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Evaluating mGlu5 knockout mice as a model of morphine addiction susceptibility

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Statement of Authentication

This thesis is submitted in fulfillment of the requirements for the degree *Master of Research* at Western Sydney University. I, Erin McLemon, hereby declare that the work submitted in this thesis, titled *'Evaluating mGlu5 knockout mice as a model of morphine addiction susceptibility'* is originally and authentically the result of my own research endeavour, under the guidance of my supervisors Dr. Rose Chesworth and Prof. Tim Karl. The work presented in this thesis, is to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

Signed

1 March 2022

Date

Dedication

I would like to dedicate this thesis to my late grandmother, who passed away in 2020 halfway through my candidature for this degree. Her support and encouragement were vital to the development of my academic abilities and my pursuit of a research career.

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Abstract

Introduction: Globally, opioid addiction causes significant health, social and economic costs. There has been an increasing trend of opioid dependence and abuse in high-income countries, and long term opioid use is associated with significant health costs. Additionally, there are substantial sex differences in opioid addiction as men report higher lifetime use of opioids; however, women are more likely to relapse during abstinence. Opioid addiction is also accompanied by significant neural adaptations that drive an abstinence-relapse cycle and the development of opioid addiction-relevant behaviours. Current treatments are limited and do not address these biological processes. The metabotropic glutamate 5 receptor (mGlu5) receptor is a potential target for treating addiction. Mice with a genetic deletion (i.e. knockout, KO) of mGlu5 exhibit addiction-like behaviour for psychostimulants and ethanol, but their response to opioids has yet to be examined. Assessing opioid addiction-like behaviour in these mice, and testing for potential sex-differences in their reward and locomotor response to morphine, will determine if mGlu5 could be a treatment target for opioid abuse.

Materials and Methods: The effects of mGlu5 deletion on morphine conditioned place preference (CPP) were assessed. Male and female mGlu5 KO and WT-like mice were conditioned to associate 5 mg/kg or 10 mg/kg morphine with a distinct environment over 4 consecutive days. Following conditioning, preference for the morphine-paired environment (i.e. time spent in the morphine environment) was assessed weekly for 4 weeks during abstinence from morphine, to test the persistence of morphine memory. A persistent preference for the morphine-paired environment during abstinence is an indicator of drug craving and risk of relapse. During all conditioning and test sessions, locomotor data was collected and assessed for morphine-induced locomotion and locomotor sensitisation.

Results: Male and female WT-like and mGlu5 KO mice acquired morphine CPP for 5 and 10 mg/kg morphine. Female mice had a higher preference for 5 mg/kg morphine than male

mice. There were no sex-differences in preference for 10 mg/kg morphine. Female mGlu5 KO had a persistent preference for a morphine-paired environment for 5 mg/kg morphine. mGlu5 KO mice, regardless of sex, showed morphine-induced hypolocomotion for 5 mg/kg morphine and hyperlocomotion for 10 mg/kg morphine. Both 5 and 10 mg/kg morphine induced hyperlocomotion in WT-like mice. Female mGlu5 KO mice developed locomotor sensitisation to 5 mg/kg morphine while all mice showed locomotor sensitisation to 10 mg/kg morphine.

Summary and Conclusion: mGlu5 modulation of morphine reward was sex-specific and dose-dependent. These results suggest that mGlu5 deletion did not affect morphine reward in male mice, but female mGlu5 KO mice are more sensitive to the rewarding effects of morphine than female WT-like mice. mGlu5 mediates sensitivity to morphine-induced locomotion in both male and female mice. These findings emphasise the importance of considering sex effects in opioid addiction research. Further investigation into the effect of mGlu5 deletion on morphine addiction-relevant should consider its impact on an operant model of addiction, like self-administration, as well as the state-dependent effect of morphine on learning. In conclusion, this thesis suggests that mGlu5 may mediate the known sex differences in opioid addiction.

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List of abbreviations

ACEC	Western Sydney University Animal Care and Ethics Committee
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	analysis of variance
Ca2+	calcium
cAMP	cyclic-AMP
CPP	conditioned place preference
CREB	cAMP response element-binding protein
CRF	corticotropin-releasing factor
D1	dopamine 1 receptor
D2	dopamine 2 receptor
DOR	δ-opioid receptor
HPA	hypothalamic-pituitary-adrenal axis
HPC	hippocampus
i.p.	intraperitoneal
K+	potassium
KO	knockout
KOR	κ-opioid receptor
LC	locus coeruleus
LTD	long term depression
LTP	long term potentiation
mGlu5	metabotropic glutamate 5 receptor
MOR	μ-opioid receptor
MPEP	2-methyl-6-(phenylethynyl)-pyridine
MSN	medium spiny neurons
MTEP	3-[(2-methyl-1, 3-thiazol-4-yl) ethynyl] pyridine
NAc	nucleus accumbens
NMDA	N-methyl D-aspartate receptor
PFC	prefrontal cortex
PKC	protein kinase C
RM	repeated measures
SEM	standard error of the mean
VTA	ventral tegmental area
WHO	World Health Organisation
WT	wild type

Chapter 1: Introduction

1.1 The "Opioid Epidemic"

Opioid addiction is a growing problem with increasing health, social and economic costs. Known colloquially as the "opioid epidemic", opioid dependence, abuse and associated deaths have significantly increased in the last thirty years. This has had a significant impact on public health systems. Between 2010 and 2019 the number of opioid users almost doubled to nearly 62 million users worldwide (World Health Organisation (WHO) 2021). This has been especially apparent in high-income countries like Australia (Degenhardt et al. 2014), where between 1992 and 2012 opioid dispensations primarily intended for pain management, increased by 15-fold (Blanch, Pearson & Haber 2014). Between 2001 and 2012 there was a significant increase in opioid related deaths in Australia, from 21.9 to 36.2 per million population, largely due to pharmaceutical opioid prescribed for pain management as opposed to illicit opioid use (Roxburgh et al. 2017).

In addition to deaths, opioid abuse is associated with the development of opioid tolerance and toxicity, and an increased risk of anxiety and depression (Cahill et al. 2016; Compton, Boyle & Wargo 2015). Nausea, constipation, sedation, dental problems, long-term hypothalamic-pituitary axis dysfunction, immunosuppression, loss of bone density in men, and predisposition to liver and respiratory diseases are common symptoms of opioid toxicity (Murnion 2012). After long term use, ceasing opioid use can cause withdrawal symptoms including increased heart rate, blood pressure and perspiration as well as fluctuating body temperature and joint and muscle aches (Scavone, Sterling & Van Bockstaele 2013). Opioid abuse also has significant economic costs, estimated to cost Australia around $15.76 billion in 2015/16 (Whetton 2016). The social and economic costs have led to changes in public health policy to increase education about opioid abuse and restrict the availability of opioid-class drugs (Armstrong et al. 2020). Public health interventions are having an impact with a reduction in non-medical

use of pain killers in Australia, from 3.6% in 2016 to 2.7% in 2019 (Australian Institute of Health and Welfare 2020). However, opioid abuse disorder remains a significant global public health concern (World Health Organisation (WHO) 2021) and there are limited options for treatment. Currently, treatment focuses on reducing the symptoms of withdrawal and relapse by replacement therapy with opioid agonists like methadone and buprenorphine (Kaplan et al. 2011; Sue Henry-Edwards, James Bell & Alison Ritter 2003). However, these treatments do not address the underlying pathophysiology that drives addiction-relevant behaviour such as relapse (Koch & Hollt 2008; Listos et al. 2019; Mazei-Robison et al. 2011). Additionally, there are significant sex differences in incidence and predisposition to opioid addiction (detailed further below (Back et al. 2010; Goetz, Becker & Mazure 2021; Kokane & Perrotti 2020; Serdarevic, Striley & Cottler 2017)). Therefore, in order to successfully treat and address the significant social and economic costs of opioid addiction, it is imperative that not only potential therapeutic targets are identified, their potential sex-dependent effects also need to be investigated.

1.1.1 Current treatment options

Current treatment for opioid abuse is focused on managing opioid dependence and withdrawal symptoms through the use of opioid agonists like buprenorphine or methadone (Murnion 2012). While these treatments can prevent withdrawal and relapse (Hurd et al. 2015) they also have the same side effects as long-term opioid use, including the development of opioid toxicity (Murnion 2012). Further, patients on replacement therapy have a higher incidence of poly-drug abuse and increased risk of catching blood-borne diseases (Murnion 2012). Importantly, current treatments do not address the neurochemical changes that result from long-term opioid abuse and the underlying pathophysiology of addiction. Greater understanding of these neurochemical changes and identification of potential treatment targets is required to improve management of the public health crisis.

1.1.2 Sex differences in opioid addiction

There are significant differences between sexes in the development and pattern of opioid use. Men report a higher lifetime use of opioids (Back et al. 2010), are more likely to use non-prescription opioid drugs (Back et al. 2010), and have a higher incidence of opioid related

deaths (Roxburgh et al. 2017). However, women are more likely to use prescription opioids (Serdarevic, Striley & Cottler 2017), which correlates with their higher incidence of chronic pain conditions (Goetz, Becker & Mazure 2021) and women with substance use disorder are more likely to report opioids as their primary drug of choice (Kokane & Perrotti 2020). In addition to these differences in patterns of use there are significant differences between the sexes in risk factors for opioid abuse. Due to chronic pain conditions women are more likely to be prescribed opioid pain relievers for long periods of time (Goetz, Becker & Mazure 2021) which increases the risks of developing opioid addiction. Additionally, women have been shown to more rapidly transition toward opioid dependence and report stronger withdrawal symptoms (Kokane & Perrotti 2020). There is evidence that this is related to sex differences in the pain regulation and symptoms of opioid withdrawal that are mediated by KOR (Chartoff & Mavrikaki 2015). Further, women are more likely to relapse after a period of abstinence and are less likely to seek treatment for opioid abuse (Kokane & Perrotti 2020). However, it is unclear whether social factors are at play since opioid abuse is more stigmatised for men than women (Weeks & Stenstrom 2020) which may encourage men to seek treatment. Overall, although men more frequently develop opioid addiction, women are more susceptible to opioid abuse patterns. Further investigation is required to isolate the specific mechanisms involved in these differences, and to understand how they impact on response to treatment for opioid abuse.

1.2 The Endogenous Opioid System

Opioid drugs act on the endogenous opioid system to mediate their rewarding effects. The endogenous opioid system is composed of receptors and peptide chains found in the central, peripheral, and enteric nervous systems. It has several important functions including pain relief, mood regulation, stress modulation and reward (Bodnar 2017) which all play a significant role in the development of addiction pathology (Trigo et al. 2010). The primary peptides of the system are ß-endorphin, met- and leu-enkephalin, dynorphins, and neo-endorphins as well as three precursor peptides proopiomelanocortin, proenkephalin, and prodynorphin (Kieffer & Gaveriaux-Ruff 2002). There are three types of opioid receptors, μ- (MOR), δ- (DOR) and κ- (KOR), which are highly expressed in the ventral tegmental area (VTA), nucleus accumbens (NAc), hypothalamus and amygdala (Ghozland et al. 2002). MOR, DOR and KOR are G-coupled

receptors that, when activated, inhibit adenylyl cyclase activity which blocks calcium (Ca^{2+}) channels and activates potassium (K^{+}) channels (Rodríguez, Mackie & Pickel 2001). While DOR has been shown to play a role in the maintenance of opioid reward (Bhargava 1991), MOR has the highest implication for opioid addiction (Kieffer & Gaveriaux-Ruff 2002) due to its involvement in opioid reward and involvement in opioid tolerance and dependence.

MOR activation is highly implicated in the rewarding effects of opioids as well as the development of opioid tolerance and dependence. Opioid class drugs have a high affinity for MOR (Law, Wong & Loh 2000; Le Merrer et al. 2009) and MOR activation can cause dopamine release in the VTA via inhibition of GABA and/or direct excitement of dopamine neurons, both of which cause the rewarding effects of opioids (Margolis et al. 2014). Additionally, MOR increases presynaptic dopamine release in the striatum (Kuschinsky & Hornykiewicz 1974) and enhances AMPA-mediated transmission of dopamine receptor 1 (D1) while reducing the excitatory input of dopamine receptor 2 (D2) in the NAc shell (Hearing et al. 2016). Common side effects of opioid drugs, like sedation, analgesia and respiratory depression are caused by an inhibitory effect of MOR within the locus coeruleus (LC) which regulates many homeostatic functions including sleep-wake cycle and norepinephrine release (Scavone, Sterling & Van Bockstaele 2013).

Repeated activation of MOR decreases the expression of the cyclic-AMP (cAMP) pathway by decreasing the phosphorylation of cAMP response element-binding protein (CREB) which leads to the up-regulation of norepinephrine synthesis (Scavone, Sterling & Van Bockstaele 2013). Symptoms of opioid withdrawal are caused by a surge in noradrenergic activity between the LC and frontal cortex related to CREB activity (Scavone, Sterling & Van Bockstaele 2013). Tolerance and dependence to opioids are associated with MOR desensitisation via elevated cAMP and g-protein decoupling rather than downregulation of MOR expression (Koch & Hollt 2008; Listos et al. 2019). However, chronic MOR activation is associated with down regulation of K^{+} channels which in turn increases the excitability of dopamine neurons in the VTA while also reducing the size of the neurons and their dopamine output to target regions (Mazei-Robison et al. 2011). Abstinence from morphine reduces expression of the immediate early

gene *c-Fos* expression in the VTA (Becker, JAJ, Kieffer & Le Merrer 2017). MOR can mediate glutamatergic activity in the NAc via Ca^{2+} and K^{+} inhibition and chronic MOR activation is linked to glutamatergic dysfunction (Chartoff & Connery 2014). As disruption of glutamate homeostasis is an important step in addiction pathology (Kalivas 2009) this provides a potential link between MOR pharmacology and the development of persistent MOR agonist abuse. Self-administration of morphine is reduced in mice with a knock out (KO) of MOR (Becker, A et al. 2000) which suggests that MOR is necessary for the motivation to take opioid drugs. While opioid drugs primarily target MOR, addiction occurs due to MOR interactions with several other receptors and neural pathways.

1.3 Addiction

Drug addiction is a complex process involving many physiological changes in the brain that correspond to addiction-associated behaviours. Humans have a natural reward system that promotes evolutionarily beneficial behaviour like food consumption, social interaction and sex (MacNicol 2017). This system has an important and beneficial function in learning, memory and habit formation that is high-jacked by the "high" produced by drugs of abuse and, for susceptible individuals, habitual drug use combines with various environmental and genetic risk factors that lead to drug addiction (Piazza & Deroche-Gamonet 2013). Piazza and Deroche-Gamonet, 2013, described a general theory of the transition to addiction based on three stages. Firstly, drug-use is sporadic and part of recreational activities and does not impact general functionality. Indeed, psychoactive drugs can be effectively used to cope with stress and improve cognitive function in most of the population (Muller 2018). However, a small percentage of drug users, 10-30% depending on the drug of choice, have a susceptibility towards problematic drug use (Piazza & Deroche-Gamonet 2013). Here, at the second stage of transition to addiction, drug use and drug seeking increase in frequency but the negative impacts are still able to be disguised (Piazza & Deroche-Gamonet 2013). At this stage environmental and social cues related to drug use can trigger drug craving and drug-seeking behaviour (Bechara et al. 2019). The third stage occurs when there is a loss of control over drug-taking behaviour (Piazza & Deroche-Gamonet 2013). There is an increase in anxiety when the drug is not available and drug seeking comes at the expense of most other activities, like work or social bonding (De Sa Nogueira, Merienne & Befort

2019). There is also an increase in risk taking in order to achieve a drug "high", which includes undertaking illegal activities and taking increasing doses of the drug which could lead to overdose (De Sa Nogueira, Merienne & Befort 2019). Addiction is primarily characterised by a cycle between drug use, abstinence from drug use, drug craving and relapse back into drug use. This cycle is also driven by physiological changes in the systems involved in reward, memory, learning and habit formation (Kalivas & Volkow 2005). Addiction is also associated with an impairment in extinction learning which makes quitting and maintaining abstinence more difficult as cue-induced drug craving and ease of reinstatement increases risk of relapse (Myers & Carlezon 2010). Therefore, understanding the effects of opioids on these systems is an important step in identifying therapeutic targets to treat opioid addiction.

Reward circuitry and the effect of opioids on neurotransmitters is key to the development of opioid addiction-relevant behaviour (Figure 1). The rewarding properties of opioids begins with the stimulation of dopaminergic neurons in the VTA, via MOR-induced via inhibition of GABA and/or direct excitement of dopamine neurons (Di Chiara & Imperato 1988). These neurons are mediated by GABAnergic interneurons in the VTA which modulate reward and aversion by the projection site of the neuron (Lammel, Lim & Malenka 2014; Lammel et al. 2012; Matsumoto et al. 2016), as rewarding stimuli activates neurons projecting into the NAc (Di Chiara & Imperato 1988; Lammel, Lim & Malenka 2014) and aversive stimuli activate those neurons projecting into the prefrontal cortex (PFC) (Matsumoto et al. 2016). The motivational value of stimuli is modulated by the medium spiny neurons (MSN) of the NAc core (Cooper, Robison & Mazei-Robison 2017) while the behavioural responses to chronic drug abuse are regulated by those of the NAc shell (Cooper, Robison & Mazei-Robison 2017). Goal-directed behaviour, like drug seeking, is mediated by glutamatergic neurons projecting from the PFC to the NAc (Kalivas & Volkow 2005) and dorsomedial striatum (Corbit, Leung & Balleine 2013; Terra et al. 2020; Yin, Knowlton & Balleine 2004) while environmental cues associated with drug taking are reinforced via glutamatergic projections from the hippocampus (HPC) to the NAc (Kalivas & Volkow 2005). Additionally, VTA dopaminergic neurons projecting to the amygdala facilitate drug reinforcement by encoding the association of rewarding stimuli with environmental cues (Kim et al. 2016). Neurons projecting from the hippocampus to the VTA respond to previously rewarding stimuli to

reinforce these drug cues (Kim et al. 2016). Additionally, there is an increased stress-response that encourages drug use due to chronic corticotropin-releasing factor (CRF) over expression in the amygdala that causes hyperactivity in the hypothalamic-pituitary-adrenal (HPA) axis (Flandreau et al. 2012). Changes to the synaptic and structural plasticity in the HPC-PFC-amygdala circuitry means that drug-associated cues acquire incentive salience, meaning that environmental cues or paraphernalia associated with drug taking increase motivation for drug reward (Luo et al. 2013), which when combined with the increased stress response drives drug-seeking behaviour (Bechara et al. 2019). Addiction is also associated with changes in neuroplasticity via the long-term potentiation (LTP) i.e. increased synaptic strength, and long-term depression (LTD) i.e. decreased synaptic strength of glutamatergic synapses in the VTA, NAc, PFC and amygdala (Saal et al. 2003). Changes in LTP and LTD are associated with the expression of addiction-relevant behaviour (Kasanetz 2010; Niehaus, Murali & Kauer 2010; Saal et al. 2003). Therefore, addiction-relevant behaviours are primarily driven by physiological changes in the dopaminergic and glutamatergic systems that encourage habit formation, cause cognitive impairments and reduce impulse control.

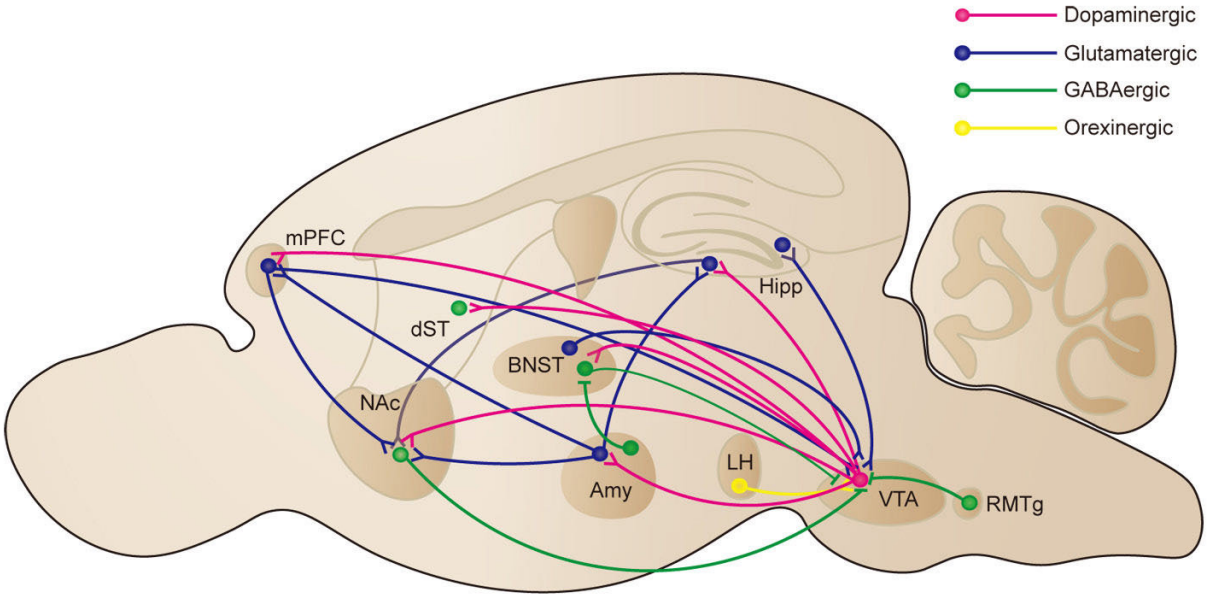

Figure 1: Diagram of the reward circuits and neurotransmitters involved in morphine reward in rodent brain taken from Kim et al. (2016). Dopaminergic projections (pink) from the ventral tegmental area (VTA) to the nucleus accumbens (NAc), medial prefrontal cortex (mPFC), hippocampus (Hipp), bed nucleus of the stria teminalis (BNST), amygdala (Amy) and dorsal striatum (dST) modulate glutamatergic (blue) and GABAergic (green) neurons. GABAergic transmission from the NAc to the VTA is modulated by glutamatergic projections from the mPFC and Amy, and VTA dopaminergic neurons are modulated by glutamatergic transmission from the mPFC and BNST. Orexinergic (yellow) neurons in the lateral hypothalamus (LH) and GABAergic neurons in the rostromedial tegmental nucleus (RMTg) also modulate VTA dopaminergic neurons.

1.3.1 Dopaminergic System Involvement in Addiction

Dopamine is the neurotransmitter that has been extensively researched in the context of substance abuse. Dopamine activates dopamine receptors which are g-coupled proteins that can be categorised as D1 receptors, that increase cAMP signalling, open sodium channels and increase excitation of neurons (Tejeda et al. 2017), or D2, that decrease cAMP, open potassium channels and increase inhibition of neurons (Tejeda et al. 2017). Acute administration of a variety of drugs is associated with an increase in the synaptic level of dopamine in the NAc from VTA afferents (Volkow et al. 2004) which is essential to encouraging subsequent drug-seeking behaviour. Activation of MOR alters the function of D1, but not D2 receptors, by inhibiting the frequency of excitatory post-synaptic currents (James et al. 2013) suggesting that D1 plays a significant role in the initial rewarding and reinforcing effects of opioids. Chronic drug use causes adaptive changes in this circuitry, such as the downregulation of VTA to NAc dopamine release (MacNicol 2017), which reduces sensitivity to natural rewards and encourages individuals to seek drug reward instead to achieve a sufficient 'high' (MacNicol 2017). Additionally, with repeated drug use, instead of simply indicating reward, dopamine signalling starts predicting reward (Volkow et al. 2012; Volkow et al. 2011). This is because repeated drug use creates an increase in responsiveness to drug-associated cues that increases the ability of those cues to increase dopamine release in the dorsal striatum and cause relapse (Sherman et al. 1980; Smith & Aston-Jones 2014; Volkow et al. 2012). Eventually, the increased dopamine activity leads to a reduction in the baseline extracellular dopamine available in the NAc and the expression of D2 in the VTA and striatum (Smith & Aston-Jones 2014; Spielewoy et al. 2000) which is associated with an increase in impulsive behaviour to obtain reward.

1.3.2 Glutamatergic System Involvement in Addiction

While the role of dopamine in addiction has been understood for a long time, research in the past decade has increasingly focused on the role of the glutamatergic system. Glutamate is the primary excitatory neurotransmitter of the central nervous system and glutamate receptors are abundant throughout addiction-relevant brain regions (Quintero 2013). Glutamate also plays a key role in learning and habit formation (Quintero 2013). The glutamatergic system is implicated in drug reward as glutamatergic activity in the VTA modulates dopaminergic activity

in the NAc (Tzschentke). Additionally, opioid use increases glutamate transmission in the NAc (Chartoff & Connery 2014) which, with chronic abuse of opioids, impairs cystine-glutamate exchange, reducing the availability of non-synaptic glutamate (Kalivas 2009) and creates a state of impaired glutamate homeostasis (Kalivas 2009). In mice, acute drug administration is associated with persistent long-term potentiation of the ionotropic glutamate α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor in the VTA (Saal et al. 2003). The glutamatergic system is of particular interest for opioid addiction as glutamatergic receptors in the NAc play an important role in the formation and maintenance of opioid memory and opioid-seeking behaviour via activation of the ionotropic glutamatergic receptor N-methyl D-aspartate (NMDA)(Peters & De Vries 2012). Activation of NMDA is also required for the increased CREB activity that occurs in response to MOR activation (Chartoff & Connery 2014), indicating that glutamatergic activity plays a key role in the acquisition of opioid reward. Chronic drug use, including opioids, also alters the expression of glutamate receptors, particularly AMPA and NMDA in the NAc and PFC (Ahmadi, Rafieenia & Rostamzadeh 2016; James et al. 2013) which is associated with susceptibility to cue-induced relapse-like behaviour. Repeated morphine administration is associated with an increase in the expression and synaptic strength of AMPA in NAc neurons (James et al. 2013), which is associated with increased LTP via an increase in GABA release from inhibitory neurons in the VTA (Nugent, Penick & Kauer 2007). Therefore, specific glutamate receptors have a significant role in the neuroplastic and other neuroadaptive changes associated with the development of opioid addiction. Investigating the role of particular glutamate receptors could provide greater insight into the role of the glutamatergic system on opioid addiction and provide targets for the development of therapies to treat opioid addiction.

