

**SEX DIFFERENCES IN THE ROLE OF PKM $\zeta$  IN DRUG SELF-  
ADMINISTRATION AND PREFRONTAL CORTEX  
SYNAPTIC PLASTICITY**

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## ABSTRACT

Neuroadaptations associated with addiction are hypothesized to involve the same glutamatergic cellular mechanisms that underlie learning and memory. Further, there are clear sex differences in both rates of diagnosis and symptom severity in substance use disorder (SUD), which may result from sexually dimorphic pathophysiology involving glutamate. However, little is known about the contribution of proteins involved in synaptic plasticity in reward related circuitry. The goal of these experiments was to determine the role of protein kinase M zeta (PKM $\zeta$ ), a protein involved in the trafficking of AMPA receptors (AMPA), in drug taking behaviors and synaptic plasticity. PKM $\zeta$  belongs to the protein kinase C family and is found exclusively in the central nervous system. PKM $\zeta$  is persistently active, making it an interesting target for persistent changes in the brain, such as memory storage. Although the exact role of PKM $\zeta$  in synaptic plasticity has been the source of controversy, there is a known role for PKM $\zeta$  in reward. Mice genetically lacking PKM $\zeta$  exhibit heightened addictive phenotypes for both ethanol and cocaine. We extended these findings to the opioid drug class and found that PKM $\zeta$  works to dampen reward in male and female mice. We next examined the role for PKM $\zeta$  in long-term synaptic plasticity in the medial prefrontal cortex (mPFC), a region critical for the expression of addictive phenotypes. We found that PKM $\zeta$  is necessary for both long-term depression and long-term potentiation in the mPFC. Finally, we investigated the role of PKM $\zeta$ , specifically in the PFC, on cocaine taking behaviors. We found that PFC PKM $\zeta$  plays a sex-specific role in cocaine cue-induced reinstatement. Taken together, these findings suggest PKM $\zeta$  has sex- and region-specific role in synaptic plasticity and reward-

related behaviors and gaining more insight into these mechanisms may provide novel routes for the treatments of SUD.

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## CHAPTER 1

### SEX DIFFERENCES IN GLUTAMATE TRANSMISSION AND PLASTICITY WITHIN REWARD AND NON-REWARD RELATED REGIONS

#### Introduction

Glutamate is a major neurotransmitter in the brain that mediates fast excitatory signals. Tight regulation of glutamate transmission is required for normal cognitive functions including learning, memory, and mood regulation. Disruption in glutamate homeostasis within mesolimbic structures contributes to various reward-related psychiatric disorders, including major depressive disorder (MDD), anxiety, and substance use disorder (SUD) (Gass & Foster Olive, 2008; C.-T. Li et al., 2019; Miladinovic et al., 2015; Niedzielska-Andres et al., 2021). Many of these disorders exhibit sex differences in both rates of diagnosis and symptom severity and therefore may result from sexually dimorphic pathophysiology involving glutamate (Green et al., 2019). Indeed, preclinical research suggests that sex differences within the glutamate system exist in many brain regions (Giacometti & Barker, 2020; Wickens et al., 2018). To understand potential sex differences in the role of glutamate in neuropsychiatric pathophysiology, it is important to first delineate baseline sex differences in glutamatergic transmission.

In addition to sex differences in baseline glutamatergic transmission, differences also exist in the capacity of the glutamate system to undergo structural remodeling and functional changes in synaptic strength. Synaptic restructuring is a form of plasticity resulting from acute or long-term changes in glutamatergic activity that can be analyzed by measuring morphological features such as spine density, dendritic branching, and receptor composition (Bernardinelli et al., 2014). Furthermore, glutamate plays a role in

the functional strengthening or weakening of synaptic connections, which may result in long-term potentiation (LTP) and long-term depression (LTD), amongst other forms of plasticity (Citri & Malenka, 2008). Therefore, in addition to differential levels of glutamatergic transmission, baseline sex differences in synaptic plasticity may also contribute the pathophysiology of the abovementioned reward-related disorders.

This review will consider preclinical rodent literature directly comparing glutamatergic transmission and plasticity in reward and non-reward related regions of males and females. This will further the understanding of how glutamate may be differentially contributing to baseline sex differences across regions. The regions considered for reward-related are the nucleus accumbens, prefrontal cortex (PFC), amygdala, and hippocampus as they are key regions in the mesolimbic pathway that are implicated in reward-related disorders (Lewis et al., 2021). Non-reward related regions will include the hypothalamus and sensory regions. Additionally, we will suggest which regions are exhibiting evidence for sexually dimorphic mechanisms, convergent mechanisms, or no sex differences in glutamatergic transmission and plasticity. Lastly, we will illuminate gaps in the literature and provide suggestions for future studies to expand the field.

### Structural and Electrophysiological measurements of Glutamate Transmission

Structural differences in glutamatergic neurons can indicate the relative synaptic strength in a specific region. Synaptic remodeling is a form of structural plasticity that refers to the physical modification of neuronal networks in an activity-dependent manner. Neurons adapt in response to stimuli or changes in an individuals' environment to undergo morphological rearrangement, affecting dendritic spines and dendritic branching. Dendritic

spines are highly dynamic structures that rapidly undergo changes in composition on a time scale from minutes to days (Bernardinelli et al., 2014). Variation in spine size, shape, and content have been shown to correlate with synaptic strength, maturity, and stability of glutamatergic signaling (Arellano et al., 2007; Hering & Sheng, 2001). Likewise, the extent of dendritic branching may also reflect the extent of glutamatergic connectivity in a specific region. Morphological analysis typically includes staining or fluorescently labeling neurons and using fluorescent or confocal microscopes in combination with imaging software to quantify various aspects of spine and dendrite properties (B.-Z. Li et al., 2023).

In addition to structural differences associated with changes in glutamatergic transmission that can be visualized in brain slices, glutamatergic transmission can be assessed using slice electrophysiology. With this technique, researchers can ascertain how neurons or populations of neurons communicate via glutamate. This technique is a powerful *ex vivo* tool used to understand the state of neurons with a high level of control over the physiological environment and spatial precision. Information regarding the properties of neurons at resting states and after stimulation can be used to understand mechanisms of glutamatergic transmission in various regions of the brain. In addition to whole cell states, highly specific protocols can further delineate differences in presynaptic and post-synaptic glutamate transmission. Together with structural analysis, electrophysiological properties give a more complete picture of glutamatergic transmission.

### Synaptic Plasticity

Along with differences in glutamatergic transmission, synaptic plasticity, or the ability for neurons to alter synaptic transmission in response to changes in electrochemical

environment, can also be measured using slice electrophysiology. There are several forms of synaptic plasticity. The most well described forms are LTP and LTD, or the long-lasting enhancement or depression of synaptic strength over time respectively. It is believed that this form of synaptic plasticity is the basis of long-lasting effects, including learning and memory. Other forms of plasticity, such as short-term plasticity, are on quicker timescales and reflect transient changes in synaptic transmission which may be critical for fast computational processes (Hennig, 2013). To investigate differences in synaptic plasticity, stimulation protocols are used which mimic naturally occurring electrochemical environments that elicit either short or long-term changes in glutamatergic signaling. In combination with glutamatergic transmission, these measurements of synaptic plasticity give insight to a region's ability to adapt to changing environments.

### Sex Differences in Glutamate Transmission and Plasticity

#### *Nucleus Accumbens*

The nucleus accumbens is part of the mesolimbic circuit and is widely recognized to play a critical role in reward and motivated behavior (Alcaro et al., 2007; Floresco, 2015). Composed mainly of GABAergic medial spiny neurons (MSNs), the nucleus accumbens receives glutamatergic inputs from the PFC, amygdala, thalamus, hippocampus, and ventral tegmental area (VTA) (Gipson et al., 2014; Z. Li et al., 2018). Understanding morphological sex differences in the nucleus accumbens therefore provides insight to differences in glutamate transmission. Several sex differences have been found when directly comparing nucleus accumbens dendritic morphology. Female rodents have greater spine density in the nucleus accumbens core compared to the nucleus accumbens core of male rodents (Forlano & Woolley, 2010; Wissman et al., 2011). A higher spine

density is thought to reflect more glutamatergic input into the region with more synaptic connections. In parallel, females also have larger spines in both core and shell (Forlano & Woolley, 2010). Spine size measurements, including head diameter have been positively correlated with synaptic strength and allow for more surface area to regulate synaptic efficacy at pre- and post-synaptic levels (Arellano et al., 2007). Although most of the work suggests higher spine density and spine size in females, contrasting evidence suggests neuronal morphology in the core and shell did not differ between males and females when estrous cycle was disregarded (Beeson & Meitzen, 2023; Meitzen et al., 2011). Discrepancies in spine density and size may in part be due to differing staining and imaging techniques or due to differences in spine density on a rostral-caudal gradient or fluctuations in gonadal hormones (Wissman et al., 2012). Overall, these studies suggest that females receive more glutamatergic input and have higher synaptic transmission in the nucleus accumbens than males.

As these morphological studies do not directly measure glutamatergic transmission, whole-cell patch clamp electrophysiology studies are needed to determine if the structural sex differences lead to functional alterations. Consistent with the structural studies, electrophysiology findings report greater synaptic strength in female rodents compared to males. Within the nucleus accumbens core, females report higher miniature excitatory post synaptic current (mEPSC) frequency (Wissman et al., 2011). Increases in mEPSC frequency could be produced either by increases in presynaptic glutamate release and/or a larger quantity of synaptic connections (Han & Stevens, 2009). This is in line with higher spine density in females and with findings showing that females have larger readily releasable pools (RRP) than males (Deutschmann et al., 2022; Knouse et al., 2023).

Together these measures indicate an overall higher presynaptic input in females in comparison to males in the nucleus accumbens. Measures of postsynaptic strength such as mEPSC amplitude and AMPA/NMDA ratio also suggest higher glutamate transmission in females (Knouse et al., 2023). When exclusively considering properties in the nucleus accumbens shell, no sex differences were found in MSN intrinsic properties or mEPSCs (Willett et al., 2016). Likewise, there are no differences between males and females on various intrinsic MSN properties when estrous cycle was disregarded or when both male and female animals are gonadectomized. However, when estrus cycle is considered, gonadal hormones seem to influence many intrinsic MSN properties suggesting that circulating hormones may contribute to many functional sex differences in this region (Proaño et al., 2018).

The nucleus accumbens of females overall show more robust pre- and post-synaptic glutamatergic transmission. However, electrophysiology studies investigating synaptic plasticity are sparse and more work is needed to delineate baseline differences in short and long-term plasticity. To date, evidence suggests females may be less plastic than males in the nucleus accumbens core. LTD in this region was harder to induce in wildtype females compared to males. This was concluded to be due to larger RRP and heightened glutamatergic activity in females (Knouse et al., 2023). Although there are differences in long term plasticity, no differences were found between males and females in short term plasticity, evidenced by similar evoked responses in a paired pulse paradigm (Wissman et al., 2011). To fully understand baseline sex differences in plasticity within the nucleus accumbens, more studies are needed that use differing stimulation protocols to elicit a full range of responses.

Unlike most other regions in the brain, in the nucleus accumbens, sex differences in glutamate transmission are consistent across neuronal morphology and electrophysiology measures. Higher presynaptic and postsynaptic markers of synaptic strength in the nucleus accumbens may underlie sex differences in reward-related disorders. This may lead females to be more vulnerable to exogenous agents that effect the mesolimbic pathway. For example, women report faster escalation from drug use to abuse and find it more difficult to quit than men, potentially due to more excitatory transmission in the nucleus accumbens (Becker & Hu, 2008). Likewise, females may exhibit less plasticity in the nucleus accumbens, which may contribute to an impaired ability to regulate drug seeking. However, men and women are similarly likely to become addicted to drugs of abuse (Anthony et al., 1994). It is likely that males and females are exhibiting sexually dimorphic characteristics in glutamate transmission in this region that may be contributing to differences in symptom expression in men and women.

#### *Prefrontal Cortex*

Much like the nucleus accumbens, there are clear sex differences in neuronal morphology within the PFC. These differences are not as stark as those found in the nucleus accumbens but underscore that the mechanisms that lead to homeostatic plasticity vary across sex. The PFC mediates higher brain functions and has been found to be incredibly plastic in response to experience (Anastasiades & Carter, 2021; Kolb & Gibb, 2015). This region is made of excitatory pyramidal neurons (70-90%) and GABAergic interneurons (DeFelipe et al., 2013). Very few studies have directly compared neuronal morphology between the sexes. Females exhibit higher synaptic density in both the infralimbic and prelimbic areas by measurement of immunofluorescent density of synaptophysin, a marker

for presynaptic sites (Carvalho-Netto et al., 2011). However, male and female animals exhibit the same number of boutons in the PFC (Drzewiecki et al., 2016). Overall higher synaptophysin immunofluorescent density in females is therefore likely due a similar quantity, but larger, spines. Indeed, females show higher levels of synaptosomal GluA1 and A2 glutamatergic receptor subunits, with no differences in overall expression between the sexes (Knouse et al., 2022). Likewise, females exhibited higher mGluR5 and NR1 expression (Wang et al., 2015). Together, this indicates greater synaptic AMPA subunit expression at the synapse, potentially contributing to spine size and heightened glutamatergic transmission in females. Another distinct factor in morphological indications of glutamatergic transmission is dendritic branching. Males have a higher mean total branches and apical dendrites in layer 2/3 pyramidal neurons (Kolb & Stewart, 1991). However, this study is dated, and no study has replicated these findings. Taken together, it is possible that males and female respond differently to presynaptic input in the PFC. Females contain larger spines indicating higher synaptic strength and males may maintain a higher level of dendritic arborization, potentially serving as a homeostatic mechanism to execute similar levels of glutamatergic transmission in this region. More work needs to be done to replicate findings suggesting sex differences in dendritic branching in this region.

Functional sex differences in glutamatergic transmission in the PFC are less clear. Many neuronal properties such as resting membrane potential, rheobase, and maturational trajectories of current-voltage relationships are the same in male and female animals, indicating similar levels of glutamate signaling (Bernabeu et al., 2020; Urban & Valentino, 2017). Likewise, no sex differences in input/output relationship are reported (Bernabeu et al., 2020). This suggests that on many levels, males and females share similar basal states

and respond to stimulation in the same manner. Measurements of synaptic strength are mixed. Females exhibit heightened spontaneous excitatory postsynaptic current (sEPSC) frequency, amplitude, and rectification index in comparison to male animals (Knouse et al., 2022). However, contradictory evidence shows higher sEPSC and mEPSC frequency in males with no differences in amplitude (Pena-Bravo et al., 2019; Urban & Valentino, 2017). Conflicting evidence may be due to differences between prelimbic and infralimbic subdivisions in the PFC and differences in methodological approach.

Similarly to the nucleus accumbens, few studies have investigated sex differences in synaptic plasticity in the PFC. A study probing the prelimbic subregion of the PFC found no sex difference in LTP and LTD. The lack of sex differences in this study was found to be related to convergent mechanisms. When probed deeper, it was revealed that a different set of receptors were employed between the sexes to produce similar LTD, suggesting sexually dimorphic mechanisms at play (Bernabeu et al., 2020). It is likely that this is true for many regions throughout the brain as steroid hormones are known neuroplasticity modulators. However, more studies evaluating the role of biological sex on synaptic plasticity are needed.

In the PFC, subtle sex differences in neuronal morphology serve as a prime example of how males and female may vary in mechanism but produce similar output. While females have larger spines in the PFC, it is possible that males have more dendritic branching, although this finding has yet to have been replicated. Functional studies utilizing slice electrophysiology are mixed. It is possible that at a resting state, functional outputs are similar, but females may have the machinery in place to have more flexibility than males and more readily response to changes in stimuli due to morphological

differences. The connection between the PFC is heavily involved in many aspects of the drug addiction cycle and is related to both reward-seeking and impulsivity (Perry et al., 2011). Understanding the baseline sex differences in this region may be a critical component in the proper treatment of the underlying pathophysiology of SUD.

### *Amygdala*

The amygdala is the central region associated with emotional regulation and plays a major role in the mesolimbic reward pathway. Unlike the nucleus accumbens and PFC, the influence of sex is less clear. This lack of clarity may be due, in part, to the many distinct nuclei within the amygdala which may not all contain the same morphological features. Males contain a higher number of dendritic shaft synapses in the amygdala compared to females in the medial nucleus (Nishizuka & Arai, 1981). Similarly, males have a higher spine density than females in the basolateral amygdala (BLA) (Rubinow et al., 2009). However, contradictory evidence suggests that females have more presynaptic sites than males in the central, basolateral, and medial amygdala compared to males (Carvalho-Netto et al., 2011). Likewise, females contain more spines in the basal and lateral nucleus and have a higher density of GluR1 expression in the lateral amygdala compared to males (Blume et al., 2017; Carvalho-Netto et al., 2011). The studies that conclude females to have heightened glutamatergic transmission are more compelling due to technical aspects and a wider array of methods and nuclei under analysis. Although there is stronger evidence for heightened glutamatergic transmission in females, no sex differences in the number of neurons in the amygdala or amount of dendritic branching between the sexes (Arpini et al., 2010; Gass & Foster Olive, 2008; Pastor & Medina, 2021; Pena-Bravo et al., 2019). Altogether, inconsistencies across studies may instead suggest differing

underlying mechanisms maintaining glutamatergic transmission in this region, potentially due to the heterogeneity of cells in the amygdala. More detailed work including both sexes throughout the amygdala is needed to better understand this region.

