# The impact of repeated opioid withdrawal on mouse behavior and microglia

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**Abstract**

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Microglia are specialized cells of the central nervous system (CNS), often regarded as the resident myeloid population or the resident immune cells of the CNS. Microglia, as well as astrocytes and oligodendrocytes, contribute to drug induced changes in CNS function, which can be investigated using powerful new omics techniques. Microglia have been shown to play a role in opioid withdrawal by modulating the actions of various signaling molecules and by releasing inflammatory agents, leading to alterations in animal behavior. We hypothesized that microglia may contribute to the behavioral signs of opioid withdrawal when an individual experiences withdrawal several times in succession. Here we present work studying the impact of multiple withdrawal experiences on mouse behavior, as well as changes to microglia morphology and microglia RNA translation, specific to the mouse striatum. We find that five withdrawal experiences lead to more intense and protracted withdrawal signs in mice. Additionally, the impact of multiple cycles of withdrawal changes microglia cells to adopt a more ameboid and inflammatory-like state, perhaps proliferating specifically in the striatum of female mice. Our RNA sequencing results also indicate that multiple cycles of withdrawal induce an inflammatory signature in the microglia translatome, and support our finding that microglia are shifted to a proliferative state in the mouse striatum.

**Chapter 1 / Introduction**

Drug overdose is currently the leading cause of injury death among U.S adults (32.6 deaths per 100,000). Incidents involving synthetic opioids, such as fentanyl, have dramatically increased since 2013 (1 per 100,000 deaths) to a record high in 2022 (22.7 per 100,000 deaths [1]). As such, there is an incredible public health pressure to develop new methods for treating opioid use disorder and avoid such unnecessary loss of life (see HHS issue brief [2]). One approach to decrease opioid use generally is to make the experience of opioid withdrawal less onerous. Currently, opioid maintenance is the most effective treatment for patients, with buprenorphine and methadone pharmacotherapy reducing the incidence of mortality 50 – 70% [3]. However, pursual of opioid withdrawal treatment through non-opioid drugs remains promising – studies of compounds acting on the dopamine, serotonin, cannabinoid, orexin/hypocretin, and glutamate systems have reported outcomes related to opioid withdrawal [4].

Opioid withdrawal itself differs in intensity and reported symptoms [5], and different opioids impact subjective experience of withdrawal according to their individual pharmacokinetics and pharmacodynamics [6]. In humans, there is overwhelming evidence that greater frequency of opioid use at a younger age is predictive of the development of opioid use disorder. However, it is unclear if greater withdrawal experiences impact the severity of withdrawal symptoms for an individual. One of the reasons addiction — and to a lesser extent, withdrawal — is so difficult to treat is that it contains both affective and pain components, which develop because of the multiple sites of action by opioids on mu opioid receptors (MORs) across the body. This leads to dramatic changes in neural circuit function which develop from the first opioid administration and progress through cycles of discontinuation and relapse.

This body of work was originally inspired by the idea (from my mentor, Dr. John F Neumaier) that microglia may play a role in opioid withdrawal through an inflammatory, signaling mechanism that is potentiated over the occurrence of multiple withdrawal experiences. Therefore, the overarching hypothesis is that, akin to the way that seizures may become more frequent and worse over time the more they are experienced (see ‘kindling’ [7], [8]), there exists a biological mechanism whereby opioid withdrawal symptoms become worse or sensitize with each individual episode of withdrawal. Microglia are certainly not the only CNS cells participating but due to their essential functions in regulating neuronal

network activity, microglia are expected to be involved in the sensitization of withdrawal symptoms over cumulative experiences.

There is evidence that microglia contribute to opioid withdrawal symptoms. For instance, Reiss et al used a microglia-selective, conditional knock-down (cKD) mouse model targeting the opioid receptor Mu 1 gene ($Cxcr1^{Cre}/Oprm1^{fl/fl}$) and found that the development of morphine induced hyperalgesia was abolished in male mice and that naloxone-induced withdrawal symptoms were attenuated in female mice [9]. This suggests that the action of opioids on microglia, via Mu opioid receptor (MOR), indirectly contributes to opioid withdrawal symptoms. Other evidence comes from Burma et al, who showed that pharmacological blockade or cKD of microglia pannexin-1 channels decreased severity of opioid withdrawal in rodents [10]. Additionally, a large body of work from Watkins, Hutchinson, and Grace has shown that an inflammation cascade involving Toll-like receptor 4 (TLR4), an key innate immune signaling receptor expressed in microglia, is responsible for some of the symptoms of opioid withdrawal and related neuropathic pain [11], [12]. While this evidence clearly implicates microglia in opioid withdrawal symptoms, glial biologists still are searching for the signaling molecules involved and physical interactions between neural cells that lead to the classical withdrawal symptoms.

Research from our lab also showed a strong role for involvement of microglia in opioid withdrawal [13]. Coffey et al found that, in animals experiencing opioid withdrawal, microglia translation and transcription of many genes involved in cAMP related signaling were increased including *Pde10a*, *Arpp21*, and *Drd1*. Pharmacologic inhibition of microglia expressing hM4Di (an inhibitory designer receptor exclusively activated by selective drugs such as clozapine-N-oxide) led to increased contractions and immobility in an open field, suggesting that general $G_i$ activity in microglia makes withdrawal worse. These intriguing results led us to speculate as to how microglia might change in their morphology and their RNA translation when animals experience withdrawal again and again.

It is important to emphasize that microglia are acting in the milieu of neuronal circuits that are affected by opioid withdrawal and that their biological role in this process is likely to be different depending on the region being studied. When opioids are used chronically [14], they change the release dynamics of dopamine neurons. This is because opioids decrease the activity of MOR expressing neurons in many regions including the striatum [15], thalamus [16] and periaqueductal grey [17], which

then indirectly increases dopamine release by cells in the substantia nigra and ventral tegmental area which innervate the dorsal striatum and nucleus accumbens [18]. MORs become internalized on neurons in response to strong activation, and this reduction of MORs at the cell surface is partially responsible for tolerance that builds with continued opioid use [19]. During opioid withdrawal, the normally depressed firing of neurons inhibited by opioid action at MORs is reversed, leading to hyperactivity of neurons innervating the nucleus accumbens, bed nucleus of the stria terminalis, and locus coeruleus. Other factors to consider include the activity of the enteric nervous system, which is responsible for the diarrhea experienced during opioid withdrawal [20], and the itching that results from opioid use, which involves both central and peripheral mechanisms [21].

In the next chapter I will present a broad overview of what is currently known about microglia and other glial cells in addictive processes, as well as state of the art findings now being revealed by experiments using omics tools. Then in chapter three I will detail our findings in studies of multiple cycles of opioid withdrawal in mice and how microglia may be participating in the worsening of symptoms with consecutive withdrawal experiences.

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## Chapter 2 / How omics is revealing new roles for glia in addiction

### Introduction

Addiction is a multifaceted disease defined by compulsion to seek and take a drug, a loss of control in limiting drug intake, and the emergence of a persistent negative emotional state (such as dysphoria) reflecting changes in motivation that can precipitate relapse [14]. Substance use disorders are human conditions, but the underlying biology of reward and reward seeking, tolerance, and withdrawal appear to be well-preserved in other species as well. In addition, the brain mechanisms underlying distinct phases of the addiction cycle (preoccupation and anticipation, binge and intoxication, withdrawal and negative affect) correspond to changes in activity of key neuronal circuits and the molecular and cellular plasticity involved with acquiring a preference for taking a drug are often distinct from the processes involved in craving and relapse to drug seeking after abstinence. Numerous animal and human studies have detected changes in neuronal structure and circuit activity in key brain regions such as the cortex, basal ganglia, and midbrain that are critical mediators of drug seeking [22]. Glia however, have usually been ignored when studying animal models of addiction even though they are intimately engaged with shaping the connectivity and regulating the activity of neural circuits.

The development of modern omics technologies that can simultaneously interrogate thousands of biological molecules in tissue samples have created new opportunities for discovery-based investigations of the underlying biology of addiction without depending on narrowly defined hypotheses. Next generation sequencing technologies and omics molecular tools for identifying systems-wide changes in biomolecules (transcriptomics, proteomics, metabolomics, et cetera) have added tremendously to our understanding of how glia participate in changes to brain function induced by drugs and provide numerous new mechanistic and therapeutic avenues to explore. These new strategies are game changers in identifying novel methods for modulating the neurochemical basis of addiction, but integrating and weighing the significance of different data sets has been challenging. This chapter will address how recent omics literature has advanced our mechanistic understanding of the roles of glia—including astrocytes, microglia, oligodendrocytes, and ependymal cells—in the biology of addiction. In general, we have

focused our review on studies using *in vivo* animal models or collections of post-mortem human tissue but, when appropriate, we cite important findings from *in vitro* studies as well.

**Glial cells involved in addiction**

The notion that dynamic neuronal circuits are involved in the encoding of drug-related cues and associated behaviors is a hallmark of contemporary thinking about addiction. The role of glial cells in the plasticity involved in developing substance use problems and maintenance of drug seeking over extended intervals has received much less attention. However, both astrocytes and microglia have been hypothesized to impact neural activity during drug taking for over twenty years [23]. Many of the studies of glia in addiction have focused on brain regions where neurons have been implicated in addiction-related behaviors. It is less clear whether adaptations of these glia are responding to local shifts in neuronal function or if they reflect more general changes in glia across the entire brain.

Astrocytes protect neurons from hyperexcitability by regulating neurotransmitter release ([24], [25]), ion gradients [26], various metabolites [27], and inflammatory signaling molecules [28] and can modulate neuronal activity over a longer time interval than typically is associated with rapid neuronal communication. Astrocytes can shape the excitability, metabolic health, and synaptic integrity of neighboring neurons over minutes to days. Astrocyte activity within [29] and across [30] brain regions plays key roles in synaptic plasticity generally and may also contribute to adaptations in functioning induced by drug seeking and relapse. One key mechanism involves astrocyte reuptake and recycling of glutamate, which is the primary excitatory neurotransmitter in the brain. Glutamate has been implicated in drug addiction and its dysregulation has been theorized as a major factor in the development of compulsive drug taking [31]. Studies of cocaine, opioids, and alcohol have all found changes in glutamate release by neurons in the prefrontal cortex projecting to the nucleus accumbens ([32], [33], [34]). Since astrocytes regulate the availability of glutamate for excitatory neurotransmission, impaired glial glutamate dynamics may contribute to synaptic dysregulation that impacts drug craving. For example, blocking glutamate uptake at the astrocytic glutamate transporter-1 using dihydrokainic acid decreased mouse consumption of alcohol in a binge-drinking model, which suggests that astrocytes may contribute to

glutamate dysregulation-mediated alcohol abuse [34]. Kalivas and colleagues have postulated that astrocyte regulation of glutamate homeostasis and synaptic excitability is a key element in stimulant and opioid seeking and relapse, and this has led to a number of potential pharmacological strategies intended to restore normal glutamate homeostasis [35]. Astrocytes have also been shown to influence cocaine-induced VTA activity by their release of GABA, which shows that both inhibitory and excitatory events are influenced by astrocyte modulation [36]. Developing astrocyte-targeted drugs that can alter neurotransmitter availability to neurons may mitigate aberrant neurotransmission that is produced during excessive intake of addictive drugs.

Microglia contribute to homeostatic regulation of neuronal network function and directly modulate circuit dynamics in response to drugs of abuse [37], [11]. Microglia can also sense the extracellular ATP released by neurons during periods of high activity via purinergic receptors expressed on their fine processes and filipodia [38]. Release of ATP is one example of signaling that can activate receptors on microglial processes and which leads to local calcium influx. Induction of calcium wave events within microglia is sensitive to both increased and decreased neuronal firing rates [39]. Thus, microglia are poised to respond to changes in neuronal function induced by drug intoxication or withdrawal and may do so either by interacting with neurons physically (such as by phagocytosis of synaptic material) or through cytokine signaling. For example, opioid withdrawal induces extracellular release of ATP in the rodent CNS [40], which in turn can lead to further ATP release from microglia via panexin-1 channels; blocking these microglial channels can reduce opioid withdrawal signs in mice [10]. Microglial calcium events drive many important cellular processes, including conversion of pro-IL1β to IL1β [41] and release of TNFα [42], both of which are proinflammatory cytokines that are elevated in response to infection or cellular damage. Many cytokines may play a role in the biology of addiction [43], including those released by microglia [44], as a response to neuronal activity changes induced during withdrawal [45]. Recent work has shown that Toll-like receptor (TLR) and MyD88 signaling, which are sensitive to intracellular calcium dynamics in microglia [46] are both necessary for some of the cytokine release that occurs in mouse models of binge drinking [47]. Microglia are also involved in maintenance and removal of dendritic spines, which is important for development [48] and may also be altered by drug exposure [49]. Indeed, diverse roles of microglia in addiction is currently an area of intense interest.

Oligodendrocytes are essential for proper development of the CNS and guide neuronal network formation well into adulthood [50]. Drug taking can alter the proper establishment of myelination, resulting in long lasting changes in neural circuitry and connectivity. One mouse study of cells in the medial prefrontal cortex, which is thought to control choices in drug taking and impulsivity, found that methamphetamine self-administration decreased the number of oligodendrocyte progenitor cells (OPCs) in a lifetime dose-dependent manner [51]. As OPCs are thought to be self-renewing across the brain, this suggests there may be a mechanistic link between myelination in the prefrontal cortex and the impaired executive function that develops in addiction, which is a hallmark of reduced cognitive flexibility and increased impulsive and compulsive behaviors. Exposure to the opioid buprenorphine during development disrupted rat brain myelination in a biphasic manner, with low doses leading to increased OPC proliferation and high doses leading to the opposite [52]. This may be due to the direct action of endogenous opioids to induce cell proliferation.

Ependymal cells, which line the ventricles of the CNS and the central canal of the spinal cord, have received less attention in models of addiction. They are responsible for sustaining circulation of cerebrospinal fluid [53] and for supporting the production of adult neural stem cells from the ventricular - subventricular zone [54]. A recent study found that opioids impair blood brain-barrier integrity, perhaps through tanycytes, which are a special class of ependymal cells that transmit signals initiated in the cerebrospinal fluid into the brain parenchyma [55]. Future studies of glia would benefit from omics methods to target ependymal cells and identify changes occurring when animals are exposed to various drugs of abuse.

**Table 2.1: Exposure to addictive drugs engages glial cells in different ways.** Here we summarize previous findings implicating glial cells in addiction as well as the results from omics studies referenced in this review.

| Drug | Glial cell involvement | Omics related findings |
|---|---|---|
| Alcohol | Microglia: induction of ER stress response, unfolded protein response | Heat-shock protein and RNA expression induced, release of cytokines, release of C1q protein in exosomes |

| | | |
|---|---|---|
| | | |
| | Astrocytes: induction of ER stress response, unfolded protein response, glutamate release and reuptake altered | Heat-shock protein and RNA expression induced, *Apoe, Grm7* and *Glast* expression changes |
| | Oligodendrocytes/OPCs: changes in cell proliferation, alteration of developmental myelination dynamics | Differential expression of *P2rx7* and other mRNAs related to calcium signaling, differential expression of myelination proteins |
| Opioids | Microglia: induction of ER stress response, unfolded protein response, TLR4 signaling activation | Changes in purinergic receptor and cAMP signaling gene expression dependent upon addiction cycle state, induction of cytokine expression and release, GR regulatory element site repression |
| | Astrocytes: induction of ER stress response, unfolded protein response, glutamate release and reuptake altered | GFAP, APOE, and extracellular matrix protein expression upregulated, increases in PI3K-Akt-mTOR and MAPK signaling |
| | Oligodendrocytes/OPCs: changes in cell proliferation, alteration of developmental myelination dynamics | Repression of unfolded protein response (UPR) and endoplasmic reticulum (ER) quality control mRNA expression |
| Nicotine | Microglia: response to changes in long term potentiation (LTP), neuronal excitability | Reduction in cell number, expression of inflammatory genes, downregulation of glutamate receptors |
| Stimulants | Astrocytes: altered glutamate reuptake | *CRF2* mRNA upregulated, *GLAST* protein decreased |

**Understanding omics techniques and their applications**

Numerous omics strategies have been applied to investigate the impact of addictive drugs (Table 2.1). RNA sequencing from bulk RNA has been used to investigate changes in specific brain regions and some genes identified by bulk sequencing can be attributed directly to glia, but a number of important molecules are produced by both neurons and glia. Furthermore, some proteins that are strongly associated with neurons are synthesized in glia as well, so this method cannot distinguish cell types without certain controls or follow up studies. For example, the mu opioid receptor and other neuroreceptors are produced in small but biologically important levels in microglia [56]. Another approach to gain focus on a specific cell type involves separating individual cells from tissue samples with enzymatic and/or mechanical dissociation followed by fluorescent activated cell sorting (FACS) or affinity

column with cell type specific antibodies conjugated to magnetic beads, followed by cell type specific acute isolation of RNA and subsequent sequencing. This approach can provide a great deal of glia-specific information, but there is some risk of shifts in RNA expression during the sorting of live cells and RNAs localized to fine cellular processes will be sheared off during preparation [57]. Single cells or single nuclei can also be prepared from fresh or fixed tissue and this can reveal diversity across a single class of cells and between distinct cell types; however, the depth of RNA detection is lower and so higher copy number genes can be detected more reliably and potentially important changes in modestly expressed genes may be missed by current single cell sequencing methods.

Translating ribosome affinity purification and sequencing (TRAP-seq [58]) and the RiboTag method [59] both allow for selective analysis of ribosome-associated RNAs. These techniques can distinguish between the transcriptome (the total pool of mRNAs) and the “translatome”– the mRNAs associated with ribosomes and actively undergoing translation at the time of sample preparation. These methods have advantages for glial cell studies as they capture RNA from the entire cell, not just the cell body as with single cell RNA sequencing methods, and they allow for isolation of RNAs in a cell-specific manner using transgenic animals crossed with inducible, Cre recombinase-driver lines. It should be noted that the translatome represents a specific subset of mRNA and may reflect shifts in protein translation at a specific moment in time, but it differs from the transcriptome and may not map onto protein levels overall at that same time. We also note that these techniques can be combined with ribosome profiling [60] to determine the translational efficiency at various open reading frames, which can differ between cell and tissue types.