1.3.3 Involvement of the Metabatropic Glutamate 5 Receptor in Opioid Abuse

The metabotropic glutamate 5 receptor (mGlu5) has been identified as a potential target for treating addiction-relevant behaviour. Indeed, low availability of mGlu5 is associated with an increased risk of developing addiction in humans (Cox et al. 2020). mGlu5 is a group 1 metabotropic glutamate receptor that, like opioid receptors, is a g-coupled protein (Brown, Mustafa, et al. 2012; Brown, Stagnitti, et al. 2012). Primarily located on postsynaptic neurons

in areas of the brain associated with learning and reward, like the NAc, dorsal striatum and hippocampus (Shigemoto et al. 1993; Valerio et al. 1997), mGlu5 activates phospholipase C and has an excitatory effect on cells via the potentiation of NMDA currents (Anwyl 1999). As it is expressed in reward learning pathways and plays a key role in synaptic plasticity mGlu5 is implicated in drug-related learning and the development of drug-seeking behaviour (Bird et al. 2008).

mGlu5 has also been implicated in several different stages of opioid abuse and addiction. For example, mGlu5-mediated protein kinase C (PKC) activation and intracellular Ca^{2+} mobilisation are critical factors for the expression of opioid reward due to modulating the excitability of dopamine neuron (Popik 2002). Further, chronic morphine use is associated with an increase in mGlu5 protein expression in the limbic forebrain (Aoki et al. 2004) and morphine administration has been shown to up-regulate mGlu5 protein expression in the NAc shell, thalamus, hypothalamus and amygdala (Zanos et al. 2016) in mice. Additionally, mGlu5 modulates locomotor activity as systemic administration of mGlu5 antagonists increase locomotor activity (Guimaraes et al. 2015) and inhibit the increase in locomotor activity seen following repeated administration of morphine (Kotlinska & Bochenski 2007). mGlu5 has strong sex-dependent effects as the dopamine-enhancing effects of the female gonadal hormone oestradiol on amphetamine reward are mediated by mGlu5 (Song et al. 2019). However, the sex-dependent effects of mGlu5 on opioid addiction-relevant behaviour have not yet been investigated. Clinical investigation of the effects of mGlu5 in addiction is difficult due to ethical issues, therefore, animal models of addiction are used instead.

1.4 Animal models of addiction

Animal models of addiction and substance abuse-like behaviour allow the establishment of a highly standardised environment to investigate genetic risk factors and investigate potential treatment targets. There are several animal behavioural models used to study addiction-relevant behaviour, which are based on behavioural similarities to humans (Muller 2018). Self-administration and conditioned place preference (CPP) are two well-established models that assess a variety of features associated with drug addiction, including the rewarding

properties of drugs and mechanisms involved in compulsive drug-seeking. Additionally, the effect of addiction on extinction learning, where animals learn that a drug-paired environment (CPP) or behaviour (self-administration) is no longer associated with the drug (Poltyrev & Yaka 2013). Importantly, extinction does not involve forgetting the previous association but learning the new context that the drug is unavailable (Nic Dhonnchadha & Kantak 2011). Self-administration and CPP can be used to assess the different and complementary mechanisms behind the development of drug-associated memories and behaviour.

1.4.1 Conditioned Place Preference

CPP is used to study the rewarding properties of drugs (Rutten, Van Der Kam, De Vry, Bruckmann, et al. 2011). It is a well-established paradigm for studying drug memory and the role of environmental cues on addiction-relevant behaviour (McKendrick & Graziane 2020). Acquisition of drug-associated memory, as well as extinction and reinstatement (a model of relapse) can be modelled in CPP. CPP involves the external administration of the drug and is, therefore, a form of Pavlovian conditioning; as the animal associates the rewarding effects of the drug with the environmental cues (Huston et al. 2013). The expression of this learning is measured by an increase in time spent in the drug-paired environment. Following acquisition animals can be put into extinction training, where repeated exposure to the apparatus without administration of the drug following conditioning is associated with a decrease in time spent in or near a drug-paired environment (Rutten, van der Kam, De Vry & Tzschentke 2011). This makes CPP effective for investigating the role of memory and learning processes in drug abuse, particularly the impact of environmental cues on the formation of habits (McKendrick & Graziane 2020). Extinction learning is also weaker than the original conditioning, and addiction-relevant behaviour can easily be reinstated via several mechanisms including the reintroduction of the drug (Mueller, Perdikaris & Stewart 2002). This association has clinical significance as human research shows there is a strong association with environmental cues and motivation to use the drug (Napier, Herrold & de Wit 2013). CPP is particularly relevant for assessing the effects of spatial learning when using visual, but not tactile, environmental cues (Cunningham, Patel & Milner 2006), and spatial learning is influenced by mGlu5 activity (Naie & Manahan-Vaughan 2004). Another key benefit to CPP is the ability to study the effect of environmental cues on drug craving which are

associated with susceptibility for relapse (McKendrick & Graziane 2020). Therefore, CPP can be used to investigate the effect of a treatment or intervention on the formation and persistence of the rewarding effects of drug memory as well as susceptibility to drug-induced relapse.

1.4.2 Self-administration

Self-administration models can be used to study the rewarding effects of drugs as well as compulsive drug-seeking behaviour. Self-administration is an operant model, where drug-reward is associated with a specific behaviour (Panlilio & Goldberg 2007), and involves the active decision to administer the drug, in contrast the CPP. Animals are trained to administer drugs orally (Elmer et al. 2010) or intravenously via a jugular vein catheter (Huang et al. 2019) by pressing a lever or by nose poking into a recessed magazine (Navarro et al. 2001; Nguyen et al. 2019). Self-administration protocols usually require a pattern of lever pressing and a discrimination between active and inactive levers to show that the drug administration is deliberate (Chistyakov & Tsibulsky 2006). Successful drug administration is often paired with an environmental cue, e.g. a light or a sound that comes on or off (Navarro et al. 2001). This cue can be used to reactivate lever pressing after a period of abstinence or extinction (Nguyen et al. 2019) where the animal learns that the drug is not available and they stop pressing the lever (Farrell, Schoch & Mahler 2018). In addition to cue-induced reinstatement, self-administration can be reinstated by exposure to stressful stimuli, like foot shock (Shalev et al. 2001), or administration of a priming dose of the drug (Ribeiro Do Couto et al. 2003). The various reinstatement protocols can be used to investigate the effect of environmental cues, stress and drug exposure on drug-craving and risk of relapse and, therefore, self-administration can be used to investigate the effect of a treatment or intervention on drug-reward and the development of addiction-relevant behaviour.

1.4.3 Pharmacological mGlu5 studies

There have been several pharmacological studies that demonstrate that mGlu5 can play a role in the development of opioid addiction-relevant behaviour in rodents summarised in Table 1. The non-competitive mGlu5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) dose-dependently inhibits morphine reward measured by CPP in rodents (Aoki et al. 2004;

Table 1. Summary of pharmacological investigation of mGlu5 and opioids in rodents

Opioid and dose	Ligand and dose	Pharmacological class	Species/Strain	Model	Effect	References
10 mg/kg Morphine	10 and 30 mg/kg MPEP	mGlu5 antagonist	Male C57BL/6J/Han/ Imp mice	CPP	dose-dependently inhibited acquisition	(Popik et al. 2003)
10 mg/kg Morphine	1, 5 and 10 mg/kg MPEP	mGlu5 antagonist	Adult male C57Bl/6J and DBA/2J mice	CPP	no effect	(McGeehan & Olive 2003)
5 mg/kg Morphine	100 nmol MPEP *intracerebroventricular administration	mGlu5 antagonist	Adult male ICR mice	CPP	attenuated acquisition	(Aoki et al. 2004)
10 mg/kg Morphine	10 mg/kg MPEP	mGlu5 antagonist	Adult male Sprague Dawley rats	CPP	expression of CPP state-dependent	(Herzig & Schmidt 2004)
30 mg/kg Morphine	1, 3 and 10 mg/kg MTEP	mGlu5 antagonist	Male C57BL/6J mice	Withdrawal	dose-dependently attenuated symptoms of naloxone-induced morphine withdrawal	(Palucha, Branski & Pilc 2004)
10 mg/kg Morphine or 37.5 mg Morphine by subcutaneous	5 and 10 mg/kg MTEP	mGlu5 antagonist	Male Swiss albino mice	Sensitisation Withdrawal	dose-dependently inhibited expression of locomotor sensitisation dose-dependently attenuated symptoms of morphine withdrawal	(Kotlinska & Bochenski 2007)
0.05 mg/kg/infusion Heroin	1.25-20 mg/kg MPEP	mGlu5 antagonist	Adult male Long Evans rats	Self-administration	dose-dependently reduced self-administration	(van der Kam, Elizabeth L., De Vry & Tzschentke 2007)
0.05 mg/kg/infusion Heroin	1 mg/kg/infusion MPEP	mGlu5 antagonist	Adult male Long Evans rats	Self-administration	self-administration stable after replacement with MPEP	(van der Kam, E. L., De Vry & Tzschentke 2009b)
0.05-0.5 mg/kg Heroin	10 mg/kg MPEP	mGlu5 antagonist	Adult male Sprague Dawley rats	CPP	acquisition potentiated extinction unaffected induced reinstatement	(van der Kam, E. L., De Vry & Tzschentke 2009a)
0.0125-0.5 mg/kg Heroin	10 mg/kg MPEP	mGlu5 antagonist	Adult male Sprague Dawley rats	CPP	potentiated acquisition	(Rutten, Van Der Kam, De Vry, Bruckmann, et al. 2011)
3 mg/kg Morphine	1, 3 and 10 mg/kg MTEP	mGlu5 antagonist	Adult male Wistar rats	CPP	attenuated acquisition no effect on locomotor sensitisation	(Veeneman et al. 2011)
0.1 mg/kg/infusion Morphine	20 mg/kg MTEP	mGlu5 antagonist	Adult male CD1 mice	Self-administration	attenuated self-administration reduced morphine-seeking following abstinence	(Brown, Stagnitti, et al. 2012)

Herzig & Schmidt 2004; Popik 2002). Additionally, a moderate dose of MPEP can reduce heroin self-administration in rats (van der Kam, Elizabeth L., De Vry & Tzschentke 2007). However, a follow-up study showed that this dose potentiated heroin reward under a place preference paradigm (van der Kam, E. L., De Vry & Tzschentke 2009a). Importantly, MPEP treatment at these doses did not induce CPP (Herzig & Schmidt 2004; Popik 2002) but did potentiate opioid reward by lowering the dose that would induce place preference (Rutten, Van Der Kam, De Vry, Bruckmann, et al. 2011). This suggests that mGlu5 modulates sensitivity to opioid reward. Interestingly, MPEP only affected place preference when tested drug-free; when tests were conducted concurrently with morphine administration there was an increase in place preference, indicating that the effects of MPEP on opioid reward are state-dependent (Herzig & Schmidt 2004). Another mGlu5 antagonist 3-[(2-methyl-1, 3-thiazol-4-yl) ethynyl] pyridine (MTEP) dose-dependently blocked morphine place preference in rats (Veeneman et al. 2011) and attenuated self-administration in mice (Brown, Stagnitti, et al. 2012). MTEP is more selective for mGlu5 and has fewer off-target effects than MPEP, particularly in the inhibition of NMDA receptors (Lea & Faden 2006). This suggests that mGlu5 increases sensitivity to opioid reward.

Morphine-induced locomotor activity was found to be unaffected by MPEP or MTEP (Herzig & Schmidt 2004; Kotlinska & Bochenski 2007; van der Kam, E. L., De Vry & Tzschentke 2009a; Veeneman et al. 2011). However, the expression of morphine locomotor sensitisation was attenuated by MTEP (Kotlinska & Bochenski 2007). MTEP also attenuated the symptoms of naloxone-precipitated morphine withdrawal (Kotlinska & Bochenski 2007; Palucha, Branski & Pilc 2004). Further, while MPEP had no effect on the extinction of heroin CPP (van der Kam, E. L., De Vry & Tzschentke 2009a), it was able to induce reinstatement of previously extinguished heroin CPP (van der Kam, E. L., De Vry & Tzschentke 2009a) and replace heroin self-administration in rats (van der Kam, E. L., De Vry & Tzschentke 2009b). However, MTEP did reduce morphine-seeeking following a period of abstinence in mice (Brown, Stagnitti, et al. 2012). Overall, these studies suggest that mGlu5 plays a significant role in the mediation of opioid reward by modulating opioid-reward and locomotor sensitisation.

Additionally, pharmacological studies have significant limitations including drug specificity, pharmacokinetics and drug interactions. MPEP is a non-competitive antagonist that is more potent than MTEP, another noncompetitive mGlu5 antagonist (Carroll 2008). Oral administration shows that these mGlu5 antagonists are rapidly absorbed and metabolised (Lindemann et al. 2011). Although MTEP is more selective for mGlu5 than MPEP (Carroll 2008), it is unclear whether their effects on opioid reward and drug-seeking behaviour described above were mediated by mGlu5 or another off-target receptor like NMDA which MTEP inhibits at high concentrations (Veeneman et al. 2011). Additionally, the doses used in these studies have been shown to affect spatial learning and memory (Naie & Manahan-Vaughan 2004). This is especially significant as spatial learning is a required component of CPP acquisition when using visual and not tactile cues (Cunningham, Patel & Milner 2006). Therefore, impairment of spatial learning by mGlu5 antagonism would also impair the acquisition of CPP. Another limitation of the available pharmacological studies is the limited data on sex differences due to a tendency to avoid testing female mice in order to increase standardisation. This is an important oversight as there is some evidence that oestrogen receptor mediation of group I mGlu receptors plays a significant role in sex differences seen in drug addiction (Tonn Eisinger et al. 2018). Overall, these studies do show there is a potential role for mGlu5 in the development of opioid addiction. However, to overcome issues of receptor specificity, drug tolerance and drug pharmacokinetics, a genetic model needs to be considered.

1.4.4 mGlu5 Knockout (KO) mice

Mice with a homozygous knockout (KO) of the mGlu5 receptor provide a genetic model to study how the mGlu5 receptor modifies addiction-relevant brain function and behaviour for opioids. Several studies indicate that these mice display addiction-relevant behavioural and brain changes for drugs such as alcohol, cocaine and methamphetamine. For example, mGlu5 KO mice showed reduced consumption of ethanol compared to wild type (WT)-like mice but an increased preference for a lower dose of ethanol compared to WT-like mice (Bird et al. 2008). This suggests that the reduced consumption may be due to an increased sensitivity to the effects of ethanol rather than reduced ethanol reward. mGlu5 KO mice also have a reduced baseline AMPA/NMDA ratio (Bird et al. 2010) and the cocaine-induced increase of the AMPA/NMDA ratio in

VTA cells is absent in these mice (Bird et al. 2010). This suggests that mGlu5 KO mice have a reduction in drug-induced neuroplasticity. mGlu5 also plays a role in the mediation of drug-induced locomotion, with mGlu5 KO mice display a delay in cocaine-induced hyperlocomotion in response to acute administration of cocaine (Bird et al. 2010). However, there is no difference in the expression of cocaine-induced locomotor sensitivity (Bird et al. 2010), which suggests that the reduced drug-induced neuroplasticity in these mice has no effect on behavioural sensitisation. Importantly these mice also show a deficit in extinction learning for psychostimulants, which has implications for the abstinence-relapse cycle that characterises addiction. mGlu5 KO mice show an impaired extinction for cocaine paired environments, increased motivation for cocaine self-administration and a stronger reinstatement of cocaine memory (Bird et al. 2014; Bird et al. 2010). These correlate with an increased susceptibility to drug-taking as well as an increased risk of drug-craving and relapse into drug use during abstinence (Farrell, Schoch & Mahler 2018). The persistence in drug-related memories in mGlu5 KO mice has also been observed for methamphetamine, suggesting that mGlu5 may play a role in reducing methamphetamine use (Chesworth et al. 2013). These studies show that mGlu5 may play an important role in drug-related learning and memory, and mGlu5 KO mice show a predisposition to addiction-relevant behaviour, like increased drug-seeking and impaired extinction learning, for several different drug classes. However, there are no studies on the behavioural or molecular responses of mGlu5 KO mice to opioid drugs, including to opioid reward, opioid memory and opioid-induced locomotions, as well as sex-differences in their response to opioid drugs.

1.5 Hypotheses

It is hypothesised that mGlu5 KO mice will be more sensitive to morphine reward, measured as an increased locomotor response and a higher preference for the morphine-paired environment compared to WT-like littermates. Additionally, due to their impaired extinction learning phenotype mGlu5 KO mice will show a persistence of morphine memory compared to WT-like littermates, similar to that observed for psychostimulants. It is also hypothesised that there will be sex differences evident in the expression of morphine reward and persistence in morphine memory, with female mice showing a higher preference for the morphine-paired environment than males.

1.6 Aims of Thesis

This thesis aimed to determine the role of mGlu5 in the underlying biology of opioid addiction, particularly its role in opioid-induced locomotion, opioid reward and opioid-environment memory. It will also assess the effect of mGlu5 deletion on the persistence of opioid memory in order to understand the impact of the receptor on the abstinence-relapse cycle characteristic of addiction. Additionally, this thesis aims to assess sex differences in opioid related learning and addiction-relevant behaviour due to the established effect of sex on susceptibility to opioid addiction.

Chapter 2: Methods

2.1 Animals

Experiment 1 used experimentally naïve adult male C57BL/6J from the Animal Resources Centre, Perth, Australia (Table 2). Experiments 2 and 3 used experimentally naïve adult male and female mGlu5 KO mice and WT-like littermates from Australian BioResources, Moss Vale, Australia (Table 3). All WT-like and mGlu5 KO mice came from a heterozygous breeding colony on a C57BL/6J background (Grm5tm1Rod; stock 003558) that was backcrossed >10 generations to the C57BL/6J background (Bird et al. 2014; Chesworth et al. 2013). Experimental subjects were genotyped by Garvan Molecular Genetics according to established genotyping protocols. Experiment 1 used only male mice as it was a validation study to find an appropriate dose for further testing in mGlu5 KO mice. Male and female mGlu5 KO mice were used to test for sex differences in mGlu5 modulation of opioid reward in Experiment 2 and Experiment 3.

Table 2. Number of mice used in Experiment 2 and 3.

Treatment Group	Number of male C57BL/6J mice
1 mg/kg morphine	12
2 mg/kg morphine	12
5 mg/kg morphine	12
10 mg/kg morphine	12

Table 3. Number of mice used in Experiment 2 and 3.

Experiment	Male WT-like mice	Male mGlu5 KO mice	Female WT-like mice	Female mGlu5 KO mice
Experiment 2	12	11	10	7
Experiment 3	14	12	8	9

At least two weeks prior to behavioural testing the mice were transported to the Western Sydney University Animal House where they were kept under a light:dark schedule with lights on 9am – 9pm and red lights during dark phase. The mice were housed in Type 1284B Tecniplast

filter top cages in groups of 2-3 per cage. They were provided with corn cob bedding, a wire lid and red domes (Bioserv, Frenchtown, USA) that provided climbing opportunities, and tissues for nesting material. Food and water were available *ad libitum*. The Western Sydney University Animal Care and Ethics Committee approved the present experiments (ACEC #A13865; biosafety #B12856) for the research and animal care procedures as per the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

2.2 Drugs

Morphine hydrochloride (In vitro technologies, Noble Park VIC, Australia) was diluted in 0.9% saline, with 0.9% saline used as the drug-free control solution. Morphine was administered in doses of 1, 2, 5 and 10 mg/kg. All doses of drugs were delivered via intraperitoneal (i.p) injection (rotating the injection site with each injection) for a volume of 10 ml/kg body weight using a 0.1 mL syringe.

2.3 Apparatus

A 43.2 x 43.2 cm Med Associates Inc (Vermont, USA) open field was modified to serve as a CPP apparatus. Two equally sized compartments (21.6 x 43.2 cm) were created using black Perspex divider with a 11 x 9 cm opening allowing access to both compartments. Each compartment was distinguished by different wall patterns: the left side of the apparatus had white walls and the right side of the apparatus had white walls with black spots on them (Figure 2). This combination has been shown not to introduce a bias at habituation (Chesworth & Karl 2020; Chesworth et al. 2021). The custom software Activity Monitor by Med Associates Inc was used to automatically record time in zones and locomotor activity using horizontal infrared beams. Software settings for the detection of locomotion were box size: 3; ambulatory trigger: 2; resting delay: 1000 ms; resolution: 100 ms (Karl et al. 2007).

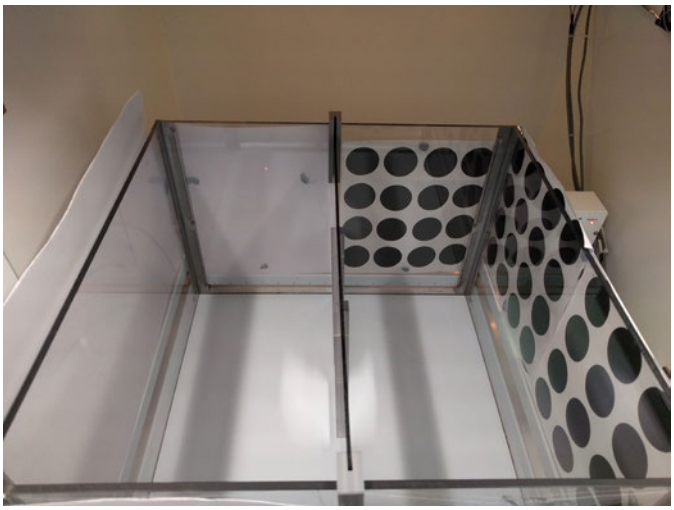

Figure 2: CPP apparatus. Modified open field box (43.2 x 43.2 cm) divided into two compartments (21.6 x 43.2 cm) with white walls on the left side and white walls with black spots on the right.

2.4 General Experimental Procedures

Figure 3 describes the timeline (days 1-6) from habituation to test. The test animals spent 30 mins in their home cages within the experimental room before each session. All sessions were 30 minutes and the apparatus was cleaned with ethanol after each mouse. All experimental procedures were adapted from established protocols (Chesworth & Karl 2020; Chesworth et al. 2021).

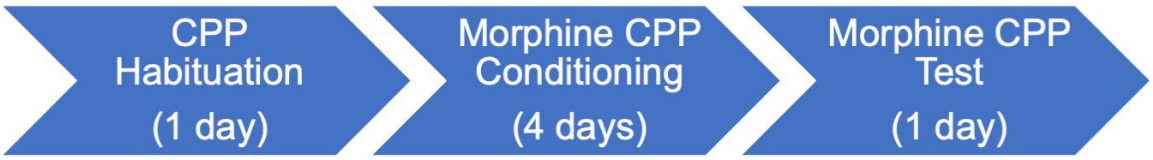

Figure 3: Timeline for general CPP procedure. During habituation mice are allowed free access to both side of the apparatus. For conditioning mice are confined to one side of the apparatus following i.p. injection of saline (am) or morphine (pm). At test mice are allowed free access to both sides.

2.4.1 Habituation

Habituation (day 1) began 30 mins after light phase onset. The opening between compartments was left open for mice to access each side freely. The mice were randomly placed in either compartment and allowed to explore for 30 mins. Between each test animal the apparatus was cleaned with 80% ethanol. The time spent in each side was used to allocate

drug pairings. Mice who showed a preference for one side (>55 % of total time spent) had the opposite side allocated as the drug paired side. Where mice expressed a neutral preference (45-55 % total time spent in either side) they were randomly allocated a drug-paired side such that 50 % of all mice had morphine paired with the left side and 50 % paired with the right (Chesworth & Karl 2020; Chesworth et al. 2021).

2.4.2 Conditioning

The opening between sides of the testing apparatus was closed for all conditioning sessions. Conditioning (days 2-5) started in the morning 30 mins after light phase onset with saline conditioning. For saline conditioning, mice were administered saline i.p. and immediately confined to the saline-paired side. There was a 5h interval between saline and drug conditioning sessions. For drug conditioning, mice were given i.p. injection of morphine and were immediately placed in the morphine-paired side. Locomotor data (distance travelled) was collected to assess for locomotor sensitisation to morphine.

2.4.3 Tests

At Test (day 6), mice were initially placed in the saline-paired compartment with the opening between the sides open and preference for the morphine compartment was assessed via a Preference Score (time in morphine-paired side – time in saline-paired side). For Experiment 2 and 3, the test protocol was repeated on a weekly interval for 4 weeks (Test 2, Test 3, Test 4, Test 5) during abstinence to assess how long preference persisted.

2.5 Experiment 1: Morphine CPP validation

To validate the morphine CPP protocol, 48 adult male experimentally naive C57BL/6J mice underwent morphine CPP using the general experimental methods outlined above (Figure 4). Following habituation mice were randomly assigned to 1, 2, 5 or 10 mg/kg treatment groups to validate morphine CPP and select an appropriate dose for the following experiments.

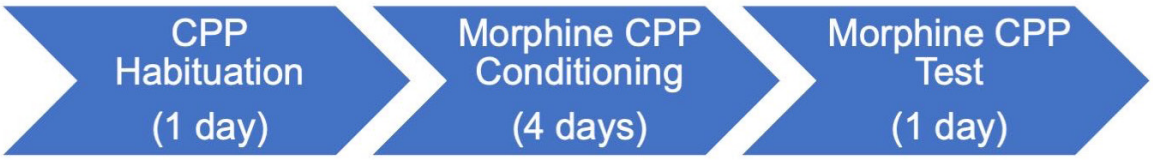

Figure 4: Timeline for Experiment 1. During habituation mice are allowed free access to both side of the apparatus. For conditioning mice are confined to one side of the apparatus following i.p. injection of saline (am) or morphine (pm). At test mice are allowed free access to both sides.

2.6 Experiment 2: Effect of mGlu5 deletion on 5 mg/kg morphine place preference

Based on the results from Experiment 1 a dose of 5 mg/kg morphine was selected to test in mGlu5 KO mice. Adult male and female experimentally naive mGlu5 KO mice and their WT-like littermates (male WT, n = 12; male mGlu5 KO, n = 11; female WT, n = 10; female mGlu5 KO, n =7) were conditioned to associate a particular environment with morphine using the general CPP protocol described above. Mice were then repeatedly tested on a weekly basis during 4 weeks of abstinence with no drug treatment during this period (days 14, 21, 28 and 35; Figure 5). Both genotypes and sexes were assessed for differences in preference and locomotor activity during all habituation, conditioning and test sessions. One female mGlu5 KO mouse was defined as a statistical outlier (> 2 S.D from mean) and was therefore excluded (Jones 2019).

Figure 5: Timeline Experiment 2. During habituation mice are allowed free access to both side of the apparatus. For conditioning mice are confined to one side of the apparatus following i.p. injection of saline (am) or morphine (pm). At test mice are allowed free access to both sides. Following conditioning, mice had free access to both sides of the apparatus and were tested on a weekly basis during abstinence from morphine.

