# TUMOR-NECROSIS-FACTOR-ALPHA-MEDIATED PLASTICITY IN BEHAVIORAL MODELS: ROLE IN COCAINE ADDICTION VERSUS MATERNAL IMMUNE ACTIVATION

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## ABSTRACT

Evidence suggests that adaptive physiological responses in the brain contribute to normal circuit function. Various mechanisms, including the brain's inflammatory response, induce compensatory changes to maintain normal circuit function after disruption caused by psychoactive drugs or environmental stressors. One such mechanism involves glial-derived tumor necrosis factor-alpha (TNF). TNF is a pro-inflammatory cytokine which mediates both inflammation and a type of compensatory plasticity known as homeostatic synaptic plasticity. Adaptive roles for TNF have been demonstrated *in vivo* in sensory cortices after blockade of sensory input, and in the striatum after chronic treatment with antipsychotics. However, dysregulated inflammatory responses in the brain, which include elevation of TNF, can be detrimental in many disease models. This doctoral thesis examines the contribution of TNF-mediated plasticity in two models where behavioral alterations are associated with both elevated TNF levels in the brain and altered synaptic function. In the first part of the thesis, we investigate the effects of TNF on the behavioral and the physiological changes occurring in mice after repeated cocaine administration. We find that by reducing synaptic strength in the nucleus accumbens (NAc), TNF counteracts cocaine-induced plasticity in the NAc which then mitigates the sensitization to repeated cocaine administration. Further, this adaptive TNF response is specifically mediated by microglia and suggests that modulation of microglia activation could be an avenue of treatment for drug addiction. We further demonstrate that TNF negatively regulates cocaine-induced dendritic spine formation in the NAc and that the basal spine density in the NAc is mediated by astrocytic TNF. We then explored whether TNF is involved in adult behavioral changes induced by prenatal immune activation, which are

relevant to neuropsychiatric disorders. We found that genetic deletion of TNF had no effect on social and anxiety behaviors induced by early immune challenge. We propose that TNF-mediated plasticity in the brain has no distinct role in models of persistent neuroinflammation where other pro-inflammatory cytokines are significantly elevated. Understanding how and when TNF-mediated plasticity is engaged by different kinds of experience is important for understanding the molecular and cellular basis of homeostatic plasticity *in vivo*.

## RÉSUMÉ

À ce jour, les données suggèrent que la physiologie du cerveau comprend des réponses adaptives qui contribuent à la fonction normale des circuits. Divers mécanismes, y compris la réponse inflammatoire du cerveau, causent des changements compensatoires aux synapses suivant une perturbation causée par des médicaments psychoactifs ou des facteurs de stress environnemental. Un tel mécanisme implique le TNF (Facteur de Nécrose Tumorale) qui est une cytokine pro-inflammatoire dérivée de cellules gliales. Le TNF fait partie du processus inflammatoire mais est aussi impliqué dans un type de plasticité compensatoire qui s'appelle « Homeostatic synaptic plasticity. » Des rôles adaptatifs du TNF ont été démontrés *in vivo* dans les régions corticaux sensoriels après blocage de l'entrée sensorielle de même que dans le striatum après un traitement chronique avec des antipsychotiques. De plus, une réponse inflammatoire dérégulée dans le cerveau, incluant l'élévation du TNF, peut avoir un effet délétère dans de nombreuses maladies. Cette thèse de doctorat examine la contribution de TNF dans deux modèles où des changements comportementaux sont associés à des niveaux élevés de TNF ainsi qu'une fonction synaptique altérée. Dans la première partie de la thèse, nous étudions les changements comportementaux et synaptiques causés par TNF chez la souris après l'administration répétée de cocaïne. Nous avons découvert qu'en réduisant la transmission synaptique dans le noyau accumbens (NAc), le TNF limite la plasticité causée directement par la cocaïne dans la NAc. Ce mécanisme atténue la réponse comportementale à la cocaïne notamment la sensibilisation locomotrice. En outre, cette réponse adaptative dirigée par TNF est spécifiquement médiée par l'activation microgliale ce qui suggère que la

modulation des cellules microgliales pourrait être une approche thérapeutique intéressante pour la dépendance. Nous démontrons aussi que le TNF dirige une réduction d'épines dendritiques suivant l'administration de la cocaïne dans le NAc et que la densité basale des épines dendritiques dans le NAc est médiée par le TNF dérivé des astrocytes. Ensuite, nous avons évalué l'impliquation de TNF dans les changements comportementaux induits par l'activation immunitaire prénatale. Ces changements comportementaux sont aussi impliqués dans certains troubles neuropsychiatriques. Nous avons constaté que la délétion génétique du TNF n'avait aucun effet sur les comportements sociaux et anxieux induits par l'activation immunitaire prénatale. Nous suggérons que la plasticité dirigée par le TNF dans le cerveau n'a pas un rôle distinct dans les modèles de neuroinflammation persistante où d'autres cytokines pro-inflammatoires sont aussi élevées. Comprendre comment et quand le TNF est impliqué dans différents processus est important pour comprendre les bases moléculaires et cellulaires de la plasticité homéostatique *in vivo*.

# DEDICATION

I dedicate this thesis to my mother Charlyn, who passed away April 27, 2014.

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## AUTHOR CONTRIBUTIONS

### CHAPTER 1

Work presented in Chapter 1 is an unpublished review written by S. Konefal of relevant scientific literature for the studies presented in Chapters 2, 3 and 4.

### CHAPTER 2

The first data chapter presented in this thesis been published as “Microglial TNF Suppresses Cocaine-induced Plasticity and Behavioral Sensitization” in Neuron, by Lewitus et al., 2016. It has been slightly modified from its published version and includes non-published results in Figure 4 (E, F & G). Author and co-author contributions are as follows:

Co-first author: Sarah Konefal

Helped conceive study design and implementation for all immunohistochemistry and qPCR experiments (Figures 2-6, 8-9). Collected and analyzed all results for IHC and qPCR experiments including flow-through from microglia purification protocol (A.G.). Performed and analysed behavioral experiments in Cre-Lox conditional knock-outs (Figure 6). Generated figures for all results except Figures 1 & 7. Contributed to writing all drafts for the final manuscript.

Co-first author: Gil Lewitus

Conceived the project, performed and analysed all electrophysiology experiments except for two done by H.P., as well as behavioral sensitization experiments in TNF-/- mice and with DN-TNF (Figures 1 & 7). Contributed to writing all drafts for the final manuscript.

Co-author: Andrew Greenhalgh

Provided feedback on study design for microglia experiments and on early drafts of the manuscript. Purified microglia from adult mouse tissue for further analysis (Figure 3A-B).

Co-author: Horia Pribiag

Performed and analysed two key electrophysiology experiments requested in revision (Figure 7D-E & Figure 8K).

Co-author: Keanan Augereau

Conducted pilot behavioural experiments measuring addictive behaviors in TNF-/- mice and established behavioral protocols in new behavioral facility.

Co-author and project leader: David Stellwagen

Conceived the project, provided feedback on study design and implementation. Also conducted pilot behavioural experiments measuring addictive behaviors in TNF-/- mice. Contributed to writing all drafts for the final manuscript.

**CHAPTER 3**

The project investigating the role of TNF in the regulation of dendritic spines in the nucleus accumbens (NAc) was conceived by Gil Lewitus and David Stellwagen, as part of the cocaine project presented in Chapter 2. The result that TNF-/- mice had a basal elevation of dendritic spine density in the NAc prompted further investigation as a separate project, and will be pursued as another manuscript to be published. The data presented in Figure 1D & E is from work done by Gil Lewitus (injection of animals with saline or cocaine) and Sabrina Chierzi (tissue preparation, diolistic labeling of neurons, and image collection). Jean-Philippe Clement analysed all images collected by S.C. and those collected by S.K. from Cre-Lox conditional knock-outs. Sarah Konefal performed the remaining experiments and final statistical analyses. Chapter 3 was written by S.K.

**CHAPTER 4**

A version of Chapter 4 has been accepted for publication as “TNF-mediated homeostatic synaptic plasticity in behavioural models: testing a role in maternal immune activation” in Philosophical Transactions B (Manuscript ID: RSTB-2016-0160.R1). Sarah Konefal conceived and implemented the study design and collected and analysed data. Both S.K. and David Stellwagen wrote the manuscript.

## LIST OF ABBREVIATIONS

| | |
|---|---|
| Akt | serine/threonine-specific protein kinase (protein kinase-B) |
| AMPA | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid |
| AMPAR | α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors |
| ALS | amyotrophic lateral sclerosis |
| ASD | autism spectrum disorder |
| ATP | adenosine tri-phosphate |
| BBB | blood brain barrier |
| cAMP | cyclic adenosine mono-phosphate |
| Cd11b | cluster of differentiation antigen-11b |
| Cdk5 | cyclin dependant kinase-5 |
| CNS | central nervous system |
| Cre | cre-recombinase |
| Cx3cr1 | Cx3 chemokine receptor-1 |
| DA | dopamine |
| DAT | dopamine transporter |
| D1R | dopamine receptor type 1 |
| D2R | dopamine receptor type 2 |
| DIV | days *in vitro* |
| E/I | excitatory/inhibitory |
| EPM | elevated plus maze |
| GABA | gamma-aminobutyric acid |
| GABAR | gamma-aminobutyric acid receptors |
| GD | gestational day |
| GFAP | glial fibrillary acidic protein |
| GluA1-4 | glutamate receptor, AMPA-binding type, subunits 1 through 4 |
| GSK-3β | glycogen kinase-3β |
| GTP | guanosine tri-phosphate |
| HSP | homeostatic synaptic plasticity |
| Iba1 | ionized calcium-binding adaptor molecule 1 |
| IHC | Immunohistochemistry |
| IL-1 | interleukin-1 |
| IL-1β | interleukin-1β |
| IL-6 | interleukin-6 |
| K+ | potassium |
| KO | knockout |
| LPS | lipopolysaccharide |
| LTD | long-term depression of synaptic strength |
| LTP | long-term potentiation of synaptic strength |
| mEPSC | miniature excitatory post-synaptic current |
| mIPSC | miniature inhibitory post-synaptic current |
| mRNA | messenger ribonucleic acid |

| | |
|---|---|
| M1/M2 | macrophage polarization – phenotype ½ |
| MIA | maternal immune activation |
| MPLA | monophosphoryl lipid A |
| MSN | medium spiny neuron |
| Myd88 | myeloid differentiation primary response gene 88 |
| NFkB | nuclear factor-kB |
| NMDA | N-methyl-D-aspartate |
| NMDAR | N-methyl-D-aspartate receptor |
| PFC | pre-frontal cortex |
| PI3K | phosphatidylinositol-4,5-bisphosphate 3-kinase |
| Poly I:C | polyinosinic:polycytidylic acid |
| qPCR | quantitative polymerase chain reaction |
| SPARC | secreted protein acidic and rich in cysteine |
| SZ | schizophrenia |
| TACE | tumor-necrosis factor-alpha converting enzyme |
| TLR-4 | toll-like receptor-4 |
| TNF | tumor necrosis factor-alpha |
| TNFR1 | tumor necrosis factor alpha receptor 1 |
| TNFR2 | tumor necrosis factor alpha receptor 2 |
| TTX | tetrodotoxin |
| vHipp | ventral hippocampus |
| VTA | ventral tegmental area |

## LIST OF FIGURES

# CHAPTER 1

# INTRODUCTION

## 1.1 – TNF: review & effects on synaptic plasticity

### *1.1.1 Introducing the pro-inflammatory cytokine, TNF*

Tumor Necrosis Factor-alpha (TNF) earned its namesake as a potent factor specifically mediating tumor cell death (Carswell et al. 1975). TNF has since been established as a pro-inflammatory cytokine and its role in the innate immune system has been well characterized. While the CNS was once thought to be immune privileged, it is now clear that immune molecules, including cytokines, have important functions in the brain under non-pathological conditions (Pribiag and Stellwagen 2014; Boulanger, Huh, and Shatz 2001; Boulanger 2009). Pro-inflammatory cytokines, including TNF, IL-1β, and IL-6, are pleiotropic and have numerous effects in the central nervous system (CNS) that are not always related to inflammation. (Vitkovic, Bockaert, and Jacque 2000; Vitkovic et al. 2001; Garay and McAllister 2010; Pribiag and Stellwagen 2014; Borsini et al. 2015). Cytokines have been implicated in both neuronal and glial plasticity and contribute to the development of neural circuits and act as an important interface between the environment and brain function (Bilbo and Schwarz 2009; Bilbo and Schwarz 2012; Dantzer et al. 2008). This thesis focuses on the role of TNF-mediated regulation of neuronal function in the brain.

While there is evidence that TNF can be produced by neuronal cells (Breder et al. 1993; Gahring et al. 1997), TNF is principally expressed and produced by glia within the CNS (Hanisch 2002; Becker et al. 2013; Balosso et al. 2005). It is produced as a 26KD transmembrane pro-protein, assembled as a trimer (Smith and Baglioni 1987), which is then cleaved by TACE (TNF alpha converting enzyme, otherwise known as ADAM17) to release a 17KD soluble fragment (Black et al. 1997). Both transmembrane TNF (tmTNF) and soluble TNF (solTNF) can bind and signal to both subtypes of TNF receptors, TNFR1 and TNFR2, which are expressed in neurons and glia (Boka et al. 1994; Cheng et al. 2010; Yang et al. 2002). TNFR1 and TNFR2 have different binding affinity for TNF and intracellular signal-transduction cascades (Tartaglia et al. 1991; Rath and Aggarwal 1999; Idriss and Naismith 2000). It was previously thought that TNFR1 activation preferentially triggers cytotoxic and apoptotic effects of TNF (Boldin et al. 1995; Shen et al. 2006), while TNFR2 activation promotes cell survival and proliferation. (Yang et al. 2002; Marchetti et al. 2004; Shen, Li, and Shiosaki 1997). However, these effects depend on the balance between TNFR1 and TNFR2 as well as the levels of TNF present (Depuydt et al. 2005; Twohig et al. 2011). TNFR1/TNFR2 signaling are both important for regulating neurogenesis, neuronal migration, and neurite outgrowth *in vivo* (Twohig et al. 2011).

Thus far, the effects of TNF on synaptic function (described below) have been found to be downstream of TNFR1 activation (Stellwagen et al. 2005; Pribiag and Stellwagen 2013; Lewitus et al. 2014b).

*1.1.2 TNF and neuroinflammation*

Before discovery of its role in the regulation of synaptic plasticity, TNF was and still is, classically implicated in neuroinflammation, which is the response of the CNS to a variety of cues, including infection, traumatic brain injury, stroke, toxic metabolites, autoimmunity and stress. In addition to these classical initiators of inflammation in the brain, elevated levels of neuronal activity can also be sufficient to trigger neuroinflammation (Xanthos and Sandkuhler 2014). A neuroinflammatory response ideally restores normal neuronal activity and brain homeostasis that has been perturbed by an intrinsic or extrinsic insult. However, unregulated neuroinflammation can result in a chronic reaction where the immune response persists and induces cellular and neuronal dysfunction. This type of chronic neuroinflammation is associated with most neurodegenerative disorders, either as cause or consequence.

Many studies have examined the effect of TNF signaling in neurodegenerative disorders. TNF is a potent activator of the immune system and can induce activation of microglial cells and astrocytes in the brain, which can contribute to neuroinflammation. TNF levels are generally increased in patients and animal models of neurodegenerative diseases, such as Huntington's Disease (HD) (Sapp et al. 2001; Ellrichmann et al. 2013; Björkqvist et al. 2008), Parkinson's Disease (PD) (Mogi et al. 1994; Mogi et al. 1999; Hirsch et al. 1998; Bessler et al. 1999), Amyotrophic Lateral Sclerosis (ALS) (Babu et al. 2008; Liu et al. 2009) and Alzheimer's Disease (AD) (Fillit et al. 1991; Álvarez et al. 2007). Elevated levels of TNF associated with neuroinflammation are also associated with the development of neuropsychiatric disorders

including autism (Chez et al. 2007; Li et al. 2009), schizophrenia (Shirts et al. 2006; Miller et al. 2011) and depression (Himmerich et al. 2008; Dowlati et al. 2010). A traditional train of thought has implied that TNF is detrimental in these pathologies and this is likely true for chronic and pathological neuroinflammation (A Frankola et al. 2011; Ferger et al. 2004; McCoy et al. 2008). However, basal expression of TNF or carefully regulated increases in expression can have beneficial roles (Lewitus et al. 2014b; Lambertsen et al. 2009; Duseja et al. 2015; A Frankola et al. 2011; Perry et al. 2002; Stellwagen 2011).

### *1.1.3 TNF-mediated homeostatic synaptic plasticity*

Neural circuits need to maintain both plasticity and stability in order to function properly. Hebbian plasticity and homeostatic synaptic plasticity (HSP) are two basic mechanisms of synaptic plasticity and are in theory, both required for normal circuit function. Hebbian plasticity generally consists of synapse-specific plasticity occurring based on correlations in firing between a pre-synaptic and post-synaptic neuron. LTP and the complementary process of LTD are synapse-specific changes at individual synapses resulting from increased or decreased efficacy of synaptic transmission (Malinow and Malenka 2002). Synaptic scaling is a key mechanism of HSP where a neuron's excitatory input is scaled up or down to compensate for significant deviations in intrinsic activity (Turrigiano and Nelson 2004) and is thought to occur in a uniform manner across all synapses (Turrigiano et al. 1998). This synaptic scaling prevents individual synapses from becoming too strong or weak, and hence unable to respond accordingly to subsequent synaptic input (Abbott and Nelson 2000;

Turrigiano and Nelson 2004). It has been hypothesized that imbalances in Hebbian and homeostatic mechanisms of synaptic plasticity may underlie cognitive deficits associated with different neurological disorders (Dickman and Wondolowski 2013; Cohen et al. 2011; Yin and Yuan 2015).

Receptor trafficking of AMPARs is an underlying mechanism of synaptic plasticity. AMPAR trafficking mechanisms underlying homeostatic plasticity have been found to be fundamentally different from those underlying Hebbian forms of plasticity such as LTP or LTD (Turrigiano 2012). TNF is one such example of a molecule that contributes specifically to homeostatic but not Hebbian plasticity (Stellwagen and Malenka 2006). Exogenous TNF was first found to regulate trafficking of AMPARs in hippocampal pyramidal cells, driving the rapid exocytosis of AMPARs (Beattie et al. 2002a; Ogoshi et al. 2005) in addition to the endocytosis of GABA-A receptors (GABARs) (Stellwagen et al. 2005). Together, these trafficking events alter excitation-to-inhibition (E/I) balance. TNF preferentially increases GluA2-lacking AMPARs (Stellwagen et al. 2005; Ogoshi et al. 2005) which are calcium-permeable and can therefore regulate additional intracellular pathways at the synapse.

Most evidence to date suggests that TNF does not affect NMDARs, as indicated by synaptic localization (Beattie et al. 2002a) or measurement of whole cell currents (He et al. 2012a). Longer term treatments with TNF may however be able to increase (Kawasaki et al. 2008; Han and Whelan 2010) or decrease (Furukawa and Mattson 1998) NMDA currents, perhaps via indirect effects. Exogenous TNF treatment on neuronal cultures also increases

mEPSC frequency (Grassi et al. 1994; Beattie et al. 2002a) and decreases mIPSC frequency (Pribiag and Stellwagen 2013). These results suggest that TNF may regulate pre-synaptic release probability of glutamate and GABA neurotransmitters, or that TNF increases glutamate release from astrocytes (Santello, Bezzi, and Volterra 2011).

Basal release of TNF in the brain has been ascertained in both dissociated neuronal cultures and acute brain slices from the hippocampus, striatum and cortex (Lewitus et al. 2014a; Beattie et al. 2002a; Kaneko et al. 2008). Constitutive production of the pro-inflammatory cytokines IL-6 and IL-1β in the brain has also been observed (Besedovsky and Rey 2008; Balschun et al. 2004; Goshen et al. 2007; Pitossi et al. 1997; Stellwagen et al. 2005). Both IL-6 and IL-1β can modulate trafficking of AMPA glutamate receptors (Lai et al. 2006; Wei et al. 2012; Stellwagen et al. 2005; Yang et al. 2005) but *in vivo* these effects seem to largely occur in inflammatory contexts (Wei et al. 2012; Nelson et al. 2012). Although exogenous application of IL-1β in neuronal culture was able to increase AMPAR surface levels, there was no effect of blocking IL-1β, suggesting it does not basally regulate surface levels of AMPARs (Stellwagen et al. 2005). Blocking TNF in culture (Stellwagen et al. 2005) and *in vivo* (He et al. 2012b) however, decreased surface AMPAR levels while exogenous IL-6 had no effect on AMPAR levels (Stellwagen et al. 2005). TNF-/- mice also have several unique neuronal phenotypes (Golan et al. 2004; Baune et al. 2008; Pickering, Cumiskey, and O'Connor 2005) suggesting that other pro-inflammatory cytokines cannot simply compensate for synaptic functions mediated by TNF.

Unlike IL-6 and IL-1β ,TNF is required for the homeostatic process of scaling up excitatory synaptic strength following chronic activity blockade (Stellwagen and Malenka 2006). In this study, action potentials in hippocampal cultures are blocked for 48 hrs using TTX, which then leads to an increase in surface AMPARs , an increase in mEPSC amplitude, a decrease in surface GABARs, and a decrease in mIPSC amplitude. Overall, this causes a compensatory increase in neuronal activity and correlates with an increase in TNF levels in culture medium. More convincingly, this HSP does not occur when TNF signaling is blocked genetically or pharmacologically. While there is evidence that IL-1β can also downregulate GABAR-mediated transmission (Wang et al. 2000; Zhu et al. 2006), there is no evidence to suggest that IL-1β-mediated GABAR trafficking occurs by activity deprivation (Stellwagen and Malenka 2006).

The above data on TNF and synaptic function were all from pyramidal cells. Importantly, TNF appears to have a differential effect on GABAergic neurons. For example TNF treatment of hippocampal interneurons does not increase the surface levels of AMPARs (Ogoshi et al. 2005). The principle cell type in the striatum are medium spiny neurons (MSNs) and are also GABAergic. These cells respond to exogenous TNF treatment with the endocytosis of synaptic AMPARs and a preferential removal of calcium-permeable AMPARs (Lewitus et al. 2014a). Overall, these results suggest that TNF is a key element in maintaining normal circuit function by differentially adjusting synaptic strength on excitatory and inhibitory neurons (Figure 1.1).

*1.1.4 Role of TNF in neurodevelopment*

TNF appears to not be essential for normal development, as mice lacking TNF or TNF receptors are largely normal in terms of cytoarchitecture and baseline behaviours (Golan et al. 2004; Baune et al. 2008; Pasparakis et al. 1996). However, perturbations of neuronal activity during development may uncover important functions for TNF-mediated plasticity. For example, TNF is required for some forms of developmental plasticity in sensory cortices (Kaneko et al. 2008; Ranson et al. 2012; Greenhill, Ranson, and Fox 2015), where it is necessary for part of the re-normalization of circuit function when sensory input is substantially altered. Therefore TNF-mediated HSP may not necessarily be required for normal development but instead be necessary when circuit activity is substantially perturbed. Inflammatory stimuli are likely to also have TNF-dependent consequences on neural development and function. Abnormal elevations in TNF can perturb normal development (Lee et al. 2010a; Gilmore et al. 2004), with reduced dendritic length and complexity in cultured neurons, and premature synapse maturation and stabilization resulting in less refinement during development *in vivo*. The contribution of TNF-mediated plasticity has not been explored in many developmental models of neurological dysfunction.

## 1.2 – Role of glia in TNF-mediated plasticity

### *1.2.1 Microglia and astrocytes as key sources of TNF in the brain*

TNF can be expressed by both neurons and glia. However, neuronal expression of TNF has only been assessed by immunoreactivity and generally occurs in severe states of brain inflammation (Liu et al. 1994; Knoblach, Fan, and Faden 1999; Ohtori et al. 2004). An increasing body of literature supports the idea that TNF is preferentially produced by microglia and astroctyes. TNF is expressed by astrocytes and microglia not just during brain injury or inflammatory processes (Hopkins and Rothwell 1995) (Renno et al. 1995; Tchelingerian et al. 1993; Bruce et al. 1996), but is also expressed by both microglia (Hanisch 2002; Kato and Kanba 2013; Bilbo and Schwarz 2009; Schwarz, Smith, and Bilbo 2013) and astrocytes (Becker et al. 2013; Balosso et al. 2005; Duseja et al. 2015) during basal conditions in the brain. TNF gene transcription and protein production is observed during development in total brain tissue extracts (Angeles Muñoz-Fernández and Fresno 1998; Zhao and Schwartz 1998). In cell culture, astrocytes secrete TNF at low levels, but cytokine secretion is significantly elevated after treatment with inflammatory stimuli (Chung and Benveniste 1990; Lieberman et al. 1989; Krasowska-Zoladek et al. 2007).

Glia are also the source of TNF required for trafficking of AMPARs. Treatment of neuronal cultures with glial-conditioned medium increases surface AMPARs, similar to TNF treatment alone (Beattie et al. 2002b). More specifically, soluble TNFR1, which blocks TNF

signaling, is able to abolish this effect of glial-conditioned medium, supporting the view that TNF is a glial-derived factor (Beattie et al. 2002b). Glia are also the source of TNF required for synaptic scaling: WT neurons cultured with TNF -/- glia are unable to scale up synaptic strength following 48hr activity blockade with TTX (Stellwagen and Malenka 2006). TNF supplied by astrocytes is likely required for homeostatic scaling up in the dentate gyrus following entorhinal denervation in hippocampal slice cultures (activity block) (Vlachos et al. 2012; Vlachos et al. 2013) (Figure 1.1).

### *1.2.2 Overview of microglia*

First identified between 1919 and 1921 by Pio del Rio-Hortega, a student of the famous Spanish histologist Ramon Y Cajal, microglia function primarily as immune cells of the CNS. Unlike neurons and astrocytes which are derived from neuroectoderm during development, microglia are derived from embryonic mesoderm originating from the same lineage as cells giving rise to the blood and immune system (Chan, Kohsaka, and Rezaie 2007). There is an embryonic invasion of these cells from the yolk sac during development that give rise to microglia in the brain (Chan, Kohsaka, and Rezaie 2007). This occurs during the middle of the first trimester and throughout the early part of the second trimester in humans and between embryonic days 10 and 19 in rodents. After invasion of the brain parenchyma, microglia form a self-renewing colony and transform into a ramified phenotype. Ramified microglia are characterized by multiple branched processes, small somata and down-regulated inflammatory properties.

In adult animals, there is generally very little exchange between the blood and brain parenchyma. This was nicely demonstrated by irradiating animals to remove monocytes and then transplanting bone marrow with GFP-labeled monocytes. While earlier reports suggested that monocytes were able to invade the brain in adulthood (Simard et al. ; Hickey, Vass, and Lassmann 1992), recent studies demonstrate that the exchange of microglia in the normal undisturbed brain is almost negligible (Mildner et al. 2007; Ajami et al. 2007). An essential consideration in the later studies was in fact the protection of the brain from irradiation which can damage the blood-brain barrier and allow a small population of monocytes to enter the brain and transform into microglia (Mildner et al. 2007).

Microglia are distributed in all brain regions with varying densities, averaging between about 500 to 2000 cells per $mm^3$ (Yang et al. 2013). Just as there are regional variations in neuronal cell populations throughout the brain, microglia also are not a homogenous population in the brain. Several recent studies have demonstrated that microglia have distinct regional variations in their transcriptional phenotypes (Grabert et al. 2016; Doorn et al. 2015; Tay et al. 2016; Yang et al. 2013). Microglial hereogeneity may underlie why certain brain regions are more sensitive to excitotoxicity (Vinet et al. 2012)or immunological insults (Yang et al. 2013).

The ramified morphology of microglia in the healthy mature CNS, including the brain and spinal cord, has been associated with a microglia “resting” state. However, this terminology can be misleading given that processes of ramified microglia are very dynamic surveyors in the

normal rodent brain (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). In addition, resting or "surveying" microglia are essential for normal synaptic function. This conclusion is supported by evidence demonstrating that microglia morphology and process motility is controlled by neuronal activity (Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010). For example, microglia motility was increased *ex vivo* by glutamatergic transmission and decreased by GABAergic transmission (Fontainhas et al., 2011). One of the most established mechanisms through which both active and resting microglia regulate synaptic plasticity is via the production of synaptic modulators such as cytokines (Hanisch 2002; Mallat and Chamak 1994; Streit and Xue 2014). Resting microglia are generally considered to have a homeostatic function via their ability to survey their environment, phagocytose debris and release synaptic modulators (Luo and Chen 2012; Morris et al. 2013). There is much more information about what factors contribute to microglia activation compared to factors that promote a resting, ramified phenotype. *In vitro* cell culture studies suggest that gliotransmitters released from astrocytes are key contributors to microglia ramification (Sievers, Parwaresch, and Wottge 1994; Wollmer et al. 2001; Schilling et al. 2001).

Microglia have the ability to detect and mount an inflammatory response to various insults. Infection, trauma, ischemia, neurodegenerative diseases, or altered neuronal activity, which all disturb brain homeostasis, evoke rapid and drastic changes in the microglial cell shape, gene expression and functional phenotype of microglia which are collectively referred to as microglia activation (Block, Zecca, and Hong 2007; Conde and Streit 2006; Davoust et al. 2008; Graeber and Streit 2010; Hanisch and Kettenmann 2007; Kreutzberg 1996; van Rossum

and Hanisch 2004). Classically, microglia activation was categorized into two main types: the cytotoxic M1 phenotype and neuroprotective M2 phenotype. This dualistic simplification of microglia activation is currently of less value given recent research *in vivo* studies of disease models (Martinez and Gordon 2014). Focus has instead shifted to characterizing changes in gene expression following specific environmental challenges (Tay et al. 2016).

Progressive stages of microglia activation are defined by morphological, molecular, and functional characteristics, whereby the complexity of the processes are reduced and microglia eventually revert to an amoeboid appearance (Colton and Wilcock 2010; Davoust et al. 2008; Hanisch and Kettenmann 2007). Microglia can then become motile and move to the site of a lesion or infection. There is evidence suggesting microglia can increase by proliferation in more extreme cases of brain injury (Kato, Takahashi, and Itoyama 2003; Koguchi et al. 2002), in order to provide more cells for the protection and restoration of tissue homeostasis. Increased expression and rearrangement of surface molecules, intracellular enzymes and immunoregulatory and proinflammatory signaling factors are additionally key elements of microglia activation. Activated microglia also increase their phagocytic activities to clear tissue debris, damaged cells or microbes, and release chemoattractive factors to recruit peripheral immune cells to the CNS.

In addition to their key role in response to brain injury or inflammation, microglia are required for normal brain development and plasticity. Microglia in the uninjured brain actively engulf synaptic material and regulate synaptic pruning – the process of synapse elimination

that occurs during post-natal development (Paolicelli, Bolasco, Pagani, Maggi, Scianni, Panzanelli, Giustetto, Ferreira, Guiducci, Dumas, et al. 2011; Schafer et al. 2012; Morris et al. 2013). In mice lacking Cx3cr1, a chemokine receptor expressed by microglia in the brain, microglia density in the developing brain is transiently reduced and synaptic pruning is delayed. This deficit in pruning results in an excess of dendritic spines, immature synapses and immature brain circuitry (Paolicelli, Bolasco, Pagani, Maggi, Scianni, Panzanelli, Giustetto, Ferreira, Guiducci, Dumas, et al. 2011). In organotypic hippocampal brain slices, depletion of microglia increased synaptic frequencies (both sEPSCs and mEPSCs) in the CA1 region, which was reversed by replenishing microglia (Ji et al. 2013). In hippocampal neuronal cultures, the chemokine CXCL1 expressed by neurons, acts via a feedback mechanism on the microglial chemokine receptor (CX3Cr1) to reduce the activity of neurons (Ragozzino et al. 2006). BDNF derived from microglia has been shown to regulate the composition of post-synaptic glutamate receptors without affecting the overall density of synapses in the cortex and hippocampus (Parkhurst et al. 2013). The microglial platelet P2Y12 receptor contributes to microglial activation, and was found to be necessary for normal activity-dependent remodelling of neuronal networks in the visual cortex in late development (Sipe et al. 2016). In conclusion, dynamic interactions between neurons and microglia influence the circuitry of the healthy brain.

*1.2.3 Overview of astrocytes*

Astrocytes were first discovered by Rudolf Virchow and later Camillo Golgi in the late 1800s. Their name literally means “star-shaped glia”, and they account for 20-40% of all glia in the rodent brain depending on the region (Verkhratsky and Butt 2013). In more advanced species, astrocytes account for a higher proportion of the total brain (Oberheim et al. 2009; Colombo 1996). For instance, human astrocytes are 20 times larger and more complex than rodent astrocytes, suggesting that they have a higher capacity to fine-tune synaptic plasticity and increase learning abilities (Han et al. 2013). Classically, astrocytes have been subdivided into two main subtypes: *protoplastmic* or *fibrous*. Protoplasmic astrocytes are found in gray matter and their morphology consists of several elaborate and complex branches which envelop synapses. Fibrous astrocytes are found in white matter and have many long fiber-like processes contacting nodes of Ranvier. Both make extensive contact with blood vessels and also form gap junctions between distal processes of neighboring astrocytes (Peters, Palay, and Webster 1991). Expression of GFAP is a typical marker used for immunohistochemical identification of astrocytes (Eng, Gerstl, and Vanderhaeghen 1970; Eng, Ghirnikar, and Lee 2000), however many mature astrocytes in gray matter do not express GFAP (Sofroniew 2009; Tong et al. 2014). Moreover, not all astrocytes are star-shaped cells, nor do they all contact blood vessels. Astrocytes are very heterogeneous: expression of astrocyte markers and neurotransmitter receptors, as well as astrocyte morphology, vary considerably within brain regions (Emsley and Macklis 2006; Höft et al. 2014; Matthias et al. 2003).

Astrocytes are essential for building the brain parenchyma, maintaining brain homeostasis, and regulating neuronal development. Astrocytes maintain homeostasis by mediating many functions that support neuronal function. These include K+ buffering (Leis, Bekar, and Walz 2005; Somjen 2004), uptake of glutamate and GABA (Sattler and Rothstein 2006; Anderson and Swanson 2000), control of cerebral blood flow (Koehler, Roman, and Harder 2009), water transport (King, Kozono, and Agre 2004), supply of energy metabolites (Magistretti 2009; Dienel and Cruz 2004) and antioxidant functions.

Astrocytes are also crucial for neuronal synapse formation. In the absence of astrocytes, neurons of all types form far less synapses than normal (Pfrieger and Barres 1997; Ullian et al. 2001; Hughes, Elmariah, and Balice-Gordon 2010; Xu, Xiao, and Xia 2010; Buard et al. 2010). In mature circuits, astrocytes respond to neuronal activity via ion channels, neurotransmitter receptors, and transporters present on their processes. Given that astrocytes are able to sense and respond to synaptic activity in many ways, they are essential for normal neuronal function. Neuronal activity results in increased intracellular calcium in astrocytes and release of gliotransmitters. These can be membrane-bound or soluble signals that direct the formation, maturation and maintenance of synapses and ultimately the development of neuronal circuits (Clarke and Barres 2013). For example, astrocytes can regulate presynaptic activity and postsynaptic strength via the secretion of different factors (Allen 2013; Allen et al. 2012; Mauch et al. 2001; Hama et al. 2004; Barker et al. 2008).

Gliotransmitters are also implicated in various forms of Hebbian or Homeostatic plasticity. ATP, glutamate, D-serine, SPARC, IL-1(Menachem-Zidon et al. 2011) and TNF are all examples of soluble signals from astrocytes that can promote co-ordinated synaptic strengthening or weakening of excitatory synapses (Yang et al. 2003; Henneberger et al. 2010; Bezzi et al. 1998; Pascual et al. 2005; Stellwagen and Malenka 2006; Jones et al. 2011). When astrocyte-mediated plasticity is compromised or dysregulated, this results in significant deficits in normal cognition, learning and memory (Lee et al. 2014; Szu and Binder 2016; Gerlai et al. 1995; Winocur, Roder, and Lobaugh 2001; Hartman et al. 2001). Moreover, many neurodevelopmental disorders such as schizophrenia and autism, are characterized by deficits in synapse formation and function (Auerbach, Osterweil, and Bear 2011; Bennett 2009; Glantz and Lewis 2000; Hutsler and Zhang 2010). Given the essential role of astrocytes in these processes, it is therefore not surprising that astrocyte dysfunction is implicated in these diseases (Jacobs and Doering 2010; Lioy et al. 2011).

