The Role of Interferon Gamma in Cortical Synaptic Plasticity and Behavioural Output

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Abstract

A previously performed gene expression screen of immune factors regulated by neuronal activity identified the pro-inflammatory cytokine Interferon gamma (IFNγ) as a potential regulator of neuronal function, perhaps as a possible opponent to TNFα to maintain circuit homeostasis. Here, we investigated the role of IFNγ, in the regulation of excitatory and inhibitory cortical synapses and in behavioural differences in a knock-out rodent model.

We examined the effect of short-term IFNγ treatment on glutamate-mediated excitation and GABA-mediated inhibition via electrophysiology, using cortical rat neuronal dissociated cultures. First, we found that acute IFNγ treatment at 100ng/ml significantly increased mEPSC frequency, amplitude, and mIPSC frequency. Additionally, acute IFNγ treatment at 10ng/ml significantly increased mIPSC amplitude but not frequency. To validate the electrophysiological changes at the synapse, we performed immunocytochemistry following IFNγ treatment on cortical pyramidal neurons to monitor receptor trafficking of AMPA and GABA-A receptors. We showed that acute IFNγ treatment increased the amount of GluA1 receptor expression on the surface of the synapse in a dose-dependent manner, with a larger expression seen at the 100ng/ml dose in comparison to the 10ng/ml treatment. IFNγ treatment at 10ng/ml, but not at 100ng/ml, was also shown to increase GABA-A receptor expression on the surface of the synapse. To identify which cell-type may be responsible for producing IFNγ in the central nervous system we performed quantitative PCR on glial subtypes with treatments to stimulate neuronal activity as IFNγ is activity regulated. Microglia were treated with fractalkine, astrocytes were treated with glutamate, and we evaluated levels of IFNγ mRNA of treated cell populations in comparison to controls. We have shown a significant fold change increase of IFNγ mRNA in treated microglia, whereas treated astrocytes show a small but insignificant increase in comparison to controls.

Lastly, we investigated the behavioural consequences of the loss of IFNγ signaling in IFN receptor knockout mice. Using the light-dark box test, our results show that IFNγ receptor KO mice experience higher baseline anxiety-like behaviour compared to WT mice. Altogether, this data reinforces the idea that the neuroimmune system can modulate synaptic plasticity and behaviour output, through multiple factors including IFNγ.

Résumé

Une précédente étude de l'expression génétique des différents facteurs immunitaires régulé par l'activité neuronal a permis d'identifier la cytokine pro-inflammatoire Interféron gamma (IFNγ) comme étant un potentiel candidat. L'objectif de cet article est de déterminer le rôle de l'IFNγ dans la régulation des synapses corticales inhibitrices et excitatrices, ainsi que son impact au niveau comportemental en utilisant d'un modèle de rongeur IFNγ-KO.

Afin d'examiner les effets à courts terme du traitement à l'IFNγ, nous avons réalisé une étude électrophysiologique des courants excitateurs glutamatergiques et des courant inhibiteurs GABAergiques sur des cultures de neurones dissociés provenant de cortex de rat. Le traitement à l'IFNγ à une concentration de 100ng/ml a considérablement augmenté la fréquence et l'amplitude des mEPSCs, et la fréquence des mIPSCs. De plus, à 10ng/ml, le traitement à l'IFNy a significativement augmenté l'amplitude des mIPSCs sans toutefois augmenter la fréquence. Pour valider les changements électrophysiologiques observés à la synapse, nous avons fait des expériences d'immunohistochimies sur les neurones pyramidaux du cortex de rat après traitement à l'IFNγ pour observer le mouvement des récepteurs GluA1 et GABA-A. Après traitement à l'IFNγ, nous avons montré que le nombre de récepteurs GluA1 présents à la synapse augmentent dépendamment de la dose, avec une augmentation plus importante pour le traitement à 100ng/ml comparativement à 10ng/ml. Le traitement à l'IFNy à 10ng/ml, et non à 100ng/ml, montre, de plus, une augmentation d'expression de récepteurs GABA-A à la surface de la synapse. Ensuite, pour identifier quel type cellulaire est responsable de la libération d'IFNγ dans le système nerveux, nous avons réalisé des PCR quantitatives sur des sous-types gliaux traités pour stimuler l'activité neuronale puisque l'IFNy est régulé par l'activité. Des microglies ont été traités par fractalkine et des astrocytes ont été traités par glutamate, et nous évalué les taux

d’IFNγ mRNA des populations de cellules traitées comparativement aux contrôles. Nous avons mis en évidence à une forte augmentation du taux d’IFNγ dans les microglies traitées alors que les astrocytes ne présentent qu’une faible augmentation comparée aux contrôles. Pour finir, nous avons mené une étude comportementale chez les souris KO pour le récepteur de l'IFNγ afin d’analyser les conséquences de la perte de la signalisation d’IFNγ sur le comportement. Pour cela nous avons utilisé le test « light-dark box » qui a mis en évidence que les souris IFNγ-KO présentent une augmentation du comportement de type anxieux. Dans l'ensemble, ces données renforcent l'idée que le système neuro-immunitaire peut moduler la plasticité synaptique et le comportement, par le biais de multiples facteurs dont l'IFNγ.

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Contribution of Authors to Research Work

CHAPTER 1

Chapter 1 is an unpublished review of scientific literature to relevant to this thesis, written by M. Abuzgaya.

CHAPTER 2

Chapter 2 is an in-depth explanation of all relevant methodology of techniques completed and discussed in chapter 3 and 4, written by M.A.

CHAPTER 3

The experiments presented in this chapter were conceived by both Malak Abuzgaya and Dr. David Stellwagen. All cultures were prepared by M.A and Zahra Abbasi, and all subsequent electrophysiology and immunocytochemistry was performed by M.A. Confocal images for Figure 2B and 3B were acquired by Z.A.

CHAPTER 4

The experiments in this chapter were developed by M.A and D.S. qRT-PCR, cultures, behavioural experiments and data analysis was performed by M.A.

CHAPTER 5

This chapter is a synthesis of data presented in Chapters 3 and 4 placed within the context of current literature, written by M.A.

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List of Abbreviations

ACSF artificial cerebral spinal fluid

AMPAR α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor

CNS central nervous system

CX3CL1 C-X3-C Motif Chemokine Ligand 1 (fractalkine)

CX3CR1 C-X3-C Motif Chemokine Receptor 1

GABAR γ-aminobutyric acid receptor

GBZ gabazine

HSP homeostatic synaptic plasticity

IFNγ interferon gamma

IFNγR1KO interferon gamma receptor subunit 1 knockout

IL-1β interleukin 1 beta

ISG Interferon stimulated genes

LDB light dark box

LTD long-term depression

LTP long-term potentiation

mEPSC miniature excitatory post synaptic current

mGluR metabotropic glutamate receptor

mIPSC miniature inhibitory synaptic current

mPSC miniature post synaptic current

mRNA messenger ribonucleic acid

NMDAR N-methyl-D-aspartate receptor

PFC prefrontal cortex

PTX picrotoxin

qPCR quantitative polymerase chain reaction

RT-qPCR reverse transcription quantitative polymerase chain reaction

TNFα tumour necrosis factor alpha

TNFR tumour necrosis factor receptor

TTX tetrodotoxin

WT wildtype

Introduction

The immune system and the central nervous system (CNS) were typically believed to be independent of one another, two distinct systems with differing functions. Classically, they were thought to be so separate that a term was created to portray how the two were kept independent: immune privilege. This was initially suggested because of the lack of conventional lymphatic vessels in the brain, proposing that the CNS is incapable of typical immune responses. However, it was discovered under certain pathological conditions that cytokines, vital moderators of the immune system, are produced in the brain (Hopkins & Rothwell, 1995). Furthermore, it is becoming apparent that many immune molecules exist not only in the nervous system, even under non-pathological conditions, but also play an active part in modulating synaptic function (Vitkovic et al., 2000).

Some immune molecules are involved in a mechanism to control the effects of the positive feedback element of Hebbian plasticity, known as synaptic scaling. If not for this plasticity, neural circuits can experience excitotoxicity or silencing of the whole network. The excitability of the neuronal network is unequivocally reliant on the delicate balance between inhibitory GABAergic (γ-aminobutyric acid) transmission and excitatory glutamatergic transmission, and this balance is vital for a fully functioning neuronal network, as demonstrated by neuropathologies where this balance is disturbed (Selten et al., 2018). Immune signals, such as the protein tumour necrosis factor alpha (TNFα) that are involved in neuroinflammation also play a role in maintaining this balance (Ibata et al., 2008), and their levels are additionally altered during neuropsychiatric disorders (Khairova et al., 2009). Since TNFα helps maintain circuit functioning during homeostatic synaptic plasticity by upscaling synapses to enhance excitatory synaptic strength overall, other factors likely have the opposite effect of TNFα to downscale

synapses and augment overall inhibitory synaptic strength to counteract circuit excitotoxicity. Determining what these factors may be is the first step towards restoring the disrupted balance of excitation and inhibition in the brain to help alleviate the debilitating symptoms of these pathologies, such as anxiety-like behaviours in mood disorders.

We have identified another immune signalling protein, the pro-inflammatory cytokine interferon-gamma (IFNγ), as a candidate for this role through a gene expression screen. We propose that IFNγ could act as an opponent to TNFα in modulating synapses. Long-term IFNγ treatment in neuronal cell culture has been previously shown to reduce the frequency of excitatory signals between neurons and to decrease the number of AMPA receptors (AMPARs) at synapses (Vikman et al., 2001). IFNγ knockout mice also exhibit increased anxiety (Campos et al., 2014). Therefore, we hypothesize that IFNγ does alter the strength of neuronal connections to decrease excitation in the brain and that loss of IFNγ function could lead to anxiety. From this, we hypothesize that IFNγ plays a substantial role in regulating synapses, and in exploring this hypothesis further, we divided our research into three aims:

Aim 1: Determine how IFNγ affects excitatory and inhibitory synapses.

1A: Examine the effect of short-term IFNγ treatment on glutamate-mediated excitation and GABA (γ-aminobutyric acid)-mediated inhibition in dissociated rat cortical cultures.

Understanding how acute IFNγ treatment physiologically affects excitatory and inhibitory signalling is the first step in fully characterizing its role at the synapse. We tested this by treating wildtype rat neuronal cultures with differing dosages of IFNγ to examine both pre-and post-synaptic current changes. We hypothesized effects opposite to those of TNFa, suggesting decreased excitatory signalling and/or increased inhibitory signalling.

1B: Quantify how IFNγ affects AMPA and GABA receptor trafficking in dissociated rat cortical cultures.

To visualize the electrophysiological changes at the synapse seen within the first aim, we monitored receptor trafficking of both GluA1 and GABA-A γ2 subunits using immunocytochemistry of rat cortical cultures to observe if any electrophysiological differences are accompanied by changes in the content of surface AMPA and GABA receptors.

Aim 2: Examine where IFNγ is released when synapses are altered in dissociated rat glial and neuronal cultures.

IFNγ has been previously shown to affect inhibitory synapses, but no evidence has shown where IFNγ is being secreted when synapses are altered. Therefore, we wanted to investigate if IFNγ was a glial factor during disrupted activity. To examine this, we looked at both astrocytic and microglial cultures treated with factors known to change signalling in these cell populations and performed qPCR with IFNγ primers to determine if IFNγ is present in either astrocytic or microglial cultures when synapses are altered.

Aim 3: Investigate the behavioural consequences of altered cortical synapse strength in IFNγ knockout mice. Understanding how IFNγ plays a role in behaviour is an important step toward creating a bigger picture of what functions IFNγ is involved in. We examined this using standardized rodent tests for anxiety on IFNγKO mice to confirm whether their behaviour is different compared to age-matched wildtype (WT) mice.

Chapter 1- Literature Review

The neurobiology of the immune system is multifaceted and involves countless cellular and molecular mechanisms to ensure normal circuit function. In the immune system, small protein molecules are secreted by cells known as cytokines that act as messengers between immune cells, regulating how they behave and interact. Any particular cytokine can be released by multiple cell types or act on multiple cell types, in which case it is deemed pleiotropic (Carswell et al., 1975). Cytokines are created in a cascade and can have redundancy in their behaviours and roles (Hopkins & Rothwell, 1995). Furthermore, these cytokines can take action cooperatively or antagonistically (Vitkovic et al., 2000). Overall, cytokine secretion and function can be exceptionally complex, specifically their molecular mechanisms and involvement in the central nervous system.

Glial Dynamics

Traditionally known as the "support-cells of the brain," the number of glial cells in the brain heavily outnumbers neurons by a factor of ten and are necessary for proper brain function. Glial cells such as astrocytes and microglia control homeostasis in the brain. Specifically, microglia are well-known as the brain's macrophages, are involved in identifying and removing pathogens, and are known to activate during differing disease states (Boche et al., 2013). Astrocytes are also involved in numerous functions regarding homeostasis and overall regulation, such as sustaining the blood-brain barrier, giving metabolic and structural support, and helping regulate neuronal communication as part of a complex known as the "tri-partite synapse" (Sofroniew & Vinters, 2010). As the information in the field develops, new evidence suggests that not just astrocytes are involved in such diverse functions. Still, microglia may also play an essential role in influencing synaptic function and maintaining overall circuit balance.