2.7 Experiment 3: Effect of mGlu5 deletion on 10 mg/kg morphine place preference

Following the results from Experiment 2, a second cohort of mGlu5 KO and WT-like mice were tested using 10 mg/kg morphine to test for genotypic differences in morphine sensitivity. Adult male and female experimentally naive mice (male WT, n = 14; male mGlu5 KO, n = 12; female WT, n = 8; female mGlu5 KO, n = 9) were conditioned using the CPP protocol outlined above and then tested weekly during abstinence for 4 weeks (Figure 6). Preference and locomotor activity was assessed for differences in genotype and sex. One male mGlu5 KO mouse was defined as a statistical outlier (> 2 S.D. from mean) and was therefore excluded (Jones 2019).

Figure 6: Timeline Experiment 3. During habituation mice are allowed free access to both side of the apparatus. For conditioning mice are confined to one side of the apparatus following i.p. injection of saline (am) or morphine (pm). At test mice are allowed free access to both sides. Following conditioning, mice had free access to both sides of the apparatus and were tested on a weekly basis during abstinence from morphine.

2.7 Statistical analysis

Behavioural data were analysed using R (Version 4.0.3) (R Core Team 2020; RStudio Team 2019). Two-, three- and four-way repeated measures (RM) analysis of variance (ANOVA) with within factors ‘days’ (conditioning days), ‘drug’ (saline vs. morphine), ‘zone’ (left vs. Right; drug-paired vs. saline-paired), ‘distance’ (distance travelled in cm) or ‘time’ (5-min blocks), and between factors ‘treatment group’ (1 vs. 2 vs. 5 vs. 10 mg/kg), ‘genotype’ (WT-like vs. mGlu5 KO) or ‘sex’ (male vs. female) were conducted (Ameijeiras-Alonso, Crujeiras & Rodriguez-Casal 2021; Coppock 2019; Dag, Dolgan & Konar 2018; Fox & Weisberg 2019; Højsgaard & Halekoh 2020; Lenth 2020; Singmann et al. 2021; Wickham 2016, 2019; Wickham et al. 2020; Wickham & Hester 2020). Where a significant interaction was detected further one-way ANOVA was conducted and Bonferroni post-hoc tests were used to identify group differences at specific time points. Additionally, in Experiment 3, the variability of treatment groups was compared using Levene’s Test, with follow up RM ANOVA on the residual and Bonferroni post hoc tests where appropriate. Variability analysis was also accompanied by Hartigan’s dip-test for unimodality to check for subgroups (Maechler 2021). Data is presented as mean ± standard error of the mean (SEM), and differences were regarded as statistically significant if $p < 0.05$. Post-hoc effects are presented in figures.

Chapter 3: Results

3.1 Experiment 1: Morphine CPP Validation

3.1.1 Habituation

There was no apparatus bias at habituation

Time spent in the left and right sides of the apparatus during habituation is shown in Figure 7A-D. There was no preference for either side [no significant effect of 'zone', $F(1,44) = 2.82$, $p = .510$] . This suggests there was no bias in the apparatus design, in any treatment group.

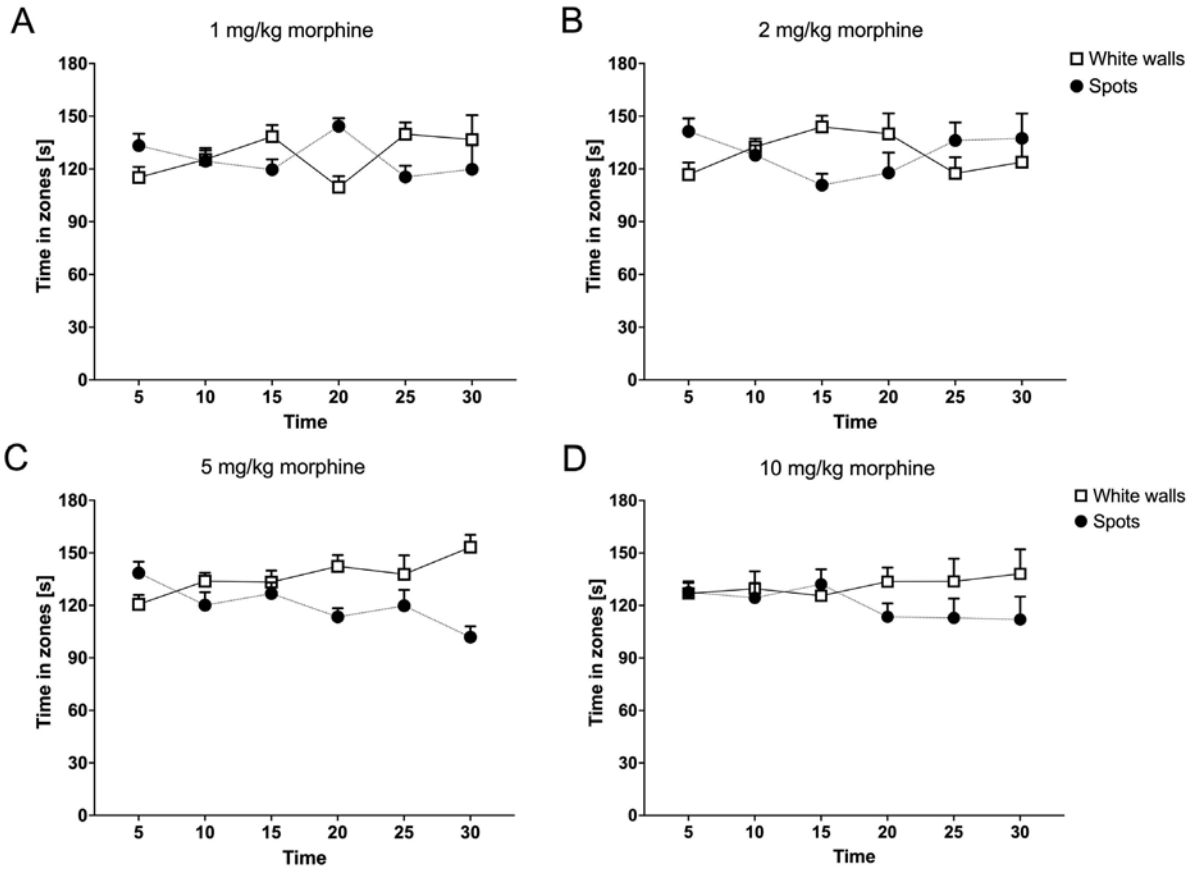

Figure 7: Time spent in zones during habituation in C57BL/6J mice. Time [s] spent in the left (white walls) and right (spots) zones of the apparatus during habituation in mice to be treated with morphine doses: (A) 1 mg/kg, (B) 2 mg/kg, (C) 5 mg/kg and (D) 10 mg/kg. Data presented as means ± SEM and analysed using three-way RM ANOVA.

Locomotor activity was also recorded at habituation. There were no to-be-treatment group differences in distance travelled at habituation [$F(3,44) = 0.91$, $p = .444$] (Figure 8). Locomotor activity decreased over the course of the test [main effect 'time', $F(5,220) = 177.15$, $p < .001$] as the novelty of the apparatus diminished. Together, this suggests that the locomotor activity of the treatment groups was balanced at habituation.

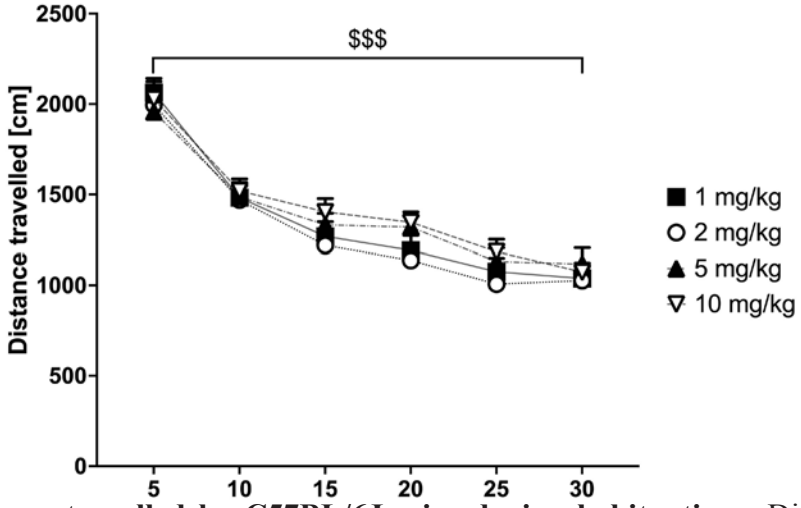

Figure 8: Distance travelled by C57BL/6J mice during habituation. Distance travelled [cm] during habituation session in mice to-be-treated with 1, 2, 5 and 10mg/kg morphine. Data presented as means ± SEM and analysed using two-way RM ANOVA. A significant main effect of 'time' is indicated ($^{\$\$\$}p < 0.001$).

3.1.2 Conditioning

5 and 10 mg/kg morphine induced hyperactivity in C57BL/6J mice

Locomotor sensitisation was measured via distance travelled under saline treatment and morphine treatment on the same day (Figure 9A-D). Morphine administration increased locomotor activity in a dose-dependent manner [main effect of 'treatment group', $F(3, 44) = 20.97$, $p < .001$; 'drug', $F(1, 44) = 54.73$, $p < .001$; significant interaction 'treatment group' and 'drug', $F(3, 44) = 29.89$, $p < .001$]. Locomotor activity increased over morphine conditioning sessions, but not saline conditioning sessions [significant interaction 'drug' and 'days', $F(3, 132) = 7.00$, $p < .001$]. Two-way ANOVA split by 'treatment group' with Bonferroni correction was used to assess dose effects, and found an increase in distance travelled after administration of morphine compared to saline at 5 mg/kg [$F(1,44) = 14.21$, $p < .001$] and 10 mg/kg [F(1,44)

= 130.07, $p < .001$] but not 1 mg/kg [F(1,44) = 0.14, $p = .713$] or 2 mg/kg [F(1,44) = 0.00, $p = .994$]. Furthermore, there was a significant interaction between 'drug' and 'day' for 5 mg/kg [F(3,132) = 2.87, $p = .039$] and 10 mg/kg [F(3,132) = 4.49, $p = .003$] but not 1 mg/kg [F(3,132) = 0.73, $p = .537$] or 2 mg/kg [F(3,132) = 0.68, $p = .564$]. Bonferroni post hoc tests found that distance travelled when administered morphine was significantly increased compared to saline on day 1, day 2, day 3 and day 4 for 5 and 10 mg/kg but not 1 and 2 mg/kg morphine. However, post hoc tests did not detect an increase in distance with repeated morphine treatment at any dose (Figure 9A-D). This suggests that 5 and 10 mg/kg morphine induces hyperactivity in C57BL/6J mice, but no morphine dose induced locomotor sensitisation.

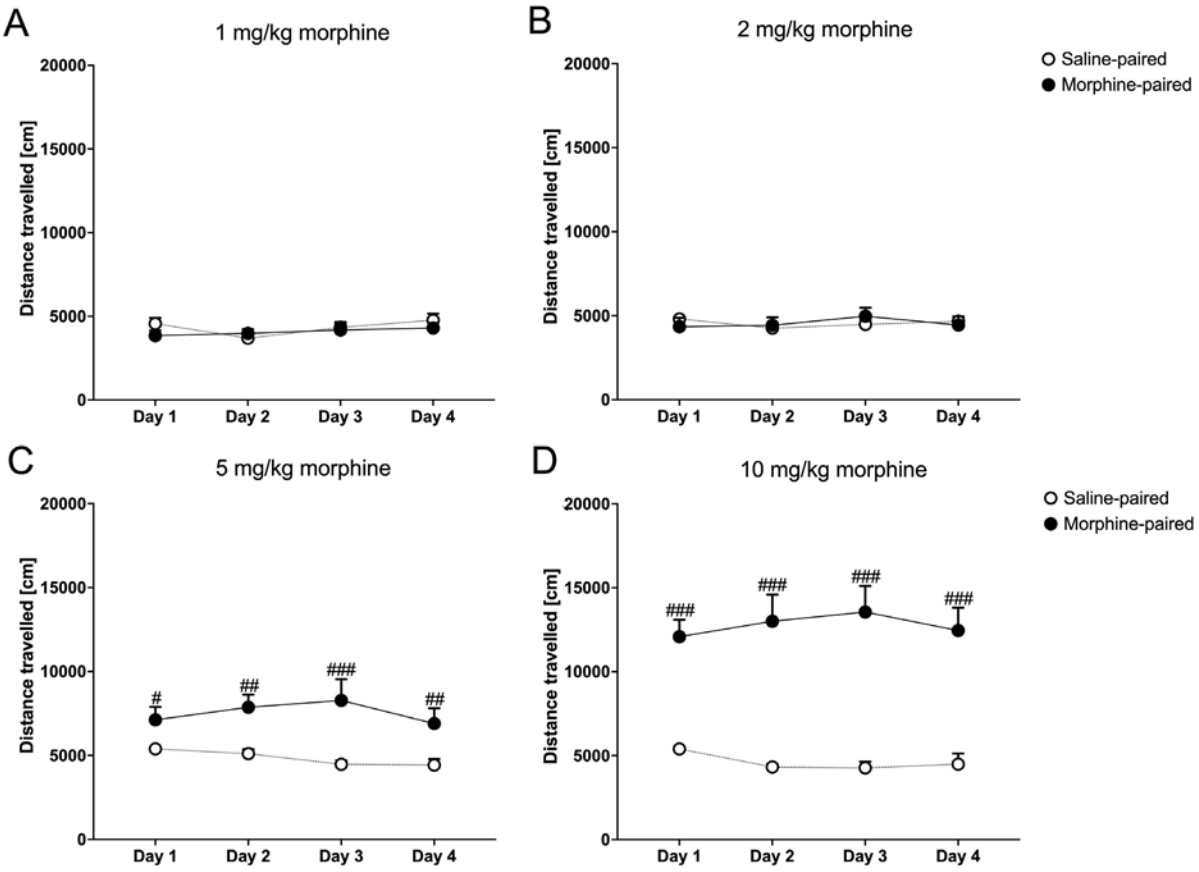

Figure 9: Locomotor response of C57BL/6J to 1, 2, 5 or 10 mg/kg morphine. Locomotor response is measured as distance travelled [cm] over 4 consecutive days during saline and morphine conditioning to (A) 1 mg/kg morphine, (B) 2 mg/kg morphine, (C) 5 mg/kg morphine and (D) 10 mg/kg morphine. Data presented as means ± SEM and analysed using three-way RM ANOVA, followed by Bonferroni post hoc tests where appropriate. Significant effects of 'drug' are indicated ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$).

3.1.3 Test

1, 2, and 5 mg/kg, but not 10 mg/kg, induced place preference in C57BL/6J mice

Place preference was assessed as a positive preference score (time in morphine-paired zone - time in saline-paired zone)(Figure 10). The habituation preference scores were negative due to the biased allocation of drug-paired side. Preference score increased in all treatment groups between habituation and test [main effect of 'day', $F(1, 44) = 20.53$, $p < .001$; but not 'treatment group', $F(3, 44) = 0.62$, $p = .608$]. There were no interactions. Figure 10 shows that 1, 2 and 5 mg/kg induced a positive preference score at test while 10 mg/kg showed a neutral preference score, suggesting that 10 mg/kg did not induce place preference. This interpretation is supported by an one-way ANOVA split by treatment group with a Bonferroni correction, which found a main effect of 'day' for 1 mg/kg [$F(1,44) = 6.71$, $p = .013$], 2 mg/kg [$F(1,44) = 6.85$, $p = .012$] and 5 mg/kg [$F(1,44) = 5.97$, $p = .019$] but not 10 mg/kg [$F(1,44) = 1.99$, $p = .165$]. This suggests that 1, 2 or 5 mg/kg were more rewarding than 10 mg/kg. This, together with the hyperlocomotion observed during conditioning, suggests that 5 mg/kg was the most appropriate dose to be tested in mGlu5 KO mice.

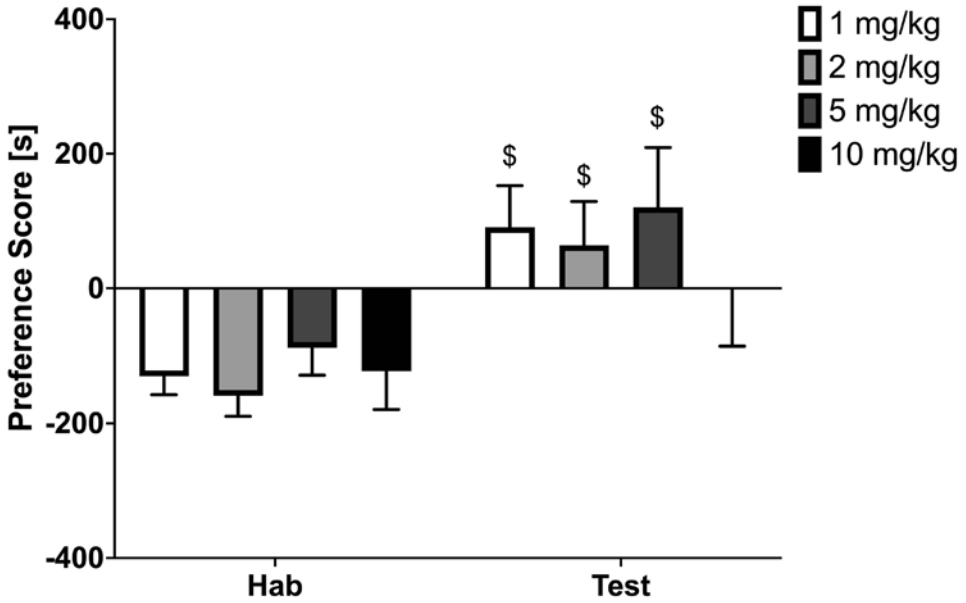

Figure 10: Preference for 1, 2, 5 or 10 mg/kg morphine in C57BL/6J mice. Preference score [s] in C57BL/6J mice following conditioning with 1, 2, 5 or 10 mg/kg morphine at Habituation and Test. Preference score is defined as (time spent in morphine-paired compartment - time spent in saline-paired compartment). Data presented as means ± SEM and analysed using two-way RM ANOVA, followed by one-way ANOVA split by treatment group. Significant effects of 'day' are indicated ($^{\$}p < 0.05$).

3.2 Experiment 2: Effect of mGlu5 deletion on 5 mg/kg morphine place preference

3.2.1 Habituation

mGlu5 KO mice displayed overall locomotor hyperactivity, but decreased exploration of apparatus at the beginning of the test

Figure 11A-D shows the time spent in the left and right sides of the apparatus at habituation. Four-way ANOVA of the variable 'time in zones' found no significant effect of 'zone' [$F(1,36) = 0.09$, $p = .762$; no interaction between 'genotype' and 'zone', $F(1,36) = 0.09$, $p = .761$], suggesting that total time spent in each side was equal (Table 4). However, time spent in each side changed over time and between genotypes and sexes [significant interaction 'genotype', 'sex', 'zone' and 'time', $F(5,180) = 3.70$, $p = .006$]. Post hoc tests with Bonferroni correction confirmed that there was a significant side preference during the first 5 min in male and female KO mice but not WT-like mice (*cf.* Figure 11B,D vs. Figure 11A,C). This preference corresponded with the side they were initially placed in, which suggest it was a respone to a novel environment. Importantly, mGlu5 KO mice returned to WT-like levels of exploration after the first 5 min of the test, indicating the effect of mGlu5 deletion on apparatus exploration was constrained to the first 5 min of the test.

Table 4: Total time spent in zones during habituation in male and female WT-like and mGlu5 KO mice.

Genotype	Sex	Left Zone	Right Zone
WT-like	Male	128.13 +- 4.04	133.04 +- 4.00
mGlu5 KO	Male	127.46 +- 7.12	137.45 +- 8.74
WT-like	Female	128.64 +- 4.79	123.77 +- 4.62
mGlu5 KO	Female	140.64 +- 9.33	117.75 +- 8.89

Locomotor activity was recorded as distance travelled (cm) in Figure 12. Male and female mGlu5 KO mice were hyperactive compared to WT-like mice [main effect 'genotype', $F(1,36) = 38.22$, $p < .001$]. A significant interaction between 'genotype' and 'time' $F(5,180) = 44.72$, $p < .001$] shows that WT-like mice decreased locomotor activity while KO mice increased locomotor activity over the course of the test. There was a 'sex' and 'time' interaction [$F(5,180) = 3.64$, $p = .016$] and an interaction between 'genotype' and 'sex' which approached significance [$F(1,37) = 4.11$, $p = .050$]. Bonferroni post hoc tests suggest that there was a larger genotype difference in distance travelled in male mice than female mice.

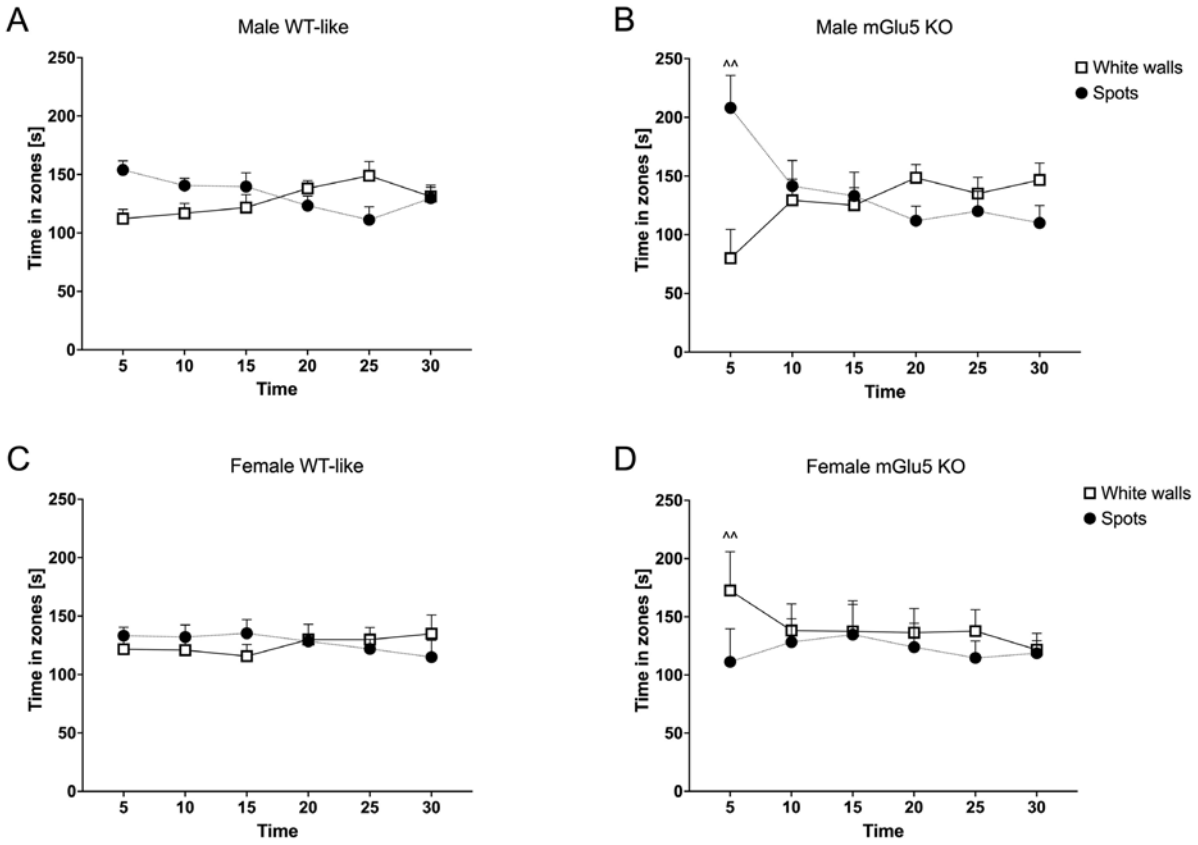

Figure 11: Time spent in zones during habituation in male and female WT-like and mGlu5 KO mice. Time [s] spent in the left (white walls) and right (spots) zones of the apparatus during habituation in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests where appropriate. Significant effects of 'zone' are indicated (^^$p < 0.01$).

3.2.2 Conditioning

mGlu5 deletion alters the locomotor response to 5 mg/kg morphine

Distance travelled during saline and morphine conditioning was used to assess locomotor sensitisation to morphine. mGlu5 KO mice were hyperactive compared to WT-like mice, regardless of sex [main effect of 'genotype', $F(1,36) = 4.20$, $p = .048$; no interactions with 'sex'], while female mice of both genotypes travelled further than male mice [main effect of 'sex', $F(1,36) = 6.02$, $p = .019$]. When averaged across days and genotypes, morphine increased locomotor activity compared to saline [main effect of 'drug', $F(1,37) = 15.80$, $p < .001$]. However, the overall increase in locomotion by morphine was driven by WT-like mice, as morphine actually reduced locomotor activity in male and female mGlu5 KO mice [significant interaction 'genotype' and 'drug' [$F(1,36) = 32.41$, $p < .001$] (Figure 13A-D). Three-way ANOVA split by

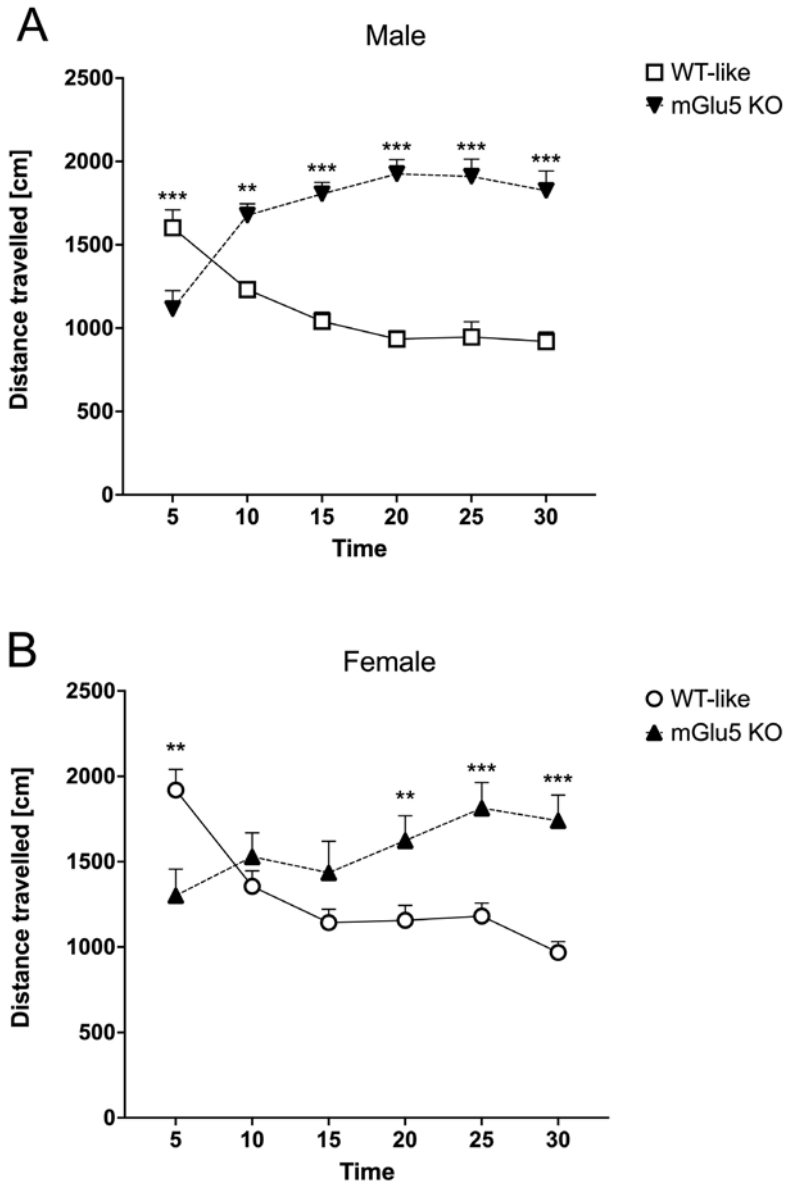

Figure 12: Distance travelled by male and female WT-like and mGlu5 KO mice during habituation. Distance travelled [cm] during habituation in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using three-way RM ANOVA, followed by Bonferroni post hoc tests where appropriate. Significant effects of 'genotype' are indicated ($^{**}p < 0.01$; $^{***}p < .001$).

