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ABSTRACT BOOK

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Investigating self-administration of delta-opioid receptor agonists in morphine withdrawn rats

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For people struggling with opioid use disorder (OUD), discontinuation of mu-opioid receptor agonists produce opioid withdrawal symptoms such as body aches, hyperalgesia, anxiety, and irritability. δ-opioid receptor (DOR) agonists produce antinociceptive, anxiolytic, and antidepressant-like effects in preclinical studies, and therefore may have potential in the treatment of opioid withdrawal. While DOR agonists are generally considered to have low abuse liability, it is important to determine whether these compounds may have abuse potential in opioid withdrawal. Thus, the present study sought to evaluate the reinforcing effects of the canonical DOR agonist SNC80 and a novel DOR agonist PN6047 in both chronically morphine-withdrawn and morphine-naive rats. Male Sprague-Dawley rats were implanted with intravenous catheters and then received twice daily saline or morphine injections for 4 days (10, 20, 20, 40 mg/kg, respectively). Then rats were allowed to self-administer remifentanil (0.0032 mg/kg/infusion) on a fixed ratio (FR) 1 schedule of reinforcement and received an injection of 40 mg/kg morphine or saline approximately 30 min after each daily self-administration session. Following stable self-administration of remifentanil, saline, different doses of PN6047 or SNC80, or cocaine were substituted for at least 7 days. Cocaine (0.32 mg/kg/infusion) maintained responding in morphine-naïve rats and some, but not all, morphine-withdrawn rats. PN6047 (0.32 mg/kg/infusion or 1 mg/kg/infusion) did not maintain responding at any PN6047 dose tested in either morphine-withdrawn or morphine-naive rats. SNC80 (0.32 mg/kg/infusion) maintained responding in morphine-naive, but not morphine-withdrawn rats. These data suggest that SNC80 and PN6047 may have differential reinforcing effects in morphine-naive animals but likely have weak reinforcing effects overall. These findings suggest that DOR agonists have low abuse liability, even during opioid withdrawal.

A photoswitchable morphinan agonist for reversible optical control of peripheral mu-opioid receptors

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Opioids, while effective for severe pain, pose challenges due to a plethora of side effects, especially during long-term use. Opioid receptors (ORs), part of the G protein-coupled receptors (GPCR) superfamily, are widely expressed in the central and peripheral nervous systems. Peripheral ORs promote analgesia without causing the most harmful side effects (respiratory depression, addiction), suggesting a potential strategy of restricting the action of opioids to peripheral receptors and/or the site of pain. Many studies indicate that light-activated ligands offer a promising approach for GPCR modulation. Here, we designed, synthesized, and assessed photochromic ligands for ORs ("opto-opioids"), that could be locally activated only in the relevant pain site and for a restricted time.

Using behavioral pharmacology, electrophysiology, and cellular imaging assays, opto-opioids were tested for agonism of ORs in cultured cells and their analgesic properties in-vivo. We found that a morphine-based compound termed “azo-morphine-3” (AM-3) has stronger agonism in the light-activated cis than in the relaxed trans configuration for the mu-opioid receptor (MOR). AM-3 shows a clear affinity (~5-fold) and efficacy shift (~3-fold) between cis and trans for the MOR, as assessed using patch clamp. An established SNAP-tagged MOR-based internalization assay revealed that in cis, but not trans, AM-3 drives robust internalization of MOR ($P<0.001$), indicating that AM-3 is a balanced agonist with both G protein and beta-arrestin coupling as morphine. In mice, intra-plantar (i.pl.) administration of cis- ,but not trans-, AM3 dose-dependently increased the paw withdrawal latency (PWL) in the radiant-heat test over time ($P<0.001$). I.pl. administration of trans-AM3 and immediate exposure to UV light in the paw also increased the PWL ($P<0.001$) compared to veh or no UV exposure. Moreover, i.pl. cis-AM3 injection following immediate exposure to visible light in the paw nullified the PWL increase ($P=0.7$). Repeated i.pl. administration of AM-3 showed a decrease in its analgesic effect over time ($P=0.167$, at day3), indicating analgesic tolerance. Noteworthy, intravenous administration of cis-AM3 failed to provide an analgesic effect in the tail-flick test, suggesting a low penetration in the CNS ($P=0.446$).

Together these findings show the feasibility of reversibly photoswitchable MOR agonism in vivo in the periphery for local analgesia.

Sex- and time-dependent shifts in cortico-striatal circuits underlying opioid self-administration in mice

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Sex- and time-dependent shifts in cortico-striatal circuits underlying opioidself-administration in mice

Canonical thinking in the addiction field posits that early drug use is controlled and mediated by goal-directed circuits such as the prefrontal cortex. With extended use, drug taking is thought to become more reliant on cortico-striatal habit-associated circuits, a phenomenon not empirically demonstrated with opioids. We recently found that self-administration of remifentanil promotes a progressive hypoactive state in the prelimbic cortical region of the mouse PFC (PrLC) that underlies impaired decision-making, develops faster in females, and aligns with an escalation of drug intake. The present study investigates whether1) with increased exposure, the PrLC becomes less involved in control of drug intake, 2) this behavior control lies within PrLC projections to the nucleus accumbens core (NACore), 3) this phenomenon occurs faster in female mice, and 4) effects of PrLC inhibition are selective for drug rewards. To do so, we virally expressed the inhibitory hM4Di DREADD or mcherry control in the PrLC or inter-sectionally within the PrLC-NACore circuit of male and female C57bl6/j mice. Mice intravenously self-administered (SA) remifentanil or orally administer a liquid reward (50% Ensure) for up to 45 days, with clozapine-n-oxide administered to all mice on day 14, 30, or 45 with drug available during all sessions. Our findings show that inhibition of the PrLC reduces drug intake in females ($p<0.01$) and males ($p<0.001$) on day 14, whereas intake was reduced in males on day 30 ($p=0.02$) but not females ($p=0.35$). Alternatively, inhibition of the PrLC reduced consumption of Ensure in males ($p<0.001$) but not females on day 14, with no effect at 30 days. Initial findings indicate that inhibition of the PrLC-NACore circuit also reduces drug intake on day 14 and 30 ($p<0.05$). These data provide the first evidence of time- and sex-specific roles for PrLC circuits in driving opioid vs appetitive reward taking. Ongoing studies will examine the role of the PrLC in development of habit-like drug seeking as well as PrLC-NACore circuit manipulation in females and non-drug reward at more protracted timepoints of self-administration.

Mapping Opioid Receptor Activation at Cellular Resolution

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Opioid receptor (OR) signaling is critical for the function of neuronal pathways involved in various physiological and behavioral processes, such as pain modulation, breathing, and reward. Therefore, elucidating the signaling pathways of ORs is crucial for understanding their roles in modulating these processes, as well as developing new therapeutic strategies with higher selectivity for receptors involved in pain modulation and lower propensity for addiction. To address these, we developed a genetically encoded fluorescent reporter, the **S**ingle-chain **P**rotein-based **O**pioid **T**ransmission **I**ndicator **T**ool for **M**OR (M-SPOTIT). This sensor detects the activation of the mu-opioid receptor (MOR), leaving a permanent green fluorescent mark quantifiable in cell cultures and *post-mortem* animal brain tissue. M-SPOTIT showed an opioid-dependent signal-to-noise ratio (S/N) of up to 12.5 in HEK293T cells and 4.6 in neuronal culture, requiring as short as 30 seconds of opioid exposure in HEK293T cells. Additionally, M-SPOTIT has been demonstrated to detect morphine in mice brains. This motif has been expanded to detect the activation of other receptors, such as the kappa-opioid receptor (KOR), the beta-2 adrenergic receptor (β2AR), and dopamine receptors (DRs). Moreover, we have developed a red fluorescent version of this motif that allows detection of the activation of two receptors simultaneously. These sensors offer a novel mechanism for opioid detection in high-throughput cell-based assays, with the potential to aid in the development of selective opioid-based therapeutics and elucidate the pathways involved in pain modulation and reward.

Evaluating the convulsant and reinforcing properties of the novel DOR agonist PN6047

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Delta opioid receptor (DOR) agonists produce antidepressant-like effects; however, their therapeutic potential is limited due to seizurogenic activity. Therefore, the goal of the present study was to evaluate potential adverse effects, such as convulsions and reinforcing effects, produced by the novel DOR agonist PN6047 in male Sprague Dawley rats. All subjects were implanted with intravenous catheters and allowed to recover. To assess convulsive properties of PN6047 or the canonical DOR agonist SNC80, drug was given subcutaneously or infused intravenously (i.v). over 30 seconds and then rats were observed continuously for 20 min. PN6047 (0.3-3 mg/kg/infusion) or SNC80 (0.1-0.56 mg/kg/infusion) were also evaluated in rats trained to self-administer remifentanil (0.003 mg/kg/infusion) on an FR10 schedule of reinforcement. PN6047 did not produce convulsions when given systemically. Intravenous administration of PN6047 produced a dose-dependent increase in the number of rats experiencing a convulsion with 56 mg/kg inducing convulsions in 100% of rats. Interestingly, convulsions induced by PN6047 occurred on average 2.72 min (0.75 - 7.1 min) following the iv bolus as compared with immediate convulsions induced by SNC80. The convulsive effects of PN6047 were blocked by pretreatment of the DOR selective antagonist, naltrindole (3.2 mg/kg subcutaneous). Unlike SNC80, PN6047 did not maintain self-administration behavior. Overall, these data demonstrate that PN6047 has DOR agonist activity that differs substantially from SNC80. These effects of PNC6047 may suggest a more advantageous profile for therapeutic potential for depression. This study was supported by UG3 DA053094.

The Gβγ inhibitor gallein produces naloxone-sensitive antihyperalgesia and potentiates the antihyperalgesic effects of morphine

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Although mu opioid receptor (MOR) agonists provide short-term pain relief, they also produce adverse effects and are frequently misused, increasing susceptibility for opioid use disorder (OUD). Thus, it is necessary to improve the safety of opioid analgesics as well as explore alternative analgesics with low abuse liability. Previous findings have shown that gallein-mediated inhibition of Gβγ signaling to a subset of effectors, such as PLCβ3, potentiates the antinociceptive effects of morphine without altering its rewarding effects *in vivo*[1,2]. Therefore, we sought to evaluate if gallein-mediated inhibition of Gβγ signaling enhances antihyperalgesic effects produced by opioid receptor activation with either endogenous or exogenous opioid ligands. We treated female and male C57BL6/N mice with the Gβγ inhibitor gallein (1, 3.2, 10, 32, or 100 mg/kg, i.p.) or vehicle 90 min after nitroglycerin (NTG, 10 mg/kg, i.p.) and measured tail withdrawal latencies from a 46^{o}C water bath. NTG decreased tail withdrawal latencies as compared with baseline, indicating a hyperalgesic state. Large doses of gallein alone reversed NTG-induced decreases in withdrawal latencies. A small, ineffective dose (3.2mg/kg) of gallein potentiated the effects of morphine as demonstrated by the leftward shift in the morphine dose response curve. Pretreatment with the non-selective opioid antagonist naloxone (NLX) (1.0mg/kg, i.p.) attenuated the effects of gallein alone and gallein-potentiated antihyperalgesic effects of morphine. Further, NTG only partially decreased tail withdrawal latencies in mice with a constitutive knockout of PLCβ3 as compared with that observed in wild-type littermates. NLX restored NTG-induced decreases in tail withdrawal latencies in PLCβ3 knockout mice comparable to that observed in wild-type littermates. In sum, the Gβγ inhibitor gallein alone produced antihyperalgesic effects that were mediated by opioid receptor activation likely induced by endogenous opioids peptides. Together, these data suggest that inhibiting a subset of Gβγ effectors, like PLCβ3, downstream of MOR activation improves the analgesic effects of MOR agonists without altering their adverse effects and may improve the effectiveness of pain relief induced by endogenous opioids. These studies were supported by NIH grant DA 0418625.

Delta opioid receptors in the insular cortex ameliorate irritable bowel syndrome-like symptoms in chronic vicarious social defeat stress mice.

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Delta opioid receptors in the insular cortex ameliorate irritable bowel syndrome-like symptoms in chronic vicarious social defeat stress mice.

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Irritable bowel syndrome (IBS) is a disorder chronically presenting gastrointestinal symptoms derived from the small and large without evidence of primary organic disease. Many IBS patients are reported to have concomitant depressive and anxiety symptoms. We previously reported that a chronic vicarious social defeat stress (cVSDS) mouse model caused by repeated exposure to psychological stress exhibits increased intestinal transit ratio and visceral pain-related behaviors without abnormal histological and inflammatory scores for more than 30 days after stress exposure (1). Based on these finding, we proposed the cVSDS as a novel animal model of IBS. Further, we recently reported that delta opioid receptor (DOP) agonists KNT-127 exert antidepressant-like in cVSDS mice (2). In this study, we evaluated the effects of KNT-127 on IBS-like symptoms in cVSDS mice. Male C57BL/6J mice aged 5-6 weeks were used. Administration of KNT-127 (10 mg/kg, s.c.) to CVSDS mice improved both intestinal transport abnormalities in the charcoal meal test and abdominal pain in the capsaicin-induced hyperalgesia test, suggesting that DOP agonists improve IBS-like symptoms observed in CVSDS mice. Local administration of KNT-127 (0.3 nmol/0.2 μL) in insular cortex (IC) suppressed the cVSDS-induced increased intestinal peristalsis. Further, extracellular glutamate levels in the IC of cVSDS mice, which were increased by re-exposure to VSDS, were significantly suppressed by KNT-127 (10 mg/kg, s.c.). We suggested that inhibition of glutamatergic neurotransmission in IC was involved in the improving effects of DOP agonist on IBS-like symptoms. We propose that DOP is a novel target for CNS-mediated IBS therapy.

Ref.1. Yoshioka et al., Front Neurosci. 2022;16:993132.2. Yoshioka et al., Neuropharmacology. 2023;232:109511.

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Interactions of isoflurane and fentanyl on opioid-induced respiratory depression in an in-vivo mouse model of repeated opioid use

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The perioperative use of opioids during surgery is a common practice recommended by the American Academy of Anesthesia. Opioids are useful in surgery by providing analgesia, having synergistic effects with adjuvant anesthetics, attenuating autonomic responses to noxious stimuli during surgeries, and suppressing gag reflex during airway manipulation. However, opioids also increase the risk for respiratory depression and apnea during both the intra-operative and post-operative period. Intra-operative respiratory depression can lead to delayed extubating and post-operative respiratory depression are associated with pulmonary complications. These perioperative complications may be complicated among individuals who suffer from opioid use disorder. Our ongoing study investigates the how repeated opioid use (ROU) impacts breathing during isoflurane anesthesia and its interactions with fentanyl. We hypothesize that tolerance to opioid induced respiratory depression is compromised during co-administration of opioids with isoflurane. To address this, we performed a series of whole-body plethysmography and fiber photometry experiments to measure breathing and locus coeruleus (LC) activity in adult mice. Mice were subjected to a ROU protocol consisting of intraperitoneal fentanyl administration for a minimum of five consecutive days. Following ROU, the majority of unanesthetized mice showed an adaptive response that was characterized by a diminished of opioid induced respiratory depression (OIRD). This adaptive response coincided with an increase in LC activity coupled to breathing during OIRD. Breathing isoflurane suppressed breathing and LC activity. Isoflurane-induced respiratory depression also decoupled the relationship between breathing and LC activity while fentanyl-induced respiratory depression did not. Co-administration of fentanyl and isoflurane produced a persistently longer respiratory depression that was greater in magnitude when compared to that caused by fentanyl alone. Moreover, respiratory depression caused by co-administration of fentanyl and isoflurane was similar prior to and following ROU. Based on these findings, we propose that the tolerance to ORID produced by ROU reflects differences in brain activity during unanesthetized and anesthetized states. These differences may be important for understanding the unique risks for co-administration of anesthesia and opioids in the clinical setting among chronic opioid users.
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Morphine treatments decreases binge drinking induced by inflammatory pain only female rats.

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The intersection between pain, alcohol use disorders (AUDs), and gender-specific responses remains poorly understood. In this study we aim to investigate the impact of inflammatory pain on binge drinking behavior and assess the efficacy of morphine in managing pain-related increase in alcohol consumption. We hypothesize that pain exacerbates alcohol intake and that morphine pre-treatment attenuates this effect, potentially through modulation of the Mesocorticolimbic System (MCLS).

Male and female rats were subjected to inflammatory pain induced by Complete Freund's Adjuvant (CFA) and assessed using the Drinking in the Dark (DID) paradigm. Morphine pre-treatment effects were evaluated through a short-access Morphine Self-Administration (MSA) paradigm. Immunohistochemistry was employed to assess neural activity in various areas of the mesocorticolimbic system.

Pain induction significantly increased high-concentration alcohol intake in female rats under DID paradigm. However, morphine pre-treatment reduced ethanol consumption in both male and female rats experiencing pain. C-Fos immunohistochemistry results suggest heterogenous alterations in neural activation as a response of the last DID session in the prefrontal cortex, nucleus accumbens and amygdala.

Our findings suggest that inflammatory pain serves as a risk factor for increased alcohol consumption in a binge drinking paradigm in female rats, while morphine pre-treatment mitigates this effect in both genders. Interestingly this effect is dose-dependent and appear only at high doses of alcohol (40% alcohol beverages). These results highlight the complex interplay between pain and alcohol consumption. Further investigation into the neurobiological mechanisms underlying these interactions is warranted, with potential implications for developing gender-specific interventions targeting AUDs in pain-afflicted populations

Negative affective states mediate the impact of pain in opioid medications misuse among chronic pain patients admitted in a hospital pain unit.

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Chronic pain is a burden of disease that reaches in some cases 30% prevalence in developed countries. Recent research has demonstrated the relationship between chronic pain, opioid use disorders, and negative affective states (such as anxiety and depression) mainly in preclinical samples. The goal of the present study was to examine pain severity in relation to opioid misuse patterns, and the role of anxiety and depression in this relationship in a Spanish clinical sample.

101 chronic pain patients were recruited from the pain unit of the *Hospital General Universitario of Valencia* (age: *M* = 57.44, *SD* = 13.88; women: 69.4%). First, the average perceived pain from the last month was obtained through a Visual Analogue Scale (VAS). Moreover, the Current Opioid Misuse Measure (COMM) was administered to assess the consumption of opioid medication. Finally, to measure anxiety and depression, Beck Anxiety Inventory (BAI) and Beck Depression Inventory (BDI) were administered.

The descriptive analysis showed an average of 13.4 score in the COMM revealing a risk of opioid misuse. Additionally, the pain perception average denoted a severe level of pain. Moreover, a moderate to strong positive correlation was found between BAI, BDI, COMM and VAS scores. However, when analyzing per gender only women maintained the correlation of the four variables whereas in the case of men, the correlations of pain perception with the other variables were dissipated. Finally, mediation models in the total sample revealed that anxiety and depression scores mediated between pain perception and COMM score.

In conclusion, these results suggest that the pain perception is not the only critical variable to increase the risk of opioid misuse, indeed the negative affective state (i.e. anxiety and depression) could be key variables to promote opioid misuse especially in female pain patients.

Rats modeling internalizing vs. externalizing temperament display different acquisition patterns of intravenous heroin self-administration as compared to cocaine

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In humans, only a minority of those exposed to addictive drugs transition to a substance use disorder (SUD). Multiple factors are thought to underlie this susceptibility including differences in temperament, with externalizing temperaments theorized to approach drugs of abuse as a result of sensation-seeking, while those with internalizing temperaments appear less likely to initially approach drugs of abuse for recreational reasons but will in response to triggers such as psychosocial stress. Our lab has used a selective breeding strategy based on exploratory locomotion in a novel environment to derive two lines of rats with distinct behavioral and neurobiological phenotypes, termed selectively-bred high-responders (bHRs) and low-responders (bLRs). We have previously shown that temperamental differences between these rats reliably predicts their propensity to seek and take psychostimulants, where sensation-seeking bHRs show a higher basal propensity to take stimulants; while internalizing bLRs appear less susceptible at baseline but will seek and take stimulants in response to psychosocial stress. However, line-specific differences in propensity to acquire opioid self-administration has never been systematically explored in this model. Here, we used a free access self-administration paradigm of diacetylmorphine (heroin) or cocaine hydrochloride to determine whether these individual differences result in differential use patterns. For both drugs, we observed the expected phenotype differences, with bHRs seeking and taking more drug than bLRs. However, the differences were less pronounced for opioids than stimulants and this was particularly pronounced in early acquisition, where bLRs take ~14% as much cocaine as bHRs, while they take ~41% as much heroin on Day 1. This discrepancy in early acquisition suggests increased preference for opioids in bLRs relative to stimulants. Interestingly, there exists a drug x sex x phenotype interaction where a line-specific (bHRs) effect of sex appears in heroin taking behavior (~1.3x greater drug taking in females), but not for cocaine in either line. These findings contribute to our understanding of the impact of individual differences as well as a potential interaction between temperament, sex, and drug choice as antecedents of addiction development.

Prenatal Opioid Exposure and Adverse Respiratory Outcomes

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The opioid epidemic has adversely affected neonates and children; but the scope and mechanisms of these affects are not well understood. Failure to understand the impact of prenatal opioid exposure on long term adverse health outcomes could lead to gaps in care for this population. The purpose of this study is to investigate the association of prenatal opioid exposure with the development of adverse respiratory health outcomes, both acute and chronic. We propose that prenatal exposure leads to increased odds of development of childhood asthma, respiratory distress, apnea, and tachypnea. This is a retrospective cohort study using the PearlDiver Mariner database and a simple match design. Records from preterm and full-term infants exposed to opioids in utero are analyzed separately for the development of adverse health outcomes. Our preliminary results indicate that opioid exposure in utero increases the incidence of preterm birth. In full-term infant births, opioid exposure leads to increased odds for developing respiratory distress (OR = 8.26), asthma (OR = 1.38), tachypnea (OR = 12.07) and apnea (OR = 1.63). Lastly, diagnoses for adverse respiratory outcomes were earlier in full-term opioid exposed infants than in their unexposed controls. The significance of this study is that it will encourage closer monitoring of full-term infants who are exposed to opioids in utero for specific respiratory outcomes and provide conceptual grounding for future mechanistic studies.