During baseline conditions, microglia exist in a resting state, consisting of a branched morphology while highly active, constantly examining their microenvironment with numerous motile processes and protrusions (Nimmerjahn et al., 2005). Once they have recognized what they consider a threat, they activate and develop an amoeboid morphology and act as a typical macrophage by initiating macrophage-like functions such as cytokine release and phagocytosis (Boche et al., 2013). Activated microglia are involved in neuropathologies such as neurodegeneration, stroke, traumatic brain injury, and neuropsychiatric conditions (Yirmiya et al., 2015; Loane & Kumar, 2016; Bachtell et al., 2017; Ma et al., 2017).

Microglia can initiate pro- and anti-inflammatory responses (Wolf et al., 2017). During these responses, pattern recognition receptors on the microglial plasma membranes identify the source of what may be disrupting homeostasis, such as endogenous proteins associated with a disease or foreign pathogens (Rodríguez-Gómez et al., 2020). Once detected, the microglia will secrete cytokines to reinstate homeostasis. In the pro-inflammatory response, they release molecules such as TNFα. Once released, this response can create a positive feedback loop and shift from neuroprotective to neurotoxic, which can aggravate or cause diseased states by the damage caused. TNFα has been shown to drive calcium-permeable AMPARs to the cell surface (Stellwagen et al., 2005). Due to this, past studies have investigated the role of TNFα -induced calcium-permeable AMPAR trafficking in promoting excitotoxic damage since calcium is the key culprit for excitotoxicity (Choi, 1992; Dong et al., 2009). Another report intriguingly demonstrated that TNFα derived from Muller glia aggravates excitotoxicity in the retina mainly due to the trafficking of calcium-permeable AMPARs to the cell surface (Lebrun-Julien et al., 2009), solidifying the importance of firm regulation and restriction of glial TNF production.

Microglia, in their repair response, will release anti-inflammatory molecules such as TGF-B and IL-10 to act as a negative feedback loop for the pro-inflammatory cytokines inflicting injury (Colton, 2009). Overall, microglial activation can have either a neuroprotective or neurotoxic phenotype dependent on the physiological context of each condition.

Microglia, just like astrocytes, can modulate circuit homeostasis at the synaptic level via regulating neuronal activity, synaptogenesis, cytokine release, chemokine release, and synaptic pruning (Wright-Jin & Gutmann, 2019; Akiyoshi et al., 2018). Most relevantly, microglia can regulate the strength of both excitatory and inhibitory synapses via the release of TNFα (Raffaele et al., 2020; Liu et al., 2017), which has an established role in homeostatic synaptic plasticity.

Tumor Necrosis Factor Alpha (TNFα)

Tumour necrosis factor (TNFα) is a pro-inflammatory cytokine produced mainly by monocytes, macrophages in the periphery, and the central nervous system microglia. However, other cells can produce it as well. TNFα is a 26kDa transmembrane protein, and the metalloprotease TNFα-converting enzyme (TACE) can break it down to a 17kDa soluble form. TNFα, in both its transmembrane and soluble form, can bind to and activate receptors (Sedger et al., 2014; Holbrook et al., 2019). It acts by binding to two receptors (MacEwan, 2002), TNFR1 and TNFR2, the first of which is the most common (Vitkovic et al., 2000; Wajant et al., 2003), and the soluble form of TNFα (sTNFα) preferentially binds to TNFR1. TNFR1 has a widespread and constitutive distribution in animal tissues, whereas TNFR2 is mainly found in immune system cells (Vandenabeele et al., 1995); however, it has also been discovered that it is expressed in neurons (Neumann et al., 2002).

TNFα role in central nervous system

TNFα was first identified as a cytokine functional only in the immune system and under pathological conditions. However, many immunological molecules, such as TNFα, are present in the nervous system even in the absence of pathology (Vitkovic et al., 2000). Many studies show that TNFα has neuroprotective effects (Marchetti et al., 2004). TNFR1 knockout mice showed decreased neurodegeneration, whereas TNFR2 knockout mice showed increased neurodegeneration (Fontaine et al., 2002). These findings suggest that the neuroprotective properties of TNFα are TNFR2-dependent, even with TNFR1 being linked to neuronal death (Swarup et al., 2007).

TNFα and synaptic plasticity

Hebbian synaptic plasticity is a positive feedback mechanism in which potentiated synapses are more likely to undergo more potentiation and get stronger once long-term potentiation (LTP) happens than before LTP induction and can reach an unstable state prone to hyperexcitation (Cooper & Bear, 2012). This is achieved through mechanisms such as changes in neuronal excitability, global changes in synaptic strengths, and the regulation of the number of synapses (Turrigiano & Nelson, 2000). The inverse occurs following long-term depression (LTD); the synapses get weaker, and unrestricted synaptic depression could lead to pathological synapse elimination (Cooper & Bear, 2012). Most studies have focused on forms of LTD that are triggered by synaptic activation of either NMDARs (N-methyl-D-aspartate receptors) or metabotropic glutamate receptors (mGluRs) (Collingridge et al., 2010). This positive mechanism can lead to excitotoxicity following too much LTP and overall network silencing following excess LTD. To bypass this possible issue, there is an additional synaptic plasticity mechanism

in the central nervous system known as homeostatic synaptic plasticity (HSP). HSP is a response to abnormal neuronal activity and serves as a negative regulator of synaptic strength to normalize circuit function (Davis, 2006; Turrigiano, 2007). An important type of synaptic plasticity called synaptic scaling adjusts the strengths of synapses on a specific cell in response to overall changes in firing rate (Turrigiano et al., 1998).

Synaptic scaling

Synaptic scaling is a homeostatic kind of synaptic plasticity that functions to reduce (downscaling) or enhance (upscaling) excitatory synaptic strength during prolonged hyperactivity or inactivity, respectively (Turrigiano et al., 1998; Turrigiano & Nelson, 2004). Inhibitory synaptic strength is regulated inversely, where synapses are strengthened during sustained hyperactivity and weakened during inactivity (Aptowicz et al., 2004; Bausch et al., 2006; Buckby et al., 2006).

TNFα, through its effects on AMPA receptor trafficking, plays a role in modulating synapses (Beattie et al., 2002). TNFα is necessary for scaling up and strengthening the excitatory synapses in response to prolonged activity blockade, as it leads to both AMPARs exocytosis and GABARs endocytosis on excitatory neurons (Pozo & Goda, 2010). Specifically, the function of glial TNFα in inactivity-induced synaptic scaling was highlighted by Stellwagen and Malenka, where blocking TNFα signalling during chronic tetrodotoxin (TTX) prevented scaling up in excitatory synapses in hippocampal neurons. Moreover, synaptic scaling was observed in neurons from TNFαKO mice and glia from wildtype (WT) mice, but not in neurons from WT mice and glia from TNFαKO mice (Stellwagen & Malenka, 2006). Additionally, mice lacking TNFα are deficient in part of the visual cortical plasticity after monocular deprivation, related to loss of homeostatic synaptic plasticity (Kaneko et al., 2008; Ranson et al., 2012). These findings

strongly suggest that TNFα is a crucial synaptic upscaling mediator, and glia are responsible for this response.

Another type of scaling, known as downscaling, happens in response to increases in the overall firing rate. After a chronic increase in neuronal activity usually mediated by GABA receptor antagonists, a compensatory mechanism reduces the AMPARs at excitatory synapses and mEPSC amplitude (Turrigiano et al., 1998). In addition to AMPAR removal, neuronal hyperactivity leads to the externalization of GABA-A Rs to the post-synaptic membrane (Rannals et al., 2011). Surprisingly, the decrease in excitatory synaptic strength in response to a prolonged increase in neuronal activity is not dependent on TNFα, implying that other signals mediate changes in opposition to TNFα. The Stellwagen group 2014 demonstrated that agrin-dystroglycan signalling is required for GABA receptors exocytosis following elevated firing activity after bicuculline (BIC) treatment (Pribiag et al., 2014). This study suggested that agrin is sufficient and required for scaling up inhibitory synapses and can directly lead to GABARs exocytosis. These reports show that TNFα is necessary for upscaling excitatory synapses during homeostatic synaptic plasticity. Other factors are involved in scaling up inhibitory synapses; both mechanisms function through receptor trafficking to modulate activity levels.

AMPARs trafficking in synaptic scaling

AMPARs are heteromultimers of the GluA1-4 subunits (Greger et al., 2007). Many studies indicate that the GluA1 subunit is necessary for activity-dependent activity-dependent AMPAR trafficking, such as one report demonstrating an increase in the GluA1 subunit after activity blockade (Stellwagen & Malenka, 2006). Other evidence showed that TNFα application led to exocytosis of GluA2-lacking AMPARs (Stellwagen et al., 2005). Moreover, other studies

corroborated these results and exhibited an increase in GluA2-lacking AMPARs after activity blockade mediated by TTX (Thiagarajan et al., 2005; Sutton et al., 2006). However, a previous lab member demonstrated that no subunit is necessarily required for synaptic scaling mediated by TTX since they could detect HSP in knockout cultures for GluA1, GluA2, and GluA3 subunits (Altimimi & Stellwagen, 2013). In 2020, one group recently showed that one tyrosine phosphorylation site (Y876) in the GluA2 subunit is necessary for AMPAR upscaling using knock-in mice (Yong et al., 2020). Another group reported that the phosphorylation site S845 in the GluA1 subunit is necessary for AMPAR upscaling (Sathler et al., 2020). There are also a few studies claiming that AMPAR exocytosis during homeostatic synaptic plasticity requires the GluA2 subunit (Gainey et al., 2009; Lu et al., 2011). These studies demonstrate that TNFα is involved in activity-dependent AMPAR trafficking and, through this process, modulates synapses during homeostatic synaptic plasticity.

GABARs trafficking in synaptic scaling

Gamma-Aminobutyric acid (GABA) is the most important inhibitory neurotransmitter within the central nervous system. The control of GABAergic synaptic strength is necessary to keep the overall firing rate within a middle range, as there are studies indicating that disruption of this mechanism results in hyperexcitability, which is a common and early symptom of a variety of brain diseases such as epilepsy (McCormick & Contreras, 2001; Saxena & Caroni, 2011). GABA receptors can be divided into two types: GABA-A and GABA-B. The majority of rapid inhibitory neurotransmission in the central nervous system is mediated by GABA-A receptors (Lorenz-Guertin et al., 2018). GABA-A receptors are pentamers that typically consist of several subunits

in the cortex and forebrain: two α, two β, and a γ2 subunit. This γ2 subunit is the most common subunit within the cortex.

The literature indicates that the γ2 subunit is necessary for GABARs trafficking. After elevated neural circuit activity mediated by glutamate receptor activation, GABA-ARs were rapidly and reversibly externalized to the plasma membrane (Muir et al., 2010). A study on murine dissociated culture neurons exposed to depolarization exhibited that increased neuronal activity led to an increase in GABA-AR γ2 subunit clusters and pre-synaptic GAD-65 (Rannals et al., 2011).

Cytokines role in Excitatory and Inhibitory Balance

Balancing excitatory and inhibitory signalling in neural circuits is critical for proper cell function, as evidenced by many neuropsychiatric conditions where this equilibrium is disrupted (Fee et al., 2017). Other cytokines are released in the brain to maintain this balance, such as the pro-inflammatory cytokine tumour necrosis factor-alpha (TNFα), which is released via glial cells in the brain to maintain this balance as it increases excitatory synaptic strength when brain activity drops (Stellwagen & Malenka, 2006) via synaptic upscaling. Another cytokine, for example, is IL-1β, where low concentration at 1–3 pM enhances LTP in hippocampal slices and facilitates hippocampal-dependent memory (Goshen et al., 2007; Ross et al., 2003); whereas pathologically-high concentrations reduce LTP at 1–3 nM (Ton et al., 2012; Ross et al., 2003) and reduces hippocampal-dependent memory (Goshen et al., 2007).

Numerous studies reported that TNF-α levels are elevated in patients with mood disorders (Elhaik et al., 2015; Liu et al., 2012; Kopschina Feltes et al., 2017; Köhler et al., 2017; Goldstein et al., 2009; Rao et al., 2010). Irregular TNF-α levels have been demonstrated to

impact the intensity of psychiatric symptoms and their resilience to treatment. One recent study indicated that elevated baseline plasma TNF-α levels in patients with major depression might predict a better improvement in the intensity of suicidal thoughts (Choi et al., 2021). Despite the majority of the literature demonstrating that modulations in inflammatory mediator levels occur in patients with mood disorders, it is important to note that some studies report opposite findings (Munkholm et al., 2013; Modabbernia et al., 2013). When TNFα is pathologically elevated in mood disorders, it can lead to over-excitation in the CNS, especially in the prefrontal cortex (PFC), thus contributing to anxiety behaviours known to be mediated by this area of the brain since clinical and basic research studies indicate that anxiety and depressive symptoms arise from synaptic deficits in the medial prefrontal cortex (PFC) and the hippocampus (Khairova et al., 2009; Dunman et al., 2016).

Since synaptic scaling helps maintain circuit homeostasis under normal conditions, there are likely other factors which have the opposite effect of TNFα to reduce excitatory signalling instead when brain activity rises; determining what these factors are is the first step towards restoring the balance of excitation and inhibition in the brain to help alleviate anxiety-like symptoms possibly.

