stop codon. *Hn1+/–* mice show reduced transcription of the wild-type (WT) *Hnrnph1* transcript, an upregulation of the mutant and total transcript levels,[17] and a two-fold increase in hnRNP H striatal synatosomal protein.[19] Therefore, *Hn1+/–* refers to the TALENs-induced indel in exon 4 that leads to a premature stop codon and not to gene haploinsufficiency.

Experimental mice were generated by mating *Hn1+/–* males with C57BL/6J females purchased from The Jackson Laboratory (Bar Harbor, ME for studies conducted at Boston University; Sacramento, CA for studies conducted at the University of California Santa Barbara; UCSB). Offspring were genotyped and, unless otherwise indicated, female and male littermates from a minimum of five different litters, ranging from 56 to 100 days of age, were employed in the studies. Mice were housed in same-sex groups of 3–5 in standard mouse cages, housed within ventilated racks under standard housing conditions. Mice involved in the fentanyl self-administration experiments were housed under a reverse light cycle (lights off: 1000 h), otherwise, all other mice were housed under a regular 12 h/12 h light/dark cycle (lights on: 0700 h at UCSB; lights on: 06:30 at Boston University). All experiments were conducted in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (NIH Publication No. 80–23, revised 2014) and approved by the IACUCs of UCSB and Boston University.

We used a previously published power analysis[19] based on our original finding of decreased MA-induced locomotor activity in *Hn1 +/–* mice[17] to guide selection of our sample size. Briefly, with an effect size of Cohen's $d = 0.9$, we used G*Power 3[34] and determined that a sample size of n = 16 per genotype is required to achieve 80% power ($p < 0.05$).

2.2 | Drugs

Fentanyl citrate was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in warm physiological saline (0.9% NaCl) for intraperitoneal (i.p.) injection or in tap water for oral consumption. Buspirone hydrochloride (Sigma-Aldrich) and naltrexone (Tocris Bioscience/Bio-Techne (Minneapolis, MN) were dissolved in sterile saline for i.p. injection.

2.3 | Fentanyl locomotor activity and sensitization

On Days 1 and 2 of locomotor testing, mice received a saline injection (10 ml/kg) and locomotor activity was recorded for 30 min. On Days 3–5, mice received a fentanyl injection (0.2 mg/kg) and locomotor activity was recorded for 30 min each day. The dose of fentanyl was chosen based on previous studies indicating a robust increase in locomotor activity in C57BL/6J mice.[25,27,28,35] Mice were recorded with infrared security cameras (Swann Communications, USA, Inc., Santa Fe Springs, CA) mounted above the Plexiglas chambers (40 cm long × 20 cm wide by 45 cm high[17]). Data analyses were performed in R (https://www.r-project.org/).

2.4 | Fentanyl CPP

Experimentally naïve mice were tested for fentanyl-CPP. The same locomotor apparatus was partitioned into two equal-sized compartments via a black, ion transparent, plastic divider with a mouse entryway (5 cm × 6.25 cm) that was flipped upside down during training and was used to confine mice to one side. Behavior was recorded using digital video-tracking (Anymaze, Stoelting Co., Wood Dale, IL). A 9-day CPP protocol was employed that included 30-min conditioning and test sessions.[17] On Day 1, initial preference was assessed whereby mice received an injection of saline (10 ml/kg, i.p.), were placed on the left side, and were provided open access to both sides. On conditioning Days 2 and 4, all mice received saline and were confined to the left side that contained smooth plastic floor inserts (Plaskolite). On conditioning Days 3 and 5, mice received either saline, 0.05 mg/kg, or 0.2 mg/kg fentanyl and were confined to the right side that contained a textured plastic floor insert. On Days 6 and 7, mice were left undisturbed in their home cages. On Day 8, mice received an injection of saline, were placed into the left side, and were provided open-access to both sides for 30 min. The difference in time spent on the drug-paired side between Day 8 and Day 1 was calculated to index the magnitude of the conditioned response. On Day 9, mice received a priming injection of either saline or their fentanyl-conditioning dose, were placed into the left side, and provided open-access to both sides. The difference in time spent on drug-paired side between Day 9 and Day 1 was calculated and indexed the magnitude of conditioning in a fentanyl-dependent state.[36]

2.5 | Baseline nociception and fentanyl antinociception

The mice used to assess the acute antinociceptive effects of fentanyl served previously as the saline control group in the CPP experiment. Thus, these mice had a history of five saline injections, but were completely opioid-naïve. For testing of baseline nociception and fentanyl antinociception, the hot plate temperature was set to 52.5°C. Mice were habituated to the testing room for at least 1 h. Mice were then placed in a Plexiglas cylinder (15 cm diameter; 33 cm in height)

on the hot plate (IITC Life Science Inc.) and two separate baseline latencies to lick the hind paw were recorded, separated by 30 min. Thirty minutes post-baseline assessment, mice were injected with cumulative doses of fentanyl every 10 min (0.1, 0.1, 0.2 and 0.4 mg/kg) at which point they were assayed for postfentanyl hot plate latencies just prior to the next injection. Thus, the final injection of 0.4 mg/kg was administered 30 min after the first fentanyl injection (0.2 mg/kg, i.p.). The 10 min time point post-fentanyl injection was chosen for assessing antinociception as previous studies have shown peak fentanyl-induced antinociception (0.9 mg/kg, s.c.) on the 55°C hot plate in CF-1 mice and peak brain and serum concentrations peaking at approximately 10 min post-injection.[30] Another study found peak fentanyl antinociception at 5 min post-fentanyl injection (0.4 mg/kg, i.p.) on the 55°C hot plate in CD-1 mice.[37] *T*max (the time to achieve *C*max, or, maximum plasma concentration) in rats following i.p. fentanyl (0.1 mg/kg) was reported to be more delayed and was achieved at approximately 28 min.[38] We previously observed that the locomotor response to 2 mg/kg i.p. fentanyl peaks at 10 min post-i.p. injection in C57BL/6J males.[35] A cut-off latency of 60 s was employed within each postinjection assessment of antinociception to avoid tissue damage. Percent maximum possible effect (% MPE) was calculated using the following formula: %MPE = (postinjection latency minus preinjection latency)/(60 − preinjection latency) * 100.

In an attempt to induce antinociceptive tolerance, we treated a separate, experimentally naïve cohort of mice with repeated injections twice daily for five consecutive days [0.8 mg/kg fentanyl (i.p.) or saline (i.p.); 0900 h and 1700 h]. On Day 6, all groups of mice were tested for baseline hot plate nociception and then administered a submaximal antinociceptive challenge dose of 0.4 mg/kg fentanyl (i.p.), beginning at 0900 h (see Supporting Information for additional details).

2.6 | Fentanyl operant conditioning

Male mice were first trained to lever press for delivery of a 10% sucrose solution under a fixed ratio 1 (FR1) schedule of reinforcement with a 20 s timeout in standard mouse operant chambers (Med Associates, St. Albans, VT). Male mice were employed exclusively in this initial operant-conditioning study because of limited availability of female mice at the time the experiment was performed. As in recent studies,[39] each right lever-press resulted in delivery of 20 μl of the sucrose solution and a 20 s presentation of a tone/light stimulus-complex. Left lever presses resulted in no programed consequences. Sucrose training proceeded for 12 days, by which point, mice of both genotypes had exceeded the minimum requirements for successful acquisition of the operant response (a minimum of 10 active lever-presses in 60 min + greater than 70% responding on the active lever for three consecutive days). Then, the sucrose solution was substituted for an unadulterated 3 mg/L fentanyl solution—a concentration showed recently to be readily consumed by mice under free-access conditions in the home-cage[39]—and mice underwent daily, 60 min testing sessions for an additional 10 days. In the initial fentanyl self-administration study, the concentration of the fentanyl reinforcer was then progressively *increased* across days (10, 30, 100, 300 and 1000 mg/L), with each concentration presented until responding stabilized (less than 15% variability across three consecutive presentations) or for a maximum of 10 days of self-administration.

Because the data from this initial fentanyl experiment suggested that the doses tested were located on the descending limb of the dose–response function (see Section 3), we trained a separate cohort of female and male mice (all sucrose-naïve) to nose-poke for 3 mg/L fentanyl during once daily (60-min) sessions. In this second study, mice of both genotypes met the acquisition criterion for self-administration training within 10 days, at which time the fentanyl concentration available was progressively *decreased* across days (1, 0.3, 0.1 and 0.03 mg/L), with each concentration presented until responding stabilized or for a maximum of 10 days of self-administration.

2.7 | Fentanyl withdrawal-induced negative affect and physical dependence

To examine the effects of *Hn1+/−* on fentanyl withdrawal-induced negative affect and physical dependence, female and male *Hn1+/−* and WT littermates were injected twice daily (0900 and 1700 h) with 0.8 mg/kg fentanyl (i.p.) or saline (i.p.) for 5 days. The next morning, mice were subjected to a 1-day behavioral test battery to assess fentanyl withdrawal-induced sensorimotor-gating deficits and negative affect. The 1-day test battery was very similar to a battery that we employed in a recent opioid study,[39] as well as prior studies of alcohol withdrawal-induced negative affect.[40-42] The battery consisted of testing for acoustic startle and prepulse inhibition (PPI) of acoustic startle, followed, in order, by testing in the light-dark shuttle box test, novel object encounter, marble-burying and the Porsolt forced swim tests. Details of these specific procedures are provided in Supporting Information. The day following the behavioral test battery, fentanyl-experienced mice were then injected in the morning (∼0800 h) with 0.8 mg/kg fentanyl. Eight hours later, mice were injected with 10 mg/kg of the opioid receptor antagonist naltrexone and were tested for physiological signs of withdrawal as described previously[39,43] and are detailed in Supporting Information. To accommodate all the mice, behavioral testing was conducted in cohorts of 8 mice, with ∼24 mice tested per day.