Comprehensive transcriptomic and anatomical mapping of mu opioid receptor-expressing neurons across mouse neocortex

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Although clinically used mu opioid receptor (MOR) agonists provide potent pain relief, their utility is hindered by adverse effects such as potential transition to addiction and respiratory depression. Resolving the molecular identity of MOR-expressing neurons could pave the way for the development of more precise pain medications that dissociate opioid analgesia and adverse effects. To elucidate the mechanism of action of opioid analgesics, we combine single cell RNA-sequencing (scRNA-seq), intersectional mouse genetic tools, neural circuit tracing, slice electrophysiology, and *in vivo* calcium imaging in behaving mice. Here, we focused on elucidating MOR distribution and function throughout neural circuits of the cerebral cortex, including cortical regions in which the distinct sensory and affective dimensions of pain are represented. First, we established the expression pattern of the gene encoding MOR, *Oprm1,* using scRNA-seq across cortical cell types and identified marker genes for the different populations of *Oprm1*+ cortical neurons. We found that *Oprm1* is highly expressed in excitatory layer 6b neurons and inhibitory *Sst*+ interneurons throughout cortical regions, with important distinctions between cortical areas. Specifically, the primary somatosensory cortex, as well other sensory cortices and primary motor cortex, contains a higher number of excitatory pyramidal L5 neurons, including *Lamp5*+ *Oprm1*+ cells and *Pvalb*+ *Oprm1*+ cells. Next, we leveraged these marker genes to develop intersectional mouse genetic tools that provide genetic access to these populations of *Oprm1*+ cortical neurons to investigate their distribution, connectivity, dynamics, and function. These experiments reveal molecularly defined *Oprm1*-expressing neuron types that demonstrate cortical region- and layer-specific distribution and connectivity patterns, and a striking divergence in the organization of MOR-mediated neuromodulation between anterior cingulate and primary somatosensory cortices. By focusing our studies on MOR-expressing cell types concordant with those detected in humans, these investigations elucidate the circuit and cell-type bases of opioid actions and the contribution of each of these cell types to opioid-induced behaviors.

Spiro[isoquinoline-4,4'-piperidin]-3-one: A Promising Scaffold for Design of Partial Opioid Agonists

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We previously reported **AT-121**, a small-molecule NOP-MOP bifunctional partial agonist of the spiroisoquinolinone chemical class, as a non-addicting analgesic devoid of opioid liabilities. The aim of the current study was to develop a broad-spectrum analgesic with NOP partial agonism comparable to **AT-121** but with higher the functional efficacy at the MOP compared to that of **AT-121**. Based on our previous SAR studies, we hypothesized that introduction of aromatic or alicyclic ring substitutions on the piperidine nitrogen or the lactam nitrogen of **AT-121** can make pi-pi stacking and/or hydrophobic interactions with key amino acid residues in the binding pockets of the NOP and MOP receptors, maintaining binding affinity at both the NOP and MOP receptors and possibly increase functional efficacy at the MOP receptor. Our SAR study indicated that replacing the 4-isopropyl cyclohexyl substitution at the piperidine nitrogen of **AT-121** with aromatic or alicyclic rings maintained binding affinity (<100 nM) but led to loss of or reduction in functional efficacy at both the receptors, except for compound **AT-661**. **AT-661** with 3,3-diphenylpropyl group instead of 4-isopropyl cyclohexyl, as in **AT-121,** at piperidine nitrogen is a potent and selective mu-opioid receptor partial agonist (EC_{50}= 46.5 ± 15.94 nM and Emax = 47.5 ± 2.6%). On the other hand, replacing the sulfamide group of **AT-121** with aryl amide groups mostly led to lower binding affinity at both the receptors than **AT-121** and loss of or reduction in functional efficacy at both the receptors. However, compound **AT-684**, which has a furan amide group instead of the sulfamide group, is a potent partial agonist at the NOP receptor (EC_{50}= 42.1 ± 4.78 nM and Emax = 24.7 ± 2.1%). Conclusion and Significance: (1) the spiroisoquinolinone scaffold is very useful core to develop partial agonists at both the NOP and MOP receptors, (2) **AT-661**, a potent MOP partial agonist, can be a potential analgesic with lower side-effects than MOP full agonists, and (3) **AT-684**, a potent NOP partial agonist, as a promising lead compound for development of analgesics for chronic pain.

Novel Fentanyl Analogs to Replace Naloxone as Reversal Agents for Fentanyl Overdose

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According to the CDC, more than 200 people are killed every day by overdoses involving synthetic opioids like fentanyl. Naloxone, the current standard of care for opioid overdose reversal, often falls short when treating overdoses involving these more potent drugs. It has been reported that multiple doses of naloxone are required to reverse overdoses with drugs like fentanyl. This is likely due to the high lipophilicity, rapid brain penetration and duration of action of fentanyl. There is clearly a need for new, more effective rescue therapies. To be effective this new therapy must be: high affinity at the mu opioid receptor (MOR) (<10 nM), a neutral antagonist, have rapid brain penetration (cLogP between 2.0 and 5.0) and a duration of action greater than naloxone. We therefore decided to peruse analogs of fentanyl. We hoped that this approach would identify a compound with nanomolar affinity at MOR and pharmacokinetic properties that mimicked fentanyl. We evaluated a series of 11 fentanyl analogs at MOR for affinity and agonist activity. The physiochemical properties of these analogs were assessed to estimate brain penetration and water solubility. Of this new catalog of compounds, we identified DRC 1 as the most promising in vitro candidate. DRC 1 binds to MOR with an affinity of 4nM and does not display agonist activity. The clogP falls within our range at 3.8 and is very similar to that of fentanyl (3.6). Based on these results we plan to move forward with the development of DRC 1 as a novel rescue compound for overdoses involving fentanyl. Supported by NIDA: R03 DA 048129, R21 DA051723, UG3 DA056884.

Mu-Opioid Receptor Neurons in the Ventral Tegmental Area Mediate Low Sociability

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Protracted opioid abstinence is characterized by negative affective behaviors such as heightened anxiety, irritability, dysphoria, and anhedonia that pose a significant risk factor for relapse. While the ventral tegmental area (VTA) and its mu-opioid receptors (MORs) are critical for opioid action, their specific contribution in mediating protracted withdrawal-induced negative affect is not fully understood. VTA MOR neurons show increased activation during morphine dependence and we hypothesized that they mediate the expression of negative affect during prolonged abstinence. Mice underwent a morphine drinking paradigm with escalating concentrations (0.3 mg/ml, 0.5 mg/ml, and then 0.75 mg/ml) or untreated water in their home cages for 13-days. Morphine-treated mice exhibit increased somatic withdrawal signs during naloxone-induced precipitated withdrawal relative to the water-drinking controls. Neuronal activity as measured by FOS was significantly increased in MOR neurons across the anterior, medial, and posterior VTA in morphine-dependent mice. We evaluated negative affect behaviors during 4-weeks of protracted withdrawal and observed increased anxiety-related, despair-like, and social deficits in morphine-treated mice. An acute morphine challenge (20 mg/kg, s.c.) in mice undergoing prolonged withdrawal induced blunted FOS expression in the anterior cingulate cortex (ACC) compared to water drinking mice. Finally, to determine if MORs in the VTA mediate protracted withdrawal behaviors, we re-expressed MORs in neurons in the VTA in MOR knockout mice prior to the morphine exposure and protracted withdrawal paradigm. MOR KO mice with VTA MOR re-expression exhibit reduced sociability during protracted withdrawal while other withdrawal behaviors remain unaltered, demonstrating the role of this cell population in mediating protracted withdrawal-induced social deficits. The re-introduction of VTA MORs also mediate increased FOS in the ACC following the morphine challenge, suggesting that VTA MOR neurons modulate ACC-specific responses to acute morphine. These findings identify VTA MOR neurons as a critical modulator of low sociability during prolonged abstinence and highlight their potential as a target to alleviate negative affective behaviors associated with withdrawal.

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Mu-opioid receptor expression and function in dopaminergic neurons of the ventral tegmental area

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The midbrain ventral tegmental area (VTA) is implicated in the modulation of opioid-related reward and addictive behaviors. Current neural circuit models suggest that opioids bind to mu-opioid receptors (MORs) on VTA GABAergic neurons and indirectly facilitate activation of VTA dopaminergic neurons via disinhibition. However, previous studies suggested that MOR agonists may also have direct effects on VTA dopaminergic neurons. Whether MOR expression in VTA is restricted to GABAergic neurons and whether opioids modulate reward exclusively through disinhibition mechanisms remains unclear. Here, we combined single-cell RNA-sequencing (scRNA-seq), fluorescence *in situ* hybridization (FISH), intersectional mouse genetic and viral strategies, slice electrophysiology, and behavior assays to clarify the mechanisms of action of opioids in VTA. Using scRNA-seq and FISH, we detected *Oprm1* RNA, which encodes MOR, in a subpopulation of dopaminergic (~5% of *Slc6a3*+ neurons) VTA neurons. To verify MOR expression and function in this subpopulation of dopaminergic neurons using slice electrophysiology, we injected an adeno-associated virus (AAV) encoding oScarlet in a Cre- and Flp-dependent manner in the VTA of *Oprm1*$^{eGFP\text{-}Cre}$::*Slc6a3*^{Flp} mice and tested the effect of the MOR agonist DAMGO on oScarlet+ neurons. We found that 7 out of 9 oScarlet+ neurons exhibited outward currents upon application of DAMGO, confirming MOR expression in this subpopulation of VTA dopaminergic neurons. To investigate the function of MOR in VTA dopaminergic neurons, we injected an AAV encoding the Gi-DREADD hM4Di in a Cre- and Flp-dependent manner in the VTA of *Oprm1*$^{eGFP\text{-}Cre}$::*Slc6a3*^{Flp} mice and mimicked MOR function in these neurons via the administration of the hM4Di agonist clozapine-N-oxide (CNO). In the open field test, CNO significantly increased locomotor activity and entries into the central zone. CNO had no effect in the elevated plus maze, suggesting that the increased activity in the open field was not attributable to a reduction in anxiety-related behavior. Additionally, CNO didn't alter pain-related behavior. Collectively, these results comprehensively establish the cell-type-specific expression MOR in VTA and unexpectedly suggest that opioid Gi/o signaling in a subpopulation of dopaminergic neurons may increase locomotor activity, potentially revealing a novel mechanism by which opioids modulate motivation and providing novel insights into opioids addictive properties.

Opioids and strategically substituted agmatine (SSA) reduce post-incisional pain in mice

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Introduction: Post-surgical pain is often managed with opioids. Significant effort has been made to replace post-surgical opioids with non-opioid analgesic alternatives due to concerns of addiction and opioid-induced hyperalgesia. The endogenous dual NMDA receptor antagonist/nitric oxide synthase inhibitor, agmatine, reduces opioid self-administration, analgesic tolerance and chronic pain. The present study assessed the effectiveness of morphine, an agmatine analog (SSA3), and their combination in a model of a post-surgical pain. To assess hypersensitivity arising from surgical incision, we used a non-reflexive assay that tracks the non-evoked animal-determined usage of their affected as well as control hindpaws. We also applied tactile stimulation (von Frey monofilaments) to measure hindpaw reflexive responses.
Methods: Post-operative hypersensitivity: An incision was made through the skin of the hindpaw of rodents. The muscle was retracted, incised, and the skin sutured. Non-reflexive monitoring: Mice were placed in a BlackBox One monitoring apparatus and their activity recorded for 5 minutes. The videos were analyzed to determine the use of both the incised and control hindpaws. von Frey tactile stimulation thresholding: A von Frey probe was applied to the incised and control hindpaws of mice prior to and after surgery to measure responsiveness to tactile stimulation.
Results: The propensity of the incised and control hindpaws to contact the glass floor of the Blackbox apparatus were assessed in the non-reflexive behavioral tracking assay. We observed significantly decreased usage of the incised hindpaw up to 8 days following surgery. Systemic delivery of SSA3 resulted in the incised hindpaws voluntarily increasing tactile contact with the glass floor surface. Morphine dose-dependently alleviated incision-induced hypersensitivity, which was absent, as expected, in mu opioid receptor knockout mice. SSA3 and morphine also demonstrated dose-dependent reversal of tactile hypersensitivity as measured by the reflexive von Frey assay.

Conclusion: These experiments demonstrate that the SSA3 non-opioid compound reverses post-surgical pain in mice with comparable efficacy to morphine.

COI

Conflict of Interest Statement: The University of Minnesota has filed a patent application on with US and European patent offices for SSA composition and use. Funding: This work was supported by NIH DA035931, DoD W81XWH-15-1-0494 and W81XWH- 19-1-0673.

Structural Relationships Between Orthosteric Antagonists and Positive Allosteric Modulators of the Mu-Opioid Receptor

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Opioid drugs are powerful analgesics, yet their usage remains limited due to on-target side effects such as abuse liability, constipation, and in the event of overdose, fatal respiratory depression. Thus, there is an unmet need for the development of safer analgesics for the treatment of pain. Positive allosteric modulators (PAMs) function by interacting with opioid receptors at binding sites distinct from the orthosteric site to increase agonist affinity, potency, and/or efficacy. PAMs for the mu-opioid receptor (MOR) have shown the ability to enhance analgesia without a corresponding increase in adverse effects, making them potential leads for opioid sparing. One PAM compound, BMS-986187, has been shown to enhance the potency and efficacy of orthosteric MOR agonists *in vitro*. Additionally, using a high sensitivity assay for MOR, BMS-986187 produces an agonist response on its own, indicating it is an allosteric agonist or allo-agonist. Our goal is to better understand the mechanism of allo-agonism exhibited by BMS-986187. In this work we sought to establish whether orthosteric MOR antagonists affect this response. Using the Promega cAMP Glo Kit, we assessed the ability of BMS-986187 to decrease forskolin-induced cAMP production in the presence of various classes of MOR antagonists. We find the traditional morphinan antagonists, naloxone and naltrexone, fully suppress the allo-agonist effect of BMS-986187. In contrast, neither the peptidic MOR antagonists CTAP and CTOP, nor the fentanyl-derived antagonist meta-methyl furanylfentanyl, had any effect of the allo-agonism of BMS-986187. Our results support a role for the orthosteric pocket of MOR in the allo-agonist activity of BMS-986187 and suggest that the way structurally distinct antagonists bind to the orthosteric pocket differentially controls this allo-agonism.
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Agmatine-based analog inhibits morphine tolerance and reduces tactile hyperalgesia through antagonism of NR2B-containing spinal NMDArs

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Previous research has shown that agmatine, a decarboxylated form of L-arginine, reduces both opioid analgesic tolerance and tactile hyperalgesia evoked by nerve injury, largely mediated by antagonism of NMDA receptors containing NR2B subunits located in the spinal cord dorsal horn. We developed a series of strategically substituted analogs of agmatine (SSA) with improved biopharmaceutical features that reduce tactile hyperalgesia in pre-clinical models of chronic pain and prevent the development of morphine analgesic tolerance. However, the molecular mechanisms of these analogs have yet to be studied. We therefore characterized the efficacy
and subunit selectivity of our lead analog at the NMDA receptor in the dorsal horn of mouse spinal cord slices. Methods: We evaluated SSA antagonism of spinal NMDArs as measured by a decrease in the excitatory postsynaptic current (EPSC) amplitude and duration. Male and female *$Na_V1.8$-ChR2*-expressing mice were used for this experiment to selectively activate $Na_V1.8$-expressing nociceptive afferents. Anesthetized mice were sacrificed and 400 µm transverse spinal cord slices taken from the lumbar spinal cord. Neurons were voltage-clamped at +40 mV to relieve Mg^{++} blockade and 470nm blue light pulses (10 ms, one per 30 s) were shone through a 60x objective onto the root entry zone and the resulting NMDAr-mediated EPSCs were recorded. Once a baseline responsiveness was established, the tissue was incubated for 3 minutes at a time with increasing concentrations of SSA. Pharmacological blockers NBQX, picrotoxin, and strychnine were present in the control and drug solutions to isolate NMDAr-mediated EPSCs. Results: SSA effectively reduced the amplitude of blue light-evoked NMDA EPSCs in a concentration-dependent manner (EC_{50} 3 mM), consistent with that seen with agmatine (Waataja et al., J Neurophysiol., 2019). SSA also concentration-dependently reduced EPSC duration (EC_{50} 10 mM) as indicated by the decay constant Tau. This reduction in EPSC duration is consistent with NR2B over NR2A subunit selectivity. Conclusion: The results of this study support the hypothesis that SSA shares a mechanism of action with agmatine. The improved biopharmaceutical features of SSA together with this subunit selectivity suggest that SSA may be a therapeutically useful opioid-sparing agent and non-opioid analgesic.

COI

The University of Minnesota has filed a patent application with US and European patent officies for SSA composition and use.

Transcranial magnetic stimulation of motor cortex produces analgesia via opioidergic descending pain control circuits

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The heavy burden of chronic pain and the opioid epidemic have prompted an urgent search for alternative methods of analgesia. One promising alternative is transcranial magnetic stimulation (TMS) of the motor cortex. While studies have shown that motor cortical TMS can reduce chronic pain in human subjects and decrease nocifensive behaviors in rodents, the underlying antinociceptive mechanisms remain elusive, preventing improvement of treatment efficacy and duration. Here, we dissected the circuit mechanism underpinning TMS-induced antinociception in a mouse model of trigeminal neuropathic pain. We first developed a mouse-scaled transcranial magnetic stimulation (miniTMS) device that we used to focally stimulate motor cortex in mice with a chronic constriction injury (CCI) of the infraorbital branch of the trigeminal nerve. We quantified mouse behavioral responses to innocuous and noxious mechanical stimuli before and after TMS. Finally, we used Neuropixels high-density electrophysiological recordings to identify neural responses to TMS protocols (*e.g.,* intermittent theta burst). Motor cortical miniTMS induced a dose-dependent decrease in reflexive and affective-motivational pain behaviors in CCI mice. Focusing first on the stimulation site, we found that layer 5 pyramidal neurons are both activated by TMS and required for TMS-induced antinociception. We traced the outputs of motor cortical neurons activated during TMS and determined they directly project to the rostral ventromedial medulla (RVM), a key node in the descending pain control pathway. We evaluated the possibility that RVM endogenous opioid signaling plays a role in TMS analgesia by injecting naloxone or opiorphin (an opioid receptor antagonist and a dual enkephalinase inhibitor, respectively). RVM intracranial naloxone injections were sufficient to prevent TMS analgesia, while opiorphin enhanced both the antinociceptive effect and duration. Finally, we recorded neural responses to TMS patterns and determined that cortical neurons are generally driven by TMS while RVM neurons are attuned to specific patterns.Together, these data elucidate the mechanism of TMS-induced analgesia by revealing that motor cortical TMS utilizes endogenous opioid signaling in medullary descending pain control pathways to produce antinociception. This knowledge paves the way to improving existing and designing novel TMS protocols.

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Serotonin neuron influence on pontine breathing circuitry impaired by opioids

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Opioid induced respiratory depression (OIRD) is due to mu opioid receptor (MOR) activation and can cause fatality in overdose. Stimulation of serotonergic raphe neurons and 5HT2A/C receptors in the brainstem have been shown to stimulate breathing in preclinical studies. Therefore, the serotonergic system may serve as a viable MOR-independent target for reversing OIRD. Despite this, the actions of opioids in raphe neurons and their inputs to OIRD-implicated lateral parabrachial neurons are poorly understood. Herein, we hypothesized that medullary serotonin neurons innervate lateral parabrachial neurons, and enhance respiratory activity, even in the presence of fentanyl. To test this hypothesis, we used mice expressing the light sensitive ion channel, channelrohdopsin-2 (ChR2), in serotonin neurons. We employed an *in situ* arterially perfused working heart-brainstem preparation to record respiratory motor output during serotonin neuron activation and fentanyl application. Optical simulation of caudal medullary raphe neurons enhanced respiratory output, which was attenuated, but not blocked by fentanyl. To characterize serotonin inputs to lateral parabrachial neurons, we used acute brain slice electrophysiology with brain slices containing the lateral parabrachial taken from mice selectively expressing ChR2 in serotonin neurons. We made whole-cell voltage-clamp recordings from lateral parabrachial neurons, and delivered paired light pulses (10 ms, 50 ms interval) to stimulate serotonin terminals. We observed optically evoked slow outward currents, that were blocked by the 5HT1A receptor antagonist WAY-100635. Surprisingly, we also observed fast inhibitory post-synaptic currents (IPSCs) that were blocked by the GABA-A receptor antagonist gabazine and persisted in the presence of the AMPA-type glutamate receptor antagonist DNQX. The opioid agonist Met-enkephalin attenuated IPSC amplitude, and in some cells, evoked a slow outward current. These data indicate that serotonin neurons may modulate breathing through complex inhibitory actions via GABA and serotonin release in lateral parabrachial neurons, some of which also express MORs. Opioid-mediated synaptic regulation of medullary raphe neurons along the rostral-caudal axis will be investigated to characterize the opioid sensitivity of this ponto-medullary circuit. Supported by NIDA R01DA047978.

An Opioid Positive Allosteric Modulator Improves Methadone-Mediated Anti-Allodynia in a Rat Model of Neuropathic Pain

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Neuropathic pain imposes a significant burden on patients, yet there are few pharmacotherapies availability for treatment. Opioids, while greatly effective for acute pain, show limited effect in neuropathic pain and are associated with a greater risk of side-effects in chronic use. Development of adjuvants to enhance analgesia while limiting side-effects could provide a critical treatment strategy for chronic pain patients. Positive allosteric modulators (PAMs) interact with agonist-occupied receptors to increase agonist affinity and/or signaling. One PAM of the μ-opioid receptor, BMS-986122, has been shown to enhance opioid analgesia *in vivo* without increasing negative effects. However, behavioral studies to date have focused on acute antinociception. Effects in a chronic pain model, such as neuropathic pain, remain unknown. Our objective was to assess the enhancement of opioid anti-allodynia by BMS-986122 in rats that have undergone spared nerve injury (SNI) to support the application of this drug class in chronic pain.

Spared nerve injury was performed in male and female Sprague-Dawley rats, and the pain state was allowed to develop for 2 weeks. All rats developed a robust and non-resolving tactile hypersensitivity. Animals then received either (R)-methadone (0.1-1 mg/kg, s.c.), BMS-986122 (10 mg/kg, s.c.), or both. Tactile thresholds were measured using von Frey filaments for 2 hours post drug-delivery. At doses of (R)-methadone where no reversal from the injury baseline was seen (0.1-0.32 mg/kg, s.c.), co-administration of BMS-986122 provided an anti-allodynic effect dependent on the dose of (R)-methadone. At doses where (R)-methadone alone elicited a full reversal of the hypersensitivity (1 mg/kg, s.c.), co-administration of BMS-986122 did not further alter tactile responses. Overall, these data suggest that opioid PAMs could serve as an opioid-sparing adjuvant for therapy in neuropathic pain patients and supports further investigation into novel modulator structures for preclinical development. Funded by R37 DA33397 (J.R. Traynor), T32 TR004764 (B.M. Clements), and DOD CDMRP W81XWH-21-1-0771 (S.W.P. Kemp).