Astrocytes are active players in neuroinflammation, and their response can be beneficial or detrimental to tissue homeostasis depending on the type of injury or inflammation. Astrocytes are immune-competent cells and respond to cytokines and chemokines, activating adaptive immune defence pathways (Rolls, Shechter, and Schwartz 2009; Farina, Aloisi, and Meinl 2007). Following CNS injury, astrocytes can form a “glial scar” – the purpose of which is to promote recovery of injured tissue but which is also detrimental because it blocks neuronal regeneration (Rolls, Shechter, and Schwartz 2009). Expression of GFAP is upregulated in immune-activated astrocytes and is a classic hallmark of many CNS pathologies involving

neuroinflammation (Sofroniew 2009; Norton et al. 1992; Crawford et al. 2015). Upon activation, astrocytes can themselves produce a variety of chemokines, growth factors and neurotrophins which may enhance or reduce neuroinflammation (Colombo and Farina 2016). Activated astrocytes can further recruit infiltrating immune cells to sites of injury either directly via these signaling factors, or indirectly by compromising the integrity of the BBB and allowing the entry of immune cells and molecules into the CNS (Colombo and Farina 2016).

## 1.3 – Synaptic plasticity & drug addiction

### *1.3.1 The mesolimbic pathway*

Addiction is a chronic disorder, characterized by compulsive drug use despite negative consequences and a high risk of relapse even after years of abstinence (Association 2013). The brain circuitry underlying addiction is complex but it is well established that the mesolimbic dopamine (DA) system plays a central role. The mesolimbic pathway is often referred to as the "reward pathway" and consists of the VTA, and the brain regions innervated by dopaminergic projections from the VTA: NAc, PFC, amygdala and hippocampus (Swanson 1982). The VTA is composed of dopamine projection neurons that are locally controlled by inhibitory GABA interneurons. The main targets of the VTA are the NAc and PFC. These brain areas mediate motivation, goal-directed behavior and habit formation and underlie motivated behavior to obtain natural rewards such as food, water and sex as well as drug rewards (Schultz 1998; Wise 2004). Drug-induced synaptic plasticity in the NAc is especially important for drug addiction

(see below) and occurs in all known forms of addiction (Nestler 2013; Angres and Bettinardi–Angres 2008; Malenka 2009; Taylor, Lewis, and Olive 2013).

All drugs of abuse act to increase DA in brain regions innervated by VTA projections which underlies acute rewarding effects. Dopamine release also reinforces reward learning and the release of dopamine by addictive drugs is important for a progressive transition to drug-seeking behaviors that are difficult to control. This increased dopamine transmission occurs through distinct cellular mechanisms depending on the drug. For example, benzodiazepines, opioids, and cannabinoids exert their effects by inhibiting GABAergic interneurons in the VTA, and subsequently reducing inhibition onto DA neurons (Johnson & North, 1992; Szabo et al., 2002; Tan et al., 2010). Psychostimulants such as cocaine and amphetamine increase extracellular DA by respectively inhibiting the reuptake of DA or enhancing non-vesicular release of DA. The increase in mesolimbic DA levels do not however explain the long-lasting behavioral abnormalities seen in addiction such as craving and relapse which occur long after drugs are cleared from the brain. There is now much evidence to show that the “synaptic trace” of addictive drugs occurs at glutamatergic synapses in the mesolimbic pathway (Lüscher 2013; Luscher and Malenka 2011). In particular, the NAc receives glutamatergic input from the prefrontal cortex (PFC), basolateral amygdala and ventral hippocampus (vHipp) which are important for driving reward seeking behavior (Schmidt and Pierce 2010; Lüscher 2013).

*1.3.2 Glutamatergic plasticity in the nucleus accumbens (NAc)*

The NAc, contained within the ventral striatum, is composed of two major types of neuronal cells: approximately 10% are interneurons, and the other 90% are medium spiny GABAergic neurons (MSNs). MSNs are the output neurons of the NAc and receive synaptic dopamine input from the VTA and glutamatergic input from the prefrontal cortex and limbic regions including the hippocampus and amygdala. MSNs integrate these signals and send projections to motor regions in the ventral pallidum and ventral mesencephalon. MSNs are separated into two largely non-overlapping populations: direct pathway MSNs primarily express D1 receptors (D1-MSNs) and indirect pathway MSNs primarily express D2 receptors (D2-MSNs) (Gerfen and Surmeier 2011). These two types of MSNs exert opposite effects on addictive behavior: optogenetic activation of D1-MSNs promotes positive reinforcement and increases the formation of reward-context associations, while activation of D2-MSNs is aversive and decreases drug reward (Kravitz, Tye, and Kreitzer 2012; Lobo et al. 2010; Pascoli, Turiault, and Luscher 2012; Caine, Stevens Negus, and Mello 1999). Synaptic changes associated with increased dopaminergic transmission also occur primarily on D1-MSNs (Dobi et al. 2011). The NAc can be further subdivided into two distinct regions, the core and shell, which respond to natural rewarding stimuli and addictive drugs differently. Hence, they are thought to mediate different aspects of addictive processes. The shell mediates the initial rewarding and reinforcing effects of addictive drugs as well as the designation of salience to rewarding stimuli, whereas the core is more associated with the expression of learned behaviors in response to addictive

drugs, such as drug seeking (Sellings and Clarke 2003; Bassareo and Di Chiara 1999; Everitt et al. 2008; Di Ciano and Everitt 2001).

Drug-induced plasticity in NAc glutamatergic transmission is primarily mediated by changes in AMPARs, making these receptors a central target for studying how drug experiences modify behaviour in animal models of addiction (Bowers, Chen, and Bonci 2010; Kalivas and Hu 2006; Kauer and Malenka 2007; Pierce and Wolf 2013). By the early 1990s, the role of glutamate was established in the psychostimulant-induced neuronal and behavioral plasticity underlying behavioral sensitization. Behavioral sensitization is the progressive and lasting enhancement of drug-induced behavioural response following repeated exposure to psychostimulants (Wolf 1998). It was subsequently reported that administration of AMPA receptor antagonists into the NAc core attenuated the expression of behavioral sensitization to psychostimulants, and that microinjection of AMPA into the core increased behavioral sensitization (Pierce et al. 1996). While the role of AMPAR transmission in behavioral sensitization is perhaps more complex (Bachtell et al. 2008; Ferrario et al. 2010; Wolf and Ferrario 2010; Kourrich et al. 2012), it has since been shown that AMPAR potentiation in the NAc is associated with the development of other addictive behaviours in rodents (Kaddis, Uretsky, and Wallace 1995; Pierce and Wolf 2013; Schmidt and Pierce 2010; Ferrario et al. 2010; Conrad et al. 2008; Britt et al. 2012).

How does increased drug-induced dopaminergic transmission lead to changes in AMPAR trafficking? Phosphorylation at serine residues 831 and 845 (S831 and S845) of AMPARs

generally increases surface localization, conductance, and open probability. Several groups have demonstrated that dopamine increases phosphorylation of GluR1 at S845 through the activation of D1-type dopamine receptors and of PKA and DARPP-32 (Snyder et al. 2000; Fienberg et al. 1998; Yan et al. 1999; Nishi et al. 2002; Surmeier et al. 2007). How these initial AMPAR trafficking events mediated by increased dopamine signaling relate to the AMPAR changes observed after short or long drug exposure is not clear.

*1.3.3 Cocaine-induced glutamatergic plasticity in the NAc*

Cocaine is a psychoactive and addictive drug used recreationally. Its use results in a loss of contact with reality, an intense feeling of euphoria and agitation. Like all drugs of abuse, it acts on the mesolimbic pathway in the brain and induces long-lasting changes at glutamatergic synapses of MSNs in the NAc (Kauer and Malenka 2007). Early reports had conflicting conclusions on the effects of cocaine exposure and withdrawal on glutamatergic synaptic strength in the NAc, which were later shown to be dependent on whether animals had recently been administered cocaine after withdrawal.

Cocaine-evoked plasticity in the NAc occurs on a slower timescale and requires a higher induction threshold compared to the VTA. This means that a single cocaine injection is not sufficient to trigger changes in synaptic transmission (Kourrich et al. 2007). Repeated cocaine administration (e.g. 5-7 days) decreases AMPA/NMDA ratios of excitatory inputs onto NAc MSNs (Thomas et al. 2001; Kourrich et al. 2007; Anderson et al. 2008), but if cocaine is followed

by a period of abstinence, AMPA/NMDA ratios are increased (Boudreau et al. 2007; Boudreau and Wolf 2005; Kourrich et al. 2007). Interestingly, these changes may only require more time to develop since extended (20-28 days) drug exposure without protracted abstinence also increases AMPA/NMDA ratios (Dobi et al. 2011). A single dose of cocaine administered after a prolonged (>1 week) period of abstinence will again decrease AMPA/NMDA ratios (Kourrich et al. 2007). In summary, opposite changes in excitatory synaptic strength are observed after short (5-7 days) or long (>2 weeks) cocaine treatments. This bidirectional AMPAR plasticity first suggests that glutamatergic plasticity in the NAc depends on previous cocaine experience, and secondly that there are two main phases of AMPAR plasticity, possibly driven by different mechanisms. Following prolonged withdrawal, enhanced AMPAR function is also attributed to a shift in AMPAR subunit composition which increases single channel conductance (Lüscher and Malenka 2011; Anderson et al. 2008; Conrad et al. 2008).

## 1.4 – Structural plasticity: dendritic spines

### *1.4.1 – Function of dendritic spines*

Dendritic spines were initially thought to be an artifact of the Golgi stain method until Ramón y Cajal suspected in 1894 that spines were in fact the main point of contact between axons and dendrites. Indeed, most excitatory synapses in the mature mammalian brain occur on dendritic spines. Dendritic spines are morphological protrusions from dendrites and occur at a linear density of 1-10 spines per µm in mature neurons (Hering and Sheng 2001). Most

neurons of either glutamate-releasing or GABA-releasing type have dendritic spines, but some classes of neuron do not, such as GABA-releasing interneurons. Spines are rarely found in lower organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans* suggesting an evolutionary advantage of spines for more advanced nervous systems.

Dendritic spines are a major site of plasticity in the developing and adult brain. Because each dendritic spine represents a single synapse, it suggests that the purpose of spines is to create a local synapse-specific compartment, and not just to expand postsynaptic surface area (Shepherd 1996). Indeed, dendritic spines provide a micro-compartment for segregating postsynaptic chemical responses such as elevated calcium (Sabatini, Maravall, and Svoboda 2001) (Volfovsky et al. 1999) (Korkotian and Segal 2000). Dendritic spines contain the PSD, a network of actin cytoskeleton, the spine apparatus, endosomal membranes and mitochondria, which all contribute to spine structure and function (Okabe 2012). The PSD is particularly important for clustering AMPARs and NMDARs, as well as cell adhesion molecules, scaffold proteins, and protein kinases and phosphatases. All of these factors are bi-directionally regulated by synaptic plasticity, and dendritic spine morphology is therefore an important factor controlling synaptic connectivity and transmission (Okabe 2012).

Changes in spine density are also a readout of synaptic connectivity. Spine density often correlates with the functional number of excitatory synapses (Lee, Huang, and Hsu 2011; Pfeiffer et al. 2010; McClelland et al. 2010; Kreple et al. 2014; Kim et al. 2011). In the hippocampus, the formation and stabilization of new dendritic spines is thought to be

associated with learning and memory. Spine density in several hippocampal regions increases during training and acquisition of spatial memory tasks (Mahmmoud, 2015; Leuner et al., 2003; Moser et al., 1994; Conrad et al., 2012; Eilam-Stock et al., 2012) and for spatial and novel object recognition tasks (Chen et al., 2010; Wang et al., 2013). In sensory cortices, spine density is reduced by sensory deprivation during early postnatal life, and is increased by an enriched environment (Valverde 1971; Micheva and Beaulieu 1995; Sadaka et al. 2003; Yang, Pan, and Gan 2009; Leggio et al. 2005). Rapid acquisition of novel motor skills are also associated with increased dendritic spine density in the motor cortex (Xu et al. 2009). The correlation between sensory experience and dendritic spine density is not however always direct and depends on the developmental age of the animal (Zuo et al. 2005). For example, spine density in the somatosensory cortex is more responsive to sensory deprivation during adolescence (Zuo et al. 2005). This is consistent with the idea that synapse elimination during adolescence, called "synaptic pruning," is important for determining adult dendritic spine density. Synaptic pruning and therefore regulation of dendritic spines, is important for the activity-dependant refinement of neural connectivity in the brain (Alvarez and Sabatini 2007; Bhatt, Zhang, and Gan 2009; Fu and Zuo 2011; Holtmaat and Svoboda 2009).

*1.4.2 –Regulation of dendritic spines*

As indicated above, dendritic spines are regulated by neuronal activity and plasticity. Key evidence for this is that dendritic spine abnormalities, either in terms of morphology, or increased or decreased density, are present in many neurological dysfunctions. Dendritic spine

density changes occur in many disease models including neurodevelopmental disorders (Irwin et al. 2001; Suetsugu and Mehraein 1980; Ferrer and Gullotta 1990; Hutsler and Zhang 2010), psychiatric disorders such as schizophrenia (Glantz and Lewis 2000; Garey et al. 1998) or depression (Norrholm and Ouimet 2001; Bedrosian et al. 2011), neurodegeneration (Knobloch and Mansuy 2008; Catala et al. 1987) and drug addiction (Lee et al. 2006; Li, Acerbo, and Robinson 2004; Norrholm et al. 2003; Shen et al. 2009). A plethora of environmental factors or manipulations also alter dendritic spine density including visual stimulation and deprivation (Globus and Scheibel 1967) (Parnavelas, Globus, and Kaups 1973), enriched environment (Rampon et al. 2000) (Moser et al. 1997), sex hormones (Mong et al. 2001; Shors, Chua, and Falduto 2001; Woolley and McEwen 1992; Yankova, Hart, and Woolley 2001), and stress (Shors, Chua, and Falduto 2001; Leuner and Shors 2013).

There has been a rapid accumulation of information describing what molecular and cellular factors affect spine morphology and density. Collectively, spine density is regulated by processes that stabilize or eliminate old spines, or produce new spines. Several key proteins that regulate synaptic plasticity also promote either the formation (Ochs et al. 2015; Lee, Huang, and Hsu 2011) or elimination (Pfeiffer et al. 2010; Bian et al. 2015) of dendritic spines. Actin dynamics and actin-remodeling proteins are key for the activity-dependent remodeling of spines. Synaptic activity can rapidly modulate actin dynamics (Star, Kwiatkowski, and Murthy 2002; Okamoto et al. 2004) and this often occurs via specialized actin-remodeling proteins (Lin and Webb 2009). Finally, both astrocytes and microglia are key regulators of dendritic spine density throughout the lifespan of animals. Astrocytes express surface receptors and soluble

factors that control synapse formation (Clarke and Barres 2013; Allen et al. 2012; Xu, Xiao, and Xia 2010; Jones et al. 2011; Chung, Allen, and Eroglu 2015) and elimination (Chung et al. 2013; Clarke and Barres 2013; Chung, Allen, and Eroglu 2015). Microglia are classically implicated in the synaptic pruning of dendritic spines during development (Paolicelli, Bolasco, Pagani, Maggi, Scianni, Panzanelli, Giustetto, Ferreira, Guiducci, and Dumas 2011; Zhan et al. 2014) and also remove dendritic spines during inflammation in the brain (Kondo, Kohsaka, and Okabe 2011; Kettenmann, Kirchhoff, and Verkhratsky 2013). More recently, microglia have been show to promote synapse formation and stabilization (Miyamoto et al. 2016; Parkhurst et al. 2013). In conclusion, the structure and number of dendritic spines is intricately co-regulated with mechanisms of synaptic plasticity.

## 1.5 – Maternal immune activation (MIA) model of neuropsychiatric disorders

Animal models of neurological disorders often involve significant alterations in synaptic connectivity and plasticity. These changes in neuronal function are also associated with alterations in astrocyte and microglia function. Maternal Immune Activation (MAI) is an animal model for several neuropsychiatric disorders, notably autism and schizophrenia, in which persistent neuroinflammation and elevation of cytokines are thought to underlie deviations in normal synaptic function and behavior.

One of the first documented examples of MIA in human populations was the rubella pandemic in 1964, where the diagnoses in offspring from exposed populations rose to 13% for

autism and 20% for schizophrenia compared to 1% in unexposed populations (Estes and McAllister 2016). Epidemiological studies have since confirmed an association between outbreaks of flu, measles, mumps, chickenpox, and polio with a subsequent increase in ASD, SZ, and mood disorders in affected populations (Reisinger et al. 2015).

In the rodent model of MIA, pregnant dames are exposed to an immunological manipulation at a specific gestational stage, and the behavior and brain structure and function of MIA offspring are compared with control offspring. The most common immunogens used in animal studies are the viral mimic polyinosinic-polycytidilic acid (Poly I:C), and the bacterial mimic lipopolysaccharide (LPS) (Meyer 2014). These immunological stimulations mimic gestational infection with real bacteria or viruses, which provided the first evidence that the behavioral changes observed in the offspring were not due to the pathogens themselves but instead to inflammatory cascades in both the mother and fetus. Immune responses in the maternal compartment are characterized by increased levels of pro-inflammatory cytokines. Consequently, the integrity of the placental barrier is compromised, allowing entry of maternally-derived cytokines in the fetal compartment and inducing inflammatory responses in the developing fetus, including the brain. Somehow this early immunological challenge leads to structural and functional disturbances which result in behavioral endophenotypes relevant to neuropsychiatric diseases.

MIA offspring display most of the core behavioral symptoms of ASD – abnormal communication, abnormal social behaviors, and increased repetitive behaviors (Patterson 2009;

Knuesel et al. 2014; Reisinger et al. 2015; Estes and McAllister 2015; Meyer 2014). They also show SZ-related behaviors including decreased sensorimotor gating (measuring the brain's ability to filter out peripheral information), deficits in working memory and cognitive flexibility, increased anxiety, and enhanced sensitivity to amphetamines (Patterson 2009; Knuesel et al. 2014; Meyer 2014). Many of these behavioral deficits can be improved by antipsychotic drugs, supporting the relevance of these MIA models to the human diseases (Meyer 2014; Reisinger et al. 2015; Knuesel et al. 2014).

There are several reports in the MIA model of alterations in dendritic spine density, synaptic transmission, and long-term plasticity (Choi et al. 2016; Meyer, Feldon, and Fatemi 2009; Coiro et al. 2015; Zhang and van Praag 2015; Giovanoli et al. 2015; Patrich et al. 2016). There is evidence to suggest that persistent upregulation of neuroinflammation in the adult MIA offspring may underlie these changes. For instance, both microglia (Krstic et al. 2012; Hao et al. 2010) and astrocytes (Hao et al. 2010; Krstic et al. 2012; Fatemi et al. 2004) appear to be activated in adult brains of MIA offspring and this correlates with an up-regulation of pro-inflammatory cytokines (Garay et al. 2013; Krstic et al. 2012). Neuroinflammation is known to dysregulate normal synaptic plasticity (Di Filippo et al. 2008; Lynch 2009; Habbas et al. 2015), and this could underlie behavioral abnormalities observed in the MIA model. For example, neuroinflammation is associated with elevated excitatory transmission (Habbas et al. 2015; El-Ansary and Al-Ayadhi 2014) and decreased dendritic spine density in the cortex (Kondo, Kohsaka, and Okabe 2011; Winston et al. 2016; Zou et al. 2016), which are consistent with

observations in the MIA model (Coiro et al. 2015; Abazyan et al. 2010). In conclusion, MIA is a model where altered inflammatory state is associated with dysregulated synaptic function.

## 1.6– Overall thesis rationale & research objectives

TNF plays an important modulatory role of excitatory and inhibitory synaptic strength, which has largely been studied in dissociated cell culture systems. It is also clear that TNF can exert both homeostatic and pathophysiological roles in the CNS but it is not yet clear how TNF-mediated homeostatic synaptic plasticity functions *in vivo*. Given the specificity of TNF to HSP and not Hebbian plasticity (Stellwagen and Malenka 2006), blocking TNF genetically or pharmacologically is a potential route to probe the role of HSP in neurodevelopment and behavior. A central goal of this thesis is to evaluate if and how TNF mediates adaptive responses in different models of behavioral perturbation, based on its role in HSP. The main study of this thesis focuses on a model of drug addiction. The rationale for examining TNF function in this model comes from a recent study suggesting that TNF-mediated plasticity is required for the development of adaptive behaviors in response to chronic psychoactive drugs.

In this previous study, TNF mediates synaptic changes within the striatum and acts to reduce the behavioural consequences of chronic administration of antipsychotics (Lewitus et al. 2014a). As noted above, TNF drives the endocytosis of AMPARs on the GABAergic medium spiny neurons (MSNs) of the striatum, reducing excitatory synaptic strength (Lewitus et al. 2014a). Chronic administration of classic antipsychotics (such as haloperidol) leads to synaptic

potentiation within the striatum, and results in the development of dyskinetic motor problems, characterized by uncontrolled movements particularly of the face and neck. However, the chronic presence of drug seems to induce a homeostatic response, where TNF is elevated in the striatum and reduces both excitatory synaptic strength on the MSNs and the severity of the dyskinetic behaviour (Lewitus et al. 2014a). Blocking TNF signaling, even acutely, will cause an increase in dyskinesic movements.

Cocaine also causes synaptic potentiation within the NAc striatum that accompanies the sensitization of the locomotor response to the drug (Luscher and Malenka 2011). In the work presented in Chapter 2, we determine whether TNF is also be part of an adaptive response to cocaine limiting drug-induced plasticity and behavior. Our research objectives for this project are to (1) determine whether blocking TNF signaling heightens the behavioral response of mice in addiction models, (2) determine if TNF signaling antagonizes cocaine-induced plasticity in the NAc, (3) determine whether microglia or astrocytes are involved in the TNF response to cocaine, and (4) determine a mechanism for how cocaine regulates TNF.

Glia play a central role in TNF-mediated plasticity. Chapter 3 presents a short investigation into how the microglia and astrocytes contribute differentially to TNF-mediated plasticity and specifically, the regulation of dendritic spine density in the NAc. The research objectives of Chapter 3 are to (1) determine whether TNF regulates dendritic spine density in the NAc in response to repeated cocaine administration, and (2) determine whether TNF from astrocytes or microglia regulate dendritic spine density in the NAc.

Both in humans and animal models, chronic cocaine exposure is associated with a mild but elevated inflammatory state (Fox et al. 2012; Guo et al. 2015). We sought to compare the role of TNF-mediated plasticity in cocaine addiction with another model where the inflammatory state of the brain is also mildly elevated – MIA. TNF likely causes pathological dysregulation of neurotransmission at very high levels in the brain as shown previously (Leonoudakis, Zhao, and Beattie 2008; Lee et al. 2010b; McCoy et al. 2008). But TNF also has a homeostatic role in adjusting synaptic strength, which has not been thoroughly been examined in models of development or inflammation. In Chapter 4, our research objective is to determine whether TNF contributes to behavioral abnormalities in MIA offspring, which could occur as part of ongoing dysfunction induced by early immune activation, or as an adaptive mechanism to offset disrupted neuronal development. Our working hypothesis is that TNF mediates an adaptive response to changes in neuronal activity via psychoactive drugs or other environmental factors that chronically alter circuit activity.

## 1.7 – References for Chapter 1


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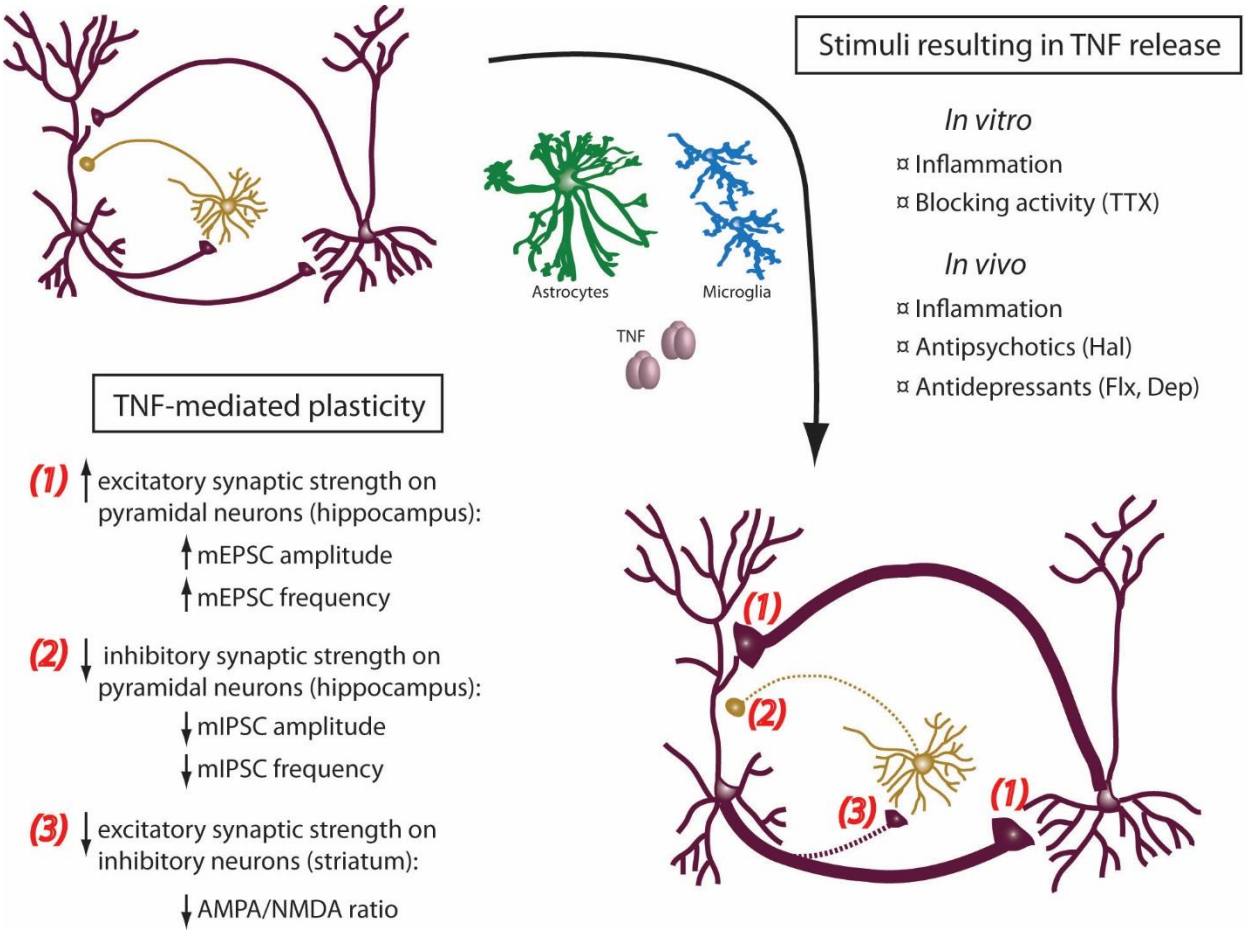
## FIGURE LEGENDS FOR CHAPTER 1

**Figure 1.1 Regulation of E/I balance in a neuronal circuit by TNF-mediated plasticity**

*In vitro*, glia (microglia and/or astrocytes) release TNF in response to prolonged activity blockade (48 hrs TTX). Inflammatory stimuli such as LPS treatment can also increase TNF release both *in vitro* and *in vivo*. Other stimuli including antipsychotics (haloperidol, see Lewitus et al., 2014) and antidepressants (fluoxetine & desipramine, see Duseja et al., 2014) have been reported to increase TNF expression in different areas of the brain. Three central mechanisms of TNF-mediated plasticity have been described which likely play a role in homeostatic-like responses or behaviors. The first (1) is that TNF increases mEPSC amplitude and frequency on excitatory neurons, notably pyramidal neurons in the hippocampus and cortex. Simultaneously (2), TNF decreases mIPSC amplitude and frequency in pyramidal neurons. Finally, in inhibitory neurons such as those found in the striatum (3), TNF acts instead to reduce synaptic AMPAR content, resulting in a reduction of synaptic strength. Overall, TNF increases in E/I balance allowing the circuit to adapt to persistent changes in activity.

## FIGURES FOR CHAPTER 1

### FIGURE 1.1



## PREFACE TO CHAPTER 2

In addition to its role in inflammation and cell survival, TNF has important neuroregulatory functions in the brain. Work from our lab recently demonstrated TNF regulates glutamatergic synaptic plasticity of MSNs in the striatum. Specifically, deleting or blocking TNF signaling results in elevated synaptic and behavioral consequences to chronic haloperidol treatment (Lewitus et al. 2014). MSN synaptic plasticity in the ventral striatum is also a central target of cocaine addiction. The study presented in this chapter describes a novel role for activity-dependent release of microglial-TNF in a model of cocaine addiction in mice.

Repeated administration of cocaine in rodents is known to decrease synaptic strength on D1-MSNs of the NAc (Kourrich et al. 2007; Thomas et al. 2001), which then slowly increases during withdrawal (Boudreau et al. 2007; Boudreau and Wolf 2005; Kourrich et al. 2007). The mechanisms underlying this bidirectional plasticity in the NAc are not fully described. While alterations in the function of microglia and astrocytes during models of addiction have been described previously (Lacagnina, Rivera, and Bilbo 2017; Miguel-Hidalgo 2009), a direct role for the contribution of glial-derived factors has not been demonstrated. In addition, whether the contribution of astrocytes and microglia to synaptic function is beneficial or detrimental is also not well defined. This study contributes two major findings to the field of drug addiction and synaptic plasticity: first that TNF signaling is a factor causing the decreased synaptic strength observed following repeated administration of cocaine, and second that moderate microglia activation has a homeostatic-type response to cocaine-mediated plasticity.

**CHAPTER 2**

# MICROGLIAL TNFα SUPPRESSES COCAINE-INDUCED PLASTICITY AND BEHAVIORAL SENSITIZATION

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## ABSTRACT

Repeated administration of cocaine results in the development of behavioral sensitization, accompanied by a decrease in excitatory synaptic strength in the nucleus accumbens (NAc) through an unknown mechanism. Furthermore, glial cells in the NAc are activated by drugs of abuse, but the contribution of glia to the development of addictive behaviors is unknown. Tumor necrosis factor alpha (TNF), an inflammatory cytokine released by activated glia, can drive the internalization of synaptic AMPA receptors on striatal medium spiny neurons. Here we show that repeated administration of cocaine activates striatal microglia and induces TNF production, which in turn depresses glutamatergic synaptic strength in the NAc core and limits the development of behavioral sensitization. Critically, following a period of withdrawal, a weak TLR4 agonist can reactivate microglia, increase TNF production, depress striatal synaptic strength, and suppress cocaine-induced sensitization. Thus, cytokine signaling from microglia can regulate both the induction and expression of drug-induced behaviors.

## INTRODUCTION

Drug addiction results in pathological and compulsive pattern of drug-seeking and drug-taking behaviours despite significant social, economic and health consequences. The NAc serves as a cross point between corticolimbic regions and motor regions, playing an important role in generating motivated behaviors to natural rewards as well as drug of abuse (Britt et al. 2012; Pierce and Wolf 2013; Lüscher and Malenka 2011). Changes in striatal processing, particularly in the NAc, are thought to be necessary for the maintenance of addictive behaviors, and repeated exposure to drugs of abuse leads to predictable changes in synaptic strength in the NAc (Lüscher and Malenka 2011). Drugs of abuse, such as cocaine, elevate dopamine levels in the striatum, and ex vivo treatment of striatal medium spiny neurons (MSNs) with D1 dopamine receptor agonists or with cocaine increases the phosphorylation and insertion of AMPA receptors (Chao et al. 2002) (Mangiavacchi and Wolf 2004) (Snyder et al. 2000). However, repeated cocaine treatment in vivo (5 days of non-contingent administration) results in an initial decrease in the AMPA/NMDA ratio on MSNs in the NAc, as measured 24 hr after the last cocaine injection (Kourrich et al. 2007) (Mameli et al. 2009). A period of withdrawal (referred to hereafter as “withdrawal”) results in a gradual elevation of AMPA/NMDA ratios and AMPAR surface expression (Boudreau and Wolf 2005; Schumann and Yaka 2009), although a challenge dose of cocaine will result in lowered ratios and surface receptor content (Boudreau et al. 2007; Kourrich et al. 2007; Thomas et al. 2001). Self-administration of cocaine also causes similar changes in the NAc, with cocaine exposure causing a loss of AMPA receptors and depressing synaptic strength on MSNs and extended withdrawal resulting in synaptic strengthening and

accumulation of surface AMPA receptors (Conrad et al. 2008; Ortinski et al. 2012) (Schramm-Sapyta, Olsen, and Winder 2005). This bidirectional plasticity suggests that other factors, in addition to dopamine, contribute to the synaptic changes induced by drug exposure.

Recently, we have shown that TNF drives internalization of AMPARs on MSNs, reducing corticostriatal synaptic strength, and reduces the aberrant changes in striatal circuit function induced by chronic blockade of D2 dopamine receptors (Lewitus et al. 2014b). Glia are the main source of TNF in the CNS, and both microglia (Sekine et al. 2008) and astrocytes (Bowers and Kalivas 2003) are activated by psychostimulants. Further, glia have been suggested to regulate drug-induced behavior (Miguel-Hidalgo 2009). Thus, glia through the release of TNF could have a mitigating effect on the circuit changes induced by cocaine. Here we demonstrate that striatal microglia are activated by cocaine, and moderate the synaptic and behavioral changes induced by the repeated administration of cocaine.

## MATERIALS AND METHODS

*Animals*

TNFα-/- (RRID: IMSR_JAX:005540) and strain-matched wildtype mice (C57Bl6/J) were acquired from Jackson Laboratories, and bred with B6.Cg-Tg(Drd1a-tdTomato) 6Calak/J (RRID: IMSR_JAX:016204) to obtain D1-MSN wildtype and D1-MSN TNFα-/- mice. Floxed TNFα mice were obtained from S. Nedospasov (Kuprash et al. 2005) and crossed with GFAP-Cre mice (Bajenaru et al. 2002) from NCI Mouse Repository (RRID: IMSR_NCIMR: 01XN3) or with Tg(Cx3cr1-cre)MW126Gsat mice (Yona et al. 2013) generated by N. Heintz (The Rockefeller

University, GENSAT) and purchased from MMRRC (UC Davis; RRID: MMRRC_036395-UCD). GFAP or Cx3Cr1-Cre expressing mice were compared with GFAP or CX3CR1-Cre non-expressing littermates. Floxed TNF and GFAP-Cre mice were on a C57/Bl6 background; Cx3Cr1-Cre mice were a mix of FVB/B6/129/Swiss/CD1. Behavioural sensitization in littermates only expressing Cre or heterozygous for the floxed allele of TNF was indistinguishable from non-Cre expressing homozygous floxed animals. Experiments only used male mice at 8-12 weeks of age (electrophysiology) or 8-16 weeks of age (behaviour). Animals were housed 2-5 per cage and maintained on a 12 hour light/dark cycle. All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and the Montreal General Hospital Facility Animal Care Committee.

*Locomotor activity*

Locomotion was monitored in 30x30cm plexiglass boxes by the EthoVision video-tracking system (Noldus) and performed near the end of the light phase of the light–dark cycle under dim red light. After habituating animals to handling, mice received IP injections of saline or cocaine and their activity was recorded for 15min. Mice were tested during habituation to injection and the activity boxes (saline; days 1–2) and 5 repeated cocaine injections (15 mg/kg; days 3–7). Following 10 days without injections (withdrawal), mice receive a challenge dose of cocaine (15 mg/kg) and locomotor activity again assessed (day 17). Total distance traveled and stereotypy were evaluated from video by EthoVision. For stereotypy, the frequency of which the mouse stops moving (#pauses/min) was measured using a threshold start velocity of 2.00/second and a threshold stop velocity of 0cm/s. The number of turns per minute (#

turns/min) was measured by calculating the frequency for which the path of the mouse turns over a minimum distance of 3.00cm. Velocity was assessed as the mean distance traveled (cm) over time (s).

*Reagents*

Reagents were acquired from Sigma, unless otherwise noted. Cocaine was acquired from Medisca Pharmaceutique, under license from Health Canada. DN-TNF (XENP1595) was a gift from Xencor, and is an engineered dominant negative variant of TNF that rapidly binds with soluble TNF to form inactive heterotrimers (Steed et al. 2003) and crosses the blood-brain barrier to affect the CNS (Lewitus et al. 2014b).