2.8 | Statistical analysis

Locomotor activity was analyzed in R (https://www.r-project.org/) using a mixed-design Genotype × Sex × Day ANOVA, with Day as the repeated measure. Locomotor habituation to saline injections was quantified as change in locomotor activity on Day 1 versus Day 2 (Day 1–Day 2). In order to take into account genotypic differences in baseline locomotion, we also quantified the acute locomotor response to fentanyl as the change in locomotor activity on Day 3 (first

fentanyl injection) versus Day 2 (second saline injection). We also quantified locomotor sensitization to fentanyl as the change in locomotor activity from Day 3 to Day 5 (i.e., the first to third fentanyl injection). These data were analyzed using a two-way Genotype × Sex ANOVA. For the CPP experiment, a three-way Genotype × Sex × Dose ANOVA was used to examine initial Day 1 preference as well as preferences in the drug-free and fentanyl state-dependent CPP. Locomotor activity in each CPP day was also analyzed with three-way Genotype × Dose × Sex ANOVA. For nociception and fentanyl antinociception, we first analyzed differences in baseline nociceptive latencies with a mixed-design Genotype × Sex × Baseline ANOVA, with Baseline as repeated measure (two baselines were recorded). Average baseline was analyzed with a two-way Genotype × Sex ANOVA. Acute fentanyl antinociception was analyzed via a mixed model ANOVA with Genotype × Sex × Cumulative Dose as the repeated measure. We analyzed post-injection latencies which allowed us to assess whether there was significant antinociception relative to baseline. We also analyzed %MPE (see above) which allowed us to take into account differences in baseline latencies in isolating potential group differences in fentanyl antinociception, while minimizing potential confounding group differences in baseline nociception. Operant studies were analyzed using SPSS v.21 statistical software (IBM, 2012). The average number of active and inactive responses, the average response allocation on the fentanyl-reinforced operandum (i.e., percentage of total responses directed at the lever or hole that resulted in reinforcer delivery) and the average intake of sucrose or fentanyl during the initial training phase and during the last 3 days of the training phase were analyzed using *t*-tests or Genotype × Sex ANOVAs, as appropriate. The data from the dose–response phase of testing were analyzed using a Genotype × Dose ANOVA (experiment 1) or Genotype × Sex × Dose ANOVA (experiment 2), with Dose as a repeated measure. For fentanyl withdrawal and physical dependence, unless otherwise indicated, the data were analyzed using a Genotype × Sex univariate ANOVA using the SPSS v.23 software. For all analyses, when appropriate, significant interactions were deconstructed prior to simple effect analyses for post hoc comparisons. For all studies, main effects and interactions in the ANOVA models were considered significant if $p < 0.05$. For post hoc simple main effects and pairwise *t*-tests, the same alpha level of 0.05 was employed after Bonferroni-adjustment for multiple comparisons as indicated.

3 | RESULTS

A schematic of the mice and the assay in which they were used are provided in Tables S1–S4. A summary of the experimental findings is provided in Table 1.

3.1 | *Hn1+/−* decreases acute fentanyl-induced locomotor activity and increases fentanyl locomotor sensitization in males

Based on our prior observation of reduced methamphetamine-induced locomotor activity in *Hn1+/−* mice,[17,19] we hypothesized that *Hn1+/−* mice would also show reduced fentanyl-induced locomotor activity. In examining habituation during saline trials (i.p.) and the subsequent fentanyl response following repeated injections (0.2 mg/kg, i.p.), we found increased habituation in *Hn1+/−* males versus WT males, decreased fentanyl-induced locomotor activity, and increased fentanyl-induced locomotor sensitization in *Hn1+/−* males.

Using a within-subjects design, in examining the effects of *Hnrnph1* deletion on the locomotor response to saline (i.p.; Days 1, 2) and fentanyl (0.2 mg/kg, i.p.; Days 3, 4 and 5), there was a significant Genotype × Sex × Day interaction ($F_{4,232} = 3.92$, $p = 0.004$) (Figure 1(A)) and thus, the interaction was deconstructed along the Sex factor to examine the source of sex differences in the effects of *Hn1+/−* on behavior.

A significant Genotype × Day interaction was detected in females ($F_{4,112} = 4.15$, $p = 0.004$; Figure 1(A)) but not males ($F_{4,120} = 2.08$; $p = 0.17$; Figure 1(B)). However, simple main effect analysis of Genotype in the females across days, adjusted for the five comparisons did not identify any significant genotypic differences that account for this interaction (p's$_{adjusted}$ >0.07).

In examining habituation to saline-induced locomotor activity (activity on Day 1–Day 2), there was a main effect of Sex ($F_{1,58} = 5.78$; $p = 0.019$) and a Genotype × Sex interaction ($F_{1,58} = 6.69$, $p = 0.012$). There was a simple main effect of Sex in WT mice ($p_{adjusted} = 0.0017$) that was explained by WT males showing less habituation than WT females ($+p_{adjusted} = 0.0018$).

In examining acute fentanyl-induced locomotor activity on Day 3 while correcting for differences in saline locomotor activity on Day 2 (Day 3–Day 2), there was a significant Genotype effect ($F_{1,58} = 4.45$, $p = 0.039$), reflecting lower fentanyl-induced locomotor activity in *Hn1+/−* versus WT mice (Figure 1(D)). There was no significant effect of Sex ($F_{1,58} = 1.65$; $p = 0.20$) or interaction ($F_{1,58} < 1$).

In examining the extent to which repeated fentanyl injections on Days 4 and 5 induced locomotor sensitization relative to the first fentanyl injection on Day 3, we examined the difference in locomotor response (Day 5–Day 3) and found a robust Genotype × Sex interaction ($F_{1,58} = 10.15$, $p = 0.002$). There was a simple main effect of Genotype in males ($^{*}p_{adjusted} = 0.028$) that reflected a significant increase in fentanyl locomotor sensitization in *Hn1+/−* males versus WT males (Figure 1(E)). In contrast, there was no simple main effect of Genotype in females ($p_{adjusted} = 0.11$). Thus, the regulation of fentanyl-induced locomotor sensitization by *Hn1+/−* varies as a function of Sex.

3.2 | Reduced fentanyl-CPP in *Hn1+/−* mice

We previously found reduced CPP to low-dose methamphetamine (0.5 mg/kg, i.p.) and increased high-dose methamphetamine CPP (2 mg/kg, i.p.) in *Hn1+/−* mice.[19] Thus, we hypothesized that *Hn1+/−* mice would show reduced low-dose fentanyl CPP (0.05 mg/kg, i.p.) and increased high-dose fentanyl CPP (0.2 mg/kg, i.p).. We found some evidence to support this hypothesis, whereby *Hn1+/−* mice showed significant high-dose fentanyl CPP relative to their *Hn1+/−* saline counterparts during the drug-free state and during state-dependent CPP assessment (Figure 2(A),(B)).

In examining drug-free expression of fentanyl reward via CPP (Day 8–Day 1), there was a main Dose effect ($F_{2,136}$ = 3.32, p = 0.039), but no Genotype effect ($F_{1,136}$ <1) or Sex effect ($F_{1,136}$ = 2.48, p = 0.12). Importantly, there was a significant Genotype × Dose interaction ($F_{2,136}$ = 3.46, p = 0.031). Post hoc pairwise t-tests adjusted for the three within-genotype comparisons indicated that *Hn1+/−* mice showed a significant fentanyl-CPP at 0.2 mg/kg fentanyl relative to their *Hn1+/−* saline (0 mg/kg) counterparts (t_{45} = 2.67; #$p_{adjusted}$ = 0.033; Figure 2(A)). Furthermore, *Hn1+/−* mice showed more fentanyl-CPP compared with WT mice at the 0.2 mg/kg dose (t_{46} = 3.07; *$p_{adjusted}$ = 0.011; Figure 2(A)).

When mice were tested for state-dependent CPP in a fentanyl-primed state (i.e., under the influence of one of three respective training doses), there was a significant Dose effect ($F_{2,136}$ = 10.98, $p = 3.8 \times 10^{-5}$) and Sex effect ($F_{1,136}$ = 4.36, p = 0.039), but no Genotype effect ($F_{1,136}$ <1) and no interactions (all p's >0.075). Post hoc pairwise t-tests adjusted for the three dose comparisons within-genotype indicated that the Dose effect was explained by *Hn1+/−* mice primed with the 0.2 mg/kg fentanyl dose exhibiting a robust place-preference, relative to their 0 mg/kg counterparts primed with a saline injection (t_{45} = 5.33; #$p_{adjusted}$ = 0.00012; Figure 2(B)).

In examining concomitant locomotor activity in these same mice during fentanyl-CPP assessment (Days 1, 8 and 9) and during fentanyl-CPP training (Days 2, 3, 4 and 5), we found little evidence for an effect of *Hn1+/−* on drug behavior in this context (details are provided in Figures S1 and S2).

