The role of tumour necrosis factor-α in synaptic and behavioural plasticity during cocaine and morphine addiction

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Abstract

The neurobiology of drug addiction has been widely investigated, due to the extensive societal cost of addiction and the need for more effective treatments. Addiction is theorized to be driven by synaptic plasticity at dopaminergic, glutamatergic, and GABAergic synapses in brain areas critical for reward, such as the nucleus accumbens (NAc). In the NAc, cocaine induces plasticity at excitatory and inhibitory synapses in a bi-phasic pattern that varies depending on the stage of addiction. The two synapse types show plasticity in opposing directions, indicating that cocaine shifts the balance of excitatory and inhibitory signalling to potentially drive drug-induced behaviour. Plasticity at glutamatergic synapses in the NAc is also associated with morphine treatment, though the pattern is more complex and not as well characterized. In the case of both psychostimulants and opioids, the molecular mediators of drug-induced plasticity are not fully known. However, one factor they have in common is altering the neuroimmune system. Cocaine and morphine both activate microglia which leads to the release of inflammatory cytokines such as tumour necrosis factor alpha (TNF). Loss of TNF signalling has been shown to exacerbate drug-induced behaviour; and in the case of cocaine, this corresponds to a change in glutamatergic synaptic plasticity in the NAc. Here we further explored TNF's role in addiction by determining which TNF receptor mediates its effects after cocaine treatment; investigating whether TNF modulates cocaine-induced plasticity at inhibitory synapses; and examining whether TNF plays a similar role in morphine administration as with cocaine. First, we found that loss of TNFR1 signalling exacerbated locomotor sensitization to cocaine, indicating that it is the receptor responsible for TNF's effects on cocaine-induced behaviour. We then showed that a lack of TNF signalling changes the direction of plasticity at inhibitory synapses caused by cocaine, confirming its role in GABAergic synaptic plasticity during cocaine treatment. Finally,

we found that morphine-induced sensitization is similarly aggravated by loss of TNF, but that the synaptic plasticity underlying this is different from our findings in cocaine. Overall, these findings have improved our understanding of how TNF functions in drug addiction, which is critical for potential development of addiction treatments in the future.

Résumé

La neurobiologie de la dépendance a fait l'objet de nombreuses recherches, en raison du coût sociétal de la dépendance et de la nécessité de trouver des traitements plus efficaces. Il est théorisé que la dépendance est dûe à la plasticité synaptique des synapses dopaminergiques, glutamatergiques et GABAergiques dans les zones du cerveau essentielles à la récompense, comme le noyau accumbens (NAc). Dans le NAc, la cocaïne induit la plasticité au niveau des synapses excitatrices et inhibitrices selon un schéma biphasique qui varie en fonction du stade de la dépendance. Les deux types de synapses montrent une plasticité dans des directions opposées, ce qui indique que la cocaïne modifie l'équilibre de la signalisation excitatrice et inhibitrice pour potentiellement causer le comportement induit par la drogue. La plasticité des synapses glutamatergiques dans le NAc est également liée au traitement avec la morphine, bien que le schéma soit plus complexe et moins bien caractérisé. Dans le cas des psychostimulants et des opioïdes, les médiateurs moléculaires de la plasticité induite par les drogues ne sont pas entièrement connus. Cependant, l'un de leurs facteurs communs est l'altération du système neuro-immunitaire. La cocaïne et la morphine activent la microglie, ce qui entraîne la production de cytokines inflammatoires telles que le TNF. Il a été démontré que la perte de la signalisation du TNF exacerbe le comportement induit par les drogues et, dans le cas de la cocaïne, cela correspond à une modification de la plasticité synaptique glutamatergique dans le NAc. Dans cette thèse, nous avons étudié le rôle du TNF dans la dépendance. Nous avons voulu déterminer

quel récepteur du TNF est le médiateur de ses effets après le traitement de la cocaïne; nous avons cherché à savoir si le TNF module la plasticité induite par la cocaïne au niveau des synapses inhibitrices; et nous avons examiné si le TNF joue un rôle similaire pendant l'administration de morphine, comme pour la cocaïne. D'abord, nous avons constaté que la perte de la signalisation du TNFR1 exacerbait la sensibilisation locomotrice à la cocaïne, ce qui indique qu'il s'agit du récepteur responsable des effets du TNF sur le comportement induit par la cocaïne. Nous avons ensuite montré que l'absence de signalisation du TNF modifie la direction de la plasticité des synapses inhibitrices provoquée par la cocaïne, confirmant son rôle dans la plasticité synaptique GABAergique pendant le traitement à la cocaïne. Enfin, nous avons constaté que la sensibilisation induite par la morphine est aggravée par la perte de TNF, comme pour la cocaïne, mais que la plasticité synaptique sous-jacente est différente de celle que nous avons observée pour la cocaïne. Dans l'ensemble, ces résultats ont amélioré notre compréhension du fonctionnement du TNF dans la dépendance, ce qui est essentiel pour le développement potentiel de traitements de la dépendance à l'avenir.

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Contribution of Authors to Research Work

Simone Valade: I performed all experiments and their corresponding analyses. I planned experiments in collaboration with Dr. David Stellwagen. Figures were made by me. The writing was done by me and edited by Dr. Stellwagen and myself.

Dr. David Stellwagen: Dr. Stellwagen supervised all aspects of the project, including elaborating experimental design and statistical analyses, addressing issues during data collection, and editing this thesis.

Hooman Salahi: Hooman performed all mouse genotyping required for the experiments.

Introduction

Addiction is a neuropsychiatric disorder that involves compulsively seeking rewarding stimuli, like drugs of abuse, to an extreme degree that becomes detrimental to physical, emotional, social, and financial well-being (Volkow and Morales 2015). Addiction also has extensive societal and economic costs beyond the individual patient; in particular, the "opioid crisis" has become a major public health issue in the last 5 years due to increasing rates of opioid-related deaths, which can be partially attributed to a lack of effective treatment options to prevent relapse (Special Advisory Committee on the Epidemic of Opioid Overdoses 2021).

Addiction is thought to be driven by aberrant synaptic plasticity at both dopaminergic, glutamatergic, and GABAergic synapses in brain areas critical for reward, such as the nucleus accumbens (NAc). All drugs of abuse facilitate dopamine neurotransmission, to produce stronger feelings of reward and pleasure upon drug consumption (Koob and Volkow 2016). However, changes at glutamatergic and GABAergic synapses last longer and follow a pattern matching the stages of addiction behaviour (Kourrich et al. 2007; Ortinski et al. 2012), suggesting that this plasticity underlies long-term maintenance of addiction. Drug-related plasticity includes traditional Hebbian plasticity like long term potentiation and depression, alongside homeostatic synaptic plasticity (HSP) which is indicated by both psychostimulants and opioids altering postsynaptic AMPA receptor trafficking (Terrier et al. 2016; Benneyworth et al. 2019; Madayag et al. 2019). Under normal conditions, HSP reins in Hebbian plasticity to maintain excitatory-inhibitory balance by adjusting synaptic strength across multiple synapses in a circuit (Turrigiano and Nelson 2004); but during addiction, HSP might underlie the dysregulation of reward circuits and thereby drive drug-seeking behaviours.

While these shifts in plasticity are well-documented, the molecular mechanisms that link drugs of abuse to altered plasticity are still being explored. One candidate mechanism is glial cell dynamics, which help maintain homeostasis in neural circuits. Both psychostimulants and opioids alter the function of glial cells, including activating microglia (Lacagnina et al. 2017), which leads to release of cytokines such as tumour necrosis factor alpha (TNF). TNF has a previously established role in HSP: it increases overall excitatory signalling by potentiating glutamatergic and reducing GABAergic inputs onto excitatory neurons (Stellwagen and Malenka 2006; Pribiag and Stellwagen 2013) and by decreasing glutamatergic synapse strength and increasing GABAergic input onto inhibitory neurons (Lewitus et al. 2014; Chambon 2020). Initially we posited that microglial activation and TNF release could contribute to addiction. We found that loss of TNF signalling exacerbated cocaine sensitization and reversed the typical pattern of cocaine plasticity at excitatory synapses; we also saw that re-inducing TNF release after withdrawal was able to reduce sensitization upon cocaine re-exposure (Lewitus et al. 2016). From this, we now hypothesize that microglial activation and TNF works via HSP to attenuate drug-induced synaptic plasticity and behaviour, rather than causing it. To further explore this hypothesis, we divided our research into three aims:

1) TNFR1 as the mediator of TNF's attenuating effects on addiction.

Understanding which TNF receptor is behind these effects is the first step to determining exactly how TNF modulates cocaine addiction. TNFR1 is the receptor responsible for TNF's role in receptor trafficking, so we hypothesized that TNFR1 mediates its role in addiction plasticity and behaviour. We tested this using cocaine-induced locomotor sensitization behaviour in the TNFR1 knockout (KO) mouse line.

2) TNF function at inhibitory synapses in cocaine addiction.

Cocaine causes biphasic plasticity at inhibitory synapses, and TNF is known to alter inhibitory synaptic strength. Therefore, we wanted to investigate whether loss of TNF signalling impacts cocaine-induced plasticity at inhibitory synapses in the same was as excitatory ones. To examine this, we looked at GABAergic synaptic plasticity induced by chronic cocaine in the TNF KO mouse model.

3) The role of TNF in morphine-induced plasticity and behaviour.

Despite the increasing prevalence of opioid addiction, there are still few avenues to prevent relapse. This is partly because the molecular mechanisms behind the rewarding effects of opioids are poorly understood. Like cocaine, morphine influences glutamatergic synaptic plasticity, but this pattern has not been fully characterized. We also know that morphine activates microglia and increases TNF levels. Therefore, we investigated whether loss of TNF signalling impacts morphine-induced sensitization and excitatory synaptic plasticity.

Chapter 1 – Literature Review

The neurobiology of addiction is complex and involves multiple cellular and molecular mechanisms. Synaptic plasticity in reward areas of the brain, such as the nucleus accumbens, is theorized to drive addiction behaviours. Drugs of abuse, such as cocaine and morphine, also alter the function of glial cells which can impact this synaptic plasticity. In particular, the release of cytokines such as tumour necrosis factor alpha (TNF) by microglia can induce homeostatic synaptic plasticity, which may act to reduce the plasticity induced by drugs of abuse. In this manner, TNF has been shown to play a role in addiction behaviours & plasticity. Exploring the role of TNF in drug addiction can improve our understanding of the neurobiology of addiction and provide insights into potential avenues for treatment of this disease.

Microglial dynamics

Glia cells are the most abundant cells in the brain and are critical to normal neuronal function, which has made them a major target in recent years for investigating neurological diseases. Homeostasis in the brain is regulated by astrocytes and microglia. Astrocytes play multiple roles to this end, including providing structural and metabolic support, maintaining the blood-brain barrier, and regulating neuronal communication as part of the tri-partite synapse (Sofroniew and Vinters 2010). Microglia are the immune cells of the brain, working to detect and eliminate pathogens and respond to various disease states (Boche et al. 2013); but emerging evidence indicates that like astrocytes, microglia play more diverse roles in maintaining circuit balance, including influencing synaptic function.

Under baseline conditions microglia exist in a resting state, with a ramified morphology consisting of many short cellular processes that continuously monitor the local environment (Nimmerjahn et al. 2005). Upon detection of a threat they enter an activated state, adopting an amoeboid morphology and taking on the functions characteristic of typical macrophages,

including phagocytosis and cytokine release (Boche et al. 2013). Many brain disorders involve microglial activation, such as stroke, neurodegeneration, traumatic brain injury, affective disorders, and addiction (Yirmiya et al. 2015; Loane and Kumar 2016; Bachtell et al. 2017; Ma et al. 2017). Activated microglia can perform both pro- and anti-inflammatory functions (Wolf et al. 2017). In the pro-inflammatory response, pattern recognition receptors on the plasma membrane of microglia detect stimuli that indicate the disruption of brain homeostasis, such as foreign pathogens or endogenous proteins associated with neurological disease (Rodríguez-Gómez et al. 2020). This leads to the release of pro-inflammatory cytokines and chemokines, such as TNF, to restore homeostasis. However, if this response becomes self-sustaining it can transition from being neuroprotective to inflicting damage and can aggravate or cause disease. In the repair response, anti-inflammatory cytokines such as IL-10 and TGF-β can be released as a negative feedback loop to pro-inflammatory cytokines (Colton 2009). Therefore, whether microglial activation has a protective effect, a destructive effect, or both is dependent on the physiological context of each condition it is involved in.

Microglia regulate circuit homeostasis and signalling balance at the synaptic level via multiple mechanisms. During development, microglia play a role in synaptogenesis, synaptic maturation, and synaptic pruning (Wright-Jin and Gutmann 2019). They are also involved in pathological synapse loss (Wilton et al. 2019). Microglia can regulate network activity; under baseline conditions direct microglial contact with a synapse increases its activity, but when lipopolysaccharide treatment is used to imitate pathological activation this function is impaired (Akiyoshi et al. 2018). Microglia can indirectly modulate the strength of both excitatory and inhibitory synapses via release of TNF (Lewitus et al. 2016; Liu et al. 2017), which has an established role in homeostatic synaptic plasticity.

Tumour necrosis factor alpha (TNF)

Tumour necrosis factor alpha is a pro-inflammatory cytokine. TNF is initially found in a 26 KDa transmembrane form, but can be cleaved from the cell surface by TNF-converting enzyme (TACE) to form a 17 KDa soluble protein which mediates inflammatory signalling (Sedger and McDermott 2014). TNF is found in a homotrimeric form which is required for binding to its homotrimeric receptors (Kalliolias and Ivashkiv 2016). TNF is expressed by a variety of immune cells, including macrophages, B cells, T cells, dendritic cells and fibroblasts (Falvo et al. 2010), while in the nervous system it is primarily expressed by glial cells (Sawada et al. 1989; Vezzani and Viviani 2015). As part of the immune system, TNF mediates inflammation and has direct anti-viral activity (Herbein and O'Brien 2000). In neurons, TNF protects against neurotoxic substances and regulates synaptic activity; and in oligodendrocytes and their precursor cells it is involved in cellular proliferation and myelin repair (Probert 2015).

TNF has two receptors, TNFR1 and TNFR2, which differ mainly in their cytoplasmic domains. TNFR1 has a cytoplasmic death domain that can mediate its apoptotic effects, which TNFR2 lacks. TNFR1 is largely responsible for pro-inflammatory signalling and apoptosis, and binds both membrane-bound TNF and soluble TNF (Sedger and McDermott 2014). TNFR2 is more involved in local homeostatic effects like cell survival and tissue regeneration, and it mainly binds membrane-bound TNF upon cell-to-cell contact (Kalliolias and Ivashkiv 2016). Another major difference is that TNFR1 is expressed ubiquitously, while TNFR2 is restricted to neurons, immune cells, and endothelial cells (Probert 2015).

