Spectrotemporal profiling of ultrasonic vocalizations during neonatal opioid withdrawal reveals a kappa opioid receptor component in female mice

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Kappa opioid receptor in neonatal morphine withdrawal in female mice

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ABSTRACT

Opioid use during pregnancy can lead to negative infant health outcomes, including neonatal opioid withdrawal syndrome(NOWS). NOWS comprises gastrointestinal, autonomic nervous system, and neurological dysfunction that manifest during spontaneous withdrawal. Current treatments involve non-pharmacological and pharmacological interventions, however, there is no one standardized approach, in part because of variability in NOWS severity. To effectively model NOWS traits in mice, we used a third trimester-approximate opioid exposure paradigm, where neonatal inbred FVB/NJ and outbred Carworth Farms White(CFW) pups were injected twice-daily with morphine(10-15mg/kg, s.c.) or saline(0.9%, 20 ul/g, s.c.) from postnatal day(P) one to P14. We observed reduced body weight gain, hypothermia, thermal hyperalgesia, and increased ultrasonic vocalizations(USVs). Neonatal USVs are emitted exclusively in isolation to communicate distress and thus serve as a model behavior for affective states. On P14, we observed altered USV syllable profiles during spontaneous morphine withdrawal, including an increase in Complex 3 syllables in FVB/NJ females(but not males) and in CFW mice of both sexes. Brainstem transcriptomics revealed an upregulation of the kappa opioid receptor(*Oprk1)*, whose activation has previously been shown to contribute to withdrawal-induced dysphoria. Treatment with the kappa opioid receptor(KOR) antagonist, nor-BNI(30 mg/kg, s.c.), significantly reduced USV emission in FVB/NJ females, but not FVB/NJ males during spontaneous morphine withdrawal. Furthermore, treatment with the KOR agonist, U50,488h(0.625 mg/kg, s.c.), was sufficient to increase USV emission on P10(both sexes) and on P14(females only) in FVB/NJ mice. Together, these results indicate a female-specific recruitment of the dynorphin/KOR system in neonatal opioid withdrawal symptom severity.

INTRODUCTION

Over the past 30 years, opioid use during pregnancy has become an increasing public health concern[1–5]. Exposure to opioids during gestation can lead to negative health outcomes for infants, such as preterm birth, growth restriction, and neonatal opioid withdrawal syndrome(NOWS). Approximately 59 infants are diagnosed with NOWS daily and are at an increased risk for admission to the neonatal intensive care unit(NICU), longer hospital stays, and greater hospital costs compared to other infants[5–10]. NOWS consists of symptoms induced by spontaneous cessation of in-utero opioid exposure and is characterized by gastrointestinal irregularities(weight loss, poor feeding, diarrhea, vomiting), disrupted autonomous system(sweating, respiratory difficulties, sneezing) and neurological dysfunction(sleep disruptions, irritability, and excessive, high-pitched crying)[9,11,12]. Current treatments for NOWS involve non-pharmacologic interventions, such as rooming-in and breastfeeding, as well as mu opioid receptor agonist therapy[7,13–16]. NOWS severity is highly variable, which can be attributed to multiple factors such as genetics, biological sex, type of opioid exposure, maternal polysubstance use, gestational age of exposure, and hospital care model[17–21]. Thus, it is challenging to investigate the impact of individual factors on NOWS severity in a clinical setting. However, understanding the factors contributing to symptom severity can inform personalized treatment strategies to improve infant health outcomes.

Rodent models for NOWS traits are used to study the influence of individual factors on withdrawal symptom onset, duration, and severity[22–24]. To effectively model NOWS traits in mice, we employ a third trimester-approximate opioid exposure paradigm, where neonatal pups are injected twice-daily with morphine(10-15mg/kg, 20ul/g, s.c.) or saline(0.9%, 20ul/g, s.c.) from postnatal day(P) one to P14, and is sufficient to induce a withdrawal state in pups[22,25–28]. P1–P14 in mice mirror the neurodevelopmental events that occur during the third trimester of human pregnancy, including oligodendrocyte maturation, myelination, synaptogenesis, and establishment of the blood-brain barrier[23,29–32]. Other models include pre- and postnatal opioid exposure to the dam; however, these models create challenges for accomplishing precise delivery of doses to individual pups which is necessary for the study of factors(e.g., genetics) underlying individual differences in neonatal opioid withdrawal presence and severity. Prenatal models necessitate maternal drug exposure, which, while valid for studying the human condition, complicates the understanding of innate characteristics underlying individual differences in neonatal opioid withdrawal severity.

Here, we implemented a third trimester-approximate NOWS model in inbred FVB/NJ and outbred Swiss Webster Carworth Farms White(CFW) mouse pups to investigate the neurological and

transcriptional adaptations resulting from chronic morphine exposure and withdrawal. We first used supervised machine learning to classify ultrasonic vocalization(USV) syllable profiles during spontaneous morphine withdrawal. Neonatal USVs are emitted exclusively in isolation to communicate discomfort and promote maternal attention[33–36]. Thus, USVs can serve to model the negative affective state associated with morphine withdrawal. Next, we examined the transcriptomic changes in the brainstem, a brain region containing multiple nuclei(e.g., locus coeruleus) that contribute to neurobehavioral adaptations during opioid withdrawal, including enhanced nociception, dysphoria, and anhedonia[37–41]. We then investigated whether kappa opioid receptors(KOR), which are known to contribute to dysphoria during opioid withdrawal, contribute to USV emissions and syllable profiles during morphine withdrawal. Together, our results identify a specific USV syllable, Complex 3, as a potential marker for withdrawal-associated dysphoria in mice, as well as a female-specific KOR component to increased USVs during spontaneous morphine withdrawal. These results support the extensive body of literature documenting sex differences in the dynorphin/KOR system in responses comprising negative affective-motivational states and emphasize the importance of biological sex in considering treatment strategies for opioid withdrawal[42–45].

METHODS

Mice

All experiments involving mice were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Animal Care and Use Committees at Boston University and Northeastern University. FVB/NJ inbred mice were purchased at 8 weeks old from The Jackson Laboratory(Strain #001800). The FVB/NJ strain was chosen based on their well-known excellent fecundity and the unexplored prospect of potentially exploiting closely related FVB substrains for mapping the genetic basis of NOWS model traits[46]. Outbred Cartworth Farm Webster(Swiss Webster) mice(8 weeks old) were purchased from Charles River Laboratories, and their historical dataset[25] was used to test for generalization of our findings as they represent a commonly used and more diverse, outbred genetic background. Mice were provided ad libitum laboratory breeding chow(Teklad 18% Protein Diet, Envigo) and tap water, and maintained on a 12h light/dark cycle(lights on at 0630h). Breeders were paired in-house after one week of acclimation to the vivarium. All breeder cages contained nestlets. Sires were removed seven days after breeder pairing to strictly control for cage environment and avoid untimed pregnancies. Phenotyping occurred during spontaneous withdrawal(16h) between 0900h and 1100h and was performed by female experimenters to control for the effect of experimenter sex on animal behavior[47,48].

124

125 **Tattooing of mice**

126 Pup tails were tattooed(ATS-3 General Rodent Tattoo System, AIMS) on postnatal day(P) 7
127 following behavioral testing and morning injections for identification, and were returned to their home
128 cage.

129

130 **Morphine administration in FVB/NJ pups from P1 to P15**

131 We employed a third-trimester approximate mouse model of opioid exposure that models the
132 first two weeks of human life[22–24,49]. Additional details on the rationale and procedure are provided
133 in **Supplementary Information**. From P1-P15, injections of either morphine sulfate
134 pentahydrate(10mg/kg, 20ul/g, s.c.; Sigma-Aldrich) or saline(0.9%, 20ul/g, s.c.) were administered
135 twice-daily at 0900h and 1700h. On P7 and P14, morning injections were administered following
136 phenotyping during spontaneous morphine withdrawal at approximately 1100h.

137

138 **Morphine administration in CFW pups from P1 to P14**

139 Morphine administration in CFW mice was performed by a separate group of female
140 experimenters in a previous study[25]. The treatment regimen was nearly identical to the FVB/NJ
141 treatment regimen, with the only exception that a higher morphine dose was used(15mg/kg instead of
142 10mg/kg). See **Supplementary Information** for additional details.

143

144 **Recording of ultrasonic vocalizations (USVs)**

145 Individual pups were each placed into a Plexiglass box(43 cm length x 20 cm width x 45 cm
146 height; Lafayette Instruments) within a sound-attenuating chamber(Med Associates). USVs were
147 recorded using the Ultrasound Recording Interface(Avisoft Bioacoustics UltrasoundGate 816H) for
148 10min (P7) or 15min (P14). Locomotor activity during all USV testing sessions was recorded using
149 infrared cameras and tracked with ANY-maze software(Stoelting).

150

151 **Thermal nociception testing on P7 and P14 in FVB/NJ pups**

152 After USV recordings, each pup was removed from the sound-attenuating chamber and placed
153 in a Plexiglass cylinder(diameter, 15 cm; height, 33 cm) on a 52.5°C hot plate(IITC Life Science). On
154 P7, the nociceptive response was defined as the latency for the pup to roll onto its back[25]. On P14,
155 the nociceptive response was defined as the latency to jump, attempt to jump, hind paw lick or attempt
156 to hind paw lick. Pups were removed from the hot plate immediately after the observation of a

nociceptive response, or after the 30s cut-off (P7) or the 60s cut-off (P14) if no pain response was observed. Upon completion of nociceptive testing, each pup was weighed and administered their morning injection and returned to their home cage.

Supervised ultrasonic vocalization classification

Labeled syllables and accompanying acoustic features were used to train a custom random forest classifier in Python. Additional classifier information is provided in the **Supplementary Material.**

Bulk RNA-seq of brainstem from morphine-withdrawn pups

The mice used for RNA-seq also underwent phenotyping on P8 and P15 while under the influence of morphine in the a.m. to assess the effect of alleviation of opioid withdrawal on USVs. Additional details are provided in **Supplementary Information**. Maintenance doses resumed for the p.m. injections on P8 and P15. During spontaneous withdrawal(16h post-morphine) on P16, mice were sacrificed by live, rapid decapitation, and brains were removed from skull. Brainstem tissue, including the pons, medulla and part of the spinal cord, was immediately collected and stored in RNAlater at 4°C for 5 days, then blotted dry and stored at -80°C for later RNA extractions. Further details on RNA extractions, RNA-seq, and analysis are provided in the **Supplementary Material.**

Effect of the kappa opioid receptor antagonist nor-BNI on ultrasonic vocalizations

A separate cohort of FVB/NJ mice was used to test the effects of the KOR antagonist nor-BNI (nor-Binaltorphimine Dihydrochloride; Sigma-Aldrich) on USVs. From P1–P14, injections of either morphine(15mg/kg, s.c) or saline(0.9%, 20ul/g, s.c.) were administered twice-daily at 0900h and 1700h**.** Given nor-BNI's extremely slow onset of KOR antagonism and its prolonged duration of action at KOR in rodents[50–52], either nor-BNI(30mg/kg, s.c.[50–52].) or saline 0.9%, 20ul/g, s.c.) was administered at 1300h on P14, approximately 4h after the morning morphine or saline injections, and 20h prior to phenotyping on P15. USVs were recorded at 0900h for 15 min.

