Discovery of Selective Orexin 1 Receptor Ligands for Regulating Motivational Processes

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The dysregulation of the orexin 1 receptor (OX_1R) signaling pathway is implicated in various motivation-related neuropsychiatric disorders such as anxiety, eating disorders, and substance use disorders (SUD). Despite advances in the development of selective orexin 2 receptor (OX_2R) antagonism (2-SORA) for treating sleep disorders, OX_1R is a severely understudied target. Currently, there are no FDA-approved OX_1R antagonists (1-SORA) or agonists. This represents a significant gap in the clinical settings, given that OX_1R hyperfunction is associated with anxiety, fear, compulsive and addictive behaviors. Extremely relevant is the therapeutic potential of 1-SORA for Substance Use Disorders (SUD) decreasing orexinergic activity in the VTA and NAc, while also modulating dopamine-dependent reward mechanisms. Moreover, stress, anxiety and panic-state represent powerful drivers for drug-seeking behaviors and relapse. 1-SORA can rebalance these emotional states by blocking orexin signaling in the amygdala, BNST and PVT, while avoiding drowsiness associated with OX_2R antagonism.

In this work our fragment-based and structure-based drug design yielded potent, subtype-selective OX_1R antagonists. We have identified new pharmacophores, key ligands conformational changes and substitution patterns leading to antagonists with >30-fold OX_1R selectivity validated in radioligand binding assays and Gq-dependent functional assays (IP1 accumulation and FLIPR Ca^{2+} mobilization assays). A critical insight revealed the importance of an azepanyl-, or a pyrrolidine-based, linker combined with sterically hindered aryl groups in driving OX_1R selectivity over OX_2R. The smaller serine residue in OX_1R (S103) relative to threonine in OX_2R (T111) confers greater tolerance for bulkier aromatic substituents, providing a reliable structural handle for engineering subtype selectivity. The successful identification of selective OX_1R antagonists represent a significant advancement in elucidating orexin pharmacology and elevate OX_1R to key therapeutic target for modulating motivational processes.

Looking further ahead, the structural and SAR framework established through this program will guide next-generation design iterations, further promoting *in silico*-guided pharmacophore optimization. Moreover, leveraging SAR findings and binding pocket insights, it might potentially offer a platform to the rational and translational design toward agonists for treating orexin hypofunction associated with anhedonia, diminished motivation, and depressive-like phenotypes where OX_1R is another central pathological driver.

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Sex Differences in Acute Pharmacokinetic and Pharmacodynamic Responses to High-Potency Δ9-Tetrahydrocannabinol in Rats.

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The consumption rates of high-potency cannabis products continue to rise, with Δ⁹-tetrahydrocannabinol (THC) concentrates surging from a 4% historical threshold in the 1990s to a 20-fold elevation of 50–95% in the United States. The health impact of these high-potency products remains, however, only partially understood. We investigated the pharmacokinetic (PK) and pharmacodynamic (PD) effects of acute high-dose THC (10 and 30 mg/kg, intraperitoneal, IP) compared to a lower dose (3 mg/kg, IP) in male and female rats. Concentrations of THC and its metabolites in plasma, brain, and white adipose tissue were quantified through short-term (24 hours) and long-term (14 days) timelines following a single administration. Ingestive behavior, pica, locomotor activity, spontaneous activity, anxiety-like and stress-coping reactions, nociception, and multiple plasma cytokine levels were measured. PK effects were sex dependent, as females had markedly greater systemic and tissue exposure with THC levels sustained for up to 14 days and extensive sequestration in adipose with exposure up to ~4-fold higher in females than in males. PD effects of THC were also dose- and sex-dependent, including suppression of body weight and food/water intake (e.g., near-minimal food intake at 30 mg/kg); temporary locomotor stimulation in females at low doses and depletions at high doses; increased pica in females at 30 mg/kg; mild anxiety-like shifts; bidirectional stress-coping changes in males; and antinociception, mostly in males at 1 hour and 24 hours, while female rats were seen to have increased proinflammatory and anti-inflammatory cytokines in a dose- and time-dependent manner. The findings show that high-dose THC induces sex-dependent nonlinear PK with extended central and peripheral retention linked to distinct behavioral outcomes as compared to a lower dose (3 mg/kg) investigated in parallel. These results emphasize the significance of potency in determining the quantity and quality of cannabis outcomes and the necessity of additional research on high-concentration products to understand the potential health hazards associated with cannabis use.

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A Replication Study of Cocaine Reinforcement in Spontaneously Hypertensive Rat Substrains: Phenotypic Expansion to F2 Offspring, Ultrasonic Vocalizations, Transcriptomics, and Reciprocal F1 Crosses

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Stimulant use disorders are on the rise. Despite high heritability, few genetic loci have been identified. Genetic mapping of addiction-relevant traits in near-isogenic rodent substrains can facilitate causal gene identification. We reported enhanced cocaine intravenous self-administration (IVSA), impulsivity, and compulsivity in SHR/NCrl vs. SHR/NHsd substrains purchased from different vendors. Here we tested for replication of cocaine IVSA differences by breeding substrains and reciprocal F2 crosses in-house to control the environment. Furthermore, we phenotyped cocaine-linked ultrasonic vocalizations (USVs) as a measure of affective state, and transcriptome analysis via bulk RNA-seq of naïve tissue from frontal cortex and striatum of SHR substrains and reciprocal F1 crosses. Adult rats from both substrains and F2 offspring were tested for acquisition of cocaine IVSA, motivation to acquire cocaine under multiple reinforcement schedules, and dose-dependent rates of responding, followed by extinction training and cue-induced reinstatement. USVs were recorded throughout testing. SHR/NCrl rats consistently earned more infusions across schedules, replicating previous findings. Both substrains showed similar extinction rates but SHR/NCrl showed greater reinstatement of lever pressing and USVs following re-exposure to cocaine-paired cues. Transcriptome analysis of striatum from substrains revealed that SHR/NCrl showed downregulated astrocyte and microglial activation (C1qa, C1qb, C1qc, Trem2, Fcgr2b, Ingr1, Ager, Cntf), predicting synaptic remodeling/wiring, upregulated HSPs and chaperone proteins involved in proteostasis/ER_stress/unfolded protein response, and upregulated TLR3->TRIF innate immune signaling (previously associated with increased cocaine reward/plasticity.Transcriptomic analysis of frontal cortex of reciprocal F1 rats identified downregulated lipid biosynthesis (e.g., cholesterol) and perineuronal nets of the extracellular matrix in frontal cortex of F1 rats from the maternal SHR/NCrl lineage. These results were corroborated in striatum, along with a downregulation of myelin, supporting a parent-of-origin effect on lipid-associated cell types/functions across multiple brain tissues. Both substrain x Sex and Cross x Sex analysis of the striatum identified a large number of overlapping RPS and RPL genes associated with “nervous system development”, “axon guidance”, and “neuronal system”, suggesting parental origin-dependent inheritance affects CNS development. To summarize, we replicated and expanded pro-addiction phenotypes of SHR/NCrl rats to new behaviors and revealed substrain- and parental origin-dependent effects on brain function and development in SHR substrains.

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Individual Differences in Stress Reactivity and Alcohol Drinking: The Role of a Dorsal BNST to Lateral Hypothalamus Circuit in Rats

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Understanding the neural basis of individual differences in stress coping and subsequent alcohol misuse may inform strategies to treat comorbid stress and alcohol use disorders. In Wistar rats, individual differences in stress coping emerge after exposure to predator odor in a place conditioning paradigm. While some rats persistently avoid a predator odor-associated context ("Avoiders"), others do not ("Non-Avoiders"). Importantly, Avoider rats display prolonged anxiety-like behavior after stress, suggesting stress vulnerability in this subpopulation. We have demonstrated that Avoider males increase their alcohol drinking after stress whereas Non-Avoiders do not. This stress-induced escalation in alcohol consumption may be driven by negative reinforcement via extended amygdala circuitry including the dorsal bed nucleus of the stria terminalis (dBNST). The overarching goal of this project is to test the role of the dBNST in supporting Avoider and Non-Avoider phenotypes in male and female rats. To test the involvement of the dBNST during predator odor stress in rats, *in vivo* fiber photometry was used to record dBNST calcium-indicated activity during odor exposure. Indeed, overall calcium signal and the number of peaks was greater during odor exposure compared to non-exposed controls. Because the lateral hypothalamus (LH) receives dense dBNST projections and regulates avoidance and arousal, we next tested whether dBNST-to-LH neurons were differentially engaged by stress in Avoiders and Non-Avoiders. Rats were given intra-LH injections of a retrograde tracer (CTB-AF647), stressed and indexed for avoidance, and re-exposed to predator odor before euthanasia for cFos immunohistochemistry. Avoider males showed greater stress-induced recruitment of dBNST-LH neurons compared to Non-Avoiders and females. These findings identify dBNST-LH projection neurons as a candidate circuit underlying Avoider/Non-Avoider phenotypic differences. Upcoming work will test whether functional modulation of dBNST-LH neurons affects stress-induced alcohol drinking and anxiety-like behavior in Avoiders and Non-Avoiders.

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Targeted lipidomics in the CNS show that acute and chronic morphine treatment differentially changes endogenous lipid signaling across the CNS: implications for CNS-based bile acid regulation and novel small molecule lipid signaling pathways

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Preclinical studies suggest that cannabinoid-based therapies targeting the endocannabinoid (eCB) system may serve as effective strategies to treat opioid use disorder, especially symptomatic aspects of withdrawal. The potential mechanisms of action of CB-based therapies and the neurophysiology of withdrawal are largely unknown. In addition to the canonical eCB signaling pathways through CB1 and CB2 with eCB ligands, Anandamide and 2-AG; eCB system-based therapeutics (e.g. CBD, FAAH and MAGL inhibitors) have broad effects on the broader endogenous lipid (endolipid) system. Our lab has shown that modulation of each of these signaling systems has broad effects on the lipidome. Here, we examine the modulation of targeted small molecule signaling endolipids after acute and chronic morphine treatment throughout the CNS and body. We found that he majority of small molecule signaling endolipids in the CNS were reduced across regions in both acute and chronic morphine treatments, though key endolipid species were differentially changed in either acute (all *N*-acyl GABA species) or chronic (all *N*-acyl taurine and *N*-acyl valine species). These differences provide novel insight into differential endolipid signaling from acute and chronic opioid use in the CNS. We also show that corticosterone levels are elevated 2-3-fold within 30 minutes of acute morphine in plasma and 5 key CNS regions and 3 CNS regions, plasma, and liver with elevated levels after chronic morphine. CNS region specificity suggests directed transport and/or local production and provides novel insight into local stress effects. Finally, CNS and plasma levels of bile acids dramatically ***decrease*** with acute morphine and ***increase*** after chronic morphine. Likewise, small molecule endolipids followed this pattern. This switch in production/metabolism, especially in the CNS, may be key in a novel understanding of molecular mechanisms of withdrawal symptoms. These data provide a foundation for understanding how dysregulation of endolipids systemically are part of the pathological shift in neurophysiology with opioid use and provide a novel framework to address endolipid modulation-based therapeutics.

Effects of Repetitive Mild Traumatic Brain Injury on Oxycodone Taking, Seeking, and Accumbal AMPAR Subunit Expression.

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Experiencing repeated mild head injuries such as concussions can alter reward processing and increase addiction liability. Consequently, individuals suffering from traumatic brain injuries (TBIs) are at heightened risk for misusing prescription opioids such as oxycodone used to treat injury-induced pain. This risk may be particularly high following repetitive mild TBIs (rmTBIs), as these individuals often experience persistent pain symptoms that may increase exposure to prescription opioids. However, the neuronal mechanisms through which rmTBI increases prescription opioid addiction and relapse vulnerability remain poorly understood. Drug-associated cues are among the most common relapse triggers. In preclinical rodent models, cue-induced oxycodone seeking progressively increases during abstinence and is widely used as a measure of relapse vulnerability. These time-dependent increases in oxycodone seeking are mediated by enhanced excitatory transmission within the nucleus accumbens (NAc) driven by the accumulation of higher-conductance, calcium-permeable AMPA receptors (CP-AMPARs). CP-AMPARs also accumulate following TBI in brain regions proximal to the injury. However, how rmTBI impacts CP-AMPAR accumulation within the mesolimbic reward circuitry remains unknown. We assessed this using a closed head controlled cortical impact model to induce 3 head injuries to mimic rmTBI. Male and female Sprague-Dawley rats underwent rmTBI or sham procedures beginning at 8–10 weeks of age and tissue was collected 48 hrs following the third and final injury (n=7) or sham surgery (n=9). Interestingly, rmTBI increased NAc GluA1 expression without altering GluA2 expression, consistent with CP-AMPAR accumulation. When a separate group of animals self-administered oxycodone (0.1 mg/kg/infusion; 6 hrs/day for 10 d) 3 days following the final injury, injured animals (n=9) exhibited greater oxycodone intake over the last five days of self-administration compared to sham controls (n=9). These same injured animals showed enhanced cue-induced oxycodone seeking during early abstinence (day 1), a time point at which seeking behavior is low in non-injured animals. Ongoing studies are determining the time course of NAc AMPAR subunit expression to better understand whether these neuroadaptations contribute to the initiation and/or maintenance of injury-induced increases in oxycodone taking and seeking. Together, these findings suggest a potential neurobiological mechanism through which rmTBI alters reward circuitry to increase oxycodone addiction vulnerability.

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Nucleus Accumbens Representation of Heroin and Methamphetamine Self-Administration

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Concurrent abuse of opioids and psychostimulants represents a significant public health concern. Preclinical models of substance use disorder often focus on a single class of drug, despite evidence that opioids and psychostimulants utilize different mechanisms of action. Electrophysiological data suggests that neural activity within canonical reward circuitry differentially represents opioid- vs. psychostimulant-seeking behavior, but these data lack cell-type specificity and longitudinal tracking of individual neurons over the entire course of self-administration. Thus, a within-subjects comparison of the neural bases of opioid vs. psychostimulant self-administration will clarify the extent to which these behaviors are driven by distinct and/or overlapping ensembles.

We used miniscopes to record activity from D1- and D2-expressing NAc neurons *in vivo* and applied machine learning-assisted analyses to characterize behavioral changes throughout concurrent self-administration of heroin and methamphetamine. Rats were trained to perform two distinct operant responses, one to self-administer heroin and the other to self-administer methamphetamine, on alternating days for twenty days, then underwent a drug preference test followed by ten days of forced abstinence and a second preference test.

Combined analysis of neural imaging data and three-dimensional pose tracking data revealed distinct subpopulations of NAc MSNs associated with either heroin lever approach or meth lever approach. The activity of these neurons appeared to correspond to the seeking of a particular drug type: heroin lever approach neurons were not active during methamphetamine lever approach and were similarly active when rats approached the heroin lever during methamphetamine sessions, when the heroin lever was unavailable (and vice-versa). These distinct patterns of neural activation were underscored by qualitative behavioral differences in heroin vs. methamphetamine self-administration. When responding for heroin, rats emitted more “redundant responses,” i.e., responses during the infusion period that had no programed consequences. This higher rate of heroin seeking was mirrored by a general preference for heroin over methamphetamine during both choice tests. In the second choice test, however, we found mixed evidence for incubation of heroin craving, whereas rats consistently demonstrated incubation of methamphetamine craving.

These data reveal subtle neurobehavioral differences between heroin and methamphetamine self-administration and highlight the need for further preclinical research on polysubstance abuse.

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Developing Selective Cannabinoid 2 Receptor Agonists

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Cannabinoid 2 (CB_2) receptors are one of the major receptors of the endocannabinoid system (ECS) and have demonstrated therapeutic promise for nociception, inflammation, and addiction treatment. While activating CB_2 receptors has no known side effects, the other major ECS receptor, cannabinoid 1 (CB_1) receptor, has undesired side effects following activation like motor impairment and addiction. Cryo-EM structures demonstrate that the active state for both CB_1 and CB_2 receptors are largely the same. As they have shared amino acid residues, pinpointing ligand selectivity during development can be difficult. Despite this shared homology, selective CB_2 agonists have been synthesized, so identifying specific parameters that cause selective activation will improve the consistent development of CB_2 selective agonists. Several studies have begun looking into potential reasons for agonist selectivity, such as studying the conformation of both receptors. During activation, the CB_1 receptor shifts into conformation, while the CB_2 receptor is more rigid. This rigidity may indicate a correlation between a constrained structure and selectivity. Furthermore, polar substituents are another area of interest, as increasing polarity seems to offer potential for CB_2 selective activation over CB_1. Through our work, we are exploring the impact of bulkiness, rigidity, and polarity on CB_2 selective agonism using two purine core scaffolds.