An initial gene expression array was conducted in a previous student's thesis (Heir, 2019) to identify possible cytokines involved in regulating synapses during activity perturbations. The expression array was performed on organotypic hippocampal slice cultures. They were treated for 48h with either 1 uM tetrodotoxin (TTX) to block activity or 20 uM gabazine (GBZ) to inhibit GABA-A receptors from elevating activity and were harvested for their mRNA. After reverse transcription, qRT-PCR was performed in order to examine the expression levels of 84 cytokines and chemokines of the array. While the array included many interesting results, the

most intriguing was how IFNγ responded to the treatments. The results of the gene expression array indicated that IFNγ drastically decreased with TTX treatment, though it was slightly below significance with a similarly large but insignificant increase with activity elevation. A post-hoc validation via qPCR was performed to verify the results found. IFNγ expression was confirmed to decrease with TTX significantly and increased with GBZ, strengthening the results obtained in the gene expression array. This was particularly interesting in light of the fact that this response acted in exact opposition to TNFα and had brought attention to the cytokine to possibly act as an opponent to TNFα in homeostatic synaptic plasticity as a potential downscaling factor.

Interferon Gamma (IFNγ)

Interferon-gamma (IFNγ) is a pro-inflammatory cytokine that classically plays a role in the response of an organism to viral and intracellular bacterial infections (Schoenborn & Wilson, 2007). In the CNS, there is some evidence that it can regulate both synaptic properties (Vikman et al., 2001; Maher et al., 2006) as well as both anxiety-like and depressive-like behaviours (Campos et al., 2014; Monteiro et al., 2016). IFN-γ binds to the heterotetrameric IFNγ receptor comprised of two subunits, IFN-γR1 and IFN-γR2, that are generally expressed in several cell types, including microglia, astrocytes, oligodendrocytes, endothelial cells, neural precursor cells and neurons (Hashioka et al., 2010; Hausler et al., 2002; Mizuno et al., 2008; Ni et al., 2014). The levels of IFN-γ receptor expression vary between cell types, brain regions, and species, between in vitro/ in vivo conditions, and can additionally change with differing external stimuli (Hashioka et al., 2010; Lee et al., 2006; Li et al., 2010; Robertson et al., 2000).

IFNγ initiates diverse signalling pathways by binding to the IFNγ receptor (IFNγR), where binding of IFNγ to the subunit IFNγR1 causes heterotetramerization of the receptor, which

then activates downstream kinases (Ramana et al., 2002; Platanias, 2005). IFNγ predominantly activates the Janus-associated kinase/signal transducer and activator of the transcription-1 (JAK/STAT) signalling pathway. The activation of this pathway results in the phosphorylation of STATs at the receptor. STAT1 is the predominant downstream effector of IFNγ. The four tyrosines contained by Jak1, Jak2, IFNγR1, and Stat1 are phosphorylated within 1 minute of IFNγ treatment (Greenlund et al., 1994; Igarashi et al., 1994). Once phosphorylated, STAT1 homodimerizes and translocates to the nucleus, where it initiates the transcription of IFNγ-stimulated genes (ISGs), with the changes acting swiftly. In fact, the first wave of IFNγ stimulated transcription occurs within 15 minutes of IFNγ treatment (Kerr et al., 1991), inducing fast-acting physiological changes. The profile of ISGs, and their uses are dependent both on cell type and on other inflammatory signals that are received by the target cell (van Boxel-Dezaire et al., 2007). Overall, the plethora and diversity of ISGs demonstrate that a phenotypic response to IFNγ varies depending upon the cell type, which coincides with the conflicting reports of neuroprotection and toxicity with IFNγ treatment (Mizuno et al., 2008; Bate et al., 2006).

On a broader scale, levels of IFN-γ in the CNS are altered during neurodegenerative, neuroinflammatory, and neuropsychiatric disorders (Arolt et al., 2000; Lee et al., 2006; Reale et al., 2011; Söderström et al., 1995; Wei et al., 2000). In addition to the vital role IFNγ plays in inflammation, growing evidence in the literature proposes that IFNγ signalling can also modulate neurological processes underlying cognitive behaviours, including neurogenesis, neuronal excitability and plasticity (Filiano et al., 2016; Li et al., 2010; Monteiro et al., 2016). Because IFNγ is a pleiotropic cytokine, alterations in IFNγ expression often affect multiple neural and immune cells, which can further impact synaptic function.

IFNγ's role in synaptic function

Many neuropsychiatric disorders, such as schizophrenia, epilepsy, autism, and anxiety, exhibit an imbalance between excitatory and inhibitory mechanisms in several brain regions, including the cortex (Yizhar et al., 2011; Marín, 2012). Thus far, little is known about the effects of IFNγ on cortical excitability. In vitro experiments have shown that the acute application of IFNγ on cultured rat hippocampal slices increased the excitability of CA3 neurons (Muller et al., 1993), while chronic IFNγ treatment decreased gamma oscillations in the CA3 (Ta et al., 2019).

In the literature, the impact of IFNγ on GABA-mediated currents in the hippocampus, a major brain region involved in cognitive function, has been investigated. It has been demonstrated that IFNγ treatment increased the frequency of both spontaneous Inhibitory Post Synaptic Currents (sIPSCs) and mini–Inhibitory Post Synaptic Currents (mIPSCs) in hippocampal CA1 pyramidal neurons (Flood et al., 2019).

Another study shows that acute application of IFNγ potentiates GABA-mediated tonic currents in mouse prefrontal cortex layer 2/3 pyramidal neurons (Fillano et al., 2016). This laboratory had shown that SCID mice, which possess a genetic autosomal recessive mutation designated $Prkdc_{scid}$ and have severe combined immunodeficiency affecting both B and T lymphocytes, exhibit social deficits and hyper-connectivity of fronto-cortical brain regions and that immune dysfunction in particular IFNγ, may play a role in disorders characterized by social dysfunction (Fillano et al., 2016).

Another group also examined the effects of IFNγ treatment on inhibitory currents in the cortex. They found that IFNγ receptors are present in the membrane of rat neocortical layer five pyramidal neurons. They performed whole-cell patch clamp electrophysiology recordings on acute slices in vivo and exogenously applied IFNγ for 20 minutes. This study shows that IFNγ

treatment increases the amplitude of spontaneous IPSCs and miniature IPSCs on neocortical layer five pyramidal neurons in P20 rats, whereas their frequency remained unchanged (Janach et al., 2020). To investigate whether IFNγ triggers a change in excitability, they examined the firing behaviour of layer five pyramidal neurons before and 20 min after direct application of IFNγ on P20 rats using acute slice electrophysiology. Interestingly, IFNγ did not alter subthreshold nor suprathreshold neuronal excitability, leading the authors to interpret this result to indicate augmented inhibitory transmission by IFNγ (Janach et al., 2020).

While the effect of IFNγ on GABAergic neurotransmission has begun to be investigated, the effect of acute IFNγ treatment on excitatory transmission has been sparsely investigated. However, to entirely understand the role of this pro-inflammatory cytokine in excitatory and inhibitory balance in the brain, a fully comprehensive study, including an analysis of both inhibitory and excitatory signalling, must be conducted.

Altogether, this evidence indicates that the neuroimmune system can modulate synaptic plasticity, with IFNγ being a possible important mediator. The evidence of IFNγ being a potential facilitator of synaptic plasticity towards inhibition is what brought us to believe that IFNγ may be a promising opponent to TNFα in homeostatic synaptic plasticity. Fully characterizing the role of IFNγ in synaptic plasticity and investigating the molecular mechanisms behind these processes is critical to potentially harnessing it as a treatment and improving our understanding of the neuroimmune system function from an excitatory and inhibitory balance lens in diseased states.

Chapter 2- General Methodology

Animals

Wild-type Sprague Dawley rats were obtained from Charles River laboratories, as were C57/BL6 wildtype mice. IFNγR1$^{-/-}$ mice were purchased from The Jackson Laboratory (Diamond et al., 2011; JAX #025394), and were kept and bred in the Montreal General Hospital animal facility. 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.

Dissociated Culture Preparation

Neural Cultures

Coverslips (Fisherbrand Microscope Cover Glass) were treated with nitric acid for 48 hours and washed 6 times for 1 hour each time with distilled deionized water (Millipore Sigma); then conserved in 90% ethanol. Coverslips were placed in 24-well plates and sterilized under UV light for 15 minutes. 450 μL of poly-Dlysine were added to each well and incubated for 1 hour; the plates were then washed 3 times with Ultrapure Distilled Water (Invitrogen) and incubated with plating media (standard neuronal media composed of 500 mL Neurobasal (Thermo Fisher), 10 mL B27 supplement (Thermo Fisher) and 1.25 mL GlutaMAX (Thermo Fisher), 10% foetal bovine serum). E17-18 embryos were removed from pregnant Sprague-Dawley rat females (Charles River Laboratories) and the cortex was dissected out. Briefly, the skull was opened with butterfly scissors and peeled back, and the brain was scooped into cold Hank's Balanced Salt Solution (HBSS) in a dish and sliced into hemispheres with a scalpel. Meninges and midbrain structures were removed, then the hippocampus was cut out to be left with only cortex. The cortex was minced, then the tissue was treated with trypsin for 20 minutes, at which point the

trypsin was removed and stop solution (2.5 mg/mL BSA in HBSS) was added. Stop-solution was replaced with standard neuronal media (composed of 500 mL Neurobasal (Thermo Fisher), 10 mL B27 supplement (Thermo Fisher) and 1.25 mL GlutaMAX (Thermo Fisher)). The tissue was triturated 2-3 times with a pipette to create a single-cell suspension. The resulting supernatant was filtered through a sterile cell strainer (Fisherbrand). Cells were counted and plated in the 24 well-plates (5 μL of supernatant for a cell density of 55 000 cells/cm^2). After two hours of plating in an incubator the media was changed to 800 μL of standard neuronal media per well.

Microglial Cultures

Mixed glial cultures were generated from P0 Sprague Dawley rat pups. Briefly, cortices were dissected in dissociated using 0.05% trypsin-EDTA and physical trituration with a pipette to create a single cell suspension. The resulting supernatant was filtered through a sterile cell strainer (Fisherbrand). Cells were plated into cell culture flasks (Sarstedt) and grown to confluence in MEM supplemented with 10% FBS. After 10 Days in vitro, flasks were placed on a shaker in a sterile incubator at 37 degrees Celsius at 200 rpm for 4 hours, essentially dislodging all microglia to allow them to collect and float in the medium. The supernatant is aspirated and plated on PDL-coated 10cm plates with glial media containing FBS and Granulocyte-macrophage colony-stimulating factor (GM-CSF) to help the microglia proliferate. After an additional 7 days, pure confluent microglial cultures are ready to be used for experiments.

Astrocytic Cultures

Mixed glial cultures were generated from P0 Sprague Dawley rat pups. Astrocyte-only cultures were generated by taking confluent mixed glial cultures post-microglial removal, and passaging cells 1-2 times over the course of 1 week to remove any remaining oligodendrocytes

that may not have been removed from the vigorous shaking. Cells were then passaged an additional time into 10cm cell culture dishes and used for experiments 2-3 days after that.

Treatments

Interferon Gamma was solubilized in PBS and applied to cultures at a concentration of either 10 ng/ml or 100 ng/ml. For all experiments, IFNγ was treated for 45 minutes to an hour maximum to evaluate the effects of acute treatment on these cell populations. Astrocytes and microglia were treated with 4μl/ml of Glutamate and 1μl/ml of fractalkine, respectively for 48 hours before harvesting cells for experiments.

Electrophysiology

Recordings

Embryonic neocortical cultures were prepared as previously described and assayed 18-23 days in vitro. Live cells were placed on an inverted microscope in circulating aCSF (115 mM NaCl; 5mM KCl; 4.2 mM HEPES; 23 mM glucose, 26 mM sucrose; 2. 5 mM CaCl2; 1.3 mM MgCl2).

During mEPSC recordings, cells were held at a membrane potential of -70mV to record AMPA receptor-mediated currents. Circulating aCSF was supplemented with 0.5 mM TTX and 100 μM PTX to block sodium channels and GABA receptor activity, respectively. Borosilicate electrodes were filled with 122mM CsMeSO4; 8 mM NaCl; 10 mM glucose; 1 mM CaCl2; 10 mM EGTA; 10 mM HEPES; 0.3 mM Na3-GTP; 2 mM Mg-ATP.

During mIPSC recordings, cells were held at a -70mV voltage-clamp, using aCSF mixed with 500 nM TTX and 50 μM NBQX to block sodium channels and AMPA receptor activity, respectively. For mIPSCs, we filled the same glass electrodes as described above with an

inhibitory internal solution containing (in mM): 122 CsCl, 8 NaCl, 10 glucose, 1 CaCl2, 10 HEPES, 10 EGTA, 0.3 Na3-GTP, 2 Mg-ATP (308-310 mOsm, pH 7.3 adjusted with CsOH).

For each cell recorded, events within one 60 second sweep were analyzed. Both mEPSC and mIPSC amplitude was calculated as the average amplitude of all events within the sweep. The mPSC frequency was determined as the number of events divided by the duration of the sweep. Pyramidal cells were identified by their morphology, input resistance, and presence of mPSCs. Only recordings with stable access resistance (6-20 MΩ), and cells with membrane resistance <150 MΩ, or very low mPSC frequency (<0.2 Hz) were excluded from further analysis. Clampfit10 was used to identify mPSCs using template matching and each mPSC was verified visually to exclude software errors.

