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The effects of synthetic glucocorticoid treatment on synaptic physiology

The effects of synthetic glucocorticoid treatment on synaptic physiology

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Abstract

Glucocorticoids are one of the most commonly prescribed classes of drug due to their potent anti-inflammatory effects. However, patients often report a number of side effects including memory impairment and mood changes, but the underlying mechanisms remain unresolved. It has recently been demonstrated that chronic treatment with the synthetic glucocorticoid methylprednisolone (MPL) disrupts the circadian regulation of clock genes in the hippocampus, and that dynamic transcription profiles of synaptic gene products are dysregulated as a result. In this thesis, by utilising extracellular electrophysiological recording techniques, I show that chronic MPL treatment also results in deficits in the circadian regulation of hippocampal synaptic plasticity in the brain slice preparation. Further to this, I demonstrate that synaptic plasticity is similarly disrupted in the perirhinal cortex. The deficits in synaptic plasticity appear to be specific to stimulation protocols that rely on activation of N-methyl-D-aspartate (NMDA) receptors, specifically those containing the GluN2B subunit. Whole-cell patch clamp recordings revealed that MPL inverted the circadian rhythms in spontaneous excitatory neurotransmission in these regions. Spontaneous glutamatergic transmission was also altered in medial prefrontal cortex (mPFC) at both the circadian peak and nadir, while inhibitory currents in all regions were much less liable to steroid-induced changes. Therefore, chronic MPL exposure led to alterations to the delicate balance between excitation and inhibition. I provide further electrophysiological evidence showing that MPL treatment alters glutamate receptor ratios in pyramidal cells of the hippocampus, perirhinal cortex, and prelimbic mPFC. These data reveal a novel role for glucocorticoids in the circadian regulation of neural activity in the brain, and provide mechanistic insight to aid the understanding of mnemonic deficits associated with chronic synthetic glucocorticoid treatment.

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Author's declaration

I declare that the work in this dissertation was carried out in accordance with the requirements of the University's Regulations and Code of Practice for Research Degree Programmes and that it has not been submitted for any other academic award. Except the RNA-sequencing (section 3.1) and RT-PCR data (section 4.1), which was carried out by Dr. Matthew Birnie and Tom Rozwaha, and where indicated by specific reference in the text, the work is the candidate's own work. Work done in collaboration with, or with the assistance of, others, is indicated as such. Any views expressed in the dissertation are those of the author.

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List of abbreviations

5-HT – 5-hydroxytryptamine

ACC – anterior cingulate cortex

aCSF – artificial cerebrospinal fluid

ACTH – adrenocorticotrophic hormone

ADX – adrenaleztomized

AF-1 – activation function-1

AF-2 – activation function-2

AHP – after-hyperpolarization

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

ANOVA – analysis of variance

AP1 – activator protein 1

AVP – arginine vasopressin

BDNF – brain derived neurotrophic factor

BLA – basolateral amygdala

BMAL1 – brain muscle ARNT-like 1

BNST – bed nucleus of the stria terminalis

CA1-3 – cornu ammonis 1-3

CaMKII – calcium/calmodulin-dependent protein kinase

cAMP – cyclic adenosine monophosphate

CBG – corticosteroid binding globulin

CeA – central amygdaloid nucleus

ChIP – chromatin immunoprecipitation

ChIP-nexus – ChIP with nucleotide resolution using exonuclease digestion, unique barcode and single ligation

CLOCK – circadian locomotor output cycles kaput

CNQX - 6-cyano-7-nitroquinoxaline-2,3-dione

CNS – central nervous system

CRE – cAMP response element

CREB – cAMP response element-binding protein

CRFR1 – CRF receptor type 1

CRH/CRF – corticotropin releasing hormone/factor

Cry1-2 – cryptochrome 1-2

CTD – carboxy-terminal domain

DAVID – database for annotation, visualisation, and integrated discovery

DBD – DNA (see below) binding domain

D-box – distal-box

DEE - developmental and epileptic encephalopathy

DEG – differentially expressed gene

DG – dentate gyrus

dHPC – dorsal hippocampus

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

EC – entorhinal cortex

Egr1 – early growth response 1

E-I balance – excitatory-inhibitory balance

Elk1 – ETS transcription factor Elk1

EPSC – excitatory post-synaptic current

EPSP – excitatory post-synaptic potential

ERK – extracellular regulated kinase

fEPSP – field EPSP

FKBP5 – FK506 binding protein 5

FRET – fluorescence resonance energy transfer

FS – forced swim

G protein – guanine nucleotide-binding regulatory protein

GABA - Gamma aminobutyric acid

GC – glucocorticoid

GO – gene ontology

GPCR – G protein coupled receptor

GR – glucocorticoid receptor

GRE – glucocorticoid response element

HFS – high-frequency stimulation

hGR – human GR

hMR – human MR

HPA axis – hypothalamic-adrenal-pituitary axis

HPC – hippocampus

HSD – 11β-hydroxysteroid hydrogenase enzyme

Hz – Hertz

I – current

IEG – immediate early gene

IL – infralimbic cortex

IPA – ingenuity pathway analysis

IPSC – inhibitory post-synaptic current

KAR – kainate receptor

K_D – dissociation constant

LA – lateral nucleus of the amygdala

LBD – ligand binding domain

LFS – low-frequency stimulation

lPFC – lateral prefrontal cortex

Lsamp – limbic system-associated membrane protein

LTD – long-term depression

LTP – long-term potentiation

MAPK – mitogen activated protein kinase

MDR – multi drug resistance

MeA – medial amygdaloid nucleus

mEPSC – miniature excitatory post-synaptic current

mGluR – metabotropic glutamate receptor

mIPSC – miniature IPSC

mPFC – medial prefrontal cortex

MPL - methylprednisolone

MR – mineralocorticoid receptor

mRNA – messenger ribonucleic acid

MSK – mitogen and stress-activated protein kinase

MTL – medial temporal lobe

NBQX - 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline

NFκB – nuclear factor kappa B

NLS – nuclear localization sequence

NMDA – N-methyl-D-aspartate

NMDAR – NMDA receptor

NO – nitric oxide

NOL – novel object location

NOR – novel object recognition

NPAS2 – neuronal PAS domain protein 2

NR3C1 – nuclear receptor subfamily 3 group C member 1

NR3C2 – nuclear receptor subfamily 3 group C member 2

NTD – amino terminal domain

NTS – nucleus of the solitary tract/nucleus tractus solitarii

P-box – proximal-box

Per1-3 – Period 1-3

PFC – prefrontal cortex

Pgp – MDR p-glycoprotein

POMC – proopiomelanocortin

PoR – Postrhinal cortex

PRH – perirhinal cortex

PrL – prelimbic cortex

PVN – paraventricular nucleus of the hypothalamus

Rec – recording electrode

RNA – ribonucleic acid

RNAseq – RNA sequencing

Ro – Ro-25-6981

RORE – retinoic acid-related orphan receptor response element

RS – rhinal sulcus

RT – room temperature

S1/S2 – stimulating electrodes

SCN – suprachiasmatic nucleus of the hypothalamus

SD – standard deviation

SEM – standard error of the mean

sEPSC – spontaneous EPSC

SGK1 – serum and glucocorticoid inducible kinase 1

sIPSC – spontaneous IPSC

sTBS – spaced theta burst stimulation

TBS – theta burst stimulation

TSST – Trier social stress test

TTX - tetrodotoxin

V – voltage

VDCC – voltage-dependent calcium channel

VEH - vehicle

vHPC – ventral hippocampus

ZT – zeitgeber time

Chapter I

Introduction

Endogenous corticosteroids are a family of hormones comprising two major groups: glucocorticoids and mineralocorticoids. Mineralocorticoids are primarily involved in the regulation of electrolyte and water balances via modulation of ion channel function in the kidneys of vertebrates (Ufferman and Schrier 1972). The primary mineralocorticoid is aldosterone in both humans and rodents. The principal glucocorticoid (GC) hormone in humans is cortisol, but corticosterone in rodents, and the abbreviation CORT will be used generically to refer to cortisol and corticosterone. Glucocorticoids, on which this thesis will focus, are adrenal hormones that are perhaps most notably recognised in neuroscience research for their prominent role in the stress response (Herman and Cullinan 1997). Stress is a complex and multifaceted phenomenon, and despite the important role of glucocorticoids in stress, the concurrent activity of other transmitters, notably noradrenaline, acts synergistically with glucocorticoids to yield a result different from that which would be achieved by activity of each individual component (Chrousos 2009). Furthermore, glucocorticoids are involved in a larger spectrum of events, indeed, these hormones are pivotal in gluconeogenesis (Exton 1979) (after which they are named), arousal (Kalsbeek et al. 2012), vasoconstriction, and anti-inflammatory processes (Schleimer 1993).

1.1. Research Statement

Glucocorticoids are intrinsic to many functions in the human body, including the co-ordination of internal clocks to external cues. They have also been shown to affect cognitive function in a multitude of ways, and despite a vast literature, the mechanisms by which they alter brain function are not well understood. Moreover, they have powerful anti-inflammatory properties, and as such, synthetic glucocorticoids are extremely commonly prescribed. Methylprednisolone is a synthetic glucocorticoid, that due to its vastly increased half-life compared to endogenous glucocorticoids, induces chronic glucocorticoid receptor (GR) activation as well as the suppression of natural corticosteroid rhythms. Patients who are prescribed these drugs often present with unwanted side effects, including memory impairment, though little is known about the underlying mechanism. Glucocorticoids have been shown to alter the expression of clock genes in multiple brain regions, including the hippocampus, and therefore methylprednisolone treatment may induce alterations the rhythmic expression of clock genes in this region. The downstream effects of disruption to hippocampal rhythms on synaptic processes is unknown. Moreover, methylprednisolone is likely to dysregulate molecular rhythms in other brain regions.

1.2. Hypothesis and Aims

Using methylprednisolone to induce chronic GR activation, while suppressing endogenous glucocorticoid rhythms, will result in dysregulation of the molecular clock and circadian

rhythms of several brain regions. I hypothesize that in health, circadian fluctuations in synaptic physiology and plasticity will be present in these brain regions that are associated with learning and memory, and that dysregulation of the molecular clock by chronic methylprednisolone treatment will alter these diurnal variations.

Chapter III – Assess circadian variation in hippocampal synaptic plasticity and physiology, and determine the interaction of MPL treatment with physiological oscillations.

Chapter IV – Investigate the effects of chronic MPL treatment on synaptic plasticity and physiology in perirhinal cortex, and determine the effects and interaction of the circadian cycle.

Chapter V – Determine the effects of MPL treatment, as well as their interaction with the circadian cycle, on synaptic physiology in the medial prefrontal cortex.

1.3. Anatomy and function of the HPA axis

The timing of glucocorticoid release is vital to the proper functioning of many bodily systems, and a complex circuit comprising of several distinct modules and feedback loops exists in order to ensure flexible and responsive hormone release in reaction to the many challenges to homeostasis that an organism may face. The primary organisers of the glucocorticoid response are the paraventricular nucleus of the hypothalamus (PVN), the anterior pituitary gland, and the adrenal glands. Thus, this network, which provides a complex and dynamic interface between the central nervous and endocrine systems, is named the hypothalamic-pituitary-adrenal axis (HPA axis).

Briefly, glucocorticoid hormones are secreted from the adrenal glands. This is initiated by the action of adrenocorticotrophic hormone (ACTH) which is secreted by the anterior pituitary gland in response to the concerted action of corticotropin releasing hormone/factor (CRH/CRF) and arginine vasopressin (AVP), hormones secreted from the PVN. Increased secretion of AVP and CRH occurs in response to stress, but notably for this thesis, their secretory levels are also under the circadian control of the suprachiasmatic nucleus of the hypothalamus (SCN) (Fig 1.1.).

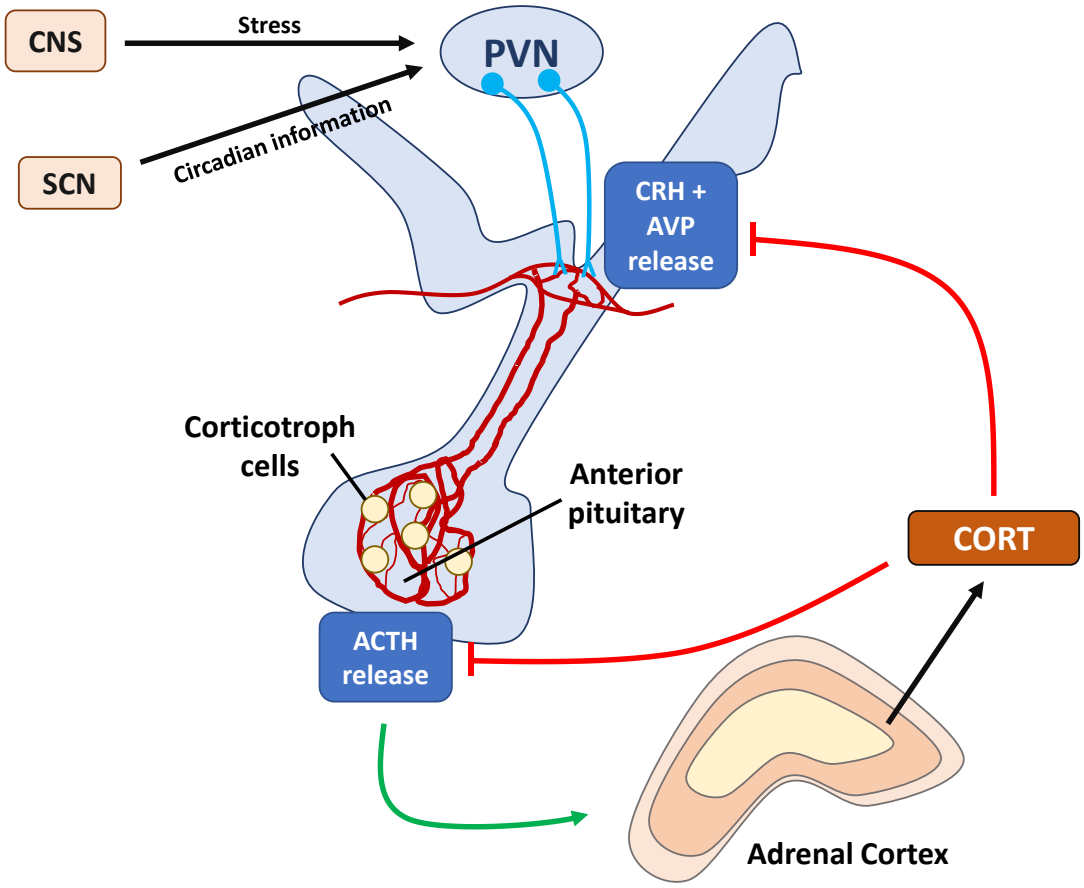


Figure 1.1. The HPA axis. *A simplified schematic diagram illustrating the multiple autoregulatory feedback loops and input to the HPA axis, which modulate the output of corticosteroid hormones from the adrenal cortex.*

1.3.1. The Paraventricular Nucleus of the Hypothalamus

The PVN is a relatively small nucleus that comprises several distinct cellular populations. Located centrally within the hypothalamus, this collection of neurons is pivotal in the maintenance of homeostasis (Swanson and Sawchenko 1980). By integrating inputs from a number of afferent fibres, sharing bidirectional projections with multiple brain regions, and implementing autonomic and neuroendocrine outputs, the PVN is able to continuously monitor and regulate the internal environment of an organism (Palkovits 1999). Classically, the neurons of the PVN are classified in to two major groups; magnocellular and parvocellular neurons, though more recently they have been defined in to three main populations.

Type I neurons are magnocellular neurons whose axons project to the posterior pituitary gland, where they release AVP and oxytocin from neurosecretory terminals into the systemic circulation. These magnocellular neurons can also be found in the supraoptic nucleus (Xi, Kusano, and Gainer 1999), which is situated adjacent to the optic chiasm. They also secrete AVP

to be transported by the systemic circulation to act on the cells of the papillary ducts in the kidneys, in order to enhance water reabsorption (Prager-Khoutorsky and Bourque 2015).

Type II neurons are neurosecretory parvocellular PVN neurons that project to the median eminence where their axons terminate at the primary capillary plexus of the hypophyseal portal system. AVP and CRH are released from nerve terminals into the portal vessels whereby they are transported in the portal circulation to stimulate the secretion of ACTH from the anterior pituitary gland (Whitnall 1993; Gillies, Linton, and Lowry 1982). Type II neurons further include thyrotropin releasing hormone cells, which act to initiate the hypothalamic-pituitary-thyroid axis.

Type III are pre-autonomic parvocellular neurons whose axons descend to sympathetic and parasympathetic nuclei within the brainstem and spinal cord (Chen and Toney 2003; Van Den Pol 1982). More recently another population of parvocellular neurons has been described, whose oxytocin releasing terminals in the spinal cord have been shown to modulate nociception (Eliava et al. 2016).

The PVN also contains neurons which project to higher order regions within the brain, such as the amygdala, where the secretion of oxytocin has been shown to attenuate learned fear responses (Knobloch et al. 2012), and the secretion of CRH was demonstrated to augment anxiety (Kalin 2018). Interestingly, the dendritic release of AVP has been shown to act as a signal within the PVN itself which may contribute to the synergistic action of neurosecretory magnocellular and parvocellular populations within the PVN and pre-autonomic neurons projecting to the brainstem (Stern 2015). It has also recently been demonstrated that neurons expressing CRF receptor type 1 (CRFR1), the principal receptor for CRH, are found within the PVN itself. This allows local CRH release to co-ordinate HPA axis activity by the activity of local microcircuits within the PVN, and selective ablation of the local CRFR1 expressing cells results in changes to the amplitude and timing of the stress response (Jiang, Rajamanickam, and Justice 2018).

1.3.2. The Pituitary Gland

The pituitary gland, or hypophysis, is often colloquially referred to as the ‘master gland’ as it controls several other glands in the body. In humans, it is a pea-sized and bean-shaped gland situated in the sella turcica, connected to the hypothalamus by the pituitary stalk.

In humans, the pituitary gland is divided in two, comprising the anterior pituitary (or adenohypophysis), which makes up around 80 % of the pituitary, and the posterior pituitary (or neurohypophysis), which is attached directly to the pituitary stalk. The anterior pituitary can be

further divided in to three lobes, the anterior lobe (pars anterior or pars distalis), the intermediate lobe (pars intermedia) and the pars tuberalis (pars infundibularis) (Holmes 1974).

CRH and AVP that is released by neurosecretory parvocellular neurons of the PVN diffuses out of the capillary bed at the base of the hypothalamic-hypophyseal portal circulation and targets the endocrine cells of the anterior pituitary, thereby regulating the release of ACTH into the systemic circulation (Gillies, Linton, and Lowry 1982). The anterior pituitary is not only involved in the regulation of stress and the HPA axis, but also in growth, reproduction and lactation via the action of other hormones like human growth hormone, thyroid stimulating hormone, luteinising and follicle stimulating hormones, and prolactin (Amar and Weiss 2003).

The posterior lobe of the pituitary is not glandular like the adenohypophysis but rather consists of neuronal projections that arise from hypothalamic cell bodies. These cells are neurosecretory and their terminals release AVP and OT directly in to the neurohypophyseal capillaries, and from here they may diffuse into the systemic circulation. The posterior pituitary additionally contains a unique class of glial cells called pituicytes, which serve to support the storage and release of AVP and oxytocin (Hatton 1988). Anatomically, the neurohypophysis can be divided into pars nervosa, which contains primarily nerve terminals, and the pituitary stalk (or infundibulum stalk), which is made up of axonal projections.

Moreover, in other species like rodents, there is a third (intermediate) lobe. In rodents, this comprises primarily melanotrophs, a class of endocrine cells also innervated by hypothalamic cells, that synthesize and secrete alpha-melanocyte-stimulating hormone and beta-endorphin as well as proopiomelanocortin (POMC).

1.3.3. The Adrenal Gland

Glucocorticoids are synthesized and released by the adrenal (or suprarenal) glands, which reside in the posterior abdomen, covering the anterior surface of each kidney in both humans (Marieb and Hoehn 2007) and rodents (Parker et al. 1993). They are made up of two primary layers: the adrenal cortex and the adrenal medulla.

The adrenal cortex is the most superficial layer of the gland and is subdivided further into three zones: the zona glomerulosa, zona fasciculata and zona reticularis, which are responsible for the production of mineralocorticoid, glucocorticoid, and androgen hormones, respectively. Accounting for almost 80% of the cortical volume, the glucocorticoid producing zona fasciculata is the largest of the three zones. Cytoarchitecturally, the cells that constitute this zone are arranged in columns that orient radially toward the medulla.

The zona fasciculata also produces androgens, which are known for their role in male development and puberty, but also play a key role in parturition among other functions in both sexes. The primary source of these hormones, though, is the deeper lying zona reticularis.

The zona glomerulosa is the most superficial of the three zones, and the mineralocorticoid (aldosterone) that is produced here is essential for the homeostatic regulation of water retention, blood pressure, and plasma mineral content by modulating the reabsorption of sodium and the excretion of potassium from the kidney. Although the products of each zone are different, the process is akin to a dividing cascade of enzymatic activity, ultimately stemming from one precursor: cholesterol (James 1979; Miller and Auchus 2011) (Fig 1.2).

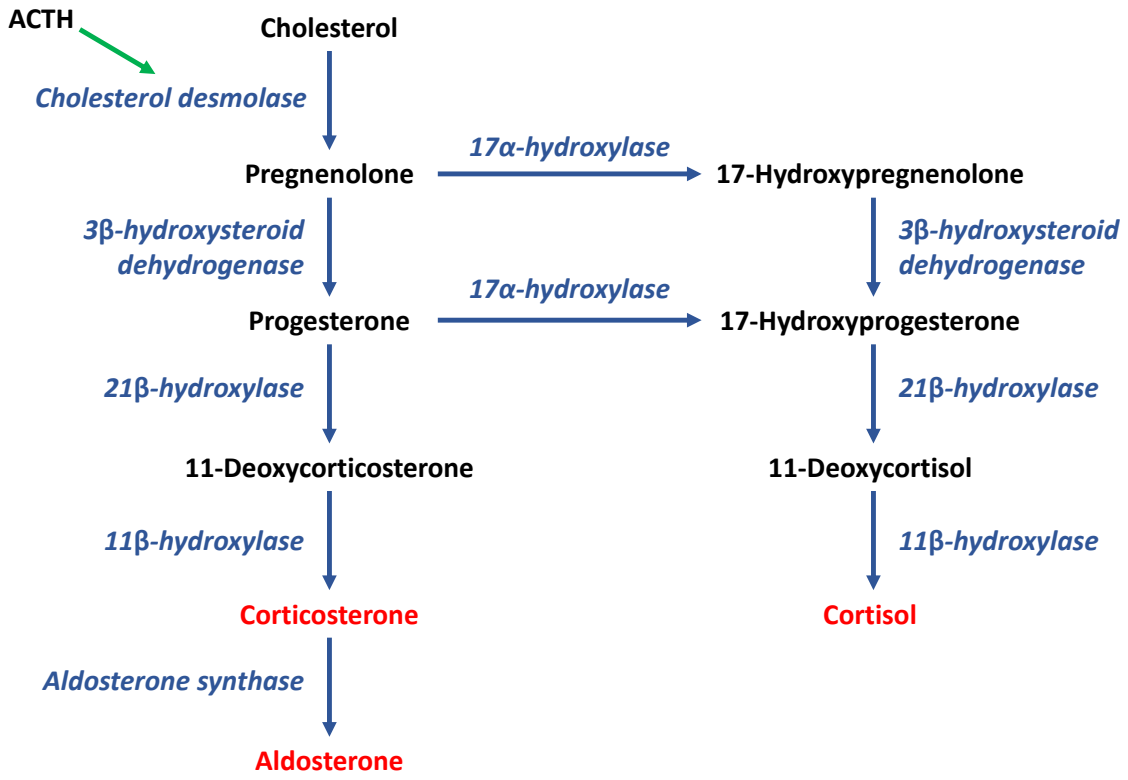


Figure 1.2. Schematic diagram illustrating biosynthetic pathways in the adrenal cortex. *The layers of the adrenal cortex are specialized to secrete different hormones due to the presence of enzymes that catalyse various modifications of the steroid hormones that stem from the precursor cholesterol, a process that is ultimately driven by ACTH.*

1.3.4. The Central Nervous System

Within the CNS are key sites that are responsible for both control of HPA axis output, as well as sites that respond strongly to stress-induced or daily fluctuations in circulating glucocorticoid levels. The anatomy, function, and interaction with the HPA axis of these regions is discussed in brief in this section.

1.3.4.1. Brainstem

The brainstem contains centres which are responsible for the stress induced modulation of HPA axis activity. For example, the nucleus of the solitary tract (NTS) is the major noradrenaline and adrenaline input to the parvocellular neurons of the PVN (Cunningham Jr and Sawchenko 1988), and by activation of α1 adrenergic receptors, provides facilitation of the HPA axis response (Plotsky 1987). The NTS may also modulate the HPA axis by action of other transmitters, like glucagon-like peptide 1 (Kinzig et al. 2003; Larsen et al. 1997), substance P (Larsen et al. 1993), somatostatin, and enkephalin (Sawchenko, Arias, and Bittencourt 1990). Neurons in the rostral ventro-lateral medulla, on the other hand, preferentially innervate magnocellular and parvoceullar pre-autonomic populations (Cunningham Jr and Sawchenko 1988). Moreover, serotonergic input from the Raphe nuclei is known to stimulate the HPA axis by the action of the 5-hydroxytryptamine (5-HT) 2A and 1A receptors in the PVN (Pan and Gilbert 1992; Van de Kar et al. 2001).

1.3.4.2. The Amygdala

The amygdala is a small structure in the medial temporal lobe that, due to its shape, owes its name to the Greek word for almond, *amygdale*. A part of the limbic system, the amygdala is cardinal in the processing of emotional information, including aversive and appetitive memories. Accordingly, the amygdala has been shown to exert top-down control over the HPA axis, particularly during stress. Indeed, artificial stimulation of this structure elicits increased glucocorticoid responses in several species (Redgate and Fahringer 1973; Mason 1958; Gallagher et al. 1987).

The amygdala is divided into several nuclei, each of which has functional specialization in the control of stress induced HPA axis activity. The basolateral amygdala (BLA) is the largest of these, and further comprises the lateral, basal, and accessory-basal nuclei. Neurons in the BLA are preferentially activated during anticipatory stress regimens (Sawchenko, Li, and Ericsson 2000), but lesions of this nucleus do not alter corticosteroid secretion in response to several other types of stressor, or basal HPA axis activity (Feldman et al. 1994). Additionally, the BLA appears to play a critical role in the maladaptive changes incurred by chronic stress, such that BLA neurons projecting to specific brain regions develop increased spine density and complexity as well as enhanced excitatory transmission following chronic restraint stress (Zhang et al. 2019). Primarily, projections from the BLA terminate in other amygdaloid nuclei, suggesting that the BLA exerts indirect influence over the PVN (Herman et al. 2003).

The central amygdaloid nucleus (CeA) is selectively responsive to inflammation and other systemic stress, whereas the medial amygdaloid nucleus (MeA) is selectively responsive to external stressors like restraint and noise (Sawchenko, Li, and Ericsson 2000; Thrivikraman, Su,

and Plotsky 1997). Both CeA and MeA send only minor projections to the PVN, and so, like the BLA, are likely to influence HPA axis activity indirectly. The HPA axis response to MeA stimulation can be truncated by lesions of the bed nucleus of the stria terminalis (BNST), and projection neurons are primarily GABAergic (Gamma aminobutyric acid, GABA, the primary inhibitory neurotransmitter). It is therefore plausible that glucocorticoid release is modulated by inhibition of the BNST GABAergic projections to the PVN. There are likely several neural circuits involved, though, as the MeA and CeA also innervate the preoptic area and NTS, respectively, which are both sites implicated in HPA axis control (Feldman, Conforti, and Saphier 1990).

1.3.4.3. The Medial Prefrontal Cortex

The prefrontal cortex (PFC) refers to the anterior portion of the frontal lobes, and further comprises the medial prefrontal cortex (mPFC) and lateral prefrontal cortex (lPFC). The mPFC has been shown to be involved in the processing of affect, value, decision making, and the formation of schema (Forbes and Grafman 2010).

Like the amygdala, the mPFC is involved in the modulation of stress induced HPA axis activation, as demonstrated by the local c-fos induction that is generated by several stress protocols (Cullinan et al. 1995; Kollack-Walker et al. 1999). Most evidence suggests that the mPFC is involved in negative feedback regulation, such that glucocorticoid activity in this region decreases stress induced HPA axis activity.

There is subregion specificity in the mPFC control of HPA axis activity, such that dorsal mPFC (anterior cingulate (ACC) and prelimbic cortices (PrL)) is involved in the inhibition of HPA axis responses to anticipatory stressors (Figueiredo et al. 2003; Diorio, Viau, and Meaney 1993). The ventral region (infralimbic cortex (IL)) seemingly has a more nuanced role; electrolytic lesions demonstrated modulation of HPA axis responses to systemic, but not restraint, stress (Crane, Ebner, and Day 2003). Conversely, ibotenate lesions incurred a reduction in PVN c-fos in response to restraint stress, suggesting that IL may augment HPA axis responses to anticipatory stressors (Radley, Arias, and Sawchenko 2006). Indeed, it was recently shown that IL stimulation increases plasma corticosterone, and local inhibition reduces corticosterone responses to anticipatory stress (Ronzoni et al. 2016). Regulation of HPA axis activity by mPFC occurs only following stressful stimuli, and this region appears to have no effect on the regulation of basal glucocorticoid levels (Diorio, Viau, and Meaney 1993; Figueiredo et al. 2003).

The influence of mPFC circuits on HPA axis activity is mediated predominantly by indirect projections to the PVN, as there are very few direct fibres. It is thought to be chiefly via IL projections to several regions known to modulate PVN activity that control is exerted. For

instance, the IL innervates the BNST, perifornical nucleus, paraventricular thalamic nucleus, amygdala, and hypothalamic nuclei, all of which provide potential routes for indirect modulation of HPA axis activity.

1.3.4.4. The Hippocampus

The hippocampus (HPC) is a structure in the medial temporal lobe (MTL) that constitutes a part of the limbic system. The curved shape of this region resulted in the name *hippocampus,* stemming from the Greek for *seahorse*. One of the most extensively studied areas in the brain, the HPC is well-known for its roles in the processing of mnemonic and spatial information, though how much of this information is actually stored within the HPC remains contentious. Unlike the neocortex, in which a laminar organization allows processing of complex information within a concentrated microcircuit, the allocortical structures within the hippocampus entail distributed networks that permit vast amounts of varied information to be collated in contextual processing.

The hippocampus is present across all mammals and consists of several subregions; the *Cornu Ammonis* (CA) regions (CA1-3), so called for the shape of the ram's horns of the Egyptian deity Amun, constitute the 'hippocampus proper', while the dentate gyrus (DG), subiculum, and CA regions constitute the hippocampal formation. Some definitions also include the presubiculum, parasubiculum, and entorhinal cortex (EC) within the hippocampal formation.

Classically, information is thought to flow through the hippocampus in a principally unidirectional manner, conducted primarily by the well-described tripartite hippocampal network. In this model, information enters the hippocampus via entorhinal projections to the DG. From here neurons relay information downstream via mossy fibre synapses on to CA3 neurons, which then project to area CA1, whose principal cells eventually direct information out of the hippocampus via the subiculum. These fibres eventually terminate in a wide variety of brain regions, including EC, perirhinal cortex, prefrontal cortex, and amygdala. However, more recent evidence suggests further circuits exist, including a disynaptic loop including CA3 and CA1, another disynaptic loop including CA2 and CA1, and the monosynaptic temperoammonic pathway which involves only CA1 (Jones and McHugh 2011).

The DG is composed of defined cell layers – the molecular layer, the principal cell layer, the polymorphic layer and, by some definitions, the hilus. The CA regions are similarly regimented in defined strata. The stratum oriens comprises inhibitory basket cell bodies and horizontal trilaminar cells. Superficial to the oriens is the stratum pyramidale, containing the cell bodies of the principal pyramidal cells of the CA fields. The pyramidal cells of CA1 are smaller than those contained in CA2 and CA3, prompting Ramón y Cajal to define the regio inferior and regio

superior. These populations were later defined further by Lorente de Nó, who noted that the cells of CA3 receive mossy fiber inputs from the DG, whereas cells in CA1 do not (Lorente de Nó 1934). Further to this, the CA2 field contains large cells like those in CA3, but these cells were not thought to receive innervation from mossy fibres, although more recent evidence suggests that the DG does form monosynaptic inputs to CA2 neurons (Kohara et al. 2014). As new evidence accumulates it paints an ever more complex picture of hippocampal connectivity. CA2 and CA3 neurons have both been found to innervate distinct subpopulations of CA1 pyramidal cells (Kohara et al. 2014), and heterogeneity is also present along the radial axis (deep vs superficial neurons). In CA1, superficial and deep neurons have distinct structures (Bannister and Larkman 1995), molecular profiles (Cembrowski et al. 2016), inputs, and outputs (Lee et al. 2014).

The hippocampus proper can also be further divided into ventral (vHPC)(septal) and dorsal (dHPC)(temporal) in rodents, and anterior and posterior in primates. Although vHPC and dHPC have broadly the same structures, they comprise unique circuits and innervate different nuclei, affording them distinct function – dHPC is typically ascribed to roles in spatial memory and navigation, and vHPC to roles in anxiety and stress processing. More recently, gene expression studies have shown the presence of multiple long-axis domains. Electrophysiological and anatomical evidence suggests a clear demarcation between the ventral two thirds and dorsal third of hippocampus, and further studies of receptor expression and place field properties indicate a gradient along the longitudinal axis (Strange et al. 2014). Together, these lines of evidence suggest that the classical divisions of vHPC and dHPC require revision.

The hippocampus plays in important role in the modulation of HPA axis activity, exerting primarily inhibitory control, as demonstrated by hippocampal lesions, which resulted in elevated basal GC levels (Fischette et al. 1980). The hippocampus acts to provide negative feedback inhibition when circulating glucocorticoids are raised. Indeed, blockade or deletion of hippocampal GR results in reduced hippocampal inhibition of HPA axis activity in response to stress or synthetic glucocorticoids (Boyle et al. 2005).

Heterogeneity along the longitudinal axis of the hippocampus is also vital in the control of the HPA axis, with lesions to vHPC and ventral subiculum resulting in enhanced PVN CRH expression in response to restraint stress, but with no effect on circadian variations in GC secretion (Herman, Dolgas, and Carlson 1998; Mueller, Dolgas, and Herman 2004). On the other hand, lesions to dHPC disrupt the diurnal rhythm and elevate basal GCs (Fischette et al. 1980).

1.3.4.5. The Perirhinal Cortex

The perirhinal cortex (PRH) is a region in the MTL best known for its role in visual perception (Buckley and Gaffan 2006) and memory processing (Brown and Aggleton 2001). The projections from PRH to EC constitute one of two major pathways for sensory information to enter the hippocampal network (Suzuki and Amaral 1994). The PRH comprises Brodmann areas 35 and 36 in primates (Amaral, Insausti, and Cowan 1987), humans (Insausti et al. 1995), and rodents (Burwell, Witter, and Amaral 1995a), lending an intermediary position between neocortex and hippocampus, and as such, an intermediary cytoarchitecture. Due to high levels of intrinsic inhibition, the PRH may act to gate information flow in to the entorhinal-hippocampal network (De Curtis and Paré 2004; Koganezawa et al. 2008).

Generally, medially directed axons (from neocortex to PRH and from PRH to EC) originate and terminate in superficial layers, with the majority of feedforward projections terminating in EC. Return projections tend to arise from deep layers and terminate in all layers of PRH, and a small number extend to neocortex, all of which are feedback projections (Lavenex, Suzuki, and Amaral 2002; Suzuki and Amaral 1994). The interconnectivity of the PRH with neocortical and entorhinal-hippocampal areas is generally well conserved between rodents and primates, as is the function (Suzuki 2009; Burwell and Amaral 1998), but little is known about what role glucocorticoids play in this brain region.

The PRH is known to contribute to several aspects of mnemonic processing. Recognition memory, in particular, is starkly affected by lesions to the PRH (Barker et al. 2007a). Crucially, in models of chronic stress this type of memory is also compromised (Franklin et al. 2018; Wang et al. 2011), suggesting a role for glucocorticoids in the modulation of recognition memory and perirhinal neurophysiology. Indeed, lesions of the PRH modulate behavioural changes incurred by stress (Schulz-Klaus 2009) and chronic stress has been shown to alter the morphology of layer V pyramidal neuron dendritic spines. Furthermore, an observed reduction in nectin-1 messenger ribonucleic acid (mRNA) in the PRH of stressed animals was dependent on CRH (Gong et al. 2018), supporting the notion that glucocorticoids are integral modulators of PRH neurons.

1.4. Glucocorticoid receptors in the CNS

The functional effects of glucocorticoids outlined in the previous sections are mediated by intracellular corticosteroid receptors. Two primary receptor types for glucocorticoids exist in the brains of rats and humans, these were originally named the Type I and Type II corticosteroid receptors. The receptors were later renamed to the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), for their roles in the maintenance of gluconeogenesis and mineral balances in the periphery, respectively.