Assessment of precipitated withdrawal and conditioned place aversion for fentanyl and xylazine in Swiss Webster mice

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The α2-adrenoreceptor agonist xylazine is becoming an increasingly common adulterant in illicit opioid supplies throughout the United States. Despite its prevalence, xylazine's effect on opioid withdrawal, and withdrawal from xylazine itself, remain largely uncharacterized. This gap in knowledge makes it difficult to treat opioid overdoses and manage withdrawal symptoms in the presence of xylazine. Here, we aimed to investigate symptoms of xylazine withdrawal and xylazine's impact on fentanyl withdrawal in Swiss Webster mice. Mice were treated with xylazine and/or fentanyl and the corresponding antagonists, idazoxan and naloxone, respectively, to induce precipitated withdrawal. Xylazine may act as a kappa-opioid receptor (KOR) agonist, so we investigated the possible kappa-opioid receptor mediated withdrawal symptoms of xylazine by pre-treatment with norbinaltorphimine (nor-BNI), a KOR antagonist. The mice were observed and scored by the presence of symptoms within the observation period. Xylazine and fentanyl withdrawal alone were shown to have different symptoms. The most prominent xylazine withdrawal symptom was scratching, whereas fentanyl withdrawal symptoms included grimacing and jumping. In fentanyl withdrawal with simultaneous xylazine treatment, there was a significant reduction in many of the classic fentanyl withdrawal symptoms including wet dog shakes ($p<0.01$) and grimacing ($p<0.01$). No jumping was observed in fentanyl withdrawal with xylazine coadministration ($p<0.0001$). Nor-BNI pretreated xylazine withdrawal mice exhibited less scratching behavior than xylazine withdrawal mice ($p<0.05$). Fentanyl precipitated withdrawal by naloxone produced significant conditioned place aversion (CPA) when compared to xylazine precipitated withdrawal by idazoxan ($F(3,28) = 11.57$, $p<0.0001$) which under the current assay conditions did not produce CPA. Taken together, these results suggest that xylazine alters fentanyl observable withdrawal symptoms, while possessing a unique set of withdrawal symptoms itself that are partially related to potential activity at KOR. This work is valuable to the understanding of xylazine's impact in the illicit drug supply and can be used to guide further research into treatment methods for the simultaneous use of fentanyl and xylazine.
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Development of Wireless Equipment for Autonomous Rodent Infusion Tasks

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Despite major advances in both the preclinical and clinical addiction fields, the number of US citizens afflicted by substance use disorders (SUDs) and the lethal overdose outcomes has continuously increased over the past two decades. To tackle this alarming health issue, intravenous self-administration procedures has been used the gold standard for translational rodent SUD models. However, despite being one of the most reliable procedures, with clear face validity, this procedure is still limited by its tethered nature, constraining its use to restricted spaces in which rodents are exposed to unenriched environments with limited or no access food, water, or social interaction. Availability for volitional social interaction and non-social rewards such as wheel running, and operant-delivered palatable foods can decrease the consumption, the escalation, and the reinstatement in drug self-administration.

To tackle those limitations, we are developing a wireless-controlled wearable drug reservoir connected to intravenous indwelling catheter and use Bluetooth mesh technology to enable homecage-based intravenous self-administration procedures.

Using a combination of 3D printed encapsulation and commercially available reprogrammed micro-pumps together with pulse oximeter, we demonstrate that wireless infusion of fentanyl (50ug.kg-1) is sufficient to produce respiratory depression in rats. Using fiber photometry to provide evidence that fentanyl infusion (20ug.kg-1) through our wireless devices is sufficient to increase ventral tegmental area activity in freely moving rodents. Further, we demonstrate that our wearable devices do not impair horizontal locomotion and performances in operant self-administration in adult male and female rats. Lastly, those newly developed wearable devices will be adapted to 1) perform home cage drug self-administration and 2) be combined with other tethered approaches to investigate neuronal circuits and ensemble responsible for consumption, escalation, and reinstatement of drug use.

Overall, we envision that these new open-source approaches will broaden the translational value of pre-clinical SUD models by enabling new experimental designs to improve current strategies aiming at developing substance use disorders treatments.

Fentanyl-Xylazine Combination Decreases Respiration and Heart Rate More than Fentanyl Alone

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Fatal opioid overdoses in the U.S. have nearly tripled since 2015, surpassing 82,000 in 2023. Over 92% of these overdoses involve a synthetic opioid such as fentanyl. The danger of illicit fentanyl has recently been exacerbated by the replacement of less harmful adulterants (i.e., mannitol) with xylazine, an α2-adrenergic receptor agonist typically used as a veterinary anesthetic. In 2023, the White House declared fentanyl-xylazine combinations as an emerging threat to the United States, with over a 1,000% increase in xylazine-positive overdoses reported in some regions. Yet, 89% of illicit opioid users do not want xylazine adulteration in their drugs. Xylazine has been shown to potentiate the lethality of fentanyl in mice, so it may increase the risk of overdose in humans. However, whether this occurs through further suppression of respiratory function or through other α2 actions, such as bradycardia, is unknown. Herein, we evaluate fentanyl, xylazine, and their combination in whole-body plethysmography (to measure respiration) and pulse oximetry (to measure blood oxygen saturation and heart rate) in male and female CD-1 mice. We show that xylazine (32 mg/kg) decreases breath rate to 30% of baseline by increasing the expiration time; in contrast, fentanyl (3.2 mg/kg) primarily inhibits the process of inspiration. The combination of xylazine and fentanyl shows a larger effect on inspiration time and air flow than fentanyl alone. As expected, 3.2 mg/kg fentanyl decreased blood oxygen levels, whereas 32 mg/kg xylazine had no effect despite reducing breathing rate to a greater extent than fentanyl. Meanwhile, xylazine and fentanyl individually decreased heart rate by 50% and 20%, respectively. Together, these results provide a basis for further research to elucidate how xylazine exacerbates opioid overdose in humans. This work was supported by NIH grants UG3 DA056884 and R21 DA051723.

Cyclic Glycopeptide Endomorphin-1 Analogs Provide Highly Effective Antinociception Without Conditioned Place Preference in Mice.

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Mu opioid receptor (MOR) agonists are the most effective treatment for moderate to severe pain relief. However, their use is limited by serious side effects, particularly the potential for abuse. The opioid epidemic has grown steadily in the past 30 years, highlighting the need for new non-addictive pain medications. Cyclic peptides have been found to be more stable *in vivo* than linear peptides. In previous studies, we showed that a cyclized analog of the natural opioid agonist endomorphin-1 provides pain relief similar to morphine while reducing or eliminating several side effects, including abuse liability. Glycosylation, the addition of sugar molecules, can enhance penetration of cellular barriers including the blood-brain barrier. Combining cyclization with glycosylation can enhance the pharmacological properties of these peptides. Here we applied this drug design approach to develop cyclic glycopeptide analogs of endomorphin as drug candidates for potent and long-lasting analgesia. We have demonstrated their serum stability *in vivo* and their penetration of the blood brain barrier by microdialysis and mass spectrometry. Two of these analogs showed selectivity for MOR and highly effective antinociception. Antinociceptive effects were tested in the tail flick and hot plate tests in male and female mice. One of the analogs (A2) provided over 5h of pain relief at a dose 5-fold lower (20-fold on a molar basis) than the dose of morphine needed for comparable antinociception. A2 was more effective than morphine in both male and female mice. To assess the abuse liability of A2, we conducted conditioned place preference (CPP) tests in male mice. Three doses of morphine and A2, that provide equiantinociceptive effects in the tail-flick test, were compared. Morphine, but not A2, produced CPP as confirmed by a 2-way analysis showing significant drug ($p< 0.01$) and drug x dose ($p<0.05$) effects. All three morphine doses, but none of the analog doses, showed CPP scores significantly greater than zero ($p<0.5$), $n=12$. The results support further study of their clinical application as non-addictive pain medications. JZ, LS and RP have patents on the subject compounds. Supported by NIDA (1U18DA052539) and Dept. of Veterans Affairs Merit Review (1 I01 BX006167-01A1) to JEZ.

The non-opioid analgesic, Agmatine, induces a recruitment of peripheral immune cells to the dorsal horn in female mice only

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Agmatine, an endogenous NMDA receptor antagonist and nitric oxide synthase inhibitor, prevents opioid self-administration, opioid analgesic tolerance, and the development of chronic hypersensitivity in mice. Although agmatine demonstrates comparable anti-hyperalgesic and anti-addictive efficacy between males and females, we observed a significant elevation in spinal cord immune cell expression after intrathecal agmatine administration in female mice only, regardless of opioid exposure or hypersensitive state. Centrally resident microglia and their peripheral counters, infiltrating macrophages/monocytes, have been implicated in the initiation and resolution of pain, respectively, though the exact contributions from either cell type to these processes remains elusive. It is unclear whether the agmatine-induced increase in immunoreactivity is due to a proliferation of resident microglial cells in the dorsal horn or a recruitment of peripheral monocytes/macrophages to the central nervous system. The objective of the study is to characterize the origin of the elevation in immune cells observed in spinal cord after agmatine administration in female mice using the resident-specific microglial marker, TMEM119.

Female ICR CD-1 mice (21-30 g) were intrathecally administered (5 ml) with either 30 nmol agmatine or saline. Half of the mice from each group were administered complete Freund's adjuvant (CFA) into the left hind paw to induce an inflammatory state. Spinal cords were extracted and prepared for immunohistochemical analysis with TMEM119. TMEM119 immunoreactivity was quantified through % area of lumbar dorsal horn fluorescence intensity.

CFA-treated female mice treated with agmatine showed a significant decrease in TMEM119 immunoreactivity in the lumbar spinal cord dorsal horn as compared to CFA-treated female mice treated with saline ($p=0.0004$) and control mice treated with saline ($p=0.0074$). Interestingly, control female mice treated with agmatine showed a lesser, but significant, decrease in TMEM119 immunoreactivity when compared with CFA-treated female mice injected with saline ($p=0.036$). No significant differences were found between saline-treated control mice and CFA-treated mice treated with saline, although there was a trend towards increase with inflammation. These data further delineate sex-differences in neuroimmune interactions under conditions of inflammation by showing that agmatine mobilizes peripheral immune cells in female mice which may contribute to the effects of agmatine in the central nervous system.

Mimicking opioid analgesia in cortical pain circuits

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The anterior cingulate cortex plays a pivotal role in the cognitive and affective aspects of pain perception. Both endogenous and exogenous opioid signaling within the cingulate mitigate cortical nociception, reducing pain unpleasantness. However, the specific functional and molecular identities of cells mediating opioid analgesia in the cingulate remain elusive. Given the complexity of pain as a sensory and emotional experience, and the richness of ethological pain-related behaviors, we developed a standardized, deep-learning platform for deconstructing the behavior dynamics associated with the affective component of pain in mice—LUPE (Light aUtomated Pain Evaluator). LUPE removes human-bias in behavior quantification and accelerated analysis from weeks to hours, which we leveraged to discover that morphine altered attentional and motivational pain behaviors akin to affective analgesia in humans. Through activity-dependent genetics and single-nuclei RNA sequencing, we identified specific ensembles of nociceptive cingulate neuron-types expressing mu-opioid receptors. Tuning receptor expression in these cells bidirectionally modulated morphine analgesia. Moreover, we employed a synthetic opioid receptor promoter-driven approach for cell-type specific optical and chemical genetic viral therapies to mimic morphine's pain-relieving effects in the cingulate, without reinforcement. This approach offers a novel strategy for precision pain management targeting a key nociceptive cortical circuit with on-demand, non-addictive and effective analgesia.

COI

G.C, K.D., C.R. and G.J.S. are listed as inventors on a provisional patent application through the University of Pennsylvania and Stanford University regarding the custom sequences used to develop, and the applications of mMORp and hMORp constructs (patent application number: 63/383,462 462 ‘Human and Murine Oprm1 Promotes and Uses There-of’).

Investigating the role of ACC SST interneurons within the opioid system

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The anterior cingulate cortex (ACC) plays a role in pain processing. This pain processing is carried out by an interaction between excitatory principal neurons and inhibitory interneurons. Principal cells and inhibitory interneurons in the ACC receive afferent inputs from various cortical and thalamic regions. The endogenous opioid system can control this pain processing by activation of opioid receptors on both afferent inputs and inhibitory interneurons in ACC circuits. Activation of opioid receptors causes a decrease in neuronal firing and neurotransmitter release resulting in excitatory:inhibitory circuit changes. Moreover, activation of opioid receptors on inhibitory interneurons results in disinhibition of nearby pyramidal cells. In the ACC, there are several subtypes of inhibitory interneurons: including parvalbumin (PV) and somatostatin (SST) -expressing interneurons. We have previously found that PV interneurons are well characterized expressing the delta opioid receptor (DOR) but not the mu opioid receptor (MOR) or kappa opioid receptor (KOR). Furthermore, PV interneurons play a functional role in thalamo-cortical feed-foward inhibitory circuits. While much is known about the role ACC PV interneurons play in the endogenous opioid system, little is understood about SST neurons and their role in the ACC endogenous opioid system. The aim of this study is to understand the expression of opioid receptors on somatostatin neurons in ACC and how it regulates the physiology of local circuits. We used a combination of brain slice electrophysiology and pharmacology in mice to characterize the effects of opioid receptors on SST neurons. We found that Met-Enkephalin activated a GIRK conductance on SST neurons. Opioid receptor specific agonists activated layer specific GIRK conductances in SST neurons with most layer ⅔ neurons functionally expressing DOR while most layer ⅚ neurons functionally expressed both MOR and DOR. These data suggest that SST neurons in the ACC exhibit differential expression of opioid receptors in a layer specific manner proposing different circuit functions across layers.

A Structure-Activity Exploration of Positive Allosteric Modulators of the Delta-Opioid Receptor

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Chronic pain and depression comprise two of the most common medical ailments experienced worldwide and often present as co-morbid. While efficacious for moderate to severe pain, medications targeting the mu-opioid receptor (MOR) are associated with severe adverse effects - namely respiratory depression and abuse liability. In recent years, the delta-opioid receptor (DOR) has become a promising target for the treatment of chronic pain and depression due to its reduced side-effect profile relative to MOR. However, the development of DOR agonists has been limited due to their propensity to cause convulsions and the rapid development of tolerance. An emerging strategy for therapeutically targeting DOR is the development of positive allosteric modulators (PAMs) that bind to a topographically distinct location from the orthosteric binding site to increase the affinity and/or efficacy of orthosteric agonists. A series of xanthene-diones, exemplified by BMS-986187, act as DOR-PAMs. However, although BMS-986187 is DOR-preferring, it also acts as a PAM at MOR *in vitro* and *in vivo*, thus precluding its future development. Here, we report a structure-activity study of BMS-986187 at both DOR and MOR to probe the DOR allosteric pharmacophore, while simultaneously improving DOR selectivity and potency. Concentration-response curves of BMS-986187 analogs were obtained using a β-arrestin recruitment assay in the presence of an EC_{20} concentration of Met-enkephalin. By altering the substitution patterns of the tolyl moiety on BMS-986187, we identified a series of compounds that were up to 5-fold more potent (EC_{50}: 16nM-40nM) than BMS-986187 (EC_{50}: 121nM) at DOR, with an equivalent ability to increase the response to Met-enkephalin. We have further identified compounds that retained DOR activity with no activity at MOR. This study will facilitate the understanding of the allosteric pharmacophore of DOR-PAMs, while generating a framework for the rational design of potent and selective opioid receptor modulators. Funded by R37 DA039997.

Bifunctional partial opioid compound effective against MUD in rodents

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Purpose: Methamphetamine (METH) use disorder (MUD) is a significant public health problem with no approved pharmacotherapy. PPL-143 and PPL-138 are non-selective opioid receptor ligands originally designed as analgesics with reduced abuse liability and side-effects as compared to standard opioid analgesics. Both compounds are bifunctional partial agonist at both nociceptin opioid peptide (NOP) and mu opioid receptors, with a higher efficacy for the NOP receptor, and antagonists at kappa receptors. **Methods:** Here, we test the efficacy of both PPL-143 and PPL-138 in reducing METH self-administration using the translational drug-vs-food choice model of self-administration in male Sprague Dawley rats. PPL-138 was also evaluated for its ability to reduce reinstatement of METH-seeking behavior, a model for MUD relapse. The compounds were administered within the same dose range (0.0, 0.1, 0.3 and 1.0 mg/kg) through the subcutaneous route of administration, prior to self-administration of multiple METH doses (0.025, 0.05, 0.1 and 0.25 mg/kg/infusion), which were offered to the rats within the same session. **Results:** Both compounds effectively decreased METH self-administration in a similar fashion at dosages that did not alter responding for food or locomotor behavior **Conclusion:** These results support the translational relevance of the drug-vs-food choice procedure for preclinical evaluation of novel medications on operant drug self-administration paradigms and the utility of developing mixed opioid partial agonists for MUD pharmacotherapy.

The Therapeutic Potential of PPL-138 as an Agent for Managing Addiction and Chronic Pain

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The Nociceptin/orphaninFQ peptide (NOP) receptor has gained interest as a novel target for managing pain and addiction. The compound PPL-138 (BU10038), derived from naltrexone, functions as a dual partial agonist at NOP and Mu opioid receptors, displaying preferential binding to NOP receptors. This profile suggests a lowered abuse potential while retaining efficacy in pain relief. Here, we investigate the analgesic and addiction management properties of PPL-138 through various animal models.

PPL-138 has been shown to exert partial antinociceptiveeffects in an acute pain setting, with a dose-dependent response observed up to 10 mg/kg. In models of chronic neuropathic pain, specifically the spared nerve injury model, PPL-138 significantly mitigated mechanical allodynia, achieving full anti-allodynicefficacy at doses as low as 0.3 mg/kg. Moreover, PPL-138 did not sustain self-administration in animals, indicating a reduced propensity for addiction relative to fentanyl. Interestingly, animals displayed a preference for PPL-138 in a conditioned place preference setup, although this preference was reversible and diminished after five days. Additionally, PPL-138 exhibited promise as a treatment for cocaine use disorder by decreasing cocaine self-administration.

Findings underscore the therapeutic potential of PPL-138 for the dual purposes of addiction and chronic pain management. The specific aims of ongoing research include elucidating the analgesic pathways engaged by PPL-138 and understanding its influence on addiction-related neural circuits. The data presented here contribute to the positioning of PPL-138 as a therapeutic for pain and addiction, with a unique pharmacological profile that may offer advantages over existing treatments.

GluN2B as an effector of inhibitory and pro-nociceptive neuromodulators

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Introduction: Spinal GluN2B-containing N-methyl-D-aspartate receptors (NMDArs) contribute to the development of opioid tolerance. Spinal agmatine, an endogenous inhibitory neuromodulator, prevents the development of morphine tolerance. Similarly, we have observed spinal immunoneutralization of the pro-nociceptive peptide TLQP-62 inhibits morphine tolerance. This study aims to determine the spinal mechanisms of both agmatine and TLQP62 using calcium imaging. We hypothesized that agmatine attenuates spinal NMDA response specific to GluN2B subunits through nNOS inhibition, and the potentiation of spinal glutamate responses by TLQP-62 requires GluN2B-containing NMDArs but not AMPA receptors. **Methods**: Calcium imaging was conducted in *ex vivo* spinal slices in ICR, C57BL/6J, and GluN2Bfl/fl mice (male and female). GluN2B in GluN2B*fl/fl* mice was deleted by intraspinal AAV9 injection containing Cre or control ΔCre. Fluo-4 was utilized to visualize spinal response in C57BL/6J and ICR mice. Fluo-4 or GCaMP6s fluorescence intensity was captured by two-photon microscopy. Slices were exposed to TLQP-62, ifenprodil, and agmatine to measure the potentiation or inhibition of glutamate, NMDA, or AMPA response, which was analyzed by Student's t-test and ANOVA. **Results**: Agmatine concentration-dependently inhibits NMDA response in mice, suggesting that agmatine is an effective spinal inhibitor of NMDArs. GluN2B-knockdown was confirmed by loss of ifenprodil inhibition of NMDA response ($P<0.01$, Student's t-test). In the GluN2B-knockdown mice, inhibition of NMDA response by 100 μM ifenprodil ($P< 0.01$, Student's t-test) was significantly reversed, but not with 3.3 mM agmatine. When agmatine was co-applied with PSD95-nNOS inhibitor, IC87201, agmatine's attenuation of NMDA response was significantly resolved ($P<0.01$, Student's t-test), suggesting agmatine's inhibition on NMDA response is mediated by intact NMDArs-PSD95-nNOS associations. In TLQP-62 studies, some spinal profiles showed potentiated glutamate response following TLQP-62 incubation in C57BL/6J mice. TLQP-62 also potentiated NMDA response, while AMPA response was not potentiated. The fold change in glutamate response amplitudes was significantly decreased in TLQP-62-exposed cells from GluN2B-knockdown male mice compared to control male mice ($p < 0.05$, Student's t-test) but not in female mice.

Conclusions: These findings showed a more detailed mechanism of agmatine and TLQP-62's involvement with spinal GluN2B-NMDArs, which will help us further understand the relationship between spinal GluN2B-NMDArs and morphine tolerance.

Pain and Chronic Morphine Exposure alter inhibitory neurotransmission in the Anterior Cingulate Cortex through opposing Sex-specific and Kir-mediated Mechanisms

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The anterior cingulate cortex (ACC) is a region involved in the processing of pain, and its output influences downstream neural circuits. ACC hyperexcitability is shown in chronic pain conditions, and direct inhibition of ACC activity via opioids provides relief of pain. However, the sites and mechanisms of opioid action in the ACC are understudied. ACC activity is regulated by endogenous opioids like enkephalins, which can act at both mu and delta opioid receptors to alter cortical function. The delta opioid receptor (DOR) is expressed in a majority of parvalbumin (PV) interneurons within the ACC. DOR activation on PVs inhibits GABA release, disinhibiting nearby pyramidal cells. While PV cells regulate a majority of inhibitory signaling in the ACC, it is unknown how PV neurotransmission adapts following opioid use or pain. To address these unknowns, we used patch clamp electrophysiology, optogenetics and pharmacology in brain slices to measure activity of Layer V PV cells. Animals were treated with morphine via an osmotic minipump, inflammatory pain via a hindpaw injection of Complete Freund's Adjuvant (CFA), or Nitroglycerin (NTG) which invokes migraine-like pain. CFA or chronic morphine exposure does not appear to change DOR action on PV interneurons, but there are changes in PV cell intrinsic properties in a treatment-dependent and sex-specific manner. PV cells from CFA-treated male animals are depolarized, have a lower rheobase and higher input resistances as compared to cells from CFA-treated females and naïve groups. These changes in PV cell function may induce disinhibition of local circuits and alter ACC output in males. Alterations in PV cell function in CFA-treated males are Kir-mediated as they are reversed with Barium Chloride, a blocker of inward-rectifying potassium channels. Additionally, these alterations are reversed when animals are treated with morphine prior to CFA. In contrast, PV cells from females are hyperpolarized following this dual treatment. These data suggests that CFA and morphine disrupt endogenous opioid signaling in the ACC in an opposing manner. Overall, these data show that PV cell function is altered in pain and morphine exposure in a treatment-dependent, sex- and synapse-specific manner which may contribute to divergent ACC output.