Immunocytochemistry

Live-feed immunofluorescence

Live cells were incubated with a 1:100 dilution of mouse monoclonal primary antibody anti- GluA1 (extracellular, Neuromab Antibodies Inc.) or a 1:100 dilution of rabbit monoclonal primary antibody anti- GABA(A)γ2 receptor (extracellular, Alomone labs) for 10 minutes, washed with Dulbecco's phosphate-buffered saline (DPBS) containing calcium and magnesium (Life Technologies) then fixed in 2% PFA for 15 minutes at 4°C on ice and washed 3 times for 5 minutes with PBS (Life Technologies). Coverslips were incubated in blocking buffer (30 mg/mL BSA, 20 μL/mL normal goat serum, PBS) for 30 minutes at room temperature; then secondary antibodies (Donkey anti-Mouse Highly Cross- Adsorbed IgG 568 nm for GluA1, Donkey anti-Rabbit Highly Cross-Adsorbed IgG 647nm for GABA, ThermoFisher) were added for 1 hour at a 1:1000 dilution in blocking buffer (from which point the cells were sheltered from light).

Coverslips were washed 3 times with PBS and mounted onto microscope slides (Fisher Scientific) with 5 μL of ProLongTM Gold antifade reagent with DAPI (Invitrogen) for 24 hours at room temperature and imaged.

Image Acquisition and Analysis

Images were acquired on an epifluorescence inverted system microscope (Olympus IX51) with a CCD camera (Hamamatsu Orca R-2) using a 60x objective. A range of 3-4 coverslips were analyzed per treatment group, with cultures being made from 3 separate rat embryonic neural culture preparations. Analysis of surface receptors was carried out using ImageJ software. Images were obtained with identical acquisition conditions. The total threshold area of fluorescence labelled surface receptors (AMPA or GABA) was measured automatically by the ImageJ software and divided by the total processed area, which was determined by setting a lower threshold level to measure background fluorescence produced by the fixed cells. The fluorescence of all cells was normalized by dividing by the average fluorescence of the untreated control cells and compared using parametric statistical tests, specifically one-way ANOVA with post-hoc Tukey's multiple comparisons test.

Quantitative PCR

In-vitro dissociated culture samples

mRNA was extracted after treatment using the RNeasy Mini kit (Qiagen), followed by reverse transcription with the Quantitect Reverse Transcriptase kit (Qiagen). The resulting cDNA was used for qPCR using the Fast Taqman master mix (ThermoFisher). The IFNγ transcript was measured using pre-validated TaqMan assays (Thermofisher, Assay ID: Rn00685059_g1). All quantification was performed using the ΔΔCt method using GAPDH (Thermofisher, Assay ID:

Rn01775763_g1) as a reference gene after verifying that the amplification efficiency was between 90-110%. To normalize the samples, ΔCt between IFN and GADPH 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. Statistical analysis was performed by comparing -ΔΔCt values using the nonparametric Wilcoxon tests.

Behavioural Testing

Our behavioural test was the light-dark box test (LDB) performed using IFNγR1$^{-/-}$ mice and C57/BL6 wildtype (WT) mice. Individual IFNγRIKO mice and WT male mice were placed in the LDB and were recorded for 10 minutes. Time spent in the light and dark box was measured and recorded using the EthoVision video analysis software. Total distance traveled and mean velocity for each mouse was also measured by EthoVision video analysis software as control variables to ensure that changes in locomotion do not account for differences in time spent between boxes.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 9 software. If the data is normally distributed (confirmed using the Shapiro-Wilk test) it was analyzed using either a student's t-test or an ordinary one-way ANOVA. If the dataset does not meet this criterion, then a nonparametric test is performed, such as Wilcoxon test. Regarding the electrophysiology data sets, we conducted ordinary one-way ANOVAs, and completed post hoc analyses where we

performed a simple main effect analysis followed by Tukey's multiple comparisons test. For the immunocytochemistry data sets, the fluorescence of all cells was normalized by dividing by the average fluorescence of the untreated control cells and compared using parametric statistical tests, a one-way ANOVA, again with a post-hoc Tukey's multiple comparisons test. For the qPCR data set, we conducted the nonparametric Wilcoxon test. Lastly, for the behavioural data set, an unpaired students t-test was conducted. All graphs were made using GraphPad Prism 9 software.

Chapter 3- The role of IFNγ signalling on excitatory and inhibitory synapses

Introduction

Homeostatic synaptic plasticity is mediated by receptor trafficking to alter signalling in response to an overall alteration in firing rate at both excitatory and inhibitory synapses (Turrigiano et al., 1998; Cooper & Bear, 2012). Many synapses in the central nervous system have been shown to be regulated by immune molecules, such as TNFα. TNFα has been shown to be necessary for strengthening excitatory synapses in response to prolonged activity blockade, as it leads to both AMPAR exocytosis and GABAR endocytosis on excitatory pyramidal cells (Pozo & Goda, 2010). TNFα, through its effects on AMPA receptor trafficking, plays a role in modulating synapses. More specifically, during inactivity-induced synaptic scaling, where blocking TNFα signalling during chronic tetrodotoxin (TTX) prevented upscaling in excitatory synapses in hippocampal neurons (Stellwagen & Malenka, 2005). This mechanism works to maintain the balance of excitatory and inhibitory signalling, and the results of this study indicate that synaptic strength is homeostatically up-regulated by TNFα, shifting neurons towards more excitation and less inhibition. Homeostatic synaptic plasticity regulates synaptic strength in responses to circuit activity and can either increase excitation following an activity blockade or promote inhibition during excessive activity, thus stabilizing the network. The fact that TNFα is only involved in upscaling suggests that there are likely to be other opposing signals pushing neurons towards less excitation and more inhibition, possibly other neuroimmune molecules that are implicated in regulating synapses already and play a role within the CNS.

In light of this, we suggested IFNγ as a possible opponent to TNFα since the literature shows some evidence that it can regulate both synaptic properties as well as behaviour, as discussed in the first chapter of this thesis. Through a gene expression array conducted to identify activity-regulated cytokines, previous findings in the lab demonstrate that IFNγ expression

significantly decreased with chronic tetrodotoxin (TTX) treatment and increased with gabazine (GBZ) treatment (Heir, 2019). TNFα is released with activity blockade, and its expression induces rapid exocytosis of AMPARs (Stellwagen et al., 2005); an interesting possibility could be that IFNγ could serve to mitigate TNFα-induced trafficking in order to evade excitotoxicity.

IFN-γ signalling can also modulate neurological processes, including neurogenesis, neuronal excitability and plasticity (Filiano et al., 2016; Li et al., 2010; Monteiro et al., 2016). Exogenous administration of IFNγ decreases AMPAR clustering in hippocampal cultures with long-lasting treatments (Vikman et al., 2001). Intraventricular injection of IFNγ into rats results in an inhibition of hippocampal LTP in vivo (Maher et al., 2006). Given the findings of the studies shown, it is clear that IFNγ has the potential to function at synapses. In this chapter, we strive to characterize IFNγ signalling on excitatory and inhibitory synapses to understand further its role in synaptic plasticity and how it acts to alter synapses during normal circuit functioning.

Results

IFNγ regulates inhibitory and excitatory synapse signalling

We have previously found that IFNγ during activity manipulations is inversely regulated in comparison to TNFα (Heir R., 2019). We examined how IFNγ regulates synaptic function. To do so, we acutely treated wildtype rat dissociated neural cultures with IFNγ to study its fast-acting effects at the synapse. We then measured mini-excitatory post-synaptic currents (mEPSC) and mini-inhibitory post-synaptic currents (mIPSCs) in cortical pyramidal neurons. Miniature post-synaptic currents are responses to spontaneous neurotransmitter release; their amplitude is classically thought to reflect post-synaptic receptor function, and their frequency is generally an indicator of release probability (Phillips et al., 2010).

We found that acute IFNγ treatment significantly increases both mEPSCs amplitude and frequency at 100ng/ml but not at the 10 ng/ml concentration level. This result indicates that acute IFNγ treatment alters excitatory synapses. Since both frequency and amplitude are increased at 100ng/ml of IFNγ, it implies that the release probability is elevated, and acute treatment of IFNγ at this concentration may result in increased post-synaptic responses. The same treatment alters mIPSCs and seems to replicate the result shown in the literature (Flood et al., 2019), where IFNγ treatment at 100ng/ml significantly increases the frequency of mIPSCs (Janach et al., 2020) (Figure 2B). However, a novel finding was a significant increase in mIPSCs amplitude at 10ng/ml in comparison to the non-treated controls and to the 100ng/ml group (Figure 2C). The effect of IFNγ at 10ng/ml has not yet been investigated in the literature; this finding indicates a possible novel mechanism of synaptic receptor trafficking at this concentration since the measure of amplitude often reflects post-synaptic receptor function.

IFNγ's effects on surface receptor trafficking

In homeostatic synaptic plasticity, one of the mechanisms of synaptic alteration is the change of surface receptor content through receptor trafficking. To visualize the electrophysiological changes seen in the aforementioned findings, we examined post-synaptic receptor content by monitoring receptor trafficking on the surface of the cells. We imaged receptor trafficking of both AMPA and GABA-A receptors via immunocytochemistry on rat wildtype dissociated neural cultures. We focused our investigation specifically on the AMPA GluA1 and GABA-A γ2 receptor subunits, as they have been found to be the most common subunits directly related to receptor trafficking implicated in synaptic plasticity (Diering & Huganir, 2018; Tyagarajan & Fritschy, 2010).

Our immunocytochemistry staining data demonstrate that acute IFNγ treatment may increase the amount of GluA1 present on synapses at both 10ng/ml and 100ng/ml in a dose dependent-manner (Figure 3). These staining results corroborate the excitatory electrophysiological data (Figure 1A) and confirm that excitatory signalling is shown to increase as IFNγ concentration increases. This suggests a dose-dependent basis for AMPA receptor trafficking in response to IFNγ treatment at the excitatory synapse. The response appears to be strong enough that the trafficking of receptors translates to increased signalling seen at the synapse.

At the inhibitory synapse, we found that the number of GABA-A receptors present on the surface significantly increased at 10ng/ml but not at 100ng/ml (Figure 4). This result suggests an interesting mechanism of receptor trafficking, as the amplitude of the mIPSCs (Figure 2C) was significantly increased at 10ng/ml since this measure generally reflects post-synaptic receptor function.

Discussion

In this chapter, we have found that IFNγ can modulate inhibitory and excitatory synaptic activity and that acute IFNγ treatment alters both mIPSCs and mEPSCs in differing ways in a dose-dependent manner. We have also shown that acute IFNγ treatment increased the amount of GluA1 receptor expression on the surface of the synapse in a dose-dependent manner, with a larger expression seen at the 100ng/ml dose in comparison to the 10ng/ml treatment. IFNγ treatment at 10ng/ml was also shown to increase GABA-A receptor expression on the surface of the synapse

mEPSCs were observed to have no change at the 10ng/ml dose. However, at 100ng/ml, both the frequency and amplitude of mEPSCs significantly increased in comparison to controls. As these currents are responses to spontaneous neurotransmitter release and their frequency is

classically considered an indicator of release probability, these results suggest that with increasing IFNγ treatment, post synaptic responses are enhanced, and release probability is likely elevated.

This data only provides a glimpse of the bigger picture at hand, and to understand what mechanisms are occurring at the synapse to create these variations, we must investigate the presence of potentially associated alterations in receptor content expressed at the surface of these pyramidal cells. In order to do that, we performed immunochemistry staining and proved that the alterations of post-synaptic receptor content do, in fact, corroborate well with the signalling data. These results show how these changes occur by monitoring receptor trafficking of both inhibitory and excitatory receptor subunits. At excitatory synapses, we observed increased surface expression of AMPARs at both concentrations, yet more strongly at the 100ng/ml dose. This data demonstrated that the amount of GluA1 at the surface increases as acute IFNγ treatment dosage increases, indicating a dose-dependent basis for AMPA receptor trafficking in response to IFNγ treatment. Since we do not see electrophysiological changes at the 10ng/ml dose, an interesting possibility could be that AMPA receptors have come to the surface but are not trafficked in sufficient quantities to affect signalling at the synapse, so no functional variation is observed in the amplitude in our mEPSCs recordings.

At inhibitory synapses, our results demonstrated that mIPSCs frequency is increased quite largely in the 100ng/ml group in comparison to both the control group and the 10ng/ml group. We had also shown no change in mIPSC amplitude between the 100ng/ml and control group. Importantly, we observed a significant effect when examining the amplitude, where the 10ng/ml group had a large increase, but once the dosage was raised to 100ng/ml, we saw a decrease back to baseline control levels. Surface GABA receptor content was only changed in the 10ng/ml group, with another increase. The change observed in the GABA-A surface receptor content indicates an

effect found only at a lower dosage of IFNγ, where there is an influx of receptors being trafficked to the surface at such a high degree that we see a large increase in signalling changes at that very same concentration. As U-shaped dose-dependent response curves are not common when examining receptor trafficking, we are uncertain as to the rationale behind such results. A potential mechanism to explain this interesting response curve could be that lower doses of IFNγ are causing exocytosis of GABA receptors to the surface, but as the dosage increases, intracellular responses are triggered to shift the GABA receptors being trafficked to AMPA receptors, as seen at 100ng/ml with an increase in mEPSC amplitude and no changes in mIPSC amplitude.