*Electrophysiology recording*

240 µm thick coronal slices containing the nucleus accumbens were prepared from brains of 8-9 week-old mice (with fluorescent D1-MSNs). After a 1 h recovery period, slices were placed in a submersion-type recording chamber and perfused (1.5-2 ml/min) at 30oC with a bicarbonate-buffered artificial CSF saturated with 95% O2/5% CO2 and containing (in mM): 119 NaCl, 2.5 KCl, 1 NaH2PO4, 1.3 MgCl2, 2.5 CaCl2, 26.2 NaHCO3, 11 glucose, and 0.05 picrotoxin. Whole-cell voltage-clamp recordings from medium spiny neurons in the nucleus accumbens core were obtained under visual control using infrared-differential interference contrast microscopy. Whole-cell electrodes (2–4 MΩ) were filled with internal solution containing the following (in mM): 120 CsMeSO3, 15 CsCl, 8 NaCl, 0.2 EGTA, 10 HEPES, 2 Mg-ATP, 0.3 Na-GTP, 10 tetraethylammonium, and 5 QX-314 [5-N-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium bromide] (308–310 mOsm). pH was

adjusted to 7.3 with CsOH. Cells with series resistance above 25 MΩ or a 20% change over time were excluded. Synaptic currents were evoked using stainless-steel bipolar stimulating electrodes placed 100–150 µm rostral to the recorded neurons. EPSCs were acquired with an Axopatch 200B (Molecular Devices), filtered at 2 kHz, and digitized at 10 kHz. Acquisition and analysis was performed using pCLAMP 10.2 (Molecular Devices). To calculate AMPA/NMDA ratios, evoked synaptic responses were collected at -70 mV to measure AMPAR-mediated EPSCs and at +40 mV to measure NMDAR-mediated EPSCs. Typically, 20 responses were averaged to generate an average response. The peak AMPA response was used to calculate the AMPAR component and the amplitude at 40 ms post the AMPAR peak response was used to calculate the slower NMDA component at +40mV to calculate the AMPA/NMDA ratio.

*Immunohistochemistry*

Mice (8-10 weeks) were perfused and their brains submerged overnight in 4% PFA, cryoprotected with 30% sucrose, and cut in 30µm thick coronal slices using a cryostat. The sections were incubated for 4 hours with blocking solution (2% normal goat serum, 3% BSA, 0.2% Triton X-100 in PBS) before incubation overnight at 4C with primary antibodies in blocking solution (rabbit anti-Iba1 antibody [019-19741, Wako; 1:800; RRID: AB_2566825], rat anti-CD11b antibody [MCA711B, Serotec, 1:500; RRID: AB_321292], chicken anti-GFAP antibody [AB5541, Millipore; 1:500; RRID: AB_177521], rabbit antiNeuN [ab104225, Abcam, 1:400; RRID: AB_10711153], and rabbit anti-TNF antibody [ab6671, Abcam, 1:100; RRID: AB_305641] or mouse anti-TNF antibody [ab1793, Abcam, 1:100; RRID: AB_302615]). Sections were washed (2 x 15 min with PBST [0.05% Tween20] and 1 x 15 min with PBS at RT) and incubated for 2 hrs at

room temperature in secondary antibodies (anti-rabbit, anti-chicken, or anti-mouse IgG-conjugated Alexa Fluorochrome [Invitrogen; 1:400]). Sections were washed and briefly (< 30 seconds) incubated with Hoechst 33342 [H1399, Molecular Probes; 1:10,000] in PBS. The stained sections were imaged using an Olympus FluoView FV1000 confocal microscope with Fluoview imaging software (FV10-ASW). Images of the ventral striatum (nucleus accumbens core and shell) were acquired with a 40x or 60x objective and stacks of 25-30 optical sections (1 µm per section) of depth were Z-projected into a single window and analysed using ImageJ software (NIH). Image analysis: The number of Iba1+ microglia were manually counted from 212 x 212 µm (60x objectives) or 317 x 317 µm (40x objectives) images. TNF intensity was measured using the mean grey value of the entire image while Iba1 intensity measurements were obtained using a thresholded area to highlight only the microglia. Cell body area ($\mu m^2$) was measured based on Iba1 staining and using the freehand selection tool. Additional analysis of microglia morphology in saline and cocaine treated mice was done using IMARIS software (Bitplane). Microglia were identified using Iba1 staining from z-stacks generated by confocal microscopy. 3D reconstruction of microglia filaments were generated by the IMARIS tool filament tracer. Tracing was done on representative microglia using the automatic detection mode, no loops allowed, start and end points manually selected. The following parameters were analyzed for 40 microglia per group: total process length (sum of length of all processes), total process volume, process diameter and number of branch points and terminals. All analyses were done blind to treatment or condition.

*RNA isolation and quantitative PCR*

For tissue, total RNA from the ventral striatum was extracted and isolated by RNAqueous-4PCR total RNA isolation kit (Ambion, AM1914), according to manufacturer's instructions. For microglia and astrocyte cultures, total RNA was isolated by RNeasy kit (Qiagen, 74136). For all sample types, 0.5 µg of total RNA was used for cDNA synthesis with Quantitect Reverse Transcription Kit (Qiagen, 205313), and quantitative RT-PCR was performed with Fast SYBR-Green Master Mix (Applied Biosystems) detected by StepOne Plus RealTime PCR system (Applied Biosystems). The sequences of qPCR primers used for mRNA quantification in this study were obtained from Integrated DNA Technologies, and are as follows: mouse TNF forward, CCAGTGTGGGAAGCTGTCTT [200 nM]; mouse TNF reverse, AAGCAAAAGAGGAGGCAACA [200nM]; mouse GAPDH forward, ATGTGTCCGTCGTGGATCTGA [300nM]; mouse GAPDH reverse, TTGAAGTCGCAGGAGACAACCT [300nM]; rat TNF forward, CCAGTGTGGGAAGCTGTCTT [600nM]; rat TNF reverse, TGGGAGTTGCTGTTGAAGTC [600nM]; rat cyclophilin forward, CCGCTGTCTCTTTTCGCC [500nM]; rat cyclophilin reverse, GCTGTCTTTGGAACTTTGTCTG [500nM]. After 40 cycles, the Ct values were determined. To normalize the samples, ΔCt between TNF and GADPH or cyclophilin Ct values was calculated. The x-fold difference in expression between the different treatments was then determined by subtraction of the ΔCt values and termed ΔΔCt. Finally, the total change was calculated as $2^{-\Delta\Delta ct}$ and the relative amount compared with control samples was deducted.

*Cell culture*

Mixed microglia-astrocyte primary cultures were prepared from the striatum of postnatal rats (day 0), essentially as described (Beattie et al. 2002b). Briefly, tissue was dissociated by titration, and cells were seeded in glia media (MEM containing 5% FBS, 1.5% high sucrose MEM and 0.1% glutamax) on culture flasks (Corning) coated with poly-D lysine (Sigma) and placed in a humidified incubator at 37C with 5% CO2. Neurons were removed by mechanical disruption and the medium was changed at day 1, and every 5-7 days after. Microglia were collected from mixed cultures at DIV 10-13 by moderate shaking (200rpm) for 4 hrs at 37C. Freshly collected microglia were plated at a density of approximately 5x10^4 cells/cm² in 6cm dishes (Corning). Drug treatments of microglia were done at DIV 2-3. Pure astrocyte cultures were generated by treating mixed glia cultures with cytosine arabinoside (8µm) for 5 days, followed by a 45min treatment with 75mM of the microglia toxin L-leucine methyl-ester (LME) (Pascual et al. 2012). Microglia and astrocyte cultures were treated for 3 hours with dopamine hydrochloride (Sigma) at 0.01µM and 0.1µM together with 0.05 mg/ml ascorbic acid (Fisher Scientific). Microglia cultures were also treated with cocaine (Medisca, 1µM), D1 agonist SKF-38393 (Sigma, 1µM), D2/3 agonist quinpirole (Sigma, 1µM), and D3 agonist pramipexole dihydrochloride (Tocris, 1µM).

*Isolation of adult microglia*

Adult male C57BL/6 mice (8-12 weeks) underwent cardiac perfusion with Hank's Balanced Salt Solution (HBSS) and whole brains were removed. Cortices and striata were dissected, cut in to small pieces and subjected to enzymatic digestion with the Neural Tissue Dissociation Kit (P)

(Miltenyi Biotech, US cat no. 130-092-628), as per manufactures instructions. Tissue was homogenised and resuspended in 35 % isotonic Percoll, overlayed with HBSS and spun at 400g for 45 mins for the removal of myelin. Pure populations of microglial cells were obtained through magnetic bead cell sorting using CD11b microbeads (Miltenyi, cat no. 130- 093-634). Aliquots from positive and negative bead selected fractions were removed for cell staining to assess cell purity (Fig S2). For staining, cells were stained with eflouro eFluor®780 viability dye (1:1000; eBioscience), blocked with FC-receptor blocked (1:200; BD Bioscience) and stained with CD11b-V450 (1:100; BD Bioscience). Cells were acquired using a BD FACS Canto II and analyzed using BD FACSDiva software. For RNA extraction, cell fractions were pelleted and lysed with 350µL of RLT buffer (Qaigen) with 1% β-ME and frozen at -80. For cell culture, pure (>95% CD11b-positive) adult microglia were plated on poly-L-lysine coated plates at a density of 2x105 mL-1 in DMEM F12 with 10% L929-cell conditioned media (rich in M-CSF), 10% FBS, 1% P/S, and 40ng/ml recombinant human TGF-β1 (Miltenyi, cat no: 130-095-067), to maintain their transcriptional profile, as previously described (Butovsky et al. 2014). Culture media were changed 3 days after plating and cells were used at 7 days.

*Statistical analysis*

Statistical analyses were performed using JMP 8 or 11 (SAS Institute). All data are presented as mean ± SEM. Data were tested for normality using the Shapiro-Wilk Test, and then compared using the two-tailed Student's t-test (unless noted); multiple comparisons were analyzed by one-way ANOVA for independent and/or repeated measures and two-way ANOVA followed by post-hoc Dunnet's or Fisher's least significant difference. For experiments where a low number

of samples were compared (n < 6), the non-parametric Wilcoxon or Steel multiple comparison with control rank sum test were utilized. In all cases, the significance level was established at $p < 0.05$. **For Figure 2.1**, the specific tests used were: Fig 2.1A: Two way-repeated-measures ANOVA (treatment, $F(1,23) = 0.38$, $p = 0.0068$; days, $F(4,20) = 6.01$, $p < 0.0001$; treatment X days, $F(4,20) = 0.07$, $p = 0.83$); and Fig 2.1B: Two way-repeated-measures ANOVA (treatment, $F(2,34) = 0.26$, $p = 0.018$; days, $F(4,31) = 3.22$, $p < 0.0001$; treatment X days, $F(4,32) = 0.31$, $p = 0.05$).

**For Figure 2.2**, the tests were: Fig 2.2C: Student's t-test, $p < 0.55$ (1 day), $p < 0.05$ (5 days), $p < 0.91$ (withdr); and Fig 2.2D: Steel multiple comparison test, $z = -0.612$, $p = 0.54$ (1 day), $z = -2.774$, $p = 0.0163$ (5 days), $z = -0.209$, $p = 0.835$ (withdr). **For Figure 2.3**, the tests were: Fig 2.3C: Steel multiple comparison test, $z = 2.99$, $p = 0.0028$; Fig 2.3D: Steel multiple comparison test, $z = -2.31$, $p = 0.0211$; and Fig 2.3E: Wilcoxon rank sum test, $p = 0.021$; $n = 4$ (each n pooled from 5 animals). **For Figure 2.4**, the tests were: Fig 2.4A: One way ANOVA: $F(5,259) = 0.986$; $p > 0.43$); Fig 2.4C: Student's one-tailed t-test, $p = 0.46$, 0.038, and 0.029, respectively; Fig 2.4D: Student's t-test, $p = 0.61$ (1 day), $p = 0.047$ (5 day), $p = 0.445$ (withdr); Fig 2.4E: Student's t-test, $p = 0.19$ (1 day), $p = 0.018$ (5 day), $p = 0.23$ (withdr); Fig 2.4G: Student's t-test, $p < 0.001$ (1 day), $p = 0.037$ (5 day), $p = 0.089$ (withdr); and Fig 2.4I: Two-way ANOVA: time, $F(1,164) = 2.663$, $p = 0.105$; drug, $F(1,164) = 4.64$, $p = 0.0327$; time X drug, $F(1,164) = 11.02$, $p = 0.0011$. **For Figure 2.5**, the tests were: Fig 2.5B: Student's t-test: 1 day, $p = 0.29$; 5 days, $p = 0.14$; withdrawal, $p = 0.77$; and Fig 2.5C: Student's t-test: 1 day, $p = 0.37$; 5 days, $p = 0.36$; withdrawal, $p = 0.46$. **For Figure 2.6**, the tests were: Fig 2.6A: two way-repeated-measures ANOVA: genotype, $F(1,26) = 0.33$, $p = 0.006$; days, $F(4,23) = 7.8$, $p < 0.0001$; genotype X days, $F(4,23) = 0.52$, $p = 0.03$ and

Student's t-test, $t(26) = 2.10$, $p < 0.04$; and Fig 2.6C: two way-repeated-measures ANOVA: genotype $F(1,38) = 0.0048$, $p = 0.67$; days, $F(7,32) = 5.8$, $p < 0.0001$; genotype X days, $F(7,32) = 0.035$, $p = 0.99$ and Student's t-test, $t(48) = 0.83$, $p = 0.4$. **For Figure 2.7**, the tests were: Fig 2.7A: One-way ANOVA: $F(5,74) = 2.84$, $p < 0.021$; Fig 1B: One-way ANOVA: $F(5,66) = 2.83$, $p = 0.022$; Fig 2.7C: One way ANOVA: $F(5,51) = 1.08$, $p = 0.38$; Fig 2.7D: Student's t-test, $t(24) = 2.16$, $p = 0.042$; Fig 2.7E: Student's t-test, $t(16) = 2.12$, $p = 0.127$; And Fig 2.7F: Student's t-test, $t(19) = 0.32$, $p = 0.75$. **For Figure 2.8**, the tests were: Fig 2.8A: One-way ANOVA: $F(2, 30) = 3.6496$, $p = 0.0390$; Fig 2.8B: one-tailed paired t-test, $p = 0.057$; Fig 2.8C: Steel multiple comparison rank sum test, $p = 0.81$; Fig 2.8D: Steel multiple comparison test, $z = -0.194$, $p = 0.847$; Fig 2.8E: one-tailed paired t-test, $p = 0.33$; Fig 2.8F: ANOVA: $F(3, 51) = 4.67$; $p = 0.0061$; Fig 2.8G: Wilcoxon Rank sum test, 1-way, $p = 0.037$; Fig 2.8H: Wilcoxon rank sum test, 1-way, $p = 0.49$; Fig 2.8I: Student's t-test, $p = 0.49$; Fig 2.8J: Wilcoxon Rank sum test, 1-way, $p = 0.0075$; and Fig 2.8K: Student's t-test, $t(15) = 2.72$, $p = 0.026$. **For Figure 2.9**, the tests were: Fig 2.9A: Student's t-test, $t(7) = -4.09$, $p = 0.006$; Fig 2.9B: Steel multiple comparison test, $z = -2.165$, $p = 0.030$ (4hrs), $z = -1.984$, $p = 0.047$ (24hrs); and Fig 2.9C: Student's t-test, $t(23) = 2.04$, $p = 0.05$. **For Figure 2.10**, the tests were: Fig 2.10A: ; Student's t-test, $t(6) = 0.067$, $p > 0.52$; Fig 2.10B: Oneway ANOVA: $F(2,19) = 14.18$, $p < 0.001$; Fig 2.10C: One-way ANOVA: $F(2,39) = 32.96$, $p < 0.0001$; Fig 2.10D: one-way ANOVA; $F(2,27) = 0.667$, $p = 0.521$; Fig 2.10E: one-way ANOVA; $F(2,30) = 12.566$, $p < 0.0001$; Fig 2.10F: one-way ANOVA; $F(2,29) = 17.729$, $p < 0.0001$; Fig 2.10G: Student's t-test, $p > 0.683$; and Fig 2.10H: Student's t-test, $p > 0.336$.

## RESULTS

*TNF antagonizes cocaine-induced behavioral sensitization*

Behavioral sensitization is a simple model of drug-induced behavioral change, which measures the progressive increase in locomotor response to psychostimulants. TNF -/- mice displayed an increased initial locomotor response to cocaine and increased sensitization, compared to WT mice (Figure 2.1A). This is similar to what has been observed with methamphetamine sensitization in TNF -/- mice (Nakajima et al. 2004). To exclude compensatory mechanisms resulting from the absence of TNF during development, we pharmacologically blocked the soluble form of TNF in WT mice using XENP1595 (a dominant-negative variant of TNF [DN-TNF]). WT mice were administered DN-TNF either during the 5 days of conditioning (to block TNF signaling during acquisition) or during the withdrawal period starting immediately after the last cocaine injection (to test the role of TNF in the maintenance of the behavior). Blocking TNF signaling during acquisition was sufficient to increase sensitization as well as maintain the elevated response on the challenge day, while blocking TNF signaling during the 10 day period of withdrawal had no effect on the response to the challenge dose (Figure 2.1B). These results suggest that TNF is active during acquisition but not during drug withdrawal. Further, the increased sensitivity observed in TNF -/- mice on the first day of cocaine is not due to an acute loss of TNF signaling and is likely to be unrelated to the increase in sensitization.

*Cocaine increases TNF levels in the Nucleus Accumbens*

To determine the effect of *in vivo* cocaine exposure on TNF levels in the NAc, we measured TNF mRNA and protein levels in mice after i.p. injections of saline or cocaine (Figure 2.2A). A single injection of cocaine had no effect on TNF levels (measured 24 hr post-injection), but 5 days of daily cocaine treatment (measured 24 hr after the final injection) increased both TNF mRNA and protein, compared to saline injected controls (Figures 2.2B-D). TNF was no longer elevated following 10 days of withdrawal from cocaine. We noticed that TNF immunoreactivity largely overlapped with the microglia marker Iba1 (Figure 2.2B), leading us to suspect that TNF levels were increasing specifically in microglia following repeated cocaine administration.

*Cocaine induces TNF expression specifically in microglia*

These results suggest that microglia are important for the adaptive TNF response to repeated cocaine administration. To verify this, we isolated microglia from the striatum of cocaine and saline treated animals by magnetic bead sorting (Figure 2.3A) and compared TNF mRNA in the microglial and non-microglial fractions. FACS analysis of microglia isolated from adult tissue yielded CD11b-positive microglia fractions of >95% (Figure 2.3B). Microglia contained the vast majority of TNF mRNA, showing over a 20-fold enrichment compared with the other striatal cell types (Figure 2.3B). Further, the TNF mRNA was increased by cocaine treatment specifically in microglia cells (Figure 2.3C) and not in other cell types (Figure 2.3D).

This result led us to suspect that repeated administration of cocaine may be modulating microglia activation.

*Microglia are activated by cocaine and antagonize cocaine-induced behavioral sensitization*

Resting microglia continuously survey the healthy brain and respond to a variety of activation signals by undergoing progressive morphological and functional changes (Kettenmann et al. 2011). Using Iba1, we labeled microglia in adult mice 24 hr after a single cocaine injection, 24 hr after 5 days of daily cocaine injections, or after 10 days of drug withdrawal (Figure 2.4B). Although the number of microglia in the NAc did not change at any time point (Figure 2.4A), Iba1 intensity was increased in microglia by 5 days of cocaine, and after a period of withdrawal (Figure 2.4C). Microglia cell body size and roundness as measured by Iba1 labeling, was increased by 5 days of cocaine (Figure 2.4D-E). We also labeled microglia in the brain with another common marker for microglia (Figure 2.4F), CD11b and observed that CD11b intensity was also increased in microglia by 5 days cocaine (Figure 2.4G). Interestingly, CD11b intensity was decreased 1 day after a single injection of cocaine (Figure 2.4G). These changes were further accompanied by a decrease in total microglial process length as measured by Iba1 (Figure 2.4H-J), and are all consistent with an activated microglia phenotype. In contrast, we did not observe any activation of astrocytes, as judged by the area or intensity of GFAP expression (Figure 2.5A-C). These data strongly suggest that microglia are the source of the cocaine-induced upregulation of TNF production in the striatum observed during sensitization.

To assess the source of TNF regulating sensitization, we utilized a Cre-loxP system to selectively delete TNF from microglia (CX3CR1-Cre; Figure 2.6B) and astrocytes (GFAP-Cre; Figure 2.Di-iii). Mice that lack microglial TNF showed significantly higher sensitization to cocaine from the second cocaine injection that was maintained through the period of withdrawal (Figure 2.6A). Conversely, mice that lack astrocytic TNF did not display a significant change in sensitization compared with littermate controls (Figure 2.6C). These results suggest that microglia are important for the adaptive TNF response to repeated cocaine administration.

*Cocaine-induced TNF causes synaptic depression on D1-MSNs*

To understand the impact of TNF on synaptic function in the NAc, we measured AMPA/NMDA ratios on MSNs in the NAc core. Alteration in NAc core AMPA receptors is involved in the expression of behavioral sensitization to psychostimulants (Kalivas 2009). We have previously shown that TNF drives internalization of AMPARs on MSNs in the dorsal striatum (Lewitus et al. 2014b). As repeated cocaine administration primarily affects direct-pathway MSNs, we tested specific subpopulations of MSNs in the NAc core for their response to TNF. Acute NAc slices were incubated with TNF and whole-cell recording made from Drd1atd Tomato (D1) positive and negative (D2) MSNs. A low dose of TNF (10 ng/ml) had no significant effect on either cell type. However, 100 ng/ml TNF significantly reduced the AMPA/ NMDA ratio on D1-MSNs, with a nonsignificant reduction in ratios on D2-MSNs (Figure 2.7A). These results suggest that D1-MSNs are more sensitive to TNF than D2-MSNs, although D2-MSNs may respond to a lesser degree. Repeated non-contingent administration of cocaine results in lower

AMPA/NMDA ratios of excitatory inputs onto the NAc specifically on D1-MSNs (Kim et al. 2011; Pascoli, Turiault, and Luscher 2012). To test whether this decrease in AMPA/NMDA ratios is due to increased TNF expression, we evaluated AMPA/NMDA ratios in the NAc core after cocaine or saline administration in WT and TNF -/- mice. As expected for WT mice, a single injection of cocaine did not significantly reduce AMPA/NMDA ratios on D1-MSNs as measured 1 day later, but five daily cocaine injections did (Figure 2.7B). Strikingly, in TNF -/- mice, a single injection of cocaine significantly increased AMPA/NMDA ratios, which remained elevated after 5 days of cocaine treatment (Figure 2.7B). No significant differences were observed in D2-MSNs for either genotype (Figure 2.7C). These results suggest that the reduction in AMPA/NMDA ratios in D1-MSNs after repeated cocaine is due to increased TNF in the NAc, and that cocaine itself increases synaptic strength. This is consistent with the exocytosis of AMPARs observed with direct stimulation of D1Rs on MSNs (Mangiavacchi and Wolf 2004). Moreover, this result suggests that, following cocaine treatment, D1-MSNs are more responsive to lower endogenous levels of TNF, perhaps because newly inserted AMPARs are more labile, as has been seen at potentiated synapses in the amygdala (Clem and Huganir 2010). To test the hypothesis that potentiated D1-MSNs are more sensitive to TNF, we treated TNF -/- mice with a single injection of cocaine and evaluated the effect of a low level of TNF (10 ng/ml) on AMPA/NMDA ratios. Although this level of TNF had no significant effect on MSNs from WT untreated animals (Figure 2.7A), it significantly reduced AMPA/NMDA ratios on D1-MSNs from cocaine treated knockout animals (Figure 2.7D). This treatment had no effect on D2-MSNs from TNF -/- mice (Figure 2.7F). To test whether cocaine-induced TNF signaling occludes further synaptic depression by TNF, we treated WT animals with cocaine for 5 days and then treated

striatal slices ex vivo with TNF (100 ng/ml). The AMPA/NMDA ratio on D1-MSNs (already reduced compared to saline treated animals; Figure 2.7A) was not further reduced by treatment with TNF (Figure 2.7E). This shows that the synaptic depression induced by repeated cocaine injections occludes the TNF-mediated reduction in AMPA/NMDA ratio. Overall, these data suggest that repeated cocaine treatment elevates TNF, which suppresses the synaptic changes directly induced by cocaine in the NAc core.

*Dopamine increases TNF mRNA in microglia through D2 receptors*

Cocaine could activate microglia directly by binding the sigma receptor (Navarro et al. 2010) or the Toll-like receptor 4 (TLR4) (Northcutt et al. 2015), or indirectly through the elevation of dopamine. To test this, we treated postnatal microglia cultures with dopamine or cocaine for 3 hr. Treatment of microglia with 0.1 μM dopamine significantly increased TNF mRNA (Figure 2.8A). This concentration of dopamine is reflective of the concentration found in the NAc in vivo following cocaine administration in rats (Hooks et al., 1992). Microglia transcriptional profiles change substantially during both development and the culturing process (Butovsky et al. 2014). We therefore cultured microglia from adult animals, in a manner that preserves an in vivo transcriptional profile (Butovsky et al. 2014). We saw a similar response to dopamine (Figure 2.8B). The same treatment applied to cultured astrocytes had no effect on TNF mRNA (Figure 2.8C). We observed no change in TNF mRNA levels in response to cocaine treatment (1 μM, 3hrs) in postnatal (Figure 2.8D) or adult microglia cultures (Figure 2.8E).

Multiple dopamine receptors are expressed on microglia (Kettenmann et al. 2011). Stimulating microglia with the D2-like agonist quinpirole increased TNF mRNA, while the D1 agonist SKF-38393 and specific D3 agonist pramipexole had no effect (Figure 2.8F). This suggests that dopamine increases TNF production in microglia through D2 receptors. To directly test the response of microglia in vivo, we treated mice with quinpirole (0.5 mg/kg, i.p.; 24 and 1 hr prior to harvest) and isolated microglia. Quinpirole treatment increased the TNF mRNA in striatal microglia cells (Figure 2.8G), but not in other striatal cell types (Figure 2.8H), nor in cortical microglia (Figure 2.8I), indicating that D2-agonism specifically increases microglial TNF in the striatum in vivo. Co-administrating cocaine with the D2 antagonist L741,626 for 5 days reduced the cocaine-induced increase in microglial TNF production (Figure 2.8J). Finally, to verify that D2-like receptor activation was required for the TNF-dependent decrease in AMPA/NMDA ratio observed on D1-MSNs, we treated animals for 5 days with cocaine and the D2 antagonist. Blocking D2 receptors (and thus preventing the activation of microglia) resulted in a large cocaine-induced increase in AMPA/NMDA ratio on D1-MSNs, similar to TNF animals (Figure 2.8K). Overall, this suggests that cocaine elevates dopamine levels, which act on D1 receptors on direct pathway MSNs to increase synaptic strength; simultaneously activates microglia through D2 receptors; and temporarily increases TNF production.

*MPLA reduces synaptic strength in the nucleus accumbens via microglia activation and TNF production*

Our data suggest that the activation of microglia limits the cocaine-induced changes to NAc circuitry, but this activation occurs only during a narrow window following cocaine exposure. Because depotentiation of MSNs reduces cocaine-induced behavioral sensitization (Pascoli, Turiault, and Luscher 2012), we tested if reactivation of microglia to increase TNF could depress NAc synapses and suppress sensitization. To do this, we utilized monophosphoryl lipid A (MPLA), a detoxified variant of LPS (Casella and Mitchell 2008). MPLA is a weak TLR4 agonist that does not induce extensive neuroinflammation or sickness behavior (Michaud et al. 2013). We first verified that MPLA activates microglia in the NAc, by injecting 10 mg MPLA IP after 10 days of withdrawal from cocaine when microglia are less activated compared to right after 5 days of cocaine administration. MPLA treatment significantly increased Iba1 intensity within 4 hr compared to saline treated controls (Figure 2.9A) and was associated with an increase in striatal TNF expression, at both 4 and 24 hr after injection (Figure 2.9B). We next tested if MPLA would depress synaptic strength in the NAc core. After 10 days of withdrawal from cocaine, mice were injected with MPLA and evaluated 24 hr later for AMPA/ NMDA ratios on D1-MSNs. MPLA treatment significantly reduced AMPA/NMDA ratio in D1-MSNs compared to saline-treated controls (Figure 2.9C). Because artificially reducing synaptic strength in the NAc can reduce behavioral sensitization (Pascoli, Turiault, and Luscher 2012), these data suggest that MPLA might suppress drug-induced behaviors.

*MPLA decreases behavioral sensitization to cocaine via TNF*

We first established that MPLA does not alter basal locomotion (Figure 2.10A). Further, MPLA (10 mg; 24 hr prior to testing) did not decrease the locomotor response to an initial dose of cocaine, as tested in saline-treated animals given an initial dose of cocaine at the challenge time point (Figure 2.10B). We then tested sensitized mice by injecting 10 mg or 50 mg MPLA or saline, 24 hr prior to the challenge dose of cocaine. Mice treated with MPLA had significantly reduced locomotor response to the cocaine challenge in a dose-dependent manner (Figure 2.10C). This suggests that MPLA reduces sensitization, rather than the locomotor response to cocaine. The effects of MPLA also did not appear to be due to an increase in sensitivity to cocaine, as we observed no increase in stereotypic behaviors in MPLA treated mice (Figures 2.10D-F). Moreover, MPLA had no effect on sensitization in TNF mice (Figure 2.10G), which suggests that MPLA acts through an elevation of TNF and not other cytokines. However, this effect is temporary, as MPLA had little impact on sensitization when tested 4 days after injection (Figure 2.10H). These observations suggest that even after a prolonged period of withdrawal from cocaine, increasing TNF can be effective in reducing the behavioral response to cocaine, although it does not revert the system to the pre-sensitized state.

## DISCUSSION

Chronic cocaine administration produces long-term neuroadaptations of glutamatergic signaling in the NAc that contribute to addiction-related changes in drug sensitivity and craving. Here we show that microglia in the NAc core are transiently activated following cocaine administration, and act to downregulate AMPARs on MSNs through TNF signaling. Our study focused on the NAc core as alterations in the core are particularly important for the expression of locomotor sensitization (Kalivas 2009), which we used as a model of drug addiction. Infusion of AMPA into the NAc core enhances locomotion in animals exposed to cocaine 2-3 weeks earlier (Bell and Kalivas 1996), while AMPAR antagonists administered into the NAc core prevent the expression of sensitization (Bell, Duffy, and Kalivas 2000; Pierce et al. 1996). The increase in NAc synaptic strength has been hypothesized to correlate with the development of craving (Conrad et al. 2008), and reducing synaptic strength reduces cue-induced self-administration (Wisor, Schmidt, and Clegern 2011).

Importantly, microglial-derived TNF signaling in the NAc core limits the development of locomotor sensitization. Although evidence for behavioral sensitization in humans is inconsistent, progressive increases in the rewarding and locomotor effects to repeated drug administration, such as potentiated eye-blink responses and mood-elevating responses, have been reported in humans (Leyton 2007; Boileau et al. 2006; Strakowski et al. 2001). In rodents, locomotor sensitization is a valuable model of drug addiction given its simplicity and relevance to more progressive animal models of addiction. For example, procedures that produce

addictive behaviors in animal models also produce sensitization, and sensitization-related plasticity in the brain are involved in the transition from casual to compulsive drug use (Robinson and Berridge 2008). Another important point is that non-contingent sensitization experiments are sufficient to produce increased motivation to obtain a drug (Vezina 2004), increased motivation to a drug-paired conditioned stimulus (Robinson and Berridge 2000) (Di Ciano 2007), cognitive impairment (Schoenbaum and Shaham 2008) and enhanced habit formation (Nelson and Killcross 2006), all of which contribute to addiction. Sensitization also changes the brain in ways related to susceptibility to relapse, such as enhanced glutamate release in the core of the nucleus accumbens (Pierce et al. 1996), and is further relevant to the incubation of cocaine craving (growing over a period of drug-free withdrawal), that increases the likelihood to relapse (Grimm et al. 2001).

Further, our results explain a perplexing feature of chronic cocaine administration—that synaptic strength on D1-MSNs decreases initially and then slowly increases during withdrawal. While the formation and subsequent unsilencing of silent synapses likely contribute (Huang et al. 2009) to the changes in AMPA/ NMDA ratios, our results suggest an additional mechanism is involved. Our data support the idea that dopamine does, as predicted by in vitro results, increase AMPA/NMDA ratios while simultaneously activating microglia to release TNF. This TNF release causes the decreased AMPA/NMDA ratios observed following repeated administration of cocaine. This suppression is temporary, as microglia slowly deactivate during withdrawal from cocaine, revealing the underlying dopamine-induced potentiation. A challenge dose would

reactivate the microglia, increase TNF release, and again suppress AMPA/ NMDA ratios as observed (Boudreau et al. 2007) (Thomas et al. 2001).

MPLA, a weak TLR4 agonist, has been shown to significantly improve cognitive function in a mouse model of neurodegeneration (Michaud et al., 2013). We found that MPLA can acutely reduce behavioral sensitization after prolonged withdrawal from cocaine, and that this is associated with reactivation of microglia, TNF expression and decreased synaptic strength in the NAc core. If MPLA is found to similarly diminish reinstatement, it would suggest that MPLA could reduce the motivation to acquire drugs and be used to prevent relapse, a significant problem in the treatment of addiction. A remaining therapeutic challenge with MPLA is that its effect is not long-lasting. When MPLA is given 4 days before a cocaine challenge, we no longer measure an attenuation of behavioural sensitization. This could be because TLR4 is downregulated after activation (Jack et al. 2005; Anwar, Basith, and Choi 2013; Bosisio et al. 2002). This results suggest that increasing TNF with mild TLR4 activation may help blunt craving or incentive sensitization.

Our results also highlight an adaptive role of striatal microglia in the response to cocaine, and suggest that their modulation could be an effective avenue of treatment. Microglia express a variety of neurotransmitters, neuropeptides, and immune receptors and have the capacity to rapidly respond to physiological changes in the brain (Kettenmann et al. 2011). Our results support the idea that moderate microglia activation has a role in a homeostatic type response to significant deviations from the basal state (Kierdorf and Prinz

2013) and has a similar beneficial response in re-establishing homeostasis following stress (Kreisel et al. 2014). Hence, augmenting the microglial response, through TLR4 or other means, might be a useful approach to treat addiction, provided it only moderately activates the microglia. This is to suggest not that astrocytes do not regulate striatal function, including the response to drugs, but merely that astrocytes do not supply the TNF that opposes the circuit and behavioral changes induced by cocaine. CX3CR1 is expressed in other cell types (including macrophages and a small number of neurons), and a TLR4 agonist like MPLA will act on astrocytes and other cell types. However, astrocytes are reported to express low levels of TLR 1, 4, 5 and 9, suggesting that TLR4 activation could preferentially affect microglia (Jack et al. 2005). Therefore, while we cannot exclude the contribution of other cell types to the TNF response, but it is difficult to argue that microglia are not the major source of the response.

Taken together, our data suggest that TNF has an adaptive role in regulating glutamatergic transmission in the NAc when circuit homeostasis is perturbed. As described in an *in vitro* model of HSP (Stellwagen and Malenka 2006), TNF-mediated plasticity is part of a mechanism that compensates for cellular alterations that destabilizes NAc output. This result suggests a framework for the aberrant synaptic plasticity underlying drug addiction (Lüscher and Malenka 2011), whereby homeostatic plasticity is unable to fully counter-balance Hebbian plasticity.

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## FIGURE LEGENDS FOR CHAPTER 2

**Figure 2.1 TNF antagonizes cocaine-induced behavioral sensitization.**

**(A)** Mean locomotor activity in response to cocaine injections in *TNF*-$\alpha^{-/-}$ and WT mice, showing higher sensitization in *TNF*-$\alpha^{-/-}$ mice, that was maintained after withdrawal (n = 12 WT, 17 *TNF*-$\alpha^{-/-}$ animals). **(B)** Blocking soluble TNF-α signaling only during the sensitization protocol (DN-TNF sensi) with DN-TNF is sufficient to sustain the elevation of the cocaine response to the challenge dose on day 15, while blocking TNF-α signaling during the withdrawal period (DN-TNF withd) had no effect on the response to the challenge dose after withdrawal (n = 16 DN-TNF sensi, 8 DN-TNF withd, 12 Control). Results are expressed as mean ± SEM, n (mice or cells) is given in bars. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

**Figure 2.2 Cocaine increases TNF levels in the Nucleus Accumbens.**

**(A)** Diagram of the time points used for experiments: 24 hr after a single injection of saline or cocaine (i.p. 15 mg/kg), 24 hr after five daily injections of saline or cocaine, and 10 days after five daily injections of saline or cocaine. **(B)** Representative confocal projection images of NAc immunostained for Iba1 (top) and TNF-α (bottom) from mice injected for 5 days with saline or cocaine (scale bar, 20 µm). **(C)** Five daily injections of cocaine increases TNF-α protein in the NAc. **(D)** Five daily injections of cocaine increases TNF-α mRNA in the ventral striatum.