GR and MR both bind cortisol and corticosterone, although MRs additionally bind aldosterone. Both receptors belong to the nuclear receptor superfamily, and thus function as ligand activated transcription factors. More recently non-genomic functions of these receptors have also been described. Despite sharing a common ligand, MRs and GRs differ in their distribution and ligand binding affinities, thereby allowing an extraordinary functional range.

1.4.1. Distribution

MRs are expressed relatively sparsely throughout the CNS, with only certain regions exhibiting notable levels of protein. For instance, there are high levels of expression in all hippocampal subfields and the lateral septum, with the highest density occurring in the subiculum, CA1 cell field, and the dentate gyrus - areas classically associated with learning and memory. MR expression has also been reported in the mPFC and amygdala (Diorio, Viau, and Meaney 1993; Mitra, Ferguson, and Sapolsky 2009), and a recent study in the *Parus major* has indicated that MR may be more widely expressed than previously thought (Senft, Meddle, and Baugh 2016).

GRs, on the other hand, are expressed relatively ubiquitously throughout the brain. There is some variation in distribution, however, with particularly enriched expression in the PVN, nucleus tractus solitarii, lateral septum, hippocampal CA1 and dentate gyrus, as well as amygdalar subregions and mPFC (Reul and Kloet 1985). GR mRNA is present but somewhat less detectable in area CA3, and has also been detected in the perirhinal and entorhinal cortices (Aronsson et al. 1988). Notably, except for a brief and transient period following birth (Rosenfeld et al. 1993), GR expression is not detectable in the SCN (Balsalobre et al. 2000).

Both MR and GR are known to be expressed by neuronal cells, and are sometimes co-expressed, for example in hippocampal pyramidal cells and dentate granule cells (Van Eekelen et al. 1988). Their expression has also been recorded in glial cells, for instance in oligodendrocytes and astrocytes (Bohn et al. 1991). There are also variations in the subcellular localisation of these receptors (Fejes-Tóth, Pearce, and Náray-Fejes-Tóth 1998). Clustering of exclusively MR or GR, or clusters of both receptor types, could confer the potential for heterodimerisation in certain cell types, a process that could allow even more complex modulation of transcriptional activity.

1.4.2. Ligand binding affinities

Another important distinction between the two corticosteroid receptors is the varying affinity for their ligands. MR has a very high affinity for glucocorticoids, with a dissociation constant (K_D) of around 0.5 nM. On the other hand, the K_D of GR is around 10-fold higher, signifying a dramatically lower affinity for corticosteroids. These differences have an important consequence for function. MRs are predominantly occupied at any given time, with percentage occupation by CORT varying very little between high and low circulating levels of CORT.

However, the markedly lower affinity of GRs for CORT means that they will only become substantially occupied when circulating levels of hormone are raised (Reul and Kloet 1985), such as during the circadian peak or stress conditions.

These differences in affinity have interesting implications; first, variations in levels of corticosteroids in the brain will result in shifts between primarily MR mediated responses to GR mediated responses. Second, combined with variations in distribution, different brain regions will respond to different ranges of hormone levels within the brain, with areas not expressing MRs having essentially no response to low CORT levels. However, recent evidence suggests that although MR may be occupied at basal CORT levels, it may not actively exert its effects until higher threshold levels of CORT are reached (Mifsud and Reul 2016), the mechanism for this threshold activity are unclear, though it may be due to epigenetic or other mechanisms affording GREs to become accessible to MR under high glucocorticoid conditions. A recent study utilising chromatin immunoprecipitation (ChIP) with nucleotide resolution using exonuclease digestion, unique barcode and single ligation (ChIP-nexus), showed that an MR mutant that could not bind to deoxyribonucleic acid (DNA), could be tethered to chromatin by GR (Rivers et al. 2019), providing a potential mechanism by which MR could modulate the transcriptional response to glucocorticoids at relatively higher levels of circulating CORT.

Recent ChIP and next generation sequencing evidence suggests that binding of both MR and GR to DNA increased following acute stress or at the circadian peak, though binding of MR at around a quarter of sites remained unchanged. Intriguingly, MR binding increased more significantly in response to circadian changes than to stress (>7-fold vs. >5-fold, respectively), whereas the opposite was true for GR binding (>8-fold vs. ~20-fold, respectively). Sequencing and pathway analysis showed that the genes corresponding to constant MR occupation encode for ciliary proteins, whereas stress/circadian responsive genes were associated with neuroplasticity, memory, and neuropsychiatric disorders. Interestingly, similar genetic pathways were regulated by stress and circadian induced glucocorticoid peaks (Mifsud et al. 2021). These recent data challenge the classical notion that MR is consistently activated by low circulating concentrations of glucocorticoids and demonstrate that MR activation is central to (at least some) circadian and stress induced changes in the brain.

1.4.3. Genetic Structure

The GR and MR are both encoded by single genes, though alternative splicing gives rise to many isoforms and facilitates disparities in distribution and function between isoforms.

The human GR (hGR) gene, *Nuclear Receptor Subfamily 3 Group C Member 1* (*NR3C1*), is located on chromosome 5q31-32 and consists of 9 exons (Encio and Detera-Wadleigh 1991), with exons

2 to 9 coding for the GR protein (Duma, Jewell, and Cidlowski 2006). Alternative splicing has been observed in exon 1, engendering variants with distinct start sites and promoter regions. Further to this, two highly homologous splice variants, α and β, are the products of alternative splicing in exon 9. hGRα and hGRβ have molecular weights of 97 and 94 kDa, respectively, and are indistinct for their first 727 amino acids, after which hGRα has an additional 50 amino acids and hGRβ has an extra 15 nonhomologous amino acids (Encio and Detera-Wadleigh 1991). hGRα is the prevalent variant of GR and hGRβ is thought to be a dominant-negative regulator of hGRα (Oakley et al. 1999). Both hGRα and hGRβ mRNA can then produce a further 8 translational isoforms owing to differences in initiation site, leading to variations in tissue and subcellular distribution as well as transcriptional activity. Further to this, each hGR protein can be subject to post translational modifications including phosphorylation, ubiquitination and sumoylation, allowing further modulation of function (Duma, Jewell, and Cidlowski 2006). There is also evidence for a β splice variant of both the mouse and rat GR, though they are formed by intron retention, rather than alternative splicing of exon 9 (DuBois et al. 2013; Hinds Jr et al. 2010).

The human MR (hMR) gene, *Nuclear Receptor Subfamily 3 Group C Member 2* (*NR3C2*), is located on chromosome 4q31.1 and spans roughly 450 kilobases (Morrison et al. 1990). *NR3C2* consists of 10 exons, including 2 exons (1α and 1β) that are untranslated. The remaining exons encode the hMR protein which is a total of 984 amino acids in length (Zennaro et al. 1995). Alternative splicing of the untranslated region yields two splice variants, hMRα and hMRβ that are differentially expressed in aldosterone target tissues (Zennaro et al. 1997). Other known variants include the use of a cryptic splice site at the exon3-intron C junction, which can induce a 4-amino acid insertion in the DNA binding domain (DBD) of the MR (Bloem, Guo, and Pratt 1995). Although this is not known to affect transcriptional activity (Bahr et al. 2004), it has been reported to be differentially expressed throughout the brain (Wickert et al. 2000). Other variants arise from skipping exons 5 and/or 6, resulting in co-expression of wild-type hMR with splice variants Δ5 or Δ5,6. The Δ5,6 hMR splice variant exhibits dramatically altered function due to the lack of a ligand binding domain (LBD) and so acts in a corticosteroid independent manner, serving to modulate the transcriptional capacity of wild type MR and GR (Zennaro et al. 2001). Similar to hGR, MRs are also subject to various post translational modifications (Pascual-Le Tallec and Lombès 2005).

1.4.4. Protein Structure

Like all steroid receptors, the GR and MR proteins themselves can be considered modular and consist of several domains that are vital for their function (Hollenberg et al. 1985; Arriza et al. 1987). The amino-terminal domain (NTD) is the area of greatest difference between the two

receptors, with MR sharing less than 15% sequence homology to GR (Pascual-Le Tallec and Lombès 2005). This domain accommodates the activation function-1 (AF-1) motif which facilitates interaction of the receptor with the basal transcriptional machinery, cofactors and chromatin modulators, thereby mediating gene activation (Govindan and Warriar 1998). The DBD is significantly more conserved between the steroid receptors, and MR shares 94% homology with GR in this domain (Pascual-Le Tallec and Lombès 2005). The DBD contains two zinc finger motifs, which facilitate binding of the receptor to glucocorticoid response elements (GREs) in the DNA via interaction with the proximal-box (P-box) in the first zinc finger. The DBD also mediates receptor dimerization (a key process for MR and GR function) via the distal-box (D-box) within the second zinc finger (Pascual-Le Tallec and Lombès 2005; Liu et al. 1995). A small and flexible hinge region separates the DBD and carboxy-terminal domain (CTD) and harbours a nuclear localization sequence (NLS), important for the translocation of receptors from the cytoplasm to the nucleus (Giguère et al. 1986). Finally, the CTD contains an additional NLS (Lombès et al. 1994; Smoak and Cidlowski 2004), a second activation function motif (AF-2) (Hollenberg and Evans 1988), and the ligand binding motif - a hydrophobic pocket formed by many α-helices and β-sheets (Fagart et al. 1998; Bledsoe et al. 2002). MR shares 57% sequence homology with GR for the LBD, and so is relatively well conserved between the two steroid receptors (Pascual-Le Tallec and Lombès 2005).

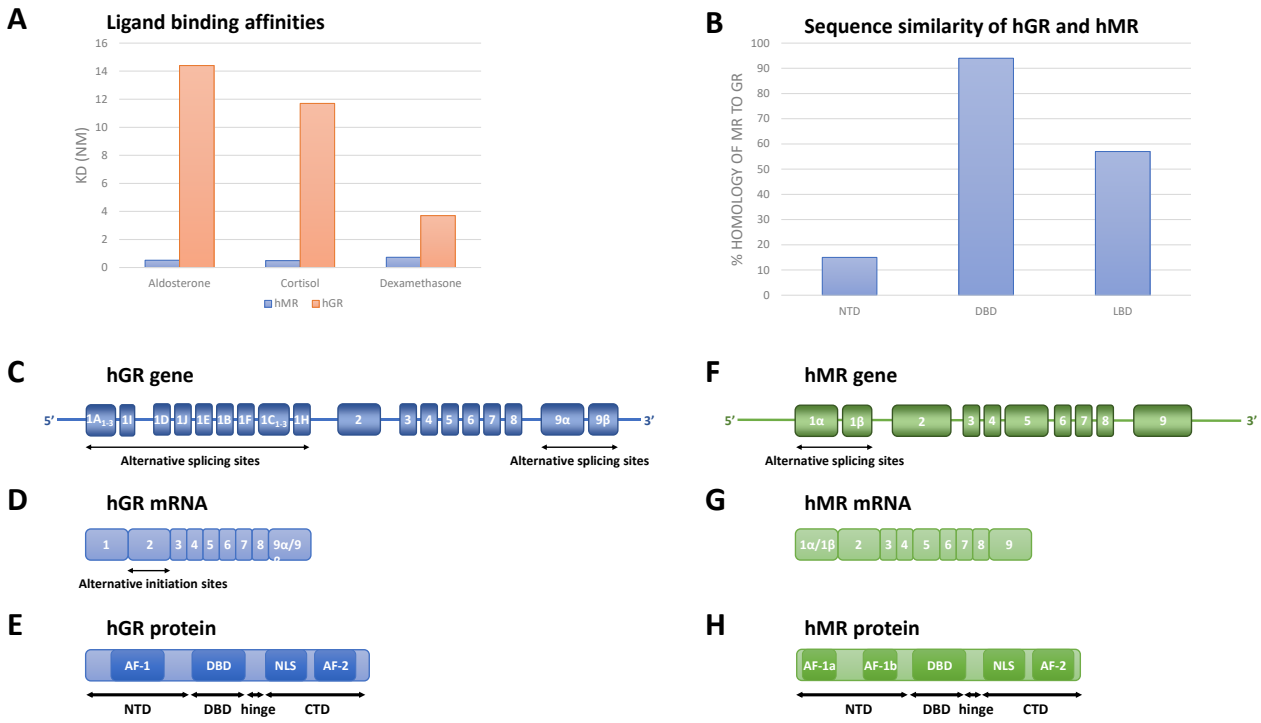


Figure 1.3. Ligand binding affinities, genetic structure, and protein structure of the human GR and MR. ***A)*** *graph showing the relative ligand binding affinities of aldosterone, cortisol and dexamethasone for human mineralocorticoid (hMR) and glucocorticoid receptors (hGR).* ***B)*** *shows the sequence homology shared by hMR and hGR in their various domains.* ***C, D, E)*** *Schematic diagrams of the hGR gene, mRNA and protein structures, respectively.* ***F, G, H)*** *Schematic diagrams of the hMR gene, mRNA and protein structures, respectively.*

1.4.5. Transcriptional regulation

One route of glucocorticoid action is via the formation of homodimers of activated GR or MR. These dimers bind to specific palindromic sequences of DNA called glucocorticoid response elements (GREs), of which there are thought to be several thousand within the genome (Reddy et al. 2009). Ordinarily, two GRE half sites are arranged as inverted palindromes (5'-AGAACA nnnnnn TGTTCT-3'), thereby facilitating dimer binding to the DNA (Beato 1993). GREs are typically found within proximity of gene promoters of 1-2% of genes, and binding of receptor dimers to these sites results in recruitment co-factors and histone modifying enzymes. Concurrent interaction with the basal transcriptional machinery results in either enhanced or, less often, suppressed transcription of the target gene (Datson et al. 2008). Although suppression of target genes in this manner is less common, its importance in regulating brain function should not be overlooked. For example, this mechanism is critical in providing the negative feedback on the HPA axis that is vital for its proper function. This occurs at the level of

hypothalamic CRH transcription (Malkoski and Dorin 1999), as well as within the pituitary where transcription of POMC, the precursor molecule to ACTH, is suppressed (Drouin, Lin, and Nemer 1989).

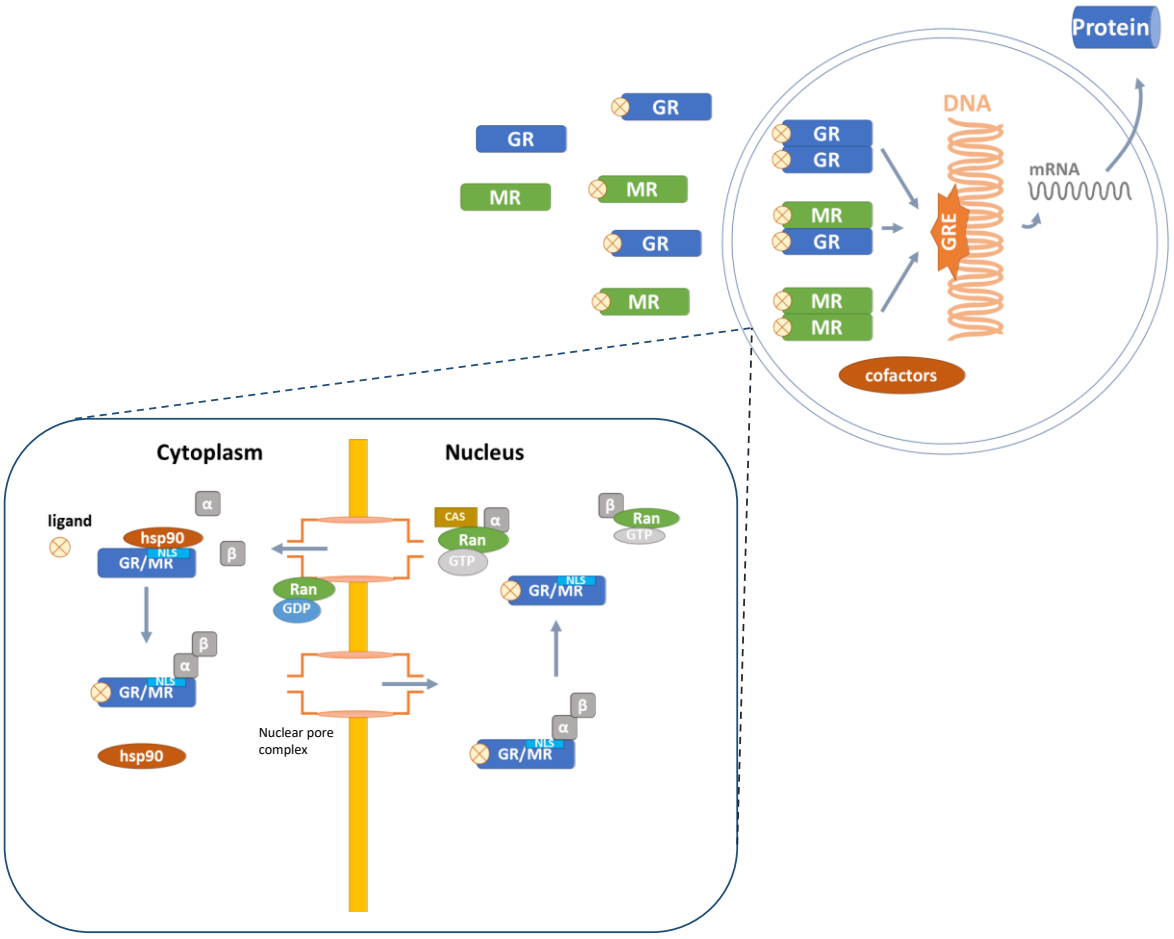


Figure 1.4. Schematic diagram of GR and MR acting as ligand activated transcription factors. *GR and MR translocate into the nucleus via the nuclear pore complex following ligand binding. Within the nucleus the receptors bind to response elements on the DNA, enabling the modulation of transcriptional processes.*

The formation of heterodimers of activated receptors (i.e. GR-MR) is thought to be one source of diversity in corticosteroid signalling (Liu et al. 1995). Intermolecular Fluorescence Resonance Energy Transfer (FRET) has been utilised to demonstrate the formation of heterodimers, which were reported to form more readily at higher corticosterone concentrations (Nishi and Kawata 2006). Transcription of the 5-HT_{1A} receptor is known to be downregulated by GRE binding, and this site appears to exhibit a preference for heterodimer over homodimer binding (Ou et al. 2001). Until recently, heterodimer formation had only been demonstrated *in vitro*. By using tandem ChIP to study GREs in the rat hippocampus, strong evidence was gained supporting the

formation of heterodimers at the *Period1* (*Per1*) and FK506 binding protein 5 (*Fkbp5*) genes, which are both known to be strongly regulated by glucocorticoids (Jääskeläinen, Makkonen, and Palvimo 2011; Rawashdeh et al. 2014). At the serum and glucocorticoid-regulated kinase 1 (*Sgk1*) gene, however, no significant evidence for heterodimerization was found, though there was evidence of both MR and GR homodimers, stressing the gene-specific nature of regulation by GR/MR homo- and heterodimers. The formation of homo- or hetero-dimers also appears to be dependent on the level of circulating GCs, such that at early morning baseline conditions primarily MR/MR homodimers are active, whereas following stress GR/GR homodimers and GR/MR heterodimers were observed (Mifsud and Reul 2016). Recent evidence shows that MR and GR co-exist at the chromatin only at the peaks of glucocorticoid oscillations, and while GR occupancy is pulsatile, MR remains bound to DNA during the inter-pulse interval. The authors also showed that GR and MR may interact in a manner more complex than previously thought, with structural modelling revealing the possibility of multimerization (Pooley et al. 2020). This is in agreement with evidence suggesting the formation of GR tetramers at DNA binding sites (Presman et al. 2016).

Activated receptors can also repress gene transcription via protein-protein interactions with transcription factors like nuclear factor kappa B (NFκB) (Ray and Prefontaine 1994), cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB) (Guardiola-Diaz et al. 1996), and activator protein 1 (AP1) (Heck et al. 1994; Pfahl 1993), which are activated by other signalling cascades. These protein-protein interactions, often referred to as 'transrepression', are thought to be responsible for the immunosuppressive effects of glucocorticoids (Almawi and Melemedjian 2002; Newton 2000). Further to this, the variation in availability of protein interacting partners between cell types is likely an important mechanism conferring specific and diverse regulation of the genome by glucocorticoid receptors.

DNA binding and protein interactions are both important for the diverse functioning of GR and MR, and this has been demonstrated by the marked effects on cognition observed in $GR^{dim/dim}$ transgenic mice. These mice express a variant of the receptor that is largely unable to homodimerize or bind to DNA, but this mutant retains capacity for protein-protein interactions. $GR^{dim/dim}$ mice exhibited deficits in spatial memory assayed by the Morris water maze, while anxiety and locomotion in the open field remained unchanged when compared to control mice (Oitzl et al. 2001). The data obtained from this study suggest that protein-protein interactions and DNA binding likely have differing significance to certain processes in the brain and body, depending on cell-type, behaviour, and context.

The functions of genes regulated by glucocorticoids are vast and varied, ranging though energy metabolism, neuronal structure, vesicle dynamics, circadian neuronal activity, plasticity

processes, and glucocorticoid regulation (Datson et al. 2001; Jääskeläinen, Makkonen, and Palvimo 2011; Rawashdeh et al. 2014; Tsai et al. 2002). This gene regulation also appears to be cell type and even cell compartment specific, and this specificity can depend on several factors including DNA composition (Datson et al. 2011) and chromatin accessibility and remodelling (John et al. 2011).

Recent evidence has also shown that GR/MR binding to GREs is highly GRE-dependent. Not only is there variability of binding to GRE sites between different genes, but also within genes. For example, within the *Fkbp5* gene exists a GRE to which GR binding is highly responsive to stress, whereas a second GRE that is merely 4-kb upstream is barely responsive (Mifsud and Reul 2016, 2018).

1.4.6. Non-genomic effects

GR and MR are now also known to function beyond their classical roles as ligand activated transcription factors. The non-genomic functions of steroid receptors were first postulated when it was observed that CORT was capable of causing changes within a time scale too short to be compatible with DNA transcription and protein synthesis. For instance, early *in vivo* electrophysiological studies in freely behaving rats showed that a 1 mg intraperitoneal injection of corticosterone was sufficient to reduce the spike firing of dorsal hippocampal neurons as soon as 10 minutes post injection, with effects lasting at least 2 hours (Pfaff, Silva, and Weiss 1974). Further evidence for non-genomic function of GR and MR comes from the demonstration that rapid effects of glucocorticoids on CRH induced ACTH secretion were not affected by the transcription inhibitor actinomycin D (Hinz and Hirschelmann 2000).

Although classically known to be cytosolically localized, the rapid actions of corticosterone led to the suggestion of membrane localized receptors. Competition experiments at the synaptic plasma membrane gave credence to this postulation (Towle and Sze 1983), and more recently electron microscopy (Johnson et al. 2005) and the use of corticosterone-BSA (Chauveau et al. 2010) (corticosterone conjugated with bovine serum albumin, a membrane impermeable molecule) have confirmed the existence of membrane bound GRs and MRs. It is possible that membrane bound receptors may partially exert their effects via interaction with guanine nucleotide-binding regulatory protein (G protein) coupled receptors (GPCRs). Indeed, a membrane bound receptor characterized in newts was shown to be key in mediating stress-induced suppression of male reproductive behaviour (Moore and Miller 1984). Subsequent radioligand binding studies demonstrated that [^{3}H]corticosterone binding was negatively modulated by guanyl nucleotides, thus indicating that the receptor is allosterically regulated by G proteins (Orchinik et al. 1992).

More recently, a non-genomic interaction between GR and the N-methyl-D-aspartate (NMDA) receptor/extracellular regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathway has been described, which bears epigenetic and transcriptional consequences, albeit via a non-classical mechanism of GR. It was reported that activated GRs bind to ERK and mitogen and stress-activated protein kinase (MSK) thereby facilitating the phosphorylation, thus activation, of these kinases. Phosphorylation of these proteins allows histone phosphorylation and acetylation so enabling the induction of the immediate early genes (IEGs) Fos and Egr1 (early growth response 1) following forced swim, Morris water maze, and other stressful memory assays. It was found that GR mediated activation of these IEGs via this non-genomic pathway was necessary for the consolidation of memory in these tasks (Gutièrrez-Mecinas et al. 2011).

1.5. Factors affecting glucocorticoid availability

1.5.1. Corticosteroid binding globulin

The availability of glucocorticoids is not just dependent on synthesis and secretion by the HPA axis, but also by the action of the 50-60 kDa plasma transport glycoprotein; corticosteroid binding globulin (CBG). 95% of all cortisol in the circulation is bound to plasma proteins, primarily CBG, maintaining an inert form of cortisol, whilst only 5% of circulating cortisol is present in its active form (Westphal 1986). CBG is a monomeric (Mickelson et al. 1982) protein with a single steroid binding site per molecule (Westphal 1986), circulating at concentrations of 175-365 nmol/L (Nenke et al. 2016) and becoming saturated at plasma cortisol levels of 400-500 nmol/L (Ballard 1979). The influence of CBG on available glucocorticoid is not simply an on-off binding event, though. The protein also acts as a thermocouple, meaning body temperatures above 39 °C will result in a threefold increase in free cortisol (Cameron et al. 2010). Further to this, the structure of CBG has evolved to allow greater delivery of the anti-inflammatory hormone to inflamed loci. Cleavage of a reactive loop in CBG by neutrophil proteases, which are recruited to sites of inflammation (Ariga et al. 2004), allows the protein to transition from a strained S conformation in to a relaxed and stable R conformation (Hammond et al. 1990; Pemberton et al. 1988), a movement which is accompanied by an increase of K_D from 32 nM to 392 nM (Chan et al. 2013), thus allowing a greater release of cortisol. The importance of CBG in determining the availability, and therefore function of glucocorticoids, is further highlighted by the studies using transgenic CBG knockout mice, in which $CBG^{-/-}$ mice display a roughly 10-fold increase in free corticosterone, a significant increase in total ACTH, indicating HPA axis overactivity, and decreased survival rates in the face of septic shock (Petersen et al. 2006).

CBG has also been implicated in regulation of glucocorticoid access to its cognate receptors within the central nervous system (CNS). It has been reported, for example, that CBG can be found in the cytosol of corticotrophs (De Kloet et al. 1984) and may explain the low uptake of corticosterone in the pituitary (De Kloet, Wallach, and McEwen 1975). CBG has also been detected in other cells of the anterior pituitary, various hypothalamic areas (Möpert et al. 2006), cerebellum, the BNST, HPC, the periaqueductal grey and the dorsal horn of the spinal cord (Jirikowski et al. 2007). The various sites of expression of CBG throughout the CNS and periphery suggests multiple functional properties and emphasises that this protein is much more than just a 'carrier'. Indeed, it has been demonstrated that following a moderate or severe, but not mild, stressor CBG was released into the circulation, consequently limiting the rising levels of free hormone (Qian et al. 2011). Therefore, CBG may play a role in modulating the amplitude of stress induced GC responses.

1.5.2. 11β-hydroxysteroid dehydrogenases

A further mechanism by which the body can regulate the action of corticosteroids is by the expression of 11β-hydroxysteroid hydrogenase enzymes (HSDs). These enzymes can modulate the bioavailability of corticosteroids by mediating the interconversion of active and inactive forms. Type 2 HSD acts as a dehydrogenase catalysing the inactivation of corticosteroids, but is not substantially expressed in the brain, bar in the NTS. Interestingly, Type 2 HSD appears to play a key role in neurodevelopment, as brain specific knockout of Type 2 HSD resulted in depression, mild memory deficits, and decreased hippocampal serotonin receptor expression in adulthood (Wyrwoll et al. 2015). Type 1 HSD acts as a reductase and is present in many peripheral tissues and throughout CNS, mediating the conversion of inactive 11-keto derivatives into active corticosteroids. In the rat brain, although HSD1 is expressed widely in both neurons and glia, there are regions of relatively higher expression including cerebellum, hippocampus, and cortex. Interestingly, HSD1 seems to be highly expressed in some cells and not others within these gross regions of high expression (Moisan, Seckl, and Edwards 1990). A similar pattern of distribution has been observed in post-mortem in situ hybridization of human brain tissue (Sandeep et al. 2004). Evidence from peripheral cells indicates that HSD1 is localized within the endoplasmic reticulum (Odermatt et al. 2006), however, immunocytochemical evidence suggests the enzyme may be located in other subcellular departments, including on the plasma membrane (Lakshmi, Nath, and Muneyyirci-Delale 1993), which could have interesting inferences for access of corticosteroids to membrane bound MRs and GRs.

Cell-specific regulation of intracellular concentrations of active glucocorticoids can be mediated by the relative expression levels of HSDs. Their importance in cognition has been highlighted by the demonstration that young HSD type 1 deficient ($HSD1^{-/-}$) mice, which cannot regenerate

active corticosterone from inactive 11-keto forms (Kotelevtsev et al. 1997), have impaired performance in both object recognition and passive avoidance assays (Wyrwoll, Holmes, and Seckl 2011), despite an increased rate in new-born cell proliferation in the DG (Yau et al. 2007). The knockout effects become distinct in later life, though, and aged HSD1$^{-/-}$ mice do not exhibit such pronounced cognitive decline as aged wild type mice (Yau et al. 2001), an effect accompanied by attenuation of the decrease in tetanus induced long-term potentiation (LTP) associated with aging in wild type mice (Yau et al. 2007). This illustrates how chronic glucocorticoid action in the brain, caused by impaired negative feedback on the HPA axis that often accompanies ageing, can result in cognitive decline (Issa et al. 1990; Yau et al. 1995). Indeed, aged HSD1$^{-/-}$ mice have lower intra-hippocampal corticosterone levels compared to wild-types (Yau et al. 2007), but they also show increased resistance to obesity (Morton et al. 2004) and hyperglycaemia (Kotelevtsev et al. 1997), which has been associated with cognitive decline in non-diabetic humans (Ravona-Springer et al. 2012) and animal models (Biessels et al. 1998), indicating that peripheral glucocorticoid action may play a role in the decline of cognition. More recently, by crossing a forebrain-specific HSD1 overexpressing transgenic line with a global HSD knockout line, it was shown that while peripheral HSD1 activity did not affect spatial memory in the Y-maze, it did appear to contribute to spatial memory deficits observed in the water maze (Caughey et al. 2017). This suggests peripheral HSD1 may contribute to age-induced deficits in cognitive function under stressful conditions.

1.5.3. Multi drug resistance (MDR) p-glycoprotein

Corticosterone is able to cross the blood-brain-barrier relatively well in both rodents and humans, however, the entry of most corticosteroids (endogenous and synthetic) to the CNS is constrained by the activity of the MDR p-glycoprotein efflux transporter (Pgp) (Ueda et al. 1996; Ueda et al. 1992). Interestingly, this means that in humans there is a higher ratio of corticosterone to cortisol in the brain (Karssen et al. 2001), despite cortisol being the primary glucocorticoid hormone. This indicates that corticosterone may play an important, and relatively unresolved, function in the human CNS.

The action of Pgp in hampering the entry of synthetic glucocorticoids like dexamethasone and prednisolone can prove problematic in the case of chronic steroid treatments. If low doses of dexamethasone are used, HPA axis release of endogenous glucocorticoids is still suppressed effectively, but Pgp allows only very poor penetration of dexamethasone into the brain, creating a hypo-corticosteroid state in the brain (Meijer et al. 1998; Karssen et al. 2002). This state, in which central MR and GR are under-activated, appears to be critical in mediating some of the negative effects of synthetic glucocorticoid treatment (Liston et al. 2013; Born et al. 1991). Co-administration of cortisol with dexamethasone appears to be effective in alleviating

psychological side effects, and this is thought to be by the re-activation of central MRs (Plihal et al. 1996). Importantly though, at high concentrations of substrate, Pgp can become saturated, and synthetic glucocorticoids remain in the CNS, thereby causing the chronic activation of brain GRs and MRs (Karssen et al. 2002).

1.6. The rhythmic nature of the HPA axis

The release of glucocorticoid hormones is known to follow a circadian pattern across many species, with glucocorticoid levels reaching their peak at the end of the resting phase and their trough at the end of the active phase (Young, Abelson, and Lightman 2004) (Fig 1.5). This is controlled principally by neurons of the suprachiasmatic nucleus in the hypothalamus (SCN) and is further enforced by projections to the neurosecretory cells in the PVN (Engeland and Arnhold 2005). Further to this circadian rhythm, an underlying ultradian oscillation exists, such that a pulse of glucocorticoid hormones is released approximately every hour in rodents and every 1.5 hours in humans (Lightman, Birnie, and Conway-Campbell 2020), with a peak amplitude dependant on the overlying circadian oscillation.

The pulsatile pattern of circulating glucocorticoids is driven by a combination of factors. It has been demonstrated that endogenous glucocorticoid pulses persist even in the presence of constant CRH infusion, and therefore are a product of the delay in the feed-forward and feedback loops of the HPA axis (Walker, Terry, and Lightman 2010; Walker et al. 2012). These oscillations in secretion and feedback inhibition are facilitated by the short half-life of endogenous glucocorticoids, such that feedback inhibition is temporary, and is quickly followed by secretion of ACTH to drive further glucocorticoid secretion.

The ultradian and circadian rhythms are maintained across the blood-brain-barrier and therefore may play important roles in regulating the physiology of GR and MR expressing brain regions, such as the hippocampus (Droste et al. 2008). This pulsatile nature of glucocorticoid release has been shown to be important in maintaining phasic GR functionality such as responsiveness to stress-induced glucocorticoid spikes (Lightman and Conway-Campbell 2010) and ultradian pulsing of gene transcription, which is necessary for normal transcriptional action in target tissues (Stavreva et al. 2009).

Disruption of the pulsatile nature of glucocorticoid action is associated with pathophysiology such as inflammation, early life stress (Shanks et al. 2000), and depression (Sonino et al. 1998). Depression, in particular, is linked with elevated glucocorticoids during the circadian nadir, resulting in a flattened pulse profile (Young et al. 1994). Major depression is a common and serious complication of Cushing's disease (Sonino et al. 1998), in which cortisol oscillations are often completely abolished, due in part to raised cortisol levels during the resting phase (Boyar

et al. 1979). It is unclear, though, whether disruptions to glucocorticoid pulsing are symptomatic or causal in the aforementioned pathologies. Moreover, how these oscillations, and alterations to them, affect neurophysiology and memory processes is not well understood.

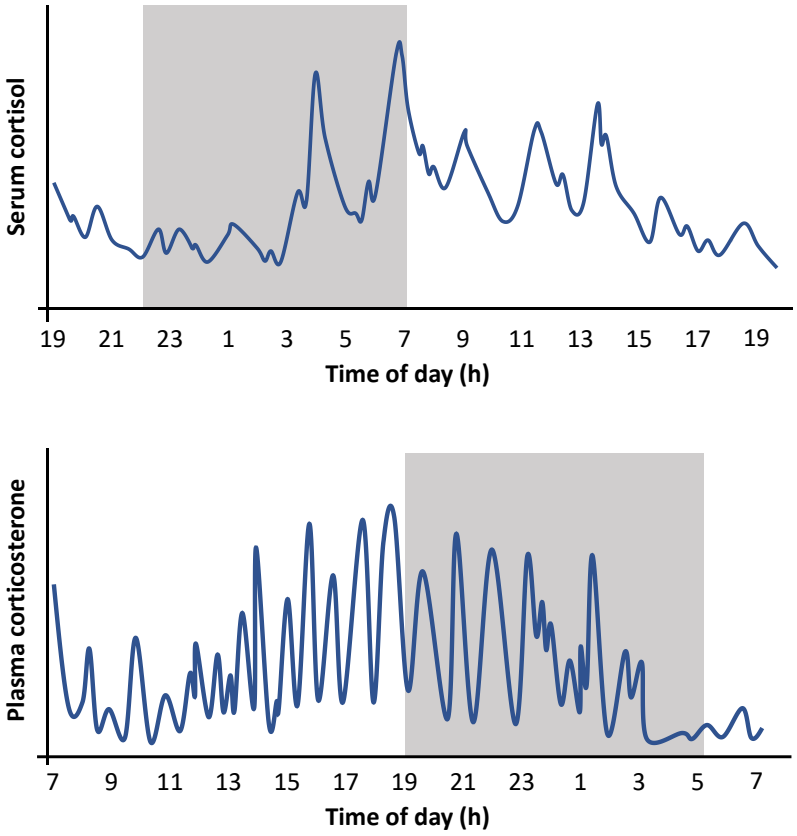


Figure 1.5. Daily glucocorticoid rhythms. Diagrams illustrating measurements of cortisol in human (above) and corticosterone in rat (below) over the course of 24 hours. Adapted from (Lightman and Conway-Campbell 2010).

1.6.1. Internal clocks

There are key aspects of behaviour in most animals that are driven by natural rhythms. The coming of night and day, as well as seasonal changes, infer changes in the environment that dictate the suitability of certain behaviours. Internal clocks have evolved that enable anticipation and adaptation to the changes occurring over the 24-hour day, granting an organism the best chance of survival.

1.6.1.1. Regulation of the HPA axis by the suprachiasmatic nucleus

The SCN is often thought of as the master clock, though it is now apparent that peripheral clocks also exist in many tissues in the body (Skene et al. 2018). Even in cell culture, AVP and vasoactive intestinal peptide are secreted in a roughly 24-hour cycle (Murakami et al. 1991;

Shinohara et al. 1994), and in hypothalamic slices neuronal firing oscillates at the same rate, peaking during the active phase (Welsh et al. 1995).