These results support our hypothesis that IFNγ regulates both inhibitory and excitatory synapses within the cortex, helps both characterize its role in excitatory-inhibitory signalling and establishes a basis for understanding its position at the synapse. These experiments also allowed us to get a glimpse at the mechanism of how IFNγ affects synapses through surface receptor trafficking. We know that TNFα treatment reduces both mIPSCs amplitude and frequency (Pribiag & Stellwagen, 2013) and increases mEPSCs amplitude and frequency (Beattie et al., 2002; Stellwagen et al., 2005). This initial data shows that, based on signalling changes, IFNγ does not seem to act in opposition to TNFα signalling, as IFNγ was not shown to reduce either mIPSCs or mEPSCs amplitude or frequency. The pre-synaptic changes observed seem to be increased at both types of synapses at the same concentration, 100 ng/ml, so IFNγ may modulate synapses by having a general effect on release probability. However, examining our post-synaptic data, we demonstrate an increase in surface GABARs at a lower concentration than AMPARs. Perhaps this increase in GABARs before AMPARs are trafficked to the surface indicates a switch from one receptor to the other. This could be that the GABARs, at a lower concentration, initiate changes in intracellular responses to induce enhanced AMPA signalling.

Given the characterization shown in this thesis of IFNγ and how it functions at the synapse, a prominent yet unanswered question is raised: where is IFNγ being released? Is it an astrocytic, microglial or neuronal factor? Additionally, how does IFNγ affect behaviour? These questions, and their solutions, will help to create a bigger picture of what functions involve IFNγ. To answer the question of origin, we can test IFNγ levels after modulating the activity of these CNS cell populations in different dissociated culture preparations. We can identify where IFNγ is being produced and characterize the role of glia concerning the previously demonstrated IFNγ-induced synaptic modulation whilst also investigating the behavioural consequences of altered cortical synapse strength in IFNγ knockout mice.

Figures

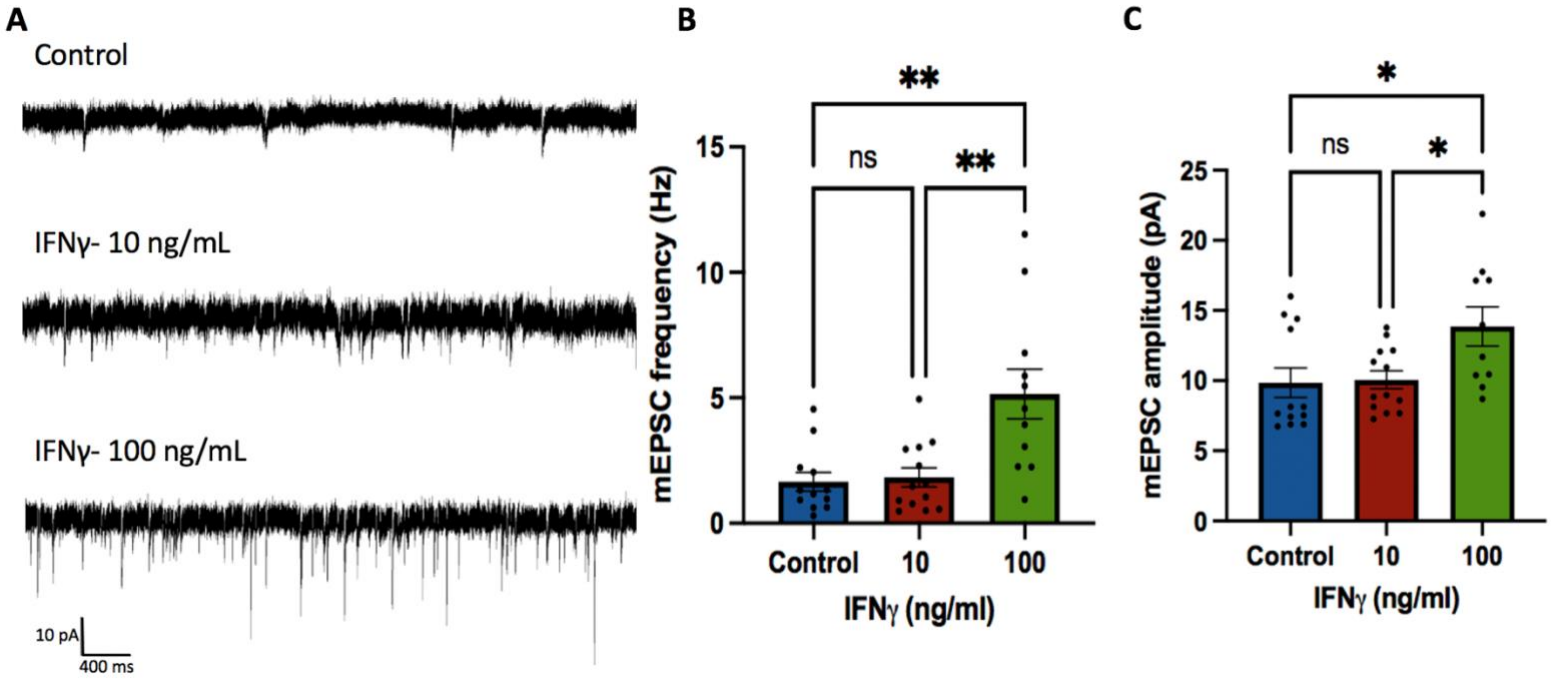

Figure 1: IFNγ treatment increases mEPSC amplitude and frequency.

(A) Sample mEPSC traces for each group. **(B)** Cortical pyramidal neuron mEPSC frequency and **(C)** amplitude after 10 ng/ml and 100 ng/ml treatment for 1 hour. Post-hoc simple main effect analysis showed that mEPSC frequency was significantly elevated after 100 ng/ml, but not 10 ng/ml treatment of IFNγ (simple main effect of treatment, $F(2, 33) = 9.847$, $P=0.0004$) N=12-13 cells per group. mEPSC amplitude also significantly increased after 100 ng/ml, but not 10 ng/ml treatment (simple main effect of treatment, $F(2, 33) = 4.5$, $P=0.0182$) N=12-13 cells per group. All groups are presented as mean ± SEM.

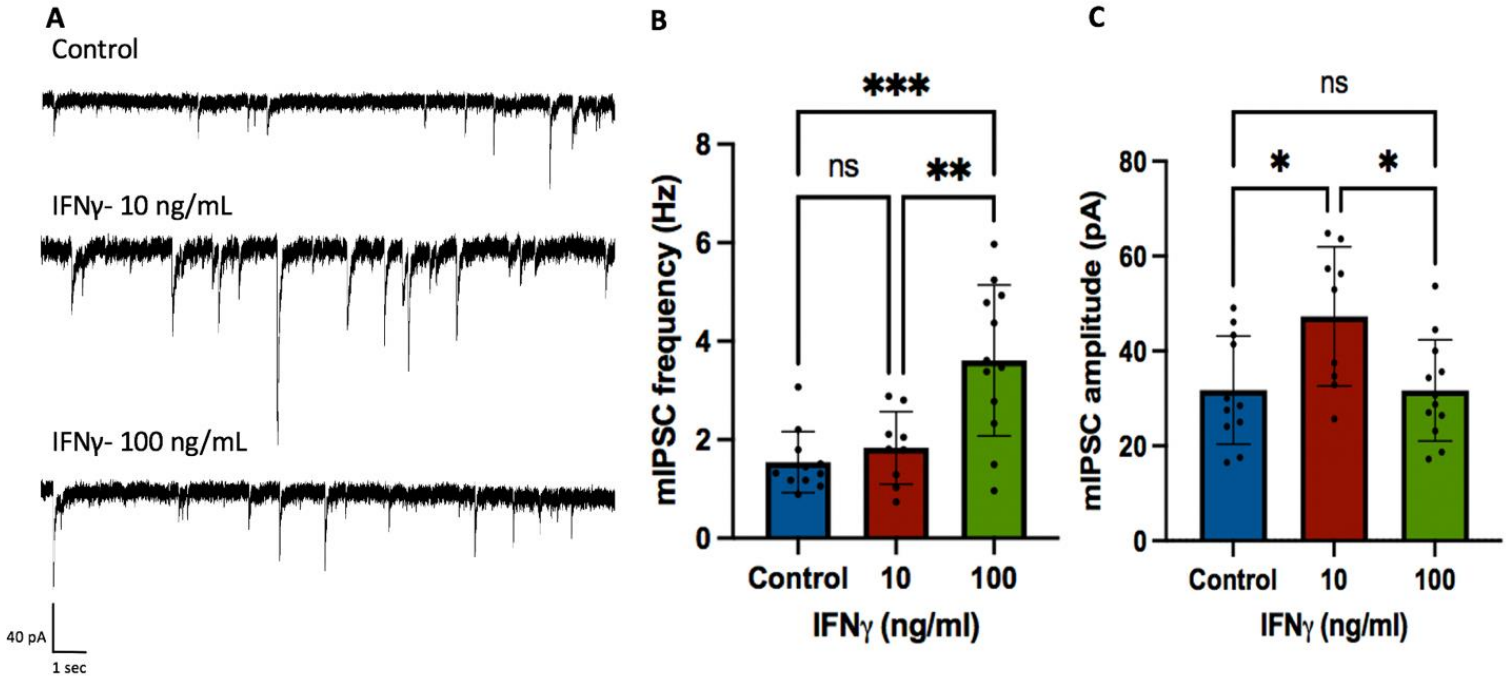

Figure 2: IFNγ treatment increases mIPSC frequency and amplitude in a dose-dependent manner. (A) Sample mIPSC traces for each group. **(B)** Cortical pyramidal neuron mIPSC frequency and **(C)** amplitude after 10 ng/ml and 100 ng/ml treatment for 1 hour. Post-hoc simple main effect analysis showed that mIPSC amplitude seems to remain unchanged at 100 ng/ml, but significantly increased at 10 ng/ml treatment of IFNγ (simple main effect of treatment, $F(2, 28) = 6.227$, $P=0.0058$). mIPSC frequency shows a significant increase at 100 ng/ml (ordinary one-way ANOVA, $F(2, 28) = 11.49$, $P=0.0002$) compared to non-treated controls, and remains unchanged at 10 ng/ml. N= 10-12 cells. All groups are presented as mean ± SEM.

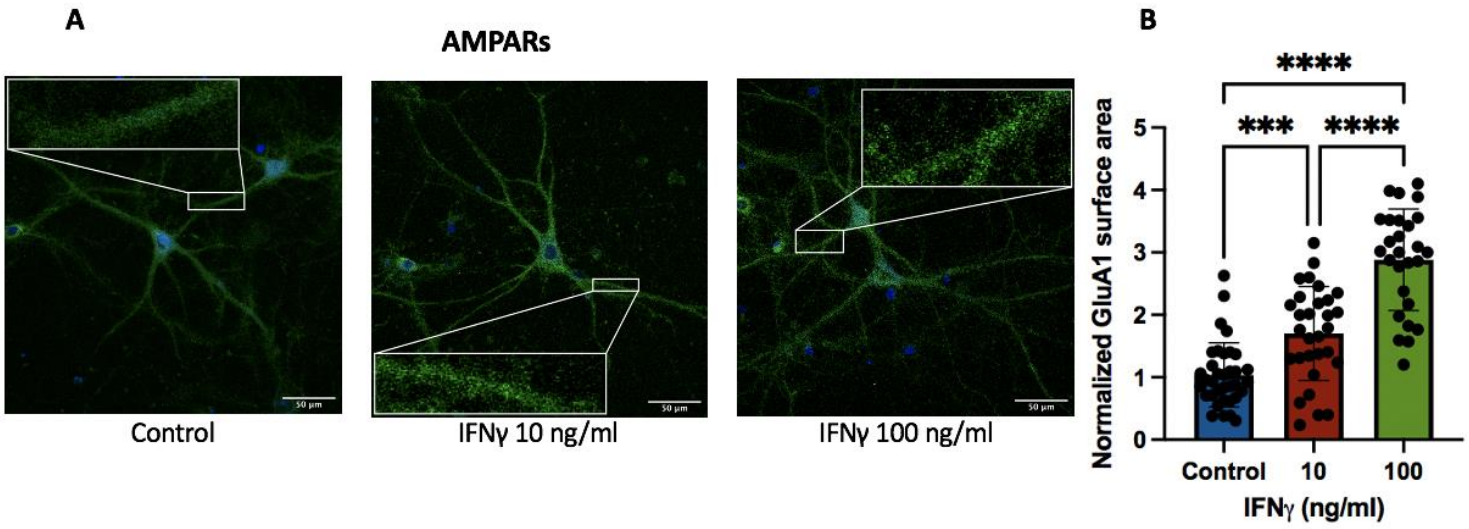

Figure 3: IFNγ treatment increases the amount of GluR1 on the surface of synapses.

(A) GluR1 immunocytochemical staining of a control pyramidal cell, and two IFNγ-treated cells at 10 ng/ml and 100 ng/ml. (B) Normalized GluA1 surface area of 10 ng/ml and 100 ng/ml (N= 35 cells) IFNγ-treated cells compared to control. One-way ANOVA was conducted for all groups followed by Tukey's multiple comparison test (ordinary one-way ANOVA, $F(2, 81) = 54$, $P<0.0001$). All groups are presented as mean ± SEM.