Epigenomic approaches that measure changes in chromatin accessibility for transcription are quickly gaining prominence and may provide mechanistic insights into how the “memory” of drug experiences are encoded in networks of altered gene expression. Other approaches including metabolomics and proteomics may reflect changes in the molecules that are directly mediating changes in cellular function compared to RNA-based approaches, in which we must infer changes in the expression of the associated proteins. The integration of multiple omics approaches at once is now feasible and we feel that it is best for these approaches to be applied to individual animals rather than pooled samples from multiple animals. Pooling samples may provide enough material for reliable

measures in some cases, but it prevents associations between individual animals' behavior and omics results. At a minimum, validation studies should be performed on samples from individual animals so that statistical inferences are more reliable. Another consideration in omics studies is that cells may react to the processing of tissue in a way that impacts the results. For example, Haimon et al ([57]) found that cell sorting introduced a number of artifacts associated with tissue dissociation and depletion of ribosome-associated RNAs. Marsh et al developed a method to prevent glia from shifting their transcriptional state induced by tissue processing that applies transcriptional and translational inhibitors to steps of the cell dissociation process, thereby limiting artifacts that arise from enzymatic treatment and single cell isolation [61]. Finally, the emergence of machine learning algorithms to manage large and complex data sets will be useful for integrating data from multi-omics experiments along with multiple indices of behavior in animal models of addiction.

**Alcohol**

Alcohol is unique as a rewarding drug. At the cellular level ethanol is a toxic metabolite and at the organismal level it is an intoxicant, capable of inducing euphoria in the short term and potentially deadly withdrawal if discontinued after chronic use. Ethanol exposure engages the unfolded protein response (UPR, [62]) and the endoplasmic reticulum (ER) stress response pathways [63] (**figure 2.1**). These cellular processes are important responses to stress and toxins that operate both as quality control features that regulate protein maturation in many tissues and as signaling systems that coordinate cellular and systemic responses upon more intense or protracted activation. While the UPR has been associated with the cellular effects of ethanol for many years, more recent unbiased omics strategies have also detected ethanol-induced UPR and ER stress related signaling changes that are shared across microglia and astrocyte transcriptomics datasets. One study using bulk RNA-seq found that astrocytes protect neurons from the effects of ethanol-induced stress [64]. They showed that conditional knock-out of the mesencephalic astrocyte neurotrophic factor (*Manf*) severely increased ER stress gene expression and death of neurons in young mice (post-natal day 7) when exposed to ethanol. Another study used chronic intermittent ethanol (CIE) vapor exposure to study the effects of repeated cycles of high ethanol doses followed by periods of abstinence in male mice; they then performed bulk RNA-seq of microglia and

astrocytes from the prefrontal cortex eight hours following the last ethanol exposure period [65]. They found that, compared to air-exposed control mice, astrocytes and microglia from ethanol-exposed mice shared increases in stress response, protein folding, and estrogen receptor signaling gene sets. These included *Hsp90ab1*, a heat shock protein involved in the UPR, as well as other heat shock proteins. In a single nucleus RNA-seq study of human post-mortem tissue from alcoholic patients and control subjects, another group found that glial cells produced the greatest number of differentially expressed genes compared to other cell types [66]. They found changes in stress response genes such as *Hsp90aa1*, which was significantly reduced in microglia and astrocytes. Furthermore, large numbers of genes associated with neuroimmune response were significantly altered, such as interferon regulatory factor 3 (*Irf3*), high mobility group box 1 (*Hmgb1*), and Toll-like receptor 2 (*Tlr2)*. Other notable genes included the astrocyte glutamate transporter GLAST (*Slc1a3*) and the apoptosis related gene B-cell lymphoma 2 (*Bcl2)*. These results were consistent across different brain regions, suggesting that gene expression changes in glia may be a global response to chronic ethanol consumption and may contribute to numerous behavioral impacts that are mediated by different brain regions. Many changes in the UPR and ER stress signaling pathways are probably context dependent and we note that individual gene changes may differ between studies of acute exposure, chronic exposure, and withdrawal from chronic administration. For example, a recent study showed that acute alcohol exposure to interneurons of the prefrontal cortex caused increased cell excitability, but when mice were allowed to access alcohol intermittently over four weeks, the same subset of PFC neurons showed decreased excitability compared to water administering control mice [67]. When assessing studies of glia, it is vital to consider how the design of animal models or the human subject characteristics impact the biological state being assessed.

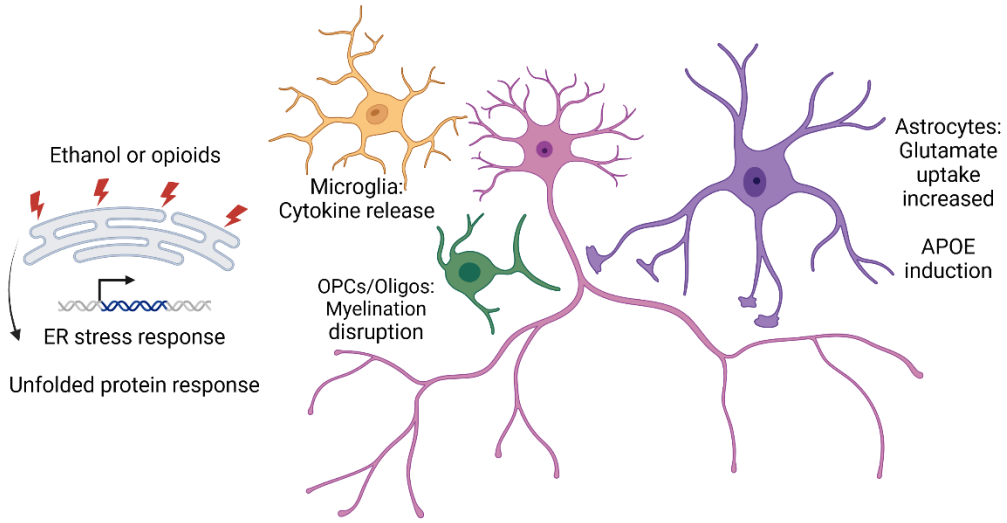



**Figure 2.1: Ethanol and opioids induce similar effects in omics investigations of glial cells.** Whether indirectly or directly via receptor signaling pathways, both ethanol and opioids activate endoplasmic reticulum (ER) stress response and unfolded protein response (UPR) events, which seem to affect specific glial cells in different ways. Microglia and astrocytes are thought to contribute to inflammatory responses to both drugs, through cytokines release and changes in glutamate uptake from the extracellular space by astrocytes. Astrocytes have also been shown to induce APOE expression specifically after ethanol exposure. Oligodendrocyte precursor cells (OPCs) and oligodendrocytes have been shown to differentially respond to low and high opioid concentrations, resulting in changes to myelination and related protein expression.

Evidence of neuroinflammation is reported in nearly all glial omics studies of alcohol dependence or models of alcohol consumption. In older studies, microarrays of cDNA probes were common for performing RNA expression analyses. These arrays are constructed with pre-selected gene targets and remain a much cheaper option compared to next generation sequencing technology but lack flexibility to discover new genes or probe gene expression for low-abundance transcripts. Astrocyte-related inflammation in the prefrontal cortex of human alcohol abuse patients was reported using such arrays, including changes in apolipoprotein-e (*Apoe)* and microglial glutathione S-transferase, which is thought to be essential for response to toxins [68]. Another microarray study using a mouse model of acute alcohol self-administration found common changes across six brain regions in astrocyte expression of glutathione S-transferase *(Mgst1)* , metallothionein 2 (*Mt2*), and cystatin 3 (*Cst3*), the latter of which is thought to be associated with CNS infection [69]. Together, these studies suggest that alcohol consumption may induce astrocyte-driven inflammation across brain regions, not just those circuits directly associated with alcohol reward, craving, and seeking.

In a proteomic study of the mouse caudate putamen and nucleus accumbens following CIE or withdrawal from alcohol, the authors saw increased farnesoid X receptor and retinoid X receptors, oxidative stress, and mitochondrial transport chain proteins in tissue from withdrawing mice, suggesting involvement of both metabolic stress and an inflammatory response in the striatum [70]. These authors proposed that their results implicated alcohol induced changes in cellular lipid and cholesterol metabolism, which may influence trafficking of receptors and transporters to the plasma membrane. Such conclusions are supported to some extent by another study which performed proteomic profiling of microglia exposed acutely to ethanol *in vitro* and found increased expression of OXPHOS-associated proteins, but decreased expression of inflammatory signaling proteins [71]. The latter may be due to acute alcohol exposure to induce anti-inflammatory signaling changes via increased sirtuin 2 (*Sirt2*) signaling [72]. Another group profiled the proteins in exosomes released by microglia from rats administered ethanol and determined that microglial complement component 1q (*C1q*) can induce apoptosis of pro-opiomelanocortin expressing neurons [73]. This recapitulated their previous results [74], suggesting that microglia might be implicated in the neurotoxic effects of ethanol via release of exosomes.

Metabolomic studies may inform treatment development and diagnostic testing for promoting abstinence or reduced drug consumption. In one example, metabolomics and pharmacogenomics were used to identify plasma markers and single nucleotide polymorphisms correlated with alcohol craving and potential for relapse among patients taking the drug acamprosate for alcohol use disorder. Using reprogramming of peripheral blood mononuclear cells from these patients, the authors were able to induce astrocyte differentiation and confirm that several genes expressed by astrocytes (metabotropic glutamate receptor 7 - *Grm7*, tyrosine phosphatase receptor type D - *Ptprd*, and autism susceptibility candidate 2 - *Auts2*) , were ethanol inducible, correlated with blood concentrations of ethanolamine (a metabolite of ethanol), and predictive of alcohol craving [75]. Another study looked at the effects of nicotine-derived nitrosamine ketone (NNK), a byproduct of tobacco smoking, as a potential deleterious agent acting synergistically with ethanol in rats [76]. They found that the combination of ethanol plus NNK caused greater demyelination in white matter and decreases in several lipid species than either agent alone.

Overall, these results on models of alcohol exposure suggest that astrocytes and microglia may be working in concert to respond to the toxic effects of ethanol that can be seen in shared activation of the UPR and ER stress pathways, as well as induction of neuroinflammation which may ultimately contribute to neurotoxicity.

**Opioids**

In opioid administration and withdrawal studies, neuroinflammation has also been implicated by changes in glial transcripts, especially in microglia and astrocytes. In a bulk RNA-seq study of microglia collected from the spinal cord of rats following 14 days of morphine administration, the authors reported that genes related to inflammation and pain were enriched during withdrawal, including the P2X purinoceptor 4 (*P2rx4*) and the triggering receptor expressed on myeloid cells 2 (*Trem2*) [77]. They also found evidence at the protein level for increases in allograft inflammatory factor 1 (*Aif1/Iba1*) and glial fibrillary acidic protein (*Gfap*) in the spinal cord using immunohistochemistry, potentially indicating activation of microglia and astrocytes, respectively. Another group performed a similar analysis with rats, collecting spinal cord and separating microglia for bulk RNA-seq one hour following the last dose of a sequence of escalating morphine treatments. They found evidence for inflammatory changes that were sex-specific, such as upregulation of interleukin-1β (*Il1b*) in male rats and downregulation of C-C chemoreceptor type 3 (*Ccr3*) in both sexes [4]. In a unique study collecting peripheral blood mononuclear cells from individuals with opioid use disorder, the authors induced forebrain organoids from the human-derived cells to study effects of opioids applied *in vitro* on the transcriptome using bulk and single cell RNA-seq. They found that oxycodone had greater effects on neuronal cells but induced changes in interferon signaling pathways in neurons, choroid plexus associated cells, and astrocytes [78]. These findings resulted from dimensionality reduction methods and separation of cell types by their gene expression using a K-nearest neighbors approach now common in bioinformatics studies. Another postmortem tissue study taking samples from human subjects with opioid use disorder performed bulk RNA-seq to correlate changes in the dorsolateral prefrontal cortex and nucleus accumbens with data from genome-wide association studies (GWAS) [79]. They found that both regions showed differentially expressed genes related to extracellular matrix remodeling, TNFα and nuclear factor-κB signaling, stress

responses, and epigenetic modifications. Additionally, both regions showed specific and significant changes in microglia transcripts, such as miRNA223, a modulator of macrophage activation. The RNA-seq results were derived from their subjects' samples and compared to previously published GWAS data and suggested that downregulated transcripts in the nucleus accumbens mapped to gene loci associated with risky behavior and various psychiatric disorders, potentially indicating a mechanistic relationship. While correspondence between risk alleles and changes in gene sets in specific brain regions is intriguing, the next step would be to determine the predicted functional impact of a particular risk allele; i.e. is it predicted to increase or decrease functional RNA and protein expression? A separate group investigated postmortem tissue from the ventral tegmental area and substantia nigra of a large sample of 95 human subjects including controls and showed that opioid use history was associated with upregulation of glial inflammatory genes [80].

The UPR and ER stress pathways have also been implicated after opioid exposure using transcriptomic methods. A study using single cell RNA-seq of tissue from the nucleus accumbens of mice four hours following a morphine or saline injection found that all glial cell types showed significant changes in gene expression, with oligodendrocytes showing the greatest number of differentially expressed transcripts [81]. By performing bulk RNA-seq of sorted oligodendrocytes in a separate experiment, the same group confirmed their single cell results and showed that the transcripts associated with the UPR and ER quality control gene sets were both repressed in oligodendrocytes after a single exposure to morphine. Such work combining multi-omics approaches highlights how one method may inform future mechanistic investigations that naturally follow from confirmatory experiments.

Our lab has used the RiboTag method to study changes to the mouse translatome during opioid administration and withdrawal. Using transgenic animals expressing a hemagglutinin (HA) tag selectively on microglial ribosomes, we found that opioid tolerance downregulated cAMP-related signaling genes in microglia from the striatum and that this pattern reversed when mice were administered naloxone to induce withdrawal following chronic morphine administration [13]. This was a surprising result because it is generally opposite of the pattern observed in the total RNA from striatum in the same animals and from neurons in particular [82]. It raises the possibility that opioid-induced gene expression changes in microglia are not regulated in response to direct opioid receptor activation as in neurons but may instead

be changing in response to the altered activity in nearby neuronal circuits, such as release of ATP or norepinephrine by neurons induced by loss of opioid tone during withdrawal (**figure 2.2**).

Transcriptomic and epigenetic methods can be combined to probe accessibility of chromatin that allows for initiation of gene expression in specific cell types. One example collected tissue from rats with a history of either cocaine taking or of opioid administration and found surprising overlap in differentially accessible transcription factor motifs and gene regulatory element expression [83]. They showed that previous oxycodone or cocaine taking downregulated many glucocorticoid receptor (GR) associated regions, including intergenic regions upstream of the FK506 binding protein 5 gene (*Fkbp5)*, and upregulated several AP1 family member motifs such as *Creb*, *Jun*, and *Fos*. Their results also specifically identified microglia, astrocytes, and oligodendrocytes with repressed GR binding site accessibility. Such work would not have located an intergenic region with older techniques such as microarrays, which usually limit transcript identification to protein coding genes and do not address the accessibility of gene regulatory elements to target genes or non-coding RNAs.

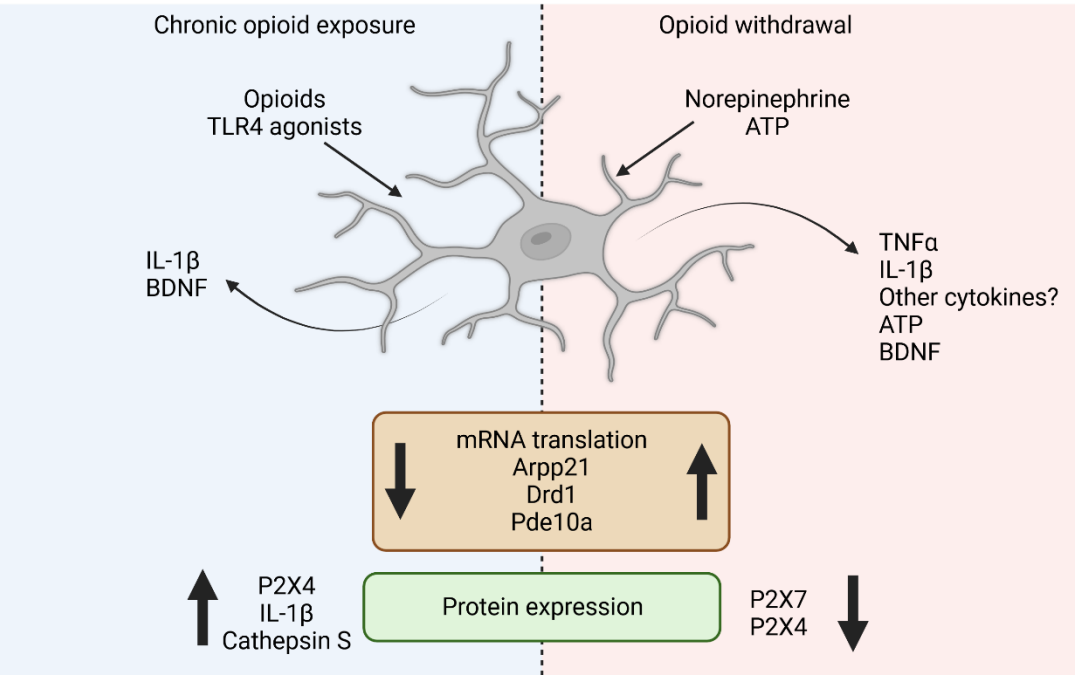



**Figure 2.2: Microglia respond in seemingly inverse ways during chronic opioid versus opioid withdrawal.** The translation of several key microglial RNAs related to cAMP related signaling is decreased in opioid tolerant mice and increased in mice undergoing opioid withdrawal [13]. On the protein level there is evidence for increases in P2X4 receptor expression [84], IL-1β production and release [84], and Cathepsin S expression [85] following chronic opioid delivery. During withdrawal, both P2X7 and P2X4 receptor expression is reported to decrease [86]. BDNF may be released both during opioid administration and during withdrawal from opioids, as a product of mu opioid receptor expression on microglia [87]. ATP [10] and cytokines such as TNFα [88] have been shown to be released by microglia during opioid withdrawal. Opioids act as TLR4 agonists, suggesting a possible mechanism for opioid-induced microglial responses [11]. Norepinephrine [89] and ATP [90] released by neurons can also act on microglia and are thought to be released by hyperactive neurons during opioid withdrawal.

In a study of postmortem human tissue from patients with opioid use disorder, the authors combined RNA-seq and proteomics methods to examine dorsolateral prefrontal cortex [91]. They found that most differentially expressed genes were explained by neurons, astrocytes, microglia, and endothelial cells. Further analysis of the RNA and protein interaction networks showed upregulation of cellular machinery related to inflammation and angiogenesis. Another proteomic study applied morphine and its metabolites to human derived primary astrocyte cultures [92]. They found that APOE and extracellular matrix proteins as well as several members of the PI3K-Akt-mTOR, MAPK, and cell cycle regulation signaling pathways were upregulated by morphine-3-beta-glucuronide exposure. A third group using proteomics to study opioid-induced hyperalgesia in rats found that oligodendrocyte myelin-related protein expression (including MBP, PLP1, MOG, MAG, and MOBP) was decreased in the prelimbic area

of the prefrontal cortex immediately following a series of acute fentanyl injections [93]. Together these results suggest that changes to RNA and protein expression are sensitive to the time course of drug exposure and whether it is voluntarily taken or administered noncontingently. Animals choosing to take drug may show changes in specific transcripts and molecules that are markedly different from animals that are administered drugs by experimenters. Furthermore, the findings of studies in one brain region may be completely distinct from those in another region of the CNS, even when taking into consideration the use of similar methods of drug delivery [94].