Upon TNF binding to TNFR1, the receptor recruits tumour necrosis factor receptor type 1-associated death domain protein (TRADD), TNF receptor-associated factors 2 and 5 (TRAF2/5), and RIPK1; then, alternate signalling complexes can be formed leading to distinct

outcomes (Dostert et al. 2019). Complex I is assembled at the receptor cytoplasmic domain and forms a ubiquitination network, including activation of p38 MAP kinase in the case of TNFR1; this network then activates the transcription factors NF-κB and AP-1. These induce transcription of genes for inflammation, host defense, cell proliferation and cell survival (Probert 2015). NF-κB can also induce transcription of TNF and TRAF2 to further amplify TNF signalling (Kalliolias and Ivashkiv 2016). Complexes IIa, IIb, and IIc are assembled in the cytoplasm: IIa and b produce a caspase cascade leading to apoptosis, while IIc induces necroptosis which triggers local inflammation (Dostert et al. 2019). TNFR2 signalling involves recruitment of TRAF2 but not TRADD due to lack of a death domain; and it uses much of the same complex I downstream pathway to influence cell survival (Vanamee and Faustman 2017).

TNF and homeostatic synaptic plasticity

Within the brain, TNF is a mediator of homeostatic synaptic plasticity (HSP). HSP involves reigning in activity-dependent forms of synaptic plasticity (such as long-term potentiation and depression) to prevent neural circuits from becoming hyper- or hypo-active, thereby maintaining the balance of excitatory and inhibitory signalling (Turrigiano and Nelson 2004). Specifically, TNF is involved in synaptic scaling, where the strength of all synapses onto a neuron are altered to compensate for changes in neural network activity (Turrigiano 2008). TNF works in scaling up, but not down, so it leads to an overall increase in excitatory signalling in the brain. It accomplishes this by having opposing effects on excitatory and inhibitory circuits. In excitatory neurons such as the pyramidal cells of the hippocampus, TNF potentiates excitatory glutamatergic inputs (Stellwagen and Malenka 2006) by increasing post-synaptic expression of GluA2-lacking AMPA receptors (AMPARs), which may be permeable to Ca^{2+} (Stellwagen et al. 2005). TNF also weakens inhibitory GABAergic inputs onto these same neurons by removing

post-synaptic GABAa receptors (GABAaRs) (Pribiag and Stellwagen 2013). In inhibitory neurons such as the medium spiny neurons (MSNs) of the dorsal striatum, TNF instead reduces the strength of glutamatergic inputs by removing GluA2-lacking AMPARs from the synapse (Lewitus et al. 2014), and increases the strength of GABAergic inputs (Chambon 2020).

TNF's effects on both synapse types in the hippocampus are mediated by TNFR1 (Stellwagen et al. 2005; Pribiag and Stellwagen 2013). Downstream of TNFR1, trafficking of both AMPARs and GABAaRs is dependent on phosphoinositide 3-kinase (PI3K), and endocytosis specifically involves protein phosphatase 1 (PP1). At excitatory synapses, PI3K activation is required for AMPAR exocytosis to occur (Stellwagen et al. 2005). At inhibitory synapses, TNF binding leads to activation of p38 MAP kinase, which activates PI3K, and this enhances the association of PP1 with the β3 subunit of GABAa receptors; PP1 then dephosphorylates the subunit to encourage endocytosis (Pribiag and Stellwagen 2013). PP1 also seems to be involved in AMPAR endocytosis in striatal MSNs. PP1 is co-localized with AMPARs at the post-synaptic density by binding to spinophilin, and is rendered inactive under baseline conditions by its association with DARPP-32 (Yan et al. 1999). TNF binding leads to dephosphorylation of DARPP-32, which dissociates it from PP1; PP1 then dephosphorylates GluA1 subunits to induce endocytosis of AMPARs (Lewitus et al. 2014). The importance of PP1 in both the hippocampal and striatal trafficking mechanisms suggests that TNFR1 is also responsible for TNF's effects on striatal neurons, but this remains to be confirmed experimentally.

Increased TNF signalling has been implicated in multiple diseases, including neurodegenerative disease, stroke, traumatic brain injury, bipolar disorder, and addiction (Sedger and McDermott 2014; Lewitus et al. 2016; Yuan et al. 2019). Many of these conditions also

involve changes in excitatory-inhibitory balance in the brain, so TNF-induced HSP could play a role in their pathology. Altered TNF-induced HSP has been found in animal models of Parkinson's disease (Lewitus et al. 2014), Huntington's disease (Chambon 2020), ALS (Franquin 2020), stress (Kemp 2021), and addiction (Lewitus et al. 2016). In the case of addiction, TNF release from microglia attenuates cocaine-induced behaviours and reverses the pattern of cocaine-induced plasticity at excitatory synapses (Lewitus et al. 2016); but it is not currently known whether TNF also impacts inhibitory synapses in addiction, as it does in the HSP experiments discussed above.

Drug addiction

Overview

Addiction is a neuropsychiatric disorder with extensive societal and economic costs: around 21% of Canadians will meet the criteria for it during their lifetime (Pearson et al. 2013), and it is estimated to cost Canadian society almost $46 billion a year (Canadian Substance Use Costs and Harms Scientific Working Group 2020). In particular, the "opioid crisis" has become a major public health issue in the last 5 years, due to increasing rates of opioid-related deaths with few options to treat the dependence driving them (Special Advisory Committee on the Epidemic of Opioid Overdoses 2021). At the most basic level, addiction is characterized by compulsively seeking a rewarding stimulus (Koob and Volkow 2016). It is essentially an aberrant form of reward learning, where addictive substances induce plasticity in the reward centers of the brain to prioritize seeking this reward at all costs. The most common and strongest triggers of addiction are drugs of abuse, such as alcohol, psychostimulants, and opioids. Addiction traps patients into an endless cycle of drug intoxication; then abstinence, typically accompanied by physical withdrawal symptoms and negative emotional states; which eventually trigger relapse

back into intoxication (Koob and Volkow 2016). Breaking this cycle by preventing relapse is the primary measure of the effectiveness of addiction treatment (Vocci and Ling 2005), but current therapies do not provide long-term relief for the majority of patients.

Animal models

Much of our knowledge of the neurobiology behind drug addiction comes from research in animal models, and animal research is also key for the development of new addiction treatments. There are three major behavioural models of addiction, primarily used in rodents: locomotor sensitization, conditioned place preference, and self-administration. Locomotor sensitization (LS) is when the locomotor response to drugs of abuse becomes increasingly heightened due to repeated exposure (Spanagel 1995). This is typically tested by placing a rodent in an open field box and measuring the total distance it travels after each drug injection over several days. LS is induced both by repeated cocaine (Tirelli et al. 2003) and repeated morphine treatment (Spanagel 1995). It can be used as an indicator of drug acquisition; if one group shows stronger sensitization than another, this is interpreted as a stronger reaction to the drug and a higher propensity towards addiction.

In conditioned place preference (CPP), one behavioural chamber is repeatedly paired with drug injections while the other is paired with a vehicle control. On the test day the rodent is given access to both chambers, and typically it will have developed a preference for spending time in the drug-associated context. The difference between the post-training and pre-training preference is a measure of the strength of drug acquisition and drug seeking behaviour (Bardo and Bevins 2000). This model is particularly useful since an extinction protocol can also be performed to approximate abstinence, and re-exposure can be tested after that as a measure of relapse.

Self-administration is an operant conditioning paradigm where a behavioural response such as a lever press or nose poke leads to delivery of a drug infusion, providing positive reinforcement for this behaviour (Scofield et al. 2016). This protocol most closely resembles addiction in humans, as it is voluntarily administered by the animal, and it can model each stage: acquisition of the drug-reward association; maintenance of drug seeking and consumption; escalation of drug seeking; abstinence/withdrawal; and relapse.

While conditioned place preference and self-administration are stronger models of human addiction behaviour, the synaptic changes behind addiction are most extensively characterized in the locomotor sensitization model. Locomotor sensitization is therefore a simple robust model of addiction that is useful as a starting point for novel addiction experiments, which can be expanded upon further with conditioned place preference and/or self-administration experiments.

Neurobiology

Motivation to seek out drugs of abuse is driven by conditioned responses which evolve from repeated feelings of reward and pleasure, and by the negative emotional and physical states of withdrawal (Volkow and Morales 2015). Therefore, several brain pathways must contribute to reward, each underlying these different aspects. Dopamine is the main neurotransmitter involved in reward and addiction, and all drugs of abuse modulate it to some degree: psychostimulants in particular block the dopamine re-uptake transporter to increase the amount of dopamine in the synaptic cleft (Ritz et al. 1990), thereby extending feelings of reward. This occurs in the mesocorticolimbic dopamine pathway, where neurons in the ventral tegmental area (VTA) of the midbrain project to limbic structures including the nucleus accumbens (NAc) and the prefrontal cortex (PFC) (Cooper et al. 2017). This pathway is critical for learning associations between stimuli and feelings of reward and pleasure (Koob and Volkow 2016).

Glutamatergic inputs to the NAc from several areas of the brain can modulate the excitability of neurons targeted by dopamine projections. Plasticity at these synapses also lasts longer than dopaminergic changes and occurs in a pattern matching the different stages of addiction (Kourrich et al. 2007; Ortinski et al. 2012), suggesting that they are largely responsible for the maintenance of addiction beyond initial consumption. The corticostriatal pathway contains glutamatergic projections from the PFC to the NAc (Scofield et al. 2016). This pathway is responsible for decision making, planning, and inhibitory control, all of which are altered to induce motivation for the drug of abuse (Volkow and Morales 2015). Excitatory inputs from the basolateral amygdala (BLA) and ventral hippocampus to the NAc are critical for conditioned responses (Stuber et al. 2011; Barker et al. 2019). The BLA also facilitates stress and mood changes involved in addiction (Sharp 2017), playing a role in withdrawal alongside the lateral habenula which mediates aversion (Meye et al. 2017).

Finally, the NAc sends out GABAergic projections to the dorsal striatum and the ventral pallidum (VP), which are responsible for motor behaviours required for drug seeking and consumption (Sesack and Grace 2010; Root et al. 2015). Drugs of abuse also affect GABAergic circuitry within the NAc and VTA (Koob and Volkow 2016). Similar to excitatory synapses, plasticity at GABAergic synapses in the NAc seems to follow the stages of addiction (Kennedy et al. 2013; Otaka et al. 2013), but they are far less well-characterized, making them a promising target for further investigation into addiction plasticity.

Many of these reward pathways center on the nucleus accumbens. The NAc is the ventral part of the striatum. It is divided into two areas: the core, surrounding the anterior commissure and continuous with the dorsal striatum; and the shell, which surrounds the core (Sesack and Grace 2010). The core is more responsible for motor behaviours involved in reward, while the

shell is more involved in the emotional states driving these behaviours (Voorn et al. 2004). Lesions to the NAc core disrupt the acquisition of stimulus-controlled cocaine seeking, (Ito et al. 2004), and inhibiting AMPAR-mediated neurotransmission specifically in the core reduces locomotor sensitization (Bell et al. 2000). The NAc shell can be further divided into lateral and medial components which have opposing effects in reward: dopamine terminals from the VTA to the medial NAc shell are excited by aversive stimuli and associated cues, while terminals in the rest of the NAc are excited by rewarding stimuli and inhibited by aversive ones (de Jong et al. 2019). Another major difference between the shell and core is that they have opposing functions in drug-associated memories triggered by either cues or contexts. The NAc core is critical for cue-induced reactivation of drug seeking (Floresco et al. 2008; Ito and Hayen 2011; Noe et al. 2019) and this is inhibited by the shell (Floresco et al. 2008); while the NAc shell promotes context-induced activation of drug seeking (Noe et al. 2019) that is inhibited by the core (Ito and Hayen 2011). There also is significant overlap and connectivity in shell and core functions; for example, optogenetic stimulation of both NAc regions can be equally reinforced in a self-stimulation task (Han et al. 2017).

While both the NAc core and shell have some level of connection with all the areas known to drive addiction behaviour, the strength and number of these connections varies between the two. Li et al. (2018) used trans-synaptic tracing to compare the inputs of the core and the shell and found that the core gets the majority of its inputs from the cortex while most of the shell afferents are from the hippocampus. This fits with the behavioural findings discussed previously where the core is involved in decision making and initiating movements, while the shell is critical for memories and contexts surrounding reward and addiction. The NAc shell projects out to the ventromedial VP, which then forms a “limbic loop” by projecting to the

medial dorsal nucleus of the thalamus, which connects to the prelimbic cortex, which synapses onto the NAc core (Zahm 1999), thus allowing the shell to enhance behaviours induced by the core. The NAc core projects to areas related to movement, such as the output nuclei of the basal ganglia (Zahm 1999; Scofield et al. 2016). Finally, there are also direct connections between the core and the shell (van Dongen et al. 2005), indicating that activity in both areas of the NAc combine to promote drug-induced behaviours.

The main cell type in the NAc is the medium spiny neuron (MSN), which are GABAergic neurons forming about 90% of the NAc population (Sesack and Grace 2010). The other 10% consists of glial cells and interneurons that modulate the behaviour of MSNs; most of the interneurons are cholinergic, with less than 1% of NAc neurons being GABAergic cells that express either somatostatin, neuropeptide Y, or neuronal nitric oxygen synthase (Kawaguchi et al. 1995). MSNs can be functionally divided based the on the type of dopamine receptor they primarily express, all of which are G-protein coupled receptors. D1 MSNs express the D1-like family of dopamine receptors. These are coupled to the $G\alpha_{s/olf}$ family of G proteins, and induce the intracellular signalling cascade involved in LTP, among other functions: the G proteins stimulate adenylyl cyclase, thereby increasing cAMP, activating PKA, and modulating CREB-mediated regulation of gene expression to increase synaptic strength (Beaulieu and Gainetdinov 2011). D2 MSNs express the D2-like family of dopamine receptors, which instead inhibit the signalling involved in LTP. They are coupled to $G\alpha_{i/o}$ G-proteins, which inhibit adenylyl cyclase to produce the opposite effects of D1 receptors (Beaulieu and Gainetdinov 2011). There are also some MSNs that express a mix of these receptor types (Gagnon et al. 2017); this must be taken into consideration when evaluating studies examining MSNs.

D1 and D2 MSNs receive inputs from generally the same areas of the brain discussed above (Li et al. 2018), but they do have distinct electrophysiological properties. D1 MSNs are overall less excitable than D2 MSNs, having a hyperpolarized resting membrane potential (Gertler et al. 2008; Cao et al. 2018) and higher action potential threshold (Cepeda et al. 2008). However, they also have stronger excitatory synapses at baseline compared to D2 MSNs, with a higher frequency and amplitude of mini excitatory post-synaptic currents (mEPSCs) (Cao et al. 2018) and a stronger current response to AMPA treatment (Cepeda et al. 2008).