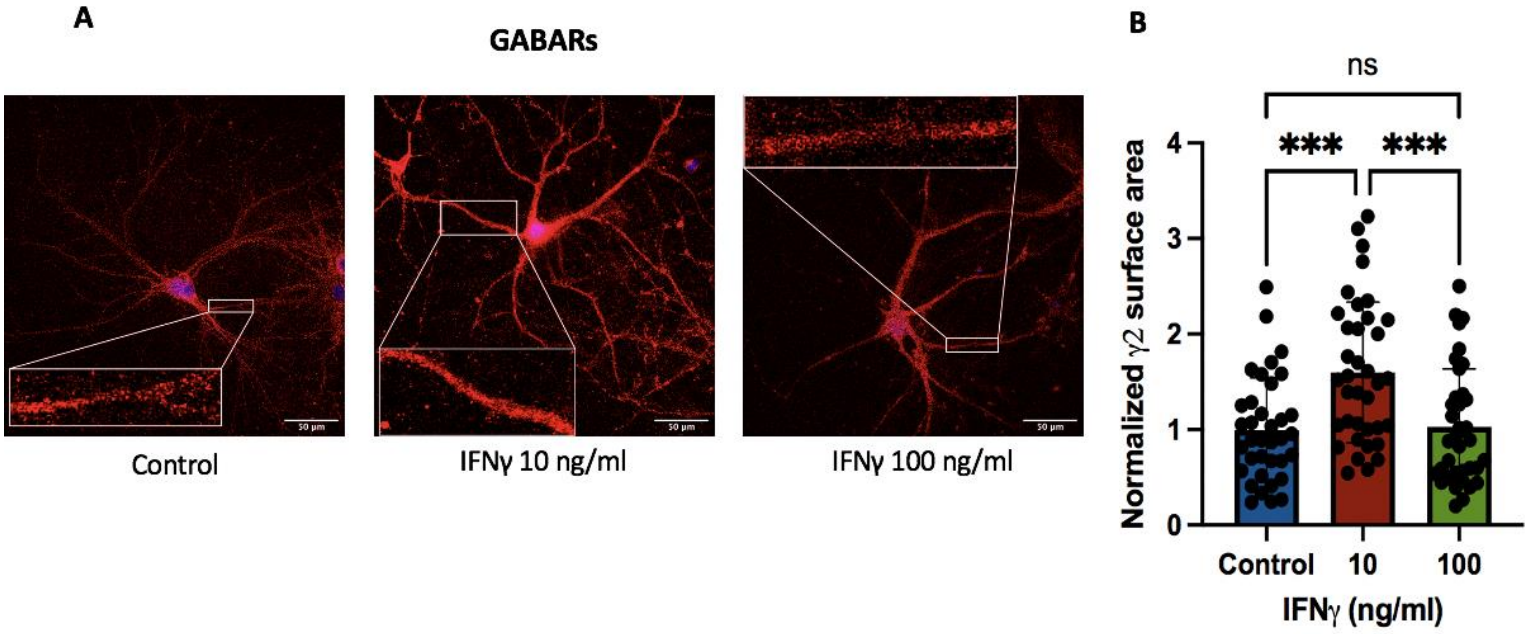

Figure 4: IFNγ treatment increases the amount of GABA-A on the surface of synapses.

(A) GABA-A immunocytochemical staining of a control pyramidal cell, and two IFNγ-treated cells at 10 ng/ml and 100 ng/ml. (B) Normalized GABA-A surface area of 10 ng/ml and 100 ng/ml (N= 37 cells) IFNγ-treated cells compared to control. One-way ANOVA was conducted for all groups followed by Tukey's multiple comparison test (ordinary one-way ANOVA, $F(2, 107) = 10.52$, $P<0.0001$). All groups are presented as mean ± SEM.

Chapter 4- Bridging the gap: The origin of IFNγ during synaptic alterations, its effects on normal functioning and behaviour

Introduction

As the field of neuroimmunology has advanced, the perception of the role of glia in the central nervous system has shifted. Initially known as passive supporters of neuronal function, the general consensus views them now as active and essential participants in many vital aspects of normal circuit function. In light of this, many studies have investigated and shown their importance in the development and removal of synapses (Chung et al., 2012; Paolicelli et al., 2011; Eroglu et al., 2010; Jones et al., 2011), and more relevantly, in forms of synaptic plasticity (Sancho et al., 2021; Ben Achour et al., 2010).

In homeostatic synaptic plasticity, it has been previously shown that glial cells are involved in synaptic scaling in response to activity deprivation (Stellwagen & Malenka, 2006). TNFα is released by glia, and increasing evidence in the literature demonstrates that TNFα is more likely to be produced by microglia and astrocytes. Notably, TNFα is also expressed by microglia (Kato & Kanba, 2013; Bilbo & Schwarz, 2009; Schwarz et al., 2013) and astrocytes (Becker et al., 2013; Balosso et al., 2005; Duseja et al., 2015) at basal conditions in the central nervous system. Astrocytes and microglia produce TNFα during brain injury and inflammatory processes (Hopkins & Rothwell, 1995; Renno et al., 1995; Bruce et al., 1996).

In dissociated cell culture during baseline conditions, astrocytes release small amounts of TNFα, and its expression is increased after treatment with inflammatory stimuli (Krasowska-Zoladek et al., 2007; Chung & Benveniste, 1990). In a previous study, it was found that glia is responsible for releasing TNFα necessary for activity-dependent AMPAR trafficking, where treating neurons in cell culture with glial-conditioned media increased AMPARs at the surface of the synapse, mimicking the effects seen when just TNFα is treated on this cell population. (Beattie et al., 2002). Moreover, soluble TNFR1, a known blocker for TNFα signalling,

eradicated this response, demonstrating that TNFα is a glial-derived factor (Beattie et al., 2002). Lastly, glial cells were found to be the origin of release for TNFα that is necessary for synaptic scaling within homeostatic synaptic plasticity, where a study found wildtype neurons cultured with TNFα knockout glia were unable to upscale synaptic strength after a 48-hour activity blockade with TTX (Stellwagen & Malenka, 2006). This brings us to the critical takeaway of these findings, that glia can be the source of cytokine secretion important for modulating synaptic activity and strength during homeostatic synaptic plasticity.

IFNγ receptors have been found to be expressed in various cell types within the CNS, such as microglia, astrocytes, neurons, endothelial cells, oligodendrocytes, and neural precursor cells (Hashioka et al., 2010; Hausler et al., 2002; Li et al., 2010; Mizuno et al., 2008; Ni et al., 2014). The IFNγ receptor subunits, IFNγR1 and IFNγR2, are expressed in hippocampal microglia (Hashioka et al., 2010; Papageorgiou et al., 2016; Ta et al., 2019). Astrocytes have also been shown to express the receptor for IFNγ (Rubio & de Felipe, 1991). IFNγ itself is found to be present at low concentrations at baseline conditions in the brain (Rady et al., 1995) and has been found to modulate neurogenesis and varying forms of synaptic plasticity (Filiano et al., 2016; Li et al., 2010; Monteiro et al., 2016). Most importantly, past studies have found that levels of IFNγ in the brain can be elevated under varying types of pathological conditions, more commonly found during neuroinflammation (Ottum et al., 2015).

During neuroinflammation activated via injury, infection or general neurological disorders, T cells that routinely examine their microenvironment enter brain tissue to secrete cytokines, including IFNγ (Filiano et al., 2017; Gemechu & Bentivoglio, 2012). Microglia have been found to alter synapses (Schafer et al., 2012) directly. Astrocytes have been shown to participate in numerous forms of plasticity (Yang et al., 2003; Perea & Araque, 2007; Gordon et

al., 2009; Henneberger et al., 2010). Both astrocytes and microglia are unmistakably involved in synaptic plasticity (Sancho et al., 2021) and immune-mediated synaptic modulation, as seen with TNFα-necessary synaptic scaling (Stellwagen & Malenka, 2006). This chapter seeks to, in part, determine which of the IFNγ-secreting glial subtypes—astrocytes or microglia—are responsible for secreting IFNγ during synaptic alterations.

Evidence in the literature shows that altered excitatory and inhibitory signalling inevitably influences neural circuits, which can promote neuropsychiatric conditions and cognitive dysfunction (Contestabile et al., 2017; Xu & Wong, 2018). These studies demonstrate that alteration of neurotransmission via IFNγ signalling may play a role in cognitive dysfunctions under neuropathological conditions. With our primary goal to fully characterize the role of IFNγ in synaptic plasticity, we must create a more comprehensive assessment to examine what part IFNγ may play at a behavioural level to understand how it may be involved in cognitive function.

IFNγ knockout animals have been studied in multiple reports. One study demonstrated that IFNγ knockout mice perform better on spatial memory tasks while observing no significant difference in anxiety behaviour compared to wildtype littermates (Monteiro et al., 2016). However, other studies show that IFNγ knockout mice have increased anxiety-like and depressive-like behaviours (Campos et al., 2014; Camas et al., 2013). Lastly, one group evaluated anxiety behaviours and found that IFNγ knockout mice demonstrate higher baseline anxiety levels compared to C57Bl/6 mice (Kustova et al., 1998). Overall, these studies results suggest that IFNγ signalling may play a role in normal CNS circuit function, with the knockout model seeming to impair cognition and memory, with conflicting results.

Clinical and basic research studies indicate that anxiety and depressive symptoms arise from synaptic deficits in the frontal cortex and hippocampus (Khairova et al., 2009; Dunman et al., 2016). More specifically, anxiety behaviours are hypothesized to be related to excitatory and inhibitory imbalance, especially in the limbic areas of the brain, such as the PFC (Nuss, 2015). To bridge the gap between cellular and molecular results within this thesis, we investigated the role of IFNγ signalling in behavioural output by examining anxiety-like behaviours in the IFNγR1 knockout model to elucidate if any changes may present themselves through apparent behavioural dysfunction, given that anxiety-like behaviours are thought to be mediated by synaptic changes in the cortex, along with many other regions, and that all experiments in Chapter 3 had been conducted on dissociated cortical pyramidal neurons.

Results

IFNγ and its glial origins

When examining microglia, we used fractalkine (CX3CL1) to manipulate the activity of this cell population. CX3CL1 is a chemokine belonging to the CX3C family. CX3CL1 is produced by neurons and is involved in modulating glial activation in the CNS once bound to its only receptor, CX3CR, which is predominantly expressed on microglia (Harrison et al., 1998), partially on astrocytes as well as neurons (Meucci et al., 2000; Chapman et al., 2000). This suggests that CX3CL1/CX3CR1 are an important bridge to connect neurons and microglia and have been shown to be an integral component in neuron-to-microglia signalling as it is necessary for cortical synaptic remodelling (Gunner et al., 2019).

Here we treated microglial dissociated cell cultures for 48 hours, as our previous gene expression screen of immune factors used a 48-hour timepoint (Heir, 2019). Quite a large

increase in IFNγ mRNA levels was observed on CX3CL1-treated microglia (Fold change CX3CL1= 4.618; $-\Delta\Delta C_t$ CX3CL1= -1.888. Wilcoxon test. $p<0.05$, n=3) (Figure 1A).

We then used glutamate to manipulate the activity of dissociated isolated astrocytic cultures, as glutamate has been known to be involved in astrocyte-synapse interactions (Blanco-Suárez et al., 2017). Glutamate has also been shown to be a candidate for astrocytes' monitoring of neuronal activity (Mahmoud et al., 2019), and glutamate levels were found to modulate astrocytic TNFα production (Heir, 2019). As performed with the dissociated microglia cultures, we treated the astrocytes for 48 hours. No significant increase in IFNγ mRNA was observed (Fold change Glutamate= 1.370; $-\Delta\Delta Ct$ = -0.75. Wilcoxon test. p= 0.1818. n=3) (Figure 1B) in glutamate-treated astrocytes, whereas a significantly larger increase in IFNγ mRNA levels was observed on CX3CL1-treated microglia (Figure 1A). The cycle number for control astrocytes (Ct average) is roughly the same as control microglia when testing for IFNg levels (Ct average astrocytes= 35.51, Ct average microglia= 35.33), indicating that the total basal amount of IFNγ is similar across cell populations.

IFNγ's involvement in anxiety-like behaviour

The light-dark box test (LDB) is a standardized and popular behavioural test to assay unconditioned anxiety responses in rodents (Khairova et al., 2009). This test is based on the innate aversion of rodents to brightly illuminated areas balanced against their innate tendency to explore, and the time spent in the light box is the main measure of anxiety. The general agreement in the literature is that the mouse feels exposed in the lightbox, whereas in the dark box, it feels more protected and safely hidden (Kulesskaya & Voikar, 2014).

It is important to note that our knockout model is, in fact, an IFNγR1KO, where the knockout is for the IFNγ receptor subunit IFNγR1. However, this acts as a complete whole body

constitutive knockout line as IFNγR1 acts as the functional receptor subunit where binding of IFNγ to IFNγR1 causes heterotetramerization of the receptor, which then activates downstream kinases (Negishi et al., 2018). We found that IFNγR1 KO mice experienced higher baseline anxiety compared to wildtype age-matched controls (Figure 2A, unpaired t-test,t=2.118, df=29, p= 0.0429) since the IFNγR1 KO mice had spent significantly less time in the lightbox. Total distance travelled (Figure 2B) and mean velocity (Figure 2C) are control variables to ensure that changes in locomotion do not account for the difference in time spent between the two boxes.