Profiling cFos Activation in the Parabrachial Nucleus after Opioid Withdrawal in Mice

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The need for effective treatment of opioid substance disorder has never been greater. Targeting highly aversive withdrawal symptoms offers a promising avenue to increase the chances that individuals may successfully enter treatment and abstain from opioid use. Therefore, it is essential to have a better understanding of the mechanisms and circuitry behind opioid withdrawal symptoms. A cell type and brain region implicated in aversive sensory processing is calcitonin gene-related peptide (CGRP), expressing neurons within the external lateral portion of the parabrachial nucleus (PBle). Because $PBle^{CGRP}$ neurons serve as a relay station between the sensory processing and its downstream regions, it is hypothesized that these neurons are recruited during opioid withdrawal and could modulate downstream regions. Our preliminary data has suggested that activity in $PBle^{CGRP}$ neurons is opioid withdrawal. To elucidate the role of these $PBle^{CGRP}$ neurons and other cells in the PBle during opioid withdrawal, we examined cFos expression in PBN neurons in male and female mice. $Calca^{cre}$ mice underwent stereotaxic surgery, where the PBle was injected with an AAV-DIO-eYFP to allow for the expression of eYFP in CGRP neurons. Two weeks post stereotaxic surgery, mice were then randomly sorted into non-dependent and opioid-dependent groups. We induced opioid dependence using twice daily escalating doses of morphine. Non-dependent mice received twice daily injections of saline. Precipitated withdrawal was then induced with naloxone. Mice were perfused 90 minutes later to capture cFos induction, a marker of neural activation. The presence of cFos in PBN neurons and PBN^{CGRP} neurons was analyzed. Our preliminary results show that withdrawal increased cFos activation in PBN cells (one-way ANOVA, $p < .05$). Analysis between sexes is ongoing. This additional information on the mechanism and neural circuitry involved in opioid withdrawal can be used for developing new treatments for opioid substance use and withdrawal symptoms.

Perinatal opioid exposure alters microglia in mice during exposure and withdrawal

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Populations affected by the opioid epidemic include pregnant women and their offspring. Infants exposed to opioids *in utero* are at risk of developing Neonatal Opioid Withdrawal Syndrome (NOWS), a combination of acute somatic withdrawal symptoms. In adult rodent models, there is evidence that morphine induces a pro-inflammatory response within the central nervous system, primarily through activation of microglia. Pharmacological suppression of microglia has also been shown to ameliorate aspects of opioid withdrawal. It is unknown how *in utero* morphine exposure impacts developing microglia, and whether microglia play a role in producing withdrawal symptoms following perinatal exposure.

We developed a mouse model of prenatal opioid exposure that encompasses the developmental equivalent of all three trimesters of human pregnancy (“three-trimester mice”) in which mice receive morphine throughout gestation and the first two post-natal weeks— a period equivalent to the third trimester of human pregnancy and includes major neurodevelopmental processes in rodents, including synaptogenesis and microglial maturation. Our model produces significant developmental delays, failure to thrive, and robust withdrawal signs. Microglia were isolated from mice at postnatal day (PND)14 and PND15, timepoints representing chronic exposure and spontaneous withdrawal, respectively. Microglial RNA sequencing revealed a large number of differentially expressed genes (DEGs) in morphine exposed mice at PND14, which largely, but not entirely, normalized by PND15. Many microglial gene expression levels were altered, and gene ontology analysis revealed enrichment of cell cycle processes. DEGs between morphine and saline were much fewer in number at PND15, but included microglial genes which regulate phenotype and response to injury, e.g. *Spp1* and *Gpnmb*. There are notable sex differences. Upstream regulators include LPS, known to act via TLR4, consistent with the known effect of opioids on microglia. Together, these data indicate that perinatal morphine exposure differentially alters the microglia transcriptome during exposure and withdrawal, suggesting the involvement of microglia in both of these processes.

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A Novel Selective G-protein Biased DOR Agonist: Analgesic Tolerance, Abuse Liability, and DOR Internalization

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Headaches are a global health concern with limited therapeutic options. The delta-opioid receptor (DOR) is a target for the treatment of pain, and has recently emerged as a therapeutic target for headache disorders. PN6047 is a novel selective G-protein biased DOR agonist that has successfully completed phase I clinical trials. The aim of this study was to determine tolerance and abuse liability associated with PN6047. In addition, we also characterized the internalization properties of this ligand. To test for tolerance, we tested repeated administration of PN6047 in the Complete Freund's Adjuvant (CFA) model of inflammatory pain. Preliminary tests in abuse liability were conducted using the conditioned placed preference (CPP) assay. The ability of PN6047 to induced DOR Internalization was determined using DOR-eGFP mice. PN6047 blocked mechanical allodynia induced by CFA, and tolerance to this effect was not observed with daily injections of PN6047 for 12 days. In contrast, twice daily injections of PN6047 did result in tolerance within 5 days. PN6047 did not produce a CPP. In DOR-eGFP mice, systemic injection of PN6047 did not produce appreciable DOR internalization. These results suggest that PN6047 could be an effective treatment for chronic pain or migraine with low adverse effects.

Alternative splicing in a mouse model of Oprm1 A118G

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There is growing evidence that alternative splicing of RNA is altered by drugs of abuse and contributes to addiction-like behavior. This is understudied with opioids, although the gene encoding the mu opioid receptor (MOR), OPRM1, is extensively spliced. The canonical protein is a 7-transmembrane GPCR, but alternative transcripts produce truncated 6TM MORs, which have reduced or lost affinity for opioid ligands. 6TM MORs are produced via usage of an alternative promoter and first exon, E11, which is upstream of the normal first exon, E1. Preliminary evidence suggests that 6TM MOR expression inversely correlates with opioid sensitivity in mice and is preferentially upregulated after opioid exposure, suggesting a role in addiction and tolerance. GWAS have associated the single nucleotide polymorphism *OPRM1* A118G, located in E1, with opioid use disorder. As E1 is excluded in 6TM MORs it is of interest to determine the role of the A118G SNP in mediating expression and splicing of *Oprm1* transcripts . A mouse strain generated with the orthologous variant, *Oprm1* A112G, displays elevated intravenous self-administration of heroin and oxycodone. GG mice also have reduced mRNA containing E1, reduced binding of radiolabeled opioid ligands, and reduced synaptic response to opioids in the hippocampus and VTA. The mechanism of altered mRNA expression has never been identified, and alternative *Oprm1* isoforms have never been quantified in these mice. We hypothesize that GG mice have reduced 7TM isoform expression and elevated 6TM isoform expression, mediating the partial loss-of-function phenotype. Using a novel method of gene-targeted long read sequencing on the Oxford Nanopore platform, we will quantify expression of alternative *Oprm1* isoforms in individual mice of both geneotypes following morphine exposure. These data will expand our understanding of the functional and regulatory roles of the A118G SNP.

Examination of the critical roles of nTS subpopulations in mediating fentanyl-induced cardiorespiratory depression

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Millions of Americans suffer from Opioid Use Disorders (OUD) and face a high risk of accidental overdose, which can cause opioid-induced respiratory depression (OIRD). Synthetic opioid opioid-related overdose deaths continue to rise, thus it is critical to understand the mechanisms by which fentanyl induces respiratory depression. We previously showed that bilateral mu opioid receptor (MOR) antagonism in the nTS significantly reduces the duration of fentanyl-induced cardiorespiratory depression, implicating the nTS as a critical site of opioid-induced dysfunction. We used transgenic MOR-Cre, tyrosine hydroxylase (TH-CRE) and glutamate decarboxylase (GAD-Cre) rats to evaluate the effects of fentanyl on these nTS subpopulations to the onset, severity, and duration of OIRD following virally mediated GqDREADD expression in the nTS. A separate group of MOR-Cre rats was bilaterally injected with a Cre-dependent retrograde virus to express GqDREADDs in MOR-expressing inputs into the nTS. Results demonstrate that chemogenetic activation (via sc injection of the DREADD agonist C21) of MOR+ nTS neurons, MOR+ afferent inputs to the nTS, and GAD+ nTS neurons evoked significant baseline decreases in heart rate and respiratory rate without affecting oxygen saturation. Activation of TH+ nTS cells evoked no significant changes in baseline cardiorespiratory parameters. We next examined the effect of activating MOR+, TH+ and GAD+ nTS cells by administering C21 30 minutes prior to intravenous fentanyl. Surprisingly, we found that pre-activation of MOR+ nTS cells did not significantly alter cardiorespiratory responses to fentanyl. However, pre-activation of MOR+ afferent inputs to the nTS significantly attenuated the duration of OIRD. Similarly, chemogenetic pre-activation of TH+ nTS cells attenuated the severity and duration of OIRD. In comparison, pre-activation of GAD+ neurons significantly increased the duration of cardiorespiratory depression after fentanyl. These data shed light on the complexities of fentanyl-MOR signaling in the nTS and the nTS subpopulations that mediate cardiorespiratory responses that are evoked by intravenous fentanyl infusion.

Screening novel G protein βγ subunit inhibitors for potentiation of morphine-induced antinociception

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Although opioid analgesics can be effective pain treatments, their clinical use has limitations, including side effects such as the development of analgesic tolerance with repeated use and abuse liability risk. It is therefore pertinent to develop therapeutic strategies to improve the safety and efficacy of opioids. Both the analgesic and unwanted effects of opioid analgesics are mediated through signaling at the μ-opioid receptor (MOR). Therefore, a novel strategy that has been employed to enhance opioid therapies for pain is to bias intracellular MOR signaling. Specifically, evidence suggests that the small molecule gallein selectively inhibits Gβγ-mediated activation of effector subsets that may oppose analgesic MOR signaling. While these findings are promising, the chemical properties of gallein make it an undesirable candidate for drug development. Consequently, we sought to develop and investigate novel compounds with preferable chemical characteristics. Here, four novel small molecule Gβγ inhibitors (compound 734, 743, 744, and 858) were screened for their ability to potentiate the antinociceptive effects of morphine (3.2 mg/kg). Prior *in vitro* studies indicate compound 744 primarily inhibits Gβγ-mediated activation of PLCβ, while 743 primarily inhibits Gβγ-mediated activation of GRK. Compound 858 inhibits activation of both effectors. Nociception was measured using the warm water tail withdrawal assay (55°C) and tail withdrawal latencies were recorded every thirty minutes after morphine administration. All Gβγ inhibitors were administered via intracerebroventricular (i.c.v.) injection 30 minutes prior to morphine. Gallein (100 nmol) and DMSO served as positive and negative controls, respectively. As expected, gallein pretreatments produced robust increases in tail withdrawal latencies compared to DMSO. Pretreatment with compound 734 or 744 (100 nmol) did not produce significant increases in tail withdrawal latencies. On the other hand, tail withdrawal latencies were significantly increased in animals receiving compound 743 (100 nmol) and 858 (50 nmol) pretreatments as compared to DMSO. These data suggest that the effectiveness of Gβγ inhibitor compounds to enhance morphine-induced antinociception lies more so in their ability to inhibit GRK activity than PLCβ. Yet, inhibiting activation of both GRK and PLCβ may provide the highest efficacy.

A novel G-protein biased δ Opioid Receptor Agonist PN6047 Attenuates Allodynia in Models of Migraine and Opioid-Induced Hyperalgesia

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Migraine is a prevalent neurological disorder characterized by recurrent episodes of severe headache, often accompanied by nausea, vomiting, and sensitivity to light and sound. Despite advances in understanding its pathophysiology, effective treatments for migraine remain limited, with many patients experiencing inadequate relief or intolerable side effects with current therapies. The δ opioid receptor (δOR) presents a promising target for migraine. PN6047, a G protein-biased δOR agonist, has been developed for the treatment of pain; and recently completed Phase I clinical trials with good safety and tolerability. The aim of this study was to determine if PN6047 was effective in models of acute and chronic migraine and opioid induced hyperalgesia. We assessed the effect of PN6047 on established acute and chronic migraine-associated cephalic allodynia evoked by nitroglycerin, a human migraine trigger. We further tested the antinociceptive effect of PN6047 in a peripheral and cephalic models of opioid-induced hyperalgesia (OIH), a condition where repetitive treatment with opioids results in paradoxical hypersensitivity to pain. PN6047 significantly inhibited established allodynia in both models and showed a dose-dependent effect. Interestingly, in the OIH model when PN6047 was co-administered with morphine it blocked the development of OIH. These results suggest that PN6047 could be an effective treatment for migraine and OIH.

Inflammatory Pain Alters Alcohol Dose-Response in a Self-Administration Paradigm in Male and Female Rats

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During the last years, clinical and epidemiological studies have revealed that the presence of chronic pain is closely related to Alcohol Use Disorder (AUD). However, there is only a limited number of preclinical studies approaching this problematic and therefore the specific effect of pain on AUD remains not fully discern. Here, we aimed to deeply explore wether the development of an inflammatory pain condition induced by the intraplantar injection of the Complete Freud Adjuvant (CFA) could impact alcohol self-administration (ASA) in animals with a previous history of alcohol exposure. After being exposed to alcohol in their homecages using the drinking in the dark protocol during 2 weeks, male and female rats were trained to self-administer 20% alcohol in a FR3 schedule of reinforcement. Once ASA was stable across days, rats were injected with CFA or saline into their hind-paws. Then, they were subjected to a dose-response test, consited of 3 consecutive sessions for each of the alcohol doses (20%, 30% and 50%), presented in a randomly assigned, ascending or descending manner. Our results show that CFA treatment shifted the alcohol dose-response to the right, in both males and female rats, although this effect was greater in the male group. These findings may contribute to the better understanding of the intersection between pain, AUD and the potential sex differences, and to the development of more individualized treatments for chronic pain patients with a history of alcohol abuse. Funding: R01 DA042499, R01 DA041781, R01 DA045463

Searching for Synthetic Opioid Rescue Agents: Identification of a Potent Opioid Agonist with Reduced Respiratory Depression

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According to the Center for Disease Control and Prevention, the number of overdoses has accumulated up to 100,000 in 2021. A significant contributor was a >1,000% increase in death due to the epidemic of synthetic opioid overdose, with an emphasis on fentanyl, from 2013 to 2019. This calls for a rescue agent with greater safety, efficacy, and long-lasting effect compared to known opioid antagonists. We unexpectedly discovered a potent new class of MOR agonists. This rare finding has led to a new class of opioid agonist based on a diazabicyclooctane scaffold. The prototype of the series, which we have termed atoxifent, possesses *in vitro* agonist activity more potent than morphine and similar to fentanyl as well as mediates G protein biased partial agonist at MOR. In our *in vivo* studies, atoxifent mimicked many fentanyl-like effects, including antinociceptive activity that was reversed by naltrexone, tolerance to the antinociceptive activity with repeated administration, naltrexone-precipitated withdrawal, and complete loss of locomotion at high doses. The antinociceptive activity of atoxifent was found to be more efficacious than buprenorphine and was more similar to fentanyl and morphine *in vivo*. Surprisingly, what made it different was its inability to produce the deep respiratory depression associated with fentanyl-induced lethality in rats. The reason why atoxifent produced less respiratory depression than fentanyl was unclear. These findings suggested that this series has a superior side effect profile compared to morphine and fentanyl with the end goal of lowering the mortality rate due to opioid intoxication.

Establishing the effect of GPR83 antagonist, Cpd25, on the systemic effects of morphine.

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Morphine is the most effective therapeutic for the treatment of pain. However, prolonged exposure to morphine activates mu opioid receptors in brain regions, including those in the reward pathway, increasing the risk for addiction. Additionally, prolonged exposure to morphine induces tolerance, necessitating increasing doses of opioids which exacerbate dependence. Patients that become tolerant to opioids and escalate their doses risk overdosing due to opioid-induced respiratory depression. In previous studies we found that the expression of the deorphanized G-protein coupled receptor, GPR83, is increased in the nucleus accumbens (NAc) following morphine conditioned place preference but not by giving morphine alone. In addition, GPR83 knockdown in the NAc attenuated morphine CPP in both male and female mice. We identified small molecule ligands, both agonists and antagonist of GPR83. In our preliminary studies, we demonstrated that GPR83 antagonist Cpd 25, administered during acquisition, blocked morphine CPP in male mice. Additionally, acute administration of Cpd 25 enhanced morphine antinociception. Our laboratory is furthering these preliminary studies by expanding our knowledge of the effect of Cpd 25 on the rewarding properties of morphine and on the unwanted side effects of tolerance, withdrawal, and respiratory depression in both male and female mice. The results will determine if Cpd 25 has potential to serve as a therapeutic to reduce the abuse liability of opioids used for pain management.

Opioid inhibition of projection-specific rat thalamic anterior paraventricular neurons

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Neurons in the rat anterior PVT (aPVT) integrate visceral and emotional signals to bias behavior toward aversive or defensive states and are sensitive to the inhibitory actions of opioids. How the heterogeneous population of neurons in this area are regulated by mu-opioid receptor (MOR) activation acutely and following chronic opioid treatment remains to be determined. This study examines neurons in the aPVT from male and female rats that project to the amygdala, nucleus accumbens, and prefrontal cortex using patch clamp electrophysiology. Application of [Met]^{5}enkephalin activated potassium conductance in more than 90% of neurons studied that projected to each area. The opioid-induced current in amygdala-projecting neurons (72.2 ± 34.9 pA) was larger than those projecting to the nucleus accumbens and medial prefrontal cortex (33.5 ± 14.6, 39.4 ± 26.0 pA respectively). Application of a saturating concentration of ME (30 µM, 10 min) resulted in a peak current that declined during the application (desensitization) in recordings from neurons that projected to each brain area. Inhibition of the G Protein Receptor Kinase (GRK2/3) with compound101 blocked desensitization indicating a phosphorylation-dependent process. In animals treated chronically with morphine, there was a decrease in sensitivity to morphine in the amygdala and nucleus accumbens but little or no tolerance in mPFC projecting neurons. During acute withdrawal, the percentage of neurons that fired spontaneously increased from 20% to 62% although there was no change in the frequency of action potentials. Further, we show that during naloxone-precipitated withdrawal and the application of a depolarizing step, the number of action potentials was increased in neurons that projected to the NAc but little or no change in the neurons projecting to the mPFC or amygdala. Taken together the results indicate that there are cell selective adaptive mechanisms that alter the activity of aPVT neurons following chronic morphine treatment. Receptor-dependent tolerance would contribute to dose escalation, while the increased excitability would contribute to signs of withdrawal.

Exploring the Effects of Tumour-Triggered Peripheral Insult on the Mesocorticolimbic System: Observations from a Lung Cancer Murine Model

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Systemic inflammatory insults are known to impact the mesocorticolimbic system (MCLS) and the opioid receptors in rodents and humans, correlating with negative affective states that not only impact quality of life but also are related with worst prognosis in several illnesses. However, no previous studies have investigated the influence of cancer as a peripheral insult on anxiety-like behaviour and the MCLS. The goal of this pilot study is to investigate the short- and long-term behavioral and molecular effects on the MCLS of the development of a tumour in a murine lung cancer model. Hereby, C57BL/6 mice were injected subcutaneously with CMT167 cells and sacrificed one (short-term) or four (long-term) weeks after this procedure. Anxiety-like behavior, opioid receptors expression and several neuroinflammatory markers were analysed in MCLS regions, including prefrontal cortex (PFC) and nucleus accumbens (NAc). Preliminary results show different alterations depending on the time of the development of the tumour. In the long-term tumour exposure experiment, while an increase in the expression of DOR was reported in the PFC in the cancer group, KOR expression in the NAc slightly tended to decrease. On the other hand, in the short-term paradigm, mice injected with cancer cells exhibited higher levels of anxiety-like behaviour. These experiments, despite their preliminary nature, were able to unmask various behavioural changes and molecular responses in the MCLS in response to the development of a tumour. Our results could represent a solid starting point for the study of the effects of peripheral insults on the brain, highlighting the importance of this brain-body communication for cancer-associated mood disorders.

Potency, dissociation kinetics and reversibility of fentanyls and nitazenes by naloxone at the μ opioid receptor

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Background and Purpose:

Fentanyls and nitazenes are μ opioid receptor agonists that are responsible for a large number of opioid overdose deaths. Here, we measured the potency, dissociation kinetics and reversibility by naloxone at the μ opioid receptor of a range of fentanyls and nitazenes and compared them to the prototypic opioid agonists morphine and DAMGO.

Experimental Approach:

To assess how fentanyls and nitazenes interact with the m receptor *in vitro* assays of G protein activation and signalling and arrestin recruitment were utilised. AtT20 cells stably expressing μ receptors were loaded with a membrane potential dye and agonist-induced changes in fluorescence that result from G protein-coupled potassium channel activation used to determine potency, dissociation kinetics and susceptibility to antagonism by naloxone. Bioluminescence Resonance Energy Transfer experiments were undertaken in HEK293T cells transiently expressing μ receptors, to assess opioid agonist signalling through Gi protein activation and β-arrestin 2 recruitment.

Key Results:

The rate of agonist dissociation from the receptor varied significantly between the opioids studied, with morphine, DAMGO, alfentanil and fentanyl dissociating rapidly whereas isotonitazene, etonitazene, ohmefentanyl and carfentanil dissociated slowly. The slowly dissociating agonists were more resistant to antagonism by naloxone. For carfentanil, the slow rate of dissociation was not due to G protein receptor kinase-mediated arrestin recruitment as its rate of dissociation was not affected by inhibition of GRKs with Compound 101. The *in vitro* relative potencies of fentanyls and nitazenes compared to morphine were much lower than that previously observed in *in vivo* behavioural assays.

Conclusions and Implications:

With fentanyls and nitazenes, that slowly dissociate from the m receptor, antagonism by naloxone is pseudo competitive. The binding kinetics of the agonist should be factored in when evaluating the nature of antagonism. In overdoses involving fentanyls and nitazenes higher doses of the antidote naloxone may be required for reversal than those normally used to reverse heroin overdose.

Endogenous opioid dynamics in the dorsal striatum promote goal-directed behavior

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Endogenous opioid signaling is uniquely poised to regulate neuronal activity and behavior across multiple timescales. Traditional studies ascribing opioid contributions to roles in behavior rely on slow exogenous ligands to alter signaling that lack spatiotemporal precision. The endogenous opioid dynorphin is highly enriched in the dorsal striatum, a region important for goal-directed behavior. This behavior relies on learning across multiple timescales - a timescale of seconds in which animals must prospectively look for causal relationships between actions and outcomes and days learning new contingencies and tracking unexpected changes to them. Although studies have posited a role for dynorphin signaling through the kappa opioid receptor (KOR) in drug-seeking across slow and sustained time periods, little is known about the role, the locus or the precise timescale of endogenous dyn-KOR signaling in goal-directed behavior. Here, we demonstrate that dynorphin neuron activity and local dynorphin release from dorsomedial striatum (DMS) neurons is essential for goal-directed behavior. In mice performing an action-outcome goal-directed behavior, we observe that DMS dyn neurons preferentially encode action and cued anticipation of the outcome via in vivo 2-photon calcium imaging using implantable microprisms allowing us access to >10,000 DMS neurons. Subsequently, using a novel dyn biosensor in conjunction with *in vivo photometry*, we show that dyn is released during the cued anticipation of an outcome in the timescale of seconds. Additionally, we show that axon terminal activity from the basolateral amygdala during action engender dynorphin release during cued outcomes using conditional deletions, optogenetics and multiplexed photometry. Our results describe an intricate feed-forward inhibition mechanism of BLA terminals by DMS dynorphin during an action-outcome behavioral sequence. Collectively, our results demonstrate a causal role for endogenous opioid release and subsequent signaling in shaping goal-directed behavior at slow and rapid timescales.