Effect of the kappa opioid receptor agonist U50,488h on ultrasonic vocalizations

A separate cohort of morphine-naïve FVB/NJ mice was used to determine whether administration of the KOR agonist U50,488h((±)-U-50488h hydrochloride; Fisher Scientific) was sufficient to induce USVs in a manner previously observed during spontaneous opioid withdrawal. From P1–P14, pups were injected with saline(0.9%, 20ul/g, s.c.) twice-daily at 0900h and 1700h to replicate the NOWS injection regimen**.** On P10 and P14, pups were injected with either U50,488h(0.625mg/kg,

s.c.) or saline and returned to their home cage 10min prior to testing. USVs were recorded at 0900h for 15min. Pups continued to receive saline injections following P10 testing until testing for the behavioral effects of U50,488h again on P14, which is when we normally record USVs. **See Supplemental Materials for U50,488h dose-response pilot and P10 justification.**

Statistical analysis

Analysis was performed in R(https://www.r-project.org/). All data are presented as the mean ± standard error of the mean(SEM), and $p<0.05$ was considered significant. Body weight, temperature, USV locomotion, and USVs over time were analyzed using linear mixed models with Saline Treatment and Female Sex as the reference variables, and Pup as a random effect(repeated measures). Sex was removed from the model if there were no interactions. Significant interactions of interest were followed up with least-square means(predicted marginal means) and Tukey's Honestly Significant Difference(HSD) tests. All other data were analyzed using linear models with Saline Treatment and Female Sex as the reference variables. For nor-BNI experiments, we were mostly interested in the effect of nor-BNI in morphine-treated females, thus Morphine Treatment(Tx1), Saline Treatment(Tx2), and Female sex were used as reference variables.

RESULTS

Morphine exposure from P1–P14 is sufficient to induce multiple signs of opioid withdrawal in neonatal FVB/NJ pups

An experimental timeline is provided in **Fig.1A**. Reduced body weight and poor body temperature regulation are commonly observed in infants diagnosed with NOWS[53–56]. Twice-daily morphine injections(10mg/kg, s.c.) from P1–P14 significantly reduced body weight(**Fig.1B**) and temperature during withdrawal(**Fig.1C**). Hyperalgesia is common in opioid withdrawal[57]. Morphine-withdrawn pups displayed thermal hyperalgesia on P7, as demonstrated by a reduced latency to elicit a nociceptive response on the hot plate(**Fig.1D).** In examining hot plate velocity on P7 during the time interval of exposure, there was no effect of morphine treatment, indicating that reduced locomotor activity did not prolong hot plate exposure and thus did not confound the group difference(**Fig.1E**). On P14, morphine-withdrawn pups displayed reduced hot plate latency(**Fig.1F**) and increased hot plate velocity(**Fig.1G**). Because increased velocity decreases paw exposure to the hot plate, we can again conclude that increased locomotor activity did not confound hyperalgesia in morphine-withdrawn pups.

222 **USV locomotor activity of FVB/NJ pups during spontaneous morphine withdrawal on P7 and**
223 **P14**

224 On P7, morphine-withdrawn pups traveled a greater distance during the first 2–5min of the USV
225 recording session compared to saline pups(**Fig.2A**). For the entire 10min, morphine-withdrawn pups
226 traveled a greater distance versus saline-treated pups(**Fig.2B**). There was no effect of morphine
227 withdrawal on P7 average velocity(**Fig.2C**). On P14, morphine-withdrawn pups also showed a time-
228 dependent increase in distance traveled during isolated USV recordings(**Fig.2D**). However, there was
229 no significant difference in total distance traveled over 15min(**Fig.2E**) nor average velocity between
230 treatment groups(**Fig.2F**).

231

232 **USV syllable profile of FVB/NJ pups during spontaneous morphine withdrawal on P14**

233 Neonatal USVs communicate negative internal states. Thus, we measured USVs as an indicator
234 of enhanced distress during spontaneous morphine withdrawal[33,36,58]. On P7, morphine-withdrawn
235 pups emitted fewer USVs during the last 5min of the recording session(**Fig.S1A**), however, there was
236 no effect of Morphine Treatment on the total number of USVs (**Fig.S1B).** On P14, there was a time-
237 dependent increase in vocalizations in morphine-withdrawn female pups(**Fig.3A**). Overall, the robust
238 morphine-withdrawn increase was completely driven by females, as there was no Treatment effect in
239 males(**Fig.3B**).

240 USVs are classified into syllable types based on their acoustic features (frequency, duration)
241 [59–61]. We used a random forest model to classify USV syllables (**Table.S1;**based on existing
242 classifications[59–61]) during neonatal morphine withdrawal. On P7, morphine-withdrawn pups emitted
243 fewer Complex 3 syllables compared to saline pups(**Fig.S1C**). On P14, morphine-withdrawn pups
244 showed the opposite, namely a robust *increase* in the percentage of Complex 3 syllables(**Fig.3C**). The
245 increase in Complex 3 syllables was completely driven by morphine-withdrawn females, as there was
246 no treatment difference in males(**Fig.3D,E**). Morphine-withdrawn females also showed a decrease in
247 percentage of Flat, Short, and Upward syllables, whereas males only showed an increase in percentage
248 of Complex syllables(**Fig.3D,E**).

249 To determine whether increased Complex 3 emissions generalize to another genetic
250 background, we also examined USV syllable subtypes in morphine-withdrawn outbred CFW stock mice
251 from a historical dataset[25] comprising a nearly identical morphine regimen(see **Supplementary**
252 **Material** for details). On P7, there was no effect of Treatment on the USV syllable profile(**Fig.S2A**). On
253 P14, the USV profile in morphine-withdrawn CFW pups showed a similar increase in the percentage of
254 Complex 3 syllables(**Fig.S2B**) except in this case, both sexes showed this effect. Additional similarities

255 between morphine-withdrawn CFW and FVB pups included a decrease in the percentage of Flat and
256 Upward syllables(**Fig.S2B**)
257
258 **Brainstem transcriptomic analysis identifies an upregulation of the kappa opioid receptor**
259 In a subset of FVB/NJ mice, we administered a maintenance dose(10mg/kg) prior to USV
260 recording on P8 and P15 to evaluate the effects of acute morphine exposure on the USV syllable
261 profile(**Fig.S3**). We collected brainstem tissue during spontaneous withdrawal on P16 to evaluate the
262 effects of chronic morphine exposure and withdrawal on gene expression(**Fig.S4**). Transcriptomic
263 analysis via RNA-seq revealed a significant upregulation of *Oprk1*($\log_2$FC=0.36, ***p*=0.0049) which
264 codes for the kappa opioid receptor(KOR) and *Slc6a3*(dopamine transporter(DAT); $\log_2$FC=3.69,
265 ***p*=0.0067) during spontaneous morphine withdrawal(**Table S2**). We observed downregulation of
266 genes associated with myelination, such as *Mbp* (myelin basic protein; $\log_2$FC= 0.41, *****p*<0.0001)
267 and *Plp1*(proteolipid protein 1; $\log_2$FC=-0.38, *****p*<0.00032)(**Table S3**). Enrichment analysis of
268 differentially expressed genes revealed processes associated with cell junction organization, glial cell
269 development, and synaptic signaling(**Table S4**). Dysregulated synaptic connectivity and increased glial
270 signaling are associated with anxiety and depressive-like behaviors observed during drug
271 withdrawal[62,63].
272
273 **The kappa opioid receptor antagonist nor-BNI reduces USVs in females during spontaneous**
274 **morphine withdrawal**
275 KOR activation contributes to the dysphoric aspects of withdrawal, such as hyperirritability,
276 anhedonia, and anxiety[40,41,64]. Furthermore, sex differences in the dynorphin/KOR system on
277 motivated behaviors in the context of drug addiction are well-documented in rodents and humans[65].
278 Therefore, we aimed to determine whether the KOR system contributes to increased USVs and
279 Complex 3 emissions during opioid withdrawal. On P15, 20h post-administration of the KOR antagonist,
280 nor-BNI, there was a significant decrease in USVs in morphine-withdrawn females, but no effect of nor-
281 BNI on USVs in males(**Fig.4A**). Furthermore, there was a reduction in USVs over time by morphine-
282 treated females, but not males, pre-treated with nor-BNI compared to morphine controls(**Fig.4B**). These
283 results identify a KOR contribution to the female-specific enhancement of USVs in morphine-withdrawn
284 pups. However, KOR activation was not specific for a particular syllable type, as nor-BNI had no effect
285 on the overall USV syllable profile(**Fig.4C**).
286
287 **The kappa opioid receptor agonist U50,488h is sufficient to induce an increase in USVs**

288 Previous studies reported that administration of KOR agonists, such as U50,488h, is aversive
289 and can induce anhedonia and decreased reward sensitivity[43,66,67]. Here, we wished to determine
290 whether KOR activation with the KOR agonist U50,488h is sufficient to increase USVs and induce a
291 syllable profile similar to morphine-withdrawn pups, particularly in females. To conduct this experiment,
292 we considered a postnatal day where saline-treated controls would show a sufficient number of baseline
293 USVs in order to detect a change in USVs. In neonates, the rate of USVs peaks around P7–P10 and
294 declines during the second postnatal week[68]. Thus, we tested the KOR agonist U50,488h on P10
295 and then again on P14, when we normally assess USVs(**Fig.3**). Based on a pilot study, the U50,488h
296 dose of 0.625mg/kg was chosen because pups displayed similar locomotor activity to saline controls
297 (**Fig. S5A**) and an effect of U50,488h on USVs(**Fig. S5B**). On P10, U50,488h increased USVs relative
298 to saline controls during the first 10min of the recording session(**Fig.5A**). Additionally, in considering
299 the entire 15min, U50,488h increased overall USVs in both sexes(**Fig.5B**). In contrast to our
300 hypothesis, there was no effect of KOR activation on the percentage of Complex 3 emissions on
301 P10(**Fig.5C**). In considering other syllable types, there was a significant decrease in the percentage of
302 Downward and Short emissions(**Fig.5C**). On P14, there was a time-dependent effect of U50,488h,
303 whereby activation of KOR increased USVs during the 5–9min of the recording session(**Fig.5D**).
304 Furthermore, on P14, U50,488h increased USVs in females, but not males(**Fig.5E**). In contrast to our
305 hypothesis, U50,488h *decreased* the percentage of Complex 3 syllables(**Fig.5F**). U50,488h also
306 decreased the emission of other multi-component syllables, including Complex 2 and Complex 4.
307

308 **DISCUSSION**

309 Consistent with previous studies, twice-daily injections of morphine from P1 to P14 was sufficient
310 to induce withdrawal-associated model traits in FVB/NJ pups on P14, including low body weight,
311 hypothermia, hyperalgesia, and increased USVs on P14[25,28,69]. Neonatal mice emit USVs
312 exclusively in isolation as a distress signal to communicate negative internal states and promote
313 maternal attention[33,36,58,70]. Rodent studies observed increased USVs during opioid withdrawal in
314 neonatal pups(mice and rats) and adult rats[25,28,71,72]. On P14, we observed increased USVs,
315 specifically in FVB/NJ female pups during spontaneous morphine withdrawal. This female-specific
316 effect depended on genetic background, as morphine-withdrawn CFW mice of both sexes showed
317 increased USVs. In humans, male neonates born with NOWS are more likely to display more severe
318 withdrawal symptoms and require more pharmacological treatment compared to female neonates
319 [73,74], which contrasts with our present and previous findings[25].