Discussion

Astrocytes and microglia have surfaced within the literature as important players in modulating synaptic physiology. They are capable of reacting to alterations in activity levels at synapses, which is one of the requirements for contributing to the mechanisms of synaptic scaling. Microglia act to evaluate synapses by directly making contact with them (Wake et al., 2009), can respond by altering their motility behaviour (Tremblay et al., 2010), and directly alter synapses (Schafer et al., 2012). Astrocytes are known to react to neural activity via calcium signalling (Cornell-Bell et al., 1990; Dani et al., 1992; Porter & McCarthy, 1996) and have been shown to participate in numerous forms of plasticity (Yang et al., 2003; Perea & Araque, 2007; Gordon et al., 2009; Henneberger et al., 2010). Consequently, both astrocytes and microglia are unmistakably involved in synaptic plasticity (Sancho et al., 2021) and immune-mediated synaptic modulations, as seen with TNFα-necessary synaptic scaling (Stellwagen & Malenka, 2006).

It has been previously shown that TNFα necessary within synaptic plasticity has glial origins (Stellwagen & Malenka, 2006), with evidence pointing towards astrocytic origins (Heir, 2019) and microglial origins when examining addiction behaviours affecting synaptic function,

not plasticity (Lewitus et al., 2014). Regulated TNFα-levels have been shown to mostly occur at the mRNA level (Tsytsykova et al., 2010), indicating that measuring mRNA levels serves as a substitute for secreted TNFα, and is a sensitive quantifier. Given this knowledge, we quantify mRNA levels in isolated astrocytic and microglial dissociated cultures using reverse-transcription quantitative polymerase chain reactions (RT-qPCR), and with its amplifying nature, can serve as an even more sensitive quantifier of IFNγ levels.

Altogether, the data presented in the first half of this chapter implicates the subtype microglia being a source of IFNγ being produced during activity manipulations. We show in two separate preparations of dissociating glial cultures that when astrocytes and microglia are induced into an altered state, microglia seem to produce IFNγ increasingly. However, it is important to note that further qPCR experiments need to be conducted to confirm the results shown. Other forms of testing are necessary to determine further which exact cell population is definitively necessary for IFNγ-induced synaptic alterations as well. Such experiments could include using a more physiologically intact cell culture model, the organotypic slice culture (Del Turco & Deller, 2007), performing a near-complete microglial depletion (Heir, 2019) and testing for IFNγ levels after modulating activity through treatments of tetrodotoxin, gabazine, glutamate and fractalkine while comparing the outcome to microglial-filled slice cultures. The results of these studies could demonstrate which glial subtype is necessary for IFNγ-induced synaptic activity modulations.

Previous studies have observed that TNFα knockout mice experience lower baseline anxiety than wildtype mice (Camas et al., 2013). Since anxiety-like behaviour in rodents is mediated by synaptic changes in the cortex (Dunman et al., 2016; Wohleb et al., 2016; Christoffel et al., 2011), investigating whether IFNγ demonstrates an opposing behavioural

phenotype could adhere to ideas of our original hypothesis indicating IFNγ and its possible role in opposing the effects of TNFα.

In the second half of this chapter, we show that IFNγR1 KO mice spent significantly less time in the lightbox in comparison to wildtype age-matched controls, suggesting that IFNγR1 KO mice experience higher baseline anxiety. These findings provide an initial measure for a possible behavioural phenotype of the synaptic changes seen in earlier experiments within cortical pyramidal neurons. However, it is important to note that the synaptic changes observed earlier in chapter 3 were caused by acute IFNγ treatment, whereas this behavioural data suggests there is basal IFNγ signalling in the brain regulation of synaptic strength.

These results help provide a behavioural consequence of IFNγ-induced synaptic modulation and how it can possibly manifest as behavioural consequences since anxiety-like behaviours are mediated by synaptic changes and deficits in many regions, including the cortex ((Martin et al., 2009). These findings raise an important question: what is the neuroanatomical basis for IFNγ signalling? We observe in this study that IFNγ is modulating anxiety-like behaviours. However, these behaviours are not solely mediated by the cortex. Brain areas such as the amygdala and the hippocampus are also implicated (Likhtik et al., 2014). Here we performed our behavioural tests using a whole body IFNγR1 KO, so where is the signalling that is regulating anxiety? A possible experiment to answer these questions could be to use a local inhibition or a conditional IFNγ knockout line to decipher where exactly is IFNγ signalling affecting basal anxiety. These findings would help provide a deeper understanding of the complex neural circuitry behind how immune-mediated synaptic modulations and altered excitatory and inhibitory signalling is implicated in cognitive dysfunction and irregular behaviours.

Figures

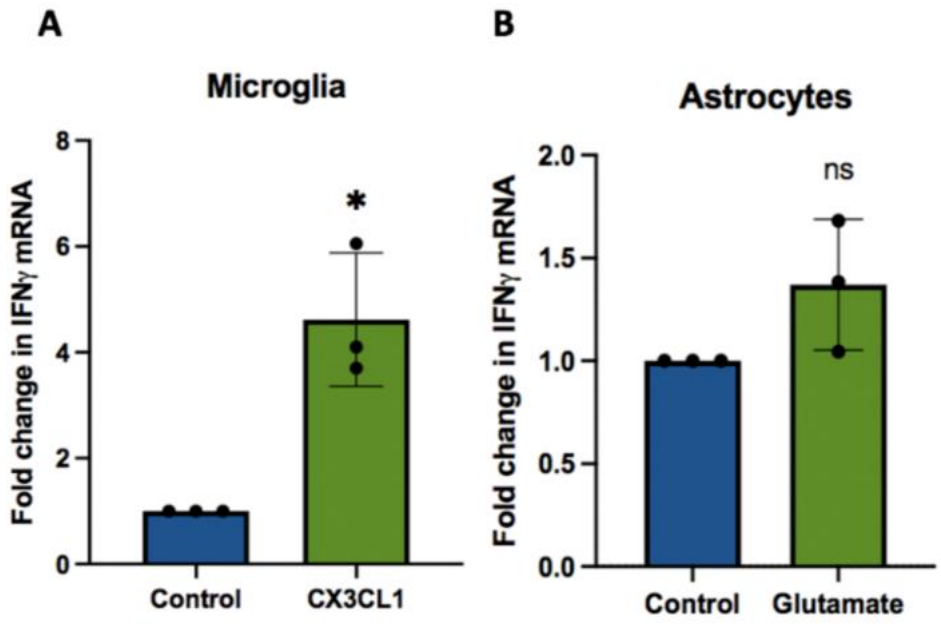

Figure 1: IFNγ -induced synaptic modulation could be a microglial factor

(A) Purified microglia from dissociated in vitro rat cultures express notably more IFNγ mRNA when treated with fractalkine (CX3CL1), a well-established microglial-neuronal communicator which alters activity levels in this cell population (Fold change CX3CL1= 4.618; $-\Delta\Delta C_t$ CX3CL1= -1.888. Wilcoxon test. $p<0.05$, n=3) . **(B)** Isolated astrocytes from dissociated in vitro rat cultures express a smaller elevation of IFNγ mRNA when treated with glutamate, a known astrocyte-neuronal modulator signal reflecting activity levels (Fold change Glutamate= 1.370; -ΔΔCt Glutamate = -0.75. Wilcoxon test. p= 0.1818. n=3).

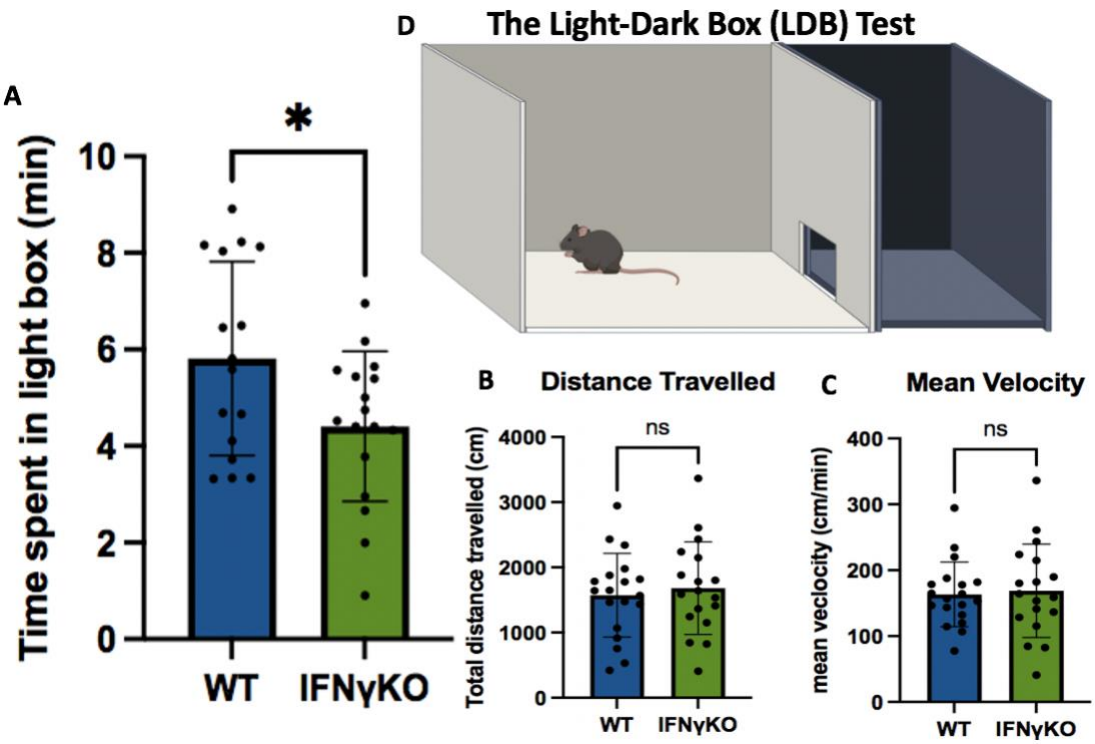

Figure 2: IFNγR1 KO mice experience higher baseline anxiety-like behaviour

(A) Duration spent in light box in IFNγR1 KO and WT mice. IFNγR1 KO mice show a significantly decreased amount of time spent in the light box (unpaired t-test, t=2.118, df=29, p= 0.0429). IFNγR1 KO mice show no difference in (B) distance travelled and (C) mean velocity compared to WT controls during the light dark box test. (WT N= 17; IFNγR1 KO N= 19). (D) A visual schematic of the behavioural test, the light dark box (LDB) created with BioRender.com.

Chapter 5- General Discussion

Immune molecules are now widely accepted to not only exist in the nervous system but play an active part in modulating synaptic function, such as TNFα, which has been shown to mediate homeostatic synaptic plasticity by upscaling excitatory synapses (Stellwagen & Malenka, 2005) and IL-1β being necessary for synaptic mechanisms important for learning and memory, such as LTP (Yirmiya & Goshen, 2011). IFN-γ levels in the CNS are altered during neurodegenerative, neuroinflammatory, and neuropsychiatric disorders (Arolt et al., 2000; Lee et al., 2006; Reale et al., 2011; Söderström et al., 1995; Wei et al., 2000). In addition to the vital role IFNγ plays in inflammation, growing evidence proposes that IFNγ signalling can modulate neurological processes underlying cognitive behaviours, including neurogenesis, neuronal excitability and plasticity (Filiano et al., 2016; Li et al., 2010; Monteiro et al., 2016). Our goal was to understand better the impact of IFNγ on synaptic function by assessing whether it influences inhibitory and excitatory synapses, how it affects surface receptor expression, which cell type is responsible for its production in the CNS and examining its role in anxiety-like behaviours to determine if IFNγ could be a possible opponent to TNFα during homeostatic synaptic plasticity.

Our findings show that acute IFNγ treatment alters pre-synaptic responses and seemingly increases release probability at both types of synapses at 100ng/ml, demonstrated by a significant increase of mEPSC and mIPSC frequency at this concentration. Observing post-synaptic responses, acute treatment of IFNγ seems to increase surface GABARs at a greater magnitude in a concentration before AMPARs are altered, indicating a possible change in intracellular responses triggered by GABARs to affect AMPAR trafficking. We also saw that IFNγ seems to be released by microglia during activity manipulations; these findings provide the basis for further investigation of the mechanisms behind IFNγ-mediated modulation of activity. Finally,

we found that loss of IFNγ signalling in mice increased time spent in the light box, reinforcing the idea that IFNγ possibly plays a role as an attenuator of anxiety-like behaviours.

These results provide further evidence for IFNγ as a regulator of synaptic function, showing that IFNγ assists in enhancing both excitatory and inhibitory signalling, is produced by glia in response to stimuli, and even is implicated in anxiety-like behaviours. We also identified one of many mechanisms behind this when observing receptor trafficking. Since IFNγ was shown to augment excitatory and inhibitory currents, we can conclude that IFNγ is unlikely to be a downscaling factor that opposes TNFα during homeostatic synaptic plasticity. Such evidence allows for the exploration of other potential immune mediators and provides a basis to investigate further the role of IFNγ in other forms of synaptic plasticity in future experiments.

IFNγ and its synaptic alterations

In chapter 3, we strove to characterize IFNγ signalling on excitatory and inhibitory synapses to further understand its role in synaptic function. Previous studies have found that IFNγ treatment increased the frequency of both spontaneous Inhibitory Post Synaptic Currents (sIPSCs) and miniature In chapter 3, we strove to characterize IFNγ signalling on excitatory and inhibitory synapses to understand its role in synaptic function further. Previous studies have found that IFNγ treatment increased the frequency of both spontaneous Inhibitory Post Synaptic Currents (sIPSCs) and miniature–Inhibitory Post Synaptic Currents (mIPSCs) in hippocampal CA1 pyramidal neurons (Flood et al., 2019). Another study shows that acute application of IFNγ potentiates GABA-mediated tonic currents in mouse prefrontal cortex layer 2/3 pyramidal neurons (Fillano et al., 2016). The evidence of IFNγ being a possible mediator in synaptic plasticity towards inhibition made us believe that IFNγ may likely oppose TNFα in homeostatic

synaptic plasticity. Therefore, we investigated IFNγ as a potential mediator of homeostatic synaptic plasticity and found that IFNγ affects excitatory and inhibitory synapses while affecting both pre-and post-synaptic responses.