**Figure 2.3 Cocaine induces TNF expression specifically in microglia.**

**(A)** Microglia cells were isolated from striatum and cortex (4-5 animals pooled per n), using CD11b microbeads. **(B)** FACS analysis of microglia isolated from adult tissue to assess efficiency of CD11b-positive microglial isolation. Representative flow cytometry plots of CD11b-negative

cell fractions (i-iii) and CD11b-positive microglial cell fractions (iv-vi) shows the hierarchical gating strategy (polygons and arrows) to assess microglial cell purity: (i,iv) P1 gates cells on their size (FSC-A; forward scatter area), and granularity (SSC-A; side scatter area); (ii, v) P2 gates live cells (negative for the eFluor780 viability dye; and (iii, vi) P3 gates CD11b-positive cells (i.e. microglia) using CD11b-V450. Only CD11b-positive microglial fractions (P3) of >95% were used for qPCR analysis. **(C)** Purified microglia (CD11b+ fraction of cells) from whole striatum tissue express significantly more TNF-α mRNA compared to other cell types (CD11b− fraction) (five mice pooled per group in each experiment). **(D)** Five daily injections of cocaine increases TNF-α mRNA in striatal microglia (five mice pooled per group per experiment). **(E)** Cocaine does not increase TNFα RNA in non-microglial cells. Five daily cocaine treatments actually resulted in a decrease in TNFα mRNA of 3.26 ± 0.21 fold; Wilcoxon rank sum test, $p = 0.021$; $n = 4$ (each n pooled from 5 animals).

**Figure 2.4 Microglia are activated by cocaine.**

**(A)** Cocaine treatment does not alter the number of microglia in the nucleus accumbens. The number of Iba1-labeled microglia in the nucleus accumbens were counted in each confocal projection image (n = 72, 55, 34, 36, 32, 31 images (respectively) from 4 animals) from adult mice 1 day after a single cocaine injection (i.p. 15mg/kg), after 5 days daily injections, or after 5 injections followed by 10 days of withdrawal. No change in the number of microglia in the NAc was observed at any time point. **(B)** Representative confocal projection images of Iba1-labeled microglia in the NAc 24 hr after a single cocaine injection, five daily injections, or 10 days' withdrawal (scale bar, 20μm). **(C)** Semiquantitative analysis of Iba1 immunoreactivity in the

NAc, normalized to the mean saline intensity for each time point. **(D)** Quantification of microglia cell body size ($\mu m^2$) measured by Iba1 immunoreactivity and normalized to the mean saline value for each time point (n = 100; 25 cells each from four animals). **(E)** Quantification of microglia cell body roundness (4πA/p^2, A=area of cell body, p=perimeter of cell body) measured by Iba1 immunoreactivity and normalized to the mean saline value for each time point (n = 100; 25 cells each from four animals). **(F)** Representative confocal projection images of CD11b-labeled microglia in the NAc 24 hr after five daily injections of cocaine or saline (scale bar, 20μm). **(G)** Semiquantitative analysis of Iba1 immunoreactivity in the NAc, normalized to the mean saline intensity for each time point. **(H)** Representative examples of microglia processes, after 5 days of cocaine or saline. **(I)** Total length of microglia processes is decreased after 5 days' cocaine by 20%, but is not significantly altered after withdrawal (n = microglia from four animals). Results are expressed as mean ± SEM, n (experiments, mice or microglia) is given in bars. $^{*}p < 0.05$, $^{**}p < 0.01$.

**Figure 2.5 Astrocytes are not activated by 5 days of repeated cocaine administration.**

**(A)** Representative confocal projection images of GFAP immunostaining in the nucleus accumbens from adult mice 1 day after a single cocaine injection (i.p. 15mg/kg), after 5 days of daily injections, or after 5 injections followed by 10 days of withdrawal (scale bar = 40μm). GFAP levels are not altered by cocaine. Quantification of the area **(B)** and **(C)** intensity of GFAP immunoreactivity.

**Figure 2.6 TNF derived from microglia antagonizes cocaine-induced behavioral sensitization.**

**(A)** Mean locomotor activity in response to cocaine in mice that lack microglial TNF (CX3CR1-Cre$^{+}$; TNF$^{flox/flox}$) and littermate controls (CX3CR1-cre negative or TNF$^{+/flox}$). The elevation was sustained for a final test dose of cocaine (n = 16 control; 12 microglia deletion).

**(B)** Validation that CX3CR1-Cre is preferentially expressed in microglia. Confocal projection images from CX3CR1-Cre x Rosa26-STOP-tdtomato mice immunostained for microglia marker Iba1 (scale bar = 40 µm). Cells expressing CX3CR1-Cre-recombinase, which removes the floxed STOP codon allowing for expression of td-tomato, are red. While no fluorescence was observed in Cre negative animals, we observed expression of td-tomato in almost all cells expressing Iba1 (arrowheads). We imaged sections from two Cre positive mice and while some brain regions (e.g. cortex, top panels) showed almost exclusive expression of td-tomato in Iba1+ microglia, some regions (including the nucleus accumbens, bottom panels) also had expression in a small subset of neurons. This is consistent with the observation that some neurons do express CX3CR1 (Hughes et al., 2002; Meucci et al., 2000). No astrocyte expression of td-tomato was observed **(C)** Mean locomotor activity in response to cocaine injections in mice that lack astrocytic TNF (GFAP-Cre$^{+}$; TNF$^{flox/flox}$ and littermate controls (GFAP-cre$^{-}$; TNF$^{flox/flox}$). GFAP-Cre mice had normal sensitization and response on the final test day (n = 25 per condition). **(Di)** Validation of GFAP-Cre expression in astrocytes and neurons in the striatum. Confocal projection images from GFAP-Cre x Rosa26-STOP-tdtomato mice immunostained for GFAP. GFAP+ cells reliably express td-tomato in the nucleus accumbens (arrowheads, scale bar = 50µm). However, only low numbers of astrocytes are observed to be GFAP-expressing in the nucleus accumbens, as is typical for grey matter astrocytes (top). More GFAP+ cells are

observed in the white matter of the commissure and also express td-tomato (scale bar = 20µm) (bottom). **(Dii)** Representative staining of GFAP and NeuN in GFAP-Cre x Rosa26-STOP-tdtomato mice in the nucleus accumbens (scale bar = 30 µm) and dorsal striatum (same scale). Some td-tomato+ cells in the nucleus accumbens are neurons (as neural progenitors typically express GFAP during development). In the nucleus accumbens core, the percent of td-tomato+ cells that are neurons (NeuN+ ) is approximately 24%. The percent of neurons (NeuN+ ) that are also td-tomato+ is ~8%. In the dorsal striatum, the percent of td-tomato+ cells that are neurons (NeuN+ ) is ~47%; the percent of neurons (NeuN+ ) that also express td-tomato is ~29%. **(Diii)** Microglia (labeled with Iba1) never displayed expression of td-tomato. Representative micrographs from the commissure and nucleus accumbens show no overlap between Iba1 staining and td-tomato expression (scale bar = 30 µm).

**Figure 2.7 Cocaine-induced TNF causes synaptic depression on D1-MSNs.**

**(A)** Representative recording of EPSCs at −70 mV and +40 mV and mean AMPA/NMDA ratios from control slices and slices treated with 10 or 100 ng/ml TNF-α in D1 (red) and D2 (green) MSNs in the NAc core. AMPA/NMDA ratios were calculated using the peak amplitude at −70 mV for AMPA and the amplitude at +40 mV taken 40 ms after the peak at −70 mV.

**(B)** Representative traces and mean AMPA/NMDA ratios from D1-MSNs in the NAc core, after 1 and 5 days of cocaine or saline in WT or TNF-α-KO mice. Ratios from mice injected with one or five daily doses of saline were not significantly different, and were combined. **(C)** AMPA/NMDA ratios on D2-MSNs are not altered by cocaine treatment. Representative traces and mean AMPA/NMDA ratios from presumptive D2-MSNs in the NAc core. Consistent with previous reports (Cepeda et al., 2008), D2-MSNs have significantly lower basal AMPA/NMDA ratios than

D1-MSNs. Treatment with 1 day or 5 days of cocaine (15 mg/kg) had no effect on AMPA/NMDA ratios in either WT or TNFα-KO mice (data from the same animals used in B). **(D)** Representative traces and mean AMPA/NMDA ratios from D1-MSNs in the NAc core from control slices or slices treated ex vivo with TNF-α. Treatment with 10 ng/ml TNF-α significantly reduced AMPA/NMDA ratios from *TNF*-$\alpha^{-/-}$ mice treated 24 hr prior with cocaine. **(E)** Ex vivo treatment with 100 ng/ml TNF-α did not further decrease AMPA/NMDA ratios from WT mice previously exposed to five daily cocaine injections. **(F)** D2-MSNs from TNFα-KO mice are not more sensitive to TNFα after a single cocaine injection. Animals were injected with cocaine 24hr prior to slicing, and incubated ex-vivo with 10ng/ml TNFα (the same animals from D). Treatment with TNFα had no effect on AMPA/NMDA ratios in D2-MSNs.

**Figure 2.8 Dopamine increases TNF mRNA in microglia through D2 receptors.**

**(A)** Primary rat microglia cultures were treated with vehicle or dopamine for 3 hr (n = biological replicates from four independent cultures). **(B)** Cultures of adult microglial cells respond similarly to cultured neonatal microglia. Microglia were isolated from adult mice (see methods) and cultured for 7 days before being treated for 3 hrs with dopamine (0.1µm). Dopamine treatment had a trend for increasing TNFα mRNA to a similar magnitude to what was observed in microglial cultures from neonatal animals, although this effect was not significant (p=0.06). **(C)** Primary rat astrocyte cultures treated with ascorbic acid alone (Control, 0.05mg/mL), or with ascorbic acid and dopamine (0.1µm) for 3 hours (n = 6 biological replicates from 2 independent cultures). Dopamine does not alter TNFα production in astrocytes. **(D)** Treatment with cocaine (1 µm, 3 hr) did not alter TNF-α mRNA levels in postnatal microglia cultures (n = replicates from three cultures) or **(E)** adult microglia cultures (n=3 animals per group). **(F)**

Normalized change in TNF-α mRNA in primary rat microglia cultures treated for 3 hr with vehicle (Control), D1-receptor agonist (SKF-38393, 1 μm), D2-receptor agonist (quinpirole, 1 μm), or D3-agonist (pramipexole, 1 μm) (n = replicates from six cultures). **(G)** Quinpirole (i.p. 0.5 mg/kg; 24 hr and 1 hr before harvesting) significantly increases TNF-α mRNA in microglia isolated from striatal tissue, compared to saline treatment (n = experiments, four mice pooled per group in each experiment). **(H)** Quinpirole does not increase TNF mRNA in non-microglia cells in the striatum. Mice were injected with quinpirole (as in Fig 3C), and the microglia separated out. The non-microglia cells (CD11b negative fraction) did not respond to quinpirole with a change in TNFα mRNA (138 ± 72% of control; n = 3 (4 mice pooled per experiment). **(I)** We also isolated microglia from the cortex from the same animals and cortical microglia do not respond to quinpirole. **(J)** Co-administration of the D2-receptor antagonist L741,626 (i.p. 3 mg/kg; 15 min before cocaine) with daily cocaine injections over 5 days significantly decreases TNF-α mRNA in ventral striatum tissue in adult mice. **(K)** Co-administration of L741,626 with cocaine results in an increase in AMPA/NMDA ratio on D1-MSNs compared with mice treated with cocaine alone. Results are expressed as mean ± SEM, n (experiments, mice or cells) is given in bars. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

**Figure 2.9 MPLA activates microglia in the nucleus accumbens, increasing TNF levels and reducing AMPA/NMDA ratios on D1-MSNs.**

**(A)** Representative confocal projection images of Iba1 immunostaining in the NAc, after MPLA (10 μg) or saline injection in mice after 10 days' withdrawal. Scale bar, 20 μm. Semiquantification of immunoreactivity reveals that Iba1 intensity was significantly increased 24 hr after a single MPLA injection. **(B)** MPLA (10 μg) significantly increases TNF-α mRNA in the

ventral striatum at 4 hr and 24 hr. **(C)** Representative traces and AMPA/NMDA ratios from D1-MSNs in the NAc core 24 hr after MPLA (10 μg) or saline injection in mice after 10 days' withdrawal.

**Figure 2.10 MPLA decreases behavioral sensitization to cocaine via TNF**

**(A)** MPLA does not alter basal locomotion. No significant differences in locomotion were seen in mice tested 24hr after an i.p. injection of 10μg MPLA compared with saline injected mice (n = 4 animals per group). **(B)** MPLA does not reduce the initial locomotor response to cocaine. Mice were given seven daily saline injections, then after 9 days of withdrawal given an injection of saline or MPLA (10 μg), followed the next day by a challenge dose of cocaine. MPLA did not alter the response to the challenge dose. This response was lower than the sensitized response in control animals given cocaine during training (n = 8 sal/sal, 7 sal/MPLA, 6 coc/sal). **(C)** MPLA did reduce the sensitized response to cocaine. After withdrawal, WT mice were injected with MPLA (10 μg or 50 μg) or saline and tested 24 hr later with a challenge dose of cocaine (n = 20 for control, 12 for 10 μg MPLA, and 10 for 50 μg MPLA). **(D)** MPLA treated animals (10μg or 50 μg) had a slight increase in pausing, and **(E)** significant decreases in turning behaviour and **(F)** in mean velocity compared with saline injected controls. We saw no evidence for an increase in stereotypic behaviours, which suggests that the decrease in locomotor response to cocaine observed following MPLA is not due to increased sensitivity to cocaine and a corresponding increase in stereotypic behaviours. **(G)** MPLA treatment had no effect on sensitization in *TNF*-$\alpha^{-/-}$ mice, as MPLA (10 μg) did not reduce the response to a challenge dose of cocaine in *TNF*-$\alpha^{-/-}$ mice (n = 10 saline, 11 MPLA). Results are expressed as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. **(H)** MPLA suppression of sensitization is temporary. The delayed effect of a

single MPLA injection (10µg) on established behavioral sensitization in WT mice. After 10 days withdrawal, mice were injected with MPLA and were then tested for their cocaine response 4 days later. MPLA did not reduce the behavioral response to cocaine after 4 days (n = 15 for saline, 12 for 10µg MPLA). ** $p < 0.01$, *** $p < 0.0001$

## FIGURES FOR CHAPTER 2

### FIGURE 2.1

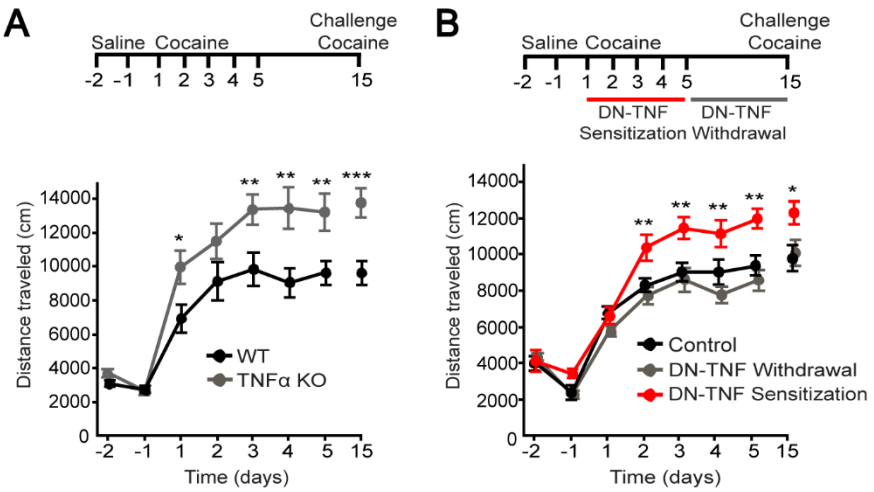


**FIGURE 2.2**

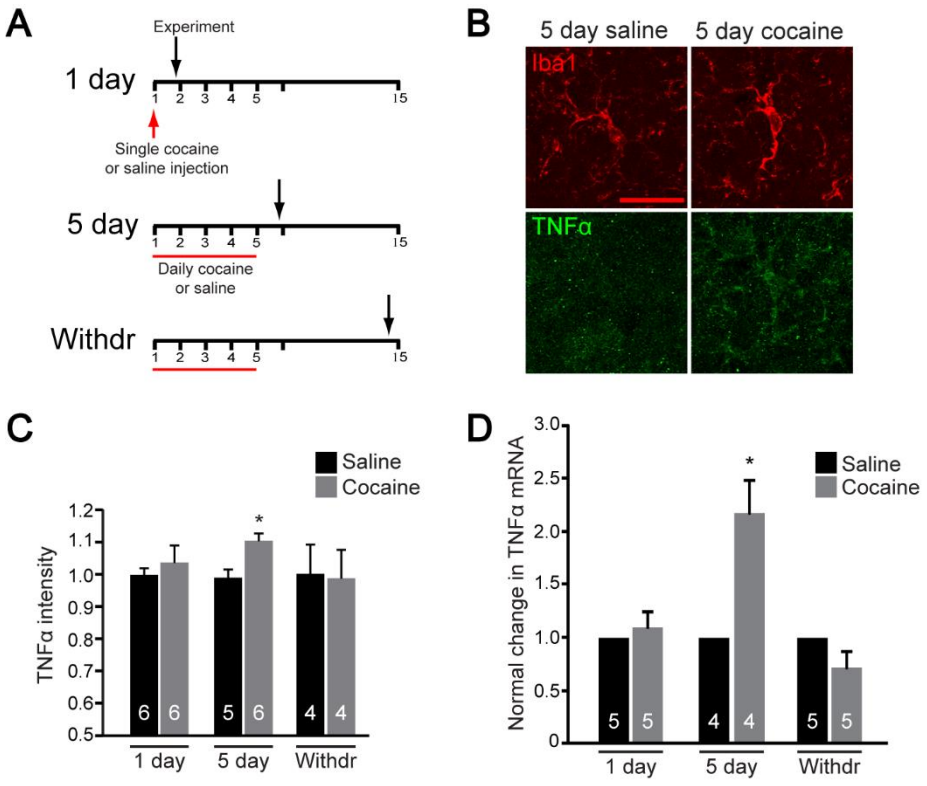

A
Experiment
1 day
1 2 3 4 5 15
Single cocaine
or saline injection
5 day
1 2 3 4 5 15
Daily cocaine
or saline
Withdr
1 2 3 4 5 15
B
5 day saline
5 day cocaine
Iba1
TNFα
C
Saline
Cocaine
TNFα intensity
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
*
6 6 5 6 4 4
1 day
5 day
Withdr
D
Saline
Cocaine
Normal change in TNFα mRNA
3.0
2.5
2.0
1.5
1.0
0.5
0
*
5 5 4 4 5 5
1 day
5 day
Withdr

**FIGURE 2.3**

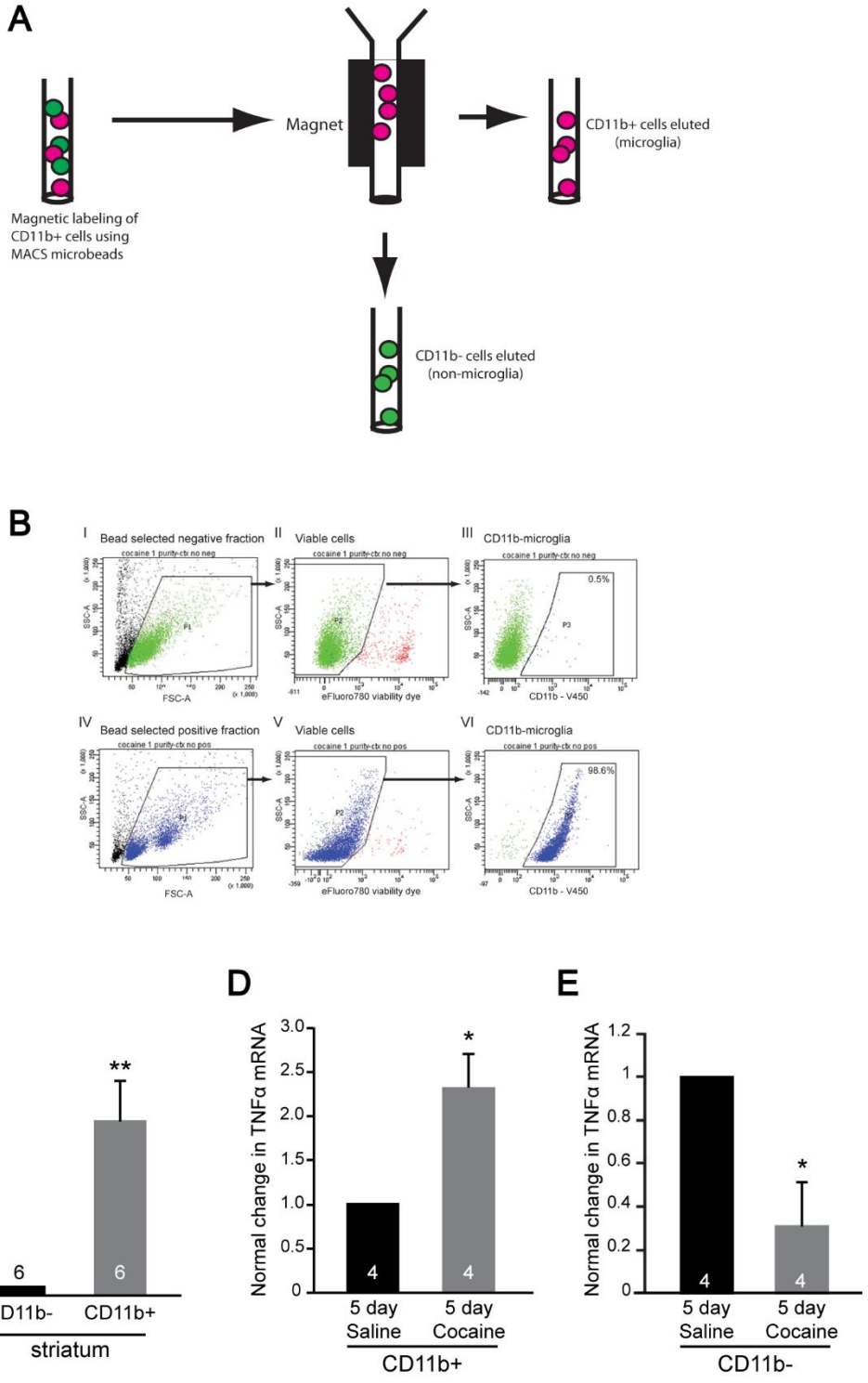

A
Magnet
CD11b+ cells eluted
(microglia)
Magnetic labeling of
CD11b+ cells using
MACS microbeads
CD11b- cells eluted
(non-microglia)
B
I Bead selected negative fraction
II Viable cells
III CD11b-microglia
0.5%
IV Bead selected positive fraction
V Viable cells
VI CD11b-microglia
98.6%
FSC-A
eFluoro780 viability dye
CD11b - V450
C
Normal change in TNFα mRNA
CD11b-
CD11b+
striatum
D
Normal change in TNFα mRNA
5 day Saline
5 day Cocaine
CD11b+
E
Normal change in TNFα mRNA
5 day Saline
5 day Cocaine
CD11b-

**FIGURE 2.4**

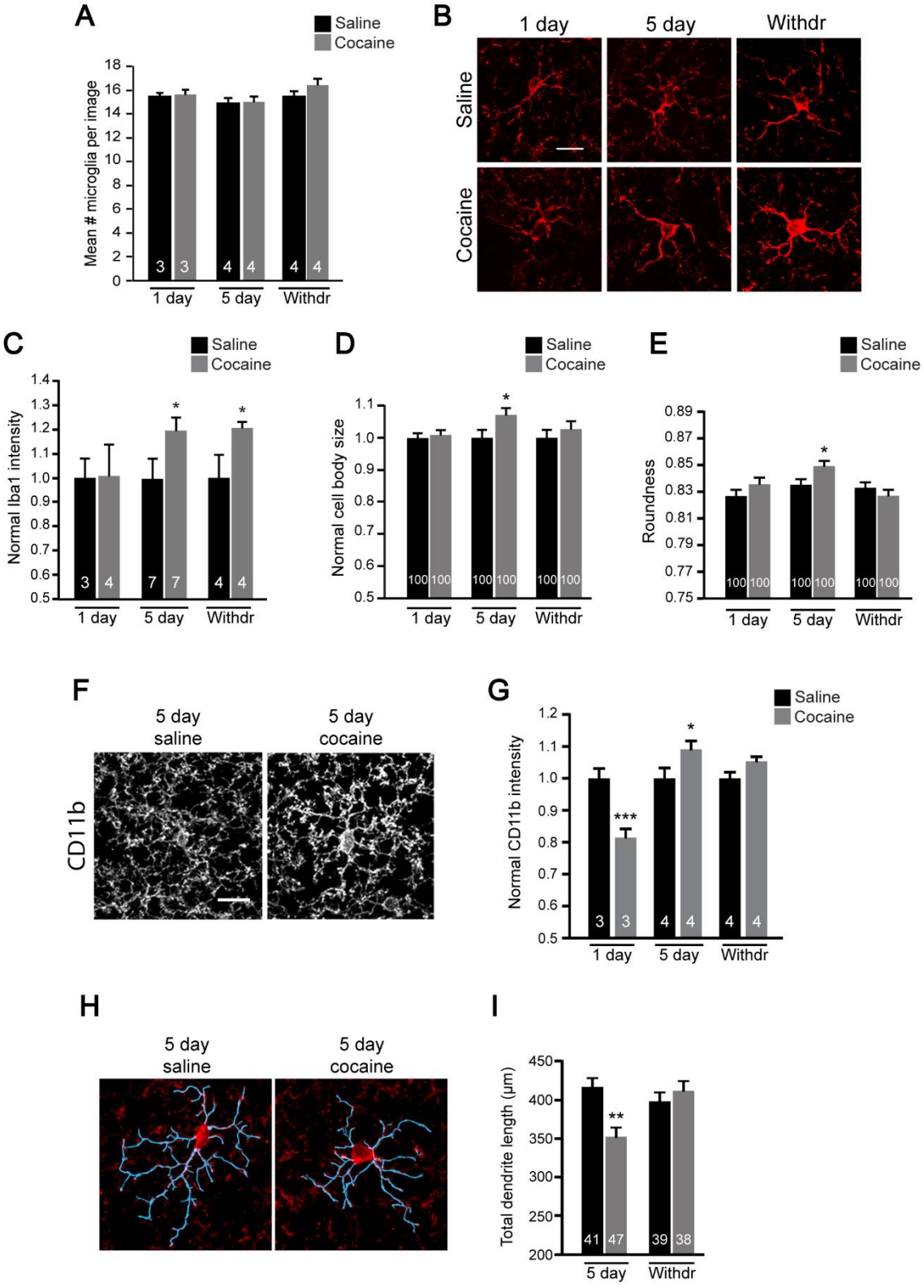

A
Saline
Cocaine
Mean # microglia per image
1 day
5 day
Withdr
B
1 day
5 day
Withdr
Saline
Cocaine
C
Normal Iba1 intensity
D
Normal cell body size
E
Roundness
F
5 day saline
5 day cocaine
CD11b
G
Normal CD11b intensity
H
5 day saline
5 day cocaine
I
Total dendrite length (µm)

**FIGURE 2.5**

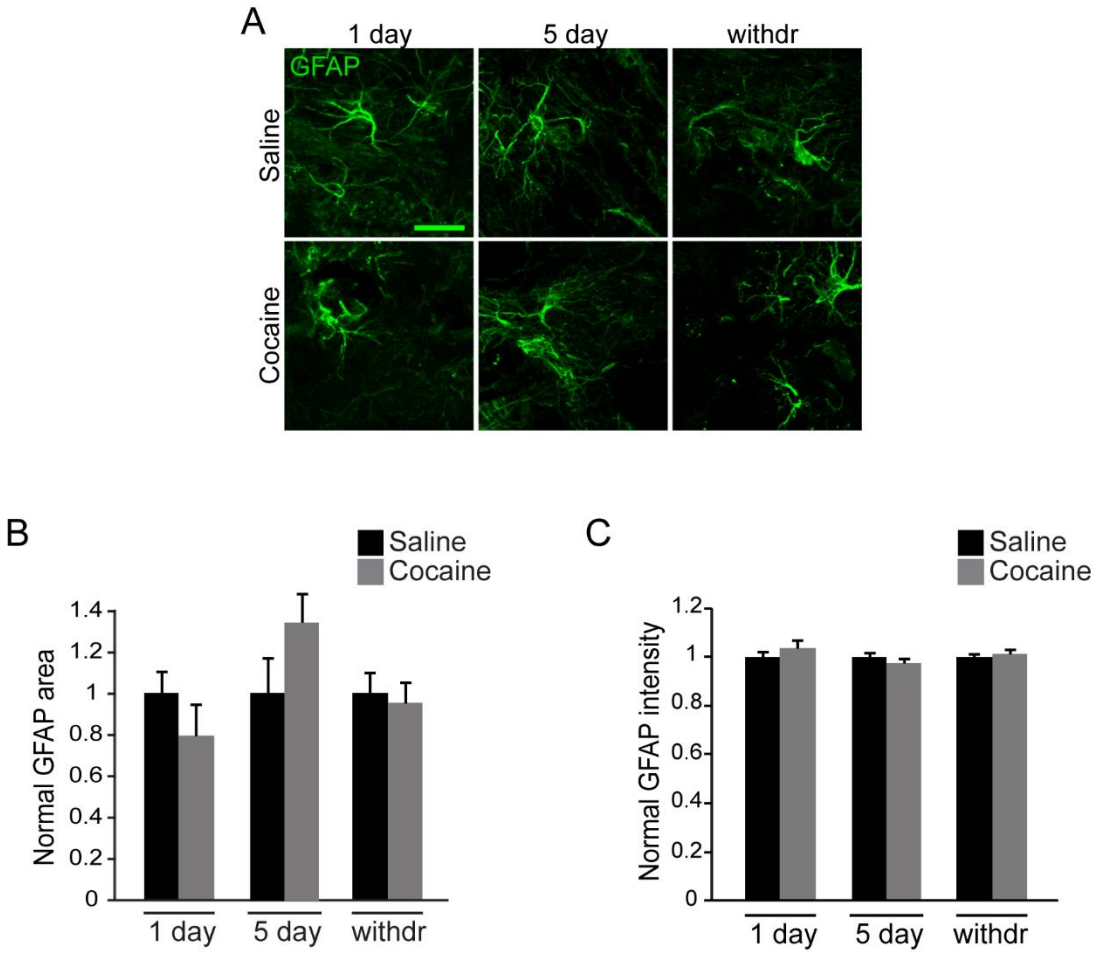

A
1 day
5 day
withdr
GFAP
Saline
Cocaine
B
Saline
Cocaine
Normal GFAP area
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1 day
5 day
withdr
C
Saline
Cocaine
Normal GFAP intensity
1.2
1
0.8
0.6
0.4
0.2
0
1 day
5 day
withdr

**FIGURE 2.6**

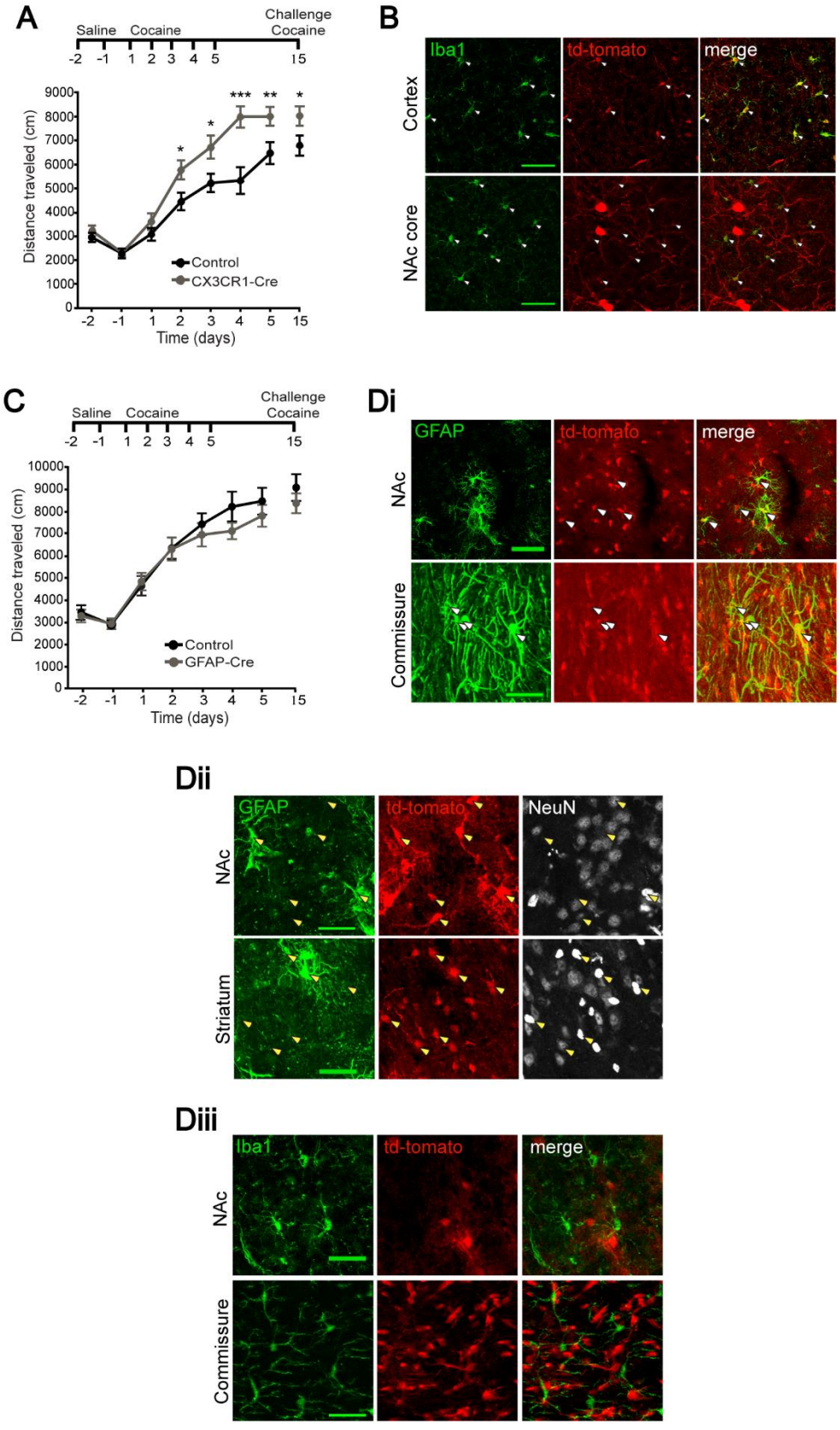

A
Saline
Cocaine
Challenge Cocaine
-2 -1 1 2 3 4 5 15
Distance traveled (cm)
Time (days)
Control
CX3CR1-Cre
B
Iba1
td-tomato
merge
Cortex
NAc core
C
Saline
Cocaine
Challenge Cocaine
Distance traveled (cm)
Time (days)
Control
GFAP-Cre
Di
GFAP
td-tomato
merge
NAc
Commissure
Dii
GFAP
td-tomato
NeuN
NAc
Striatum
Diii
Iba1
td-tomato
merge
NAc
Commissure