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Avoidance of Stress-Related Stimuli Predicts Negative Affect and Alcohol Sensitivity

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Traumatic events may lead to post-traumatic stress disorder (PTSD) in some individuals, characterized by avoidance of stimuli associated with their trauma and negative affect. PTSD is highly comorbid with alcohol use disorder (AUD), and individuals displaying more avoidance symptoms are more likely to escalate their alcohol drinking after stress. Rats show similar individual differences in stress reactivity, with some animals exposed to a “traumatic” predator odor stress showing avoidance of a stress-paired context (Avoiders), while others do not (Non-Avoiders). Following traumatic stress, we have shown that Avoider rats increase their alcohol drinking, recapitulating findings in humans.

The goals of this study are to test if Avoider rats show heightened negative affect-like behaviors after traumatic stress as seen in humans with PTSD, to test the effects of alcohol on these behaviors in Avoiders and Non-Avoiders. In this study, we examined negative affective behaviors in male Avoider and Non-Avoider rats after stress using novelty-suppressed feeding (NSF) and forced swim tests (FST). In the following experiment, we treated animals with a low dose of alcohol (0.5g/kg) prior to NSF testing. Avoiders indeed had more negative affect-like behavior than Non-Avoiders after traumatic stress, and Non-Avoiders were sensitive to the anxiolytic effects of alcohol. The effects of alcohol on negative affect-like behaviors and neuronal activation (as measured via c-Fos expression and fiber photometry) in the lateral habenula (LHb) of Avoiders and Non-Avoiders will be investigated in the future.

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Mechanistic Characterization of Recycling Pathways for Buprenorphine and Structurally Related Opioids

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Buprenorphine is a cornerstone medication for opioid use disorder, yet its clinical use is complicated by variable systemic exposure, narrow safety margins, and potential drug–drug interactions. Although this variability is commonly attributed to absorption, metabolism, and transporter activity, the contribution of recycling pathways remains unclear. Classical enterohepatic recycling (EHR), in which hepatically formed glucuronides are excreted into bile and potentially deconjugated in the intestine, may prolong systemic exposure. Hepatoenteric recycling (HER), where glucuronides are first formed in the intestine, taken up by the liver, and secreted into bile, has been less explored. This study aimed to determine whether buprenorphine and structurally related opioids undergo EHR, HER, or both, and to define mechanisms relevant to opioid exposure and therapeutic optimization.

We integrated in vitro, in situ, and in vivo approaches to define the mechanistic basis of recycling. Caco-2 permeability studies assessed whether parent opioids could cross the intestinal epithelium, supporting intestinal exposure and potential recycling. Hepatic and intestinal microsomal incubations compared tissue-specific glucuronidation, while in situ intestinal perfusion directly evaluated intestinal glucuronide formation under physiologically relevant conditions. Most parent opioids showed high intestinal permeability, consistent with passive diffusion. Among the tested opioids, buprenorphine was the only compound that generated detectable glucuronide in intestinal perfusate, with buprenorphine glucuronide accounting for 8.85% of the perfused dose. This supports intestinal glucuronidation as a key initiating step for HER.

Portal vein infusion studies were then used to determine whether opioid glucuronides undergo hepatic uptake and biliary excretion. Opioid glucuronides showed higher hepatic extraction than their corresponding aglycones, including buprenorphine glucuronide versus buprenorphine (80.0% vs. 18.1%), naloxone glucuronide versus naloxone (67.5% vs. 12.4%), and morphine-6-glucuronide versus morphine (40.5% vs. 25.1%). Rifampicin co-infusion reduced biliary excretion of buprenorphine glucuronide, morphine-3-glucuronide, and naloxone glucuronide, supporting transporter-mediated hepatic uptake. In vitro uptake studies further identified OATP1B3 as a major transporter involved in opioid glucuronide uptake.

Together, these findings demonstrate that buprenorphine disposition involves integrated HER and EHR pathways mediated by intestinal glucuronidation, hepatic uptake, and biliary excretion. These recycling mechanisms may represent underrecognized determinants of buprenorphine exposure, safety, and therapeutic response.

Neurometabolic and synaptic correlates of heroin addiction vulnerability: a PET study in rats.

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Background: Only a subset of heroin users develop addiction, yet determinants of addiction vulnerability remain unclear. We developed a rat model featuring choice between heroin and social interaction that produces substantial individual variability in heroin intake, binge-like taking, and preference. We combined this model with PET imaging to assess relationship between addiction vulnerability and brain metabolism (using FDG), and structural changes in synaptic density (using SynVesT-1).

Methods: In Exp. 1, we trained rats to self-administer heroin (or saline) and social interaction, then tested heroin- and social-seeking and heroin-vs.-social choice. During 3-5 abstinence weeks, we used FDG and scanned awake rats at rest and during social- and heroin-seeking tests. In Exp. 2, using the same model, we scanned rats before heroin self-administration (SA), after choice, and after 3 abstinence weeks using FDG and SynVesT-1.

Results: In Exp. 1, addiction severity was primarily associated with metabolic alterations at rest, rather than during reward-seeking. At rest, severity was associated with lower uptake in piriform cortex and higher uptake in ventral hippocampus. During heroin seeking, severity was associated with lower uptake in postsubiculum and cerebellum. Severity was not associated with social SA and social seeking at early and late abstinence, or FDG uptake during social seeking. In a pilot study, we scanned high- and low-severity rats from Exp. 1 with SynVesT-1 and found reduced whole-brain uptake in high-severity rats. Data from Exp. 2 will be presented at the meeting.

Conclusions: Addiction severity is primarily associated with resting metabolic and synaptic alterations, rather than reward-seeking brain activity.

STRUCTURE-BASED DISCOVERY OF NOVEL GPR34 CHEMICAL PROBES THROUGH VIRTUAL SCREENING AND IN-VITRO EVALUATION.

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Lysophosphatidylserine (LysoPS) has emerged as a bioactive lipid of significant interest due to its involvement in a range of physiological and pathological processes. Efforts to elucidate the molecular mechanisms underlying LysoPS signaling led to the identification of GPR34 as its cognate receptor, mediating LysoPS-dependent signaling pathways. GPR34 is widely expressed across immune cells in the nervous system and peripheral tissues, where its activation regulates key processes including phagocytosis of Aβ fibrils, pain modulation, resolution of inflammation, and tissue repair. Notably, dysregulated GPR34 signaling has also been implicated in proinflammatory and pathogenic roles in disorders such as neuropathic pain, cancer, and multiple sclerosis. Accordingly, both selective GPR34 agonists and antagonists represent promising therapeutic strategies, with potential applications spanning immune modulation, inflammation control, and analgesia. Here, we report a structure-based discovery of novel small-molecule GPR34 ligands with distinct modes of action, providing a foundation for the therapeutic targeting of GPR34.

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Optimization of a Translationally Relevant Mouse Model for Binge Eating and Binge Drinking Comorbidity in Outbred Stocks of Mice

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Although eating disorders and alcohol use disorder (AUD) are heritable, few genetic loci have been identified that influence their comorbidity. To address this gap, the Szumlinski Lab developed a behavioral model to investigate cross-sensitization between binge-like eating (BE) and binge-like drinking (BD) in mice, whereby C57BL/6NJ mice showed cross-sensitization in a sequential BE-BD paradigm. Here, we sought to optimize the model and its generality by examining how BE history influences subsequent BD in two outbred mouse stocks.

CD-1 and CFW mice underwent a sequential BE-BD paradigm comprising 2 weeks of BE followed by 2 weeks of BD (“short timeline”), 4 weeks of BE followed by 4 weeks of BD (“intermediate timeline”), or 6 weeks of BE followed by 4 weeks of BD (“long timeline”; CFW only). BE was induced using a limited access procedure where mice were presented with sweetened palatable food (SPF) pellets for 30 min, 5 days/week. BD was subsequently assessed using a modified drinking-in-the dark (DID) procedure. Mice underwent 9 (short timeline) or 19 (intermediate timeline) daily (Mon-Fri) 2-h sessions followed by 1 final 4-h session. An intermittent access DID paradigm was used for the long timeline where CFW mice underwent weekly 2-h sessions on Monday and Wednesday, and a 4-h session on Friday for 4 weeks.

In the short timeline, CFW mice consumed more SPF but drank less ethanol than CD-1 mice. During the intermediate timeline, CFW mice escalated SPF intake more than CD-1 mice, and CFW females displayed enhanced ethanol intake compared to all other mice. In the long timeline, female and male CFW mice continued escalating SPF intake across sessions. Surprisingly, we did not observe immediate enhancement of BD after 6 weeks of BE. These findings indicate that CFW mice, especially females, are more prone to BE and subsequent enhancement of ethanol drinking. Cross-sensitization between BE and BD is contingent on the number of BE sessions, and 4 weeks of BE testing may be optimal for this behavioral model in outbred mice. Future studies will use this procedure to test pharmacotherapeutics and screen additional genetic backgrounds with the goal of conducting quantitative genetic studies.

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Purinergic P2X7 Receptor Antagonist Reduces Oxycodone Self-Administration and Relapse in Rats

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The only FDA-approved treatments available for opioid use disorder (OUD) - methadone and buprenorphine – are opioids with addiction liability, highlighting a need to investigate non-opioid therapeutic targets. One such candidate is the P2X7 receptor, a purinergic receptor activated by extracellular ATP. Activation of P2X7 increases release of pro-inflammatory cytokines and dopamine, both of which facilitate drug reinforcement and relapse. However, the role of P2X7 in substance use disorders is poorly understood and limited to psychostimulants, where blocking P2X7 reduces rewarding effects of methamphetamine and 3,4-methylenedioxypyrovalerone (MDPV). We determined if a selective P2X7 receptor antagonist (A438079) (10, 30 mg/kg) would attenuate oxycodone self-administration (SA) and relapse behaviors in rats. Under a fixed-ratio (FR1) schedule, A438079 (30 mg/kg) reduced oxycodone infusions across multiple doses of oxycodone (0.007, 0.01, and 0.056 mg/kg/inf), causing a downward shift of the dose-response curve without affecting inactive lever responding. Following 14 d of forced abstinence, A438079 (30 mg/kg), administered before a cue-induced relapse test, reduced active lever responding for oxycodone seeking. A438079 (30 mg/kg) also reduced sucrose pellet SA under FR1 conditions but did not reduce sucrose relapse. Finally, the existence of P2X7 on neurons in the mesolimbic system has not been properly characterized. Immunohistochemistry on neurons show that P2X7 is on dopaminergic neurons and GABAergic neurons in the VTA. Our results provide evidence that P2X7 receptor antagonism reduces opioid SA and relapse, potentially through antagonism of P2X7 receptors in the VTA.

Neutral CB1 Receptor Antagonism Suppresses Alcohol Seeking and Effort-Based Motivation.

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Motivational dysregulation and excessive alcohol seeking are central features of alcohol use disorder (AUD), with converging evidence implicating cannabinoid type-1 receptor (CB1R) signaling in AUD. However, the role of the endocannabinoid system in alcohol-related motivation and effort expenditure remains largely unexplored. Here, we examined the behavioral effects of the neutral CB1R antagonist AM6527 on spontaneous behavior, ethanol seeking, and effort-based responding in male mice.

Using machine learning–based 3D motion sequencing (MoSeq), AM6527 affected spontaneous behavior, increasing self-directed actions and suppressing locomotor-related syllables. In operant paradigms using 20% ethanol reinforcement, AM6527 robustly suppressed alcohol-directed responding across all doses tested, decreasing center-port head entries, total rewards earned, and correct nose-poke responses under a fixed ratio-3 (FR3) schedule. These effects closely paralleled suppression observed under milk reward reinforcement, indicating a generalized reduction in motivated reward seeking rather than reinforcer-specific effects. Ongoing work uses c-Fos immunohistochemistry to identify neural circuits engaged by AM6527 during ethanol and non-ethanol operant behavior.

Together, these findings demonstrate that the CB1R-mediated inhibition of the endocannabinoid system robustly suppresses alcohol seeking and effort-based motivation, providing mechanistic insight into cannabinoid regulation of alcohol-related behavior and identifying CB1R-neutral strategies as potential modulators of AUD-relevant motivational processes.

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Design and Evaluation of Dual FAAH/ABHD6 Inhibitors

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The endocannabinoid system is an expansive network associated with several physiological processes, making it an intriguing therapeutic target for various applications relating to reward signaling, neuropathic pain, and mood disorders among others. The endocannabinoids anandamide (AEA) and 2-arachadonylglycerol (2-AG) act on cannabinoid receptor 1 (CB1) and cannabinoid receptor 2 (CB2) to mediate signaling primarily in the central nervous system and peripheral nervous system respectively. The major enzymes responsible for regulation of this signaling are fatty acid amide hydrolase (FAAH) which degrades AEA and monoacylglycerol lipase (MGL) which degrades 2-AG. Inhibition of FAAH and subsequent increase in AEA levels has been shown to have therapeutic potential in reducing neuroinflammation, anxiety, depression, and pain. Similarly, inhibitors of MGL have been studied as well as dual FAAH and MGL inhibitors for the treatment of pain. While MGL degrades 85% of 2-AG, another enzyme, α/β-hydrolase domain-containing 6 (ABHD6), is responsible for 4% of 2-AG degradation. MGL inhibitors have been shown to cause CB1 desensitization due to the large increase in 2-AG concentration which may result in adverse psychological effects. Additionally, MGL has a presynaptic expression while ABHD6 has a postsynaptic expression which suggests the potential for spatial and temporal control of 2-AG levels. ABHD6 is highly expressed in the cerebellum, frontal cortex, hippocampus, and striatum. There is especially promise for modulation of cannabinoid signaling in the cerebellum, as ABHD6 is the dominant enzyme responsible for 2-AG degradation in this brain region. Overall, inhibition of ABHD6 offers opportunities for modulating the endocannabinoid system which are unique from inhibition of MGL. For these reasons, FAAH and ABHD6 were selected as targets for inhibition to increase signaling of both CB1 and CB2 in the specified brain regions by decreasing degradation of AEA and 2-AG. Novel small molecules were designed and characterized for inhibitory activity and selectivity.

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Preclinical Evaluation of Nr4a1 as a Therapeutic Target for Cocaine Use Disorder.

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Cocaine use disorder (CUD) is a chronic neuropsychiatric disorder affecting approximately 2.4 million Americans in 2019[1-3], yet there are currently no FDA-approved pharmacological treatments. Previous work from the Heller Lab demonstrated that activation of Nr4a1 (Nur77) by the naturally occurring small molecule Cytosporone B (Csn-B) attenuates cocaine-seeking behavior in cocaine-primed mice[4,5]. This occurs through epigenetic regulation of the Cartpt gene, which encodes cocaine- and amphetamine-regulated transcript (CART). As a key regulator of mesolimbic dopamine signaling within the striatum, Nr4a1's regulation of Cartpt represents a promising therapeutic target for CUD. However, the therapeutic potential of Csn-B is limited by poor bioavailability resulting from rapid metabolic degradation.

To improve the pharmacological properties of Csn-B, this project focuses on the design, synthesis, and characterization of novel Csn-B derivatives that retain Nr4a1 agonist activity while exhibiting enhanced drug-like properties. Preliminary in silico modeling predicts that KMH-0046, possesses greater Nr4a1 binding affinity, improved pharmacokinetic properties, and enhanced blood-brain barrier permeability relative to Csn-B. In addition, 6-hour treatment of mouse Neuro-2a cells with select Csn-B analogs at nontoxic concentrations significantly upregulated Nr4a1 and Cartpt expression, while Nr4a2 expression remained unchanged, suggesting selective activation of Nr4a1. Together, these findings identify KMH-0046 and related Csn-B derivatives as promising lead compounds for further optimization and preclinical evaluation as potential therapeutics for cocaine use disorder.

REFERENCES

1. Kerridge, B. T. et al. Changes in the prevalence and correlates of cocaine use and cocaine use disorder in the United States, 2001–2002 and 2012–2013. Addictive Behaviors 90, 250–257 (2019).
2. Butler, A. J., Rehm, J. & Fischer, B. Health outcomes associated with crack-cocaine use: Systematic review and meta-analyses. Drug Alcohol Depend 180, 401–416 (2017).
3. National Institutes of Drug Abuse. How is cocaine addiction treated? nida.s://nida.nih.gov/publications/research-reports/cocaine/what-treatments-are-effective-cocaine-abusers (2020).
4. Carpenter, M. D. M. D. et al. Nr4a1 suppresses cocaine-induced behavior via epigenetic regulation of homeostatic target genes. Nat Commun 11, 504 (2020).
5. Fischer, D. K., Krick, K. S., Han, C., Woolf, M. T. & Heller, E. A. Cocaine regulation of Nr4a1 chromatin bivalency and mRNA in male and female mice. Sci Rep 12, (2022).