Unfortunately, very little work has been done to understand sex differences in physiology within the amygdala. A whole-cell patch clamp electrophysiology study reports higher firing rate and higher mEPSC frequency and amplitude in female animals compared to male animals in the BLA (Blume et al., 2017). This study also reported female tissue used glutamate more effectively than males when using iontophoretic glutamate application techniques (Blume et al., 2017). However, the opposite was found in the posterior division of the medial nucleus of the amygdala, showing that males had a higher firing rate than females (Dalpian et al., 2019). While the BLA contributes to reward, the medial division of the amygdala is involved in copulatory behaviors (Kondo, 1992). Here, differential levels of excitatory transmission are expected due to necessary sex specific copulatory behaviors. However, not all electrophysiological studies have revealed sex differences, Corbett et al reports no difference between male and female animals in sEPSC frequency and sEPSC amplitude (Corbett et al., 2023). Likewise, no differences in passive membrane properties of these cells were found between the sexes (Dalpian et al., 2019). Perhaps, differing nuclei containing stronger or weaker connectivity may balance one another and lead to overall similar glutamatergic transmission across the region. This would suggest that males and females have similar overall functional output but have differing patterns of connectivity that lead to sex specific behavioral outputs.

Few studies have looked at sex differences in synaptic plasticity in the amygdala. Synaptic plasticity in the BLA plays a role in rodent behaviors such as freezing and

components of fear conditioning (Maren & Fanselow, 1995). Female rats exhibit heightened levels of both cued fear freezing behavior and LTP in the LA. The lower levels of LTP and freezing are mediated by testosterone, whereas ovarian hormones are loosely associated with heightened LTP and freezing in females (L.-S. Chen et al., 2014). However, this is the only study found to investigate sex differences in synaptic plasticity, and similarly to other regions, more is needed to understand the differences in plasticity and the potential contribution to differences in behavior.

The amygdala plays a key role in integrating information from various sources and contributes to emotion, learning and memory, reward, and motivation (Murray, 2007). Hyperactivity in the amygdala is linked to susceptibility of stress-related psychiatric disorders due to the close parallel regulation of the hypothalamic pituitary adrenal (HPA) axis. Stress related psychiatric disorders are more prevalent in women. Many different functional sex differences have been examined in the amygdala in human populations. For example, women have enhanced amygdala responding during aversive stimuli and exhibit more negative emotions (Domes et al., 2010; Stevens & Hamann, 2012). This may be partially explained by heightened glutamatergic transmission in females and greater synaptic plasticity. However, this is based on very few studies that do not all align with this conclusion. Additionally, sex differences men and women could be explained by differences in areas of recruitment during the evaluation of emotional stimuli, and less so explained by baseline sex differences in glutamatergic transmission and plasticity.

### *Hippocampus*

Decades of research has provided substantial information on hippocampal function and circuitry (Amaral & Witter, 1989; Chauhan et al., 2021). Clear differences have

emerged in glutamatergic tone between males and females. Females have a greater spine density in the CA1 region and higher expression of glutamate receptor subunits such as mGluR2, mGluR3, mGluR5, NR2B compared to males (*Y. Wang et al., 2015*). Females also have larger spines along dendrites of pyramidal neurons and higher levels of NMDA1 and NMDAR2 receptor expression (*Brandt et al., 2020*). However, no differences were found in the total number of synapses between mossy fibers and apical dendrites (*Madeira et al., 1991*). In opposition to the sex difference in spine morphology, male neurons in the CA3 region have longer dendrites and more dendritic volume than females (*Isgor & Sengelaub, 2003*). Together, this suggests although females may have a higher level of structural connectivity and glutamatergic transmission and that males may compensate this difference by having more advanced dendritic arborization in the CA1 and CA3 subregions. In other subregions of the hippocampus, no sex differences are found. A study looking specifically at the dentate gyrus found no sex differences in spine volume or density (*Niiyama et al., 2020*), an effect that may be specific to the dentate gyrus. Therefore, sex differences within the hippocampus in spine morphology and glutamatergic transmission may be subregion specific.

The sex differences in electrophysiology properties of glutamatergic neurons in the hippocampus suggest greater transmission in male animals. Males exhibited a higher input-output curve in response to increasing stimulus intensity while also showing greater activity at excitatory synapses than females (*Harte-Hargrove et al., 2015; Sertel et al., 2021*). This robust effect can be seen in fEPSC slope, amplitude, and parallels a sex difference in NMDA receptor activation and more efficient use of vesicle pool recycling and stronger local translation at the synapse (*Maren, 1995; Monfort et al., 2015*). However,

not all neuronal properties are stronger in males. Under basal conditions, females showed larger AMPA receptor-mediated synaptic response (Monfort et al., 2015), and in cultured neurons, females presynaptic terminals exhibited a higher number of synaptic vesicles compared to males (Kim et al., 2023). Here, no sex differences were found in presynaptic protein expression, vesicle endo- or exocytosis, or presynaptic calcium alternation (Kim et al., 2023). Although not all parameters of glutamatergic transmission are greater in males, most studies suggest heightened glutamatergic transmission in male rodents.

The hippocampus was the first region used to study synaptic plasticity, with major focus in delineating mechanisms for learning and storage of memory (Avshalumov & Mandyam, 2021). To date, much of this work was exclusively done in male animals. With the recent inclusion of female animals, it is clear that sex differences in synaptic plasticity exist. Across varying electrophysiology protocols, male animals exhibit a higher magnitude of LTP in the CA1 region and the perforant pathway-dentate gyrus synapse (Maren, 1995; Monfort et al., 2015; Safari et al., 2021; Sertel et al., 2021). Likewise, male animals can respond to a broader range of tetanic stimuli for the induction of LTP compared to females. However, further investigation studying mechanisms underlying this plasticity are sexually dimorphic. Females and males have differences in mechanisms and thresholds for field CA1 LTP, with different kinase activation and NMDA receptor association (W. Wang et al., 2018). LTP in males requires NMDARs while LTP in females occurs independent to NMDAR activation. Additionally, estradiol induced LTP recruits a different set of kinases between the sexes, with only females requiring cAMP-activated protein kinases. Females were found to utilize both L-type calcium channels and internal calcium stores whereas in males, either resource is sufficient to permit potentiation (Jain et

al., 2019). These studies are some of the first to show mechanistically different processes that produce the same endpoint. It is possible therefore that many regions may share these sexually dimorphic synaptic plasticity mechanisms that have yet to be determined.

#### *Non-Reward-Related Regions*

Very few regions of the brain aside from the mesolimbic circuitry have been investigated for sex differences in glutamate transmission and plasticity. The hypothalamus is the most well documented due to its involvement in endocrine and autonomic nervous systems that regulate reproductive behaviors, appetite, motivational states, energy balance and circadian rhythms (Schröder et al., 2020). Clear sex differences in this region have been found, specifically relating to the larger size of the sexually dimorphic nucleus of the preoptic area (SDN-POA) in males compared to females (Gorski et al., 1978). However, subtle sex differences in spine morphology can be found throughout the hypothalamus (van den Pol & Trombley, 1993). Male animals contain larger and more synaptically dense hypothalamic nuclei than females. The ventral-medial hypothalamus in male rodents is approximately 1.25 times larger than females (Dugger et al., 2007; Dulce Madeira et al., 2001). Likewise, males display a higher number of axo-spinous synapses and spine density in the ventral-lateral and ventromedial hypothalamus (Larriva-Sahd et al., 1995; Matsumoto & Arai, 2008; Pozzo Miller & Aoki, 1991), with only one study reporting the opposite finding (Dulce Madeira et al., 2001). Higher spine density and more synaptic connections suggests higher glutamatergic transmission in males in the hypothalamus. However, this may not be uniform across all subregions in the hypothalamus, for example females exhibited higher presynaptic markers in the paraventricular nucleus of the

hypothalamus (Carvalho-Netto et al., 2011). Thus far, no work has been done to delineate sex differences in synaptic plasticity within the hypothalamus.

Concerning other non-reward related regions, females report lower spine density in the visual cortex due to fewer stubby and mushroom spines (Parker et al., 2020). Similarly, young adult male rats display a higher dendritic tree and spine density in the anterior cingulate cortex in comparison to females (Markham & Juraska, 2002). Although limited, it is possible that in non-reward related regions, there is a pattern higher glutamatergic transmission in females, evidenced by higher structural plasticity in these regions. Studies utilizing electrophysiology in cortical regions to understand functional differences in synaptic plasticity generally report a lack of robust sex differences. No sex differences were found in the magnitude of LTP within the anterior cingulate cortex or the recruitment of silent synapses (Liu et al., 2020). Likewise, males and female neurons do not differ in active or passive membrane properties in the insular cortex (Iezzi et al., 2023). Although there are no differences in measures of LTP were found in the anterior cingulate cortex, LFS was more likely to induce LTD in male mice in comparison to females. This subtle difference implies a sex difference in network plasticity (Iezzi et al., 2023). This work highlights that although males exhibit higher baseline structural plasticity markers, most neuronal properties are similar in males and females throughout these regions. More work is needed to investigate properties of glutamatergic transmission that may differ between the sexes.

Even more so than the amygdala, the hypothalamus is part of the HPA axis and extensive literature has found sex differences this region in response to stress (Heck & Handa, 2019). These sex differences exist in hormonal responses to stress, in feedback

mechanisms, and stress induced receptor mobilization. Varying neuroendocrine responses contribute to sex differences in stress-related psychiatric disorders. Less work has been done to elucidate the influence of biological sex on glutamatergic transmission and how these differences may contribute to the overall sex differences in behavior. The trend towards heightened glutamatergic transmission in males is also true for non-hypothalamic regions and may continue to be a pattern elsewhere in the brain. However, limited studies have looked into the functional consequences for differences in glutamatergic transmission in these regions.

### Conclusion

Here, we synthesized data on glutamatergic transmission and synaptic plasticity from male and female rodents, both within the mesolimbic reward system and non-reward related brain regions to understand the influence of biological sex on reward related circuitry. We found that sex differences in glutamatergic transmission and synaptic plasticity are not uniform across the brain or even within the reward circuit. Instead, evidence for convergent and divergent mechanisms underlying these differences exists between the sexes. Although the limited availability of data on synaptic plasticity throughout the brain makes it difficult to capture the complete picture, glutamate signaling differences exist in many regions. In some cases, the differences may “cancel each other out” leading to similar levels of glutamate transmission (functional convergence) whereas others may result in sex differences (functional divergence; Figure 1).

In this first category, the functional convergence often results from mechanistic sex differences that produce similar levels of synaptic plasticity across biological sex. This may be the case for many regions beyond those discussed here but there is a dearth of published

work examining these potential convergent mechanisms. The PFC falls into this category with similar levels of glutamatergic transmission across biological sex that exist on a background of sex differences within the glutamate signaling system. While females exhibit a higher number of dendritic spines, males recruit more efficient receptors, possibly contributing to similar levels of LTP and LTD. This serves as a prime example of a region that maintains consistent levels of glutamatergic signaling via different mechanisms. The PFC plays a central role in an individual's experience of reward-related disorders such as SUD, largely controlling impulsive, motivated, and relapse behaviors (Renard et al., 2017). When developing treatments, subtle differences in glutamatergic transmission mechanisms between the sexes are paramount. Although the region may display similar levels of glutamate transmission, targeting a specific aspect may necessarily have to be sex specific. Continuing to investigate the mechanisms underlying similar levels of synaptic plasticity between the sexes is therefore critical to the ongoing development of neurological treatments.

Unlike the PFC, which may display converging mechanisms and similar levels of glutamatergic transmission between the sexes, other regions such as the hippocampus and the nucleus accumbens exhibit sexually dimorphic mechanisms. The nucleus accumbens shows higher glutamatergic transmission and synaptic plasticity in females. This true baseline sex difference is the strongest out of the regions discussed and therefore should be considered heavily when translating findings to human populations. The hippocampus similarly exhibits sexually dimorphic mechanisms of glutamatergic transmission and synaptic plasticity that instead favors male animals. As this region is well studied for physiological properties, divergence in mechanism can be attributed partially due to

hormonal modulation and differential receptor recruitment. Continuing to delineate mechanistic differences in glutamate transmission within both the nucleus accumbens and the hippocampus may provide insight to sex specific treatments or understanding of specific vulnerability.

Still other regions of the brain have limited research on the influence of biological sex in glutamatergic signaling. While the hypothalamus and other regions display evidence for morphological markers of higher synaptic plasticity in males, there is a lack of functional data to support this claim. Lack of clear sex differences in these regions may represent little to no influence of biological sex on glutamatergic transmission. Discrepancies in sub regions or in methodological approaches may contribute to inconsistencies in reports. However, literature purposefully comparing male and female animals on levels of synaptic plasticity across the brain are sparse. In combination across all regions discussed, articles containing direct comparison of males and females on levels of structural analysis included only 30 articles; functional measures of glutamatergic transmission were limited to 20 articles. Further, when considering a major mechanism of synaptic plasticity, LTP, only 10 articles discuss sex differences. Uncovering sex differences in these key features of glutamate transmission and synaptic plasticity is crucial for the understanding of the brain across reward and non-reward related regions. Therefore, it is clear there is much more work to be done in this space to fully elucidate the influence of biological sex on glutamate transmission and synaptic plasticity throughout the brain.

Understanding sex differences in baseline levels of glutamatergic transmission may be critical for the development of treatments that target this system. For example, chronic stress may lead to cases of depression and anxiety in which there are alterations in synaptic

plasticity. In the PFC and hippocampus, this results in deficits in LTP and facilitation in LTD (Marsden, 2013). To treat males and females with the highest efficacy, understanding the different mechanism that may be underlying baseline synaptic plasticity is necessary. With the increasing specificity of drug development, it may be possible to target region specific transmission and therefore, mechanistic differences between males and females will need to be understood. Likewise, treatments that may target overall glutamatergic transmission may need to consider baseline differences between males and females. A general increase in glutamate function may have drastically different effects between the sexes and have differential associated risks due to different starting points. However, by continuing to investigate the glutamatergic system and biological sex differences, we can attempt to identify targets that may benefit both men and women.

Reward-related	Synaptic structure	Synaptic strength	Synaptic plasticity
Nucleus accumbens	♀ > ♂	♀ > ♂	♂ ↑ LTD
Prefrontal cortex	♀ > ♂ Spine density    ♀ < ♂ Branching	Mixed Evidence	♀ = ♂
Amygdala	♀ > ♂	-	♀ ↑ LTP
Hippocampus	♀ > ♂ Spine density    ♀ < ♂ Branching	♀ < ♂	♂ ↑ LTP
Non-reward related	Synaptic structure	Synaptic strength	Synaptic plasticity
Hypothalamus & other cortices	♀ < ♂	-	♀ = ♂

Figure 1. Summary table representing sex differences in synaptic structure, strength, and plasticity in reward related and non-reward related regions in male and female rodents.

## CHAPTER 2

# PKM $\zeta$ ALTERS OXYCODONE-TAKING IN A DOSE- AND SEX-SPECIFIC MANNER

### Introduction

Altered glutamate transmission underlies many aspects of the substance use disorder (SUD) cycle, with disruptions to glutamate homeostasis driving relapse (Gardner, 2011; Hearing, 2019; Knackstedt & Kalivas, 2009). Trafficking of glutamatergic AMPA receptors (AMPA) specifically underlies learning and drug use (Malenka, 2003; Malinow & Malenka, 2002). PKM $\zeta$ , an atypical isoform of Protein Kinase C (PKC), is an AMPAR trafficking protein. It potentiates N-ethylmaleimide-sensitive factor (NSF)-mediated insertion of GluA2-containing AMPARs to the cell membrane (Yao et al., 2008a). This makes PKM $\zeta$  an interesting target for studies on the synaptic plasticity underlying learning, memory, and drug use.

Early studies demonstrated a role for PKM $\zeta$  in long-term potentiation (LTP). PKM $\zeta$  levels increase during and after LTP induction and it was proposed to be sufficient to maintain LTP (Hsieh et al., 2021; Ling et al., 2002; Sacktor et al., 1993). In learning and memory, PKM $\zeta$  levels increase following spatial conditioning and during memory reconsolidation (Bernabo et al., 2021; Hsieh et al., 2021). Further, an infusion of zeta inhibitory peptide (ZIP), a proposed PKM $\zeta$  inhibitor, into the hippocampus prevented rats from exhibiting a previously-learned fear response (Pastalkova et al., 2006). Relevant to substance use, ZIP was also shown to block morphine conditioned place preference (Y. Li et al., 2011). Altogether, these studies demonstrated a role for PKM $\zeta$  in memory formation, memory preservation, and learning.

Recently, the specificity of ZIP has been called into question. It likely targets both PKM $\zeta$  and another atypical PKC isoform, PKC $\delta/\lambda$ . PKM $\zeta$  knockout mice exhibit normal learning, memory, and LTP and ZIP still induces learning and memory deficits in these animals (Lee et al., 2014; Volk et al., 2013). This makes studies that utilized ZIP to block PKM $\zeta$  difficult to interpret. These findings could indicate PKM $\zeta$  is not involved in LTP, learning, and memory. Another explanation, however, is that there are compensatory mechanisms at play in PKM $\zeta$  knockout mice. As evidence of this, PKC $\delta/\lambda$  is recruited for LTP in animals that lack PKM $\zeta$  (Tsokas et al., 2016a). Regardless of the controversy surrounding ZIP, PKM $\zeta$  does alter spine density.

There is evidence for this as PKM $\zeta$  knockout increases ethanol consumption in an intermittent access paradigm in male mice (Lee et al., 2014). We previously extended these findings to cocaine where we demonstrated PKM $\zeta$  knockout potentiates cocaine taking and seeking in both male and female mice (McGrath et al., 2018a). Together, these studies indicate PKM $\zeta$  works to dampen drug reward. Cocaine craving is promoted by changes in spine density within the nucleus accumbens core (Lüscher & Bellone, 2008). As opioids and cocaine generally have opposing effects on spine density in the nucleus accumbens (Robinson et al., 2002; Robinson & Kolb, 1999), and PKM $\zeta$  plays a critical role in spine maturation (Hernández et al., 2014; Ron et al., 2012), we were interested in exploring how PKM $\zeta$  knockout may alter opioid-taking behaviors.