**Food rewards as potential addictive substances**

The term addiction is sometimes applied to abnormal patterns of excessive or binge-purge, palatable food intake that individuals find difficulty in controlling. For example, a high fat diet has been associated with changes in motivation and mesolimbic dopamine system plasticity which mirrors administration of certain drugs of abuse [95]. A metabolomic study of mice found that markers of oligodendrocyte progenitor cells in the spinal cord were reduced after twelve weeks of voluntary, high fat diet consumption. Further RNA-seq profiling showed that several ER stress-related genes were upregulated in the spinal cord and that ER stress and mitochondrial dysfunction metabolites were associated with oligodendrocyte cell death or dysfunction [96]. Given that opioids have been shown to impact oligodendrocytes and myelin biology, this highlights the power of metabolomics to identify the mechanisms by which glia are involved in changes induced by administration of drugs or highly palatable food rewards. Since food has both nutritional and motivational domains, distinguishing metabolic from behavioral impacts in omics results is not straightforward, but by comparing across different reinforcers we may be able to draw inferences regarding which changes relate to the behavioral adaptations that are most closely aligned with habitual and problematic reward seeking.

**Nicotine**

Several studies have used omics methods to study nicotine as a drug of abuse ([97], [98], [99]) but, as elsewhere in this review we have limited the scope of our comments to include only those studies

which directly discuss changes ascribed to glial cells or glial signaling. In a study of nicotine exposure to pregnant rats combining single nucleus RNA-seq and ATAC-seq, Chen et al found that total microglia were reduced in the developing brain and that the offspring were more susceptible to cerebral ischemic injury, which was correlated to increased expression of inflammatory genes and downregulation of glutamate receptors [100].

**Amphetamine, cocaine, and other stimulants**

TRAP-seq was used to examine astrocyte changes in response to cocaine or methamphetamine in the mouse substantia nigra and ventral tegmental area [101]. The authors found that corticotropin releasing factor type 2 receptor (*Crhr2*) transcripts were increased and GLAST was decreased at protein level by stimulant exposure. This work demonstrated that CRHR2 is produced by astrocytes in the midbrain, suggesting a greater role for astrocytes in the response to CRF as a neuromodulator.

A meta-analysis of RNA-seq data from mice administered palatable food or cocaine found that both food and stimulant exposure caused gene expression changes related to cAMP signaling pathways [102]. The authors also found evidence for involvement of glia, with cAMP regulatory genes such as protein phosphatase 1 regulatory subunit 1B (*Ppp1r1b*), regulator of G-protein signaling 9 (*Rgs9*), and phosphodiesterase 10a (*Pde10a*) changing across neurons, astrocytes, oligodendrocytes, and endothelial cells.

**Future work can benefit from multi-omics approaches**

We hope to see greater adoption of multi-omics methods to leverage precious tissue generated for hypothesis testing. For example, the ability to combine RNA-seq methods with either chromatin accessibility assays or proteomics/metabolomics methods allows for cross correlation of results and may allow for better resolution of mechanisms regarding glia in addiction biology. Additionally, questions remain as to if and how glia may differ in the biology of addiction between distinct regions of the brain. Clearly, certain components of addiction-related behaviors will be mediated by different brain regions yet most addictive substances impact the entire brain and can alter glial biology globally. The extent to which

changes in glia are observed across brain regions vs. within specific brain regions may provide insights into whether the drugs are affecting the glia directly or whether the glia are responding to changes in the function of neural circuits within a particular brain region. Given our current understanding of how drug taking alters neural communication between and within the basal ganglia, the extended amygdala, and the prefrontal cortex over time, it is worth dissecting how and when glia are participating and their relevant effects are mediated. Some of this is now coming to light, such as how neuroinflammation and cellular stress responses are engaged by microglia and astrocytes in several regions, including the prefrontal cortex and the striatum. Still, more work needs to be done to understand if microglia (for example) differ significantly in their response to a given drug of abuse across the brain and across stages of the addiction cycle.

We also propose a few best practices regarding interpretation of omics data. First, controls for negative conditions must be carefully chosen as they represent the reference state for biomolecules under question. This is particularly salient for RNA-seq studies, where small differences in how samples are handled or how animals are tested in different behaviors can give rise to potentially significant changes in RNA expression. Glial biology especially can be impacted by loss of cell tissue during cell sorting (microglial processes, astrocyte processes, et cetera). Second, for studies involving immunoprecipitation of a biomolecule from tissue homogenate (such as is done for RiboTag or TRAP-seq), it is of vital importance to always compare paired input and immunoprecipitated samples. Purification methods are not perfect and large changes in the input tissue may contaminate the immunoprecipitated fraction, resulting in neuronal or other cell genes being present in glial-specific results. We have attempted to estimate, model, and correct for such contamination in some cases [13]. Third, the use of a sufficient number of biological replicates is of greater importance than technical replicates when performing omics studies to contend with the degree of variability that exists between individuals. Since animals vary a great deal in their drug seeking behaviors, correlating individual subjects' omics results with their behavior may provide clues as to which gene changes alter a particular feature of addiction-related behaviors, such as intensity of tolerance or withdrawal, craving, and likelihood to relapse after abstinence. Using distinct methods to validate and verify differentially expressed biomolecules in separate samples can increase our confidence in the results of an omics study, such as

by validating changes in glial cells using fluorescent *in situ* hybridization experiments to target RNAs. Potential pitfalls exist as well—true changes in the transcriptome may or may not be reflected in the translatome or the proteome, and the functional state of cells in individuals going through tolerance and withdrawal may change rapidly, making it difficult to compare results from two different time points or across drugs with different biological half-lives. Finally, rigorous studies must incorporate enough male and female subjects for adequate power, whenever possible, as sex remains a poorly studied variable in addiction science and biomedical science generally.

**Conclusions**

Harnessing the power of omics techniques can now give scientists great precision to answer hypotheses regarding timing of drug-induced changes without having to limit data collection to a single cell type or a single portion of the CNS. From our review of the literature, we can draw several overarching conclusions about the current state of glial omics work on addiction. While much has been learned about astrocytes and microglia, ependymal cells remain the least studied of the glial cell types. Drug-induced changes to oligodendrocytes can impact the developmental trajectories of neural circuits, thereby altering function over the lifespan. The UPR and ER stress pathways are differentially engaged with astrocytes and microglial, often in similar directions when studying multiple brain regions. This may point to a common effect of drugs of abuse generally, such as the toxic effects of the substances themselves or the action that they induce on circuit function across the CNS. Neuroinflammation is a common response to drug exposure and seems to be driven primarily by astrocytes and microglia but is not necessarily restricted to these cells and remains poorly described except as a general response. Finally, some changes in glial cells may relate to effects of the drugs on the glial cells directly whereas other changes to glia are likely to be induced as responses to alterations in neighboring neurons. These distinctions are important as they may have implications for the development of novel therapeutics that target glial biology selectively, while attempting to avoid off-target effects on neurons that respond differently from glia to drug exposure.

## Chapter 3 / Repeated opioid withdrawal increases mouse anxiety and hyperalgesia, and induces an inflammatory microglia profile

### Introduction

Based on previous work from our laboratory and others, we designed a series of experiments to study the effects of multiple cycles of opioid withdrawal on mouse behavior. We believe this is the first study of its kind to investigate the notion that the subjective experience of withdrawal may worsen with repeated withdrawal episodes in an individual's life. We also were inclined to study how microglia may contribute to the etiology of withdrawal by performing RNA sequencing of the microglia RNAs undergoing translation at 16 hrs into the development of spontaneous withdrawal, looking specifically at microglia in the mouse striatum.

There are several reasons for studying microglia in the striatum. The striatum is a complex hub of information processing, integrating inputs from the cortex, thalamus, and the basal ganglia [103]. The ventral striatum, comprising the nucleus accumbens, has major roles in reward processing, learning, and emotional responses [104] and receives strong innervation from the dopamine-releasing neurons of the substantia nigra and ventral tegmental area [105]. The dorsal portion is characterized by patch (also known as striasome) and matrix components and can further be subdivided into functionally meaningful dorsolateral and dorsomedial domains [106]. The expression of mu opioid receptors (MORs) on medium spiny neurons of the striatum [107] implicates microglia in the effects of long term opioid administration and its effects on neuronal circuit changes and animal behavior [108], as well as the effects other drugs of abuse [109]. Additionally, basic responses to food rewards in certain states depend on MOR expression in the ventral striatum specifically, giving strong rationale for investigating how microglia might be impacting striatal activity in the context of opioid action [110].

### Materials and methods

Animals

Transgenic mice were generated by crossing tamoxifen-inducible Cx3cr1-CreERT2 hemizygous mice (Cx3cr1-Cre$^{+/-}$) with homozygous, floxed RiboTag mice (Ribotag$^{+/+}$) on a C57BL/6 background. Mice received tamoxifen (75 mg/kg dissolved in corn oil) via intraperitoneal injection (ip) for 5 days at starting at 4 weeks of age to induce Cre-mediated recombination. Males and females were housed in separate cages of two to five total animals for all strains used. Mice ranged in size from 18 to 32 g and were between 8 and 14 weeks old at the time of placement into experimental groups. Mice were group housed in a temperature- and humidity-controlled vivarium with a 14–10 light–dark cycle with ad libitum access to food and water. Most experiments were performed during the light phase except for the sucrose preference test described below, which was performed overnight beginning at 5 PM over several days. All experiments were performed in compliance with the Guide for the Care and Use of Laboratory Animals (NRC 2011) and were approved by the Institutional Animal Care and Use Committees for both the University of Washington and for the Veteran's Affairs, Puget Sound Health Care System. Heterozygous Ribotag$^{+/-}$ :: Cx3cr1-Cre$^{+/-}$ offspring were chosen for RNA immunoprecipitation and sequencing following the behavioral experiments while Cre-null Ribotag$^{+/-}$:: Cx3cr1-Cre$^{-/-}$ cage mates were chosen for perfusion and immunohistochemistry (IHC). Live decapitation was used for RiboTag RNA isolation procedures while cardiac perfusion using paraformaldehyde (PFA) was conducted for tissue preservation and performance of IHC.

Drugs

For all experiments involving fentanyl treatment, ip injection was performed twice a day for five days to induce opioid tolerance. Animals in the fentanyl treatment group were exposed to increasing fentanyl concentrations each day; the dose doubled each day, starting at 200 μg/kg on day one and increasing to 3200 μg/kg on day five. For animals in the saline control groups, saline vehicle of equal volume was matched for fentanyl solution. For comparison of treatment groups, animals exposed to five cycles of withdrawal repeated the injection protocol a total of five times. Between each cycle of injections, animals in both the saline and fentanyl conditions were allowed to rest in the home cage without any injections for four days. Tamoxifen solution was prepared by dissolving 20 mg/mL tamoxifen in corn oil and stirring

overnight at room temperature, protected from light. Prior to IP injections, both fentanyl and tamoxifen solutions were sterile filtered (Thermo Scientific, 0.2 μm pores).

## Sucrose Preference Testing

The sucrose preference test procedures were carried out following on the work of Meng-Ying Liu et al [111]. Briefly, animals were first exposed to two 50 mL BioServ bottles in their home cages for a period of two days. The bottles contained either water or 2% mass/volume sucrose in water (~0.058 M). Bottles were washed each day, the contents refilled, and the side of the cage assigned to each bottle was swapped from left to right or vice versa. After two days of training with their littermates, animals were isolated to individual cages at 5 PM the following night (3 hours prior to the beginning of the dark cycle) and again exposed to both bottles with cage sides randomized by a coin flip for the water and sucrose positions. Masses of the bottles were recorded and mice were allowed ad lib access to the bottles until 9 AM the following day (16 hours total). The following morning, bottle masses were again recorded and the difference was determined.

## Tail Flick Testing

The tail flick testing procedure was performed following the methods of Reichard et al [112]. Briefly, animals were first handled for at least three days before any measures were taken. Using a hot plate, a beaker filled to 1L with water was brought to 52°C while under constant stirring with a stir bar. Mice were then held gently in the experimenter’s hand, using a disposable paper towel as a restraint, and their tails submerged into the water bath. The time for each animal to flick their tail from the water was recorded using a stopwatch. The median result of three consecutive trials was recorded for all animals tested.

## Open field recordings

Animals were recorded for thirty minutes in a novel environment made of 0.9 cm thick, white acrylic (dimensions: 22.86 cm tall x 35.56 cm wide x 33.91 cm wide) using webcams placed above the open field (resolution 1920 x 1080 pixels, Spedal) and lit by bright white light (usb LED ring lights, 10 inch diameter,

Amazon) from above. Based on previous experiments in our own laboratory and others, we chose to examine 16 hours and 196 hours following the final fentanyl dose for video capture of spontaneous behaviors. Experimenters visually watched the mice for escape jumps and recorded these. Following video capture, recordings were analyzed using EthoVision XT software (Noldus) to determine mobility, distance traveled, and time spent in the edges of the open field compared to the center (defined by 50% of the floor area split between each).

Tissue collection and Immunohistochemistry (IHC)

For collection of RNA for sequencing, Ribotag$^{+/-}$ mice were taken immediately after the last behavioral experiment and euthanized by decapitation using sharp scissors. The head was then immersed in ice cold PBS for ~10-15 seconds and the brain then carefully extracted from the skull. Half of each brain was dissected following sagittal separation along the midline and the striatum was homogenized in supplemented homogenization buffer (50 mM Tris HCl, 100 mM MgCl2, 1% NP-40, 1 mM DTT, 1 x protein inhibitor cocktail (Sigma), 200 U/mL RNasin (Promega), 100 ug/mL cycloheximide (Sigma) and 1 mg/mL heparin). All homogenates were centrifuged, and supernatant was collected. From each sample, 10% was set aside as the whole transcriptome sample (input, IN), and the remaining sample was processed to isolate ribosome bound mRNA (immunoprecipitated, IP). All aspects of tissue processing, immunoprecipitation, and RNA-Seq library generation are described in detail in work by Lesiak et al [113]. In the present study, Ribotag$^{-/-}$ animals were used to generate the negative control samples (Ctrl NA) for sequencing.

For collection of PFA fixed tissue, mice were injected with a fatal dose of Euthasol (pentobarbital sodium 390 mg/mL and phenytoin sodium 50 mg/mL, diluted with an equal volume of saline) according to their body mass (0.1 mL of diluted solution per 10 g). After reaching sufficient analgesic depth, the chest cavity was opened, the heart was pierced with a needle, and the body was perfused with ice-cold PBS to remove circulating blood and then by 4% PFA (prepared in PBS) to induce tissue fixation. The brain from each animal was carefully extracted from the skull following perfusion and post-fixed in 4% PFA for 16 hours at 4 °C and then transferred to a 30% sucrose solution (made in PBS as well). Sections were then

sliced using a cryostat to 30 - 40 µm and stored in PBS (with 0.01% sodium azide as a preservative) until IHC was performed.

Free floating sections were prepared for IHC by incubating in blocking buffer (4% normal donkey serum, 1% bovine serum albumin, 0.5% Triton-x 100, 0.5% Tween-20, 0.5% sodium deoxycholate, in Tris buffered saline) for 1 hour at room temperature using a nutating shaker. Sections were then moved to the primary antibody solution (blocking buffer with primary antibodies as described below) for incubation at 4 degrees C, shaking, for 72 hours. We used the following primary antibodies: Rabbit anti-Iba1 (1:500, Fujifilm Cellular Dynamics 019-19741), Rat anti-C1q (1:1000, Abcam ab11861), and Goat anti-PSD95 (1:500, Abcam ab12093). Following primary antibody incubation, sections were washed three times for five minutes each in blocking buffer followed by incubation in the secondary antibody solution for 1 hour, shaking at room temperature. We used the following secondary antibodies: Donkey anti-Rabbit AlexaFluor 555 (1:1000, Abcam ab150062), Donkey anti-Rat AlexaFluor 488 (1:1000, Invitrogen A-21208), and Donkey anti-Goat AlexaFluor 647 (1:1000, Invitrogen A-21447). Following the secondary antibody incubation, sections were moved to TBS with one drop of DAPI solution (NucBlue Fixed Cell ReadyProbes Reagent, Invitrogen R37606) for thirty minutes, followed by five washes in TBS for ten minutes each before being mounted on slides using Prolong Diamond Antifade Mountant (Invitrogen P36965).

RNA Sequencing and Data Analysis

We prepared cDNA libraries using the SMARTer Stranded Total RNA-Seq Kit v2 – Pico Input Mammalian (Takara). These were sent for quality control testing and sequencing using a NovaSeq two-lane chip on the Illumina system at the Northwest Genomics Center facilities at the University of Washington (https://nwgc.gs.washington.edu). The raw fastq files were concatenated (see supplemental materials for the Python script) to combine the data from two lanes, preserving separate forward and reverse runs from the paired-end samples. Quantification of transcripts was performed via Salmon [114] using the mouse transcriptome downloaded from Ensembl (script also available in the supplemental materials). Determination of differentially expressed genes was performed using R (DESeq2 package,

[115]), followed by generation of gene modules based on Weight Gene Coexpression Network Analysis (WGCNA, [116]). Briefly, the gene count matrix from the differential expression analysis for all IP samples that was generated using DESeq2 was filtered to remove zero-variance genes and genes below a minimum sequence count of five in at least two of the biological replicates. A signed topological overlap matrix was generated for clustering the genes. Module membership was assigned using a dynamic tree cut, and highly correlated modules were merged by reclustering module eigengenes. Gene module names were left as R color values to avoid introducing experimenter bias. Scripts for all RNA sequencing data processing and analysis are available in the supplementary materials.