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Quantitative Confocal Mapping of Dopamine D1 and Muscarinic M4 Receptor Coexpression in Corticostriatal Brain Regions

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Disruption of dopaminergic and cholinergic signaling is implicated in numerous neuropsychiatric and basal ganglia disorders, including Parkinson's disease, schizophrenia, and substance use disorders. Dopamine D1 receptors (D1R) and muscarinic M4 receptors (M4R) regulate overlapping corticostriatal signaling pathways, but the extent to which these receptors are expressed within the same neuronalpopulations across relevant brain regions has not been fully defined. Clarifying the cellular distribution of D1R and M4R is an essential step toward understanding where dopaminergic and cholinergic signaling may converge in the brain.

This study uses confocal microscopy to map D1R and M4R reporter expression in fixed brain sections from double-positive fluorescent reporter mice expressing drd1-tdTomato and chrm4-eGFP. We focus on anterior cortical and striatal regions, including dorsal striatum and nucleus accumbens, to assess regional patterns of D1R-positive, M4R-positive, and D1R/M4R double-positive cells. Images were acquired across anatomically matched regions of interest and analyzed using a standardized quantitative cell-counting workflow developed to identify and compare receptor reporter expression and coexpression across brain areas. Reporter-based findings were further evaluated using complementary histological validation approaches, including fluorescence in situ hybridization using hybridization chain reaction amplification (HCR-FISH) and immunofluorescence staining, to confirm receptor-associated signal and support interpretation of the observed expression patterns.

Our results reveal region-specific patterns of D1R and M4R expression across corticostriatal regions. In striatal and accumbens regions, D1R- and M4R-expressing cells are abundant and include a measurable population of double-positive cells, supporting a cellular context for dopaminergic-cholinergic integration in circuits involved in action selection, motivation, and reward. By combining double-reporter confocal imaging, histological validation, and region-resolved cell counting, this work provides a novel anatomical framework for identifying where D1R and M4R signaling may converge in vivo and informs future studies of dopaminergic-cholinergic integration in basal ganglia-related disease states.

Novel Phenyl- and Fluoro-Substituted Chiral Anandamide Probes with Exceptional Potency and Metabolic Resistance.

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Building upon our chiral lipid technology, we report the development of novel anandamide analogs carrying chiral centers, fluorine atoms, and phenyl rings in judiciously chosen positions within the eicosanoid template of the endogenous *N*-arachidonoylethanolamine (AEA). Key successful analogs were found to exhibit remarkably high binding affinity and potency for cannabinoid receptors along with excellent stability against metabolizing enzymes.

One of our advanced molecules, namely 20,20,20-trifluoro-(*R*)-*N*-(1-Methyl-2-hydroxyethyl)-13-(*S*)-methyl-arachidonamide (**AM12814**), is the first endocannabinoid analog exhibiting unpresented affinity for both the CB1 and CB2 receptors. In further in vitro functional characterization, **AM12814** behaves as a potent, partial CB1 and CB2 agonist, thus resembling the functional profile of the physiological ligand. Our SAR results are supported by docking studies of the novel anandamide probes on the crystal structures of cannabinoid receptors. When tested in vivo, **AM12814** behaves as a very potent and efficacious CB1 agonist.

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Evaluating the Role of the MX Helix on the Chaperone Actions of NACHO and RIC3 for Alpha7 Nicotinic Receptor Assembly

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How multi-subunit receptors fold and assemble in the endoplasmic reticulum is not known. Alpha7 nicotinic receptors (α7nAChRs) are pentameric proteins requiring assistance of the chaperones Resistance to Inhibitors of Cholinesterase 3 (RIC3) and/or Nicotinic Acetylcholine Regulator (NACHO) to express in Bosc23 or HEK cells. In contrast, a chimeric receptor consisting of the human α7nAChR extracellular domain, the transmembrane domains of mouse serotonin 5HT3a receptors (5HT3ARs) and the α7nAChR intracellular domain (ICD: consisting of linker L1, the MX helix, linker L2 and the MA helix that terminates in transmembrane domain 4) does not need these chaperones to assemble. Both α7nAChR and 5HT3AR possess an ICD MX helix. In oocytes, RIC3 inhibits 5HT3AR assembly. Do and Jansen (J. Gen. Physiol. 2023 Vol. 155:e202213305) report that alanine substitutions in the MX helix of 5HT3AR alter this inhibition suggesting this is a site for RIC3/5HT3AR interaction. Since the role of the MX helix in RIC3 chaperone effects on α7nAChR is not known, we tested the effect of substituting the α7 MX helix with that of 5HT3A on the ability of RIC3 and NACHO to assemble pentameric receptors on the surface of Bosc23 cells that bind fluorescent α-bungarotoxin.

Results: Substituting the a7 MX helix and surrounding amino acids in the L1 linker with the 5HT3 MX helix completely blocks the ability of both NACHO and RIC3 to assemble α7nAChR-5HT3MX construct as demonstrated by fluorescent toxin binding. Further substitutions suggest the effect is not the 5HT3MX helix but rather the surrounding linker. AlphaFold did not find any major changes in predicted protein structure due to these mutations.

Conclusions: The MX helix may be a site of interaction between both RIC3 and NACHO during the folding and assembly of α7nAChRs. We are constructing other α7/5HT3 MX combinations to see if we can localize discrete sites of interactions between α7 receptor subunits and the chaperones that are needed to allow these receptors to fold and assemble.

Feto-maternal PK-PD and Translational Insights into Neonatal Withdrawal Following Mitragynine and Oxycodone Exposure.

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Kratom (*Mitragyna speciosa*) is widely used as a self-prescribed remedy for pain, mood disorders, and opioid withdrawal, particularly among women of childbearing age in the United States. Its principal alkaloid, mitragynine, a partial μ-opioid receptor agonist, has attracted interest as a potential alternative to conventional opioids. The rapid expansion of the kratom market has been accompanied by increasing kratom-related exposures, hospitalizations, deaths, and poison center calls in the United States. This concern is further highlighted by reports of pregnant women with oxycodone dependence switching to mitragynine during early gestation to manage opioid withdrawal symptoms, raises significant safety concerns regarding fetal exposure. There is an urgent need to characterize the feto-maternal pharmacokinetics of mitragynine and its metabolites to determine their placental transfer and potential to induce neonatal abstinence syndrome. In this context, the present study investigated maternal and fetal disposition following in utero exposure to oxycodone and mitragynine in pregnant rat models. Metabolite profiling revealed that 9-hydroxycorynantheidine was the most abundant metabolite followed by 7-hydroxymitragynine in mitragynine-dosed dams. In oxycodone-treated dams, noroxymorphone was the major metabolite. Neonates displayed withdrawal behaviors of similar magnitude in mitragynine, oxycodone, and morphine-exposed groups following naltrexone administration. Importantly, withdrawal severity was more pronounced in offspring with prenatal exposure to oxycodone prior to pregnancy, followed by a switch to mitragynine during gestation. These findings indicate that prenatal exposure to mitragynine is associated with neonatal withdrawal, demonstrating that kratom is not a safe alternative to opioids during pregnancy. Furthermore, transition from oxycodone to mitragynine does not mitigate neonatal risk, underscoring the need for clinical guidelines and cautionary use during gestation.

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Probing Ligand-Pocket Interactions of Synthetic Cannabinoids at the CB1 Receptor by Mutagenesis

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Over the past decade, synthetic cannabinoid receptor agonists (SCRAs) have emerged as a predominant structural class of new psychoactive substances. As drugs of abuse, SCRAs pose a substantial global health threat, eliciting severe effects including psychosis, seizures, and even rare cases of lethality at low doses.

Unlike (−)-trans-Δ^9-tetrahydrocannabinol (THC), a partial agonist, SCRAs function as full agonists with markedly elevated potency and efficacy at the cannabinoid 1 receptor (CB1R), among the most abundantly expressed G protein–coupled receptors in the central nervous system. Through Gαi signaling, CB1R reduces intracellular cyclic AMP, modulating downstream gene transcription and synaptic remodeling. This study characterizes how structural substituents at the head, core, and tail moieties of SCRAs influence CB1R Gαi engagement, the step in which the Gαi subunit docks into the intracellular cavity of CB1R to initiate signaling.

Ligand-dependent Gαi engagement was monitored over a course of time by bioluminescence resonance energy transfer (BRET), with Renilla luciferase (RLuc) fused to the receptor as the donor and Venus to the Gαi as the acceptor. Energy transfer arising from donor–acceptor proximity yielded BRET ratios, from which dose–response curves were generated to determine EC_{50} and E_{max}. Guided by prior structural studies and molecular dynamics simulations, residues F268 and F379 were predicted to form π–π interactions with the core moiety, while F108 and Y275 were predicted to engage the head and tail moieties through hydrophobic contacts within the CB1R binding pocket. Site-directed mutagenesis was performed to obtain alanine, tyrosine, and tryptophan substitutions (F268A/Y/W, F379A/Y/W, F108A/Y/W, Y275A/F), which were tested against a set of novel SCRAs specifically differ in head (cumyl and L-tert-leucine methyl ester), core (indole, 5-bromoindazole, azaindole, and pyridoindolone), and tail (5-fluoropentyl, pentyl-4-en, and 4-fluorobenzyl) moieties.

By mapping how selected residues in the CB1R pocket govern recognition of structurally diverse SCRAs, this study contributes to our understanding of CB1R activation by novel SCRAs.

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Mutational Analysis of NACHO, a Chaperone for alpha7 Nicotinic Receptors

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NACHO (gene name TMEM35A) is a protein chaperone that allows folding and expression of α7 nicotinic receptors (α7nAChR) in cell lines such as HEK or Bosc23. NACHO is a 167 amino acid protein with four transmembrane domains in the endoplasmic reticulum (ER). Transfecting NACHO into Bosc23 cells allows α7nAChR expression as determined by cell surface expression detected by fluorescent α-bungarotoxin binding. In contrast, a similar gene (TMEM35B) has 154 amino acids (26% identical, 48% similar according to BLAST) and according to AlphaFold, folds up virtually identically to NACHO except for the C-terminal tail. Unlike NACHO, TMEM35B does not allow surface expression of α7 nicotinic receptors in Bosc23 cells. Therefore, we investigated important regions of NACHO for chaperone activity by swapping out regions of TMEM35B. Previous experiments suggested that NACHO's first two transmembrane domains are important for activity because replacing them with TMEM35B sequences blocked chaperone activity. These studies focused on the C-terminal tail region of NACHO. In one construct, the EQPS sequence in the C-terminal is replaced with STFK from TMEM35B (STFK-NACHO). In a second construct, the RKPE sequence at the base of transmembrane 4 was replaced with LLAQ from TMEM35B (LLAQ-NACHO). Both constructs allowed surface expression of α7nAChRs in Bosc23 cells in 3 out of 4 experiments to date. Furthermore, AlphaFold predicted that both constructs fold very similarly to wild-type NACHO. These results suggest that substituting regions of NACHO with TMEM35B may provide useful information about the location of interactions between NACHO and α7nAChRs during the assembly of α7 receptors

Evolutionary Conservation of the human α7 SAP motif across species and nicotinic receptor sub-units

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Background: How surface receptors fold and assemble is not understood even though they bind neurotransmitters and a wide variety of drugs. Improperly folded receptors can lead to epilepsy and are implicated in other neurological conditions such as Alzheimer's disease and schizophrenia. α7 nicotinic receptor (Hα7 nAChR, including the α7 nAChR-FamA fusion protein variant) is unique among human nicotinic receptor subunits to have transmembrane 4 (M4) terminate in an SAP (Serine-Alanine-Proline) motif before a helical C-terminal called the latch. However, the SAP motif is highly conserved among both α7 nAChR homologs and other nicotinic subunits in multiple species. For instance, in *C. elegans* the SAP motif is found in 7 of all known 27 nicotinic subunits (including both αs and βs) and SAP variants (e.g. RAP, TAP) are found in another 11 (In *D. melanogaster*, SAP variants are in 90% of subunits). The conserved SAP motif implies a possible mechanistic role.

Hypothesis: Hα7 nAChRs require chaperones NACHO and RIC3 for proper folding and assembly and we hypothesized that they may be required to fold subunits with SAP motifs in other species as well. We previously found that NACHO and RIC3 fold Hα7 chimeric proteins with the latch replaced with that of flies or liver flukes. In these experiments, we tested whether human NACHO and RIC3 can fold α7 chimeras with the entire M4 domain and C-terminal replaced with those of other species.

Methods: α7 folding was indirectly measured in BOSC23 cells using fluorescent α-bungarotoxin binding after transfection as an indication of surface receptor expression.

Results: We found that Hα7-M4 chimeras from C. elegans Unc29 (SAP) and Unc38 (RAP) do not express with NACHO and RIC3. The RAP motif in Unc38 is not the cause, as Hα7-nAChR with a S489R (RAP) mutation expresses well. Further experiments will determine if incompatibility between mutant M4 and Hα7 M2 and M3 causes failure to express or if the length of the latch is an issue. NACHO and RIC3 co-evolve with nicotinic receptors in individual species. Thus, changes in chaperone function across species may also be involved.

Enantiomerically-Selective Activation and Inactivation of the Serotonin 5-HT6 Receptor by New 2-Aminotetralins and Characterization of Their Pro-Cognitive Effects in Rats

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The serotonin (5-hydroxytryptamine, 5-HT) 6 $G\alpha_s$ protein-coupled receptor (5-HT_6R) is exclusively expressed within the CNS. It plays a significant role in cognitive function due to its ability to modulate general brain activity and promote neuronal plasticity. This makes the 5-HT_6R a promising target for the development of pro-cognitive therapeutics to treat neurological and neuropsychiatric disorders, including those comorbid with substance use disorders. Interestingly, both activation and inactivation of the 5-HT_6R have been reported to enhance cognition and several 5-HT_6R antagonists have undergone clinical trials for Alzheimer's disease as well as schizophrenia but did not meet endpoints in phase III. This gap in clinically relevant 5-HT_6R ligands highlights the need to discover and develop potent and selective 5-HT_6R agonists and inverse agonists to better understand the pharmacotherapeutic potential of the 5-HT_6R.

Our lab develops novel chemotypes based on the 2-aminotetralin scaffold that is drug-like regarding oral bioavailability, brain penetration, and lack of inherent toxicity. We have synthesized new 7-**s**ubstituted-2-**a**mino**t**etralins (**7-SATs**) that are enantiomerically-selective for activation or inactivation of the human 5-HT_6R, with very high affinity and functional potency (e.g., K_i <0.5 nM; EC50/IC50 <10 nM). We are using a structure-based drug design strategy incorporating molecular docking studies based on a 5-HT_6R model built from the cryo-EM structure and utilizing 1 **µ**sec molecular dynamics simulations to optimize 5-HT_6R–7-SAT affinity and agonist vs. inverse agonist function. Behavioral studies of lead 7-SATs indicate that they robustly produce pro-cognitive effects in rat models. *In vivo* pharmacological (ph) MRI studies in awake rats after peripheral administration of 7-SAT leads show activation of brain regions associated with cognitive processes, providing mechanistic clues regarding 5-HT_6R pharmacotherapeutic relevance. In addition to molecular and behavioral pharmacological as well as phMRI data, stereoselective synthetic strategies to realize new 7-SAT-type ligands with enantioselective function will be reported at the conference.

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Psilocybin Treatment During Opioid Exposure Attenuates Spontaneous Withdrawal in Morphine-Dependent Rats

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Preclinical and clinical studies indicate that the psychedelic compound psilocybin may have utility to reduce opioid-induced phenotypes, including use and seeking behaviors. However, the impact of psilocybin on opioid-induced withdrawal behaviors has not been described. Here, we assessed this critical knowledge gap by evaluating the impact of psilocybin on spontaneous- and naloxone-precipitated withdrawal from morphine in rats. Adult male Sprague-Dawley rats were implanted with morphine pellets (75 mg) to induce opioid dependence. Two different time points (using independent cohorts) were chosen for the spontaneous withdrawal (SW) studies, 11 days and 15 days post-implantation of the pellets were used to investigate timeline effects of psilocybin treatment. Following pellet implantation, rats received (IP) injections of 0.3 or 3 mg/kg psilocybin, or saline, every other day with a total of 5 injections for SW and 4 injections for naloxone-precipitated withdrawal (NPW). SW behavior was assessed for 45 min on days 10, 11, and 15 and elevated plus maze was performed to measure anxiety-like behavior on days 11 and 15. Spontaneous withdrawal was detected on days 11 and 15, but not day 10. Both doses of psilocybin significantly reduced global withdrawal scores (GWS) on day 11 compared to saline-treated rats. SW-induced weight loss was more prominent in rats administered psilocybin on days 10 and 11, but the effect was normalized on day 15. SW did not induce anxiety-like effect on both days 11 and 15, however excessive rearing, another measure of anxiety-like effect, was significantly reduced in rats received psilocybin. To assess NPW, naloxone (3 mg/kg) was injected on day 8 and rats were observed for 30 min. Psilocybin did not reduce GWS in NPW, but significantly diminished teeth chattering. These data suggest that psilocybin may have additional therapeutic utility for reducing withdrawal symptoms associated with cessation of chronic opioid use.