The frequency of miniature post-synaptic currents has been classically associated with alterations in pre-synaptic function, developing from interactions between release probability and the pool of releasable quanta (Glasgow et al., 2019). Observing excitatory and inhibitory synapses at 100 ng/ml, the frequency of mEPSCs and mIPSCs significantly increased compared to controls. These results suggest that with acute IFNγ treatment at this concentration, release probability is likely elevated. However, a mixture of techniques is necessary to accredit a singular mechanism to changes in pre-synaptic function. Interpreting frequency changes cannot be stated as an absolute reflection of alterations in release probability. It could be that the number of synapses made on the post-synaptic cell has increased at this concentration. A possible experiment to account for this would be to stain dissociated neural cultures that have been acutely treated with IFNγ for a synaptic marker, such as PSD-95, and measure the number of synapses of both treatment groups in comparison to non-treated neurons.

Additional possible explanations behind frequency alterations could be the un-silencing of synapses. A silent synapse is defined as a synapse in which a post-synaptic current (PSC) is absent at the resting membrane potential but becomes evident on depolarization (Kerchner & Nicoll, 2008). However, this process would not show up in any experiments conducted throughout this thesis, as all mPSC recordings were performed under a -70mV voltage clamp. To observe for potential un-silencing of synapses, we could perform electrophysiology on the same preparations shown and discussed in chapter 3 but instead place a voltage clamp at +40mV to depolarize the cell and measure mPSCs. These future experiments would allow us to demonstrate

whether the frequency changes seen are due to increased release probability, the un-silencing of synapses, increased number of synapses, or potentially a combination of these mechanisms.

Frequencies of mPSCs have been used as proxies for synapse number and release probability, and the amplitude of mPSCs has been used as a measure for the relative strength of synapses made onto the post-synaptic cell. More specifically, the amplitude of mPSCs is the neuronal response to the unitary release of neurotransmitters. It is traditionally assumed to be reflective of the receptor content of the post-synaptic cell. In previous studies, acute treatment of IFNγ has been shown not to affect the amplitude of mPSCs. However, past studies examining mIPSCs had not investigated IFNγ treatment at the dosage of 10ng/ml (Flood et al., 2019; Fillano et al., 2016). Additionally, the effect of IFNγ treatment on excitatory currents has been scarcely investigated. During these two conditions, mIPSCs at 10ng/ml and mEPSCs at 100ng/ml, we observe significant increases in mPSC amplitude. Given these results and how amplitude is generally interpreted as a measurement of post-synaptic receptor content, we thus examined if acute IFNγ treatment affects surface receptor trafficking of both AMPA and GABA receptors.

At excitatory synapses, we observed increased surface expression of AMPARs at both concentrations, yet more strongly at the 100ng/ml dose. This data demonstrated that the amount of GluA1 at the synapse increases as acute IFNγ treatment dosage increases, indicating a dose-dependent basis for AMPA receptor trafficking in response to IFNγ treatment at the excitatory synapse. As discussed in chapter 3, a mechanism to explain this result could be that AMPA receptors have come to the surface but are not trafficked in sufficient quantities to the synapse to cause any signalling changes, so no functional variation is observed in the amplitude in our mEPSC recordings.

When examining amplitude, the 10ng/ml group significantly increased, but once the dose rose to 100ng/ml, we saw a decrease back to baseline control levels of surface GABA receptor content. The change observed in the GABA-A γ2 surface receptor subunit content indicates an effect found only at a lower dosage of IFNγ, where there is an influx of receptors being trafficked to the surface at such a high degree that we see a significant increase in signalling changes at that very same concentration. However, surface GABA receptor content declines back to baseline at the 100ng/ml group, where the amplitude is also shown to be equivalent to control levels. A potential mechanism to explain this interesting U-shaped response curve could be that lower doses of IFNγ are causing exocytosis of GABA receptors, but as the dosage increases, the receptors being trafficked to the surface switch to AMPA receptors, as seen at 100 ng/ml with an increase in mEPSC amplitude and no changes in mIPSC amplitude. GABA receptors, at a lower concentration, could initiate some sort of change in intracellular responses to then induce enhanced AMPA signalling via rapid exocytosis to traffic these receptors to the surface.

Although, it is important to note that the measure of mPSC amplitude being reflective of ionic flux through post-synaptic receptors comes with a baseline assumption that the amount of neurotransmitter within a singular synaptic vesicle stays the same. There is some variability between synapses that can impact alterations in typical quantal amplitudes and increases in the number of releasable quanta at a particular set of synaptic inputs or release probability. This variability has been described to skew the distribution of post-synaptic currents towards these inputs (Glasgow et al., 2019; Segal, 2010). This provides evidence that changes in mPSC amplitude may not absolutely reflect changes in post-synaptic receptors and demonstrates a possible, although unlikely, mechanism of variability to explain the lack of increase in surface GABA-A receptor content in the 100 ng/ml group.

A general limitation of this study is regarding our investigation of receptor trafficking. We observe surface changes and infer signalling changes from the results. To truly visualize the electrophysiological modifications seen in the aforementioned findings and to strengthen our comparison of surface receptor content and synaptic changes, we could monitor GABA and AMPA receptor trafficking on the surface of the cell while also staining with a synaptic marker such as Synaptophysin, Synapsin1, PSD95, or VAMP2. This would allow us to colocalize our receptor staining with synapses to accurately view if these receptors are being trafficked to the surface of a synapse, thereby measuring the number of receptors at the synapse to compare better our receptor content increase to synaptic signalling changes seen in the electrophysiological experiments conducted in chapter 3.

Another possible future experiment to further examine IFNγ's role at the synapse would be to conduct a classical loss of function study. Our constitutive IFNγR1KO mouse line could be used to create post-natal mouse dissociated neuronal cultures to conduct electrophysiology and immunocytochemistry to understand better how the loss of IFNγ signalling affects the synapse. The results of these studies could demonstrate the necessity of the role that IFNγ plays at the synapse, how its absence affects surface receptor trafficking by staining for GluA1 and GABA-A receptors, and how activity manipulations could affect HSP. This could be achieved by treating cell cultures with tetrodotoxin and gabazine for 48 hours to block and enhance overall neuronal activity, respectively and by recording spontaneous post-synaptic currents in comparison to WT cultures.

Throughout this study, we demonstrate that IFNγ is not an immune mediator of homeostatic synaptic plasticity, let alone an opponent to TNFα as a downscaling factor. Although we did not directly test IFNγ in a loss of function study paired with induced scaling

experiments, the synaptic changes induced are inconsistent with IFN playing the role of an immune mediator in HSP. IFNγ has acted at both excitatory and inhibitory synapses to enhance signalling, which does not occur during HSP. This is an exciting finding, as it points towards IFNγ possibly playing a role in Hebbian forms of plasticity. Studies investigating immune molecules within synaptic plasticity examine the homeostatic type (Stellwagen & Malenka, 2005; Goshen et al., 2007; Ton et al., 2012). Many reports examine immune molecules within Hebbian plasticity, such as TNFα, which is found to, at high concentrations, inhibit LTP in both the CA1 and dentate gyrus of the hippocampus (Maggio & Vlachos, 2018; Singh et al., 2022). IFNγ has been found to modulate inhibitory signalling (Filiano et al., 2016), and one study found that acute IFNγ local administration produced LTP impairment (Maher et al., 2006). The findings in this thesis, placed in context with past outcomes in the literature investigating IFNγ's role, provide a basis for future experiments working towards fully understanding the impact of IFNγ in synaptic function and examining its potential as a participant in Hebbian plasticity.

IFNγ's origins and its impact on behaviour

In chapter 4, we explored the role of IFNγ on behaviour and demonstrated where it is being produced in the CNS during activity manipulations. IFNγ receptors are expressed in various cell types within the CNS, such as microglia, astrocytes, neurons, endothelial cells, oligodendrocytes, and neural precursor cells (Hashioka et al., 2010; Hausler et al., 2002; Li et al., 2010; Mizuno et al., 2008; Ni et al., 2014). IFNγ itself is found to be present at low concentrations at baseline conditions in the brain (Rady et al., 1995) and has been found to modulate neurogenesis and varying forms of synaptic plasticity (Filiano et al., 2016; Li et al., 2010; Monteiro et al., 2016). The presence of IFNγ receptor subunits, IFNγR1 and IFNγR2, have

been shown to be expressed in hippocampal microglia (Hashioka et al., 2010; Papageorgiou et al., 2016; Ta et al., 2019). Astrocytes have also been shown to express the receptor for IFNγ (Rubio & de Felipe, 1991). IFNγ-induced MHC class II expression by astrocytes has been shown in the literature, both in vitro and in vivo (Fierz et al., 1985; Pulver et al., 1987). However, in vivo levels are low in comparison to those detected in microglia (Benveniste, 1992). We, therefore, sought to determine which glial subtype produces IFNγ under activity manipulations—astrocytes or microglia.

We found that microglia were shown to increasingly produce IFNγ, in comparison to astrocytes, where no significant fold change was observed. These results suggest that microglia are producing IFNγ during activity modulations. It is important to note that other forms of testing are necessary to accurately determine which cell population is responsible for IFNγ-induced synaptic changes. These future experiments could use a more physiologically intact cell culture model, the organotypic slice culture (Del Turco & Deller, 2007), perform a near-complete microglial depletion (Heir, 2019) and test for IFNγ levels after altering activity through treatments of tetrodotoxin, gabazine, glutamate and fractalkine while comparing the outcome to microglial-filled slice cultures. The results of these studies could demonstrate which glial subtype is necessary for IFNγ-induced synaptic activity alterations by measuring which cell population produces the most IFNγ when modulated.

These results from the first half of chapter 4 provide a critical piece of information in understanding the role of glial cells in immune-mediated synaptic modulation. IFNγ has been involved in numerous neuropathologies (Lee et al., 2006; Reale et al., 2011; Wei et al., 2000), highlighting the importance of understanding the underlying cellular and molecular mechanisms

of IFNγ-induced synaptic alterations. Our results identify an active cellular player within this process, allowing further dissection of the mechanisms underlying IFNγ-mediated regulation of activity levels. Understanding these pathways can provide insight into the basic functioning of neural circuits and their maintenance and allow us to elucidate how these mechanisms are dysregulated within neuropathologies.

We also show that IFNγR1 KO mice spent significantly less time in the lightbox than WT controls, suggesting that IFNγR1KO mice experience higher baseline anxiety. These findings offer a behavioural effect of IFNγ-induced synaptic modulation and how it can manifest as behavioural consequences. A limitation to this experiment observed is that our IFNγR1KO is a constitutive whole body knockout line. Anxiety behaviours are mediated by the cortex and many other regions, such as the amygdala and the hippocampus (Likhtik et al., 2014). A future experiment to address these concerns could be to use a local inhibition or a conditional IFNγ knockout line to decipher which exact brain region is responsible for the IFNγ signalling affecting basal anxiety. These findings would help provide a deeper understanding of the complex neural circuitry behind how immune-mediated synaptic modulations and altered excitatory and inhibitory signalling can be implicated in cognitive dysfunction and irregular behaviours.

When examining behaviour, it is imperative to include female mice in behavioural experiments, as emerging evidence demonstrates sex-based differences within neurodegenerative and neuropsychiatric conditions significant differences (Chen et al., 2021; Stroud et al., 2021). Most relevantly, there is concrete evidence of sex-based differences in depression and anxiety (McLean et al., 2011). A future experiment should investigate possible sex-based differences and monitor the estrous cycle via vaginal smear cytology to control for cyclical changes, as cyclical

changes in hormonal levels have been found to influence anxiety and depression-like behaviours (Kokras et al., 2015). To minimize the effects of hormones on behaviour, the experimenter must analyze behavioural differences during all parts of the estrous cycle. Such experiments have already begun in our laboratory to create a sex-based behavioural analysis protocol. Protocols such as these are essential to make since investigating the role of sex would develop a deeper understanding of these disease states.

Conclusion

Neuroinflammation is a potential connector between mood disorders and the synaptic plasticity that underlies it. Our project helps establish the inflammatory cytokine IFNγ as a synaptic modulator. We found that IFNγ modulates both excitatory and inhibitory synapses and enhances both signals via its effects through surface receptor trafficking. These findings provide more substantial evidence that IFNγ is an important mediator of synaptic function. We also showed that IFNγ is produced by microglia when activity is altered, which is the first step to proving which cell type is responsible for the release of IFNγ during IFNγ-induced synaptic alterations. Finally, we established a role of IFNγ in anxiety-like behaviours, our results indicating that loss of IFNγ is associated with increased anxiety-like behaviours. We demonstrated how altered excitatory and inhibitory signalling in the brain could be implicated in neuropsychiatric conditions. This can allow us to understand better how immune signalling molecules involved in inflammation impact the neuronal circuitry behind these disorders. Overall, this work improved our understanding of the role of IFNγ in synaptic function, its connection to behavioural phenotypes seen in mood disorders, and the neurobiology of the immune system.

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