Here, we examined the role of PKM $\zeta$  in oxycodone-taking, motivation, and locomotor sensitization in response to oxycodone. We tested two doses of oxycodone self-administration and used a progressive ratio paradigm to further our understanding of PKM $\zeta$ 's role in opioid use. We found that, similarly to cocaine experiments, PKM $\zeta$

knockout potentiates oxycodone self-administration at the moderate dose. Interestingly, we see a sex-specific effect at a low dose oxycodone. Likewise, we found that PKM $\zeta$  has a sex-specific effect on oxycodone induced locomotion and sensitization. These data indicate that while PKM $\zeta$  does work to blunt opioid-taking and seeking, its role in these processes is sex-specific. This highlights the possibility that while PKM $\zeta$  does dampen drug reward across multiple drug classes, the sensitivity may differ across sex.

## Methods

### *Subjects*

The current study utilized male and female PKM $\zeta$  knockout (KO) mice as described previously (Volk et al., 2013). Heterozygous PKM $\zeta$  KO mice on a C57BL/6J background were mated resulting in mutant and wildtype littermates. After weaning, tail snips were taken and genotyped using quantitative polymerase chain reaction (qPCR). Mice (2–6 months old, age matched across group) were group housed until the start of the behavioral experiments at which point they were individually housed. All animals were housed in a temperature and humidity-controlled animal care facility with a 12 h reverse light/dark cycle (lights on a 7:00 p.m.). All procedures were approved by the Temple University Animal Care and Use Committee.

### *Drugs*

Oxycodone was obtained from the National Institutes of Drug Abuse Drug Supply Program (Bethesda, MD) and dissolved in sterile 0.9% saline.

### *Jugular Catheterization Surgery*

Prior to surgery, mice were anesthetized with 80mg/kg ketamine and 12 mg/kg xylazine. An indwelling silastic catheter was placed into the right jugular vein and sutured

in place. The catheter was then threaded subcutaneously over the shoulder blade and was routed to a backmount platform (Instech Laboratories, Inc.) that secured the placement. Catheters were flushed daily with 0.1ml of an antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline. Mice recovered in their home cage 3 days prior to the start of our experiments.

### *Operant Sucrose Training*

Following catheterization, mice were trained to perform an operant response for sucrose pellets. The mice were placed in operant chambers (Med-Associates) and trained to press a lever to receive a sucrose pellet. Mice performed 2 days of fixed ratio 1 (FR1) responding where only the active lever was available. A compound cue stimulus consisting of a cue light above the active lever, a 2900-Hz tone, and house light off was concurrent with each pellet administration, followed by an additional 8 s time-out when responding had no programmed consequences and the house light remained off. Mice were allowed to self-administer a maximum of 50 pellets per 60 min operant session. The mice were food restricted to approximately 90% of their free feeding weight throughout the course of the operant training. They returned to ad libitum feeding 6 days into oxycodone self-administration.

### *Oxycodone Self-Administration*

Oxycodone self-administration was measured over 2 h sessions (7 days per week) in the same chamber used for operant sucrose training. Mice were trained to self-administer oxycodone for 3 days using an active and inactive wheel. Following the training phase, the active and inactive wheels were replaced with levers and mice continued oxycodone self-administration for 12 days on a fixed ratio 1 (FR1) schedule. Throughout all of oxycodone

self-administration, responding on the active manipulandum delivered an intravenous oxycodone injection (0.25 or 0.125 mg/kg/infusion) paired with the same cues as food training. Following the self-administration phase, mice were tested for 1 day on a progressive ratio (PR) schedule, where the response requirement for each infusion increased until the subject did not fulfill the requirement. The response requirement was defined as  $R(i)=[5e0.15i-5]$  and the session ended if the animal took longer than 30 min to meet the requirement. The breakpoint is defined as the final ratio completed.

#### *Oxycodone Sensitization*

Mice were tested for 5 consecutive days in a white plastic arena (35 x 35 x 24cm) under red light conditions (8.4-9.5lx). Each day animals were first placed in the chamber for 30 minutes to habituate. Directly following habituation, mice were given an injection of oxycodone (5mg/kg, i.p.) and placed back in the chamber. Locomotor activity was recorded for an additional 60 minutes following the injection. Total distance travelled was tracked during habituation and testing using the ANY-Maze Video Tracking System (Stoelting Co., Version 7.4).

#### *Data Analysis*

All analyses were performed using GraphPad Prism 10 software (GraphPad Software). Self-administration data were analyzed using two-way ANOVA with Sidak's post hoc tests or linear mixed-effects models (Verbeke and Molenberghs, 2000) as appropriate. Oxycodone behavioral sensitization data were analyzed using a repeated measures three-way (raw locomotor activity) and two-way ANOVAs (following area under the curve calculations). Statistical significance for all tests was set at  $\alpha= 0.05$ .

## Results

### *PKM $\zeta$ Knockout Potentiates 0.25 mg/kg/inf Oxycodone Self-Administration in Both Sexes*

Wildtype and PKM $\zeta$  knockout mice self-administered 0.25 mg/kg/infusion oxycodone for 12 days. Both male and female PKM $\zeta$  knockout mice earned more infusions [effect of genotype; females:  $F(1,41)=5.64$ ,  $p=.022$ , Fig. 2A,  $n=8$ /group); males:  $F(1,42)=18.4$ ,  $p<.001$ ; Fig. 2B,  $n=8-10$ /group]. Males responded more for oxycodone [effect of genotype: males:  $F(1,42)=7.81$ ,  $p=.008$ ; Fig. 2D] and had significantly lower inactive presses [effect of genotype;  $F(1,42)=5.80$ ,  $p=.021$ ; Fig 2F] than wildtype conspecifics. There was no effect of PKM $\zeta$  knockout in females on either active responding [effect of genotype;  $F(1,41)=3.61$ ,  $p=.065$ ; Fig 2C] or inactive responding [effect of genotype;  $F(1,41)=.33$ ,  $p=.57$ ; Fig 2E].

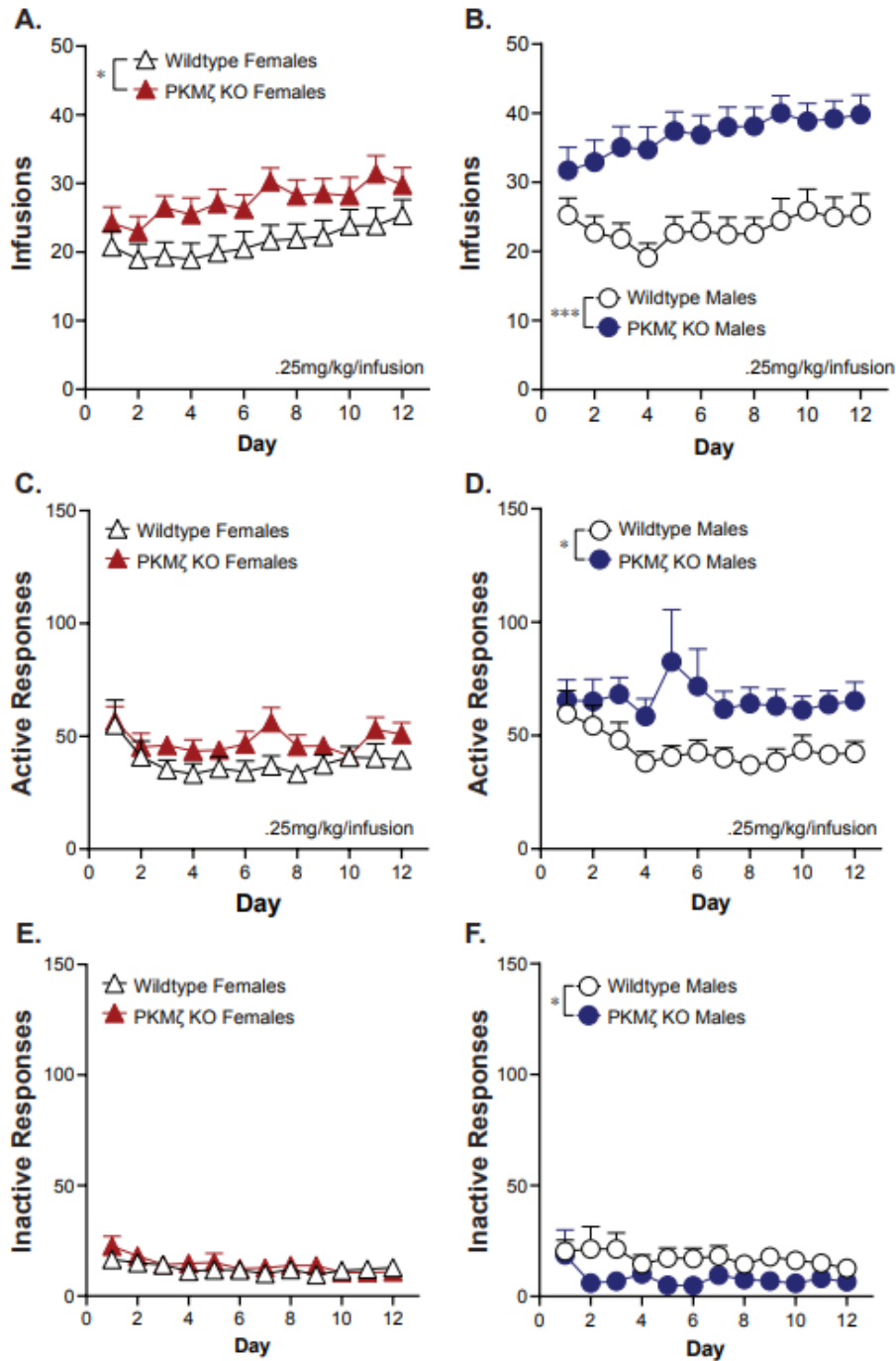


Figure 2. Effects of constitutive PKMζ deletion on oxycodone self-administration. PKMζ deletion leads to an increase in the number of oxycodone infusions earned in both females and males. Male PKMζ knockout mice also exhibit an increase in the number of active responses across 12-days of oxycodone self-administration (D), effect that is not seen in female PKMζ knockout mice (C). While there were no significant effects of PKMζ knockout on inactive responding in females (E), there was a decrease in inactive responding in male knockout mice (F). \* $p < .05$ , \*\*\* $p < .001$  main effect of genotype.

*PKMζ Knockout Alters Motivation for 0.25 mg/kg/inf Oxycodone in Males and Females*

We next examined whether PKMζ knockout affects motivation to obtain oxycodone as measured by a progressive ratio paradigm. At this dose of oxycodone, we found a main effect of genotype, indicating that PKMζ knockout led to higher breakpoints [ $F(1,30)=7.70, p=.009$ ; Fig. 3].

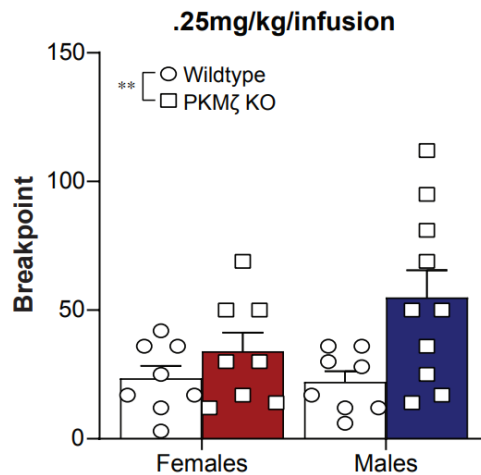


Figure 3. Effects of constitutive PKMζ deletion on motivation for oxycodone. PKMζ deletion leads to an increase in breakpoint for oxycodone at the 0.25mg/kg/inf dose in male and female animals. \*\* $p < .01$  main effect of genotype.

*PKMζ Knockout Potentiates 0.125 mg/kg/inf Oxycodone Self-Administration Exclusively in Female Animals*

Wildtype and PKMζ knockout mice self-administered 0.125 mg/kg/inf oxycodone for 12 days. Female PKMζ knockout animals earned more infusions [effect of genotype;  $F(1,16)=7.23, p=.016$ ; Fig. 4A] and responded significantly more for oxycodone [effect of genotype;  $F(1,16)=9.039, p=.008$ ; Fig. 4C] than wildtype conspecifics. In contrast, while male PKMζ knockout animals exhibit a trend towards a higher number of infusions [ $F(1,15)=4.28, p=.056$ ; Fig. 4B], they did not exhibit a higher number of active responses compared to wildtype conspecifics [ $F(1,15)=1.013, p=.33$ , Fig. 4D]. There was no

significant effect of genotype on inactive lever responding [females:  $F(1,16)=4.18, p=.057$ ; Fig. 4E; males:  $F(1,15)=0.037, p=.85$ ; Fig. 4F].

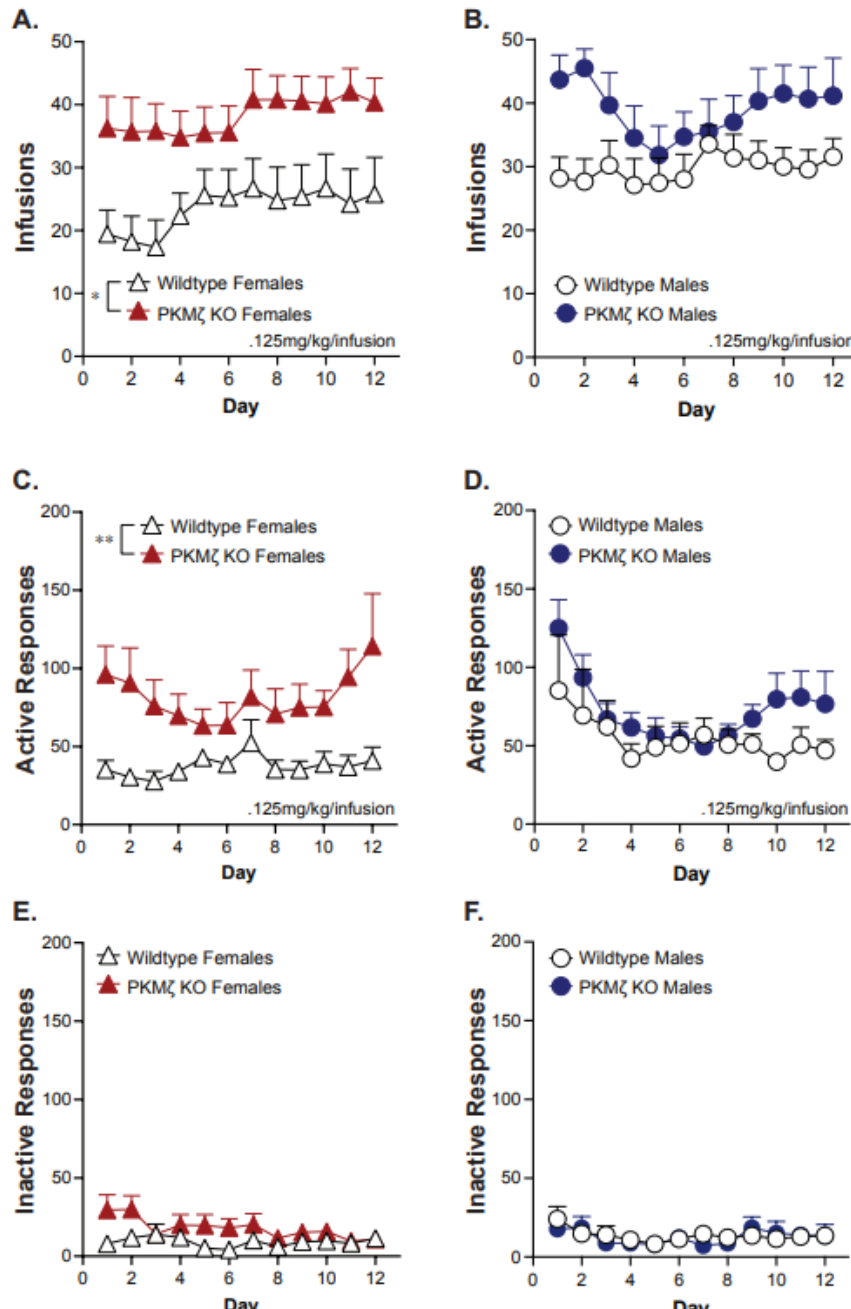


Figure 4. Effects of constitutive PKMζ deletion on oxycodone self-administration at low dose. Constitutive PKMζ deletion leads to an increase in the number of oxycodone infusions earned in females (A;  $n=9$ /group) but not males (B;  $n=6-11$ /group). Female PKMζ knockout mice also exhibit an increase in the number of active responses across 12-days of oxycodone self-administration (C), effect that is not seen in male PKMζ knockout mice (D). There were no significant effects of PKMζ knockout on inactive responding in either females (E) or males (F). \* $p<.05$ , \*\* $p<.01$  main effect of genotype.

*PKMζ Knockout Increases Motivation for 0.125 mg/kg/inf Oxycodone Exclusively in Female Animals*

When we examined responding for this dose of oxycodone on a progressive ratio schedule, we found that PKMζ deletion led to an increase in breakpoint in female mice but not males [effect of genotype:  $F(1,31)=8.56$ ,  $p=.006$ ; effect of sex:  $F(1,31)=2.35$ ,  $p=.14$ , interaction:  $F(1,31)=6.46$ ,  $p=0.016$ ; Sidak post-hoc: wildtype females vs. PKMζ KO females,  $t(31)=4.02$ ,  $p<.0007$ ; Fig. 5].

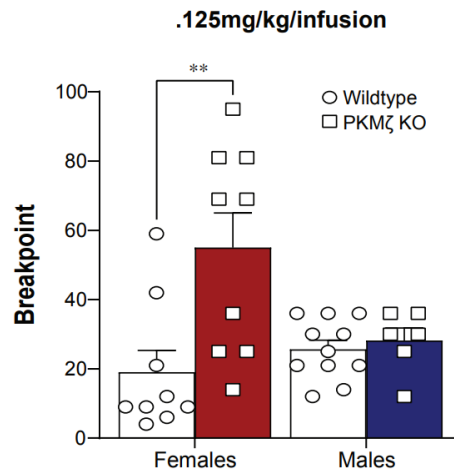


Figure 5. Constitutive PKMζ deletion leads to an increase in the breakpoint for oxycodone at the 0.125 mg/kg/inf dose in female, but not male, mice. \*\* $p<.01$ , pairwise comparison wildtype females vs. PKMζ knockout females.