TABLE 1 Summary of key findings of fentanyl-associated behaviors in *Hn1+/−* mice

Assay	Dependent variable	Sex-dependent effect	*Hn1+/−* versus WT
Locomotor activity	Distance traveled (m)	↓ in *Hn1+/−* females versus WT females (Day 5, p_{adj} = 0.068)	
	Habituation: D1–D2	↓ in WT males versus WT females (p_{adj} = 0.002) ↑ in *Hn1+/−* males versus WT males (p_{adj} = 0.093)	No difference
	Acute: D3–D2	No difference	No difference
	Sensitization: D5–D3	↓ in *Hn1+/−* females versus WT females (p_{adj} = 0.088) ↑ in *Hn1+/−* males versus WT males (p_{adj} = 0.045)	No difference
CPP	Drug-free	No difference	↑ in *Hn1+/−* versus WT at 0.2 mg/kg (p_{adj} = 0.011)
	Fentanyl-state	No difference	No difference
Fentanyl acute antinociception	Baseline and postinjection latency and % MPE	No difference	No difference
Fentanyl tolerance	Baseline and postinjection latency and % MPE	No difference	No difference
Operant conditioning: sucrose reinforcement	Mean active lever-presses	No difference	↓ in *Hn1+/−* versus WT
	Mean inactive lever-presses	No difference	↓ in *Hn1+/−* versus WT
	Active lever allocation (%)	No difference	No difference
	Sucrose intake (g/kg)	No difference	No difference
Operant conditioning: Low dose fentanyl reinforcement without sucrose training	Active lever allocation (%)	↑ in *Hn1+/−* males versus WT males	No difference
	Fentanyl intake (mg/kg)	↓ in *Hn1+/−* males versus WT males	No difference
Withdrawal: ligh-dark shuttle box	Number of light-side entries	↑ in *Hn1+/−* males versus WT males	No difference
Withdrawal: novel object	Latency to 1st contact (s)	↓ in *Hn1+/−* males versus WT males	No difference
	Number of contacts with novel object	↑ in *Hn1+/−* males versus WT males	↓ in WT mice only
Withdrawal: marble-burying	Marbles buried	↑ in *Hn1+/−* males versus WT males	No difference
Withdrawal: forced swim test	Latency to float (s)	No difference	No difference
	Time spent floating (s)	No difference	No difference
	Number of floating episodes	No difference	No difference
Withdrawal reversal by buspirone: forced swim test	Latency to float (s)	No difference	No difference
	Time spent floating (s)	No difference	No difference
	Number of floating episodes	No difference	No difference

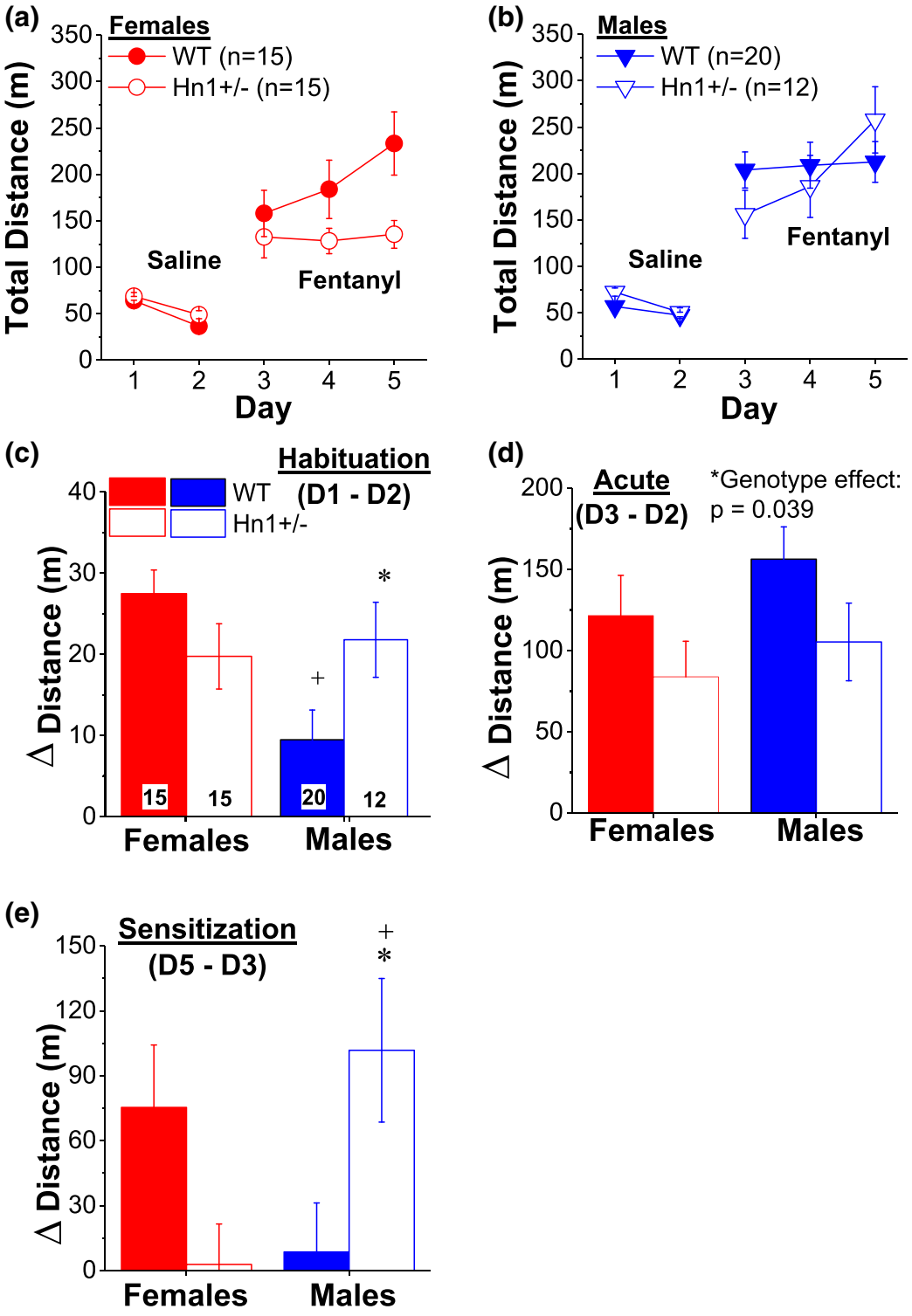

FIGURE 1 Sex-dependent modulation of fentanyl-induced locomotor activity and sensitization in *H1*+/− mice. (A) A significant Genotype × Day interaction was detected in females ($p = 0.004$). In response to saline (i.p.), there was no significant difference in Day 1 or Day 2 activity in *Hn1*+/− females versus WT females when correcting for the five comparisons across days (p's >0.14). Furthermore, in response to repeated fentanyl (2 mg/kg, i.p.), there were no significant genotypic differences in locomotor activity across Days 3, 4 and 5 when correcting for 5 days of comparison (p's >0.07). (B) There was no significant Genotype × Day interaction in males ($p = 0.17$). (C) In examining habituation to locomotor activity following saline injections (i.p.) from Day 1 to Day 2 (Day 1–Day 2), WT males showed less habituation than WT females ($+p_{adjusted} = 0.0018$), while no sex difference was apparent in *Hn1*+/− mice. (D) When taking into account genotypic differences in baseline locomotor activity (following the 2nd saline injection on Day 2; Day 3–Day 2), *Hn1*+/− mice showed less robust acute fentanyl-induced locomotor activity (0.2 mg/kg, i.p.) compared with WT mice (Genotype effect: $^*p = 0.039$). (E) In examining fentanyl locomotor sensitization from Day 3 to Day 5 (Day 5–Day 3), there was a significant Genotype × Sex interaction ($F_{1,58} = 10.15$, $p = 0.002$). A simple main effect of Genotype in males ($^*p_{adjusted} = 0.028$), but not females ($p_{adjusted} = 0.11$) reflected a significant increase in fentanyl locomotor sensitization in *Hn1*+/− males versus WT males. Data represent the mean ± *SEM*. Ns are included in the figure legends and the sample sizes employed in Panels D and E are the same as Panel C