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Endocannabinoid Modulation as a Therapeutic Strategy for Opioid-Induced Hyperalgesia

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Opioid-induced hyperalgesia (OIH) is a paradoxical consequence of repeated opioid exposure characterized by enhanced responses to painful stimuli and pain evoked by normally innocuous mechanical or thermal stimuli. OIH complicates long-term opioid therapy, may contribute to opioid dose escalation, and remains difficult to treat. The endocannabinoid system offers a promising therapeutic target through modulation of pain processing and neuroinflammatory pathways. We hypothesized that enhancing endocannabinoid signaling through monoacylglycerol lipase (MAGL) inhibition or cannabinoid receptor type 1 (CB1) positive allosteric modulation would attenuate OIH, supporting the development of strategies to both prevent and reverse opioid-induced pain sensitization.

Male and female C57BL/6J mice (n = 8–10/group) received morphine via osmotic minipumps (64 mg/mL; 1 µL/hour). Mechanical and acetone-induced cold allodynia developed reliably by day 5 of morphine administration, accompanied by splenomegaly ($p = 0.012$). The acute effects of the MAGL inhibitor JZL184 (40 mg/kg) and the CB1 positive allosteric modulator ZCZ011 (40 mg/kg) were evaluated. The effects of repeated JZL184 treatment (0.6, 2.5, 10, and 40 mg/kg) were subsequently assessed, and cannabinoid receptor involvement was examined by co-administering the selective CB2 receptor antagonist SR144528 (3 mg/kg) with repeated JZL184.

Acute ZCZ011 and JZL184 reduced both mechanical ($p = 0.047$; $p = 0.009$) and cold allodynia ($p < 0.001$; $p = 0.009$). Repeated JZL184 dose-dependently attenuated mechanical ($F(12,123) = 7$, $p < 0.001$) and cold allodynia ($F(12,126) = 3$, $p < 0.001$). SR144528 failed to block the anti-allodynic effects of JZL184 ($p = 0.619$), suggesting a CB2-independent mechanism.

Together, these findings demonstrate that enhancing endocannabinoid signaling attenuates opioid-induced pain sensitization and supports the continued development of endocannabinoid-based therapies for preventing and reversing opioid-induced hyperalgesia.

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Incubation of discriminative stimulus-controlled cocaine craving is associated with brain-wide neuronal activation in rats

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Background: Cues previously associated with drug-taking can precipitate relapse long after the last drug-taking experience. Discriminative stimuli (DSs) signal drug availability (DS+) or unavailability (DS-), and guide drug seeking-behavior. We previously reported that DS-controlled cocaine seeking progressively increases (*or incubates*) during abstinence and persists up to 300 days (Madangopal, eLife, 2019). Here we used the activity marker Fos to quantify brain-wide neuronal activation associated with DS-controlled cocaine seeking during early and late abstinence.

Methods: We used our trial-based procedure to train rats to self-administer cocaine (0.75 mg/kg/infusion) during DS+ trials (cocaine available) and suppress responding during DS- trials (cocaine unavailable) during the same session (2 x 3 h/d; 30 each DS+/DS- trials). Next, we tested rats for cocaine seeking after 1 or 21 days of abstinence (1.5 h; 15 each DS+/DS- trials; extinction conditions) and extracted brains either immediately after the test (day 1 or day 21 test groups) or directly from the homecage (no-test group).

Results: We observed reliable DS-controlled cocaine self-administration during training and time-dependent increases in DS-controlled relapse to cocaine seeking during abstinence. Fos-based activity mapping identified multiple cortical, striatal, and thalamic brain regions that showed (1) time-independent increased activation following DS-controlled relapse versus no-test controls and (2) time-dependent increase in activation during relapse test on abstinence day 1 versus day 21.

Conclusions: Incubation of DS-controlled cocaine seeking is associated with widespread activation beyond those previously implicated in incubation of food or drug craving. In ongoing analyses we are integrating region-wise activation data with behavioral measures to test whether this widespread engagement reflects a general increase in brain-wide activation or a reorganization of specific co-activation networks during abstinence, and to identify candidate hub regions associated with coordinated network recruitment during incubation of cocaine seeking.

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Proof of Concept for Dual FAAH/ABHD6 Inhibition: Antinociception Without Cannabimimetic Effects

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An extensive body of evidence demonstrates that concomitant inhibition of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), the principal hydrolytic enzymes for anandamide (AEA) and 2-arachidonoylglycerol (2-AG), respectively, produces greater antinociceptive efficacy than selective inhibition of either enzyme alone. However, dual FAAH/MGL blockade also elicits the full spectrum of cannabimimetic effects, as demonstrated in the drug discrimination paradigm and tetrad assay, thereby limiting the therapeutic potential of this approach. In contrast, dual inhibition of FAAH and α/β-hydrolase domain-containing protein 6 (ABHD6), a minor 2-AG catabolic enzyme, may preserve analgesic efficacy while minimizing cannabimimetic side effects.

To test this hypothesis, we evaluated the highly selective FAAH inhibitor AM11994 and the ABHD6 inhibitor AM12100, administered alone or in combination, in an established paclitaxel (PAC)-induced mouse model of neuropathic pain and in the tetrad assay. Although neither compound alone attenuated PAC-induced hypernociception, their combined administration produced robust antinociceptive effects without eliciting the full complement of cannabimimetic behaviors. These findings prompted the development of AM12216, a prototype dual FAAH/ABHD6 inhibitor with IC_{50} values of 43.7 nM and 6.8 nM for FAAH and ABHD6, respectively. Consistent with the pharmacological combination, AM12216 significantly attenuated PAC-induced hypernociceptive responses without producing cannabimimetic effects in the tetrad assay.

Collectively, these findings establish proof of concept for dual FAAH/ABHD6 inhibition as a strategy for treating neuropathic pain while avoiding the dose-limiting cannabimimetic side effects associated with dual FAAH/MGL inhibition. Moreover, these compounds provide valuable pharmacological tools for mechanistic studies and for evaluating the therapeutic potential of dual FAAH/ABHD6 inhibition in additional preclinical models of pain and disease.

An Examination for Glutamate Receptor Correlates of Alcohol-Stress Cross-Sensitization

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Problematic drinking in females has been attributed to coping with stress and negative affect regulation. While repeated unpredictable stress has been well-characterized to influence AUD-related behaviors in male laboratory rodents, studies investigating stress-alcohol interactions in female subjects are lacking. Based on clinical and preclinical evidence that the propensity to drink is inversely related to alcohol behavioral sensitivity, we employed an 11-day repeated, unpredictable, mild stress (RUMS) procedure in CFW mice to examine for sex differences in stress-alcohol behavioral cross-sensitization, followed by immunoblotting to examine glutamate receptor expression and kinase activation within the extended amygdala. RUMS lowered body weights in mice of both sexes, with earlier-onset effects observed in females. RUMS increased alcohol-induced locomotion and reduced the latency to right in both females and males, indicative of behavioral cross-sensitization. When examined 3 h following injection with 5 g/kg ethanol, male-selective alcohol effects were detected for GluN1 (increase) and both total and relative p(Ser729)-PKCε expression (decrease) within the nucleus accumbens. No alcohol-related changes in protein expression were detected in females, although RUMS lowered the relative expression of p(Ser473)-Akt selectively in the female nucleus accumbens. Despite evidence of behavioral cross-sensitization, we failed to detect stress-alcohol cross-sensitization of protein expression within the nucleus accumbens of either sex. These data argue against changes in excitatory glutamate signaling within the nucleus accumbens as a key driver of stress-alcohol behavioral cross-sensitization.

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A History of Adolescent Oxycodone Self-Administration Reshapes Excitatory and Inhibitory Neur

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Adolescence and young adulthood are crucial neurodevelopmental periods associated with nonmedical use of prescription opioids and maturation of the prefrontal cortex (PFC), with the former having key potential impacts on the latter. Cortical maturation during adolescence is characterized, in part, by a shift in the excitation-inhibition balance; alterations in the excitation-inhibition balance have drastic consequences for higher-order cognitive processes. Adolescent oxycodone (OXY) self-administration induces persistent dose-dependent impairments in neurobehavioral and neurocognitive development. To date, however, if and/or how adolescent prescription opioid use disorder (APOUD) alters excitatory and/or inhibitory neurons in the PFC has not been systematically evaluated. Male and female F344/N rats voluntarily self-administered oral OXY (0, 2, 5, or 10 mg/kg) or water via a two-bottle choice experimental paradigm for ten weeks (i.e., Postnatal Day 35 to 105); rats returned to oral OXY self-administration for five days before euthanasia. First, structural alterations in excitatory pyramidal neurons, and their associated dendritic spines, in layers II-III of the PFC were examined using a DiOlistic labeling technique and sophisticated neuronal reconstruction software. Adolescent OXY self-administration induced a profound sex- and dose-dependent population shift in the morphological parameters of dendritic spines relative to control rodents. Crucially, the profound dendritic spine dysmorphology observed following a history of adolescent OXY self-administration supports prominent synaptic dysfunction in excitatory pyramidal neurons. Second, the transcriptomic expression of inhibitory interneurons was evaluated in cortical tissue using quantitative polymerase chain reaction techniques. Independent of biological sex, a history of adolescent OXY self-administration induced a selective increase in the mRNA expression of *Sst*, a gene encoding a peptide hormone for somatostatin interneurons; *Pvalb*, a gene encoding a calcium-binding protein expressed in parvalbumin interneurons, was not significantly altered by adolescent OXY self-administration. In a consistent manner, mRNA expression of *Vgat* (Vesicular GABA Transporter), the primary vesicular transporter in somatostatin interneurons, was significantly upregulated in rodents with a history of adolescent OXY self-administration relative to their control counterparts. Collectively, results support alterations in both excitatory and inhibitory neurons in the PFC. Identifying the neural mechanisms underlying APOUD-induced neurocognitive impairments affords a key target for the development of novel therapeutics.

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Functional Mechanisms Linking Zhx2 Loss-of-Function with Increased Brain Oxymorphone Levels and Oxycodone Addiction Model Behaviors in Females

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Overprescription of oxycodone (**OXY**; active ingredient of OxyContin®) is a major historical contributor to the ongoing opioid epidemic. OXY is metabolized into oxymorphone **(OMOR)**, a much more potent and efficacious metabolite at the mu-opioid receptor associated with increased OXY locomotion and reward in BALB/cJ females vs. BALB/cByJ mice. We previously mapped and validated loss-of-function in zinc-fingers and homeobox 2 **(Zhx2)** as a quantitative trait gene and genetic mechanism underlying increased brain [OMOR] in BALB/cJ females. We are currently testing two potential functional mechanisms linking Zhx2 loss-of-function with increased brain [OMOR] and OXY behaviors, including 1) increased blood brain barrier **(BBB)** permeability and 2) increased metabolism of OXY to OMOR.

We previously found differences in gene enrichment for extracellular matrix, cell-to-cell adhesion, and lipid metabolism via bulk RNA-seq in Zhx2 knockout **(KO)** mice. Recent analysis further shows a reduction in the tight-junction protein, Claudin5, expression in KO females, further supporting Zhx2 as necessary for BBB integrity. Furthermore, following systemic administration, KO females showed greater BBB permeability of sodium fluorescein, but not Evans blue in the hippocampus, supporting region-specific BBB disruption at the tight-junction level. *In-vivo* diffusion tensor imaging **(DTI)** revealed reduced diffusion of water at baseline. Functional magnetic resonance imaging **(fMRI)** revealed increased OXY-induced negative BOLD signal throughout striatal and sub-cortical midbrain regions of KO females, indicating an enhanced OXY-induced neural response within opioid addiction-relevant brain regions that we hypothesize is due to increased brain OMOR. In addition to the BBB permeability hypothesis, we found an increase in both plasma and brain [OMOR] in KO females following high-dose OXY, implicating increased liver metabolism of OXY to OMOR underlying these observations. Analysis of liver-derived microsomes is underway to test the hypothesis that KO females show increased metabolism of OXY to OMOR via phase I metabolism. Finally, KO females show reduced liver UGT2b37 expression supporting a potential reduction in phase II metabolism and decreased clearance of OMOR as yet an additional mechanism underlying increased brain OMOR. The results support both BBB disruption and pharmacokinetic/metabolic mechanisms underlying increased brain OMOR in KO females.

AM6527 Reduces Binge-Like Ethanol Consumption and Attenuates Ethanol-Associated Dopamine Release in the Nucleus Accumbens

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Alcohol use disorder (AUD) remains a significant public health challenge, underscoring the need for effective pharmacological treatments. AM6527 is a novel cannabinoid type-1 (CB1) receptor neutral antagonist developed by the Center for Drug Discovery at Northeastern University that reduces alcohol consumption without the adverse side effects associated with CB1 receptor inverse agonists such as rimonabant. Previous studies have demonstrated that AM6527 decreases ethanol intake in multiple drinking paradigms in both male and female mice. Additionally, a previous generation CB1 receptor neutral antagonist, AM4113, has been shown to attenuate ethanol-induced dopamine release in the nucleus accumbens (NAc) of rats, suggesting that modulation of mesolimbic dopamine signaling may contribute to the therapeutic efficacy of this class of compounds. The present study investigated whether AM6527 reduces binge-like ethanol consumption and alters ethanol-associated dopamine signaling in the NAc of mice.

Adult male C57BL/6J mice received intra-NAc injections of an adeno-associated virus encoding the dopamine sensor dLight1 and implantation of an optical fiber for real-time dopamine measurements using fiber photometry. Mice were trained in a drinking-in-the-dark (DID) paradigm until they achieved binge-like ethanol intake (2–4 g/kg in 2 h). Ethanol consumption was monitored using a capacitive lickometer, and blood ethanol concentrations were measured using an ethanol colorimetric enzymatic assay. Following baseline recordings, mice received vehicle or AM6527 (3 mg/kg, i.p.) 20 min prior to ethanol access, and dopamine dynamics were recorded throughout drinking sessions.

AM6527 significantly reduced binge-like ethanol consumption during DID sessions. Event-based fiber photometry analyses revealed that AM6527 attenuated ethanol-associated dopamine signaling, as evidenced by reduced dopamine responses during both drinking-bout initiation and post-bout periods. Notably, the magnitude of the drug effect was greatest in mice exhibiting higher baseline alcohol consumption and more context-independent drinking behavior. AM6527 did not alter overall locomotor activity, indicating that reductions in drinking were not attributable to nonspecific motor impairments.

Together, these findings demonstrate that AM6527 reduces binge-like ethanol consumption while attenuating ethanol-associated dopamine signaling in the NAc. These results support neutral CB1 receptor antagonism as a promising therapeutic strategy for AUD and suggest that disruption of alcohol-associated dopamine signaling may contribute to its therapeutic efficacy.

Beyond the Open Field: A Deep Learning Approach for Decoding Latent Rodent Behaviors in Structured Reinforcement Tasks

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Quantitative analysis of behavior offers a critical window into the brain mechanisms underlying learning, sensory processing, and decision-making. However, understanding how animals adapt their behavior in structured, cognitively demanding environments remains a central challenge. Although recent machine learning approaches have advanced behavioral classification in open-field settings, these methods often fall short in reinforcement learning (RL) tasks, where actions are shaped not only by immediate sensory cues but also by past experiences and future expectations. This gap highlights the need to evaluate whether most recent modeling approaches can capture task-relevant actions in structured experimental contexts.

To address this, we analyzed videos of mice performing RL tasks combined with the systemic injection of AM6527, a neutral CB1 receptor antagonist. Using DeepLabCut, we first extracted body parts as model inputs and then compared Deep Behavior Mapping (DBM) and Keypoint-MoSeq (KPMS). This comparison revealed that DBM detected behavioral syllables but depended heavily on spatial location, lacked intuitive visualization, and required extensive syllable definition. Similarly, although KPMS provided motif visualization, it failed to distinguish task-specific actions and, because of stationary HMM assumptions, could not capture temporal or contextual changes.