In the striatum, D1 MSNs are generally considered part of the direct motor output pathway of the basal ganglia, which facilitates movement, while D2 MSNs are part of the indirect pathway, which suppresses it (Calabresi et al. 2014). D1 MSNs in the NAc core generally project to direct basal ganglia output structures like the substantia nigra and also the VTA in the midbrain, while D2 MSNs project to indirect pathway structures like the VP and the subthalamic nucleus (Soares-Cunha et al. 2016). However, the direct-indirect dichotomy does not hold as well in the NAc compared to the dorsal striatum, since D1 MSNs can also project to the VP (Smith et al. 2013; Kupchik et al. 2015). Furthermore, cocaine treatment can change the strength of connections from the ventral hippocampus or the BLA to shell D1 MSNs that project to either the VTA or the VP respectively (Baimel et al. 2019), indicating that these pathways can become further complicated during drug addiction.

Following from these functional differences, D1 and D2 MSNs in the NAc have different roles in drug addiction. Optogenetic activation of D1 MSNs promotes psychostimulant-induced motor sensitization and drug seeking behaviour while activating D2 MSNs inhibits it (Lobo et al. 2010; Soares-Cunha et al. 2020); in D2 MSNs, this occurs via inducing inhibitory post-synaptic currents (IPSCs) on neighbouring MSNs (Song et al. 2014). Increased excitatory synapse

strength onto D1 MSNs in both the NAc shell and core underlies drug-seeking behaviour and sensitization (Pascoli et al. 2012; Roberts-Wolfe et al. 2018), while increased synaptic strength onto D2 MSNs occurs after extinction training and is likely reflective of drug-refraining behaviour (Roberts-Wolfe et al. 2018). Cocaine increases the strength of excitatory inputs onto D1 MSNs but not D2 (Lewitus et al. 2016; Terrier et al. 2016), and acquisition of morphine CPP reduces D2 receptor expression (Jiang et al. 2020), suggesting that an imbalance in signalling between these two populations contributes to addiction.

Synaptic plasticity in addiction

Cocaine-induced plasticity

Cocaine addiction is primarily driven by blockade of the dopamine re-uptake transporter, which increases the amount of dopamine in the synaptic cleft (Ritz et al. 1990) and induces stronger feelings of reward and pleasure. However, plasticity at excitatory and inhibitory synapses in the NAc is more associated with long-term maintenance of drug-seeking behaviour, drug withdrawal, and relapse. After extended cocaine access, the critical signalling pathway for addiction phenotypes seems to shift from dopaminergic to glutamatergic pathways (Doyle et al. 2014; Ramôa et al. 2014; Lynch et al. 2020). Cocaine induces synaptic plasticity in both the NAc core and shell, where the direction of synaptic strength alternates depending on the stage of addiction. Initially, cocaine conditioning decreases excitatory synapse strength onto MSNs, via both presynaptic (Ortinski et al. 2012; Liu et al. 2014) and post-synaptic mechanisms (Kourrich et al. 2007; Ortinski et al. 2012; Vaquer-Alicea et al. 2018). After abstinence from cocaine, synaptic strength is now increased compared to controls (Dobi et al. 2011; Rothwell et al. 2011; Britt et al. 2012; Jedynak et al. 2016). And when cocaine is re-introduced, glutamatergic synaptic strength returns to the same reduced level as before withdrawal (Spencer et al. 2017; Ebner et al.

2018; Benneyworth et al. 2019). Additionally, if cocaine access is removed again after re-exposure, glutamatergic synaptic strength increases once again (Spencer et al. 2017). Cocaine also induces a homeostatic cross-talk between synapses and the cell membrane, where changes in NMDA signalling after cocaine conditioning decrease intrinsic membrane excitability; this then leads to a strengthening of excitatory synapses over long-term withdrawal (Wang et al. 2018). In studies where MSNs were divided by subtype, these effects were specific to D1 MSNs (Lewitus et al. 2016; Terrier et al. 2016), thereby skewing the ratio of signalling between the two subtypes (Roberts-Wolfe et al. 2019). Overall, these findings point towards synaptic plasticity in the NAc being an important mediator of cocaine addiction.

This plasticity is specifically driven by trafficking of calcium-permeable, GluA2-lacking AMPA receptors. Withdrawal from cocaine increases the proportion of GluA2-lacking AMPA receptors in the NAc shell (Terrier et al. 2016) while re-exposure leads to their endocytosis (Benneyworth et al. 2019), corresponding to increased and decrease synaptic strength respectively. This trafficking might involve activation of various protein kinases (García-Pardo et al. 2016); in particular, multiple studies indicate that it is dependent on protein kinase C (PKC) activation, which can phosphorylate GluA2 AMPAR subunits to allow for their internalization. Inhibiting PKC function prevents cocaine-induced increases in excitatory synapse strength in the VTA and decreased strength in the NAc (Briand et al. 2016; Vaquer-Alicea et al. 2018; Deutschmann et al. 2019). Metabotropic glutamate receptors have also been implicated in cocaine-induced synaptic plasticity (Mameli et al. 2009; Benneyworth et al. 2019; Deutschmann et al. 2019), as have a variety of transcription factors (Teague and Nestler 2021). The question of exactly how cocaine is altering glutamate receptor trafficking and function is still being explored.

Importantly, a direct causal link has been established between cocaine-induced synaptic plasticity in the NAc and addiction behaviours. Optogenetic stimulation of the infralimbic cortex to NAc shell pathway in vivo in the absence of cocaine produces reinstatement of previously-learned cocaine CPP and the associated decrease in synaptic strength, while inhibiting this pathway prevents cocaine-induced reinstatement of both CPP and locomotor sensitization (Pascoli et al. 2012; Benneyworth et al. 2019). Disrupted AMPAR trafficking via PKC inhibition has been associated both with reduced (Ortinski et al. 2015; Deutschmann et al. 2019) and potentiated reinstatement of cocaine seeking (Briand et al. 2016). As well, blocking the homeostatic loop between glutamatergic synaptic strength and membrane excitability prevents the incubation of cocaine craving after long-term withdrawal from self-administration (Wang et al. 2018).

Further evidence for HSP in cocaine addiction comes from plasticity at inhibitory synapses onto NAc MSNs. Cocaine conditioning initially increases GABAergic neurotransmission in the NAc shell (Kennedy et al. 2013). Abstinence then decreases synaptic strength, thereby increasing the excitatory-inhibitory ratio (Otaka et al. 2013). Finally, cocaine re-exposure increases GABAergic neurotransmission once again, bringing the excitatory-inhibitory ratio back to baseline levels (Otaka et al. 2013). This is the exact opposite pattern of synaptic plasticity seen at excitatory synapses, so that we see an overall decrease in circuit activity after repeated cocaine treatment, an increase after abstinence, and a decrease once again upon re-exposure to cocaine. Since the balance of excitatory and inhibitory signalling is being adjusted by plasticity across multiple synapse types onto MSNs, homeostatic synaptic plasticity seems to be at work. Like excitatory plasticity, inhibitory cocaine-induced plasticity is driven by post-synaptic receptor trafficking. Cocaine conditioning increases gene expression of the α and β

subunits of GABAa receptors (Kennedy et al. 2013) and increases the amount of GABAa receptor protein at the cell surface, then withdrawal reduces this protein expression back to baseline (Purgianto et al. 2016). Acute cocaine treatment also increases the amount of GABA in the synaptic cleft by reducing the function and expression of GABA transporter type 1 (Kubrusly et al. 2020). Furthermore, there is evidence that increased inhibitory synapse strength at the conditioning and re-exposure stages works to reduce drug-associated behaviours rather than causing it. Activating GABAb receptors reduces initial cocaine self-administration and re-instatement (Gawlińska et al. 2020). Increasing the amount of GABA in the synaptic cleft during cocaine conditioning or re-exposure reduces cocaine sensitization (Filip et al. 2006). As well, activating extrasynaptic GABAa receptors on D1 MSNs decreases cocaine CPP (Maguire et al. 2014).

From these findings, it is clear that changes at multiple synapse types on the same neurons work in tandem to alter the excitability of NAc MSNs in a bi-phasic manner during cocaine addiction, which could drive drug-seeking behaviour.

Morphine-induced plasticity

Compared to psychostimulants, the synaptic plasticity underlying opioid addiction is not well understood. Opioids do not directly alter dopamine neurotransmission, so it is not immediately clear how opioids induce reward. Morphine acts primarily on the mu opioid receptor, which is distributed throughout reward circuits and seems to mediate the reinforcing activity of morphine by indirectly affecting dopaminergic VTA neurons and their NAc targets (Contet et al. 2004). One theory is that opioids can disinhibit dopamine neurons in the VTA by hyperpolarizing interneurons (Johnson and North 1992), thereby increasing dopamine release in the NAc. Another possible facilitator of morphine reward is plasticity at glutamate synapses in the NAc.

Like with dopamine, morphine plasticity at these synapses is more complex than cocaine, having different effects on the core vs. the shell and in D1 vs. D2 MSNs.

In the NAc shell, repeated morphine treatment decreases excitatory synapse strength in D2 MSNs with no effect on D1 MSNs (McDevitt et al. 2019). This is accompanied by an increase in intrinsic membrane excitability, showing a similar homeostatic response as discussed above with cocaine. Abstinence from morphine increases synaptic strength onto D1 MSNs in the shell both pre- and post-synaptically (Hearing et al. 2016), and upon re-exposure to morphine D1 MSNs now show a decrease in synaptic strength (Madayag et al. 2019). D2 MSNs remain depressed at both the abstinence and re-exposure stages in the NAc shell, so only plasticity at D1 MSNs reflects the stages of addiction. It is also notable that when MSN types are pooled together, abstinence produces an increase in synaptic strength (Wu et al. 2012). Taken together, it is possible that plasticity at D1 MSNs play a more influential role in morphine-induced plasticity in the shell, reflecting findings in cocaine addiction. But in the NAc core, it is the D2 MSNs that change with the stage of addiction: abstinence decreases glutamatergic synaptic strength onto D2 MSNs, and re-exposure increases it, whereas D1 MSNs exhibit a reduction in strength at both stages (Madayag et al. 2019).

Morphine withdrawal coincides with the upregulation of surface GluA2-lacking AMPARs in the NAc (Russell et al. 2016), and downregulation of mGluR2/3 which leads to increased glutamate release (Wu et al. 2012; Qian et al. 2019). These mediators are also directly connected to morphine-induced behaviours. Blocking AMPARs and kainate receptors makes extinction of morphine CPP occur faster (Siahposht-Khachaki et al. 2017). Inhibiting AMPAR endocytosis in D1 MSNs of the NAc shell prevents the typical decrease in synaptic strength and exacerbates reinstatement of morphine CPP (Madayag et al. 2019), while stimulating the ILC-

NAc shell inputs reduces synaptic strength onto D1 MSNs and blocks reinstatement of morphine CPP (Hearing et al. 2016). This morphine-induced decrease in synaptic strength could therefore have an attenuating effect on drug-seeking behaviour.

There is no data on plasticity at the morphine conditioning stage in the NAc core, and all of these electrophysiology findings come from only a few papers, so more research is required to fully characterize morphine-induced plasticity. There is also evidence for morphine withdrawal reducing glutamate transmission in the lateral habenula (Valentinova et al. 2019), and morphine treatment altering excitatory synapses in the hippocampus (Billa et al. 2010; Xia et al. 2011; Elahi-Mahani et al. 2018), which could impact opioid addiction in a more indirect fashion that is not yet fully elucidated.

The neuroimmune system in drug addiction

While psychostimulants and opioids produce different patterns of synaptic plasticity, one effect they do have in common is altering glial cell function (Lacagnina et al. 2017), which can regulate synapses. Activation of microglia in the NAc and striatum is induced by both cocaine (Lewitus et al. 2016; Cotto et al. 2018; Periyasamy et al. 2018) and morphine (Zhang et al. 2012; Campbell et al. 2013). This pro-inflammatory phenotype seems to be a driver of addiction (Zhang et al. 2012; Eriksen et al. 2016; Kashima and Grueter 2017; Brown et al. 2018), so we hypothesized that microglial activation and TNF release would exacerbate cocaine-induced behaviour. Instead, we found that re-activating microglia after cocaine withdrawal prevents locomotor sensitization upon cocaine re-exposure, alongside reversing withdrawal-induced plasticity (Lewitus et al. 2016). Therefore, microglial activation could be a homeostatic mechanism to compensate for drug-induced plasticity, and thereby dampen drug-seeking behaviour. Additionally, mice lacking toll-like receptor signalling, which is a critical driver of microglial activation, take longer to

unlearn morphine-seeking behaviour and show greater reinstatement of it with re-exposure (Rivera et al. 2019). This suggests that morphine-induced microglial activation can reduce drug-induced behaviours similar to cocaine.

Microglial activation by cocaine or morphine can alter neuronal function through the release of cytokines, including TNF (Niwa et al. 2007; Campbell et al. 2013; Liao et al. 2016). Through its previously established role in homeostatic synaptic plasticity, TNF could link microglial activation with NAc synaptic plasticity and subsequent drug-seeking behaviours during addiction. This is supported by findings that TNF knockout mice show exacerbated cocaine locomotor sensitization (Lewitus et al. 2016) and morphine-induced CPP (Niwa et al. 2007). Furthermore, these behaviours can be attenuated by increasing TNF levels, either via injecting mice with TNF (Niwa et al. 2007) or inducing microglial TNF release (Wu et al. 2014; Lewitus et al. 2016). With TNF signalling absent, cocaine conditioning reverses the pattern of plasticity seen in wild-type (WT) controls; while WT mice show a decrease in excitatory synapse strength after cocaine treatment, these synapses are instead strengthened in TNF KO mice (Lewitus et al. 2016). Combined with the exacerbated addiction behaviour, this suggests that TNF release is a homeostatic response to counteract cocaine-induced synaptic plasticity in the NAc, rather than causing it. In morphine-treated animals, inducing TNF release from microglia mimics withdrawal-induced synaptic depression in the lateral habenula, and TNFR1 signalling is required for this to occur (Valentinova et al. 2019); but whether TNF plays a similar role in the NAc during morphine addiction is unknown.