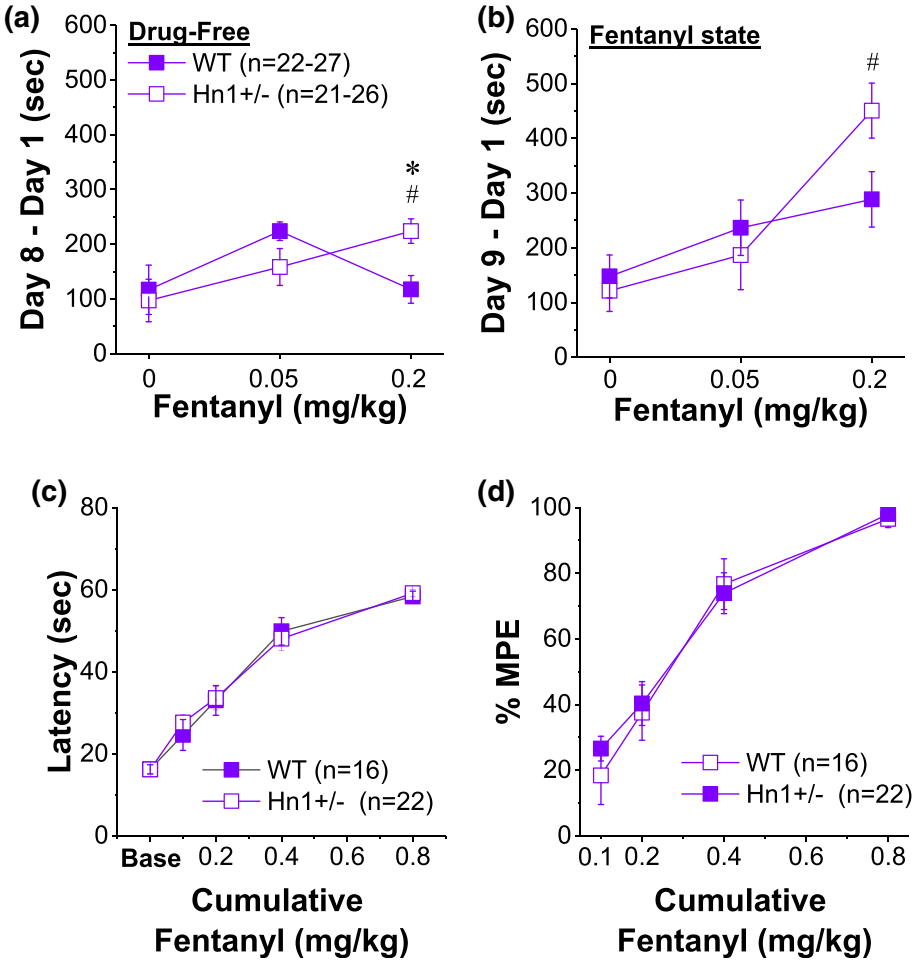

FIGURE 2 Modulation of fentanyl reward but not antinociception in *H1+/−* mice. (A) When assessed in a drug-free state, only *Hn1+/−* mice exhibited significant CPP at the 0.2 mg/kg dose relative to their Hn1+/− SAL controls (vs. 0 mg/kg: $\#p_{adjusted} = 0.033$). Additionally, *Hn1+/−* mice showed greater fentanyl-CPP compared with WT mice at the 0.2 mg/kg dose ($^{*}p_{adjusted} = 0.011$). (B) When tested under the influence of their conditioning dose, only *Hn1+/−* mice exhibited a significant CPP at the 0.2 mg/kg fentanyl dose compared with their saline control Hn1 +/− counterparts (vs. 0 mg/kg; $\#p_{adjusted} = 0.00012$). (C) No genotypic differences were apparent for the latency to lick the hind paw at baseline or in fentanyl antinociception following treatment with a cumulative fentanyl-dosing regimen. (D) Likewise, no genotypic differences were noted in the percent maximum possible effect (%MPE) of fentanyl antinociception. Data represent the mean ± *SEM.* of the number of mice indicated in the figure legends. "Base" = baseline latency, averaged across two separate measurements

A retrospective analysis of initial preference for the right (drug)-paired side indicated that there was a random but significant effect of Initial Treatment Assignment whereby the SAL groups showed lower initial preference for the drug-paired side (details of statistical results are provided in Figure S3; further data are provided in Table S5). This observation likely explains why we saw a slight preference for the drug-paired side in SAL controls (~100 s). Given our extensive experience with this CPP assay configuration and in particular, given the lack of preference under saline conditions using the same pure C57BL/6J background[19,36] or in testing C57BL/6 substrains,[36] it is unlikely that the effect of random treatment assignment leading to lower initial preference can account for our current set of results with fentanyl treatment, nor does it confound the interpretation. If anything, the small preference because of random treatment assignment in the SAL treatment groups underestimates the true effect size of treatment effects and genotypic differences.

3.3 | No change in baseline thermal nociception or fentanyl antinociception following acute or repeated fentanyl administration in *Hn1+/−* mice

Given the genotypic effects of *Hn1+/−* on fentanyl-induced locomotor activity and reward, we wondered if the *Hn1+/−* genotype might also affect pain-related behaviors and the therapeutic (antinociceptive) response to fentanyl. We did not observe significant evidence for altered baseline thermal nociceptive sensitivity or fentanyl-induced antinociception following acute or repeated administration in *Hn1+/−* mice.

Mixed-effects ANOVA of the two baseline measurements that were collected just prior to the cumulative-dosing experiment indicated a main effect of Baseline as the repeated measure ($F_{1,34} = 8.71$; $p = 0.006$) but no effect of Genotype or Sex ($F_{1,34} < 1$), nor any interactions (p's >0.079). Similarly, two-way ANOVA of the average baseline indicated no effect of Genotype ($F_{1,34} < 1$), Sex ($F_{1,34} = 1.90$; $p = 0.18$), or interaction ($F_{1,34} = 1.36$; $p = 0.25$). Therefore, we defined the baseline as the average of the two measurements and included these values in the ANOVA involving postfentanyl hot plate latencies (Figure 2(C)).

In examining fentanyl-induced antinociception following cumulative dosing and using the average baseline values and post-injection latencies, there was a main effect of Dose ($F_{4,136} = 147.16$; $p = 3.34 \times 10^{-47}$) but no effects of Genotype, Sex or Genotype × Sex interaction ($F_{1,34} < 1$), nor was there any interaction with Dose ($F_{4,136} < 1$; Figure 2(C)). Pairwise t-tests correcting for the 10 comparisons among doses indicated that the simple effect of Dose ($F_{4,148} = 146.75$; $p = 1.87 \times 10^{-50}$) was explained by a significant

increase in postinjection latency from baseline to 0.1, 0.2, 0.4, and 0.8 mg/kg doses (all $p_{adjusted}$'s <0.0001). Furthermore, increased antinociception was observed from the 0.1 mg/kg dose to the 0.2, 0.4 and 0.8 mg/kg doses as well as the increase from 0.2 to 0.4 and 0.8 mg/kg doses, and 0.4 to 0.8 mg/kg dose (all $p_{adjusted}$'s <0.0001; Figure 2(C)).

In examining fentanyl antinociception following cumulative dosing as %MPE, similar results were observed in that there was no effect of Genotype or Sex ($F_{1,34}$ <1), but there was a main effect of Dose ($F_{3,102}$ = 95.68; p = 1.05 × 10–28) and a Dose × Sex interaction ($F_{3,102}$ = 2.89; p = 0.039; Figure 2(D)). Pairwise t-tests correcting for the six comparisons among %MPE doses indicated that the effect of Dose ($F_{3,111}$ = 93.15, p = 3.47 × 10^{-30}) on %MPE was explained by the significant increase from the 0.1 mg/kg dose to the 0.2, 0.4 and 0.8 mg/kg doses (all p's$_{adjusted}$ <0.0001) as well as the increase from 0.2 dose to 0.4 and 0.8 mg/kg doses, and 0.4 to 0.8 mg/kg dose (all p's$_{adjusted}$ <0.0001; Figure 2(D)).

Similarly, in a separate cohort of mice, in examining baseline hot plate latencies and post-fentanyl antinociception following repeated injections of either saline (i.p.) or fentanyl (0.8 mg/kg, i.p.) and a single fentanyl challenge dose (0.4 mg/kg, i.p.), there were no significant genotypic differences in baseline nociception or fentanyl antinociception (details of statistical results are provided in Figure S4).

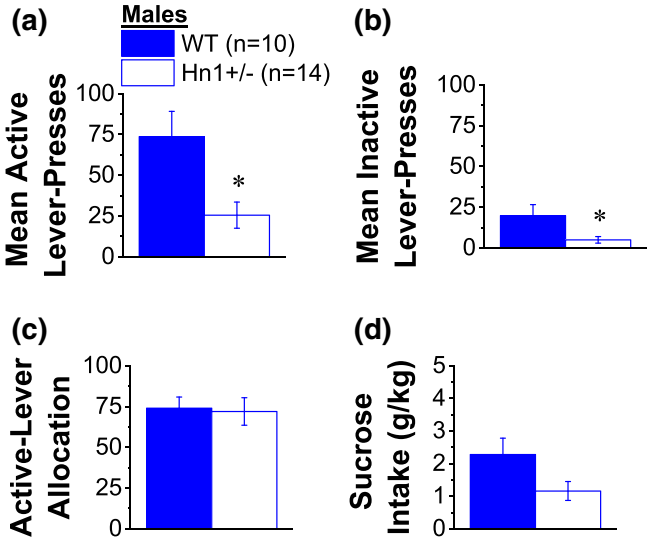

FIGURE 3 Blunting of operant-responding for sucrose reinforcement in male *Hn1+/–* mice. When trained to lever-press for 10% sucrose, *Hn1+/–* males exhibited fewer active (A: *p = 0.008) and inactive (B: *p = 0.02) lever-presses, than WT male controls. However, both genotypes directed a similar percentage of their responses toward the sucrose-reinforced lever, indicating that the mutation did not alter fentanyl-directed responding (C). (D) While sucrose intake was lower in *Hn1+/–* males versus WT males, this genotypic difference was not statistically significant. Data represent the mean ± *SEM*. Ns are indicated in the figure legends and in Table #. *p <0.05 versus WT

3.4 | Decreased sucrose operant responding in *Hn1+/–* males

We next determined the effect of *Hn1+/–* on responding for sucrose, a naturally rewarding stimulus. In this experiment, only males were available for testing and we found a decrease in sucrose responding in *Hn1+/–* males.

In examining operant-responding for reinforcement for 10% (w/v) sucrose, on average, *Hn1+/–* males showed significantly fewer active lever-presses than WT males during the 12-day training period (Figure 3(A)) (t_{22} = 2.93, *p = 0.008). Although both genotypes met the acquisition criterion for sucrose self-administration by the end of the training period, active lever-responding was still significantly lower in *Hn1+/–* males versus WT males over the last 3 days of training (t_{22} = 2.99, p = 0.008; WT = 73.73 ± 15.50 vs. *Hn1+/–* = 25.57 ± 8.01). Similarly, *Hn1+/–* males also emitted fewer inactive lever-presses during the sucrose phase of the study, as evidenced both in terms of the average overall number of inactive lever-responses (Figure 3(B): t_{22} = 2.56, *p = 0.02) and the average number of inactive lever-presses during the last 3 days of sucrose training (t_{22} = 2.63, p = 0.02; WT = 19.87 ± 6.48 vs. *Hn1+/–* = 4.55 ± 1.78). Importantly, there were no genotypic differences in the allocation of responding toward the sucrose-reinforced lever, indicating that *Hn1+/–* deletion did not significantly impair sucrose reinforcement, at least in males (Figure 3(C)) (for average of the 12-day training period: t_{22} = 0.12, p = 0.90; for the last 3 days of sucrose training: t_{22} = 0.09, p = 0.93; WT = 73.18 ± 6.83% vs. *Hn1+/–* = 72.21 ± 8.42%). Finally, while the average sucrose intake was lower in *Hn1+/–* males versus WT males, this difference was not statistically significant (Figure 3(D); for average of the 12-day training period: t_{22} = 1.89, p = 0.12; for the last 3 days of sucrose training: t_{22} = 1.57; p = 0.23; WT = 2.51 ± 0.62 g/kg vs. *Hn1+/–* = 1.87 ± 0.45 g/kg). These data provide new evidence that *Hnrnph1* gene products regulate behavioral output of male mice under operant-conditioning procedures but are less involved in regulating the appetitive and consummatory aspects of sucrose reinforcement.