The SCN receives inputs from the retina, midbrain raphe nuclei, and intergeniculate leaf, which act to drive the core clock (Levine et al. 1986; Dudley, Dinardo, and Glass 1999; Moore, Speh, and Leak 2002). Retinal input from photosensitive retinal ganglion cells via the retinohypothalamic tract allows the SCN to respond to changes in the light-dark cycle, which is additionally modulated by serotonergic input from the midbrain raphe (Rea, Glass, and Colwell 1994). The intergeniculate leaf is thought to mediate non-photic circadian entrainment of SCN, despite receiving retinal input (Harrington 1997).

Indirect projections from SCN to the PVN are responsible for regulating circadian glucocorticoid oscillations, such that CRH and ACTH release is inhibited by SCN activity during the inactive phase (Buijs et al. 1993; Watts and Swanson 1987). The SCN is also able to indirectly regulate the sensitivity of the adrenal cortex to ACTH via the splanchnic nerve (Jasper and Engeland 1994). Moreover, the adrenal gland has its own innate clock, which acts to regulate ACTH sensitivity throughout the circadian cycle (Ishida et al. 2005; Oster et al. 2006).

1.6.1.2. Molecular mechanisms

Circadian gene expression is controlled by two major transcriptional feedback loops involving core clock genes Circadian Locomotor Output Cycles Kaput (CLOCK) and Brain Muscle ARNT-Like 1 (BMAL1). BMAL1 heterodimerizes with CLOCK (or the paralogue NPAS2 [Neuronal PAS domain protein 2]) and these complexes bind at enhancer boxes (E-boxes) to promote transcription of PERIOD 1-3 (*Per1, Per2 and Per3*) and CRYPTOCHROME 1-2 (*Cry1 and Cry2*). PER and CRY proteins then dimerise and translocate to the nucleus where they bind to the CLOCK:BMAL1 dimer, inhibiting the transcription of all clock-controlled genes, forming the principal autoregulatory feedback loop.

The accessory loop involves CLOCK:BMAL1 heterodimer induced transcription of retinoic acid-related orphan nuclear receptors REV-ERBα and RORα. These proteins also translocate to the nucleus and competitively bind to the retinoic acid-related related orphan receptor response elements (ROREs) in the *Bmal1* promoter region. RORα acts to promote expression of *Bmal1* while REV-ERBα inhibits RORα thereby repressing *Bmal1* expression (Buhr and Takahashi 2013; Takahashi 2017).

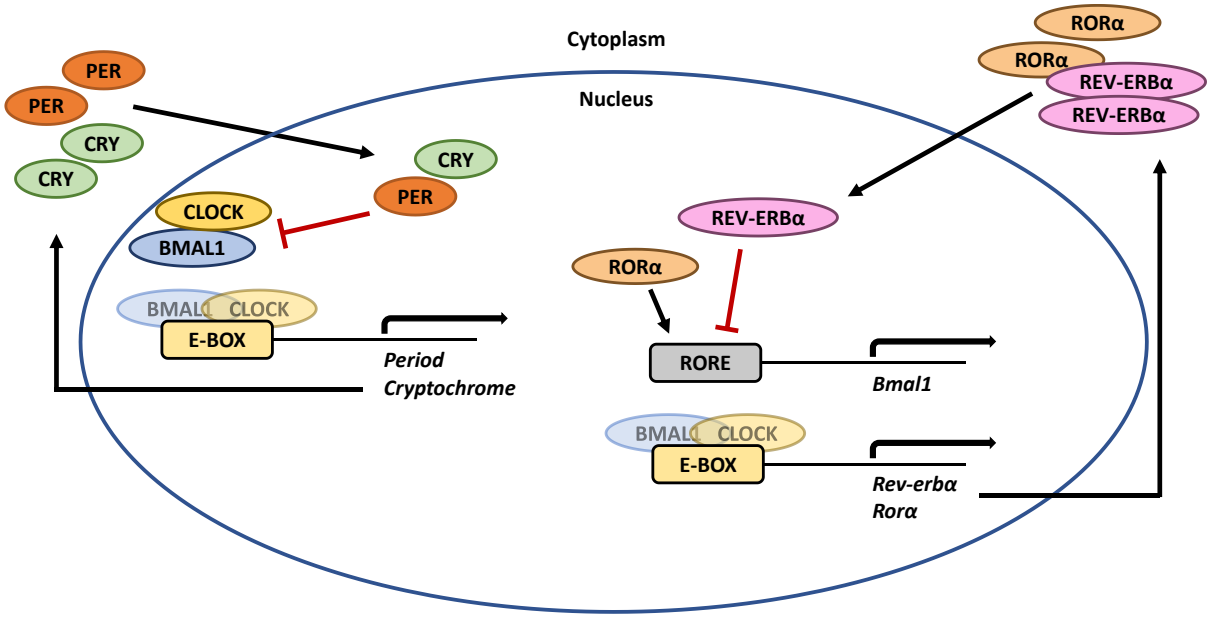


Figure 1.6. Diagram of the primary and accessory loops of the molecular clock. CLOCK:BMAL1 dimers bind to E-boxes to promote transcription of *Per 1-3, Cry, Rev-erbα*, and *Rorα*. PER and CRY proteins then competitively bind upstream of *Per* and *Cry* to inhibit their expression, forming the primary loop of the molecular clock. RORα binds at ROREs upstream of the *Bmal1* gene to promote expression of BMAL1, REV-ERBα competitively binds at ROREs to block *Bmal1* transcription, forming the accessory loop. Adapted from (Udoh et al. 2015).

These feedback loops are further refined by post-translational modifications that ultimately culminate in a roughly 24 hour cycle (Yoshitane et al. 2009; Stojkovic, Wing, and Cermakian 2014). The facilitation of circadian expression of other genomic pathways is also promoted, as rhythmic CLOCK:BMAL1 binding enables rhythmic chromatin opening and remodelling (Menet, Pescatore, and Rosbash 2014).

Interestingly, PER has been shown to have a number of non-clock functions, including the regulation of cell growth (Relles et al. 2013) as well as being crucial in the formation of long-term memory (Sakai et al. 2004).

1.6.1.3. Clocks in the brain

Circadian oscillations in electrical activity can also be observed in several brain regions peripheral to the SCN, including the ventromedial hypothalamus and substantia nigra (Abe et al. 2002). SCN lesions led to the ablation of rhythmicity in extra-SCN regions, leading to the notion that the SCN is the central oscillator responsible for driving periodicity in the rest of the brain.

Indeed, the SCN exerts circadian control over other regions by both neural and humoral signals (Guo et al. 2005). Distinct systems appear to be activated by different mechanisms, for example, circadian oscillations in luteinizing hormone secretion are driven by neural input from the SCN to luteinizing hormone-releasing hormone neurons (Horacio, Meyer, and Schwartz 2003), whereas circadian oscillations in locomotor activity are driven by a humoral signal (Silver et al. 1996).

The SCN master clock is not the only oscillator in the brain, though, and other hypothalamic nuclei also utilise the molecular clock to drive circadian changes in processes like metabolism, hormone secretion and the sleep-wake cycle (Lu et al. 2002; Lu et al. 2000). Circadian oscillations of clock genes have been observed outside the hypothalamus too, including in neo- and cerebellar-cortex, albeit with a 6-hour delay compared to the SCN, reinforcing the notion that the SCN master clock drives these extra-SCN oscillators (Rath, Rovsing, and Møller 2014). The olfactory bulb, on the other hand, was shown to retain rhythmic expression of *Per1* following lesion of the SCN, inferring that other regulatory systems may play a role (Granados-Fuentes et al. 2004; Granados-Fuentes, Tseng, and Herzog 2006).

In the hippocampus, ultradian glucocorticoid pulses have been shown to drive rhythmic *Per1* expression, suggesting that HPA axis control of clock genes is a crucial mechanism driving rhythmicity in this brain region (Conway-Campbell et al. 2010). The properties of glucocorticoid signalling facilitate the synchronization of the hippocampal clock with the 24-hour cycle in several ways. The short half-life of endogenous glucocorticoids, the low affinity of ligand binding, and the rapid and dynamic nature of GR binding to GREs, allow for the GR-driven, pulsatile transcription of *Per1* (Conway-Campbell et al. 2011; Conway-Campbell et al. 2007; Reddy et al. 2012). A pulse of *Per1* transcription driven by GR can consequently synchronize the autoregulatory molecular clock to external cues.

Moreover, unpublished evidence suggests that the perirhinal cortex also rhythmically expresses clock genes in a manner similar to the hippocampus (Rozwaha 2017). Oscillations in reward-related behaviours have also been shown to follow a roughly 24-hour period, and reward system associated regions like the ventral tegmental area and prefrontal cortex exhibit rhythmic activity in clock gene expression (Chun et al. 2015). Diurnal rhythms in mPFC also appear to be driven by adrenal glucocorticoids, as adrenalectomized rats exhibit phase-shifted expression of *Per1* and *Per2*, as well as an apparent lack of a 24-hour rhythm in *Bmal1* expression in this region (Woodruff et al. 2016).

The role of the SCN, rather than setting the rhythms of body clocks, is more likely to synchronise peripheral clocks throughout the body and brain to photic cues from the environment, and

glucocorticoids are a humoral mediator of this synchrony. Indeed, when glucocorticoid rhythms are chronically elevated or are out of phase with environmental light cues, peripheral clocks are affected (Girotti, Weinberg, and Spencer 2009; Sage et al. 2004; Kino and Chrousos 2011).

1.6.2. Circadian effects on learning and memory

These endogenous circadian systems are not only intricate and impressive, but necessary for the co-ordination of physiology and behaviour with fluctuations in the external world. As well as being able to associate time of day with appropriate behaviour, there appears to be daily variation in the efficacy of learning and memory processes. For instance, mice and rats learn maze navigation more effectively during the active phase (Hoffmann and Balschun 1992; Valentinuzzi, Menna-Barreto, and Xavier 2004; Hauber and Bareiss 2001). Interestingly, tone-cued fear conditioning memory in mice is formed more effectively during the inactive phase, indicating that there may be stimulus specificity (Chaudhury and Colwell 2002). In more complex, operant conditioning tasks, time of day effects on learning are less clear, and there is evidence that tasks with a high cognitive demand can themselves serve as zeitgebers – in that they are sufficient to shift the circadian phase (Ghiselli and Patton 1976; Gritton et al. 2013). The importance of circadian oscillations in cognitive function is further highlighted by the memory deficits incurred in rats by the repeated phase-shift of circadian rhythms (Craig and McDonald 2008; McDonald et al. 2013).

The mechanisms underlying these circadian variations in performance are yet to be fully understood. The hippocampus is one region that is ideally positioned to mediate circadian effects on memory, given that it is critical in the formation of many forms of memory, and is a site of oscillatory clock rhythms that are co-ordinated by the SCN via neural and humoral signals. Rhythmic transcription of certain genes may play a role in facilitating the time-of-day effects on learning. Indeed, *Per1* expression has been shown to be vital in hippocampus dependent learning assays (Kwapis et al. 2018), potentially by promoting the nuclear translocation of CREB kinase, thereby altering CREB-dependent gene expression (Rawashdeh et al. 2016). Moreover, *Per2* knockout mice exhibit deficits in hippocampal plasticity and memory processing (Wang et al. 2009), and both *Bmal1* and *Clock* mutant mice display impaired performance in hippocampus-dependent memory tasks (Garcia et al. 2000; Snider et al. 2016; Wardlaw et al. 2014). However, the role of local clocks and extra-SCN oscillators in these global knockout induced impairments is yet to be resolved. Moreover, while these global knockout studies reinforce the importance of intact circadian rhythms and the molecular clock in enabling learning and memory at appropriate times, the mechanisms of their action on brain processes, and ultimately behaviour, remain unclear. Given their influence over hippocampal rhythmicity

and their diverse roles in learning and memory, glucocorticoid hormones are potential mediators of the circadian influence over learning and brain physiology.

1.7. Glucocorticoid effects on learning and memory

1.7.1. Memory Consolidation

Memory consolidation is an important cognitive process known to involve gene expression and protein synthesis (Kandel 2001), thereby facilitating the conversion of labile short-term memories into more stable long-term memories. Long-term synaptic and structural plasticity events are thought to be key manifestations of consolidation processes (Nabavi et al. 2014). Pioneering behavioural studies in rats demonstrated the importance of glucocorticoid action in memory processing when adrenalectomized (ADX) rats were found to exhibit spatial memory impairment (Roozendaal, Portillo-Marquez, and McGaugh 1996; Oitzl and De Kloet 1992). This impairment could be replicated by injection of specific GR (Roozendaal, Portillo-Marquez, and McGaugh 1996; Oitzl and De Kloet 1992), but not MR (Oitzl and De Kloet 1992), antagonists into adrenally intact rats. Other lines of evidence have confirmed the importance of corticosteroids in memory consolidation. For example, delivery of the 11β-hydroxylase inhibitor metyrapone blocked corticosterone synthesis, consequently impairing memory retention in a water maze assay. This deficit could be rescued by systemic administration of dexamethasone immediately following training. Further to this, it was later shown that administration of exogenous corticosterone immediately after training facilitated the consolidation stage of spatial and object recognition memory (Akirav et al. 2004; Roozendaal et al. 2006).

Emotionally salient events are typically remembered better than neutral events, a phenomenon thought to be reliant on temporal coincidence of arousal and GR activation (Finsterwald and Alberini 2014; de Quervain, Schwabe, and Roozendaal 2017). The importance of emotional arousal in GR-mediated facilitation of memory consolidation may have initially been overlooked due to the arousal and stress that is implicit in handling and testing animals in assays like the water maze, which has been shown to significantly increase circulating corticosteroid levels. By lesioning the BLA, Roozendaal et al. demonstrated that this region is critical for GR-mediated facilitation of spatial memory in the water maze (Roozendaal, Portillo-Marquez, and McGaugh 1996).

Placing animals in to unfamiliar environments like testing apparatus is known to cause arousal, but habituation markedly reduces the elicited arousal (Cerbone and Sadile 1995). In a novel object recognition task, one group of rats was habituated to the training environment, while one group was not. Post-training injection of corticosterone facilitated consolidation of object recognition memory in only the aroused, non-habituated group (Okuda, Roozendaal, and

McGaugh 2004), suggesting that arousal and GR activation must occur simultaneously to mediate the consolidation enhancing effects of corticosteroids. It must be noted, though, that these experiments were performed during the inactive phase when endogenous GCs and arousal state are minimal. Whether these facilitatory effects required simultaneous arousal from lack of habituation during the active phase, when arousal would be increased naturally, remains unclear. Nevertheless, It has been shown that emotional arousal is associated with the release of noradrenaline in the BLA (Quirarte et al. 1998), suggesting that corticosteroids may interact with the adrenergic system to exert their effects on cognition. Indeed, β-adrencoceptor blockade in the BLA abolished the enhanced consolidation elicited by GR activation (Roozendaal et al. 2006; Quirarte, Roozendaal, and McGaugh 1997; Roozendaal et al. 1999), while simultaneous delivery of corticosterone and yohimbine, which acts to release noradrenaline, was sufficient to augment consolidation of an emotionally neutral memory (Roozendaal et al. 2006).

Similar observations have been made in human studies wherein exogenous cortisol (Buchanan and Lovallo 2001), hydrocortisone (Rimmele et al. 2003), and elevated endogenous cortisol (Abercrombie, Speck, and Monticelli 2006) have all been shown to enhance consolidation of emotionally arousing material. The specificity for consolidation above other phases of memory processing was also demonstrated as the enhancing effects of cortisol were observed only with delayed, and not immediate, recall in a verbal memory task (Lupien et al. 2002). The importance of concurrent arousal and corticosteroid signalling observed in rodent models has also been demonstrated in humans (Maheu et al. 2004), though, some studies have reported augmented consolidation for emotionally neutral stimuli. One study, for example, used post-learning anticipation of public speaking as a stressor, which causes increased salivary cortisol levels. In a verbal memory task where only neutral stimuli were presented, the stressed group performed significantly better (Beckner et al. 2006), and similar results have been observed when using a cold-pressor stress test and neutral information (Andreano and Cahill 2006). Since only neutral information was presented, though, it leaves the possibility that emotionally stimulating material may have been better remembered. It has also been reported that, using the Trier Social Stress Test (TSST), which causes more marked elevations in salivary cortisol than anticipatory social stressors, memory consolidation was enhanced for only neutral, and not emotionally positive or negative stimuli (Preuß and Wolf 2009). A potential explanation for the differences observed is that a ceiling effect was induced in the latter study, such that emotional memories were acquired more effectively (improved immediate recall when compared to neutral images), a further improvement in memory due to post-learning cortisol increases was

less feasible. This notion is supported by the fact that there was no forgetting between immediate and delayed recall for these images.

As well as the importance of the emotional salience of the memory and the concurrent activation of the adrenergic system, the consolidation enhancing effects of corticosteroids follow an inverted U shaped dose-response curve, and this has been demonstrated in both animal (Akirav et al. 2004) and human models (Roozendaal et al. 2006; Andreano and Cahill 2006). Yerkes and Dodson first reported this as early as 1908, and since it has been shown to be the case for both multiple doses of exogenous corticosteroids (Akirav et al. 2004; Lupien et al. 2002) and endogenous corticosteroid release (Andreano and Cahill 2006). This could somewhat explain differences in the literature regarding the GC induced augmentation of memory for neutral vs. emotive information. For example, it could be that these effects can only be induced by an optimal arousal state induced by a mild stressor (i.e. cold-pressor stress (Andreano and Cahill 2006)) combined with emotive learning material. Conversely, a more profound stressor (TSST (Preuß and Wolf 2009)) combined with emotive information exceeds this optimal arousal level and the enhancing effects of GCs are nullified. Although the complex mechanisms and interactions remain unclear, it is certain that the effects of GCs are dependent on many factors including the nature of the stressor, the duration, the intensity, and the timing of GC secretion.

1.8. Glucocorticoid effects on synaptic physiology

This section aims to explore the changes that GC hormones elicit in relevant brain regions that underly the phenotypic changes described above.

1.8.1. Hippocampus

1.8.1.1. Rapid/non-genomic effects

The rapid effects of GCs on neuronal activity in different brain regions have been studied to varying degrees, with the majority of work being done in the hippocampus.

Rapid effects of GCs on hippocampal function seem to fall in to 2 sub domains of time. Within minutes (<10 minutes), there seems to be an augmentation of hippocampal activity, and this is followed by slightly more delayed (10-60 minutes) effects which act to suppress hippocampal transmission, both of which are separable from the more delayed (>1 hour) genomic effects.

1.8.1.1.1. Molecular pathways

In learning assays that are known to be modulated by GCs, memory consolidation has been shown to depend on activation of the MAPK pathway (Blum et al. 1999). Concurrent activation of the MAPK pathway and GR has also been reported during learning (Guzowski et al. 2001; Hall, Thomas, and Everitt 2001). In mouse hippocampus, GR activation induces the expression

of the IEG Egr1 in a process dependent upon ERK phosphorylation, thereby depending on MAPK-pathway activity (Revest et al. 2005).

Specifically, forced swim (FS) evoked activation of ERK1/2 in DG neurons leads to rapid (within 15 minutes) activation of MSK1/2 and Elk1 (ETS transcription factor Elk1), thereby resulting in phosphorylation and acetylation of the histone H3 in the promoter region of the IEGs *c-fos* and *Egr-1.* Because GR and activated ERK1/2 have been shown to rapidly associate following FS stress, it has been proposed that GR acts as a scaffold, facilitating the phosphorylation of MSK1/2 and Elk1(Gutièrrez-Mecinas et al. 2011). The FS-induced induction of these IEGs has also been shown to depend upon demethylation of the promotor region and/or the 5' untranslated region (Saunderson et al. 2016), as well as CREB binding to CREs (cAMP response elements) (Naqvi, Martin, and Arthur 2014), reinforcing the highly specific and complex nature of GC modulation of signalling cascades.

Moreover, GR activation causes a rapid increase in steady-state levels of Ras and Raf-1 mRNA and protein, presumably due to binding to GREs identified on Ras and Raf family promoters (Strawhecker et al. 1989; Lee et al. 1996). This, as well as the timescale of expression, indicates that Egr-1 expression is being modulated by non-genomic effects of GR. Pharmacological blockade of ERK phosphorylation eliminated the memory enhancing effects of GCs in a contextual fear conditioning assay in mice (Revest et al. 2005), suggesting that this mechanism is critical for mediating GR effects on at least certain types of memory consolidation.

1.8.1.1.2. Passive membrane properties

While glucocorticoids do not appear to greatly alter the passive and active membrane properties of hippocampal neurons, some effects on voltage-gated ion channels have been documented. For example, L-type and N-type Ca^{2+} channels are inhibited rapidly by high concentrations of cortisol, and less effectively by corticosterone, in dissociated hippocampal cells (Ffrench-Mullen 1995). In CA1 pyramidal cells, corticosterone (acting via MRs) shifted the voltage dependency of activation for transient K^{+}-currents such that the channel is less activated by small depolarizations (Olijslagers et al. 2008).

1.8.1.1.3. Effects on glutamatergic transmission

On the other hand, numerous studies have reported GC mediated effects on amino acid transmission. In CA1 pyramidal cells the frequency of spontaneous glutamatergic transmission (as quantified by miniature excitatory post-synaptic currents (mEPSCs)) is increased within minutes in response to corticosterone, and promptly returns to baseline upon washout. The amplitude of these events, though, is not rapidly altered by GCs (Karst et al. 2005). The timescale, as well as evidence using protein synthesis inhibitors and membrane impermeable

ligands, infers a non-genomic modulation of glutamatergic transmission mediated by membrane bound receptors. These effects were found to be mediated primarily by MR and to depend on the presence of limbic system-associated membrane protein (Lsamp) (Qiu et al. 2010). Interestingly, the concentrations of corticosterone required to induce this MR-mediated effect were higher than those required to incur genomic MR actions, indicating that these effects are likely sensitive to stress or rhythms of GCs. Curiously, conditional GR knockout in hypothalamic nuclei indicated that cytosolic GR was required to mediate the rapid effects of GCs on glutamate and GABA transmission (Nahar et al. 2015).

Single quantum dot imaging of rat hippocampal cultured neurons revealed that exposure to 100 nM corticosterone led to increased GluA2 and GluA1 subunit containing AMPAR (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) surface diffusion as soon as 7 minutes post-application. The same effect could be reproduced by application of both BSA-corticosterone and aldosterone, but not RU-28362 (a selective GR agonist), implicating a membrane bound MR. Moreover, it was shown that corticosterone treated cells showed a greater increase in GluA2 containing AMPAR density in the synaptic compartment following a chemical LTP stimulus when compared to untreated cells (Groc, Choquet, and Chaouloff 2008), suggesting that increased AMPAR motility elicited by glucocorticoid action on membrane bound MR is a potential mechanism for the rapid facilitation of LTP. Indeed, intracerebroventricular infusion of pep2m, a peptide that inhibits GluA2 synaptic trafficking, impaired the performance enhancing effects of stress in a water maze task (Conboy and Sandi 2010), suggesting that this process is key in mediating GC effects on learning and memory. Moreover, *in vivo* and *in vitro* experiments have indicated that mitochondrial GR can reduce AMPAR trafficking by decreasing microtubule stabilization, leading to memory deficits in an elevated Y-maze assay (Choi, Oh, et al. 2018).

In contrast to this, other studies examining GC effects within this time frame (10-60 minutes post administration) have reported suppression of hippocampal activity. Single unit activity in dorsal hippocampus recorded *in vivo* (Pfaff, Silva, and Weiss 1971), amplitude of EPSP (excitatory post-synaptic potential) and slow IPSP (inhibitory post-synaptic potential) events, firing probability of synaptically evoked action potentials (Joëls and de Kloet 1993), and population spike amplitude (Vidal, Jordan, and Zieglga 1986) in CA1 neurons are all reduced by corticosterone in this medial time frame.

In cultured hippocampal neurons, application of extracellular corticosterone (50 nM) was sufficient to increase the motility of GluN2B containing NMDARs, ultimately inducing an increased GluN2B-NMDA receptor synaptic content, within 5 minutes. Interestingly, these alterations to NMDAR (NMDA receptor) dynamics were found to be requisite for the

aforementioned GC-induced potentiation of synaptic AMPAR signalling (Mikasova et al. 2017). Application of 100 nM corticosterone significantly enhanced NMDAR fEPSP (field EPSP) slope in the CA1 region of adult hippocampal slices within 30 minutes of administration, as well as increasing the NMDAR:AMPAR ratio within an hour, in an effect mediated by GR (Tse et al. 2011).

Conversely, it was reported that co-delivery of CORT and NMDA to the soma of cultured hippocampal neurons led to a linearly concentration dependent decrease in NMDA-elicited currents. Interestingly, this could not be blocked by GR antagonism and intracellular delivery of CORT did not reproduce this effect, implying a role for membrane-bound MR (Liu et al. 2007). Research from the same group later showed that this reduction in NMDA-elicited current was dependent on G-protein, protein kinase C and inositol-1,4,5-trisphosphate (Zhang et al. 2012).

1.8.1.1.4. Effects on GABAergic transmission

As well as the effects on excitatory synaptic transmission, there have been reported alterations to GABAergic transmission. Intracellular recordings using high-resistance sharp electrodes revealed a GR dependent decrease in inhibitory post-synaptic potentials in CA1 pyramidal cells (Zeise et al. 1992), though this could not be observed when recording in whole-cell patch clamp conditions (Teschemacher, Zeise, and Zieglgänsberger 1996), potentially due to wash out of cytosolic factors following the formation of a whole-cell configuration. Application of dexamethasone caused a rapid (within 10 minutes) increase in spontaneous IPSC (inhibitory post-synaptic current, sIPSC), but not miniature IPSC (mIPSC), frequency without effecting IPSC amplitude. A similar effect was observed following acute restraint stress, and this was found to depend on retrograde nitric oxide (NO) signalling altering the activity of cholecystokinin and parvalbumin hippocampal interneurons (Hu et al. 2010).

1.8.1.2. Hippocampal slow/genomic mechanisms

As well as the rapid non-genomic mechanisms described above, GCs exert influence over the hippocampus by their action as ligand-activated transcription factors, promoting and repressing the transcription of many target genes, altering the neurophysiology and molecular profile of this region.

It is likely that GC mediated genomic effects on mnemonic and synaptic processes are dependent on many factors including cell-type, context, and type of memory. Given that the vast majority of genomic analysis has been conducted in the hippocampus, there may remain large gaps in the knowledge of how GCs can alter the expression of many important genes in other brain regions.

1.8.1.2.1. Molecular pathways

Due to the vast and varied effects of GCs on cognition there are no doubt many effector proteins and pathways that are transcriptionally regulated and poised to influence learning and memory. For example, Synapsin is a protein involved in controlling neurotransmitter release, acting to alter the readily releasable pool of vesicles and thereby modulate synaptic plasticity processes (Ferreira and Rapoport 2002). The presence of Egr-1 response elements in the *Syn1* gene identified this as a potential downstream effector for GC modulation of memory. Indeed, inhibition of synapsin-1 expression eliminated GC-induced memory augmentation (Valtorta, Benfenati, and Greengard 1992). Further to this, substantial evidence points toward neural cell adhesion molecule of the immunoglobulin superfamily, known to be critical in plasticity processes, being a key mediator of the genomic effects of GR on memory consolidation (Bisaz, Conboy, and Sandi 2009).

GR and MR ChIP of rat hippocampus has revealed that binding to well-known target genes *Fkbp5*, *Per1*, and *Sgk1* is significantly increased following stress or at the circadian peak compared to the circadian nadir, resulting in increased expression of these genes (Mifsud and Reul 2016). Although it has been known for some time that these genes are critically regulated by GCs, the way that they may affect neurological processes is not well understood. For example, only recently has it been shown how *Fkbp5* expression alters CRH neuron activity in the PVN, acting to tune the HPA axis response (Häusl et al. 2021).

A recent study utilised a combination of ChIP and RNA-sequencing techniques in hippocampal tissue to reveal several gene ontology (GO) pathways that are responsive to stress and circadian changes in GCs. Crucially, a number of genes associated with synaptic function were found to be GC responsive, including calcium/calmodulin-dependent protein kinase genes (*CaMKIId* and *CaMKIIa*), AMPAR subunit genes *Gria1* and *Gria2*, Kainate receptor subunit gene *Grik4*, and NMDA receptor subunit genes *Grin2a* and *Grin2b*. Further to this, "Ingenuity Pathway Analysis" (IPA) software revealed that pathways involving synaptic transmission, LTP and LTD (long-term depression) were affected by GC fluctuations (Mifsud et al. 2021). The details of how changes in the expression of these target genes affects synaptic processes in the hippocampus, or in other brain regions, remains unclear. Moreover, this study utilized only the circadian nadir and peak of untreated rats, so the relative contribution of circadian cues versus glucocorticoid effects on the transcription of these key target genes is also yet to be fully resolved.

1.8.1.2.2. Membrane properties

As well as the molecular evidence for GC-induced genomic changes in the hippocampus, a number of physiological studies have revealed alterations to membrane properties and amino acid transmission that are slowly induced and rely on *de novo* protein synthesis (Karst et al.

2000). For example, CA1 L-type calcium channel mediated currents are augmented by GCs in a manner dependent on GR binding and sensitive to protein synthesis blockers (Chameau et al. 2007). Potassium and sodium ion channels seem to be somewhat insensitive to the effects of GCs (Werkman, Van der Linden, and Joels 1997), however inward rectifier current was increased by concurrent activation of MR and GR, but not by selective activation of either receptor type alone (Karst, Wadman, and Joe 1993). Consistent with this, a U-shaped dose-response of the GC-mediated effects on Ca^{2+} current amplitude was observed in CA3 (Kole et al. 2001). These dose-dependent effects on Ca^{2+} conductance ultimately result in modulation of action potential firing, such that at low GC levels, selective MR activation leads to a reduced frequency accommodation and after-hyperpolarization (AHP) amplitude, thereby maintaining excitability of dHPC neurons. Elevated GC levels resulting in the activation of GR increase Ca^{2+} influx and reduce efflux, leading to increased frequency accommodation and AHP amplitude, thereby reducing prolonged excitatory transmission in dHPC (Joëls and De Kloet 1990; Joels and De Kloet 1989).

However, a differential response to corticosterone between dorsal hippocampus and the ventral-most 20% has been demonstrated, such that in dHPC input resistance and membrane time constant was reduced, resulting in leakier, less excitable cells. In the most ventral fifth, action potential threshold was reduced, resulting in increased excitability (Maggio and Segal 2009).

1.8.1.2.3. Amino acid neurotransmission

Unlike the rapid effects of GCs, mEPSC amplitude, but not frequency, was increased in CA1 and cultured HPC neurons, depending on GR activation (Karst and Joëls 2005). GR also mediates a concurrent increase in GluA2 surface expression (Groc, Choquet, and Chaouloff 2008), potentially accounting for the increased mEPSC amplitude. Interestingly, after a further 21 hours following CORT exposure, GluA1 expression was relatively increased compared to controls (Martin et al. 2009). In this time frame, AMPAR mediated components of evoked EPSCs (excitatory post-synaptic currents) were found to be significantly increased by GCs, while the NMDAR mediated component was not changed (Karst and Joëls 2005). On the other hand, an increase in the synaptic expression of GluN2A and GluN1 subunits was observed 1-2 hours after corticosterone application to hippocampal slices (Tse et al. 2011).

The genomic effects of GCs on inhibitory transmission in hippocampus are not well described, but appear to be sub-region specific, such that sIPSC frequency is reduced in ventral, but not dorsal, pyramidal cells mediated by an MR-dependent mechanism. Conversely, GR activation led to an increase in mIPSC amplitude in both sub-regions (Maggio and Segal 2009).

1.8.1.2.4. Aminergic neurotransmission

Both the 5-HT and cholinergic system were found to respond in a similar way to GCs (Hesen and Joëls 1993; Hesen and Joels 1996). Activation of GRs as well as MRs, by raised GC levels, induced increased synaptic responses in response to both amines. In slices from ADX animals, where no GC receptors were active, synaptic responses to 5-HT were also small, indicating a U-shaped dose-response curve. The effects on 5-HT transmission were found to be mediated particularly by the 5-HT_{1A} receptor and were dependent on GR homodimer binding to DNA (Karst et al. 2000). Moreover, they could be replicated following a physical stressor (Hesen and Joels 1996). However, the impact of these changes on hippocampal excitability or information processing remains unclear (Hesen et al. 1998).

1.8.1.3. Synaptic plasticity

Synaptic plasticity refers to the bidirectional alteration of synaptic efficacy and is known to be a critical mediator of learning and memory. LTP and LTD have been shown to persist for days, and even weeks (Abraham and Williams 2003), and provide a cellular and neurophysiological substrate that can be manipulated in order to assess how changes in molecular processes alter a physiological event known to be critical for learning (Nabavi et al. 2014).

1.8.1.3.1. Rapid effects

Corticosterone induces an enhanced increase in GluA2-AMPAR density in the synaptic compartment following a chemical LTP stimulus, suggesting that increased AMPAR motility elicited by GCs is a potential mechanism for the rapid facilitation of LTP (Groc, Choquet, and Chaouloff 2008). Crucially, the rapid effects of GCs on plasticity are highly dependent of the temporal relationship between GC receptor activation (or GC administration) and delivery of the plasticity inducing stimulus, as well as GC concentration. For example, LTP in CA1 was enhanced if CORT was applied during, but not after, HFS (high-frequency stimulation) and this effect was only apparent with 30 or 100, but not, 3 nM corticosterone (Tse et al. 2011; Wiegert, Joëls, and Krugers 2006). CORT delivery during LFS (low-frequency stimulation) was also found to facilitate the induction of LTD in area CA1 (Tse et al. 2011).

In contrast, LTP recorded in CA1 *in vivo* was found to be markedly reduced by 100 nM corticosterone delivered 30 minutes prior to HFS (Zhang et al. 2012). It is possible that differences in pulse frequency in the LTP induction protocol could be responsible for these discrepancies. Facilitatory effects were found with 100 pulses at 100 Hz and 90 pulses at 10 Hz, and impairment with 3 trains of 20 pulses at 200 Hz with a 30 second inter-train interval. It is also a possibility that the intact nature of the in the *in vivo* recordings caused this discrepancy. Moreover, it is unclear at which Zeitgeber time (ZT, time in relation to the light/dark cycle) all of

the recordings were made, so it may be that basal circulating corticosterone concentrations of the rats at the time of death were different.

A similar conflict in evidence is apparent in the DG, where *in vitro* plasticity experiments revealed a corticosterone induced increase in the early phase of bicuculline facilitated LTP (Pu, Krugers, and Joëls 2007), but *in vivo* evidence points to a decrease in LTP (Filioini et al. 1991). Again, different stimulation protocols increase the complexity of interpretating these conflicting results, as well as ADX vs. circadian nadir conditions.

Interestingly, it has been reported that antagonists of the intracellular MR and GR could not abolish the rapid effects of GR on LTP (Wiegert, Joëls, and Krugers 2006), and so it is plausible that membrane-bound MRs are implicated in rapid alterations to plasticity, in a process that also depends on activation of the ERK1/2 signalling pathway (Olijslagers et al. 2008). Importantly, the corticosterone binding affinity for this membrane bound variant appears to be significantly lower than the intracellular MR, meaning that it can be involved in mediating stress responses (Joels et al. 2008). As well as being pivotal in glutamatergic transmission, Lsamp has been shown to be critical for the GC induced changes to synaptic plasticity. Plasticity deficits in Lsamp knockout mice were accompanied by a reduction in MR expression (Qiu et al. 2010), further indicating the importance of this receptor in mediating the rapid effects of GCs.

1.8.1.3.2. Delayed effects

1.8.1.3.2.1. CA1

As well as the rapid effects described above, plasticity is differentially regulated by glucocorticoids in a longer time domain. In the CA1 region of the hippocampus, the delayed effects of GCs have been shown to diminish NMDAR dependent synaptic plasticity (Alfarez et al. 2002), which can be selectively elicited by using mild stimulation protocols (Raymond and Redman 2002). By using a strong stimulus protocol that is known to induce both NMDAR- and voltage-dependent calcium channel (VDCC)-dependent LTP (Grover and Teyler 1990), Krugers et al were able to establish that different forms of LTP were affected differently by the slow actions of corticosterone in acute mouse brain slices. By separate application of 30 μM L-type calcium channel antagonist nifedipine and 50 μM NMDAR antagonist APV, the authors were able to identify the components of the LTP contributed by either route of calcium entry to the neurons. It was shown that application of corticosterone at least 1 hour prior to recording facilitated VDCC-dependent LTP while simultaneously supressing NMDAR-dependant LTP (Krugers et al. 2005a). In line with this, a separate study demonstrated that the weakening of LTP in CA1 mediated by stress is dependent on NMDARs (Kim, Foy, and Thompson 1996). Interestingly, the latter study also demonstrated a facilitation of LTD in stressed animals. More

recently, it has been reported that stress induced modulation of LTP in CA1 can be blocked by selective blockers of GR, NMDAR and Phosphatidylinositol 3-kinase signalling (Yang et al. 2008).