The severity of NOWS and the need for treatment is determined by observer-rated scales using the Finnegan Neonatal Abstinence Scoring Tool(FNAST)[53]. Thus, NOWS assessment is subjective and may contribute to unnecessary treatment interventions and lengthy hospital stays[6,11,75]. Therefore, unbiased methods for assessing withdrawal symptoms may lead to better prediction of optimal treatment plans, improved infant outcomes, and reduced hospital costs. NOWS infants present with excessive, high-pitched cries that can be differentiated from non-NOWS cries[76]. Again, the severity of NOWS cries is subjective and can be interpreted differently between listeners. A recent study revealed that infants with NOWS emit cries with distinct features unrecognizable by humans, such as altered frequency formants, utterances, and amplitude[77]. Thus, alterations in acoustic features of cries may be associated with the severity of NOWS, which can be useful for predicting the need for pharmacological treatment, compared to subjective evaluation.

A major goal here was to identify a USV spectrotemporal profile associated with the negative affective state of neonatal morphine withdrawal. For P14, we identified a robust increase in Complex 3 syllables in two genetic backgrounds, which together implicate Complex 3 syllables as a potential preclinical biobehavioral marker for symptomatic severity and the negative affective state associated with morphine withdrawal. Furthermore, morphine-withdrawn FVB/NJ females emitted a greater proportion of Complex 3 syllables compared to morphine-withdrawn males, suggesting female pups experience a more severe affective withdrawal state. However, sex differences in Complex 3 emissions during neonatal opioid withdrawal depended on genetic background, as both female and male outbred CFW pups emitted more Complex 3 syllables compared to saline-treated pups.

We analyzed the transcriptome of brainstem tissue due to its role in morphine withdrawal and production of vocalizations [78–82]. On P16 during morphine withdrawal, there was an upregulation of *Oprk1,* which codes for kappa opioid receptors(KOR). KORs and their endogenous ligand, dynorphin[83], mediate withdrawal-associated dysphoria in rodents and humans[40,41,65,84]. Thus, we assessed KOR antagonism as a potential target for reducing negative affect in the morphine-withdrawn state. Pre-treatment with the KOR antagonist, nor-BNI on P14(a known postnatal day for morphine withdrawal-induced increase in USVs), decreased USVs exclusively in morphine-treated females during withdrawal on P15(20h pre-treatment) but had no effect on Complex 3 syllables, which suggests a female-specific component of KOR in mediating USVs and by extension, aversive states. Several rat studies have shown that the KOR antagonist nor-BNI reduces withdrawal symptoms(wet dog shakes, jumps, weight loss, and conditioned place aversion) in morphine-dependent rats[85], attenuates ethanol withdrawal-induced decreases in immobility during the Forced Swim Test[86], and blocks decreases in open arm explorations in the elevated plus maze during ethanol withdrawal[87].

Most importantly, nor-BNI can block increases in 22-kHz USVs, which indicate a negative affective state in rats, during ethanol withdrawal and following administration of U50,488[88].

Administration of KOR agonist U50,488h was sufficient to increase USVs on P10 in both sexes and on P14, specifically in females. In contrast to our prediction, U50,488h induced a decrease in Complex 2, -3, and -4 syllables on P14. Rodent studies have observed enhanced USVs in neonatal rats following U50,488h administration[69,89,90]. Together, these data are consistent with a female-specific role of KOR activation on P14–P15 in mediating overall USVs but not Complex 3 emissions, indicating that changes in USV profiles during morphine withdrawal involve additional mechanisms.

Interestingly, there was a significant, upregulation of *Slc6a3*(dopamine(DA) transporter, DAT) in the brainstem. Activation of KOR at DAergic terminals inhibits DA release and can promote negative affective states that drive drug-seeking behavior[91,92]. Accordingly, increased DAT expression would be expected to increase synaptic DA uptake and further support a negative affective state. In neonatal rats, KOR activation with U50,488h increases USV emissions[69,89,90], which has been shown to be attenuated by DA receptor agonists[90]. Thus, increased *Oprk1* and *Slc6a3* expression could contribute to the aversive state during withdrawal and mediate enhanced USV emission.

Sex differences in reward and stress-related pathways mediated by the dynorphin/KOR system have been observed in rodents and humans[42,43,93,94]. In adult rats, U50,488h-mediated KOR activation decreases reward motivation and greater thermal analgesia in females compared to males[43,94,95]. Human studies have also observed increased analgesic efficacy of KOR agonists in females versus males[96,97]. Female rats are less sensitive to the DA-reducing and depressive-like effects of KOR agonists[43,94,98]. These discrepant results in adult rodents versus neonatal females in our study could potentially be due to differences in the immature and rapidly evolving circuitry in neonates versus adults. Rodent studies have shown spatiotemporal variations in KOR expression during development in rats and mice[99–101]. Additionally, adult human studies have shown sex differences in KOR availability and expression[93]. Time-dependent sex differences in the dynorphin/KOR circuitry might also explain why U50,488h increased USVs in both sexes on P10 but only in females on P14.

There are several limitations to this study. First, the third trimester-approximate model involves neonatal injections, potentially affecting maternal care quality between morphine- and saline-treated pups. Second, although we collected brainstem tissue for RNA-seq, analyzing other brain regions involved in pain and affective states(e.g., anterior cingulate cortex) and vocalizations(e.g., periaqueductal grey(PAG))[102–104] could offer additional insights into the molecular mechanisms involved in the induction of morphine withdrawal-induced USVs. Lastly, we used the KOR antagonist,

386 nor-BNI, which has a delayed onset of effect[50,52], requiring modifications to our NOWS paradigm.
387 Future studies will implement faster-acting KOR antagonists, such as CERC-501(LY2456302)[105] to
388 assess USVs on P14 rather than P15. Additionally, using KOR-knockout mouse models could further
389 elucidate the relationship between the dynorphin/KOR system and USVs during neonatal morphine
390 withdrawal.

391 We observed robust sex differences in USVs and syllable profiles in a third trimester-
392 approximate treatment regimen for NOWS model traits. The enhanced USVs in morphine-withdrawn
393 FVB/NJ females has a KOR component, suggesting that females may experience greater withdrawal
394 distress and dysphoria compared to males. More broadly, we provide a quantitative, machine learning-
395 based approach to studying negative internal states associated with neonatal opioid withdrawal in mice,
396 identifying specific USV profiles as indicators of opioid withdrawal. Examining spectrotemporal features
397 of vocalizations provides a useful approach for assessing affective states during NOWS in the clinic
398 and for assessing potential experimental therapeutics in reducing emotional symptom severity.
399

400 **REFERENCES**

401 1. Hales CM, Martin CB, Gu Q. Prevalence of Prescription Pain Medication Use Among Adults:
402 United States, 2015-2018. NCHS Data Brief. 2020:1–8.
403 2. VanHouten JP, Rudd RA, Ballesteros MF, Mack KA. Drug Overdose Deaths Among Women
404 Aged 30–64 Years — United States, 1999–2017. Morb Mortal Wkly Rep. 2019;68:1–5.
405 3. Ko JY, D'Angelo DV, Haight SC, Morrow B, Cox S, Essen BS von, et al. Vital Signs: Prescription
406 Opioid Pain Reliever Use During Pregnancy — 34 U.S. Jurisdictions, 2019. Morb Mortal Wkly
407 Rep. 2020;69:897.
408 4. Goetz TG, Becker JB, Mazure CM. Women, opioid use and addiction. FASEB J Off Publ Fed Am
409 Soc Exp Biol. 2021;35:e21303.
410 5. Haight SC. Opioid Use Disorder Documented at Delivery Hospitalization — United States, 1999–
411 2014. MMWR Morb Mortal Wkly Rep. 2018;67.
412 6. Milliren CE, Gupta M, Graham DA, Melvin P, Jorina M, Ozonoff A. Hospital Variation in Neonatal
413 Abstinence Syndrome Incidence, Treatment Modalities, Resource Use, and Costs Across
414 Pediatric Hospitals in the United States, 2013 to 2016. Hosp Pediatr. 2018;8:15–20.
415 7. Wachman EM, Werler MM. Pharmacologic Treatment for Neonatal Abstinence Syndrome: Which
416 Medication Is Best? JAMA Pediatr. 2019;173:221–223.
417 8. Tolia VN, Patrick SW, Bennett MM, Murthy K, Sousa J, Smith PB, et al. Increasing Incidence of
418 the Neonatal Abstinence Syndrome in U.S. Neonatal ICUs. N Engl J Med. 2015;372:2118–2126.
419 9. Strahan AE, Guy GP Jr, Bohm M, Frey M, Ko JY. Neonatal Abstinence Syndrome Incidence and
420 Health Care Costs in the United States, 2016. JAMA Pediatr. 2020;174:200–202.
421 10. Winkelman TNA, Villapiano N, Kozhimannil KB, Davis MM, Patrick SW. Incidence and Costs of
422 Neonatal Abstinence Syndrome Among Infants With Medicaid: 2004–2014. Pediatrics.
423 2018;141:e20173520.
424 11. Jansson LM, Velez M, Harrow C. The Opioid Exposed Newborn: Assessment and
425 Pharmacologic Management. J Opioid Manag. 2009;5:47–55.