'genotype' with Bonferroni correction found a main effect of 'drug' in male and female WT-like mice [$F(1,36) = 53.15$, $p < .001$] but not male and female mGlu5 KO mice [$F(1,36) = 1.32$, $p = .259$]. There was an interaction between 'drug' and 'day' in mGlu5 KO mice [$F(3,108) = 9.32$, $p < .001$] but not WT-like mice [$F(3,108) = 2.22$, $p = .089$]. Bonferroni post hoc tests indicate no change to morphine-induced locomotion between day 1 and day 4 in male mice regardless of genotype, suggesting no morphine locomotor sensitisation. However, repeated administration

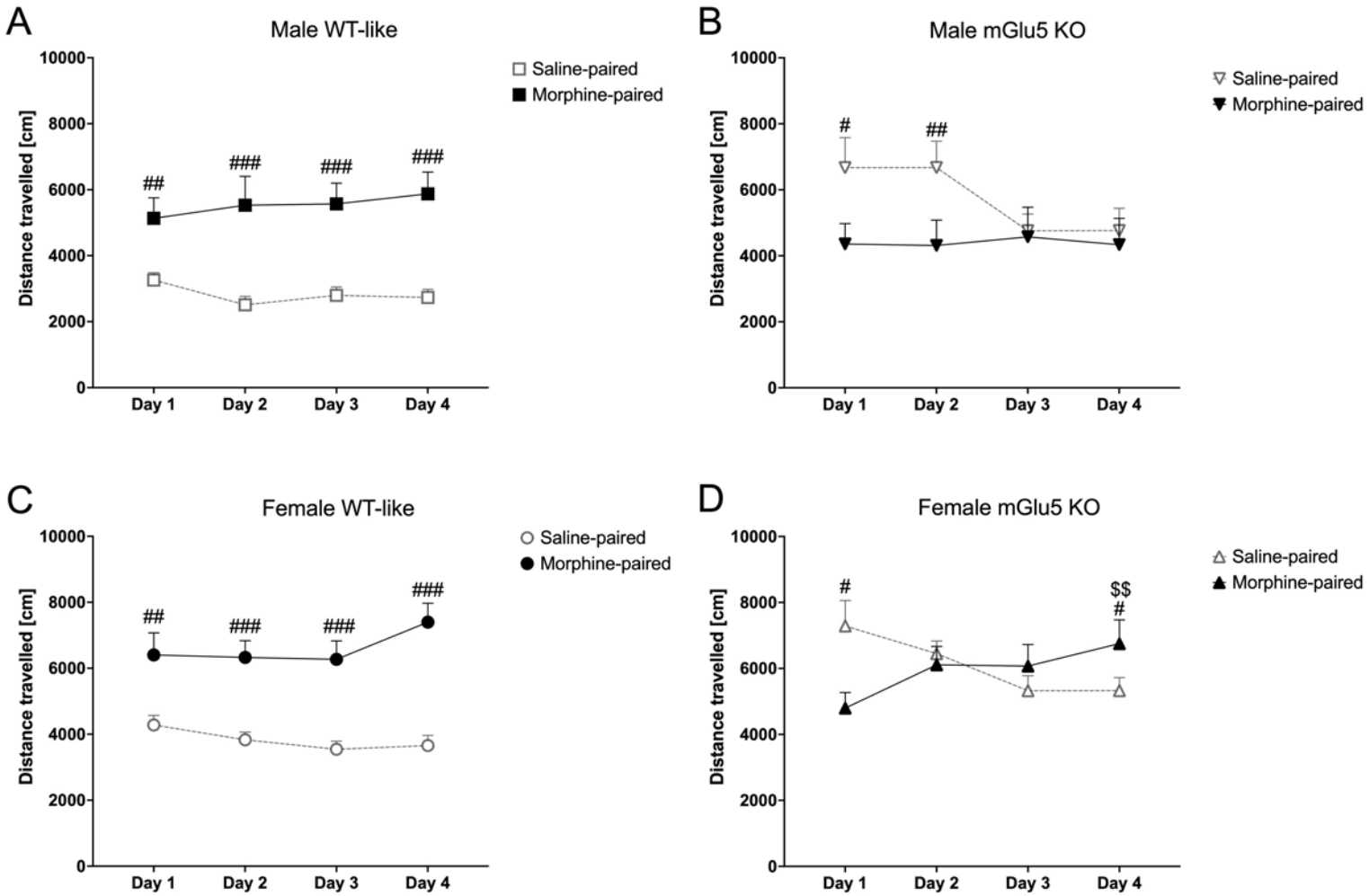

Figure 13: Locomotor response of WT-like and mGlu5 KO mice to repeated 5 mg/kg morphine. Locomotor response is measured as distance travelled [cm] over 4 consecutive days during saline and morphine conditioning to (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant post hoc effects of 'drug' are indicated ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). Significant post hoc effects of 'day are indicated ($^{\$\$}p < 0.01$).

of morphine increased locomotor activity from day 1 to day 4 in female mGlu5 KO mice, but not female WT-like mice. This suggests that mGlu5 deletion inhibits morphine-induced hyperlocomotion for 5 mg/kg morphine in male and female mice but promotes morphine sensitisation in female, but not male, mice.

3.2.3 Test

Female, but not male mGlu5 KO mice show persistent morphine place preference during abstinence

Figure 14 shows that preference score increased from habituation in all treatment groups [main effect of 'day', $F(5,180) = 12.79$, $p < .001$; no significant effect of 'genotype', $F(1,36) = 0.73$, $p = .399$, or 'sex', $F(1,36) = 0.19$, $p = .665$]. However, a significant interaction between 'genotype' and 'sex' [$F(1,36) = 4.22$, $p = .047$] suggest that mGlu5 has a sex-specific effect on the acquisition and continued expression of morphine place preference. This was explored using post-hoc tests. Female mGlu5 KO mice showed a persistent preference for the drug-paired side during abstinence while female WT-like mice did not on most test days (Figure 14B), despite there being no difference at habituation. Post hoc tests indicate female mGlu5 KO mice preference score was higher than habituation at Test 1 and this persisted for all tests. In contrast female WT-like mice had an increase in preference score at Test 2 compared to habituation, but not at any other Tests. Male mGlu5 KO mice showed a similar pattern in preference score to male WT-like mice and did not show a persistent place preference during abstinence. In males, preference increased from habituation at Test 2 in both genotypes and Test 5 in mGlu5 KO mice only. This suggested that mGlu5 deletion did not affect the development of preference for 5 mg/kg morphine in male mice.

Interestingly, preference for morphine was highest at test 2 for male and female WT-like and male mGlu5 KO mice but consistent across all tests in female mGlu5 KO mice. This suggested that mGlu5 deletion promotes morphine preference and persistence of morphine-associated memories in female mice. Time-course data from the test sessions was assessed to further explore the increase in preference observed from Test 1 to Test 2.

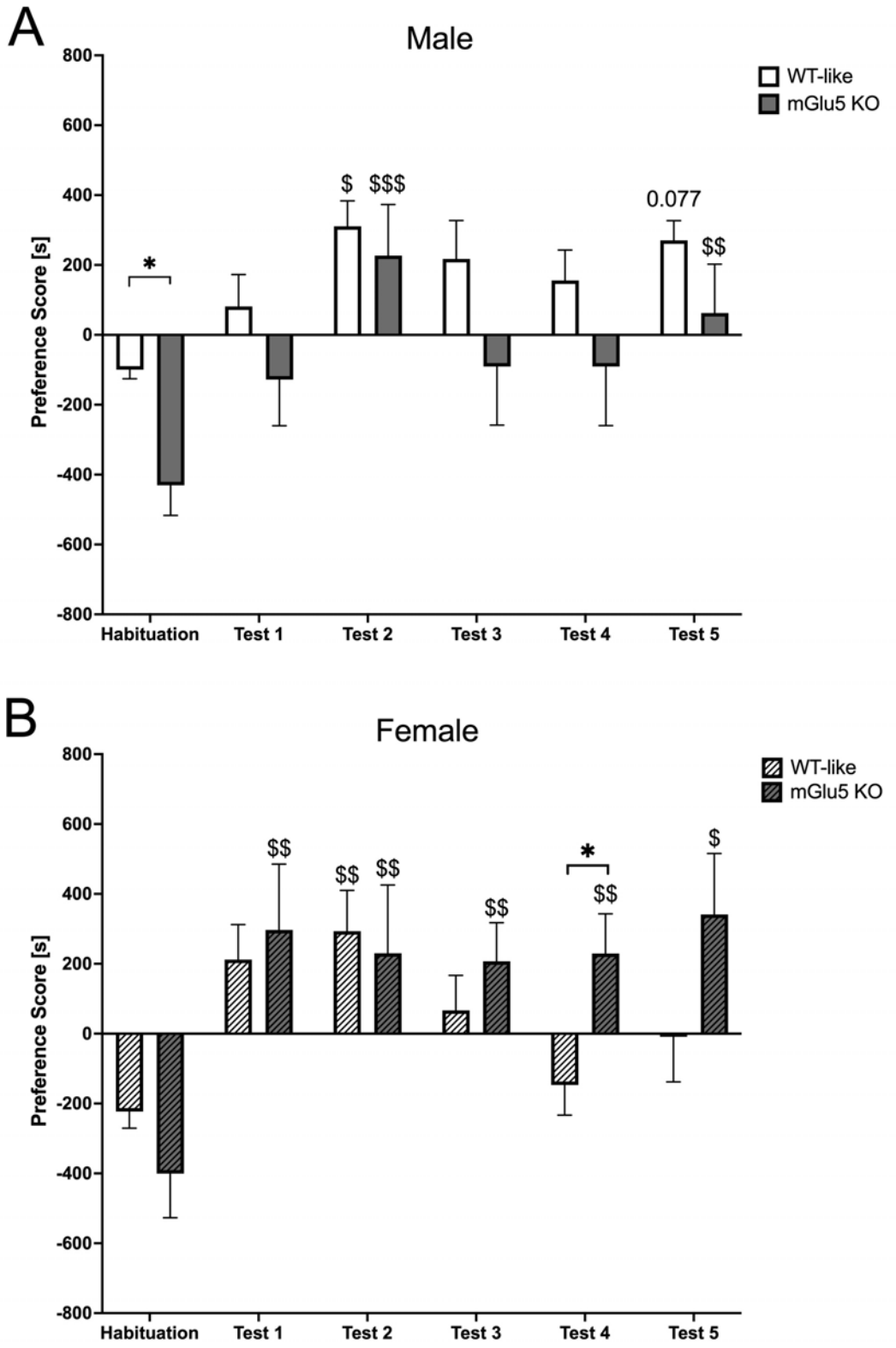

Figure 14: Preference following conditioning with 5 mg/kg morphine in male and female WT-like and mGlu5 KO mice. Preference score [s] is defined as (time spent in morphine-paired compartment - time spent in saline-paired compartment). Data presented as means ± SEM and analysed using three-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'day' compared to habituation are indicated ($^{\$}p < 0.05$; $^{\$\$}p < 0.001$). Significant effects of 'genotype' are indicated ($^{*}p < 0.05$).

3.2.4 Test Time-course

Female mGlu5 KO mice spent more time in the morphine-paired side and showed a conditioned locomotor response to 5 mg/kg morphine

Time-course data from Test 1 was analysed to further probe morphine preference immediately following conditioning. Time-course data for test 1 (Figure 15) shows female mice, but not male mice, had a preference for the morphine-paired side [no effect of 'zone', $F(1,36) = 2.95$, $p = .095$; significant interaction between 'sex' and 'zone', $F(1,36) = 4.35$, $p = .044$]. Three-way ANOVA split by 'sex' with Bonferroni correction found a 'zone' and 'time' interaction in male mice [$F(5,180) = 9.75$, $p < .001$] and female mice [$F(5,180) = 3.72$, $p = .003$]. There was also a significant interaction between 'genotype', 'zone' and 'time' in male [$F(5,180) = 3.08$, $p = .011$], but not female [$F(5,180) = 0.59$, $p = .710$], mice. Bonferroni post hocs show that male mGlu5 KO mice spent significantly more time in the saline-paired side during the first 5 min. As mice were placed in the saline-paired side at the start of the test this appears to reflect the genotype effect on initial exploration seen at habituation. Over time, as mGlu5 KO mice explored the apparatus further, they increased their time in the morphine-paired side.

Mice travelled further in the morphine-paired side [main effect 'zone', $F(1,36) = 11.71$, $p = 002$] (Figure 16) with female mice travelling further than male mice [main effect 'sex', $F(1,36) = 7.72$, $p = .009$; significant interaction between 'sex' and 'zone', $F(1,36) = 4.51$, $p = .041$] and mGlu5 KO mice were hyperactive in the morphine-paired side compared to WT-like mice [significant interaction between 'genotype', 'zone' and 'time', $F(5,180) = 3.25$, $p = .017$]. Further analysis with Bonferroni post hoc tests suggested that locomotor activity increased over time in the morphine-paired side but not the saline-paired side in female mGlu5 KO mice, but this did not occur in female WT-like mice. This suggests that female mGlu5 KO, but not WT, mice showed a conditioned locomotor response to 5 mg/kg morphine. Male WT-like and mGlu5 KO mice did not show this increased locomotor activity in the morphine-paired side compared to the saline-paired side, indicating they did not have a conditioned locomotor response to 5 mg/kg morphine.

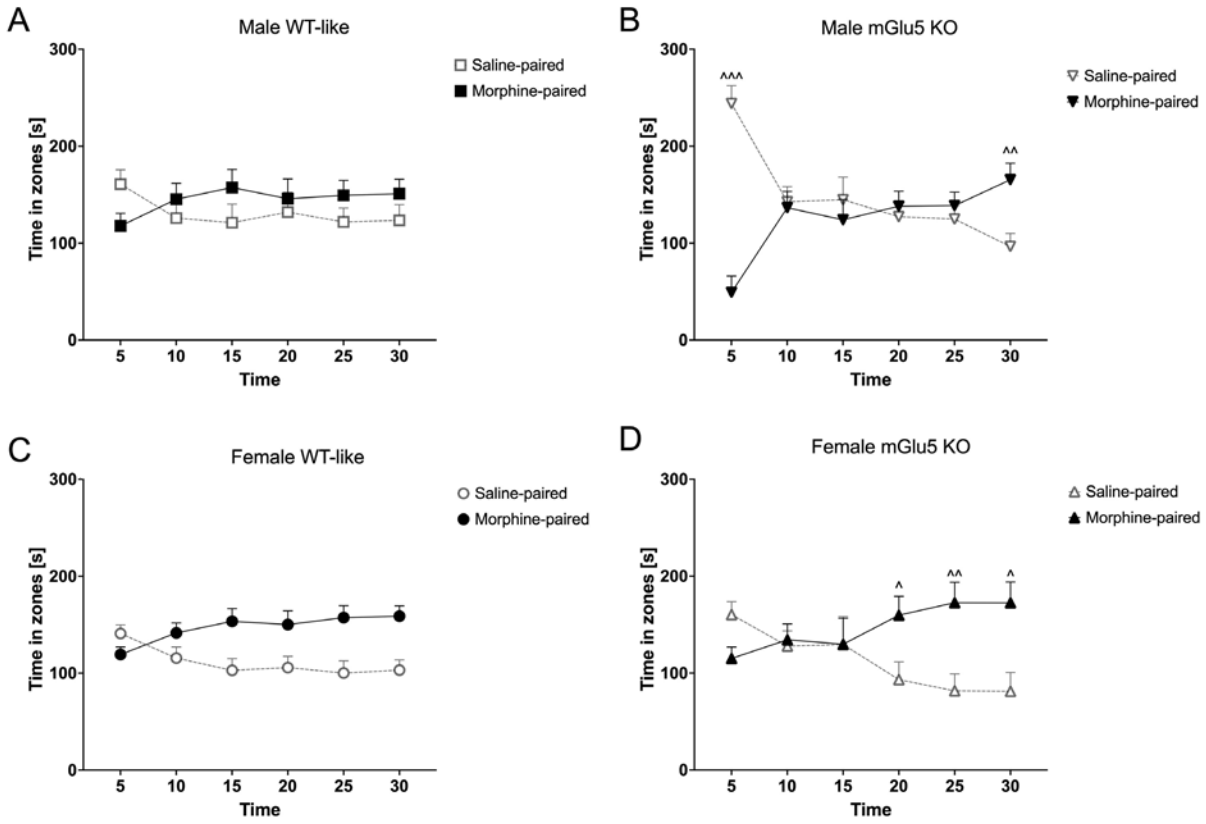

Figure 15: Time spent in zones during Test 1 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 1 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge\wedge}p < 0.001$).

During Test 2 male and female mGlu5 KO and WT-like mice spent more time in the morphine-paired zone [main effect 'zone', $F(1,36) = 16.81$, $p < .001$; no significant interactions] (Figure 17) and travelled further in the morphine-paired zone [main effect 'zone' $F(1,36) = 13.94$, $p < .001$] (Figure 20). mGlu5 KO mice travelled further than WT-like mice [main effect 'genotype', $F(1,36) = 5.70$, $p = .022$; significant interaction between 'genotype' and 'time, $F(5,180) = 24.52$, $p < .001$] (Figure 18). This supports the finding that morphine preference score was highest at Test 2.

Timecourse data from Tests 3, 4 and 5 showed no significant differences in the time spent and distance travelled between the morphine- and saline-paired sides (data included in Appendix A).

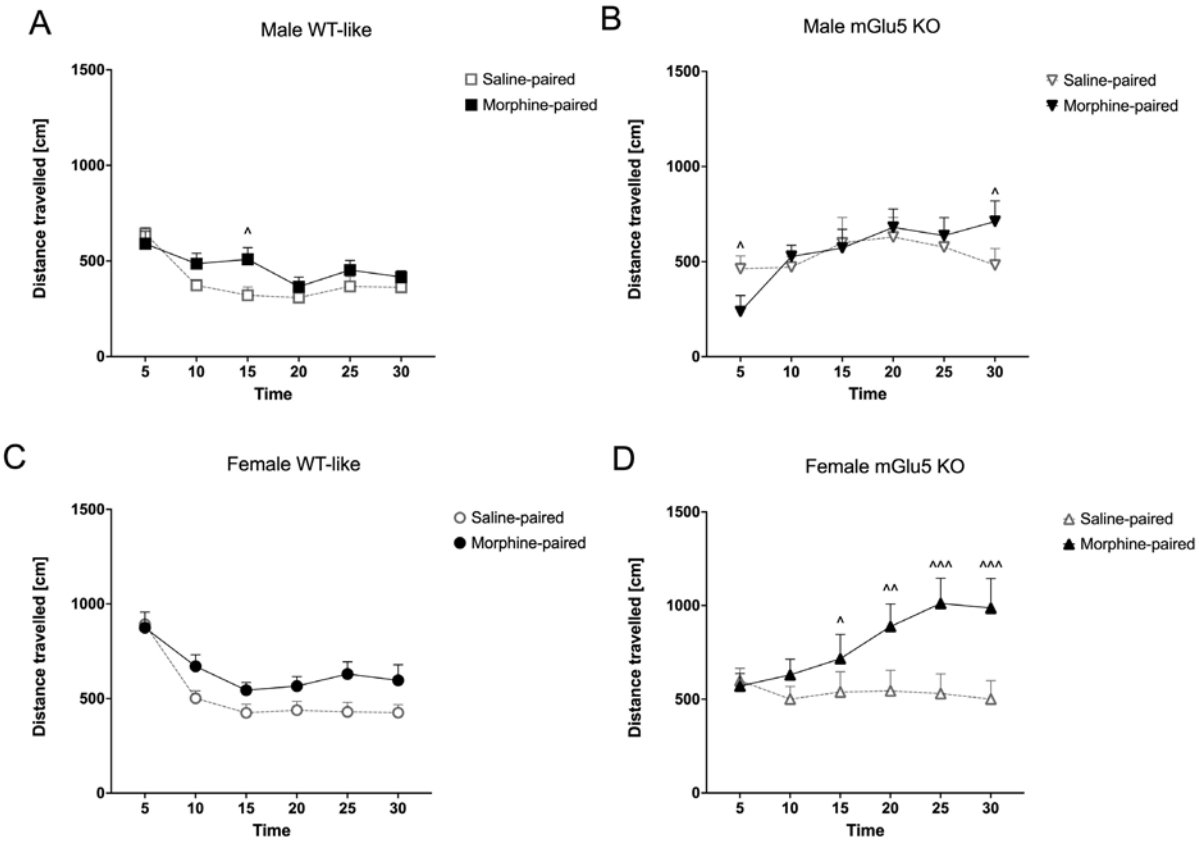

Figure 16: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 1. Distance travelled [cm] in the morphine- and saline- paired zones during Test 1 in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$; $^{\wedge\wedge\wedge}p < 0.001$).

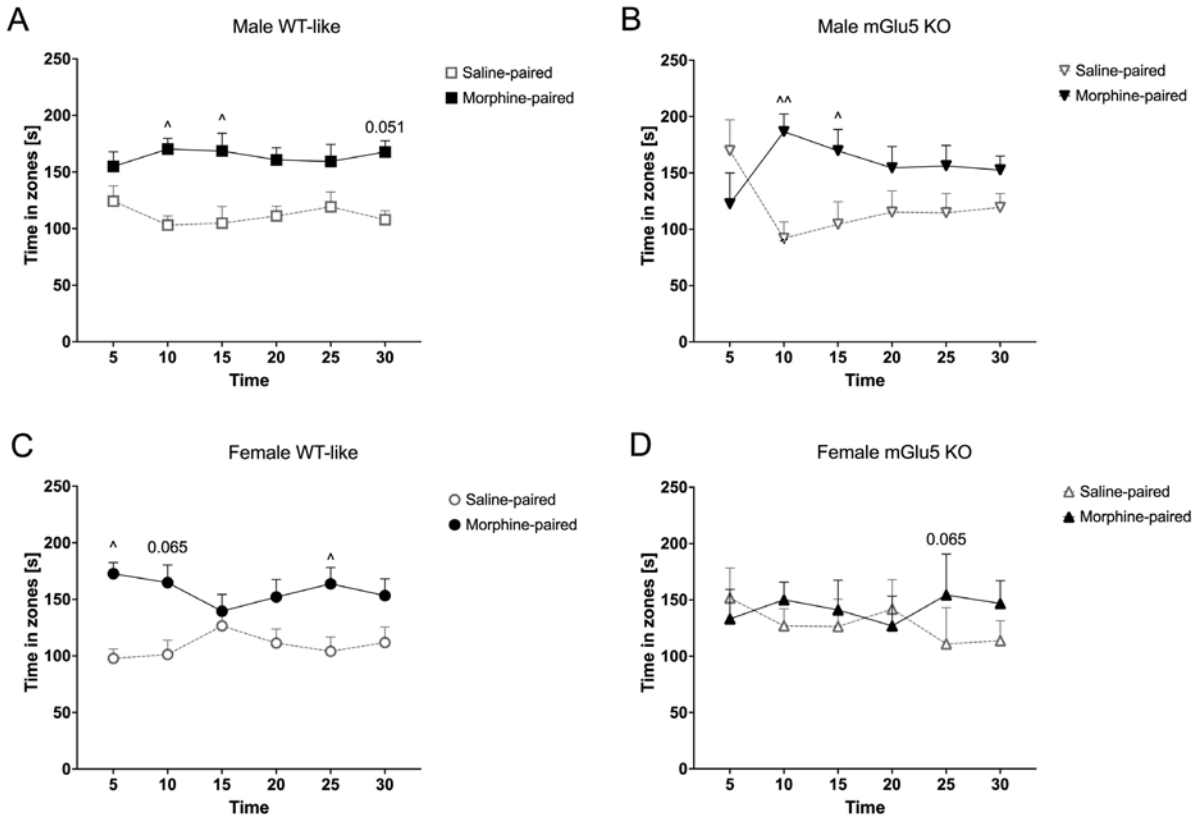

Figure 17: Time spent in zones during Test 2 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 2 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).