The Functional Diversity of G Proteins in Opioid Receptor Signaling

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G protein heterotrimers (Ga, Gb, and Gg) are important signal transducers upon activation of G protein coupled receptors (GPCRs). Opioid receptors belong to the class A GPCRs and play major roles in pain, mood, reward, and motivation. Opioids are used for pain management despite the side effects that contribute to the opioid crisis. The pursuit of non-addictive opioid analgesics remains unattained due to the unresolved intricacies of opioid actions, receptor signaling cascades, and neuronal plasticity. Opioid receptor activation couple to up to seven Ga subtypes (Gai1, Gai2, Gai3, GaoA, GaoB, Gaz, and/or Gustducin). The molecular basis for the G protein subtype selectivity are largely unclear; the functional consequence of individual G protein signaling remains unknown. We thus determined the active-state structures of kappa opioid receptor (KOR) in a complex with multiple G-protein heterotrimers using cryo-electron microscopy. Comparisons of these structures reveal molecular determinants critical for KOR-G-protein interactions as well as key elements governing Gi/o-family subtype selectivity. Furthermore, the G-protein subtypes display an intrinsically different allosteric activity on agonist activity at KOR. These results provide insights into the actions of opioids and G-protein-coupling specificity at KOR and establish a foundation to examine the therapeutic potential of pathway-selective agonists of KOR.

Development of Orally Bioavailable HSP90β Inhibitors for Opioid Dose Reduction Therapy

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In our work, we've found that Hsp90 inhibition in the spinal cord enhances the potency of opioid pain relief by 2-3 fold, while strongly reducing tolerance, rescuing already established tolerance, and without altering the potency of opioid constipation and reward. This points the way to use Hsp90 inhibitors to improve the therapeutic index of opioids. However, non-selective Hsp90 inhibitors given systemically also inhibit brain Hsp90, which leads to the loss of opioid pain relief. To surmount this difficulty, we examined the active Hsp90 isoforms in brain vs. spinal cord, finding that in the brain, Hsp90α alone regulated opioid anti-nociception. In contrast, in the spinal cord, we determined that Hsp90α, Hsp90β, and Grp94 all regulate anti-nociception. These data led to our hypothesis that systemic Hsp90β and Grp94 isoform-selective Hsp90 inhibitors will selectively block spinal cord Hsp90 activity, resulting in enhanced anti-nociception and reduced side effects. We validated this hypothesis with the Hsp90β-selective inhibitor KUNB106, which enhanced pain relief and reduced and rescued tolerance, without altering respiratory depression. However, KUNB106 is a poor clinical candidate, with low solubility, poor metabolic stability and undesirable pharmacokinetic profile, and no oral bioavailability. We thus engaged in a medicinal chemistry effort to improve the drug-like features of KUNB106 while retaining Hsp90β isoform selectivity. We report here the creation of multiple selective HSP90β inhibitors with low nanomolar affinity/potency, high Hsp90β selectivity, and promising ADME features. Further, lead compounds such as NDNB-01 and NDNB-25 showed oral bioavailability in enhancing opioid anti-nociception at a 10 mg/kg dose in male and female CD-1 mice. These advances point the way to further lead development and pre-IND testing for our Hsp90β inhibitors and their eventual use as a therapy for opioid dose reduction.

Effects of fentanyl on VTA dopamine neurons and in opioid associative learning using conditioned place preference in mice

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The opioid epidemic is a nationwide condition that is affecting more than 2.5 million people in the United States. The latest opioid overdose wave is driven by synthetic opioids like fentanyl, which has high potency and lethality. We must understand the effects of fentanyl on the reward circuits and its relationship with the progression of opioid use disorders. Recent studies have uncovered recruitment of functionally diverse populations of ventral tegmental area (VTA) dopamine neurons to opioids such as heroin and morphine. Yet, fentanyl's effects on these populations remains unclear. We use genetic strategies to isolate VTA dopamine neurons that are anatomically and functionally diverse and which have been demonstrated to regulate distinct aspects of food reward. Here, we seek to elucidate how fentanyl impacts opioid reward learning and the recruitment of subpopulations of VTA dopamine neurons. We used a conditioned place preference (CPP) behavioral paradigm to study the opioid-associative learning behavior of mice using different fentanyl concentrations (0.2 and 0.05 mg/kg) and cFos immunohistochemistry to identify activated neurons. Our results indicate that mice develop fentanyl-associative learning with both fentanyl doses when compared to their saline counterparts (t-test 0.2 mg/kg dose $t(34)= 2.595$, *p= 0.0139; 0.05 mg/kg dose $t(17)= 3.611$, **p= 0.0022). However, an increased opioid preference is seen on the 0.05 mg/kg fentanyl dose when compared to the 0.2 mg/kg dose (one-way ANOVA $F(2, 41)= 13.21$, ****p= <0.0001). Our cFos data will be analyzed using confocal imaging and Qu-Path quantification of cFos + cells. T-test statistical analyses will be performed. This research is important to study fentanyl's impacts on opioid associative learning while creating a foundation to study polysubstance use disorders.

Profiling of nociceptive neurons enables synergistic pharmacology against pain unpleasantness

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Pain is a complex experience with sensory, affective, and cognitive dimensions. An ensemble of neurons in the amygdala encodes, and is causally necessary for, the unpleasant quality of pain. To determine the molecular identity of nociceptive amygdalar neurons, we combined activity-dependent fluorescent labeling (TRAP2) and single-cell RNA sequencing (scRNA-seq) to profile the gene expression of individual neurons active during nociception. These analyses revealed 17 types and 28 subtypes of amygdalar neurons. We next established a comprehensive neuron atlas by mapping the spatial organization of the marker genes defining these amygdalar cell types. Additionally, we integrated our scRNA-seq results with prior findings about amygdalar cell-types and their functions to guide the discovery of analgesic drugs that exert their effects by modulating amygdala circuits. Specifically, we searched for G-protein-coupled receptors (GPCRs) that were enriched on amygdalar neuron types involved in pain processing. We identified more than a dozen G-protein-coupled receptors (GPCRs) differentially expressed on amygdalar neuron types involved in pain processing, including specific serotonin, vasoactive intestinal peptide, and neurotensin receptors. We tested drugs engaging these GPCR targets using preclinical pain assays and identified multiple amygdalar GPCR agonists with analgesic properties. Based on prior knowledge, we predicted that the tested drugs modulate multiple pain-related pathways, we devised a combination strategy of three drugs that enhance analgesia specifically against pain unpleasantness both across acute pain models and in a preclinical model of orofacial chronic pain. Together, our findings establish the molecular structure of the amygdala and identify highly druggable targets to treat pain unpleasantness across pain types. Our results support the concept that precisely modulating GPCR drug targets to inhibit ensembles of neurons while activating others in functionally bidirectionally organized circuits such as the amygdala result in significant analgesic benefits. We anticipate that this combination approach may improve the treatment outcomes of chronic pain patients.

COI

G.S. is a cofounder of Epiodyne, a drug discovery company, an inventor on a US patent application (#20210220489) related to imaging of neural dynamics to discover analgesics, and a member of the National Institutes of Health Preclinical Screening Platform for Pain (PSPP) External Consulting Board.

Selective targeting of mu opioid receptors to primary cilia

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Opioid receptors comprise a subfamily of GPCRs activated by endogenously produced opioid peptides and various non-peptide analgesic drugs. Opioid receptors have long been recognized to partition between different subdomains of the plasma membrane, but a mechanistic basis for opioid receptor targeting to any particular surface domain remains elusive. Here we show for the first time that the mu-opioid receptor (MOR) localizes to the primary cilium, a discrete microdomain of the plasma membrane. This discovery was enabled by observations in MOR-Venus mice and validated in wildtype animals, in both intact brain slices and cultured habenula neurons. In a ciliated cell model, we show that the propensity for MOR to be targeted to cilia is a specific capability of MOR but not other opioid receptors. We determine that a 17-residue sequence in the cytoplasmic tail, that overlaps with a motif previously shown to sort receptors from endosomes into the recycling pathway, is necessary for cilia targeting. We further find TULP3, a ciliary membrane delivery protein, promotes MOR targeting to primary cilia. Our results reveal novel localization of MOR to neuronal primary cilia and support a model by which MOR cilia targeting is mediated by a C-terminal sequence in *cis* and TULP3 in *trans* via the recycling pathway.

Identifying the anti-nociceptive mechanism of action for Cannabis sativa terpenes in mouse chemotherapy-induced peripheral neuropathy

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Chronic pain is a debilitating condition that affects 20.5% of the United States adult population. Limited efficacy and/or harsh side effects limit many pain drugs, which has driven the search for alternate analgesics. Our past research has shown that the terpenes geraniol, linalool, β-pinene, α-humulene, and β-caryophyllene, small hydrocarbons found in *Cannabis sativa*, are cannabimimetic and capable of blocking pain in multiple mouse models and work via multiple receptor targets. Moreover, we have shown that this anti-nociception in chemotherapy-induced peripheral neuropathy (CIPN) is the result of the activation of spinal cord Adenosine A2a receptors (A2aR), shown by utilizing both spinal cord-specific CRISPR knockdown of the A2aR and the A2aR antagonist istradefylline (3.2 mg/kg, IP). To identify a circuit mechanism of action underlying this spinal A2aR anti-nociception, we performed immunohistochemistry to identify the neurons where the A2aR is located in mouse (CD-1, male and female) spinal cords. We have stained for the A2aR and colocalized this signal with markers for neurons using NeuN, microglia using IBA1, and astrocytes using GFAP. We additionally colocalized the A2aR with neuronal population markers such as CGRP and pro-dynorphin. We then performed cell segmentation analysis with QuPath to identify the cell types most associated with the A2aR. Based on this work, we hypothesize that the A2aR is likely localized to inhibitory interneurons in the spinal dorsal horn and inhibits neuronal pain transmission. To further support this hypothesis, we stained for the neuronal activity marker C-Fos, finding it upregulated in CIPN and colocalized with the A2aR; this colocalization was abolished by terpene treatment. Together, these results demonstrate our progress in identifying a circuit mechanism for terpene-A2aR pain relief in CIPN, which helps illuminate the therapeutic mechanism of these potential new therapeutics. Future work will use cell-type selective CRISPR knockdown of the A2aR to demonstrate that the receptor works through the identified cell populations.

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Identification of GPR63 and GPR153 as Novel Modulators of Opioid-Induced Antinociception in Mouse Models of Post-Operative and Neuropathic Pain

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Treatments for chronic pain disorders are currently dominated by opioid drugs which lose efficacy over time and yield various undesirable side effects. Thus, the need for identifying novel targets for modulating pain is critical. In this work, we have identified two potential targets for non-opioid analgesics - the orphan GPCRs GPR63 and GPR153. This was achieved by *in vivo* transfection of targeted CRISPR-Cas9 DNA constructs in the spinal cords of adult male and female CD-1 mice and evaluating changes in the response to morphine in post-operative and neuropathic pain models. The post-operative pain model consisted of longitudinally incising the flexor digitorum brevis muscle of the left hindpaw. Neuropathic pain, modeled by chemotherapy-induced peripheral neuropathy (CIPN), was induced by 2 mg/kg paclitaxel via intraperitoneal (IP) injection resulting in the development of mechanical allodynia. Both models were followed by administration of 3.2 mg/kg morphine SC and a three-hour von Frey time course to evaluate changes in opioid-induced antinociception. Another cohort of animals received a non-targeted universal negative control (NC) CRISPR construct for all experiments. Receptor knockdown ablated the analgesic effect of morphine in both pain models. These receptors' effects on opioid-induced antinociception were also evaluated in an acute tail flick pain model, but no difference was observed. These findings suggest that these receptors are not involved in direct neurotransmission of pain signals but instead play roles in the neuropathology of chronic pain and/or in altering cellular responses to opioids in chronic pain states. To further investigate this finding, we performed RNAScope *in situ* hybridization in the spinal dorsal horn to colocalize RNA transcripts of mouse *Gpr63* and *Gpr153* with markers for microglia or astrocytes which are known to contribute to the development and maintenance of neuropathic pain. Together this work identifies completely novel pain modulators which could be exploited to develop new pain therapies.

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COI

John Streicher is an equity holder in Botanical Results, LLC and Teleport Pharmaceuticals, LLC, but not company products or interests were tested in this study.

10-I-Akuammicine and 10-Br-Akuammicine, Novel Selective KOR agonists, Produced Anti-Scratch effect and CPA but no Hypolocomotion or KOR Phosphorylation in mouse brains

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Akuammicine (AKC), an indole alkaloid isolated from the seeds of *Picralima nitida*, has been used in West African traditional medicine for treatment of many ailments, including pain and fever. AKC was demonstrated to be a kappa opioid receptor (KOR) agonist with a K_i of 89 nM (doi:10.1021/acs.jnatprod.0c01036). In this study, *in vitro* and *in vivo* pharmacology of two AKC derivatives, 10-Iodo-AKC (I-AKC) and 10-Bromo-AKC (Br-AKC), were characterized. Affinity of I-AKC and Br-AKC to opioid receptors were determined by competitive inhibition of [^{3}H]diprenorphine binding to the MOR, DOR, or KOR in membranes of CHO cells stably expressing each receptor. The Ki values of I-AKC and Br-AKC for the KOR were determined to be 2.4 and 5.1 nM, respectively, and I-AKC and Br-AKC showed 164x and 130x selectivity for the KOR over MOR. Both had low affinity for the DOR (K_i > 1 mM). Br-AKC and I-AKC inhibited compound 48/80-induced scratching in mice in dose-dependent manner, with IC_{50} values of 3.4 and 5.7 mg/kg (s.c.), respectively, indicating anti-pruritic activities. At 5 mg/kg (s.c.), Br-AKC and I-AKC produced significant conditioned place aversion (CPA) following three 30-min conditioning, comparable to U50,488H (2 or 5 mg/kg, s.c.); however, neither Br-AKC or I-AKC reduced novelty-induced hyperlocomotion. Thirty min following injection of Br-AKC or I-AKC at 5 mg/kg (s.c.), KOR in the mouse brain was not phosphorylated at S369, whereas U50,488H at 5 mg/kg greatly enhance KOR phosphorylation. We previously found that the KOR agonists U50,488H and MOM-SalB caused hypolocomotion, CPA and KOR phosphorylation, but nalfurafine did not produce any of these effects, whereas 42B produced hypolocomotion and KOR phosphorylation, but no CPA (doi:10.3389/fphar.2022.835809; doi:10.3389/fnins.2022.964724). Taken together, our current results support the notion that among the KOR agonists, there is a correlation between hypolocomotion and KOR phosphorylation, but not between CPA and KOR phosphorylation. Thus, CPA and hypolocomotion appear to have different signaling bases. [supported by NIH grants R01 DA041359, R01 DA056581, and P30 DA013429 (L-YL-C) and R35GM147005 (APR)]

Characterization of the Opioid System in the Pacific Hagfish, a Vertebrate/Invertebrate Transition Species

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The opioid system plays a role in controlling a myriad of functions in mammals with opioid drugs influencing reward processing, gut function, and respiration. Opioid receptors and peptides have been found in all vertebrate species examined, however, their behaviors have been less studied in non-mammalian vertebrates. Previously, a mu-like opioid receptor (esMOR) and a proenkephalin-like peptide (esPENKL1) were found in Pacific Hagfish (Huang et al., Journal of neuroscience research vol. 100,1 (2022): 19-34, PMID:32830380), which is often considered a transitional species between vertebrate and invertebrate lineages. This established that a functional opioid system existed before the divergence of the cyclostome lineage over 400 mya. Here we extend this analysis with the identification of 4 opioid-like receptors and 7 opioid precursors sequences in the Pacific Hagfish. Sequences were identified from Hagfish genome databases and compared with opioid genes from human and mouse genomes. All 4 hagfish receptor-like sequences have shared intron-exon boundaries and conserved sequence motifs with all four human opioid receptors. Sequence alignment of the four different hagfish opioid receptors showed matching regions of high and low sequence homology which paralleled the different mammalian mu, delta, kappa, and ORL-1 opioid receptors. Seven different opioid precursor-like genes were identified in the hagfish genome by the presence of consensus pro-hormone processing sites, signal peptides, and Tyr-Gly-Gly-Phe motifs (YGGFM, YGGFL, YGGFI). Many sequences of interest were found, however, only four were predicted to be secreted based on signal peptide prediction. We have also performed additional analysis of the previously cloned mu-like opioid receptor, which was ectopically expressed in HEK293 cells. Gi G protein activation assays were carried out to test the response of MOR with different ligands. esMOR responded to endogenous opioids, synthetic opioids, natural opioids, and several of the proposed hagfish opioid peptides. However, each agonist has a lower potency of action with esMOR compared to human MOR, suggesting less coupling efficiency or lower binding affinity in mammalian cells. Though genomic analysis has still to clearly identify opioid systems in invertebrates, our analysis clearly demonstrates that the cyclostome genome has the components of multiple functional opioid systems.

The beta-lactam derivative MC-100093 does not reduce cue-induced reinstatement following extinction to oxycodone self-administration in male and female rats

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Reduction of the membrane associated glutamate transporter GLT-1 protein expression within the nucleus accumbens (NAc) has been well established in preclinical models of cocaine use disorder. Restoring membrane associated GLT-1 by treatment with beta-lactam antibiotics such as ceftriaxone is associated with reduced reinstatement to cocaine seeking. For better translation, a beta-lactam derivative, MC-100093 (093), was developed lacking antibiotic properties that recapitulate the effects of ceftriaxone on cocaine-seeking and GLT-1. However, it has not been established if 093 is also capable of reducing opioid-seeking. Here we determined the effects of 093 on oxycodone use. Rats underwent intravenous oxycodone self-administration for 14 days followed by 10 days of extinction. During the final 6 days of extinction, at the end of each session, rats were intraperitoneally injected with either saline or 50mg/kg 093. We found that 093 did not reduce cue-induced reinstatement following extinction from oxycodone self-administration. In addition, Western blot analyses revealed no differences in membrane associated GLT-1 protein expression within the NAc of 093-treated rats compared to saline that self-administered oxycodone. Previous work with beta-lactams such as ceftriaxone has shown that they do not increase GLT-1 expression in naïve animals and require a previous experience dependent decrease in GLT-1 expression to have an effect. In ongoing studies, using a conditioned place preference (CPP) model, we will determine if oxycodone treatment also reduces NAc GLT-1 expression as observed with cocaine as this has not been demonstrated. So far, we have found that consistent with previous findings, GLT-1 expression downregulates following cocaine CPP. Next, we aim to determine if this reduction is also observed with oxycodone treatment. If we do not observe changes in GLT-1 expression following CPP for oxycodone, this would suggest that oxycodone does not cause the initial decrease in GLT-1 needed for beta-lactam molecules to rescue its expression. Taken together, our results will highlight potential differences in 093's ability to reduce seeking to different drug classes. Further studies will be needed to elucidate the underlying mechanisms governing these differences between psychostimulants and opioids.

Influence of Endogenous Opioids on Breathing Development

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Birth is a major transition for the respiratory system. After spending several months underwater in the womb, newborns must breathe air for the first time. Breathing is irregular in newborns and breathing irregularity can be more severe for premature infants. It has been hypothesized, but not directly tested, that elevated endogenous opioid levels contribute to breathing variability during the perinatal period. To begin to uncover the role of endogenous opioids in breathing development, I measured breathing in newborn transgenic mouse pups that lack functional mu-opioid receptors (MOR-KO pups, n = 3) and wild-type control pups (n =3). Breathing was measured in postnatal day 0 (P0) mouse neonates using a small piezoelectric sensor placed underneath the chest. Breathing rate and inter-breath interval (IBI) were compared. Wild-type mouse pups exhibited ~50% longer maximum inter-breath intervals (average max IBI: 1.8 ± 0.1 s) compared to MOR-KO pups (average max IBI: 0.9 ± 0.1 s). Consistent with this, wild-type pups also tended to breathe more slowly than MOR-KO pups (WT: 86 ± 7 breaths per min; MOR-KO: 143 ± 8 breaths per min). The average coefficient of variation of IBI, which is an indicator of breathing variability, was not different for wild-type mice compared to MOR-KO mice (WT: 0.5 ± 0.1; MOR-KO: 0.4 ± 0.1), which was contrary to our hypothesis. These preliminary results suggest that endogenous mu-opioid receptor activity leads to longer respiratory pauses and slower breathing in newborn pups. The contribution of endogenous opioid peptides to respiratory pauses and slower breathing rates remains to be determined. Understanding the role of endogenous opioids in newborn breathing is important since modulating the endogenous opioid system could be an effective way to improve breathing regularity and prevent life-threatening oxygen desaturating events, especially in premature infants.

NOP Receptor Involvement in Migraine Circuitry

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Headache disorders are extremely disabling and are among the most common neurological disorders worldwide. Migraine is highly sex dependent, with roughly 70% of migraineurs being women. New classes of medications for cephalic pain, with novel targets and mechanisms of action, are greatly needed. One potential component crucial to the circuitry of cephalic pain is the NOP receptor. This receptor is highly expressed in dorsal root ganglia, spinal cord, and in brain regions involved in pain and drug reward with very high expression levels of NOP receptors in the trigeminal ganglia and trigeminal nucleus caudalis (TNC). Our histochemical studies with NOP-eGFP knock-in mice indicate that NOP receptors are on the majority of cells in the trigeminal ganglia. Activation of trigeminal ganglia and TNC are thought to be involved in the etiology of headache, and therefore inhibition of this neuronal pathway might be expected to reduce migraine and other cephalic pain symptoms. Experiments using two different migraine models have demonstrated that the selective NOP receptor agonist Ro 64-6198 can reduce periorbital allodynia induced by systemic administration of both nitroglycerin (NTG) and calcitonin gene-related peptide (CGRP). Although it is generally well established that migraine pain is associated with activation of nociceptors travelling from the dura through the trigeminal ganglia to the TNC and then higher order locations, cellular activation has not been extensively determined at the neuronal level. Experiments were conducted to identify neuronal circuitry activated during cephalic pain and to examine the requirement for this circuitry to mediate cephalic pain. Using Fos2A-iCreER/Ai9 (TRAP2/Ai9) mice, we have identified cells in various brain regions activated by both NTG and CGRP. In particular, CGRP increases activated cells more than 50-fold in lamina I and II of the TNC, the site of primary afferent innervation coming from dural activation. In some regions, the number of activated cells is reduced by administration of the NOP receptor agonist Ro 64-6198, consistent with its effect on migraine pain. Because in TRAP2 mice cellular activation deposits cre recombinase in activated cells, future experiments will use DREADDs to examine whether these cells are necessary and sufficient to induce migraine-like pain.