To overcome these limitations, we developed a semi-supervised Bidirectional Long Short-Term-Memory (Bi-LSTM) model. By incorporating both past and future time points, this architecture mirrors the way prior actions and anticipated outcomes shape animal behavior. Specifically, we integrated behavioral events and timestamps into a small set of pre-defined behaviors, training and generalizing these across the entire set of collected videos. As a result, in comparison to DBM and KPMS, the Bi-LSTM achieved higher classification accuracy and lower training loss across behavioral categories, outperforming all baseline models. Therefore, the results indicate that the Bi-LSTM, by combining supervised and unsupervised strategies, is well suited to capture subtle, temporally structured, and context-sensitive behavioral microstates that previous methods failed to detect.

Our efforts underscore the promise of expressive sequence modeling for decoding behaviors in structured, cognitively demanding environments and provide a foundation for linking behavioral microstates more directly with neural activity, paving the way for a deeper understanding of integrative behavioral and neural dynamics influenced by medicinal compounds.

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In-Vitro Evaluation of β-substituted-γ-lactones as Potential Substance Use Disorder Therapies

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Substance use disorder (SUD) remains a major public health challenge, with cocaine use disorder (CUD) representing a significant unmet medical need due to the lack of FDA-approved pharmacotherapies. The development of novel therapeutics capable of modulating the neurobiological pathways underlying addiction is therefore of considerable interest. Previous work in our laboratory has focused on the design, synthesis, and pharmacological evaluation of functionalized α-substituted γ-lactones as biogenic amine-inspired scaffolds for central nervous system drug discovery. Through systematic medicinal chemistry optimization and structure-activity relationship (SAR) studies, this scaffold has produced compounds with low-nanomolar affinity and selectivity for the serotonin 5-HT_7 receptor and the σ_2 receptor, both of which have been implicated in addiction-related neuroplasticity, mood regulation, and neuroinflammation. These studies have yielded promising 5-HT_7 antagonists as lead candidates for the treatment of CUD.

More recently, this scaffold has unexpectedly produced highly potent and selective σ_1 receptor ligands. Importantly, the σ_1 receptor shares pathological overlap with the other targets of interest (σ_2 and 5-HT_7). The convergence of these receptors across related disease pathways suggests that ligands capable of engaging multiple targets, or selectively modulating one over the others, may offer enhanced therapeutic benefits. The pharmacophore consists of a γ-lactone headgroup, a flexible linker, a piperazine moiety, and an aryl ring system, providing a versatile framework for tuning receptor affinity, selectivity, and drug-like physicochemical properties.

In an effort to expand upon these findings, we have extended the medicinal chemistry efforts to include the exploration of β-substituted-γ-lactones. This series examines how β-substitution, in combination with systematic modifications in steric bulk and polarity, alters receptor-binding profiles. Herein, we report the synthetic methodology, structural characterization, and preliminary *in-vitro* pharmacological evaluation of these analogs. Early results indicate that β-functionalization preferentially enhances σ_1 receptor affinity and selectivity, whereas α-substitution favors 5-HT_7 and σ_2 receptor activity. This observation presents a unique opportunity to modify molecular features of the γ-lactone class of compounds with the goal of selectively targeting one of the individual receptors, thus improving therapeutic specificity and creating an adaptable scaffold for the discovery of next-generation therapeutics for SUD and related neuropsychiatric diseases.

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Dual Fatty Acid Amide Hydrolase (FAAH) and Monoacylglycerol Lipase (MGL) Inhibitors Produce Radio-Dependent Cannabinoid Activity In Vivo

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Cannabinoid 1 (CB_1) receptor agonists like Δ9-THC have shown therapeutic potential in pain management, nausea and appetite loss, and several neuropsychiatric and neurodegenerative disorders. Despite encouraging pre-clinical and clinical results, CB_1 agonists have well characterized side effect profiles that limit their therapeutic utility. Inhibition of fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGL), the enzymes primarily responsible for the breakdown of the endogenous cannabinoids anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), has emerged as an alternative cannabinoid system drug target. Dual FAAH/MGL inhibitors have been shown to reproduce some therapeutic effects of CB_1 agonists with efficacy and side-effect profiles varying by species and inhibition ratio. The present studies aimed to characterize the in vivo behavioral pharmacological effects of four novel dual inhibitors (MAK2375, MAK2376, MAK2386, and MAK2388) with variable FAAH:MGL selectivity ratios of 1:10, 1:43, 1:242, and 1:83 respectively in mice and rats. In CD-1 mice, the CB_1 full agonist AM8936 or enzyme inhibitors were evaluated in both catalepsy and warm water tail withdrawal experiments. MAK2375 and MAK2376 produced significant antinociceptive effects at doses roughly 3 to 5-fold lower than those that produced cataleptic effects, providing a similar therapeutic window to AM8936. MAK2386 and MAK2388 produced virtually no cataleptic effects but also provided only partial antinociceptive effects. Rats were trained to discriminate between 0.1 mg/kg of AM8936 and saline. The results of the discrimination studies showed that MAK2375 and MAK2376 produced full substitution for AM8936, while MAK2386 and MAK2388 only produced partial substitution. These results were consistent with the increased FAAH inhibition bias in MAK2386 and MAK2388 relative to MAK2375 and MAK2376. Taken together, these findings indicate that MGL inhibition is the primary driver of CB_1-like effects in vivo. They also suggest that more balanced FAAH/MGL inhibitors may provide similar therapeutic effects to CB1 agonists but may also have similar drawbacks, while more FAAH-biased dual inhibitors reduce CB_1-like effects but may not fill the same therapeutic roles as CB_1 agonists.

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SHR/NCrl Rats Show Increased Fentanyl Locomotor Sensitivity Compared to SHR/NHsd Rats

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Fentanyl contributes to over one-half of overdose deaths in the US. Although opioid use disorder (OUD) and its comprising traits are heritable, few significant, genome-wide associations have been identified. Quantitative genetic studies in near-isogenic rodent strains differing in opioid behaviors can facilitate the discovery of novel genes influencing OUD model traits. We previously showed that the spontaneously hypertensive rat strain (SHR) from Charles River Laboratory (SHR/NCrl) exhibits a pro-addiction phenotype in cocaine intravenous self-administration (IVSA) versus the closely related, near-isogenic SHR substrain from Harlan-Sprague Dawley (Innotiv; SHR/NHsd).

Here, we sought to determine whether SHR/NCrl rodents' pro-addiction phenotype extends to the addictive and highly potent mu opioid receptor agonist fentanyl. Adult rats underwent 10 days of testing for two weeks (Monday-Friday). Prior to each experimental session, rats were acclimated to the room for 30-min, followed by injections and a 2h assessment of locomotor activity in an open field (46.1 cm x 33.0 cm x 40.6 cm) enclosed in a sound-attenuating chamber (MedAssociates). Video recordings (Arducam Day and Night,1080P, CMOS camera) and video tracking/quantification was done via AnyMaze software (Boston, MA). On Days 1 and 2, rats were injected intraperitoneally (IP) with saline to habituate them and assess response to novelty. On Days 3-9, rats were injected IP with either 0.05 or 0.1 mg/kg fentanyl to examine acute and sensitized locomotor responses to fentanyl. On Day 10, rats were injected IP with saline to examine drug-free, conditioned locomotion. Each cohort contained at least two control rats injected with saline every day during the 10-day experimental period. SHR/NCrl rats showed greater fentanyl-induced locomotion than SHR/NHsd rats at both fentanyl doses, further supporting a more general pro-addiction phenotype. Across both substrains, females displayed greater locomotion than males. Females displayed a greater novelty response than males. The enhanced fentanyl locomotor sensitivity in SHR/NCrl versus SHR/NHsd suggests that they may also show greater opioid reward and reinforcement, which when subjected to genetic analysis via a cross between these near-isogenic strains, could greatly facilitate quantitative trait locus mapping and identification of the causal genes.

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Design, Synthesis and Preliminary *In Vivo* Target Profiling of Psychedelic Tryptamine-based Photoaffinity Labeling Probes

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Psychedelics have demonstrated rapid and sustained therapeutic effects in neuropsychiatric disorders, yet these same compounds are used recreationally, raising concerns about abuse. However, their molecular mechanisms remain poorly defined. One strategy for understanding the neuropharmacological mechanisms involved is through a comprehensive study of their target proteins and receptor interactions. Here, we employed a zebrafish model to systematically profile the in vivo target interactome of psychedelic tryptamines using an integrated approach that combines in vivo photoaffinity labeling (PAL), behavioral assays, and quantitative proteomics.

We have synthesized a focused library of PAL probes based on dimethyltryptamine (DMT) and 5-methoxy-DMT (5-MeO-DMT) to validate structure-activity relationships. As anticipated, in vitro receptor-binding assays and monoamine reuptake studies showed that the probes bind to relevant monoamine targets, similar to the parent tryptamines. A PAL version of 5-methoxy-*N*-methyltryptamine (Photo-5-MeO-NMT) showed affinity for the 5-HT_{2A} (Ki = 0.65 μM) and 5-HT_{1A} (K_i = 1.5 μM) receptors, while displaying greater affinity at 5-HT_{1D} (Ki = 0.1 μM) and Sigma 1 (K_i = 0.02 μM) receptors. Reuptake inhibition studies in rat brain synaptosomes showed that Photo-5-MeO-NMT was non-selective across transporters for dopamine, norepinephrine, and 5-HT (SERT) with IC_{50} =8.6 μM, 5.8 μM and 3.3 μM, respectively. Photo-5-MeO-DMT displayed inhibition at SERT (IC_{50} =2.6 μM). In pilot behavioral studies using a larval zebrafish photomotor response assay, both Photo-5-MeO-DMT and Photo-DMT reduce locomotor activity when administered at 10 and 30 μM. In vivo PAL studies revealed specific protein labeling when analyzed by in-gel fluorescence of SDS-PAGE gels, including labeling in the molecular weight region of the consensus targets of these agents (e.g., 5-HT receptors), as well as significant labeling at other molecular weights, which may reflect novel targets. Follow-up studies are ongoing to identify and profile the enriched *in vivo* binding partners using proteomics, and the resulting data will be analyzed with bioinformatics tools to map signaling networks. Collectively, these studies establish a first-in-class vertebrate platform that integrates synthetic chemistry, behavioral neuroscience, and proteome-wide target study to examine psychedelic pharmacology with the goal of accelerating the discovery of novel neuropsychiatric therapeutics.

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Pharmacological Characterization of Cannabinoid Ligands Across the Efficacy Spectrum at CB1 and CB2 Receptors

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Cannabinoids act primarily through two G protein coupled receptors, CB1 and CB2, and to a lesser extent through other non-canonical receptors. The psychoactive and abuse-relevant central effects of cannabinoids are mediated primarily by CB1, whereas CB2 is associated largely with peripheral and immunomodulatory functions. The cannabinoid research community has characterized CB1 and CB2 ligands spanning the full range of efficacy: inverse agonists, neutral antagonists, partial agonists, full agonists, and superagonists. Among agonists, Δ^9-tetrahydrocannabinol (Δ^9-THC) acts as a partial agonist whereas many synthetic cannabinoid receptor agonists (SCRAs) show full or higher efficacy. Among antagonists, inverse agonists and neutral antagonists have been linked to different therapeutic applications, with neutral antagonists generally preferred for neuropsychiatric indications. A complete account of cannabinoid action requires resolving how ligands engage its two major signaling pathways: Gi1 and β-arrestin 2. Yet prior characterizations have typically examined narrow subsets, often across differing cell systems and assay conditions, making efficacy difficult to compare across compounds; a panel of this efficacy and structural breadth has not been evaluated within a well-controlled uniform assay system at both CB1 and CB2.

In this study, we use in vitro bioluminescence resonance energy transfer (BRET) assays, established in house, to evaluate Gi1 engagement and β-arrestin 2 recruitment at CB1 and CB2 for a panel of cannabinoid ligands spanning the full range of efficacy, including endocannabinoid analogues, Δ^9-THC, SCRAs, and various antagonists. For every compound, dose-response curves are determined for each transducer and receptor, enabling direct comparison of the efficacies and potencies of structurally and functionally diverse ligands within a uniform assay system. Unlike fragmented sets of reports, this approach will provide a useful reference for cannabinoid pharmacology.

Opposite Regulation of Heteromeric GIRK1/2 Channels and Homomeric GIRK2 Channels by Cholesterol

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Drugs of abuse, while causing substance use disorders through a variety of mechanisms, share a common feature in the increased release of dopamine (DA) in the brain's reward pathways. Some of these drugs, acting at $G_{i/o}$-coupled receptors in GABAergic neurons, reduce GABA release to dopaminergic neurons in the ventral tegmental area (VTA), thus disinhibiting DA release associated with induction of addiction.

G protein coupled inwardly rectifying potassium (GIRK) channels are effectors of $G_{i/o}$ signaling that are unevenly distributed in the neuronal populations involved in the brain's reward pathways. Heteromeric GIRK1/GIRK2 (GIRK1/2) channels are present in GABAergic neurons, mediating both antinociceptive and pro-addictive effects of $G_{i/o}$ agonist drugs, including opioids and cannabinoids. Homomeric GIRK2 channels are expressed in dopaminergic neurons. Selective agonism of GIRK2 is proposed to prevent induction of addiction by hyperpolarizing dopaminergic neurons in the VTA and preventing excessive DA release. The pursuit of this therapeutic strategy has been limited by a lack of understanding of structural and functional differences between the GIRK2 homomer and GIRK1/2 heteromer channels. Our group has recently solved the cryo-EM structure of GIRK1/2 allowing computational analyses to study dynamics and targeted electrophysiology to measure channel function. The structure-guided paradigm has enabled us to characterize differences between the heterotetramer as compared to the previously published GIRK2 structure. We found that the single-subunit difference between GIRK1/2 and GIRK2 results in functional changes, including opposite regulation by cholesterol. Following cholesterol binding, rotation of the transmembrane domains occurs in opposite directions in GIRK1/2 and GIRK2, constricting or dilating the channel pore, respectively. We propose that the Met187 residue is critical for this effect, which in GIRK1/2 moves towards the helix bundle crossing (HBC) gate in response to cholesterol binding. This movement brings HBC residues closer together, constricting the conduction pore and limiting the permeation of potassium ions. We aim to leverage the novel structure of GIRK1/2 and improved understanding of functional differences between GIRK isoforms to allow for the rational design of GIRK2 specific activators that have to potential to limit abuse liability of otherwise addictive drugs.

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Structure-Based Design of Positive Allosteric Modulator of The Mu Opioid Receptor

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Traditional approaches that target the orthosteric site of mu opioid receptors (MOR) provide effective pain relief but can also lead to receptor overactivation and severe side effects, such as addiction and life-threatening respiratory depression. A promising alternative is to target the allosteric sites of opioid receptors by developing positive allosteric modulators (PAMs). PAMs bind outside the orthosteric pocket and modulate the activity of orthosteric agonists by enhancing endogenous pain-relief pathways and temporal selectivity, thus reducing side effects and abuse potential.

We screened a DNA-encoded library (DEL)from Hitgen to identify novel chemotypes with PAM activity at MOR. Through iterative medicinal chemistry focused on improving physicochemical properties such as cLogP, topological polar surface area (TPSA), and CNS multiparameter optimization (CNSMPO) score, we optimized our first generation hit and synthesized a library of ~40 analogs. Structure-activity relationship studies, using radioligand binding and GTP turnover assays at MOR, led to the identification of a potent candidate, **AP58**. AP58 showed MOR dependent PAM actions in vitro in biochemical as well as cell-based G-protein assays as well as ex-vivo slice electrophysiology assays. The cryo-EM structure of AP130, an iodo analog of **AP58**, in its G-protein-bound active state was elucidated with met-enkephalin and it showed a distinct allosteric binding site. **AP58** displayed effective anti-allodynic properties in mice models of chronic constriction injury (CCI), and chemotherapy-induced peripheral neuropathy (CIPN), when administered alone in mice. In an acute thermal pain model, **AP58** alone was inactive, but pretreatment potentiated the efficacy of a subtherapeutic dose of morphine. Unlike traditional MOR agonists such as morphine and fentanyl, **AP58** did not produce conditioned place preference (CPP), respiratory depression, or hyperlocomotion administered alone. While **AP58** enhanced the antinociceptive effect of morphine, it did not increase side effects associated with orthosteric ligands, such as CPP, hyperlocomotion, or self-administration, suggesting that **AP58** can separate analgesia from adverse effects.

In summary, we developed a novel MOR-selective PAM that may serve as a foundation for safer analgesics, and this concept could potentially be extended to other GPCRs.

Targeting Kappa Opioid Receptor Subpockets to Fine Tune Receptor Pharmacology

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The Kappa opioid receptor (KOR) is a viable target for new analgesic therapeutics without the deleterious effects associated with the mu opioid receptor (MOR). Further understanding of how to fine tune KOR pharmacology is needed.