12. Ko JY, Wolicki S, Barfield WD, Patrick SW, Broussard CS, Yonkers KA, et al. CDC Grand Rounds: Public Health Strategies to Prevent Neonatal Abstinence Syndrome. Morb Mortal Wkly Rep. 2017;66:242–245.
13. Abdel-Latif ME, Pinner J, Clews S, Cooke F, Lui K, Oei J. Effects of Breast Milk on the Severity and Outcome of Neonatal Abstinence Syndrome Among Infants of Drug-Dependent Mothers. Pediatrics. 2006;117:e1163–e1169.
14. Howard MB, Schiff DM, Penwill N, Si W, Rai A, Wolfgang T, et al. Impact of Parental Presence at Infants' Bedside on Neonatal Abstinence Syndrome. Hosp Pediatr. 2017;7:63–69.
15. Kraft WK, Adeniyi-Jones SC, Chervoneva I, Greenspan JS, Abatemarco D, Kaltenbach K, et al. Buprenorphine for the Treatment of the Neonatal Abstinence Syndrome. N Engl J Med. 2017;376:2341–2348.
16. Wachman EM, Schiff DM, Silverstein M. Neonatal Abstinence Syndrome: Advances in Diagnosis and Treatment. JAMA. 2018;319:1362–1374.
17. Liu AJW, Jones MP, Murray H, Cook C-M, Nanan R. Perinatal risk factors for the neonatal abstinence syndrome in infants born to women on methadone maintenance therapy. Aust N Z J Obstet Gynaecol. 2010;50.
18. O'Connor AB, O'Brien L, Alto WA. Are there gender related differences in neonatal abstinence syndrome following exposure to buprenorphine during pregnancy? J Perinat Med. 2013;41:621–623.
19. Seligman NS, Salva N, Hayes EJ, Dysart KC, Pequignot EC, Baxter JK. Predicting length of treatment for neonatal abstinence syndrome in methadone-exposed neonates. Am J Obstet Gynecol. 2008;199:396.e1-396.e7.
20. Wachman EM, Hayes MJ, Brown MS, Paul J, Harvey-Wilkes K, Terrin N, et al. Association of OPRM1 and COMT Single-Nucleotide Polymorphisms With Hospital Length of Stay and Treatment of Neonatal Abstinence Syndrome. JAMA. 2013;309:1821–1827.
21. Wachman EM, Hayes MJ, Sherva R, Brown MS, Davis JM, Farrer LA, et al. Variations in opioid receptor genes in neonatal abstinence syndrome. Drug Alcohol Depend. 2015;155:253–259.
22. Richardson KA, Yohay A-LJ, Gauda EB, McLemore GL. Neonatal Animal Models of Opiate Withdrawal. ILAR J. 2006;47:39–48.
23. Byrnes EM, Vassoler FM. Modeling prenatal opioid exposure in animals: Current findings and future directions. Front Neuroendocrinol. 2018;51:1–13.
24. Ferrante JR, Blendy JA. Advances in animal models of prenatal opioid exposure. Trends Neurosci. 2024;0.
25. Borrelli KN, Yao EJ, Yen WW, Phadke RA, Ruan QT, Chen MM, et al. Sex Differences in Behavioral and Brainstem Transcriptomic Neuroadaptations following Neonatal Opioid Exposure in Outbred Mice. eNeuro. 2021;8.
26. Dunn AD, Robinson SA, Nwokafor C, Estill M, Ferrante J, Shen L, et al. Molecular and long-term behavioral consequences of neonatal opioid exposure and withdrawal in mice. Front Behav Neurosci. 2023;17:1202099.
27. Ferrante JR, Dunn AD, Mengaziol J, Noreck J, Lemchi CO, Creus-Muncunill J, et al. Neonatal Opioid Withdrawal Syndrome in Mice: Acute Molecular and Long-Term Behavioral Consequences. FASEB J. 2022;36.
28. Robinson SA, Jones AD, Brynildsen JK, Ehrlich ME, Blendy JA. Neurobehavioral Effects of Neonatal Opioid Exposure in Mice: Influence of the OPRM1 SNP. Addict Biol. 2020;25:e12806.
29. Semple BD, Blomgren K, Gimlin K, Ferriero DM, Noble-Haeusslein LJ. Brain development in rodents and humans: Identifying benchmarks of maturation and vulnerability to injury across species. Prog Neurobiol. 2013;106–107:1–16.
30. Craig A, Ling Luo N, Beardsley DJ, Wingate-Pearse N, Walker DW, Hohimer AR, et al. Quantitative analysis of perinatal rodent oligodendrocyte lineage progression and its correlation with human. Exp Neurol. 2003;181:231–240.

31. Rice D, Barone S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect. 2000;108:511–533.
32. Saunders N, Liddelow S, Dziegielewska K. Barrier Mechanisms in the Developing Brain. Front Pharmacol. 2012;3.
33. Branchi I, Santucci D, Alleva E. Ultrasonic vocalisation emitted by infant rodents: a tool for assessment of neurobehavioural development. Behav Brain Res. 2001;125:49–56.
34. Caruso A, Marconi MA, Scattoni ML, Ricceri L. Ultrasonic vocalizations in laboratory mice: strain, age, and sex differences. Genes Brain Behav. 2022;21:e12815.
35. Scattoni ML, Gandhy SU, Ricceri L, Crawley JN. Unusual Repertoire of Vocalizations in the BTBR T+tf/J Mouse Model of Autism. PLOS ONE. 2008;3:e3067.
36. D'Amato FR, Scalera E, Sarli C, Moles A. Pups Call, Mothers Rush: Does Maternal Responsiveness Affect the Amount of Ultrasonic Vocalizations in Mouse Pups? Behav Genet. 2005;35:103–112.
37. Basinger H, Hogg JP. Neuroanatomy, Brainstem. StatPearls, Treasure Island (FL): StatPearls Publishing; 2023.
38. Downs AM, McElligott ZA. Noradrenergic circuits and signaling in substance use disorders. Neuropharmacology. 2022;208.
39. Bruchas MR, Land BB, Chavkin C. The dynorphin/kappa opioid system as a modulator of stress-induced and pro-addictive behaviors. Brain Res. 2010;1314:44–55.
40. Land BB, Bruchas MR, Lemos JC, Xu M, Melief EJ, Chavkin C. The Dysphoric Component of Stress Is Encoded by Activation of the Dynorphin κ-Opioid System. J Neurosci. 2008;28:407–414.
41. Shippenberg TS, Zapata A, Chefer VI. Dynorphin and the pathophysiology of drug addiction. Pharmacol Ther. 2007;116:306–321.
42. Becker JB, Chartoff E. Sex differences in neural mechanisms mediating reward and addiction. Neuropsychopharmacology. 2019;44:166–183.
43. Conway SM, Puttick D, Russell S, Potter D, Roitman MF, Chartoff EH. Females are less sensitive than males to the motivational- and dopamine-suppressing effects of kappa opioid receptor activation. Neuropharmacology. 2019;146:231–241.
44. Fox HC, Sinha R. Sex Differences in Drug-Related Stress-System Changes: Implications for Treatment in Substance-Abusing Women. Harv Rev Psychiatry. 2009;17:103.
45. Madurai NK, Jantzie LL, Yen E. Sex differences in neonatal outcomes following prenatal opioid exposure. Front Pediatr. 2024;12.
46. Bryant CD, Smith DJ, Kantak KM, Nowak TS, Williams RW, Damaj MI, et al. Facilitating Complex Trait Analysis via Reduced Complexity Crosses. Trends Genet. 2020;36:549–562.
47. Driel KS van, Talling JC. Familiarity increases consistency in animal tests. Behav Brain Res. 2005;159:243–245.
48. Sorge RE, Martin LJ, Isbester KA, Sotocinal SG, Rosen S, Tuttle AH, et al. Olfactory exposure to males, including men, causes stress and related analgesia in rodents. Nat Methods. 2014;11:629–632.
49. Barr GA, McPhie-Lalmansingh A, Perez J, Riley M. Changing Mechanisms of Opiate Tolerance and Withdrawal during Early Development: Animal Models of the Human Experience. ILAR J. 2011;52:329–341.
50. Kishioka S, Kiguchi N, Kobayashi Y, Yamamoto C, Saika F, Wakida N, et al. Pharmacokinetic evidence for the long-lasting effect of nor-binaltorphimine, a potent kappa opioid receptor antagonist, in mice. Neurosci Lett. 2013;552:98–102.
51. Marchette RCN, Gregory-Flores A, Tunstall BJ, Carlson ER, Jackson SN, Sulima A, et al. κ-Opioid receptor antagonism reverses heroin withdrawal-induced hyperalgesia in male and female rats. Neurobiol Stress. 2021;14:100325.

52. Munro TA, Berry LM, Van't Veer A, Béguin C, Carroll FI, Zhao Z, et al. Long-acting κ opioid antagonists nor-BNI, GNTI and JDTic: pharmacokinetics in mice and lipophilicity. BMC Pharmacol. 2012;12:5.
53. Finnegan LP, Connaughton JF, Kron RE, Emich JP. Neonatal abstinence syndrome: assessment and management. Addict Dis. 1975;2:141–158.
54. Hudak ML, Tan RC, THE COMMITTEE ON DRUGS, THE COMMITTEE ON FETUS AND NEWBORN, Frattarelli DAC, Galinkin JL, et al. Neonatal Drug Withdrawal. Pediatrics. 2012;129:e540–e560.
55. Jansson LM, Velez M. Neonatal abstinence syndrome. Curr Opin Pediatr. 2012;24:252–258.
56. Patrick SW, Barfield WD, Poindexter BB, COMMITTEE ON FETUS AND NEWBORN COSUAP, Cummings J, Hand I, et al. Neonatal Opioid Withdrawal Syndrome. Pediatrics. 2020;146:e2020029074.
57. Corder G, Castro DC, Bruchas MR, Scherrer G. Endogenous and Exogenous Opioids in Pain. Annu Rev Neurosci. 2018;41:453–473.
58. Ehret G. Infant Rodent Ultrasounds ? A Gate to the Understanding of Sound Communication. Behav Genet. 2005;35:19–29.
59. Portfors CV. Types and Functions of Ultrasonic Vocalizations in Laboratory Rats and Mice. J Am Assoc Lab Anim Sci. 2007;46:28–34.
60. Grimsley JMS, Monaghan JJM, Wenstrup JJ. Development of Social Vocalizations in Mice. PLOS ONE. 2011;6:e17460.
61. Heckman J, McGuinness B, Celikel T, Englitz B. Determinants of the mouse ultrasonic vocal structure and repertoire. Neurosci Biobehav Rev. 2016;65:313–325.
62. Koob GF, Volkow ND. Neurobiology of addiction: a neurocircuitry analysis. Lancet Psychiatry. 2016;3:760–773.
63. Lacagnina MJ, Rivera PD, Bilbo SD. Glial and Neuroimmune Mechanisms as Critical Modulators of Drug Use and Abuse. Neuropsychopharmacology. 2017;42:156–177.
64. Margolis EB, Karkhanis AN. Dopaminergic Cellular and Circuit Contributions to Kappa Opioid Receptor Mediated Aversion. Neurochem Int. 2019;129:104504.
65. Chartoff EH, Mavrikaki M. Sex Differences in Kappa Opioid Receptor Function and Their Potential Impact on Addiction. Front Neurosci. 2015;9:466.
66. Huang P, Gentile TA, Muschamp JW, Liu-Chen L-Y. The kappa opioid receptor agonist U50,488H did not affect brain-stimulation reward while it elicited conditioned place aversion in mice. BMC Res Notes. 2020;13:384.
67. Vonvoigtlander PF, Lahti RA, Ludens JH. U-50,488: a selective and structurally novel non-Mu (kappa) opioid agonist. J Pharmacol Exp Ther. 1983;224:7–12.
68. Elwood RW, Keeling F. Temporal organization of ultrasonic vocalizations in infant mice. Dev Psychobiol. 1982;15:221–227.
69. Carden SE, Barr GA, Hofer MA. Differential effects of specific opioid receptor agonists on rat pup isolation calls. Dev Brain Res. 1991;62:17–22.
70. Dirks A, Fish EW, Kikusui T, van der Gugten J, Groenink L, Olivier B, et al. Effects of corticotropin-releasing hormone on distress vocalizations and locomotion in maternally separated mouse pups. Pharmacol Biochem Behav. 2002;72:993–999.
71. Vivian JA, Miczek KA. Ultrasounds during morphine withdrawal in rats. Psychopharmacology (Berl). 1991;104:187–193.
72. Barr GA, Wang S. Tolerance and withdrawal to chronic morphine treatment in the week-old rat pup. Eur J Pharmacol. 1992;215:35–42.
73. O'Connor AB, O'Brien L, Alto WA. Are there gender related differences in neonatal abstinence syndrome following exposure to buprenorphine during pregnancy? J Perinat Med. 2013;41.