Again, the relative activation of MR vs GR is important in determining the direction of these slower effects. By subcutaneously injecting ADX rats with either RU28362 (a selective GR agonist) or aldosterone (both 100 µg/kg body weight), slow modulation of LTP by MR and GR activation could then be determined. Recordings in *ex vivo* brain slices were made at least two hours following injection. Aldosterone application enhanced LTP in the CA1 field, whereas RU28362 diminished the potentiation induced by HFS, compared to vehicle injected animals (Pavlides et al. 1996). Interestingly, MR mediated facilitation of LTP appears to be particularly potent in the ventral portion of the rat hippocampus (Maggio and Segal 2007).

1.8.1.3.2.2. Dentate gyrus

Early *in vivo* studies in the DG of ADX rats revealed that like CA1 effects, selective GR activation 1 hour before delivery of HFS results in weakened LTP. Strikingly, in the DG, CORT resulted in a 100 Hz HFS protocol, that would ordinarily induce LTP, instead causing LTD (Pavlides et al. 1995). On the other hand, selective MR activation in ADX rats *in vivo* prolonged the period for which LTP could be observed (Pavlides et al. 1994). Another study, however, reported enhanced LTP following injection of 3 mg/kg corticosterone, an effect accompanied by augmented consolidation in a fear memory task (Abrari et al. 2009). Moreover, *In vitro* data has indicated that no direct slow effects of corticosterone could be observed on LTP induced by theta burst stimulation (TBS) in the DG. However, corticosterone administration at least 2 hours prior to TBS abolished the facilitating effect on LTP by a β-adrenoceptor agonist (isoproterenol). Interestingly, concurrent application of corticosterone and isoproterenol immediately before TBS produced an even greater facilitation of LTP (Pu, Krugers, and Joëls 2007). The interactions between glucocorticoids and other signalling systems, particularly the noradrenergic system, as well as cellular context, likely go some way to explaining the complex and conflicting evidence regarding the effects of stress and GCs on synaptic plasticity in the dentate gyrus (Shors and Dryver 1994; Korz and Frey 2003; Vouimba et al. 2004). Additionally, interaction with the BLA has been shown to be important in mediating the effects of stress on plasticity in the DG (Korz and Frey 2005; Akirav and Richter-Levin 2002).

A contributing factor to the different observed responses to glucocorticoids in the DG could be the neurogenesis that is almost exclusive to this area (Opendak and Gould 2015). Because of this continual addition of young neurons in the subgranular layer, the granule cells in the DG differ in age to each other and the rest of the neuronal population, and therefore may have different electrophysiological properties. Both neurogenesis and apoptotic cell death are enhanced in the DG of ADX animals, therefore resulting in altered population age (Sloviter et al.

1989). This enhanced neurogenesis was later shown to be accompanied by diminished synaptic plasticity (Joëls, Stienstra, and Karten 2001) as well as dendritic atrophy (Wossink et al. 2001). The impairments in synaptic plasticity could be completely recovered by *in vitro* administration of aldosterone or corticosterone (Krugers et al. 2007), suggesting that glucocorticoids are necessary for the maintenance of synaptic activity and neuronal health in the DG, presumably via MR activation. The differences in GC-induced changes between CA1 and the DG could be further explained by the observation that GR activation increased L-type calcium channel mediated currents in the CA1 and not the DG. Both areas, however, exhibited a similar increase in mRNA levels for the calcium channel-β4 subunit, though an increase in the protein level could only be observed in CA1 (Van Gemert et al. 2009). This illustrates a divergence between the transcriptional and translational changes induced by glucocorticoids in these hippocampal regions.

1.8.2. Amygdala

Fewer studies have been conducted investigating the effects of glucocorticoids on the electrical activity of amygdalar pathways, however, it seems that these hormones exert distinct effects in this region. Mice subjected to restraint stress were shown to have enhanced levels of LTP in the projection from the lateral nucleus of the amygdala (LA) to the BLA, when compared to control mice. By using specific antagonists, MR, GR and β-adrenergic receptor activation were shown to be necessary for the full effect (Sarabdjitsingh et al. 2012). These differences could be explained by more recent findings showing that the delayed effects of corticosterone acts to enhance amygdalar excitatory transmission, unlike in CA1. *In vitro* application of 100 nM corticosterone induced a depolarized resting potential, increased input resistance and a decrease in $GABA_A$-mediated IPSP amplitude due to a positive shift in the $GABA_A$ reversal potential (Duvarci and Paré 2007). Another contributing factor to the divergence from the slow suppression of excitatory transmission that is observed in CA1 is the fact that the slow AHP of EPSPs, which is slowly increased by corticosterone in CA1 pyramidal neurons, is not affected in principal neurons of the BLA (Liebmann et al. 2008). These mechanisms may be in place to prolong the window in which emotionally salient material can be processed in amygdalar circuits and stored into memory. Moreover, in BLA principal neurons, glucocorticoid administration resulted in increased L-type calcium channel mediated Ca^{2+} currents. This effect was apparent after a 1-4 hour delay and was the consequence of upregulation of the α1 subunit (Karst et al. 2002)

1.8.3. Prefrontal cortex

The response to acute glucocorticoid challenge in the prelimbic prefrontal cortex is similar to that of the CA1 region of the hippocampus. Recent evidence shows that corticosterone induces

increased amplitudes of AMPAR and NMDAR mediated EPSCs in the pyramidal cells of layer V, over 2 hours following corticosterone administration. Such an increase was found to be accompanied by increased surface expression of both receptor types and occurred via a serum- and glucocorticoid-inducible kinase and Rab4 dependent mechanism (Yuen et al. 2011a; Liu, Yuen, and Yan 2010). Interestingly, these effects could be reversed by dopamine D_4 receptor activation (Yuen et al. 2013), suggesting a modulatory role for this neurotransmitter in the responses of the PFC to glucocorticoids. AMPAR mediated currents were also found to be altered by acute stress in Layer III neurons of mPFC. spontaneous EPSC (sEPSC) amplitude was increased in stressed animals, and the rise and decay times of evoked EPSCs were both increased in these cells (Musazzi et al. 2010a).

GABA receptor mediated currents were also found to be modulated by the delayed effects of GCs. The frequency of mIPSCs recorded from layer V pyramidal neurons was decreased in slices treated with 100 nM corticosterone 1 hour prior to recording, an effect that could be blocked by antagonism of the cannabinoid 1 receptor (Hill et al. 2011a).

In summary, the acute effects of GCs are still largely unknown in the prefrontal cortex, though from the limited number of studies it seems that they generally act to increase glutamatergic transmission while simultaneously suppressing inhibitory transmission.

1.8.4. Effects of chronic stress/GC exposure

There have been many studies investigating the effects of chronic stress, typically using protocols of around 3 weeks of restraint or psychological stress. It must be considered, though, that stress and glucocorticoids are not equivalent entities, and as such a small number of studies have investigated the effects of chronic glucocorticoid exposure specifically. However, glucocorticoids are important mediators in the stress response and are in no doubt responsible for many of the actions, albeit in concert with other stress mediators, so chronic stress studies remain somewhat relevant.

Some of the most striking changes in the hippocampus induced by chronic stress or glucocorticoid exposure are the modulations of dendritic complexity and spine density. In hippocampal CA3 and infralimbic PFC dendritic complexity and spine density are markedly decreased, and to a lesser extent in CA1 (McEwen and Magarinos 1997; Li et al. 2011). Interestingly, dendritic complexity and spine density have been reported to be increased in the BLA and orbitofrontal cortex (Roozendaal, McEwen, and Chattarji 2009a; Mitra et al. 2005). As well as inducing plastic changes in dendritic structure, chronic stress also suppresses the three stages of neurogenesis in the dentate gyrus; proliferation, differentiation and survival (Leuner and Gould 2010).

Accompanying the above, prominent increases in neuronal activity have been reported following chronic stress paradigms. In the CA3 (Kole, Czéh, and Fuchs 2004), CA1 (Kerr et al. 1991) and DG (Karst and Joëls 2003) regions of the hippocampus, chronic stress was found to augment excitatory synaptic transmission, possibly caused indirectly by a decrease in GABAergic activity (Holm et al. 2011). In CA1, increased Ca^{2+} current amplitude due to chronic stress could be alleviated by RU38486 treatment for the final 4 days of chronic stress, implicating GR activation (Karst and Joëls 2007). However, in ventromedial PFC pyramidal neurons GluA1 and GluN1 subunit degradation mediated a reduced synaptic efficacy following a week of unpredictable stress (Yuen et al. 2012).

Despite increases in excitability, inputs to dorsal CA1 exhibit a reduced potential for LTP and increased tendencies for the induction of LTD (Bodnoff et al. 1995; Gerges, Stringer, and Alkadhi 2001; Von Frijtag et al. 2001; Krugers et al. 2006). Similar observations have been made for CA3 (Maggio and Segal 2011; Pavlides, Nivón, and McEwen 2002; Gerges, Stringer, and Alkadhi 2001) and PFC (Zhang et al. 2011) but observations in the DG have been somewhat varied (Pavlides, Nivón, and McEwen 2002; Gerges, Stringer, and Alkadhi 2001; Spyrka and Hess 2010; Alfarez, Joëls, and Krugers 2003). Calcineurin and Ca^{2+}/calmodulin -dependent protein kinase II (CaMKII) (Aleisa et al. 2006a; Gerges et al. 2004), GluN2B (Christoffel et al. 2011; Li et al. 2011) and brain derived neurotrophic factor (BDNF) (Aleisa et al. 2006b; Smith et al. 1995) have all been shown to be critical for these changes to occur.

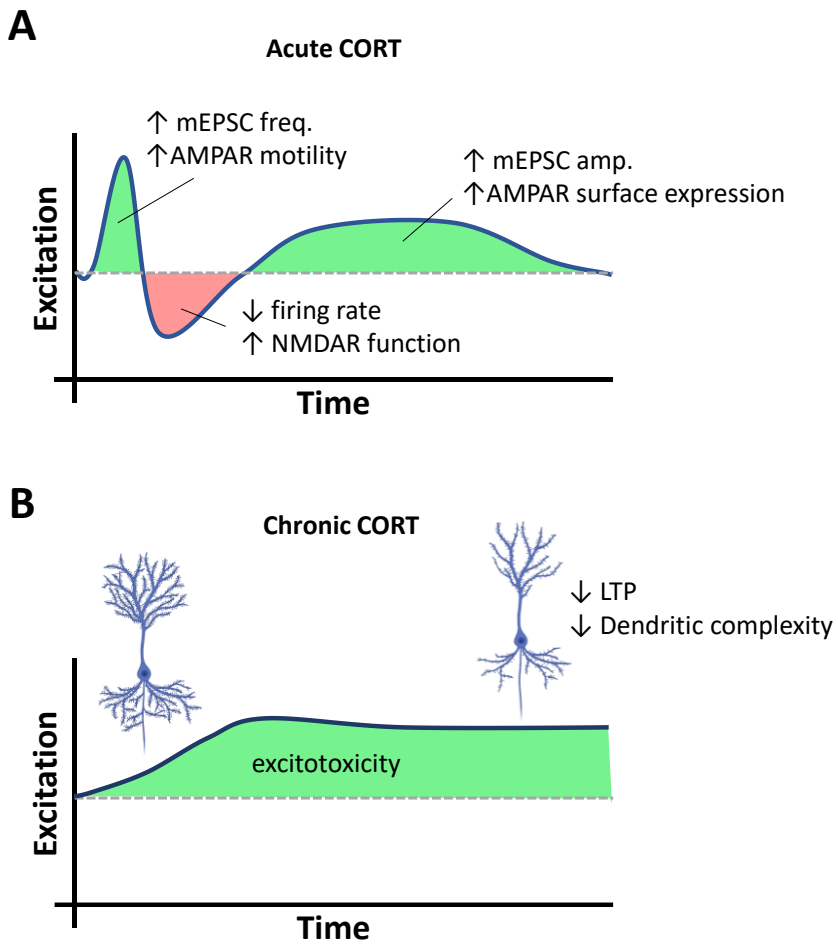


Figure 1.7. Effects of acute and chronic CORT exposure in the hippocampus. *A)* *Hippocampal excitation is enhanced rapidly (< 10 minutes) by the increase of AMPAR motility and the frequency of mEPSCs. Excitation is suppressed in the intermediate timeframe and NMDAR function is promoted. Over an hour post-CORT, excitation is enhanced as increased AMPAR surface expression facilitates increased mEPSC amplitudes.* ***B)*** *In the case of chronic exposure, excitotoxicity results in a decrease in dendritic complexity as well as the ability to induce LTP.*

An important aspect to consider, and one that is often overlooked in chronic models of stress and glucocorticoid application, is the development of effects over time. Spyrka and Hess (Spyrka and Hess 2010) conducted electrophysiological studies of acute slices from mice exposed to 1, 3, 7, 14 or 21 days of consecutive restraint stress, in order to investigate the development of effects on DG LTP over different time courses of chronic stress. They found impaired LTP after 3 days of restraint, an effect that could be blocked by administration of RU38486 prior to each restraint, implicating the importance of GR. However, following 7 days of restraint, LTP had returned to normal, and at 14 and 21 days LTP was found to be enhanced. This effect could not be blocked by pre-restraint RU38486, but an MR antagonist was able to completely abolish LTP.

It should be noted, though, that this time course may be specific for the DG, as well as for restraint stress. Indeed, in the CA1 region, the abolishment of LTP incurred by chronic stress could be prevented by treatment of RU38486 for 4 days prior to recording (Krugers et al. 2006), indicating distinct effects and mechanisms between the two regions.

Another factor that is undoubtedly important to consider is how the response to acute glucocorticoid challenge is modulated by prior chronic stress or glucocorticoid exposure. *In vitro* application of corticosterone to slices from chronically stressed animals decreased Ca^{2+} current amplitude of CA1 neurons, whereas treatment of slices from naïve animals revealed increased Ca^{2+} current amplitude (Karst and Joëls 2007). This is at odds with data from the DG granule cells of animals with chronic stress history, which show enhanced calcium and AMPAR currents following corticosterone administration, an effect not observed in slices from control animals (Karst and Joëls 2003). Transcriptional responses to corticosterone in the DG have also been shown to depend on the stress history of the animal (Gray et al. 2014).

Much less explored are the effects of GCs on the Kainate receptor (KAR), although an increase in the expression of KAR subunit (KA1, GluR6 and GluR7) mRNA in hippocampal neurons 7 days after adrenalectomy, which could be reversed by CORT, has been reported. Moreover, chronic stress or chronic corticosterone exposure resulted in increased KA1 mRNA in the dentate gyrus (Hunter et al. 2009).

1.8.5. Effects of glucocorticoid rhythms on synaptic physiology/plasticity

Given the rapid effects of glucocorticoids on both neuronal excitability and plasticity discussed above, it stands to reason that the pulsatile nature of glucocorticoid release, and the preservation of such rhythms in key brain regions like the hippocampus, that pulsatile changes in neural excitability and plasticity may result.

One study utilised two photon microscopy to demonstrate that circadian glucocorticoid rhythms facilitate structural plasticity in the mouse brain following motor learning. Mice were trained either during the circadian peak or nadir, and further groups were administered corticosterone immediately following training. It was shown that dendritic spine formation is facilitated by circadian peaks, in a non-genomic MR mediated process involving the LIM kinase-cofilin pathway, while nascent spine stability is promoted during the circadian nadir (Liston et al. 2013). In rats, circadian modulation of Ca^{2+} currents in CA3 (Kole et al. 2001), synaptic responses in hippocampal granule cells (Cauller, Boulos, and Goddard 1985), and LTP in the DG (Dana and Martinez Jr 1984) and of optic nerve input to SCN neurons (Nishikawa, Shibata, and Watanabe 1995) has been reported. Despite this, how circadian fluctuations interact with

glucocorticoid signalling to affect synaptic physiology that underlies learning and memory has not been fully resolved.

Very little research has been done regarding the effect of ultradian glucocorticoids on synaptic plasticity. Sarabdjitsingh and colleagues have recently demonstrated that the augmentation of LTP induced by 90 seconds of stimulation at 10 Hz by a single pulse of corticosterone is reduced if 2 pulses are administered separated by one hour, mimicking an ultradian pattern (Sarabdjitsingh et al. 2014). This was more recently elaborated on by the same group, showing that a third and fourth pulse of corticosterone further diminished the LTP in response to this paradigm, even reducing it compared to vehicle administration (Sarabdjitsingh et al. 2016a).

As mentioned previously, in the CA1 region, glutamatergic transmission is enhanced rapidly and reversibly by corticosterone acting on MRs, and in the BLA a similar enhancement is rapidly induced. However, this change is persistent and is responsive to a second pulse of corticosterone, which depotentiates the excitatory transmission to below baseline levels, in a process that has been termed 'metaplasticity'. This metaplastic switch from excitation to inhibition depends on GR instead and can therefore provide a powerful modulation of amygdalar circuitry depending on recent experiences of stress (Karst et al. 2010). The same authors recently built on this by applying four pulses of CORT at a high concentration. The first two pulses elicited the same result as above, but pulses 3 and 4 incurred no changes to mEPSC frequency. Four pulses of CORT of increasing or decreasing concentration were also applied in order to mimic the ultradian pulsing during the active and inactive phases. Pulses of increasing concentration steadily increased mEPSC frequency over the course of the four pulses and the opposite was true for the pulses of decreasing concentration (den Boon et al. 2019). These results indicate that glucocorticoid pulsing over the course of the day acts to gradually induce changes in excitability of BLA neurons corresponding to the internal glucocorticoid driven clock.

Thus, glucocorticoid rhythms may be serving to maintain the stability of key brain regions and facilitate readiness to change in response to challenges presented by the environment. Disruptions to these endogenous rhythms could therefore have implications for synaptic physiology resulting in maladaptive behaviours.

1.9. Synthetic glucocorticoids

Synthetic glucocorticoids are a commonly prescribed class of drugs, with nearly 2% of the adult UK population using oral glucocorticoid treatment at any given time. They are useful in treating a variety of conditions including Crohn's disease, rheumatoid arthritis, dermatitis, and systemic lupus erythematosus. They are also often administered as replacement therapy in patients with adrenal deficiencies, or in the case of congenital adrenal hyperplasia and glucocorticoid

resistance, they are used to induce adrenal suppression (Overman, Yeh, and Deal 2013; Van Staa et al. 2000).

Synthetic glucocorticoids suppress endogenous glucocorticoid oscillations (Kalafatakis et al. 2018) (Fig 1.8A), this is in part due to increased binding affinity to the GR, as is the case for dexamethasone. Methylprednisolone (MPL, on which this thesis will focus), is similar in structure to prednisolone, with the addition of a methyl group at position 6 which renders it water soluble, facilitating intravenous use. MPL binds to GR and MR with similar affinity to endogenous glucocorticoids, but the binding duration is roughly 6 hours. In humans, the plasma cortisol half-life is roughly 60 minutes, whereas plasma methylprednisolone half-life is considerably longer, as it cannot be metabolized by Type 2 HSD, ranging from 130-250 minutes (Uhl et al. 2002; Al-Habet and Rogers 1989). The enhanced binding of synthetic glucocorticoids leads to chronic GR activation, in place of the circadian and ultradian activation dynamics achieved by the endogenous hormones (Fig 1.8B).

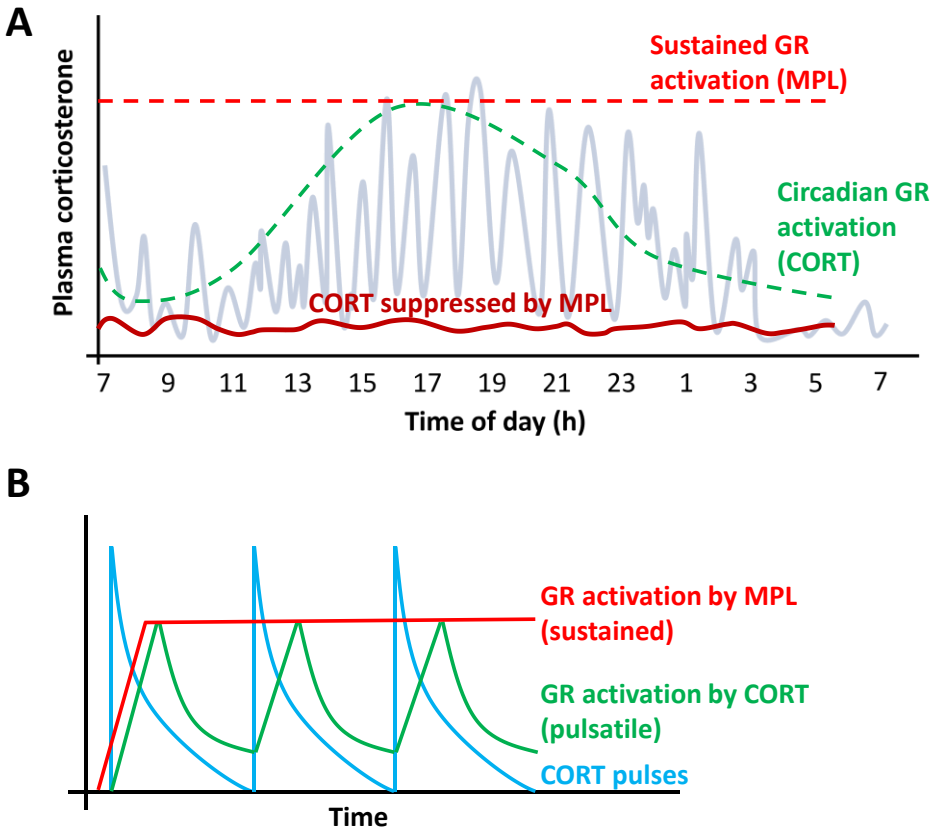


Figure 1.8. Synthetic glucocorticoids suppress the HPA axis and cause chronic GR activation. ***A)*** *Representative graph showing the suppression of endogenous corticosterone rhythms (greyed out) by synthetic glucocorticoids, leaving only a flattened corticosterone profile (red line).* ***B)*** *Representative graph showing that while endogenous glucocorticoid pulses incur*

rhythmic GR activity, the prolonged binding of synthetic glucocorticoids like methylprednisolone result in sustained GR activity. ***B*** *is adapted from (Stavreva et al. 2009).*

Despite their efficacy and undoubted clinical usefulness, chronic treatment with high doses of synthetic glucocorticoids can lead to severe side effects including cognitive deficits, depression, hypertension and obesity (Judd et al. 2014; Brown and Chandler 2001). One patient study even reported a sevenfold increase in suicide or suicide attempts in patients using glucocorticoid therapies (Fardet, Petersen, and Nazareth 2012). Even at low doses, the sustained inhibition of endogenous glucocorticoid release by synthetic steroid treatment can cause psychological outcomes by putting the brain in a hypocortisolic state (Meijer and de Kloet 2017).

1.10. Research statement

Glucocorticoids are intrinsic to many functions in the human body, including the co-ordination of extra-SCN clocks to external cues. They are also vital in mediating the stress response and have been shown to affect cognitive function in a multitude of ways, and despite a vast number of studies, the mechanisms by which they alter brain function are not well understood. They also have powerful anti-inflammatory properties, and as such, synthetic glucocorticoids are extremely commonly prescribed. Methylprednisolone is a synthetic glucocorticoid, that due to its vastly increased half-life compared to endogenous glucocorticoids, induces chronic GR and MR activation as well as the suppression of natural corticosteroid rhythms. Patients who are prescribed these drugs often present with unwanted side effects, including memory impairment, though little is known about the underlying mechanism. Glucocorticoids have been shown to alter the expression of clock genes in multiple brain regions, including the hippocampus, and therefore methylprednisolone treatment may induce alterations the rhythmic expression of clock genes in this region. The downstream effects of disruption to hippocampal rhythms on synaptic processes is unknown. Moreover, methylprednisolone is likely to dysregulate molecular rhythms in other brain regions.

1.11. Hypothesis and aims

Using methylprednisolone to induce chronic GR and MR activation, while suppressing endogenous glucocorticoid rhythms, will result in dysregulation of the molecular clock and circadian rhythms of several extra-SCN brain regions. I hypothesize that in health, circadian fluctuations in synaptic physiology and plasticity will be present in extra-SCN brain regions associated with learning and memory, and that dysregulation of the molecular clock by chronic methylprednisolone treatment will alter these diurnal variations.

Chapter III – Assess circadian variation in hippocampal synaptic plasticity and physiology, and determine the interaction of MPL treatment with physiological oscillations.

Chapter IV – Investigate the effects of chronic MPL treatment on synaptic plasticity and physiology in perirhinal cortex, and determine the effects and interaction of the circadian cycle.

Chapter V – Determine the effects of MPL treatment, as well as their interaction with the circadian cycle, on synaptic physiology in the medial prefrontal cortex.

Chapter II

Materials and methods

2.1. Animals and treatments

Adult male Lister hooded rats (8-12 weeks, 225 – 350 g) (Envigo, UK) were used in all experimental procedures. Animals were housed in pairs in Techniplast 1500 conventional containers in standard housing conditions under a 12:12 light/dark cycle. Animals to be used for experiments examining the active period were housed in reverse lighting (lights off 08:00/lights on 20:00) and for experiments examining the inactive period animals were housed in standard lighting (lights on 08:00/lights off 20:00). Food and water were available *ab libitum*. Rats were allowed to acclimatize to the facility for >1 week before any experimental manipulation. All procedures described here were conducted in accordance with the UK Animals (Scientific Procedures) Act, 1986.

2.1.1. Administration of methylprednisolone

For use in chronic experiments, sterile Methylprednisolone (MPL) (Solu-Medrone, Pfizer, UK) was prepared in accordance with the supplier's directions and subsequently diluted with tap water to yield a final concentration of 1 g/L (20 mg/day) (optimized in (Demski-Allen 2014) to yield brain GR activation). In order to preserve the stability of MPL, opaque drinking bottles were used to combat light sensitivity. Normal drinking water was replaced with 1 g/L MPL solution, to which animals had ad-libitum access. MPL drinking solution was replaced every two days. Drinking water was then monitored to check and confirm consumption of MPL solution and body weight was also recorded daily as weight change is a known effect of steroid treatment (Azcue et al. 1997). MPL was administered via the means described above in order to reduce the stress that could be caused by other means of delivery such as subcutaneous/intraperitoneal injections or oral gavage. Rats were housed in pairs or threes to avoid changes in adrenocortical reactivity linked with individual housing (Brain 1975), however, a caveat incurred by this was a variation in the duration of treatment by up to 2 days, resulting in a treatment of 4-7 days in all animals.

2.2. Brain slice preparation

Adult male rats were anaesthetized by inhalation with gaseous isoflurane combined with medical grade air with all pertinent Home Office guidelines until no reflex response was observed upon pinching of both hind paws, and quickly decapitated with a guillotine. The cranium was carefully cut and the brain swiftly removed and placed in to ice cold artificial cerebrospinal fluid (aCSF) (containing (in mM): 124 NaCl, 3 KCl, 26 $NaHCO_3$, 1.4 NaH_2PO_4, 1 MgSO4, 10 D-Glucose, 2 $CaCl_2$) if used for field electrophysiology recordings or modified sucrose aCSF (containing (in mM): 52.5 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 5 $MgCl_2$, 25 D-Glucose,

100 Sucrose, 2 $CaCl_2$, 0.1 Kynurenic acid) if used for patch-clamp electrophysiology. In both cases solutions had been saturated with 95% O_2 and 5% CO_2 (carbogen). Slices were prepared at a thickness of 300 μm for patch-clamp or 400 μm for field recordings.

Brain slices for the analysis of neurophysiology during the active period (ZT12 onwards, ZT12 group) were prepared around 30 minutes after lights off. Electrophysiological recording then commenced at around the corresponding ZT14. Brain slices for the analysis of neurophysiology during the inactive period (ZT0 onwards, ZT0 group) were prepared around 30 minutes after lights on. Electrophysiological recording then commenced at roughly the corresponding ZT2 Figure 2.1.).

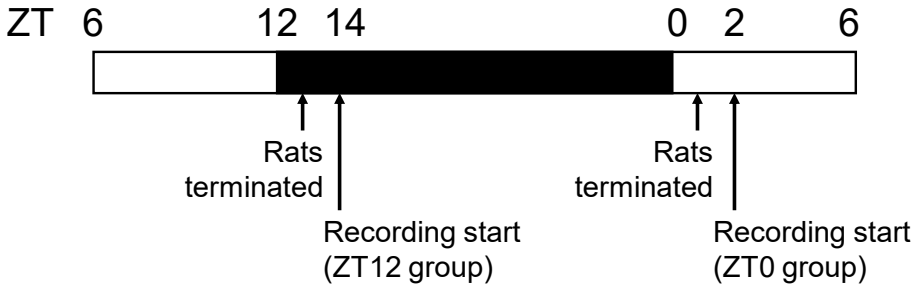


Figure 2.1. Schematic diagram demonstrating timing of slice preparation and electrophysiological recording. *Schematic depicts the time when rats were terminated, and brain slices prepared. Electrophysiological recordings typically commenced 1.5 hours later, at ZT14 for the analysis of the active period and ZT2 for the analysis of the inactive period.*

2.2.1. Hippocampal slice preparation

The brain was removed from solution and quickly blocked on filter paper on the lid of a glass petri dish filled with ice. The brain was then hemisected by the sagittal midline and fixed (using cyanoacrylate adhesive) to a metal cutting platform so that the medial surface was facing upwards, and a supporting piece of 2% w/v agar was glued underneath the ventral surface of the brain. The brain was immediately submerged in ice cold sucrose/aCSF within the slicing chamber parasagittal slices cut using a vibratome (Leica VT1000S). Slices were transferred to a petri dish containing room temperature (RT) sucrose/aCSF, wherein the hippocampus was isolated and excess tissue discarded. For field electrophysiology experiments, hippocampal slices were then transferred to the holding chamber filled with fresh RT aCSF and allowed to recover for > 1 hour before electrophysiological recording. If slices were to be used for patch clamp recordings, they were incubated immediately following dissection at 32 °C for 30 minutes and then rested at RT for at least another 30 minutes before being transferred to the recording chamber.

2.2.2. Perirhinal cortex slice preparation

The preparation of perirhinal slices is very similar to that of hippocampal slices. The brain was removed from solution and quickly blocked on filter paper on the lid of a glass petri dish filled with ice. The brain was hemisected along the sagittal midline and a 45° cut was made from the caudal end to the dorsal edge of each hemisphere. The halves were then glued (cyanoacrylate adhesive) by this cut side to a metal vibratome stage and supported with a piece of 2% w/v agar. 300 or 400 µm slices were then cut using a vibratome (Leica VT1000S) in an identical manner to that described in the previous section, resulting in slices cut 45° rostral to the coronal plane as shown in figure 2.2. Slices were transferred to the holding chamber filled with fresh RT aCSF and allowed to recover for > 1 h before electrophysiological recording. If slices were to be used for patch clamp recordings they were incubated immediately following dissection at 32 °C for 30 minutes and then rested at RT for at least another 30 minutes before being transferred to the recording chamber.

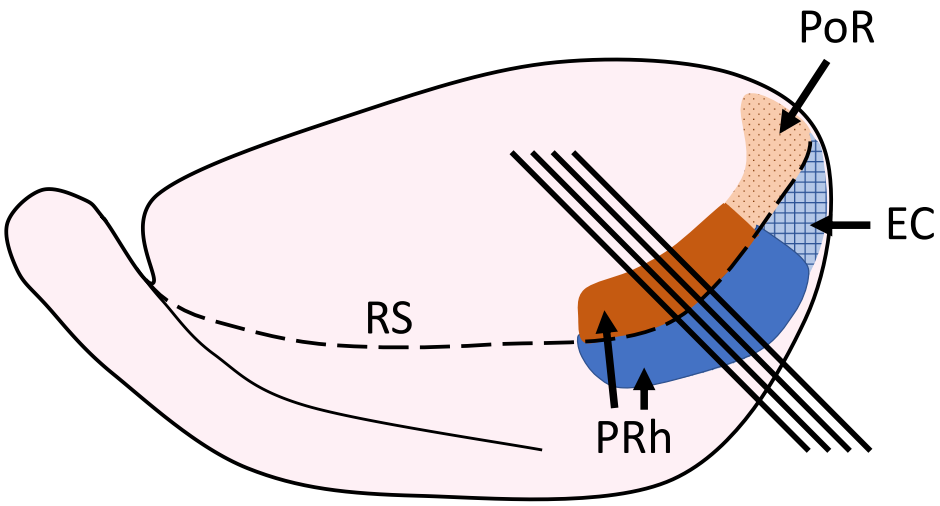


Figure 2.2. Schematic diagram showing perirhinal cortex slice orientation. *Schematic also depicts the positions of Postrhinal (PoR), Entorhinal (EC), and Perirhinal (PRh) cortices and the Rhinal Sulcus (RS). Adapted from (Burwell, Witter, and Amaral 1995b). The 4 lines represent the 45° cutting angle used to obtain perirhinal slices.*

2.2.3. Medial prefrontal cortex slice preparation

The preparation of prefrontal cortex slices was performed by placing the extracted rat brain in to a brain matrix, allowing the brain to be cut at the correct angle to produce coronal slices. The cut surface of the frontal cortex was then glued with cyanoacrylate adhesive to the vibratome stage and 300 µm slices were cut (Leica VT1000S). Slices were transferred to the holding

chamber filled with fresh RT aCSF and incubated immediately following dissection at 32 °C for 30 minutes and then at RT for at least another 30 minutes before being transferred to the recording chamber.

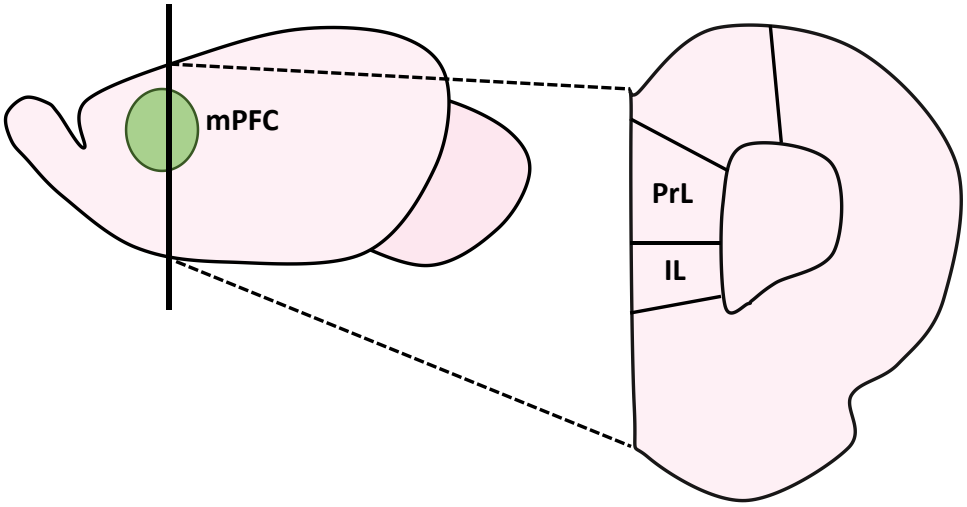


Figure 2.3. Schematic diagram illustrating the orientation and regional divisions of prefrontal cortex coronal slices. *Prelimbic (PrL) and infralimbic (IL) subdivisions of the mPFC are shown on the diagram, as these are the sites of recording in this thesis*.

2.3. Field Electrophysiology

2.3.1. Submerged field electrophysiology recordings

For all field excitatory post-synaptic potential (fEPSP) recordings, slices were placed in a modified Haas style recording chamber, initially in an interface configuration. The chamber was maintained at 30 °C and aCSF was continuously saturated with 95% O_2 and 5% CO_2 and perfused into the recording chamber at a rate of 2 ml/min, via a peristaltic pump (Masterflex C\L, Cole-Parmer, UK).

A nylon net was used to hold slices in place and following the placement of recording and stimulating electrodes, slices were submerged in aCSF. Recording then commenced after a 30-minute acclimatisation period.

All fEPSP data are presented as mean ± standard error of the mean (SEM). Data analysis was performed using the WinLTP software (Anderson and Collingridge 2007a). Briefly, average traces were calculated over 2 minutes (n = 4, consecutive sweeps) of stimulation and plotted. These averaged traces were normalized to the mean peak amplitude that was recorded over the 30-minute baseline.

2.3.2. Recording electrodes

Recording electrodes were prepared by pulling borosilicate capillary tubes with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, USA). Capillary tubes of 1.2 mm outer diameter and 0.69 mm inner diameter (Harvard Apparatus, Cambridge, UK) were pulled to a tip resistance of 2-6 MΩ and backfilled with aCSF before use in all field experiments.

2.3.3. Stimulation

Platinum/Iridium concentric bipolar electrodes (FHC, Bowdoin ME, USA) that terminate with a flexible wire which directly contacts the surface of the slice were used to stimulate axonal fibres. Each electrode was positioned precisely using a manual manipulator (Narishige, Japan) and attached via gold pin connections to wires leading to a DS3 constant current stimulation unit (Digitimer, Letchworth Garden City, UK) which delivered square wave pulses, triggered by the WinLTP software (Anderson and Collingridge 2007b). In all experiments, two stimulating electrodes were positioned either side of the recording electrode, in order to evoke independent responses at a frequency of 0.033 Hz. Responses were ensured to be independent by conducting a brief paired-pulse experiment prior to each recording. One input was then considered the experimental input, which would be manipulated by the induction of synaptic plasticity, and the other independent input served as an internal control, to ensure that any changes in the experimental input were not a consequence of a decline in slice health or electrode drift. The internal control inputs are not plotted.