We have designed ligands with a morphinan backbone and an arm that targets a subpocket made up of transmembrane domain 5 (TM5) and extracellular loop 2 (ECL2); balanced ligand MP1104 targets TM2/3. Using bioluminescence resonance energy transfer (BRET) assays we have determined that lead compounds VRB37 and VRB42I display high G protein efficacy with minimal GRK3 recruitment and β-arrestin2 recruitment. Using confocal microscopy, we show that VRB37 and VRB42I display minimal receptor internalisation. Via phospho-immunoblotting and immunoprecipitation, we show that VRB37 and VRB42I display minimal C-tail phosphorylation, no significant p38 phosphorylation and display early but minimal late ERK phosphorylation. In all assays MP1104 displays a high efficacy profile.

We solved a cryo-EM structure which confirmed that VRB37 binds in the TM5/ECL2 subpocket, a distinct subpocket from MP1104. We changed the heteroatom in VRB37 to show that a salt bridge interaction in this subpocket is needed to maintain minimal efficacy in the β-arrestin2 pathway. Using molecular dynamics simulations, and point mutations, we determine that targeting residues E209, S211 and D223 are important to drive down β-arrestin activity.

Using in vivo (C57/BJ6 mice) we show that VRB42I displays KOR mediated partial antinociception, and no sedation in the rotarod assay (at 10 and 30 mg/kg s.c.). VRB42I displays conditioned place aversion only at high doses (30 mg/kg s.c.) and blocks cocaine (10 mg/kg s.c.) conditioned placed preference at lower doses (3 and 10 mg/kg s.c.).

Together, we show that a TM5/ECL2 subpocket in the receptor can be targeted to rationally design G protein biased kappa opioid receptor ligands with minimal efficacy in the β-arrestin2 pathway.

Targeting Cryptic Pocket in Cannabinoid Receptor 1 Drives Peripheral and Functional Selectivity

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Chronic pain impacts 10% of the US population and leads to considerable disability. Current pharmacotherapies for chronic pain rely heavily on opioids, and overprescription and increase in illicit use of fentanyl has contributed to the ongoing opioid epidemic in the US. The CB1 receptor is considered an alternative therapeutic target for chronic pain. However, centrally mediated side effects and analgesic tolerance curb its usage. We used structure-based approach on CB1 receptor by targeting $D^{2.50}$ residue present in cryptic pocket. We designed a series of positively charged derivatives of the potent CB1 agonist, MDMB-FUB. Among the bitopics, VIP-36 in cell assays is a G-protein-biased drug with reduced βarrestin-2 recruitment and is CB1 selective. Cryo-EM structure predicted the guanidinium linker is involved in a cation-π interaction with the "toggle switch" aromatic residues $W356^{6.48}$ and $F200^{3.36}$ present in cryptic pocket. Multiple distinct conformations of the guanidinium linker are observed via MD simulations, wherein the VIP36 linker can sample both longer-range (similar distance as seen in the structure), water-mediated interactions with $D^{2.50}$ while also directly engaging $D^{2.50}$ via short range (< 3.5Å) charge-charge interactions. SAR developed by substituting the polar and charged guanidino group with other neutral but polar residues as well charged residues suggest that the positive charge of tail group dictates arrestin efficacy and may be a mechanism for G-protein bias. A pharmacological study shows that VIP36 does not penetrate the brain, and analgesic in multiple pain models. CB1 related catalepsy, hypothermia and locomotion effects were not observed with VIP36 at analgesic doses, it has 100-fold dose separation between analgesic efficacy and centrally mediated tetrad effects compared to the parent, FUB which showed no separation between analgesia and tetrad effects. VIP-36 has reduced tolerance and maintains analgesia for longer periods. In conclusion, targeting the $D^{2.50}$ residue in the cryptic pocket led to the development of a G-protein-biased drug, peripheral selectivity, and desired in vivo pharmacology with reduced side effects.

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(2R,6R)-Hydroxynorketamine Reduces Δ^9-tetrahydrocannabinol Withdrawal in Mice

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Δ9-tetrahydrocannabinol (THC) is the primary psychoactive component of cannabis and contributes to the drug dependence underlying cannabis use disorder (CUD). Currently, no pharmacological treatments are approved to address CUD, including withdrawal following cannabis cessation. (2R,6R)–Hydroxynorketamine (HNK) is a nonpsychedelic ketamine metabolite that has been found to reduce facets of opioid use disorder in rodents, in addition to possessing antidepressant and analgesic properties. However, HNK has not yet been assessed in a model of THC withdrawal. Thus, the goal of this study was to determine whether HNK can reduce somatic and affective signs induced by precipitated THC withdrawal in mice.

Male and female C57Bl/6J mice were administered THC (10 mg/kg, s.c.) BID for 6 days. HNK (1, 5, or 10 mg/kg, s.c.) or saline vehicle was injected either 1 or 24 hrs before THC withdrawal. After the final AM injection of THC on day 6, the CB1 antagonist SR141716A (3 mg/kg, i.p.) was administered to precipitate THC withdrawal. Mice were immediately placed in activity chambers and video-recorded for 1 hr. Immediately afterward, they were removed from the testing chambers and assessed for time spent struggling in the tail suspension test (TST). The number of somatic paw tremors and head twitches, and the time struggling in the TST were manually quantified by a blinded observer.

THC withdrawal *per se* induced paw tremors ($p<0.001$), head twitches ($p<0.001$), and struggling ($p<0.001$) compared to saline control. HNK (10 mg/kg) pretreatment at either 1 or 24 hrs reduced THC withdrawal-induced paw tremors ($p<0.05$) and head twitches ($p<0.05$). HNK dose-dependently (1 or 5 mg/kg) reduced THC withdrawal-induced struggling in the TST.

Administration of HNK reduced somatic and affective signs of THC withdrawal in mice. HNK possesses high clinical potential for substance use disorders like CUD due to its lack of psychedelic properties and emerging therapeutic profile. Ongoing work will establish the dose-related effects of HNK on THC withdrawal-induced somatic signs and probe potential receptor mechanisms, such as the AMPA receptor.

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Targeted Naltrexone Use During Dry January: A Mixed-Methods Pilot Study of Feasibility, Acceptability, and Alcohol Use Outcomes

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Background: There is growing interest in Dry January, a temporary abstinence challenge (TAC) in which individuals reduce or stop alcohol use for one month. Naltrexone, an FDA-approved medication for alcohol use disorder (AUD) may have utility beyond AUD treatment, including short-term alcohol reduction. Limited evidence supports as-needed naltrexone for mild AUD or at-risk drinking, but no prior trials have evaluated this approach during TAC. This pilot study evaluated the feasibility, acceptability, and preliminary outcomes of targeted naltrexone use during Dry January.

Methods: Mixed-methods pilot study recruited adults planning to participate in Dry January 2026. Those with moderate/severe AUD, receiving medications for AUD, or contraindications to naltrexone were excluded. Participants received a 31-day supply of naltrexone for as-needed use (≤1 tablet/day). Alcohol use was assessed using Timeline Follow-Back, and phosphatidylethanol (PEth) served as a biomarker of alcohol exposure. Follow-ups occurred in February and March. Semi-structured interviews explored medication experience and analyzed using thematic analysis.

Results: Seventeen participants enrolled. Mean drinks per drinking day decreased from 2.43 (SD=1.55) at baseline to 1.65 (SD=1.05) during Dry January and remained below baseline at follow-up (2.34, SD=1.00). Mean PEth levels decreased from 45.24 ng/dL (SD=38.07) to 28.00 ng/dL (SD=30.49) and rebounded at follow-up (36.11 ng/dL, SD=38.52). 70.6% reduced alcohol use and 11.8% achieved abstinence during January.

Qualitative analyses identified five key themes. Many experienced reduced rewarding effects from alcohol, feeling satisfied with fewer drinks and finding it easier to stop. Most viewed naltrexone as a supportive tool used before anticipated drinking occasions rather than a primary intervention. Common barriers to use included no cravings, forgetting the medication, and wanting alcohol's full effects. Acceptability was high, with most reporting no or mild side effects; a few experienced fatigue, nausea, or emotional blunting. Participants broadly supported greater accessibility, with most endorsing over-the-counter availability and willing to recommend it to others with higher-risk drinking patterns.

Conclusions: Targeted naltrexone during Dry January was feasible and well tolerated. Findings suggest variable perceived pharmacologic effects alongside high acceptability and strong support for increased accessibility. These results support further study of targeted use of naltrexone among individuals participating in TAC such as Dry January.

Behavioral Assessment of the Abuse Liability of the Novel Atypical Dopamine Transporter Inhibitor UR-1-1-8

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Dopamine transporter (DAT) inhibitors often possess reinforcing properties that contribute to their abuse liability, making preclinical evaluation of abuse potential an important step during drug development. UR-1-1-8 is a recently developed DAT inhibitor with high affinity for DAT (15 nM), marked selectivity over the serotonin and norepinephrine transporters, and previously reported anti-inflammatory and neuroprotective effects in experimental models of multiple sclerosis. The present study examined the abuse-related behavioral profile of UR-1-1-8 using drug discrimination and intravenous self-administration procedures in Sprague-Dawley rats.

Rats were trained to discriminate 0.32 mg/kg methamphetamine. Methamphetamine produced dose-dependent increases in drug-lever responding with an ED_{50} of 0.146 (0.117–0.175) mg/kg, whereas cocaine fully substituted for methamphetamine with an ED_{50} of 1.703 (0.412–3.526) mg/kg. Modafinil also substituted for methamphetamine, exhibiting an ED_{50} of 113.7 (84.72–175.4) mg/kg. Although UR-1-1-8 was evaluated at doses that reduced response rates to 48.1% of vehicle, it produced a maximum of only 25% methamphetamine-lever responding and did not significantly alter the discriminative stimulus effects of methamphetamine or cocaine. In intravenous self-administration studies, UR-1-1-8 alone failed to maintain responding above saline levels, indicating an absence of reinforcing effects. Moreover, co-administration of 0.1 mg/kg/infusion UR-1-1-8 with methamphetamine did not significantly alter the methamphetamine dose-effect function. Collectively, these findings demonstrate that UR-1-1-8 lacks methamphetamine-like discriminative stimulus effects and reinforcing efficacy, suggesting that UR-1-1-8 has substantially lower abuse liability than classical psychostimulants while remaining a promising therapeutic candidate.

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Unilateral Transcranial Photobiomodulation for Opioid Use Disorder: A Non-Pharmacological, Hemispherically-Targeted Intervention with FDA Breakthrough Designation

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Opioid use disorder remains a public health emergency despite available pharmacotherapies. Roughly half of buprenorphine-treated patients relapse within a year, and stigma plus diversion concerns limit broader uptake of medication-assisted treatment. A non-pharmacological intervention that reduces cravings without receptor-level action, drug interactions, or diversion risk would meaningfully expand the treatment landscape.

We developed unilateral transcranial photobiomodulation (utPBM): a 4-minute application of 810 nm near-infrared light at 250 mW/cm² to the forehead over the dorsolateral prefrontal cortex of the hemisphere identified by a lateral visual field test as having more positive hemispheric emotional valence. The targeting rationale derives from Dual-Brain Psychology, which posits that one cerebral hemisphere in a given individual is more affected by past maltreatment and more prone to craving and negative affect, while the other is healthier. This lateralization is individual rather than fixed by side. The framework is supported by fMRI activation studies, structural MRI showing lateralized differences in nucleus accumbens, hippocampus, and amygdala volumes that correlate with valence, and two studies using lateralized testing to predict rTMS antidepressant response.

Two randomized, double-blind, sham-controlled trials have been completed. The within-subject trial (N=22) showed a 51% reduction in Opioid Craving Scale scores one week post-active treatment versus 16% after sham (Cohen's d = 0.73; depression and anxiety also improved significantly). The two-site NIDA/HEAL-funded RCT (N=39, MindLight and McLean Hospital) showed a 71% craving reduction in the active group versus 35% in sham, effect size 1.5 at final follow-up, with significant reductions in TimeLine FollowBack opioid use and benefit observed in both buprenorphine-treated and untreated participants. A case series of 42 OUD patients receiving utPBM in clinical practice over 382 treatments showed remarkable response in 62% and positive response in 19%. No serious adverse events were observed across studies. A Phase II NIDA/SBIR trial in participants using fentanyl has completed enrollment; analysis is underway.

utPBM is a safe, rapid-acting, non-pharmacological intervention for OUD with FDA Breakthrough Device Designation, supported by two positive RCTs and converging mechanistic evidence for hemispheric lateralization in addiction vulnerability.

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Characterizing Allosteric Modulation of N-Acylethanolamine Acid Amidase (NAAA) Active Site by Endogenous Phospholipids

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N-acylethanolamine acid amidase (NAAA) is a lysosomal N-terminal nucleophile enzyme that has emerged as a pharmacological target for treating chronic pain and inflammation. NAAA overexpression reduces the amount of available palmitoylethanolamide (PEA) in cells, triggering sustained pro-inflammatory responses.

NAAA features an allosteric binding site for phospholipids in the lysosomal membrane to open the active binding site and allow for N-acylethanolamine (NAE) hydrolysis. The exact effects of different phospholipids on the conformation of the NAAA active site or the enzyme kinetics have not been determined. Furthermore, published crystal structures of NAAA have only been calculated with Triton-X, a non-endogenous lipid detergent, occupying the allosteric site. These factors could be biasing drug design efforts towards scaffolds that do not fit an enzyme conformation observed *in vivo*.

To acquire information regarding the effects of different biolipid modulators, we tested three phospholipids commonly found in lysosome membranes for their effects on NAAA activity and substrate affinity. We incubated NAAA with each of the biolipids and measured the rates at which they hydrolyzed the fluorogenic substrate N-(4-methyl coumarin) palmitamide (PAMCA).

We also developed a new HPLC-based assay that allows us to monitor PEA hydrolysis over time. We incubated the activated enzyme in each biolipid buffer and stopped the hydrolysis over time by precipitating the enzyme out of solution with acetonitrile. By measuring the changes in peak area over time, we could calculate the amount of PEA hydrolyzed over time in the different buffer conditions, allowing for more accurate replication of *in vivo* conditions.

We present our findings on the biochemical differences induced by each biolipid modulator and how those changes could be exploited for future NAAA inhibitor generations.

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Fentanyl-Naloxone Conditioned-Place Aversion and the Effects of α2-Adrenoreceptor Agonists

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The synthetic opioid fentanyl is the primary opioid associated with overdose deaths in the United States. Illicit fentanyl supplies are frequently contaminated with adulterants, often producing deleterious effects in addition to fentanyl's own risks. Currently, the α2-adrenoreceptor agonists xylazine and medetomidine are highly prevalent adulterants in areas of the United States. Reports have indicated that these drugs may exacerbate withdrawal from fentanyl. This is clinically relevant, as withdrawal can be a driver of the drug taking cycle whereby people who use drugs will continue using a drug to avoid symptoms of withdrawal. To investigate fentanyl withdrawal and the effect of medetomidine and xylazine on fentanyl withdrawal, we established a conditioned-place aversion protocol in male, Swiss Webster mice. Mice received four days of conditioning, alternating between drug and saline treatments. On drug conditioning days, mice first received either saline, fentanyl, xylazine, dexmedetomidine, or a combination of fentanyl with xylazine or dexmedetomidine, and were then returned to their home cage. Mice were then injected 2 h later with saline, naloxone, idazoxan, atipamezole, or a combination to precipitate withdrawal. After the antagonist injection, mice were immediately placed into the corresponding chamber for conditioning.

We found that precipitated withdrawal from fentanyl produced a conditioned-place aversion, but that precipitated withdrawal from xylazine and dexmedetomidine did not. In fact, when conditioning of xylazine alone was delayed to 4 h after administration, a significant place preference was observed when compared to the 2 h timepoint. Coadministration of either xylazine or dexmedetomidine with fentanyl did not alter the conditioned-place aversion when withdrawal was precipitated by naloxone. However, if withdrawal from the fentanyl and xylazine combination was precipitated by both naloxone and idazoxan, the conditioned-place aversion was significantly reduced. These results suggest that precipitated withdrawal from α2-adrenoreceptor agonists is not aversive, and their mixture with fentanyl are not significantly increasing the negative affective state of fentanyl withdrawal in mice.

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The monoclonal antibody CSX-1004 blocks fentanyl discrimination in squirrel monkeys.