*PKMζ Knockout Alters Oxycodone-Induced Locomotion and Sensitization in a Sex-Specific Manner*

When we examined the impact of PKMζ deletion on the locomotor response to a novel environment during the habituation phase of the sensitization experiment, we did not see any effect of genotype or sex on locomotor activity on day 1 of habituation, prior to any oxycodone administration (3-way ANOVA: effect of genotype:  $F(1,24)=0.0003$ ,  $p=.99$ ; effect of sex:  $F(1,24)=3.36$ ,  $p=.08$ ). Examining the area under the curve over the five daily habituation sessions, we did not see any impact of genotype over time in either

the male mice or the female mice [males: 2-way ANOVA: effect of genotype:  $F(1,60)=0.03$ ,  $p=.87$ ; Fig. 6A; females: 2-way ANOVA: effect of genotype:  $F(1,60)=2.10$ ,  $p=.15$ ; Fig. 6B].

We next assessed oxycodone-induced locomotor activity by measuring distance traveled in an open field for 60 minutes. Data was binned in 5-minute intervals. On days 1 and 2, along with the main effect of time, we found a significant time x genotype interaction [Day1, Fig 6C: effect of time:  $F(2.298,55.15)=40.9$ ,  $p<.0001$ ; time x genotype interaction:  $F(11,264)=2.04$ ,  $p=.025$ ; Day 2, Fig. 6D: effect of time:  $F(3.080,73.92)=44.1$ ,  $p<.0001$ ; time x genotype interaction:  $F(11,264)=2.77$ ,  $p=.002$ ]. On day 3, we detected an effect of time, sex and significant interactions between time and genotype, time and sex, and genotype and sex [Day 3, Fig. 6E: effect of time:  $F(3.433,82.38)=31.2$ ,  $p<.0001$ , effect of sex:  $F(1,24)=7.18$ ,  $p=.013$ ; time x genotype interaction:  $F(11,264)=3.89$ ,  $p<.0001$ ; time x sex interaction:  $F(11,264)=2.46$ ,  $p=.006$ ; genotype x sex interaction:  $F(1,24)=4.27$ ,  $p=.049$ ]. On day 4, there was only a main effect of time on distance travelled [Day 4, Fig. 6F: effect of time:  $F(2.834,68.02)=24.6$ ,  $p<.0001$ ]. Finally, on day 5 we detected significant effects of time along with genotype by sex and time x genotype x sex interactions [Day 5, Fig. 6G: effect of time:  $F(2.456,58.95)=9.97$ ,  $p<.0001$ ; genotype x sex interaction:  $F(1,24)=9.66$ ,  $p=.0048$ ; time x genotype x sex interaction:  $F(11,264)=2.31$ ,  $p=.010$ ].

To further characterize these complicated interactions, we examined the area under the curve during the 60min following oxycodone injection over the course of the 5 daily injections. We found that PKM $\zeta$  deletion led to an overall decrease in oxycodone-induced locomotor activity in male mice [effect of genotype:  $F(1,60)=22.44$ ,  $p<.001$ ; Fig. 6H]. In contrast, PKM $\zeta$  deletion in females potentiated the locomotor response to oxycodone over

time [effect of genotype:  $F(1,60)=9.16$ ,  $p=.004$ ; effect of day:  $F(4,60)=0.32$ ,  $p=.87$ ; interaction:  $F(4,60)=1.69$ ,  $p=.17$ ; Fig. 6I].

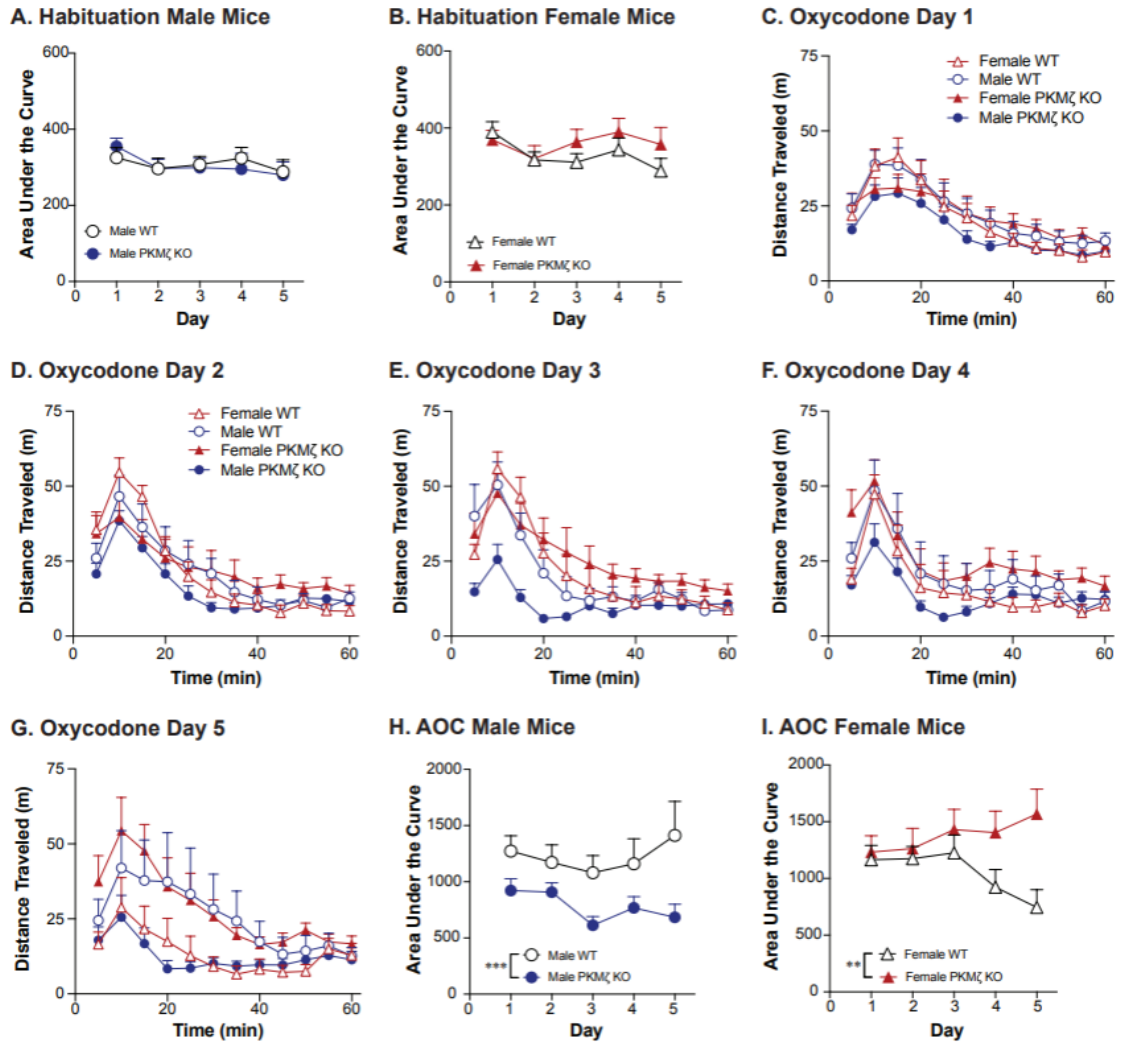


Figure 6. Constitutive PKM $\zeta$  deletion does not alter the locomotor response to the testing chamber during the habituation phase prior to oxycodone injection. Shown for both male (A) or female (B) mice ( $n=6-8$ /group). For 60 min following each daily oxycodone injections (5 mg/kg, i.p.) we measured distance travelled in meters (m) and binned the data in 5-minute intervals (C-G). The influence of genotype on oxycodone-induced locomotor behavior varied by sex, an effect that is more clearly illustrated when we examined area under the curve across the five days. Male PKM $\zeta$  knockout mice exhibit a decrease in oxycodone-induced locomotion across all five days (H,  $***p<.001$ , effect of genotype). In contrast, female PKM $\zeta$  knockout mice exhibit greater oxycodone-induced locomotion, particularly during the final two days of injections, where the wildtype mice appear to exhibit habituation (I,  $**p<.01$ , effect of genotype).

## Discussion

Glutamatergic AMPAR activity underlies much of the SUD cycle (Malenka, 2003; Malinow & Malenka, 2002). PKM $\zeta$ , an atypical isoform of Protein Kinase C, potentiates NSF-mediated insertion of GluA2-containing AMPARs to the cell membrane (Patel & Zamani, 2021). This made PKM $\zeta$  a popular target for studies on synaptic plasticity, mainly LTP (Hsieh et al., 2021; Ling et al., 2002; Sacktor et al., 1993). Despite some controversy surrounding the role of PKM $\zeta$  in synaptic plasticity and behavior (Lee et al., 2014; Tsokas et al., 2016b; Volk et al., 2013), PKM $\zeta$  can alter dendritic spine density and is involved in cocaine and ethanol-taking behaviors (Lee et al., 2014; McGrath et al., 2018a; Ron et al., 2012). As cocaine and opioids alter spine density in opposing manners, we were interested in whether PKM $\zeta$  knockout would affect opioid self-administration in a different manner than we previously found with cocaine (Robinson et al., 2002). In these experiments we found biological sex can influence the effect of PKM $\zeta$  knockout on oxycodone-taking and motivation. Our results indicate PKM $\zeta$  works to dampen drug reward across multiple drug classes and PKM $\zeta$  may play a role in sex differences in the dose response for oxycodone.

### *PKM $\zeta$ Blunts Oxycodone-Taking and Motivation for Oxycodone in Both Sexes at a Moderate Dose of Oxycodone*

In our first experiment, we found PKM $\zeta$  knockout potentiates oxycodone self-administration and motivation in both sexes at a dose of 0.25 mg/kg/infusion. We first examined this dose as it is a moderate dose of oxycodone that is readily self-administered by mice (Zhang et al., 2009). Our data show significant potentiation in both the number of infusions earned for both sexes and the number of active responses in male PKM $\zeta$  knockout animals compared to wildtype controls across 12 days of self-administration. This is in

line with previous data showing PKM $\zeta$  works to dampen ethanol- and cocaine consumption (Lee et al., 2014; McGrath et al., 2018a). We do not see an increase in the number of responses on the inactive lever, indicating potentiated responding for oxycodone is not due to increases in perseverative responding. PKM $\zeta$  knockout animals also showed a significant increase in motivation for oxycodone, as indicated by their increased breakpoint on the progressive ratio schedule. Although not statistically significant, it is noteworthy that this effect of genotype on breakpoint was most likely driven by male animals.

PKM $\zeta$  potentiates insertion of GluA2 AMPARs to the cell membrane. Previous data show disrupting GluA2 trafficking through mechanisms other than PKM $\zeta$  alters cocaine reinstatement (Briand et al., 2014; Famous et al., 2008; Wickens et al., 2019, 2021b). Though there are fewer studies examining opioid self-administration, opioid exposure does alter GluA2 trafficking (Hearing et al., 2018). Here, our findings further the data that disrupting GluA2 trafficking alters drug-taking behaviors by demonstrating a role for PKM $\zeta$  in opioid self-administration. Further, we found the effect of PKM $\zeta$  knockout on opioid-taking is present in both sexes. The results from our first experiment highlight the ubiquitous role of GluA2 trafficking in drug use in both males and females. In combination with previous studies, these data specifically further the finding that GluA2 trafficking via PKM $\zeta$  broadly works to dampen drug reward in both sexes.

In addition to its role in GluA2 AMPAR insertion, PKM $\zeta$  may also play a role in spine morphology. PKM $\zeta$  induces a maturation of dendritic spines in cultured cortical neurons (Ron et al., 2012). Though the role of PKM $\zeta$  in spine density is still being explored, this highlights the possibility knocking out PKM $\zeta$  would lead to less stable spines. As opiates, including oxycodone, lead to decreased dendritic spine density in the nucleus

accumbens (Robinson et al., 2002), prefrontal cortex (Robinson et al., 2002), and hippocampus (Wan et al., 2023), PKM $\zeta$  knockout could potentiate this spine loss. However, cocaine and opioids affect spine density in the nucleus accumbens in opposing manners (Diana et al., 2006; Dumitriu et al., 2012; Kasture et al., 2009; Leite-Morris et al., 2014; Norrholm et al., 2003; Robinson et al., 2002, 2001; Robinson and Kolb, 1999; Spiga et al., 2005) and we found the same behavioral phenotype in PKM $\zeta$  knockout animals across both drugs. This indicates that while PKM $\zeta$  may play a role in spine maturation, its role in drug-taking may not be due explicitly to its role in spine density.

*PKM $\zeta$  Plays a Sex-Specific Role in Oxycodone-Taking and Motivation at a Low Dose of Oxycodone*

When examining responding for a lower dose of oxycodone (0.125mg/kg/infusion), we found PKM $\zeta$  knockout potentiates oxycodone self-administration and motivation exclusively in female animals. While we did not see any significant effect of sex on oxycodone-taking at the moderate dose, there are known dose-dependent sex differences in drug-taking behaviors (Becker & Hu, 2008). We chose this lower dose as it may elicit more responding and capture group differences better than higher doses (Zhang et al., 2009). We conclude PKM $\zeta$  knockout blunts opioid reward in both sexes at multiple doses. Nonetheless, we do not see any effects of genotype on either the number of infusions earned or active responding in the male animals. Our data indicate there is a sex-specific effect of PKM $\zeta$  knockout on low dose oxycodone self-administration.

We saw a similar effect with PKM $\zeta$  knockout enhancing the final breakpoint in female animals at the .125mg/kg/inf dose and male animals at the .25mg/kg/inf dose. Our current data indicate there is a role for PKM $\zeta$  in motivation to acquire oxycodone that

varies based on dose. Biological sex can modulate behavioral responses to different doses of the same drug. Females acquire drug-taking at lower doses and when given a choice will choose a higher dose of cocaine than males (Becker & Hu, 2008). Thus, varying the dose may elicit effects of sex not previously seen at other doses. PKM $\zeta$  can also be modulated by biological sex. There are sex differences in expression within the hippocampus and nucleus accumbens following drug exposure (Avila et al., 2018; McGrath et al., 2018a). This indicates that while many behaviors involving PKM $\zeta$  may present in a similar manner, PKM $\zeta$  activity may not explicitly be the same between the sexes. Previous studies demonstrate this, where constitutive PKM $\zeta$  knockout potentiates cocaine taking in both sexes but site-specific knockout in the nucleus accumbens potentiates cocaine taking exclusively in males (McGrath et al., 2018). Altogether, these data indicate that PKM $\zeta$  does work to dampen drug reward, but its role is not explicitly the same in males and females.

Oxycodone suppresses glutamatergic input in the hippocampus of male rats (Lu et al., 2020). In addition to there being region specific differences between the sexes, perhaps PKM $\zeta$  plays a differential homeostatic role in maintaining excitatory balance within learning and reward regions by stabilizing synapses. There is additional evidence that the effects of opioids on glutamatergic synapses may be sex specific. Chronic exposure to oxycodone was found to have differential effects on synaptic protein gene expression in the hippocampus of male and female rats (Randesi et al., 2019), including kinase expression. Other reward related regions may also show sex specific alterations in synaptic proteins after opiate administration. Our results show a sex-specific effect of oxycodone at the lower dose. This may be due to specific synaptic protein changes that occur after oxycodone self-administration. These mechanisms may compensate for lack of PKM $\zeta$  in

males but not females at a lower dose of oxycodone. A previously proposed mechanism suggests that PKC $\delta$ /λ may compensate for LTP deficits in the absence of PKM $\zeta$ . This was only studied in male mice and further work is needed to investigate whether these compensatory mechanisms are the same in female animals.

#### *PKM $\zeta$ Plays a Sex-Specific Role in Oxycodone-Induced Locomotion*

In our final experiment, we examined oxycodone-induced locomotion to see if PKM $\zeta$  influences either the acute locomotor response to oxycodone or locomotor sensitization over time. While the current dose of oxycodone did not produce behavioral sensitization in our wildtype mice, we did detect effects of PKM $\zeta$  on oxycodone-induced locomotion. While in male mice, PKM $\zeta$  deletion led to a decrease in oxycodone-induced locomotor behavior, in female mice we see increased sensitivity. This supports our hypothesis that PKM $\zeta$  deletion may potentiate the effects of oxycodone at lower doses in females.

#### Conclusion

Here, we found PKM $\zeta$  knockout potentiates oxycodone-taking in a dose- and sex-dependent manner. Additionally, PKM $\zeta$  has sex-specific effects on oxycodone sensitization. This adds to the literature demonstrating PKM $\zeta$  works to dampen drug-taking across multiple drug classes. Additionally, this work suggests that PKM $\zeta$  plays a differential role in homeostatic mechanisms between the sexes where females may be more sensitive to opioid induced changes in excitatory transmission. These experiments highlight the importance of including both sexes in biomedical research. Recent evidence indicates men and women do not always respond to SUD treatments in the same manner (Agabio et al., 2016; Arevalo et al., 2015; Huhn et al., 2019). Further investigation into the

neural mechanisms driving SUD in both sexes could aid in the development of more effective pharmacotherapies for males and females.