3.5 | Decrease in operant fentanyl intake in sucrose-trained *Hn1+/–* males

Following training for sucrose responding, we subsequently trained the same sucrose-experienced males to lever-press for oral fentanyl reinforcement (3 mg/L) and found evidence for reduced indices of fentanyl reinforcement in the same *Hn1+/–* males during the 10-day training phase of the study (Figure S5A–D). However, no significant effect of *Hn1+/–* was observed for the dose–response functions related to high-dose (3–1000 mg/L) fentanyl reinforcement (-Figure S5E–H). As we also detailed in Supporting Information Results, when fentanyl reinforcement was assessed a second time in sucrose-naïve female and sucrose-naïve male mice, there were no significant genotypic differences in drug self-administration or intake during the training phase of the study (i.e., when 3 mg/L fentanyl served as the

reinforcer), although males emitted more active nose-pokes and exhibited greater fentanyl intake than females (Figure S6). These negative findings suggest that the decrease in fentanyl self-administration observed in sucrose-trained males (Figure S5A–D) could have been influenced by prior sucrose experience.

3.6 | Operant fentanyl intake in sucrose-naïve *Hn1+/−* mice

Given the evidence for reduced fentanyl reinforcement in sucrose-experienced *Hn1+/−* males as described above, we wanted to further test whether these significant results depended on prior sucrose exposure. Thus, in the next experiment, we tested a new cohort of experimentally naïve female and male mice (no prior sucrose training) and again, we found evidence for male-selective effects of *Hn1+/−* on fentanyl self-administration across a range of lower drug doses (0.01–3 mg/L) but no significant effect in females.

Neither Sex nor Genotype influenced the dose–response functions for active or inactive nose-poking (statistical results provided in Supporting Information; see Figure S7). However, there was a sex-dependent effect of *Hn1+/−* on the allocation of responding in the fentanyl-reinforced hole (Genotype × Sex: $F_{1,135}$ = 7.02, p = 0.01). This interaction was not reflected in females (Figure 4(A); test for simple main effect: p >0.05); rather, *Hn1+/−* males showed greater fentanyl-appropriate responding than WT males (Figure 4(B); test for simple main effect: *p <0.05). While the allocation of responding in the active hole also decreased as a function of fentanyl dose (Dose effect: $F_{4,140}$ = 7.41, p <0.0001), neither Sex nor Genotype influenced the shape of this dose–response function (Figure 4(A),(B); Dose interactions, p's >0.75). Overall, males consumed more fentanyl than females during dose–response testing (Figure 4(C),(D); Sex effect: $F_{1,35}$ = 11.14, p = 0.002; Sex × Dose: $F_{4,140}$ = 9.61, p <0.0001). Moreover, there was a Genotype × Sex interaction with respect to the dose–response function of fentanyl intake (Genotype × Sex × Dose: $F_{4,140}$ = 2.44, p = 0.049). Deconstructing this three-way interaction along the Sex factor indicated no genotypic difference in fentanyl intake in females (Figure 4(C); Genotype effect and interaction, p's >0.88). In contrast, the dose–response function was shifted downward in *Hn1+/−* males, relative to their WT male counterparts (Genotype × Dose: $F_{4,60}$ = 3.67, p = 0.01), with *Hn1+/−* mice showing less fentanyl intake at the 3 mg/L concentration (Figure 4(D): test for simple main effect: *p <0.05) and a trend toward lower intake at 1.0 mg/L (test for simple main effect: p >0.05). Together, these latter findings indicate that *Hn1+/−* increases the reinforcing efficacy of fentanyl in males (as indicated by the shift upward in fentanyl-appropriate responding), which likely reduces their propensity to consume fentanyl.

3.7 | Fentanyl withdrawal does not induce obvious sensorimotor-gating deficits in *Hn1+/−* or WT mice

We next turned to examining the effect of *Hn1+/−* on behavioral measures of spontaneous opioid withdrawal. First, we started with

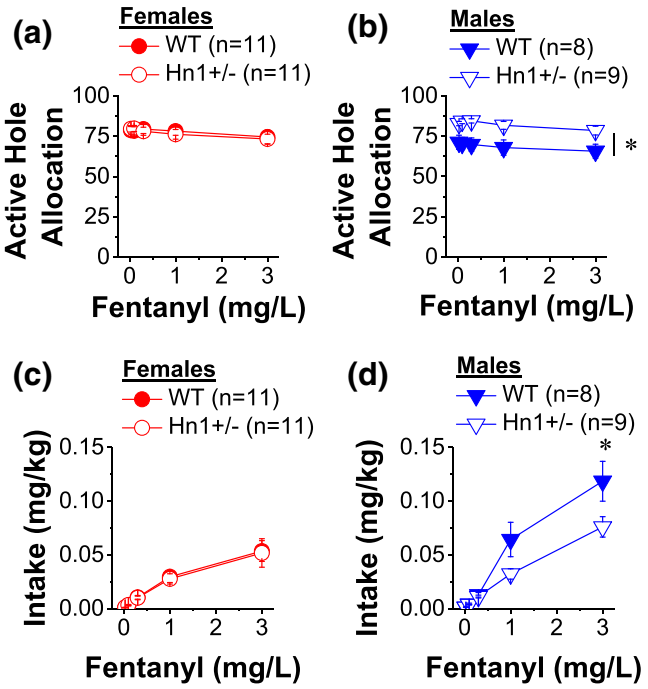

FIGURE 4 Sex-dependent effects of *Hn1+/−* on fentanyl reinforcement during acquisition of self-administration in sucrose-naïve mice. (A) When assessed across a range of low fentanyl doses (0.03–3.0 mg/L), no genotypic differences were apparent in experimentally naïve females for the allocation of responding in the active, fentanyl-reinforced hole. (B) *Hn1+/−* males directed more nose-pokes toward the fentanyl-appropriate active lever, overall, than did their WT controls (*p = 0.004). (C) For females, there was no significant genotypic difference in low-dose FEN intake. (D) In contrast, *Hn1+/−* males consumed less FEN than WT males at 3 mg/L (*p = 0.05). Data represent the mean ± *SEM*. of the number of mice indicated in the figure legends. *p <0.05 versus WT; * denotes main effect of Genotype

sensorimotor gating in the acoustic startle test as opioid withdrawal has been shown to modulate this behavior.[44] Details of the procedure are provided in Supporting Information. We found that neither *Hn1+/−* nor fentanyl withdrawal significantly altered startle amplitude (-Figure S8A) or the PPI of startle amplitude (Figure S8B). However, fentanyl-withdrawn males showed an overall modest impairment in PPI at both decibel levels (Figure S8C).

3.8 | Sex-dependent modulation of behavior in light-dark shuttle box in *Hn1+/−* mice

The light–dark shuttle box apparatus is used to index anxiety-like behaviors[45,46] and at least one prior study has showed behavioral effects of opioid withdrawal in this paradigm.[47] Thus, following startle testing, we tested the same mice for shuttle behavior and found that repeated fentanyl administration (twice daily injections of 0.8 mg/kg for 5 days) did not alter the number of light-side entries exhibited by either male or female WT or *Hn1+/−* mice (Drug effect and interactions, p's >0.13). However, a significant Genotype × Sex interaction

was detected for this measure ($F_{1,100}$ = 4.68, p = 0.03), that reflected a greater number of light-side entries in *Hn1*+/− males versus WT males (Figure 5(A): test for simple effects: *p <0.05), but no genotypic difference in female subjects (p >0.05). None of the independent variables influenced the latency to enter, the time spent in, nor the distance traveled in, the light-side (data not shown; Genotype × Sex × Drug ANOVAs,