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Fentanyl is a synthetic opioid that is routinely abused for its pleasurable psychoactive effects, which can lead to opioid use disorder (OUD) and subsequent overdose. While μ-opioid receptor antagonist-based treatments for fentanyl overdose exist, they can be ineffective and are short-acting, and the currently available treatments for OUD, the μ-opioid receptor agonists (methadone, buprenorphine) and antagonist (naltrexone) show variable and unsatisfactory long-term efficacy in preventing relapse. Thus, there is an unmet need for novel non-opioid receptor medications for the treatment and prevention of OUD. Antibody-based therapeutics have emerged as a new approach to prevent fentanyl overdose and for the management of fentanyl-related OUD. We previously demonstrated the fully human monoclonal antibody CSX-1004, which has picomolar affinity for fentanyl, dose-dependently prevents the antinociceptive and respiratory effects of fentanyl in rodents and squirrel monkeys; these effects were observed up to 28 days post administration. Here, we investigated the ability of subcutaneously (SC) administered CSX-1004 to block fentanyl's discriminative-stimulus effects in opioid (oxycodone)-discriminating squirrel monkeys (n=4-6). We found that SC administration of 10, 40, and 80 mg/kg CSX-1004 dose-and time-dependently blocked fentanyl substitution for the training dose of 0.18 mg/kg oxycodone. The blockade of fentanyl's discriminative-stimulus effects was most pronounced on day 2 post-CSX-1004 treatment, producing approximately 14-, 31-, and 71-fold rightward shift in the fentanyl dose-response function, respectfully. This protection was long-lasting as significant rightward shifts in fentanyl's ED50 were detectable 35 days after CSX-1004. Moreover, consistent with our prior work, CSX-1004 did not modify the discriminative-stimulus effects of other opioids (oxycodone, altentanil). Collectively, these studies provide further evidence that, in addition to blocking fentanyl overdose, the monoclonal antibody CSX-1004 may be a promising therapeutic strategy for blocking fentanyl's abuse-related behavioral effects while not disrupting the efficacy of other opioids prescribed for pain management.

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Persistent vulnerability to heroin relapse after more than one year of abstinence in rats

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Background: Relapse to opioid use during abstinence is often triggered by drug-associated cues, but the persistence of this vulnerability across prolonged abstinence is unknown. We previously reported that rats' response to cocaine-associated discriminative stimuli (DSs) progressively increases, or incubates, during the first 60 abstinence days and persists up to 300 days. Here, we used the same trial-based discrimination procedure to assess relapse vulnerability and persistent DS control following exposure to heroin-availability DSs over more than one year of abstinence.

Methods: We trained rats to self-administer heroin during DS+ trials (heroin available upon lever press; 0.025 mg/kg/infusion; no drug-paired CSs), but not DS− trials (heroin unavailable) in the same session (2 x 3 h/d; 30 each DS+/ DS− trials). In Experiment 1, we repeatedly tested rats for DS-controlled heroin seeking (3 h; 30 each DS+/DS− trials; extinction conditions) after 1, 21, 60, 120, 200, 300 and 385 abstinence days. In Experiment 2, we tested contributions of DS+/DS− to heroin seeking after 21 abstinence days (3 h; 15 each *no-DSs*, *DS+*, *DS−*, and *both-DSs* trials; extinction conditions). We also evaluated DS control of heroin-primed reinstatement after prolonged abstinence.

Results: In Experiment 1, DS-controlled relapse to heroin seeking was higher after 21 and 60 days of abstinence than after 1 day and persisted for up to 385 days, with greater responding during DS+ than DS− trials. In Experiment 2, responding on abstinence day 21 was low during DS− trials, intermediate during *no-DSs*, and *both-DSs* trials, and maximal during *DS+* trials.

Conclusions: Incubation of DS-controlled drug seeking generalizes to heroin and relapse vulnerability under DS-control persists across more than half the adult rat lifespan. Additionally, DS+ and DS- maintained independent control over expression and suppression of heroin seeking during prolonged abstinence. These data highlight the enduring ability of drug-availability signals to precipitate opioid relapse and suggest that treatments that reduce the motivational impact of drug-availability cues or strengthen behavioral control using non-drug-availability stimuli, may help reduce relapse vulnerability during abstinence.

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Discovery of a Novel Class of Allosteric Modulators Acting at a New Druggable Adjacent Site in Human Monoacylglycerol Lipase (hMGL)

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Drug discovery is facing a productivity crisis as costs continue to climb while nearly 90% of candidates ultimately fail, frequently for a lack of demonstrated efficacy in late-stage clinical trials. A major contributor to this trend is the field's reliance on potency-driven optimization in an absence of mechanistic understanding, leading to ultimately unsuitable candidates being advanced without a clear picture of how such hits actually engage and functionally alter their targets. To address this, we implement an advanced mechanism-first discovery pipeline that couples high-throughput virtual screening with extensive biophysical validation, resolving binding mode and functional effect prior to significant financial investment and late-stage pharmacological studies. We apply this pipeline to monoacylglycerol lipase (hMGL), a serine hydrolase of the endocannabinoid system with broad therapeutic relevance in multiple pathologies. Virtual screening of large commercial libraries identified a novel class of pyrrolidine carboxamide scaffolds predicted to bind a large, conformationally flexible pocket adjacent to the canonical active site. Michaelis–Menten kinetic analysis revealed mixed-type inhibition for both the hit compounds and several known orthosteric inhibitors. Multinuclear ($^{1}H/^{19}F$) NMR spectroscopy further supports that this adjacent site constitutes a true allosteric pocket, demonstrating ligand co-occupancy, catalytic activation, and engagement through conformational selection. Binding proceeds via slow and progressive engagement that culminates in a conformationally trapped ligand and catalytically incompetent conformation. Together, these findings define a novel scaffold and pharmacophore for allosteric inhibition of hMGL and establish a general framework for front-loading mechanistic insight in early-stage drug discovery.

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An Integrated Zebrafish Platform for Synthetic Opioid Reversal Screening and Associated Protein-Network Analysis.

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Synthetic opioid overdose, particularly from fentanyl, remains a major public health challenge and motivates therapeutic strategies that extend beyond μ-opioid receptor (MOR) antagonism. We developed an integrated larval zebrafish (*Danio rerio*) platform that combines a high-throughput photomotor response (PMR) assay for phenotypic screening with *in vivo* photoaffinity labeling (PAL) to investigate fentanyl-associated protein networks. Fentanyl produced biphasic, concentration-dependent suppression of stimulus-evoked locomotion. Naloxone shifted the low-dose phase but had limited efficacy against the high-dose phase, supporting the search for complementary reversal mechanisms.

Screening approximately 1,000 ChemBridge compounds identified nine candidate reversal agents, including several sharing a common scaffold. Resynthesis and preliminary structure–activity relationship studies introduced systematic modifications at three scaffold regions. Individual analogs produced distinct PMR profiles and differential reversal across fentanyl exposure conditions, demonstrating that the assay can guide early hit validation.

For mechanistic studies in the model, we evaluated Photofent, a clickable aryl-azide photoaffinity labeling fentanyl analog. Photofent retained measurable MOR affinity (Ki = 167 nM), produced fentanyl-like concentration-dependent PMR suppression, and labeled larval proteins in vivo in a concentration- and UV-dependent manner. Fentanyl co-treatment substantially reduced Photofent labeling, whereas naloxone produced little or inconsistent competition. After streptavidin enrichment, proteomic analysis, and vehicle subtraction, 78 proteins were identified with Photofent, 72 with Photofent plus naloxone, and 40 with Photofent plus fentanyl. Comparative set analysis defined a 27-protein fentanyl-competitive, naloxone-retained subset. STRING analysis organized these candidate proteins into networks involving metabolic and translational stress responses, among other processes. MOR was not detected in the proteomic dataset; accordingly, these results are interpreted as candidate interaction networks rather than a complete target map.

Together, these studies establish a unified zebrafish platform for identifying novel reversal chemotypes while generating testable hypotheses about protein networks associated with fentanyl exposure. Ongoing work will refine the hit series and validate prioritized candidate interactions using multi-omic analysis and orthogonal assays.

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Psilocybin Reduces Opioid Seeking Behavior and Modulates Both mRNA and Cytokine Expression in the Prefrontal Cortex in a Pre-Clinical Model of Fentanyl Self-Administration

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Opioid use disorder (OUD) leads to thousands of overdose deaths annually. FDA-approved treatments for OUD are methadone, buprenorphine and naltrexone. While these medications reduce mortality and provide symptomatic relief, they are associated with risk of overdose, precipitated withdrawal, and inadequate suppression of craving. Individuals living with OUD need alternative prospects to pharmacologically treat and support their recovery. Clinical studies have demonstrated psilocybin has therapeutic potential for treatment of substance use disorders, with a low abuse potential and safe therapeutic profile. Data published from our lab support these findings; we have demonstrated that an acute administration of psilocybin following opioid abstinence, but prior to relapse testing, reduces heroin seeking behavior in a rodent opioid self-administration (SA) model. Due to the rise of human use of synthetic opioid agonists, we evolved our rodent opioid SA model from heroin to fentanyl SA. We hypothesized that an acutely administered dose of psilocybin that reduced heroin seeking behavior would be efficacious for reducing fentanyl craving behavior. We tested this by administering psilocybin following three weeks of forced abstinence from fentanyl SA, prior to a cue-induced relapse test. Rats in the psilocybin treatment group responded significantly less on the cue-paired lever during relapse testing compared to vehicle-treated rats. Additionally, we report psilocybin may accelerate extinction of opioid seeking and reduce seeking behavior following a fentanyl re-exposure event during abstinence. Bulk RNA sequencing from the prefrontal cortex (PFC) and cytokine analysis from both the nucleus accumbens and PFC of rats that underwent fentanyl SA with psilocybin treatment was performed to begin to identify mechanisms through which psilocybin may reduce fentanyl seeking behaviors. We report significant biological pathways of genes regulated in the PFC following psilocybin and fentanyl and highlight key gene and cytokine expression signatures that may be evaluated in future studies to understand the molecular consequences of psilocybin use in the context of reduction of opioid seeking behaviors.

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D2.50 Residue in Class A G Protein-Coupled Receptors Acts a Functional Efficacy Switch

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The aspartate (D) residue located at position 50 of Transmembrane Helix 2 (D2.50) is highly conserved across 330 class A G protein-coupled receptors (GPCRs). This residue in some but not all GPCRs is part of the sodium binding site. Functionally, sodium ions are conceptualized as negative allosteric modulators for GPCRs. We previously employed a bitopic approach by functionalizing the well-known orthosteric ligands with a flexible straight-chain linker bearing a positively charged guanidino group that interacts with the key D2.50 within the sodium ion-binding site, yielding partial or biased agonists at various class A GPCRs. In this study, we first dissected the sodium site effect on μ-opioid receptor activation by linker rigidification, to limit the number of possible conformations and hence increase the interaction frequency of the bitopic ligands with the D2.50. We generated a strong structure-activity relationship on C6guano through in vitro cell-based signaling assays with ligands that gradually decrease receptor efficacy, correlating well with the rigidity of the linker and ultimately yielding a functional antagonist effect. Our lead ligand derived from fentanyl, BT139, reversed morphine-induced analgesia in the tail-flick assay but did not affect locomotion or respiratory depression when both were administered subcutaneously, suggesting BT139 is a peripherally restricted functional antagonist in vivo. We successfully validated our approach with another class A GPCR, cannabinoid receptor 1 (CB1), rigidifying the flexible straight-chain linker of bitopic CB1 ligand VIP36 also yielded functional antagonist phenotype. We thereby have developed a platform for structure-based design of antagonists by targeting the D2.50 residue at class A GPCRs.

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Profiling of G-Protein Subtype Activation by Novel Allosteric Modulators at the Dopamine D1 Receptor

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The dopamine D1 receptor (D1R) is a therapeutically well-established G protein-coupled receptor involved in motor control, cognition, and reward processing. While orthosteric agonists directly activate D1R signaling, their therapeutic utility can be limited by a lack of signaling selectivity and dose-limiting adverse effects. Positive allosteric modulators (PAMs) represent an alternative strategy that enhances receptor activity by stabilizing specific receptor conformations while preserving endogenous dopamine signaling. Recent structural studies have revealed multiple allosteric binding sites on D1R, raising the possibility that modulation at distinct receptor locations may produce unique signaling outcomes.

To investigate this hypothesis, four novel unpublished D1R PAMs were evaluated alongside LY3154207, a well-characterized D1R PAM used here as a positive control, across three signaling readouts: Gs, Golf and β-Arrestin2. Compounds were selected to engage distinct allosteric regions of the receptor and were assessed for their ability to modulate D1R-mediated signaling in the presence and absence of orthosteric stimulation. Preliminary findings indicate that PAMs targeting different allosteric sites produce distinct signaling profiles, suggesting that allosteric site location influences which transducer is preferentially activated in a context-dependent manner. In several cases, compounds showed differential G-protein subtype activation, selectively potentiating Gs or Golf engagement to varying degrees, with β-arrestin2 recruitment evaluated as an additional signaling endpoint, supporting the idea that individual allosteric sites stabilize unique receptor conformations that favor distinct downstream signaling outcomes.

Ongoing studies are focused on defining the molecular basis of these transducer-selective differences and determining whether site-selective allosteric modulation can be leveraged to improve signaling specificity at D1R. Future work will also investigate whether PAMs acting at distinct receptor sites can be rationally combined to further enhance receptor signaling and expand the range of signaling profiles achievable through allosteric modulation. Together, these studies aim to establish a mechanistic framework linking allosteric site location to biased transducer activation and may provide new strategies for the development of more selective and efficacious dopaminergic therapeutics.

Projections Expressing PACAP from the PBN Induce Sex-Specific Changes in ex vivo BNST Neuronal Activity

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Clinical studies show greater alcohol-related dysregulation of pituitary adenylate cyclase-activating peptide (PACAP) in females than males. The bed nucleus of the stria terminalis (BNST) receives PACAP input and is a sexually-dimorphic region involved in abstinence-induced anxiety. Preclinical studies show sex-specific differences in BNST PACAP expression at baseline and after ethanol exposure. However, whether PACAP differentially regulates BNST activity in males and females is unknown.

The parabrachial nucleus (PBN) is the primary source of PACAP input in the BNST. To test if PACAP sex-specifically modulates BNST activity, we chemogenetically manipulated PACAP-expressing PBN projections (PBN[PACAP]) while recording ex vivo BNST calcium activity. Female and male $PACAP^{Cre+/-}$ (*Adcyap1*) mice received bilateral viral infusions to express Cre-dependent hM3D(Gq) DREADDs in the PBN and the calcium sensor GCaMP in the BNST. *ex vivo* BNST slices were treated with bath-applied Clozapine N-Oxide (CNO) to activate PBN(PACAP) projections or CNO with PACAP receptor antagonist PACAP6-38 to dissect PACAP receptor signaling. Changes in GCaMP fluorescence was used to measure BNST firing frequency.

CNO decreased firing frequency in females during and after application. In males, CNO decreased firing frequency only after application. Bath application of CNO + PACAP6-38 decreased firing frequency after application in females. In males, CNO + PACAP6-38 increased firing frequency during application and decreased firing frequency after application. Comparing males vs females demonstrates the decrease in activity induced by CNO and CNO + PACAP6-38 was potentiated in females.

These data demonstrate that activation of PACAP-expressing PBN projections suppressed BNST activity in both sexes, with a more pronounced effect in females. Future studies will determine whether sex differences in PACAP-induced neurotransmission persist in abstinence. Given that PACAP-targeting therapies are being investigated clinically, understanding sex-specific PACAP signaling may inform therapeutic strategies for alcohol-induced disruptions in anxiety and neurotransmission.

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Chronic delivery of buprenorphine during abstinence decreases incubation of heroin seeking and neuronal activation in medial prefrontal cortex and striatum in male and female rats.

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Rationale and Objective: Buprenorphine is an FDA-approved medication for opioid addiction, but the brain regions underlying its therapeutic effects remain unknown. We previously found that chronic buprenorphine treatment decreases several relapse-related behaviors in rats. Here, we tested whether chronic buprenorphine decreases the time-dependent increase (incubation) in heroin seeking during abstinence. We also used the activity marker Fos to test whether buprenorphine's inhibition of incubation of heroin seeking is associated with decreased activation of cortical and striatal regions.