**FIGURE 2.7**

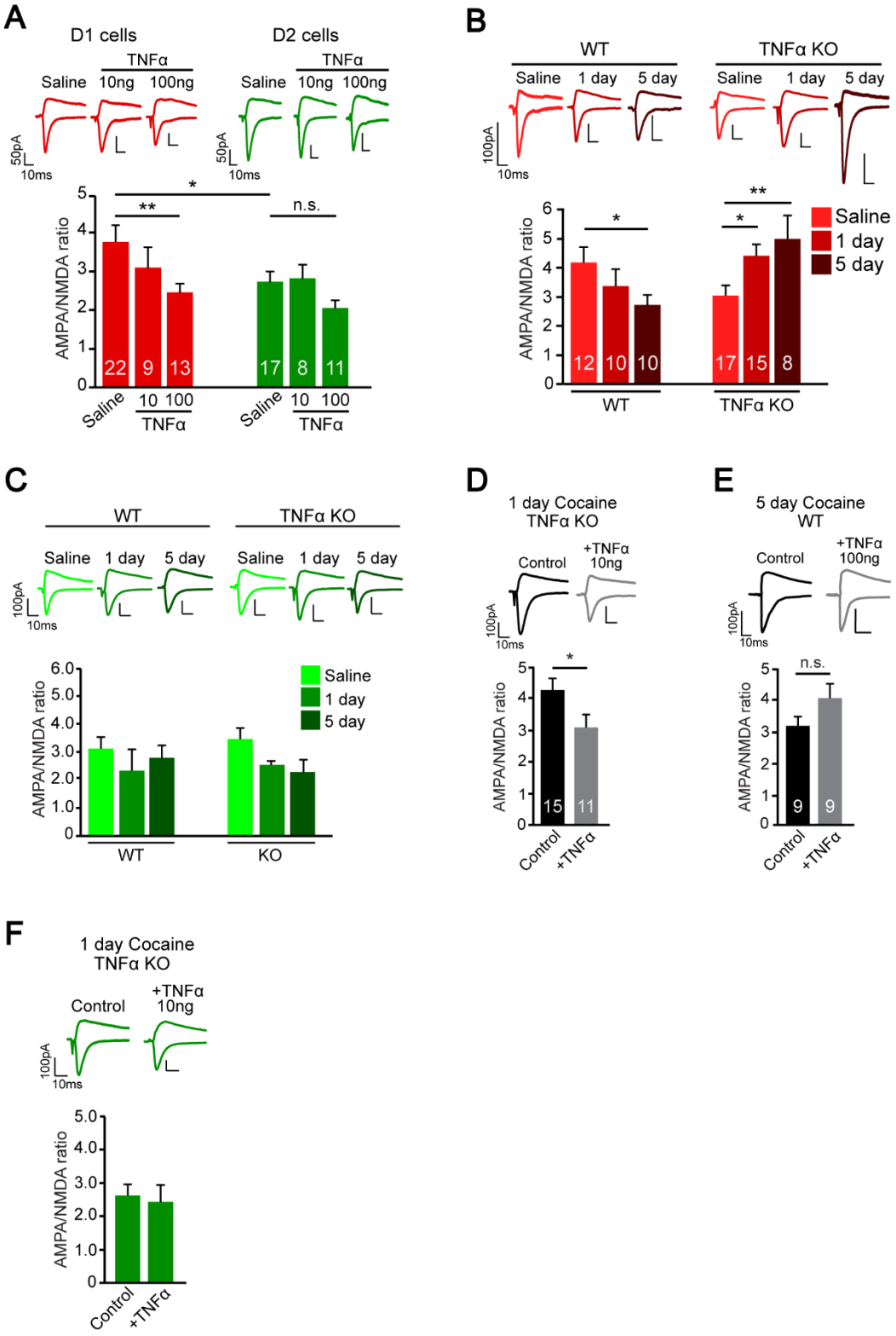

A
D1 cells
D2 cells
TNFα
Saline
10ng
100ng
50pA
10ms
AMPA/NMDA ratio
*
**
n.s.
22
9
13
17
8
11
Saline
10
100
TNFα
B
WT
TNFα KO
Saline
1 day
5 day
100pA
10ms
AMPA/NMDA ratio
*
**
12
10
10
17
15
8
C
WT
TNFα KO
Saline
1 day
5 day
100pA
10ms
AMPA/NMDA ratio
KO
D
1 day Cocaine
TNFα KO
Control
+TNFα
10ng
100pA
10ms
AMPA/NMDA ratio
15
11
+TNFα
E
5 day Cocaine
WT
Control
+TNFα
100ng
100pA
10ms
AMPA/NMDA ratio
n.s.
9
9
F
1 day Cocaine
TNFα KO
Control
+TNFα
10ng
100pA
10ms
AMPA/NMDA ratio
+TNFα

**FIGURE 2.8**

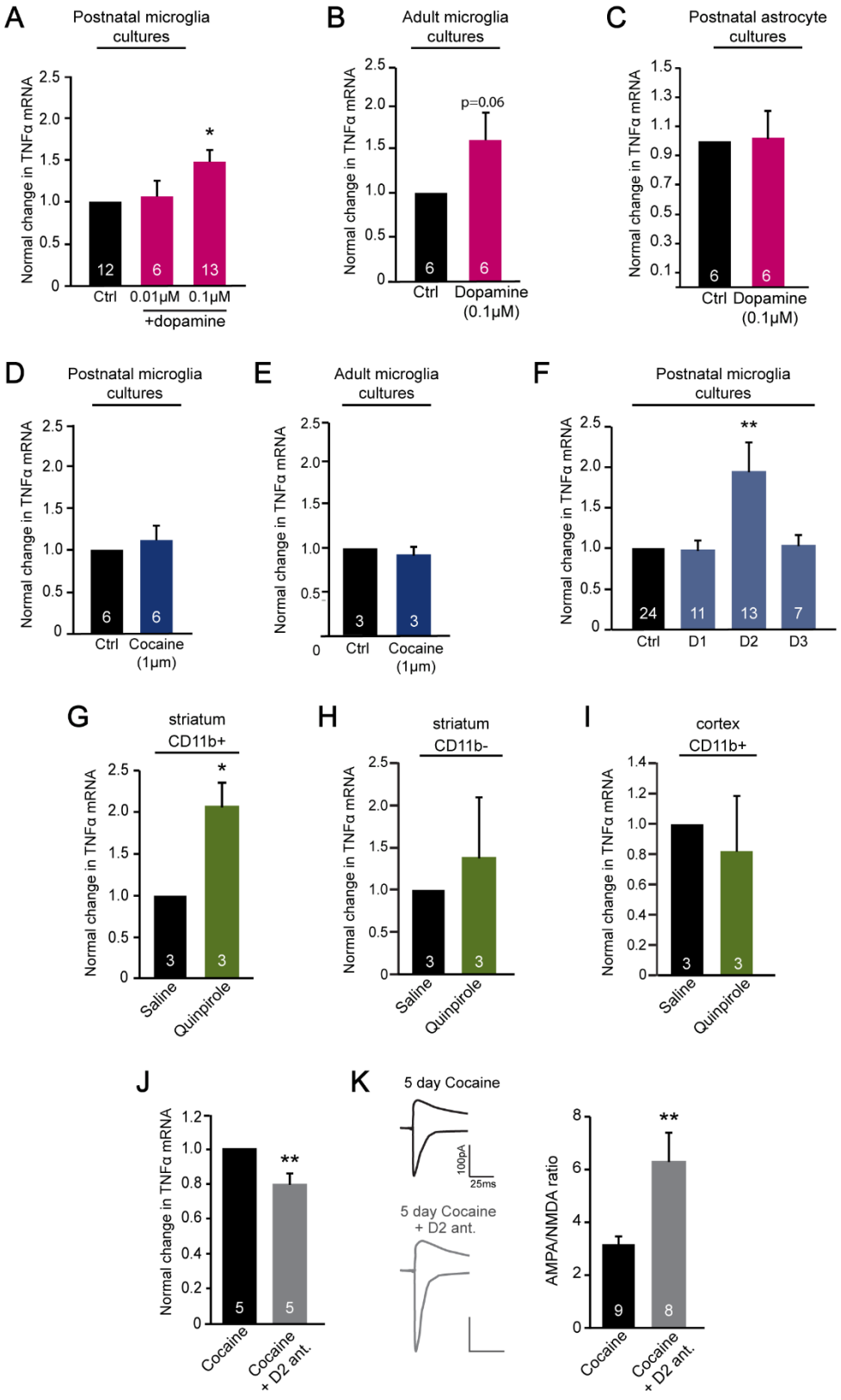

A
Postnatal microglia cultures
Normal change in TNFα mRNA
2.5
2.0
1.5
1.0
0.5
0
*
12
6
13
Ctrl
0.01μM
0.1μM
+dopamine
B
Adult microglia cultures
Normal change in TNFα mRNA
p=0.06
6
6
Ctrl
Dopamine (0.1μM)
C
Postnatal astrocyte cultures
Normal change in TNFα mRNA
1.5
1.3
1.1
0.9
0.7
0.5
0.3
0.1
6
6
Ctrl
Dopamine (0.1μM)
D
Postnatal microglia cultures
Normal change in TNFα mRNA
6
6
Ctrl
Cocaine (1μm)
E
Adult microglia cultures
Normal change in TNFα mRNA
3
3
0
Ctrl
Cocaine (1μm)
F
Postnatal microglia cultures
Normal change in TNFα mRNA
**
24
11
13
7
Ctrl
D1
D2
D3
G
striatum CD11b+
Normal change in TNFα mRNA
*
3
3
Saline
Quinpirole
H
striatum CD11b-
Normal change in TNFα mRNA
3
3
Saline
Quinpirole
I
cortex CD11b+
Normal change in TNFα mRNA
1.4
1.2
1.0
0.8
0.6
0.4
0.2
3
3
Saline
Quinpirole
J
Normal change in TNFα mRNA
**
5
5
Cocaine
Cocaine + D2 ant.
K
5 day Cocaine
100pA
25ms
5 day Cocaine + D2 ant.
AMPA/NMDA ratio
**
8
6
4
2
0
9
8
Cocaine
Cocaine + D2 ant.

**FIGURE 2.9**

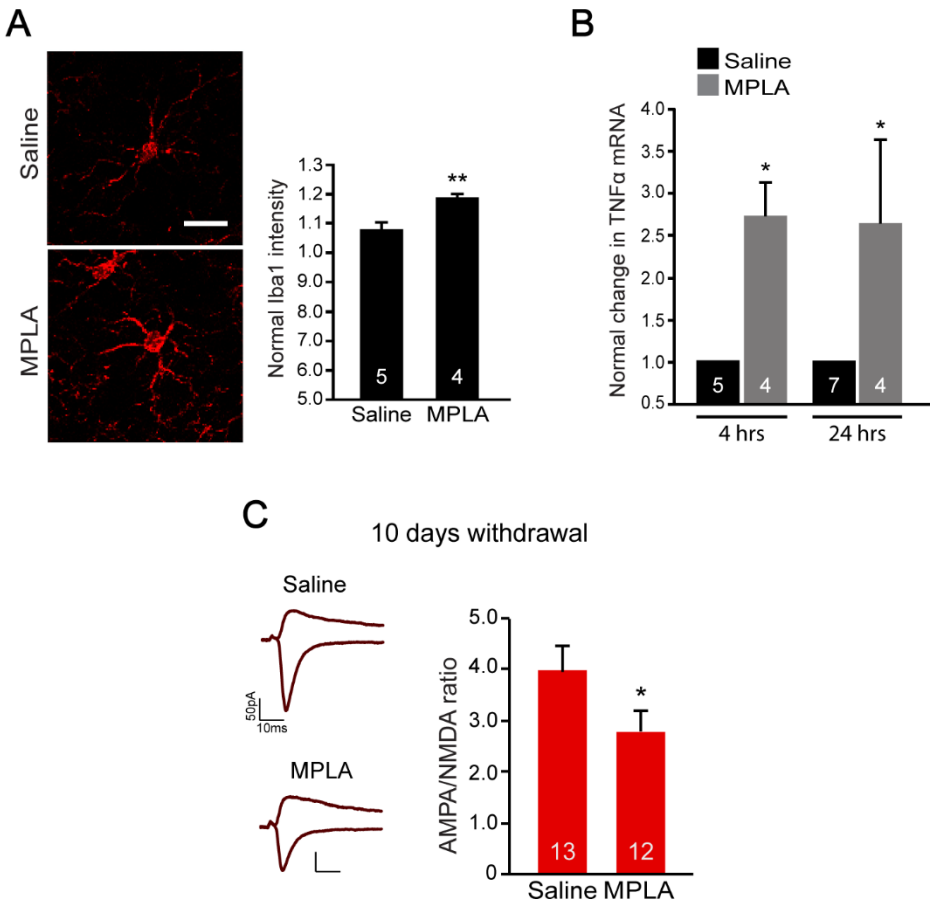
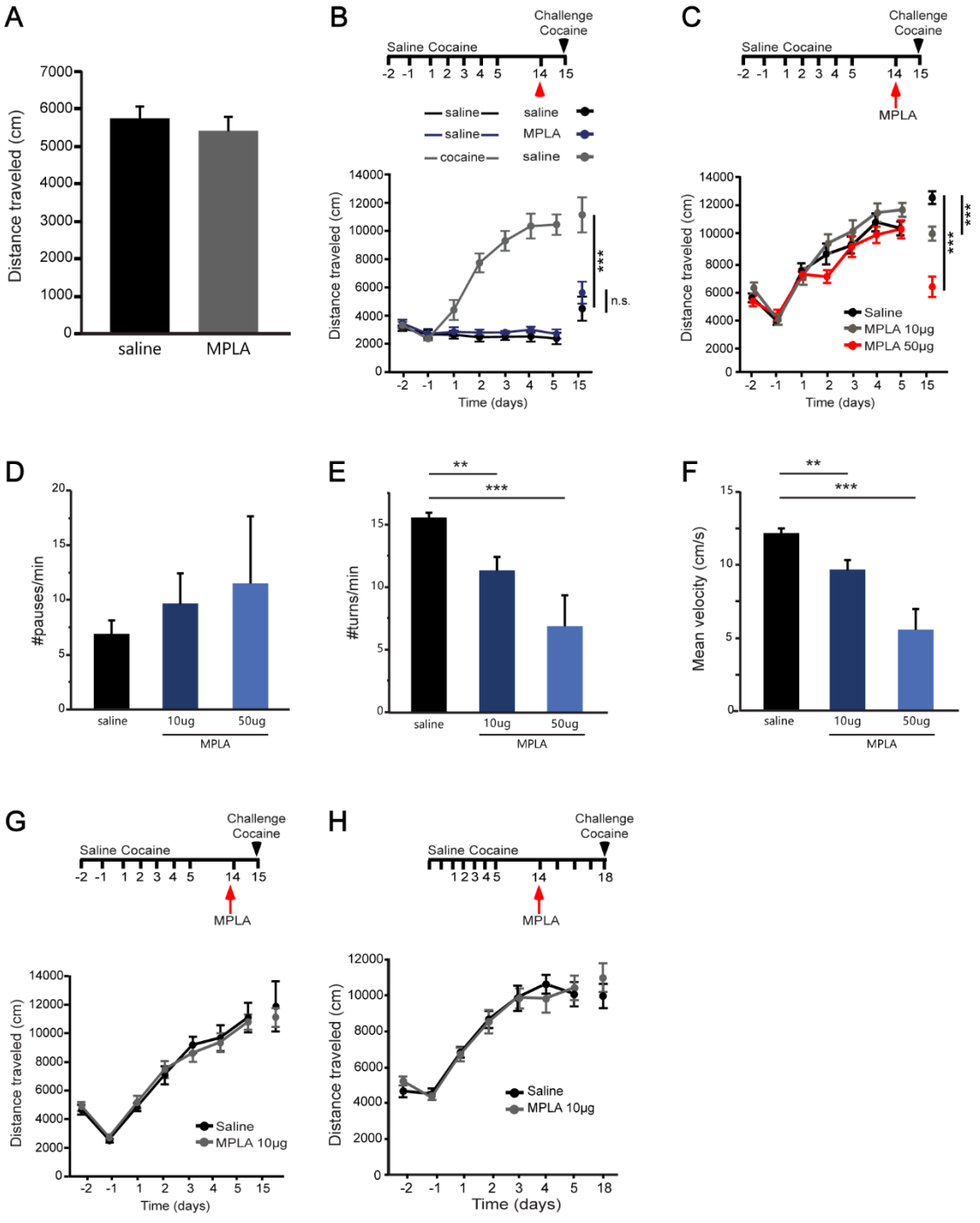

A
Saline
MPLA
Normal Iba1 intensity
1.3
1.2
1.1
1.0
9.0
8.0
7.0
6.0
5.0
**
5
4
Saline
MPLA
B
Saline
MPLA
Normal change in TNFα mRNA
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
*
*
5
4
7
4
4 hrs
24 hrs
C
10 days withdrawal
Saline
50pA
10ms
MPLA
AMPA/NMDA ratio
5.0
4.0
3.0
2.0
1.0
0
*
13
12
Saline MPLA

**FIGURE 2.10**


A
Distance traveled (cm)
saline
MPLA
B
Challenge Cocaine
Saline Cocaine
saline saline
saline MPLA
cocaine saline
Distance traveled (cm)
***
n.s.
Time (days)
C
Challenge Cocaine
Saline Cocaine
MPLA
Distance traveled (cm)
Saline
MPLA 10µg
MPLA 50µg
Time (days)
D
#pauses/min
saline
10ug
50ug
MPLA
E
#turns/min
**
***
F
Mean velocity (cm/s)
G
Challenge Cocaine
Saline Cocaine
MPLA
Distance traveled (cm)
Saline
MPLA 10µg
Time (days)
H
Challenge Cocaine
Saline Cocaine
MPLA
Distance traveled (cm)
Saline
MPLA 10µg
Time (days)

## PREFACE TO CHAPTER 3

Dendritic spines represent the majority of excitatory synapses on a neuron, and spine density is therefore an approximation of how much excitatory input a given neuron receives (Lai et al. 2016). TNF regulates synaptic strength on both excitatory (Pribiag and Stellwagen 2013; Stellwagen and Malenka 2006) and inhibitory neurons (Lewitus et al. 2014) via GABAR and AMPAR trafficking. We showed in Chapter 2 that TNF-mediated plasticity at NAc MSNs antagonized cocaine-induced plasticity. Therefore, we postulated that TNF may also antagonize cocaine-induced formation of dendritic spines, which increases MSN dendritic spine density in the NAc. In order to increase our understanding of TNF-mediated plasticity, we sought to determine whether TNF is able to regulate structural plasticity, specifically the density of dendritic spines. The main results of this chapter describe for the first time a role for TNF in the regulation of dendritic spine density both in basal conditions in the brain and following repeated cocaine administration. Our finding that astrocyte-derived TNF regulates dendritic spine density is a novel example of how endogenous levels of TNF in the brain regulate synaptic physiology.

**CHAPTER 3**

# GLIAL TNFα REGULATES DENDRITIC SPINE DENSITY IN THE NUCLEUS ACCUMBENS

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Department of Neurology & Neurosurgery, Centre for Research in Neuroscience, Research Institute of McGill University Health Centre, Montreal, QC, Canada H4G 1A4

## ABSTRACT

The nucleus accumbens (NAc) plays an important role in generating motivated behaviors to natural rewards and drugs of abuse. We have previously reported that TNF decreases excitatory synaptic strength in the NAc core following repeated cocaine administration via the endocytosis of AMPA-type glutamate receptors on medium spiny neurons (MSNs). TNF is produced by glia in the brain, the two main types of which are microglia and astrocytes. Both cell types have been shown to regulate the structural plasticity of dendritic spines on neurons. We show that glia-derived TNF regulates the density of dendritic spines on neurons in the NAc core. Adult TNF -/- mice have an increased baseline spine density in MSNs and, following repeated administration of cocaine, also have an increased spine density as compared to WT cocaine treated mice. Interestingly, mice lacking TNF only in microglia do not have altered spine density compared to WT controls, but mice lacking TNF in astrocytes show the same baseline increase in spine density as full TNF -/- mice. Interestingly, deletion of TNF in microglia does regulate cocaine-induced spinogenesis, but in the opposite direction as full TNF-/- mice. We suggest this occurs indirectly via TNF-regulation of astrocyte function. This work demonstrates a novel role for TNF in the regulation of dendritic spine density in the NAc core and is a component of TNF-mediated plasticity following repeated cocaine exposure.

## INTRODUCTION

Structural plasticity is a critical element of synaptic function and plasticity of neural circuits in the brain, and involves changes in dendritic spine morphology and density. Activity-dependant structural plasticity is implicated in long-term adaptations underlying drug addiction. Repeated administration of cocaine and other psychostimulants induces a persistent increase in the dendritic spine density and complexity of dendritic branching in NAc MSNs (Lee et al. 2006; Li, Acerbo, and Robinson 2004; Norrholm et al. 2003; Robinson and Kolb 1999; Shen et al. 2009). Chronic cocaine also increases spine density in the VTA and PFC (Robinson and Kolb 1999; Sarti et al. 2007). In the NAc, these structural changes last for at least 4 weeks after drug exposure ends (Shen et al. 2009; Robinson and Kolb 2004). The increased spine density is more prevalent in D1-expressing MSNs and is only maintained in D1-expressing MSNs 30 days after chronic cocaine (Lee et al. 2006). Cocaine administration also affects the morphology of individual dendritic spines: the enlargement of spines or an increased number of “mushroom”-type spines is commonly reported (Shen et al. 2009). Studies in the hippocampus and cortex indicate that changes in spine morphology correlate with increased surface expression of AMPARs and persist for months (Zuo et al. 2005; Matsuzaki et al. 2004). It is not clear how altered dendritic spine density and morphology contribute to addictive behavior, but it has been hypothesized that structural changes may solidify the initial changes of synaptic strength and underlie the prolonged changes in neural function observed in addiction (Robinson and Kolb 2004).

We previously reported that TNF plays an adaptive role in drug-induced plasticity, at the level of synaptic strength measured by AMPA/NMDA ratios on MSNs in the NAc core, but had not yet tested whether TNF could contribute to changes in dendritic spine density. The increased spine density following cocaine exposure has also been found to correlate with an increased number of functional synapses as assessed by an increase in mEPSC frequency (Kim et al. 2011). We predicted that TNF, either from microglia or astrocytes, may also regulate the formation and maintenance of dendritic spines on medium spiny neurons (MSNs) in the NAc core. There is previous evidence to suggest that TNF can regulate dendritic spines. TNF -/- mice have reduced spine density on both the basal and apical dendrites of cortical pyramidal cells (Meissner et al. 2015). Increased TNF is also associated with abnormally high turnover of dendritic spines in an EAE model (Yang et al. 2013). Similarly, peripheral elevations of TNF by a single LPS injection in mice destabilizes spines and ultimately results in a reduction in total spine density (Kondo, Kohsaka, and Okabe 2011). This was accompanied by a persistent activation of microglia (Kondo, Kohsaka, and Okabe 2011).

Both microglia (Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Paolicelli et al. 2011) and astrocytes (Risher et al. 2014; Chung et al. 2013) contribute to the activity-dependent elimination of dendritic spines. Astrocytes are also implicated in dendritic spine formation (reviewed in (Chung, Allen, and Eroglu 2015). Microglia and astrocytes release cytokines which can also alter spine morphology and affect synaptic function (Bitzer-Quintero and González-Burgos 2012; Chung, Allen, and Eroglu 2015). Given that microglia and astrocytes are both potential sources of TNF, that TNF regulates cocaine-induced plasticity and is also

implicated in neuroinflammation, we measured the dendritic spine density in the NAc in TNF-/- mice, and in mice where TNF was selectively deleted from astrocytes or microglia. We further examined whether TNF regulates cocaine-induced spinogenesis on MSNs in the NAc.

## MATERIALS AND METHODS

*Mice*

TNFα-/- (RRID: IMSR_JAX:005540) and strain-matched wildtype mice (C57Bl6/J) were acquired from Jackson Laboratories. Floxed TNFα mice were obtained from S. Nedospasov (Kuprash et al. 2005) and crossed with GFAP-Cre mice (Bajenaru et al. 2002) from NCI Mouse Repository (RRID: IMSR_NCIMR: 01XN3) or with Tg(Cx3cr1-cre)MW126Gsat mice (Yona et al. 2013) generated by N. Heintz (The Rockefeller University, GENSAT) and purchased from MMRRC (UC Davis; RRID: MMRRC_036395-UCD). GFAP or Cx3Cr1-Cre expressing mice were compared with GFAP or CX3CR1-Cre non-expressing littermates. Floxed TNFα and GFAP-Cre mice were on a C57/Bl6 background; Cx3Cr1-Cre mice were a mix of FVB/B6/129/Swiss/CD1. Experiments only used male mice at 8-10 weeks of age. Animals were housed 2-5 per cage and maintained on a 12 hour light/dark cycle. All animal procedures were performed in accordance with the guidelines of the Canadian Council for Animal Care and the Montreal General Hospital Facility Animal Care Committee.

*Immunohistochemistry*

Mice (8-10 weeks) were perfused and their brains submerged overnight in 4% PFA, cryoprotected with 30% sucrose, and cut in 30µm thick coronal slices using a cryostat. The

sections were incubated for 4 hours with blocking solution (2% normal goat serum, 3% BSA, 0.2% Triton X-100 in PBS) before incubation overnight at 4C with primary antibodies in blocking solution (rabbit anti-Iba1 antibody [019-19741, Wako; 1:800; RRID: AB_2566825]. Sections were washed (2 x 15 min with PBST [0.05% Tween20] and 1 x 15 min with PBS at RT) and incubated for 2 hrs at room temperature in secondary antibodies (anti-rabbit IgG-conjugated Alexa Fluorochrome [Invitrogen; 1:400]). Sections were washed 3x in PBS for 5min incubations. The stained sections were imaged using an Olympus FluoView FV1000 confocal microscope with Fluoview imaging software (FV10-ASW). Images of the ventral striatum (nucleus accumbens core and shell) were acquired with a 40x objective and stacks of 25-30 optical sections (1 µm per section) of depth were Z-projected into a single window and analysed using ImageJ software (NIH). Image analysis: The number of Iba1+ microglia were manually counted from 317 x 317 µm (40x objectives) images. TNF intensity was measured using the mean grey value of the entire image while Iba1 intensity measurements were obtained using a thresholded area to highlight only the microglia. The measurement of Iba1 area per microglia was determined as the total thresholded (automatic setting) area of Iba1 divided by the number of microglia in each image.

*Cocaine injections*

Mice were treated with either daily saline or cocaine in sensitization boxes (30cm x 30cm) for 7 consecutive days in the latter phase of the light cycle (20mg/kg i.p.). At 10 days after the last daily injection, the animals were anesthetized and transcardially perfused with 1.5% PFA. Cocaine was acquired from Medisca Pharmaceutique, under license from Health Canada.

*Diolistics*

After perfusion with 1.5% PFA (40mL, 10mL/minute), brains were kept in 1.5% PFA solution for 1 hour and then left in PBS. 300µm coronal slices containing ventral the ventral striatum were created using a McIllwain tissue chopper (Stoelting, Kiel, WI). Tungsten particles (1.3 µm) (Bio-Rad, Hercules, CA) carrying the lipophilic dyes DiI (Invitrogen, Carlsbad, CA), were prepared as described previously (Gan et al., 2000). Particles were coated onto the inner lining of plastic cartridges, loaded into a Helios Gene Gun (BioRad), and propelled into coronal slices at 100 psi. The dye was allowed to transport within slices for 24 h in PBS before post-fixing for 1 hour in 4% PFA. Slices were kept free floating in PBS until imaging where they were mounted directly for imaging in PBS using glass spacers.

*Image collection & analysis*

Slices were imaged within 7 days of diI labeling using an Olympus FluoView FV1000 confocal microscope with Fluoview imaging software (FV10-ASW). DiI was excited using the Helium 543 laser line. Profiles of DiI-positive MSNs from the nucleus accumbens core were acquired with a 60x objective and stacks of 30-70 optical sections (0.4-0.5µm per section) of depth were acquired. Each image a frame size of 800 x 800 pixels and field size of 212 x 212µm. For quantitative analysis, a three-dimensional perspective was rendered by the Surpass module of Imaris software (Version 7.6, Bitplane). Spine density was counted semi-automatically using the Filament Tracer plugin, based on a protocol described previously (Shen et al. 2008). The minimum end segment diameter (spine head) was set at 0.2µm, and fluorescence in each dendritic segment quantified was thresholded manually so that all discernible protuberances were identified. Only cells localized to the nucleus accumbens core were quantified, and spine

quantification occurred on dendrites at >50µm distal to the soma, and after the first branch point. Measurements were made out to a maximum of 150µm from the soma, and the length of dendrite quantified was 20-40µm. For each neuron, 1-3 dendrites was analysed. When >1 dendrite was measured, the values were averaged within each individual neuron. For each animal, at least 15 neurons were analyzed.

*Statistics*

Spine density was determined by summing the total number of spines per dendritic segment length (spines/µm). Statistics were performed using JMP 11. For gene x treatment effects, differences were detected with a two-way analysis of variance. Following two-way ANOVA, *post hoc* differences were resolved using the Fisher's Least Significant Differences (LSD) test. A *p*-value of <0.05 was considered significant. All other differences between two groups were determined using a student's t-test. All values are expressed as mean ± SEM.

## RESULTS

*TNF negatively regulates dendritic spine density and cocaine-induced spinogenesis in NAc core MSNs*

We used dil labeling of MSNs in the NAc core (representative image of an MSN shown in Figure 1A) to examine the effect of genetic deletion of TNF both on baseline and cocaine-induced spine density. Repeated cocaine administration results in an increased density of dendritic spines on NAc MSNs (Li, Acerbo, and Robinson 2004; Robinson et al. 2001; Robinson and Kolb 1999, 2004; Kim et al. 2011; Lee et al. 2006; Ferrario et al. 2005). These studies have typically measured cocaine-induced spinogenesis after a long period of withdrawal (>1 week),

but a lengthy period of abstinence after repeated cocaine is not necessarily required to increase dendritic spine density (Lee et al. 2006; Ren et al. 2010). We administered a daily dose (i.p. injection) of 20mg/kg of cocaine for 7 consecutive days, and removed brains to examine spine density after 10 days of abstinence (schematic in Figure 1B). Control mice were given daily injections of saline for 7 days and brains were removed after 10 days of no injection. We analysed dendritic spine density in both TNF -/- and WT mice following repeated cocaine or saline administration. Representative dendrites from control TNF-/- and WT animals are shown in Figure 1C. As expected, we observe a significant effect of cocaine treatment, which increases dendritic spine density on NAc core MSNs in both WT and TNF-/- mice (Figure 1D). We also report a significant effect of genotype on MSN dendritic spine density as TNF -/- have a higher baseline spine density in the NAc core (Figure 1D). Interestingly, the magnitude of spinogenesis following repeated cocaine appears to be greater in TNF-/- mice (20.3% increase compared to 11.6% increase in WT mice; Figure 1E). To further examine this, we normalized within both genotypes the spine density value of each cocaine-treated mouse to the average density of saline controls. We report a strong trend that TNF-/- animals have a larger increase in spine density following cocaine administration (student's one-way t-test, $p=0.06$). Cocaine-induced spinogenesis occurs predominantly in D1-MSNs (Lee et al. 2006; Kim et al. 2011). Moreover, TNF-mediated plasticity in the NAc following cocaine has been shown to occur predominantly on D1-MSNs (Lewitus et al. 2016). We therefore suspect that our study underestimates the effect of cocaine on spine density, both in WT and TNF-/- animals, because we have randomly sampled both D1 and D2-expressing MSNs in the NAc core.

*Microglia in the NAc of TNF-/- mice have no gross abnormalities in microglia numbers or activation*

To begin understanding why TNF -/- mice have a higher density of dendritic spines on NAc MSNs, we examined whether TNF -/- mice had a reduced density of microglia in the NAc core. It was previously shown that TNF -/- mice have a reduced number of microglia in the cortex (Lambertsen et al. 2009). Since microglia are important for synaptic pruning during development (Paolicelli et al. 2011; Schafer et al. 2012) and adult plasticity (Tremblay, Lowery, and Majewska 2010), a reduced number of microglia in the NAc of TNF-/- mice could result in an increased density of dendritic spines. In TNF -/- mice, we observed only a trend for a reduced number of microglia in the NAc core and shell (Figure 2B). To further rule out differences in microglial activation between WT and TNF-/- mice, we briefly assessed whether or not there were differences in microglia activation in the NAc of TNF-/- mice. Microglia activation is a graded process, where processes retract and thicken, and cell body size increases. We first measured the mean area of Iba1 area of each microglia (both cell body and processes) and found no significant difference in the area of Iba1 staining per microglia in either the NAc core or shell (Figure 2C). Iba1 is specifically expressed in microglia and strongly upregulated by microglia following activation. We then measured the intensity of Iba1 immunostaining and again found no significant difference in the NAc core or shell (Figure 2D). There was however a trend, especially in the NAc shell, for TNF-/- microglia to express less Iba1, which could indicate minor differences in microglia motility or their ability to detect certain signals in their environment (Kanazawa et al. 2002). Overall, we conclude that the increased spine density in TNF -/- MSNs is not associated with significant abnormalities in microglia function (density and activation state), which is important for regulating spine density.

*Role of astrocytic versus microglial TNF in the regulation of MSN spine density*

Given our previous results where microglia-derived TNF was shown to antagonize cocaine-induced potentiation of synaptic strength on D1-MSNs, we asked whether TNF regulation of dendritic spines depended differentially on the two major sources of TNF in the brain: microglia and astrocytes. Our genetic approach to this question utilized a Cre-loxP system to selectively delete TNF from microglia (CX3CR1-Cre) and astrocytes (GFAP-Cre). When TNF expression is genetically deleted in astrocytes, we observed a significant increase in dendritic spine density in NAc core MSNs (Figure 3A; GFAP-Cre negative (n=6), 1.90 +/-0.043 spines/µm; GFAP-Cre positive (n=6), 2.08 +/-0.048 spines/µm). This is in line with previous studies showing the importance of astrocyte-specific factors in regulating dendritic spine density (Allen et al. 2012; Christopherson et al. 2005; Hughes, Elmariah, and Balice-Gordon 2010; Ullian et al. 2001). Conversely, when TNF is specifically deleted in microglia, there is no significant change in dendritic spine density in NAc core MSNs (Figure 3B; CX3CR1-Cre negative (n=5), 1.85 +/-0.12 spines/µm; CX3CR1-Cre positive (n=6), 1.91 +/-0.061 spines/µm). Hence, microglial and astrocytic-derived TNF can have distinct roles in structural and synaptic plasticity. We report in Chapter 2 that repeated cocaine administration increases TNF production in microglia. Because TNF reduces cocaine-induced spine formation, we hypothesized that microglial-derived TNF likely underlies this effect of cocaine on MSN dendritic spines. Interestingly, we observed that in mice where TNF was genetically deleted in microglia, cocaine-induced spinogenesis was reduced (Figure 4).

## DISCUSSION

Our results show that TNF negatively regulates dendritic spine density in MSNs in the core of the NAc. Changes in dendritic spine density have been found to correlate with the functional number of excitatory synapses on neurons (Zhou et al. 2008), but this can depend on the type of plasticity occurring and the timescale of the plasticity (Soares et al. 2013). Manipulations that increase dendritic spine density can also increase mEPSC frequency in hippocampal cultures (Lee 2011), hippocampal slice culture (Pfeiffer 2010), and the nucleus accumbens (Kreple, 2014; Kim et al., 2011), and correlations between decreases in dendritic spine density and mEPSC frequency are also reported in hippocampal neurons (Lee 2010) and cortical slice cultures (McClelland 2010). The most relevant information for our study is that cocaine-induced increases in spine density on D1-MSNs are accompanied by an increase in mEPSC frequency, indicating that cocaine-induced increases in dendritic spines corresponds to a functional increase in excitatory synapse number (Kim et al. 2011). However, increased density of dendritic spines does not always correlate with an increase in functional synapses due to the generation of silent synapses. These are newly generated glutamatergic synapses expressing post-synaptic NMDARs but lacking functional post-synaptic AMPARs, and are therefore unable to mediate synaptic transmission under basal conditions (Hanse, Seth, and Riebe 2013). Cocaine-induced formation of silent synapses has been suggested to be involved in some addictive behaviors (Brown et al. 2011; Lee et al. 2013) and may contribute to the increase in spine density following repeated administration of cocaine (Dong and Nestler 2014; Brown et al. 2011). Nevertheless, an increase of functional synapses certainly does not exclude the possibility for an increase of silent synapses as well.