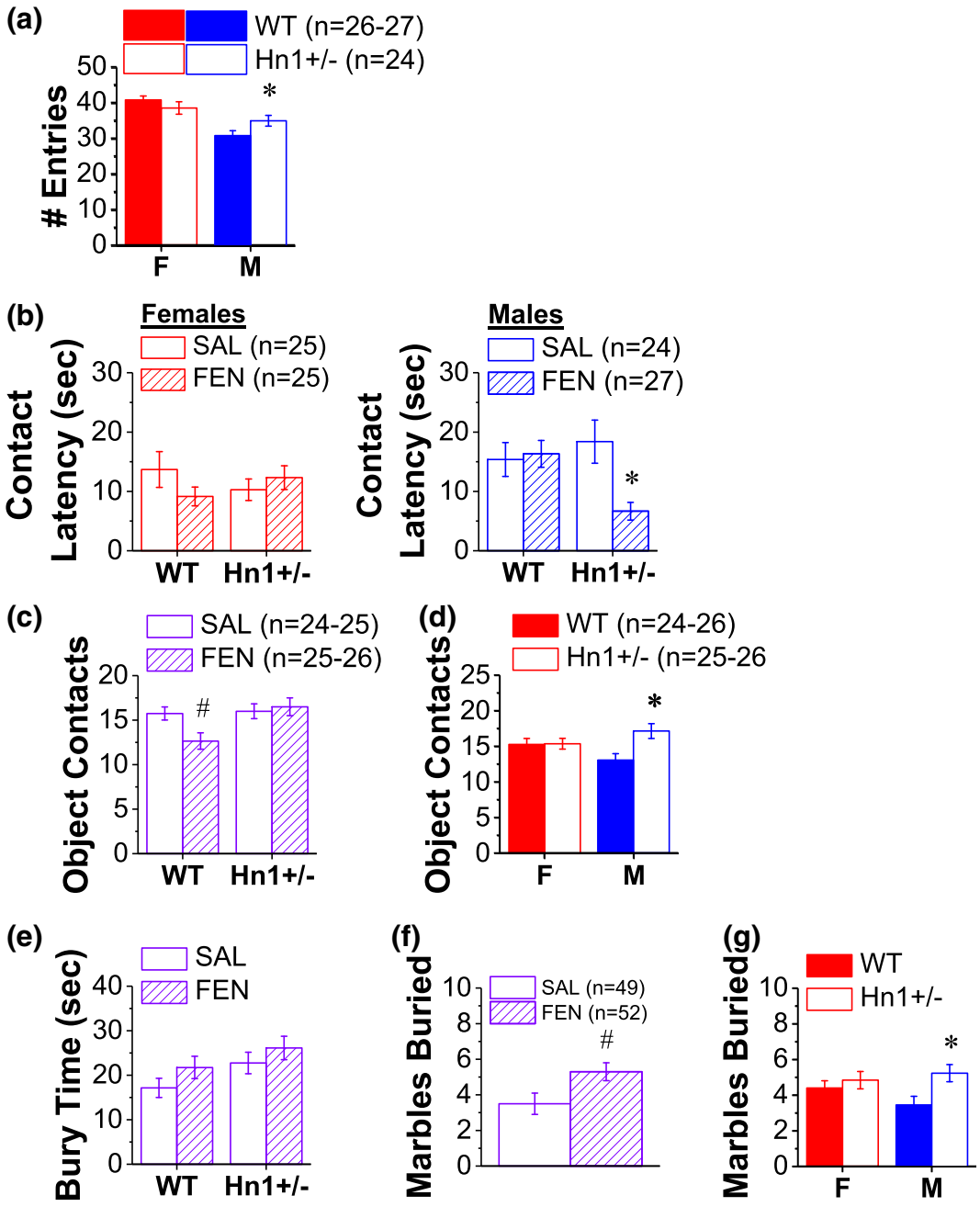

FIGURE 5 Sex-dependent effects of *Hn1*+/− on behavior in assays of anxiety-like behavior in naïve and fentanyl-withdrawn mice. (A) In the light–dark shuttle-box test, *Hn1*+/− males exhibited a greater number of light-side entries than WT males (*p = 0.04). (B) In the novel object encounter, fentanyl (FEN) withdrawal decreased the latency to first make contact with the object compared with saline (SAL) controls, but this effect was observed only in *Hn1*+/− males (right panel, versus WT males: *p = 0.002). (C) Irrespective of sex, fentanyl withdrawal reduced the number of novel object contacts in WT mice (#p = 0.01), but this effect was not apparent in *Hn1*+/− mice. (D) *Hn1*+/− males exhibited a greater number of contacts with the novel object than WT males (*p = 0.005), with no genotypic difference observed in females. (E) *Hn1*+/− mice trended toward spending more time burying marbles than WT mice and fentanyl withdrawal tended to increase the time spent burying. Neither of these main effects were statistically significant. (F) FEN withdrawal increased marble burying, irrespective of Genotype (#p = 0.01). (G) *Hn1*+/− males buried more marbles than WT males, irrespective of Treatment (*p = 0.01), with no genotypic difference observed in females. Data represent the mean ± *SEM*. of the number of mice indicated in the figure legends. Note that the sample sizes in Panel E are the same as in Panel C and the sample sizes in Panel G are the same as in Panel D

all *p*'s >0.14). These data from the light–dark shuttle box do not support an increase in anxiety-like behavior or locomotor activity during early fentanyl withdrawal under this treatment regimen, but suggest that *Hn1+/–* reduces some signs of anxiety-like behavior in male mice only.

3.9 | Increased novel object approach behavior during fentanyl withdrawal in *Hn1+/–* males

To index genotypic differences in neophobia-related anxiety during opioid withdrawal,[48,49] we next tested these same mice in a novel object encounter assay. We found evidence for increased approach behavior in fentanyl-withdrawn *Hn1+/–* males.

Examination of the latency to first approach a novel object showed a significant Genotype × Sex × Drug interaction (Figure 5(B); $F_{1,100} = 8.06$, $p = 0.006$). Deconstruction of the interaction along the Sex factor failed to indicate any effect of Genotype or Drug on this measure in females (Figure 5(B), left panel; Genotype × Drug interaction: *p*'s >0.13). However, a significant Genotype × Drug interaction was detected in males (Figure 5(B), right panel; $F_{1,150} = 5.87$, $p = 0.02$). As illustrated, this male-specific interaction reflected a shorter latency of fentanyl-treated *Hn1+/–* males to make contact with the novel object compared with WT males (Figure 5(B), right; test for simple effects: $^{*}p < 0.05$), while no genotypic difference was apparent for the contact latencies of saline control mice ($p > 0.05$). A significant Genotype × Drug interaction was also detected for the number of contacts with the novel object ($F_{1,100} = 4.06$, $p = 0.05$) that reflected a fentanyl withdrawal-induced reduction in novel object contacts in WT mice (Figure 5(C), left: test for simple effects: $\#p < 0.05$), but no fentanyl effect in *Hn1+/–* mice ($p > 0.05$). There was also a significant Genotype × Sex interaction in the number of novel object contacts ($F_{1,100} = 4.88$, $p = 0.03$) that reflected a significant increase in *Hn1+/–* males relative to their WT male counterparts (Figure 5(D); test for simple effects: $^{*}p < 0.05$), but no genotypic difference in females ($p > 0.05$). None of our independent variables influenced the time in contact with the novel object (data not shown; Genotype × Sex × Drug ANOVAs, all *p*'s >0.08). Lastly, an examination of the distance traveled during the novel object test failed to indicate any group differences (Genotype × Sex × Drug ANOVA, all *p*'s >0.19). These data provide limited evidence that fentanyl withdrawal alters behavioral signs of negative affect in the novel object encounter assay in a manner that can be dissociated from locomotor activity. Further, these data provide additional evidence that *Hn1+/–* can increase approach behaviors in males, particularly those with a history of repeated fentanyl exposure.

3.10 | Fentanyl withdrawal increases marble burying behavior irrespective of genotype and *Hn1+/–* increases the number of marbles buried in males, irrespective of prior treatment

The marble burying test is proposed to measure aspects of both compulsive-like and anxiety-like behavior[50,51] and a prior study reported an increase in marble burying during opioid withdrawal in mice.[52] Therefore, we examined this phenotype in the same batch of mice that were run through our opioid withdrawal behavioral test battery and found no group differences in the latency to start marble-burying (data not shown; Genotype × Sex × Drug ANOVA, all *p*'s >0.08).

Overall, fentanyl withdrawal induced a modest, nonsignificant increase in time spent marble-burying (Figure 5(E); Drug effect: $F_{1,100} = 2.89$, $p = 0.09$). While this fentanyl effect did not vary as a function of Genotype (Genotype × Drug: $p = 0.71$), *Hn1+/–* mice tended to spend more time burying marbles, overall, than their WT counterparts (Figure 5(E); Genotype effect: $F_{1,100} = 3.79$, $p = 0.06$). Mice in fentanyl withdrawal buried a significantly greater number of marbles than saline-injected controls (Figure 5(F); Drug effect: $F_{1,100} = 6.61$, $p = 0.01$), but this fentanyl effect did not vary as a function of either Sex or Genotype (Dose interactions, *p*'s >0.14). However, there was a significant Genotype × Sex interaction observed for the number of marbles buried ($F_{1,100} = 6.84$, $p = 0.01$), which reflected a greater number of marbles buried by *Hn1+/–* males versus their male WT controls (Figure 5(G); test of simple effects: $^{*}p < 0.05$), but no genotypic difference in females (Figure 5(G); $p > 0.05$). Thus, as observed for alcohol withdrawal (e.g., Lee et al.[40,53]) the marble-burying test appears to be particularly sensitive at detecting an effect of early fentanyl withdrawal. However, in contrast to our other assays of anxiety-like behavior presented above, the data from this assay indicate an *anxiogenic-like* (or perhaps "compulsive-like) effect of *Hnrnph1* mutation that is selective for males.

3.11 | Fentanyl withdrawal induces a buspirone-reversible increase in swimming behavior in the forced swim test: No effect of *Hn1+/–* on fentanyl withdrawal signs or the buspirone treatment response

Opioid withdrawal can increase forced swim test behavior in mice.[54] We previously showed a buspirone-reversible increase in forced swim behavior during ethanol withdrawal.[53] Therefore, we examined changes in forced swim behavior during opioid withdrawal in the same mice as above that were used in our opioid withdrawal behavioral test battery. Following the observation of significant behavioral effects of fentanyl withdrawal on forced swim behaviors, we then ran a separate cohort of mice to test for the ability of buspirone to reverse this behavior.