In total, this evidence indicates that the neuroimmune system can modulate addiction, with TNF being a key mediator. Drugs of abuse induce synaptic plasticity in brain areas critical for addiction, which is theorized to drive addiction behaviour, and TNF can modulate both. Fully

characterizing the circuit-level and molecular mechanisms behind this effect is critical to potentially harnessing TNF as a treatment, and to improve our understanding of addiction neurobiology overall. To this end, we further investigated the role of TNF in addiction through three experimental aims. First, we determined which TNF receptor is responsible for its effects in cocaine addiction, which is an important first step in understanding the molecular mechanisms involved. We then tested whether TNF impacts cocaine-induced plasticity at inhibitory synapses like excitatory ones, to fully establish that TNF is involved in homeostatic synaptic plasticity in addiction. Finally, we looked at the effects of losing TNF signalling on morphine-induced plasticity and behaviour, expanding our previous findings to opioid addiction, and providing more evidence for microglial activation as a homeostatic regulator of addiction.

Chapter 2 – General Methodology

Animals

D1 MSN mice, which are C57Bl/6 mice expressing the tD-Tomato fluorescent protein under the dopamine D1 receptor promoter, were acquired from Jackson Laboratories (B6Cg-Tg(Drd1a-tdTomato)6Calak/J [RRID: IMSR_JAX:016204]). These mice were bred with $TNF^{-/-}$ (RRID: IMSR_JAX:005540) and strain-matched wildtype mice (C57Bl6/J) to obtain D1-WT mice and D1-TNF KO mice respectively. Genotyping of animals for D1 tD-Tomato expression was performed after weaning (around 28 days of age). $TNFR1^{-/-}$ mice with a C57Bl/6 background were obtained from Jackson Laboratories (RRID: ISMR_JAX:003242) with C57Bl/6 mice used as wild-type controls.

All experiments were performed on mice aged 8 to 12 weeks. Animals were housed in standard laboratory conditions, with free access to food and water. All experiments were performed in accordance with the guidelines of the Canadian Council for Animal Care (CCAC) and the Montreal General Hospital Animal Care Facility.

Drug treatment

Cocaine hydrochloride (Medisca) was dissolved in saline at a concentration of 2 mg/mL. Morphine sulfate (Medisca) was also dissolved in saline at this same concentration. For both drug treatments, mice were injected intra-peritoneally at a dose of 15 mg/kg. Most mice were treated daily in the morning for 7 days with electrophysiology performed 24 hours after the final injection, while a subset was treated for a single day with electrophysiology performed 24 hours later.

Behavioural testing

A locomotor sensitization behavioural paradigm was used as the drug conditioning protocol. Locomotion was monitored in 30 x 30 cm plexiglass boxes via video recording and performed under dim red light, around the same time each day in the late morning or early afternoon. Once daily for 7 days, mice received injections of saline or drug (either cocaine or morphine) and their motor activity was recorded. Mice were first given two days of saline injections to habituate them to the protocol (days -2 to -1) and tested after each injection to establish a locomotion baseline. Then mice were tested after each repeated drug or saline injection (days 1 to 5). In cocaine experiments, motor activity was recorded for 15 minutes each day. In morphine experiments, activity was recorded for 30 minutes total but only movement during the last 15 minutes was analyzed. Total distance traveled for each mouse during each day was measured by EthoVision video analysis software.

Electrophysiology

Acute slice preparation

24 hours after the final drug or saline injection, mouse brains were dissected and placed in oxygenated, ice-cold slicing solution containing (in mM): 92 choline chloride, 2.5 KCl, 1.2 NaH_2PO_4, 30 HEPES, 25 glucose, 30 $NaHCO_3$, 5 sodium ascorbate, 3 sodium pyruvate, 10 $MgSO_4$, 0.5 $CaCl_2$. We obtained 240 µm thick coronal slices containing the NAc using a vibratome. Slices recovered for at least 30 minutes in artificial cerebrospinal fluid (aCSF), which was saturated with 95% O_2/ 5% CO_2 and contained (in mM): 119 NaCl, 2.5 KCl, 1 NaH_2PO_4, 1.3 $MgCl_2$, 2.5 $CaCl_2$, 26.2 $NaHCO_3$, 11 glucose. Slices were then transferred to a recording chamber, perfused with oxygenated aCSF at around 1.5 to 2 mL/min.

Recordings

Whole-cell voltage clamp recordings were acquired using a MultiClamp 700B amplifier (Molecular Devices), filtered at 2 kHz and digitized at 10 kHz using a Digidata 1440A (Molecular Devices). All signals were recorded using Clampex 10.7 (Molecular Devices) and used to monitor passive membrane properties of the patched cell. Visual was obtained using an infrared differential interference contrast microscope (Olympus BX51WI) with an attached camera (Jai, CV-A50). MSNs were identified using fluorescence, but a minority were identified by morphological differences: D1 MSNs typically had triangular or heart-shaped cell bodies and were found in large clusters, while D2 MSNs were more elongated and less densely populated. Each day of recording typically included slices from two different mice of the same genotype, one treated with drug and one saline control, to account for any variation in the saline baseline.

Miniature inhibitory post-synaptic currents (mIPSCs)

mIPSCs were recorded using whole-cell glass electrodes (2-4 MΩ tips) filled with an inhibitory internal solution containing (in mM): 122 CsCl, 8 NaCl, 10 glucose, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 Na_3-GTP, 2 Mg-ATP (280-290 mOsm, pH 7.3). Recordings were performed under a -70mV voltage-clamp, using aCSF mixed with 500 nM tetrodotoxin and 50 µM NBQX to block sodium channels and AMPA receptor activity, respectively. For each cell recorded, events within one 60 second sweep were analyzed. The mIPSC amplitude was calculated as the average amplitude of all events within the sweep. The mIPSC frequency was determined as the number of events divided by the duration of the sweep.

AMPA/NMDA ratios

Excitatory postsynaptic currents (EPSCs) were evoked using a stainless steel, bipolar stimulating electrode placed dorsally near the recorded neuron. We filled the same electrodes previously

described with an excitatory internal solution containing (in mM): 122 cesium methanesulphonate, 8 NaCl, 10 glucose, 1 $CaCl_2$, 10 HEPES, 10 EGTA, 0.3 Na_3-GTP, 2 Mg-ATP (280-290 mOsm, pH 7.3). Recordings were performed in the presence of 100 µM picrotoxin (dissolved in aCSF) to block GABAa receptor function. We recorded the evoked synaptic response at -70 mV to measure the AMPA receptor-mediated EPSC, and at +40 mV for NMDA receptor-mediated EPSC. 15 responses per recording were averaged for analysis. AMPA/NMDA ratio was obtained as a ratio between the amplitude of the AMPAR-mediated EPSC, measured at its peak; and the amplitude of the NMDAR-mediated EPSC, measured 40 ms post the AMPAR current peak to ensure only NMDA receptors are still open.

Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed using GraphPad Prism 6 and JASP software. If the data is normally distributed (confirmed using the Shapiro-Wilk test) it was analyzed using a two-way ANOVA, either ordinary or repeated measures depending on experimental design. If the dataset does not meet this criterion, then a nonparametric test was performed. The assumption of equality of variances was checked using Levene's test. If this is significant ($p < 0.05$) an alternate test or post-hoc test to correct for unequal variances is performed; in the case of an ordinary two-way ANOVA we used the Games-Howell post-hoc test. For the repeated measures ANOVA the assumption of sphericity was assessed using Mauchly's test. If Mauchly's test is significant ($p < 0.05$), we applied either the Greenhouse-Geisser correction if $\varepsilon <= 0.75$, or the Huynh-Feldt correction if $\varepsilon > 0.75$. For post-hoc analyses we initially performed a simple main effect analysis followed by Tukey's multiple comparisons test.

Chapter 3 – Influence of TNF signalling on cocaine-induced plasticity and behaviour

Introduction

Reward learning is mediated by synaptic plasticity at dopaminergic, glutamatergic, and GABAergic synapses in the nucleus accumbens (NAc). Addiction therefore involves modification of these synapses to distort circuit balance and produce the cycle of addiction behaviours: intoxication, then abstinence, then relapse (Koob and Volkow 2016). While the effect of cocaine at dopamine synapses is well understood, it is unclear exactly how cocaine modifies glutamatergic and GABAergic synapses. One potential pathway is cocaine-induced microglial activation, leading to the release of cytokines such as TNF which can modulate drug-induced synaptic plasticity (Lewitus et al. 2016).

In cocaine addiction, plasticity at both excitatory and inhibitory synapses in the NAc occurs in a pattern matching the behavioural stages of addiction: we see distinct changes after initial drug treatment, abstinence, and re-exposure. Repeated cocaine treatment over several days initially decreases excitatory synaptic strength (Kourrich et al. 2007; Ortinski et al. 2012); withdrawal increases it (Rothwell et al. 2011; Britt et al. 2012); and re-exposure decreases it once again (Spencer et al. 2017; Ebner et al. 2018). Inhibitory synapses show the exact opposite pattern of plasticity to excitatory ones; the overall balance of excitatory and inhibitory signalling is altered by adjusting the strength of multiple synapse types onto MSNs, which indicates homeostatic synaptic plasticity is likely taking place. Cocaine conditioning increases the frequency of spontaneous inhibitory post-synaptic currents (sIPSCs) alongside increases in the expression of GABAa receptor subunits (Kennedy et al. 2013) and surface protein expression (Purgianto et al. 2016). Abstinence from cocaine instead decreases miniature IPSC (mIPSC) amplitude, which increases the excitatory to inhibitory ratio; re-exposure then increases mIPSC frequency, continues the decrease in amplitude, and brings the excitatory-inhibitory ratio back to

baseline (Otaka et al. 2013). Overall, the pattern seems to fit with the changes in glutamatergic synaptic plasticity, occurring in the opposite direction to shift in the balance of excitatory and inhibitory signalling which could drive drug-seeking behaviour.

One regulator of plasticity during cocaine addiction is the pro-inflammatory cytokine TNF. TNF is elevated in patients with substance abuse disorders (Chen et al. 2012; Narvaez et al. 2013; Heberlein et al. 2014) and cocaine induces TNF release from microglia in animal models (Liao et al. 2016; Periyasamy et al. 2018). TNF is also involved in homeostatic synaptic plasticity, where it increases overall activity in hypoactive circuits by increasing excitatory input onto glutamatergic neurons and dis-inhibiting GABAergic ones. In glutamatergic pyramidal cells from the hippocampus, TNF treatment increases the excitatory-inhibitory ratio by increasing the proportion of GluA2-lacking AMPA receptors and decreasing GABAa receptors at the cell surface (Stellwagen et al. 2005; Pribiag and Stellwagen 2013). In GABAergic cells such as MSNs in the dorsal striatum, TNF treatment instead decreases post-synaptic GluA2-lacking AMPA receptors (Lewitus et al. 2014), alongside an increase in inhibitory synapse strength (Chambon 2020).

Based on this, we previously examined the role of TNF in cocaine-induced behaviours and synaptic plasticity. First, we found that TNF KO mice exhibit stronger locomotor sensitization over 5 days of cocaine treatment compared to wild-type (WT) controls (Lewitus et al. 2016). This was accompanied by a pattern of excitatory synaptic plasticity opposite to the WT mice, where cocaine treatment increased AMPA/NMDA ratio in the NAc core of the TNF KO group but decreased it in the WT mice. We also found that after 10 days of abstinence, inducing TNF release by activating microglia reduced locomotor sensitization upon cocaine re-exposure, and this effect was absent in the TNF KO group. We concluded that instead of contributing to

cocaine addiction, TNF has an attenuating effect on drug-induced behaviours, perhaps mediated by TNF's effects on cocaine synaptic plasticity.

To fully confirm this hypothesis, it is critical to determine whether TNF impacts cocaine-induced plasticity at inhibitory synapses as well, thereby altering the balance of excitatory and inhibitory signalling in a homeostatic manner. Furthermore, we do not know which of the two TNF receptors (TNFR1 and TNFR2) is behind its effects in cocaine addiction, which is the first step to determining the downstream mediators of TNF's effects on cocaine addiction. In this chapter, we test whether our previous behavioural findings in TNF KO mice can be replicated by loss of TNFR1 and investigate how loss of TNF signalling could influence cocaine-induced plasticity at GABAergic synapses.

Results

TNFR1 as the mediator of TNF's role in addiction.

To discern which TNF receptor mediates its role in cocaine addiction, we assessed locomotor sensitization in TNFR1 KO mice and WT controls treated with either cocaine or saline, comparing these findings to our previous results in TNF KO mice. TNFR1 is the receptor responsible for its pro-inflammatory properties and apoptosis induction (Sedger and McDermott 2014), and it mediates TNF-induced synaptic plasticity in the hippocampus (Stellwagen et al. 2005; Pribiag and Stellwagen 2013). We also previously found that the soluble form of TNF is responsible for cocaine-induced plasticity (Lewitus et al. 2016), which is primarily bound by TNFR1 rather than TNFR2. This made TNFR1 the most likely candidate for us to target in our experiments.

Locomotor sensitization (LS) is when the locomotor response to drugs of abuse becomes increasingly heightened due to repeated exposure (Spanagel 1995). It can be used as an indicator of drug acquisition; if one group shows stronger sensitization than another, we interpret this as a stronger reaction to the drug and a higher propensity towards addiction. We employed this measure because it was modulated by TNF in our previous experiments (Lewitus et al. 2016) and, for our later experiments, because it matches protocols for drug conditioning typically used in electrophysiological studies.

We found that TNFR1 KO mice exhibit stronger locomotor sensitization compared to WT controls. The initial response to cocaine on the first day of treatment was the same, but over the next several days of cocaine treatment there was a significantly greater increase in movement in the TNFR1 KO group (Figure 1B). Since this matches our previous findings in TNF KO mice, this supports TNFR1 as the receptor mediating TNF's attenuating effect on cocaine-induced behaviour.

TNF function at inhibitory synapses after cocaine treatment.

Since our knowledge of cocaine-induced plasticity at GABAergic synapses is lacking compared to excitatory synapses, especially regarding molecular mediators, we wanted to examine TNF's role in inhibitory synaptic plasticity after cocaine treatment. We also wanted to further establish our hypothesis that TNF is involved in homeostatic synaptic plasticity (HSP) during addiction; if HSP is occurring, we would predict changes in inhibition as well as excitation, so we need to examine inhibitory synaptic plasticity. To determine whether loss of TNF signalling impacts cocaine-induced plasticity at GABAergic synapses, we recorded mIPSCs, a measure of inhibitory synaptic function, in the NAc core of TNF KO and WT mice treated with either cocaine or saline. We focused on the NAc core because it is the area responsible for locomotor

sensitization and for expression of reward-induced motor behaviours in general (Sesack and Grace 2010). Additionally, TNF modulates excitatory synaptic plasticity here during cocaine addiction (Lewitus et al. 2016), but cocaine-induced inhibitory plasticity has not been characterized in the NAc core before. We also recorded in D1 MSNs to start with, since cocaine-induced plasticity at excitatory synapses (Terrier et al. 2016) and TNF-induced plasticity at both synapse types (Lewitus et al. 2016; Chambon 2020) occurs exclusively in D1 MSNs.