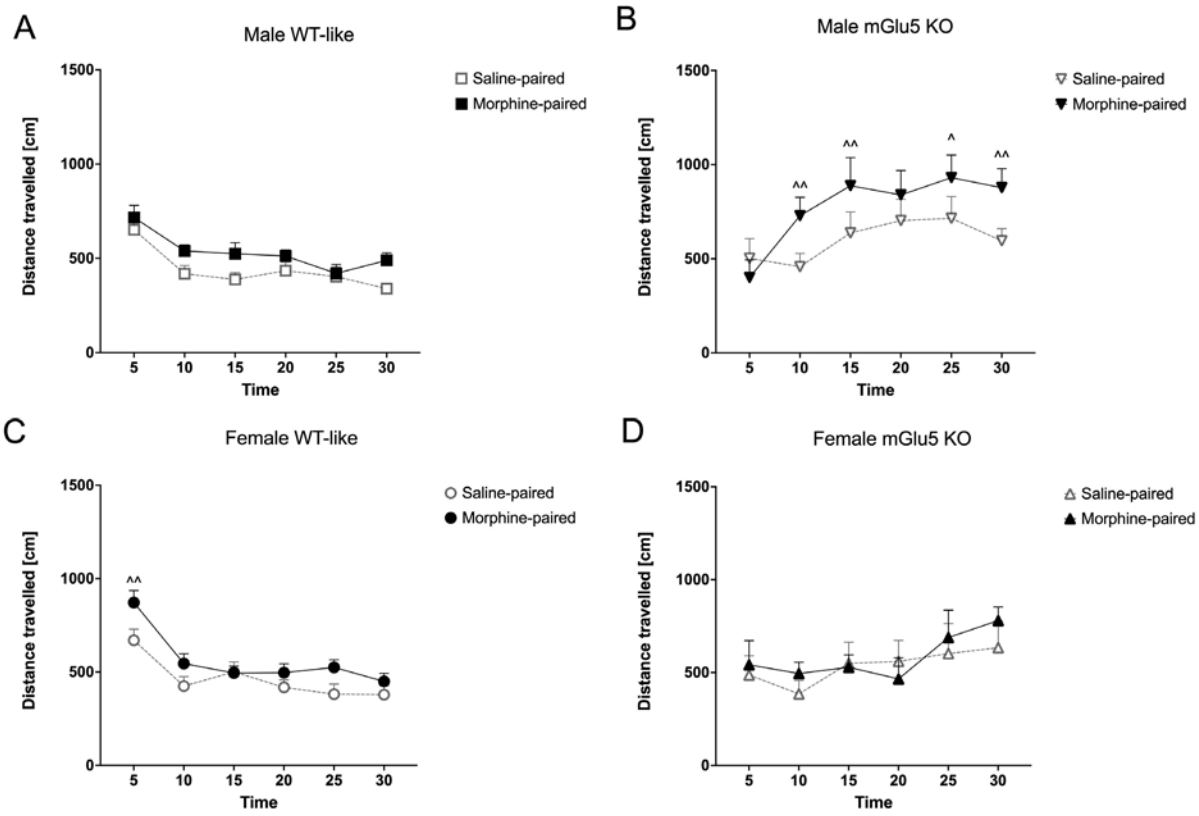

Figure 18: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 2. Distance travelled [cm] in the morphine- and saline- paired zones during Test 2 in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).

3.3 Experiment 3: Effect of mGlu5 deletion on 10 mg/kg morphine place preference

3.3.1 Habituation

Male, but not female, mGlu5 KO mice showed decreased initial exploration of the apparatus

There was no overall side preference at habituation as seen in Figure 19 [no main effect 'zone', $F(1,36) = 0.05$, $p = .833$; no interaction between 'genotype' and 'zone', $F(1,36) = 0.01$, $p = .982$]. There was a significant interaction between 'genotype', 'zone' and 'time' [$F(5,180) = 3.83$, $p = .009$] suggesting that side preference changed over the course of habituation differently between genotypes. Bonferroni post hoc tests indicate that male mGlu5 KO mice had a side preference for the right chamber during the first 5 min of the test and there was a trend for preference for the left chamber at 15 min. The side preference in the initial 5 minutes corresponded with the side they were initially placed, similar to mGlu5 KO mice of both sexes in Experiment 2. There was no effect of genotype on side preference in female mice.

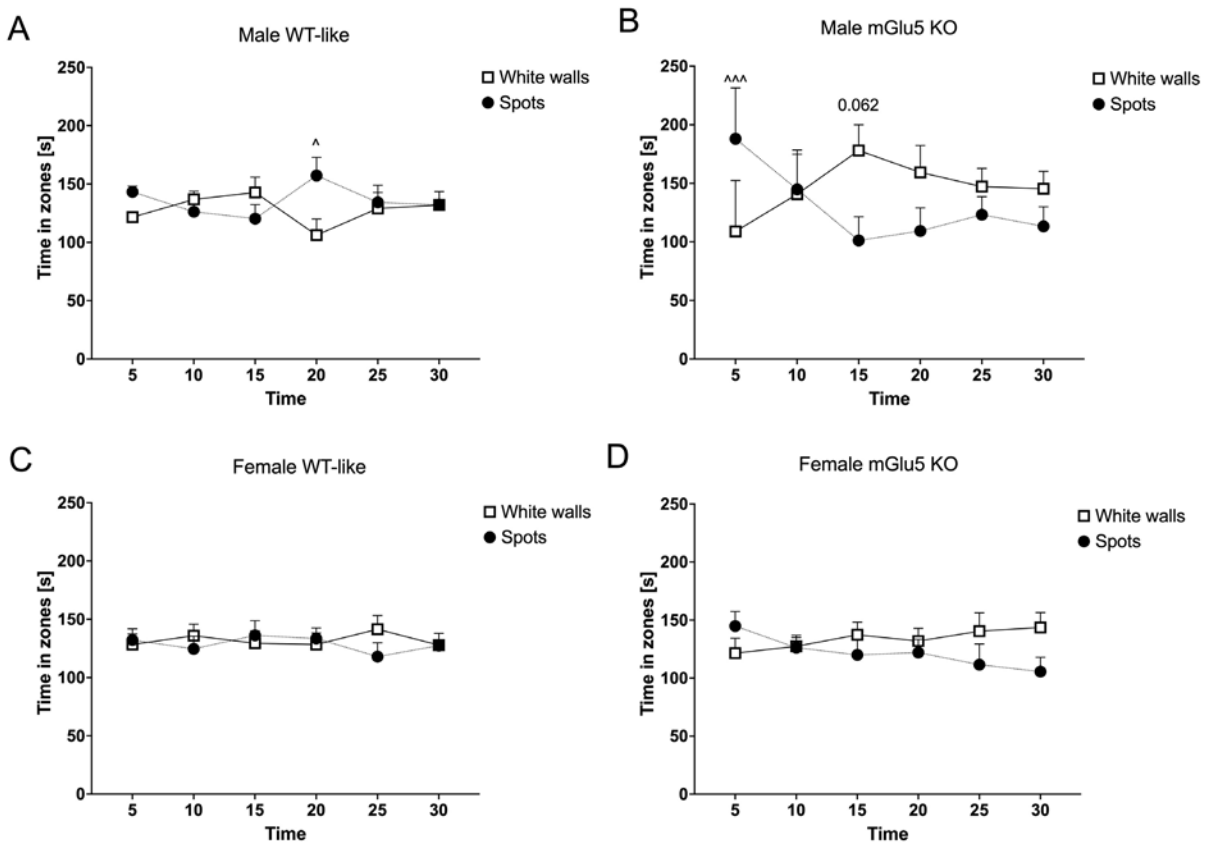

Figure 19: Time spent in zones during habituation in male and female WT-like and mGlu5 KO mice. Time [s] spent in the left (white walls) and right (spots) zones of the apparatus during habituation in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge\wedge}p < 0.001$).

As shown in Figure 20 male and female mGlu5 KO mice were hyperactive compared to WT-like mice [main effect 'genotype', $F(1,36) = 39.26$, $p < .001$]. Female mice of both genotypes travelled further than male mice [main effect 'sex', $F(1,36) = 13.95$, $p < .001$; no interaction 'genotype' and 'sex', $F(1,36) = 2.41$, $p = .130$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 60.03$, $p < .001$] indicating that locomotor activity decreased in WT-like mice but increased in mGlu5 KO mice across the test. Bonferroni post hoc tests show that male mGlu5 KO mice had a very low locomotor activity in the first 5 min of habituation which corresponds with the initial side preference seen above.

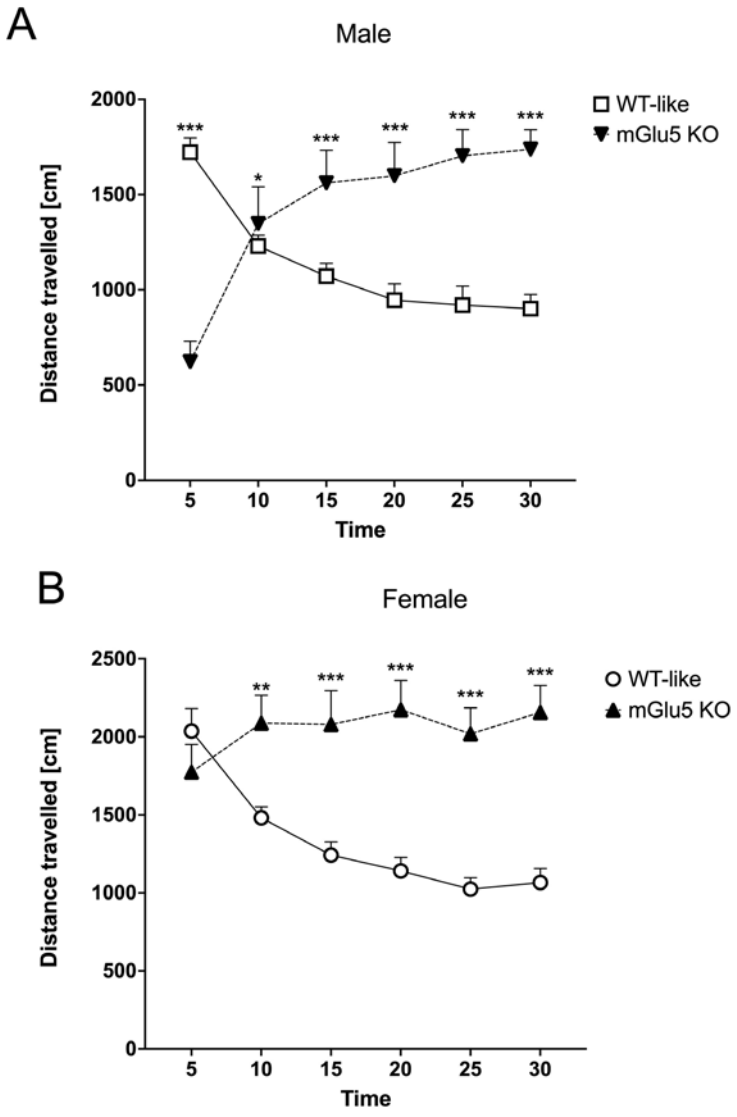

Figure 20: Distance travelled by male and female WT-like and mGlu5 mice during habituation. Distance travelled [cm] during habituation in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using three-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'genotype' are indicated ($^{**}p < 0.01$; $^{***}p < .001$).

3.3.2 Conditioning

10 mg/kg morphine induced locomotor hyperactivity in mGlu5 KO mice

Figure 21 shows total distance travelled by male and female mGlu5 KO mice and their WT-like littermates when conditioned with saline and 10 mg/kg morphine over 4 days. Morphine increased locomotor activity [main effect 'drug', $F(1,37) = 73.06$, $p < .001$] regardless of sex or genotype, though the effect of morphine was more pronounced in female mice than male mice [significant interaction between 'sex' and 'drug', $F(1,36) = 12.01$, $p = .001$]. Locomotor activity during morphine conditioning increased over days [significant interaction between 'drug' and 'day', $F(3,108) = 40.66$, $p < .001$], suggesting there was locomotor sensitisation to 10 mg/kg morphine. mGlu5 deletion did not affect the locomotor response to 10 mg/kg morphine [no effect 'genotype', $F(1,36) = 0.14$, $p = .715$; no interactions with 'genotype']. However, there was significant interaction between 'sex', 'drug' and 'day' [$F(3,108) = 3.06$, $p = .035$], suggesting that female mice were more susceptible to locomotor sensitisation. Bonferroni post hoc tests show that female WT-like and mGlu5 KO mice both sensitised to 10 mg/kg morphine.

3.3.3 Tests

Female, but not male, mGlu5 KO mice had an increased variability in preference score to 10 mg/kg morphine that persisted through abstinence

Preference score for 10 mg/kg morphine (Figure 22) increased from habituation across all test days [main effect 'day', $F(5,180) = 4.8235$, $p = .002$] mainly driven by the male data (*cf.* Figure 22A vs. Figure 22B). This was consistent across both genotypes and sexes [no effect 'genotype', $F(1,36) = 0.87$, $p = .356$; no effect 'sex', $F(1,36) = 0.003$, $p = .957$; no interactions involving 'genotype' or 'sex']. This suggests that 10 mg/kg morphine increased preference in male and female mGlu5 KO and WT-like mice.

It should be noted that there was a large variability in preference score during abstinence for 10 m/kg (Figure 22) that was not seen for 5 mg/kg. A small number of mice (n=3) appeared to have an aversion to the morphine-paired side and so additional analysis was performed to investigate this further. Levene's test confirms that there were significant differences in variability

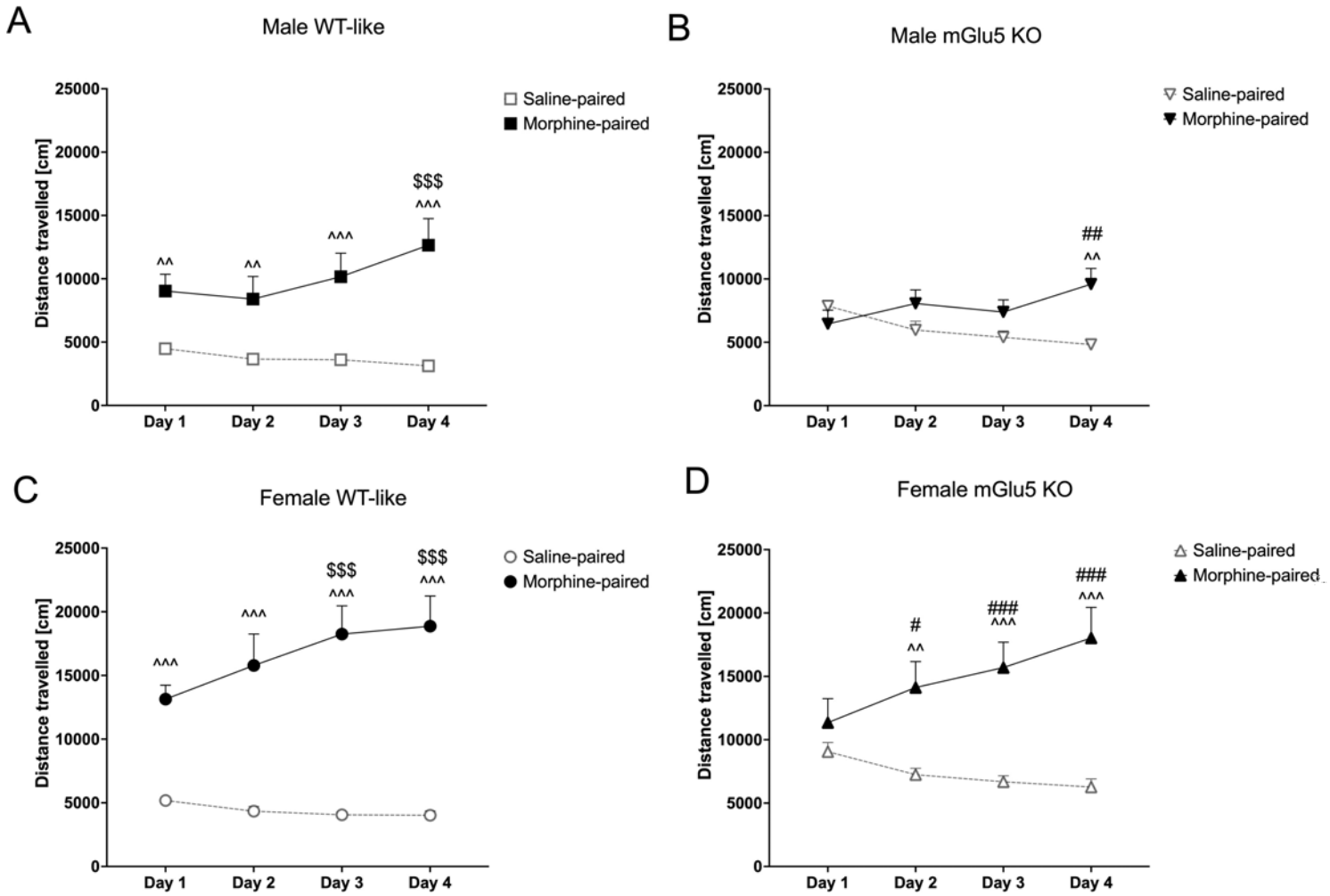

Figure 21: Locomotor response of male and female WT-like and mGlu5 KO mice to 10 mg/kg morphine. Locomotor response is measured as distance travelled [cm] over 4 consecutive days during saline and morphine conditioning to (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests where appropriate. Significant effects of 'drug' are indicated ($^{\#}p < 0.05$; $^{\#\#}p < 0.01$; $^{\#\#\#}p < 0.001$). Significant effects of 'day' are indicated ($^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.001$). Significant effects of 'sex' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$; $^{\wedge\wedge\wedge}p < 0.001$).

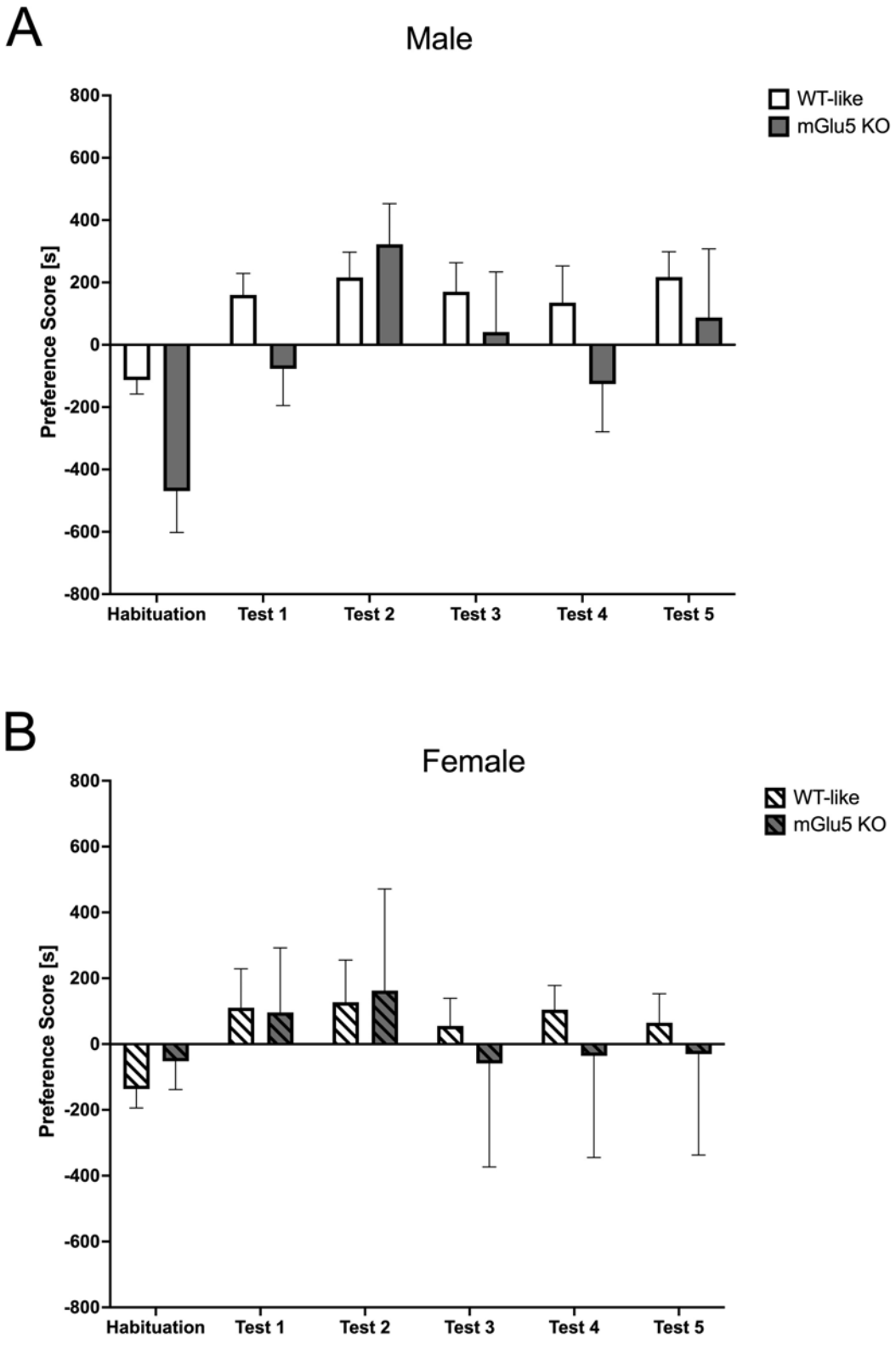

Figure 22: Preference following conditioning with 10 or mg/kg morphine in male and female WT-like and mGlu5 KO mice. Preference score [n] is defined as (time spent in morphine-paired compartment - time spent in saline-paired compartment). Data presented as means ± SEM and analysed using three-way RM ANOVA.

between groups [F(23,215) = 4.35, $p < .001$]. Three-way ANOVA of the residuals (Figure 23) found that mGlu5 KO mice had an increased variability compared to WT-like mice [main effect 'genotype', F(1,36) = 18.91, $p < .001$] and variability increased following conditioning [main effect 'day', F(5,180) = 6.9, $p < .001$; significant interaction between 'genotype' and 'day', F(5,180) = 3.38, $p = .012$]. Two-way ANOVA split by 'genotype' showed that mGlu5 KO but not WT-like mice showed a significant interaction between 'sex' and 'day' [F(5,180) = 2.28, $p = .048$]. Bonferroni post hoc tests indicate that female mGlu5 KO mice had significantly more variability than habituation at Test 2, Test 3, Test 4 and Test 5, but not Test 1. Male mGlu5 KO mice tended to have increased variability in test 5 only. It also suggests that the effects of 10 mg/kg morphine, whether rewarding or aversive, persist during abstinence in female, but not male,

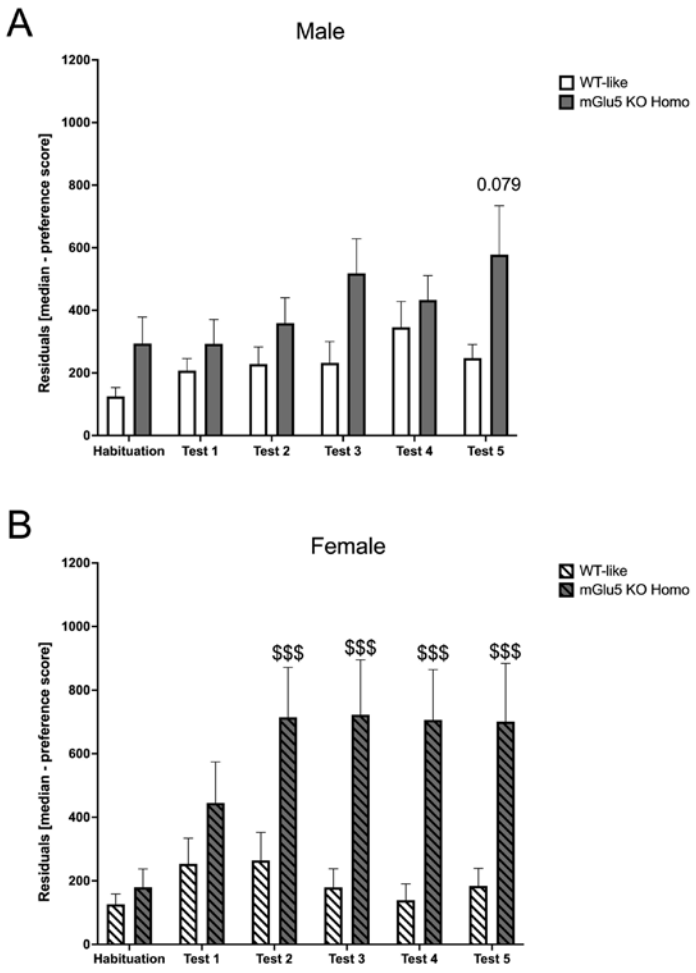

Figure 23: Residuals following conditioning with 10 or mg/kg morphine in male and female WT-like and mGlu5 KO mice. Residuals are calculated as (median preference score - individual preference score). Data presented as means ± SEM and analysed using three-way RM ANOVA, followed by Bonferroni post hoc tests where appropriate. Significant effects of 'day' are indicated ($^{\$}p < 0.05$; $^{\$\$\$}p < 0.001$).

mGlu5 KO mice. Shapiro-Wilk normality test was used to test for multimodality to determine if there were any subgroups but no subgroups were found [$p = .182$].

3.3.4 Test Time-course

mGlu5 KO mice showed behavioural conditioning to 10 mg/kg morphine

The time course data from test 1 (Figure 24) shows that there was no overall preference for the morphine-paired side [no effect 'zone', $F(1,36) = 1.03$, $p = .317$] but preference changed over the course of the test [significant interaction between 'zone' and 'time', $F(5,180) = 8.64$, $p < .001$]. Bonferroni post hoc tests suggested that this was due to an initial preference for the saline-paired zone during the first 5 min in mGlu5 KO mice, which was the side mice were initially placed in in the Test.

In contrast, distance travelled was higher in the morphine-paired side compared to the saline-paired side, and this became more pronounced as the test progressed [main effect 'zone',

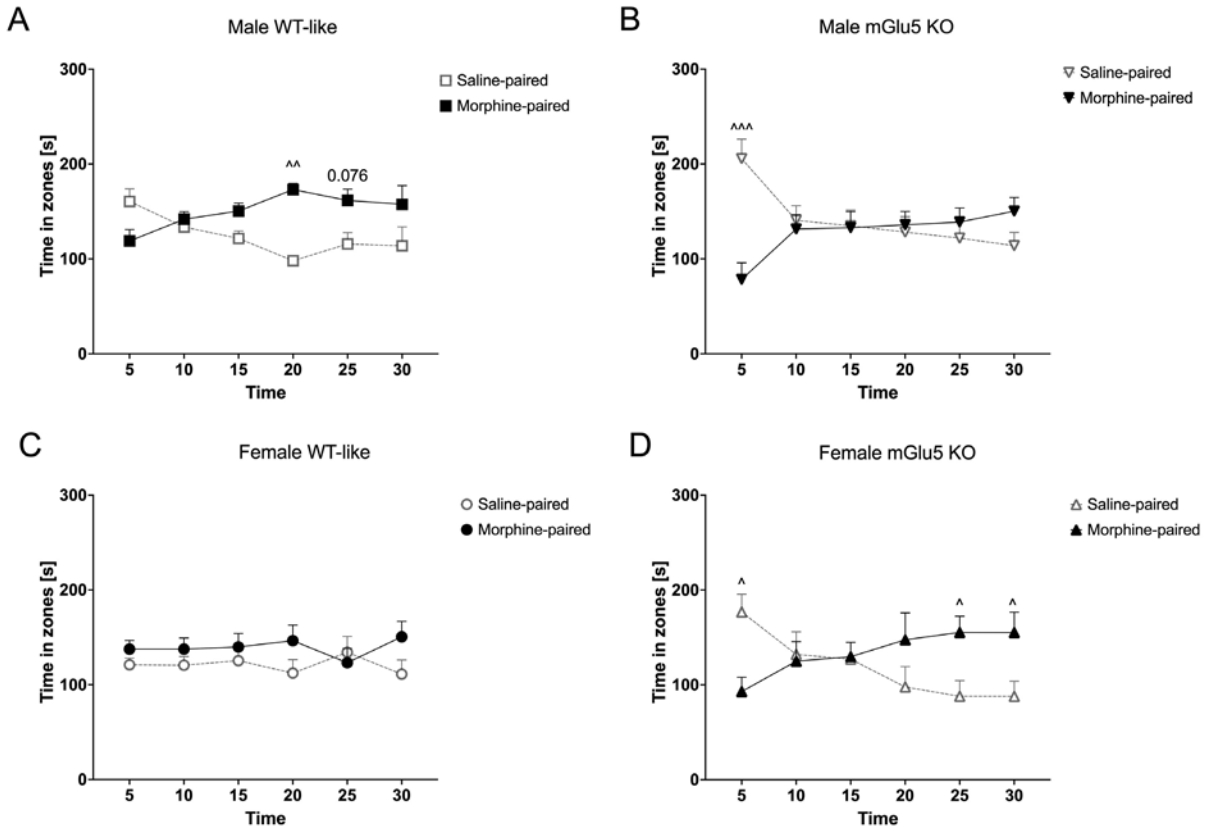

Figure 24: Time spent in zones during Test 1 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 1 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge\wedge}p < 0.001$).