In the forced swim test, fentanyl withdrawal increased the latency to first float, irrespective of the Genotype or Sex (Figure 6(A); Drug effect: $F_{1,100} = 6.87$, $^{*}p = 0.01$; all other *p*'s >0.14). Fentanyl withdrawal also significantly reduced the time spent floating in a manner independent of Genotype or Sex (Figure 6(B); Drug effect: $F_{1,100} = 12.44$, $^{*}p = 0.001$; other *p*'s >0.28), and a similar result was observed for the number of immobile episodes (Figure 6(C); Drug effect: $F_{1,100} = 16.74$, $^{*}p < 0.0001$; other *p*'s >0.45). These data indicate that, similar to alcohol withdrawal,[40,41,55] early withdrawal from repeated fentanyl administration induces a robust increase in

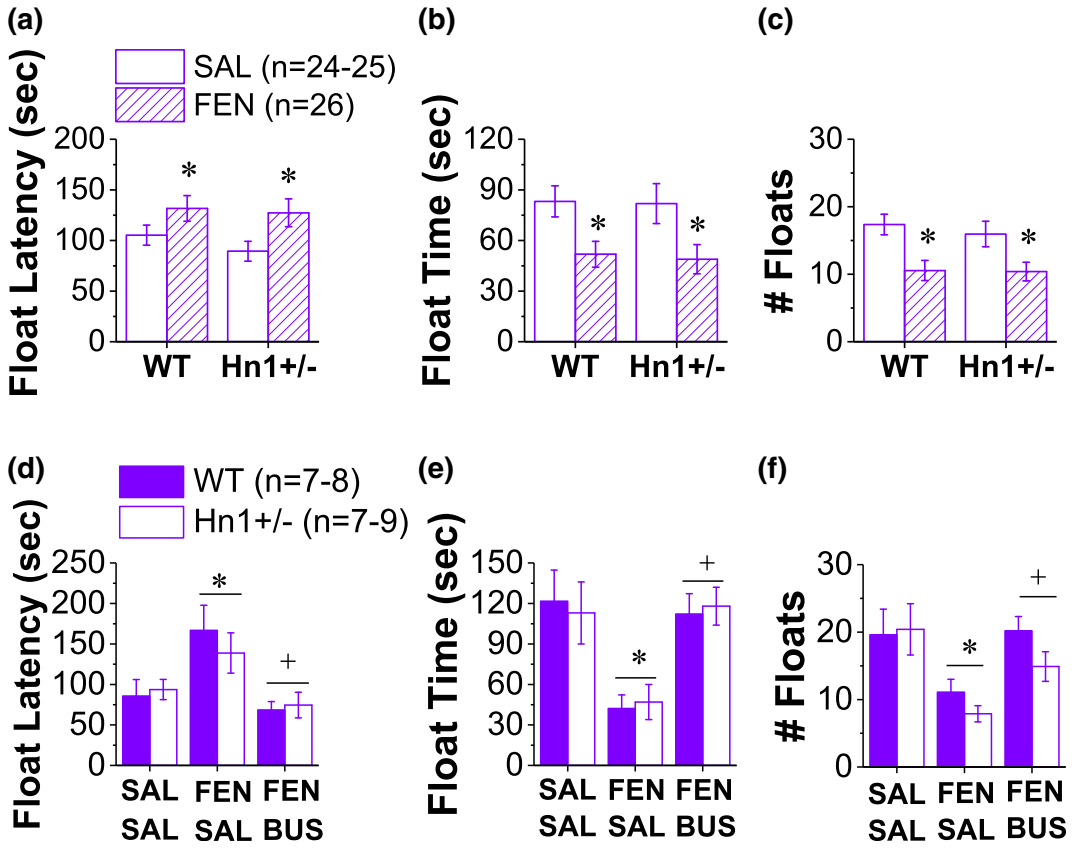

FIGURE 6 Buspirone pretreatment reverses the increase in swimming behavior in the forced swim test exhibited by WT and *Hn1+/−* mice during fentanyl withdrawal. Irrespective of Genotype, fentanyl (FEN)-experienced mice exhibited (A) a longer latency to first float, (B) reduced time spent floating, and (C) reduced number of floats, relative to their saline (SAL)-injected controls. In a separate cohort of mice, we replicated the effect of repeated fentanyl treatment upon swimming behavior (SAL-SAL vs. FEN-SAL). Importantly, pretreatment of a separate cohort of fentanyl-treated mice with 5 mg/kg (i.p.) of buspirone (FEN-BUS) prior to testing reversed the fentanyl effect upon the latency to first float (D), the time spent floating (E), and the number of floats (F), when compared with their saline-pretreated controls (FEN-SAL). Data represent the mean ± *SEM*. **p* <0.05 versus SAL or SAL-SAL; +*p* <0.05 versus FEN-SAL

swimming behavior in the forced swim test. However, *Hn1+/−* mutation does not alter this fentanyl withdrawal behavior in this assay.

We next tested the hypothesis that the fentanyl withdrawal-induced increase in swimming observed in the forced swim test might reflect increased anxiety-like behavior by pretreating mice with an effective dose of the anxiolytic buspirone[53] using a new cohort of experimentally naïve mice. Replicating our above findings (Figure 6 (A)–(C)), mice in early fentanyl withdrawal exhibited increased swimming, irrespective of Genotype or Sex (*Figure 6(D)–(F)). As reported previously for alcohol withdrawal,[40] acute pretreatment with 5 mg/kg buspirone reversed the increased swimming behavior that was present in fentanyl-withdrawn mice (Figure 6(D)–(F)). This pattern of effects was statistically significant for the latency to first float (Figure 6(D); Group effect: $F_{1,45}$ = 7.57, +p = 0.002; LSD post hoc tests: saline-saline vs. fentanyl-saline, p = 0.007; fentanyl-saline versus fentanyl-buspirone, p = 0.001; no other main effects or interactions, p's >0.40), the time spent floating (Figure 6(E); Group effect: $F_{1,45}$ = 6.58, +p = 0.004; LSD post hoc tests: saline-saline vs. fentanyl-saline, p = 0.001; fentanyl-saline vs. fentanyl-buspirone, p = 0.002; no other main effects or interactions, p's >0.34) and the number of floating episodes (Figure 6(F); Group effect: $F_{1,45}$ = 6.29, +p = 0.005; LSD post hoc tests: saline-saline vs. fentanyl-saline, p = 0.001; fentanyl-saline vs. fentanyl-buspirone, p = 0.009; no other main effects or interactions, p's >0.25). These data replicate our original observation that opioid withdrawal-induced behaviors can manifest in the forced swim test as an increase in swimming and provide pharmacological validation that this swimming reflects anxiety-like behavior. However, *Hn1+/−* does not affect either the withdrawal or buspirone response.

3.12 | No changes in signs of fentanyl dependence in *Hn1+/−* mice

Opioid withdrawal induces multiple behavioral and physical signs of dependence in mice, including jumping, wet dog shakes, ptosis, piloerection, rears, fecal boli output and paw tremors.[56] In the final experiment involving the mice that were run through the behavioral battery of opioid withdrawal as described above, we examined a subset of these signs of opioid dependence in fentanyl-withdrawn mice.

Although a modest sex difference was observed for some measures of physical dependence, we detected no effects of *Hn1+/–* on naltrexone-precipitated withdrawal signs (Figure S9).

4 | DISCUSSION

We previously linked *Hnrnph1* (*Hn1*) polymorphisms to alterations in several methamphetamine-induced addiction-relevant behaviors in mice.[17-19] Furthermore, polymorphisms in *OPRM1* (mu opioid receptor) have been linked to changes in hnRNP H1 binding to and post-transcriptional processing of *OPRM1* (gene coding for mu opioid receptor) and heroin addiction severity in humans.[22] Here, we show that one copy of a frameshift deletion in the first coding exon of *Hnrnph1* (*Hn1 +/–*) can also alter opioid addiction-relevant behaviors in mice. Specifically, *Hn1+/–* mice showed reduced acute fentanyl-induced locomotor activity, sex-interactive differences in fentanyl-induced locomotor sensitization (trending decrease in *Hn1+/–* females, significant increase in *Hn1+/–* males), and increased high-dose fentanyl reward (Figures 1 and 2). Furthermore, *Hn1+/–* males showed reduced operant responding for sucrose (Figure 3) and subsequent reduced operant fentanyl intake (-Figure S5). Sucrose-naïve *Hn1+/–* males showed evidence for increased efficacy of fentanyl reinforcement by demonstrating an increase in active hole allocation, while at the same time showing reduced fentanyl intake (Figure 4; Figures S5 and S6). In contrast, no genotypic differences in fentanyl intake or reinforcement were observed in sucrose-naïve females (Figure 4; Figure S6). Together, these results provide support that *Hn1* dysfunction can alter behavioral responses to multiple classes of drugs of abuse.

We previously observed a reduction in methamphetamine-induced locomotor activity, reward, reinforcement, and dopamine release in *Hn1+/–* mice.[17,19] In this study, the behavioral effects of *Hn1+/–* on fentanyl-induced addiction-relevant behaviors were generally less pronounced, were sometimes sex-dependent, and unlike methamphetamine behaviors, were sometimes displayed as an increase in fentanyl-induced behavior. Specifically, although the reduction in fentanyl-induced locomotor activity was in the same direction as with methamphetamine, significant differences in the acute fentanyl response were only observed after correcting for individual differences in baseline locomotion from the prior saline injection (main effect of Genotype: Figure 1(D)). High-dose fentanyl CPP was also increased in *Hn1+/–* mice (Figure 2) which was similar to our previous result with high-dose methamphetamine.[19] Focusing on the fentanyl operant-conditioning findings for sucrose-naïve mice, we observed a male-specific reduction in overall fentanyl intake (Figure 4)—a finding that partially aligns with our report of a sex-independent reduction in methamphetamine intake in *Hn1+/–* mice.[19] However, in the present study, reduced fentanyl intake did not reflect lower responding in the drug-reinforced hole, as reported for methamphetamine.[19] Instead, *Hn1+/–* males allocated a greater proportion of their total responding to the active, fentanyl-reinforced hole (Figure 4), which may be interpreted as reflecting greater, rather than less, fentanyl reinforcement in *H1+/–* mice.