We used both a 1-day cocaine treatment protocol and a 5-day protocol (Figure 1A; for details, refer to Chapter 2). We previously found that in TNF KO mice, a single cocaine injection produces an increase in AMPA/NMDA ratio that is intermediate between the saline treated group and the 5 day group (Lewitus et al. 2016); so we thought it would be interesting to also examine how inhibitory synaptic plasticity changes between acute and repeated treatment.

After a single cocaine injection, we found that D1 MSNs in WT mice showed a significant increase in the frequency of mIPSCs, which did not occur in the KO group (Figure 2A). There was a difference in the response to single cocaine treatment between genotypes, but due to unequal variances between the WT cocaine and KO cocaine groups (Levene's test $p < 0.001$) we were unable statistically conclude this from the two-way ANOVA. Instead, we performed a Games-Howell post-hoc test (Figure 2A) which is non-parametric and does not assume equal variances between groups; this test was also significant, confirming that there was an effect of genotype on how mIPSC frequency is altered by acute cocaine treatment. However, mIPSC amplitude remained unaffected by cocaine treatment (Figure 2B).

5 days of cocaine treatment reversed this pattern: now the TNF KO mice showed a decrease in mIPSC frequency which did not occur in the WT group (Figure 3A), and amplitude was once again not changed (Figure 3B). Both findings indicate that lack of TNF signalling

alters GABAergic synaptic plasticity during cocaine addiction. Changes in mIPSC frequency tend to reflect pre-synaptic neurotransmitter release probability, while mIPSC amplitude indicates post-synaptic receptor function (Phillips et al. 2010), so only seeing changes in mIPSC frequency but not amplitude suggests that TNF is mainly influencing pre-synaptic mechanisms of plasticity. Additionally, the differing patterns between the 1-day and 5-day timepoints suggests that build-up of TNF levels from chronic microglial activation is important for it to alter cocaine-induced plasticity.

Locomotor sensitization was also measured to confirm cocaine conditioning and to see if we could replicate our previous behavioural findings. In this case, the TNF KO group had slightly weaker sensitization than the WT mice (Figure 4), showing a smaller increase in movement over each day of drug injections; however, there was no statistically significant difference between genotypes.

Discussion

In this chapter, we found that loss of TNF signalling alters the response of inhibitory synapses to cocaine treatment. This supports our hypothesis that TNF is involved in shifts in excitatory-inhibitory balance caused by cocaine and helps us better understand the attenuating role TNF plays in cocaine addiction. We also determined that TNFR1 is the receptor responsible for TNF's effect on cocaine-induced behaviour, since TNFR1 KO mice show the same exacerbation of locomotor sensitization as previously seen in TNF KO mice. This is the first step into looking at the downstream mediators of TNF's effects, and it provides a basis for performing further experiments using a TNFR1 conditional KO mouse. We can use this model to remove TNF signalling with higher specificity, such as in the NAc core vs. shell or in D1 vs D2 MSNs; but this could not be done without first confirming that loss of TNFR1 function can replicate loss of

TNF function in the context of cocaine treatment. Overall, these findings have strengthened our theory of microglial activation and TNF release as a homeostatic regulator in cocaine addiction and provide a strong foundation for future experiments exploring TNF in addiction.

We predicted TNFR1 would be responsible based on it mediating the effects of TNF on synaptic plasticity in the hippocampus (Stellwagen et al. 2005), but whether it played the same role in the striatum and NAc was not yet known. Since locomotor sensitization behaviour is dependent on synaptic plasticity in the NAc, it is likely that TNFR1 is required for TNF-induced plasticity here in general and in the context of cocaine addiction. Examining AMPA/NMDA ratios in MSNs of TNFR1 KO mice after cocaine treatment could confirm this, like in our previous TNF KO experiments. Another important point would be to examine some potential pathways downstream of TNFR1 that could be involved in drug addiction. One approach is to inhibit molecules known to mediate TNF's effects on synaptic plasticity and receptor trafficking, such as PP1, PI3K, and DARPP-32 (Stellwagen et al. 2005; Pribiag and Stellwagen 2013; Lewitus et al. 2014), and look at whether this produces a similar effect on cocaine behaviour and plasticity as loss of TNFR1 signalling. We could also target compounds associated with cocaine plasticity such as protein kinases (García-Pardo et al. 2016) and examine their function in TNF or TNFR1 KO mice.

At inhibitory synapses, we found that both WT and TNF KO mice showed different patterns of plasticity between 1 day and 5 days of cocaine treatment. After a single cocaine treatment, WT mice showed an increase in mIPSC frequency, but this was not present after 5 days of treatment; whereas TNF KO mice showed no change after a single treatment, but repeated treatment reduced mIPSC frequency. This could be interpreted as TNF reining in cocaine-induced synaptic plasticity as it builds up over time in the WT mice; but when this

signalling is lost, we instead see cocaine driving inhibitory synaptic strength in the opposite direction over time. It is also interesting that we saw a change in pre-synaptic plasticity (frequency) rather than post-synaptic (amplitude); even though this fits the previous findings of the effect of repeated cocaine at inhibitory synapses (Kennedy et al. 2013), it does not match the idea that TNF mainly affects synaptic plasticity via post-synaptic receptor trafficking. However, we previously found that in hippocampal cultures, TNF treatment reduces both mIPSC amplitude and frequency (Pribiag and Stellwagen 2013). The mechanism behind this was not determined, though a change in GABA release sites or the production of GABA was ruled out by immunostaining for GAD65, the rate-limiting enzyme in the production of GABA from glutamate. However, it could still be valuable to compare this measure in cocaine-treated WT and TNF KO mice, since these findings could differ in the context of cocaine addiction. Additionally, changes in GABA release probability could be investigated by biochemically measuring SNARE protein phosphorylation or performing paired-pulse recordings; an increase in phosphorylation and/or a lower paired-pulse ratio would indicate a higher release probability in the WT mice, and we would expect the opposite in the TNF KO mice.

Despite finding that locomotor sensitization is exacerbated in TNFR1 KO mice, we were not able to replicate our previous behavioural findings in TNF KO mice. There was a relatively low number of mice in the KO groups in the TNF KO experiment compared to the TNFR1, below the level we would typically use to analyze a behaviour experiment, so testing more mice could give a more accurate picture. This also limits our ability to conclusively interpret our current electrophysiology results, but there still seems to be a strong pattern that could potentially be further reinforced by increasing the number of mice.

Figures

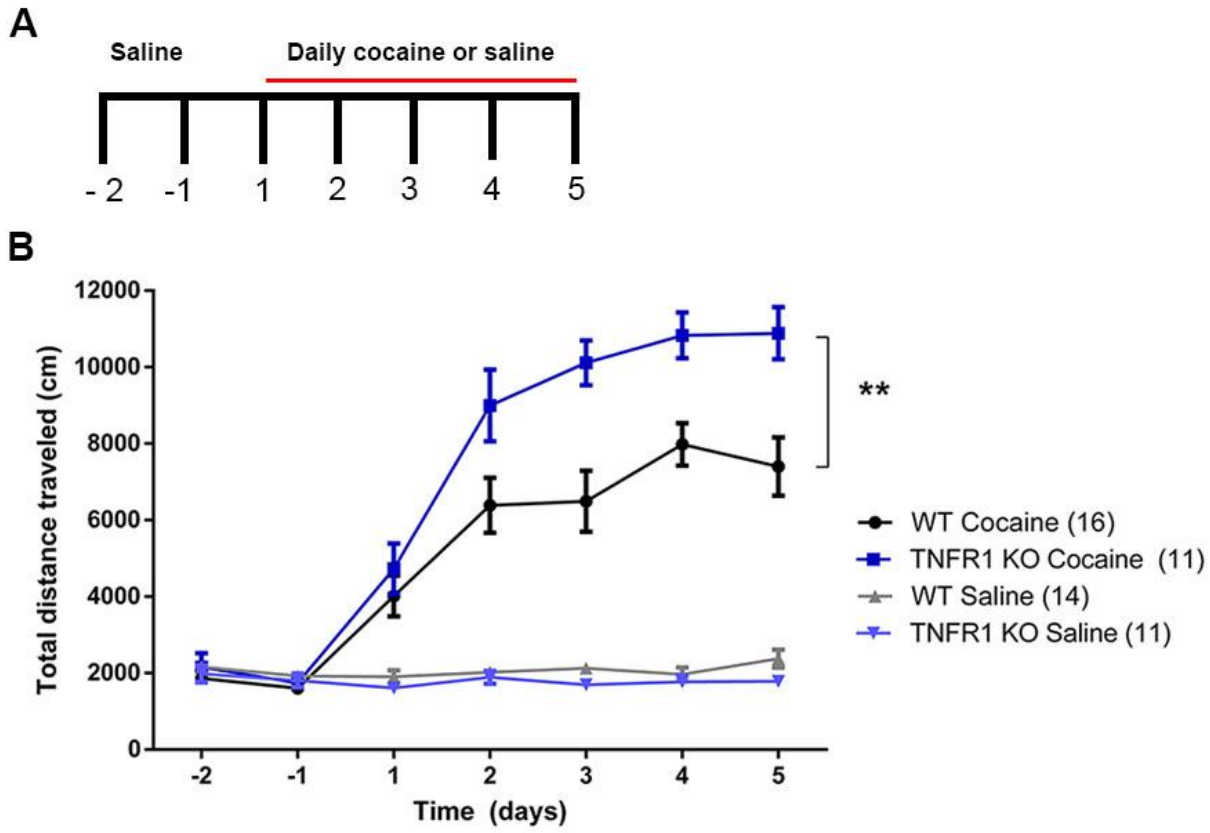

Figure 1. Cocaine locomotor sensitization in TNFR1 KO mice. Experimental timeline for cocaine conditioning (A) and total distance traveled of WT and TNFR1 KO mice during 5 days of treatment (B). All cocaine-treated mice exhibited progressively increasing locomotion with repeated injections (two-way repeated measures ANOVA with Greenhouse-Geisser correction [Mauchly's test $p < 0.001$], main effect of time, $F(4,104) = 96.55$, $p < 0.001$) and this effect was exacerbated in TNFR1 KO mice (time X genotype interaction, $F(4,104) = 4.825$, $p = 0.001$). TNFR1 KO mice also responded stronger to cocaine treatment regardless of test day (main effect of genotype, $F(1,27) = 9.761$, $p = 0.004$). Points are presented as the means for each day ± SEM.

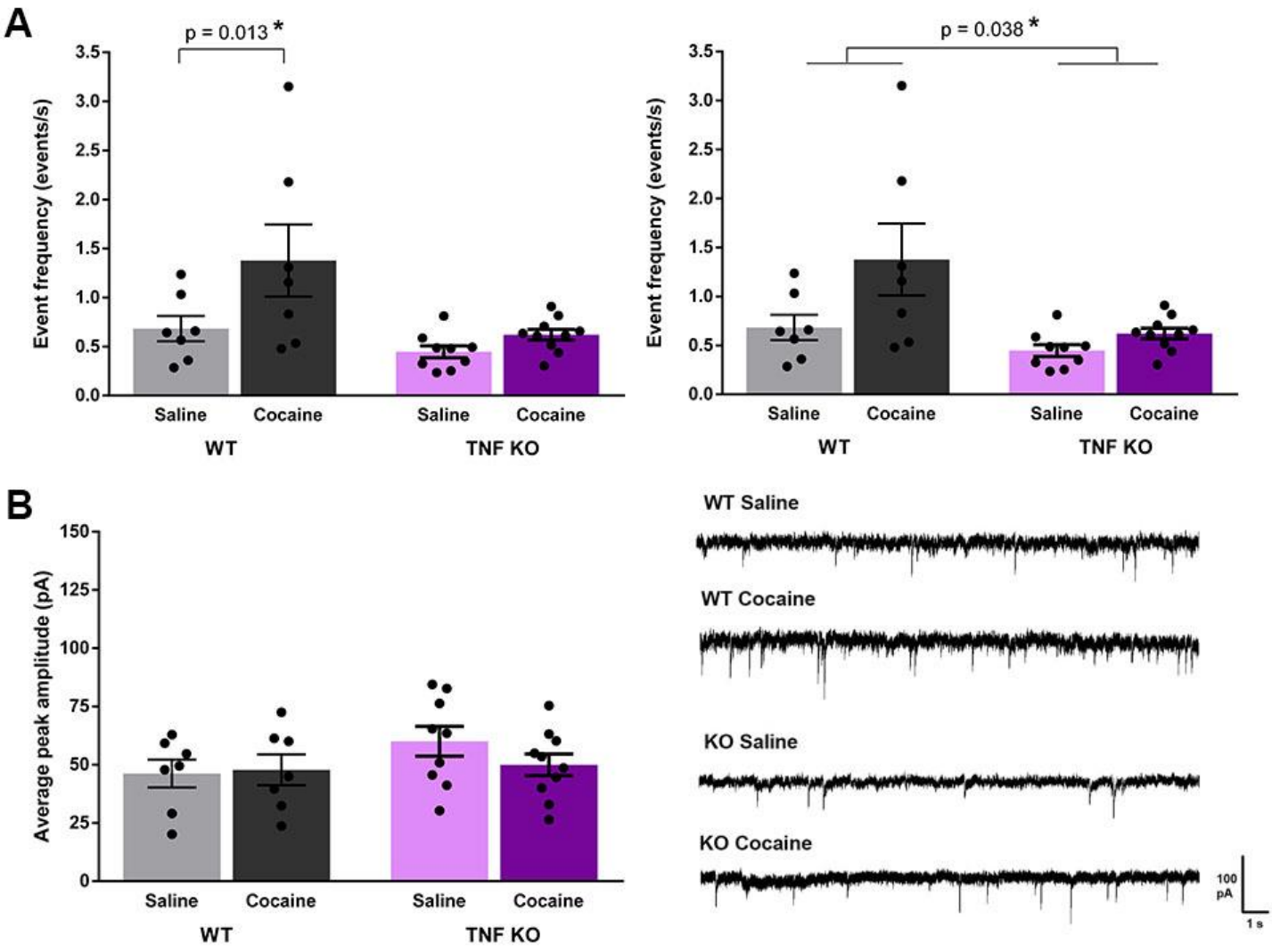

Figure 2. Effect of TNF KO on inhibitory synaptic plasticity after a single cocaine treatment. Frequency (A) and amplitude (B) of mIPSCs in NAc core D1 MSNs of WT and TNF KO mice. A single cocaine injection produced a significant change in mIPSC frequency between the two genotypes (ordinary two-way ANOVA, main effect of genotype $F(1,29) = 8.314$, $p = 0.007$; genotype X treatment interaction $F(1,29) = 6.358$, $p = 0.017$). Simple main effect analysis showed that cocaine significantly increased frequency within the WT group (simple main effect of treatment, $F(1,29) = 7.076$, $p = 0.013$). A Games-Howell post-hoc test was performed to correct for unequal variances between the WT cocaine and TNF KO cocaine groups, and it confirmed that the mIPSC frequency was significantly changed between genotypes ($t(14.18) = -2.285$, $p = 0.038$). Amplitude was unchanged. All groups are presented as the mean ± SEM.