The role of astrocytes-selective TLR4 in morphine physical dependence in mice

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There is an increased risk of dependence and death with higher daily opioid doses for increasing analgesic effect. Opioid dependence is a chronic and relapsing disease and is characterized by compulsive drug seeking and use. Astrocytes equipped with several toll-like receptors (TLRs) have been implicated in pathological processes of opioid use disorders. In the preliminary study, we hypothesized that astrocytes-derived TLR4 plays important role in mice with morphine withdrawal (MW). Naloxone-precipitated MW was induced by chronic morphine for 5 days and followed by naloxone; spontaneous MW induced by chronic morphine for 5 days and observed for 7 days since opioid abstinence. Aldh1l1CreERT2::TLR4$^{fl/fl}$ mice were produced using tamoxifen-inducible astrocytes-selective Aldh1l1CreERT2 mice breeding with mice containing TLR4$^{fl/fl}$ sequences in the study. We found that MW increased TLR4 expression using western blots, and that TLR4 was mainly expressed in astrocytes using immunostaining in the mice brainstem PAG. In naloxone-precipitated MW, astrocytic knockout of TLR4 induced by tamoxifen reduced bodyweight loss, jump, and global scores. In spontaneous MW, astrocytic knockout of TLR4 reduced MW scores of teeth chat, ptosis, digging, tremor, jump, wet-dog shake, and total global scores. The main difference focuses on the time points of 24, 30, 48, 54, 72 hours after morphine. The effect of astrocytic knockout of TLR4 in suppressing MW worked in both sex mice. The preliminary data suggests that astrocytic TLR4 plays an important role in morphine physical dependence.

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A novel negative allosteric modulator at the μ-opioid receptor potentiates naloxone-mediated antagonism

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The μ-opioid receptor (μOR) is a well-established target for analgesia, yet conventional opioid receptor agonists such as fentanyl cause serious adverse effects, notably respiratory depression, addiction and overdose. μOR negative allosteric modulators (NAMs) may serve as powerful tools in preventing opioid overdose deaths, but promising chemical scaffolds remain elusive. We screened a large DNA-encoded chemical library against inactive μOR, counter-screening with active, G-protein and agonist bound receptor to “steer” selections toward conformationally- selective modulators. We discovered a NAM compound, **368**, with high and selective enrichment to inactive μOR that both inhibits receptor-mediated G-protein activation in a biochemical assay and enhances μOR affinity for the key opioid overdose reversal molecule, naloxone. NAM **368** works cooperatively with naloxone in vitro to potently antagonize opioid agonist-induced signaling, perturbing orthosteric ligand kinetics in therapeutically desirable ways and to cooperatively potentiate the activity of low doses of naloxone. Using cryoEM, we demonstrated that the NAM accomplishes this by binding to a site on the extracellular vestibule in direct contact with naloxone, while also making a series of inactive-state specific interactions across the entire vestibule, stabilizing a unique inactive conformation of the extracellular portions of the second and seventh transmembrane helices. When administered intravenously (10 mg/kg) in mice, **368** enters the brain with moderate penetration and displays a observed half-life of 0.51 hr, though this is still longer-lived than naloxone. Subcutaneous administration of **368** resulted in cooperative, dose-dependent potentiation of low doses of naloxone in behavioral assays in vivo, enhancing antagonism of morphine- and fentanyl-induced antinociception in the 55^{o}C warm-water tail-withdrawal assay, agonist-induced respiratory depression and morphine-conditioned place preference, but did not likewise potentiate naloxone-precipitated withdrawal behaviors. Our results provide detailed structural insights into the mechanism of negative allosteric modulation of the μOR, and through NAM **368**, demonstrate how this might be therapeutically exploited.

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Xylazine Exacerbates Fentanyl-induced Respiratory Depression in Awake Mice

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Xylazine is an increasingly widespread adulterant in the illicit opioid supply. The sedative effects of xylazine, an α-2 adrenergic receptor agonist and common veterinary anesthetic, are viewed as potentiating the intensity and duration of opioid reward. Consequently, xylazine-opioid coadministration has become increasingly common. Despite suggestions that combining xylazine with μ-opioid agonists may increase the risk of fatal overdose in humans, existing preclinical work does not establish whether the coadministration of xylazine with opioids increases the risk or severity of opioid-induced respiratory depression, the primary cause of death associated with opioid overdose.

Here we use whole body plethysmography in awake and unrestrained C57BLJ6 mice of both sexes to evaluate the respiratory effects of xylazine in the presence and absence of the potent synthetic μ-opioid agonist fentanyl. We additionally evaluate the efficacy of naloxone, a μ-opioid antagonist used clinically to reverse opioid-induced respiratory depression, in reversing xylazine-fentanyl coadministration.

Xylazine, when administered alone, produced rapid, persistent, and severe respiratory depression at sub-anesthetic doses after intraperitoneal injection. Xylazine decreased minute ventilation, respiratory frequency, tidal volume, and peak inspiratory flow, while increasing end expiratory pause in comparison to vehicle controls. These effects were prevented by coadministration of the α-2 adrenergic receptor antagonist atipamezole. Animals exposed to combinations of xylazine and fentanyl exhibited more severe respiratory effects than those exposed to the same dose of fentanyl, but only when the mixture included a dose of xylazine that showed intrinsic effects on respiration. Furthermore, the respiratory effects of xylazine-fentanyl coadministration were only partially attenuated by subsequent injections of naloxone; respiratory parameters reflected the intrinsic effects of the administered xylazine dose rather than restoring breathing to baseline levels.

Together, these results suggest that xylazine administration depresses breathing through interactions with α-2 adrenergic receptors, and that exposure to xylazine both exacerbates opioid-induced respiratory depression and prevents its full reversal by naloxone.

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The missing rung? Using ex vivo electrophysiology to bridge the causal gap for mu opioid ligands with different effects in vivo but similar effects in vitro

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A persistent challenge of developing treatments for CNS disorders is that reductive heterologous systems often fail to model complex *in vivo* effects. To bridge this causal gap, we explore the feasibility of assaying pharmacology in acutely isolated rodent brain slices. Slice electrophysiology allows straightforward control of the treatment dose, while leaving intact endogenous cellular signaling pathways. Crucially, using a multielectrode extracellular array (MEA) enables recording from ~4-60 neurons per slice for several hours, vastly increasing the number of samples that can be collected compared to whole cell recording per slice. To facilitate the analysis, we have developed the opensource Multielectrode Extracellular Array Pharmacology (MEAP) computational pipeline to pick and cluster units and estimate treatment effects using Bayesian non-linear regression across neuronal populations.

As case study, we compared the effects of ZH853 vs. DAMGO in behaviorally relevant brain regions. ZH853 is a cyclized analog of endomorphin I that shows high selectivity for MOR in heterologous systems and produces analgesia *in vivo*. Strikingly, however, unlike DAMGO, ZH853 does not produce locomotor activation or IV self-administration at analgesic doses, and only produces respiratory depression at supra-analgesic doses. To explore the biology underlying this discrepancy, we measured treatment effects on spontaneously firing neurons in the ventral tegmental area (VTA), lateral habenula, and Kolliker Fuse (KF) in acute brain slices from male SD rats. Excitingly, we found a subset of neurons where responses varied between DAMGO (1 uM) and ZH853 (1 uM). In a subset of slices, the MOR selective antagonist CTAP (500 nM) was applied and blocked responses to both agonists. Together, these results demonstrate that we can ground differences in behavioral responses to ligands in neurobiological responses with this medium-throughput assay, which can guide future studies to characterize the *in vivo* effects of new treatments.

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COI

JEZ is an inventor on the patents for ZH853

Increased remifentanil self-administration in susceptible mice following chronic social defeat stress

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Stress and stressful environments lead to maladaptive behaviors that can increase vulnerability to substance use disorders (SUDs). Individuals' response to stress varies with some showing resilience, while others show susceptibility. While the relationship between stress and SUDs is well documented, there exists a significant gap in knowledge regarding the role of stress phenotype in mediating opioid-taking. Using a tractable and translationally relevant mouse model of social stress we determined that susceptible mice self-administered significantly more remifentanil than resilient and non-stress controls. Specifically, increases in acquisition (Fixed Ratio [FR] 1) of drug taking behavior in susceptible mice occurred at days 1,2,3, and 6 compared to no stress controls. Cumulative intake was also increased in susceptible mice compared to resilient and no stress controls. One brain region implicated in both stress and drug reward is the paraventricular nucleus of the thalamus (PVT). The PVT is rich in mu-opioid receptors (MORs), which may play an integral role in mediating stress induced changes in drug taking behavior. To determine if these cells are active following CSDS, we exposed mice to 10d CSDS, used the social interaction test to determine susceptibility and resilience, and then exposed each mouse to an acute defeat. Brains were collected and cFOS was used to assess neural activity. We found increases in cFOS in susceptible brains compared to resilient and no-stress controls. Further, we found cFOS was enriched in MOR+ cells in the PVT. Given the prominent activation of MORs in the PVT of susceptible mice following CSDS, we used our recently developed MOR-Cre mouse line to investigate the function of MOR+ cells in the PVT. To determine the functional importance of MOR+ cells, we injected a Cre-recombinase dependent inhibitory DREADD virus (pAAV5-hSyn-DIO-hM4D(G_i)-mCherry; AddGene) in the PVT of MOR-Cre mice. In a locomotor activity assay, the DREADDs + CNO group had significantly decreased activity after morphine injection compared to the DREADDs plus saline group. These data support the notion that MOR+ cells in the PVT are responsive to morphine and future studies will used this model to determine if the same cells mediate susceptible or resilient phenotypes following CSDS.

Efficacy modulation by semi-synthetic diversification of mitragynine pseudoindoxyl

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Clinical treatment of acute to severe pain involves the use of opioids. Drugs targeting the μ-opioid receptor (MOR), are effective analgesics. Although they still show considerable adverse effects such as fatal respiratory depression and addiction. Mitragynine pseudoindoxyl (MP), a minor metabolite of kratom derived indole alkaloid mitragynie, shows low efficacy at G-protein biased agonism for MOR and demonstrates improved pharmacological properties. Owing to the partial agonistic property and reduced arrestin recruitment, MP also demonstrates slower antinociceptive tolerance over morphine and shows reduced respiratory depression at equianalgesic doses when compared with morphine (***J Med Chem*** 2016;59(18):8381-97). Recently we reported the cryo-EM structures of MOR-Gi1 complex with MP (PDB: 7T2G). The structure demonstrates that 9-methoxyaromatic ring of the indole moiety of MP occupies an extracellular outlet and creates a unique subpocket 2 not occupied by other high efficacy agonists related to opioid peptides or fentanyl and indicates a unique binding approach in the active-state conformation on the intracellular side of the receptor (***Nat Chem Biol.*** 2023;19(4):423-430). Herein, we map out the molecular determinants of efficacy by diversifying the aromatic ring of MP. Specifically, a library of analogs where positions 9, 10 and 12 on indole moiety were looked at with substituents such as aromatic/hetrocyclic/amide/halogens/aliphatic attachments. Pharmacological characterization of analogs has allowed us to develop a structure activity relationship with different efficacy ranges at MORGi1. We have identified analogs with varying efficacies (60%-0%) with some lower than the parent natural product as well as some which are antagonists at the receptor. We hypothesize that the lower efficacy agonists, specially with intrinsic efficacy lower than 20% may be interest in developing safer analgesics. We achieved an analog having efficacy in between an agonist and an antagonist and showing in-vivo antinociception in both thermal and neuropathic pain models with reduced opioid related adverse effects such as respiratory depression, locomotion, CPP. Overall, this work will emphasize that MP template can be used for efficacy modulation and to develop novel partial agonists having lower efficacy at the receptor and showing comparable analgesic effect as found in morphine but with minimized life-threatening side effects.

Validating Zhx2 as a quantitative trait gene underlying oxycodone metabolite (oxymorphone) brain concentration and behavior: Insights from brain multi-omics analysis

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Opioid Use Disorder (OUD) maintains epidemic proportions in the U.S. with limited pharmacological treatments. Sensitivity to opioid reward/reinforcement has a genetic component and can predict addiction liability of opioid compounds. We identified Zhx2 as a candidate gene underlying increased oxycodone (OXY) metabolite brain concentration in BALB/cJ (J) vs. BALB/cByJ (By) females. The metabolite, oxymorphone (OMOR), is more potent than OXY and could explain the enhancement of state-dependent learning of OXY conditioned place preference (CPP) in J vs. By females. A structural intronic variant robustly reduced Zhx2 expression in J vs. By mice, which could enhance OMOR levels and OXY addiction-model behaviors. We tested this hypothesis in Zhx2 knockout mice by measuring OXY metabolite levels via mass-spectrometry, OXY-induced locomotor activity, and OXY-CPP. Consistent with our hypothesis, Zhx2 KO females showed an increase in brain OMOR levels and OXY-induced locomotor activity compared to WT females. However, contrary to our hypothesis, state-dependent expression of OXY-CPP was actually decreased in KO females and increased in males. We followed up with -omics analyses in naïve brains between genotypes using liquid-chromatography/mass-spectrometry for proteomics and RNA-sequencing for transcriptomics. Proteomic analysis identified multiple proteins implicated in small-molecule metabolism and inflammatory processes between genotypes, while transcriptomic analyses identified multiple genes implicated in extracellular matrix formation, particularly in females. Further analysis suggested a disruption between integrin and collagen/laminin binding medicated by astrocytes between genotypes that could compromise blood brain barrier integrity and thus increase metabolite and behavioral differences. These results support validation of Zhx2 as a quantitative trait gene underlying brain OMOR concentration and behavior and implicate novel quantitative trait mechanisms that could increase our understanding of Zhx2 brain function and OXY addiction liability in humans.

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The involvement of mu opioid receptor in binge eating, food devaluation and food reward.

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Hypothesis: The mu opioid receptor (MOP) is involved in binge eating, food devaluation and food reward.

Materials & Methods: We used male mice lacking MOP and their wildtype littermates. Mice were singly housed and allowed to habituate for a week. Regular chow diet (RCD) intake was then recorded for 24h. Mice were then exposed to a high-fat diet (HFD) for 24h. The HFD intake was recorded for 1 h and 24h. Mice were then exposed to RCD for four weeks, and then tested for binge eating, in which mice were given access to the HFD for 1h and the amount of HFD intake was recorded. Mice were then maintained on HFD for 7 days and then tested food devaluation, in which HFD was replaced with RCD. Food intake was recorded for 24 h and compared to their initial 24h RCD consumption. Mice were then tested for HFD-induced reward using the place conditioning paradigm. Mice were tested for baseline preference toward the place conditioning chambers and then conditioned with RCD or HFD for 16 h (5 pm - 9 am). This alternate-day conditioning lasted for 4 consecutive days and mice were then tested for place preference.

Results: We found no difference in the initial HFD consumption between mice of the two genotypes ($P>0.05$). Mice of both genotypes showed increased HFD intake on the second exposure, suggesting that mice of both genotypes exhibited binge eating. However, wildtype mice consumed significantly more HFD when compared to knockout mice ($P<0.0001$). Correspondingly, the food devaluation was significantly higher in wildtype than the knockout mice ($P<0.001$). Intriguingly, HFD induced conditioned place preference (CPP) in wildtypes but not in knockouts.

Conclusions: MOP is essential for HFD-induced CPP and contributes at least in part in binge eating and food devaluation in male mice. Our results support the notion that MOP could be a potential target to develop medications to treat binge eating, food reward and food devaluation.

Gap of the study: We need to define which opioid peptide is stimulating MOP to mediate HFD-induced reward, binge eating, and food devaluation.

Intracellular pocket conformations determine signaling through the μ opioid receptor

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The relationship between the binding of a ligand to a receptor and activation of biological signaling pathways has remained unclear. The challenge is compounded by functional selectivity, in which a single ligand binding to a single receptor can activate multiple signaling pathways at different levels. Spectroscopic studies show that in the largest class of cell surface receptors, 7 transmembrane receptors, activation is associated with shifts in the equilibria of intracellular pocket conformations. We hypothesized that signaling through the μ opioid receptor, a prototypical 7TMR, is linearly proportional to the equilibrium probability of observing intracellular pocket conformations. Here we show that a machine learning model based on this hypothesis accurately calculates the efficacy of both G protein and β-arrestin-2 signaling within 9 and 19% or experimental medians, respectively. Structural features that the model associates with activation are intracellular pocket expansion, toggle switch rotation, and sodium binding pocket collapse. Distinct pathways are activated by different arrangements of the ligand and sodium binding pockets and the intracellular pocket. Our study provides the first identification of specific ligand-induced intracellular conformations of a 7TMR that adequately explains signaling events. While recent work has categorized ligands as active or inactive (or partially active) based on binding affinities to two conformations, our approach accurately computes signaling efficacy along multiple pathways, which could provide tremendous insight to opioid pharmacology.

COI

David Alvin Cooper and David Do Le Minh are co-inventors of the methodology described in this work and the Illinois Institute of Technology has filed a provisional patent on their behalf. They have started a company, Biagon Inc., to commercialize the technology.

The effects of opioids on PACAP modulation of vlPAG neurons.

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The ventrolateral periaqueductal gray (vlPAG) is a key brain area within the descending pain modulatory pathway and an important region in opioid-mediated analgesia. The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) promotes migraine via the PACAP receptor (PAC1) that is densely expressed in the vlPAG. The goal of this project is to determine the effects of PACAP on activity and intrinsic membrane properties of vlPAG neurons and GABAergic synaptic transmission within the vlPAG. Whole cell patch clamp recordings from ex vivo brain slices containing the vlPAG from C57/B6 male and female mice were performed. PACAP 1-38 (10 nM) depolarized most neurons and increased spontaneous firing. The PAC1 specific inhibitor M65 (100 nM) did not reverse this effect of PACAP suggesting non-PAC1-mediated effects, possibly via VPAC receptors.

The effects of PACAP 1-38 on GABAergic synaptic transmission were determined using bipolar stimulating electrodes and NBQX (5 μM) to block glutamatergic synaptic transmission. PACAP 1-38 enhances evoked GABA release which is reversed in the presence of M65 (100 nM). In addition, PACAP 1-38 significantly increased spontaneous inhibitory postsynaptic currents and miniature inhibitory postsynaptic current frequency in the vlPAG in a PAC1-dependent manner. Increased GABA release in the vlPAG is consistent with promoting hyperalgesia. Given that delta-opioid agonists reverse behavioral effects of PACAP (Mangutov, et al., 2023), we are currently testing the ability of delta opioid agonists to reverse PACAP-mediated potentiation of GABAergic inhibitory postsynaptic currents in the vlPAG. Understanding PACAP signaling in the descending pain modulatory circuit may identify new therapeutic targets for migraine.

Exploring Corticosterone-Driven Adaptations in the Endocannabinoid System Following Chronic Inflammatory Pain in the vlPAG

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Chronic pain represents an enormous personal and economic burden, affecting over 30% of the world's population, and its clinical management remaining a pressing challenge. The ventrolateral periaqueductal gray (vlPAG), a pivotal brain region involved in the modulation of pain through opioids and endocannabinoids, integrates inputs from various brain regions associated with the perception of nociceptive, cognitive, and affective aspects of chronic pain. Both opioids and endocannabinoids exert their effects by reducing GABA release via their respective presynaptic receptors (MOR and CB1R). Chronic inflammation induced by injections of Complete Freund's adjuvant (CFA) into a hind paw result in increased pain sensitivity as well as CB1R desensitization and elevation of endocannabinoid in the vlPAG. Interestingly, CB1 desensitization develops faster in females than in males, indicating a sex-specific modulation of the endocannabinoid system. The current studies investigate the hypothesis that CFA-induced elevation of corticosterone mediates the observed adaptations in CB1Rs and explain the difference in the onset of CB1R desensitization observed between males and females. Using whole-cell patch-clamp recordings from vlPAG neurons in ex vivo slices of male and female Sprague-Dawley rats, we initially observed that corticosterone, activation of glucocorticoid receptors (GRs), augments endocannabinoid tone and inhibits presynaptic GABA release via CB1R activation. Corticosterone effects are abolished by inhibition of 2-arachidonoylglycerol (2-AG) synthesis, and its rapid effects suggest a non-genomic action of corticosterone, possibly mediated through membrane receptors. In CFA-treated animals, despite CB1R desensitization, endocannabinoid-mediated activation of CB1R remains intact, as evidenced by depolarization-induced suppression of inhibition (DSI). The alterations in 2-AG tone observed in slices from CFA-treated rats and corticosterone-exposed naive rats are dependent on PKA and reversed by inhibiting DAGL (2-AG synthesis enzyme). In summary, our findings indicate that corticosterone regulates the endocannabinoid system in the vlPAG in a sex-dependent manner. We are currently examining corticosterone/CB1R modulation of pain.

Isolating the role of endogenous μ-opioid activity in the Ventral Tegmental Area during natural reward

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Opioid use disorder and opioid overdose death rates in the United States reached unprecedented levels during the COVID-19 pandemic. Understanding the critical role of endogenous opioid activity in natural reward behaviors is essential for understanding opioid use disorder, yet the fundamental mechanisms by which opioids affect these behaviors in the brain remain elusive. Here, we used a combination of genetic and molecular tools to isolate the role of μ-opioid receptor (MOR) activity in the ventral tegmental area (VTA) and identified a source of endogenous opioid release onto these receptors in the VTA. To assess the role of endogenous opioid activity on VTA^{GABA} neurons, we used *ex vivo* 2-photon slice imaging. Preliminary results demonstrate that endogenous opioid peptides produced heterogenous effects on VTA^{GABA} neurons which can be reversed following the bath application of tetrodotoxin. Next, using *in situ* hybridization and viral tracing methods, we identified enkephalin-containing neurons in the lateral hypothalamus (LH) that project to the VTA. We observed endogenous enkephalin release from the LH in the VTA using chemogenetics and a newly developed mu-opioid biosensor, μMASS. Using fiber photometry and $Oprm1^{fl/fl}$ mice, we recorded dopamine release in the nucleus accumbens (NAc) and found decreases in dopamine activity during Pavlovian conditioning in the absence of VTA MORs. These results provide insight into the role of endogenous opioids in the VTA and consequently, on dopamine activity and motivated behavior. Current work is focused on further elucidating the role of endogenous μ-opioid activity in the VTA on striatal dopamine release and determining how enkephalinergic projections from the LH to the VTA modulate natural reward. Ultimately, these studies lay the groundwork for understanding how opioid drugs of abuse elicit maladaptive changes in endogenous opioid systems to drive opioid use disorder.

Antinociceptive and discriminative stimulus effects of fentanyl and nitazene analogs in rats

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In concert with the recent increase in opioid overdoses in the United States, law enforcement and forensic laboratories have reported increased seizures of novel synthetic opioid substances. Due to the immediate public health threat associated with these substances, several new compounds have been placed under schedule I restrictions pursuant to the Controlled Substances Act (CSA). The objective of these studies is to characterize pharmacological effects of novel fentanyl related substances and nitazene analogs in comparison to more widely studied opioids: morphine, fentanyl, and etonitzene. The effects of ten compounds (protonitazene, metonitazene, etodesnitazene, para-methylcyclopropyl fentanyl, isovaleryl fentanyl, piperidylthiambutene, metodesnitazene, alpha'-methyl butyryl fentanyl, flunitazene, 3'-4'-dimethoxy fentanyl) were compared to the effects of the opioid standards in two assays of opioid effect in male rats. First the antinociceptive effects of the drugs were assessed using cumulative dosing procedures in a warm-water tail-withdrawal assay (water held at 54±0.5°C). Drugs with antinociceptive effects were advanced for further study in rats trained to discriminate 3 mg/kg morphine from saline using a standard, 2-lever, food-maintained operant task. Results show that, except for 3'-4'-dimethoxy-fentanyl, the drugs tested had antinociceptive effects (order of potency as listed above) and these effects were antagonized following pretreatment with 0.1 mg/kg naltrexone. All compounds with antinociceptive effects also had morphine-like discriminative stimulus effects. The order of potency of the drugs in generalizing from the morphine discriminative stimulus cue largely mirrored that of their antinociceptive effects although comparisons of ED50 values across the two procedures reveals that the drugs were approximately 5- to 10-fold more potent in producing morphine-like discriminative stimulus effects than antinociception in the warm water tail-withdrawal assay. Three drugs - metonitazene, protonitazene, and piperidylthiambutene - resulted in unusual convulsant-like or catalepsy-like behaviors during the tests for antinociception; these effects were largely reversed following injection of naltrexone. Together, data from these studies reveal that nine of the novel drugs tested (all except for 3'-4'-dimethoxy-fentanyl) share interceptive stimuli and other pharmacological features with standard opioids, and thus likely also have abuse potential.