Methods: we trained Oprm1-Cre rats and their wild-type littermates to self-administer heroin for 12 days (6 h/day). On abstinence Day 1, we tested for heroin seeking under extinction conditions. On Day 14, we implanted osmotic minipumps containing saline or buprenorphine (6 mg/kg/day). On abstinence Days 21-22, we tested the rats (or not tested, baseline condition) for incubation of heroin seeking, after which brains were collected for Fos immunohistochemistry. **Results:** Oprm1-Cre rats did not differ from wild-type littermates in heroin self-administration or 'non-incubated' heroin seeking on abstinence Day 1. In both genotypes, chronic buprenorphine decreased incubated heroin seeking on Days 21-22. Buprenorphine also decreased incubation-related neuronal activation in several cortical areas (anterior cingulate and dorsal peduncular, but not prelimbic, infralimbic, or orbitofrontal cortex) and striatal areas (dorsolateral and dorsomedial striosomes and nucleus accumbens core, but not shell). **Conclusions:** Chronic buprenorphine decreased incubation of heroin seeking, supporting the predictive validity of the rat model. This effect was associated with decreased neuronal activity in specific subregions of the medial prefrontal cortex and striatum.

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Optimization of Pyrrole-based Positive Allosteric Modulator–Antagonists of the D_3 Dopamine Receptor.

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Dopamine receptors (DARs) are G protein-coupled receptors (GPCRs) which are classified as either D_1-like (D_1R and D_5R) or D_2-like (D_2R, D_3R and D_4R) based on structural homology and pharmacological properties. The D_3R has been a target of pharmacotherapeutic interest due to its localized expression within brain regions associated with reward and motivation. Modulation of D_3R is therefore an attractive approach for the treatment of substance abuse disorders. The development of ligands with sub-type selectivity for D_3R has proven challenging due the high degree of homology within the orthosteric binding sites of the D_2-like receptors. However, the development of ligands that interact at less conserved allosteric sites provides an opportunity for enhanced sub-type selective molecules.

To identify novel D_3R-selective antagonist scaffolds, we conducted a high-throughput screen of a small molecule library using a β-arrestin recruitment assay. The lead hit compound, **MLS6357**, displayed unprecedented D3R selectivity as well as unusual positive allosteric modulator (PAM)–antagonist activity in that it increased agonist affinity for the D_3R while decreasing efficacy of signaling. This pharmacological profile may confer unique therapeutic advantages to this scaffold. Iterative medicinal chemistry was used to synthesize and characterize over 150 analogues, with several demonstrating both high D_3R selectivity and improved D_3R potency in β-arrestin recruitment and G protein activation assays. Two of the more promising analogues with 10-fold or greater improvements in potency were further characterized and found to recapitulate both the allosteric PAM–antagonism and global D_3R selectivity of **MLS6357**. The optimized analogues also demonstrated favorable pharmacokinetics in mice suggesting that these compounds may serve as both research tools and therapeutic leads for the treatment of neuropsychiatric disorders, including substance use disorder.

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Extraction and Isolation of Mitragynine and Semisynthetic Development of Low-Efficacy Mitragynine Analogs

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Kratom, also known as *Mitragyna speciosa*, is a plant native to Southeast Asia. Traditionally, it has been used for medicinal purposes, including pain relief and the treatment of opioid withdrawal. However, kratom is also used recreationally; it may be smoked, ingested directly, brewed into tea, taken as capsules, or consumed as liquid concentrates. Its use has been associated with adverse effects such as vomiting, seizures, and drowsiness. These effects are linked to its opioid-like activity, which also underlies its contemporary use as an analgesic, mood enhancer, and stimulant. Kratom contains more than 40 biologically active alkaloids, and the isolation, characterization, and evaluation of these compounds represent an important step toward the development of improved analgesics.

In this study, we extracted compounds from moon kratom powder by dissolving the plant material in methanol. After filtration and removal of undesired nonpolar components through hexane washing, an acid-base workup was performed to obtain the crude extract. Following purification, mitragynine was isolated as the major alkaloid, comprising approximately 1–2% of the total dry leaf material, along with paynantheine and speciogynine. Crude kratom is better described as exhibiting mu-opioid receptor (MOR) activity with some degree of functional preference rather than strict selectivity. MOR ligands have effective pain-relieving properties, but they also cause respiratory depression and addiction. We hypothesized that lower-efficacy agonists may be promising candidates for safer analgesic development at MOR with reduced adverse effects. Based on this rationale, we designed and synthesized a library of analogs using a semisynthetic approach starting from mitragynine. Pharmacological characterization using cell-based assays of the synthesized analogs enabled us to develop a structure-activity relationship, revealing distinct efficacy ranges at MOR. Cell-based characterization of these analogs using a bioluminescence resonance energy transfer (BRET) assay targeting MOR showed extremely low-efficacy candidates relative to DAMGO, a peptide-based full agonist. Our designed analogs exhibited even lower efficacy than previously reported partial agonists such as mitragynine pseudoindoxyl (MP) and buprenorphine.

Overall, this study describes the extraction of mitragynine from kratom leaves and the design of novel low-efficacy analogs to produce analgesic effects with reduced MOR–related side effects.

Discovery of a Novel Synthetic Opioid with Improved Safety Profile

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Atoxifent is a potent, biased mu-opioid receptor (MOR) agonist that produces antinociception with reduced respiratory depression. Structurally, it contains a conformationally constricted piperazine core. We sought to explore the importance of the piperazine core as the unconstrained precursor acts as an antagonist. This indicates that the piperazine core is a key modulatory feature that can be used to gain further insight into ligand recognition at the MOR. The size and position of the bridge were explored on the piperazine core to modulate the angle of the substituents on the core and alter the distance between the basic nitrogen and other key interactions in the binding pocket. These variations resulted in mostly equipotent MOR agonists, in reference to Atoxifent, however varying levels of □-arrestin recruitment as well as differing kappa and delta opioid receptor activity were observed in-vitro.

Further modification led to the discovery of KSW-88, a GPCR-biased MOR agonist with fentanyl-like antinociception that was reversible with naltrexone in mice. In rats, KSW-88 demonstrated reduced levels of respiratory depression compared to fentanyl and maintained the righting reflex. These results indicate a novel synthetic opioid class that provides a more favorable safety profile compared to fentanyl.

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Troriluzole: Dual Glutamate Release Inhibitor and Glutamate Reuptake Enhancer as Therapeutic for Methamphetamine Use Disorder

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Riluzole, approved for amyotrophic lateral sclerosis (ALS), acts through a unique dual mechanism by reducing neuronal glutamate release and enhancing astrocytic glutamate reuptake, offering advantages over agents that only increase glutamate reuptake. Despite this favorable anti-glutamatergic profile for mitigating methamphetamine (METH) addiction, riluzole has pharmacokinetic (PK) liabilities that limit repurposing. To overcome these limitations, the prodrug troriluzole (TRLZ) was designed and prepared. TRLZ retains riluzole's mechanistic profile but with optimized metabolic and PK properties (*e.g.*, longer half-life and less PK variability). Since glutamatergic dysfunction promotes continued METH use and relapse, we tested the hypothesis that TRLZ would inhibit METH reward, reinforcement, and relapse-like behaviors in male and female adult Sprague-Dawley rats using self-administration (SA) assays. We also investigated effects of TRLZ on food SA and METH conditioned place preference (CPP). TRLZ was administered intraperitoneally (IP) in a dose range of 1-16 mg/kg.

TRLZ dose-dependently reduced acquisition of METH SA and intake under fixed ratio (FR-1) conditions and reduced reinforcing efficacy under a progressive-ratio (PR) schedule. TRLZ dose-dependently facilitated extinction of METH SA behaviors, and reduced both cue- and METH prime-induced reinstatement of seeking behaviors, with greater efficacy observed in males. In an extended METH abstinence model following 2 weeks of chronic METH SA, TRLZ reduced cue-induced seeking. TRLZ also reduced food SA, but at higher doses than those required to reduce METH SA. TRLZ also reduced acquisition and expression of METH CPP and disrupted maintenance of an established METH CPP. *In vitro* screening studies showed that TRLZ (riluzole) lacked binding affinity and functional activity at dopamine D1, D2, and D3 receptors, as well as at vesicular monoamine transporter 2 (VMAT2) and trace amine-associated receptor 1 (TAAR1).

In summary, TRLZ attenuated multiple core features of METH use disorder, including drug taking, motivation to obtain drug, drug seeking, and conditioned reward, with greater efficacy observed in male rats. Together with our recent demonstration of robust efficacy for TRLZ in opioid-related rat models and the current testing of TRLZ in a clinical trial for METH use disorder, these findings identify TRLZ as a potential broad-spectrum therapeutic candidate across multiple substance use disorders.

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Xylazine: Evidence for Kappa Opioid Mechanisms and Methamphetamine Interactions

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Xylazine, an adulterant in the illicit drug supply, worsens opioid-related adverse effects. Two recent findings have emerged regarding xylazine pharmacology. First, xylazine co-use with methamphetamine (METH) is increasing; for example, xylazine was detected in 30% of human METH samples in a toxicology report. Second, xylazine is not only an α2-adrenoceptor agonist but also a full κ-opioid receptor agonist. Here, we investigated effects of xylazine, administered alone or with methamphetamine (METH), across multiple endpoints in male rats. Contributions of κ-opioid receptors and α2-adrenoceptors were investigated.

For studies with xylazine alone, xylazine (1, 2.5 mg/kg) was administered daily for 15 days. Locomotor activity was measured on days 1, 5, and 15, and anxiety-like behavior was assessed using the elevated plus maze (EPM) 24 h after the last xylazine exposure. In the EPM, rats treated with 2.5 mg/kg xylazine spent less time in the open arms than drug-naïve rats, indicating anxiety-like behavior during abstinence. Xylazine-induced locomotor suppression increased with chronic exposure, with significant reductions on day 15 and after a xylazine challenge following 9 days of abstinence, indicating sensitization. Acute xylazine exposure (1, 2.5, 5 mg/kg) induced dose-dependent diuresis that was attenuated by a κ-opioid receptor antagonist, 5′-guanidinonaltrindole (0.01-0.1 mg/kg), or an α2-adrenoceptor antagonist, yohimbine (0.3-1 mg/kg), suggesting involvement of both receptors.

During co-administration with METH, xylazine (1, 2.5, 5 mg/kg) reduced METH-induced hyperlocomotion, an effect attenuated by pretreatment with yohimbine (1 mg/kg) or the κ-opioid receptor antagonist LY2456302 (30 mg/kg). In self-administration studies, rats with stable METH (0.056 mg/kg/inf) infusions under a fixed-ratio (FR-1) schedule were assigned to METH alone or METH + xylazine (10, 30 µg). The higher xylazine dose reduced METH infusions without altering inactive lever responding. After 2 weeks of abstinence, rats previously exposed to xylazine + METH showed greater cue-induced seeking than rats previously exposed to METH alone. In a FR-1 choice assay, rats preferred METH alone over METH + xylazine.

Our results demonstrate significant xylazine–METH interactions, as xylazine co-administration reduced METH self-administration and hyperlocomotion but exacerbated cue-induced seeking after abstinence. We also demonstrated that both κ-opioid and α2-adrenergic receptors contribute to xylazine's in vivo pharmacological effects.

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Interpretable Prediction of Protein–Ligand Unbinding Kinetics

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The rate at which a drug dissociates from its target—the off-rate k_{off}, or residence time $\tau = 1/k_{off}$—often predicts *in vivo* efficacy better than binding affinity, because drug–target complexes rarely reach equilibrium between doses and slow dissociation is what sustains target occupancy. However, it is expensive and time-consuming to determine k_{off} by experiments. A machine learning model that can predict k_{off} from protein sequences and chemical structures will benefit k_{off}-based compound screening and accelerate drug discovery. Nevertheless, accurate and scalable prediction of k_{off} poses two coupled difficulties. Physically, k_{off} is governed by a few specific contacts in the binding pocket. It is challenging for a machine learning model to infer them from data. Computationally, experimentally determined k_{off} is scarce and imbalanced across the kinetic range—a regime in which machine learning models collapse toward the mean. At the same time, the abundant unlabeled protein–ligand space goes unused. We develop an interpretable semi-supervised deep learning model to address above challenges. There are several innovations in our method. First, we pair embeddings from protein and chemical foundation models (ESM-2 for residues, Uni-Mol for atoms) to improve model's generalizability. Secondly, we apply quantile-classification for regression to counter mean-regression under imbalance. Thirdly, we develop a confidence-gated self-training algorithm that converts unlabeled pairs into filtered pseudo-labels for data efficiency. Finally, we utilize an additive encoder to make per-token attribution exact and faithful, allowing us pinpoint key residues responsible for unbinding kinetics. Our model achieves Pearson's correlation of 0.682 when tested on novel chemotypes. When validated against experimental co-crystal structures for over 90 proteins, the key residues identified by our model are significantly enriched in binding pockets. The deep learning developed here represents a significance advance towards high-throughput predictive modeling of ligand unbinding kinetics.

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PET Imaging of Orexin Receptors: A Novel Tool for Advancing Drug Abuse Research and Therapeutics

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Drug addiction is a chronic, relapsing neuropsychiatric disorder affecting nearly 300 million individuals worldwide, characterized by compulsive drug-seeking behavior, persistent use despite adverse consequences, and long-lasting neurobiological alterations. Central to addiction is the dysregulation of the brain reward system, particularly dopaminergic signaling, which is also modulated by circadian rhythms. Emerging evidence suggests that the orexinergic system, comprising orexin peptides (orexin A and B) and their receptors (OX1R and OX2R), plays a critical role in integrating arousal, motivation, and reward processing, thereby contributing to substance use disorders. Orexin signaling has been implicated in drug-seeking behaviors, relapse, and stress responsiveness. Specifically, OX1R is strongly associated with cue-induced drug-seeking across multiple substances, including stimulants and alcohol, whereas OX2R is more closely linked to arousal and stress-related mechanisms. Importantly, the orexin system interacts with key neurochemical pathways involved in addiction, including dopaminergic, opioid, and endocannabinoid systems, highlighting its potential as a therapeutic target.

To enable in vivo investigation of orexin receptors in the human brain and accelerate drug development, we report the development of a novel positron emission tomography (PET) radioligand, [^{11}C]CW24. This probe demonstrates good affinity for OX1R and OX2R, with high selectivity over a broad panel of central nervous system targets. In preclinical evaluations, [^{11}C]CW24 exhibited favorable brain uptake, metabolic stability, and pharmacokinetic properties in both rodents and nonhuman primates, supporting its suitability for brain PET imaging. Importantly, PET imaging provides a non-invasive, quantitative platform to measure receptor availability, enabling the characterization of neurobiological mechanisms underlying drug abuse and the assessment of target engagement for novel therapeutics.

In conclusion, [^{11}C]CW24 represents a valuable lead compound for the development of next-generation PET tracers targeting orexin receptors. Advancing such imaging tools will facilitate translational research, improve our understanding of addiction neurobiology, and support the development and evaluation of orexin-based therapeutics for substance use disorders.

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C2 Reduction of Kratom Alkaloids Increases μ-Opioid Activity and Dissociates Reinforcement from Respiratory Depression

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Semi-synthetic kratom-derived opioids are emerging in the U.S. market, exemplified by MGM-15 (dihydro-7-hydroxymitragynine), which has been found in commercial products. To understand how a defined structural change alters receptor pharmacology and abuse-related behavior, we examined a matched modification, stereospecific reduction of the C2 double bond of the alkaloid core, applied to both principal kratom scaffolds, mitragynine and 7-hydroxymitragynine. Radioligand binding and cAMP functional assays were performed at human μ-opioid receptors (MOR) in CHO-K1 cells. Reinforcing effects were measured by intravenous self-administration in rats trained on remifentanil using a multi-component FR5 procedure. Respiratory effects were measured by whole-body plethysmography after intravenous bolus infusion, referenced to morphine. Compounds studied were mitragynine (MG) and its 2,7-dihydro derivative; 7-hydroxymitragynine (7-HMG) and its 1,2-dihydro derivative, MGM-15. Reduction of the C2 double bond enhanced MOR agonist activity on both scaffolds. Parent MG bound MOR weakly (K_i 183 nM), was a partial agonist (71% of DAMGO), did not maintain self-administration, and did not depress respiration but instead increased respiratory frequency; its 2,7-dihydro derivative had higher affinity (Ki 39 nM), was a high-efficacy MOR agonist (E_{max} = 89%), maintained self-administration, and produced only transient respiratory depression or stimulation. Parent 7-HMG was a MOR full agonist (K_i 14 nM, E_{max} = 97%), maintained self-administration, and produced dose-dependent respiratory depression comparable to morphine; MGM-15 retained high MOR affinity and efficacy (K_i 18 nM, E_{max} = 98%) and was more potent than 7-HMG at MOR (EC_{50} = 6.6 vs. 19 nM), maintained self-administration, yet produced only transient respiratory effects without 7-HMG's sustained depression. Thus, reduction of the C2 double bond appears to enhance MOR agonist activity and confer or preserve reinforcing effects while sparing sustained respiratory depression, identifying MGM-15 as a potent emerging reinforcer with a distinct abuse-related and respiratory profile.

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