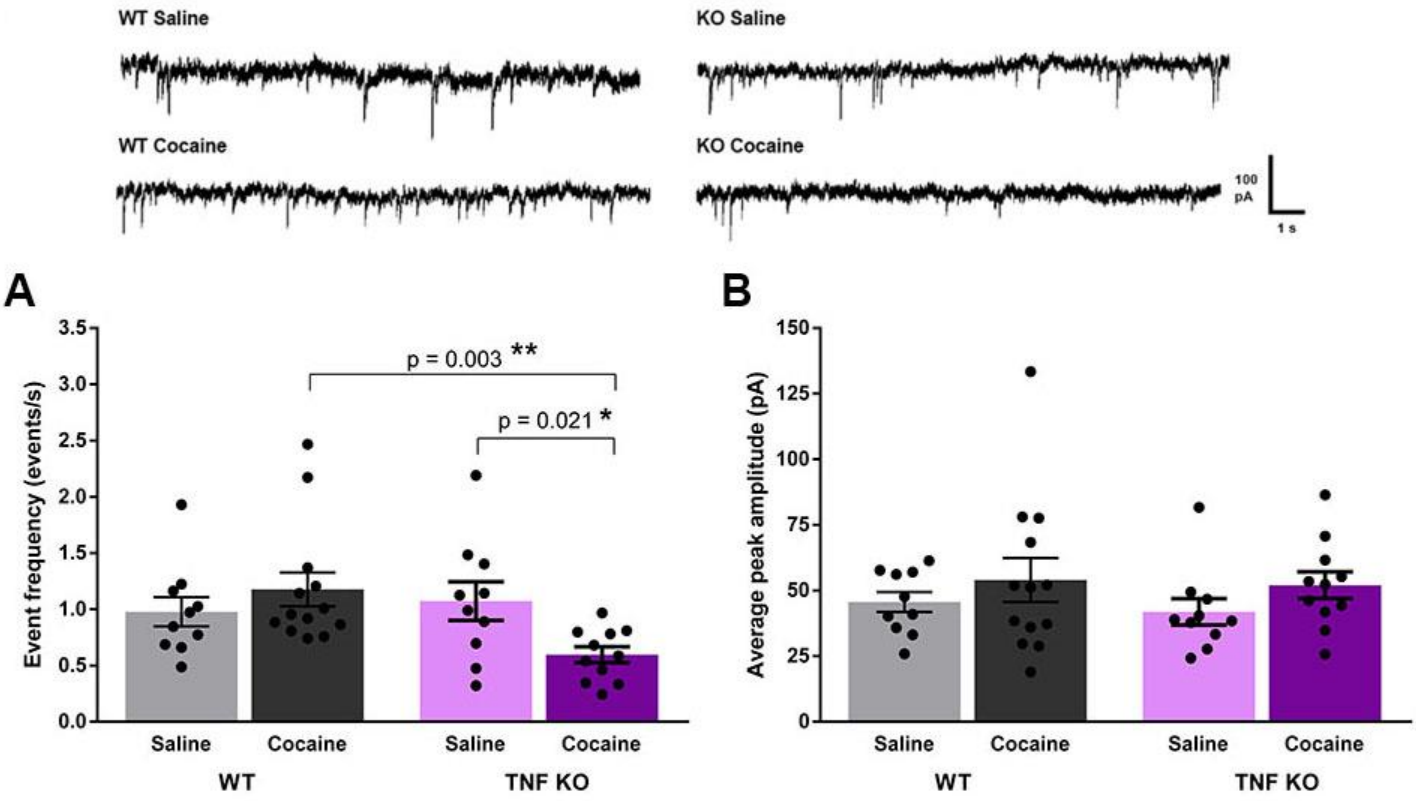

Figure 3. Loss of TNF signalling impacts inhibitory synaptic plasticity induced by repeated cocaine treatment. Frequency (A) and amplitude (B) of mIPSCs in NAc core D1 MSNs of WT and TNF KO mice. 5 days of cocaine treatment significantly altered the frequency of mIPSCs differentially between the two genotypes (ordinary two-way ANOVA, genotype X treatment interaction $F(1,40) = 6.071$, $p = 0.018$). Post-hoc simple main effect analysis showed that cocaine significantly reduced frequency within the TNF KO group (simple main effect of treatment, $F(1,29) = 5.813$, $p = 0.021$), and that this phenotype was not present in the cocaine treated WT mice (simple main effect of genotype, $F(1,40) = 9.832$, $p = 0.003$). Amplitude was not significantly changed. All groups are presented as mean ± SEM.

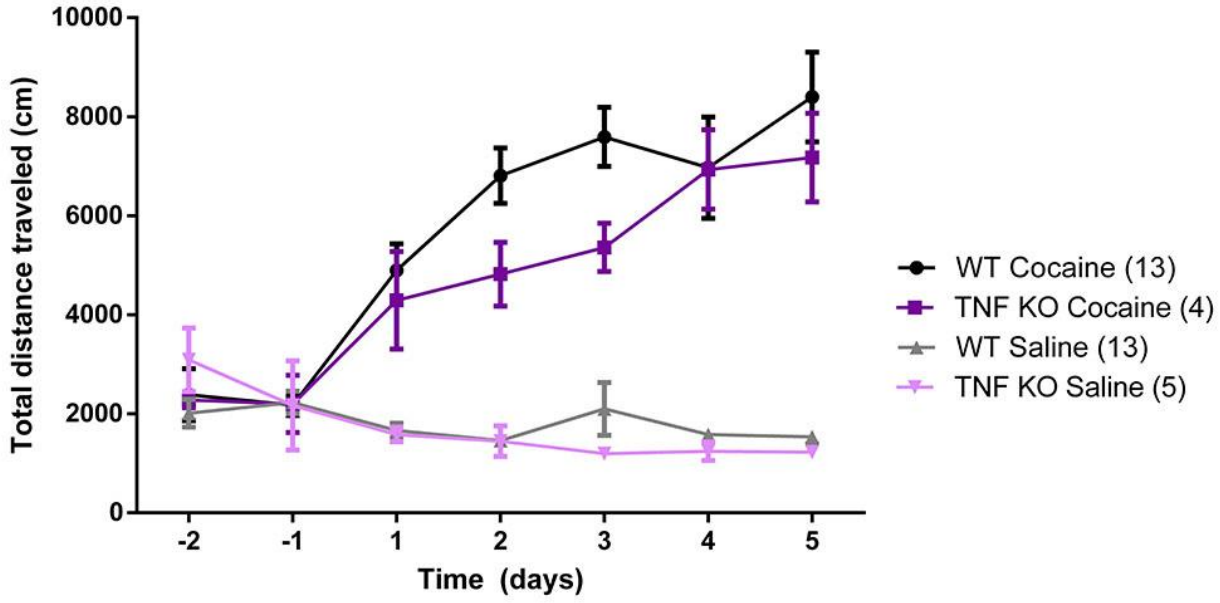

Figure 4. Cocaine locomotor sensitization in TNF KO mice. Total distance travelled over 5 days of cocaine treatment in WT and TNF KO mice. See Figure 1A for treatment timeline. Both groups of cocaine-treated mice showed locomotor sensitization to repeated treatments of cocaine (two-way repeated measures ANOVA, main effect of time, $F(6,6) = 7.943$, $p = 0.012$), but there was no significant effect of genotype. Points are presented as the means for each day ± SEM.

Chapter 4 – The role of TNF signalling in morphine-induced behaviour and synaptic plasticity

Introduction

The molecular mechanisms behind the rewarding effects of opioids are not entirely known. Unlike psychostimulants, opioids such as morphine do not directly affect dopaminergic neurotransmission. What psychostimulants and opioids do have in common is the induction of glutamatergic synaptic plasticity and neuroinflammation, which could interact to trigger behaviours associated with opioid addiction. Morphine treatment produces a diverse pattern of synaptic plasticity at excitatory synapses in the NAc, with varied effects between these the NAc core and shell and among D1 and D2 MSNs. D1 MSNs tend to reinforce drug-seeking behaviours, while D2 MSNs inhibit them (Lobo et al. 2010), so morphine seems to alter the excitatory drive onto these two populations to shift the balance of their signalling and drive drug-induced behaviours.

In the NAc shell, repeated morphine treatment decreases excitatory synaptic strength onto D2 MSNs, as seen by reduced frequency of mEPSCs, with no effect on D1 MSNs (McDevitt et al. 2019); this is accompanied by an increase in intrinsic membrane excitability, which is perhaps a homeostatic response to the reduction of excitatory inputs. D2 MSNs then remain depressed throughout both the abstinence and re-exposure stages (Hearing et al. 2016; Madayag et al. 2019). Abstinence from morphine increases excitatory drive onto D1 MSNs in the shell, indicated by an increase in AMPA/NMDA ratio. This is accompanied by a higher rectification index, demonstrating that the increase in synaptic strength is driven by trafficking of GluA2-lacking AMPA receptors to the synapse (Hearing et al. 2016). Re-exposure then decreases excitatory synapse strength once again (Madayag et al. 2019). While in the NAc core, it is the D2 MSNs that change with the stage of addiction. Abstinence decreases the frequency of mEPSCs onto D2 MSNs, which is then increased by morphine re-exposure, whereas D1 MSNs have

reduced mEPSC amplitude through both stages (Madayag et al. 2019). In total, it seems that glutamatergic synaptic plasticity is altered throughout the course of morphine addiction, although this pattern is complex and not fully characterized; it is unclear what occurs in the NAc core after repeated morphine treatment, and more investigation is required.

To fully understand morphine's effects on reward, it is also important to determine how these synaptic changes translate onto addiction behaviour. Morphine treatment induces microglial activation and release of the pro-inflammatory cytokine TNF, which are connected to drug-induced behaviours and could potentially mediate synaptic changes. Patients with opioid addiction have elevated inflammatory markers, including TNF (Chen et al. 2012), and morphine activates microglia in animal models (Zhang et al. 2012; Taylor et al. 2016; Valentinova et al. 2019). There are conflicting findings as to whether preventing this activation reduces morphine addiction behaviours (Zhang et al. 2012; Eriksen et al. 2016) or aggravates them (Rivera et al. 2019). In particular, loss of TNF signalling reduces the dose threshold for morphine conditioned place preference (Niwa et al. 2007), which matches our previous findings with cocaine where TNF KO mice show exacerbated locomotor sensitization behaviour (Lewitus et al. 2016). TNF also seems to be involved in morphine-induced plasticity in the lateral habenula (LHb), an area involved in the aversive aspects of addiction such as withdrawal symptoms (Volkow and Morales 2015). Valentinova et al. (2019) found that conditional knockout of the TNF receptor TNFR1 prevents the reduction of AMPA/NMDA ratio in the LHb after morphine withdrawal. Additionally, activating microglia using the TLR4 agonist MPLA after repeated morphine treatment can produce this same plasticity, and this effect requires intact TNF signalling.

These findings suggest that TNF plays a role in morphine-induced plasticity and behaviour, supporting the theory of microglial activation as a link between the two. However, we

still do not know how TNF is involved in morphine plasticity in the NAc, the main reward center of the brain. Additionally, we do not know how the loss of TNF signalling impacts other models of opioid addiction behaviour, such as locomotor sensitization. In this chapter, we use TNF KO mice to examine TNF's role in morphine locomotor sensitization and in NAc plasticity produced by repeated morphine treatment.

Results

TNF's involvement in morphine-induced behaviour

We previously found that TNF KO mice show stronger locomotor sensitization in response to cocaine (Lewitus et al. 2016), so we wanted to examine whether TNF has the same attenuating effect with morphine to fully determine whether it plays a similar role in morphine-related behaviours. To do this, we measured locomotor sensitization in TNF KO mice and WT controls treated with either morphine or saline using the same basic protocol (Figure 1A) as our previous cocaine experiments. We used this as a measure of drug acquisition, where stronger sensitization means a stronger reaction to the drug and enhanced development of addiction.

We found that TNF KO mice have exacerbated locomotor sensitization to morphine; while the response to the first morphine treatment was the same between groups, there was then a stronger increase in movement over the next four days in the TNF KO mice compared to the WT controls (Figure 1B). This indicates that, like in cocaine addiction, TNF signalling works to reduce morphine-seeking behaviour rather than facilitating it.

Interaction of TNF with synaptic plasticity after repeated morphine treatment

The synaptic plasticity underlying opioid addiction is only beginning to be characterized. It is unknown whether TNF is involved in this plasticity in the NAc, which is critical for morphine

seeking and withdrawal. Since loss of TNF signalling impacts morphine sensitization, we wanted to investigate the changes at the circuit level that might drive this behavioural change. We examined excitatory synaptic strength in the NAc by recording AMPA/NMDA ratios in D1 MSNs, in WT and TNF KO mice treated with morphine or saline. We focused our investigation on D1 MSNs since TNF treatment affects glutamatergic synaptic plasticity in D1 MSNs in the NAc but not D2 MSNs (Lewitus et al. 2016). The NAc core was targeted initially because it is not known how repeated morphine treatment affects synaptic plasticity in this area. This is also where we saw TNF-dependent changes with cocaine (Lewitus et al. 2016), and the core underlies the more motor aspects of addiction such as locomotor sensitization (Ito et al. 2004).

In the NAc core, we saw no effect of either loss of TNF signalling or morphine treatment on AMPA/NMDA ratio in D1 MSNs (Figure 2). From this, we can conclude that repeated morphine treatment does not impact excitatory synaptic plasticity in D1 MSNs in the NAc core, and therefore loss of TNF signalling also has no effect. Because we did not find any changes in the core, we decided to extend our investigation to D1 MSNs in the NAc shell, as MSNs in this area also exhibit plasticity at excitatory synapses after morphine abstinence and re-exposure. So far, we have only obtained preliminary data for this experiment (Figure 3). While there are no significant differences statistically, there is a slight trend toward an increase in AMPA/NMDA ratio in the morphine-treated WT mice which is not present in the TNF KO group. There also may be a slight difference in the saline baseline between the two groups. If these differences are reinforced by collecting more data, it would indicate that TNF signalling is required for morphine-induced glutamatergic plasticity in D1 MSNs in the NAc shell.