In addition to the increase in mEPSC frequency, repeated cocaine (15mg/kg dose per day, 5 days, brains removed 24hrs after last dose) decreased the amplitude and frequency of mIPSCs in D1-MSNs (and not D2-MSNs) (Kim et al. 2011). These changes in mEPSC and mIPSC frequency occur at a time when overall synaptic strength on D1-MSNs is decreased compared to naïve animals (Kourrich et al. 2007; Mameli et al. 2009; Lewitus et al. 2016). Given that many studies have detected increased spine density soon after discontinuing cocaine administration (Golden and Russo 2012), this suggests that the increased excitatory input onto MSNs precedes the overall increase in synaptic strength, as measured by AMPA/NMDA ratios. Increased surface AMPARs measured after prolonged abstinence (Wolf and Ferrario 2010; Boudreau and Wolf 2005; Conrad et al. 2008), may result from the stabilization of cocaine-induced thin spines (Cahill et al. 2016; Dietz et al. 2012) into mushroom spines with enlarged PSDs and more AMPAR surface expression, along with the un-silencing of silent synapses (Brown et al. 2011; Dong and Nestler 2014). TNF therefore antagonizes both cocaine-induced synaptic (e.g. AMPA/NMDA ratios) and structural plasticity (e.g. formation of new spines).

We argue that negative regulation of dendritic spines by TNF is largely independent of any effects that genetic deletion of TNF may have on microglial number or function, given that we do not observe any major differences in the number of microglia or the activation of microglia (Figure 2). It was previously reported that TNF-/- mice have reduced densities of microglia in the cortex (Lambertsen et al. 2009). Reduced microglia density in the hippocampus is associated with elevated dendritic spine density (Paolicelli et al. 2011; Ji et al. 2013), and LPS-induced increases in microglia number was associated with reduced spine density in the

somatosensory cortex (Kondo, Kohsaka, and Okabe 2011). Hence, a plausible explanation for why we observed an increased spine density on neurons in the NAc was that there were less microglia present. Non-activated microglia actively modulate the number of dendritic spines and functional synapses (Ji et al. 2013; Tyler and Boulanger 2012; Schafer et al. 2012; Paolicelli et al. 2011). Altered microglia activation, measured by expression of cell-surface markers and morphological changes, can therefore also have consequences for the regulation of dendritic spine density. We did not however observe any overt abnormalities in Iba1 expression or total area of microglial cells. Possibly, there could be other TNF-dependent effects that secondarily modulate microglia function. These effects could occur via subtle alterations in microglial gene expression in TNF-/- mice or via TNF regulation of signals from other cell types. For example, TNF was shown to stimulate fraktalkine expression in astrocytes, which is an important chemokine influencing microglial activity (Yoshida et al. 2001). Further analysis of microglia phenotypes in the TNF-/- mice are required to consolidate our understanding for how TNF regulates dendritic spine density.

Astrocytes are closely associated with dendritic spines, and respond to neuronal activity via neurotransmitter receptors and ion channels which then lead to the expression or release of many different factors able to regulate synaptic function (Haber, Zhou, and Murai 2006; Theodosis, Poulain, and Oliet 2008). Astrocytes control spine formation, stabilization and elimination, both during development and experience-dependent plasticity (Clarke and Barres 2013). Of particular relevance for our study is that astrocytes control basal synaptic transmission, which is the release of neurotransmitters from a single action potential (Panatier

et al. 2011). Our results demonstrate that astrocytic TNF inhibits the number of dendritic spines on NAc MSNs. Together with our previous findings that TNF regulates MSN synaptic strength (Lewitus et al. 2016; Lewitus et al. 2014), we propose that TNF derived from astrocytes may regulate basal synaptic transmission in the NAc. We have not yet characterized whether genetic deletion of TNF specifically in microglia (CX3CR1-Cre x TNF-flox) or astrocytes (GFAP-Cre x TNF-flox) affects microglia density or activation. There is the possibility that, given the important role for microglia in synaptic pruning (Zhan et al. 2014; Schafer et al. 2012), astrocytic TNF may indirectly affect the ability of microglia to prune dendritic spines. Future experiments will assess changes in morphology or expression of markers such as Iba1 and CD11b to verify whether microglia parameters in GFAP-Cre x TNF-flox mice are significantly altered.

Given that the TNF response in microglia is upregulated following repeated administration of cocaine, we initially hypothesized that TNF derived from microglia may negatively regulate cocaine-induced spine formation. This would predict that cocaine-induced spinogenesis in CX3CR1-Cre x TNF-flox mice would be significantly larger compared to Cre negative controls, similar to what we see in full TNF-/- mice. However, we observed the opposite result: less spines formed following repeated cocaine and abstinence in mice lacking microglial TNF compared to controls. We speculate that this result could be due to an indirect effect resulting from the deletion of TNF in microglia. In particular, microglial release of TNF can modulate astrocyte functions (Steelman et al. 2013; Santello, Bezzi, and Volterra 2011; Phulwani et al. 2008). There is also the possibility that compensatory increases in TNF production occurs in astrocytes, which would then reduce cocaine-induced spine formation as

observed. Characterization of TNF expression in astrocytes from CX3CR1-Cre x TNF-flox mice are needed to evaluate this possibility. Overall, our results reveal an additional mechanism of structural and functional connectivity in the brain, and how this is altered in drug-induced plasticity.

The neuronal mechanism through which TNF may regulate dendritic spines remains unknown. The dynamics of actin and cell adhesion molecules are both central players determining the formation and elimination of dendritic spines. TNF modulation of spines likely occurs via signaling proteins involved in cocaine-induced spine formation such as Rho GTPases (Russo et al. 2010; Mathew et al. 2009), Akt (Cahill et al. 2016; Wajant, Pfizenmaier, and Scheurich 2003), Cdk5 (Norrholm et al. 2003; Utreras et al. 2011), Nf-Kβ (Russo et al. 2009) and DARPP-32 (Norrholm et al. 2003; Lewitus et al. 2014). Importantly, many of these factors are known to be regulated by TNF in other contexts (Lewitus et al. 2014; Wajant, Pfizenmaier, and Scheurich 2003; Utreras et al. 2009; Sabio and Davis 2014). Investigating whether these proteins are modulated by TNF in the NAc core will provide more insight into activity-dependent structural plasticity.

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## FIGURE LEGENDS FOR CHAPTER 3

**Figure 3.1 TNF negatively regulates dendritic spine density and cocaine-induced spinogenesis in NAc core MSNs.**

**(A)** Diolistic labeling of MSNs in the NAc (scale bar 30μm). **(B)** Protocol for cocaine-induced spine formation in WT and TNF-/- mice. Adult mice were injected daily with either saline or cocaine (20mg/kg) for 7 days, which is known to produce an enduring increase in dendritic spine density in WT mice. Brains were removed for DiI labeling after a 10 day period of abstinence (no treatment). **(C)** Representative confocal images of secondary dendrites on MSNs in the NAc core from WT or TNF-/- adult mice (scale bar 10 μm). **(D)** Cocaine treatment significantly increases dendritic spine density on NAc core MSNs in both WT and TNF-/- mice (WT saline n=9, WT cocaine n=11, KO saline n=11, KO cocaine n=7; 2-way ANOVA effect of treatment: $F_{(1, 34)} = 25.05$, $p<0.0001$) in both WT mice (Fisher's LSD post-hoc $p=0.018$) and TNF -/- mice (Fisher's LSD post-hoc $p<0.0001$). TNF -/- have a higher baseline spine density in the NAc core (2-way ANOVA effect of genotype: $F_{(1, 34)} = 23.51$, $p<0.0001$; Fisher's LSD post-hoc $p=0.023$). **(E)** The magnitude of spinogenesis following repeated cocaine is larger in TNF-/- versus WT mice (normalized to mean saline-treated values for each genotype, n=11 WT and n=7 TNF-/-; Student's one-way t-test, $t(16)= -1.63$, $p=0.06$).

**Figure 3.2 Microglia in the NAc of TNF-/- mice have no gross abnormalities in microglia numbers or activation.**

**(A)** Coronal slices of the NAc were obtained from WT and TNF-/- and immunostaining with the microglia marker Iba1 was used to quantify the number of microglia per image in the NAc core and shell (317 x 317 μm and 800 x 800 pixels, 8-10 images collected per animal). **(B)** The average number of microglia (Iba1 positive cells) per field of view (317 x 317 μm). No significant difference is observed between WT and TNF-/- mice in the NAc core (WT (n=4), 26.6 +/-1.44 microglia/image; TNF -/- (n=4), 23.96 +/-1.25 microglia/image; student's t-test, t(6)=1.39, p=0.21) and shell (WT, 22.82 +/-1.42 microglia/image; TNF -/-, 21.44 +/-2.34 microglia/image; student's t-test, t(6)=1.04, p=0.34). **(C)** The total Iba1 area per image is divided by the total number of microglia to obtain an estimate of microglia size (both cell body and processes). No significant change occurs in TNF-/- mice compared to WT in the NAc core (WT, 344.63 +/-20.58 Iba1 area (um^2)/microglia; TNF -/-, 342.94 +/-16.99 Iba1 area (um^2)/microglia; student's t-test, t(6)=0.064, p=0.95) or shell (WT, 370.65 +/-22.95 Iba1 area (um^2)/microglia; TNF -/-, 377.99 +/-22.16 Iba1 area (um^2)/microglia; student's t-test, t(6)=0.23, p=0.83). **(D)** Overall Iba1 intensity in all the microglia for each image in WT and TNF-/- mice from the core (WT, 344.63 +/-20.58 Iba1 area (WT, 1.00 +/-0.055 normal Iba1 intensity; TNF -/-, 0.86 +/-0.065 normal Iba1 intensity; student's t-test, t(6)=1.70, p=0.14) and shell (WT, 1.00 +/-0.034 normal Iba1 intensity; TNF -/-, 0.90 +/-0.035 normal Iba1 intensity; student's t-test, t(6)=1.99, p=0.093).

**Figure 3.3 Role of astrocytic versus microglial TNF in the regulation of MSN spine density.**

**(A)** Floxed TNF mice were crossed with GFAP-Cre mice and compared with non-expressing littermates. Genetic deletion of TNF in astrocytes (GFAP expressing cells) results in an increased spine density on NAc core MSNs (n=6 Cre positive and n=6 Cre negative mice; Student's t-test, $t(11)=2.75$, $p=0.021$). **(B)** Floxed TNF mice were crossed with CX3CR1-Cre mice and compared with non-expressing littermates. There was no effect of TNF deletion in microglia on spine density on NAc core MSNs (n=6 Cre positive and n=5 Cre negative; Student's t-test, $t(9)=0.44$, $p=0.67$).

**Figure 3.4 Role of astrocytic versus microglial TNF in the regulation of MSN spine density.**

The magnitude of spinogenesis following repeated cocaine administration and abstinence is smaller in CX3CR1-Cre negative (control) littermates compared to CX3CR1-Cre positive animals, normalized to mean non-treated values from Figure 3B (n=4 Cre positive and n=4 Cre negative, Student's t-test, $t(6)=-2.29$, $p=0.67$).

## FIGURES FOR CHAPTER 3

### FIGURE 3.1

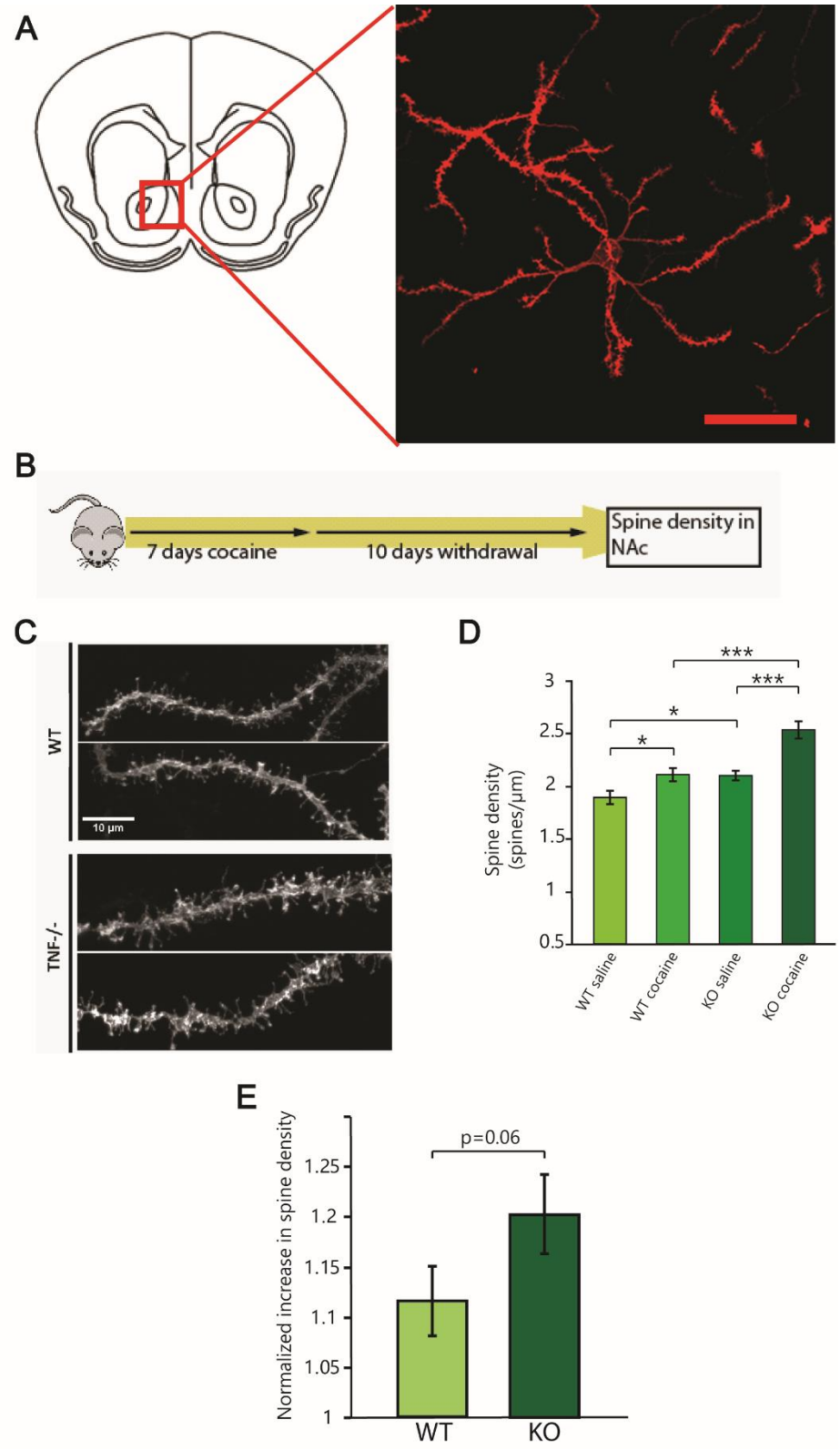


**FIGURE 3.2**

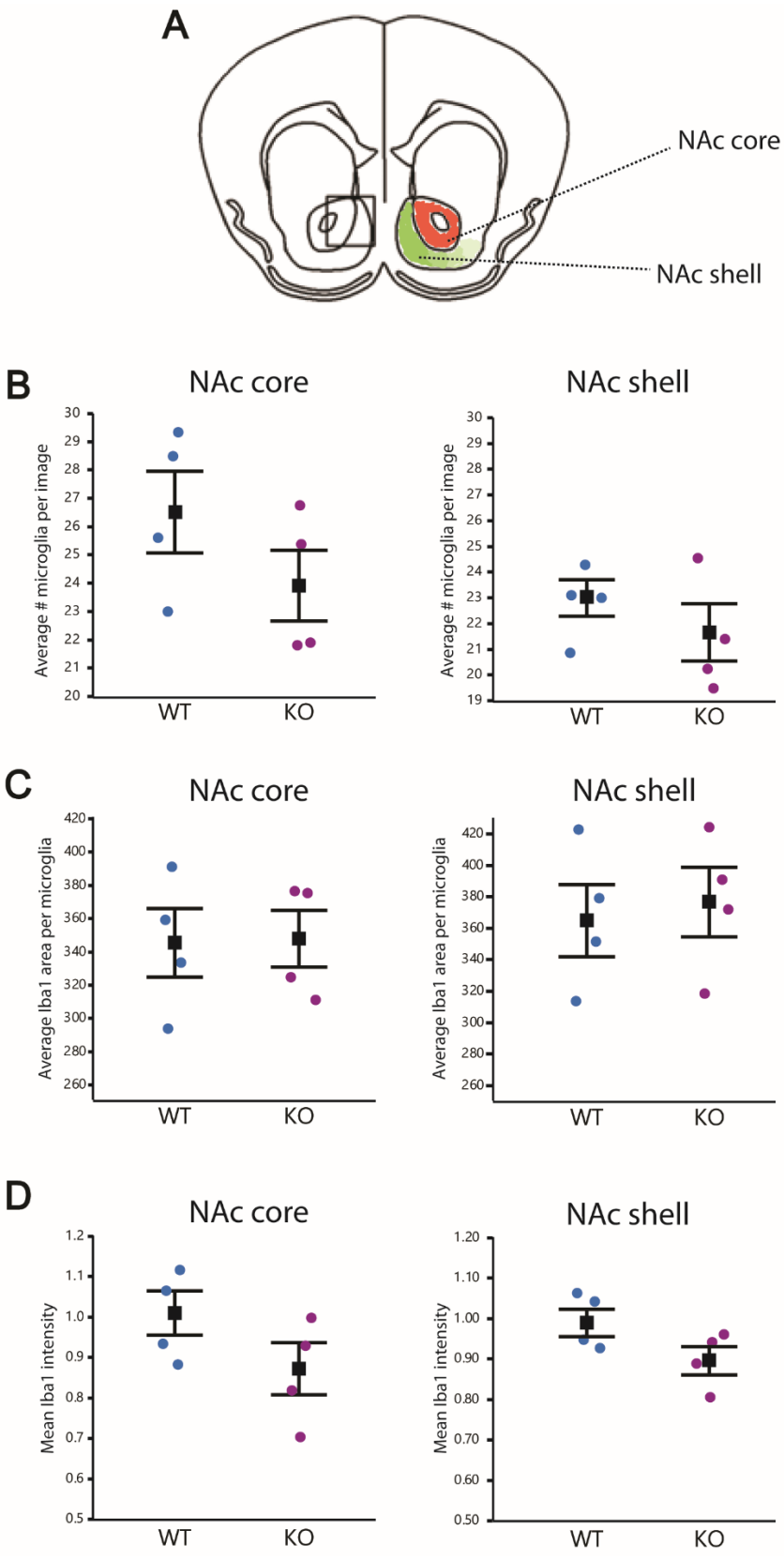

A
NAc core
NAc shell
B
NAc core
NAc shell
Average # microglia per image
WT
KO
C
NAc core
NAc shell
Average Iba1 area per microglia
WT
KO
D
NAc core
NAc shell
Mean Iba1 intensity
WT
KO

**FIGURE 3.3**

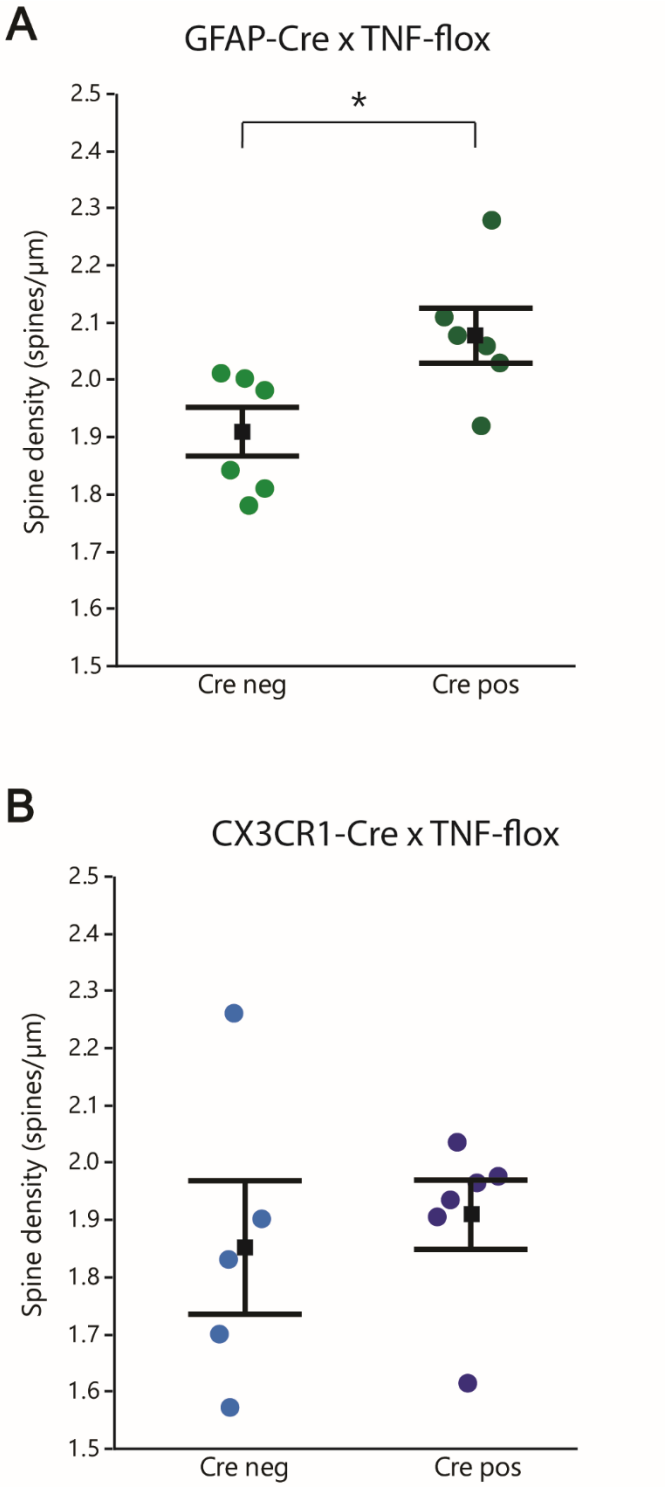
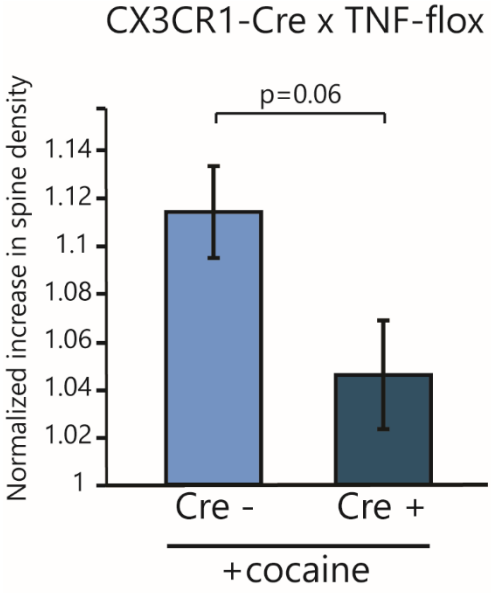

A
GFAP-Cre x TNF-flox
*
Spine density (spines/µm)
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
Cre neg
Cre pos
B
CX3CR1-Cre x TNF-flox
Spine density (spines/µm)
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
1.7
1.6
1.5
Cre neg
Cre pos

**FIGURE 3.4**


CX3CR1-Cre x TNF-flox
p=0.06
Normalized increase in spine density
1.14
1.12
1.1
1.08
1.06
1.04
1.02
1
Cre -
Cre +
+cocaine

## PREFACE TO CHAPTER 4

TNF-mediated homeostatic plasticity is an example of intrinsic regulation of neuronal function by an immune signaling molecule. An adaptive response mediated by TNF, such as that described in Chapter 2, could play a role in mitigating behavioral changes induced by Maternal Immune Activation (MIA). MIA causes region-specific changes in the expression of cytokines in the brain, including TNF which persist throughout adulthood (Garay et al. 2013; Krstic et al. 2012). It is thought that a general increase in brain neuroinflammation underlies abnormal synapse and circuit formation (Knuesel et al. 2014) which then lead to dysregulated behaviors consistent with neuropsychiatric disorders such as autism (Malkova et al. 2012; Smith et al. 2007) and schizophrenia (Patterson 2011).

The contribution of TNF to MIA-induced behavioral deficits has not been examined to date and this is the first investigation using TNF-/- mice in an MIA model. While we do not observe any behavioral differences in TNF-/- mice in behavioral tests assessing social and anxiety related behavior, these results are important for delineating the effects of different cytokines or other immune signaling factors on the abnormal developmental trajectory of MIA offspring.

**CHAPTER 4**

# TNF-MEDIATED HOMEOSTATIC SYNAPTIC PLASTICITY IN BEHAVIORAL MODELS: TESTING A ROLE IN MATERNAL IMMUNE ACTIVATION

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## ABSTRACT

The pro-inflammatory cytokine tumor necrosis factor alpha (TNF) has long been characterized for its role in the innate immune system but more recently has been found to have a distinct role in the nervous system that does not overlap with other pro-inflammatory cytokines. Through regulation of neuronal glutamate and GABA receptor trafficking, TNF mediates a homeostatic form of synaptic plasticity, but plays no direct role in Hebbian forms of plasticity. As of yet, there is no evidence to suggest that this adaptive plasticity plays a significant role in normal development, but it does maintain neuronal circuit function in the face of several types of disruption. This includes developmental plasticity in primary sensory cortices, as well as modulating the response to anti-depressants, chronic anti-psychotics, and drugs of abuse. TNF is also a prominent component of the neuroinflammation occurring in most neuropathologies, but the role of TNF-mediated synaptic plasticity in this context remains to be determined. We tested this in a maternal immune activation (MIA) model of neurodevelopmental disorders. Using TNF-/- mice, we observed that TNF is not required for the expression of abnormal social or anxious behavior in this model. This indicates that TNF does not uniquely contribute to the development of neuronal dysfunction in this model, and suggests that during neuroinflammatory events, compensation between the various pro-inflammatory cytokines is the norm.

## INTRODUCTION

*TNF-mediated synaptic plasticity in the brain*

Receptor trafficking is an underlying mechanism of synaptic plasticity, which comes in two basic forms – Hebbian plasticity and homeostatic synaptic plasticity (HSP). Hebbian plasticity is thought to be the synaptic mechanism of learning and memory, and involves activity-dependent changes at individual synapses. HSP is part of a circuit-level negative feedback system, where glutamatergic and GABAergic synaptic strengths are inversely regulated to stabilize circuit function. HSP and Hebbian plasticity are theoretically both required for normal circuit function: HSP is designed to maintain circuit function while Hebbian plasticity is designed to change circuit function so that the same input will now lead to a different output/behaviour (a minimalistic framing of learning). These processes must be balanced – pure HSP would prevent any learning, while if Hebbian processes dominate, the network becomes very unstable. Given the specificity of TNF to HSP and not Hebbian plasticity (Stellwagen and Malenka 2006), this is a potential route to probe the role of HSP in neurodevelopment and behaviour.

TNF appears to not be essential for normal development, as mice lacking TNF or TNF receptors are largely normal in terms of cytoarchitecture and baseline behaviours. A number of subtle behavioural changes have been reported in TNF and TNF receptor knockout mice, with inconsistent results. For example, spatial learning is either better (Golan et al. 2004), worse (Baune et al. 2008), or unchanged (Yamada et al. 2000) in TNF mutant animals. Similarly, changes in baseline anxiety and depressive-like behaviours (such as the open field, elevated

plus maze, light/dark box, and forced swim test) are sometimes (Yamada et al. 2000; Patel, Siegel, and Zalcman 2010) (Simen et al. 2006) but not always observed (Duseja et al. 2015; Fursenko et al. 2016). Overall, the behavioural phenotype is quite mild, which suggests that HSP (at least the TNF-mediated form of it) is not required for the majority of normal development, and perhaps only becomes engaged when circuit function is substantially perturbed. Such perturbations can reveal roles for TNF in development. For example, TNF is required for some forms of developmental plasticity in sensory cortices (Kaneko et al. 2008b; Ranson et al. 2012b), where it is necessary for part of the re-normalization of circuit function when sensory input is substantially altered. During monocular deprivation in adolescent (but not adult) mice, there is an initial loss of closed eye responses in the primary visual cortex that presumably occurs through Hebbian mechanisms. There is a subsequent gain of response to the open eye, which is at least partially homeostatic in nature and requires TNF signaling (Ranson et al. 2012b; Kaneko et al. 2008b). Further, abnormal elevations in TNF can perturb normal development (Lee et al. 2010b; Gilmore et al. 2004b), with reduced dendritic length and complexity in cultured neurons, and premature synapse maturation and stabilization resulting in less refinement during development in vivo. This would suggest that inflammatory stimuli during development could have consequences for later neural function.

TNF also appears to be required for the development of adaptive behaviours in the adult, particularly in response to chronic psychoactive drugs. We have recently shown that TNF is required for the behavioral response to antidepressants, as measured by the ability of fluoxetine and desipramine to reduce immobility in the forced swim and tail suspension tests (Duseja et al. 2015). This suggests a role for cytokines in regulating depressive behaviours.

Further, TNF mediates synaptic changes within the striatum and acts to reduce the behavioural consequences of chronic administration of antipsychotic (Lewitus et al. 2014b) and drugs of abuse (Lewitus et al. 2016). As noted above, TNF drives the endocytosis of AMPARs on the GABAergic medium spiny neurons (MSNs) of the striatum, reducing excitatory synaptic strength (Lewitus et al. 2014b). Chronic administration of classic antipsychotics (such as haloperidol) leads to synaptic potentiation within the striatum, and results in the development of dyskinetic motor problems, characterized by uncontrolled movements particularly of the face and neck. However, the chronic presence of drug seems to induce a homeostatic response, where TNF is elevated in the striatum and reduces both excitatory synaptic strength on the MSNs and the severity of the dyskinetic behaviour (Lewitus et al. 2014b). Blocking TNF signaling, even acutely, will cause an increase in dyskinesic movements. Similar changes are seen during the repeated administration of cocaine (Lewitus et al. 2016), which likewise causes synaptic potentiation within the striatum that accompanies the sensitization of the locomotor response to the drug (Lüscher and Malenka 2011). Cocaine also activates striatal microglia, which increase TNF production that in turn reduces excitatory synaptic strength on MSNs and decreases the locomotor sensitization to cocaine (Lewitus et al. 2016). Importantly, increasing the microglial response can reduce previously established cocaine-induced sensitization (Lewitus et al. 2016). As the development of addiction is thought to be an aberrant form of learning (Lüscher and Malenka 2011), these data suggest that Hebbian and homeostatic plasticity can act to counter-balance each other, with a TNF-mediated homeostatic response acting to limit the changes induced by overactive Hebbian-type learning.

Thus, TNF can actively modify behaviour in the adult animal, which could suggest that abnormal elevation of TNF in an adult could likely have behavioural consequences. We have explored the role of TNF in a variety of behaviours without specific immune activation; here, we sought to test if TNF was essential for the behavioural changes induced by an inflammatory state. To do this, we utilized the maternal immune activation model of neurodevelopmental disorders.

*Implications for TNF in an MIA model of neurodevelopment disorders*

Maternal infection during pregnancy, referred to as maternal immune activation (MIA), is an environmental risk factor for both schizophrenia and autism (Meyer 2014; Knuesel et al. 2014). In animal models, MIA is typically achieved by injecting pregnant mice with the viral mimic and immunostimulant, Polyinosinic:polycytidylic acid (Poly I:C), although bacterial mimic lipopolysaccharide (LPS) is also used. Offspring from injected pregnant mice develop key behavioral phenotypes as well as molecular and cellular signatures that are indicative of neurodevelopmental disorders including schizophrenia and autism. The phenotype of the MIA offspring depends on whether the mother's immune system is activated mid or late gestation, which can shift the model from schizophrenia to autistic phenotypes (Knuesel et al. 2014). However, the behavioral and cellular phenotypes of these disorders overlap greatly in animal models.

MIA triggers long-term behavioural alterations in the offspring. Importantly, infectious agents do not act directly on the developing fetal brain, but instead activate several immune signaling pathways and lead to primed immune function in the offspring via increased

expression of cytokines and other immune factors, which is maintained in adulthood (Onore et al. 2014; Han et al. 2011; Garay et al. 2013; Hsiao et al. 2012). This altered immune status may contribute to abnormal neurodevelopment and/or continued synaptic dysfunction in MIA offspring. Moreover, changes in immune function are prominent in both autism and schizophrenia-related disorders (Knuesel et al. 2014; Onore et al. 2014; Patterson 2009), including elevated levels of TNF (Patterson 2009; Hope et al. 2013; Goldsmith, Rapaport, and Miller 2016; Chez et al. 2007; Li et al. 2009; Saetre et al. 2007). In the MIA animal model, the exact immune signature in MIA offspring depends on the type of stimulation and timing of the immune activation in the mother (Knuesel et al. 2014). Importantly, though, most pro-inflammatory cytokines, including TNF, are elevated at various stages of postnatal development and in adulthood in most versions of MIA (Meyer 2014). This is observed in both serum (Han et al. 2011; Basta-Kaim et al. 2012; Krstic et al. 2012) and the brain (Garay et al. 2013; Krstic et al. 2012), and in response to immune stimulation (Hsiao et al. 2012; Krstic et al. 2012; Giovanoli et al. 2016; Schwartzer et al. 2013).

The elevated levels of TNF in this model suggest that TNF could contribute to the behavioural abnormalities of MIA offspring. This could occur as part of the ongoing dysfunction induced by the immune activation or as an adaptive mechanism to offset disrupted neuronal development. Further, glial activation has been observed in MIA models, with both microglia (Schwartzer et al. 2013; Krstic et al. 2012) and astrocyte function (Borrell et al. 2002; Fatemi et al. 2002) being affected. Increased expression of inflammatory modulators and morphological changes indicative of an activated state are observed in microglia from adult MIA offspring (Krstic et al. 2012; Giovanoli et al. 2013; Juckel et al. 2011). Because TNF is released by activated

microglia and astrocytes depending on the context (for example, see (Duseja et al. 2015; Lewitus et al. 2016)), altered glia function in MIA offspring would also suggest that TNF-mediated plasticity could be involved in an MIA model. Behavioural abnormalities (in MIA or other models) are presumed to result from synaptic changes that change neuronal circuit function. Reports do indeed indicate that MIA leads to alterations in synaptic transmission and plasticity in the brain (Giovanoli et al. 2016; Ducharme et al. 2012; Escobar et al. 2011; Lowe, Luheshi, and Williams 2008; Oh-Nishi et al. 2010; Roumier et al. 2008; Patrich et al. 2016). TNF could provide a mechanism for these changes, perhaps acting as a dysregulated version of HSP. TNF is critical for maintaining E/I balance following changes in circuit activity (Stellwagen and Malenka 2006), resulting from activity-dependant trafficking of AMPAR and GABAR receptors. Altered synaptic transmission in MIA models has been reported as an increase in mEPSC amplitude and decrease in mEPSC frequency (Ito et al. 2010). An MIA-induced reduction in neural connectivity has also been reported. In cultured cortical neurons from MIA offspring, spine density and mEPSC frequency is decreased (Elmer et al. 2013); similarly, the number and turnover of dendritic spines in vivo has also been demonstrated (Coiro et al. 2015).Importantly, the altered structural and functional synaptic connectivity in MIA offspring is prevented by early anti-inflammatory treatment with ibudilast for the first two post-natal weeks (Coiro et al. 2015).

As both a pro-inflammatory cytokine and mediator of HSP, TNF provides an avenue to study the potential role of HSP in a neurodevelopmental model of psychiatric disorders. Importantly, previous studies have indicated that TNF does not play a role in the early transmission of inflammatory signaling to the fetus (Smith et al. 2007; Ratnayake et al. 2014)

before post-natal development. Additionally, in the Poly I:C model, TNF levels are reported to decrease initially in the fetal brain (Gilmore et al. 2004b; Garbett et al. 2012; Ratnayake et al. 2014). Hence if TNF-mediated plasticity regulates the neurodevelopment of the MIA offspring, this regulation would likely occur after the animal was born.

Core symptoms of both autism and schizophrenia include deficits in social interaction (Patterson 2009; Schwartzer et al. 2013; Malkova et al. 2012; Zuckerman and Weiner 2005; Ozawa et al. 2006; O'Tuathaigh et al. 2009). In mouse models, this is investigated by measuring the preference mice typically exhibit for an unfamiliar mouse over an unfamiliar object or empty chamber (Silverman et al. 2010). This preference is significantly reduced in Poly I:C offspring. Investigations into gene x environment interaction have frequently used the social approach test and found interactions between genotype and maternal immune activation (e.g. (Schwartzer et al. 2013; Abazyan et al. 2010; Ibi et al. 2010)) although some studies have focused on other behavioral read-outs (Vuillermot et al. 2012; Wu et al. 2015). Previous evidence has suggested that TNF signaling does contribute to this behavior as TNF-/- mice showed a baseline preference for a stranger mouse as compared to an empty containment cup in the 3-chamber paradigm, as measured by total interaction time (74). Interestingly, the same authors did not report behavioral differences for TNFR1-/- or TNFR2-/- mice.