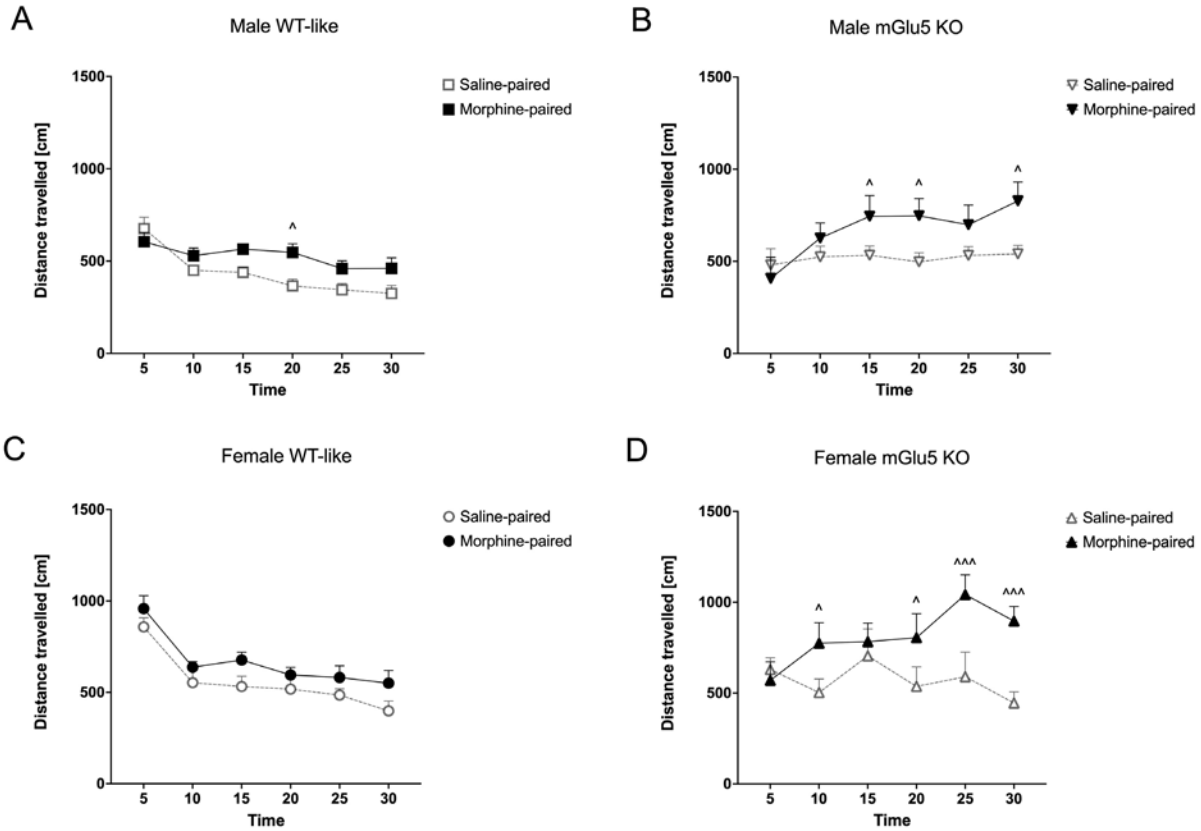

Figure 25: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 1. Distance travelled [cm] in the morphine- and saline- paired zones during Test 1 in (A) male WT-like and mGlu5 KO littermates and (B) female WT-like and mGlu5 KO littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).

F(1,36) = 15.48, $p < .001$; significant interaction 'zone' and 'time', F(5,180) = 6.81, $p < .001$] (Figure 25). mGlu5 KO mice showed hyperlocomotion compared to WT-like mice [main effect 'genotype', F(1,36) = 5.75, $p = .022$].

The test 2 time course data (Figure 26) shows that there was an overall preference for the morphine-paired side [main effect 'zone', F(1,36) = 6.24, $p = .017$]. This corresponds with the peak in preference score seen at Test 2 (Figure 24).

Figure 27 shows that mice travelled further in the morphine-paired side compared to the saline-paired side at test 2 [main effect 'zone', F(1,36) = 8.92, $p = .005$]. Male mGlu5 KO mice were hyperactive compared to male WT-like mice, while female WT-like mice were more active than female mGlu5 KO mice [significant interaction between 'genotype' and 'time',

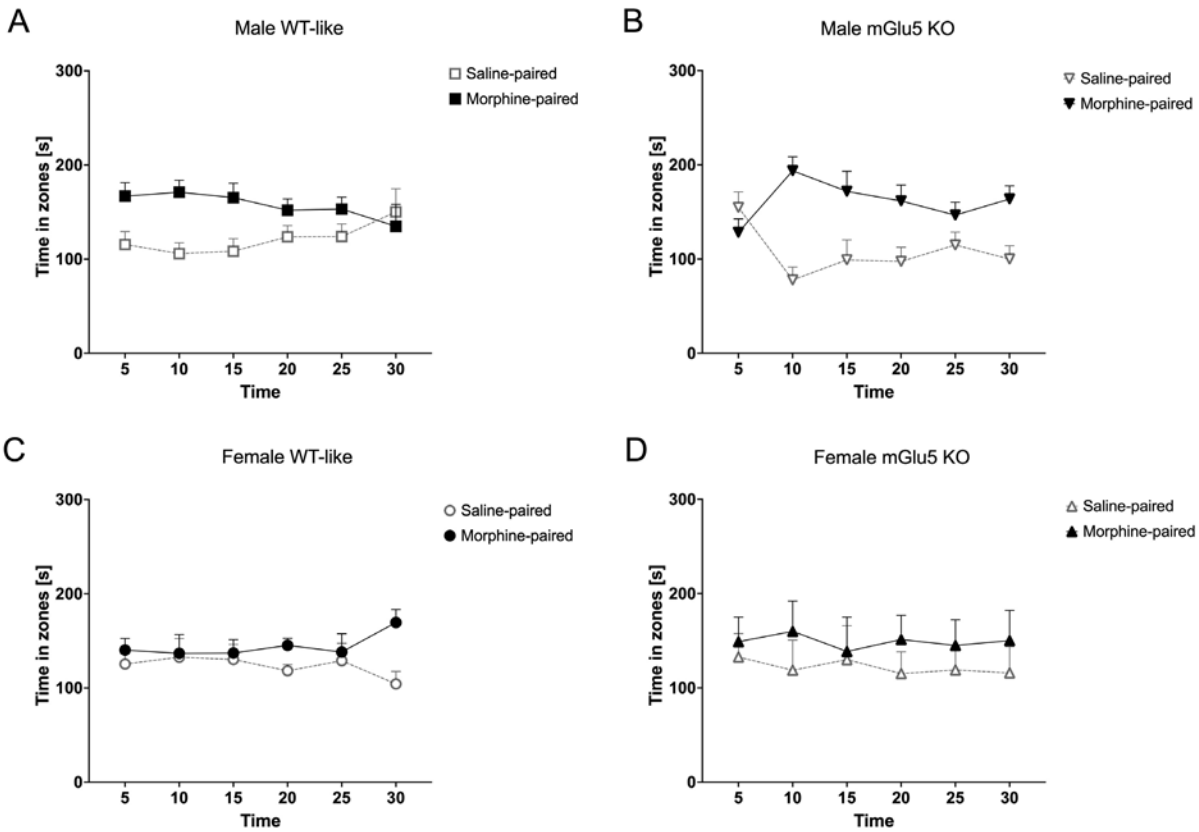

Figure 26: Time spent in zones during Test 2 in male and female WT-like and mGlu5 mice. Time [s] spent in the morphine- and saline- paired zones during Test 2 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA.

$F(5,180) = 35.84$, $p < .001$; significant interaction between 'genotype' and 'sex', $F(1,36) = 8.95$, $p = .005$; significant interaction between 'sex' and 'time', $F(5,180) = 6.04$, $p < .001$]. Three-way ANOVA split by 'sex' with Bonferroni correction show there was a significant interaction between 'zone' and 'time' in male [$F(5,180) = 2.933$, $p = .014$], but not female [$F(5,180) = 1.10$, $p = .362$], mice. This suggests that male mice increased distance travelled in the drug-paired side than the saline-paired side over the test while female mice locomotor activity changed consistently in both zones.

There were no significant effects of 'zone' for time or distance in Tests 3, 4 or 5 (data included in Appendix B).

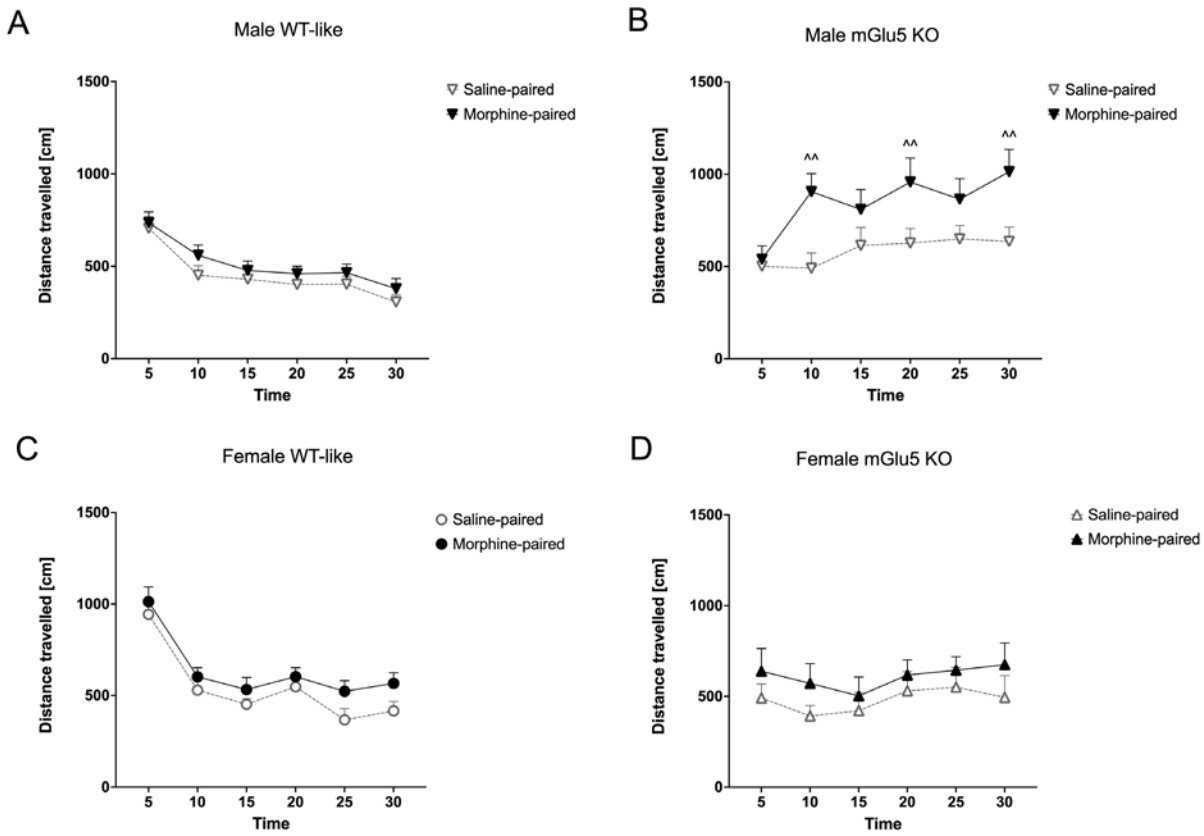

Figure 27: Distance travelled in zones by male and female WT-like and mGlu5 mice during Test 2. Distance travelled [cm] in the morphine- and saline- paired zones during Test 2 in (A) male WT-like and mGlu5 KO littermates and (B) female WT-like and mGlu5 KO littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).

Chapter 4: Discussion

4.1 Summary of results

This is the first study of the effect of mGlu5 germline deletion on the expression of morphine reward and morphine-induced locomotion in male and female mice. Significant effects of genotype, sex and morphine dose were found. The acquisition of morphine place preference was unaffected by mGlu5 deletion. However, there was a significant effect of dose on the acquisition of morphine memory, as 5 mg/kg induced a higher preference score in male and female mice regardless of genotype. Genotype and sex also impacted on persistence of morphine reward memory, as female mGlu5 KO mice showed a persistent preference for 5 mg/kg morphine during abstinence compared to female WT-like mice. There was significant variability in preference for 10 mg/kg morphine in female mGlu5 KO mice that likewise persisted during abstinence and was not observed in female WT-like mice. Male mGlu5 KO mice had a similar reward response to male WT-like mice to 5 and 10 mg/kg morphine, indicating sex-specific effects of mGlu5 deletion on morphine reward. Overall, 5 mg/kg morphine was more rewarding for female mice than male mice, while 10 mg/kg morphine was similarly rewarding in male and female mice.

In addition to the effects of genotype and sex on reward, a significant effect of genotype was found on morphine-induced locomotion. Deletion of mGlu5 reduces the acute locomotor response to 5 mg/kg morphine administration in male and female mice. Despite this female mGlu5 KO mice expressed locomotor sensitisation to 5 mg/kg morphine. However, 10 mg/kg morphine increased locomotor activity in male and female mGlu5 KO and WT-like mice. Further, female WT, female mGlu5 KO mice and male WT, but not male mGlu5 KO mice expressed locomotor sensitisation to 5 and 10 mg/kg morphine. Thus, these results suggest that mGlu5 plays a key role in opioid reward and memory processes, and these findings are dependent on sex.

4.2 Genotype and sex effects on morphine reward

A key feature of addiction pathology is the cycle of abstinence and relapse to drug use. Finding treatment targets that promote abstinence is an important step to developing effective treatments for opioid addiction. Exposure to environmental cues associated with drug-taking can increase drug-craving and risk of relapse (Myers & Carlezon 2010). Therefore measuring the persistence of the memory of these cues in abstinence provides insight into their potential impact on the risk of relapse. CPP is an effective method of assessing the effect of environmental cues morphine memory as these environmental cues are an effect of Pavlovian conditioning that can drive drug-seeking, similar to that seen in CPP. Reducing the effect of these cues is achieved by extinction which is, importantly, not a process of forgetting the conditioned response. Extinction is another learning process that requires repeated exposure to the conditioned stimulus in the absence of the unconditioned stimulus (Myers & Carlezon 2010). However, often extinction training does not occur clinically, and drug-seeking behaviour occurs following a period of abstinence. Here we modelled the effects of abstinence seen in clinical settings with weekly testing during abstinence to observe the persistence of morphine preference under a pattern of intermittent testing. Previously, spontaneous loss of morphine CPP, where morphine-preference reduces without exposure to the conditioned stimulus, has been reported within 5-7 days in rats (Wang et al. 2000). However, other studies have shown morphine CPP is resistant to extinction when undertaken on an intermittent schedule of testing every 2-6 weeks (Mueller, Perdikaris & Stewart 2002; Sakoori & Murphy 2005) with morphine CPP persisting up to twelve weeks (Mueller, Perdikaris & Stewart 2002). In the current study, this persistence was only observed in female mGlu5 KO mice at 5 mg/kg morphine. There are various protocol differences that may explain the different findings here, such as species differences (Mueller, Perdikaris & Stewart 2002; Sakoori & Murphy 2005), the time between conditioning and initial test of preference (Sakoori & Murphy 2005; Wang et al. 2000) and the testing schedule. Mueller *et al* did not find any differences in preference due to testing every 2 or 6 weeks for a total of 12 weeks but Sakoori *et al* noted that mice initially tested after 6 days abstinence tended to increase preference when tested again after 28 days abstinence compared to mice initially tested the day following conditioning which may be related to the increased preference score seen from Test

1 to Test 2 (discussed below). Therefore, longer gaps in the intermittent testing schedule may be reinforcing because they permit spontaneous recovery and a weekly testing schedule may be frequent enough to encourage extinction learning (Chesworth & Corbit 2017; Conklin & Tiffany 2002).

Previously, mGlu5 antagonism with systemic MPEP has been shown to impair extinction learning when there are no changes to the conditioned context but has no effect when there is a change to the context (Andre, Gunturkun & Manahan-Vaughan 2015). However, MPEP administration during conditioning has no effect on passive extinction of morphine CPP in male rats, which is when animals are given free access to the entire apparatus in the absence of the drug (van der Kam, E. L., De Vry & Tzschentke 2009a). The passive extinction used by van der Kam *et al* is similar to the methods used in this thesis, however, this current study had a longer interval between extinction sessions (weekly as opposed to daily). Importantly, MPEP was not administered during extinction training (van der Kam, E. L., De Vry & Tzschentke 2009a) and the lack of effect on extinction may be due to the state-dependent effect that MPEP has on learning. This interpretation is supported by the finding that MTEP attenuated self-administration following 3 weeks of abstinence in mice when MTEP was administered prior to extinction training (Brown, Stagnitti, et al. 2012). In this thesis, mGlu5 deletion did not affect extinction learning in male mice which suggests that the impact of mGlu5 antagonism is due to pharmacological effects. However, these previous studies have been conducted in male mice and this thesis was the first investigation of mGlu5 modulation of extinction learning in both male and female mice. As female mGlu5 KO mice had a persistent preference for morphine while female WT-like mice extinguished morphine preference by Test 3, mGlu5 deletion appears to impede extinction learning of morphine reward in female mice. This suggests that mGlu5 modulates extinction learning, at least on an intermittent schedule, in a sex-dependent manner.

An unexpected finding was the increase in preference score between Test 1 and Test 2 in male mGlu5 KO and WT-like and female WT-like but not female mGlu5 KO mice. Supporting our findings of an increase in preference after initial preference testing, it was found that mice tended to increase preference for 4 mg/kg morphine 28 days following conditioning when they

were initially tested 6 days, but not 1 day, post-conditioning (Sakoori & Murphy 2005). This thesis reports the first finding of an increase in morphine preference following a 7-day period of abstinence. The peak in preference at Test 2 is potentially due to an incubation of drug-craving, where increasing time in abstinence of a drug increases the strength of cue-induced drug seeking following abstinence from the drug (Pickens et al. 2011). The timing of this increase following 1 week of abstinence is similar to an increase in self-administration lever pressing when extinction training commenced after 6 days of abstinence, compared to 1-66 days, seen in rats (Shalev et al. 2001). That female mGlu5 KO mice were the only group that did not show this increase in preference could suggest that mGlu5 modulates the incubation of drug-craving in female but not male mice. This may be due to oestradiol-mediated DA release discussed below. Another explanation for the increase in preference may be due to morphine-withdrawal induced anxiety- and depression-like symptoms (Zanos et al. 2016). Abstinence from morphine has been associated with an increase in anxiety and depressive symptoms in mice (Zanos et al. 2016). These emotional negative effects as a response to morphine abstinence can persist for up to 4 weeks, long after the cessation of morphine withdrawal (Zanos et al. 2016) and morphine-conditioned environments can temporarily reduce withdrawal symptoms in rats (Numan et al. 1976). However, the protocol used to induce spontaneous withdrawal in C57BL/6J mice involves an escalating schedule of much higher doses (up to 100 mg/kg morphine vs 10 mg/kg morphine used here), twice daily administration as opposed to the once daily administration here as well as a longer period of treatment of 6 days (Papaleo & Contarino 2006; Zanos et al. 2016). The protocol used in this thesis is unlikely to induce spontaneous withdrawal as the dose is low and the length of treatment is short. Further, the lack of genotypic differences in males suggests that this increase in preference was not a symptom of severe negative effect.

This thesis also found sex-differences in mGlu5 modulation of sensitivity to morphine reward. Female mGlu5 KO mice had the highest initial preference for 5 mg/kg morphine which suggests they may be more predisposed to the rewarding effects of morphine. However, they had a lower preference for 10 mg/kg morphine. Previously, mGlu5 has been shown to regulate sensitivity to opioid reward, as the mGlu5 antagonist MPEP reduces the rewarding dose of heroin in male rats (Rutten, Van Der Kam, De Vry, Bruckmann, et al. 2011; van der Kam,

E. L., De Vry & Tzschentke 2009a), and mGlu5 deletion in male mice has been shown to increase sensitivity to ethanol (Bird et al. 2008). However, 5 mg/kg morphine also induced CPP in WT-like mice which suggests that this dose is not low enough to say if mGlu5 deletion potentiated morphine reward. Further testing using very low doses of morphine in mGlu5 KO mice would be required to establish if mGlu5 deletion regulated morphine sensitivity; however, the present results do support previous findings that MPEP and MTEP do not affect preference for morphine doses that were sufficient to induce place preference in the absence of mGlu5 antagonism in males (van der Kam, E. L., De Vry & Tzschentke 2009a).

This was the first study to investigate sex-differences in mGlu5 modulation of opioid reward which is an important consideration due to well established sex-differences in sensitivity to opioid reward (Cicero, Aylward & Meyer 2003; Cicero et al. 2000; Karami & Zarrindast 2008) and mGlu5 KO mice display sex-specific differences (Joo et al. 2020). For example, morphine has been shown to be rewarding at higher doses in female rats than males (Cicero et al. 2000) and female rats have a greater magnitude of preference for morphine than males (Karami & Zarrindast 2008). While male rats displayed an inverse U-shaped dose response curve there was no upper limit to doses of heroin (up to 30 mg/kg) that would produce CPP in females (Cicero, Aylward & Meyer 2003). Female rats also consumed more and worked harder to obtain morphine in a self-administration model (Cicero, Aylward & Meyer 2003). It has been suggested that male mice are more susceptible to the negative effects of morphine (Cicero et al. 2000), and while the current study found no sex differences between male and female WT-like mice for 10 mg/kg, it may be possible that sex-differences are evident at higher morphine doses. The increased variability during abstinence seen in the female mGlu5 KO mice for 10 mg/kg suggests that mGlu5 deletion may modulate sensitivity to the negative effects of morphine in female mice. This variability was a result of a small number mice with very low preference scores (n = 3) and it is possible that 10 mg/kg morphine can be either rewarding or aversive in female mGlu5 KO mice, and this is the source of variability in this genotype. The female sex hormone oestradiol has previously been implicated sensitivity to morphine reward. In female mice, ovariectomisation attenuated acquisition of morphine CPP to low doses of morphine and this was reversed by administration of oestradiol benzoate (Mirbaha et al.

2009). Additionally, overectomisation enhanced morphine preference for 10 mg/kg compared to intact females (Mirbaha et al. 2009). While the effects of estradiol on morphine sensitivity are primarily believed to be due to its action on the mesolimbic DA system (Kokane & Perrotti 2020) mGlu5 may help modulate this action in a similar manner to oestradial-mediated DA release in response to amphetamines (Song et al. 2019).