One reason for the less pronounced decrease in fentanyl-induced locomotor activity in *Hn1+/–* mice could relate to the cellular mechanism proposed to underlie reduced methamphetamine-induced behaviors in *Hn1+/–* mice. *Hn1+/–* induced a profound reduction in methamphetamine-induced dopamine release in the nucleus accumbens as evidenced by in vivo microdialysis in the absence of any change in baseline extracellular dopamine levels, dopamine uptake, transporter expression or transporter function.[19] *Hn1+/–* also induced a two-fold increase in synaptosomal hnRNP H protein and proteomic analysis identified a highly enriched set of mitochondrial proteins that were perturbed at baseline and showed opposite methamphetamine-induced changes in synaptosomal expression/localization in *Hn1+/–* versus WT mice.[19] Based on these observations, we proposed that increased synaptic hnRNP H in *Hn1+/–* mice alters mitochondrial gene expression and function to disrupt dopamine release and behavior in response to dopamine release-provoking stimuli. Methamphetamine as a stimulus acts directly at the site of action (dopamine transporters and vesicular monoamine transporters of the presynaptic dopaminergic neuron terminals) to cause a surge in extracellular dopamine levels. In contrast, like other opioids such as morphine,[57] fentanyl is thought to disinhibit midbrain dopaminergic neurons to increase excitability, depolarization and dopamine release in the nucleus accumbens.[58] Thus, if decreased dopamine release is the parsimonious mechanism that underlies reduced methamphetamine- and fentanyl-induced behaviors in *Hn1+/–* mice, a more pronounced behavioral effect would be expected in response to a psychostimulant compared with an opioid. We should also note that we previously found indirect evidence for modulation of hnRNP H immunohistochemical staining in cultured rat primary neurons in response to D1 but not D2 receptor activation that was reversed by a D1 receptor antagonist[20]; thus, because our more recent study did not distinguish between the pre- and postsynaptic synaptosome,[19] an alteration in postsynaptic D1 receptor signaling in *Hn1+/–* mice could also comprise a molecular mechanism underlying both psychostimulant and opioid behaviors.[59]

What is the mechanism underlying the sex-interactive effects of *Hn1+/–* on behavior, in particular fentanyl-induced locomotor effects (Figure 1) and fentanyl reinforcement (Figure 4; Figure S6)? *Hnrnph2* is a gene homolog of *Hnrnph1 and is* located on the X chromosome in both rodents and humans. Human mutations in both *HNRNPH2* and *HNRNPH1* have been linked to a rare, x-linked neurodevelopmental disorder in females.[60-62] Because males only have one copy of *Hnrnph2* whereas females have two, it is possible that *Hn1+/–* could lead to sex-dependent changes in *Hnrnph2* expression and behavior (in particular if *Hnrnph2* undergoes variable X-inactivation) that in turn, lead to sex-dependent differences in fentanyl-related behaviors. *Hn1+/–* is known to be associated with a dysregulation of dopaminergic function[19]; thus, it is conceivable that a sex-specific change in *Hnrnph2* expression could alter the dopamine system and fentanyl-induced behaviors. Besides sex chromosome effects, a separate mechanism could involve sex steroids, which are known to modulate exogenous opioid-induced behaviors, including the discriminative stimulus and locomotor stimulant effects of morphine,[63,64] as well as heroin and oxycodone self-administration.[65]

In agreement with our extensive previous behavioral battery in *Hn1+/−* mice,[19] the behavioral effects of *Hn1+/−* in this study appear to be quite selective for drug-induced behaviors relevant to reward and reinforcement, with little evidence for effects on baseline behaviors. *Hn1+/−* mice showed unaltered nociception and fentanyl-induced antinociception after acute or chronic administration (Figure 2; Figure S4). Furthermore, with the exception of a small but significant enhancement of marble burying behaviors in *Hn1+/−* males (Figure 5), *Hn1+/−* did not significantly alter several other behavioral measures, either in control mice or in mice receiving chronic fentanyl injections, including forced swim test behavior (Figure 6), startle (-Figure S8), or precipitated withdrawal (Figure S9). These results provide further support that *Hn1+/−* exerts selective effects on drug-induced behaviors indicative of mesolimbic dopaminergic dysfunction.

Another key set of novel findings of this study relates to the demonstration of altered affective-like behavioral states during natural, spontaneous withdrawal from a repeated fentanyl injection regimen in mice on a C57BL/6J background. In humans, opioid withdrawal is associated with a number of affective or subjective signs, including dysphoria, irritability and anxiety.[66] For several decades, an opioid withdrawal-induced negative affective state has been successfully recapitulated in rodent models of morphine dependence using a variety of different behavioral assays.[67-70] More recently, genetic variability in the manifestation of oxycodone and fentanyl withdrawal-induced negative affect was highlighted through studies of different 129 mouse substrains.[39,43] Consistent with prior results for both opioid[39,43,68] and alcohol[41,55] withdrawal, the manifestation of the fentanyl withdrawal-induced negative affective state herein is task-specific. When assessed at 8–12 h following the last injection, fentanyl pre-exposed C57BL/6J mice made fewer novel objects contacts (Figure 5(C)), buried more marbles (Figure 5(F)) and exhibited more swimming behavior in the forced swim test (Figure 6) than saline-treated animals. In contrast, no fentanyl effect was apparent for acoustic startle (Figure S8) or behavior expressed during the light-dark shuttle-box test in this study or that previous for 129 substrains.[39] The congruent findings across assays of neophobia/agoraphobia,[39] coupled with the reversal of the heightened swim reactivity in the forced swim test by the anxiolytic buspirone (Figure 6(D)–(F)), argues that our repeated fentanyl injection regimen is sufficient to elicit an anxiety-like state during early withdrawal.

Buspirone has a complex pharmacology in that it acts as a partial agonist for 5-HT1A receptors to modulate monoamine release, in addition to antagonizing D2, D3 and D4 dopamine receptor subtypes.[71-73] Furthermore, its metabolite acts as an alpha-2 adrenergic receptor antagonist.[74] The ability of acute buspirone to reverse the increased swimming behavior observed in opioid-withdrawn animals aligns with our prior results for alcohol withdrawal using this assay.[53] Together, such parallels in results suggest that opioid and alcohol withdrawal engage a common neurobiological mechanism that promotes maladaptive coping strategies related to augmented monoamine neurotransmission, which should be pursued in future work.

There are several limitations to this study. First, with regard to statistical power, we powered most of the experiments based on the effect size of the sex-combined effect of *Hn1+/−* deletion on methamphetamine-induced locomotor activity (Cohen's $d = 0.9$; minimum of n = 16 per genotype to achieve 80% power, $p < 0.05$).[17] In hindsight, the fentanyl effects were not as robust but nevertheless, we observed several instances of Sex × Genotype and Sex × Genotype × Treatment interactions. The reliability of these observations will need to be tested in a replication study with a larger, independent cohort. A second limitation is that our chronic fentanyl regimen did not induce robust signs of opioid tolerance. The lack of robust tolerance could be because of the rapid onset/offset of fentanyl physiological effects that dictate the time course of physiological recovery from opioid exposure and thus, the optimal time point for measuring physiologically perturbed behaviors. The lack of tolerance and subtle physical dependence we observed is unlikely to be explained by insufficient dosing as 0.8 mg/kg of systemic fentanyl is considered quite high of a dose. Behavioral signs of fentanyl dependence (e.g., changes in elevated plus maze behavior) have been observed following repeated fentanyl dosing with 0.3 mg/kg in C57BL/6J mice but with the exception that a longer treatment regimen of two to 4 weeks was used.[75] Thus, a longer treatment regimen with 0.8 mg/kg fentanyl could induce more reliable opioid tolerance and additional behavioral signs of opioid withdrawal. Future studies will employ a different fentanyl regimen (longer treatment and/or shorter interval between fentanyl injections and tolerance assessment) and additional opioids (morphine, oxycodone) to test for potential effects of *Hn1+/−* on opioid tolerance. A final limitation is that we do not know if there are differences in pharmacokinetics (transport, metabolism) that could explain the main effects and interactive effects of Genotype and Sex. If pharmacokinetics alone explained our results, we would have expected to observe consistent main effects or trends in the same direction across a majority of the phenotypes but this was not the case. In our previous study, we did not observe any genotypic difference in brain concentration of methamphetamine or its metabolites at 30 min postadministration (2 mg/kg, i.p.). However, we only examined brain drug concentration at one time point and furthermore, the mechanisms of fentanyl versus methamphetamine transport are likely to differ and so we cannot rule out pharmacokinetics as an explanation.

To summarize, we found complex, and sometimes sex-dependent effects on fentanyl-induced locomotor activity, sensitization, reward, and reinforcement efficacy in *Hn1+/−* mice. In the context of our prior studies, these observations support a role for *Hn1* function in the behavioral response to both psychostimulants and opioids. By extension, we suspect that the behavioral and neurochemical effects of drugs of abuse from other classes and stimuli that are capable of robustly stimulating dopamine release will also be affected by *Hn1* dysfunction.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILTY STATEMENT

All data in raw and processed forms will be made available upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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