74. Charles MK, Cooper WO, Jansson LM, Dudley J, Slaughter JC, Patrick SW. Male Sex Associated With Increased Risk of Neonatal Abstinence Syndrome. Hosp Pediatr. 2017;7:328–334.
75. Timpson W, Killoran C, Maranda L, Picarillo A, Bloch-Salisbury E. A Quality Improvement Initiative to Increase Scoring Consistency and Accuracy of the Finnegan Tool: Challenges in Obtaining Reliable Assessments of Drug Withdrawal in Neonatal Abstinence Syndrome. Adv Neonatal Care. 2018;18:70.
76. Blinick G, Tavolga WN, Antopol W. Variations in birth cries of newborn infants from narcotic-addicted and normal mothers. Am J Obstet Gynecol. 1971;110:948–958.
77. Manigault AW, Sheinkopf SJ, Silverman HF, Lester BM. Newborn Cry Acoustics in the Assessment of Neonatal Opioid Withdrawal Syndrome Using Machine Learning. JAMA Netw Open. 2022;5:e2238783.
78. Wei XP, Collie M, Dempsey B, Fortin G, Yackle K. A novel reticular node in the brainstem synchronizes neonatal mouse crying with breathing. Neuron. 2022;110:644-657.e6.
79. Geva R, Sopher K, Kurtzman L, Galili G, Feldman R, Kuint J. Neonatal brainstem dysfunction risks infant social engagement. Soc Cogn Affect Neurosci. 2013;8:158–164.
80. Bush NE, Ramirez J-M. Latent neural population dynamics underlying breathing, opioid-induced respiratory depression and gasping. Nat Neurosci. 2024;27:259–271.
81. Tschida K, Michael V, Takatoh J, Han B-X, Zhao S, Sakurai K, et al. A Specialized Neural Circuit Gates Social Vocalizations in the Mouse. Neuron. 2019;103:459-472.e4.
82. LaGasse LL, Neal AR, Lester BM. Assessment of infant cry: Acoustic cry analysis and parental perception. Ment Retard Dev Disabil Res Rev. 2005;11:83–93.
83. Mansour A, Hoversten MT, Taylor LP, Watson SJ, Akil H. The cloned μ, δ and κ receptors and their endogenous ligands: Evidence for two opioid peptide recognition cores. Brain Res. 1995;700:89–98.
84. Leconte C, Mongeau R, Noble F. Traumatic Stress-Induced Vulnerability to Addiction: Critical Role of the Dynorphin/Kappa Opioid Receptor System. Front Pharmacol. 2022;13.
85. Kelsey JE, Verhaak AMS, Schierberl KC. The kappa-opioid receptor antagonist, nor-binaltorphimine (nor-BNI), decreases morphine withdrawal and the consequent conditioned place aversion in rats. Behav Brain Res. 2015;283:16–21.
86. Jarman SK, Haney AM, Valdez GR. Kappa opioid regulation of depressive-like behavior during acute withdrawal and protracted abstinence from ethanol. PLOS ONE. 2018;13:e0205016.
87. Valdez GR, Harshberger E. Kappa opioid regulation of anxiety-like behavior during acute ethanol withdrawal. Pharmacol Biochem Behav. 2012;102:44–47.
88. Berger AL, Williams AM, McGinnis MM, Walker BM. Affective Cue-Induced Escalation of Alcohol Self-Administration and Increased 22-kHz Ultrasonic Vocalizations during Alcohol Withdrawal: Role of Kappa-Opioid Receptors. Neuropsychopharmacology. 2013;38:647–654.
89. Carden SE, Davachi L, Hofer MA. U50, 488 increases ultrasonic vocalizations in 3-, 10-, and 18-day-old rat pups in isolation and the home cage. Dev Psychobiol. 1994;27:65–83.
90. Nazarian A, Rodarte-Freeman AL, McDougall SA. Dopaminergic modulation of kappa opioid-mediated ultrasonic vocalization, antinociception, and locomotor activity in the preweanling rat. Behav Neurosci. 1999;113:816–825.
91. Zachry JE, Nolan SO, Brady LJ, Kelly SJ, Siciliano CA, Calipari ES. Sex differences in dopamine release regulation in the striatum. Neuropsychopharmacology. 2021;46:491–499.
92. Shippenberg TS, Bals-Kubik R, Herz A. Examination of the neurochemical substrates mediating the motivational effects of opioids: role of the mesolimbic dopamine system and D-1 vs. D-2 dopamine receptors. J Pharmacol Exp Ther. 1993;265:53–59.
93. Vijay A, Wang S, Worhunsky P, Zheng M-Q, Nabulsi N, Ropchan J, et al. PET imaging reveals sex differences in kappa opioid receptor availability in humans, in vivo. Am J Nucl Med Mol Imaging. 2016;6:205–214.

94. Russell SE, Rachlin AB, Smith KL, Muschamp J, Berry L, Zhao Z, et al. Sex Differences in Sensitivity to the Depressive-like Effects of the Kappa Opioid Receptor Agonist U-50488 in Rats. Biol Psychiatry. 2014;76:213–222.
95. Bartok RE, Craft RM. Sex Differences in Opioid Antinociception. J Pharmacol Exp Ther. 1997;282:769–778.
96. Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KVS, Lariviere WR, et al. The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. Proc Natl Acad Sci. 2003;100:4867–4872.
97. Gear RW, Miaskowski C, Gordon NC, Paul SM, Heller PH, Levine JD. Kappa–opioids produce significantly greater analgesia in women than in men. Nat Med. 1996;2:1248–1250.
98. Laman-Maharg A, Williams AV, Zufelt MD, Minie VA, Hao R, Carroll FI, et al. Sex Differences in the Effects of a Kappa Opioid Receptor Antagonist in the Forced Swim Test. Front Pharmacol. 2018;9.
99. Tan KZ, Cunningham AM, Joshi A, Oei JL, Ward MC. Expression of kappa opioid receptors in developing rat brain – Implications for perinatal buprenorphine exposure. Reprod Toxicol. 2018;78:81–89.
100. Zhu Y, Hsu M-S, Pintar JE. Developmental Expression of the μ, κ, and δ Opioid Receptor mRNAs in Mouse. J Neurosci. 1998;18:2538–2549.
101. Georges F, Normand E, Bloch B, Le Moine C. Opioid receptor gene expression in the rat brain during ontogeny, with special reference to the mesostriatal system: an in situ hybridization study. Dev Brain Res. 1998;109:187–199.
102. Loyd DR, Wang X, Murphy AZ. Sex Differences in μ-Opioid Receptor Expression in the Rat Midbrain Periaqueductal Gray Are Essential for Eliciting Sex Differences in Morphine Analgesia. J Neurosci. 2008;28:14007–14017.
103. Jürgens U. The Neural Control of Vocalization in Mammals: A Review. J Voice. 2009;23:1–10.
104. Vivian JA, Miczek KA. Interactions between social stress and morphine in the periaqueductal gray: effects on affective vocal and reflexive pain responses in rats. Psychopharmacology (Berl). 1999;146:153–161.
105. Rorick-Kehn LM, Witkin JM, Statnick MA, Eberle EL, McKinzie JH, Kahl SD, et al. LY2456302 is a novel, potent, orally-bioavailable small molecule kappa-selective antagonist with activity in animal models predictive of efficacy in mood and addictive disorders. Neuropharmacology. 2014;77:131–144.

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AUTHOR CONTRIBUTIONS

K.K.W.: Data collection, analysis, and writing of manuscript; T.M.: Data collection; K.J.: Data collection; C.S.M.: Data collection; N.M.A.; Data collection; K.T.R.: Data collection; M.B.R.; Data collection; J.A.B.: Data collection and RNAseq analysis; S.A.M.: Data collection; E.J.S.: Data collection; B.M.B.: Data collection; K.N.B.; CFW Data collection; E.J.Y.; CFW Data collection; E.M.W.: Writing of manuscript; C.D.B.: Experimental design, writing of manuscript.

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COMPETING INTERESTS

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FIGURE LEGENDS

FIGURE 1. Morphine exposure from P1–P14 is sufficient to induce withdrawal-associated phenotypes in neonatal FVB/NJ pups. Data are plotted as the mean ± SEM. **A. Experimental Timeline. B. Body Weight**: The effect of Morphine Treatment on body weight was dependent on Postnatal day (β = -0.30, SE = 0.0055, t(611) = -54.38, ****p < 0.0001), where morphine-treated pups weighed significantly less than saline-treated pups from P3 (**p = 0.0013) – P14 (P4 – P14: ****p < 0.0001). **C. Temperature**: The effect of Morphine Treatment on body temperature was dependent on Postnatal day (β = -0.087, SE = 0.041, t(611) = -2.12, *p = 0.035), where morphine-treated pups displayed hypothermia compared to saline-treated pups from P3–P11 and P13 – P14 (all *p ≤ 0.026). SAL, n = 25 (11F, 14M); MOR, n = 22 (11F, 11M). **D. P7 Hot Plate Latency**: There was no effect of Sex (β = 0.49, SE = 2.64, t(46) = 0.19, p = 0.85). The simplified model revealed that Morphine Treatment was associated with a decrease in hot plate latency compared to saline controls (β = -12.88, SE = 1.86, t(48) = -6.91, ****p < 0.0001). **E. P7 Hot Plate Velocity**: There was no effect of Sex (β = 0.00037, SE = 0.005, t(39) = 0.076, p = 0.94). The simplified model revealed that Morphine Treatment had no effect on hot plate velocity (β = 0.001, SE = 0.0034, t(41) = 0.27, p = 0.79). SAL, n = 23–26 (11–12F, 12–14M); MOR, n = 22–24 (12–13F, 10–11M). **F. P14 Hot Plate Latency**: There was no effect of Sex (β = 1.089, SE = 6.085, t(35) = 0.18, p = 0.86). The simplified model revealed that Morphine Treatment was associated with decreased hot plate latency compared to saline controls (β = -15.50, SE = 4.42, t(37) = -3.51, **p = 0.0012). **G**. **P14 Hot Plate Velocity**: There was no effect of Sex (β = -0.0047, SE = 0.0041, t(33) = -1.15, p = 0.23). The simplified model revealed that Morphine Treatment was associated with increased hot plate velocity compared to saline controls (β = 0.011, SE = 0.0030, t(35) = 3.67, ***p = 0.00081). SAL, n = 20–21 (8–9F, 12M); MOR, n = 17–18 (9F, 8–9M).