## CHAPTER 3

### PKM $\zeta$ IS NECESSARY FOR LONG-TERM DEPRESSION AND LONG- POTENTIATION IN THE MEDIAL PREFRONTAL CORTEX

#### Introduction

Post-synaptic AMPARs mediate nearly all fast excitatory neurotransmission and are composed of four types of subunits (GluA1-GluA4), which combine to form tetramers (Gan et al., 2015; Henley et al., 2011; Henley & Wilkinson, 2016; Hollmann & Heinemann, 1994; Morise et al., 2019). The dynamic alteration of both the composition and amount of AMPARs at the synapse determines the state of potentiation at the synapse (de León-López, Carretero-Rey, and Khan 2025). The atypical protein kinase C, PKM $\zeta$ , is poised to play a unique role in this trafficking due to its ability to remain constitutively active (Hernandez et al., 2003b). This is due to the lack of a regulatory N-terminal domain commonly found in other protein kinase C (PKC) isoforms, such that once the substrate is bound PKM $\zeta$  it is not removed (Hernandez et al., 2003a). PKM $\zeta$  maintains GluA2-containing AMPARs at the synaptic membrane by preventing AMPAR endocytosis and lateral diffusion (Patel & Zamani, 2021; Yao et al., 2008a).

At the synapse, AMPARs number and composition are dependent on the rate of endocytosis and exocytosis (Anggono & Huganir, 2012; Turrigiano, 2000). More specifically, GluA2-containing AMPARs have been shown to be the most associated with these changes. GluA2-containing AMPARs are typically calcium impermeable (CI-AMPARs) ion channels that make up the majority of AMPARs in mature neurons (Greger et al., 2003; Man, 2011). Generally, during long-term depression (LTD), there is enhanced AMPAR endocytosis, while during long-term potentiation (LTP), more AMPARs are

inserted or maintained at the synapse (Beattie et al., 2000; Carroll et al., 1999; Makino & Malinow, 2009). The bidirectional changes in receptor density during LTD and LTP implies that the relative activity of PKM $\zeta$  should impact both forms of plasticity.

PKM $\zeta$  knockout modulates synaptic plasticity in the nucleus accumbens (Knouse et al., 2022). Male mice genetically lacking PKM $\zeta$  exhibited blunted LTD in response to low frequency stimulation in the accumbens, an effect not seen in female mice. Similarly, altering GluA2 trafficking via glutamate receptor-interacting protein (GRIP) abolishes LTD in the nucleus accumbens (Briand et al., 2014). As opposed to the clear role for GluA2 trafficking in the accumbens, studies in the hippocampus indicate that PKM $\zeta$  is not necessary for synaptic plasticity (Volk et al., 2013). Mice lacking PKM $\zeta$  displayed normal LTP, suggesting either a different role for PKM $\zeta$  in this form of plasticity or this region.

The current paper aims to examine both forms of plasticity in the same brain region to determine if the effects of PKM $\zeta$  are region-specific and or if the role of PKM $\zeta$  differs based on form of plasticity. The medial prefrontal cortex (mPFC) is a region that displays both LTD and LTP and receives input from both the nucleus accumbens and hippocampus, making it an interesting target for investigation. Using a constitutive PKM $\zeta$  knockout model, we recorded LTD and LTP from the mPFC of wildtype and PKM $\zeta$  knockout male and female mice.

## Methods

### *Subjects*

The current study used wildtype male and female mice and constitutive PKM $\zeta$  knockout mice as previously described (Volk et al., 2013). Heterozygous PKM $\zeta$  KO mice on a C57BL/6J background were mated, resulting in mutant and wildtype littermates.

Animals were group housed throughout the experiments with food and water available *ad libitum*. All animals were housed in a temperature and humidity-controlled animal care facility. All procedures were approved by the Temple University Animal Care and Use Committee.

### *Slice preparation*

Mice were decapitated following cervical dislocation. The brain was removed and coronal slices (350  $\mu\text{m}$ ) containing the prefrontal cortex were cut with a Vibratome (VT10000S, Leica Microsystems) in an ice-cold artificial cerebrospinal fluid solution (ACSF), in which NaCl was replaced by an equimolar concentration of sucrose. ACSF consisted of (in mM): 128.2 NaCl, 2.9 KCl, 1.2  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.25  $\text{NaH}_2\text{PO}_4$ , 28.8  $\text{NaHCO}_3$ , 2  $\text{CaCl}_2$ , 10 glucose (pH 7.2–7.4 when saturated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$ ). Slices were incubated in ACSF at 32–34  $^\circ\text{C}$  for 25 min and kept at 22–25  $^\circ\text{C}$  thereafter, until transferred to the recording chamber. The osmolarity of all solutions was 300–310 mOsm. Slices were viewed using an infrared differential interference contrast optics under an upright microscope (Slice Scope Pro, Scientifica) with a 10x objective.

### *Electrophysiology*

The recording chamber was continuously perfused (1–2  $\mu\text{l}/\text{ml}$ ) with oxygenated ACSF. Picrotoxin (100  $\mu\text{M}$ ) was added to all solutions to block the  $\text{GABA}_A$  receptor-mediated currents. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 1–2  $\text{M}\Omega$  when filled with extracellular solution. All recordings were conducted with MultiClamp700B amplifier (Molecular Devices).

### *Field recordings*

A glass capillary electrode filled with ACSF was placed within 100-300  $\mu\text{m}$  from the recording electrode used to simulate excitatory afferents in layer 2/3 and recorded from layer 5 of the mPFC in the prelimbic area.

### *Long-term depression*

The amplitude of current pulses was set at the intensity to evoke a 50% maximal response. After 10 minutes of stable responding, LTD was induced using a low frequency stimulation (LFS), paired-pulse protocol (50 ms inter-pulse interval) consisting of a 1-Hz train of paired stimuli for 10 min. Both field EPSP (fEPSP) slope (calculated over 1 ms after peak) and fEPSP amplitude were measured (graphs depict slope) from fEPSPs recorded at 0.05 Hz for 60 min following the pairing protocol. *Input-output curves.* An input-output (I/O) curve was constructed by increasing the stimulation magnitude until a maximal fEPSP was obtained.

### *Long-term potentiation*

The amplitude of current pulses was set at the intensity to evoke a 50% maximal response. After 10 minutes of stable responding, LTP was induced using a theta burst stimulation induction protocol (5 trains of 4 pulses with an inter-pulse interval of 10 seconds). Both field EPSP (fEPSP) slope (calculated over 1 ms after peak) and fEPSP amplitude were measured (graphs depict slope) from fEPSPs recorded at 0.05 Hz for 60 min following the theta burst protocol.

### *Data analysis*

All analyses were performed using GraphPad Prism 9.0 software (GraphPad Software). Data were analyzed using two-tailed Student's t-test or two-way ANOVA with Sidak's post hoc tests as appropriate. Statistical significance for all tests was set at  $\alpha = 0.05$ .

## Results

### *PKM $\zeta$ Knockout Alters LTD in Female and Male Mice in the mPFC*

We examined LTP in layer V pyramidal neurons in slice preparations from mouse mPFC. These neurons receive glutamatergic inputs and give rise to major cortical and subcortical projections to many regions in the brain. We utilized a paired pulse LFS protocol that produced consistent, long-lasting reduction in signal in female ( $n=10$ ) and male ( $n=11$ ) wildtype mice [females:  $t(9) = 4.945$ ,  $p = 0.0008$ ; males  $t(6) = 4.816$ ,  $p = 0.003$ , Fig 7A-B]. There were no significant differences between female and male wildtype mice [effect of sex:  $F(1,15) = 1.201$ ,  $p = 0.29$ ]. We found that neither female ( $n=10$ ) nor male ( $n=7$ ) PKM $\zeta$  knockout mice exhibited a reduction in fEPSC slope following the LFS protocol [females; effect of genotype:  $F(1,17) = 8.968$ ,  $p = 0.008$ , Fig 7A-B; males; effect of genotype:  $F(1,16) = 4.914$ ,  $p = 0.042$ ; Fig 7D-F]. Representative traces are shown in Fig 7C and 7F.

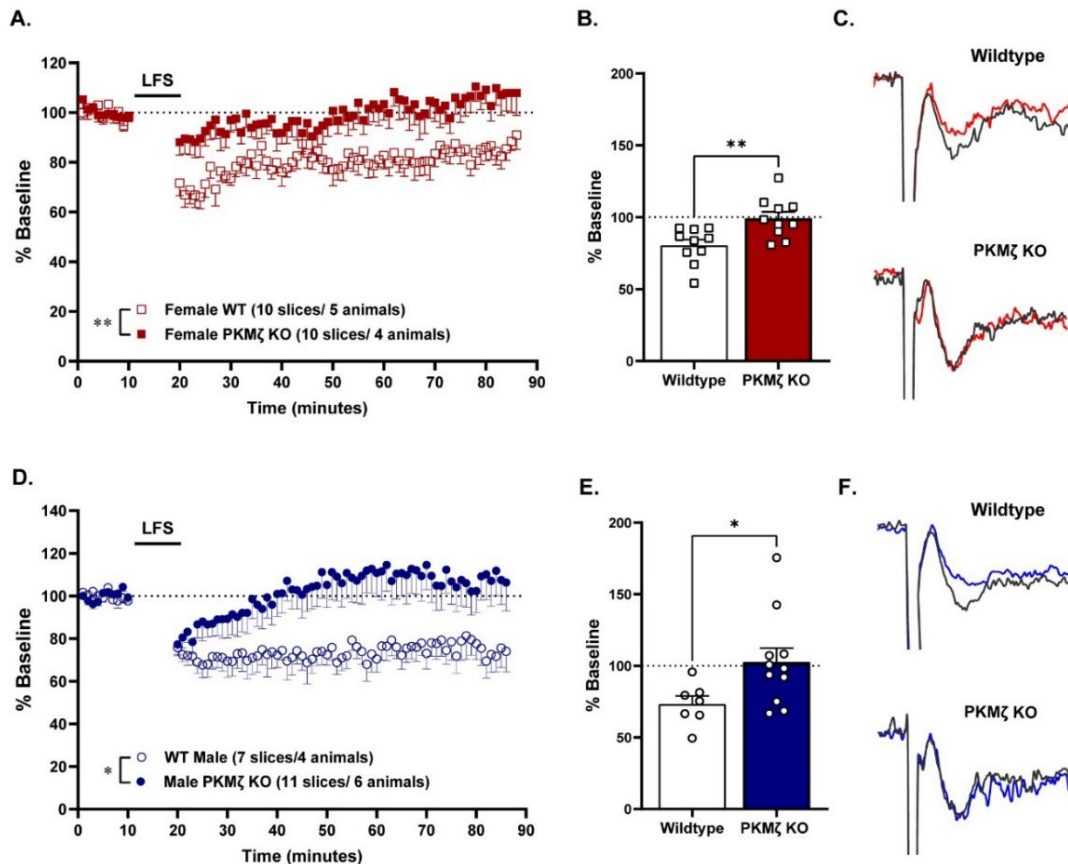


Figure 7. Constitutive PKM $\zeta$  knockout mice have blunted LTD in the mPFC. LTD induction by LFS (1 Hz train of paired stimuli for 10 minutes) is blunted in female (A;  $n=10$ /group) and male (B;  $n=7-11$ /group) PKM $\zeta$  knockout animals compared to wildtype controls. Change in fEPSP slope over 1-hour post-LFS represented as % change from baseline shows LFS facilitates LTD in wildtypes and does not in PKM $\zeta$  knockouts. Representative pre- and post-LFS traces (C, F). \* $p < .05$ , \*\* $p < .01$  effect of genotype.

#### *PKM $\zeta$ Knockout Alters LTP in Female and Male Mice in the mPFC*

In a separate cohort of mice, using the same slice preparation as used for LTD, we used a theta burst stimulation (TBS) protocol found to consistently produce a robust, long-lasting potentiation of fEPSCs in female ( $n=10$ ) and male ( $n=13$ ) wildtype mice [females:  $t(9) = 2.686$ ,  $p = 0.25$ ; males:  $t(12) = 7.662$ ,  $p < 0.0001$ ]. As with LTD, female and male wildtype mice did not significantly differ in level of potentiation after TBS [effect of sex:  $F(1,21) = 2.196$ ,  $p = 0.153$ ]. To examine the role of PKM $\zeta$ , we used slices from female ( $n=8$ ) and male ( $n=8$ ) constitutive PKM $\zeta$  knockout mice. The magnitude of LTP was lower

in both female [effect of genotype:  $F(1,16) = 7.912$ ,  $p = 0.013$ ; Fig 8] and male [effect of genotype:  $F(1,19) = 26.66$ ,  $p < 0.0001$  Fig 8] PKM $\zeta$  knockout mice, evidenced by a significantly reduced fEPSC slope compared to wildtype counterparts. Representative traces shown in Fig 8C and 8F.

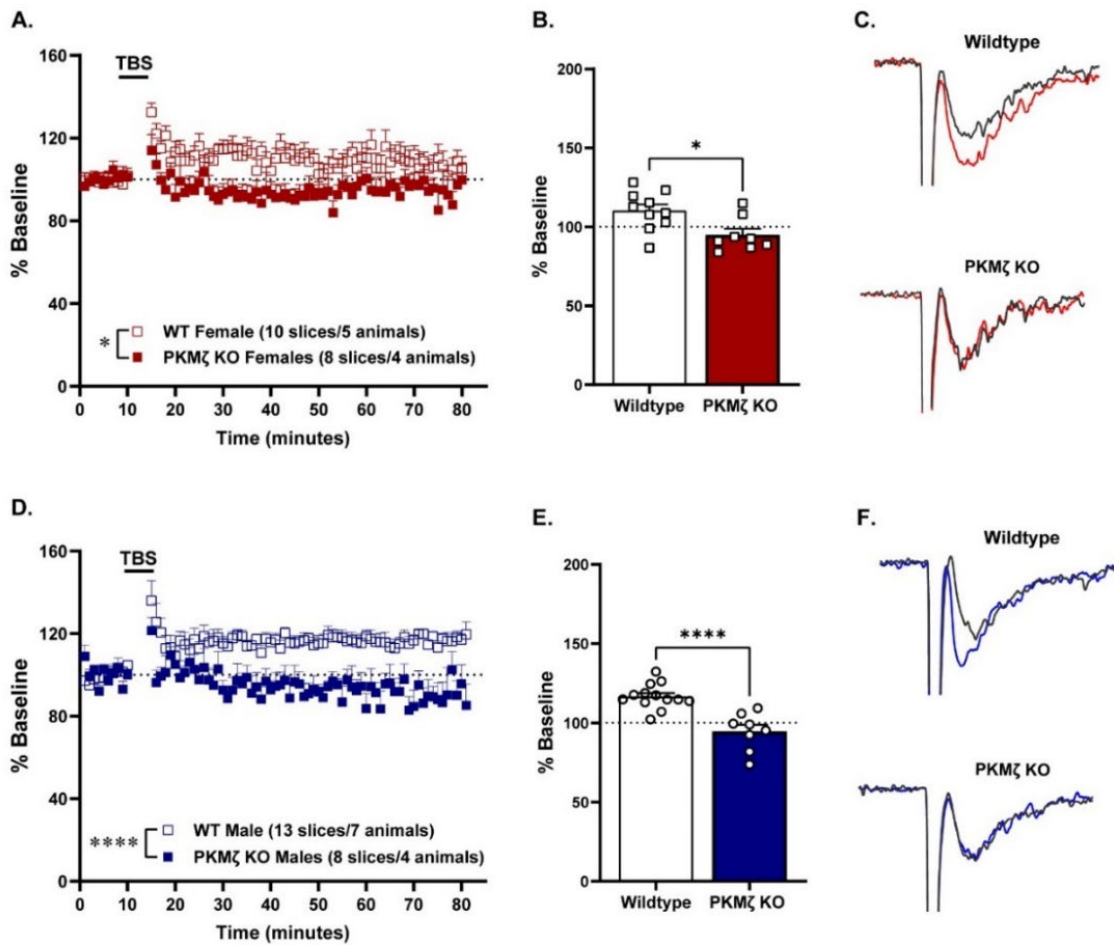


Figure 8. Constitutive PKM $\zeta$  knockout mice have blunted LTP in the mPFC. LTP induction by TBS (5 trains of 4 pulses with an inter-pulse interval of 10 seconds) is blunted in female (A;  $n=8-9$ /group) and male (B;  $n=8-13$ /group) PKM $\zeta$  knockout animals compared to wildtype controls. Change in fEPSC slope after 1-hour post-TBS is represented as % change from baseline and shows TBS facilitates LTP in wildtypes but not PKM $\zeta$  knockouts. Representative pre- and post-LFS traces (C, F). \* $p < .05$ , \*\*\*\* $p < .0001$  effect of genotype.

### Male PKMζ Knockout Mice Have Reduced Neuronal Excitability

Using slices from the experiments above, we also examined excitability by taking input/output (I/O) curves by steadily increasing stimulation intensity once a stable field was found. No differences were found in I/O curve between female and male WT mice [effect of sex:  $F(1,29) = 0.56, p = 0.46$ ]. However, a sex specific effect of PKMζ knockout was revealed. While no differences were found between wildtype and PKMζ knockout female mice [effect of genotype:  $t(14) = 0.087, p = 0.932$ , Fig 9A], male PKMζ knockout mice had significantly lower I/O curves compared to wildtype males [effect of genotype:  $F(1,18) = 5.119, p = 0.036$ , Fig 9B]. This indicates that PKMζ knockout mice required a higher threshold for excitation.