It is less clear whether or not TNF signaling basally contributes to anxiolytic behavior in the elevated plus maze. Some reports demonstrate no difference in TNF-/- mice (Fursenko et al. 2016; Camara et al. 2015) but TNF-/- mice are also reported to be generally less exploratory and more anxious in the EPM test (Yamada et al. 2000). However, TNFR1 and TNFR2 KO mice have lower levels of anxiety as judged by an increased time in the center of the open field test and in

the light compartment of the light-dark box test (Patel, Siegel, and Zalcman 2010). Interestingly, anxiety behaviors are hypothesized to be related to E/I imbalance, especially in the limbic areas of the brain (Nuss 2015). Further, maternal immune activation increases limbic expression of GABA receptors, which correlates with observed decreased exploratory behavior (Nyffeler et al. 2006). MIA also results in decreased GABAergic transmission in the medial prefrontal cortex (Canetta et al. 2016).

Here we tested the contribution of TNF to the MIA-induced behavioural changes in social approach and the EPM. However, TNF-/- mice had similar MIA-induced changes as their wildtype counterparts, suggesting that TNF signaling is not essential to the development or expression of these immune-induced behavioural changes.

## MATERIALS AND METHODS

All animal procedures were approved by the Montreal General Facility Animal Care Committee, in accordance with the guidelines of the Canadian Council for Animal Care.

*Breeding*

All mouse lines were initially acquired from Jackson Labs. Females used for timed mating experiments were 8-12 weeks old (on the C57BL/6J background; the TNF-/- mice were back-crossed for minimum of 6 generations by Jackson Labs). Breeding pairs for TNF-/- mice (in-house colony) are maintained by back-crossing to wild-type C57BL/6J mice. At the time of the experiments performed here, TNF KO colony had been in-house for 3 generations, and

backcrossed to an in-house C57BL/6J colony every 2nd generation. Timed pregnancies were set up by placing bedding from a male mouse in cages for 4-5 females 2-3 days before mating. Groups of 4-5 females were then placed overnight in a cage with one male. Successful mating was verified the next morning by the presence of a vaginal plug, and the day after was referred to as gestational day 0.5. Because a plug does not always indicate that a female is pregnant, female mice were also weighed at gestation day (GD) 12.5 and only those with significant weight gain were injected with saline or Poly I:C. Pregnant dames on GD 12.5 received either a single intraperitoneally injection of 5mg/kg Poly I:C (potassium salt at 1mg/ml; Sigma-Aldrich) or vehicle (sterile saline). The dose of Poly I:C was chosen based on previous studies in C57BL/6J mice (39, 78, 79). All solutions were freshly prepared on the day of administration and injected with a volume of 5ml/kg. Animals were returned to their home cages immediately after the injection procedure. Resulting offspring are weaned at postnatal day 24-28. Behavioral data was obtained from male offspring from two separate cohorts. The social approach test was performed at 8 weeks of age, and the elevated plus maze was subsequently performed at 12 weeks of age. The first cohort contained n=4 WT dames (Poly I:C, n=2; Saline, n=2) and n=4 TNF KO dames (Poly I:C, n=2; Saline, n=2). This cohort comprised 12 WT males and 15 KO males (WT Saline, n=7; WT Poly I:C, n=5; KO Saline, n=8; KO Poly I:C, n=7), and all males from the first cohort were put through the social approach test only. The second cohort contained n=3 WT dames (Poly I:C, n=1; Saline, n=2) and n=4 TNF KO dames (Poly I:C, n=2; Saline, n=2). This cohort comprised 12 WT males and 14 KO males (WT Saline, n=8; WT Poly I:C, n=5; KO Saline, n=5; KO Poly I:C, n=7), and all males from the second cohort were put through the social approach test and the elevated plus maze.

*Behavioral testing – social interaction*

The social interaction test apparatus was made of Plexiglas and consisted of two identical chambers (20cm x 20cm) connected to each other by a third chamber (7cm x 20cm). The Plexiglas walls of the apparatus are covered with different patterns (stripes or polka dots) on each side. We first established that mice had no preference for either side. All animals were habituated to the test apparatus on the day of testing to reduced novelty-related locomotor hyperactivity. Two plastic containment cups (with holes to allow permeation of olfactory cues) were left empty during habituation. Each test mouse was gently placed in the middle chamber and allowed to explore the apparatus for 5 minutes. Following habituation, the test mouse was isolated (using Plexiglas sliders) in the middle chamber while an unfamiliar male mouse of the same age (8 weeks) and background (C57BL/6J) from a non-experimental cage was placed inside one of the plastic containment cups located in one of the chambers. The plastic containment cup in the opposite chamber was left empty. Subjects were allowed to explore the 3-chamber apparatus for 10 minutes. Each mouse received one trial and all testing chambers were cleaned thoroughly with 30% ethanol between testing session. Experiments were conducted in a dim lighting provided by two incandescent red lights on either side of the testing arena approximately 6 feet from the ground.

*Behavioral testing – elevated plus maze*

One month after the social approach test, the second cohort of mice from saline or Poly I:C treated mothers were tested in the elevated plus maze. The apparatus used for the elevated

plus maze test comprises two open arms (25 x 5 x 0.5cm) across from each other and perpendicular to two closed arms (25 x 5 x 16cm) with a centre platform (5 x 5 x 0.5cm). The open arms have a very small (0.5cm) wall to decrease the number of falls, whereas the closed arms have a high (16cm) wall to enclose the arm. The entire apparatus is 50cm above the floor, and made of light grey plexiglass. During testing, the mouse is placed in the centre area of the maze with its head directed toward a closed arm and allowed to move freely in the maze for 5 minutes. Each mouse received one trial. Experiments were conducted in a dim lighting provided by two incandescent red lights on either side of the testing arena approximately 6 feet from the ground. Mice that fell off the elevated plus maze (2 KO Poly I:C mice) were excluded from the analysis. The trials were recorded using a video camera attached to a computer, and the number of entries into each arm and the time spent in the open arms manually scored. These measurements serve as an index of anxiety-like behavior.

*Behavioral analysis*

Behavioural tests were monitored and recorded with a camera placed above the 3-chamber apparatus and EPM. Videos were analysed with Ethovision XT video tracking software from Noldus. Behavioral data was coded by personalizing Arena Tracking settings. In the social approach test, the animal was measured for the time spent in each chamber (e.g. the centre, and the chambers with the stranger mouse or empty containment cup), as well as a proximal circular zone around both containment cups in the opposite corners of the chambers. Because the Ethovision software measures only the centre point of the mouse, we selected a distance from the cup where that if the mouse's head was next to the cup, its centre point would be

within the proximal zone, which was approximately a distance of 3cm. The total time and entries (frequency) in different zones as well as the total distance traveled during the test was collected. For the EPM test, we collected the total time and entries into the open and closed arms as well as the centre zone.

*Statistical analysis*

Data were analyzed in JMP11 using a two-way (factorial) analysis of variance (ANOVA) to compare the main effects of genotype (WT or KO) and treatment (saline or Poly I:C), and the interaction between genotype and treatment. Pairwise comparisons were made using a post-hoc Fisher LSD test. $P < 0.05$ was used for the significance level.

## RESULTS

*TNF is not required for loss of social preference in MIA offspring*

To investigate the role of TNF in the behavioural changes in adult MIA offspring, we tested social approach in TNF mutant mice, using a Poly I:C MIA model. Abnormalities in social approach and social novelty in the 3-chamber test are some of the most reported and validated behavioral changes in offspring from Poly I:C injected mothers (Meyer 2014). Initially, we verified that we were able to reproduce these findings, showing that wild-type mice from saline treated mothers (controls) exhibit normal social preference for a novel mouse over an empty chamber (Figure 4.1A-D). We calculated a preference ratio for a proximal zone around the cup with the mouse versus the empty cup (Figure 4.1A, B). The preference ratio takes into account that both the time spent proximal to the cup with mouse and proximal to the empty cup. MIA is

known to reduce the normal social preference in the offspring (Smith et al. 2007; Malkova et al. 2012). This social behavior deficit is consistent in offspring from immune-stimulated rodents (single LPS or Poly I:C injection) across different time-points during gestation (Onore et al. 2014; Patterson 2009; Schwartzer et al. 2013; Smith et al. 2007). We were able to demonstrate that the WT adult offspring from Poly I:C treated mothers displayed a reduced social preference towards un unknown stranger mouse, consistent with previous results (Figure 4.1C, 2-way ANOVA effect of treatment: $F(1, 46) = 4.51$, $p<0.05$). Social preference was also measured by the time spent in the chamber with the mouse, as a fraction of the total testing time (10 min) spent exploring the entire apparatus. The main effect of Poly I:C treatment was again significant ($F(1,46) = 19.94$, $p<0.0001$), with the WT Poly I:C mice spending significantly less time with the stranger mouse compared to saline controls.

We then tested the contribution of TNF to the MIA-induced loss of social preference, using TNF-/- mice. We did observe a significant effect of genotype for both measurements of sociability (proximal approach: $F(1, 46) = 4.23$; chamber time: $F(1, 46) = 10.11$, $p<0.01$), but this was due to differences in the Poly I:C groups and not from differences in baseline social preference between WT and TNF-/- mice. But critically, we did not observe a genotype-dependent response to the Poly I:C treatment. For both the proximal preference ratio (Figure 4.1C) and fraction of time in the mouse chamber (Figure 4.1D), the interaction effect of genotype and treatment was insignificant (proximal approach: $F(1, 46) = 0.995$, $p=0.32$; chamber time: $F(1, 46) = 0.58$, $p=0.45$), demonstrating that KO offspring responded in the same way to MIA as WT offspring in the 3chamber social approach test. As in WT offspring, the KO Poly I:C group exhibited significantly lower preference ratio for the stranger mouse ($p=0.045$)

and spent less time in the chamber with the stranger mouse (p=0.0087). These results indicate that TNF is not required for the development and expression of social preference in adult mice, nor is it essential for the loss of normal social preference in MIA offspring.

We expected that the decreased time spent exploring and/or approaching the containment cup with the mouse would be accompanied by an overall decrease in frequency to enter the proximal zone, compared with entries into the proximal zone of the empty containment cup. We computed the ratio of social to non-social approaches (Figure 4.1E), and observed an overall shift to non-social approaches for MIA-treated animals (2-way ANOVA, effect of treatment: $F(1,45) = 4.97$; $p=0.031$), as well as slightly higher numbers of approaches in the KO animals (effect of genotype: $F(1,45) = 6.07$; $p=0.018$). But while we observed similar shifts in both WT and KO animals from Poly I:C treated backgrounds, none of the individual changes were significant in post-hoc analysis. So overall, MIA had similar effects on WT and TNF-/- mice and we do not observe any significant interaction between TNF and maternal Poly I:C treatment ($F(1, 45) = 0.097$, $p=0.76$).

We also analysed the locomotor behavior of all the groups tested in the social approach paradigm and did not observe a change in locomotion based on treatment. We did observe a slight difference in baseline locomotion between WT and KO mice (from saline treated mothers) in the 3-chamber apparatus (Figure 4.1F, $p=0.012$). We have not previously observed a decreased locomotor phenotype in TNF-/- mice (as measured in the open field test (Lewitus et al. 2016)), and also do not observe a baseline change in exploratory behavior in TNF KO mice in the EPM test (Figure 4.2G), which suggests that the baseline exploratory difference in KO mice is specific to the 3-chamber apparatus.

*TNF is not required for the change in anxiety-related behaviour induced by MIA*

We next compared the effect of MIA on the expression of anxiety-like behaviours in WT and TNF-/- mice, which typically is increased in adult offspring from immune-stimulated mothers (Canetta et al. 2016; Babri, Doosti, and Salari 2014). We observed no significant differences in baseline anxiolytic behavior between saline and TNF-/- mice in any of the measurements, looking at time or frequency in the different zones of the EPM (Figure 4.2C-F). In contrast to previous findings on the effect of maternal immune activation on anxiolytic behavior in the elevated plus maze, we observed a very pronounced and significant effect of Poly I:C treatment on exploration time in the EPM open arms (2-way ANOVA effect of treatment: $F(1, 18) = 39.87$, $p<0.0001$), accompanied by a significant decrease in time spent in the EPM closed arms (2-way ANOVA effect of treatment: $F(1, 18) = 33.6$, $p<0.0001$). This was significant for both WT and TNF-/- offspring and there was no evidence for an interaction between maternal immune activation and genotype (2-way ANOVA effect of interaction open arm time: $F(1, 18) = 0$, $p=0.997$; closed arm time: $F(1, 18) = 0.020$, $p=0.89$). TNF-/- offspring from Poly I:C mothers may become slightly more exploratory as they spend significantly more time in the centre (Figure 4.2E, $p=0.015$). However, both genotypes demonstrate the same frequency to the open arms, as well as a similar locomotor response to Poly I:C (Figure 4.2G-H), and there is no evidence of an interaction between genotype and maternal immune activation for time in centre, frequency to open arms and total locomotion (Respectively, effect of interaction $F(1,18) = 0.69$, $p=0.42$; $F(1, 18) = 0.16$, $p=0.88$; $F(1, 18) = 0.31$, $p=0.59$). Overall, we conclude that maternal immune activation results in decreased anxiety-like behaviour in the

elevated plus maze, and that TNF is dispensable for the development of this immune-driven behavioural change.

## DISCUSSION

TNF is known to have important neuroregulatory functions (Stellwagen et al. 2005b; Beattie et al. 2002b; He et al. 2012b), and modulates homeostatic plasticity in vitro (Stellwagen and Malenka 2006) and in vivo (Kaneko et al. 2008b; Ranson et al. 2012b). But further work is needed to establish the role of TNF-mediated plasticity in different models of disease, under both non-inflammatory and inflammatory contexts. Previously we have shown that TNF-mediated plasticity is required for normal behavioral response to antidepressants (Duseja et al. 2015), and that TNF mediates several adaptive responses in the striatum when circuit activity is acutely perturbed by drugs of abuse (Lewitus et al. 2016) or antipsychotics (Lewitus et al. 2014b). Our goal here was to evaluate the role of TNF-mediated regulation of circuit function during inflammatory neurodevelopment conditions, using the MIA model of neuropsychiatric disorders.

We saw no differential MIA-induced behavioral deficits in TNF-/- mice compared with WT mice, and conclude that TNF-mediated signaling is not critical for the long-term alterations in neurodevelopment caused by MIA. Specifically, our results indicate that neither the social indifference nor the changes in anxiety seen in MIA models require TNF signaling at the time-point and dose of Poly I:C investigated. At least by these measures, TNF is dispensable for the development or maintenance of MIA-induced behavioural change in this model.

We should note that in our hands, offspring of MIA mothers displayed a lower level of anxiety, as measured in the EPM. Although studies to date largely indicate that MIA offspring exhibit higher levels of anxiety (Abazyan et al. 2010; Canetta et al. 2016; Babri, Doosti, and Salari 2014), the genetic background of mice plays a large role in the behavioral outcome of MIA (Schwartzer et al. 2013), particularly for anxiety-related phenotypes (Babri, Doosti, and Salari 2014). Specifically, MIA was found to increase anxiety-like behavior of offspring in NMRI mice but not in C57BL/6J mice (as used here) (Babri, Doosti, and Salari 2014). Interestingly, NMRI offspring displayed behaviour consistent with elevated anxiety only in the elevated plus-maze but not in other paradigms such as the open field and light-dark box (Babri, Doosti, and Salari 2014). Strain-treatment interactions were also observed between C57BL/6J and BTBRT+tf/J mice for other behavioural deficits in the MIA model including decreased sociability and increased repetitive/stereotyped behavior (Schwartzer et al. 2013). Importantly, offspring from both strains respond to maternal Poly I:C injections with increased exploratory behavior in the EPM. Other studies have reported no change in anxiety behavior in the elevated plus maze with maternal Poly I:C treatment alone (Giovanoli et al. 2013; Abazyan et al. 2010; Li, Chang, et al. 2014),which could be due to differences in genetic background. However, we again report no differences in the way wildtype and TNF-/- offspring respond to MIA (Figure 4.2). Our interpretation is that TNF-mediated plasticity does not play a unique adaptive role in the dysregulated neurodevelopment of MIA offspring.

Another finding from our study was in fact that TNF-/- showed a slight but significantly decreased baseline locomotor behavior in the social approach task, which was not significantly

altered by MIA. However, TNF-/- have no differential locomotion in the open field test (Lewitus et al. 2014b; Lewitus et al. 2016); nor did we see this difference in the EPM. This result might indicate that TNF-/- mice have more exploratory behavior when social cues are present, although the number of approaches to the "social" containment cup and the "non-social" containment cup did not differ from WT mice. Most studies do not report any alteration in spontaneous locomotor activity in the offspring of Poly I:C treated rodents as measured in the open field test (Zuckerman and Weiner 2005; Ozawa et al. 2006; Zuckerman et al. 2003), although both increased (Howland, Cazakoff, and Zhang 2012) and decreased (Van den Eynde et al. 2014) locomotor activity in Poly I:C offspring have been reported.

Differences in behavioral abnormalities do critically depend on the timing of Poly I:C treatment, which may be due to differences in the fetal brain cytokine response at earlier stages of development (e.g. GD9) versus later stages (e.g. GD17) (Meyer et al. 2006; Meyer et al. 2008). Cytokine expression and TNF in particular, seems to be more elevated after an acute Poly I:C injection at GD17 compared to GD9, however GD9 injection favored an overall more pro-inflammatory cytokine profile (Meyer et al. 2006). From a behavioral perspective, we also did not want to bias our behavioral analysis towards neither autism (later time-point) or towards schizophrenia (earlier time-point) (Meyer 2014). For example, some behaviors of interest are not present in animals from immune-activated mothers at later gestation times including Poly I:C-induced decreases in exploratory behavior, observed at GD9 and GD12 but not GD17 (Meyer 2014). Deficits in social approach have also been consistently established by mid-gestation MIA (Schwartzer et al. 2013; Smith et al. 2007; Malkova et al. 2012) but are also

seen at with MIA at GD9 (Abazyan et al. 2010) and at GD17 (Van den Eynde et al. 2014; Labouesse, Langhans, and Meyer 2015; Bitanihirwe et al. 2010).

It is important to note that the timing and intensity of the MIA, as well as additional genetic factors, can have an effect on cellular and behavioral phenotypes (Knuesel et al. 2014), so investigation of further paradigms (e.g. with LPS at a later stage of gestation) are required to confirm our results. Nonetheless, many MIA-induced behavioral changes, especially deficits in social approach and interaction, have been replicated in several laboratories despite the use of different species (rats or mice), different ages of gestational activation, and different doses and routes of administration (reviewed in (Meyer 2014; Knuesel et al. 2014; Patterson 2009)). Investigation of further MIA-induced behavioral abnormalities will further elucidate whether TNF function contributes uniquely to neurodevelopmental abnormalities resulting from prenatal neuroinflammation. For instance, peripheral levels of TNF in adult MIA offspring was positively correlated with repetitive, compulsive-like behavior in the marble-burying test (Onore et al. 2014). Unfortunately, we were not able to experimentally obtain reliable behavioral readouts in the marble-burying test used in other studies. MIA has also been shown to affect both the acute locomotor response to drugs of abuse (Labouesse, Langhans, and Meyer 2015; Zager, Mennecier, and Palermo-Neto 2012) and drug-induced place preference (Labouesse, Langhans, and Meyer 2015; Richtand et al. 2012).

Acutely, MIA causes a strong inflammatory response in both the mothers and fetuses, although the cytokine profile depends both on whether LPS or Poly I:C is used (Meyer 2014) and when the immune stimulant is administered (Meyer et al. 2006; Meyer et al. 2008). In the Poly I:C model, an increase in IL-1B is observed in the fetal brain 24 hours after maternal

injection (Arrode-Brusés and Brusés 2012) but TNF in the fetal brain is actually decreased 24 hours after maternal Poly I:C injection (Ratnayake et al. 2014) and also in the neonatal brain (Gilmore et al. 2004b). This may be a fetal mechanism to adapt to the maternal immune activation and occurs through alpha- and beta-crystallin downregulation of TNF (Garbett et al. 2012). Importantly, TNF is significantly elevated in adult MIA offspring in the Poly I:C model, both in the brain (Garay et al. 2013) and periphery (Han et al. 2011). In an LPS rat model however, TNF is generally increased in the fetal brain (Urakubo et al. 2001; Ashdown et al. 2006; Cai et al. 2000). MIA increases the levels of other pro-inflammatory cytokines in the periphery (38, 46) and in the fetal brain including IL-6 (Krstic et al. 2012; Pratt et al. 2013). However, the acute changes in TNF do not likely affect the long-term consequences of MIA since IL-6 alone reproduces the effect of Poly I:C (Smith et al. 2007) and because neonatal challenge with TNF produces no apparent behavioral alterations in adult offspring (Nawa and Takei 2006). These findings again support our rationale to use TNF-/- mice to specifically examine the contribution of TNF-mediated plasticity during development to behavioral abnormalities caused by MIA.

Overall, the long-term neuroinflammatory effects of MIA in adolescent and adult offspring is quite mild (Meyer et al. 2008; Smolders et al. 2015). Adult MIA offspring do have elevated levels of peripheral TNF (Han et al. 2011; Basta-Kaim et al. 2012) and an elevated TNF response to immune challenges which depends on genetic background (Schwartzer et al. 2013). But this is not a consistent finding (Onore et al. 2014) and TNF expression in the adult brain does not seem to be elevated in the MIA model (Garay et al. 2013). In the mouse Poly I:C model however, both IL-1B and IL-6 are increased in the adult offspring (Garay et al. 2013; Schwartzer

et al. 2013; Meyer et al. 2006; Meyer et al. 2008; Golan et al. 2006) but see (Onore et al. 2014)) and these other cytokines may play a more predominant role in the long-term behavioral consequences of the MIA model.

Outside of an inflammatory context, TNF has established roles in the development of the nervous system, including regulation of progenitor proliferation and neurogenesis (Iosif et al. 2006), sympathetic innervation (Kisiswa et al. 2013) and normal development of the hippocampus (Golan et al. 2004). Increasing TNF levels during development has been shown to dysregulate dendrite and synapse formation (Lee et al. 2010b; Golan et al. 2004). Importantly, these effects also occur with increased levels of other pro-inflammatory cytokines IL6 and IL1B. In the context of neurodevelopment IL-6, IL-1B and TNF have all been shown to have the same effect on primary dendrite number, dendritic nodes and total dendrite length during the development of neurons (Golan et al. 2004). And so during MIA, other cytokines may largely be able to compensate for the deletion of TNF. Therefore, two possibilities appear to be explanations for why genetic deletion of TNF has no effect on the behavioral changes observed in MIA offspring. The first is that TNF has no role in the neurodevelopment of the circuits underlying the behaviors we examined, either by not being elevated in those tissues or not impacting the circuit function. In favor of this idea is that development is largely normal in TNF and TNFR knockout mice, though subtle differences are detected, and the examined behaviours were largely normal in offspring from saline-treated TNF-/- mothers compared with those from saline-treated WT mothers. However, other studies have demonstrated that MIA can reveal functions of gene deletions or mutations that do not cause behavioral phenotypes on their own

(Schwartzer et al. 2013; Abazyan et al. 2010; Ibi et al. 2010; Vuillermot et al. 2012; Wu et al. 2015).

The second possibility is that during even mild models of neuroinflammation, pro-inflammatory cytokines are co-regulated and act in conjunction with each other. In this model, no single cytokine has a unique role and the other cytokines can compensate for the loss of one. For example, artificial elevation of TNF during development dysregulates dendrite and synapse formation (Lee et al. 2010b; Gilmore et al. 2004b), but these effects also occur with increased levels of other proinflammatory cytokines IL6 and IL1B. Thus, interfering with TNF under conditions when several pro-inflammatory cytokines are elevated will not have distinct effects. Although current data do not allow us to distinguish between these possibilities, we favor the second option and propose that the impact of TNF signaling will vary due to context. Under basal non-inflammatory conditions, TNF acts as the sole cytokine mediator of HSP, and thus interfering with TNF signaling will prevent the circuit normalization we have observed under other experimental paradigms. However, under inflammatory conditions (such as MIA), TNF is part of a suite of cytokine signaling with overlapping functions. Interfering with TNF signaling under these conditions would not have distinct effects, as we observed here. Clearly, elucidating the role of TNF-mediated plasticity in other behavioral models of neuroinflammation and neurodevelopment are needed to expand on these findings.

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## FIGURE LEGENDS FOR CHAPTER 4

*Figure 4.1 TNF is not required for loss of social preference in MIA offspring.*

**(A)** Mice were tested in a 3-chambered social approach task. An unfamiliar stranger mouse was enclosed in an aerated enclosure in one main chamber, with an empty enclosure in the opposite chamber. The separate enclosures are weighed down to prevent escape. The subject mouse is placed in the smaller middle chamber and can freely explore. The red track is a representative Ethovision trace collected over 10 minutes from a mouse from the WT saline group. **(B)** Comparison with a track from a mouse from the WT Poly I:C group. A shift in preference to the chamber and proximal zone (green circle) with the empty enclosure is visible. **(C)** A preference ratio was calculated for the time spent in close proximity (green circles in A & B) with the enclosure containing the stranger mouse as compared to an empty enclosure for WT and KO offspring from saline or Poly I:C treated mothers. Preference ratio was calculated as: (time proximal to mouse)/ (time proximal to mouse & empty). Both wildtype ($p=0.0022$) and KO ($p=0.045$) offspring from saline-injected mothers spend significantly more time exploring the enclosure with the stranger mouse than offspring from Poly I:C injected mothers (2-way ANOVA: effect of Poly I:C yielded $F(1,46) = 4.51$, $p<0.05$). The interaction effect of genotype and treatment was insignificant: $F(1, 46) =0.995$, $p=0.32$. N for each group is indicated in the corresponding bar of the graph. **(D)** Data are means of the fraction of 10 minutes spent in the chamber containing the stranger mouse. The main effect of Poly I:C treatment was again significant: $F(1, 46) = 19.94$, $p<0.0001$. Further comparisons between groups showed that WT Poly I:C mice spent proportionally less time in the chamber with the mouse ($p=0.0009$) than

their saline treated counterparts, as did the TNF KO Poly I:C mice (p=0.0087). There was again no significant effect of genotype x treatment interaction: F(1, 46) = 0.58, p=0.45. **(E)** Ratio of the frequency of approaches to the containment cup with the mouse to the frequency of approaches to the empty containment cup. A number greater than 1 indicates an overall larger number of "social" approaches. A 2-way ANOVA yields an effect of both treatment (F(1,45) = 4.97; p=0.031) as well as genotype (F(1,45) = 6.07; p=0.018), but no interaction effect between treatment and genotype (F(1,45) = 0.097, p=0.76). Post-hoc pairwise analysis however shows no significant decrease in ratio of social approaches with Poly I:C in either WT or KO mice. **(F)** Locomotor values during the 10 minute duration of the social approach test measured as the total distance traveled (cm). A 2-way ANOVA showed neither a significant effect of genotype (F(1, 45) = 2.41, p=0.13) or treatment (F(1, 45) = 0.033, p=0.86) on total locomotion but a trend for genotype x treatment interaction was evident whereby Poly I:C increased locomotion in KO offspring but decreased locomotion in WT offspring (F(1, 45) = 4.04, p=0.051). There was a significant decrease in baseline locomotion in the KO saline group compared to the WT saline group (p=0.012).

*Figure 4.2 TNF is not required for the change in anxiety-related behaviour induced by MIA.*
**(A)** The diagram shows the experimental methods to examine a mouse staying in the open arms (thick lines) vs. closed arms (thin lines) in an elevated plus-maze. Red track is representative Ethovision trace collected over 5 minutes in a mouse from the WT saline group. **(B)** Red track representative of a mouse from the WT Poly I:C group. An obvious increase in exploratory behavior in the open arms is visible. **(C)** Open arm time in seconds measured over 5 minutes of testing for WT and KO offspring from saline or Poly I:C injected mothers. Maternal injection

with Poly I:C significantly increases open-arm time, with no significant differences between genotype and no interaction between Poly I:C and genotype (2-way ANOVA, treatment: $F(1, 18) = 39.87$, $p<0.0001$; genotype: $F(1, 18) = 0.59$, $p=0.45$; interaction: $F(1, 18) = 0$, $p=0.997$). Open arm time increases 4-fold in the WT Poly I:C group ($p=0.0002$) and 5-fold in the KO Poly I:C group ($p=0.0004$). **(D)** Closed arm time in seconds measured over 5 minutes of testing for WT and KO offspring from saline or Poly I:C injected mothers. Maternal injection with Poly I:C significantly correspondingly decreases closed-arm time, with no significant differences between genotype and no interaction between Poly I:C and genotype (2-way ANOVA, treatment: $F(1, 18) = 33.6$, $p<0.0001$; genotype: $F(1, 18) = 0.43$, $p=0.52$; interaction: $F(1, 18) = 0.020$, $p=0.89$). Closed arm time decreases approximately 50% in both the WT Poly I:C group and KO Poly I:C group as compared to respective saline groups (WT: $p=0.0006$; KO: $p=0.0008$). **(E)** Time in centre of EPM over 5 minutes. Maternal injection with Poly I:C significantly increases exploratory time in the centre of the maze, with no significant differences between genotype and no interaction between Poly I:C and genotype (2-way ANOVA, treatment: $F(1, 18) = 9.66$, $p=0.0061$; genotype: $F(1, 18) = 0.16$, $p=0.70$; interaction: $F(1, 18) = 0.69$, $p=0.42$). There was only a significant increase in KO mice with Poly I:C ($p=0.015$) and not in WT mice ($p=0.11$). **(F)** The total number of entries into the open arms, as measured by the location of the centre of the mouse with Ethovision. The frequency of open arm entries was significantly increased in the Poly I:C groups from both WT ($p=0.012$) and KO mice ($p=0.030$). 2-way ANOVA effect of treatment: $F(1,18) = 3.63$, $p=0.0019$; effect of genotype: $F(1,18) = -1.33$, $p=0.20$; effect of interaction: $F(1,18) = -0.16$, $p=0.88$. **(G)** Locomotor values during the 5 minute duration of the EPM test measured as the total distance traveled (cm). Poly I:C treatment significantly

increased locomotion in the WT offspring (p=0.019), but not the KO offspring (p=0.12). 2-way ANOVA effect of treatment: $F(1, 18) = 8.74$, $p=0.0084$; effect of genotype: $F(1, 18) = 1.92$, $p=0.18$; effect of interaction: $F(1, 18) = 0.31$, $p=0.59$.

## FIGURES FOR CHAPTER 4

### FIGURE 4.1

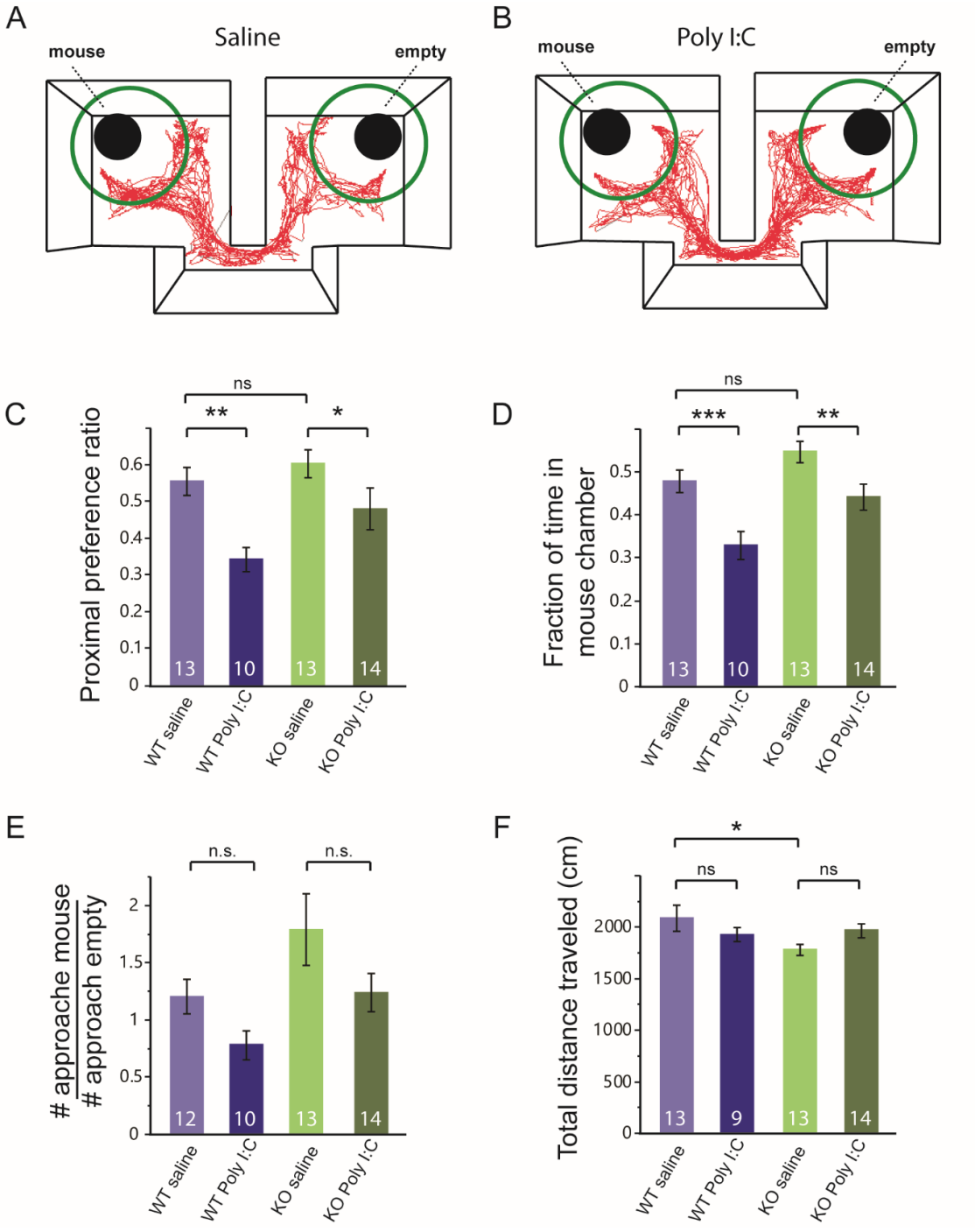
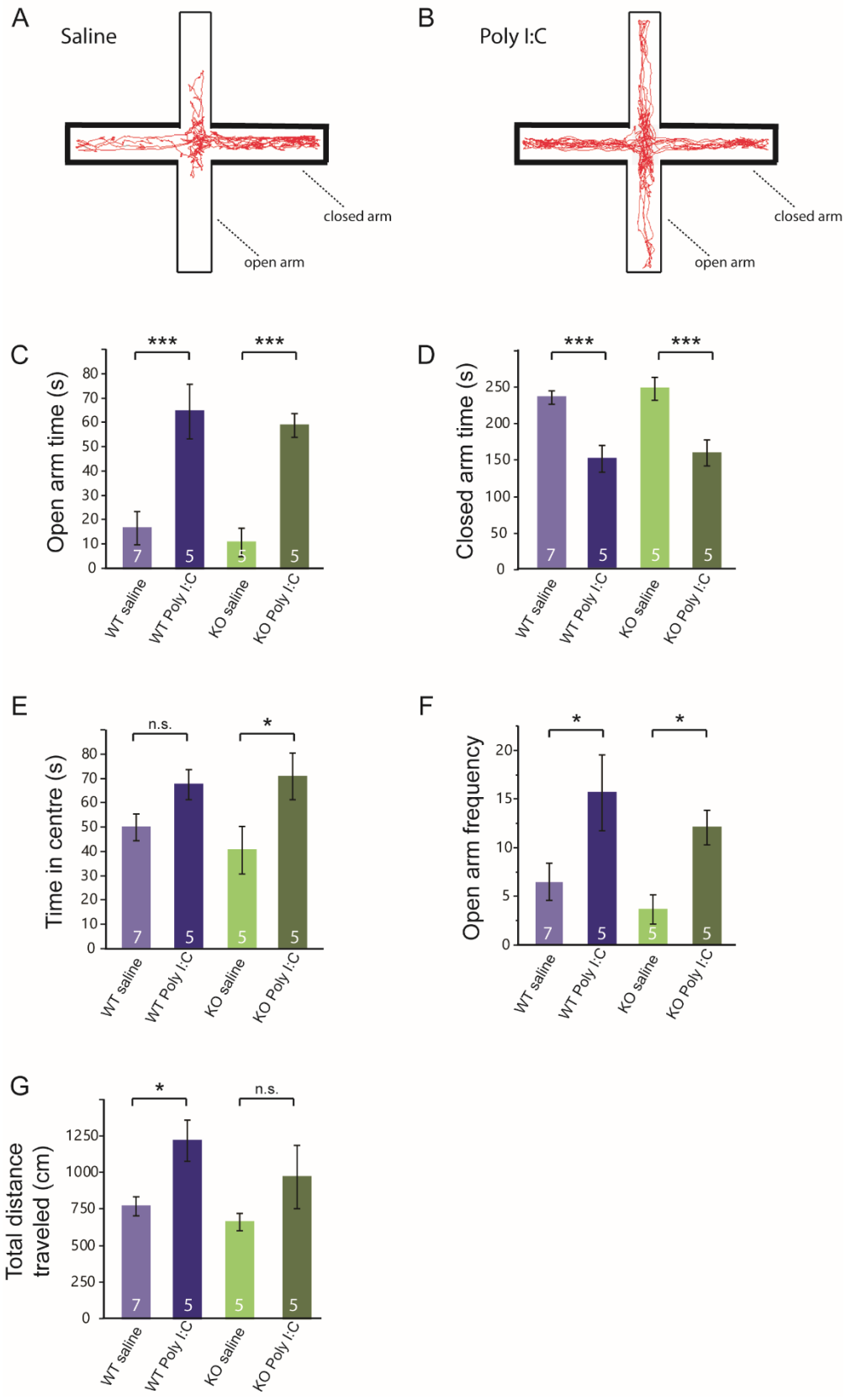


**FIGURE 4.2**


A
Saline
closed arm
open arm
B
Poly I:C
closed arm
open arm
C
Open arm time (s)
***
***
WT saline
WT Poly I:C
KO saline
KO Poly I:C
D
Closed arm time (s)
***
***
E
Time in centre (s)
n.s.
*
F
Open arm frequency
*
*
G
Total distance traveled (cm)
*
n.s.