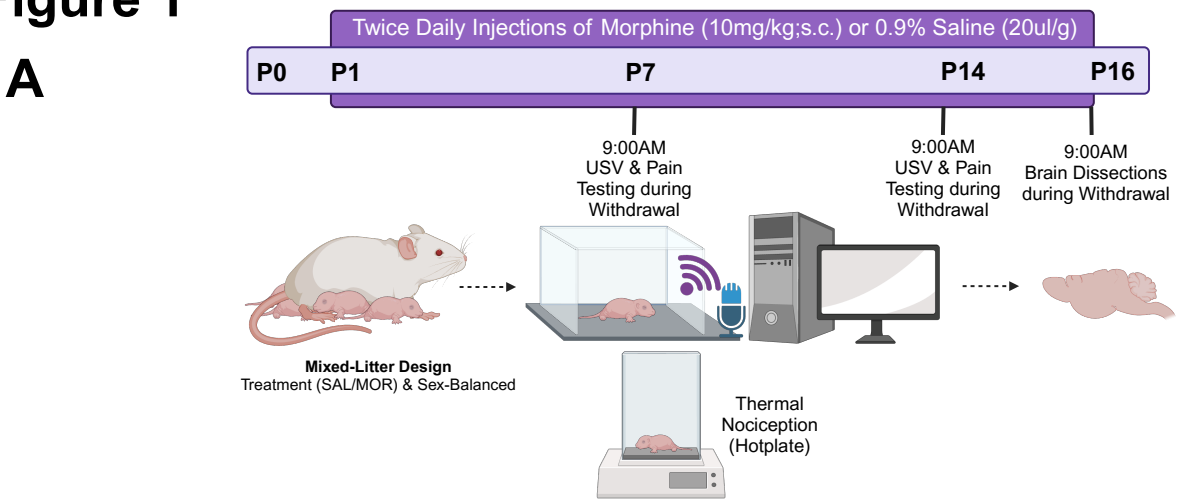
FIGURE 2. Locomotor activity during USV assessment of FVB/NJ pups undergoing spontaneous morphine withdrawal on P7 and P14. Data are plotted as the mean ± SEM. **A. P7 Distance**: The effect of Morphine Treatment on USV distance was dependent on Time (β = -0.030, SE = 0.0056, t(378) = -5.34, ****p < 0.0001), where morphine-treated pups traveled a greater distance than

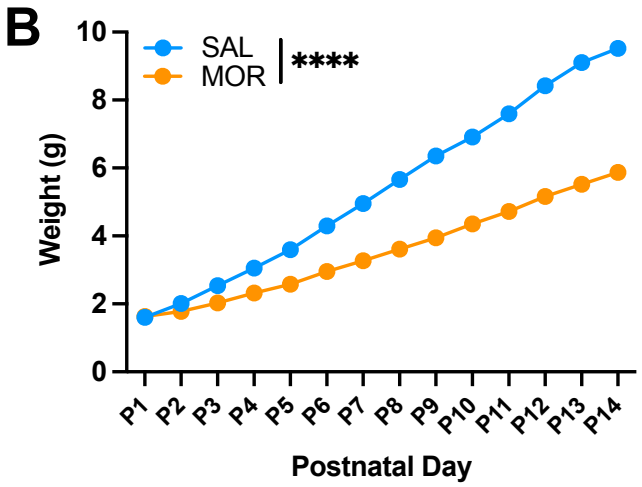
699 saline controls from 2 – 5 min of the recording session (all *$p \leq 0.031$). **B. P7 Total Distance:** There
700 was no effect of Sex (β = -0.11, SE = 0.58, t(38) = -0.20, $p = 0.85$). The simplified model revealed that
701 Morphine-treated pups traveled a greater distance during USV recordings compared to saline-treated
702 pups (β = 1.24, SE = 0.40, t(40) = 3.12, **$p = 0.0033$). **C. P7 Average Velocity:** There was no effect of
703 Sex (β = 0.0022, SE = 0.0040, t(38) = 0.59, $p = 0.56$). The simplified model revealed that Morphine
704 Treatment had no effect on USV velocity (β = -0.0031, SE = 0.0027, t(40) = -1.17, $p = 0.25$). SAL, n =
705 21 (9F, 12M); MOR, n = 21 (11F, 10M). **D. P14 Total Distance:** There was no Morphine Treatment x
706 Time interaction (β = 0.0029, SE = 0.012, t(518) = 0.24, $p = 0.81$), so data was collapsed across the 15
707 min recording session. There was no effect of Sex (β = -0.93, SE = 3.84, t(33) = -2.42, $p = 0.81$). The
708 simplified model revealed that Morphine Treatment had no effect on the total distance traveled (β =
709 5.20, SE = 2.81, t(35) = 1.85, $p = 0.073$). **E. P14 Average Velocity:** There was no effect of Sex (β =
710 0.019, SE = 0.016, t(33) = 1.20, $p = 0.24$). The simplified model revealed that Morphine Treatment had
711 no effect on USV velocity (β = 0.00020, SE = 0.012, t(35) = 0.017, $p = 0.99$). SAL, n = 21 (9F, 12M);
712 MOR, n = 13 (7F, 9M).
713
714 **FIGURE 3. USV profiles of FVB/NJ pups during spontaneous morphine withdrawal on P14.** Data
715 are plotted as the mean ± SEM. **A. P14 USV Emission:** There was a Morphine Treatment x Sex x Time
716 interaction, where morphine females vocalized more than saline females (β =4.66, SE = 0.75, t(518) =
717 6.21, ****$p < 0.0001$) over time, specifically during the 4 – 15 min intervals (all *$p \leq 0.035$) . **B. P14 Total**
718 **USV Emission**: The effect of Morphine Treatment on USV emission was dependent on Sex (β = 919.6,
719 SE = 180.7, t(33) = 5.088, ****$p < 0.0001$). Specifically, morphine-treated females vocalized more than
720 saline females (β = 937.0, SE = 181, t(33) = 5.19, ****$p < 0.0001$). **C. P14 Syllable Profile:** Morphine
721 Treatment was associated with an increase in the percentage of Complex 3 syllables emitted (β = 0.14,
722 SE = 0.033, t(35) = 4.19, ***$p = 0.00018$), and a decrease in the percentage of Flat (β = -0.051, SE =
723 0.017, t(35) = -3.0, **$p = 0.0051$), and Short (β = -0.030, SE = 0.014, t(35) = -2.08, *$p = 0.045$) syllables
724 emitted compared to saline controls. There were Morphine Treatment x Sex interactions, where
725 morphine females emitted more Complex 3 (β = 0.14, SE = 0.044, t(33) = 3.13, **$p = 0.0036$) and less
726 Upward (β = -0.11, SE = 0.038, t(33) = -2.92, **$p = 0.0063$) syllables compared to morphine males, so
727 we investigated the syllable profile for each sex separately. **D. Female P14 Syllable Profile:** Morphine
728 Treatment was associated with an increase in the proportion of Complex 3 syllables (β = 0.21, SE =
729 0.046, t(15) = 4.8, ***$p = 0.00030$) and a decrease in Flat (β = -0.051, SE = 0.020, t(15) = -2.55, *$p =$
730 0.022), Short (β = -0.055, SE = 0.020, t(15) = -2.69, *$p = 0.017$), and Upward syllables (β = -0.097, SE
731 = 0.031, t(15) = -3.13, **$p = 0.0069$). **E. Male P14 Syllable Profile:** Morphine Treatment was associated

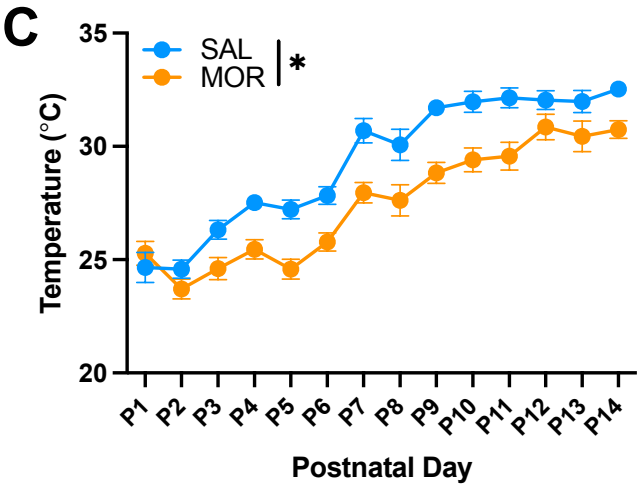
732 with an increase in the proportion of Complex syllables (β = 0.017, SE = 0.0072, t(18) = 2.41, *p =
733 0.027). SAL, n = 20 (8F, 12M); MOR, n = 17 (9F, 8M).
734
735 **FIGURE 4. The kappa opioid receptor antagonist nor-BNI reduces USV emissions in FVB/NJ**
736 **female pups during spontaneous withdrawal on P15.** Data are plotted as the mean ± SEM. **A. P15**
737 **Total USV Emissions**: There was a Morphine Treatment x norBNI Treatment x Sex interaction, where
738 Morphine-treated females pre-treated with nor-BNI (MOR/norBNI) emitted fewer USVs compared to
739 morphine-treated females pre-treated with saline (MOR/SAL) (β = -272.35, SE = 127.11, t(143) = -2,143
740 *p = 0.034). **B. Female P15 USV Emissions**: Given the three-way interaction, we analyzed males and
741 females separately across time to simplify the linear mixed model. There was a time-dependent
742 decrease in USVs in morphine-treated females pre-treated with nor-BNI (MOR/norBNI) compared to
743 morphine-treated females pre-treated with saline (MOR/SAL)(β = -1.42, SE = 0.52, t(1148) = -2.72, **p
744 = 0.0068), specifically during the 4–8 and 10min intervals (all *p ≤ 0.037). **C. P15 Syllable Profile:**
745 There was no effect of Sex on any Syllable emitted (all p ≥ 0.20), so Sex was removed from the models.
746 Morphine pups pre-treated with nor-BNI emitted fewer Chevron syllables compared to morphine-treated
747 pups pre-treated with saline (β = -0.036, SE = 0.017, t(147) = -2.065 *p = 0.041). SAL/SAL, n = 40 (22F,
748 18M); SAL/norBNI, n = 33 (17F, 16M); MOR/SAL, n = 39 (21F, 18M); MOR/norBNI, n = 39 (22F, 17M).
749
750 **FIGURE 5. Kappa-opioid receptor agonism increases USV emission in morphine-naïve FVB/NJ**
751 **pups on P10 and P14. A. P10 USV Emission**: The effect of U50,488h Treatment depended on Time
752 (β = -5.89, SE = 0.52, t(812) = -11.42, ****p < 0.0001). U50,488h-treated pups vocalized more than
753 saline pups during the 2–9min intervals (all *p ≤ 0.020). **B. P10 Total USV Emission**: There was no
754 effect of Sex (β = -167.71, SE = 205.43, t(54) = -1.34, p = 0.19). The simplified model revealed that
755 U50,488h Treatment was associated with increased USV emission compared to saline controls (β =
756 406.2, SE= 148.9, t(56) = 2.73, **p = 0.0085). **C. P10 Syllable Profile:** There was no effect of Sex on
757 the percentage of syllables emitted (all p ≥ 0.42). The simplified model revealed that U50,488h
758 Treatment was associated with a decrease in the percentage of Downward syllables (β = -0.021, SE =
759 0.0070, t(56) = -3.04, **p = 0.0036) and Short syllables (β = -0.015, SE = 0.0072, t(56) = -2.11, *p =
760 0.039) compared to saline controls. SAL, n = 29 (18F, 11M); U50,488h, n = 31 (17F, 12M). **D. P14 USV**
761 **Emission:** The effect of U50,488h on USV emission depended on Time (β = -0.56, SE = 0.025, t(966)
762 = -2.25, *p = 0.025), where U50,488h-treated pups vocalized more than saline pups during the 5–9min
763 intervals (all p ≤ 0.0087). **E. P14 Total USV Emission:** There was a U50,488h Treatment x Sex
764 interaction, where U50,488h Treatment was associated with increased USV emission in females