Quantitative PCR (qPCR) analyses

The mRNA expression levels for input (IN) and immunoprecipitated (IP) samples were assessed via qPCR on a Quant Studio Pro 7 using Power Sybr (ThermoFisher). cDNA libraries were standardized across samples and targets with Ct values being normalized to that of PPIA. Primer sequences were designed using the IDT PrimerQuest tool (https://www.idtdna.com/pages/tools/primerquest). Relative quantification and fold enrichment was obtained using the standard curve Pffafl method [117].

| Primer Set | Forward | Reverse |
|---|---|---|
| Beta Actin | CACCAGTTCGCCATGGAT | TCACACCCTGGTGCCTA |
| CD68 | CATCCTTCACGATGACACCTAC | AACTGTGACATTTCCGTGACT |
| P2RX4 | GCACACTACACACAATGGATTTC | CGTGTCTCTGCTCCCATATTC |
| P2RY6 | AAACAACGAGGAACACCAAATC | TAACTGCCTGTCAGCCTTTC |
| PDEB3 | GCTACCGGGACATTCCATATC | ATCTGAGGTAAGCCAGGAATTG |
| PPIA | TGGCAAGACCAGCAAGAA | CTCCTGAGCTACAGAAGGAATG |
| RPL22 | GAGCTGCGTTACTTCCAGATTA | ATAGGGATAGCCCGCATAGT |
| Siglec1 | GGACTTTCTTGGTGGGTTAGAG | CTCTGTTAGAGCAACCTGACTG |
| TMEM119 | GGCAGTGACATCTACTTTCCA | CTCCTCATTGGCTAGCTGTATC |
| TNFRSF1a | GTCTGGAACCAGTTTCGTACAT | ACACTCGGTTCTGCTGTTTAG |

Confocal microscopy and Image analysis

Following IHC, slices were imaged using either a Leica SP8 or a Nikon A1R confocal microscope, both part of the microscopy core services as the VA Puget Sound Health Care System. The SP8 imaging

used an HC PL APO CS2 objective (20x, 0.75 NA). Slides were illuminated using a 470 – 670 nm tunable white light laser with power no greater than 30% for each channel. The average voxel size was 0.135 x 0.135 x 0.684 µm and an image size of 2048 x 2048 pixels (pixel dwell time 0.375 µs) using 4x line averaging. The A1R imaging used a CFI Plan Apo Lambda objective (40x, 0.95 NA). Slides were illuminated from a white light source with four filter cubes (DAPI, FITC, TRITC, and Cy5), with power no greater than 9 for each channel. The average voxel size was 0.156 x 0.156 x 0.650 µm and an image size of 1024 x 1024 pixels using 16x line averaging. A bit-depth of 8 or 16 and a zoom factor of two was used for all images.

Statistical Methods and figure generation

Because our multi-factor ANOVA results did not show a significantly effect of sex on any of our behaviors, we pooled data from male and female unless otherwise specified. Statistical comparisons were first made for experimental conditions in all data analyzed using either multi-factor ANOVA (figures 3.2, 3.5, 3.6) or a general linear model (GLM, 3.3, 3.4) unless otherwise specified. All pairwise comparisons or adjusted p-values were computed using Tukey's HSD (figures 3.2, 3.5, 3.6) or the method of Benjamini-Hochberg (figures 3.3, 3.4). Figure generation was performed using R and Python software, except for figure 3.1 which was performed using Biorender.com. R and Python scripts for statistical analyses and figure generation are available in the supplementary materials.

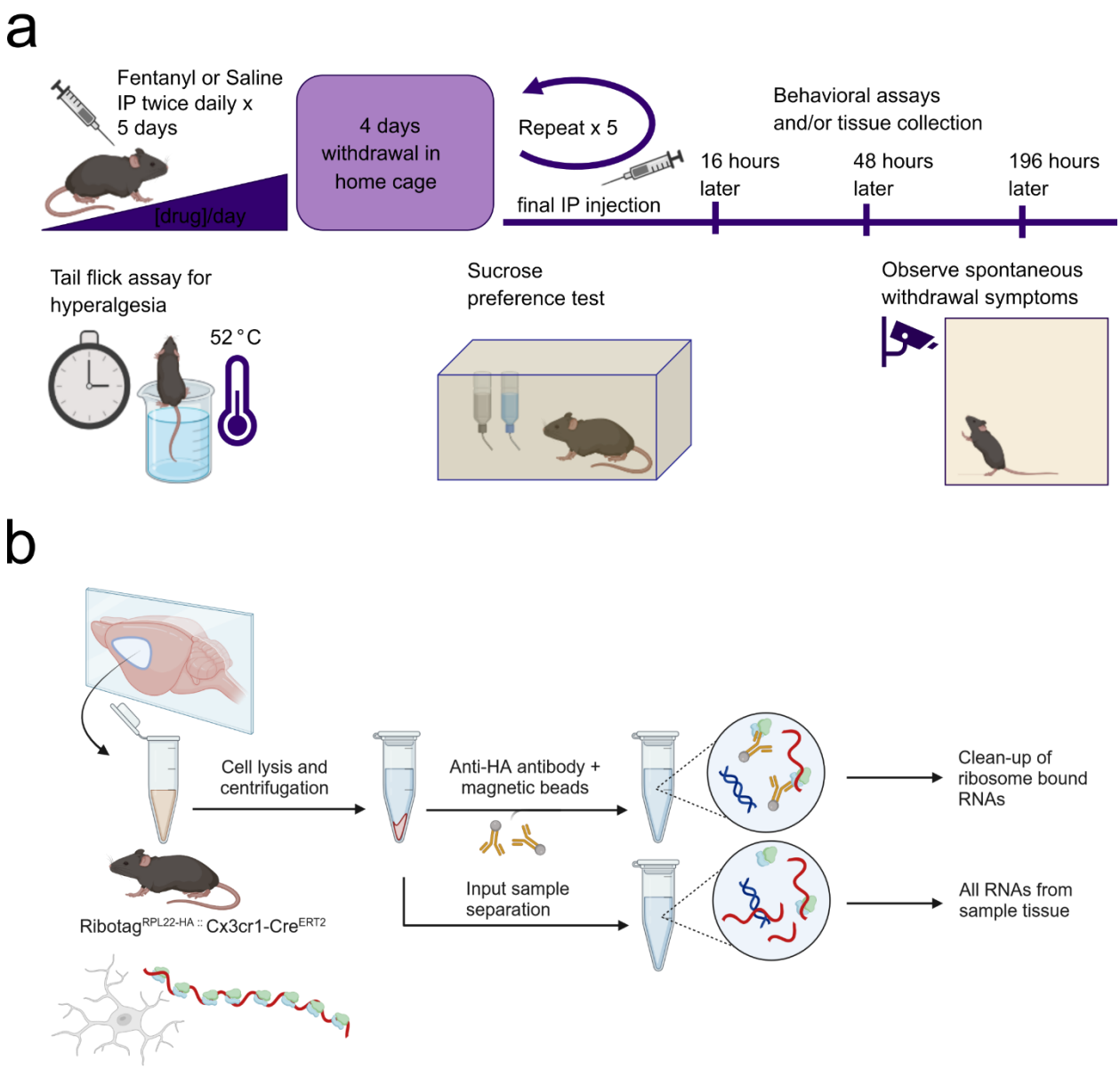



**Figure 3.1**: Mice were treated with fentanyl or saline for five days followed by measurement of withdrawal behaviors (a). For animals in the one cycle of withdrawal group, behaviors were measured at 16, 48, or 196 hrs following the final injection on day five. For animals in the five cycles of withdrawal group, behaviors were measured at the same time points, but following the fifth and final cycle of fentanyl delivery. For measurement of translatome changes induced in microglia, Ribotag+/- :: Cx3cr1-Cre+/- mice were euthanized following the 16hrs of spontaneous withdrawal and the striatum was isolated from one sagittal half of the brain (b). Immunoprecipitation of the ribosome bound RNAs was performed using anti-HA antibodies and magnetic beads while 10% of the input sample was collected to perform whole transcriptome sequencing of bulk RNAs from the striatum.

**Results**

We first wanted to determine how the experience of opioid withdrawal multiple times would impact animal behavior. Mice were grouped into four experimental groups based on drug treatment (Saline - Sal or Fentanyl - Fent) and number of withdrawal cycles (One or Five). Animals in the one cycle condition were injected twice daily for five consecutive days with saline or fentanyl (ip) and then behavioral measures were made at 16, 48 or 196 hrs later. Animals in the five cycle condition were also injected twice daily for five days, and then allowed to undergo withdrawal in their home cage for four days before undergoing four additional cycles of injection (figure 3.1a).

We used the sucrose preference test to determine if withdrawal cycles induced a change in affective state. We found that greater withdrawal experiences led to a significant decrease in total sucrose consumed as well as the percent of sucrose consumed as a portion of total fluid intake between the water and sucrose bottles (**figure 3.2a**). Multi-factor ANOVA showed a significant effect of drug treatment ($F = 11.4$, $p = 0.001$) as well as a significant interaction between treatment and withdrawal cycle ($F = 7.37$, $p = 0.007$). The animals in the Fent Five group drank significantly less sucrose solution than animals in the Sal Five group at 16 hours (-9.0%, $p = 0.02$) as well as at 48 hours following the final fentanyl injections (-14.3%, $p = 0.003$). Animals in the Fent One group also drank significantly less sucrose solution compared to animals in the Sal Five group (-7.6%, $p = 0.02$) but not compared to the Sal One group as a percentage of sucrose consumed (compare 3.2a to figures 3.2b and 3.2c). These data suggest that the Fent Five animals experienced anhedonia and lack of motivation to consume a palatable reward compared to the Fent One animals.

We used the tail flick test to determine if hyperalgesia, normally induced during opioid withdrawal [118], was different between animals experiencing multiple cycles of withdrawal. Mice in the tail flick test showed greater hyperalgesia with more fentanyl withdrawal experiences (**figure 3.2d**). Multi-factor ANOVA showed a significant effect of drug treatment ($F = 132.0$, $p < 0.001$), a significant effect of withdrawal cycles ($F = 33.2$, $p < 0.001$) and a significant interaction of treatment and cycle ($F = 8.8$, $p = 0.003$). The hyperalgesia was more pronounced for animals in the Fent Five group compared with the Fent One group (-0.16 s, $p < 0.001$) at sixteen hours following the final fentanyl injection and both the Fent Five and Fent One groups were significantly more hyperalgesic than animals in either saline group

at the sixteen-hour timepoint (Fent Five vs Sal Five: --0.27 s, $p < 0.001$, Fent Five vs Sal One -0.32 s, $p < 0.001$, Fent One vs Sal Five -0.11 s, $p < 0.001$, Fent One vs Sal One -0.17 s, $p < 0.001$). By forty-eight hours since the final fentanyl injection, the Fent Five and Fent One groups did not showing significant differences in the tail flick test, but were still significantly more hyperalgesic than the saline groups (Fent Five vs Sal Five -0.27 s, $p < 0.001$, Fent Five vs Sal One -0.22 s, $p < 0.001$, Fent One vs Sal Five -0.20 s, $p < 0.001$, Fent One vs Sal One – 0.15 s, $p < 0.001$). At seven days (or 196 hrs) following the last fentanyl injection, the Fent One group did not show differences in the tail flick test compared to the Sal One group, but did show less hyperalgesia compared to the Sal Five (+0.09 s, $p = 0.006$) and Fent Five groups (+0.16 s, $p < 0.001$). These results demonstrate that hyperalgesia can be long lasting and more severe for animals that have experienced multiple opioid withdrawal episodes.

We used the open field test to determine if greater experiences of opioid withdrawal had an impact on general locomotion and anxiety-like behavior. When tested in the open field chamber for anxiety-like behavior at sixteen hours after the last fentanyl injection (see **figure 3.2e**), animals in the Fent Five group spent less time in the center of the open field than animals in the saline groups (vs Sal Five: -7.6% total time, $p < 0.001$, vs Sal One: -20.4% total time, $p < 0.0001$) as well as compared to the Fent One animals (-27.75% total time, $p < 0.0001$). The Fent One animals showed an unusual increase in time spent in the center of the open field when compared with either saline group (vs Sal Five: +20.17 % total time, $p = 0.002$, vs Sal One +7.31, $p = 0.03$). This may be due to the propensity of these animals to try to escape from the open field chamber. At 7 days after the final fentanyl dose, the Fent Five animals spent significantly less time in the center of the open field compared to the Sal One animals (-16.5% total time, $p = 0.005$), but did not show a significant difference in time spent in the open field compared to the Fent One or Sal Five groups. Multi-factor ANOVA showed a main effect of treatment ($F = 5.34$, $p = 0.02$) and cycle ($F = 31.3$, $p < 0.0001$). We also noticed that mice in the Fent One and Fent Five groups showed a great number of escape attempts from the open field 16 hours after the last fentanyl injection, but not at 7 days after the last fentanyl injection (**figure 3.2f**). Here ANOVA sowed a main effect of treatment ($F = 8.93$, $p = 0.003$).

Together these data suggest that mice who experience a greater number of extended withdrawal episodes also experience an increase in the severity of the withdrawal symptoms, especially at 16 hours

following their last fentanyl dose, which appears to be the most significant point where differences emerge between animals in the one cycle and five cycle groups.

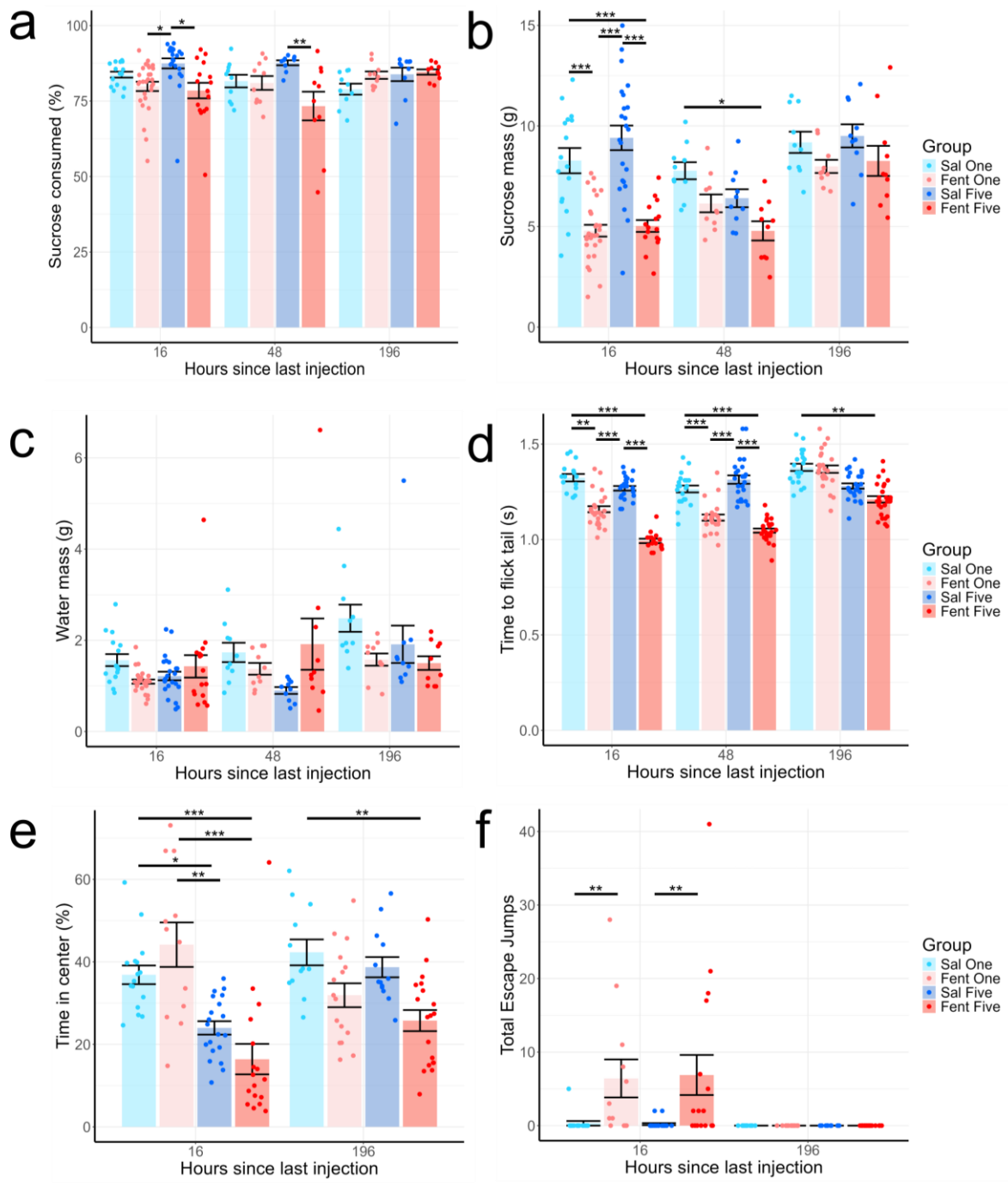



**Figure 3.2**: Fentanyl withdrawal symptoms are exacerbated by repeated opioid withdrawal experiences. (a) Mice in the Fent One and Fent Five groups consumed significantly less sucrose solution by percent of total fluid intake 16 hours following their last injection when compared with the Sal Five animals, but not the Sal One animals. (b) The total mass of sucrose solution consumed by Fent One and Fent Five animals was significantly lower than both Sal groups at the 16 hour time point. At 48 hours following their

final injection, Fent Five animals (n = 10, 5M 5F) show decreased sucrose consumption compared with the Sal One group (n = 10, 5M 5F) but not the Sal Five (n = 10, 5M 5F) group or the Fent One Group (n = 10, 5M 5F). (c) Total mass of water consumed by each group was not significantly different at any time point tested. (d) In the tail flick test, Fent One (n = 28 ,15M 13F) and Fent Five (n = 16, 8M, 8F) groups show significantly greater hyperalgesia from the Sal One (n = 16, 7M 9F) and Sal Five (n = 23, 16M, 8F) groups at 16 and 48 hours following the last injection, but Fent One (n = 25, 13M 12F) animals return to baseline tail flick time by 196 hours while Fent Five (n = 27, 12M 15F) animals still show significantly faster time flick time. Note the number of Sal One (n = 21, 10M, 11F) and Sal Five (n = 25, 13M 12 F) animals tested in the tail flick test at 48 and 196 hrs. (e) Fent Five animals spent significantly less time in the center of the open field arena 16 hours after their last injection compared with either Sal group and compared with the Fent One group. Seven days (196 hrs) following the last fentanyl injection, there is still some evidence for anxiety-like behavior in the Fent Five animals, but only when compared to animals in the Sal One group. (f) Mice in both Fent groups showed significantly greater escape jump from the open field arena than either Sal group at 16 hours but none of the groups showed escape jumps at 196 hours. Asterisks indicate significant effects of pairwise comparisons with adjusted $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

Sucrose preference test animal breakdown

| Time point | Group | **N (sex = male, female)** |
|---|---|---|
| **16 hrs** | Sal One | 16 (7, 9) |
| | Fent One | 28 (15, 13) |
| | Sal Five | 23 (16, 7) |
| | Fent Five | 16 (8, 8) |
| **48 hrs** | Sal One | 10 (5, 5) |
| | Fent One | 10 (5, 5) |
| | Sal Five | 10 (5, 5) |
| | Fent Five | 10 (5, 5) |
| **196 hrs** | Sal One | 10 (5, 5) |
| | Fent One | 10 (5, 5) |
| | Sal Five | 10 (5, 5) |
| | Fent Five | 10 (5, 5) |

Tail flick test animal breakdown

| Time point | Group | **N (sex = male, female)** |
|---|---|---|
| **16 hrs** | Sal One | 16 (7, 9) |
| | Fent One | 28 (15, 13) |
| | Sal Five | 23 (16, 7) |
| | Fent Five | 16 (8, 8) |
| **48 hrs** | Sal One | 21 (10, 11) |
| | Fent One | 25 (13, 12) |
| | Sal Five | 25 (13, 12) |
| | Fent Five | 27 (14, 13) |
| **196 hrs** | Sal One | 21 (10, 11) |
| | Fent One | 25 (13, 12) |
| | Sal Five | 25 (13, 12) |
| | Fent Five | 27 (14, 13) |

Open field test animal breakdown

| Time point | Group | **N (sex = male, female)** |
|---|---|---|
| **16 hrs** | Sal One | 16 (7, 9) |
| | Fent One | 12 (5, 7) |
| | Sal Five | 20 (13, 7) |

| | | |
|---|---|---|
| | Fent Five | 17 (10, 7) |
| **196 hrs** | Sal One | 12 (7, 5) |
| | Fent One | 16 (9, 7) |
| | Sal Five | 13 (7, 6) |
| | Fent Five | 19 (9, 10) |

RNA Sequencing results

Following the sixteen hour time point, we dissected out the striatum (both ventral and dorsal regions) from one hemisphere of each Ribotag$^{+/-}$ : Cx3cr1-Cre$^{+/-}$ mouse, homogenized the tissue, and isolated the HA-tagged ribosomes to perform sequencing of the microglia-specific RNAs undergoing translation (**figure 3.1b**). This portion, which we refer to as the immunoprecipitate (IP), represents the microglia translatome during spontaneous opioid withdrawal and can be compared to the other RNAs from the striatum. The latter we refer to as the input (IN), and we also sequenced a fraction of this material to determine the extent of microglia specificity in our results. The sequenced RNA represents a total of 30 mice as shown in Table 3.1 below.