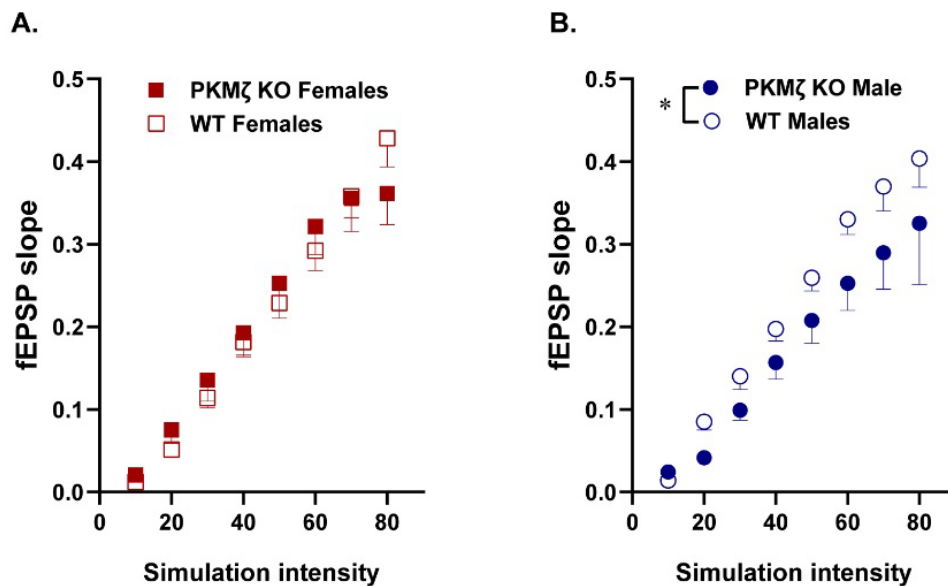


Figure 9. Input/output curves plotted for PKMζ knockout compared to wildtype counterparts. No differences in genotype were found in fEPSP slope in females (A). Male PKMζ knockout mice exhibited a reduced neuronal excitability compared to wildtype counterparts (B) ( $n=8-19$  slices/group). \* $p<0.5$

## Discussion

Glutamatergic pyramidal neurons in the mPFC are a key target for experience-dependent plasticity in response to both local and far-reaching input (DeFelipe & Fariñas, 1992; van Aerde & Feldmeyer, 2015). Our results demonstrate that PKM $\zeta$  plays a critical role in synaptic plasticity in both males and females. Although PKM $\zeta$  is critical for both LTP and LTD in the mPFC, this effect is not uniform amongst all brain regions and more work is needed to delineate the mechanism of action of PKM $\zeta$  (Knouse et al., 2023; Volk et al., 2013, p. 20213).

### *PKM $\zeta$ is Necessary for LTD in the mPFC*

We find that mice genetically lacking PKM $\zeta$  have significantly blunted LTD in the mPFC, regardless of sex. This is the first experiment to examine the role of PKM $\zeta$  in LTD in the mPFC and our results point to a clear role in the trafficking of GluA2 AMPAR subunits in LFS-induced LTD. One other study has described AMPA-mediated effects on LTD in the mPFC. Disrupting the interaction between the GluA2 AMPAR subunit and clathrin adaptor protein AP2, disrupts endocytosis mechanisms. This results in reduced ability for AMPAR endocytosis and effectively blocks LTD induction (Zhong et al., 2008). Our results further highlight the role of AMPAR trafficking in LTD in the mPFC.

LTD is a result of decrease synaptic strength, generally resulting from a reduction in AMPAR synaptic transmission at the post-synaptic membrane (Cao et al., 2023; O'Leary & Wyllie, 2008). AMPAR trafficking proteins are involved in the alteration of AMPAR composition or number at the synapse (Anggono & Huganir, 2012; Malinow & Malenka, 2002). Glutamate receptor interacting protein (GRIP) anchors GluA2 containing AMPAR at the synapses and works similarly to PKM $\zeta$ . GRIP is necessary for LTD in the

nucleus accumbens (Osten et al., 2000). Further, disrupting AMPAR trafficking by altering the function of protein interacting with C-kinase (PICK1) results in disrupted plasticity in the hippocampus (Terashima et al., 2008). PICK1 is involved in AMPAR internalization and is a key player in the recycling of AMPARs (Hanley, 2008; Lu & Ziff, 2005). Together, this shows the balance of AMPAR trafficking, in and out of the synapse, is critical in this form of plasticity. Our work builds on this body of literature indicating that PKM $\zeta$  also plays a critical role in LTD in the mPFC.

Our findings are distinct from previous studies examining the role of PKM $\zeta$  in LTD in the nucleus accumbens (Knouse et al., 2023). There, the role of PKM $\zeta$  in accumbal LTD is not uniform across sex. Male PKM $\zeta$  knockout mice exhibit blunted accumbal LTD while female PKM $\zeta$  knockout mice exhibited the opposite. However, both the effect of genotype and sex were abolished using a longer stimulation protocol, indicating PKM $\zeta$  modulates the ability for synaptic connections to undergo LTD, as opposed to eliminating it altogether (Knouse et al., 2023). This effect might be specific to the nucleus accumbens as our results do not indicate a sex difference in the mPFC. Our study in combination with abovementioned study implies that PKM $\zeta$  is not uniform across brain region. It has been shown that the level of PKM $\zeta$  expression varies across brain region, which may be contributing differential roles in the nucleus accumbens vs mPFC however more work is needed to delineate the exact mechanism of PKM $\zeta$  in LTD.

#### *PKM $\zeta$ is Necessary for LTP in the mPFC*

Consistent with a general role for PKM $\zeta$  in synaptic plasticity, we found that mice lacking PKM $\zeta$  exhibit blunted LTP in the mPFC. As PKM $\zeta$  is an AMPAR trafficking protein, this provides evidence that AMPAR trafficking is a critical component of LTP

in the mPFC. This result is in direct opposition to hippocampal recordings done with PKM $\zeta$  knockout mice (Volk et al., 2013). Within the hippocampus, mice genetically lacking PKM $\zeta$  exhibit normal synaptic transmission and LTP at the Schaffer collateral-CA1 synapses. In addition to conventional genetic knockout strategies, an inducible PKM $\zeta$  knockdown model that results in approximately an 80% reduction in PKM $\zeta$  in the dorsal hippocampus also reveals intact hippocampal LTP (Volk et al., 2013). This rules out the possibility that full PKM $\zeta$  knockout models induced an increase in other PKC isoforms that occlude the results.

In combination with our results, this raises questions about region-specific role for PKM $\zeta$  in LTP. Synaptic transmission in the mPFC is extremely complex. The mPFC receives layer-specific inputs from cortical and subcortical structures and is a central hub for information integration that is reliant on local circuits and long-range connections (Le Merre et al., 2021). Field recordings within the mPFC capture this non-specific summation of inputs, most likely in pyramidal neurons (Xu et al., 2019). The hippocampus functions as a closed circuit and has a less diverse set of functions (Naber et al., 2001). Plasticity within the hippocampus is incredibly robust and may be a much more redundant system. This would enable a wider range of stimulation protocols to elicit LTP (H.-X. Chen et al., 1999; Nicoll, 2017). This fundamental difference between the hippocampus and mPFC may underlie the differences in necessity of PKM $\zeta$  driven LTP. However, it is likely that PKM $\zeta$  is playing at least a supporting role in hippocampal synaptic plasticity. Inducing LTP in the hippocampus increases expression of PKM $\zeta$  and is thought to maintain synaptic potentiation (Sacktor, 2008, p. 20008). Similarly, overexpression of PKM $\zeta$  is sufficient to induce LTP (Schuette et al., 2016). Therefore, the role of PKM $\zeta$  in LTP in the mPFC and

hippocampus may be different, or there may be more redundancy in the plasticity mechanism in the hippocampus that prevent PKM $\zeta$  knockout from disrupting LTP.

#### *Sex Differences in the Role of PKM $\zeta$*

The sex-specific role of PKM $\zeta$  is complex and spans many areas of research. PKM $\zeta$  plays a sex-specific role in visceral pain models. Global knockout of PKM $\zeta$  reduces neuropathic pain in male rodents but has no effect on females (Nasir et al., 2016). Similarly, in a spatial memory task, male rats exhibit a positive correlation of synaptic PKM $\zeta$  level with performance whereas stable levels of synaptic PKM $\zeta$  were found in females (Sebastian et al., 2013). Various addiction studies have shown a sex-specific role for PKM $\zeta$  in drug self-administration. Knocking out PKM $\zeta$  in the nucleus accumbens produces an addictive phenotype in male mice but not females (McGrath et al., 2018). There is also evidence to suggest that PKM $\zeta$  plays a sex-specific role in motivation for oxycodone (Knouse et al., 2024).

We found no differences in the role of PKM $\zeta$  between male and female mice in LTD or LTP recordings in the mPFC. This indicates that regardless of the mechanisms that LTD and LTP, this response is disrupted when PKM $\zeta$  is absent. These results are surprising due to the literature suggests PKM $\zeta$  plays a more critical role in males in comparison to females. As mentioned above, PKM $\zeta$  knockout blunts LTD in males but not females in the nucleus accumbens using a less intense stimulation protocol. Female PKM $\zeta$  knockout mice expressed the opposite, a reduced thresholds for LTD compared to wildtypes (Knouse et al., 2023). Together, this shows that the role of PKM $\zeta$  in synaptic plasticity is region-specific.

Our data on glutamatergic transmission may correlate with more profound role for PKM $\zeta$  in male mice. We found that male PKM $\zeta$  knockout mice had significantly reduced basal glutamatergic transmission compared to wildtypes as shown in the input output curve with no differences found in females. This result was interesting due to a lack of sex differences in LTD and LTP measures. It implies that PKM $\zeta$  is a critical component of maintaining glutamatergic function in males but not females. A possibility is that females have additional systems in place that regulate AMPA receptor trafficking at basal levels of input such that PKM $\zeta$  disruption does not alter glutamatergic transmission. Since most of the research and stimulation protocols have been developed in male rodents, it is possible we are missing a critical mechanism that is exclusive to females that has not been captured.

### Conclusion

LTD and LTP are both critical components in the brain's ability to adapt to a changing environment. Together, these mechanisms maintain glutamatergic homeostasis and sculpt neural circuits that allow for learning and memory. While LTP is responsible for the strengthening of synaptic connections with repetitive input, LTD is essential for selectively weakening specific connections to allow others to persist. Dysregulation in mPFC synaptic plasticity has been implicated in the pathophysiology of various illnesses and understanding the neurobiological underpinnings of these disorders may help aid the development of treatments (Kalivas, 2009). AMPAR trafficking is a critical component of many forms of synaptic plasticity (Malinow & Malenka, 2002). Here we show that AMPAR trafficking governed by PKM $\zeta$  is not consistent across brain region in that it is necessary for LTD and LTP in the mPFC. Our work coincides with the modulatory role of PKM $\zeta$  in the nucleus accumbens but opposes work in the hippocampus indicating PKM $\zeta$

is not necessary for LTP. This lack of consistency poses an obstacle for the development of pharmacological treatments targeting PKM $\zeta$  and/or AMPAR trafficking and highlights the necessity of using multiple brain regions in the study of synaptic plasticity.

## CHAPTER 4

# PREFRONTAL CORTEX PKM $\zeta$ ALTERS COCAINE CUE-INDUCED REINSTATEMENT IN A SEX-SPECIFIC MANNER

### Introduction

A major clinical problem in the treatment of substance use disorder (SUD) is the management of relapse (Volkow & Blanco, 2023; Weiss, 2005). Individuals with SUD report powerful memories of drug experience following drug-related cues, even after long periods of abstinence, and report higher levels of “drug craving”. Accumulating evidence suggests that men and women may not experience SUD in the same manner (Becker & Hu, 2008; Brady & Randall, 1999; Knouse & Briand, 2021). While more men than women suffer from SUD, women report higher drug craving, exhibit a higher rate of drug use escalation, and have more frequent relapse events (Becker et al., 2017; Fonseca et al., 2021). Understanding underlying sex differences that may be contributing to these behavioral patterns is critical in the development of treatment of SUD in both sexes.

Altered glutamate transmission plays a role in the development and symptomology of the SUD cycle (Giacometti & Barker, 2020; Miladinovic et al., 2015). Shifts in neuronal plasticity within the prefrontal cortex (PFC) are associated with an increased responsiveness to drug related cues and reduced inhibitory control over drug seeking (Goldstein & Volkow, 2011). Additionally, cocaine self-administration alters glutamatergic transmission and AMPAR composition in the PFC (Kalivas et al., 2005). PKM $\zeta$  is an AMPAR trafficking protein that maintains GluA2-containing AMPARs at the synaptic membrane through NSF-mediated mechanisms and prevents AMPAR endocytosis and lateral diffusion (Henley et al., 2011; Miguez et al., 2010). This makes

PKM $\zeta$  an interesting target for the study synaptic plasticity underlying learning, memory, and drug use.

There is evidence that suggests PKM $\zeta$  dampens drug reward across drug class. Constitutive deletion of PKM $\zeta$  leads to increase ethanol consumption in male mice (Lee et al., 2013). Similarly, PKM $\zeta$  knockout mice exhibit potentiated cocaine taking and seeking behaviors (McGrath et al., 2018). These findings were extended to opioids in a previous study done with oxycodone reporting that PKM $\zeta$  knockout mice earned more oxycodone infusions and were more motivated to take oxycodone (Knouse et al., 2024). However, when challenged with a lower dose, only female mice exhibited this phenotype, indicating a role for biological sex in the influence of PKM $\zeta$  (Knouse et al., 2024). In addition to this sex difference using global knockouts, regional differences can be seen using site-specific knockdown of PKM $\zeta$ . While global knockout enhanced cocaine taking and seeking behaviors in both sexes, knocking down PKM $\zeta$  specifically in the nucleus accumbens recapitulated these results in male mice only (McGrath et al., 2018a). This poses an interesting question regarding where in the brain PKM $\zeta$  may be exerting an effect in female mice.

The PFC is positioned to modulate reward related behaviors through glutamatergic projections to the nucleus accumbens (Gipson et al., 2014; Kalivas et al., 2005). With the known role of PKM $\zeta$  in altering drug reward, we are interested in the specific influence of PKM $\zeta$  within the PFC in cocaine taking and seeking behaviors. Here, we examined the role of PFC PKM $\zeta$  knockout in cocaine taking, motivation, and cue-induced reinstatement. We tested both male and female mice and used an inducible genetic deletion model to selectively knockout PKM $\zeta$  in the PFC during adulthood prior to cocaine self-

administration. We found that PKM $\zeta$  in the PFC does not influence cocaine taking on self-administration fixed ratio schedule or motivation on a progressive ratio schedule. We do, however, see a sex specific effect in that female PFC PKM $\zeta$  knockout mice display blunted cue-induced reinstatement behavior. This indicates a region-specific role of PKM $\zeta$  between the sexes and highlights the need for the inclusion of females in drug use studies.

## Methods

### *Subjects*

Mice homozygous for the Cre/lox-conditional allele of PKM $\zeta$  (flox/flox) were bred on a C57/BL6 background. Adult male and female mice (2-6 months old, age matched across group) were group housed until the start of the behavioral experiments at which point they were individually housed. All animals were housed in a temperature and humidity-controlled animals care facility with a 12-hour light/dark cycle (lights on at 7am). All procedures were approved by the Temple University Animal Care and Use Committee.

### *Drugs*

Cocaine was obtained from the National Institutes of Drug Abuse Drug Supply Program (Bethesda, MD) and dissolved in sterile 0.9 % saline.

### *Intra-Prefrontal Cortex Microinjections*

PKM $\zeta_{\text{flox/flox}}$  mice (6-8weeks) were anesthetized with isoflurane and adeno-associated virus (AAV) expressing Cre recombinase (AAV2/9.CMV.PI.CRE, titer  $2.84 \times 10^{13}$  gc/ $\mu$ l) or the AAV expressing green fluorescent protein (eGFP) (AAV2/9.CMV.PI.CRE, titer  $2.84 \times 10^{13}$  gc/ $\mu$ l) diluted in sterile phosphate-buffered saline (PBS) were injected (.2 $\mu$ l volume) into the PFC through a 30 gauge needle at the rate of 0.1 $\mu$ l/min. Stereotaxic coordinates for the PFC are (from Bregma) anterior-posterior 1.9,

lateral +/- 0.25, dorso-ventral -1.05. Following recovery, mice were housed in the home cage for 6-weeks prior to behavioral testing.

#### *Jugular Catheterization Surgery*

Prior to surgery, mice were anesthetized with 80mg/kg ketamine and 12mg/kg xylazine. An indwelling silastic catheter was placed into the right jugular vein and sutured in place. The catheter was then threaded subcutaneously over the shoulder blade and was routed to a backmount platform (Instech Laboratories, Inc.) that secured the placement. Catheters were flushed daily with 0.1 ml of antibiotic (Timentin, 0.93 mg/ml) dissolved in heparinized saline.

#### *Cocaine Self-Administration*

Mice were given 3-4 days to recover before the initiation of behavioral testing. The cocaine self-administration behavior was tested in 2-hour sessions (7 days a week). Active wheel spin or lever press delivered an intravenous cocaine infusion (0.6 mg/kg/infusion), paired with light and tone cues. The first 3 days, animals trained to self-administer using an active wheel with an inactive wheel present but not providing any consequences. For the following behavioral days, mice self-administered on a fixed ratio schedule using levers for with escalating difficulty (3 days FR1, 5 days FR3, 3 days FR5). After 11 days of FR schedules, mice were tested for 1 day on a progressive ratio (PR) schedule, where the response requirement for each infusion increased until the subject did not fulfill the requirement in 30 minutes. The response requirement was defined as  $R(i)=[5e^{0.2i}-5]$ . The breakpoint is defined as the total number of responses or the final ratio completed. Following PR, cocaine self-administration resumed for 3 days to normalize cocaine responses before mice underwent extinction training. During extinction, cocaine seeking is

extinguished by replacing cocaine with 0.9% saline and removing the light and tone cue, previously paired with cocaine delivery. Daily 2-hour extinction sessions occurred until animals performed <30% of their self-administration responding average (last 3 days). Twenty-four hours after meeting this extinction criterion, animals underwent a cue-induced reinstatement session. The light and tone cues were presented non-contingently for 20 s, every 2 min during the first 10 min of the session. For the remainder of the session, the cues were presented contingent with operant responding, as they were during the cocaine self-administration phase but received saline infusions following active lever responses. Catheter patency was tested following PR testing and animals were removed from the study if found to have lost patency.