4.3 Genotype and sex effects on morphine locomotion and sensitisation

A significant genotype-dose interaction for the acute administration of morphine suggests that mGlu5 modulates sensitivity to morphine-induced locomotor activity. We observed dose-dependent effects of morphine on locomotor activity in both genotypes and sexes, consistent with well-established effects of morphine on rodent locomotion (Heidari et al. 2006; Koek 2014, 2016; Koek, France & Javors 2012; Loggi et al. 1991; Patti et al. 2005; Saito 1990). The findings in Experiment 1 where 5 and 10 mg/kg, but not 1 and 2 mg/kg, of morphine increased locomotor activity in male C57BL/6J mice, and the results in Experiments 2 and 3 where 5 and 10 mg/kg morphine increased locomotor activity in male and female WT-like mice are consistent with the literature (Heidari et al. 2006; Koek 2014, 2016; Koek, France & Javors 2012; Loggi et al. 1991; Patti et al. 2005; Saito 1990). In contrast, the finding that acute administration of 5 mg/kg morphine induced hypolocomotion in male and female mGlu5 KO mice while 10 mg/kg morphine induced hyperlocomotion in male and female mGlu5 KO mice indicates a dose-genotype interaction. Previous studies have shown that morphine-induced hypolocomotion is biphasic, with low doses of morphine associated with a decrease in locomotor activity while high doses are associated with an increase in locomotor activity (Patti et al. 2005; Saito 1990). The present findings suggest that mGlu5 may modulate sensitivity to morphine-induced locomotor activity. This is in contrast to previous pharmacological studies that have shown that mGlu5 antagonists MPEP and MTEP did not affect morphine- and heroin-induced hyperlocomotion in rats (Herzig & Schmidt 2004; van der Kam, E. L., De Vry & Tzschentke 2009a; Veeneman et al. 2011) and mice (Kotlinska & Bochenski 2007). Considering the impact of mGlu5 deletion on morphine-induced locomotion in the present thesis, it is possible that the doses of MPEP and MTEP used in these pharmacological studies were not sufficient to affect morphine-induced locomotion (Kotlinska & Bochenski 2007). Additionally, although systemic mGlu5 antagonism

promotes hyperlocomotion, selective blockage in the ventral striatum is associated with hypolocomotion (Guimaraes et al. 2015). As morphine-induced hyperlocomotion is associated with increased DA release in the ventral striatum (Murphy, Lam & Maidment 2001) the present data suggests that mGlu5 deletion may modulate morphine-mediated dopamine activity in the ventral striatum where mGlu5 is strongly expressed.

mGlu5 deletion had a sex-dependent effect on the expression of morphine-induced locomotor sensitisation. Locomotor sensitisation occurs when locomotor activity increases following repeated administration of same dose of a drug. Female mGlu5 KO mice sensitised to both 5 and 10 mg/kg morphine while female WT-like mice only sensitised to 10 mg/kg morphine, indicating mGlu5 deletion potentiates morphine sensitisation. However, as male mGlu5 KO and WT-like mice showed sensitisation to 10 mg/kg morphine but not 5 mg/kg morphine, the potentiation of morphine sensitisation appears to be sex-dependent. A previous study showed that the mGlu5 antagonist MTEP did not affect the development morphine sensitisation in rats when 1 mg/kg MTEP was administered 30 min before 3 mg/kg morphine conditioning (Veeneman et al. 2011). Additionally, there was no effect of prior MTEP on the expression of morphine sensitisation when rats were challenged with morphine 3 weeks later. However, 10 mg/kg, but not 5 mg/kg, MTEP inhibited the expression of 10 mg/kg morphine locomotor sensitisation in mice (Kotlinska & Bochenski 2007) so the lack of effect seen in Veenemen *et al* could be due to the MTEP dose being insufficient to affect locomotor sensitisation. As mGlu5 deletion did not prevent the acquisition of locomotor sensitisation in this study, this suggests that the effect of MTEP on locomotor sensitisation may be due to inducing locomotor sensitisation on its own (Herzig & Schmidt 2004; Kotlinska & Bochenski 2007) or via an off target effect, potentially due to a low affinity to the NMDA receptor (Guimaraes et al. 2015), as NMDA antagonism blocks the acquisition of morphine-induced locomotor sensitisation (Wolf & Jeziorski 1993).

mGlu5 deletion appears to increase susceptibility for morphine sensitisation in female mice. Powel and Holtzman (Powell & Holtzman 2001) have suggested that there is a narrow range of morphine doses that induce locomotor sensitisation, due to a biphasic sensitisation

response to morphine that peaked at 3 mg/kg (1-10 mg/kg morphine tested) in rats. There are differences between mouse and rat metabolism of morphine (Way & Adler 1962) and therefore, there may be a different dose-response curve in mice. However, these results indicate that mGlu5 deletion may extend the range of morphine doses that induce locomotor sensitisation in female mice. Female mice are more sensitive to the locomotor effects of morphine (Craft et al. 2006) and oxycodone (Collins et al. 2016). While this could be attributed to sex differences in striatal dopamine release (Arvidsson et al. 2014; Laakso et al. 2002; Riccardi et al. 2011), estradiol has been shown to increase cocaine locomotor sensitisation (Martinez et al. 2014). Opioids and psychostimulants like cocaine have different mechanisms of action, but the receptors and pathways involved in locomotor sensitisation are similar and there is evidence that cross-sensitisation is common (Valjent et al. 2010). The increased sensitisation in female mGlu5 KO mice indicated that mGlu5 is a potential point of divergence in these pathways as oestradiol-mediated facilitation of cocaine locomotor sensitisation requires mGlu5 activation (Martinez et al. 2014) while in this study mGlu5 deletion potentiated morphine locomotor sensitisation. This suggests that mGlu5 activity may have attenuated the oestradiol-associated increases to morphine locomotor sensitisation.

4.4 No genotype effect on the acquisition of morphine CPP

Data on acquisition of morphine CPP suggested that mGlu5 is not necessary for the expression of morphine reward. This contrasts with previous pharmacological findings where MPEP and MTEP dose-dependently attenuate the acquisition of morphine and heroin self-administration, morphine and heroin CPP in rats and mice (Aoki et al. 2004; Brown, Stagnitti, et al. 2012; Popik 2002; van der Kam, Elizabeth L., De Vry & Tzschentke 2007; Veeneman et al. 2011). The contrary finding here suggests that pharmacological studies are not sufficient to understand the effect of a receptor on drug-relevant pathology and behaviour as adaptive changes in the mGlu5 KO gremline also have to be considered. A potential explanation for the null effect of mGlu5 deletion on morphine CPP include such adaptive changes. The attenuation of morphine CPP by localised administration of MPEP was associated with an attenuation of morphine-mediated upregulation of PKC-γ (Aoki et al. 2004) which has been shown to be necessary for the expression of morphine reward. An increase in expression of PKC-ß proteins

in the hippocampus has been observed in mGlu5 KO mice (Gonzalez-Lozano et al. 2021) which suggests a potential change in PKC activity in mGlu5 KO mice to compensate for the lack of mGlu5 signalling. mGlu5 KO mice do not show the pilocarpine-induced expression of PKC-ß and PKC-γ in pyramidal neurons in the hippocampus (Liu et al. 2008). Together, this suggests that mGlu5 KO mice may have an altered drug-induced expression of PKC-ß and PKC-γ. Additionally, Go6976, a specific PKC-α and PKC-ß inhibitor, HBDDE and PKC-α and PKC-γ inhibitor, facilitated morphine-induced MOR internalisation (Ueda, Inoue & Matsumoto 2001) suggesting there is a potential PKC-ß and PKC-γ interaction in the development of morphine tolerance. This indicates that changes in PKC isoform expression may explain the lack of genotype on the acquisition of morphine CPP. Further investigation is required to see if mGlu5 KO mice also have an increase in the expression of PKC isoforms following morphine administration.

4.5 Methodological considerations

There was a strong bias for the compartment that mGlu5 KO mice were initially placed in at habituation. The apparent bias at habituation could have an impact on the interpretation of our findings. It should be noted that this apparatus has previously been used with no side preference in C57BL/6J mice (Chesworth & Karl 2020; Chesworth et al. 2021). Additionally, WT-like mice did not show a side bias at habituation, indicating this is a confound with the mGlu5 KO mice rather than the apparatus. There was a strong correlation between the initial compartment preference and distance travelled during the first 5 min of habituation, which suggests that this initial preference was due to low exploration during the first 5 min. It is important to note that there was no side bias during the remainder of the habituation sessions when locomotor activity was higher so this initial preference appears to be a consequence of hypolocomotion as a response to a novel envrionment. Further analysis of the first saline and morphine conditioning sessions showed no initial hypolocomotion (data not shown). However, this did lead to low preference scores at habituation in the mGlu5 KO male and female mice for 5 mg/kg and male mGlu5 KO mice for 10 mg/kg morphine. Therefore, there is a possibility that an increase in preference score may have been due to morphine overcoming the animals' natural aversion to the environment rather than a measurement of the rewarding effects of morphine, or these two

things may both be occurring. While habituation side bias can determine whether ethanol CPP is acquired as animals paired with the preferred side do not acquire CPP with those paired with the non-preferred side do acquire CPP (Cunningham, Ferree & Howard 2003; Cunningham, Gremel & Groblewski 2006) it has been established that the magnitude of preference following morphine conditioning is unaffected by a biased or completely randomised side allocation in rats (Blander et al. 1984). This suggests that initial side preference may not have significantly impacted the acquisition of place preference. The successful acquisition of morphine CPP in both male and female mGlu5 KO mice despite different initially preferred sides supports this proposition. Therefore, the bias at habituation is a product of the mGlu5 KO mice but has not prevented the acquisition and expression of morphine CPP.

4.6 Limitations

The current study was limited to looking at the effect of mGlu5 deletion on morphine locomotion, CPP and the persistence of preference during abstinence. While CPP is effective at measuring the effect of environmental cues on drug-seeking behaviour it involves the external administration of the drug under a form of Pavlovian conditioning, which does not reflect all of the varied and complex processes involved in addiction pathology. A self-administration model would be required to more fully assess the role of mGlu5 in morphine addiction-like behaviour, because an operant model can evaluate a wider degree of addiction-relevant behaviour including drug reinforcement, motivation for drug-seeking, extinction behaviour and relapse-like behaviour. Such a model was not included in this thesis due to the significant time required to implement, e.g. it would have taken 2-3 months to test a single cohort and would require a minimum of 2 cohorts to test both sexes and reach sufficient statistical power. The findings of this thesis nonetheless are a foundation for future investigation of the sex effects of mGlu5 on morphine addiction-relevant behaviour using a self-administration model.

4.7 Conclusion

This thesis shows that mGlu5 modulates morphine addiction-relevant behaviour in a sex-dependent manner. This emphasises the importance of testing for sex-differences in addiction research in general and opioid addiction in particular. The potentiation of morphine reward

and the persistence of morphine memory seen in female mGlu5 KO mice suggest that this receptor plays a significant role in sex differences in susceptibility to opioid addiction. mGlu5 also mediates sensitivity to morphine-induced hyperlocomotion and locomotor sensitivity. This thesis emphasises the role of mGlu5 in the strength of morphine-associated contextual cues that could increase the risk of relapse and supports the further investigation of this receptor as a target for the development of opioid addiction therapies. Potential areas to follow from this study include the effects of mGlu5 deletion on the state-dependent effect of morphine learning, by comparing Test data in the presence and absence of morphine, and the acquisition and persistence of morphine self-administration.

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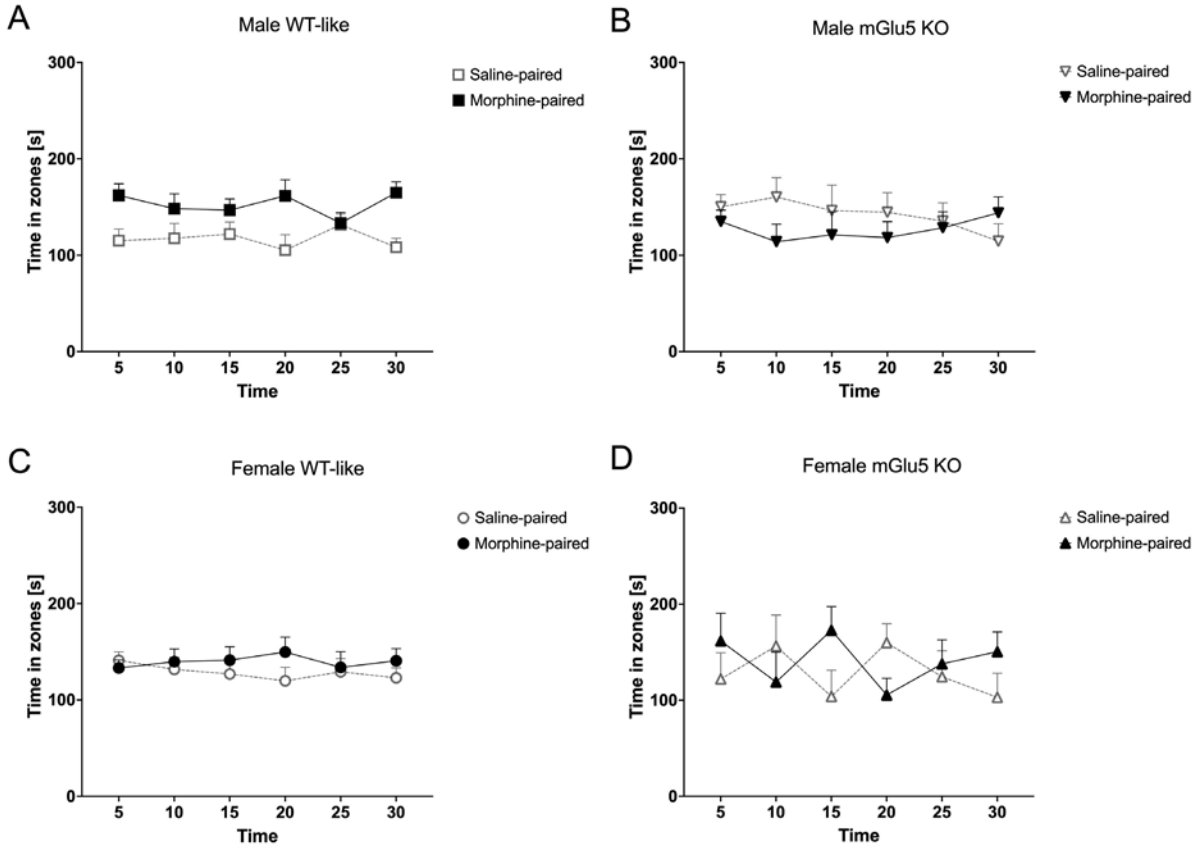
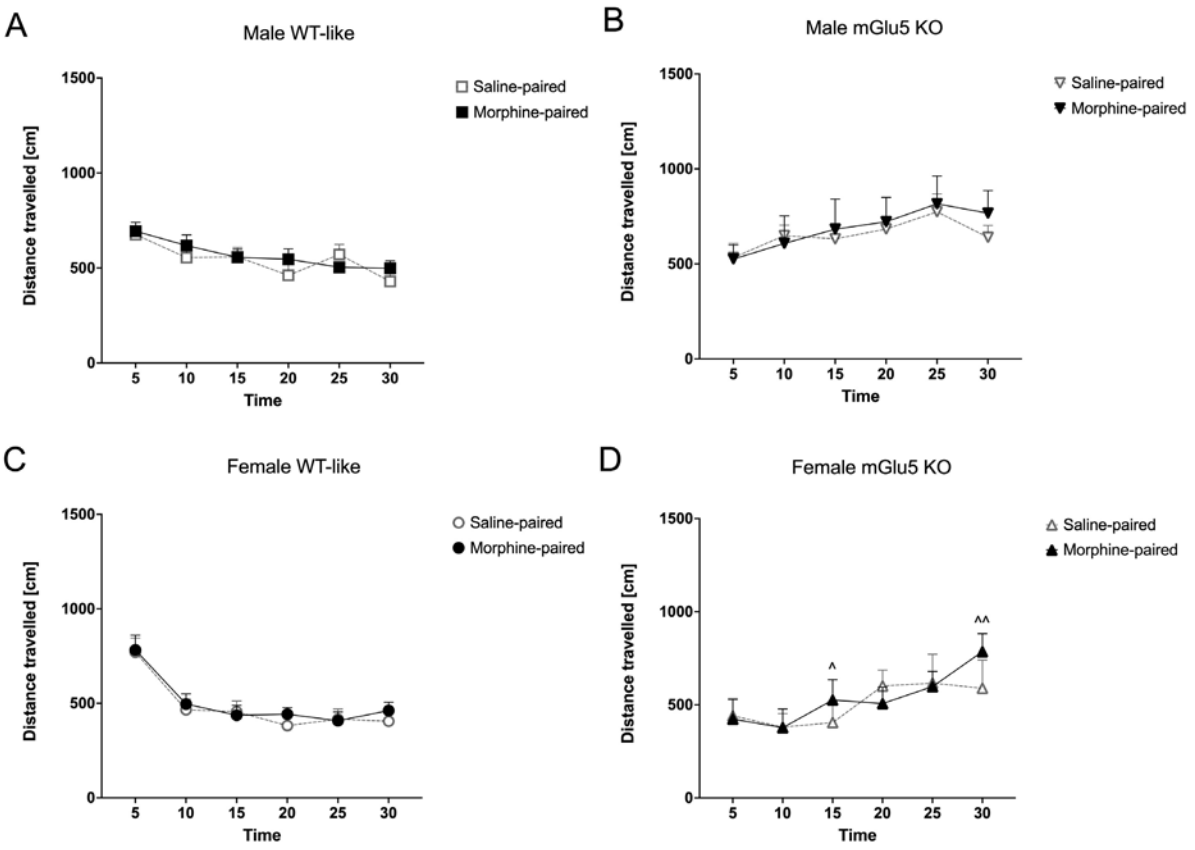
Appendices

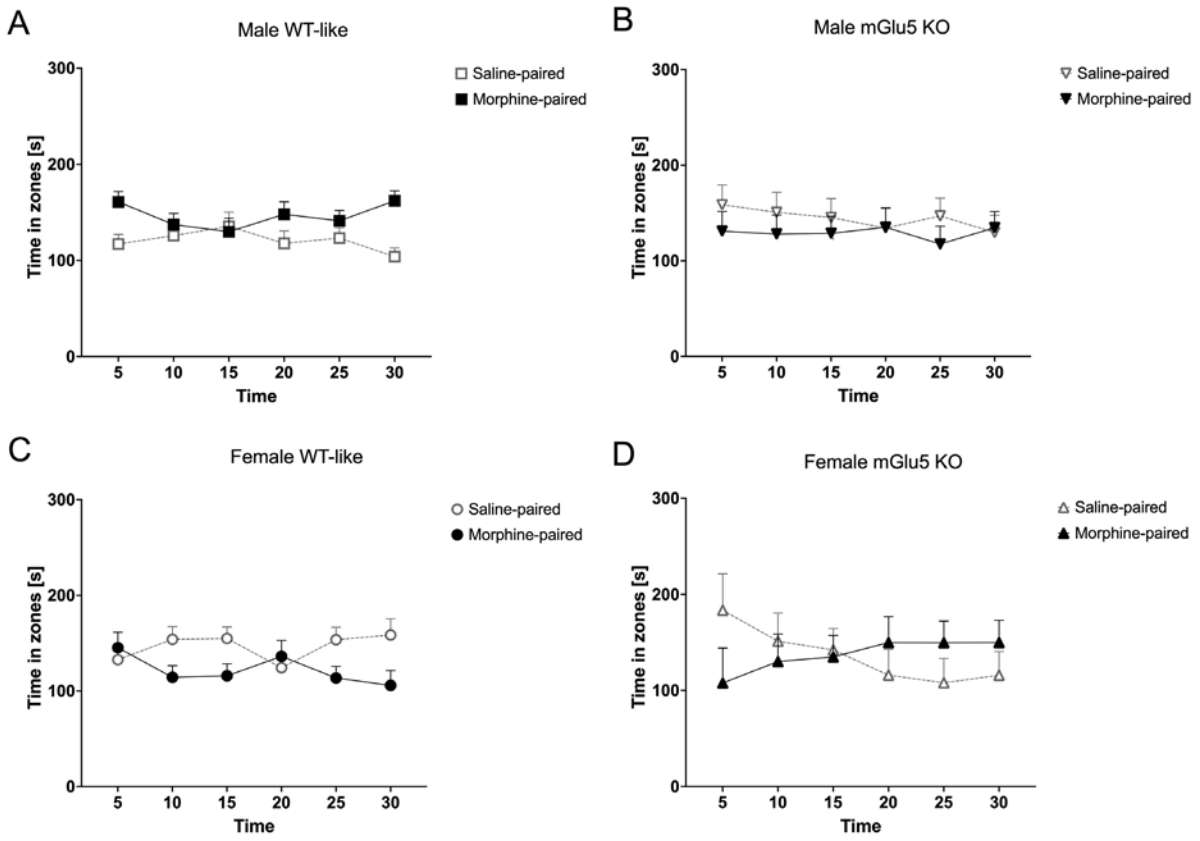
Appendix A: Timecourse data for Tests 3, 4 and 5 for Experiment 2



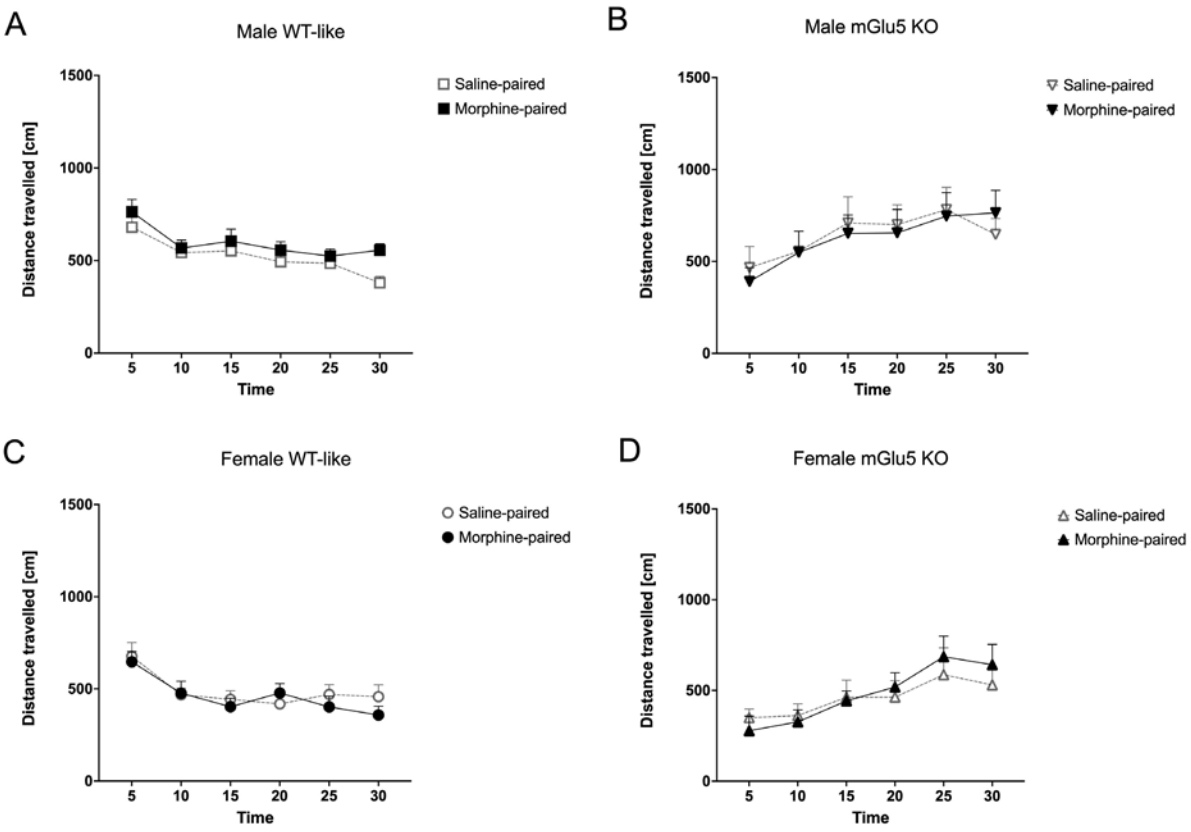
Supplementary Figure 1: Time spent in zones during Test 3 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 3 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA. Four-way RM ANOVA found no main effect of 'zone' [$F(1,36) = 2.26$, $p = .142$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 6.27$, $p < .001$].



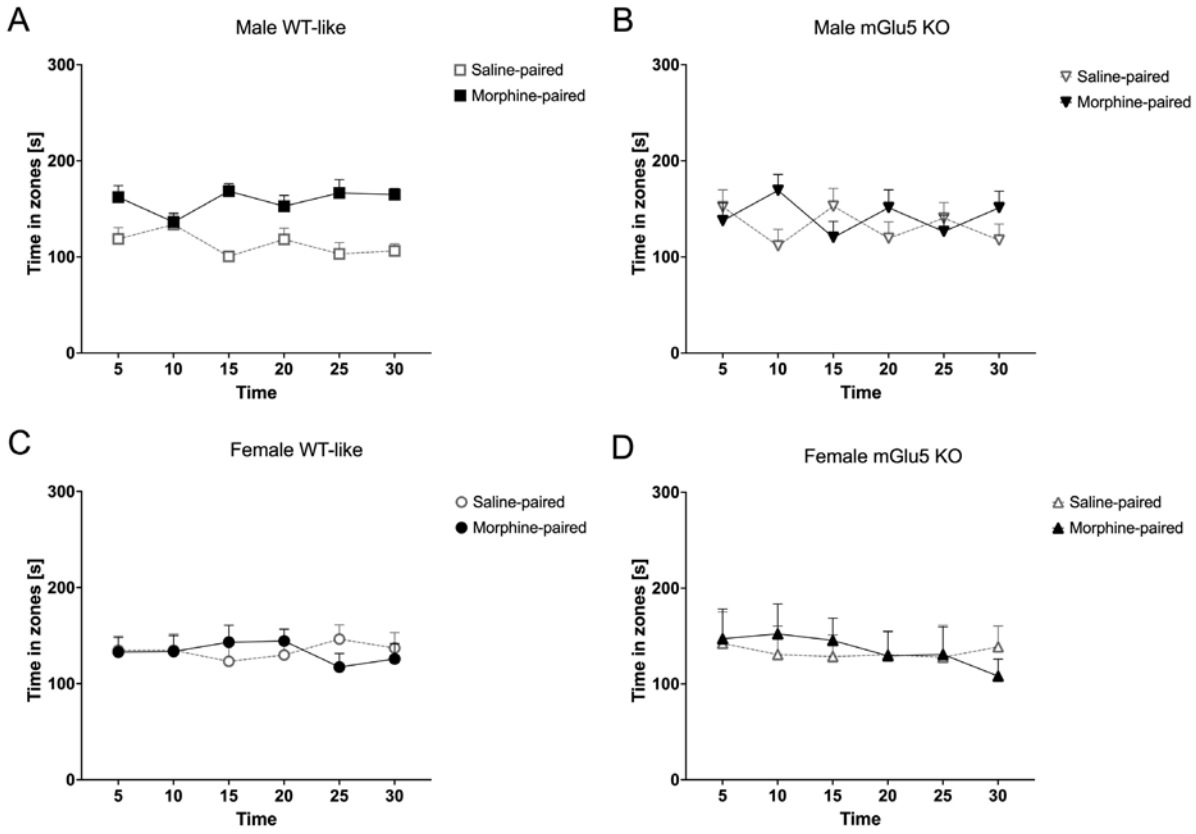
Supplementary Figure 2: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 3. Distance travelled [cm] in the morphine- and saline- paired zones during Test 3 in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found a main effect of 'sex' [$F(1,36) = 6.04$, $p = .019$] and 'time' [$F(5,180) = 3.66$, $p = .004$] but not 'genotype' [$F(1,36) = 2.29$, $p = .139$] or 'zone' [$F(1,36) = 3.23$, $p = .081$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 20.47$, $p < .001$], 'sex' and 'time' [$F(5,180) = 4.38$, $p = .004$], 'zone' and 'time' [$F(5,180) = 2.57$, $p = .039$] and 'genotype', 'zone' and 'time' [$F(5,180) = 3.29$, $p = .012$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).