**Table 3.1**: Mice from the experimental groups represented in the RNA sequencing data

| Drug | Withdrawal Cycles | N (sex = male, female) |
|---|---|---|
| Saline | One | 10 (5, 5) |
| Fentanyl | One | 9 (4, 5) |
| Saline | Five | 4 (2, 2) |
| Fentanyl | Five | 7 (3, 4) |

We analyzed the RNA sequencing data and determined differential gene expression using DESeq2 [115]. A principal components analysis (PCA) of all the genes showed that the IN samples separate completely from their paired IP samples along the axis of the first PC (see **figure 3.3a**). A great number of the most enriched genes in the IP samples are microglia specific (**figure 3.3b**) and microglia transcripts are more abundant compared to other cell type genes in the IP samples (**figure 3.3c**), which strongly suggests that our enrichment of microglia specific RNAs using the Ribotag method was successful. Comparing IP samples by experimental treatment group suggests that the gene expression is similar for the Fent One and Sal One groups, which generally segregate along a combination of the first and second PC axes (**figure 3.3d**). However, there is no clear separation between drug treatment, suggesting that a combination of withdrawal experience and drug exposure made the largest difference in the gene expression among groups. This is further supported when looking at the number of differentially

expressed genes as determined by DESeq2. Comparing the Sal Five to the Sal One group, we find 45 significantly differently expressed transcripts (**figure 3.3 e**), whereas comparing the Fent Five and Fent One groups, we find 5614 transcripts meeting our significance thresholds (adjusted p-value < 0.01 and absolute log2(fold change) > 0.6, **figure 3.3f**). These data suggest that multiple withdrawal experiences induce more robust changes in microglia gene translation compared to a single withdrawal experience following chronic fentanyl administration.

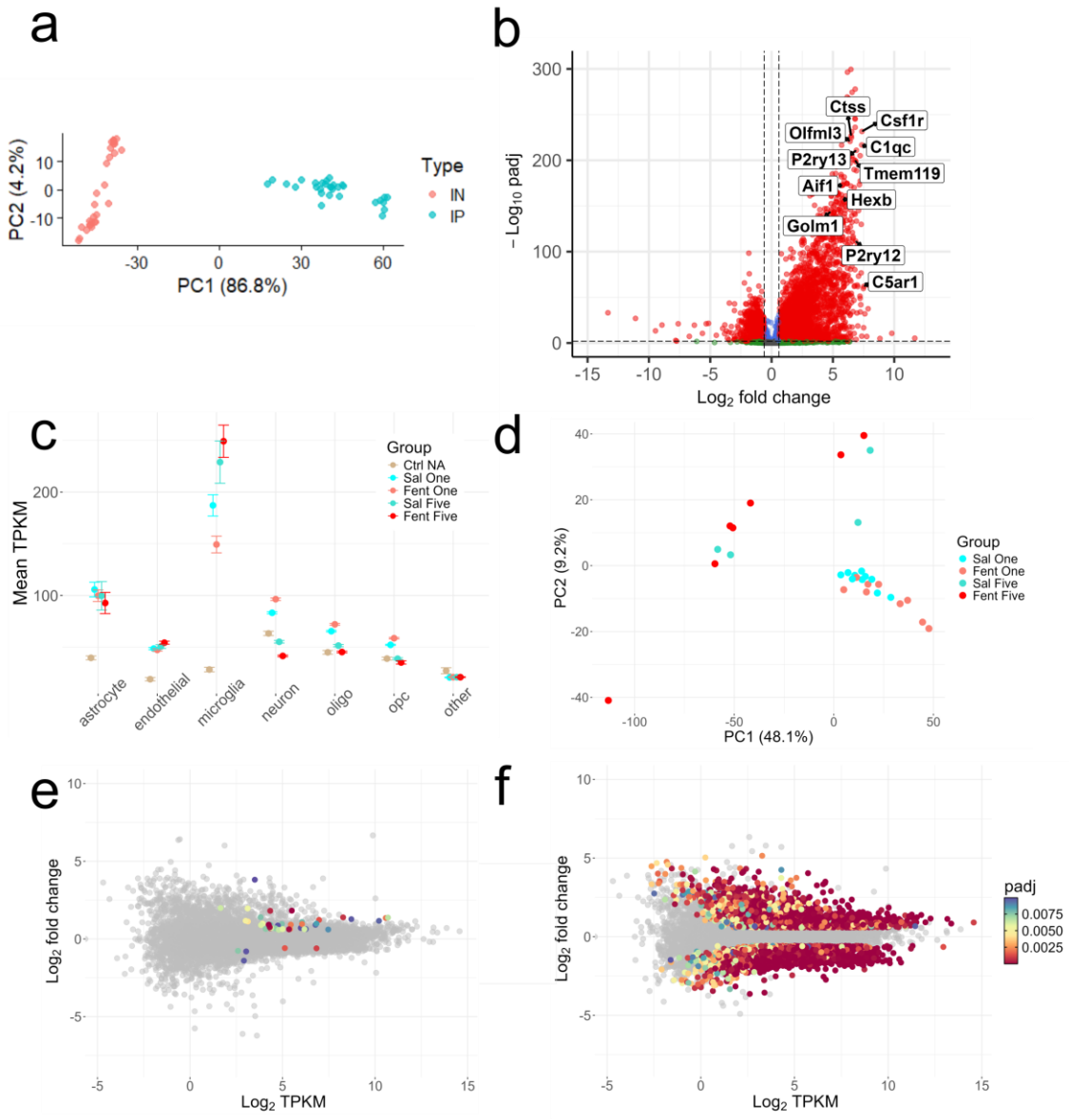



**Figure 3.3**: Principal component analysis (PCA) of the IN and IP samples together shows clear discrimination (a). Volcano plot of adjusted p-values for each gene in IP vs IN groups versus the corresponding foldchange for that gene. Comparison of IP vs IN in differential gene expression gives

many microglia genes as significantly enriched, such as *Ctss, Csf1r, Olmf3, P2ry13, Tmem119, Aif1, Hexb, Golm1, P2ry12, and C5ar1* (b). Comparison of IP vs IN cell type classification clearly indicates microglia genes are enriched compared to other cell types (c). PCA of the IP samples alone does not clearly differentiate groups, however the Sal One and Fent One groups do segregate from the Sal Five and Fent Five groups (d). DESeq2 analysis reveals that Sal Five vs Sal One gives 43 significantly differentially expressed genes (e) whereas Fent Five vs Fent One gives 5486 differentially expressed genes (f). Note that differentially expressed genes were filtered to those with an adjusted p-value < 0.01 and abs(Log2FoldChange) > 0.6.

Following determination of differential gene expression using DESeq2, we chose to use an unbiased approach to find gene sets of interest in the data by applying weighted gene co-expression network analysis (WGCNA, [116]). This determines a correlation between each gene and every other gene sequenced using the DESeq2 differential expression data. Modules of genes are then assigned based on hierarchical clustering of similar gene co-expression. These color-coded modules represent potential biological networks that can be explored further and tested with specific hypotheses. WGCNA revealed several interesting modules with overall increased mean expression of genes involved in microglia function, such as the brown and orangered4 modules, as well as one module with decreased gene expression related to synaptic transmission and communication, the steelblue module (**figure 3.4a**). A great number of genes in the brown module (**figure 3.4b**) were related to purine or nucleotide sensing and/or metabolism, such as *P2ry12*, *P2rx7,* and *P2ry13* which were significantly increased in the Fent Five animals compared to the Fent One animals. Other significantly increased genes were related to innate immunity or regulation of inflammation, such as *Rnaset2b* (an RNase), *Ly86* (also known as MD1, a regulator of Tlr4), and *Tnfrsf13b* (aka *Taci*, a Tnf receptor that signals through the AP-1/NF-kappa-B pathway).

We further investigated the brown, steelblue, and orangered4 modules using clusterProfiler and the KEGG PATHWAY database [119] to perform overrepresentation analysis of genes that were significantly changed between the Fent Five and Fent One groups (figure **3.4c - e**). This can be useful for determining what kinds of genes are more common in each module, as the KEGG database organizes genes into categories by their known molecular interactions, reactions, and relational networks. Surprisingly, the brown (**figure 3.4c**) and steelblue (**figure 3.4d**) modules resulted in only one significantly enriched, descriptive category of genes each: neuroactive ligand-receptor interaction. The orangered4 module however showed many enriched gene categories (**figure 3.4e**). When digging into these genes to

investigate further, we found that many of the shared hits were histone related (*H2ac13*, *H2bc8*, *Hcbc18*, and others, see supplementary table). Many of the transcripts were present at low mean count values in the IP data, suggesting that they may be differentially expressed but not meaningful for further investigation. However a few stood out, including *Tlr2*, *Clec7a*, *Ccl12*, *Bcl2a1b*, *Cenpe*, and *Ifi204*. These genes are significantly upregulated in the Fent Five group compared to the Fent One group and, importantly, not significantly different (adjusted p-value > 0.01) in expression between the Sal Five and Sal One groups. These results suggest that the orangered4 module represents an inflammation-related gene set that is more engaged in the animals that underwent five cycles of withdrawal.

**Table 3.2**: orangered4 module genes related to inflammatory and disease response related KEGG PATHWAY categories enriched in the Fent Five vs Fent One IP samples

| Gene | Log2(Fold Change) – Fent Five vs Fent One | Log2(Fold Change) – Sal Five vs Sal One | Base mean count |
|---|---|---|---|
| *Tlr2* | 2.06 | 0.929 | 104 |
| *Clec7a* | 2.14 | 1.14 | 45.3 |
| *Ccl12* | 3.03 | 0.257 | 43.4 |
| *Bcl2a1b* | 2.05 | 0.649 | 60.6 |
| *Cenpe* | 2.20 | 0.611 | 47.0 |
| *Ifi204* | 2.66 | 0.929 | 100 |

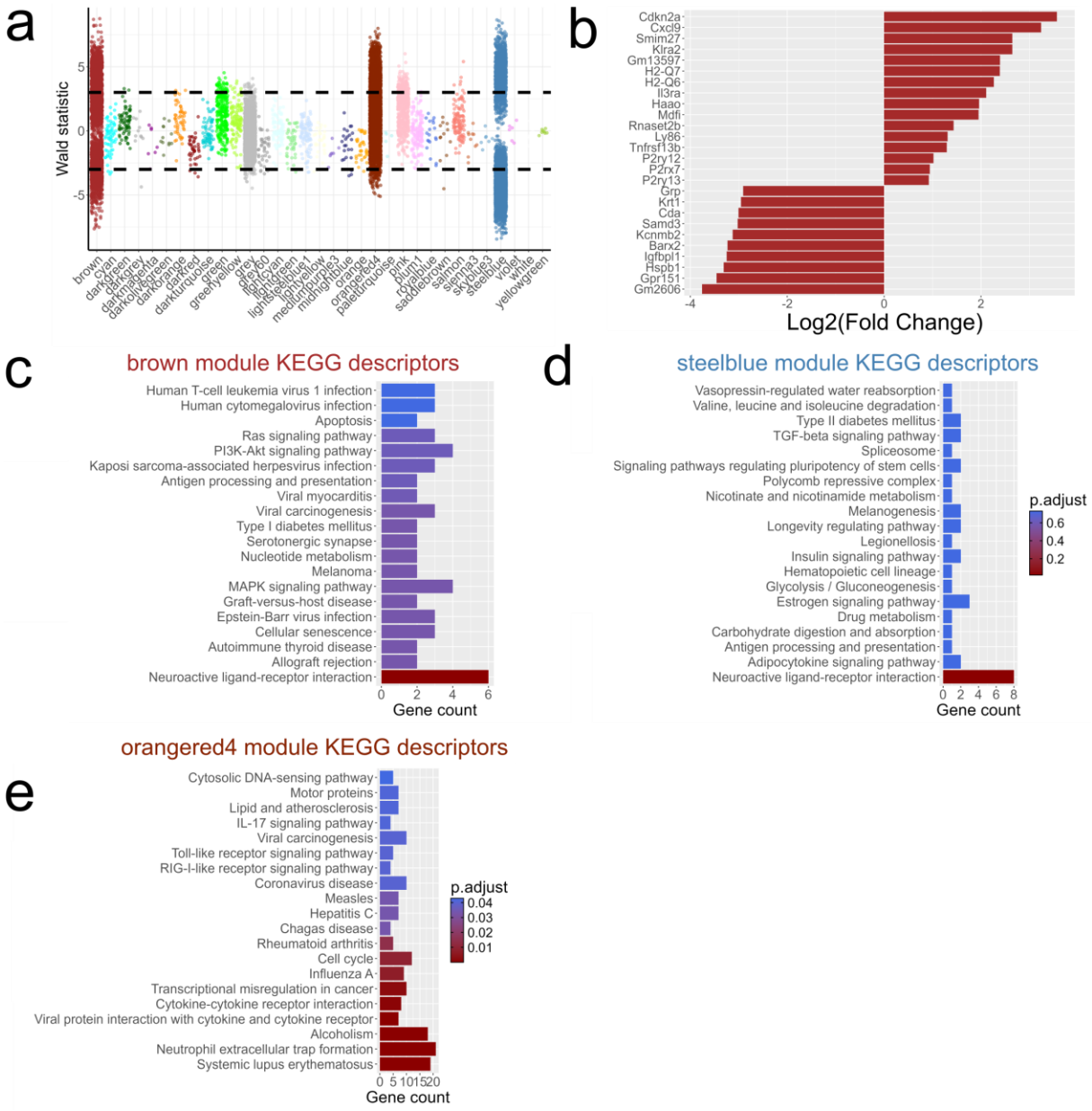



**Figure 3.4**: Genes in the Fent Five versus Fent One groups organized into modules according to WGCNA (a), with dark black dotted lines indicating the genes above and below significant Wald statistic values (p-adjusted < 0.01 and absolute log2foldchange >= 2) from DESeq2 results. Select genes in the brown module showing significant differential expression (b). KEGG PATHWAY overrepresentation analysis for genes in the brown (c), steelblue (d), and orangered4 (e) modules. Adjusted p-values are shown for c-e based on enrichment relative to other genes present in the module.

Following up on the results from RNA sequencing, we decided to corroborate our findings by performing qPCR of several genes of interest that showed differences in expression when comparing the Fent Five and Fent One groups. Some of our targets did not replicate our DESeq2 results: *Tmem119, Siglec1,* and *Pdeb3* (**figure 3.5a - c**) were not significantly different in expression between any of the experimental groups when looking at normalized relative starting quantity of RNA (NRStQ). We did see a sex effect on expression of two transcripts. *Tnfrsf1a* (**figure 3.5d**) showed a significantly higher amount of expression in the Fent Five males compared to the Sal Five and Fent One groups, but in the females this effect was not significant. *P2yr6* (**figure 3.5e**) showed a significant difference in expression between the female Fent Five and Fent One groups, and between the male Sal Five and Fent One groups. For three other transcripts that we investigated (*CD68*, *P2rx4*, and *Actb*, **figure 3.5f - h**) we did not find an effect of sex, but did find overall effects of treatment, with Fent Five animals having significantly greater expression compared to Fent One animals. These results demonstrate that many of our initial findings from the sequencing of the microglia translatome are supported by follow-up studies to validate individual gene changes.