## Results

### *Site-Specific Knockout of PKM $\zeta$ in the PFC Does Not Alter Cocaine Taking During Fixed-Ratio Self-Administration*

No differences were seen in the number of cocaine infusions earned over the course of cocaine self-administration between the GFP injected controls or the Cre injected PKM $\zeta$  KO<sup>PFC</sup> mice [2-way ANOVA, females: effect of viral injection,  $F(1,21)=0.95$ ,  $p=0.34$ ; males: effect of viral injection,  $F(1,18)=0.03$ ,  $p=0.86$ ; Fig. 10A,B]. Additionally, while responding increased as the fixed-ratio requirements increased, we did not see an effect of prefrontal PKM $\zeta$  KO on active lever presses over the course of the 13 days of self-administration [mixed effects analysis, females: effect of session,  $F(3.362,70.04) = 45.01$ ,  $p<0.001$ ; effect of viral injection,  $F(1,21)=0.64$ ,  $p=.43$ ; males: effect of session,  $F(2.593,46.67)=32.3$ ,  $p<0.001$ ; effect of viral injection,  $F(1,18)=0.09$ ,  $p=.77$ ; Fig. 10C,D]. No differences were seen between the groups in inactive responding [2-way ANOVA,

females: effect of viral injection,  $F(1,20)=0.37$ ,  $p=.55$ ; mixed effects model, males: effect of viral injection,  $F(1,18)=0.23$ ,  $p=.64$ ; Fig. 10E,F].

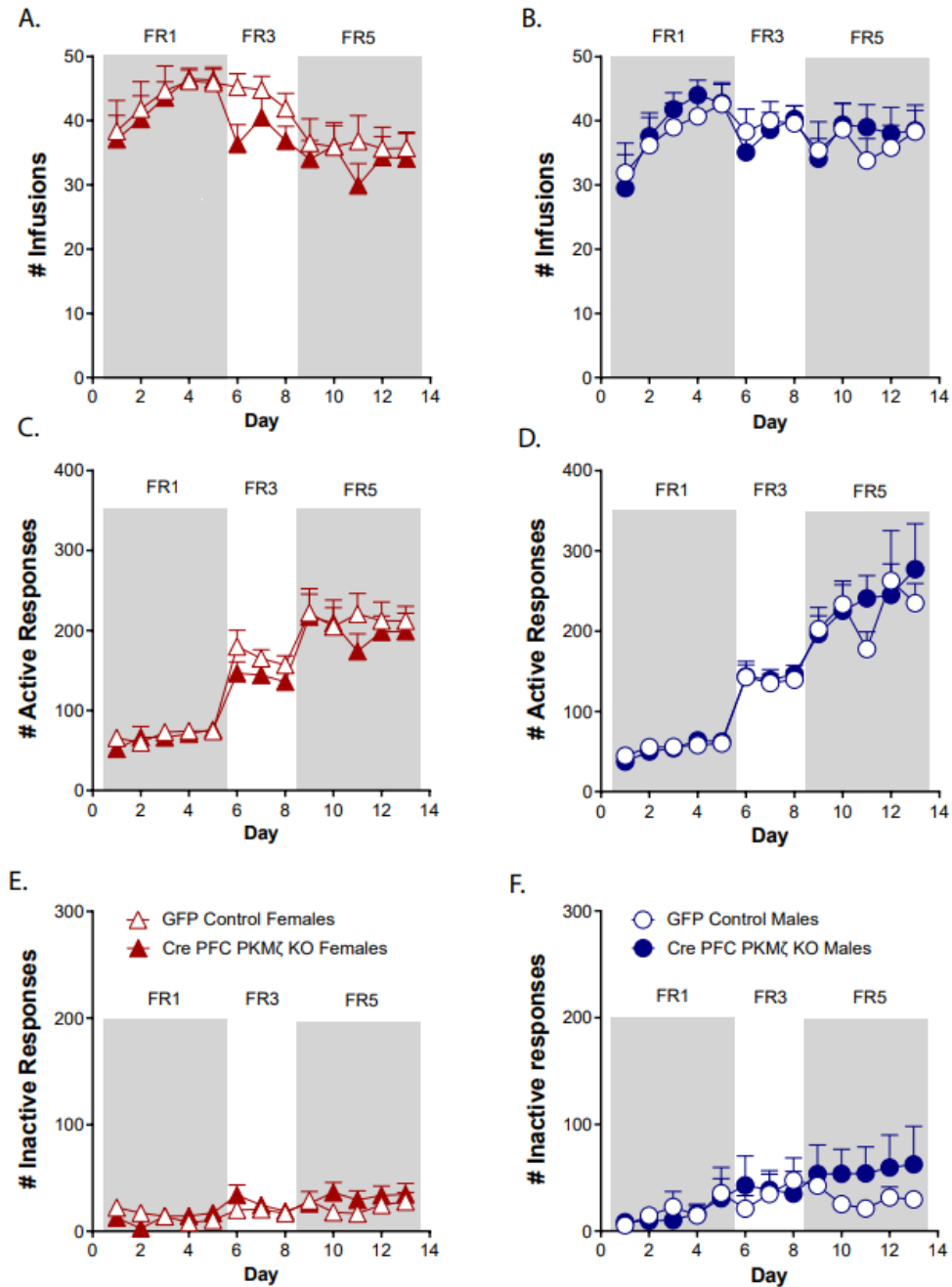


Figure 10. Effect of viral prefrontal PKM $\zeta$  knockout on cocaine self-administration. No effect of viral prefrontal PKM $\zeta$  knockout (Cre PKM $\zeta$  KO) in female ( $n=11-12$ /group) or male ( $n=10$ /group) mice on number of infusions earned (A, B), active lever responses (C, D), or inactive lever responses (E, F). Fixed ratio schedules were used with increasing criteria over 13 days of self-administration, represented by alternating grey bars labeled with fixed ratio schedule.

*Site-Specific Knockout of PKMζ in the PFC Does Not Alter Motivation for Cocaine on a0 Progressive Ratio Schedule*

To determine if prefrontal PKMζ KO affected the motivation for cocaine, we tested mice on a progressive ratio schedule of reinforcement following their final day of self-administration on FR5. While we did not see any effect of prefrontal PKMζ KO, there was an overall effect of biological sex such that male mice in both groups were willing to work harder for cocaine than their female counterparts [2-way ANOVA, effect of viral injection,  $F(1,39)=0.018$ ,  $p = 0.893$ , effect of sex,  $F(1,39) = 10.4$ ,  $p = 0.003$ ; Fig 11].

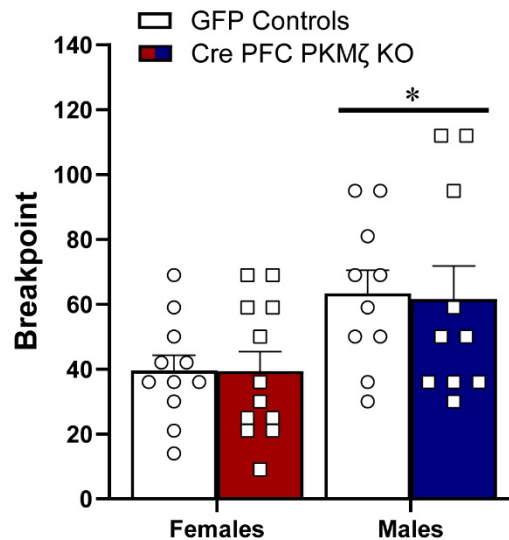


Figure 11. Effect of viral prefrontal PKMζ knockout on motivation for cocaine. No effect of viral prefrontal PKMζ knockout (Cre PKMζ KO) in females ( $n=11-12$ /group) or males ( $n=10$ /group) on breakpoint using a progressive ratio schedule. Male mice exhibited significantly higher breakpoint than females regardless of viral injection.  $p < .05$ .

*Site-Specific Knockout of PKMζ in the PFC Decreases Cue-Induced Reinstatement in Female Mice but Not Male Mice*

Following extinction of responding to less than 30% of their responding during self-administration on an FR5 schedule, mice underwent a cue-induced reinstatement test.

Female PKM $\zeta$  KO<sub>PFC</sub> mice exhibited diminished cue-induced reinstatement behavior compared to GFP injected control counterparts [effect of session,  $F(1,22) = 9.55, p = 0.005$ ; effect of viral injection,  $F(1,22)=4.93, p=.037$ ; interaction,  $F(1,22)=4.88, p=0.038$ ; Sidak's multiple comparisons, Cre vs. GFP RI test, adjusted  $p=0.0097$ ; Fig 12A]. However, in male mice, PFC knockout of PKM $\zeta$  had no effect on cocaine cue-induced reinstatement [effect of session,  $F(1,20) = 6.473, p = 0.019$ , effect of viral injection,  $F(1,20)=0.055, p=0.82$ , interaction,  $F(1,20)=0.20, p=0.66$ ; Fig 12B].

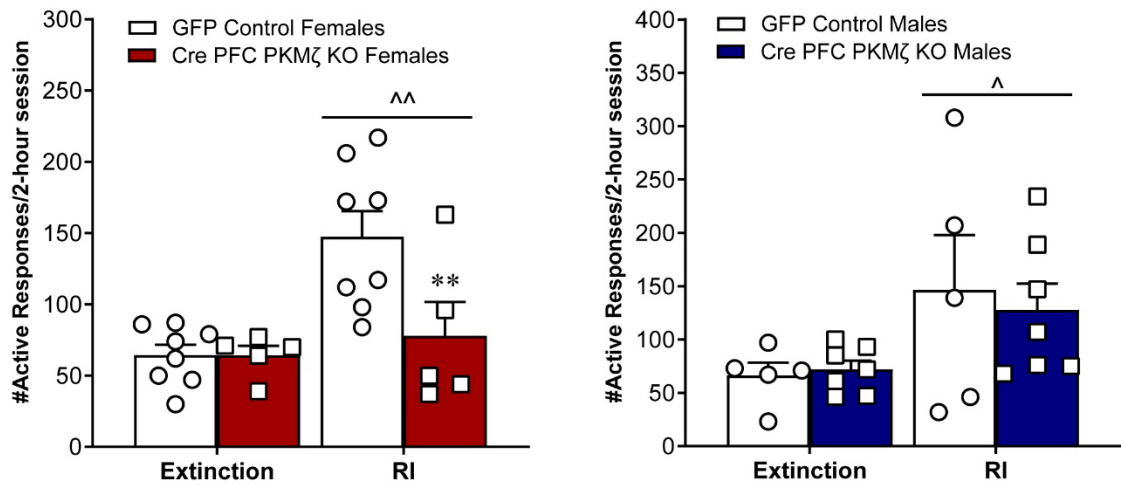


Figure 12. Effect of viral prefrontal PKM $\zeta$  knockout on cue-induced reinstatement. Female ( $n=5-7$ /group) and male ( $n=5-7$ /group) viral prefrontal PKM $\zeta$  knockout (PFC PKM $\zeta$  KO) displayed significant higher active lever responses during cue-induced reinstatement (RI) compared to last day of extinction training. PFC PKM $\zeta$  KO led to significant decrease in active responses during reinstatement compared to wildtype counterparts (A), no effect of genotype on cue-induced reinstatement in males (B).  $\wedge p < 0.5$ ,  $\wedge\wedge p < 0.1$  main effect of session,  $**p < 0.1$  Sidak's multiple comparisons.

## Discussion

Altered glutamatergic transmission is thought to underline many aspects of SUD, including relapse behaviors (Kalivas, 2009). The behavioral effects of cocaine are in part mediated by long term plasticity in mesocorticolimbic regions (Gipson et al., 2014). PKM $\zeta$  mediates the insertion of GluA2-containing AMPARs to the synaptic membrane and is

involved in synaptic plasticity. Here, we provide evidence that PKM $\zeta$  plays a sex-specific role in PFC driven reward related behaviors. Selectively knocking out PKM $\zeta$  in the PFC blunted cue-induced reinstatement behaviors in female mice exclusively and did not impact cocaine taking or motivation in either sex.

#### *PKM $\zeta$ in the PFC Does Not Impact Cocaine Self-Administration*

In our first experiment, we found that PFC PKM $\zeta$  knockout does not impact cocaine taking on a fixed ratio schedule in either sex. Our results reflect other studies manipulating AMPAR trafficking proteins in the PFC. Glutamate receptor interacting protein 1 (GRIP1) is an AMPAR anchoring protein that facilitates the maintenance of GluA2 containing AMPARs at the synapse and therefore, plays a similar role as PKM $\zeta$  (Anggono & Huganir, 2012; Dong et al., 1997; Tan et al., 2015). Knocking out GRIP in the PFC does not induce changes in cocaine taking in self-administration paradigms in male or female mice (Wickens et al., 2019, 2021b). Likewise, AMPAR trafficking protein PICK1 plays an opposing role in AMPAR trafficking in that it facilitates the endocytosis of GluA2-containing AMPARs (Citri et al., 2010; Hanley, 2008; Ramsakha et al., 2023). Knockout of PICK1 in the PFC has no effect on cocaine taking in mice (Wickens et al., 2021a). Together, these data suggest that AMPAR trafficking in the PFC is not critical for cocaine taking behaviors, including the AMPAR trafficking protein PKM $\zeta$ .

#### *PKM $\zeta$ in the PFC Does Not Impact Motivation for Cocaine*

In addition to cocaine taking, we did not see an effect of PFC PKM $\zeta$  knockout on motivation for cocaine in either sex, as indicated by no changes in breakpoint on a progressive ratio schedule. This is surprising because glutamatergic signaling both within the PFC and projections to the nucleus accumbens have been found to regulate this

behavior (Kalivas et al., 2005; Knackstedt & Kalivas, 2009). Further, altering AMPAR trafficking in the PFC with knockdown of GRIP results in increased motivation for cocaine (Wickens et al., 2019). GRIP and PKM $\zeta$  are thought to play similar roles in GluA2 trafficking and therefore it was hypothesized that PKM $\zeta$  knockout in the PFC would produce a similar phenotype as previous GRIP studies. Our results point to the engagement of potential compensatory mechanisms following PKM $\zeta$  knockdown in the PFC that are not engaged following knockdown of GRIP. Some have suggested that other PKC isoforms could act similarly to PKM $\zeta$  when PKM $\zeta$  is knocked out, however this has not been investigated in the context of motivation for cocaine or in the PFC.

Alternatively, it could be that under normal conditions PKM $\zeta$  does not play the described role in synaptic plasticity or AMPAR trafficking in the PFC and therefore is not involved in the motivation for cocaine. Motivation for cocaine has not been investigated using a global knockout. However, it seems that brain wide, there is a role for PKM $\zeta$  in motivation because looking across drug class, constitutive PKM $\zeta$  knockout increases motivation for oxycodone, suggesting a role for PKM $\zeta$  in this phenotype (Knouse et al., 2024). In this study, higher doses of oxycodone resulted in non-sex specific increases in motivation while lower doses induced this behavioral effect in female mice only. Thus, leading to the idea that females may be more sensitive to disruptions in PKM $\zeta$ . Our study uses a dose of cocaine known to drive self-administration and reinstatement behaviors, but it is possible that the dose we are using occludes the role of PKM $\zeta$  for motivation to take cocaine or that there is a distinct role for PKM $\zeta$  in the PFC that does not influence this behavior (Ebner et al., 2018; Highfield et al., 2002).

*PKM $\zeta$  in the PFC Plays a Sex-Specific Role in Cue-Induced Reinstatement for Cocaine*

Finally, we examined cue-induced reinstatement following extinction training in our PFC PKM $\zeta$  knockdown mice. Cocaine seeking behavior is driven by alterations in glutamate signaling in the PFC (Kasanez et al., 2013; Knackstedt & Kalivas, 2009; Schmidt & Pierce, 2010). Therefore, we expected that selective PKM $\zeta$  knockout in the PFC to impact cocaine seeking. Surprisingly, we found that male PFC PKM $\zeta$  knockout mice exhibited normal cue-induced reinstatement in comparison to wildtypes, indicating that in males PFC PKM $\zeta$  does not have a role in this behavior. In female mice, we see that PFC PKM $\zeta$  knockout induced a decrease in cue-induced reinstatement. No differences were found between groups on the number of days to reach extinction criteria or survival rates. These results suggest that PKM $\zeta$  within the PFC plays a sex-specific role in cue-induced reinstatement such that females require PFC PKM $\zeta$  to express this behavior.

Other studies also show a sex-specific effect of PKM $\zeta$  in reinstatement behaviors. In male mice, global knockout of PKM $\zeta$  induced a potentiated cue-induced reinstatement response (McGrath et al., 2018a). This effect is recapitulated with a site-specific knockout of PKM $\zeta$  in the nucleus accumbens showing that PKM $\zeta$  is critical for expression of cue-induced reinstatement behaviors in male mice. We show that PKM $\zeta$  in the PFC does not influence cue-induced reinstatement in males which is somewhat surprising. Either PKM $\zeta$  is not playing the same role in plasticity in the PFC as it does in the accumbens, or other mechanisms are able to compensate for the loss of PKM $\zeta$  in the PFC that are not found in the accumbens. GRIP1 functions similarly to PKM $\zeta$  by increasing GluA2 at the synapse and PICK1 is involved with the removal of GluA2 AMPARs (Hanley, 2008; Ramsakha et al., 2023). GRIP1 knockout in the PFC increases reinstatement behaviors while PICK1 knockout reduces cue-induced reinstatement in male mice (Wickens et al., 2019).

Differences in results between GRIP1 and PKM $\zeta$  studies indicate different net effects on GluA2 AMPARs. We suspect that there are other roles for PKM $\zeta$  that have not yet been described that are potentially presynaptic. Cue induced reinstatement is mainly driven by postsynaptic alterations in the PFC that lead to phenotypes driven by altered accumbal transmission. This explains why PKM $\zeta$  knockout in the accumbens may still produce this phenotype. However, it is possible that GluA2 trafficking mechanisms are more redundant in the PFC and can compensate for the loss of PKM $\zeta$ . Perhaps there is an upregulation of GRIP1 and downregulation of PICK1 in the absence of PKM $\zeta$  maintains cue-induced reinstatement behaviors in males similarly to mice with PKM $\zeta$ .