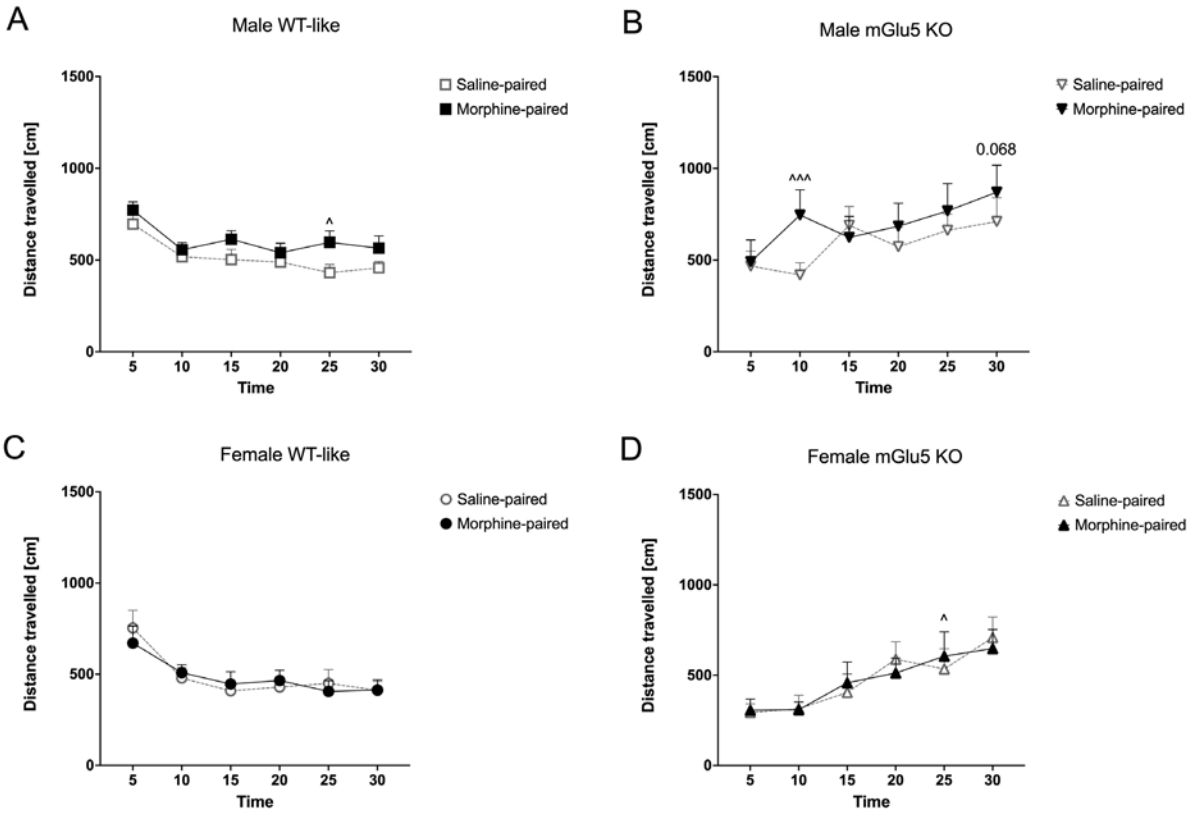
Supplementary Figure 3: Time spent in zones during Test 4 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 4 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA. Four-way RM ANOVA found no main effect of 'zone' [$F(1,36) = 0.27$, $p = .605$]. There was an interaction between 'genotype', 'sex' and 'zone' [$F(1,36) = 6.42$, $p = .016$] and 'genotype' and 'time' [$F(5,180) = 4.90$, $p < .001$].



Supplementary Figure 4: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 4. Distance travelled [cm] in the morphine- and saline- paired zones during Test 4 in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA. Four-way RM ANOVA found a main effect of 'sex' [$F(1,36) = 4.53$, $p = .040$] but not 'genotype' [$F(1,36) = 0.50$, $p = .483$], 'zone' [$F(1,36) = 1.42$, $p = .241$] or 'time' [$F(5,180) = 2.17$, $p = .093$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 24.79$, $p < .001$].

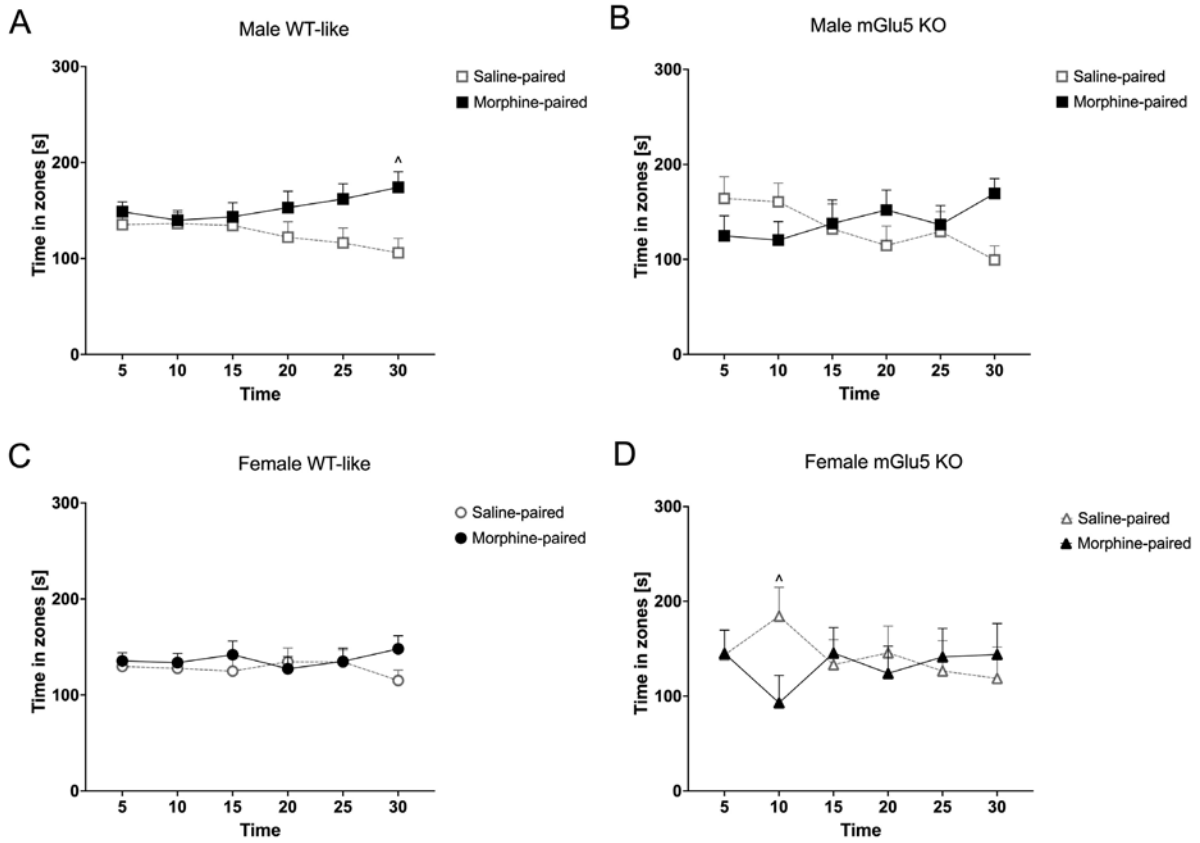


Supplementary Figure 5: Time spent in zones during Test 5 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 5 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA. Four-way RM ANOVA found no main effect of 'zone' [$F(1,36) = 2.90$, $p = .097$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 9.09$, $p < .001$], 'sex' and 'time' [$F(5,180) = 2.74$, $p = .032$] and 'genotype', 'sex' and 'time' [$F(5,180) = 2.46$, $p = .049$].

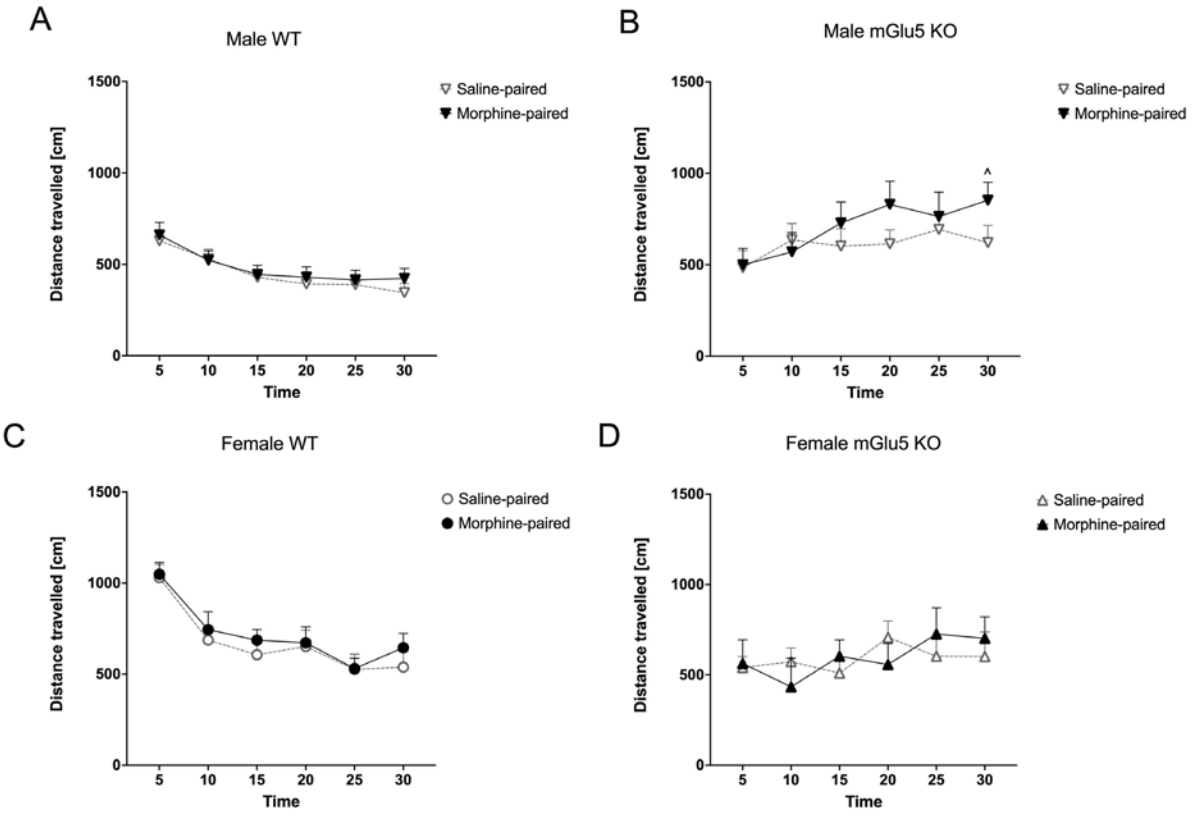


Supplementary Figure 6: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 5. Distance travelled [cm] in the morphine- and saline- paired zones during Test 5 in (A) male mGlu5 KO and WT-like littermates and (B) female mGlu5 KO and WT-like littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found a main effect of 'sex' [$F(1,36) = 5.43$, $p = .026$], 'zone' [$F(1,36) = 4.19$, $p = .048$] and 'time' [$F(5,180) = 2.75$, $p = .041$] but not 'genotype' [$F(1,36) = 0.80$, $p = .377$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 19.13$, $p < .001$] and 'genotype', 'sex', 'zone' and 'time' [$F(5,180) = 2.86$, $p = .031$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).

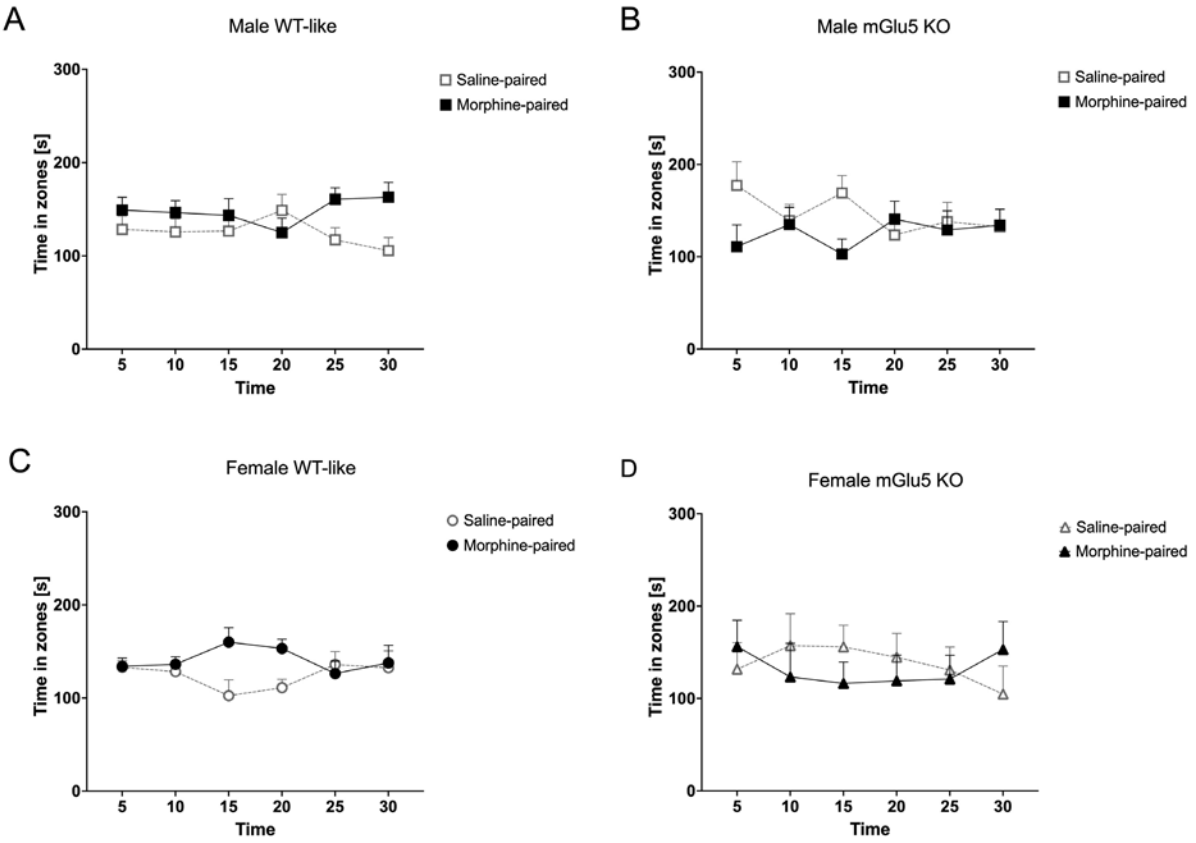
Appendix B: Timecourse data for Tests 3, 4 and 5 for Experiment 3



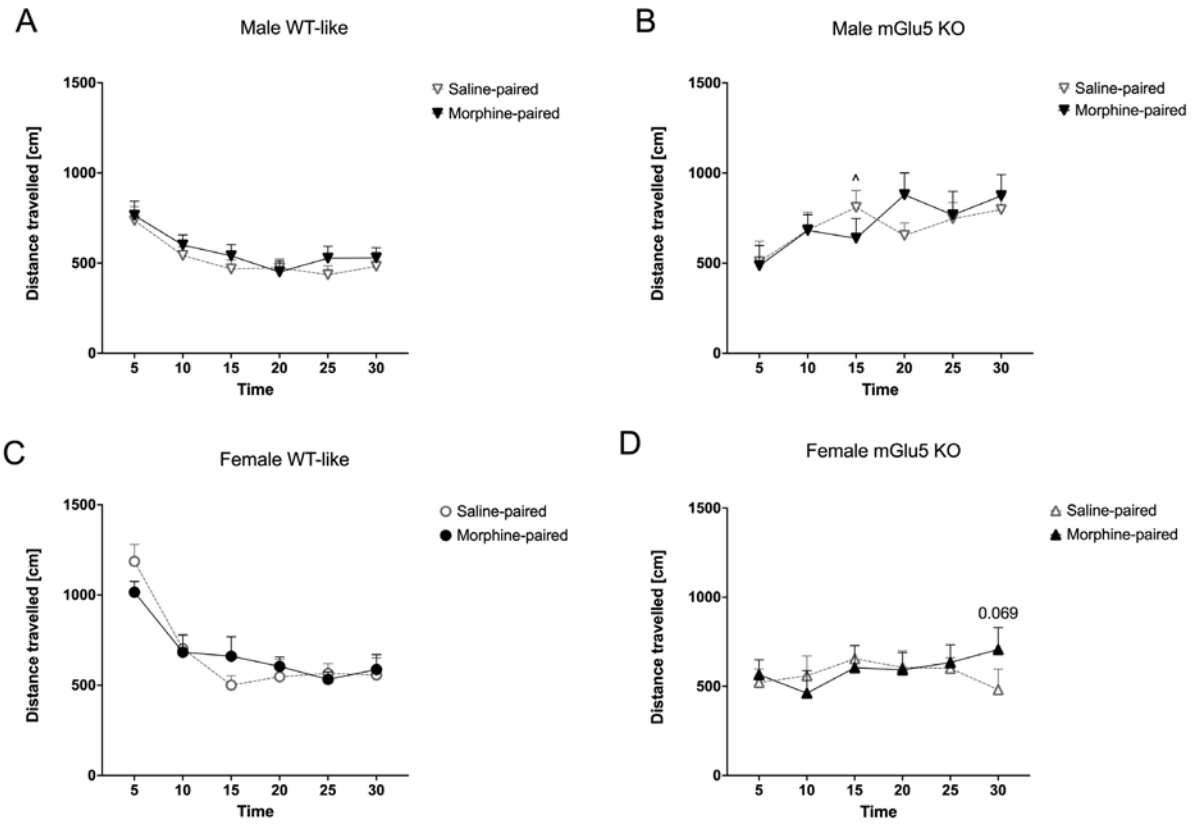
Supplementary Figure 7: Time spent in zones during Test 3 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 3 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found found no main effect of 'zone' [$F(1,36) = 0.18$, $p = .675$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 6.89$, $p < .001$] and 'zone' and 'time' [$F(5,180) = 3.58$, $p = .012$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).



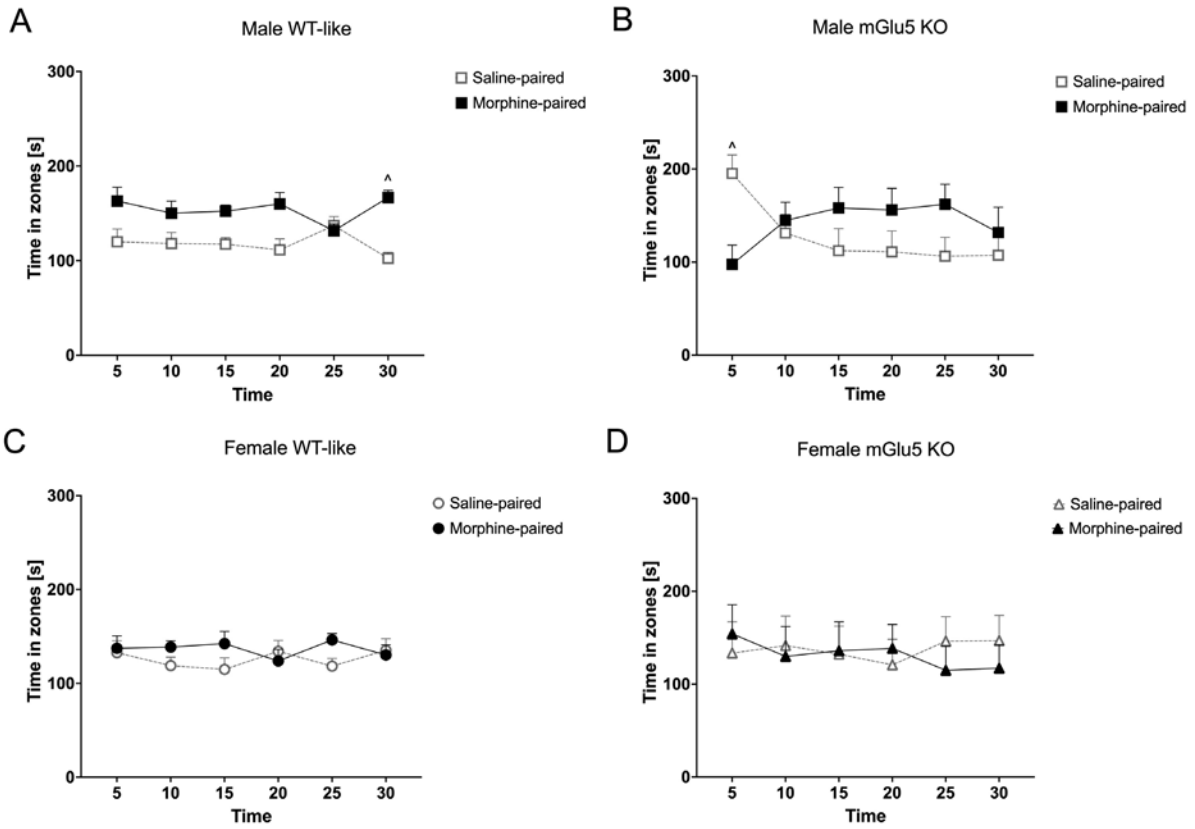
Supplementary Figure 8: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 3. Distance travelled [cm] in the morphine- and saline- paired zones during Test 3 in (A) male WT-like and mGlu5 KO littermates and (B) female WT-like and mGlu5 KO littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found a main effect of 'sex' [$F(1,36) = 6.04$, $p = .019$] and 'time' [$F(5,180) = 3.66$, $p = .004$] but not 'genotype' [$F(1,36) = 2.29$, $p = .139$] or 'zone' [$F(1,36) = 3.23$, $p = .081$]. There was an interaction between 'genotype' and 'time' [$F(5,180) = 20.47$, $p < .001$], 'sex' and 'time' [$F(5,180) = 4.38$, $p = .004$], 'zone' and 'time' [$F(5,180) = 2.57$, $p = .039$] and 'genotype', 'zone' and 'time' [$F(5,180) = 3.29$, $p = .012$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).



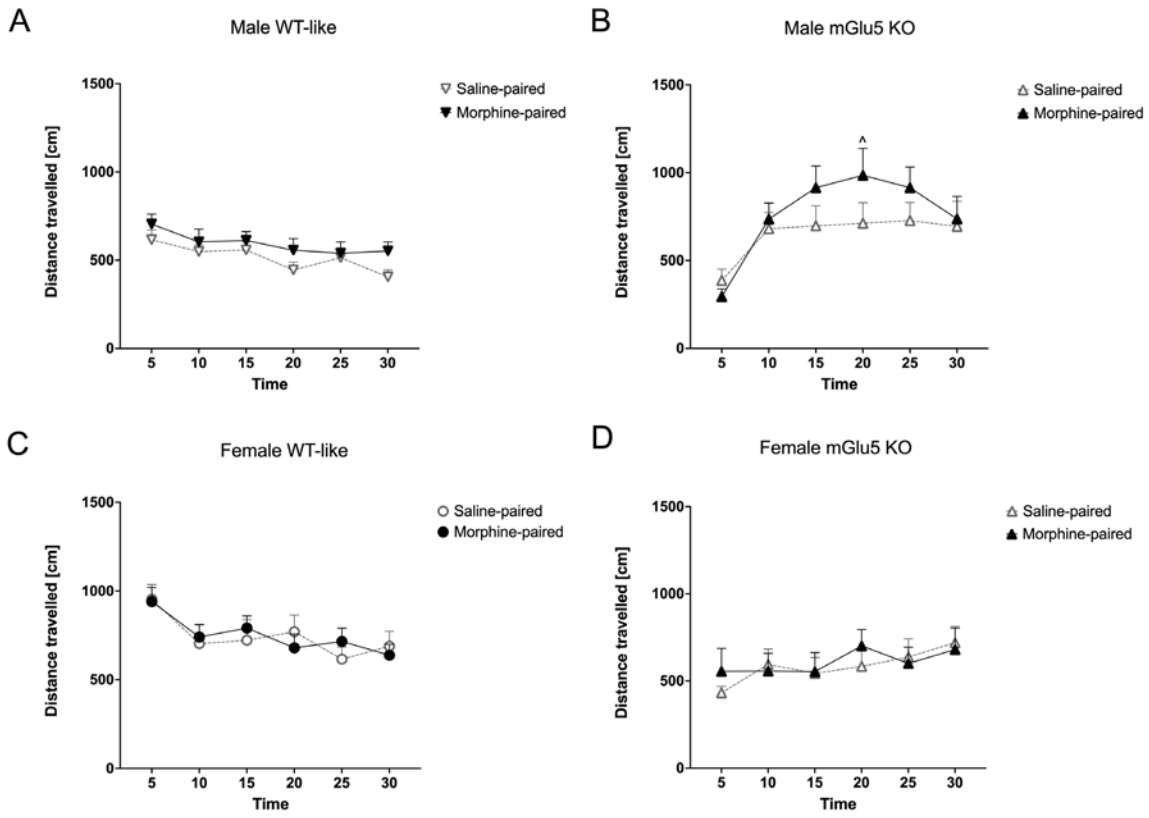
Supplementary Figure 9: Time spent in zones during Test 4 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 4 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA. Four-way RM ANOVA found no main effect of 'zone' [$F(1,36) = 0.01$, $p = .908$].



Supplementary Figure 10: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 4. Distance travelled [cm] in the morphine- and saline- paired zones during Test 4 in (A) male WT-like and mGlu5 KO littermates and (B) female WT-like and mGlu5 KO littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found a main effect of 'time' [$F(5,180) = 6.66$, $p < .001$] but not 'genotype' [$F(1,36) = 0.24$, $p = .626$], 'sex' [$F(1,36) = 0.39$, $p = .843$] or 'zone' [$F(1,36) = 0.08$, $p = .781$] . There was an interaction between 'genotype' and 'sex' [$F(1,36) = 5.27$, $p = .028$], 'genotype' and 'time' [$F(5,180) = 26.78$, $p < .001$], 'sex' and 'time' [$F(5,180) = 4,20$, $p = .012$] and 'genotype', 'zone' and 'time' [$F(5,180) = 3.65$, $p = .005$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).



Supplementary Figure 11: Time spent in zones during Test 5 in male and female WT-like and mGlu5 KO mice. Time [s] spent in the morphine- and saline- paired zones during Test 5 in (A) male WT, (B) male mGlu5 KO, (C) female WT-like and (D) female mGlu5 KO mice. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found no main effect of 'zone' [$F(1,36) = 0.75$, $p = .391$]. There was an interaction between 'genotype', 'sex' 'zone' and 'time' [$F(5,180) = 3.62$, $p = .014$]. There were no other interactions. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).



Supplementary Figure 12: Distance travelled in zones by male and female WT-like and mGlu5 KO mice during Test 5. Distance travelled [cm] in the morphine- and saline- paired zones during Test 5 in (A) male WT-like and mGlu5 KO littermates and (B) female WT-like and mGlu5 KO littermates. Data presented as means ± SEM and analysed using four-way RM ANOVA, followed by Bonferroni post hoc tests. Four-way RM ANOVA found no main effect of 'genotype' [$F(1,36) = 0.06$, $p = .803$], 'sex' [$F(1,36) = 1.18$, $p = .284$], 'zone' [$F(1,36) = 0.99$, $p = .326$] and 'time' [$F(5,180) = 1.48$, $p = .221$]. There was an interaction between 'genotype' and 'sex' [$F(1,36) = 1.81$, $p = .010$], 'genotype' and 'time' [$F(5,180) = 24.22$, $p < .001$] and 'sex' and 'time' [$F(5,180) = 5.61$, $p > .001$]. Significant effects of 'zone' are indicated ($^{\wedge}p < 0.05$; $^{\wedge\wedge}p < 0.01$).