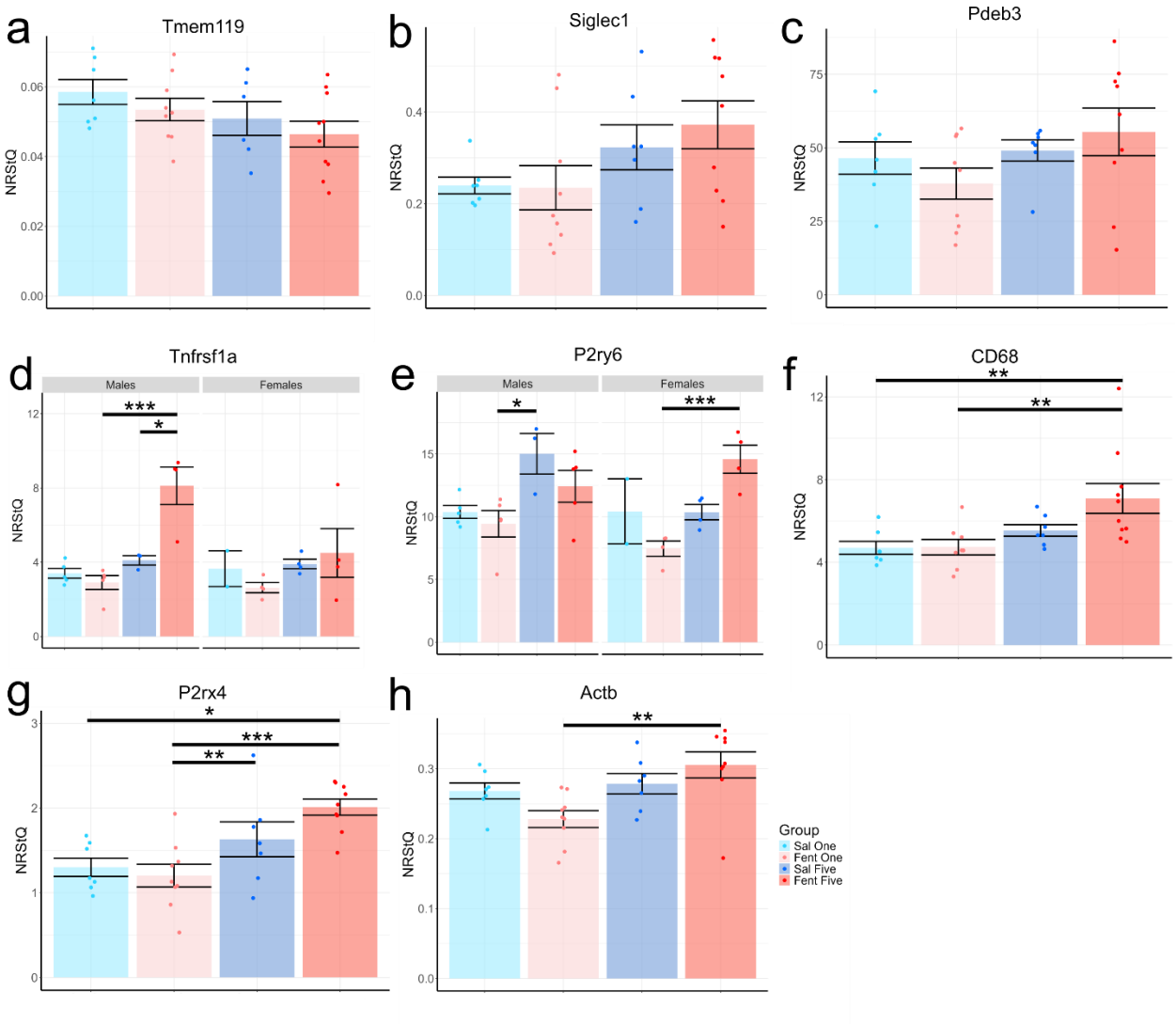
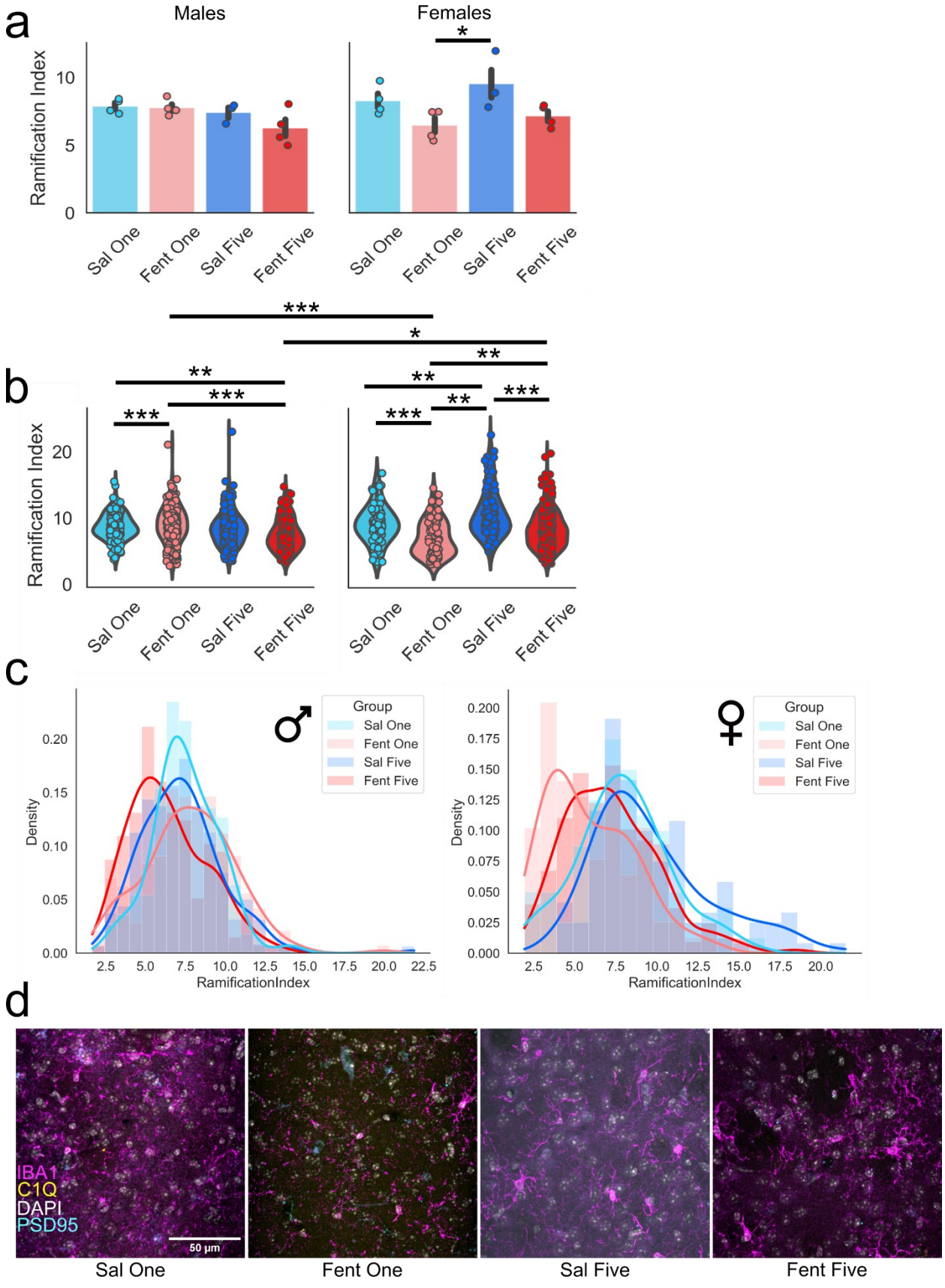



**Figure 3.5**: Quantification of IP samples of transcripts by qPCR for *Tmem119* (a), *Siglec1* (b), and *Pdeb3* (c) show no significant effects of treatment, cycle, or sex by multi-factor ANOVA. Results for *Tnfrsf1a* (d) showed significant main effects of cycle ($F = 21.5$, $p = 0.0001$) and sex ($F = 5.79$, $p = 0.02$) and an interaction effect between treatment and cycle ($F = 9.31$, $p = 0.006$). Results for *P2ry6* (e) showed a main effect of cycle ($F = 23.2$, $p < 0.0001$) and an interaction effect of treatment, cycle, and sex ($F = 9.34$, $p = 0.005$). Results for *Cd68* (f) showed main effects of treatment ($F = 4.41$, $p = 0.04$) and cycle ($F = 13.5$, $p = 0.001$), as well as an interaction effect between treatment and cycle ($F = 4.51$, $p = 0.04$). Results for *P2rx4* (g) showed a main effect of cycle ($F = 20.9$, $p = 0.0001$). Results for *Actb* (h) showed a main effect of cycle ($F = 10.9$, $p = 0.003$) and an interaction effect between treatment and cycle ($F = 4.54$, $p = 0.04$). Asterisks indicate significant effects of pairwise comparisons with adjusted $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

We were also interested in determining if microglia in the mouse striatum exhibited altered morphology following multiple experiences of opioid withdrawal. By staining for Iba1, we were able to quantify certain characteristics of microglia cells in the striatum. Analysis of the ramification index of each cell measured (ratio of territorial volume to cell volume) showed that the Fent Five group had significantly lower cell ramification index on average than the three other groups (**figure 3.6a-c**). An analysis of variance comparing average cell ramification index per subject (per animal versus per cell) showed a main effect of treatment ($F = 10.6$, $p = 0.004$) and an interaction between cycle and sex ($F = 7.10$, $p = 0.02$). However, individual pairwise comparisons between groups did not suggest any significant differences, probably due to there being no main effect of cycle. Following up on the effect of sex, we found that the female animals showed a significant difference in ramification index between Fent One and Sal Five groups compared to the male animals (**figure 3.6a**). There appears to be an effect of more cycles of withdrawal driving the microglia treated with fentanyl toward a more ameboid and less ramified state, which is evident in the main effect of treatment (**figure 3.6c**), which was validated by testing the ramification index as a result of group using mixed linear model regression (z values Sal One = 16.9, Fent One = 15.0, Sal Five = 16.9, Fent Five = 15.6, all $p < 0.0001$). This suggests that greater withdrawal experiences induce a more inflammatory microglia cell state, and that this is more pronounced in female mice.


a
Males
Females
Ramification Index
10
5
0
*
Sal One
Fent One
Sal Five
Fent Five
b
***
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Ramification Index
20
10
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Group
Sal One
Fent One
Sal Five
Fent Five
Density
0.20
0.15
0.10
0.05
0.00
0.200
0.175
0.150
0.125
0.100
0.075
0.050
0.025
0.000
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5.0
7.5
10.0
12.5
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20.0
22.5
RamificationIndex
d
IBA1
C1Q
DAPI
PSD95
50 µm
Sal One
Fent One
Sal Five
Fent Five

**Figure 3.6:** Comparing the ramification index of microglia and averaging all cells by mouse in each group (n = 8, 4 males and 4 females, in the images analyzed) via multi-factor ANOVA there is a main effect of treatment ($F = 10.6$, $p = 0.003$) and an interaction effect between cycle and sex ($F = 5.79$, $p = 0.02$) with a significant pairwise comparison between female Fent One and Sal Five groups (a). There are many significant comparisons when treating each cell (b) as an individual data point, but these disappear when averaged together by biological replicates (compare a and b). Kernel density estimate histograms (normalizing per cell in each group) show that males and females in the Fent Five group generally have microglia with a smaller ramification index, and this is more pronounced for the females (c). Total cells analyzed per group Sal One = 224, Sal Five = 328, Fent One = 264, Fent Five = 510. Maximum projection z-stacks from the mouse striatum are shown to give representative examples of images analyzed in each group (d) with the protein Iba1 marking microglia (magenta channel), C1q (yellow), DAPI marking nuclei (white), and PSD-95 (cyan).

## Discussion

Overall our behavioral results show that at least in mice, enduring multiple opioid withdrawal experiences results in more severe and unpleasant symptoms. To our knowledge, this is the first set of experiments that has investigated this possibility directly. In one recent study Lefevre et al studied mice receiving morphine by osmotic mini-pump over 6 days, where one group was given saline injections twice daily and the other was given naloxone [120]. In this way, they induced a kind of repeated withdrawal that could be considered similar to our paradigm and found greater psychomotor sensitization to the effects of morphine and greater changes in the expression of unfolded protein response genes in the striatum, for animals given the naloxone treatment. Another study by Schulteis, Morse, and Liu showed that when rats were trained to lever press for a food reward, naloxone induced a more significant decrease in responding when the rats had previously received four episodes of morphine treatment followed by naloxone [121]. They argued that this repeated experience of withdrawal may induce a conditioning effect on subsequent animal behavior. In human subjects, there is some anecdotal evidence that the experience of opioid withdrawal is indeed more difficult, after multiple episodes of opioid use and subsequent abstinence. However, there is little published empirical evidence of these symptoms and their magnitude in the literature. We propose that this worsening of opioid withdrawal symptoms over time is a general phenomenon and that it warrants further investigation in both animal models of opioid use disorder and in human subjects.

The RNA sequencing data from microglia are surprising, as they suggest that fentanyl animals and saline animals may both experience a strong effect from the stress of ip injections when these are

repeated many times. This is striking because we originally hypothesized that saline injections alone would not have a meaningful impact on gene translation, given that the saline animals would be handled in the same way as the fentanyl animals. Some of the differences in the translatome data may be due to physical adaptations and expectations regarding handling by experimenters over the course of 41 days, in the case of the five cycle group animals, compared to 6 days in the case of the one cycle group animals. We did control for age in this study, with animals in the five cycle and one cycle groups both between 8 and 14 weeks of age at the beginning of their injections. However another explanation is that our animal replicates were underpowered in the Sal Five group (two males and two females) to detect changes compared to our other experimental groups. This is a possibility and we originally planned to perform the experiment with additional animals in that group (five males and five females). We are confident, however, that the overall trends we observed in the sequencing analysis are consistent with the results from our confirmatory qPCR experiments. This gives weight to our conclusions in the analysis of RNA sequencing results and suggests that the majority of our findings are biologically valid.

In general, we see that microglia expression of immune function related genes is increased in the Fent Five group compared to the Fent One group. These results are in line with findings from other groups in the field of addiction neuroscience. There is evidence that both alcohol and opioid withdrawal states share key inflammatory signaling changes that are mechanistic in the unpleasant symptoms experienced. For instance, Ahlstrom et al found that chronic morphine exposure induced microglial expression (specifically in the spinal cord) of genes related to inflammation in rats [4]. Another study by Cuitavi et al showed that direct opioid agonist delivery to the nucleus accumbens of rats induced local release of cytokines (IL-1α, IL-1β, and IL-6) and greater expression of microglia Iba1 protein in the ventral tegmental area [56]. We were also able to recapitulate results from our previous study [13], where we compared microglia translation of RNAs after morphine administration versus during naloxone-precipitated withdrawal. In both this study and the former, we found expression of cAMP related genes increased during withdrawal (see supplementary materials for genes in the steelblue module). It may be the case that the release of microglia cytokines and driving of inflammatory gene expression is a result of the increased signaling through cAMP dependent mechanisms within microglia and this warrants further study as well.

With regard to the involvement of microglia in opioid withdrawal, we cannot make claims of exactly how the RNA sequencing changes in the translatome are directly impacting neuronal circuit function or directly impacting behavior. However, our work strongly implies that microglia in the mouse striatum are driven into an inflammatory signaling state involving release of interferons when opioid withdrawal is experienced multiple times. This process is likely to involve release of cytokines, purines, and perhaps even results in microglia cell proliferation, based on our RNA sequencing results and our measurements of microglia cells in IHC.

Future studies may benefit from examining how individual components of microglia inflammatory signaling impact animal behaviors. For example, is release of TNFα or other pro-inflammatory cytokines sufficient to drive hyperalgesia or anhedonia (decreased sucrose preference)? Furthermore, there are other brain regions where we hypothesize that microglia may be playing particularly important roles during opioid withdrawal. For instance, Chaudun et al recently found that MORs are expressed on neurons of the central amygdala and were necessary for some of the aversive symptoms of opioid withdrawal, suggesting that this region in particular is hyperactive during opioid withdrawal [122].

Chapter 4 / Conclusions

Our work in mice demonstrates that repeated opioid withdrawal, when experienced multiple times sequentially, induces more severe withdrawal signs than a single withdrawal experience. To our knowledge, this is the first set of experiments that has investigated this possibility directly. No human or other animal studies have yet observed if the withdrawal experience changes when individuals experience it again and again. Future studies by our group will attempt to replicate these results in mice. Other researchers in the field are encouraged to study this phenomenon in more animal models and to determine if humans experience more discomfort and difficulty with each successive experience of opioid withdrawal. In the alcohol literature, repeated withdrawal from alcohol produces more severe anxiety-like behaviors in rats [123] as well as more withdrawal-induced seizures [124] when compared to a single episode of withdrawal. The risk of severe alcohol withdrawal increases for human patients with a more extensive history of past episodes of alcohol withdrawal [125]. In one study of human subjects with a history of opioid use, Azorlossa et al found that opioid users (4+ years of use, 3 – 7 uses per week) reported more liking of an opioid dose than opioid naïve individuals, but the authors saw no differences in precipitated withdrawal symptoms when each group was administered naloxone [126]. Thus, there is not enough evidence yet to conclude that our results are a general phenomenon of opioid withdrawal.

The results shown in Chapter 3 show that repeated opioid withdrawal impacts microglia morphology and RNA translation. Previous work from our laboratory by Coffey et al found that a number of cAMP signaling related genes were upregulated during withdrawal from morphine in the microglia of the mouse striatum [13]. In the RNA sequencing data presented in this work we found many of the same cAMP related genes (located in the steelblue module) were downregulated in the Fent Five versus Fent One animals. In the Coffey et al study, morphine withdrawal was precipitated by naloxone infusion whereas in this study the mice proceeded into spontaneous fentanyl withdrawal; however, the similarities in the sequencing results in the present study replicate our previous work, as a number of the same differentially expressed genes were identified by applying WGCNA to a new set of data from animals undergoing opioid withdrawal.

The brown module we identified strongly suggests that translation of cell-type specific, homeostatic microglia genes change dramatically at 16 hours into withdrawal. Included in this module are

*Tmem119*, *P2ry12*, *P2ry13*, *Hexb*, *Csf1r*, and *Cx3cr1*, all which were upregulated in the Fent Five vs Fent One comparison. However, we also see some disease-associated genes, such as *Trem2* which has been widely implicated in mouse models of neurodegenerative diseases [127], and *P2rx7*, which is hypothesized to play a mechanistic role in neuroinflammation via release of cytokines [128]. As expected, we detected very few differentially expressed genes relating to the age of experimental animals. We did however see a change in *Cd63,* which was upregulated in the Fent Five vs Fent One comparison and showed up in our orangered4 module. This gene has previously been identified in disease-associated microglia (DAM) populations as well as studies of age-related changes in microglia RNA expression [129]. We saw increased expression of many DAM genes including *Lyz2* and *Ctss* (both in the brown module), *Ctsb* (orangered4), *Ctsz* (orangered4), *H2-D1* (brown), *H2-K2* (steelblue) and *Tyrobp* (steelblue) when comparing the Fent Five and Fent One groups.

The orangered4 module contains a number of the genes ascribed to DAM (*Ctsb*, *Ctsl*, *Csf1*); these as well as several interferon-related signaling genes were upregulated in the Fent Five versus Fent One group. For instance, we see increased expression of *Tlr7* in this module, which has been shown to be increased in rodents and humans following chronic alcohol exposure [130]. TLR7 binds to damage associated RNAs that become endocytosed and activate the interferon regulatory factor 7 (IRF7) transcription factor, ultimately leading to interferon production or proinflammatory cytokine production ([131], [132]). In the brain, TLR7 is predominately expressed in microglia and infiltrating or resident macrophages [133], which makes it a curious and a potential therapeutic target for both alcohol and opioid withdrawal. Other interferon related genes upregulated in the orangered4 module include *Myd88*, *Ifit2*, *Ifit3*, *Ifi30*, *Ifi204*, *Slfn2*, and *Aim2*, however we also see some interferon related genes upregulated in the steelblue module such as *Irf1* and *Tyk2*.