# CHAPTER 5 GENERAL DISCUSSION AND CONCLUSIONS

## 5.1 – Summary

The publications presented in Chapter 2 and Chapter 4 report on the role of TNF in two different models of behaviors where TNF levels in the brain are elevated – cocaine addiction (Chapter 2) and MIA (Chapter 4). We investigated whether TNF contributes to a homeostatic-like response in these two models, based on the unique contribution of TNF to HSP in vitro (Stellwagen and Malenka 2006) and in vivo (Kaneko et al. 2008; Greenhill, Ranson, and Fox 2015; Ranson et al. 2012; Li et al. 2014). We find that genetic deletion of TNF exacerbates cocaine-induced plasticity in the ventral striatum and cocaine-induced behavioral sensitization. The adaptive function of TNF in cocaine sensitization is likely related to its ability to decrease excitatory synaptic strength on neurons in the NAc. There are several reports demonstrating a correlation between increased synaptic strength in the NAc and cocaine-induced locomotion (Britt et al. 2012) and sensitization (Pascoli, Turiault, and Luscher 2012). TNF production in microglia following repeated administration of cocaine occurs through activation of D2 receptors on microglia, indicating the importance of dopaminergic transmission for this adaptive mechanism. However, TNF levels and effects on NAc synaptic strength could also be manipulated via TLR4 activation. In the second model, we found that TNF-/- offspring exposed to prenatal inflammation had no differential expression of reduced social and anxiety related behaviors. This suggests that TNF is not uniquely involved in contributing to dysregulated neuronal function or to adaptive mechanisms in this model. In Chapter 3, we investigated if TNF

regulates the formation and maintenance of dendritic spines in the ventral striatum and if TNF played a role in cocaine-induced changes in dendritic spine density. We describe a novel role for TNF in the negative regulation of dendritic spines in the NAc both in the unperturbed brain and following repeated cocaine administration. We further conclude that the basal regulation of NAc dendritic spine density is due to astrocytic expression of TNF. Taken together, our results highlight novel roles for glial TNF in the brain, both in normal development and function, and in response to drug-induced plasticity.

**5.2 – The dual nature of TNF-mediated plasticity in the brain**

Arguably, the majority of literature on the role of TNF in the brain has focused on how excess production of the pro-inflammatory cytokine is a cause or contributor to disease pathology. Indeed, many neurodegenerative and neuropsychiatric disorders are associated with increased brain and CSF levels of TNF, including Alzheimer's disease (Tarkowski, Andreasen, et al. 2003; Tarkowski, Liljeroth, et al. 2003), fronto-temporal dementia (Sjögren et al. 2004), Parkinson's disease (PD) (Mogi et al. 1994), Amyotrophic Lateral Sclerosis (ALS) (Babu et al. 2008; Liu et al. 2009), schizophrenia (Wilson et al. 1997; Hänninen et al. 2005), autism (Chez et al. 2007; Li et al. 2009) and depression (Kaster et al. 2012; Dantzer 2004). In addition, there are also several examples for co-segregation of TNF gene or promoter polymorphisms causing elevated TNF expression, and brain disorders including schizophrenia (Boin et al. 2001; Czerski et al. 2008) AD (Laws et al. 2005; Yang et al. 2009) and PD (Wu et al. 2007; Nishimura et al. 2001). Gene polymorphisms causing elevated TNF production can also be protective as is the

case for dyskinesia (Wang et al. 2012). However, these associations are often not consistent (Elahi et al. 2009), and it is still unclear whether elevated levels of TNF play a direct role in disease processes or an indirect role due to dysregulated neuroimmune responses generally observed in these disorders. (Hirsch et al. 1998; Müller et al. 2015; Heppner, Ransohoff, and Becher 2015; McDougle and Carlezon 2013). The association of elevated TNF levels in some neurodegenerative diseases spurred clinical trials for TNF inhibitors in several neurodegenerative diseases (Tweedie, Sambamurti, and Greig 2007). Trials have been conducted in patients with Multiple Sclerosis (MS) (Van Oosten et al. 1996; Stommel et al. 2009), Alzheimer's disease (AD) (Tobinick et al. 2006; Tobinick and Gross 2008) and Amyotrophic Lateral Sclerosis (ALS) (Gowing et al. 2006). Significant cognitive and behavioral improvements were only observed in AD patients. In an animal model, blocking soluble TNF signaling in the brain may be a viable therapeutic approach for PD (McCoy et al. 2008). These mixed results highlight that TNF cannot be considered only as a contributing factor to diseases involving neuroinflammation. While TNF may largely function as a homeostatic factor in the normal brain, it can be concluded however that excess or prolonged TNF levels in the brain may be detrimental.

Excess or prolonged TNF can result in abnormal circuit function via its effects on the major excitatory (Grassi et al. 1994; Beattie et al. 2002; Kawasaki et al. 2008; Stellwagen et al. 2005; Han and Whelan 2010) and inhibitory (Pribiag and Stellwagen 2013; Stellwagen et al. 2005) neurotransmitter receptors. On glutamatergic neurons, TNF application not only results in increased levels of post-synaptic AMPARs, but preferentially targets calcium-permeable

AMPARs for exocytosis (Ogoshi et al. 2005; Stellwagen et al. 2005). Correspondingly, TNF preferentially targets calcium-permeable AMPARs for endocytosis on GABAergic neurons (Lewitus et al. 2014). Calcium-permeable AMPARs, while important for synaptic plasticity, are implicated in excitotoxic cell death and several disease states (Dong, Wang, and Qin 2009). For instance, TNF-induced trafficking of calcium-permeable AMPARs increase the susceptibility of neurons to excitoxicity of kainite treatment (Leonoudakis, Zhao, and Beattie 2008) and spinal cord injury (Ferguson et al. 2008). Elevated TNF can also increase extracellular glutamate concentrations (Pickering, Cumiskey, and O'Connor 2005; Hermann et al. 2001) and increased mEPSC frequency (Grassi et al., 1994; Beattie et al., 2002), further increasing the risk of glutamate excitotoxicity. During development, increases in TNF levels can impair normal dendrite and synapse formation (Lee et al. 2010; Golan et al. 2004). Therefore, detrimental effects of TNF can occur via dysregulation of the same mechanisms underlying its homeostatic function.

Indeed, the work presented in Chapter 2 of this thesis, as well as previous publications from our lab and other groups, have highlighted a protective role of TNF in the brain in response to injury, inflammation and other environmental challenges. In these models, detrimental effects occur when TNF levels are either absent or below a threshold homeostatic range. In response to various excitotoxic insults, TNF has been shown to have neuroprotective effects (Tamatani et al. 1999; Cheng, Christakos, and Mattson 1994; Carlson et al. 1998; Carlson et al. 1999; Liu, Xu, and Barks 1999). These beneficial effects of TNF are mediated by astrocytes and microglia. For instance, following focal cerebral ischemia, microglial-derived TNF promotes

survival of endangered neurons in the cortex, which was associated with improved sensory motor function (Lambertsen et al. 2009). In another study, astrocyte-specific overexpression of TNF was found to attenuate seizure sensitivity in a mouse model (Balosso et al. 2005). The neuroprotective effects of TNF in these studies may be unrelated to its effect on synapses (Heir and Stellwagen 2015).

Recent published work from our lab suggests that TNF's beneficial effects on brain physiology and behavior can in fact be mediated by its role in AMPAR trafficking. Here, TNF reduces corticostriatal synaptic strength by endocytosing AMPARs, improving the impaired locomotor movements associated with prolonged treatment of typical antipsychotics (Lewitus et al. 2014). As discussed in Chapter2, TNF mediates a similar adaptive response to repeated administration of cocaine (Lewitus et al. 2016). TNF also attenuates addictive behaviours and dopaminergic neurotoxicity to other drugs of abuse (Nakajima et al. 2004; Niwa, Nitta, Yamada, Nakajima, Saito, Seishima, Noda, et al. 2007; Niwa, Nitta, Yamada, Nakajima, Saito, Seishima, Shen, et al. 2007). When TNF is deleted genetically or blocked pharmacologically, this compromises the brain's ability to regulate circuit function in response to certain stimuli. Understanding what stimuli these are and how the homeostatic function of TNF works in different models of disease will yield useful insights into how neuroinflammation intersects with synaptic plasticity mechanisms in the brain.

**5.3 – Role of neuroinflammation and TNF in MIA versus drug addiction**

As previously introduced in Chapter 1, neuroinflammation is at its core a homeostatic response to intrinsic or extrinsic factors that perturb neuronal function. Increased production of cytokines by astrocytes and microglia play a central role in mediating subsequent adaptations in neural circuitry and behavior. IL-1β, IL-6 and TNF are arguably the most studied pro-inflammatory cytokines, while IL-4 and IL-10 are well characterized anti-inflammatory cytokines (Kim et al. 2016). They are released by activated glial cells (Muñoz-Fernández and Fresno 1998; Sheng et al. 2011; Lacagnina, Rivera, and Bilbo 2017) and can subsequently modulate neuronal function. Generally, pro-inflammatory cytokines increase neuronal E/I balance which is the case for TNF (Lewitus et al. 2014; Pribiag and Stellwagen 2013; Stellwagen et al. 2005; Stellwagen and Malenka 2006), IL-6 (Garcia-Oscos et al. 2012; Wei et al. 2012) and IL-1β (Kawasaki et al. 2008; Vezzani et al. 1999). One report also suggests that the pro-inflammatory cytokine IFN-γ, also enhances glutamate transmission via AMPARs (Mizuno et al. 2008). Less is known about how anti-inflammatory cytokines regulate neurotransmission, but one study reports that IL-10 decreases mIPSC frequency and current (Suryanarayanan et al. 2016), suggesting that anti-inflammatory cytokines may have similar effects on E/I balance as pro-inflammatory cytokines. Unlike IL-1β and IL-6, TNF is required for HSP, which increases E/I balance in a manner that depends on the activity of neuronal networks (Stellwagen et al. 2005; Stellwagen and Malenka 2006). Hence, investigating the role of TNF in animal models of drug addiction and neuroinflammation provides a mechanistic link between two different types of homeostasis in the brain: neuroinflammation and HSP. We investigate this mechanistic link in two models:

cocaine-induced sensitization and MIA and argue that TNF is part of a homeostatic-like response to cocaine-induced plasticity & sensitization but not to MIA-induced deficits in social and anxiety related behaviors. Two main differences could explain why TNF plays a role in one model and not the other. The first is the timescale of neuroinflammation in the two models (acute adult exposure versus developmental exposure) and the second is the compensatory role of other pro-inflammatory cytokines such as IL-6 and IL-1β.

First, our results from Chapter 2 are consistent with previous evidence that psychostimulants activate immune responses in the brain (Clark, Wiley, and Bradberry 2013; Guo et al. 2015). These responses are characterized by activation of microglia and astrocytes, and up-regulation of pro-inflammatory cytokines. Indeed, we find that repeated administration of cocaine results in the activation of microglia and increased microglial production of TNF. Microglia activation and upregulation of inflammatory markers was observed after acute (1 day) administration of methamphetamine (Asanuma et al. 2004) and after chronic (7 days) administration of cocaine (Renthal et al. 2007). An acute dose of methamphetamine increases mRNA expression of microglial IL-6 and TNF in the striatum and hippocampus (Gonçalves et al. 2008), while TNF mRNA levels were also found significantly elevated in the mouse striatum 4 hrs after a single dose of cocaine (25mg/kg) (Piechota et al. 2010). The latter study also reports that both common and distinct transcriptional networks are activated (acutely) by different drugs of abuse (Piechota et al. 2010). Hence, elevation of microglial TNF may not be a characteristic response for all drugs of abuse. Collectively, our results along with previous findings indicate that cocaine administration is associated with microglia activation and TNF

expression in the brain. There is some evidence to suggest that cocaine also elevates other pro-inflammatory cytokines in the brain. Acute cocaine was found to increase IL-1β in the NAc and cortex (Cearley et al. 2011) as well as the VTA (Northcutt et al. 2015), and it is suggested that this occurs in microglia (Liao et al. 2016; Northcutt et al. 2015). A study in human tissue isolated microglia and astrocytes from 20-week old human fetal cerebral cortex and measured increased levels of microglial IL-1β after in vitro treatment with 100uM cocaine for 24hrs (Lee et al. 2009). In human cocaine users, there is also a report that serum levels of IL-6 are significantly elevated (Moreira et al. 2016). It is however not clear what mechanisms are initiated by IL-6 and IL-1β in the brain following cocaine administration.

We argue that the TNF component of drug-induced immune activation is also homeostatic. There are several lines of evidence for this argument. The first line of evidence is that in TNF -/- mice there is a unique neuronal phenotype in NAc MSNs where synaptic strength is elevated both at baseline (Lewitus et al. 2014) and in response to cocaine. This suggests that there is no compensation by other cytokines which mediate the TNF-dependent synaptic response to cocaine. The next evidence is that microglia activation and TNF production mitigate cocaine-induced locomotor sensitization by reducing synaptic strength on NAc MSNs. Contradicting results have suggested that blocking microglia activation and TNF production with the antibiotic minocycline prevents the development of cocaine sensitization (Chen, Uz, and Manev 2009). However, minocycline did not affect sensitization once it had already been established (Chen, Uz, and Manev 2009). Another study shows that minocycline reduced cocaine-induced place preference (Northcutt et al. 2015). A potential explanation for this

discrepancy is that minocycline's mechanisms of action in the brain have not been fully characterized (Grieco et al. 2014) and can for example modulate synaptic function by reducing MMP activity (Bilousova 2009). Importantly, reactivation of microglia and TNF with MPLA is able to reduce sensitization that was already established, and this appeared to be mediated by TNF-mediated trafficking of AMPARs to reduced MSN synaptic strength. This TNF effect echoes the TNF-dependent homeostatic response occurring in neuronal cultures following chronic activity manipulation (Stellwagen and Malenka 2006).

The other key piece of evidence for a homeostatic-like response is that given a limited exposure to cocaine, the levels of microglia activation and TNF production return to baseline after a period of abstinence. Our results suggest that microglia activation induced by 5 days of cocaine does not last longer than 10 days. We also observe that TLR4 activation by MPLA does not mitigate behavioral sensitization after a delay of 4 days. Similarly, levels of microglia activation in the striatum induced by acute methamphetamine returned to baseline within 7 days (Thomas et al. 2004). An acute dose of cocaine was found to have a similar pattern of microglia activation but was non-significant, possibly because the dose used (10mg/kg) was much lower compared to many cocaine studies (Thomas et al. 2004). This effect however also returned to baseline levels after 7 days (Thomas et al. 2004). The homeostatic response of microglia and TNF may no longer function after prolonged exposure to drugs of abuse, perhaps due to a general dysregulation of the brain's immune response. For example, a post-mortem comparison of human tissue from chronic cocaine abusers (n=10) with non-addict controls (n=9) showed a significant increase both in activated macrophages and microglia in the

midbrain of chronic cocaine abusers (Little et al. 2009). In animal models of drug addiction we would therefore predict that chronic cocaine administration may result in dysregulated, chronic microglia activation, as well as infiltration of peripheral immune cells. This conclusion is also consistent with the hypothesis that drug abuse may be a contributing factor to the development of several neurodegenerative disorders (Guilarte 2001; Byars et al. 2012; Vicente-Rodríguez et al. 2013; Miksys and Tyndale 2010). This conclusion highlights that regulation of immune responses in the brain is important for determining whether the end result is adaptive or pathological.

MIA can persistently increase immune signaling in the adult brain, including activation of microglia, astrocytes and elevated levels of pro-inflammatory cytokines. The degree of immune activation in the adult brain depends on the type and dose of immune agent used (e.g. LPS vs Poly I:C, high or low dose), and whether the immune agent is given acutely or chronically (Patterson 2009; Borrell et al. 2002; Knuesel et al. 2014). Increased immunoreactivity of microglial immune markers such as CD11b and morphological alterations were observed in adult MIA brains and are indicative of microglia activation (Krstic et al. 2012; Hao et al. 2010). Elevated expression of GFAP further indicates that astrocytes are also activated in adult MIA brains (Hao et al. 2010; Krstic et al. 2012; Fatemi et al. 2004). MIA also leads to long-lasting, region-specific alterations in the levels of cytokines in the brain (Garay et al. 2013). Most pro-inflammatory cytokines, including TNF, are elevated in brains of adult MIA offspring (Garay et al. 2013; Krstic et al. 2012). Permanent immune dysregulation is a hallmark of both schizophrenia and autism, diseases for which MIA is used as a model. Upregulation of immune

genes in the schizophrenic brain, and microglia and astrocyte activation in addition to increased expression of cytokines in the autistic brain (Vargas et al. 2005; Arion et al. 2007; Chez et al. 2007; Saetre et al. 2007) are all consistent with the framework that there is some degree of neuroinflammation in the MIA model.

In this model, we did not gather any evidence that TNF has a role in the normalization of social and anxiety related behaviors in MIA offspring. Nor did we find evidence that TNF is a contributing factor in these behavioral abnormalities occurring in MIA offspring. We can conclude that in this model, circuit development and neuronal function is not uniquely affected by TNF-mediated plasticity. As mentioned previously, we propose that this is because TNF does not play a homeostatic role when circuit activity is altered over very long periods of time. In the MIA model, persistent neuroinflammation is measured during the entirety of development (Bilbo and Schwarz 2009; Bilbo and Schwarz 2012; Knuesel et al. 2014). In our cocaine model, TNF-mediated plasticity is activated by a comparably acute perturbation of circuit activity in adult animals. Therefore a transition from an acute elevation of TNF signaling to a chronic level of TNF signaling may underlie more neuroinflammatory instead of homeostatic effects of TNF. In line with this framework, other pro-inflammatory cytokines, notably IL-6 and IL-1β, may have a more prominent role in directing the development of abnormal behaviors resulting from MIA. Indeed, both IL-6 (Smith et al. 2007) and the IL-1 receptor (Girard et al. 2010) have been shown to be a critical mediators of many behavioral changes in MIA offspring (Smith et al. 2007). Interestingly, mice overexpressing IL-6 exhibit many autistic-like behavioral and neuronal

phenotypes including decreased sociability and anxiety and increased E/I balance (Wei et al. 2012).

**5.4 – Dendritic spine structural plasticity as a mechanism of HSP**

Dendritic spines are far from static structures and both spine density and morphology can change rapidly in response to activity-dependant synaptic plasticity (Trachtenberg et al. 2002; Tropea et al. 2010; Yang, Pan, and Gan 2009; Sadaka et al. 2003) and several neuromodulatory signals (Jones et al. 2009; Woolfrey et al. 2009; Liston et al. 2013; Srivastava et al. 2008; Clarke and Barres 2013). Hebbian and homeostatic plasticity both contribute to activity-dependent changes at synapses and have therefore both been implicated in the regulation of spine morphology and density. LTP and LTD are well-studied forms of Hebbian plasticity. Increasing synaptic strength via LTP increases dendritic spine density in dissociated hippocampal neurons (Lin, Huganir, and Liao 2004; Park et al. 2006) and hippocampal slices (Engert and Bonhoeffer 1999; Maletic-Savatic, Malinow, and Svoboda 1999). LTP induction also increases spine size (Park et al. 2006; Lang et al. 2004; Kopec et al. 2006) which precedes the observed increase in post-synaptic AMPAR abundance (Kopec et al. 2006; Harris and Stevens 1989; Matsuzaki et al. 2004). LTP-inducing glutamate uncaging at single spines was also found to stabilize newly-formed spines (Hill and Zito 2013). Overall, LTP can both generate new spines and stabilize newly-formed spines, and the subsequent increase in spine density is associated with learning and memory in vivo (Xu et al. 2009; Yang, Pan, and Gan 2009; Mahmmoud et al. 2015; Leuner, Falduto, and Shors 2003; Moser, Trommald, and Andersen 1994). As expected,

LTD-like plasticity is generally accompanied by smaller and fewer dendritic spines (Okamoto et al. 2004; Zhou, Homma, and Poo 2004; Nägerl et al. 2004).

Dendritic spine density, and by extension connectivity of neural circuits, is not only mediated by Hebbian-like changes, but also by HSP. Structural dynamics of HSP are observed under chronically altered conditions of neuronal activity and therefore occur on slower timescales (Fauth and Tetzlaff 2016; Butz, Wörgötter, and van Ooyen 2009). In terms of spine morphology, activity-dependent scaling up of synapses are correlated with increased spine volume both *in vitro* (Murthy et al. 2001) and *in vivo* (Keck et al. 2013). In terms of spine density, chronically blocking neuronal activity yields a homeostatic increase in the number of spines, which occurs on a much slower timescale than classic LTP and LTD (Dalva, Ghosh, and Shatz 1994; Rocha and Sur 1995; McAllister, Katz, and Lo 1996; Kirov and Harris 1999). Neuronal deprivation after 4 days of monocular deprivation similarly leads to a homeostatic increase in dendritic spine density in the binocular cortex of adult mice (Hofer et al. 2009). Conversely, persistent depolarization of neurons induces loss of dendritic spines (Müller et al. 1993; Drakew et al. 1996). In animal models of epilepsy, circuits are hyperactive yet there appears to be a homeostatic reduction in the number of dendritic spines (Paul and Scheibel 1985; Geinisman, Morrell, and de Toledo-Morrell 1990; Isokawa 1998; Isokawa and Levesque 1991). Therefore, spine density is bi-directionally regulated in an activity-dependent and homeostatic manner.

Cocaine-induced structural plasticity is characterized by an increase in dendritic spine density in brain regions innervated by the VTA, including excitatory, glutamatergic neurons in the PFC and inhibitory, GABAergic neurons in the NAc (Golden and Russo 2012). The findings presented in Chapter 2 and 3 suggest a model where cocaine-induced structural plasticity results in part from an imbalance between Hebbian and homeostatic mechanisms so that homeostatic mechanisms are unable to completely correct or compensate for drug-induced synaptic plasticity. Early drug-induced plasticity has previously been suggested to be Hebbian in nature (Ungless et al. 2001), and many molecular factors underlying cocaine-induced spinogenesis are classically associated with Hebbian LTP (Russo et al. 2010).

Moreover, the main evidence for this Hebbian/homeostatic imbalance model is that TNF acts as a homeostatic signal which antagonizes drug-induced changes in synaptic strength and spine density i.e. in TNF-/- mice, repeated cocaine administration results in both a larger increase in AMPA/NMDA ratios and a larger increase in spine density on NAc MSNs. Importantly, this cocaine-induced synaptic and structural plasticity correlates with the level of behavioral sensitization, and by extension, addictive behavior. Cocaine-induced spinogenesis has in fact been found to correlate with cocaine-induced behavioral responses (Russo et al. 2009; Li, Acerbo, and Robinson 2004; Maze et al. 2010). These findings are consistent with what we observe in TNF-/- mice, where cocaine-induced spinogenesis is elevated, along with an increase in cocaine sensitization. However, the direct relationship between MSN spine density and addictive behaviors are more complex because contradicting studies show that blocking cocaine-induced spinogenesis enhance sensitization (LaPlant et al. 2010; Kiraly et al. 2010;

Pulipparacharuvil et al. 2008). These discrepancies could be due to off-target effects of the proteins studied, and focusing on glial-derived factors such as TNF may offer new insights into how structural plasticity in the NAc affects addictive behaviors. Overall, our findings with regard to TNF suggest that cocaine-induced structural plasticity favors Hebbian mechanisms over homeostatic mechanisms, contributing to maladaptive circuit re-wiring during addiction.

**5.5 – Differential roles of microglial and astrocytic TNF**

In Chapter 2, we show that 5 days of daily cocaine administration activates microglia and that microglia specifically provide the TNF which then decreases excitatory synaptic transmission and AMPAR abundance in NAc neurons (Lewitus et al. 2016). Previously, both astrocytes (Bowers and Kalivas 2003; Blanco-Calvo et al. 2014; Ortiz et al. 1995; Pu, Broening, and Vorhees 1996; Hebert and O'Callaghan 2000) and microglia (Thomas et al. 2004; Sekine et al. 2008; Blanco-Calvo et al. 2014; Yamamoto, Moszczynska, and Gudelsky 2010; Guo et al. 2015; Liao et al. 2016) have been shown to be activated by drugs of abuse, including cocaine (Bowers and Kalivas 2003; Blanco-Calvo et al. 2014). Other drugs, including some antidepressants and antipsychotics are also known to modulate microglia (Cotel et al. 2015; Tynan et al. 2012; A Kato et al. 2011; Kreisel et al. 2014) and astrocyte activity (Duseja et al. 2015; Konopaske et al. 2013; Quincozes-Santos et al. 2009). Given the differential contribution of astrocytes and microglia to cocaine-induced TNF expression, it is likely that various drugs can differentially activate TNF in astrocytes or microglia depending on expression of neurotransmitter receptors and intracellular signaling pathways (discussed below in 5.6).

Clearly, different stimuli can results in TNF production in either microglia or astroctyes. Our results suggest that TNF release is specific to microglia following repeated cocaine administration, and are still consistent with the idea that astrocyte activation occurs on a much slower timescale in response to cocaine (Bowers and Kalivas 2003). Studies on other drugs of abuse have also shown that drug-induced microglia activation lasts for only a short period of abstinence (Zhao et al. 2013; Thomas et al. 2004). This suggests that certain drugs or environmental changes which stimulate microglial TNF may not be long lasting. Indeed, stress-induced microglia activation was found to persist for up to four days only (Kreisel et al. 2014). Transient activation of microglia may however, release signaling cues that may then alter astrocytic responses. One example of such a cue is TNF itself, which was found to control the rate of glutamate release from astrocytes (Santello, Bezzi, and Volterra 2011; Bezzi et al. 2001). Other microglial cues found to mediate astrocyte function are ATP (Pascual et al. 2012; Shinozaki et al. 2014) and the pro-inflammatory cytokines IL-6 and IL-1β (Lee et al. 1993; Clark, Wiley, and Bradberry 2013; Liberto et al. 2004). These results suggest that TNF derived from microglia can have an important modulatory role for astrocytes, which may be relevant for later stages of cocaine addiction.

Previously, it was not clear whether microglia activation was a detrimental effect of cocaine or if it served a homeostatic purpose. The results from our cocaine study place TNF as a key regulator an early homeostatic response in microglia, and further highlight that microglia activation can be beneficial in the context of addiction. A homeostatic role for microglia was recently demonstrated in a model of stress-induced depression, a study which also highlighted

how reduced microglial activation status can be associated with detrimental behavioral effects (Kreisel et al. 2014). While the specific contribution of microglial TNF was not examined there, microglial TNF was shown to be beneficial and neuroprotective in animal models of stroke (Clausen et al. 2016; Lambertsen et al. 2009). Taken together, these findings support the idea of TNF as a homeostatic regulator in many conditions of dysregulated brain function.

Astrocytes are also homeostatic regulators in the brain, and can release a plethora of "gliotransmitters," including TNF, given different stimuli. TNF derived from astrocytes is implicated in models of HSP in neuronal culture (Stellwagen and Malenka 2006) and slice culture (Becker et al. 2013). Our molecular and behavioral studies presented in Chapter 2 suggest that astrocytic TNF does not seem involved in early phases of cocaine addiction. However, in Chapter 3 we find that basal expression of TNF by astrocytes in the NAc regulates dendritic spine density on MSNs (Chapter 3). Interestingly, we see no effect of microglial derived TNF on basal MSN spine density nor do we find that microglial TNF underlies the increased cocaine-induced spinogenesis observed in TNF-/- mice. This finding is surprising given that basal expression of TNF in astrocytes (non-microglial cells) is almost 25x less than basal expression in microglia (Figure 2.3C). However, our measurement of TNF mRNA in microglial and non-microglial fractions were performed in adult animals, and so it is possible that astrocytes express much higher levels of TNF at different stages of development. An increased level of TNF expression in astrocytes during adolescence would suggest that astrocytic TNF plays a role in synaptic pruning – the process of synapse elimination occurring at this stage of brain development. Nonetheless, regulation of dendritic spines by astrocyte-derived TNF is

consistent with a growing field of literature demonstrating an instructive role for astrocytes in determining the number of excitatory synapses on neurons (Allen et al. 2012; Christopherson et al. 2005; Hughes, Elmariah, and Balice-Gordon 2010; Kucukdereli et al. 2011; Pfrieger and Barres 1997; Ullian et al. 2001). The close proximity of astrocytic processes near dendritic spines is thought to regulate the positioning of key molecules secreted by astrocytes (Bernardinelli, Muller, and Nikonenko 2014; Clarke and Barres 2013), and in some cases is necessary in order for neurons to receive certain signals from astrocytes (Hama et al. 2004; Barker and Ullian 2008). While our study shows no direct role for astrocytic TNF in cocaine-induced plasticity and sensitization, it is still likely that astrocytes contribute in some way to drug addiction (Miguel-Hidalgo 2009). Soluble factors released by astrocytes applied *in vivo* to the NAc core increase the rewarding effects of methamphetamine and morphine (Narita et al. 2006; Narita et al. 2005). Whether or not cocaine-induced spine formation is negatively regulated by astrocytic TNF remains an important question and will shed further light on the role of astrocytic TNF in the plasticity events of cocaine addiction.

**5.6 – D2 Receptor activation: a novel molecular and cellular pathway regulating TNF synthesis in microglia**

TNF is known to be activated in microglia via the TLR4-Myd88 pathway which leads to increased TNF transcription via NF-kB (Akira and Takeda 2004). Several types of receptors can signal through the same pathways downstream of TLR4 and these activate TNF transcription in microglia (Means et al. 2000; Nadeau and Rivest 2000). These include receptors for various

growth factors (Lull and Block 2010; Lee, Schrader, and Kim 2000; Tang et al. 2007) and receptors for other cytokines (DiDonato et al. 1997; Hoogland et al. 2015). Both microglia (Kettenmann et al. 2011) and astrocytes (Verkhratsky 2009) also express various neurotransmitter receptors, several of which have been shown to be able to increase or decrease TNF expression in these glia (Pocock and Kettenmann 2007). Glutamate especially, is an important signal for activity-dependant TNF production in glia (Stellwagen and Malenka 2006; De, Krueger, and Simasko 2005). Recent work from our lab demonstrated a role for serotonin in increasing TNF expression in astrocytes (Duseja et al. 2015). Interestingly, while dopamine has been reported to modulate TNF expression in astrocytes (Nakagawa and Schwartz 2004; Ding et al. 2015), no direct role of dopamine in regulating microglial TNF expression has been established (Pocock and Kettenmann 2007). In cultured microglia, we observed that both dopamine and the D2-receptor agonist quinpirole were able to increase TNF mRNA without further stimulation. Our results in the NAc (ventral striatum) demonstrate that dopamine is able to increase TNF production in microglia through D2 receptors. Accordingly, D2 receptors are expressed in striatal microglia (Schwartzer et al. 2013) and striatal astroctyes (Bal et al. 1994; Miyazaki et al. 2004). We found that *in vivo* administration of the D2 agonist quinpirole increased TNF mRNA in striatal but not cortical microglia. This suggests that there are regional differences in microglia (Grabert et al. 2016; Doorn et al. 2015; Yang et al. 2013) in terms of neurotransmitter receptors that they express. These differences could underlie differential responses of microglia to various drugs or environmental stressors. Indeed, it was observed that microglia within the NAc core but not the hippocampus express both D1 and D2 dopamine receptors (Schwartzer et al. 2013), suggesting that striatal microglia may be more

sensitive to changes in dopamine transmission compared to hippocampal microglia. Our results indicate first that dopamine receptor expression on microglia are a mechanism by which microglia respond to the activity of surrounding neurons and second, that TNF expression plays a role in this response. These results further suggest that TNF expression by microglia is associated with microglia activation by dopamine.

Our results also indicated that D2 agonism has no effect on astrocytes, despite evidence that striatal astrocytes express D2 receptors in culture (Bal et al. 1994) and *in vivo* (Miyazaki et al. 2004). This could be due to D2R-mediated inhibition of αβ-crystallin in astrocytes, which promotes inflammatory phenotypes in astrocytes (Shao et al. 2013). In addition, repeated cocaine administration could result in a specific upregulation of D2 receptors on microglia, which occurs in a model of stroke (Huck et al. 2015), leading to a robust TNF response in microglia but not astrocytes. Signaling pathways downstream of D2 receptors do seem to be associated with increased TNF expression, which is what we expect from our results. D2 receptors are coupled with Gi alpha subunits which inhibit the production of the second messenger cAMP (Beaulieu et al. 2011). cAMP is known to inhibit the expression of TNF (Pober et al. 1993; Spriggs, Deutsch, and Kufe 1991) so this is consistent with D2-receptor mediated increases in microglial TNF following repeated cocaine administration. There is also a G-protein independent pathway where D2 receptors activate Akt/GSK3-β via the multifunctional adaptor protein beta-arrestin (Beaulieu et al. 2011). Akt/GSK3-β activation is upstream of TNF production in astrocytes and microglia (Cortés-Vieyra et al. 2012; Green and Nolan 2012). Interestingly, there is a report suggesting that acute cocaine activates GSK3-β, and that this is

contingent upon D2 receptor activation (Miller et al. 2014). However, it is not clear whether this activation occurs in neurons or glia. In conclusion, TNF production mediated by microglial D2 receptors following repeated cocaine administration represents a novel signaling pathway leading to TNF expression in microglia, which is likely important in several disease models.

**5.7 – Conclusion**

The work presented in this thesis investigated the contribution of TNF in two animal models implicating altered synaptic plasticity and neuroinflammation as causes underlying dysregulated behaviors. First, we investigate the effects of glial-derived TNF on the behavioral and the physiological changes occurring in mice after cocaine administration. We demonstrate a novel adaptive role for microglial-derived TNF in its ability to limit cocaine-induced plasticity in the NAc and behavioral sensitization. Our results suggest that modulation of microglia or TNF signaling could be an avenue of therapy in more advanced models of drug addiction. We also report that negative regulation of dendritic spines in the NAc by astrocyte-derived TNF may be implicated in the adaptive response to cocaine. Second, we investigate the role of TNF in social and anxiety related behaviors altered by prenatal immune activation (MIA). We did not observe any behavioral differences in WT and TNF-/- strains following immune activation and conclude that TNF-mediated plasticity does not uniquely contribute to synaptic changes MIA-induced behavioral changes. These studies advance our understanding of how TNF, as an effector of neuroinflammation and synaptic plasticity, modulates behavioral responses in animals after different environmental stimuli.

## 5.8 – References for Chapter 5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