Discussion

Here we demonstrated that TNF has the same attenuating effect on morphine-induced behaviour as with cocaine, since loss of TNF signalling aggravates morphine locomotor sensitization. This provides us with further evidence for our theory of microglial activation and TNF release as a homeostatic regulator of addiction. Unexpectedly we found that morphine, in both the presence and absence of TNF, has no effect on excitatory synapses onto D1 MSNs in the NAc core; but this still provides the first characterization of plasticity in the NAc core after repeated morphine treatment. This also indicates that despite having the same behavioural findings as our previous cocaine experiment, there must be different synaptic changes underlying this drug-seeking behaviour, such as plasticity at a different set of synapses, onto different neurons, or in different areas. In our preliminary recordings in D1 MSNs of the NAc shell, we saw a trend towards an increase in excitatory synapse strength after morphine treatment which was not present in the KO group, meaning that this could be the mechanism behind TNF's effect on locomotor sensitization behaviour. Overall, these experiments not only helped us establish TNF's role in opioid addiction but improved our understanding of how exactly it is working at the synaptic level, which is vital for potentially utilizing TNF therapeutically in the future.

One major question remaining is why these synaptic mechanisms are different from our cocaine studies, despite the same behavioural outcome. Glutamate signalling in the NAc core mediates the sensitizing effects of cocaine (Bell et al. 2000), so we expected synaptic plasticity in this area to accompany both cocaine and morphine-induced locomotor sensitization. We can further explore this issue using a TNFR1 conditional KO mouse. Specifically, we have obtained a TNFR1 floxed mouse, and because we established in chapter one that TNFR1 is responsible for TNF's effects on locomotor sensitization we can now use it to remove TNF signalling in a much

more precise manner. We would first like to determine whether the NAc is the critical area for TNF's effects on locomotor sensitization, or whether TNF signalling in other brain areas could also be driving it; this could explain why we might not see plasticity after morphine treatment in the NAc the same way it is shown in our cocaine experiments. This can be examined via stereotaxic injection of a viral vector (like AAV) containing Cre recombinase into the NAc and then performing the same locomotor sensitization experiments as we have previously. If the NAc is the primary or sole area responsible, we would expect to see the same increase in locomotor sensitization behaviour previously exhibited by TNF KO mice; whereas if TNF signalling in other areas can make up for loss of TNF in the NAc, we might see no difference between the TNF KO and WT groups. We can also take this further by removing TNF signalling in the core or shell only, which is especially relevant to morphine addiction where the pattern of plasticity differs between the two areas. For example, if TNF is modulating morphine-induced plasticity in the shell only, deleting TNFR1 here would replicate the locomotor sensitization finding, but removing it in the core would not. Finally, we can look at TNF's effects on D1 vs D2 MSNs in morphine addiction by crossing our TNFR1 floxed mouse with a D1-Cre mouse line, so that TNFR1 is only knocked out in D1 MSNs (and other D1 receptor-expressing cells). We can then repeat our morphine locomotor sensitization experiment to confirm whether TNF signalling onto D1 MSNs is somehow driving this behaviour, perhaps via plasticity in the NAc shell.

Further recordings in D1 MSNs in the NAc shell are necessary to fully determine if there is morphine-induced plasticity. It is notable that the only publication that has examined this so far found that morphine conditioning did not affect mEPSCs or mIPSCs in D1 MSNs in the NAc shell (McDevitt et al. 2019). This plasticity may still be important to look at since the NAc is the main area responsible for locomotor sensitization (Bell et al. 2000; Ito et al. 2004) but we did not

find anything in the NAc core, and because TNF only affects D1 MSNs. If we can confirm a change, it would be important to measure rectification index or input-output curves to determine whether changes to AMPA receptors are driving the TNF effect. If no significant change in excitatory synaptic plasticity is found in the shell, as it is in the core, recording mIPSCs in core D1 MSNs may be an interesting next step. This would be relevant since in chapter 3 it was found that TNF can affect these in the context of cocaine addiction. Additionally, the current findings on inhibitory synapses in morphine addiction are limited. Morphine can alter LTP and LTD at GABAergic synapses in the VTA (Nugent et al. 2007; Dacher and Nugent 2011), and it increases cholinergic modulation of inhibitory synapses in the NAc (De Rover et al. 2005), but further investigation in the NAc especially is needed. Despite TNF not affecting D2 MSNs, it would still be relevant to measure AMPA/NMDA ratios in NAc core D2 MSNs in WT mice, since glutamatergic synaptic plasticity after repeated morphine treatment has not been characterized yet in D2 MSNs in the NAc core.

Figures

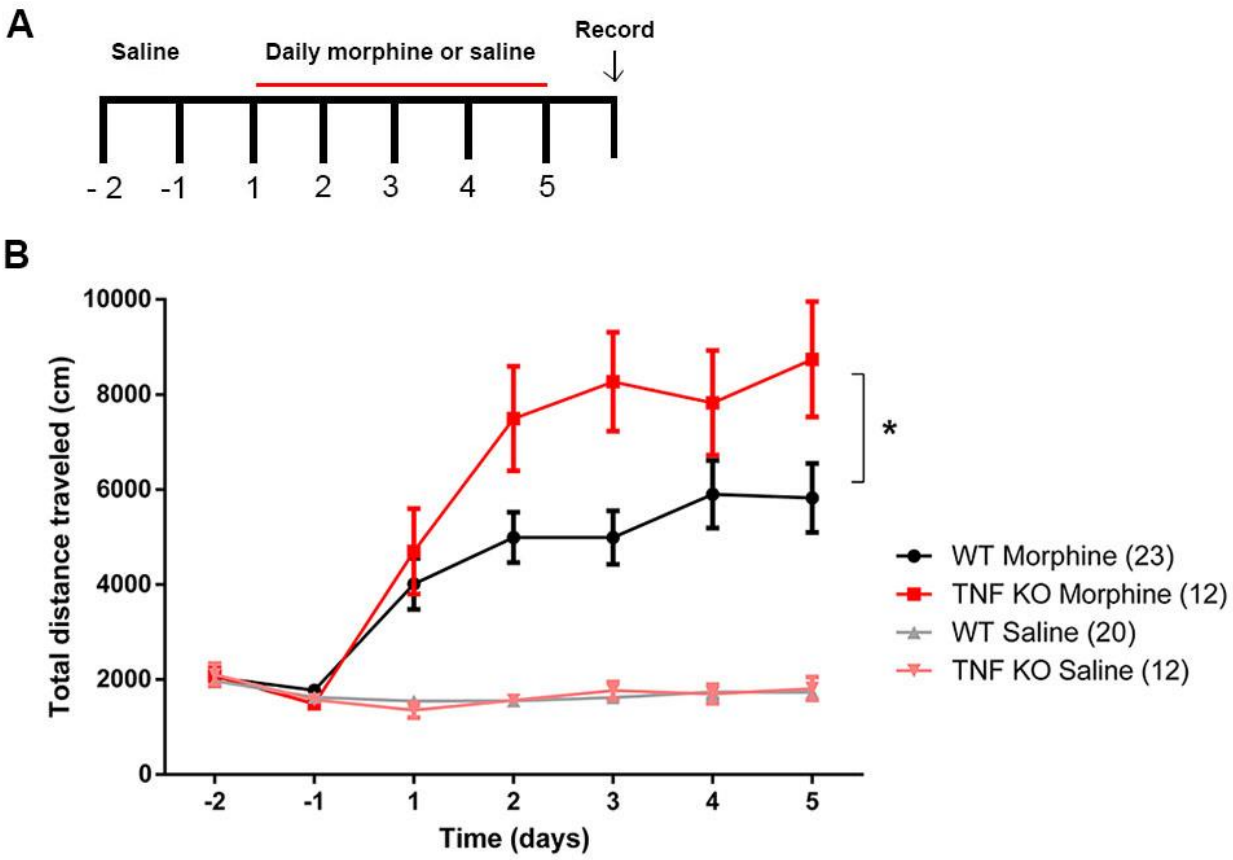

Figure 1. Morphine locomotor sensitization in TNF KO mice. Experimental timeline for repeated morphine treatment (A) and total distance traveled of WT and TNF KO mice during 5 days of treatment (B). Both groups of cocaine-treated mice showed an increase in locomotion over repeated injections (two-way repeated measures ANOVA with Greenhouse-Geisser correction [Mauchly's test $p < 0.001$], main effect of time, $F(3,112) = 42.53$, $p < 0.001$) and this effect was stronger in TNF KO mice (time X genotype interaction, $F(3,112) = 3.94$, $p = 0.008$). As well, TNF KO mice had more locomotion regardless of the test day during the morphine treatment period (main effect of genotype, $F(1,33) = 4.83$, $p = 0.035$). Points are presented as the means for each day ± SEM.

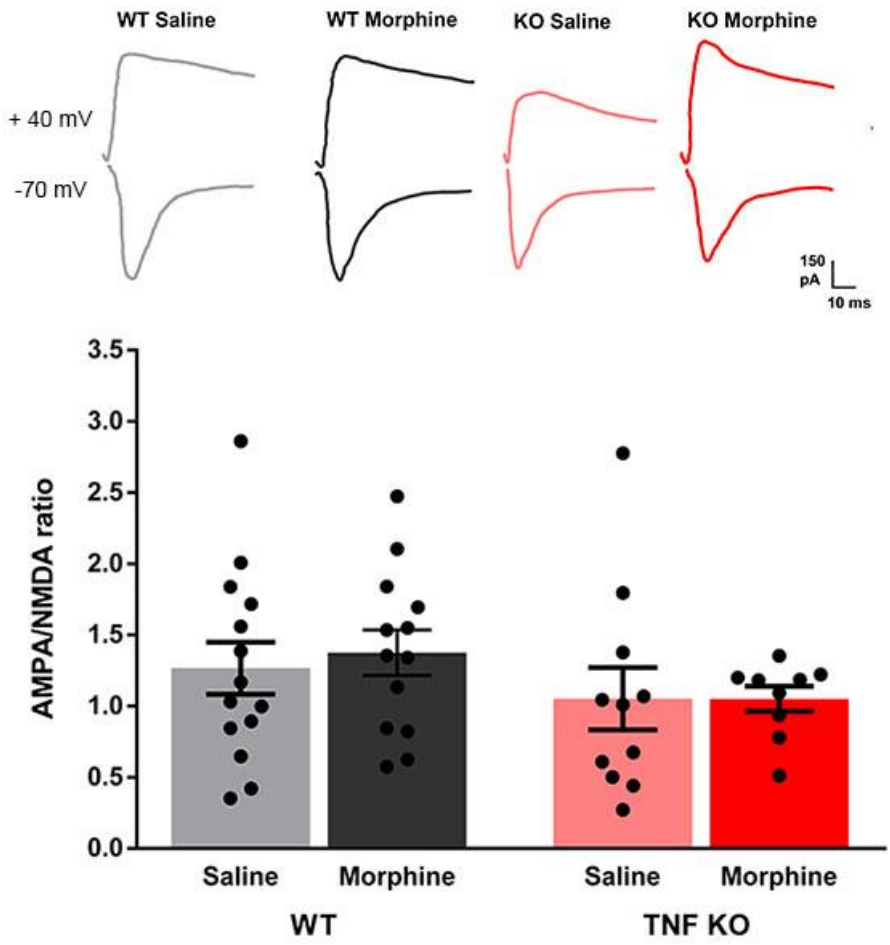

Figure 2. Excitatory synaptic plasticity after repeated morphine treatment in the NAc core. AMPA/NMDA ratios in D1 MSNs in the NAc core of WT and TNF KO mice after 5 days of morphine treatment. There was no significant effect of either genotype (ordinary two-way ANOVA, $F(1,43) = 2.242$, $p > 0.05$) or morphine treatment ($F(1,43) = 0.094$, $p > 0.05$) on AMPA/NMDA ratio, a measure of excitatory synaptic strength. All groups are presented as mean ± SEM.

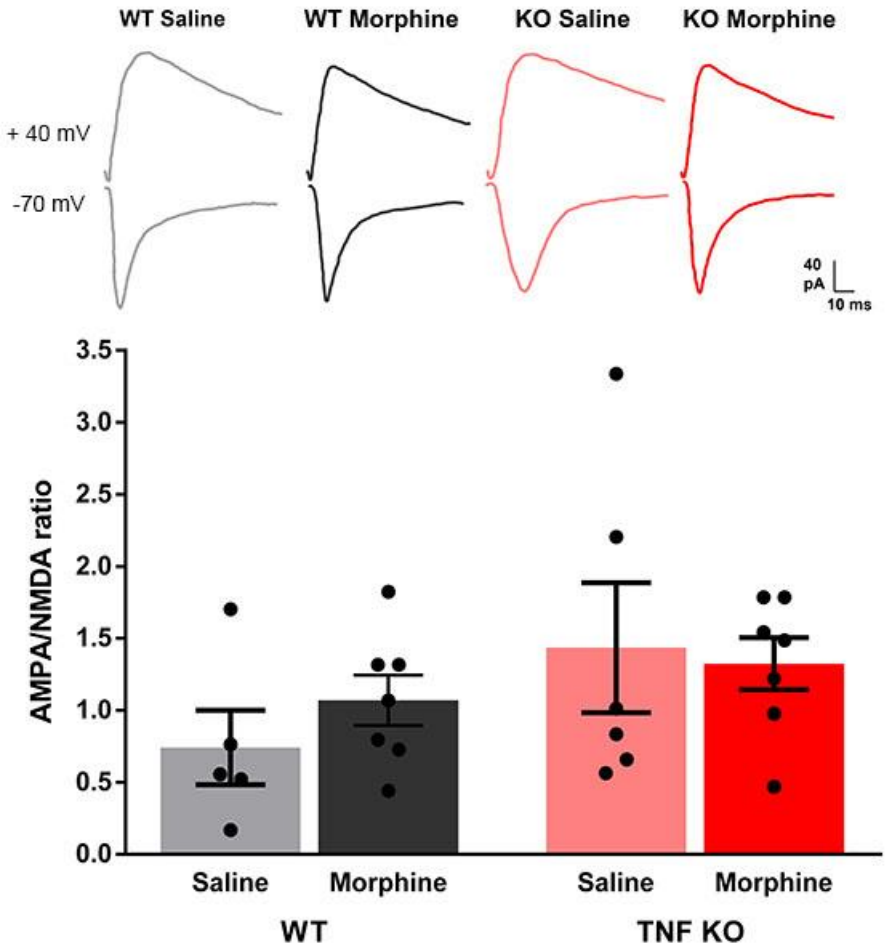

Figure 3. Excitatory synaptic plasticity after repeated morphine treatment in the NAc shell. AMPA/NMDA ratios in D1 MSNs in the NAc shell of WT and TNF KO mice after 5 days of morphine treatment. There was also no significant effect of genotype (ordinary two-way ANOVA, $F(1,21) = 2.86$, $p > 0.05$) or treatment ($F(1,21) = 0.15$, $p > 0.05$), but there seemed to be a trend towards an increase in AMPA/NMDA ratio in the morphine-treated WT mice. All groups are presented as mean ± SEM.