2.4. Whole cell patch clamp electrophysiology

2.4.1. Submerged patch clamp recordings

Slices were transferred to the recording chamber, held in place with a nylon net and perfused with aCSF saturated with carbogen at 30 ± 2 °C (TC-344B temperature controller, Warner Instruments, Hamden, CT, USA), before being visualised using a Zeiss upright microscope (Zeiss, Germany) with differential interface contrast optics (ORCA camera system, Hamamatsu photonics, Japan). For all recordings, signals were amplified using an MultiClamp 700B amplifier (Molecular Devices, LLC, California, USA). Signals were Bessel filtered at 6 KHz and digitized at 10 KHz using a BNC-2110 interface (NI, Austin, Texas, USA). Data was acquired and analysed online using WinLTP software (Anderson and Collingridge 2007a).

2.4.2. Recording electrodes

Recording electrodes were prepared by pulling borosilicate capillary tubes with a P-97 Flaming/Brown micropipette puller (Sutter Instrument Co., Novato, USA). Capillary tubes of 1.6 mm outer diameter and 0.69 mm inner diameter (Harvard Apparatus, Cambridge, UK) were

pulled to a tip resistance of 2-6 MΩ and backfilled with intracellular solution passed through a 0.2 μM filter.

2.4.3. Intracellular solution

Intracellular (internal) solution was prepared containing (in mM): 8 NaCl, 130 CsMeSO4, 10 HEPES, 0.5 EGTA, 4 MgATP [Adenosine 5'-triphosphate magnesium salt], 0.3 NaGTP [Guanosine 5'-triphosphate sodium salt hydrate], 5 QX-314, and adjusted to pH 7.2-7.3 using CsOH. Osmolality was checked to be within 10 mOsmoles/kg of the external solution.

2.4.4. Stimulation

For evoked EPSC studies, Platinum/Iridium concentric bipolar electrodes (FHC, Bowdoin ME, USA) that terminate with a flexible wire which directly contacts the surface of the slice were used to stimulate axonal fibres. A stimulating electrode was positioned precisely using a manual manipulator (Narishige, Japan) and attached via gold pin connections to wires leading to a DS3 constant current stimulation unit (Digitimer, Letchworth Garden City, UK) which delivered square wave pulses, triggered by the WinLTP software.

2.4.5. Evoked EPSCs in voltage clamp

Recordings were taken at a sampling frequency of 10 KHz and Bessel filtered online at 6 KHz. Cells were held in voltage clamp mode at a holding potential appropriate to the current recorded, as indicated in each section, using the Axon™ MultiClamp™ 700B Commander software (Molecular Devices, LLC, California, USA). Series resistance was monitored throughout the course of the experiment and any experiments in which RS changed by over 10% were not included in the final analyses. EPSCs were re-analysed again offline using the WinLTP software (Anderson and Collingridge 2007a) before being included in final datasets.

2.4.6. Spontaneous and miniature post-synaptic currents

For all recordings of spontaneous events data was sampled at 10 KHz, Bessel filtered online at 6 KHz and subsequently Bessel filtered at 2 KHz offline prior to analyses. R_S was monitored throughout the experiment and was not compensated for due to the small amplitude of currents being recorded, but, if R_S changed by 10% then recordings were not selected for analysis.

Spontaneous EPSCs and IPSCs were recorded in the absence of tetrodotoxin (TTX), a potent voltage-gated sodium channel blocker. TTX acts to inhibit the propagation of action potentials, and therefore eliminates any action potential generated neurotransmitter release. sEPSCs and sIPSCs are therefore recorded when action potential generated release is viable. mEPSCs and mIPSCs are recorded in the presence of TTX, and therefore any neurotransmitter release is purely "spontaneous".

Analyses were performed offline using Clampfit 9.2. (Molecular Devices, LLC, California, USA). A 5-minute epoch was selected for analysis for each condition, and the first 5 minutes of the recording were never used to allow the cell to acclimatise to the whole-cell configuration. From these 5-minute periods the frequency and amplitude of all accepted spontaneous events were calculated. Events were accepted for analysis if the rise time was faster than the decay time and the event had a peak amplitude of over 6 pA.

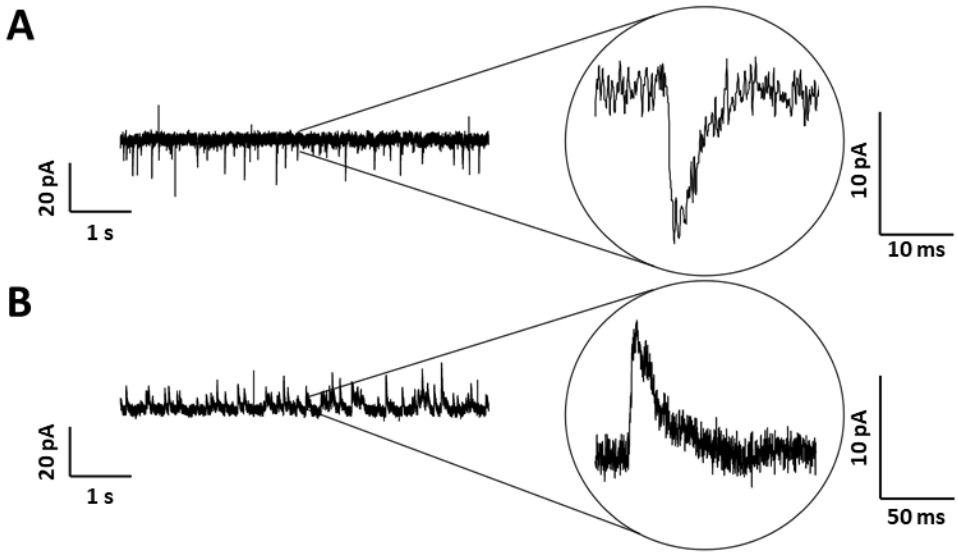


Figure 2.4. Spontaneous and miniature post-synaptic currents analysis. ***A)*** *Representative trace from a recording containing spontaneous EPSCs and a representative sEPSC event.* ***B)*** *Representative trace from a recording containing spontaneous IPSCs and a representative sIPSC event.*

2.5. Trunk blood collection and plasma isolation

During slice preparation, immediately following decapitation, the trunk blood was collected from the body of the rat and mixed with 100 µL EDTA and temporarily stored on ice to prevent coagulation. Blood samples were then centrifuged for 10 mins at 2,000 x g at 4 °C. Plasma supernatant was then collected in 500 µL aliquots and stored at -20 °C for later analysis.

2.6. Radioimmunoassay for Corticosterone

Radioimmunoassay was carried out using an automated gamma counter (PerkinElmer, US), in order to assess corticosterone levels in blood samples collected from trunk blood. An 11-point standard curve of known corticosterone concentrations was prepared in B-buffer (25 mM tri-sodium citrate, 50 mM sodium dihydrogen orthophosphate, 1 mg/mL bovine serum albumin: pH3). Plasma obtained from trunk blood was diluted in triplicate at a ratio of either 1:10 or 1:50 in B-buffer, respectively. A specific corticosterone antibody (kindly provided by G. Makara,

Institute of Experimental Medicine, Budapest, Hungary) was diluted at a ratio of 1:50 in B-buffer and 50 µl added to 100 ul standards, unknown samples, and Quality Control (QC20 and QC100) tubes. Tracer (Izotop, Institute of Isotopes, Hungary) (Zelena et al. 2003) was diluted in B-buffer to give total counts of 3750cpm in 50 µl and added to all tubes (50 µl/tube), which were then incubated overnight at 4 °C. Charcoal suspension (5 g charcoal added to 0.5 g Dextran T70 dissolved in 1 L B-buffer) was prepared and 500 µl added to all tubes and briefly vortexed. Blocks were centrifuged at 4000 rpm at 4 °C and the resulting supernatant aspirated off. Unknown samples were determined from interpolation of the standard curve.

Radioimmunoassay was performed on selected plasma samples to ensure methylprednisolone treatment via drinking water had been successful. Chronic treatment with methylprednisolone led to suppressed endogenous corticosterone at the circadian peak (Figure 2.5), indicating successful treatment.

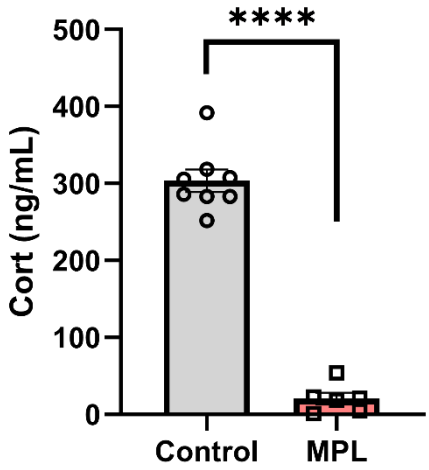


Figure 2.5. Corticosterone radioimmunoassay at the circadian peak. *Chronic treatment with methylprednisolone resulted in significantly reduced circulating corticosterone levels at the circadian peak compared to control animals (t_{12} = 15.56, P < 0.0001, unpaired t-test).*

2.7. Statistical Analysis

Graphs and statistical analyses were generated using GraphPad Prism software (version 8.4.3.) (GraphPad Software Inc., California, USA). Data were tested for normality using the D'Agostino-Pearson omnibus normality test, and then an appropriate statistical test was performed. For comparing two groups of normally distributed data with similar standard deviations (SDs), unpaired t-tests were performed. In the case of dissimilar SDs, Welch's t-test was used. If data did not pass the normality test, then a Mann-Whitney test was performed. For multiple comparisons, analysis of variance (ANOVA) with post-hoc tests were used, as specified in the

text. In each experiment the experimental unit (i.e. a single animal) will represent n = 1, in the case that multiple subsamples were recorded from an experimental unit, the n will be indicated as n = X, Y), where X = subsamples and Y = experimental units. This will be clarified where appropriate in each figure legend.

2.8. Materials

For electrophysiology, standard laboratory salts were obtained from Thermo Fisher Scientific (Waltham, Massachusetts, USA). All drugs except methylprednisolone were obtained from Hello Bio (Bristol, UK). Drugs were all prepared in purified water, except for picrotoxin which was dissolved in dimethyl sulfoxide (DMSO), and added to the perfusing aCSF when administered to the brain slice, unless otherwise stated.

Methylprednisolone (MPL) as the sodium succinate (Solu-Medrone, Pfizer) was obtained from University Hospitals Bristol pharmacy stores.

Chapter III

Methylprednisolone disrupts the circadian regulation of hippocampal synaptic physiology

3.1. Introduction

Circadian rhythms, derived from the Latin "circa" (about) and "diem" (day), are conserved across many species and in many biological systems, and the rhythmic nature of the HPA axis is vital in maintaining the body's readiness to challenges at appropriate times. The circadian undulation in circulating adrenal glucocorticoids is composed of ultradian pulses of variable amplitude, occurring every 60-90 minutes. The circadian and ultradian glucocorticoid secretory rhythm can be observed in many species and is thought to enable the system to remain responsive to stress, as well as serving an important role in cognition and gene transcription, though relatively little is known about the function of these pulses in the brain.

Glucocorticoid rhythms are maintained across the blood-brain-barrier, meaning that neurons are subject to rhythmic changes in glucocorticoid concentration (Droste et al. 2008). It has been shown that transcription of target genes in the hippocampus is dynamically regulated by CORT pulses (Lightman, Birnie, and Conway-Campbell 2020), interestingly, this effect seems to be tissue dependent (George et al. 2017). Circadian rhythms are known to be important in the formation and stabilization of dendritic spines in the formation of motor memory (Liston et al. 2013) and the physiological rhythms of CORT secretion have been shown to be vital in working memory, sleep quality and emotional face recognition in humans (Kalafatakis et al. 2018).

In contrast to the rhythmic oscillations in endogenous glucocorticoids, in both ultradian and circadian timeframes, synthetic glucocorticoids are significantly longer lasting in the circulation. Methylprednisolone (the focus of this thesis) induces prolonged GR activation (> 3 hours) compared to endogenous GCs which cause considerably less sustained activation (< 10 minutes) (Stavreva et al. 2009). This chronic GR activation causes a flattening of the endogenous CORT profile and alterations to daily activity, sleep, memory, and mood have all been reported following long-term use, and in some cases depression and even mania (Brown and Chandler 2001). Understanding the action of synthetic glucocorticoids like methylprednisolone has important clinical significance because they are one of the most commonly prescribed classes of drugs, with 2% of the general population taking oral GCs at any given time (Overman, Yeh, and Deal 2013; Van Staa et al. 2000). Often, patients continue to use these drugs for long periods, which can incur unwanted side effects.

Ribonucleic acid-sequencing (RNA-seq) of whole rat hippocampus taken every 4 hours over 24 hours (ZT2, ZT6, ZT10, ZT14, ZT18, ZT22, as indicated in the experimental design schematic in figure 3.1A) identified 485 genes that were regulated by time of day (Fig 3.1B), and of these, 56 were confirmed to be directly regulated by glucocorticoids in an independent experiment where adrenalectomized rats were treated with an acute infusion of corticosterone during the circadian inactive phase. Gene ontology analysis of the genes found to be differentially

expressed in the comparison between the beginning of the active phase (ZT10) and the inactive phase (ZT2), revealed that circadian, calcium signalling, and long-term potentiation pathways (amongst others) were regulated by time of day (Fig 3.1C). Differentially expressed genes (DEGs) in the comparison between ZT14 and ZT2 included *Per1-3, Bhlhe40, CaMKII, Grin2a* and *Grin2c* (Fig 3.1D). *Per1, Grin2a* and *Grin2b* were found to be directly regulated by glucocorticoids at the timepoint investigated (60 minutes after a 30 minute infusion) while *Per1, Grin2a* and *Grin2b, Bhlhe40, CaMKII* contained proximal GR binding sites, reported in a similar experimental design of adrenalectomized rats infused with an acute corticosterone dose during the circadian nadir. Clustering of ZT10 DEGs by known association revealed an interaction between core clock genes (*Per1, Per3, Dbp, Bhlhe40* and *Aanat*) and synaptic genes (*Grin2a, Grin2c* and *CaMKIIa*) (Fig 3.1E) (Data from Birnie, M., Claydon, M. D. B. et al. Circadian regulation of hippocampal function is disrupted during chronic glucocorticoid treatment, *in submission*).

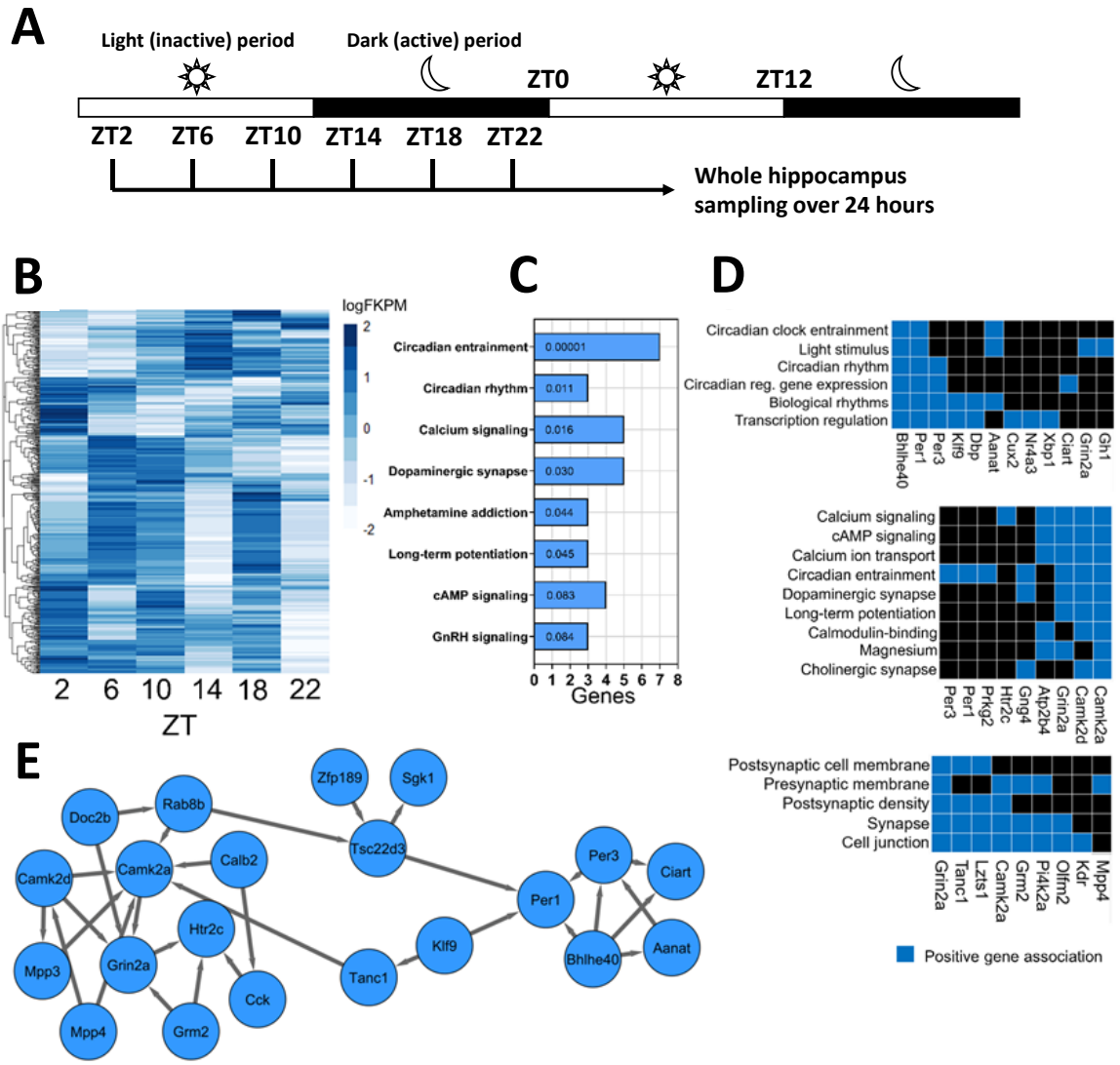


Figure 3.1. Circadian regulation of the hippocampal transcriptome. ***A)*** *Schematic diagram showing experimental timepoints of hippocampus extraction in relation to the light/dark cycle and zeitgeber time.* ***B)*** *Differentially expressed genes (DEGs; P < 0.05) across time (n = 24, 4 per time point). Each row corresponds to a single gene (485 DEGs).* ***C)*** *Top associated (P < 0.085) gene ontology terms among DEGs at ZT10.* ***D)*** *Functional clustering of DEGs and associated pathways at ZT10.* ***E)*** *Representation of a circadian regulated signalling network enrichment analysis including interacting clock, glucocorticoid and synaptic DEGs. (Data from Birnie, M., Claydon, M. D. B. et al. Circadian regulation of hippocampal function is disrupted during chronic glucocorticoid treatment, in submission).*

Interestingly, while acute glucocorticoid administration can result in the augmentation of memory consolidation, chronic exposure is known to impair mnemonic function. Although BDNF (Smith et al. 1995; Aleisa et al. 2006b), CaMKII (Gerges et al. 2004), and GluN2B (Li et al.

2011) have all been found to play a pivotal role, the molecular basis of this detriment remains unresolved. Chronic treatment with the synthetic glucocorticoid methylprednisolone (MPL), suppresses endogenous adrenal glucocorticoid secretion and replaces the transient (pulsatile) activation of hippocampal GRs, with prolonged (constant) activation, leading to increased *Per1* transcription that remains elevated during the circadian nadir. RNA-sequencing of whole rat hippocampus taken every 4 hours over 24 hours (ZT2-22) revealed 512 genes regulated by time of day in MPL treated rats (Fig 3.2A) and GO pathway analysis showed that different pathways were regulated at ZT10 (vs. ZT2) compared to controls (Fig 3.2B), though more DEGs were identified at this time in the MPL group (Fig3.2C). Crucially, circadian entrainment and rhythm pathways were no longer regulated by time following MPL treatment, indicating a loss of hippocampal rhythms that are mediated by GR driving the molecular clock. The peak amplitude of all DEGs across the light/dark cycle was shifted from ZT5-7 in controls to between ZT22-1 following MPL treatment (Fig 3.2F). The expression pattern of clock regulating, GC target, and synaptic plasticity associated genes were characterized and striking differences in the peak and rhythm of expression were apparent (Fig 3.2G). (Data from Birnie, M., Claydon, M. D. B. et al. Circadian regulation of hippocampal function is disrupted during chronic glucocorticoid treatment, *in submission*).

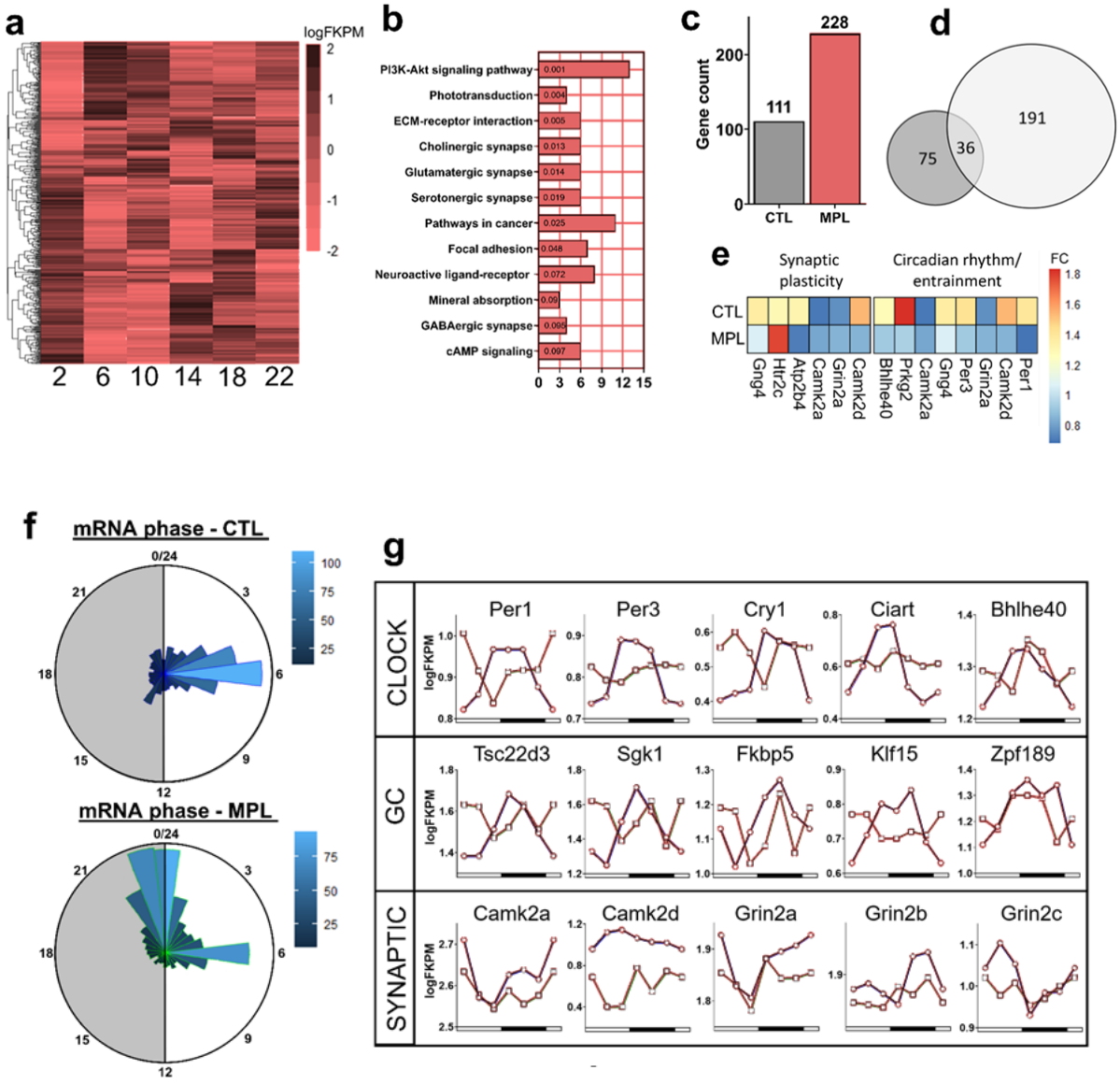


Figure 3.2. Methylprednisolone disrupts circadian regulation of hippocampal transcriptome.

A) *Differentially expressed genes (DEGs; P < 0.05) across time (n=24, 4 per time point). Each row corresponds to a single gene (512 DEGs).* ***B)*** *Top associated (P < 0.1) gene ontology terms of DEGs at ZT10.* ***C)*** *Total number of DEGs at ZT10 in control and MPL treated groups.* ***D)*** *Overlap of the DEGs at ZT10 in control and MPL.* ***E)*** *Expression of circadian regulating and synaptic function genes at ZT10* ***F)*** *Distribution of peak expression of DEGs in control and MPL groups.* ***G)*** *RNA-seq expression pattern data of clock-regulating, glucocorticoid-target, and synaptic plasticity genes. (Data from Birnie, M., Claydon, M. D. B. et al. Circadian regulation of hippocampal function is disrupted during chronic glucocorticoid treatment, in submission).*

Further to this, chronic MPL treatment was shown to impair novel object location (NOL) memory, which is known to depend on the hippocampus. Interestingly, this impairment could only be observed at 6 and 24, but not 1, hours post-learning (Fig 3.3) (Demski-Allen 2014), indicating a deficit in memory consolidation. Therefore, I expected to see a deficit in plasticity incurred by chronic MPL treatment.

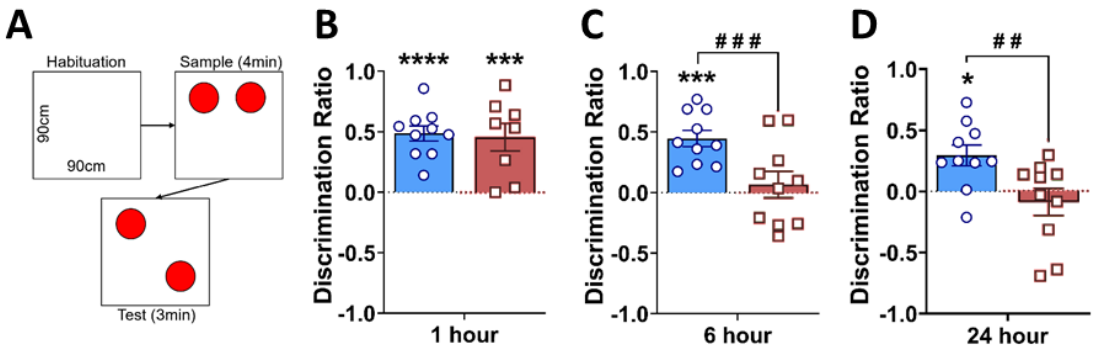


Figure 3.3. Methylprednisolone impairs spatial memory consolidation. ***A)*** *Schematic of Novel Object Location (NOL) task.* ***B)*** *NOL memory 1 hour post acquisition phase in control and MPL treated rats.* $F_{1,32} = 59.52$, $P < 0.0001$, *for learning, two-way ANOVA.* $^{****}P < 0.0001$. ***C)*** *NOL memory 6 hours post acquisition phase in control and MPL treated rats.* $F_{1,36} = 15.87$, $P = 0.0003$, *for memory, and* $F_{1,36} = 8.727$, $P = 0.0055$, *for treatment, two-way ANOVA.* $^{***}P = 0.0001$, $^{###}P = 0.0010$, *Tukey's multiple comparisons.* ***D)*** *NOL memory 24 hours post acquisition phase in control and MPL treated rats.* $F_{1,36} = 7.646$, $P = 0.0089$, *for treatment, by two-way ANOVA.* $^{*}P = 0.0245$, $^{##}P = 0.0021$, *Tukey's multiple comparisons. Data are mean ± SEM,* $^{*}P < 0.05$. *(Data from Birnie, M., Claydon, M. D. B. et al. Circadian regulation of hippocampal function is disrupted during chronic glucocorticoid treatment, in submission).*

3.1.2. Aims & hypotheses

This chapter aims to address the functional consequences of dynamic regulation of the hippocampus at different times of day, and how disruption to the circadian regulation of GR and Per1 by administration of MPL, affects synaptic physiology in the hippocampus. Because the hippocampus exhibits pulsatile and circadian expression of known synaptic function genes, I hypothesised that there would be concomitant differences in LTP and synaptic properties between the circadian peak and nadir, and for this circadian regulation of synaptic processes to be significantly affected following MPL treatment. Although the effects of chronic stress on synaptic plasticity have been examined, the effects of chronic exposure to the synthetic glucocorticoid MPL have not been investigated. In this chapter, I explore the effects and

interaction of time of day (ZT) and treatment (acute and chronic MPL treatment) on synaptic physiology in the hippocampal slice preparation.

3.2. Methods

3.2.1. Hippocampal field recordings

Brain slices were prepared (as described in chapter II) roughly 30 minutes after lights off (ZT12) or 30 minutes after lights on (ZT0) for analyses of the active and inactive phases, respectively (Fig 3.4A). Field excitatory post-synaptic potentials (fEPSPs) were evoked at 0.033 Hertz (Hz) by placing bipolar stimulating electrodes on the Schaffer collateral fibres with the recording electrode positioned in CA1 stratum radiatum, stimulating electrodes were placed on opposite sides of the recording electrode, stimulating different fibres, and generating independent responses (Fig 3.4B). Signals were amplified using an AxoClamp 2B amplifier (Axon Instruments) and 50/60 Hz noise eliminated by a Hum Bug (Quest Scientific), data was acquired and analysed using WinLTP software (Anderson and Collingridge 2007a). At the beginning of each experiment the stimulus intensity was calculated which evoked roughly half-maximal fEPSP amplitude, and this intensity was used for the remainder of the experiment. A stable baseline recording of 30 minutes was acquired before experimental manipulations were applied.

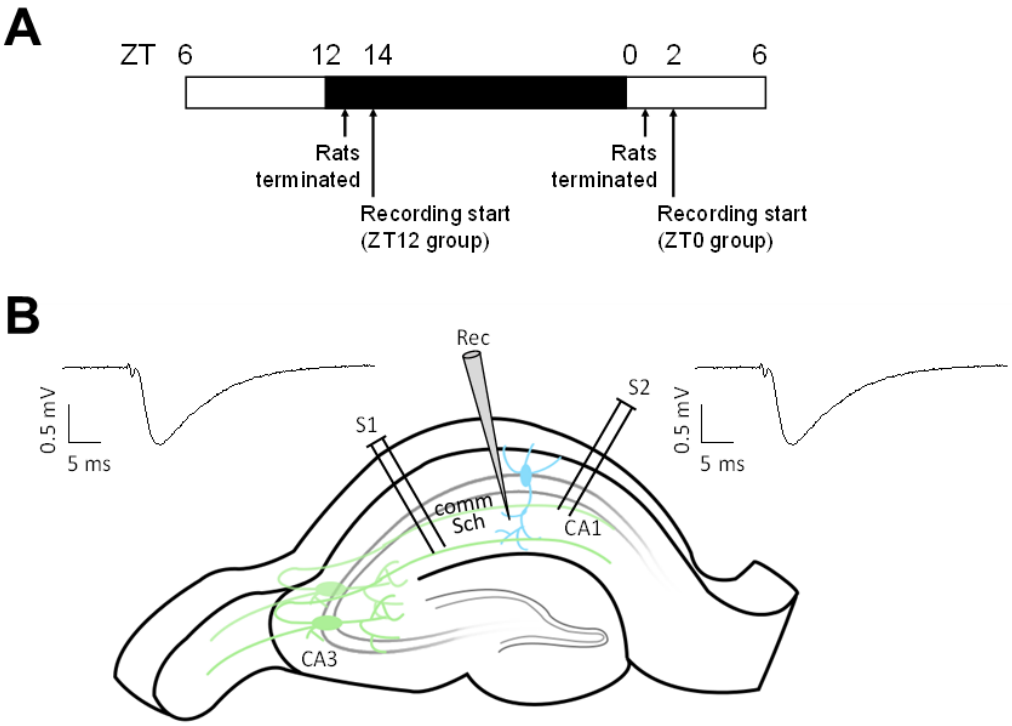


Figure 3.4. Electrophysiological recordings in area CA1. A) Schematic illustrating the timing of brain slice preparation and the commencement of electrophysiology experiments for analysis of neurophysiology during the active (ZT12 group) and inactive (ZT0 group) phases. **B)** Schematic of the hippocampal slice. Stimulating electrodes (S1 and S2) are positioned on Schaffer collateral fibres and the recording electrode (Rec) is placed in the stratum radiatum around CA1 pyramidal cell dendrites. Representative fEPSP traces from both S1 and S2, recorded in the CA1 stratum radiatum, with the stimulus artefact removed.

3.2.2. Evoked EPSC recordings

Evoked EPSCs were elicited by placing a bipolar stimulating electrode in the stratum radiatum towards area CA3, to produce action potentials at 0.067 Hz and whole cell voltage clamp recordings were taken from pyramidal neurons in the stratum pyramidale in area CA1.

For the calculation of input/output (Boltzmann) curves and current/voltage (I/V) curves, 50 µM picrotoxin was perfused throughout the experiments. Briefly, for Boltzmann curves, following a 5-minute acclimatization to the whole-cell configuration, cells were held at -70 mV, and EPSCs were elicited using a series of stimulation intensities. For I/V curves, following a 5-minute acclimatization, EPSCs were elicited using a fixed stimulation intensity using a series of holding potentials from -70 to +40 mV. In each case, a total of four signals were averaged for each condition, and averaged signals used for analysis offline.

For AMPAR:NMDAR ratios, AMPAR mediated EPSCs were evoked at a holding potential of -70 mV, 5 μM 2,3-dioxo-6-nitro-7-sulfamoyl-benzo[f]quinoxaline (NBQX, an AMPAR antagonist) was added to the perfusion and NMDAR mediated EPSC amplitude was measured at a holding potential of -40 mV. Following acquisition of NMDAR-mediated EPSCs, while the cell was held at -40 mV, 5 μM ifenprodil was perfused in aCSF for a duration of 25 minutes to determine the GluN2A:GluN2B ratio. 50 μM picrotoxin and 1 μM glycine were included in the aCSF for the duration of EPSC recordings to block $GABA_A$ receptor mediated currents and ensure presence of NMDAR co-substrate, respectively. Peak amplitudes were used to calculate the AMPAR:NMDAR ratio.

3.2.3. Miniature EPSC and IPSC recordings

Whole-cell voltage clamp recordings were made to ascertain the properties of mEPSCs and mIPSCs in CA1 pyramidal cells. 100 μM D-AP5 ((2R)-amino-5-phosphopentanoic acid) was added to the perfusing aCSF during all recordings to eliminate NMDA receptor mediated currents. mEPSCs were recorded at a holding potential of -70 mV when GABAR mediated transmission is negligible and mIPSCs were recorded at a holding potential of 0 mV when AMPAR mediated transmission is negligible.

3.3. Results

3.3.1. Modulation of synaptic plasticity by time of day and acute MPL treatment

LTP in the hippocampus has previously been shown to be modulated by administration of corticosterone in a protocol dependent manner. Stimulation with 900 pulses at 10 Hz (10 Hz 90 s HFS) was found to be sensitive to CORT (Krugers et al. 2005b). Given that CORT is secreted in a circadian profile and that this pattern is maintained in brain regions including the hippocampus, it follows that hippocampal plasticity processes might also change with natural oscillations in CORT concentration. However, there are many other factors, besides circulating CORT levels, that are modulated by time of day, and these may also give rise to changes in brain physiology. By using the mixed MR/GR agonist MPL, the interactions of acute doses of this drug with time of day can be observed, which highlight the importance of the interaction between glucocorticoid receptor activation and time of day.

Parasagittal hippocampal slices were prepared from animals terminated at the onset of the active (ZT12) or inactive phase (ZT0) (Fig 3.5A), and then pre-treated with 1 μM MPL or vehicle (aCSF), at least 1 hour prior to recording. In ZT0 control slices, LTP could not be elicited by delivery of 10 Hz 90 s HFS ($n = 6$, $t_5 = 1.140$, $P = 0.3058$, paired t-test vs. baseline, Fig 3.5B), whereas in ZT12 control slices, robust LTP of 121.8 ± 4.8% could be measured 50-60 minutes following HFS ($n = 6$, $t_5 = 4.514$, $P < 0.01$, paired t-test vs. baseline, Fig 3.5C). Conversely,

application of 1 μM MPL >1 hour prior to recording facilitated the induction of 10 Hz 90 s LTP of 112.5 ± 1.9% in ZT0 slices (n = 5, t_4 = 6.653, P < 0.01, paired t-test vs. baseline, Fig 3.5D) but inhibited the induction of LTP in ZT12 slices (n = 8, t_7 = 1.676, P = 0.1376, paired t-test vs. baseline, Fig 3.5E). Two-way ANOVA revealed a significant main effect of time ($F_{1, 21}$ = 6.384, P = 0.0196), but not treatment. The interaction between time and treatment was also found to be significant ($F_{1, 21}$ = 22.44, P = 0.0001, Fig 3.5).