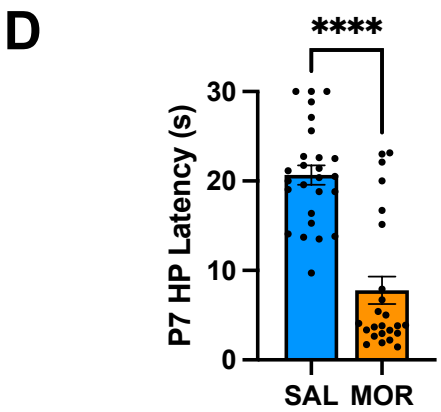
765 compared to saline-treated females (β = 164.85, SE = 67.34, t(65) = 2.45, *p = 0.017). **F. P14 Syllable**
766 **Profile**: There was no effect of Sex on the percentage of syllables emitted (all $p \geq 0.12$). The simplified
767 model revealed that U50,488h Treatment was associated with a decrease in Complex 2 (β = -0.021,
768 SE = 0.0082, t(67) = -2.57, *p = 0.012), Complex 3 (β = -0.078, SE = 0.018, t(67) = -4.43, ****p <
769 0.0001), and Complex 4 (β = -0.0080, SE = 0.0028, t(67) = -2.91, **p = 0.0049) syllables. SAL, n = 29–
770 32 (18–19F, 11–13M); U50,488h, n = 31–37 (17–21F, 12–16M).

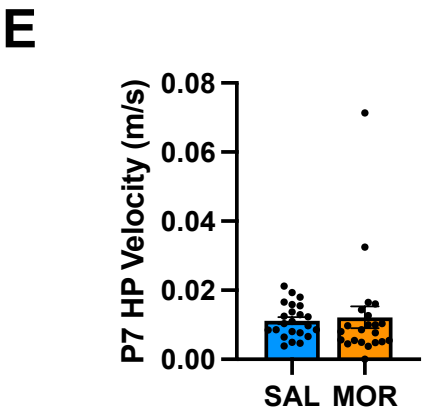
Figure 1

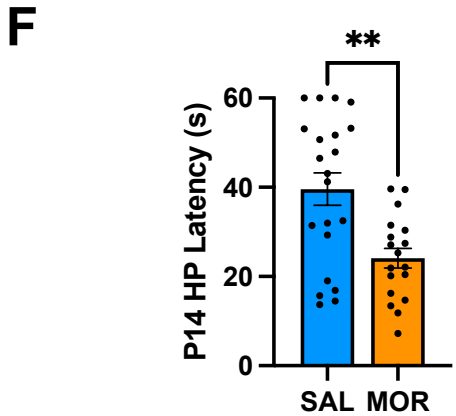

A
Twice Daily Injections of Morphine (10mg/kg;s.c.) or 0.9% Saline (20ul/g)
P0
P1
P7
P14
P16
9:00AM
USV & Pain
Testing during
Withdrawal
9:00AM
USV & Pain
Testing during
Withdrawal
9:00AM
Brain Dissections
during Withdrawal
Mixed-Litter Design
Treatment (SAL/MOR) & Sex-Balanced
Thermal
Nociception
(Hotplate)


B
SAL
MOR
****
Weight (g)
0
2
4
6
8
10
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
Postnatal Day


C
SAL
MOR
*
Temperature (°C)
20
25
30
35
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
P11
P12
P13
P14
Postnatal Day


D
****
P7 HP Latency (s)
0
10
20
30
SAL
MOR


E
P7 HP Velocity (m/s)
0.00
0.02
0.04
0.06
0.08
SAL
MOR


F
**
P14 HP Latency (s)
0
20
40
60
SAL
MOR

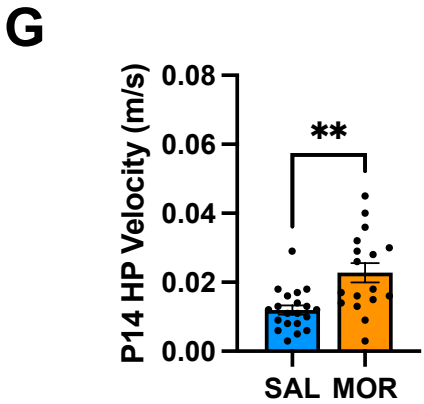

G
**
P14 HP Velocity (m/s)
0.00
0.02
0.04
0.06
0.08
SAL
MOR

Figure 2

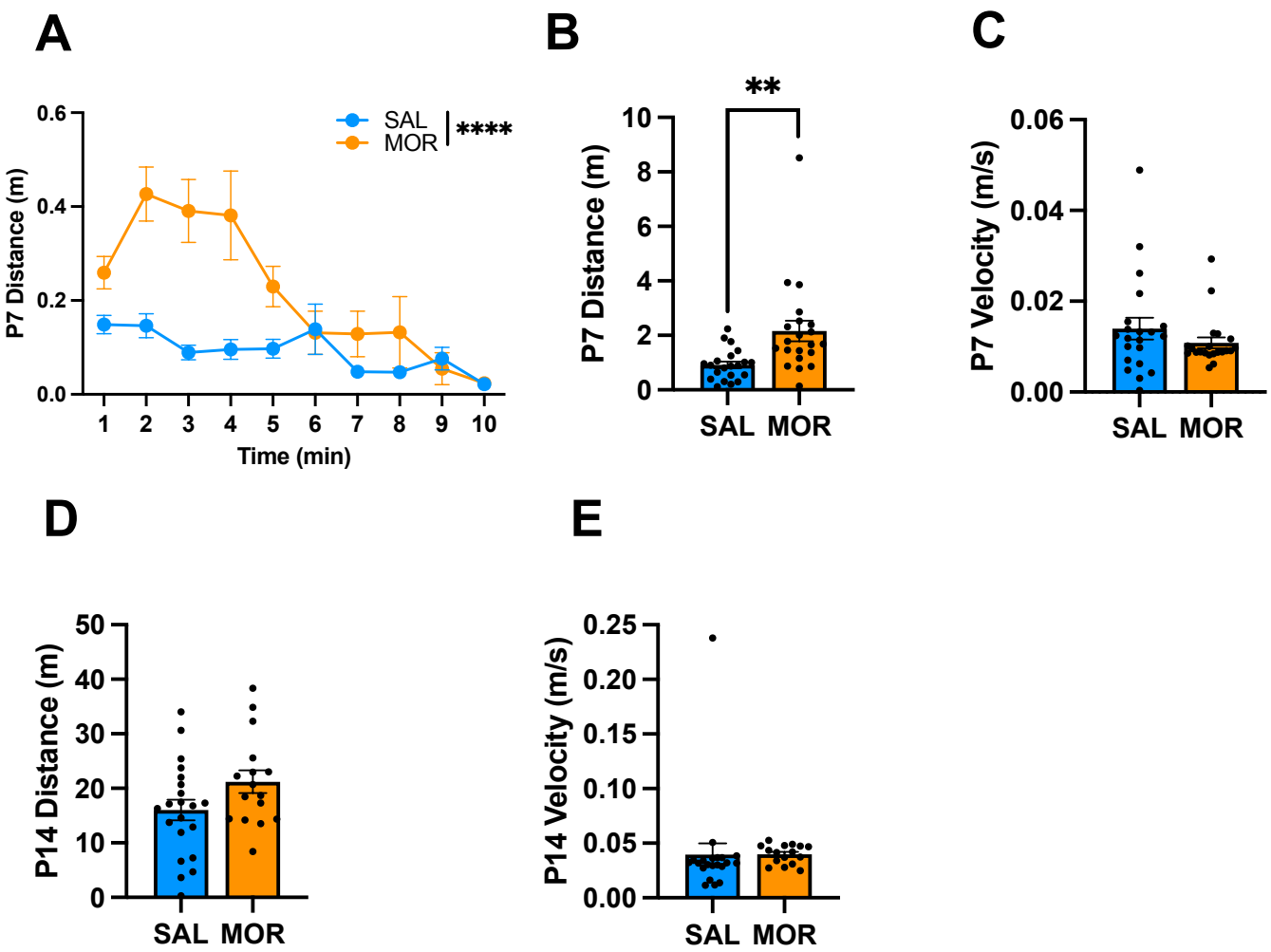

A
B
C
D
E
SAL
MOR
****
**
P7 Distance (m)
Time (min)
P7 Velocity (m/s)
P14 Distance (m)
P14 Velocity (m/s)
SAL
MOR