Chapter 5 – General Discussion

The processes defining the different aspects of addiction are not entirely known, and understanding this could be key to developing therapies that break the cycle of addiction. One common feature triggered by different drugs of abuse is inflammation. Patients with addiction exhibit elevated levels of inflammatory cytokines (Chen et al. 2012; Narvaez et al. 2013; Heberlein et al. 2014), and both cocaine and morphine induce microglial activation and release of cytokines such as TNF in animal models (Zhang et al. 2012; Liao et al. 2016). TNF has been shown to influence addiction behaviours and synaptic plasticity in brain areas critical for addiction (Niwa et al. 2007; Lewitus et al. 2016; Valentinova et al. 2019) but its role in addiction is still not fully characterized. Our goal was to better understand TNF's impact on addiction behaviours and plasticity by determining which receptor is responsible for its effects, assessing whether it influences inhibitory synaptic plasticity, and examining its role in morphine-induced behaviour and plasticity.

In total, our findings provide further evidence for TNF as a homeostatic regulator of addiction, showing that TNF helps to reduce the effects of drugs of abuse rather than causing them. We also identified some of the mechanisms behind this, which is the first step to potentially harnessing TNF signalling in a therapeutic manner. Since the loss of TNFR1 replicates the effect of losing TNF on cocaine-induced behaviours, we can conclude that TNFR1 is the receptor involved in its attenuating effect on sensitization. This allows for the exploration of downstream molecular mediators, in addition to providing a basis to use a conditional TNFR1 KO mouse to remove TNF signalling in a more specific manner for future experiments. We also saw that loss of TNF signalling alters inhibitory synaptic plasticity caused by cocaine addiction. After repeated cocaine treatment, TNF seems to strengthen GABAergic neurotransmission, while weakening glutamatergic synapses as established previously; this is evidence for TNF inducing

homeostatic synaptic plasticity, where plasticity occurs at multiple synapse types across a neuron to alter the equilibrium of excitatory and inhibitory signalling in response to circuit activity levels. Finally, we found that loss of TNF signalling exacerbates morphine-induced behaviour, cementing its role as an attenuator of addictive behaviours. These findings provide a strong basis for future experiments working towards fully understanding TNF in addiction and harnessing it to alleviate symptoms or prevent relapse.

There are a few general limitations and remaining questions that could be addressed with future research. Our TNF KO model has always been constitutive, so we have just assumed that TNF function in the NAc is the main mediator of its effects on behaviour, since NAc plasticity has been shown to cause drug-induced behaviours (Pascoli et al. 2012) and it is critical for locomotor sensitization (Ito et al. 2004). However, it is still possible that loss of TNF signalling in other areas of the brain could contribute to its behavioural effects. To address this, we can use a conditional TNFR1 KO mouse to stop TNF signalling in the NAc exclusively, and then repeat our locomotor sensitization behavioural testing and our electrophysiology experiments. We also cannot be entirely certain in our differentiation of D1 and D2 MSNs, since there is a minority third subpopulation of NAc MSNs that express both D1 and D2 dopamine receptors (Gagnon et al. 2017) alongside multiple types of interneurons that do not express dopamine receptors (Scofield et al. 2016). Therefore, our D1 MSN recordings could include some mixed receptor MSNs, or interneurons when identifying some cells morphologically. To better address this D1-D2 divide, we could target deletion of TNFR1 specifically to D1 receptor expressing cells.

It is also important to address the role of TNF at the abstinence and relapse stages of addiction. Locomotor sensitization is a basic measure of addiction behaviour, that only assesses a general motor response to acquiring drug dependence and does not measure drug-seeking

behaviour itself, so it is not the best model for the later phases of addiction. The two other standard behaviour paradigms, conditioned place preference (CPP) and self-administration (SA), directly measure drug seeking and better encapsulate the patterns seen in addiction patients. We previously found that inducing microglial activation also reduces the development and relapse of CPP and SA in cocaine addiction, presumably by elevating TNF levels (Athanassiou 2018); however the synaptic changes behind this effect remain to be determined.

TNF and TNFR1 in cocaine addiction

In chapter 3, we wanted to determine which TNF receptor is responsible for its role in cocaine addiction and examine the effect of TNF on cocaine-induced plasticity at GABAergic synapses. We previously found that loss of TNF signalling strengthens locomotor sensitization to cocaine, and reverses the initial change in cocaine-induced plasticity at glutamatergic synapses (Lewitus et al. 2016). We also know that TNFR1 is the receptor responsible for TNF's effects on synaptic plasticity in the hippocampus (Stellwagen et al. 2005; Pribiag and Stellwagen 2013); additionally, the soluble form of TNF was the specific mediator of our cocaine findings, and this form primarily binds to TNFR1 (Sedger and McDermott 2014). Therefore, we targeted TNFR1 as the potential mediator of TNF's role in cocaine addiction and found that TNFR1 KO mice show the same exacerbated locomotor sensitization to cocaine as in our previous experiment with TNF KO mice. This allows us to now use a conditional TNFR1 KO mouse to remove TNF signalling more specifically from areas of the brain or specific cell types, like the NAc core and shell and D1 or D2 MSNs. This model will be the basis for many of our future experiments and can improve our understanding of how TNF works overall. Moreover, we can also look at proteins associated with TNFR1's effect on post-synaptic receptor trafficking to find potential mediators of TNF's effects in cocaine addiction.

TNF treatment has been shown to alter GABAergic synaptic plasticity in the hippocampus and striatum in a direction opposite to plasticity at glutamatergic synapses (Pribiag and Stellwagen 2013; Chambon 2020) and cocaine induces a pattern of inhibitory synaptic plasticity that changes with the stages of addiction in the opposite direction of excitatory synapses (Kennedy et al. 2013; Otaka et al. 2013). We thus aimed to determine if loss of TNF reverses cocaine-induced plasticity at inhibitory synapses the same way it does at excitatory ones. In WT mice, we found that a single day of cocaine treatment increased the frequency of inhibitory post-synaptic currents, while at 5 days of treatment there was no change. We predicted there would be an increase based on previous findings after 5 days of treatment (Kennedy et al. 2013), but seeing this only at 1 day and not 5 was unexpected. Interestingly, TNF KO mice show a decrease in inhibitory synaptic strength after 5 days of treatment, essentially opposing this previously found pattern. A potential explanation is that TNF building up over time in WT mice opposes the cocaine-induced reduction in inhibitory synaptic strength that we saw in TNF KO mice; which fits with TNF treatment increasing inhibitory synaptic strength onto striatal MSNs (Chambon 2020).

Loss of TNF signalling worsens locomotor sensitization behaviour (Lewitus et al. 2016) and increasing GABAergic neurotransmission can reduce cocaine-induced behaviour (Filip et al. 2006; Gawlińska et al. 2020). Therefore, we expected that along with a reduction in inhibitory synapse strength, we would see stronger locomotor sensitization in TNF KO mice as observed previously. We did not see any significant difference between the two groups, which makes it more complicated to interpret our electrophysiology findings and could potentially challenge their validity. Since we previously found a behavioural phenotype in TNF KO mice that was associated with changes in glutamatergic synaptic plasticity (Lewitus et al. 2016), seeing altered

plasticity at inhibitory synapses with no change in cocaine-induced behaviour could call into question whether the difference within the TNF KO group is truly due to the cocaine treatment. It could instead be influenced by some artefact of losing TNF or other unknown factors. We did see locomotor sensitization to morphine in the TNF KO mice and to cocaine in the TNFR1 KO mice using the same protocol, and there were far fewer mice used in the TNF KO cocaine experiment than we would normally use to make conclusions from a behaviour experiment. Perhaps more trials must be done to get a full picture of this behaviour, making sure we can replicate the previous phenotype and thus better interpret our synaptic findings.

One important facet of our findings was that changes in mIPSC frequency but not amplitude are suggestive of presynaptic plasticity, but TNF typically works via altering postsynaptic receptor trafficking. Inhibitory synapses onto MSNs can be from other MSNs, GABAergic interneurons, or from other areas like the VP or VTA (Scofield et al. 2016), and MSNs have been shown to synapse back onto themselves in vitro (Shi and Rayport 1994). Perhaps one or more of these subsets are exhibiting post-synaptic plasticity but we just cannot see it in the larger dataset because our mIPSC recordings would sample from all types. For synapses from the VTA or VP, we could potentially stimulate these specific axons in the NAc using optogenetics, then record inhibitory currents in MSNs to assess the plasticity of these specific synapses. It is also possible that there is a switch to post-synaptic mechanisms at the abstinence or re-exposure stages, since mIPSC amplitude is changed at these stages in WT mice (Otaka et al. 2013). Additionally, TNF can affect mIPSC frequency alongside amplitude in the hippocampus (Pribiag and Stellwagen 2013), so it would be important to further explore what pre-synaptic mechanisms could be driving these changes in the NAc. This includes increased GABA production potentially leading to a larger number of GABA vesicles released, an increase

in the ready releasable pool, or higher release probability. We should also examine D2 MSNs to assess whether cocaine does not affect inhibitory synapses onto them, since it does not impact excitatory synapses onto D2 MSNs. It would also be interesting to look at MSNs in the NAc shell. This is because all the previous cocaine GABAergic plasticity findings were in the NAc shell and not the core, and perhaps this is why our results after 5 days cocaine treatment in the WT group do not fully match up with previous data.

Impact of TNF on morphine addiction

In chapter 4 we explored the role of TNF in morphine synaptic plasticity and behaviour, to assess if it has similar effects on behaviour and plasticity as in our cocaine experiments. Morphine treatment activates microglia which subsequently elevates TNF (Pan et al. 2016: 201; Amri et al. 2018), and TNF KO mice have a lower threshold dose for development of morphine CPP (Niwa et al. 2007). Additionally, TNF regulates synaptic plasticity triggered by morphine in the lateral habenula (Valentinova et al. 2019), but whether it can also affect neurons in the NAc had not been previously examined. We therefore sought to determine whether loss of TNF signalling alters morphine-induced synaptic plasticity in the NAc, alongside locomotor sensitization. We found that TNF KO mice have stronger sensitization to morphine, which replicates our previous finding with cocaine; this indicates that TNF plays a similar homeostatic role in regulating drug-induced behaviour by morphine as with cocaine. However, morphine did not alter excitatory synaptic strength in D1 MSNs in the NAc core, which means the mechanism behind TNF's effect on cocaine vs. morphine locomotor sensitization is probably different. This is assuming that the NAc plasticity we see after loss of TNF signalling in cocaine treatment is solely responsible for this behaviour, but we would need to eliminate TNF from the NAc specifically to prove causation here, as previously discussed. Additionally, our preliminary findings suggest that

the NAc shell could be the critical area for morphine behaviour instead of the core, but further research is required to confirm this.

Why are the synaptic mechanisms behind TNF's effect on cocaine and morphine behaviours different? It seems that if repeated morphine treatment is not affecting synaptic plasticity in the NAc core with TNF signalling intact, then there is also no change when TNF signalling is lost. Perhaps TNF release triggered by morphine is not enough to alter synaptic plasticity if morphine does not have a baseline effect. This is different from cocaine, where we still see a baseline effect on D1 MSNs in the core when TNF signalling is absent. A critical missing piece to our theory is how exactly morphine or cocaine induce synaptic plasticity if TNF is working to counteract it rather than causing it. After abstinence and re-exposure to morphine, there are different patterns of plasticity in D1 and D2 MSNs; therefore, there must be other factors aside from the possibility of TNF, since TNF can only affect D1 MSNs. Examining dopamine signalling more closely is one option, specifically how activating D1 vs D2 receptors could lead to different outcomes for glutamatergic synaptic plasticity, since D1 receptors generally induce LTP while D2 receptors oppose it (Beaulieu and Gainetdinov 2011). Additionally, there could be other mediators of AMPA receptor trafficking involved, such as PKA and PKC (García-Pardo et al. 2016), and several transcription factors and immediate early genes have also been implicated in synaptic plasticity during addiction (Chandra and Lobo 2017; Teague and Nestler 2021). All of these could be worth investigating to determine the initial cause of drug-induced plasticity, which TNF could then oppose to reduce drug-induced behaviour.

Looking back at TNF, it is possible that TNF is somehow differentially released in the core vs shell during morphine treatment, or that there are differences in receptor function and signalling further downstream. This could be addressed using the TNFR1 conditional KO mouse

to target the loss of TNF signalling to core or shell specifically. We also have assumed that TNF elevation is occurring in the NAc, due to our behavioural findings and previous research on the effects of morphine on microglia. Therefore, we should independently verify that morphine is inducing microglial TNF release like we have previously with cocaine. We could also test whether activating microglia to induce TNF release could reduce morphine locomotor sensitization after re-exposure to morphine, to complement our finding that losing TNF strengthens this behaviour. Furthermore, it is possible that TNF-induced plasticity in an entirely different area of the brain is responsible for TNF's effects in morphine addiction, as morphine-induced plasticity also occurs in the VTA, LHb, hippocampus, and cortex (Elahi-Mahani et al. 2018; Sun et al. 2019; Valentinova et al. 2019; Yang et al. 2020). If removing TNFR1 in the NAc alone does not replicate the effect of a constitutive TNF KO on behaviour, then these other areas could be examined next. Inhibitory plasticity in the core is another candidate, since we showed a role for this in cocaine addiction, but there is very little data on this for morphine so far. Overall, there are several potential avenues to fully determine why our synaptic plasticity data was so divergent between our morphine and cocaine experiments.

Conclusion

Neuroinflammation is a potential connector between addiction behaviour and the synaptic plasticity that underlies it. Our project helps establish that the inflammatory cytokine TNF is a homeostatic regulator of drug addiction, which reduces drug seeking behaviour via its effects on homeostatic plasticity in the nucleus accumbens. We found that TNFR1 is the receptor responsible for TNF's effects, which is the first step to further exploring some potential molecular mechanisms in drug addiction. This also provides a basis for using a conditional TNFR1 KO mouse to expand our findings with future experiments. We also showed that TNF

alters inhibitory synaptic plasticity after chronic cocaine treatment, providing stronger evidence for TNF mediating homeostatic synaptic plasticity during addiction. Finally, we established the role of TNF in morphine addiction, determining that it has the same attenuating effect on morphine-induced behaviour as with cocaine, but that the underlying synaptic plasticity differs between the two drugs. Overall, this work improved our understanding of TNF's role in the response to drugs of abuse and of the neurobiology of addiction as a whole, which is critical to developing new therapies to treat this disease.

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