This indicates that time of day modulates hippocampal LTP, such that LTP is more readily induced during the active vs. inactive phase of the circadian cycle. GR/MR activation by MPL when circulating CORT is low (ZT0) is sufficient to facilitate the induction of LTP to levels similar to those found in ZT12 control slices. However, MPL agonism of GR/MR when endogenous CORT is already high abolishes the induction of LTP by 10 Hz 90 s HFS, potentially due to an inverted U-shaped dose-response curve, such that at very high levels of receptor occupation LTP is disrupted.

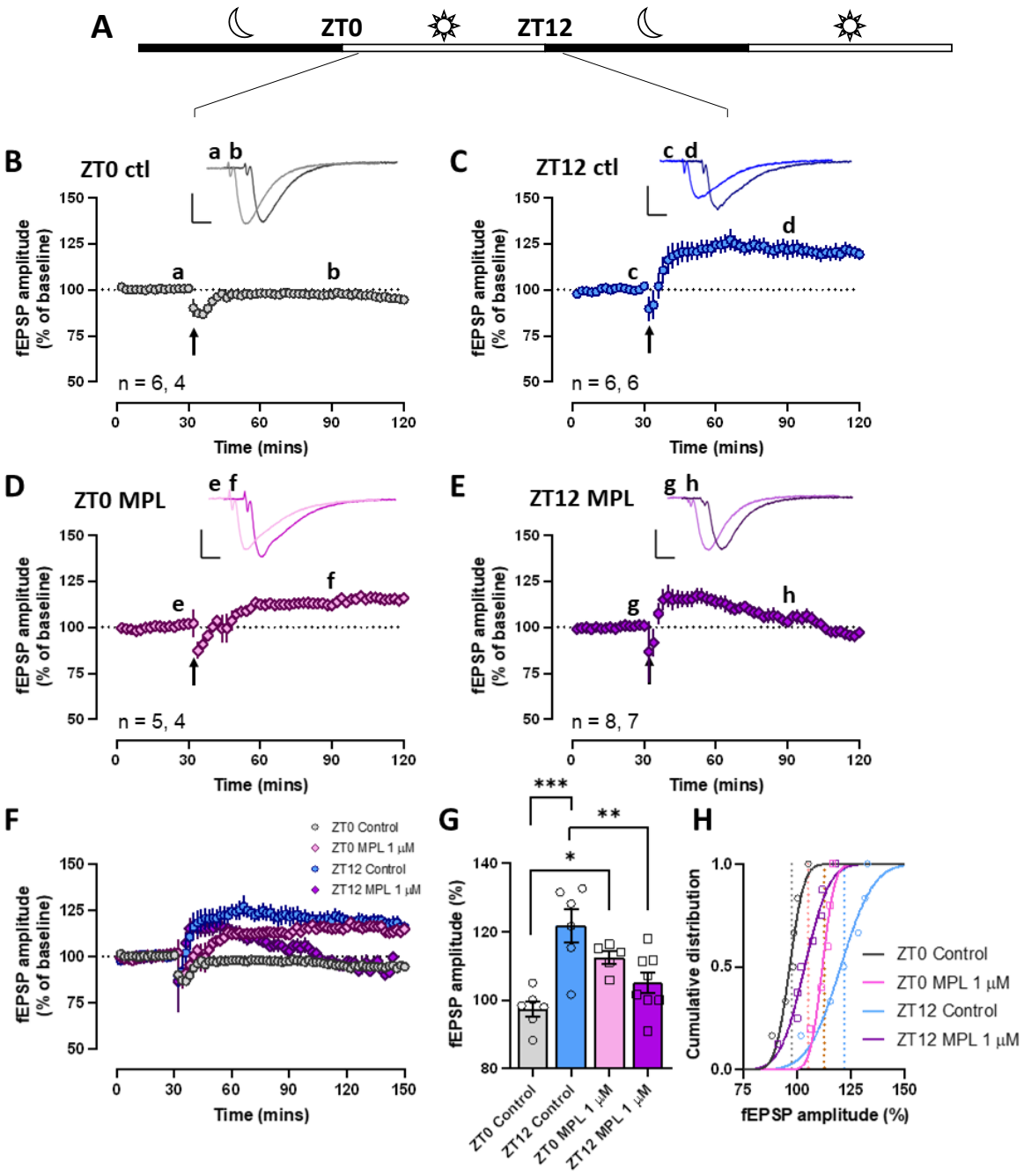


Figure 3.5. The effect of time of day and acute MPL (1 µM) treatment on LTP of CA1 fEPSPs. ***A)*** *Schematic illustrating the timing of slice preparation for analysis of neurophysiology and the interaction of time with acute MPL treatment during the inactive phase (ZT0 group) and the active phase (ZT12 group).* ***B)*** *LTP is not induced by stimulation at 10 Hz for 90 seconds in slices taken from animals terminated at the onset of the inactive phase (ZT0) (n = 6, 4) (slices, animals).* ***C)*** *In slices from control animals terminated at the beginning of the active phase (ZT12), 10 Hz 90 s stimulation is sufficient to induce LTP (n = 6, 6).* ***D)*** *10 Hz 90 s stimulation is sufficient to elicit LTP in slices taken at ZT0 that have been incubated with 1 µM MPL for at least 1 hour prior*

to recording (n = 5, 4). ***E)*** *LTP is not elicited in slices taken at ZT12 that are treated with 1 μM MPL (n = 8, 7). Traces in each graph represent an average fEPSP before (light) and after (dark) 10 Hz 90 s stimulation. Traces are identified to corresponding time points by the indication of lower-case letters. Scale bars represent x = 5 ms and y = 0.5 mV.* ***F)*** *Overlay of previous graphs to illustrate the differences between groups.* ***G)*** *Bar graph representing average fEPSP amplitude 50-60 minutes after 10 Hz 90 s stimulation. Individual experiments are plotted as points.* ***H)*** *Cumulative distributions of fEPSP amplitude during the 50-60 post-stimulation period are plotted for each dataset. Dotted lines represent mean values in* ***H*** *and baseline in other graphs. All data are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to check for statistical significance, which is indicated as P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***).*

3.3.2. Circadian oscillations in excitatory and inhibitory spontaneous activity

Spontaneous activity can be measured by miniature EPSCs/IPSCs in the presence of tetrodotoxin, when action potential evoked transmission is blocked, or measured by spontaneous EPSCs/IPSCs with network activity (at least within the slice) preserved. Although the precise function of spontaneous neurotransmission remains unclear, it is thought to be important in the maintenance and plasticity of synaptic connections (Kavalali et al. 2011; Gonzalez-Islas, Bülow, and Wenner 2018). Changes in the frequency of spontaneous events are typically due to pre-synaptic alterations to the rate of neurotransmitter release, whereas changes in the amplitude of spontaneous events are usually ascribed to changes in the post-synaptic density of certain receptor types. These measures can also provide a useful proxy of the excitatory-inhibitory (E-I) balance, which is known to be perturbed in several psychopathologies (Sohal and Rubenstein 2019).

In slices from control animals terminated at either ZT0 or ZT12 a clear modulation of the frequency but not amplitude of spontaneous events was observed. mEPSC frequency was significantly higher at ZT12 (1.842 ± 0.5032 Hz, n = 13) and lower at ZT0 (0.5897 ± 0.0845 Hz, n = 10) ($t_{12.67}$ = 2.454, P < 0.05, Welch's t-test, Fig 3.6A), while mEPSC amplitude was not different between ZT12 (13.61 ± 1.265 pA, n = 13) and ZT0 (15.98 ± 2.065 pA, n = 10) (U = 50, P = 0.3758, Mann-Whitney, Fig 3.6C). Interestingly, the circadian variation in the rate of miniature excitatory events was reflected in a circadian regulation of mIPSC frequency ($t_{14.31}$ = 4.261, P < 0.001, Welch's t-test, Fig 3.6E), such that at ZT12 mIPSC frequency was raised (4.588 ± 0.6953 Hz, n = 13) and lowered at ZT0 (1.484 ± 0.6953 Hz, n = 12), presumably to maintain the balance between excitation and inhibition and prevent epileptiform activity. Like mEPSCs, there was no

change in mIPSC amplitude between ZT12 (15.21 ± 1.543 pA, n = 13) and ZT0 (14.40 ± 0.9655 pA, n = 12) ($t_{19.91}$ = 0.4443, P = 0.6616, Welch's t-test, Fig 3.6G).

This striking circadian modulation of spontaneous activity in the hippocampus may act to enable better processing of information and support synaptic processes, at a time relevant to learning.

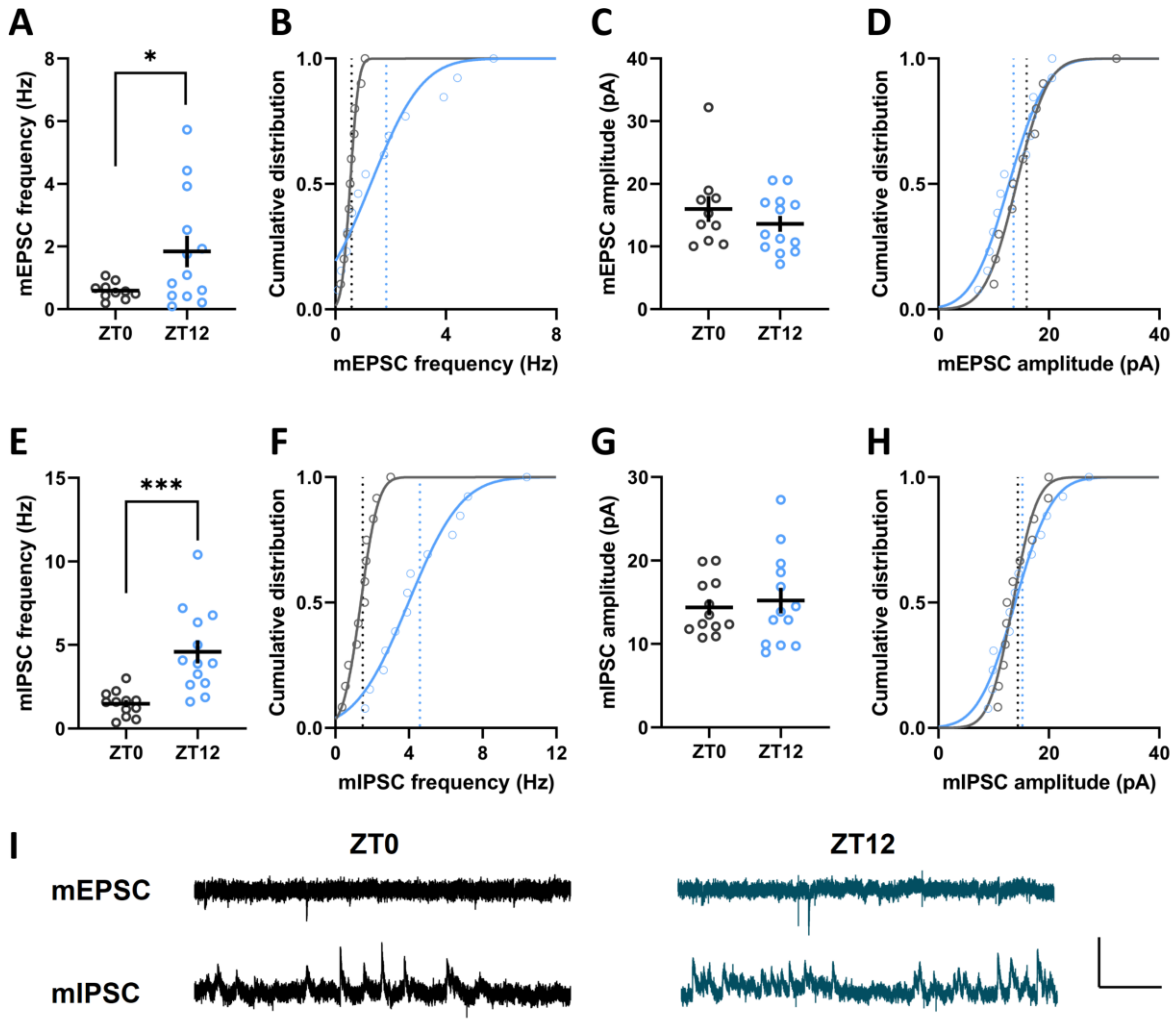


Figure 3.6. The effect of time of day on spontaneous activity in CA1 pyramidal neurons. ***A)*** *mEPSC frequency is significantly raised at ZT12 (n = 13, 6, 4) (cells, slices, animals) compared to ZT0 (n = 10, 6, 4).* ***B)*** *cumulative distribution of mEPSC frequency.* ***C)*** *mEPSC amplitude was not significantly altered between ZT12 (n = 13, 6, 4) and ZT0 (n = 10, 6, 4).* ***D)*** *Cumulative distribution of mEPSC amplitude.* ***E)*** *mIPSC frequency was significantly increased at ZT12 (n = 13, 6, 4) compared to ZT0 (n = 12, 6, 4).* ***F)*** *Cumulative distribution of mIPSC frequency.* ***G)*** *mIPSC amplitude was not different between ZT12 (n = 13, 6, 4) and ZT0 (n = 12, 6, 4).* ***H)*** *Cumulative distribution of mIPSC amplitude.* ***I)*** *Representative traces of recordings made at ZT0 and ZT12 for mEPSC and*

*mIPSC analyses. Scale bar represents x = 1 s and y = 20 pA. Dotted lines in cumulative distribution graphs represent means. Data in bar graphs are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). A Welch's t-test was used in **A**, **E**, and **G**, and a Mann-Whitney test in **C**. Statistical significance is indicated at P < 0.05 (*) and P < 0.001 (***).*

3.3.3. Circadian regulation of LTP is abolished in MPL treated animals

Animals treated chronically (between 4 and 7 days) with the synthetic glucocorticoid MPL exhibit a flattened endogenous CORT profile, and elevated GR activity during the circadian nadir, and this is reflected in changes to the dynamic nature of the hippocampal transcriptome.

Unlike in control animals, 10 Hz 90 s HFS was not sufficient to induce LTP in both ZT0 slices (n = 6, t_5 = 1.291, P = 0.2530, paired t-test vs. baseline, Fig 3.7A) and ZT12 slices (n = 7, t_6 = 0.4006, P = 0.7026, paired t-test vs. baseline) (Fig 3.7B) from MPL treated animals. Because no LTP could be elicited at either ZT0 or ZT12, the circadian regulation of hippocampal LTP reported in Fig 3.5 was lost in MPL treated animals (t_{11} = 0.9646, P = 0.3555, unpaired t-test, Fig 3.7D).

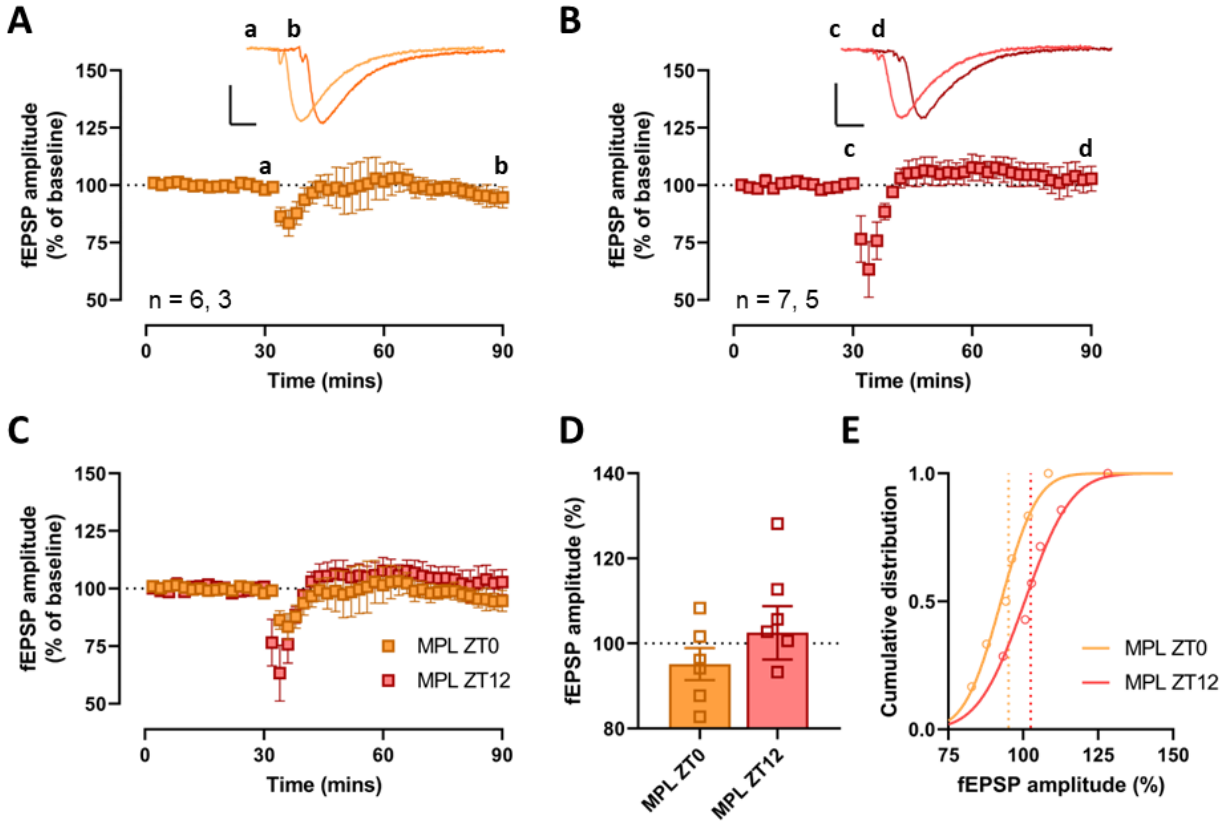


Figure 3.7. The effect of time of day on 10 Hz 90 s LTP in hippocampal slices from MPL treated animals. ***A)*** *10 Hz 90 s stimulation was not able to induce LTP in slices taken from animals terminated at ZT0 (n = 6, 3) (slices, animals).* ***B)*** *10 Hz 90 s stimulation was not able to induce LTP in slices taken from animals terminated at ZT12 (n = 7, 5). Representative fEPSP traces for before (light) and after (dark) HFS are shown. Traces are identified to corresponding time points by the indication of lower-case letters Scale bars represent x = 5 ms and y = 0.5 mV.* ***C)*** *Overlay of ZT0 and ZT12 groups.* ***D)*** *Bar graph representing the average fEPSP amplitude during the 50-60 minute post-HFS period. An unpaired t-test was used to test for statistical significance. Individual experiments are plotted as points.* ***E)*** *Cumulative distribution of fEPSP amplitude during the 50-60 minute post-HFS period. Dotted lines represent means in* ***E*** *and baseline in other graphs. Data in* ***A***, ***B***, ***C***, *and* ***D*** *are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals).*

3.3.4. MPL disrupts the circadian regulation of spontaneous activity in the hippocampus

Control animals exhibit a robust circadian regulation of mEPSC and mIPSC frequency in the hippocampus (Fig 3.6), in a balanced manner, presumably to promote synaptic maintenance and plasticity at times of day that require learning, cognitive flexibility, and remembering.

GO pathway analysis predicted that the glutamatergic synapse was differentially regulated between control and MPL conditions, so I hypothesized that changes in mEPSC properties might occur in addition to the reported deficits in synaptic plasticity (Fig 3.7).

Indeed, in MPL treated animals mEPSC frequency was raised at ZT0 (2.755 ± 0.0845 Hz, n = 10) and reduced at ZT12 (0.7857 ± 0.2529, n = 10). This significant increase of mEPSC frequency at ZT0 compared to ZT12 (U = 15, P < 0.01, Mann-Whitney, Fig 3.8A) represents an inversion of the rhythm observed in control animals (Fig 3.6). On the other hand, mEPSC amplitude – like controls – was not different between ZT0 (12.31 ± 0.9743 pA, n = 10) and ZT12 (14.76 ± 1.425 pA, n = 10) (t_{19} = 1.388, P = 0.1812, unpaired t-test, Fig 3.8C).

Despite the circadian inversion of mEPSC frequency, the frequency of mIPSC events in CA1 cells was not different between ZT0 (3.821 ± 0.6953 Hz, n = 10) and ZT12 (3.216 ± 0.4123 Hz, n = 12) (t_{20} = 0.9804, P = 0.3386, unpaired t-test, Fig 3.8E). Consistent with the other results, mIPSC amplitude remained unchanged between ZT0 (13.56 ± 0.9375 pA, n = 10) and ZT12 (12.93 ± 0.5976 pA, n = 12) (t_{20} = 0.5858, P = 0.5645, unpaired t-test, Fig 3.8G). Thus, chronic treatment with synthetic glucocorticoids incurs a change in the balance between excitatory and inhibitory spontaneous neurotransmission, a phenomenon that is regulated by time of day in control animals.

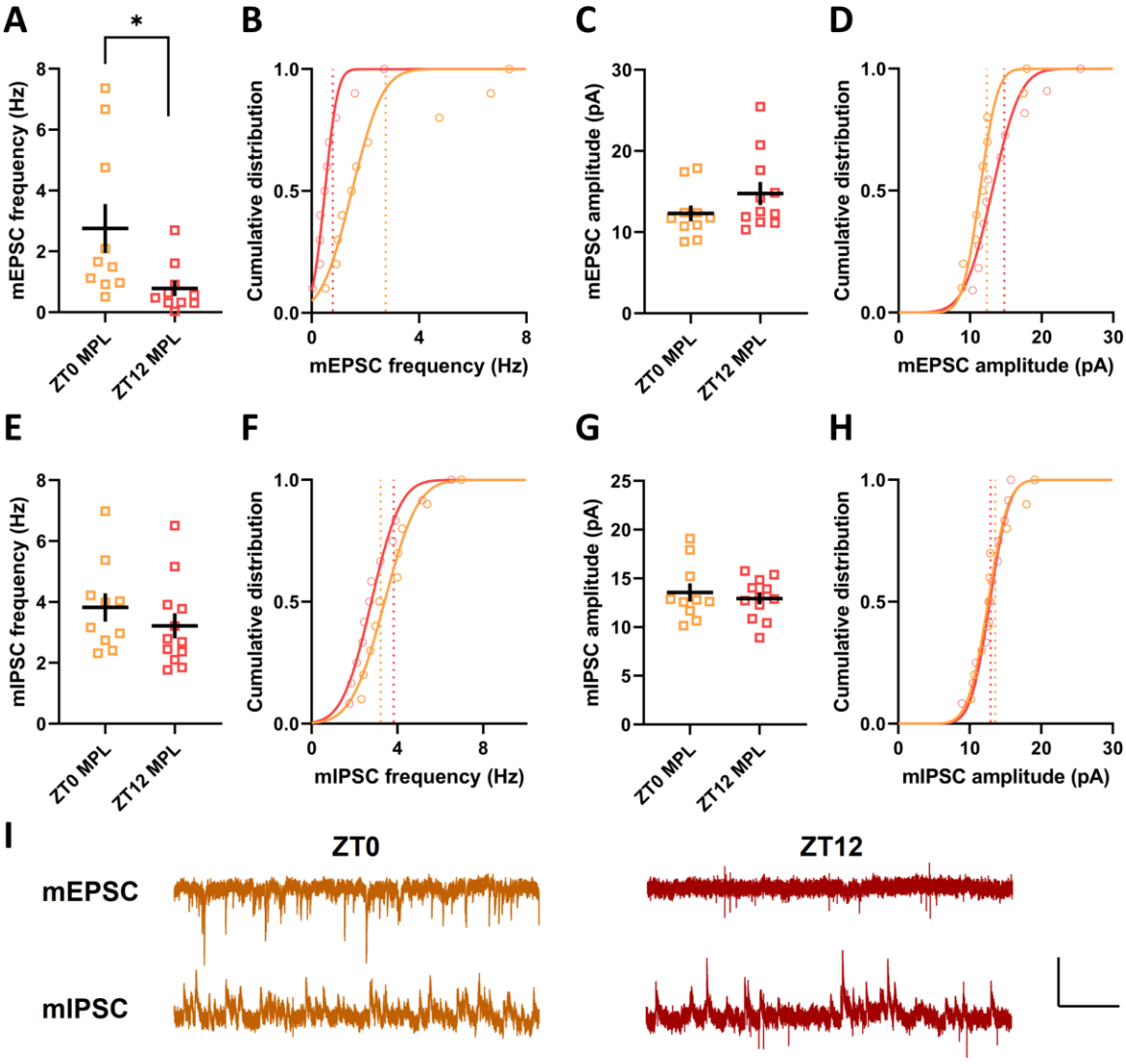


Figure 3.8. The effect of time of day on spontaneous activity in CA1 pyramidal neurons in slices from animals treated with MPL. ***A)*** *mEPSC frequency is significantly decreased at ZT12 (n = 10, 7, 4) (cells, slices, animals) compared to ZT0 (n = 10, 6, 4).* ***B)*** *cumulative distribution of mEPSC frequency.* ***C)*** *mEPSC amplitude was not significantly altered between ZT0 (n = 10, 6, 4) and ZT12 (n = 10, 7, 4).* ***D)*** *Cumulative distribution of mEPSC amplitude.* ***E)*** *mIPSC frequency was not significantly different between ZT0 (n = 10, 6, 4) and ZT12 (n = 12, 7, 4)* ***F)*** *Cumulative distribution of mIPSC frequency.* ***G)*** *mIPSC amplitude was not different between ZT0 (n = 10, 6, 4) and ZT12 (n = 12, 7, 4).* ***H)*** *Cumulative distribution of mIPSC amplitude.* ***I)*** *Representative traces of recordings made at ZT0 and ZT12 for mEPSC and mIPSC analyses. Scale bar represents x = 1 s and y = 20 pA. Dotted lines in cumulative distribution graphs represent means. All data in bar graphs are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). Mann-*

*Whitney test was used in **A**, unpaired t-test used in **C**, **E**, and **G**. Statistical significance is indicated at P < 0.05 (*).*

3.3.5. MPL induces deficits in LTP during the active phase

Data from our lab (Fig 3.3) indicate that chronic MPL treatment induces deficits in the NOL task, which is known to rely on hippocampal functioning. These behavioural experiments were conducted during the active phase (ZT12-24). Interestingly, deficits were only observed at 6 and 24, but not 1, hours post-acquisition, indicating a deficit in the consolidation phase of learning. Therefore, I hypothesized that deficits in synaptic plasticity in the hippocampus may underlie the behavioural changes observed at this time.

Robust LTP (117.2 ± 3.553% at 2 h post-HFS) could be induced by 10 Hz 90 s HFS and remained stable for at least 3 hours in slices from control animals (n = 6, t_5 = 4.829, P < 0.01, paired t-test vs. baseline, Fig 3.9B), whereas LTP could not be generated in slices from MPL treated animals (n = 7, t_5 = 0.3812, P = 0.7611, paired t-test vs. baseline (Fig 3.9C), indicating a significant difference between controls and MPL groups in the LTP observed at 140-150 minutes post-HFS (t_{11} = 2.943, P < 0.05, nested t-test, Fig 3.9E). These deficits in synaptic plasticity during the active phase could be the cause of the memory deficits observed in the NOL task following chronic steroid treatment.

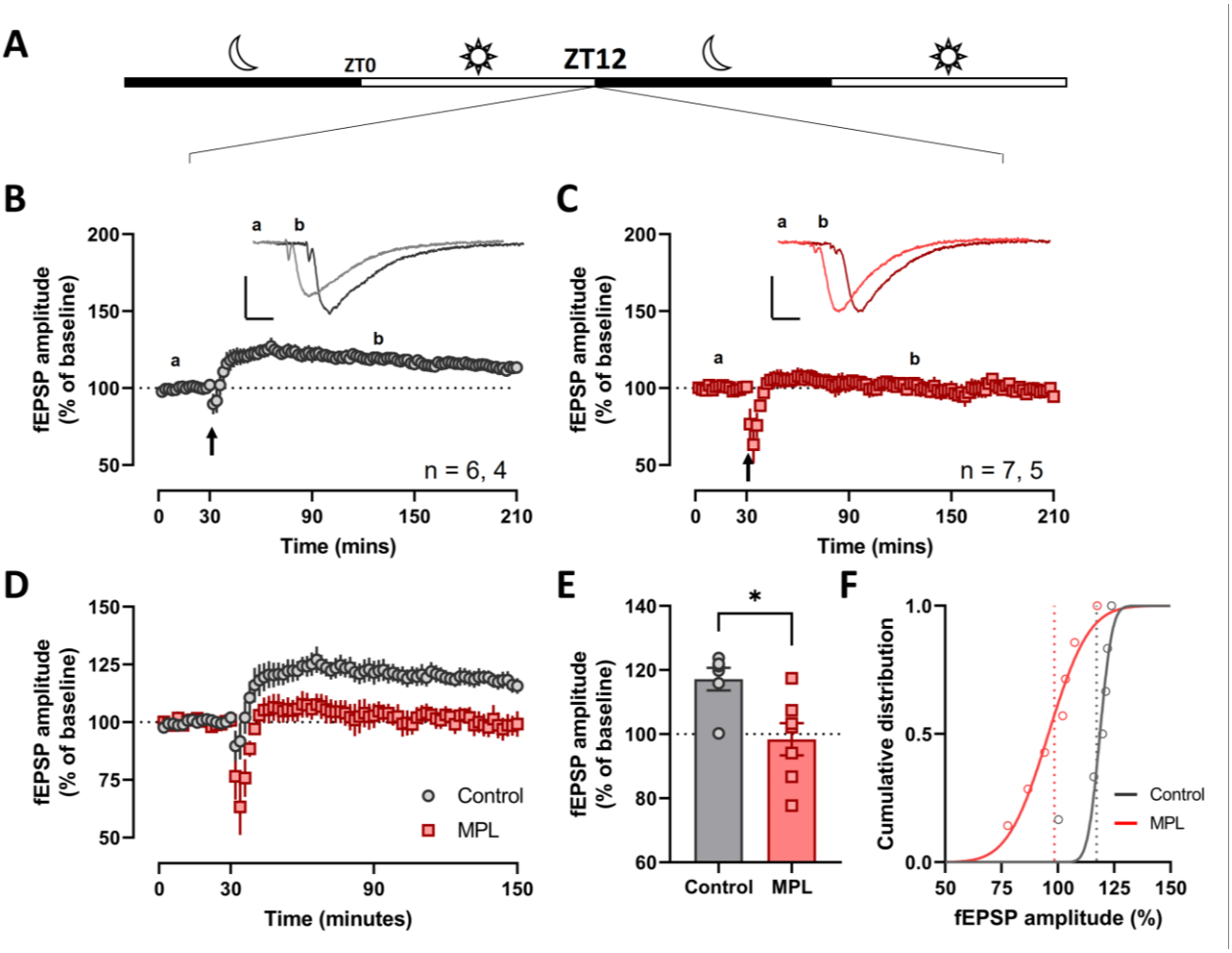


Figure 3.9. The effect of chronic MPL treatment on hippocampal LTP during the active phase. ***A)*** *Schematic of the light/dark cycle illustrating that these recordings were made following the onset of the dark/active period of the rat (ZT12).* ***B)*** *Robust LTP of CA1 fEPSPs can be elicited in slices taken from control animals by delivery of 10 Hz 90 s stimulation (arrow) (n = 6, 4) (slices, animals).* ***C)*** *10 Hz 90 s stimulation does not induce LTP in hippocampal slices taken from animals treated chronically with MPL (n = 7, 5) (slices, animals). Representative traces for before (light) and after (dark) HFS. Scale bars represent x = 5 ms and y = 0.5 mV.* ***D)*** *Overlay of data from control and MPL treated animals illustrates the difference in LTP induction.* ***E)*** *Bar graph representing the average fEPSP amplitude over the period 110-120 minutes following LTP induction. Mean fEPSP amplitude from single experiments is plotted.* ***F)*** *Cumulative distributions of the fEPSP amplitude over the 110-120 minute post-HFS period. Dotted lines represent mean values in* ***E*** *and baseline in other graphs. All data in* ***B***, ***C***, ***D***, *and* ***E*** *are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals). A nested t-test was used in* ***E.*** *Statistical significance is represented as P < 0.05 (*). Data sets used in this graph are the same as the data presented in figures 3.5 and 3.7, but are*

shown in extended format and are used to illustrate the difference between MPL and vehicle treated groups at a time relevant to the novel object location data.

3.3.6. The MPL and time-of-day sensitive LTP protocol depends on GluN2B-containing NMDA receptors

RNA-seq analysis revealed disruption to the circadian expression of NMDA receptor subunits, as well as LTP-pathway protein CaMKIIα, whose association with GluN2B subunits is known to be critical during the successful induction of synaptic plasticity (Halt et al. 2012). It is therefore possible that disruption to the rhythmic expression of these proteins prevents their association at a time critical to learning.

GluN2B has also been shown to be critical in mediating neural deficits associated with chronic stress and is known to conduct more effectively during stimulation at lower stimulation frequencies than GluN2A containing receptors (Erreger et al. 2005). Thus, to investigate in greater detail the mechanism by which MPL induces plasticity and memory deficits, experiments using the selective GluN2B antagonist Ro-25-6981 (Ro) were conducted. It should be noted, though, that at the concentration of Ro used (5 μM), blockade of tri-heteromeric receptors is possible.

Under control conditions stable LTP (121.8 ± 4.838%) could be recorded 50-60 minutes post-HFS ($n = 6$, $t_5 = 4.533$, $P < 0.01$, paired t-test vs. baseline, Fig 3.10A) whereas 20 minute application of Ro prior to HFS blocked the induction of LTP ($n = 6$, $t_5 = 0.4484$, $P = 0.4484$, paired t-test vs. baseline, Fig 3.10B). Consequently, blockade of GluN2B containing NMDARs with Ro significantly reduced LTP compared to control conditions ($t_{10} = 3.442$, $P < 0.01$, nested t-test, Fig 3.10D), indicating that the protocol sensitive to modulation by chronic MPL treatment and time of day relies on activation of GluN2B containing NMDARs. It is, therefore, tempting to speculate that the disruption to rhythmic expression of NMDA receptor subunits and downstream communicators like CaMKII interrupts their association at a time critical to learning, thereby incurring deficits in memory and learning. The dependency of this protocol on GluN2B may be due to the relatively low stimulation frequency (10 Hz), and the relatively long train duration (90 seconds), as GluN2A and GluN2B desensitise at different rates (Erreger et al. 2005). The dependency of this protocol on GluN2B activation may therefore explain why it is sensitive to GCs.

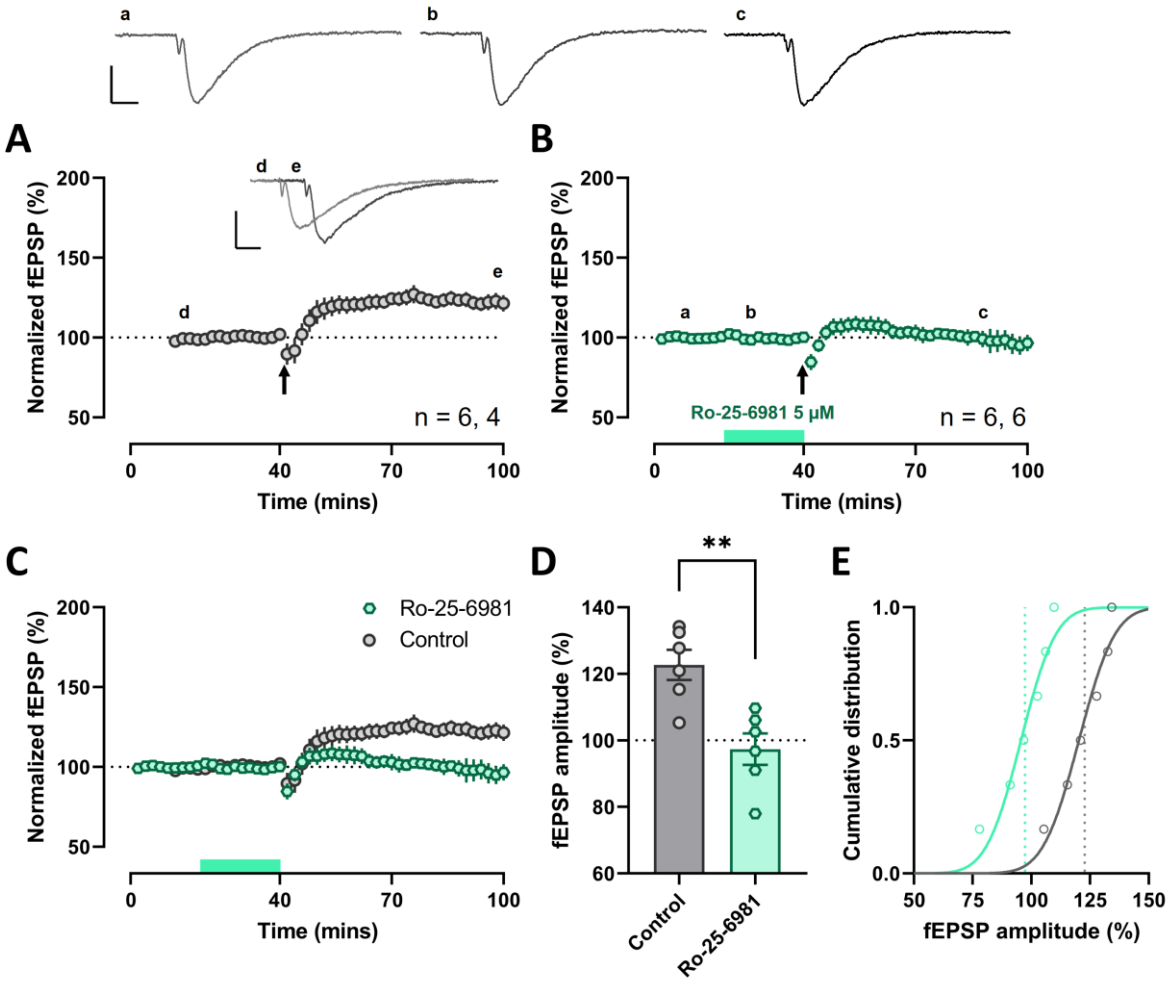


Figure 3.10. The effect of selective GluN2B antagonist Ro-25-6981 (5 µM) on 10 Hz 90 s LTP. ***A)*** *Robust LTP of CA1 fEPSPs can be induced by 10 Hz 90 s stimulation in hippocampal slices from control animals terminated at the onset of the active phase (ZT12) (n = 6, 4) (Data from figure 3.5B). Representative traces from individual experiments before (light) and after (dark) HFS.* ***B)*** *Perfusion of 5 µM Ro-25-6981 for 20 minutes prior to delivery of HFS abolishes LTP induction (n = 6, 6). Representative traces a, b, and c (above) correspond to baseline, baseline + drug, and post-HFS periods.* ***C)*** *Overlay of control and treated experiments for comparison.* ***D)*** *Bar graph representing average fEPSP amplitude over the 50-60 minute post-HFS period. Mean fEPSP amplitude from individual experiments is plotted.* ***E)*** *Cumulative distributions of fEPSP amplitude for the 50-60 minute post-HFS period. Dotted lines represent mean values in* ***E*** *and baseline in other graphs. All data in* ***A***, ***B***, ***C***, *and* ***D*** *are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals). Scale bars are x = 5 ms and y = 0.5 mV. A nested t-test was used in* ***D****. Statistical significance is indicated at P < 0.01 (**).*

3.3.7. LTP induced by a strong protocol is not affected by MPL

Because GO pathway analysis indicates a specific disruption to the NMDA receptor at the glutamatergic synapse, I hypothesized that stronger induction protocols that invoke calcium influx via other sources, like VDCCs, would not be affected by chronic MPL treatment. Spaced theta burst stimulation (sTBS) is a strong, PKA-dependent, induction protocol that produces an extremely robust form of LTP in the hippocampus (Park et al. 2016). Three trains of theta burst activity are delivered with a spacing of 10 minutes between trains.