Overall, the steelblue module seems to contain neuronal signaling-related genes and genes involved in communication between microglia and neurons. For instance, we see a great number of genes related to the axon guidance gene set from the KEGG pathway database, most of which are downregulated in the Fent Five versus Fent One groups. Other significant KEGG gene sets that were identified within the steelblue module include those related to glutamatergic synapses, morphine addiction, GABAergic synapses, cAMP signaling, endocannabinoid signaling, and oxytocin signaling. The

hub genes in the steelblue module, identified by their centrality and coexpression with other genes in the module, include *Dlg4* (the gene for PSD-95 aka SAP-90) and *Prkn*, which were surprising at first glance. Microglia do indeed express PSD-95 as a functional protein [134], but it is not known how PSD-95 contributes to microglia biology. Krishnan et al (see previous reference) hypothesize that PSD-95 may act as a signaling mediator which allows for cell-cell communication between microglia, neurons, and other glia. PSD-95 has been implicated in neuropsychiatric disorders ([135], [136], [137], [138]) as well as drug-induced synaptic plasticity ([139], [140]), which is not surprising given that PSD-95 is essential for certain aspects of NMDA receptor and AMPA receptor localization at the cell membrane ([141], [142]). Prkn is an E3 ubiquitin ligase and is involved in maintaining proper autophagy of misfolded proteins around mitochondria. Interestingly we saw decreased expression of *Pink1*, a serine/threonine kinase encoding mRNA that is important for triggering mitophagy. We also saw upregulation of many mitochondrial related genes in our results, but not restricted to the steelblue module. These changes together suggest that the steelblue module is encapsulating the potential metabolic and signaling changes induced by repeated opioid withdrawal which are important for microglia-neuron communication.

For future studies, it will be important to consider how targeting microglia with specific drugs could potentially alleviate painful withdrawal symptoms, making it easier for patients to stop taking opioids chronically. One promising class of molecules are agonists of the adenosine A3 receptor (*Adora3* gene, A3AR). *Adora3* RNA was upregulated in the brown module comparing our Fent Five vs Fent One groups and A3AR is expressed by neurons and glia. Work by Durante et al. and others in the Salvemini group has showed that A3AR agonists induce release of IL-10 and reduce neuropathic pain via decreased excitability of neurons in the dorsal root ganglia [143]. Currently at least two A3AR agonists (piclidenoson [144] and namodenoson [145]) are undergoing clinical trials and have shown early success. GPR34 (also upregulated in the brown module) is a receptor for cleaved phosphatidylserine [146] that is expressed by microglia in the spinal cord and also shows promise as a target for neuropathic pain [147]. Less is known about the mechanism by which GPR34 may decrease pain; the receptor appears to activate Akt, ERK, and NF-κB signaling, which increase inflammatory responses of microglia such as release of TNFα [148]. Both TLR7 and TLR9 have been implicated in neuroinflammatory pain and the corresponding mRNAs were upregulated in our data comparing Fent Five and Fent One groups. Pre-clinical work on TLR7/9

antagonists has shown exciting success at reducing inflammatory signaling *in vitro* by reducing MAP kinase and NF-κB induced gene expression [149]. *Tlr2* and *Tlr4* were also upregulated in our data and seem to be potential targets for antagonist development. Chronic inhibition of TLR4 has been shown to reduce cue-induced, heroin seeking [150] and a dual TLR2/TLR4 antagonist has been shown to reduce neuropathic pain [151]. Finally, we would like to investigate *P2rx7* (P2X7 receptor) and *P2rx4* (P2X4 receptor) as potential targets for mitigating opioid withdrawal, based on our data and data in the literature connecting them to microglial release of inflammatory cytokines. Both receptors were increased at the RNA level in our Fent Five versus Fent One groups and both receptors have been implicated in pain related to inflammation ([152], [153]). Additionally, P2X7 receptor has been shown to couple with Pannexin 1 channels, causing release of large molecules (~900 Da) from cells when exposed to prolonged stimulation ([154], [155]). Currently there are two P2X7 antagonists in clinical trials for treatment of depression and bipolar disorder ([156], [157]). Development of P2X4R antagonists has been more difficult and less fruitful; a single selective allosteric antagonist exists [158] and some compounds are being developed to target peripheral pain specifically [159], suggesting that blood brain barrier penetrance of these drugs may be of concern for drug development.

In summary, we have developed a new model that captures the impact of repeated cycles of opioid withdrawal on mouse behavioral signs of withdrawal as well as allows us to explore the impact of withdrawal on the biology of microglia in any brain region of interest. This has led us to predict that drugs targeting specific receptors and proteins involved in the inflammatory signaling in microglia may be effective for treating the behavioral and biochemical consequences of opioid withdrawal. More work remains to be done to flesh out how microglia participate in a single experience of withdrawal versus multiple experiences. Brain regions such as the amygdala, the prefrontal cortex, the thalamus, and the bed nucleus of the stria terminalis are likely to be engaged during withdrawal as well. Further work exploring if microglia morphology changes in those regions may be worth investigating first, before beginning time and resource intensive RNA sequencing studies. It will be straightforward to apply the current set of behaviors to future studies, even when changing the length of time to allow animals to experience opioid withdrawal or the opioid being administered. Therefore, this work sets a strong precedent for future experimental designs.

Bibliography


1. Spencer, M.R.G., Matthew F.; Minino, Arialdi M., *Drug Overdose Deaths in the United States, 2002-2022*, in *NCHS Data Briefs*. 2024, National Center for Health Statistics (U.S.).
2. Services, D.o.H.a.H., *Opioid Abuse in the U.S. and HHS Actions to Address Opioid-Drug Related Overdoses and Deaths*. 2015.
3. Sordo, L., et al., *Mortality risk during and after opioid substitution treatment: systematic review and meta-analysis of cohort studies.* Bmj-British Medical Journal, 2017. **357**.
4. Ahlstrom, F.H., et al., *The effects of chronic high-dose morphine on microgliosis and the microglial transcriptome in rat spinal cord.* Mol Pain, 2023. **19**: p. 17448069231183902.
5. Harris, A.C. and J.C. Gewirtz, *Acute opioid dependence: characterizing the early adaptations underlying drug withdrawal.* Psychopharmacology (Berl), 2005. **178**(4): p. 353-66.
6. Pergolizzi, J.V., Jr., R.B. Raffa, and M.H. Rosenblatt, *Opioid withdrawal symptoms, a consequence of chronic opioid use and opioid use disorder: Current understanding and approaches to management.* J Clin Pharm Ther, 2020. **45**(5): p. 892-903.
7. McIntyre, D.C., M.O. Poulter, and K. Gilby, *Kindling: some old and some new.* Epilepsy Res, 2002. **50**(1-2): p. 79-92.
8. Goddard, G.V., *Development of Epileptic Seizures through Brain Stimulation at Low Intensity.* Nature, 1967. **214**(5092): p. 1020-+.
9. Reiss, D., et al., *Mu opioid receptor in microglia contributes to morphine analgesic tolerance, hyperalgesia, and withdrawal in mice.* J Neurosci Res, 2022. **100**(1): p. 203-219.
10. Burma, N.E., et al., *Blocking microglial pannexin-1 channels alleviates morphine withdrawal in rodents.* Nat Med, 2017. **23**(3): p. 355-360.
11. Hutchinson, M.R., et al., *Evidence that opioids may have toll-like receptor 4 and MD-2 effects.* Brain Behavior and Immunity, 2010. **24**(1): p. 83-95.
12. Ellis, A., et al., *Morphine amplifies mechanical allodynia via TLR4 in a rat model of spinal cord injury.* Brain Behav Immun, 2016. **58**: p. 348-356.
13. Coffey, K.R., et al., *A cAMP-Related Gene Network in Microglia Is Inversely Regulated by Morphine Tolerance and Withdrawal.* Biol Psychiatry Glob Open Sci, 2022. **2**(2): p. 180-189.
14. Koob, G.F. and N.D. Volkow, *Neurocircuitry of Addiction.* Neuropsychopharmacology, 2010. **35**(1): p. 217-238.
15. Jiang, Z.G. and R.A. North, *Pre- and postsynaptic inhibition by opioids in rat striatum.* J Neurosci, 1992. **12**(1): p. 356-61.
16. Goedecke, L., et al., *micro-opioid receptor-mediated downregulation of midline thalamic pathways to basal and central amygdala.* Sci Rep, 2019. **9**(1): p. 17837.
17. Vaughan, C.W., et al., *How opioids inhibit GABA-mediated neurotransmission.* Nature, 1997. **390**(6660): p. 611-4.
18. Bontempi, B. and F.R. Sharp, *Systemic morphine-induced Fos protein in the rat striatum and nucleus accumbens is regulated by mu opioid receptors in the substantia nigra and ventral tegmental area.* J Neurosci, 1997. **17**(21): p. 8596-612.
19. Williams, J.T., et al., *Regulation of mu-opioid receptors: desensitization, phosphorylation, internalization, and tolerance.* Pharmacol Rev, 2013. **65**(1): p. 223-54.
20. Gao, H., et al., *mu-Opioid Receptor-Mediated Enteric Glial Activation Is Involved in Morphine-Induced Constipation.* Mol Neurobiol, 2021. **58**(7): p. 3061-3070.
21. Wang, Z., et al., *Central opioid receptors mediate morphine-induced itch and chronic itch via disinhibition.* Brain, 2021. **144**(2): p. 665-681.
22. Koob, G.F. and N.D. Volkow, *Neurobiology of addiction: a neurocircuitry analysis.* Lancet Psychiatry, 2016. **3**(8): p. 760-773.

23. Peterson, P.K., T.W. Molitor, and C.C. Chao, *The opioid-cytokine connection.* Journal of Neuroimmunology, 1998. **83**(1-2): p. 63-69.
24. Tanaka, K., *Epilepsy and exacerbation of brain injury in mice lacking glutamate transporter GLT-1 (vol 276, pg 1699, 1997).* Science, 1997. **278**(5335): p. 21-21.
25. Lewerenz, J., et al., *The Cystine/Glutamat Antiporter System x(c)- in Health and Disease: From Molecular Mechanisms to Novel Therapeutic Opportunities.* Antioxidants & Redox Signaling, 2013. **18**(5): p. 522-555.
26. Song, D., et al., *Comparison between drug-induced and $K^+$-induced changes in molar acid extrusion fluxes ($J^+$) and in energy consumption rates in astrocytes.* Neurochemical Research, 2013. **38**(11): p. 2364-2374.
27. García-Cáceres, C., et al., *Astrocytic Insulin Signaling Couples Brain Glucose Uptake with Nutrient Availability.* Cell, 2016. **166**(4): p. 867-880.
28. Pulvirenti, L., R. Maldonadolopez, and G.F. Koob, *Nmda Receptors in the Nucleus-Accumbens Modulate Intravenous Cocaine but Not Heroin Self-Administration in the Rat.* Brain Research, 1992. **594**(2): p. 327-330.
29. Perea, G., et al., *Optogenetic astrocyte activation modulates response selectivity of visual cortex neurons.* Nature Communications, 2014. **5**.
30. Lines, J., et al., *Astrocytes modulate sensory-evoked neuronal network activity.* Nature Communications, 2020. **11**(1).
31. Kruyer, A. and P.W. Kalivas, *Astrocytes as cellular mediators of cue reactivity in addiction.* Curr Opin Pharmacol, 2021. **56**: p. 1-6.
32. Kalivas, P.W., *The glutamate homeostasis hypothesis of addiction.* Nature Reviews Neuroscience, 2009. **10**(8): p. 561-572.
33. LaLumiere, R.T. and P.W. Kalivas, *Glutamate release in the nucleus accumbens core is necessary for heroin seeking.* Journal of Neuroscience, 2008. **28**(12): p. 3170-3177.
34. Smith, K.L., et al., *Exploring the Role of Central Astrocytic Glutamate Uptake in Ethanol Reward inMice.* Alcoholism-Clinical and Experimental Research, 2014. **38**(5): p. 1307-1314.
35. Neuhofer, D. and P. Kalivas, *Metaplasticity at the addicted tetrapartite synapse: A common denominator of drug induced adaptations and potential treatment target for addiction.* Neurobiol Learn Mem, 2018. **154**: p. 97-111.
36. Yang, J.H., et al., *Ventral tegmental area astrocytes modulate cocaine reward by tonically releasing GABA.* Neuron, 2023. **111**(7): p. 1104-+.
37. Lewitus, G.M., et al., *Microglial TNF-alpha Suppresses Cocaine-Induced Plasticity and Behavioral Sensitization.* Neuron, 2016. **90**(3): p. 483-91.
38. Badimon, A., et al., *Negative feedback control of neuronal activity by microglia.* Nature, 2020. **586**(7829): p. 417-+.
39. Umpierre, A.D., et al., *Microglial calcium signaling is attuned to neuronal activity in awake mice.* Elife, 2020. **9**.
40. Sperlagh, B. and P. Illes, *Purinergic modulation of microglial cell activation.* Purinergic Signal, 2007. **3**(1-2): p. 117-27.
41. Hanamsagar, R., V. Torres, and T. Kielian, *Inflammasome activation and IL-1beta/IL-18 processing are influenced by distinct pathways in microglia.* J Neurochem, 2011. **119**(4): p. 736-48.
42. Hide, I., et al., *Extracellular ATP triggers tumor necrosis factor-alpha release from rat microglia.* J Neurochem, 2000. **75**(3): p. 965-72.
43. Frank, M.G., L.R. Watkins, and S.F. Maier, *Stress- and glucocorticoid-induced priming of neuroinflammatory responses: potential mechanisms of stress-induced vulnerability to drugs of abuse.* Brain Behav Immun, 2011. **25 Suppl 1**(Suppl 1): p. S21-8.

44. Schwarz, J.M. and S.D. Bilbo, *Adolescent morphine exposure affects long-term microglial function and later-life relapse liability in a model of addiction.* J Neurosci, 2013. **33**(3): p. 961-71.
45. Parekh, S.V., et al., *Hippocampal TNF-α Signaling Mediates Heroin Withdrawal-Enhanced Fear Learning and Withdrawal-Induced Weight Loss.* Molecular Neurobiology, 2021. **58**(6): p. 2963-2973.
46. Jeon, S., et al., *Clozapine reduces Toll-like receptor 4/NF-kappaB-mediated inflammatory responses through inhibition of calcium/calmodulin-dependent Akt activation in microglia.* Prog Neuropsychopharmacol Biol Psychiatry, 2018. **81**: p. 477-487.
47. Holloway, K.N., et al., *Ethanol Induces Neuroinflammation in a Chronic Plus Binge Mouse Model of Alcohol Use Disorder via TLR4 and MyD88-Dependent Signaling.* Cells, 2023. **12**(16).
48. Paolicelli, R.C., et al., *Synaptic pruning by microglia is necessary for normal brain development.* Science, 2011. **333**(6048): p. 1456-8.
49. Wong, E.L., et al., *Dynamics of microglia and dendritic spines in early adolescent cortex after developmental alcohol exposure.* Dev Neurobiol, 2021. **81**(6): p. 786-804.
50. Taylor, K.R. and M. Monje, *Neuron-oligodendroglial interactions in health and malignant disease.* Nat Rev Neurosci, 2023.
51. Mandyam, C.D., et al., *Methamphetamine self-administration and voluntary exercise have opposing effects on medial prefrontal cortex gliogenesis.* J Neurosci, 2007. **27**(42): p. 11442-50.
52. Eschenroeder, A.C., et al., *Oligodendrocyte responses to buprenorphine uncover novel and opposing roles of mu-opioid- and nociceptin/orphanin FQ receptors in cell development: implications for drug addiction treatment during pregnancy.* Glia, 2012. **60**(1): p. 125-36.
53. Worthington, W.C., Jr. and R.S. Cathcart, 3rd, *Ependymal cilia: distribution and activity in the adult human brain.* Science, 1963. **139**(3551): p. 221-2.
54. Wang, H., et al., *Gli3 repressor controls cell fates and cell adhesion for proper establishment of neurogenic niche.* Cell Rep, 2014. **8**(4): p. 1093-104.
55. Zhu, Y., et al., *Opioid-induced fragile-like regulatory T cells contribute to withdrawal.* Cell, 2023. **186**(3): p. 591-606 e23.
56. Cuitavi, J., et al., *Focal mu-opioid receptor activation promotes neuroinflammation and microglial activation in the mesocorticolimbic system: Alterations induced by inflammatory pain.* Glia, 2023. **71**(8): p. 1906-1920.
57. Haimon, Z., et al., *Re-evaluating microglia expression profiles using RiboTag and cell isolation strategies.* Nat Immunol, 2018. **19**(6): p. 636-644.
58. Doyle, J.P., et al., *Application of a Translational Profiling Approach for the Comparative Analysis of CNS Cell Types.* Cell, 2008. **135**(4): p. 749-762.
59. Sanz, E., et al., *RiboTag Analysis of Actively Translated mRNAs in Sertoli and Leydig Cells.* Plos One, 2013. **8**(6).
60. Ingolia, N.T., et al., *Genome-wide analysis in vivo of translation with nucleotide resolution using ribosome profiling.* Science, 2009. **324**(5924): p. 218-23.
61. Marsh, S.E., et al., *Dissection of artifactual and confounding glial signatures by single-cell sequencing of mouse and human brain.* Nat Neurosci, 2022. **25**(3): p. 306-316.
62. Navarro-Tapia, E., A. Querol, and R. Perez-Torrado, *Membrane fluidification by ethanol stress activates unfolded protein response in yeasts.* Microb Biotechnol, 2018. **11**(3): p. 465-475.
63. Er, H., et al., *Acetyl-L-carnitine attenuates chronic ethanol-induced oxidative stress, ER stress and apoptosis in rat gastric tissue.* Alcohol, 2023. **112**: p. 51-59.