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Deep spectrotemporal profiling of ultrasonic vocalization profiles during neonatal opioid withdrawal reveals a kappa opioid receptor component in female mice

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Opioid use during pregnancy can lead to negative infant health outcomes, including neonatal opioid withdrawal syndrome (**NOWS**). NOWS refers to the set of symptoms due to spontaneous cessation of opioid exposure during gestation, including low body weight, body temperature dysregulation, hyperirritability, and excessive, high-pitched crying. Current treatments involve non-pharmacological and pharmacological interventions; however, there is no standard care approach due to variability in NOWS symptom severity. To effectively model NOWS-associated traits in mice, we use a third trimester-approximate opioid exposure paradigm, where neonatal inbred FVB/NJ and outbred Carworth Farms White (CFW) pups are injected twice-daily with morphine (10 mg/kg, s.c.) or saline (20 ul/g, s.c.) from postnatal day (P) one to P14. We assess several phenotypes during spontaneous morphine withdrawal (16h post-morphine) on P7 and P14, including nociception, ultrasonic vocalization (**USV**) emission, and locomotor activity. Neonatal USVs are emitted in isolation to communicate distress and promote maternal attention. Thus, USVs can model negative affective states. Mouse USV syllables are classified into different syllables based on acoustic features; however, the connection between certain syllables and affective states, specifically morphine withdrawal, remains unknown. We implemented a custom supervised machine learning model to automatically classify USVs. During spontaneous morphine withdrawal on P14, we observed an increase in the proportion of Complex 3 syllables in FVB/NJ and CFW pups. Additionally, morphine-treated female FVB/NJ pups vocalized more and emitted a greater proportion of Complex 3 syllables than males. Brainstem transcriptomics revealed upregulation of the kappa opioid receptor (*Oprk1;* **KOR***)*, which is associated with dysphoria observed during withdrawal. With our current sample size, pre-treatment (44h) with the selective KOR antagonist, norBNI (30 mg/kg, s.c.) was sufficient to reduce Complex 3 emission specifically in female pups during spontaneous morphine withdrawal ($t(63) = -2.38$, $p^{adj} = 0.02$; Tx1:MOR/Tx2:SAL = 10F, 7M; MOR/norBNI = 10F, 10M; SAL/SAL = 7F, 7M; SAL/norBNI = 12F, 9M). These results implicate Complex 3 syllables as a potential biobehavioral marker for the dysphoric internal state associated with neonatal opioid withdrawal and suggest a female-specific involvement of the KOR system in mediating withdrawal symptom severity in neonates.

Translating human drug taking patterns into rat models: investigating spontaneous interindividual differences via refined self-administration procedure

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Background: During their drug-use history, cocaine and heroin users gain mastery and control over their drug consumption. Indeed, they self-regulate the dosage, route, speed, and frequency of administration as a function of the expected effects (e.g., avoid withdrawal, experiencing euphoria, etc.). Counterintuitively, most preclinical self-administration and choice procedures use discrete dimension strategies, featured by experimenter-imposed unit-doses interspersed by timeouts, which prevent the experimental animal to self-select the appropriate dose-time relationship of administration and result in standardized patterns of drug taking across different addictive drugs. Here, we contrasted discrete to continuous dimension strategies (i.e., self-selected doses without timeout) that allow to do so.**Methods**: We analyzed the drug-taking patterns and modeled drug-brain levels (PK profiling) under distinct self-administration training conditions, featured by the presence or absence of time-out between consecutive drug injections. We further assessed the motivation to take and seek drugs across training conditions and in the context of drug-vs-social choice procedures. **Results**: The drug-taking patterns, and related PK profiling, were profoundly different across both training conditions and drug under examination. Continuous dimension strategy resulted in an increased heroin intake and promoted the emergency of drug-taking patterns characterized by the injection of spaced and large doses of drug, resulting in high and fast-rising brain levels of heroin. By contrast, cocaine intake was only slightly increased and there were no differences in the drug taking patterns. Notably, we did not observe overdoses in rats trained without a timeout, contrary to what the literature would have anticipated. Rather, the lack of timeout was associated with stronger motivation to take and seek drugs. Finally, by employing a continuous dimension strategy we described, for the first time, social withdrawal after heroin, but not cocaine self-administration in rats. **Conclusions**: Here, we provide evidence advocating for the implementation of continuous, rather than discrete dimension strategies in self-administration and choice procedures because more accurately mirror human-drug-related behaviors (and likely the neural adaptations).

Central amygdala Protein Kinase C-δ neurons are required for fentanyl withdrawal in mice

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Agonists for the μ-opioid receptor, such as fentanyl, remain a frontline option for moderate to severe pain management. However, their propensity to produce dependence and withdrawal limit their long-term clinical use. Preclinical studies have associated both chronic pain and opioid withdrawal with increased activity in the central nucleus of the amygdala (CeA). In the context of chronic pain, this is seen largely in neurons expressing Protein Kinase C-δ ($CeA^{PKC\delta}$). *The CeA cell types impacted by opioid withdrawal, on the other hand, are not fully understood*. We hypothesized that $CeA^{PKC\delta}$ neurons also show increased activity during fentanyl withdrawal, and that their activity contributes to the behavioral correlates of dependence. Mice were given fentanyl (0.02 mg/mL) or untreated water in their homecage drinking water supply for 8 days. Fentanyl-drinking mice developed significantly more classic murine somatic withdrawal signs after forced abstinence or naloxone administration (3 mg/kg) compared to untreated water-drinking controls (n=16/group, unpaired t-test), indicating the development of fentanyl dependence. Immunohistochemistry revealed that this fentanyl withdrawal induces robust FOS expression in $CeA^{PKC\delta}$ neurons (n=4/group, two-way ANOVA). Fiber photometry in the CeA of PKCδ-Cre mice confirmed that these neurons show increased activity during fentanyl withdrawal, compared to opioid-naïve conditions (n=4-6/group, two-way repeated measures ANOVA). Finally, we chronically inhibited $CeA^{PKC\delta}$ neurons via overexpression of the potassium channel Kir2.1 in the CeA of PKCδ-Cre mice during the development of fentanyl dependence. Kir2.1-overexpressing mice exhibited fewer somatic withdrawal signs compared to fluorophore-expressing control mice during spontaneous fentanyl withdrawal (n=10-12/group, unpaired t-test). Collectively, these data support our hypothesis that the activity of $CeA^{PKC\delta}$ neurons is both impacted by fentanyl withdrawal, and that this activity drives the behavioral correlates of withdrawal.

“Anti-inflammatory” cannabinoid CB2 receptors enhanced high-fat diet evoked neuroinflammation in mice

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It is widely known that cannabinoid type 2 (CB2) receptor deficiency enhances inflammatory response and further symptoms in various animal models of inflammation, allergy, or cancer. As CB2 receptors are inhibitory Gi/Go G-protein coupled receptors and as major expression site of CB2 receptors are immune cells, it is no wonder that lack of CB2 receptors lead the exacerbated inflammation. We therefore hypothesized that lack of CB2 receptor might also enhance the high fat diet (HFD)-induced peripheral neuroinflammation.

However, surprisingly, CB2 receptor knockout animals (CB2-KOs) showed the significant resistance to the HFD-induced neuroinflammation. Namely, 5-week feeding of HFD induced substantial hypersensitivity in WT mice, while tactile sensitivity of HFD-fed CB2-KO animals remained intact. Alongside to the tactile allodynia, we further found that HFD will lead 1) the robust upregulation of infiltrated macrophages and 2) chemokine receptor CXCR4 overexpression at the peripheral nervous tissue, together with 3) modified differentiation of splenic CD11b+ myeloid subsets only in WT animals, but not in CB2 knockout mice. Note that standard diet had no effect to the nervous macrophage infiltration, CXCR4 expression and splenic myeloid subsets in either WT or CB2-KO animals. In addition, CD-fed, nerve-injured CB2-KO showed significant increase of macrophage infiltration and CXCR4 overexpression, which supports past reports suggesting the anti-inflammatory role of CB2 receptors, but contradict to the present observation with HFD exposure.

Based on these results, we will propose that CB2 receptors might have the bipolar regulatory role to the chemokine receptor-mediated inflammatory response. To be anti-inflammatory or pro-inflammatory could be regulated through the modification of splenic myeloid differentiation, which in the end enhance or inhibit the neuroinflammation development depending on its initial cause.

Fine mapping and multi-level functional analysis in C57BL/6 substrains identify Atp1a2 and Kcnj9 as candidate genes underlying oxycodone behavioral sensitivity and withdrawal

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Opioid use disorder is heritable, yet its genetic etiology is largely unknown. Utilizing rodent models of addiction, such as opioid behavioral sensitivity and withdrawal traits, offers a promising avenue for genetic and mechanistic discovery. Closely related C57BL/6J and C57BL/6NJ substrains show extremely limited genetic diversity, yet reliable phenotypic diversity in behavioral traits which can facilitate gene discovery. In this study, we compared the sensitivity to oxycodone (OXY) and spontaneous anxiety-like withdrawal behavior of C57BL/6J and C57BL/6NJ mice using locomotor activity, conditioned place preference (CPP), and elevated plus maze (EPM). We then used a quantitative trait locus (QTL) analysis in an F2 cross followed by F2 founder recombinant lines refinement to identify a candidate gene. Our findings suggest that C57BL/6NJ mice exhibit less sensitivity to oxycodone (OXY) and show lower levels of spontaneous anxiety-like withdrawal behavior compared to C57BL/6J mice. Through the F2 QTL, we identified a distal chromosome-1 QTL that accounted for 7-12% of the variance in OXY locomotor sensitivity and 7-10% of the variance in withdrawal. Additionally, we detected a chromosome 5 QTL located near Gabra2 (alpha-2 subunit of GABA-A receptor) that explained 9% of the variance in open-arm entries. The F2 founder recombinant lines allowed us to capture and fine-map the QTL for OXY sensitivity and withdrawal to a 2.45-Mb region (170.16-172.61 Mb) of the distal chromosome 1 locus (163-181 Mb). Within this region, we identified five striatal cis-eQTL transcripts (Pcp4l1, Ncstn, Atp1a2, Kcnj9, Igsf9), two of which (KCNJ9 and ATP1A2) were confirmed at the protein level. KCNJ9 (GIRK3) codes for a potassium channel that plays a significant role in mu opioid receptor signaling, while ATP1A2 codes for a subunit of a Na+/K+ ATPase enzyme that regulates neuronal excitability and responds to chronic opioid administration.

To summarize, we identified genetic sources of opioid behavioral differences in C57BL/6 substrains, which are the most widely used strains in opioid addiction research.

Inhibition of CeA-dynorphin neurons is reinforcing in mice with chronic pain

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Chronic pain is a debilitating condition that affects more than 50 million Americans annually. The underlying neurobiological circuits that mediate chronic pain states are not fully elucidated, contributing to the limited treatment options. The dynorphin neurons in the central amygdala (CeA) are known to contribute to pain-induced aversion and the CeA is a central hub for integrating pain and stress states. We investigted if inhibiting the CeA dynorphin neurons was sufficient to produce negative reinforcement using chemogenetics and conditioned place preference. We hypothesized that mice in a chronic pain state will prefer the chamber associated with clozapine-N-oxide (CNO), a designer drug used to inhibit dynorphin neurons expressing an inhibitory designer receptor. We transfected male and female transgenic mice (dyn-cre+ and dyn-cre-) with AAV-hSyn-DIO-hM4D to express the inhibitory designer receptor in dynorphin neurons or AAV-hSyn-DIO-mCherry as a control. For the dyn-cre- mice, we transfected non-cre dependent AAVs of the same constructs. Two weeks after the viral injection, we conducted either a chronic constriction injury or sham surgery to induce neuropathic pain. Two weeks following nerve injury, mice were conditioned with either CNO or saline using a three-chamber conditioned place preference unbiased paradigm counter-balanced for day and chamber. Mice were conditioned for six days followed by a post condition test (drug free) and a state-dependent test (drug onboard). We found that mice in chronic pain preferred the chamber associated with CNO, irrespective of their genotype. This suggests that inhibiting the CeA is negatively reinforcing and alleviates the negative affect induced by chronic pain state and more specifically, that dynorphin neuron inhibition in the CeA is sufficient to produce this phenotype. Moreover, we found that mechanical allodynia was not changed, as CNO did not alter mechanical threshold compared to baseline values using von frey. Further studies are planned to investigate the effects of activating these dynorphin neurons in the CeA using optogenetics and real-time place preference. This study adds to the knowledge of the underlying circuits of chronic pain and the potential for the kappa/dynorphin system to be a neurobiological target for its treatment.

Fentanyl inhibits neuronal activity in dissociated striatal medium spiny neurons co-cultured with glia through a non-opioid receptor dependent mechanism

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Fentanyl can be 50-200-fold more potent than morphine depending on the bioassay, refractory to opioid antagonists, such as naloxone, and its misuse can result in opioid-induced respiratory depression and death. Our limited understanding of fentanyl's action is a major barrier in combating opioid use disorder. We hypothesized that besides direct interactions with μ-opioid receptors and other opioid receptors, fentanyl would affect striatal function through non-ORs. We used electrophysiological recordings from dissociated cultures of striatal medium spiny neurons (MSNs) co-cultured with glia to test whether fentanyl inhibits neuronal activity by activating α_1-adrenoceptors. Acute exposure to 100 nM fentanyl decreased the number of spontaneous action potentials from 104.1 ± 16.7 to 58.6 ± 15.6 ($p < 0.01$, $n = 24$). Overnight exposure of co-cultures to 100 nM fentanyl severely reduced the proportion of MSNs with spontaneous action potentials from 24 out of 39 in controls to 5 out of 45 in the fentanyl-treated group ($p < 0.001$, z-score = 4.848). This suppression of spontaneous activity was unaffected by co-exposure to the opioid antagonist naloxone (10 μM) (3 out of 45 neurons, $p > 0.05$, z-score = 0.741), but fully negated by co-exposure to the pan-α_1-adrenoceptor, inverse agonist prazosin (100 nM) (24 out of 45 neurons, $p < 0.001$, z-score = 4.286) and partially reversed by the selective $\alpha_{1A/C}$ adrenoceptor antagonist RS 100329 (300 nM) (16 out of 43 neurons, $p < 0.01$, z-score = 2.871). In *ex vivo* striatal slice recordings, the acute application of 100 nM fentanyl modestly reduced the frequency of action potentials at ≥ 500 pA stimuli and caused firing rate adaptations in dopamine type 2 (D2) receptor-expressing, but not type 1 (D1), MSNs. Prolonged (2 - 5 h) fentanyl application dramatically attenuated firing rates in both D1 and D2 MSNs at mid-range stimuli. $\alpha_{1A/C}$-adrenoceptor expression was identified in subpopulations of astroglia and striatal interneurons, but not MSNs, by immunocytochemistry and *in situ* hybridization. We conclude that, besides actions at μ-opioid receptors, sustained fentanyl exposure can inhibit striatal MSN activity via a non-opioid, α_1 adrenergic-dependent pathway that may, at least in part, be mediated by astroglia.

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Escalation of intravenous fentanyl self-administration and assessment of withdrawal behavior in male and female mice

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Background: The rise in overdose deaths from synthetic opioids, especially fentanyl, necessitates the development of preclinical models to study fentanyl use disorder (FUD). While there has been progress with rodent models, additional translationally relevant models are needed to examine excessive fentanyl intake and withdrawal symptoms.

Methods: The study performed intravenous self-administration (IVSA) of fentanyl in male and female C57BL/6J mice for 14 days. Mechanical pain sensitivity during withdrawal was assessed using the von Frey test. Anxiety-like behavior was evaluated via the open field test one week into abstinence and incubation of craving for fentanyl was assessed four weeks into abstinence.

Results: Both male and female mice demonstrated a significant escalation in fentanyl intake over the 14 days of self-administration, with significant front-loading observed in the final days of self-administration. Increased mechanical pain sensitivity was present from 36- to 48-hour into withdrawal and increased anxiety-like behavior was found 1 week into abstinence. Four weeks into abstinence, mice showed significantly higher active lever pressing than the final self-administration session before abstinence.

Conclusions: The study establishes a translationally relevant mouse model of IVSA of fentanyl, effectively encapsulating critical aspects of FUD, including escalation of drug intake, front-loading behavior, withdrawal symptoms, and prolonged craving for the drug into abstinence. This model offers a robust basis for further exploration into behavioral and neurobiological mechanisms involved in fentanyl dependence and potential therapeutic strategies.

In vitro molecular pathway analysis evaluating pregabalin effects on morphine signaling to implement a Quantitative Systems Pharmacology platform for chronic pain therapy

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Chronic pain severely reduces patients' quality of life and their socio-economic contribution to society, representing a significant global burden. As a modality of chronic pain management, medications are often prescribed in combination. However, the clinical practice of combinatorial treatment has been based on clinical experiences due to the incomplete understanding of pain mechanisms, the variability in pain intensity among individuals, and the absence of data guiding the choice of pain syndrome-specific drug combinations. Novel technologies, such as in silico pharmacology, pharmacogenetics, and systems biology, merged into Quantitative Systems Pharmacology (QSP), use computational models to guide decision-making in areas such as dose optimization, precision medicine, and drug efficacy and safety. To implement a QSP-based platform for identifying, assessing, and predicting alternative combinations of existing opioid/non-opioid drugs eliciting analgesic synergy and reduced adverse effects, we aim to evaluate pregabalin co-administration effects on morphine-mediated signaling in different neuronal cell models.

Molecular pathway analysis was performed in primary cultures of brain region-specific rat and mouse neuronal cells and differentiated SH-SY5Y under basal conditions and after exposure to morphine, pregabalin, and morphine+pregabalin. Morphine's activity was determined by measuring the inhibition of forskolin-stimulated cAMP accumulation via ELISA assay. The expression of seven pain/analgesia-related targets was quantified via qPCR. Receptor density (B_{max}), a receptor expression readout, was determined via radioligand binding assay.

Co-administering 1 μM pregabalin significantly enhanced adenylyl cyclase inhibition by morphine in rat cortical (IC_{50}=0.052±0.6 nM;p<0.001;n=6) and mouse cortical primary neurons (IC_{50}=0.18±0.06 nM;p<0.001;n=6). In a model of human mature neuron-like cells, 0.1 nM and 10 nM pregabalin potentiated morphine potency (IC_{50}=0.18±0.06 nM and 0.1086±0.046 nM, respectively;p<0.001;n=6). After MOR desensitization, morphine's ability to inhibit adenylyl cyclase is dampened. In the same experimental condition, co-administering 1 μM pregabalin partially rescued morphine's activity (IC_{50}=0.034±0.071 nM;p<0.001;n=6). In the same cell model, the drug combination increased mRNA level of OPRM1 and CACNA2D1, encoding for morphine and pregabalin pharmacological target, respectively. We found that pregabalin affects morphine signaling at the μ receptor, increasing adenylyl cyclase inhibition and rescuing morphine's activity following MOR desensitization, suggesting that it might be promising as an opioid-sparing agent for chronic pain treatment. Supported by QSPainRelief (H2020GAn.848068)

In vitro characterization of novel endomorphin-1 analogues to develop innovative opioid ligands with distinct binding profiles and improved pharmacological activity

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Mu opioid receptor (MOR) agonists are still among the most widely used analgesics, despite their limited efficacy in chronic pain, relevant adverse effects and abuse liability. G protein-biased or peripherally-restricted agonists at kappa opioid receptor (KOR) have been sought as innovative opioids with improved pharmacology, and ligands simultaneously modulating multiple opioid receptors have been recently attracting increasing interest for their potentially enhanced effectiveness and reduced side effects [Li et al., ACS Chem Neurosci. 2022].

Endomorphin-1 (EM-1) is an endogenous, MOR-selective agonist inducing strong analgesic effects also in chronic pain albeit displaying poor metabolic stability. Modifying EM-1 structure has proven effective to develop innovative opioids with higher stability and improved activity towards distinct opioid receptors [De Marco et al., J Med Chem. 2018]. Here we characterize a small library of EM-1 analogues containing modified urea (urea-EM-1) to identify innovative opioid ligands with distinct binding profile to one or more opioid receptors and possibly improved pharmacology. Receptor affinity was assessed via competition binding assays in HEK-293 cells selectively overexpressing human MOR, DOR or KOR; ligands ability to inhibit forskolin-induced cAMP accumulation in the same cell models was employed as readout for activity. Ligands-mediated effects on expression of inflammatory mediators were evaluated via qPCR in PMA-differentiated SH-SY5Y cells (a human neuron-like model). Tetrapeptide DME36 displayed high affinity and selectivity to MOR (k_i=0.18±0.08 nM), without inhibiting adenylyl cyclase in HEK-293/hMOR cells. Tetrapeptide DME49 showed high affinity to KOR (k_i=0.38±0.05 nM) and DOR (k_i=0.11±0.03 nM), but not to MOR; moreover, DME49 inhibited adenylyl cyclase in HEK-293/hKOR (EC_{50}=0.20±0.05 nM; E_{max}=53±6 %) but not in HEK-293/hDOR. Interestingly, in PMA-differentiated SH-SY5Y cells 1μM DME49 significantly down-regulated TNF-a mRNA levels by 80% in a KOR-dependent way at 12 and 24h of exposure (effect similar to that elicited by U50,488); in the same cell model DME49 significantly reduced IL-1β mRNA levels by 90% in a DOR- and KOR-dependent way at 12 and 24h of exposure (effect greater than that elicited by U50,488). DME49 emerges as a high-affinity KOR-DOR divalent ligand displaying partial agonism at KOR, and downregulating pro-inflammatory cytokines expression in human neuron-like cells.