Disrupting GluA2 AMPAR insertion is thought to increase cocaine seeking, in part, by leading to a corresponding increase in GluA2-lacking AMPARs that have greater conductance. This explains why knocking down GRIP1 in the PFC could potentiate cocaine seeking since a general increase in glutamate transmission is needed for this phenotype. This is consistent with sex-specific role in PICK1 studies in the PFC (Wickens et al., 2021a). Altering AMPAR trafficking with PICK1 knockout in the PFC induces increased cue-induced reinstatement in females. However, our results investigating PKM $\zeta$  do not follow this pattern. In female mice, PFC PKM $\zeta$  knockout decreases cue induced reinstatement. The direction of this effect in females is interesting and suggests a reduction in glutamatergic drive. Females may be more vulnerable to PKM $\zeta$  knockout in the PFC because they lack compensatory mechanisms to maintain proper glutamatergic signaling. Therefore, our sex differences may be due to differences in a secondary mechanism that becomes activated with the absence of PKM $\zeta$ .

The PFC is divided into prelimbic and infralimbic subregions that have unique circuits and modulate specific behaviors. The prelimbic region drives cocaine seeking behaviors in male rats (B. T. Chen et al., 2013; Kasanetz et al., 2013; Mihindou et al., 2013). Pharmacological inactivation of the prelimbic subregion, but not the infralimbic, attenuates cocaine seeking (Limpens et al., 2015). Similarly, prelimbic neurons show enhanced activity during cocaine seeking and under extinction procedures, resulting in cocaine seeking behaviors following cocaine associated cues (West et al., 2014). However, all these studies were done using male rodents. It is possible that our sex differences found in cue-induced reinstatement are derived from differential involvement or sensitivities in the prelimbic region in females. Further, the absence of PKM $\zeta$  may shift this circuit in favor of infralimbic system which sends inhibitory signals for goal-seeking behavior, resulting in reduced cue-induced reinstatement in females. It is also possible that AMPAR trafficking may differ between the subregions. Baseline sex differences in other features exists between male and female rodents. Differences such as level of GluA1 and A2 AMPAR subunits and dendritic spine morphology have been documented and may all play a role in the apparent bidirectional role of PKM $\zeta$  in cocaine seeking (Knouse et al., 2022).

### *Sex Differences*

With the inclusion of females in preclinical research, many sex differences have emerged in rodent studies of drug self-administration (Radke et al., 2021). We find that males overall are more motivated to take cocaine than females, however this may be cohort specific and not hold true across studies. Most strikingly, we found a bidirectional effect of PKM $\zeta$  on cue-induced cocaine seeking. Male mice were unaffected by PFC PKM $\zeta$  knockout whereas females had reduced cocaine seeking. This shows a greater role for

PKM $\zeta$  in female mice compared to males. This directional effect is not typical for literature surrounding sex differences in PKM $\zeta$ . Male mice have higher levels of PKM $\zeta$  expression than females in the nucleus accumbens after cocaine experience (McGrath et al., 2018a). Male rodents also display a higher correlation between PKM $\zeta$  expression and memory performance, indicating a more pronounced role for PKM $\zeta$  (Sebastian et al., 2013). Our sex differences may have been driven by a lack of compensatory mechanisms in females such that PKM $\zeta$  disruption did not result in upregulation of other PKC isoforms. Studies that have shown these compensatory mechanisms have exclusively been in male rodents (Tsokas et al., n.d.). Alternatively, as PKM $\zeta$  knockout in the nucleus accumbens has no effect on cue induced reinstatement in females, it is possible the same is true for the PFC in male behavior.

### Conclusion

The PFC is highly plastic and the proper strengthening, or weakening, of synapses allows for the fine tuning of experience-dependent behavior (Meunier et al., 2017). Indeed, dysregulation of the PFC is associated with the psychopathology of various psychiatric illnesses including depression, anxiety, schizophrenia, and substance use disorder (Goto et al., 2010; Yan & Rein, 2022). Glutamatergic projections from the PFC to the nucleus accumbens drive cue-induced reinstatement, the animal model for relapse, however this has been primarily studied in male rodents (Van den Oever et al., 2010). We find that in females, cue-induced reinstatement of cocaine relies on the presence of PKM $\zeta$ . This indicates that PKM $\zeta$  may be playing a different role in AMPAR trafficking and synaptic plasticity in females. In males, there may be compensatory mechanisms that prevent the reduced reinstatement phenotype as seen in females. Similarly, reduced plasticity due to

PKM $\zeta$  knockout may preferentially drive signaling in infralimbic regions, resulting in decreased reinstatement. These findings in combination with previous studies suggest that the role of GluA2 AMPAR trafficking is not as straightforward as previously thought.

## CHAPTER 5

### CONCLUSION

Protein kinase M zeta (PKM $\zeta$ ) belongs to the protein kinase C family. Once activated, PKCs regulate many cellular processes including signal transduction, cell growth, survival, and differentiation (Newton, 2010). PKM $\zeta$  is found exclusively in the central nervous system and is formed by alternative splicing and autocatalytic cleavage of PKC $\zeta$ . This removes the regulator domain, thus making PKM $\zeta$  persistently active (Yao et al., 2008a). The discovery of PKM $\zeta$  and its constitutively active form made it an interesting target for persistent changes in the brain, such as memory storage.

PKM $\zeta$  is in an AMPA receptor (AMPA) trafficking protein. One of the functions of PKM $\zeta$  is through NSF-mediated insertion of GluA2 containing AMPARs to the synapse (Yao et al., 2008). The upregulation of AMPARs is associated with increases in synaptic strength, leading to long-term potentiation (LTP). Similarly, removal of AMPARs from the synapse is the basis for long-term depression (LTD), resulting in weakened synaptic transmission (Anggono & Huganir, 2012; Cao et al., 2023). Therefore, PKM $\zeta$  is situated to impact synaptic plasticity. PKM $\zeta$  is expressed abundantly in grey matter in the neocortex, hippocampus, and thalamus and is involved in the formation and stabilization of dendritic spines (Hernández et al., 2014). This is in line with the idea that PKM $\zeta$  aids in the insertion of AMPARs at post-synaptic sites.

The role of PKM $\zeta$  in cellular and behavioral models has been the source of many controversies (Patel & Zamani, 2021). Early studies demonstrate a role for PKM $\zeta$  in memory formation and claimed that PKM $\zeta$  was the cellular substrate sufficient to induce and maintain long-term memories via long term potentiation (LTP) (Ling et al., 2002).

PKM $\zeta$  expression increases during and after LTP and is localized at the post-synaptic site (Hsieh et al., 2021; Sacktor et al., 1993). Further, studies showed that the inhibition of PKM $\zeta$  could reverse already established LTP and disrupt long-term memories in rodents (Pastalkova et al., 2006). However, these studies utilized zeta inhibitor peptide (ZIP), which was proposed to be an inhibitor for PKM $\zeta$ . Later it was discovered that ZIP interacts with other PKC isoforms and has excitotoxic off target effects (Sadeh et al., 2015). Further, PKM $\zeta$  knockout mice exhibit normal learning and memory (Lee et al., 2013; Volk et al., 2013), making researchers take a second look at claims surrounding PKM $\zeta$ .

Neuroadaptations associated with addiction are hypothesized to involve the same glutamatergic cellular mechanisms that underlie learning and memory (Kalivas, 2009; Kalivas & Volkow, 2005). Further, drugs of abuse hijack mechanisms of synaptic plasticity, making PKM $\zeta$  an interesting target in addiction research (Lüscher & Malenka, 2011). PKM $\zeta$  has indeed shown to play a role in reward. Male mice genetically lacking PKM $\zeta$  take more ethanol in an intermittent access paradigm than wildtype counterparts (Lee et al., 2013). This behavioral phenotype is also seen in cocaine self-administration studies. Both male and female PKM $\zeta$  knockout mice demonstrated potentiated cocaine taking and seeking behaviors (McGrath et al., 2018). Together, this indicates that PKM $\zeta$  works to dampen drug reward in male and female mice. We extended this line of research to opioids and found that male and female mice exhibited a similar phenotype. PKM $\zeta$  knockout increased oxycodone taking and motivation for oxycodone in both sexes. Using a lower dose of oxycodone, we did see that the effect of PKM $\zeta$  was sex specific. Female PKM $\zeta$  knockout mice had increased motivation at the lower dose of oxycodone whereas the higher dose yielded increased responding in both male and female PKM $\zeta$  knockouts

(Knouse et al., 2024). This work further solidifies that PKM $\zeta$  dampens drug reward, although maybe not explicitly the same across sex, and underscores the role of the glutamatergic system in substance use disorder (SUD).

In these behavioral experiments, we used a global knockout to investigate complex behaviors. However, we are also interested in what PKM $\zeta$  is doing on a physiological level. PKM $\zeta$  knockout modulates synaptic plasticity in the nucleus accumbens in a sex-specific manner (Knouse et al., 2023). Male PKM $\zeta$  knockout mice exhibit blunted accumbal LTD, the main form of plasticity in this region. This data supports the role of PKM $\zeta$  in synaptic plasticity. However, female PKM $\zeta$  knockout mice show the opposite effect in that knockout mice have a reduced threshold to induce LTD. Similarly to our oxycodone behavioral effect, different “doses,” in this case, of stimulation, induce different LTD patterns with PKM $\zeta$  knockout male and female mice. In the hippocampus, a region known for a robust LTP response, PKM $\zeta$  is not necessary for LTP (Volk et al., 2013). Further, these two studies looked at different forms of plasticity, which may imply that PKM $\zeta$  has a role in LTD but not LTP or, it could imply that PKM $\zeta$  plays distinct roles in synaptic plasticity depending on region.

We furthered this line of work by looking at the role of PKM $\zeta$  in both LTD and LTP in the medial prefrontal cortex (mPFC). We were interested in the mPFC due to the direct role in addictive behaviors (Goto et al., 2010; Van den Oever et al., 2010; Yan & Rein, 2022). Neurons from the mPFC project to the accumbens as part of the mesocorticolimbic reward circuitry and play a critical role in drug seeking behaviors (Cooper et al., 2017). Using slice electrophysiology in PKM $\zeta$  knockout and wildtype mice, we found that PKM $\zeta$  is necessary for both LTD and LTP in the mPFC. This was the first

study done investigating PKM $\zeta$  in the mPFC and our results add to an interesting growing body literature.

We show that PKM $\zeta$  does not function uniformly across the brain. Our results directly contradict finding in the hippocampus that show that PKM $\zeta$  is not necessary for LTP (Volk et al., 2013). This entirely shifts the picture of what is known about PKM $\zeta$ . In the hippocampus, it was shown that other protein kinases were upregulated in the absence of PKM $\zeta$ , including PKC $\alpha/\lambda$ . It is possible that this compensatory upregulation does not occur in the mPFC, resulting in blunted LTP. Further, the role of PKM $\zeta$  in hippocampal LTP is unknown in females and it is possible that females may lack this compensatory mechanism. It is well documented that LTP is influenced by sex and hormonal status (Gall et al., 2023; Koss & Frick, 2017; Simpson & Kelly, 2012; Yagi & Galea, 2019). Males exhibit LTP in response to a broader range of induction protocols and have higher levels of potentiation (Yang et al., 2004). Although we did not see quantifiable sex differences in LTP, inducing LTP in males in the mPFC produced a less variable potentiation than in female slices. This is most likely due to protocol optimization occurring in male tissue as opposed to female, but could also be due to variable hormonal stages in female mice at the time of brain extraction as estrogen levels have been found to alter LTP response (Smith & McMahon, 2005). Regardless, our LTP results indicate PKM $\zeta$  is critical for LTP in the mPFC, potentially due to a lack of redundant mechanisms found in the hippocampus. Further studies should investigate the role of PKM $\zeta$  in females in the hippocampus and track estrous cycle in females to delineate the role for PKM $\zeta$  with cycling hormones, as it is unclear if PKM $\zeta$  is modulated by hormonal status.

The picture is not as simple in the nucleus accumbens. There it was found that PKM $\zeta$  plays a sex-specific role in LTD. Shorter LFS protocols elicited LTD in male but not female mice, and PKM $\zeta$  knockout blunted this effect in males and potentiated it in females (Knouse et al., 2023). This effect was hypothesized to be due to possible presynaptic role of PKM $\zeta$  in female mice such that PKM $\zeta$  is dampening synaptic plasticity. Thus, without PKM $\zeta$ , LFS was able to induce LTD in females. To further evaluate the hypothesis that there is a presynaptic role for PKM $\zeta$  in females, future studies utilizing a viral knockdown of PKM $\zeta$  could be used to uncover potential presynaptic effects. Although this sex differences is a possibility, we do not see sex differences in LTD in the mPFC. Both male and female PKM $\zeta$  knockout mice had blunted LTD. However, the protocol used to probe this effect was the longer, more intense stimulation protocol adopted from accumbal studies. It is possible that sex differences in the role of PKM $\zeta$  in LTD the mPFC were occluded due to this high intensity protocol. Using a less intense protocol in this region may tease apart more subtle differences in both the role of PKM $\zeta$  and potential sex differences in the region. It is known that females at baseline have more GluA1 and GluA2 subunits present at the synapse in the mPFC (Knouse et al., 2022). It is possible that this baseline difference may make it harder to induce LTD at lower stimulation intensities in females. Nonetheless, our results in combination with studies done in the accumbens and hippocampus indicate that PKM $\zeta$  is not functioning entirely the same across brain region. The role of PKM $\zeta$  in synaptic plasticity is much more nuanced than previously described and may be play separate roles in plasticity across region.

The finding that PKM $\zeta$  is necessary for long-term plasticity in the mPFC poses interesting questions for behaviors that are modulated by the PFC. In the context of

addiction, glutamatergic dysfunction in this region is associated with maladaptive behaviors, including cocaine craving and relapse (Knackstedt & Kalivas, 2009). Therefore, we wanted to see how PKM $\zeta$  in the mPFC impacts cocaine taking and seeking behaviors. Using a flox/cre genetic model, we knocked out PKM $\zeta$  selectively in the mPFC in male and female mice. Interestingly, we found different effects of PKM $\zeta$  knockout in the PFC on cue-induced reinstatement in males and females. Male PFC PKM $\zeta$  knockout mice behaved the same as control mice and showed cue-induced cocaine seeking behaviors. In female mice, PFC PKM $\zeta$  knockout resulted in a blunted cue-induced cocaine seeking phenotype. There was no difference between PFC PKM $\zeta$  knockout mice and wildtypes in cocaine intake during the fixed ratio sessions. Likewise, we did not see an effect of PKM $\zeta$  knockout in measures of motivation for cocaine.

This differential effect of PFC PKM $\zeta$  knockout in cocaine cue-induced reinstatement implies differential roles for PKM $\zeta$  in males and females. However, we do not find there to be any sex specific effects of global PKM $\zeta$  knockout on LTP or LTD in the mPFC. Together, these experiments indicate that cue-induced reinstatement in female mice may require PKM $\zeta$ -mediated synaptic plasticity, whereas in males it may not. Our electrophysiology results utilize global knockouts while cocaine behavioral experiments utilize site specific knockout of PKM $\zeta$  in adulthood. Global knockout mice entirely lack PKM $\zeta$  and therefore lack of PKM $\zeta$  in presynaptic sites as well as post-synaptic sites. This difference between global and viral knockout may contribute to differences in synaptic vs behavioral phenotypes. In our cocaine behavioral experiments, our site-specific knockout does not impact PKM $\zeta$  in presynaptic terminals. This could indicate that presynaptic PKM $\zeta$  is playing a role in these synaptic phenotypes such that presynaptic PKM $\zeta$  is driving the

reduced cocaine seeking behaviors in females. One major limitation of this study was the lack of circuit specificity, as the prelimbic and infralimbic subregions project to the core and shell of the accumbens, respectively, and have distinct roles in drug-seeking behaviors. As we did not isolate one of these circuits, we cannot comment on which circuit is being engaged in this study. Future studies could utilize a retrograde virus and inject into the core or shell to induce a circuit specific change in PKM $\zeta$ . Further, our electrophysiology studies utilized global PKM $\zeta$  knockouts and therefore develop without PKM $\zeta$ . Future studies could utilize viral PKM $\zeta$  knockouts in electrophysiology experiments to determine if the synaptic phenotype is driven by pre- or post-synaptic PKM $\zeta$ .

In conclusion, these studies highlight the complexity of PKM $\zeta$  at the synaptic level and the role of PKM $\zeta$  in reward-related behaviors. We show that PKM $\zeta$  dampens reward for oxycodone, extending the literature to opioids. In addition, we are the first to demonstrate the necessity of PKM $\zeta$  in LTP and LTD in the mPFC in both sexes. This result is critical to the understanding of PFC synaptic plasticity. Finally, we show that PKM $\zeta$  in the PFC is not functioning uniformly across biological sex in the role of cocaine seeking behaviors. Instead, females present the opposite behavioral phenotype when challenged with cue-induced reinstatement compared to global PKM $\zeta$  knockout females. This result potentially changes the way we should think about the role of glutamatergic signaling and reward in females. Our studies highlight the critical need for neuroscientist to "redo" basic studies involving the glutamate system in reward using female subjects. Our work with PKM $\zeta$  is further evidence that most systems in the brain are complex and developing a single drug targeting a synaptic plasticity protein will not produce the same effects in males and females. To target PKM $\zeta$  to treat the underlying glutamatergic dysregulation in SUD,

more work is needed to delineate the sex-specific, region-specific, and potentially presynaptic mechanisms in reward-related circuitry. Although this makes PKM $\zeta$  a difficult target for the treatment of SUD, understanding the role of synaptic plasticity in addictive behaviors remains a critical piece of the puzzle.

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