64. Wang, Y., et al., *MANF is neuroprotective against ethanol-induced neurodegeneration through ameliorating ER stress.* Neurobiol Dis, 2021. **148**: p. 105216.
65. Erickson, E.K., et al., *Glial gene networks associated with alcohol dependence.* Sci Rep, 2019. **9**(1): p. 10949.
66. Brenner, E., et al., *Single cell transcriptome profiling of the human alcohol-dependent brain.* Hum Mol Genet, 2020. **29**(7): p. 1144-1153.
67. Thompson, S.M., et al., *Acute alcohol and chronic drinking bidirectionally regulate the excitability of prefrontal cortex vasoactive intestinal peptide interneurons.* Neuropharmacology, 2023. **238**: p. 109638.
68. Liu, J., et al., *Altered gene expression profiles in the frontal cortex of cirrhotic alcoholics.* Alcohol Clin Exp Res, 2007. **31**(9): p. 1460-6.
69. Mulligan, M.K., et al., *Molecular profiles of drinking alcohol to intoxication in C57BL/6J mice.* Alcohol Clin Exp Res, 2011. **35**(4): p. 659-70.
70. Ayers-Ringler, J.R., et al., *Label-Free Proteomic Analysis of Protein Changes in the Striatum during Chronic Ethanol Use and Early Withdrawal.* Front Behav Neurosci, 2016. **10**: p. 46.
71. Guergues, J., et al., *Deep proteome profiling reveals novel pathways associated with pro-inflammatory and alcohol-induced microglial activation phenotypes.* J Proteomics, 2020. **220**: p. 103753.
72. Gandhirajan, A., et al., *SIRT2-PFKP interaction dysregulates phagocytosis in macrophages with acute ethanol-exposure.* Front Immunol, 2022. **13**: p. 1079962.
73. Mukherjee, S., et al., *Alcohol Increases Exosome Release from Microglia to Promote Complement C1q-Induced Cellular Death of Proopiomelanocortin Neurons in the Hypothalamus in a Rat Model of Fetal Alcohol Spectrum Disorders.* J Neurosci, 2020. **40**(41): p. 7965-7979.
74. Shrivastava, P., et al., *Mu-opioid receptor and delta-opioid receptor differentially regulate microglial inflammatory response to control proopiomelanocortin neuronal apoptosis in the hypothalamus: effects of neonatal alcohol.* J Neuroinflammation, 2017. **14**(1): p. 83.
75. Ho, M.F., et al., *Genetic variants associated with acamprosate treatment response in alcohol use disorder patients: A multiple omics study.* British Journal of Pharmacology, 2022. **179**(13): p. 3330-3345.
76. Yalcin, E.B., et al., *Differential Sphingolipid and Phospholipid Profiles in Alcohol and Nicotine-Derived Nitrosamine Ketone-Associated White Matter Degeneration.* Alcoholism-Clinical and Experimental Research, 2015. **39**(12): p. 2324-2333.
77. Jokinen, V., et al., *Differential Spinal and Supraspinal Activation of Glia in a Rat Model of Morphine Tolerance.* Neuroscience, 2018. **375**: p. 10-24.
78. Ho, M.F., et al., *Single cell transcriptomics reveals distinct transcriptional responses to oxycodone and buprenorphine by iPSC-derived brain organoids from patients with opioid use disorder.* Mol Psychiatry, 2022.
79. Seney, M.L., et al., *Transcriptional Alterations in Dorsolateral Prefrontal Cortex and Nucleus Accumbens Implicate Neuroinflammation and Synaptic Remodeling in Opioid Use Disorder.* Biol Psychiatry, 2021. **90**(8): p. 550-562.
80. Wei, J., et al., *Single nucleus transcriptomics of ventral midbrain identifies glial activation associated with chronic opioid use disorder.* Nat Commun, 2023. **14**(1): p. 5610.
81. Avey, D., et al., *Single-Cell RNA-Seq Uncovers a Robust Transcriptional Response to Morphine by Glia.* Cell Rep, 2018. **24**(13): p. 3619-3629 e4.
82. Nestler, E.J., *Reflections on: "A general role for adaptations in G-Proteins and the cyclic AMP system in mediating the chronic actions of morphine and cocaine on neuronal function".* Brain Res, 2016. **1645**: p. 71-4.

83. Duttke, S.H., et al., *Glucocorticoid Receptor-Regulated Enhancers Play a Central Role in the Gene Regulatory Networks Underlying Drug Addiction.* Front Neurosci, 2022. **16**: p. 858427.
84. Liang, Y., et al., *Morphine enhances IL-1beta release through toll-like receptor 4-mediated endocytic pathway in microglia.* Purinergic Signal, 2016. **12**(4): p. 637-645.
85. Ye, L., et al., *Cathepsin S in the spinal microglia contributes to remifentanil-induced hyperalgesia in rats.* Neuroscience, 2017. **344**: p. 265-275.
86. Metryka, E., et al., *The expression of purinergic P2X4 and P2X7 receptors in selected mesolimbic structures during morphine withdrawal in rats.* Brain Res, 2019. **1719**: p. 49-56.
87. Takayama, N. and H. Ueda, *Morphine-induced chemotaxis and brain-derived neurotrophic factor expression in microglia.* J Neurosci, 2005. **25**(2): p. 430-5.
88. Yang, T., et al., *Withdrawal from spinal application of remifentanil induces long-term potentiation of c-fiber-evoked field potentials by activation of Src family kinases in spinal microglia.* Neurochem Res, 2018. **43**(8): p. 1660-1670.
89. Fox, M.E., N.T. Rodeberg, and R.M. Wightman, *Reciprocal Catecholamine Changes during Opiate Exposure and Withdrawal.* Neuropsychopharmacology, 2017. **42**(3): p. 671-681.
90. von Kugelgen, I., et al., *Co-release of noradrenaline and ATP from cultured sympathetic neurons.* Neuroscience, 1994. **61**(2): p. 199-202.
91. Mendez, E.F., et al., *Angiogenic gene networks are dysregulated in opioid use disorder: evidence from multi-omics and imaging of postmortem human brain.* Mol Psychiatry, 2021. **26**(12): p. 7803-7812.
92. Dozio, V., et al., *Deep proteomics and phosphoproteomics reveal novel biological pathways perturbed by morphine, morphine-3-glucuronide and morphine-6-glucuronide in human astrocytes.* J Neurosci Res, 2022. **100**(1): p. 220-236.
93. Wang, X.X., et al., *Inhibition of Oligodendrocyte Apoptosis in the Prelimbic Medial Prefrontal Cortex Prevents Fentanyl-induced Hyperalgesia in Rats.* Journal of Pain, 2022. **23**(6): p. 1035-1050.
94. Penrod, R.D., et al., *Differential Roles of Oxytocin Receptors in the Prefrontal Cortex and Nucleus Accumbens on Cocaine Self-Administration and Reinstatement of Cued Cocaine Seeking in Male Rats.* Int J Neuropsychopharmacol, 2023. **26**(12): p. 817-827.
95. Sharma, S. and S. Fulton, *Diet-induced obesity promotes depressive-like behaviour that is associated with neural adaptations in brain reward circuitry.* International Journal of Obesity, 2013. **37**(3): p. 382-389.
96. Langley, M.R., et al., *High fat diet consumption results in mitochondrial dysfunction, oxidative stress, and oligodendrocyte loss in the central nervous system.* Biochimica Et Biophysica Acta-Molecular Basis of Disease, 2020. **1866**(3).
97. Henley, B.M., et al., *Transcriptional regulation by nicotine in dopaminergic neurons.* Biochem Pharmacol, 2013. **86**(8): p. 1074-83.
98. Silva, C.P., et al., *The influence of adolescent nicotine exposure on ethanol intake and brain gene expression.* PLoS One, 2018. **13**(6): p. e0198935.
99. Silva, J.P., et al., *Epigenomic and metabolic responses of hypothalamic POMC neurons to gestational nicotine exposure in adult offspring.* Genome Medicine, 2016. **8**.
100. Chen, Z., et al., *Single-nucleus chromatin accessibility and RNA sequencing reveal impaired brain development in prenatally e-cigarette exposed neonatal rats.* iScience, 2022. **25**(8): p. 104686.
101. Sharpe, A.L., et al., *Repeated cocaine or methamphetamine treatment alters astrocytic CRF2 and GLAST expression in the ventral midbrain.* Addiction Biology, 2022. **27**(2).

102. Navandar, M., et al., *Transcriptional signatures in prefrontal cortex confer vulnerability versus resilience to food and cocaine addiction-like behavior.* Scientific Reports, 2021. **11**(1).
103. Hunnicutt, B.J., et al., *A comprehensive excitatory input map of the striatum reveals novel functional organization.* Elife, 2016. **5**.
104. He, J., et al., *Transcriptional and anatomical diversity of medium spiny neurons in the primate striatum.* Curr Biol, 2021. **31**(24): p. 5473-5486 e6.
105. Haber, S.N. and N.R. McFarland, *The concept of the ventral striatum in nonhuman primates.* Ann N Y Acad Sci, 1999. **877**: p. 33-48.
106. Gruber, A.J. and R.J. McDonald, *Context, emotion, and the strategic pursuit of goals: interactions among multiple brain systems controlling motivated behavior.* Front Behav Neurosci, 2012. **6**: p. 50.
107. Miura, M., M. Masuda, and T. Aosaki, *Roles of micro-opioid receptors in GABAergic synaptic transmission in the striosome and matrix compartments of the striatum.* Mol Neurobiol, 2008. **37**(2-3): p. 104-15.
108. Severino, A.L., et al., *mu-Opioid Receptors on Distinct Neuronal Populations Mediate Different Aspects of Opioid Reward-Related Behaviors.* eNeuro, 2020. **7**(5).
109. Ben Hamida, S., et al., *Mu opioid receptors in GABAergic neurons of the forebrain promote alcohol reward and drinking.* Addict Biol, 2019. **24**(1): p. 28-39.
110. Castro, D.C., et al., *An endogenous opioid circuit determines state-dependent reward consumption.* Nature, 2021. **598**(7882): p. 646-651.
111. Liu, M.Y., et al., *Sucrose preference test for measurement of stress-induced anhedonia in mice.* Nature Protocols, 2018. **13**(7): p. 1686-1698.
112. Reichard, K.L., et al., *Regulation of Kappa Opioid Receptor Inactivation Depends on Sex and Cellular Site of Antagonist Action.* Mol Pharmacol, 2020. **98**(5): p. 548-558.
113. Lesiak, A.J., et al., *Sequencing the serotonergic neuron translatome reveals a new role for Fkbp5 in stress.* Mol Psychiatry, 2021. **26**(9): p. 4742-4753.
114. Patro, R., et al., *Salmon provides fast and bias-aware quantification of transcript expression.* Nat Methods, 2017. **14**(4): p. 417-419.
115. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2.* Genome Biol, 2014. **15**(12): p. 550.
116. Langfelder, P. and S. Horvath, *WGCNA: an R package for weighted correlation network analysis.* BMC Bioinformatics, 2008. **9**: p. 559.
117. Pfaffl, M.W., *A new mathematical model for relative quantification in real-time RT-PCR.* Nucleic Acids Res, 2001. **29**(9): p. e45.
118. Wala, E.P. and J.R. Holtman, Jr., *Buprenorphine-induced hyperalgesia in the rat.* Eur J Pharmacol, 2011. **651**(1-3): p. 89-95.
119. Yu, G.C., et al., *clusterProfiler: an R Package for Comparing Biological Themes Among Gene Clusters.* Omics-a Journal of Integrative Biology, 2012. **16**(5): p. 284-287.
120. Lefevre, E.M., et al., *Interruption of continuous opioid exposure exacerbates drug-evoked adaptations in the mesolimbic dopamine system.* Neuropsychopharmacology, 2020. **45**(11): p. 1781-1792.
121. Schulteis, G., A.C. Morse, and J. Liu, *Repeated experience with naloxone facilitates acute morphine withdrawal: potential role for conditioning processes in acute opioid dependence.* Pharmacol Biochem Behav, 2003. **76**(3-4): p. 493-503.
122. Chaudun, F., et al., *Distinct micro-opioid ensembles trigger positive and negative fentanyl reinforcement.* Nature, 2024. **630**(8015): p. 141-148.
123. Overstreet, D.H., D.J. Knapp, and G.R. Breese, *Accentuated decrease in social interaction in rats subjected to repeated ethanol withdrawals.* Alcohol Clin Exp Res, 2002. **26**(8): p. 1259-68.

124. Spanagel, R. and S.M. Hölter, *Long-term alcohol self-administration with repeated alcohol deprivation phases:: An animal model of alcoholism?* Alcohol and Alcoholism, 1999. **34**(2): p. 231-243.
125. Goodson, C.M., B.J. Clark, and I.S. Douglas, *Predictors of severe alcohol withdrawal syndrome: a systematic review and meta-analysis.* Alcohol Clin Exp Res, 2014. **38**(10): p. 2664-77.
126. Azorlosa, J.L., M.L. Stitzer, and M.K. Greenwald, *Opioid Physical-Dependence Development - Effects of Single Versus Repeated Morphine Pretreatments and of Subjects Opioid Exposure History.* Psychopharmacology, 1994. **114**(1): p. 71-80.
127. Zhao, Y., et al., *TREM2 Is a Receptor for beta-Amyloid that Mediates Microglial Function.* Neuron, 2018. **97**(5): p. 1023-1031 e7.
128. Monif, M., et al., *Interleukin-1beta has trophic effects in microglia and its release is mediated by P2X7R pore.* J Neuroinflammation, 2016. **13**(1): p. 173.
129. Keren-Shaul, H., et al., *A Unique Microglia Type Associated with Restricting Development of Alzheimer's Disease.* Cell, 2017. **169**(7): p. 1276-1290 e17.
130. Coleman, L.G., Jr., J. Zou, and F.T. Crews, *Microglial-derived miRNA let-7 and HMGB1 contribute to ethanol-induced neurotoxicity via TLR7.* J Neuroinflammation, 2017. **14**(1): p. 22.
131. Chen, C.Y., et al., *Beyond defense: regulation of neuronal morphogenesis and brain functions via Toll-like receptors.* J Biomed Sci, 2019. **26**(1): p. 90.
132. Petes, C., N. Odoardi, and K. Gee, *The Toll for Trafficking: Toll-Like Receptor 7 Delivery to the Endosome.* Front Immunol, 2017. **8**: p. 1075.
133. Michaelis, K.A., et al., *Persistent Toll-like receptor 7 stimulation induces behavioral and molecular innate immune tolerance.* Brain Behav Immun, 2019. **82**: p. 338-353.
134. Krishnan, M.L., et al., *Integrative genomics of microglia implicates DLG4 (PSD95) in the white matter development of preterm infants.* Nat Commun, 2017. **8**(1): p. 428.
135. Tsai, N.P., et al., *Multiple autism-linked genes mediate synapse elimination via proteasomal degradation of a synaptic scaffold PSD-95.* Cell, 2012. **151**(7): p. 1581-94.
136. Fernandez, E., et al., *Arc Requires PSD95 for Assembly into Postsynaptic Complexes Involved with Neural Dysfunction and Intelligence.* Cell Rep, 2017. **21**(3): p. 679-691.
137. Purcell, S.M., et al., *A polygenic burden of rare disruptive mutations in schizophrenia.* Nature, 2014. **506**(7487): p. 185-90.
138. Fromer, M., et al., *De novo mutations in schizophrenia implicate synaptic networks.* Nature, 2014. **506**(7487): p. 179-84.
139. Camp, M.C., et al., *A novel role for PSD-95 in mediating ethanol intoxication, drinking and place preference.* Addict Biol, 2011. **16**(3): p. 428-39.
140. Hemby, S.E., *Morphine-induced alterations in gene expression of calbindin immunopositive neurons in nucleus accumbens shell and core.* Neuroscience, 2004. **126**(3): p. 689-703.
141. Kim, E. and M. Sheng, *PDZ domain proteins of synapses.* Nat Rev Neurosci, 2004. **5**(10): p. 771-81.
142. Elias, G.M. and R.A. Nicoll, *Synaptic trafficking of glutamate receptors by MAGUK scaffolding proteins.* Trends Cell Biol, 2007. **17**(7): p. 343-52.
143. Durante, M., et al., *Adenosine A3 agonists reverse neuropathic pain via T cell-mediated production of IL-10.* J Clin Invest, 2021. **131**(7).
144. Papp, K.A., et al., *Efficacy and safety of piclidenoson in plaque psoriasis: Results from a randomized phase 3 clinical trial (COMFORT-1).* J Eur Acad Dermatol Venereol, 2024. **38**(6): p. 1112-1120.
145. Stemmer, S.M., et al., *Namodenoson in Advanced Hepatocellular Carcinoma and Child-Pugh B Cirrhosis: Randomized Placebo-Controlled Clinical Trial.* Cancers (Basel), 2021. **13**(2).

146. Sugo, T., et al., *Identification of a lysophosphatidylserine receptor on mast cells.* Biochem Biophys Res Commun, 2006. **341**(4): p. 1078-87.
147. Sayo, A., et al., *GPR34 in spinal microglia exacerbates neuropathic pain in mice.* J Neuroinflammation, 2019. **16**(1): p. 82.
148. Wang, W., P. Ji, and K.E. Dow, *Corticotropin-releasing hormone induces proliferation and TNF-alpha release in cultured rat microglia via MAP kinase signalling pathways.* J Neurochem, 2003. **84**(1): p. 189-95.
149. Haseeb, M., et al., *Discovery of Novel Small Molecule Dual Inhibitor Targeting Toll-Like Receptors 7 and 9.* J Chem Inf Model, 2024. **64**(13): p. 5090-5107.
150. Theberge, F.R., et al., *Effect of chronic delivery of the Toll-like receptor 4 antagonist (+)-naltrexone on incubation of heroin craving.* Biol Psychiatry, 2013. **73**(8): p. 729-37.
151. Kwilasz, A.J., et al., *Toll-like receptor 2 and 4 antagonism for the treatment of experimental autoimmune encephalomyelitis (EAE)-related pain.* Brain Behav Immun, 2021. **93**: p. 80-95.
152. He, W.J., et al., *Spinal P2X(7) receptor mediates microglia activation-induced neuropathic pain in the sciatic nerve injury rat model.* Behav Brain Res, 2012. **226**(1): p. 163-70.
153. Tsuda, M., et al., *Fibronectin/integrin system is involved in P2X(4) receptor upregulation in the spinal cord and neuropathic pain after nerve injury.* Glia, 2008. **56**(5): p. 579-85.
154. Locovei, S., et al., *Pannexin1 is part of the pore forming unit of the P2X(7) receptor death complex.* FEBS Lett, 2007. **581**(3): p. 483-8.
155. Pelegrin, P. and A. Surprenant, *Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor.* EMBO J, 2006. **25**(21): p. 5071-82.
156. Recourt, K., et al., *Characterisation of the pharmacodynamic effects of the P2X7 receptor antagonist JNJ-54175446 using an oral dexamphetamine challenge model in healthy males in a randomised, double-blind, placebo-controlled, multiple ascending dose trial.* J Psychopharmacol, 2020. **34**(9): p. 1030-1042.
157. Bhattacharya, A., et al., *Neuropsychopharmacology of JNJ-55308942: evaluation of a clinical candidate targeting P2X7 ion channels in animal models of neuroinflammation and anhedonia.* Neuropsychopharmacology, 2018. **43**(13): p. 2586-2596.
158. Ase, A.R., et al., *Identification and characterization of a selective allosteric antagonist of human P2X4 receptor channels.* Mol Pharmacol, 2015. **87**(4): p. 606-16.
159. Di Salvo, C., et al., *The pharmacological blockade of P2X4 receptor as a viable approach to manage visceral pain in a rat model of colitis.* J Drug Target, 2024: p. 1-11.