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Effects of morphine and THC co-administration to human and rodent neuronal cell models in vitro: evaluation of MOR-CB1 heterodimerization and intracellular signaling

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Chronic pain is a debilitating and longstanding condition. Although opioid analgesics are widely used in chronic pain treatment, many patients report insufficient pain relief and/or relevant adverse effects [PMID: 37111650], thus highlighting the need for analgesics or drug combinations with improved efficacy/safety. Combining of mu-opioid receptor (MOR) and cannabinoid type-1 (CB1) receptor agonists has been proposed as an improved analgesic treatment for chronic pain. Indeed, research has been conducted on MOR-CB1 crosstalk and heteromerization [PMID: 28327548] but molecular determinants are yet largely unknown. Therefore, our study aimed at (i) validating possible MOR/CB1 heteromerization interfaces in MOR-CB1-HEK cells, under basal conditions and following morphine and THC administration, and (ii) investigating ∆9-tetrahydrocannabinol (THC)-mediated effects on morphine-induced signaling in several human and rodent primary neuronal cells and HEK293 cells co-expressing Flag-MOR and HA-CB1 (MOR-CB1-HEK cells). Site-directed mutagenesis was performed to mutate MOR residues computationally predicted as relevant for heteromerization. Confocal microscopy was employed to quantify MOR-CB1 heteromerization in HEK-293 cells co-expressing MOR (either wild-type (wt) or mutated) and wt-CB1. Morphine's (10^{-4}-10^{-12}M) ability to inhibit forskolin-induced cAMP production was quantified by ELISA, in MOR-CB1-HEK-293 cells, PMA (16nM, 5 days)-differentiated SH-SY5Y human cells, rat and mouse primary neurons, both under basal condition and after cotreatment with THC (10-100nM). Regarding MOR-CB1 heteromerization, THC reverted reduced wt-MOR-wt-CB1 colocalization (Pearson's coefficient=0.5341, $p<0.001$) induced by morphine (Pearson's coefficient=0.3795) in transfected HEK293 cells. In V82A-MOR-wt-CB1-HEK293 cells, morphine did not alter heterodimerization (Pearson's coefficient=0.6066), while THC increased receptor colocalization (Pearson's coefficient=0.7011, $p<0.001$). Under basal condition, THC co-administration significantly potentiated morphine-mediated inhibition of adenylyl cyclase in rat cortical primary neurons ($IC50_{morphine}$ =1.63±0.31nM vs $IC50_{morphine+THC}$ =1.46±0.62, $p<0.05$). Following prolonged MOR stimulation, in rat cortical, striatal primary neurons, and differentiated SH-SY5Y cells THC was able to rescue the diminished response to morphine. Our results indicate that co-administration of THC improves morphine effects, both under basal conditions and following prolonged MOR stimulation; moreover, the analysis of MOR-CB1 heterodimerization has highlighted residues important for receptor colocalization. Thus, supporting that an optimal stimulation of the CB1 receptor in combination with morphine may improve chronic pain management. Supported by QSPainRelief (H2020 grant agreement n.848068).

Sex differences in opioid-induced tolerance and hyperalgesia.

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Although opioid analgesics exhibit potent antinociceptive effects, various side effects including antinociceptive tolerance limit their effective clinical use. Our previous studies show that receptor transporter protein 4 (RTP4), one of the receptor chaperone proteins, contributes to the mechanism of development of tolerance to morphine. Interestingly, studies suggest that opiate exposure can paradoxically induce sustained hyperalgesia that may manifest as antinociceptive tolerance. Not much is known about any role for RTP4 in opiate induced hyperalgesia. Here, we describe studies examining changes in the pain threshold of male and female mice, and in levels of RTP4 mRNA as well as of several cytokines in the spinal cord or dorsal root ganglion (DRG).

We found that repeated administration of morphine (10 mg/kg, s.c. daily for 10 days) leads to antinociceptive tolerance in both male and female mice. We also observe a decrease in mechanical pain threshold (i.e., hyperalgesia) only in female but not in male mice. Interestingly, we find that RTP4 mRNA levels in the hypothalamus tend to increase in male but not in female mice. Moreover, levels of IL-1beta and TNF-alpha mRNA in DRG and spinal cord tend to increase in female but not in male mice.

These findings suggest that there are sex differences in the mechanism of development of opiate-induced tolerance or hyperalgesia. Future studies will comprehensively evaluate the role of RTP4 and the inflammatory cytokines including IL-1beta and TNF-alpha in opiate-induced tolerance or hyperalgesia.

Perinatal Fentanyl Exposure in a Rat Model: Investigating Long-Term Neurobehavioral Consequences and Implications for Neonatal Opioid Withdrawal Syndrome

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Background: Neonatal Opioid Withdrawal Syndrome (NOWS) is associated with long term neurobehavioral deficits and is currently increasing in prevalence due to opioid use by pregnant women. There is an urgent need for preclinical models that accurately recapitulate perinatal drug use. Fentanyl, a highly potent synthetic opioid, is particularly understudied in this context, especially considering that it is the leading driver of the opioid epidemic. Here, we describe a novel rat model of perinatal fentanyl exposure which will allow us to examine long-term neurobehavioral deficits and ultimately characterize the neural mechanisms that govern these consequences.

Methods: Adult male and female heterogeneous stock rats were genetically characterized as high responders for opioid intake. Dams were implanted with an osmotic minipump containing fentanyl solution prior to establishing breeding pairs. Control dams were implanted with identical minipumps containing saline. Minipumps remained in the dams throughout the entire pregnancy and until pups were weaned at P21. Beginning at P21, pups were assayed for various physiological and behavioral deficits that resulted from perinatal fentanyl exposure including body weight, spontaneous withdrawal symptoms, sucrose splash test, mechanical nociceptive Von Frey test, tail immersion test, and fentanyl self-administration.

Results: Fentanyl-exposed pups were severely underweight at P35 and onward when compared to controls. They also exhibited severe somatic withdrawal symptoms, as well as behavioral deficits in the sucrose splash test and elevated plus maze, and allodynia in the Von Frey mechanical sensitivity test. Fentanyl-exposed pups demonstrated dysregulated addiction-like behaviors when allowed to self-administer fentanyl as adults, including significantly increased fentanyl intake and fentanyl-seeking behaviors in both drug- and cue-induced reinstatement tests, as well as dysregulated motivation for fentanyl in a progressive ratio test.

Conclusions: Our findings demonstrate that perinatal fentanyl exposure via osmotic minipump serves as a reliable model for human opioid use during pregnancy. Fentanyl-exposed pups exhibit behavioral withdrawal symptoms and long-term behavioral deficits like those observed in humans with NOWS.

Mu opioid receptors are expressed in mouse islet of Langerhans and exhibit basal activation.

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Exogenous and endogenous opioids have been shown to dynamically influence circulating metabolic hormones like insulin and glucagon. Glucagon and insulin are hormones that are produced in the endocrine pancreas by alpha and beta cells respectively. The insulin producing beta-cells are particularly noteworthy, as these are the cells that become damaged/dysfunctional in Type 1 and Type 2 Diabetes Mellitus. While systemic effects of opioids are known to impact glucose homeostasis, there is also evidence showing that mu opioid receptors (MOPRs) have a direct mechanism of action on pancreatic islets themselves. For example, selective antagonism of MOPRs has been shown to increase glucagon secretion from human islets.

Despite data indicating a role for MOPRs on islets, almost nothing is known regarding the mechanism. Further, while islet secretion data indicates functional MOPRs are expressed on islets, protein expression has yet to be authenticated. Similarly, functional cellular signaling assays have yet to be established in islets.

Through western blotting, I provide compelling evidence for MOPR (and other opioid receptor subtypes) in addition to enkephalin protein expression in isolated murine islets. Furthermore, ongoing work using C-tail phosphorylation site specific antibodies directed at pT370, pS375, pT376 and pT379 will be utilized to survey MOPR phosphorylation through immunoblotting under a variety of conditions on ex vivo murine islets. This will include treatments of selective MOPR agonists (DAMGO) and antagonists (CTAP) at low (1mM) and high (11mM) glucose concentrations. To support immunoblotting data, a FRET based cAMP accumulation assay was utilized to investigate how MOPR recruits Gαi signaling pathways in murine islets. Preliminary data suggests antagonism through pre-treatment of 10μM CTAP increases cAMP accumulation 2-fold, while agonism through 10μM DAMGO treatment has no effect, suggesting endogenous activation in this cellular context.

Neuropathic pain is associated with approximately 50% of diabetes mellitus patients and is frequently treated with MOPR agonists as analgesics. Given the potential interaction between MOPR on endocrine pancreatic function and the frequent use of opioid analgesics in diabetic neuropathic pain, understanding the role opioid pharmacology has in this cellular context has potential clinical significance.

Modulation of kappa opioid receptor efficacy by targeting subpockets

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Introduction: Chronic pain affects 1 in 5 Americans and is largely treated with agonists that act on the μ opioid receptor (MOR), however, misuse of MOR drugs has led to the opioid epidemic in the US. Drugs targeting the κ opioid receptor (KOR) may be a viable alternative to MOR agonists as analgesia can be achieved without respiratory depression; additionally, there is evidence that the β-arrestin2 pathway may mediate KOR dysphoria through p38 MAPK. We hypothesize that KOR ligands displaying low efficacy and minimal β-arrestin2 recruitment may give analgesia with decreased KOR side effects.

Methods: A novel morphinan-based KOR ligand, VRB37, was designed using the previously solved structure of MP1104 bound KOR and characterized using a G_{i1} protein activation bioluminescence resonance energy transfer (BRET) assay and a β-arrestin2 recruitment BRET assay in HEK293T cells. A complex of KOR-Gαβγ-ScFv16-VRB37 was expressed in Sf9 cells, purified using size exclusion chromatography and a cryo-EM structure was determined. Further VRB37 analogues were made to probe the ligand-receptor interaction and characterized using BRET.

Results: VRB37 displayed high potency for G protein activation, and efficacy 80% of standard agonist U50,488h. VRB37 displayed very low potency and efficacy (25%) for β-arrestin2 recruitment, whilst MP1104 displayed robust high efficacy β-arrestin2 recruitment (102%). Our cryo-EM structure of VRB37 bound to KOR revealed that VRB37's amidophenyl arm was positioned towards transmembrane (TM) domain 5 and extracellular loop 2 (ECL2) with potential interactions with $SER211^{ECL2}$ and $ASP223^{5.35}$. Overlay with the crystal structure of KOR-MP1104 highlighted that this arm was binding in a distinct subpocket that MP1104 does not occupy. Further VRB37 analogues revealed that substituting VRB37's nitrogen in the amidophenyl arm to an oxygen or carbon led to an increase in β-arrestin2 recruitment (76% and 142% respectively) with no change to G protein activation.

Conclusions: We hypothesize that ligand interaction with TM5/ECL2 and decreased TM2/3 interaction leads to decreased β-arrestin2 recruitment efficacy. We are currently exploring the binding positions of the VRB37 analogues using docking and molecular dynamics, as well as investigating downstream of the β-arrestin2 pathway by measuring mTOR and p38 phosphorylation.

Exploring the Pharmacodynamic Properties of Fentanyl: A Comparative Analysis of Intraperitoneal Injection and Vapor Self-Administration in mice.

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Abstract: Amidst the ongoing opioid crisis, there is a critical need for research to comprehend the underlying mechanisms of substance use disorders and investigate potential treatments. While intravenous fentanyl administration stands as a gold standard in mouse studies, it presents challenges such as difficulty in placing catheters in mice and frequent catheter failure, hindering experimental progress. This study introduces an innovative vapor self-administration model for fentanyl delivery, aiming to evaluate the pharmacodynamic properties of fentanyl when administered passively through vapor.

Methods: A series of experiments were conducted involving three groups of mice: intraperitoneal injection of fentanyl, passive vapor self-administration using equivalent doses, and a control group for each experimental group. The analgesic effect of fentanyl was assessed through the hot plate test over a period ranging from 5 to 120 minutes. Mice were placed on a hot plate, and specific signs were evaluated within a 30-second window following fentanyl administration.

Results: The analgesic effect of fentanyl was examined using the hot plate test, and a dose-response curve was constructed, revealing comparable responses in mice for both intraperitoneal injection and passive vapor self-administration of fentanyl.

Conclusions: Utilizing the vapor self-administration model represents a promising advancement in the field research of substance use disorders. Our data sheds light on this method from a pharmacological perspective, demonstrating a robust analgesic effect of fentanyl through vapor self-administration, comparable to intraperitoneal administration. Further experiments are warranted to explore this methodology's full range of properties.

Key words: Fentanyl; Vaopr-self administration; Opioids; Substance Use disorders.

Fos-TRAP reveals distinct brain-wide neural activation patterns after initial morphine exposure and after morphine sensitization within the same animal

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Fos-TRAP reveals distinct brain-wide neural activation patterns after initial morphine exposure and after morphine sensitization within the same animal

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Morphine is widely used for the management of pain in clinic. It is also highly rewarding, and its repeated use can lead to tolerance, dependence and addiction. Repeated, intermittent morphine exposure produces behavioral sensitization that is thought to contribute to drug craving, compulsive use, and relapse. Thus, gaining a greater understanding of morphine sensitization offers valuable insights into the neural mechanisms driving addiction. To better understand the neural dynamics underlying morphine-induced sensitization, we used Fos-TRAP technology to capture the brain-wide neural activation pattern elicited during the initial morphine exposure on Day1, i.e., capturing neural activation by tdTomato reporter gene expression in the living animal. Mice with this Fos-TRAP labeling were then allowed to go on to experience the full course of morphine-induced sensitization. Following morphine challenge in sensitized mice on Day 14, we evaluated brain-wide neural activation by classic cFos protein immunohistology. Using the high-throughput cell analysis of NeuroInfo software, we examined brain-wide neural activation patterns during both acute morphine exposure and morphine-induced sensitization in the same animal.

Our preliminary results reveal distinct brain-wide patterns of neural activation following the initial morphine exposure on Day 1 and after the morphine challenge in sensitized mice on Day 14, suggesting that there are not only quantitative but qualitative changes in network activity induced by sensitization. To test this idea, we are using correlation-based seed node analyses to identify specific brain-regions that act as primary neural coordinating sites (nodes) under acute morphine vs. sensitization. Our evidence shows that, indeed, different seed brain regions are involved in coordinating network activity under acute morphine vs. sensitized conditions. This demonstrates that morphine sensitization alters network connectivity and offers a glimpse of the dynamic circuit reorganization that occurs during morphine sensitization.

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Multiplexed pharmacological targeting of prefrontal cortex-projecting locus coeruleus neurons drives antinociception

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Optogenetic and chemogenetic approaches targeting discrete neural circuits are powerful research tools. The required genetic access to critical neurons for these tools, however, hinders translational potential. In this vein, we propose a framework for identifying the genetic tool-independent, pharmacological strategies for neural circuit-selective modulation. As a testbed for this approach, we use the mouse locus coeruleus (LC) due to its diverse physiological functions through the widespread, modular-organized norepinephrinergic (NE) efferent as well as the rich expression of wide variety of endogenous GPCRs and other cell-surface receptors. First, we applied agonists targeting 18 different GPCRs to reveal the pharmacological profile of LC neurons. Using a multiplexed pharmacological approach leveraging techniques from calcium imaging and machine learning-based deconvolution in brain slices, we found that the application of selective agonists yielded differential effects on the firing rate between anatomically-defined LC populations, in particular, LC neurons with efferent projections to the medial prefrontal cortex (mPFC). Multiple ligands convergently shifted the neural activity away from mPFC-projecting LC module, including the mu opioid agonist (MOR) DAMGO, 5HTR1a agonist 8OH-DPAT, and mAChR1 agonist McN-A-343. One of the physiological roles of NE release in mPFC is pronociception elicited by the activation of mPFC-projecting LC neural population. We next test whether our slice results could extend in vivo by determining whether a local infusion of pharmacological cocktails derived from the slice experiment could elicit an antinociceptive outcome in thermal nociception by dampening the NE release in mPFC. Surprisingly, the application of cocktail A (DAMGO : 8OH-DPAT = 1:4) caused a synergistic antinociception compared to single compound infusions; and cocktail B (DAMGO : 8OH-DPAT : McM-A-343 = 1:4:5) resulted in a greater antinociceptive effect than other infusions. Moreover, modular knockout of MOR in mPFC-projecting LC neurons disrupted the enhancement of antinociception driven by cocktail B, while not affecting cocktail C (8OH-DPAT : McN-A-343 = 4:5). Here we demonstrate a novel antinociceptive approach through multiplexed pharmacological strategies. This framework has a board utility for selective targeting of other brain regions under different physiological and pathological states, hence facilitating translational applications from circuit neuroscience.

Development of PACAP and PAC1 targeting therapies for the Treatment of Opioid-Induced Hyperalgesia/Medication Overuse Headache

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Opioids are commonly prescribed for the treatment of pain and headache disorders. However, chronic opioid use can result in a paradoxical increased sensitivity to pain as well as an extension of pain area known as opioid-induced hyperalgesia (OIH) or medication overuse headache (MOH). Using an unbiased proteomic screen, our lab has identified the pituitary adenylate cyclase activating polypeptide (PACAP) as a possible target for OIH/MOH.

PACAP binds to the excitatory G protein coupled receptor, PAC1, and previous studies from our lab have demonstrated the effectiveness of a peptide PAC1 antagonist M65 in preclinical models of MOH.

An antibody targeting PACAP has recently shown promise in a Phase II clinical trial for the treatment of migraine. In addition, novel small molecule PAC1 antagonists have also been recently characterized. In this study, we tested a PACAP targeting antibody and a novel small molecule PAC1 antagonist, in models of chronic migraine and opioid-induced MOH.

We found that PACAP antibody effectively blocked established cephalic allodynia in MOH models, and pretreatment also prevented the development of MOH/OIH to chronic opioid treatment. Additionally, the PAC1 antagonist blocked migraine and MOH-associated allodynia, and co-treatment of the PAC1 antagonist with morphine also prevented the development of MOH.

Together, our results suggest that chronic opioids lead to allodynia by PACAPergic mechanisms, and that PACAP or PAC1 targeting therapies may be an effective therapeutic target for opioid-induced hyperalgesia/medication overuse headache.

Investigating the role of locus coeruleus mu opioid receptors in drug reward

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Activity of the locus coeruleus noradrenergic (LC-NE) system is upregulated during opioid withdrawal and is responsible for shifting cognitive resources towards salient stimuli. As such, the LC has a complicated role in drug seeking behavior. The LC densely expresses mu opioid receptors (MORs). While these intra-LC MORs have not been directly attributed to drug reward, LC MORs are activated with the cessation of stress. We also know that stress potentiates the likelihood of drug relapse. Taking this together, we sought to study the role of intra-LC MORs in opioid reward. To do this, we bred a MOR conditional knockout (Dbh-Cre x oprm1$^{fl/fl}$; MOR cKO) mouse where MOR expression is significantly reduced in the LC. These mice have increased nociception, but otherwise do not appear to have altered learning, memory, motivation, or baseline anxiety-related behaviors. To determine whether noradrenergic MORs alter opioid reward, we performed a morphine conditioned place preference (CPP) test. Results from our initial CPP experiment suggest MOR expression in the LC is critical for behavioral responses to drug intake. However, it is unknown whether intra-LC MORs gate sensitivity to drug reward or conversely, promote opioid withdrawal-induced negative affect. To further disentangle this, we are performing a two-bottle choice paradigm, comparing ingestion of fentanyl with water consumption over five days to assess capacity to experience opioid reward. If MOR expression in the LC gates sensitivity to drug reward, we expect the MOR cKO mice to demonstrate a greater preference for fentanyl. We will also evaluate affective behavior over a three-day withdrawal period following fentanyl ingestion. For this we will observe canonical somatic phenotypes associated with withdrawal and use assays for pain (hot plate test) and avoidance behavior (open field test). If intra-LC MORs promote opioid withdrawal-induced negative affect, we would expect the MOR cKO to display more withdrawal phenotypes, higher thermal sensitivity, and more avoidance behavior. Results from these experiments will inform our understanding of the role of intra-LC MORs in opioid intake.

A Decrease in Perineuronal Nets in the Mesencephalic Reticular Formation is Observed in Mouse Models of Migraine and Opioid Induced Hyperalgesia.

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The development of novel therapeutics for migraine has been hindered by the incomplete understanding of the mechanisms that regulate headache disorders. A better understanding of the molecular and cellular contributors to the development of headache could lead to new therapeutic targets. Specialized condensed extracellular matrix structures known as perineuronal nets (PNNs) have recently been implicated in the modulation of inflammatory and neuropathic pain. Our lab has identified a cluster of PNNs surrounding a population of parvalbumin (PV) cells in the mesencephalic reticular formation (MRF), a brain region that innervates regions involved in pain-processing. The present study investigated the effect of two different mouse headache models on PNN integrity: a nitroglycerin model of chronic migraine, and a model of opioid-induced medication overuse headache (MOH)/opioid induced hyperalgesia. Male and female C57BL6/J mice were tested in these two models, and brains were collected 18-24 h after the final injection in each paradigm, a time at which maximal cephalic allodynia is observed. Immunohistochemistry was performed to visualize PNNs and PV neurons. The number and fluorescence intensities of PNNs and PV+ cells were analyzed in the somatosensory cortex (SSC), insular cortex, and MRF. PNN numbers did not change following NTG or MOH treatment in any brain region analyzed. In both models, PNN and PV intensity significantly decreased in the MRF in both sexes. MOH also resulted in a significant decrease in PNN intensity in the insula of both sexes with no changes observed in the SSC. Future studies will determine the mechanistic role of MRF PNNs in nociceptive and headache pain processing.

Delta opioid receptor agonists facilitate neuroexcitability in the infralimbic cortex via the PI3K-mTOR pathway in inhibitory neurons to exert antidepressant-like effects

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The delta opioid receptor (DOP) is an attractive target for novel antidepressants due to its potential for rapid action with minimal adverse effects; however, the functional mechanism underlying this action remains unclear. In this study, we investigated the mechanism underlying the antidepressant-like effects of DOP agonists in mice. In the forced swimming test, which is widely used in the screening of antidepressants, an intracerebroventricular administration of a selective mechanistic (or mammalian) target of rapamycin (mTOR) inhibitor, rapamycin, and a phosphatidylinositol-3 kinase (PI3K) inhibitor, LY294002, reversed the decreased immobility counts of mice by a selective DOP agonist, KNT-127. In a highly validated animal model of depression, chronic vicarious social defeat stress mice model, KNT-127 also alleviated the social interaction deficits and decreased sucrose preference thorough the PI3K and mTOR signaling. In immunoblotting assays, KNT-127 increased the phosphorylation level of the mTOR signal-related proteins, Akt and p70S6 kinase, in the medial prefrontal cortex. Moreover, a bilateral microinfusion of KNT-127 and another DOP agonist, SNC80, in the infralimbic cortex (IL-PFC) of the medial prefrontal cortex attenuated the immobility counts in the forced swimming test, which were abolished by rapamycin and LY294002. In whole-cell voltage-clamp recordings, the frequencies, but not amplitudes, of miniature excitatory and inhibitory postsynaptic currents in the IL-PFC increased and decreased, respectively, with the perfusion of KNT-127, which were inhibited by pretreatment with either rapamycin or LY294002. Furthermore, in an immunohistochemical evaluation, 99% of DOP-positive neurons were classified into the parvalbumin-positive interneurons in the IL-PFC. Altogether, these findings indicate that DOP agonists exert antidepressant-like actions through the facilitation of neuronal excitability in the mouse IL-PFC, which is implicated in the PI3K-Akt-mTORC1-p70S6 kinase signal transduction in parvalbumin-positive interneurons. We propose that this study constitute the first step in elucidating the complete mechanical functions of DOPs as a target for novel antidepressants and have a huge impact on the future medication strategy for major depressive disorder.

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