A long lasting (at least 6 hour) LTP of fEPSP amplitude (128.7 ± 8.820%) could be elicited in slices taken from control ZT12 animals 350-360 minutes post sTBS ($n = 5$, $t_4 = 3.256$, $P < 0.05$, paired t-test vs. baseline, Fig 3.11A) as well as slices taken from MPL treated ZT12 animals (130.4 ± 7.103%) ($n = 4$, $t_3 = 4.275$, $P < 0.05$, paired t-test vs. baseline, Fig 3.11B). LTP elicited was not different between control and MPL groups ($t_7 = 0.1397$, $P = 0.8928$, paired t-test, Fig 3.11D). This data reinforces the supposition that chronic steroid treatment incurs memory deficits primarily through disruption to the NMDA receptor and its downstream effectors.

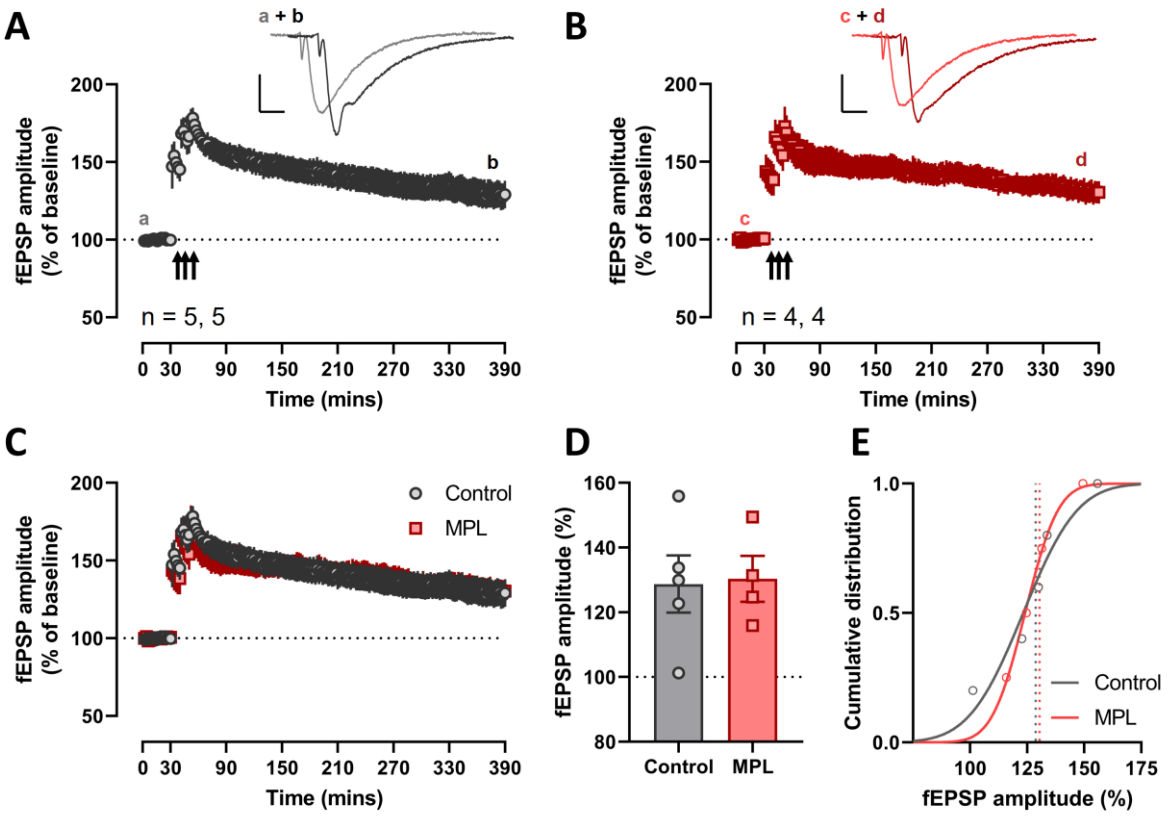


Figure 3.11. The effect of chronic MPL treatment on LTP induced by spaced theta burst stimulation (sTBS) in CA1 Schaffer collateral fibres. ***A)*** *Robust LTP of CA1 fEPSPs is elicited by delivery of sTBS (three TBS episodes spaced by 10 minutes) in slices from control animals 6 hours post-stimulation (n = 5, 5).* ***B)*** *Robust LTP is also elicited in slices taken from animals treated with MPL for up to 5 days (n = 4, 4). Representative traces for before (light) and after (dark) HFS. Scale bars are x = 5 ms and y = 0.5 mV.* ***C)*** *Overlay of control and MPL groups for comparison.* ***D)*** *Bar graph representing average fEPSP amplitude for the 350-360 minute period post sTBS. All data in* ***A***, ***B***, ***C***, *and* ***D*** *are plotted as mean ± SEM.* ***E)*** *Cumulative distribution of fEPSP amplitude during the 350-360 minute post-sTBS period. Dotted lines represent mean values in* ***E*** *and represent baseline in other graphs. Experimental n is indicated as (n = slices, animals). An unpaired t-test was used to test for statistical significance.*

3.3.8. MPL treatment does not affect input/output or I/V curves of CA1 neurons

To investigate whether MPL induced any changes in the currents passed by ionotropic glutamate receptors Boltzmann (or input/output) curves were prepared by stimulating hippocampal slices with incremental currents at a holding potential of -70 mV, allowing AMPAR-mediated currents to be generated using increasing stimulation intensity (Fig 3.12A). A significant main effect of stimulus intensity was found ($F_{9, 88} = 11.25$, $P < 0.0001$, Two-way

ANOVA) but not for treatment ($F_{1,88}$ = 0.4662, P = 0.4965) or interaction ($F_{9,88}$ = 0.0906, P = 0.9997). No significant difference between treatment groups were found at any given stimulus intensity (Sidak's) (Fig 3.12A). This type of experiment has many limitations, however, as many extraneous factors could affect the amplitude of evoked EPSCs besides treatment with MPL, though it provides an easy initial screening for the health of AMPAR-mediated responses.

This was further investigated by recording EPSCs evoked by a fixed (half-maximal) current at different holding potentials (10 mV increments between -70 and +40 mV). From this data an I/V (current/voltage) relationship was plotted. Because the voltage dependent Mg^{2+} block of NMDA receptors is removed at holding potentials of -40 mV (or more depolarized), these curves can provide information regarding the currents passed by AMPAR and NMDAR receptors, as well as the reversal potential. However, the scope of conclusions that can be drawn from these experiments is still quite limited. Two-way ANOVA revealed a significant main effect of holding potential was found ($F_{11,71}$ = 26.53, P < 0.0001) but not for treatment ($F_{1,71}$ = 0.0042, P = 0.9483) or interaction ($F_{11,71}$ = 0.9084, P = 5371). Sidak's multiple comparison test showed no significant differences between control and MPL groups at any given holding potential (Fig 3.12C).

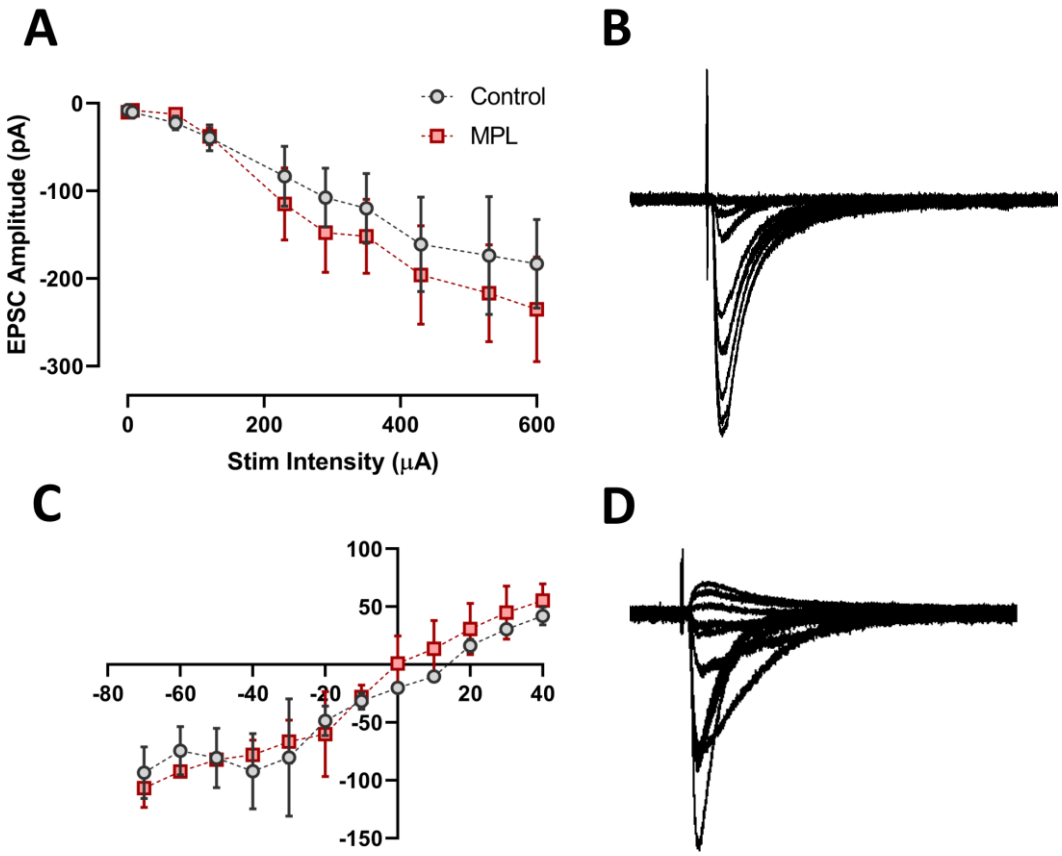


Figure 3.12. Chronic MPL treatment does not alter input/output or I/V curves. ***A)*** *Overlay of input/output relationship of CA1 neurons voltage-clamped at -70 mV, stimulated with incremental current steps, from control (n = 5, 5) and MPL (n = 6, 6) treated animals.* ***B)*** *Representative traces showing EPSCs from each different stimulation intensity.* ***C)*** *Overlay of I/V relationships of CA1 neurons stimulated with half-maximal current at incremental holding potentials from control (n = 4, 4) and MPL (n = 4, 4) treated animals. X axis is holding potential (mV) and y axis is EPSC amplitude (pA).* ***D)*** *Representative traces of EPSCs recorded at each holding potential between -70 and +40 mV. All data are mean ± SEM. Experimental n is indicated as (n = slices, animals). Two-way ANOVA with Sidak's multiple comparisons test was used in* ***A*** *and* ***C****.*

3.3.9. MPL treatment alters AMPAR:NMDAR ratio in CA1 pyramidal neurons

Specific deficits in GluN2B-NMDAR dependent LTP in MPL treated animals indicate that there may be changes to the post-synaptic glutamate receptor composition in CA1 pyramidal cells. To test this, I conducted AMPAR:NMDAR ratio experiments under voltage-clamp conditions in the whole-cell patch clamp configuration. Holding cells at -70 mV in the presence of picrotoxin to block $GABA_A$ mediated currents allows for the AMPAR mediated current to be recorded, then

application of NBQX and holding at -40 mV to relieve Mg^{2+} blockade allows recording of NMDAR mediated currents. Recordings were made at -40 mV rather than +40 mV because this allowed a more stable recording configuration for the further application of GluN2B selective antagonist Ifenprodil, thereby facilitating calculation of the GluN2A:2B ratio (Fig 3.13A).

A significant decrease in AMPAR:NMDAR ratio was seen in slices from MPL treated animals (2.811 ± 0.2119, n = 13) compared to controls (3.624 ± 0.2348, n = 14) ($t_{24.9}$ = 2.57, P < 0.05, Welch's t-test, Fig 3.13B). However, how these changes in channel conductance are manifest in molecular changes cannot be concluded without additional experiments. Because GluN2B is known to be vital in mediating deficits induced by chronic stress, I hypothesized that the ratio between GluN2A and GluN2B-containing NMDAR mediated currents would be affected by MPL treatment. Ifenprodil application showed a non-significant trend for an increased GluN2A:2B ratio in MPL treated animals (1.188 ± 0.0367, n = 9) compared to controls (1.057 ± 0.0923, n = 3) (U = 5, P = 0.1455, Mann-Whitney, Fig 3.13E). More experiments may reveal a significant effect but due to changes in R_S over the course of the experiment many control experiments had to be discarded.

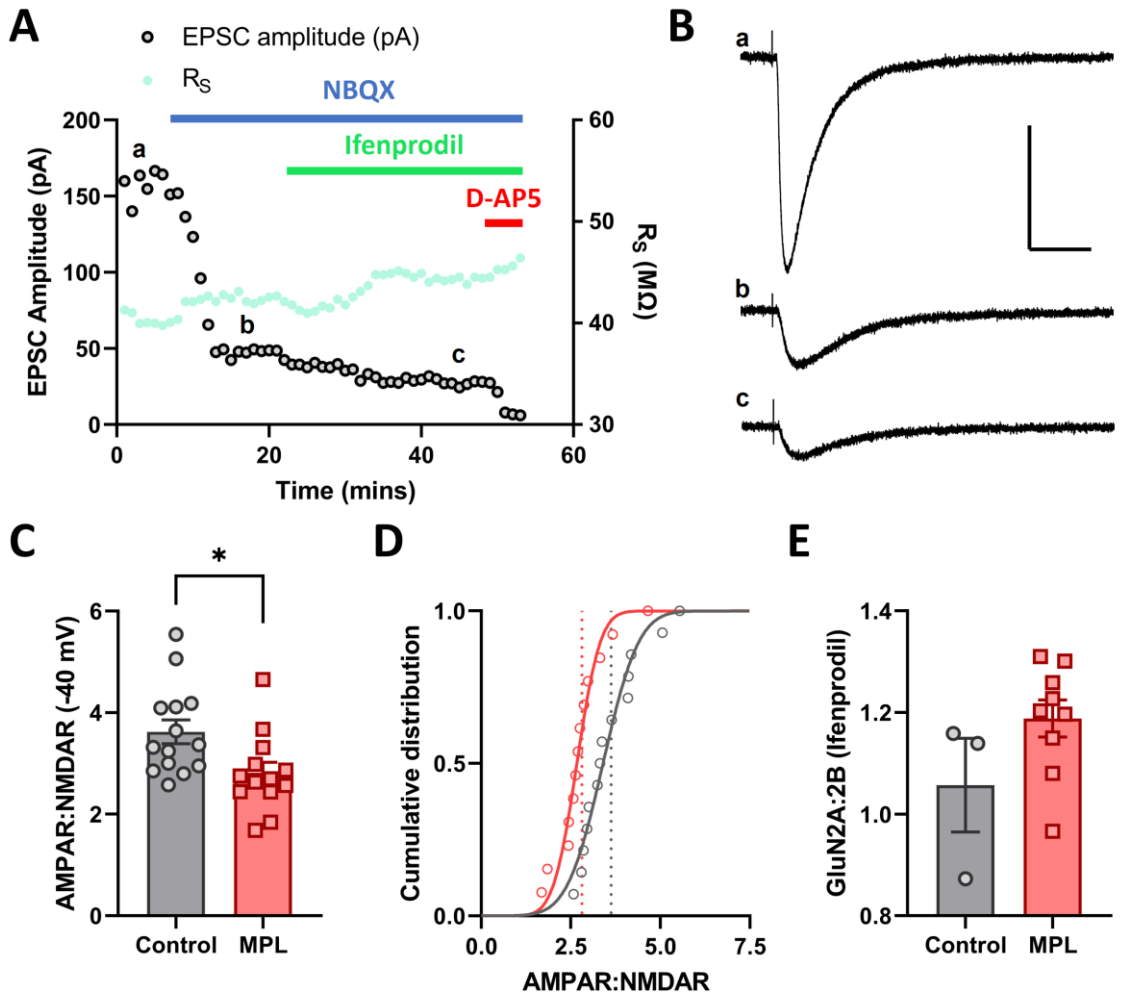


Figure 3.13. Chronic MPL treatment decreases the AMPAR:NMDAR ratio in CA1 neurons. ***A)*** *An example experiment detailing how drugs were applied through the experiment to isolate the NMDAR-dependent (following NBQX 5 µM) and GluN2A-dependent (following Ifenprodil 5 µM) currents. D-AP5 (100 µM) was added to the perfusion to ensure EPSCs were mediated by NMDARs only.* R_S *was monitored throughout the experiment and if it changed by over 10% then recordings were not selected for analysis.* ***B)*** *Representative traces for AMPAR-mediated EPSCs at -70 mV (top), NMDAR-mediated EPSCs at -40 mV (middle) and GluN2A-mediated EPSCs at -40 mV (bottom). Scale bar represents x = 5 ms and y = 100 pA.* ***C)*** *AMPAR:NMDAR ratio was significantly reduced in CA1 neurons of MPL treated animals (n = 13, 8) (slices, animals) compared to controls (n = 14, 9). Data are mean ± SEM.* ***D)*** *Cumulative distribution of AMPAR:NMDAR ratios. Dotted lines represent means.* ***E)*** *No significant difference was found between control (n = 3, 3) and MPL groups (n = 9, 5) in GluN2A:2B ratio. Data in* ***C*** *and* ***E*** *are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals). Welch's t-test was used to test for statistical significance in* ***C*** *and a Mann-Whitney test was used in* ***E****. Statistical significance is indicated at* $P < 0.05$ *(*).*

3.6. Discussion

The hippocampus has been one of the most studied brain regions since the advent of neuroscience. The significance of LTP and LTD in this area is known to be vital for memory processing, and these synaptic plasticity processes are also known to be disrupted in neurological disorders like Alzheimer's disease (Di Lorenzo et al. 2016). Furthermore, circadian rhythms are thought to be instrumental in learning and memory, with peak performance during the beginning of the active phase, gradually declining throughout the day (Hoffmann and Balschun 1992; Valentinuzzi, Menna-Barreto, and Xavier 2004; Hauber and Bareiss 2001). The master molecular transcriptional clock in the hypothalamic SCN is vital for regulating these rhythms, with glucocorticoids acting as one of the major humoral signals influencing peripheral clocks and extra-SCN oscillators in the brain, including the hippocampal clock (Birnie 2018).

In this chapter, I demonstrate the circadian regulation of NMDA receptor dependent LTP in the hippocampus, providing a physiological substrate for the improved cognitive performance reported during the active phase. Moreover, acute administration of the synthetic glucocorticoid MPL at ZT0 leads to an LTP similar to that observed at ZT12 in control conditions, suggesting that glucocorticoids are mediators of hippocampal circadian regulation. The blockade of LTP by acute MPL at ZT12 is in agreement with the idea that GCs affect synaptic function in the hippocampus with an inverted U-shape dose-response curve (Akirav et al. 2004), although there are many other variables besides circulating concentration that can change the effects of glucocorticoid hormones, for instance chromatin accessibility and receptor distribution.

This circadian regulation of hippocampal LTP is underscored by the dynamic regulation of mEPSC and mIPSC frequency. Spontaneous synaptic transmission is thought to be important for facilitating plasticity and synaptic maintenance (Gonzalez-Islas, Bülow, and Wenner 2018), so the dynamic changes in mEPSC frequency during the active and inactive phases may be important in regulating plasticity processes to allow for increased capacity for learning and cognitive function at the appropriate time of day, leaving other processes such as synaptic pruning and maintenance to occur during the inactive phase.

The importance of the circadian regulation of the hippocampus by glucocorticoid hormones is underscored by the disruption to the hippocampal transcriptome induced by chronic MPL treatment. Here, I have shown that chronic MPL treatment leads to an ablation of the dynamic regulation of hippocampal LTP, indicating that GR regulation of the molecular clock, is a key driver of circadian variation in CA1 plasticity. My thesis that hippocampal function is regulated by the molecular clock, and influenced further by glucocorticoids, is supported by evidence showing that expression of REV-ERBα is critical for proper LTP induction during the active

phase (Choi, Kim, et al. 2018), and Per1 is critical in successful memory formation (Sakai et al. 2004).

Further to this, the dynamic regulation of spontaneous synaptic transmission is also perturbed following chronic MPL treatment. Changes in mEPSC and mIPSC frequency were not balanced, and so the delicate equilibrium between excitation and inhibition in the hippocampus is disturbed by chronic GC treatment. Imbalances in the E-I ratio are thought to be critical in the development of certain psychiatric disorders like autism spectrum disorder (Lee, Lee, and Kim 2017). Recent evidence has shown that early-life stress can accelerate developmental shifts in the E-I balance (Karst et al. 2020), reinforcing the idea that GCs are vital in modulating this phenomenon.

RNA-seq of whole hippocampus showed that the rhythmic expression of certain synaptic genes, including the *Grin2* family and *CaMKII,* was disrupted by chronic MPL treatment. Moreover, chronic MPL treatment resulted in NOL memory deficits. The association of GluN2B and CaMKII is known to be critical for the successful induction of LTP and memory formation (Halt et al. 2012). GluN2B has been shown to anchor activated CaMKII at the synapse, thus primed to activate downstream effectors (Barcomb et al. 2016). Recent evidence has demonstrated that calcium-influx induced phase separation of GluN2B and CaMKII co-segregates AMPARs and neuroligin into a phase-in-phase assembly (Hosokawa et al. 2021), thereby forming an activity-dependent mediator for the association of other plasticity-associated proteins . In this chapter I have shown that during the active phase, hippocampal LTP is abolished in MPL treated animals, compared to controls. Therefore, given the disruption to the rhythmic expression of GluN2 subunits and CaMKII, it is plausible that LTP is abolished by blocking their association at a time critical to learning. This highlights the importance of endogenous glucocorticoid rhythms in maintaining the dynamic molecular profile of the hippocampus in a state appropriate for the challenges relevant to that time, for instance, learning during the active phase. This is in agreement with data from the motor cortex of mice showing that learning and plasticity are more readily induced during circadian peaks, while maintenance of those new connections is promoted during the circadian nadir (Liston et al. 2013). However, it must be noted that the same control data has been used for comparison against multiple datasets (in Figure 3.5, Figure 3.9, and Figure 3.10). This makes the interpretation of these data more difficult, and the use of interleaved controls for each data set would make the conclusions drawn from these data significantly more robust.

In line with GO pathway analysis, which predicted a specific loss of rhythmic regulation of the NMDAR in the glutamatergic synapse (leaving VDCCs, KARs, and AMPARs unaffected by treatment) (Fig 3.14), I showed that a sTBS LTP induction protocol could still induce late LTP in

slices from MPL treated animals. This supports the notion that disruption to the rhythmic expression of NMDAR subunits is critical for the deficits induced by chronic steroid use. Further evidence comes from the fact that the MPL/time of day sensitive protocol is dependent on GluN2B-containing NMDARs. GluN2B membrane trafficking has been shown to be rapidly regulated by endogenous GCs (Mikasova et al. 2017), and GluN2B containing NMDARs are known to be crucial in mediating the dendritic atrophy and synaptic impairments induced by chronic stress (Li et al. 2011). Recent evidence also shows that GluN2B expression is regulated by stress and circadian glucocorticoid dynamics (Mifsud et al. 2021). Therefore, the data I present here suggest that the physiological rhythmic expression of GluN2 NMDA receptor subunits is critical for maintaining proper hippocampal function.

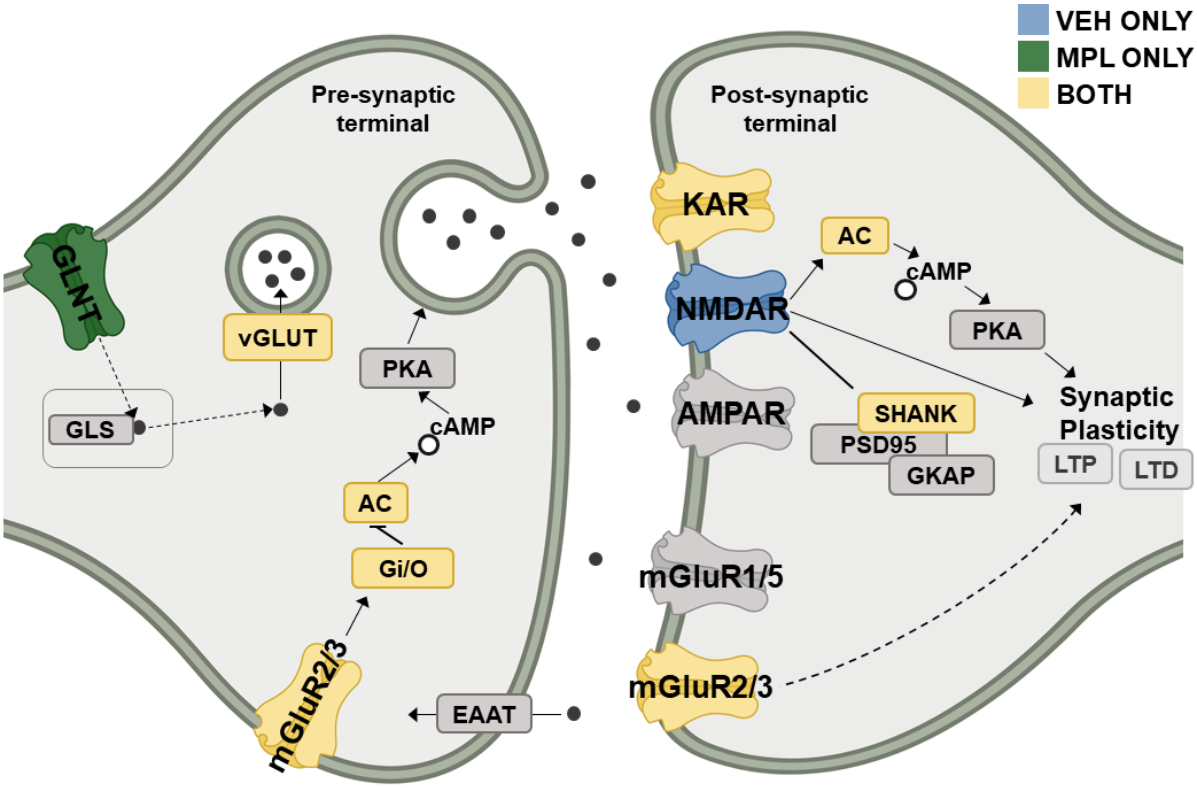


Figure 3.14. Representation of MPL-induced LTP pathway changes predicted by functional pathway analysis. *DAVID (Database for Annotation, Visualisation, and Integrated Discovery) analysis of RNA-seq data predicts specific loss of circadian regulation of the NMDAR. Gene products regulated by time are indicated by different colours; those significantly regulated over one circadian cycle in both MPL and control groups are shaded in yellow, while those significantly regulated over one circadian cycle in controls (vehicle) only are blue, MPL only in green, and neither in grey. Abbreviations: GLNT, glutamine transporter; GLS, glutaminase; vGLUT, vesicular glutamate transporter; PKA, protein kinase A; cAMP, cyclic adenosine monophosphate;*

AC, adenylate cyclase; $G_{i/0}$, G_i protein alpha subunit; mGluR, metabotropic glutamate receptor; EAAT, excitatory amino acid transporter; KAR, kainate receptor; NMDAR, N-methyl-D-aspartate receptor; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; SHANK, SH3 and multiple ankyrin repeat domains protein; PSD95, post-synaptic density protein 95; GKAP, guanylate kinase-associated protein; LTP, long-term potentiation; LTD, long-term depression; VEH, vehicle; MPL, methylprednisolone.

In agreement with data showing that chronic corticosterone reduces the AMPAR:NMDAR ratio of field EPSPs in temporoammonic-CA1 synapses (Kvarta et al. 2015), I have shown that chronic MPL treatment reduces the AMPAR:NMDAR ratio of evoked EPSCs in Schaffer collateral-CA1 synapses, recorded in whole-cell voltage clamp. Unlike recording in the extracellular set-up, whole cell recording allowed me to unmask a reduction after a much shorter course of GC treatment (< 7 days compared to 4 weeks), as well as providing a more reliable comparison, given the very small NMDAR fEPSPs that were isolated in the aforementioned study. Unfortunately, the attempted exploration of NMDA receptor subunit composition was not completed, due to technical issues resulting in a number of control studies being eliminated from analysis. Although it is tempting to speculate that there is a non-significant trend towards an MPL-induced increase in GluN2A:GluN2B ratio, more experiments would be needed to assess this.

Together, these data illustrate the significance of the GR driven intrahippocampal molecular clock in driving synaptic plasticity and spontaneous synaptic transmission. Dysregulation of these molecular rhythms by chronic GR activation with the synthetic GC MPL resulted in deficits in learning that were consequential of disruption to hippocampal LTP during the active phase, a time that is crucial for learning. Further disruption to spontaneous synaptic transmission may reduce the maintenance and plasticity of CA1 synapses, and MPL-induced imbalances to the E-I ratio in the long term are a potential route for the onset of psychopathologies. Furthermore, one of the many novel findings arising from this chapter is that NMDARs, in particular GluN2B-containing receptors, appear to play a pivotal role in the changes that I have characterised in my studies. What still remains unknown, however, is how time of day and glucocorticoid treatment affects the molecular clock and synaptic physiology in other brain regions. Therefore, in the following chapter I will explore the effects of the circadian cycle, chronic MPL treatment, and their interaction, in the perirhinal cortex – a region in which the effects of glucocorticoids remain largely unknown.

Chapter IV

Methylprednisolone treatment disrupts perirhinal cortex function

4.1. Introduction

The perirhinal cortex (PRH) is a small region in the MTL situated between the entorhinal and parahippocampal/postrhinal cortices, in a discrete area of the cortex that immediately surrounds the rhinal sulcus. Generally, PRH neurons receive highly processed sensory information of many modalities, but there is also a great dopaminergic input to this region (Richfield, Young, and Penney 1989; Berger et al. 1988), which is thought to be involved in the reward associated with visual stimuli (Liu and Richmond 2000).

4.1.1. The role of perirhinal cortex in memory processing

Far from a passive relay station, PRH neurons are known to receive inputs from many sensory modalities (Suzuki and Amaral 1994) and are able to function as a site of integration, thereby playing an important role in learning and memory (Squire, Stark, and Clark 2004). Recognition memory, particularly single item recognition, has been the most extensively studied in the PRH in both rodents and primates. Initially, lesion studies suggested a central role for PRH in single-item recognition memory (Aggleton, Kyd, and Bilkey 2004) and it has since been demonstrated that neurons of the PRH are differentially activated by familiar and novel stimuli (Zhu et al. 1995). It is now widely accepted that the PRH can function to discern the familiarity of an object, and the mechanisms are relatively well understood (Brown and Aggleton 2001).

Reversibly blocking AMPAR and Kainate receptor mediated transmission with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) in PRH induces impairments in novel object recognition, and blockade of local NMDAR or metabotropic glutamate receptors (mGluRs) impairs the consolidation of long-term novel object recognition memory (Barker, Warburton, et al. 2006; Winters and Bussey 2005). Further evidence from synaptic studies has demonstrated the importance of NMDAR activation in the plasticity of PRH synapses (Cho et al. 2000; Ziakopoulos et al. 1999), a process known to be important in learning and memory (Bliss and Collingridge 1993). Familiarity of an item is represented by a reduction in the neuronal response to the presentation of that item, mediated by synaptic dampening (Bogacz and Brown 2003). Indeed, it has been shown that NMDA receptor dependent LTD of PRH synapses is essential for the functional expression of visual recognition memory in rodents (Griffiths et al. 2008). It should be noted, though, that other receptors have also been shown to play crucial roles in recognition memory (Warburton et al. 2003; Barker, Bashir, et al. 2006).

Although this phenomenon, often referred to as repetition suppression, is considered to be the neural correlate of recognition memory, it can be observed in many cortical areas along the ventral visual pathway. However, the endurance of the memory is considerably longer in the PRH than elsewhere (Xiang and Brown 1998).

Besides its role in single item recognition (Brown and Aggleton 2001), the PRH also serves complex roles in associative mnemonic processes. Single unit recordings in macaque monkeys have demonstrated the forward propagation of information from area TE (part of the temporal association cortex, adjacent to PRH) to PRH, and that a significantly higher population of neurons in PRH than area TE respond selectively to learned paired items. Moreover, due to the rapidity of adaptation, encoding in PRH was permitted with little or no feedback input from higher cortical regions (Naya, Yoshida, and Miyashita 2003).

Evidence from rodents, primates and humans suggests that the PRH functions as part of a wider network including hippocampus, EC, prefrontal cortex and certain thalamic nuclei that facilitates associative memory function(Barker and Warburton 2020; Miyashita 2019; Suzuki and Naya 2014). Superficial neurons of PRH layers 2/3 receive projections from various neocortical areas, allowing the PRH to act as a site of integration, coding various aspects of the associated features of an object (Miyashita 1993), associations between objects (Miyashita 1988), and even behavioural contexts associated with an object (Mogami and Tanaka 2006; Tamura et al. 2017). The PRH further distinguishes itself from other areas of MTL by its building on associative information encoded in hierarchically lower cortical areas (i.e. area TE), allowing flexible coding of associations (Hirabayashi et al. 2013).

More recent evidence from primate studies has highlighted a role for PRH in memory recollection as well as consolidation. Using MRI-assisted electrophysiology, it was demonstrated that the projections originating in the infragranular layer (layers 5 and 6) and terminating in neocortex, are critical in the retrieval of the associated features of an object (Koyano et al. 2016), potentially by influencing the local circuits contained within area TE (Takeda et al. 2015).

4.1.2. Effects of glucocorticoids on perirhinal cortex

Although little work has been done to investigate the effects of glucocorticoids on perirhinal cortex function, studies have shown that recognition memory is compromised by chronic stress (Franklin et al. 2018; Wang et al. 2011), which is known to depend on perirhinal cortex function. In agreement with this, lesions to PRH alter stress-induced behavioural changes (Schulz-Klaus 2009). Moreover, chronic stress has been shown to alter the morphology of dendritic spines and results in a reduction in nectin-1 mRNA in the PRH. These changes were dependent on CRH (Gong et al. 2018), reinforcing the importance of GC regulation of this brain region.

Stress and GCs are not equivalent, though, as many other effectors are important in mediating the stress response. Recent data (Birnie 2018) shows that chronic treatment with the synthetic glucocorticoid MPL results in deficits in a novel object recognition (NOR) task (Fig 4.1).

Interestingly, these deficits could only be observed at the 6 and 24, but not 1, hour post-training time points, indicating that consolidation of memory is impaired.