Figure 3

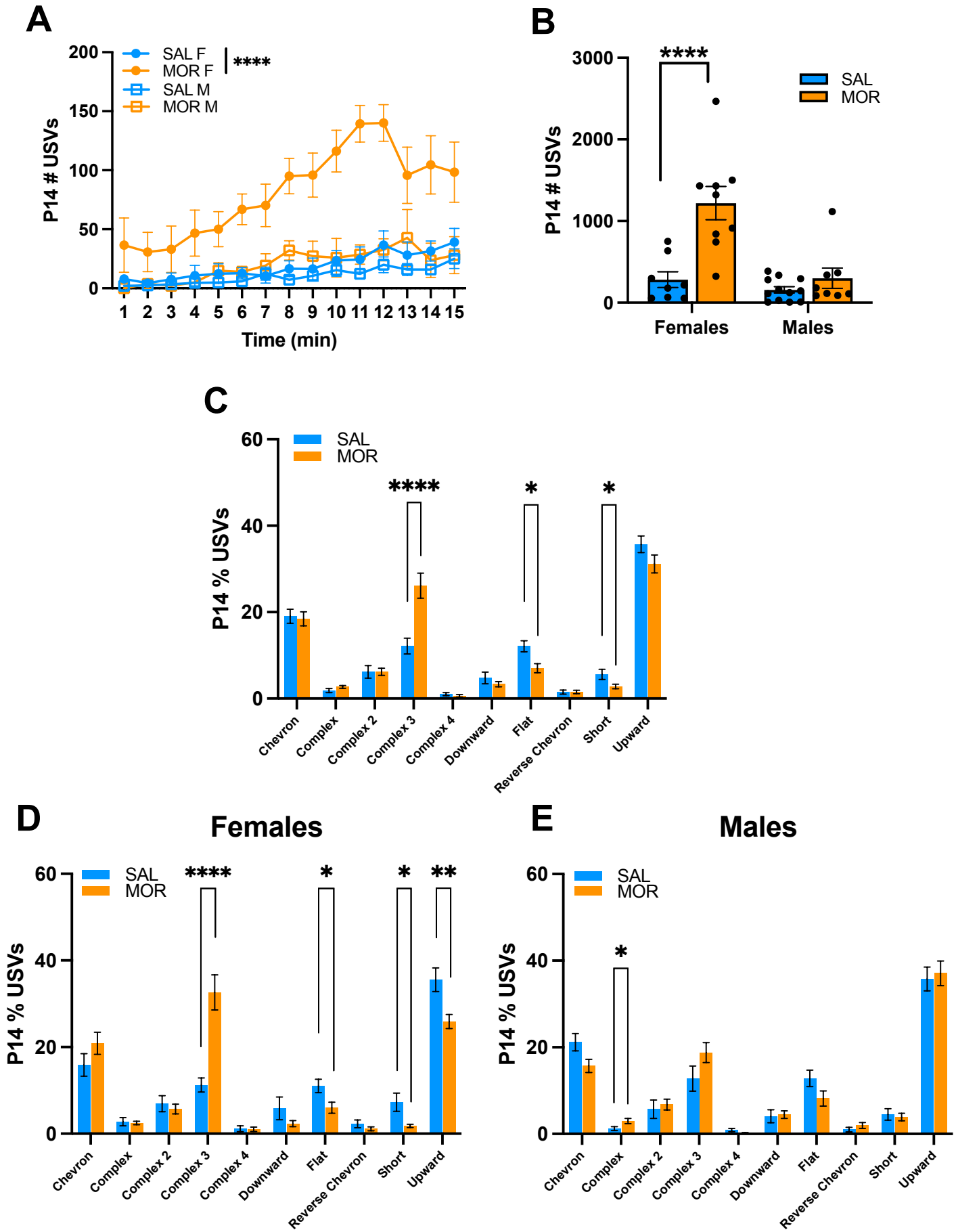

A
SAL F
MOR F
SAL M
MOR M
****
P14 # USVs
Time (min)
B
****
SAL
MOR
P14 # USVs
Females
Males
C
SAL
MOR
****
*
*
P14 % USVs
Chevron
Complex
Complex 2
Complex 3
Complex 4
Downward
Flat
Reverse Chevron
Short
Upward
D
Females
SAL
MOR
****
*
*
**
P14 % USVs
E
Males
SAL
MOR
*
P14 % USVs

Figure 4

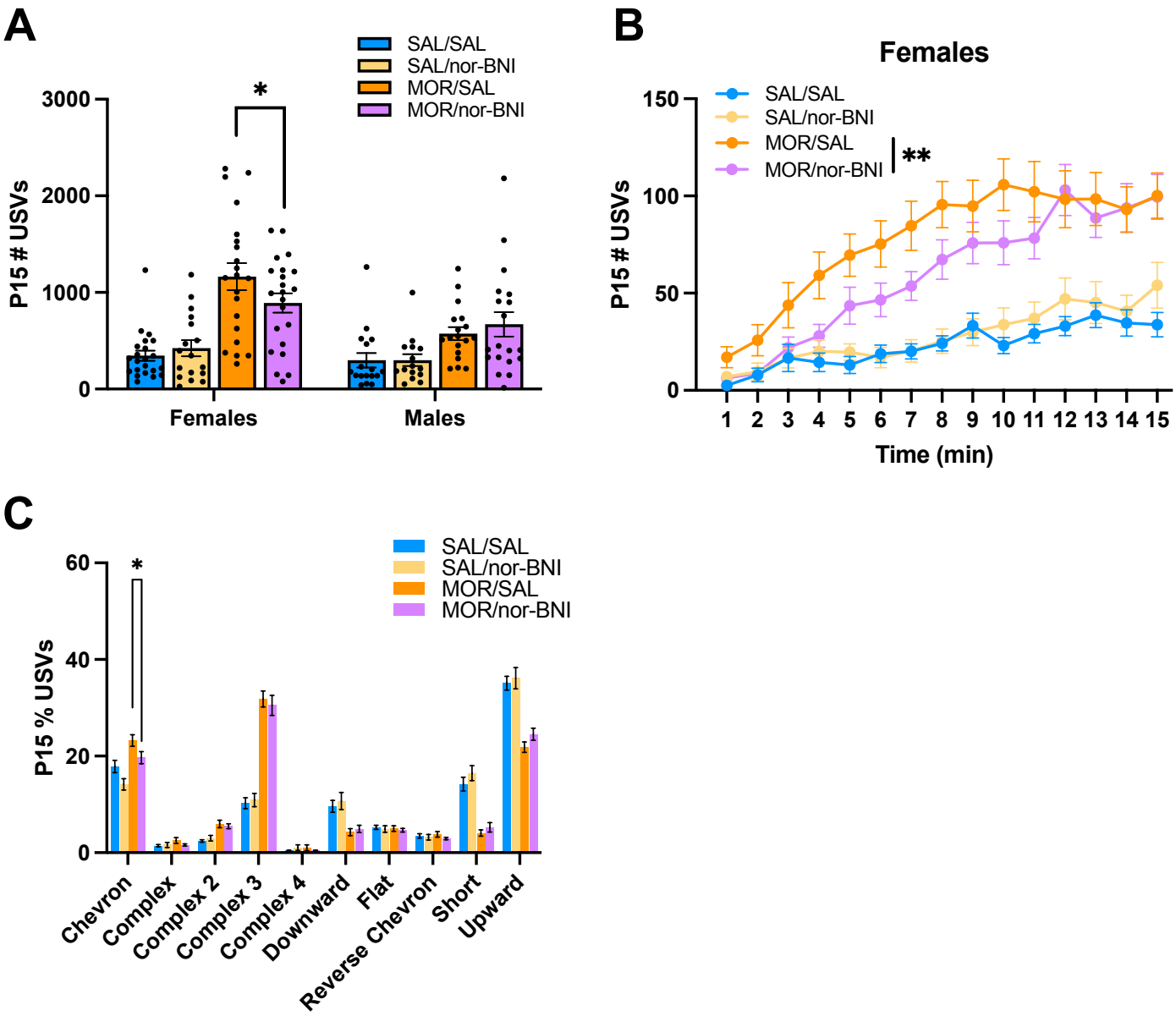
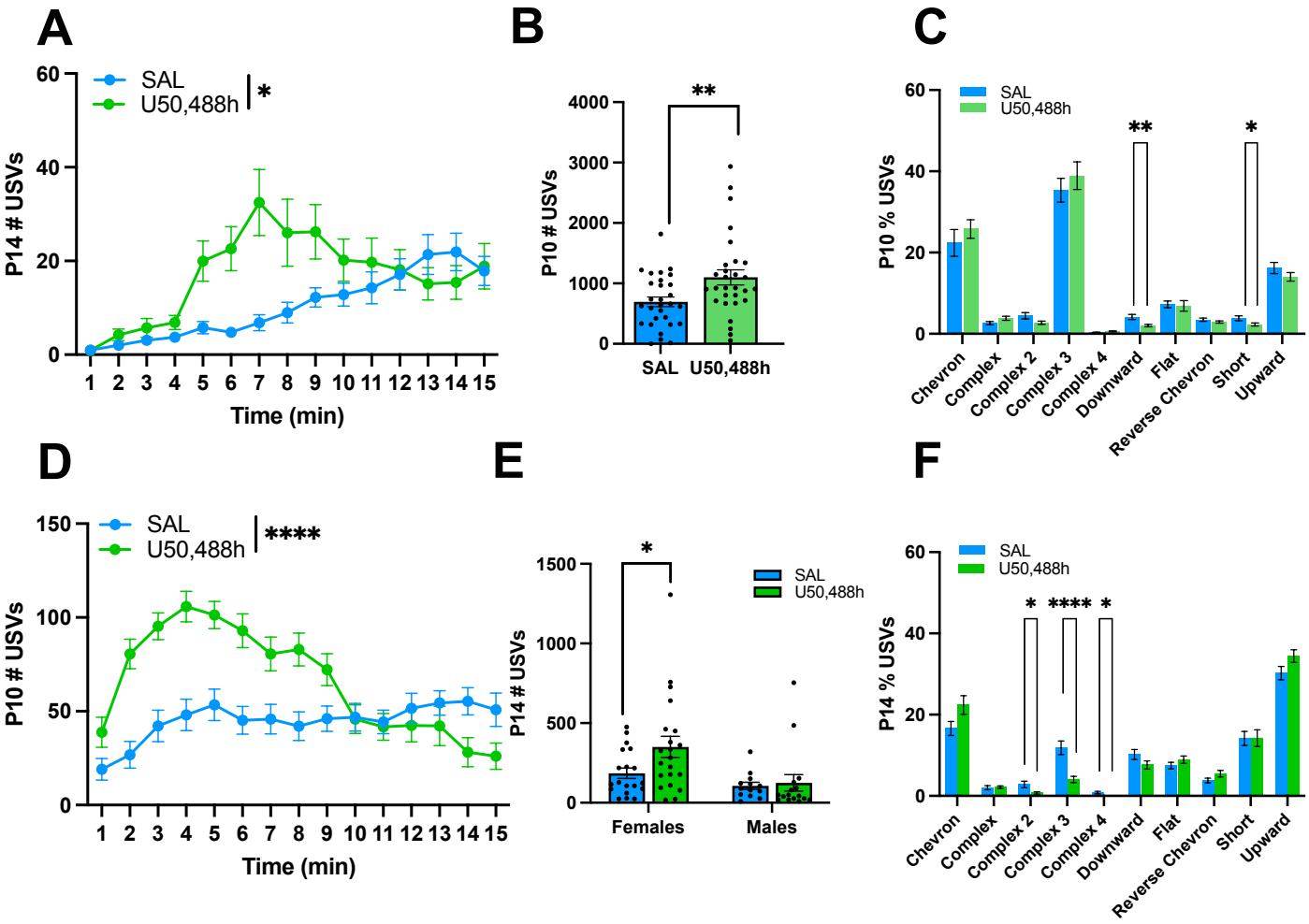

A
SAL/SAL
SAL/nor-BNI
MOR/SAL
MOR/nor-BNI
P15 # USVs
3000
2000
1000
0
*
Females
Males
B
Females
SAL/SAL
SAL/nor-BNI
MOR/SAL
MOR/nor-BNI
**
P15 # USVs
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (min)
C
SAL/SAL
SAL/nor-BNI
MOR/SAL
MOR/nor-BNI
P15 % USVs
60
40
20
0
*
Chevron
Complex
Complex 2
Complex 3
Complex 4
Downward
Flat
Reverse Chevron
Short
Upward

Figure 5


A
SAL
U50,488h
*
P14 # USVs
Time (min)
B
**
P10 # USVs
SAL
U50,488h
C
SAL
U50,488h
**
*
P10 % USVs
Chevron
Complex
Complex 2
Complex 3
Complex 4
Downward
Flat
Reverse Chevron
Short
Upward
D
SAL
U50,488h
****
P10 # USVs
Time (min)
E
*
SAL
U50,488h
P14 # USVs
Females
Males
F
SAL
U50,488h
* **** *
P14 % USVs
Chevron
Complex
Complex 2
Complex 3
Complex 4
Downward
Flat
Reverse Chevron
Short
Upward