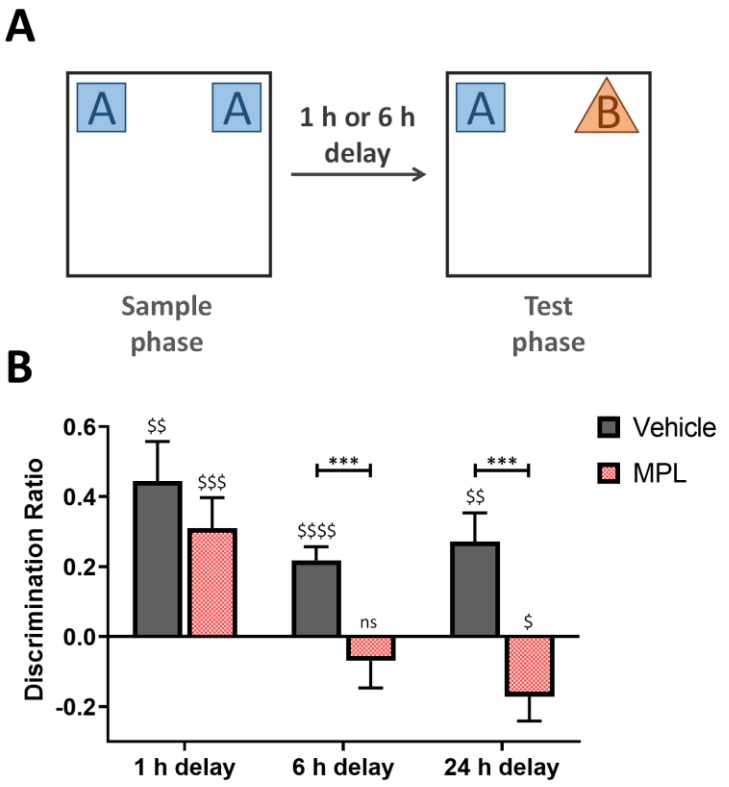
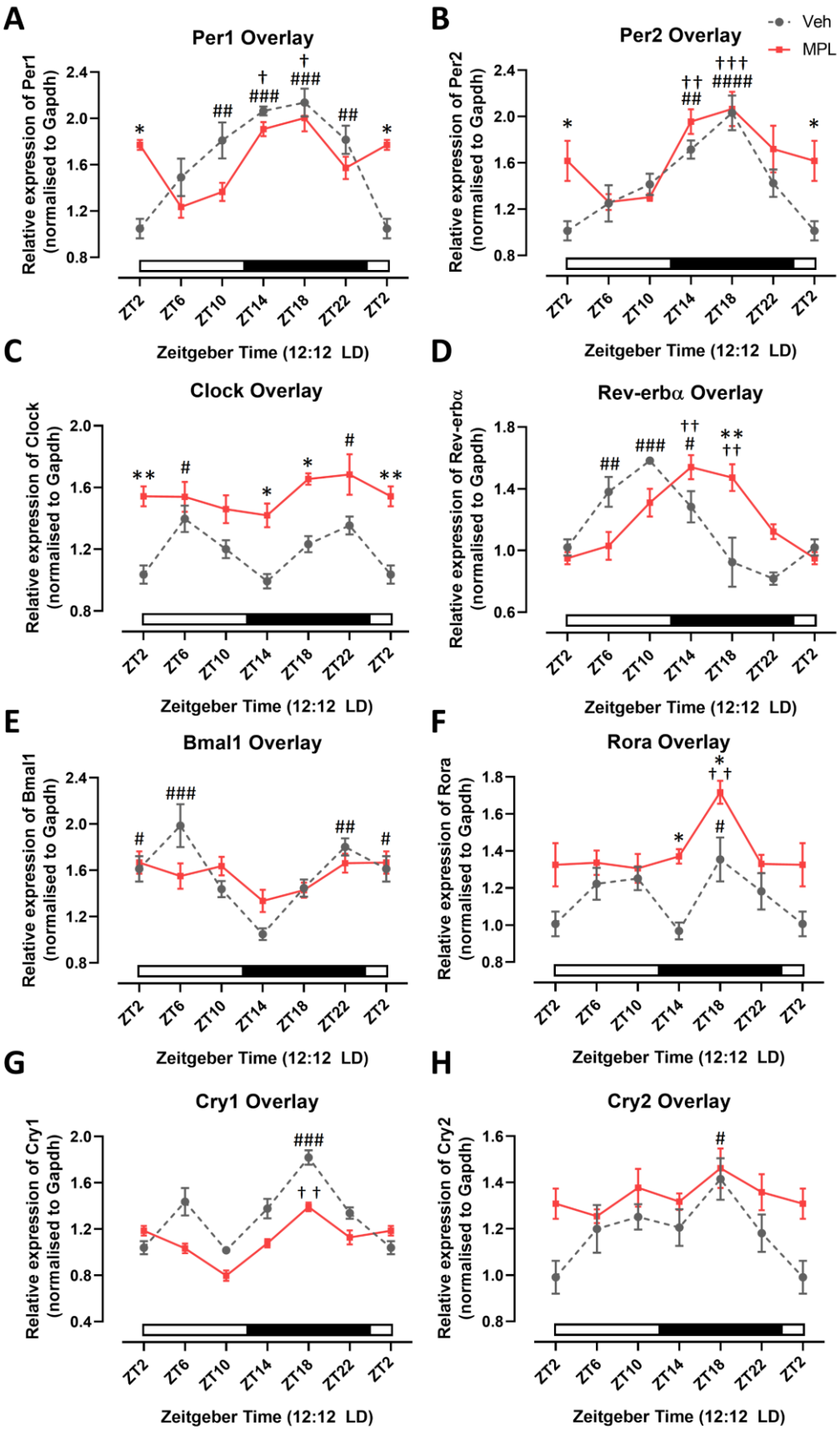


Figure 4.1. MPL treatment induces deficits in a novel object recognition task. ***A)*** *Diagram of the NOR test in which one of two objects used in the sample phase is replaced with a novel object during the test phase, and the proportion of time spent investigating the novel object is used to calculate a discrimination ratio.* ***B)*** *Rats treated with methylprednisolone (n = 7) exhibited impaired performance at 6 h compared to the vehicle treated group (n = 10). At a 1 h delay there was no difference between MPL (n = 10) and Vehicle (n = 11) treated rats. The MPL treated group showed no preference for the novel object at delays longer than 1 h. $ denotes significant difference to 0 and * denotes significant difference between treatment groups. All data are shown as mean ± SEM. All statistical significance indicated at p < 0.05 (*/$), p < 0.01 (**/$$), p < 0.001 (***/$$$) and p < 0.0001 (****/$$$$). Adapted from (Birnie 2018).*

The rhythmic action of GCs is known to be important in memory processing (Liston et al. 2013; Kalafatakis et al. 2018), and in the previous chapter I showed that the glucocorticoid driven molecular clock in the hippocampus appears to be crucially involved in regulating time of day dependent hippocampal function, including synaptic plasticity and spontaneous neurotransmission. Perturbations to the natural glucocorticoid driven rhythms in the

hippocampus by the chronic action of the synthetic glucocorticoid methylprednisolone led to impairments in several aspects of hippocampal neurophysiology. In turn, this ultimately led to memory deficits in a hippocampus dependent memory task (Birnie 2018). Recent data has shown that chronic MPL administration results in desynchronization of the molecular clock in the perirhinal cortex, as well as the hippocampus (Rozwaha 2017) (Fig 4.2).

I performed statistical analysis of data that was collected during an MSc project (from (Rozwaha 2017)) and two-way ANOVA revealed a significant main effect of Time on expression of Per1 ($F_{5,30} = 7.059$, $p < 0.001$, Fig 4.2A), Per2 ($F_{5,30} = 11.89$, $p < 0.0001$, Fig 4.2B), Clock ($F_{5,30} = 3.327$, $p < 0.05$, Fig 4.2C), Rev-erbα ($F_{5,30} = 6.889$, $p < 0.001$, Fig 4.2D), Bmal1 ($F_{5,30} = 4.855$, $p < 0.01$ Fig 4.2E), Rorα ($F_{5,30} = 5.196$, $p < 0.01$, Fig 4.2F) and Cry1 ($F_{5,30} = 7.345$, $p < 0.001$, Fig 4.2G). Cry2 ($F_{5,30} = 2.196$, $p > 0.05$, Fig 4.2H) was the only transcript studied that did not exhibit a significant main effect of time. A main effect of treatment was revealed by two-way ANOVA for the mRNA expression of Per2 ($F_{1,6} = 6.640$, $p < 0.05$), Clock ($F_{1,6} = 42.30$, $p < 0.05$), Rorα ($F_{1,6} = 22.48$, $p < 0.01$), Cry1 ($F_{1,6} = 32.05$, $p < 0.01$) and Cry2 ($F_{1,6} = 7.397$, $p < 0.05$), but not Per1 ($F_{1,6} = 0.7244$, $p > 0.05$), Rev-erbα ($F_{1,6} = 0.8049$, $p > 0.05$) or Bmal1 ($F_{1,6} = 0.01346$, $p > 0.05$). Two-way ANOVA showed a significant interaction of time and treatment for only Per1 ($F_{5,30} = 3.185$, $p < 0.05$) and Rev-erbα ($F_{5,30} = 5.324$, $p < 0.01$). There was no significant interaction between factors for all remaining transcripts measured.


A
Per1 Overlay
Relative expression of Per1 (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
B
Per2 Overlay
Veh
MPL
Relative expression of Per2 (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
C
Clock Overlay
Relative expression of Clock (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
D
Rev-erbα Overlay
Relative expression of Rev-erbα (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
E
Bmal1 Overlay
Relative expression of Bmal1 (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
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Rora Overlay
Relative expression of Rora (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
G
Cry1 Overlay
Relative expression of Cry1 (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
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Cry2 Overlay
Relative expression of Cry2 (normalised to Gapdh)
Zeitgeber Time (12:12 LD)
ZT2
ZT6
ZT10
ZT14
ZT18
ZT22
ZT2

Figure 4.2. Circadian expression of clock gene mRNA in PRH of MPL or vehicle treated rats. ***A-H)*** *mRNA expression patterns of clock genes (from left to right) Per1, Per2, Clock, Rev-erbα, Bmal1, Rorα, Cry1, and Cry2 from qPCR analysis of perirhinal cortex tissue taken at 4-hour intervals across the light dark cycle (n = 24, 4 per time point). Data are plotted as mean ± SEM. Two-way ANOVA was used to test for statistical significance with Dunnett's multiple comparisons to test for significance between each time point and the circadian nadir within a treatment group (# in control, † in MPL) and Sidak's multiple comparisons to test for significance between treatment groups at each time point (*). Statistical significance is indicated at P < 0.05 (*/#/†), P < 0.01 (**/##/††), P < 0.001 (***/###/†††), and P < 0.0001 (****/####/††††). Data was collected by Tom Rozwaha during an MSc project and I have subsequently statistically analysed and presented this data for the purpose of this thesis.*

Given the disruption to the rhythmic expression of clock genes in PRH, it is likely that, similar to the hippocampus, the rhythmic transcription of synaptic gene products is also perturbed by chronic steroid exposure. I therefore hypothesised that MPL induced deficits in synaptic plasticity, as well as disrupting circadian and/or ultradian aspects of neurophysiology in the PRH. These changes may provide a molecular mechanism to explain the behavioural deficits in the NOL task induced by chronic MPL administration.

4.2. Methods

4.2.1. Perirhinal field recordings

fEPSPs were evoked at 0.033 Hz by placing bipolar stimulating electrodes on either side of the recording electrode which was positioned directly beneath the rhinal sulcus at the juxtaposition of layer I and layers II/III (figure 4.3). One stimulating electrode was placed superficial and one deep to ensure independent responses. Signals were amplified using an AxoClamp 2B amplifier (Molecular Devices, LLC, California, USA) and 50/60 Hz noise eliminated by a Hum Bug (Quest Scientific), data was acquired and analysed using WinLTP software (Anderson and Collingridge 2007b). At the beginning of each experiment the stimulus intensity was calculated which evoked roughly half-maximal fEPSP amplitude, and this intensity was used for the remainder of the experiment. A stable baseline recording of 30 minutes was acquired before experimental manipulations were applied. Compounds were applied by addition to the perfusing aCSF, or/as well as being applied while slices were resting in the holding chamber.

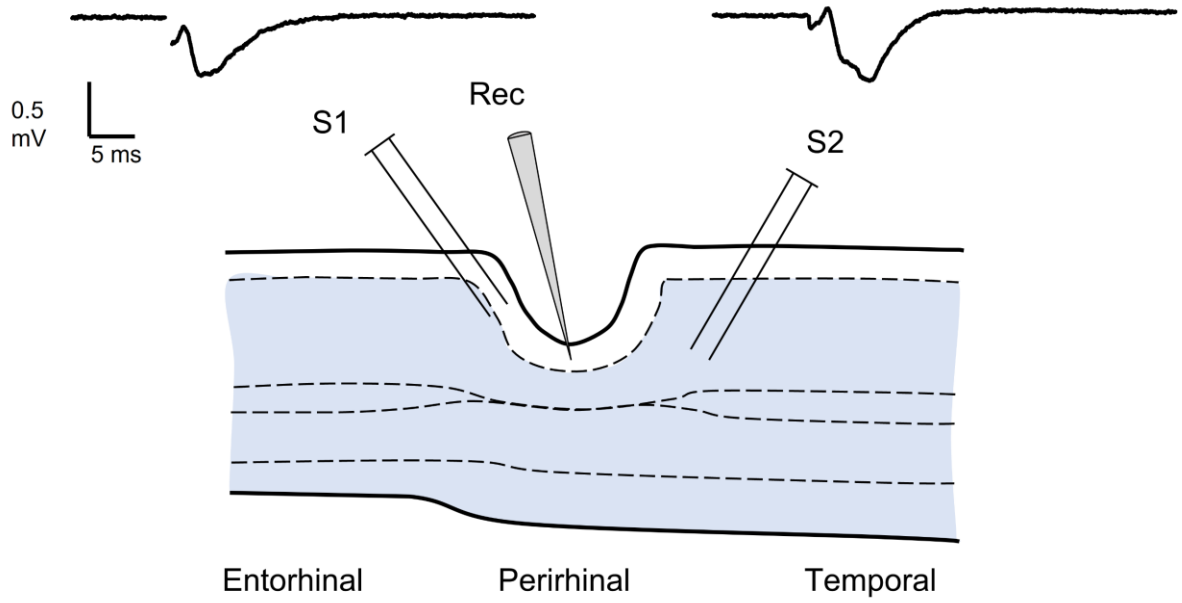

Fig 4.3. Diagram illustrating perirhinal recording set up. *Stimulation electrodes (S1/S2) are placed either side of the recording electrode (Rec), which is positioned directly beneath the rhinal sulcus at the junction between layer I and layer II/III. S1 is placed superficial to this on the entorhinal side, while S2 is placed deeper on the temporal side. Representative traces resulting from stimulation of either side are shown, each trace is an averaged signal from 4 recorded fEPSP sweeps.*

4.2.2. AMPAR:NMDAR ratios

Evoked EPSCs were elicited by placing a bipolar stimulating electrode in fibres of layers II/III to produce action potentials at 0.07 Hz and whole cell voltage clamp recordings were taken from pyramidal neurons located in layer II/III.

For AMPAR:NMDAR ratios, AMPAR mediated EPSCs were evoked at a holding potential of -70 mV, 5 µM NBQX was added to the perfusion and NMDAR mediated EPSC amplitude was measured at -40 and +40 mV holding potentials. While the cell was held at +40 mV, 5 µM Ro-25-6981 (a selective GluN2B antagonist) was perfused in aCSF for a duration of 25 minutes to determine the GluN2A:GluN2B ratio. 50 µM picrotoxin and 1 µM glycine were added to the aCSF for the duration of EPSC recordings to block $GABA_A$ receptor mediated currents and ensure presence of NMDAR co-substrate, respectively. Peak amplitudes were used to calculate the AMPAR:NMDAR ratio.

4.2.3. spontaneous and miniature PSCs

Whole-cell voltage clamp recordings were made in layer III pyramidal cells directly beneath the rhinal sulcus. For sEPSC recordings, 100 µM D-AP5 and 50 µM Picrotoxin were added to the

perfusion to block NMDAR and $GABA_A$ mediated currents, respectively. For mEPSC recordings 1 μM TTX was also added to the perfusion to block action-potential induced neurotransmission. In both instances the holding potential was maintained at -70 mV.

For spontaneous and miniature IPSCs, cells were instead held at +10 mV, and 100 μM D-AP5 and 5 μM NBQX were added to the perfusion to block NMDAR and AMPAR mediated currents, respectively. For mIPSC recordings, 1 μM TTX was included in the perfusion.

4.3. Results

4.3.1. Perirhinal cortex LTD is abolished following chronic MPL treatment

Because NOR memory is known to depend on NMDA receptor dependent LTD in the perirhinal cortex, which can be induced by 3000 pulses at 5 Hz (Griffiths et al. 2008), and chronic MPL treatment results in NOR deficits, I assessed whether this specific form of synaptic plasticity was impaired by chronic steroid treatment. In perirhinal cortex slices from control animals (n = 5), stable LTD of fEPSPs to 86.95 ± 1.572% of baseline could be induced by 3000 pulses at 5 Hz (t_4 = 8.304, P < 0.01, paired t-test vs. baseline, Fig 4.4A), however, in slices from animals chronically treated with MPL, this stimulation protocol could not induce LTD (t_7 = 0.4519, P = 0.665, paired t-test vs. baseline, Fig 4.4B). Therefore, MPL treatment resulted in the abolition of perirhinal LTD, compared to controls (t_{11} = 4.754, P < 0.001, unpaired t-test, Fig 4.4D). This indicates that MPL-induced disruption of synaptic plasticity could underlie the behavioural changes in the NOR task.

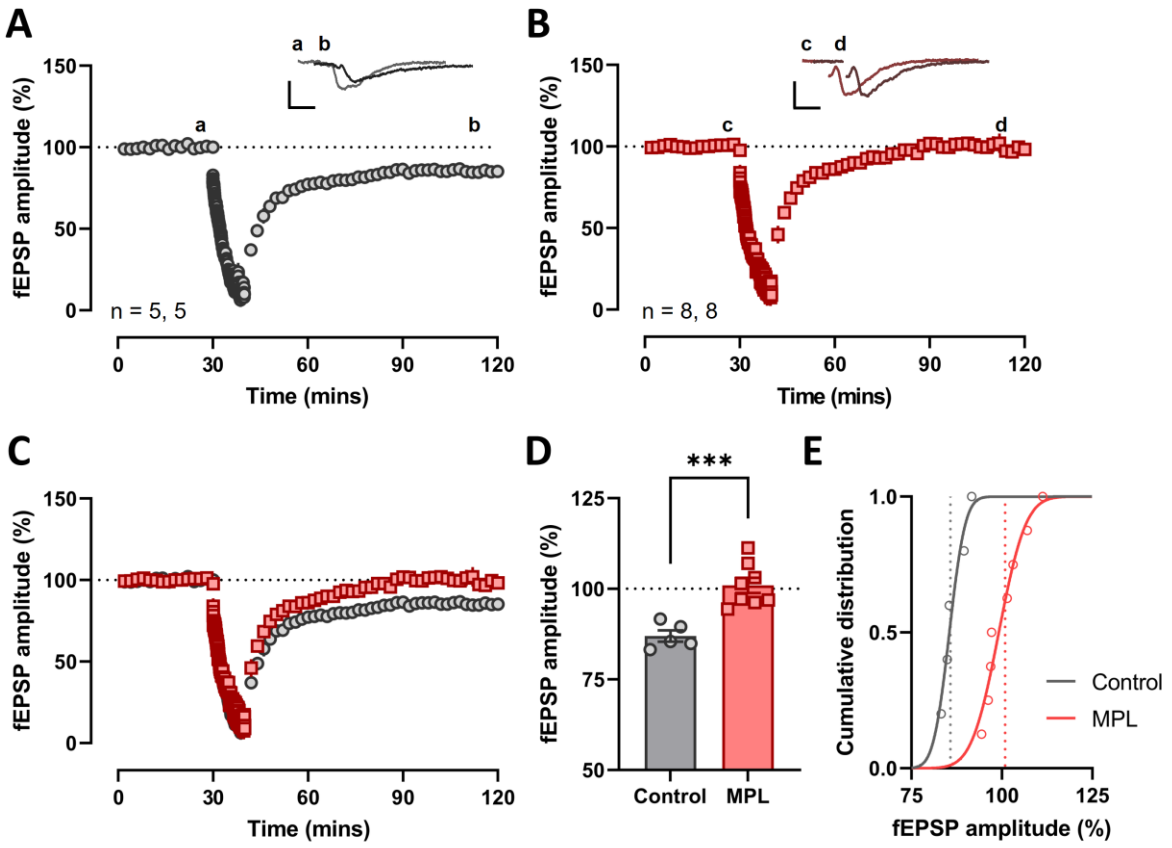

Figure 4.4. The effect of chronic MPL treatment on perirhinal LTD during the active phase. ***A)*** *In PRH slices taken from vehicle treated animals 10 minutes of 5 Hz stimulation reliably and robustly induces depression of fEPSPs recorded at the interface of layers I/II (n = 5, 5) (slices, animals).* ***B)*** *The same stimulation protocol is unable to induce LTD of these synapses in slices taken from rats chronically treated with MPL (n = 8, 8). Scale bars represent x = 5 ms and y = 0.5 mV.* ***C)*** *Overlay of MPL and vehicle treated groups.* ***D)*** *Bar graph illustrating the difference in the degree of fEPSP depression during the final 10 minutes of recording. An unpaired t-test was used to test for statistical significance.* ***E)*** *Cumulative distributions of fEPSP amplitude during the final 10 minutes of recordings. All data are shown as mean ± SEM. Experimental n is indicated as (n = slices, animals). Statistical significance is indicated at P < 0.0001 (****).*

4.3.2. The MPL sensitive LTD protocol is NMDA receptor dependent

In the hippocampus I showed that steroid induced disruption to the dynamic transcription of NMDA receptor subunits resulted in impairments in plasticity and behaviour. Therefore, I hypothesized that in the perirhinal cortex, steroid induced memory and synaptic plasticity deficits also depend on disruption to NMDA receptor-dependent processes. Indeed, blockade of NMDA receptors with 100 µM D-AP5 prevented the induction of LTD by delivery of 3000 pulses at 5 Hz (t_3 = 0.9614, P = 0.4073, paired t-test vs. baseline, Fig 4.4A). D-AP5 application thusly

yielded a significant blockade of LTD when compared to the degree of LTD induced by the same protocol following an hour washout of D-AP5 within the same experiment ($t_3 = 8.265$, $P < 0.01$, paired t-test, Fig 4.4B). Therefore, the protocol that is compromised by chronic MPL exposure is dependent on the activation of NMDA receptors.

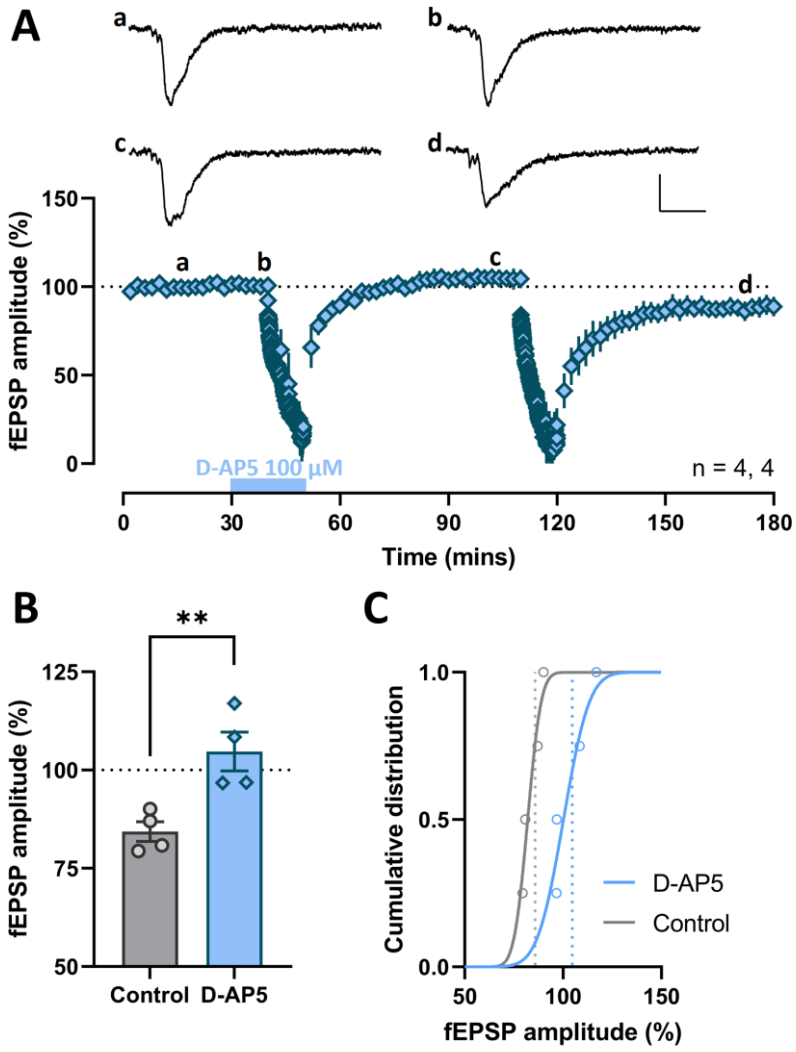

Figure 4.5. Perirhinal LTD depends on NMDA receptor activation. ***A)*** *Addition of D-AP5 (100 µM) to the perfusion system blocks the induction of PRH LTD by 10 minutes of 5 Hz stimulation. Washout of D-AP5 for an hour meant that LTD could be induced once more under control (washout) conditions (n = 4, 4) (slices, animals). Representative traces correspond to letters indicated on the graph. Scale bar represents x = 10 ms and y = 0.2 mV* ***B)*** *Bar graph comparing the average fEPSP amplitude during the 50-60 minute post-LFS period between D-AP5 (n = 4, 4) and washout/control (n = 4, 4) conditions. A paired t-test was used to test for statistical significance, which is indicated at P < 0.01 (**).* ***C)*** *Cumulative distributions of the 50-60 minute fEPSP*

*amplitude average of D-AP5 and washout groups, dotted line represent means. Data in **A** and **B** are plotted as mean ± SEM. Experimental n is indicated as (n = slices, animals).*

4.3.3. Pharmacological characterization of the MPL sensitive LTD protocol

Previous studies have shown the importance of the GluN2B subunit of the NMDA receptor in mediating the neurological effects of chronic stress (Li et al. 2011; Kiselycznyk et al. 2011), and in the previous chapter I showed that this subunit may be critical in MPL-induced hippocampal deficits. Further to this, GluN2B is known to play an important role in the successful expression of synaptic plasticity and memory consolidation (Halt et al. 2012). Since the expression of LTD induced by 3000 pulses at 5 Hz was dependent on NMDA receptor activation (Fig 4.5), specific blockade of GluN2B-containing NMDARs using Ro-25-6981 may also prevent the expression of this form of LTD. However, PRH infusions of the cholinergic muscarinic receptor antagonist Scopolamine have been shown to have variable effects on recognition memory (Winters et al. 2007, 2010), raising the question of the importance of the acetylcholine system in the expression of this form of memory, and introducing another potential mechanism by which MPL could induce memory deficits.

Administration of the selective GluN2B antagonist Ro-25-6981 (5 μM) for 10 minutes prior to, and during, LFS (3000 pulses at 5 Hz) did not prevent an LTD of 17.33 ± 3.784% from being produced (Fig 4.6A). Consequently, 20-minute wash-in of Ro did not produce an LTD different to that seen under control conditions (U = 6, P = 0.1225, Mann-Whitney, Fig 4.6B).

Because a short application of Ro was insufficient to block PRH LTD, and the expression of perirhinal LTD induced by 200 pulses at 1 Hz (in PRH neurons voltage clamped at -70 mV or -40 mV) can be blocked by 20 μM scopolamine, I investigated whether 3000 pulse 5 Hz LTD could be similarly blocked by the muscarinic antagonist. Perfusion of scopolamine (20 μM) for 10 minutes prior to (and during) LFS did not successfully block the induction of LTD (t_4 = 6.840, P < 0.01, paired t-test vs. baseline) (Fig 4.6D). Moreover, the degree of LTD (9.36 ± 1.368%) induced following scopolamine was not significantly different to that induced under control conditions (13.05 ± 1.572%) (U = 3, P = 0.0556, Mann-Whitney, Fig 4.6E).

The combined perfusion of 5 μM Ro and 20 μM scopolamine did not block (t_5 = 5.586, P < 0.01, paired t-test vs. baseline, Fig 4.6G), but rather amplified the degree of LTD (31.84 ± 5.7%), which was significantly greater than in controls (U = 0, P < 0.01, Mann-Whitney, Fig 4.6H).

Because Ro can take a relatively long time to achieve blockade of GluN2B containing NMDA receptors, I assessed whether a longer administration of this drug prior to LFS would be effective at blocking PRH LTD. Ro wash-in for 40 minutes led to a blockade of LTD in perirhinal slices (t_5 = 0.8022, P = 0.4589, paired t-test vs. baseline, Fig 4.6J). However, due to the large

degree of variance in the data, this was also not significantly different to control LTD (U = 12, P = 0.6623, Mann-Whitney, Fig 4.6K).

It should be noted that in the bar graphs in Figure 4.6 multiple datasets have been compared to one set of controls taken from Figure 4.4. This makes the interpretation of the differences in these conditions compared to control conditions more difficult, and an interleaved set of control experiments for each pharmacological manipulation would have been a more appropriate experimental design and the conclusions drawn from such a dataset would be considerably more robust.

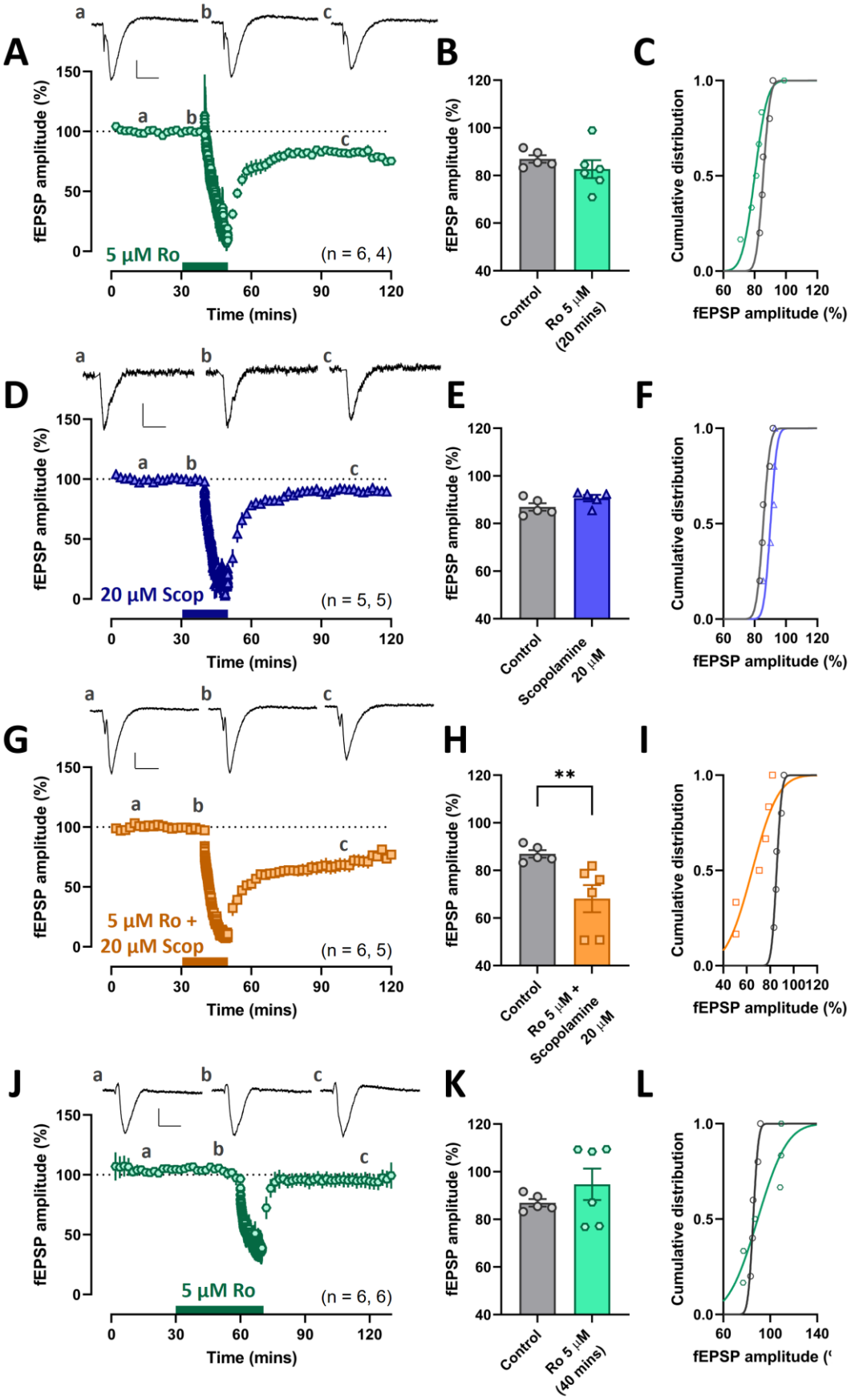
A
B
C
D
E
F
G
H
I
J
K
L
a
b
c
fEPSP amplitude (%)
Time (mins)
5 µM Ro
(n = 6, 4)
20 µM Scop
(n = 5, 5)
5 µM Ro +
20 µM Scop
(n = 6, 5)
5 µM Ro
(n = 6, 6)
Control
Ro 5 µM
(20 mins)
Scopolamine
20 µM
Ro 5 µM +
Scopolamine
20 µM
Ro 5 µM
(40 mins)
**
Cumulative distribution
fEPSP amplitude (%)

Figure 4.6. Pharmacological characterization of perirhinal LTD. ***A)*** *Perfusion of Ro-25-6981 (5 μM) for 10 minutes prior to, and during the 10-minute LFS, did not block the induction of LTD by 3000 pulses at 5 Hz (n = 6, 4) (slices, animals). Scale bar represents x = 10 ms and y = 0.2 mV.* ***B)*** *Bar graph representing the average fEPSP amplitude over the 50-60 minute post-LFS period. A Mann-Whitney test was used to test for statistical significance.* ***C)*** *Cumulative distributions of 50-60 minute fEPSP amplitude averages of drug and control groups.* ***D)*** *Perfusion of Scopolamine (20 μM) for 10 minutes prior to, and during the 10-minute LFS, did not block the induction of LTD by 3000 pulses at 5 Hz (n = 5, 5). Scale bar represents x = 10 ms and y = 0.1 mV.* ***E)*** *Bar graph representing the average fEPSP amplitude over the 50-60 minute post-LFS period. A Mann-Whitney test was used to test for statistical significance.* ***F)*** *Cumulative distributions of 50-60 minute fEPSP amplitude averages of drug and control groups.* ***G)*** *Perfusion of Ro-25-6981 (5 μM) and Scopolamine (20 μM) for 10 minutes prior to, and during the 10-minute LFS, did not block the induction of LTD by 3000 pulses at 5 Hz (n = 6, 5). Scale bar represents x = 10 ms and y = 0.2 mV.* ***H)*** *Bar graph representing the average fEPSP amplitude over the 50-60 minute post-LFS period. A Mann-Whitney test was used to test for statistical significance.* ***I)*** *Cumulative distributions of 50-60 minute fEPSP amplitude averages of drug and control groups.* ***J)*** *Perfusion of Ro-25-6981 (5 μM) for 30 minutes prior to, and during the 10-minute LFS, did block the induction of LTD by 3000 pulses at 5 Hz in 50% of experiments (n = 6, 6). Scale bar represents x = 10 ms and y = 0.2 mV.* ***K)*** *Bar graph representing the average fEPSP amplitude over the 50-60 minute post-LFS period. A Mann-Whitney test was used to test for statistical significance.* ***L)*** *Cumulative distributions of 50-60 minute fEPSP amplitude averages of drug and control groups. Representative traces from each set of experiments are shown with letters corresponding to their positions on the fEPSP graph, with a, b, and c representing baseline, drug, and post-LFS. All data in fEPSP and bar graphs are presented as mean ± SEM. Experimental n is indicated as (n = slices, animals). Statistical significance is indicated at P < 0.01 (**).*

4.3.4. Time of day does not affect AMPAR:NMDAR ratio in PRH principal neurons

In the previous chapter I showed that time of day modulated various aspects of hippocampal function, mediated by the GR driven local molecular clock. Therefore, I investigated whether some aspects of perirhinal synaptic function were also modulated by time of day, given that this region is also subject to dynamic circadian control, which is disrupted by glucocorticoid treatment (Rozwaha 2017). Because expression of the *Grin2* family is known to be modulated by glucocorticoids and time of day (Birnie 2018; Mifsud et al. 2021), in hippocampus at least, I assessed whether AMPAR:NMDAR ratios were liable to change over the course of the day, in line with physiological circadian glucocorticoid rhythms. However, Brown-Forsythe ANOVA did not reveal any significant effect for time of day on AMPAR:NMDAR ratios ($F_{3, 20.71} = 0.9214$, P =

0.4479, Fig 4.7C), and Dunnett's T3 test for multiple comparisons revealed no significant difference between any groups.

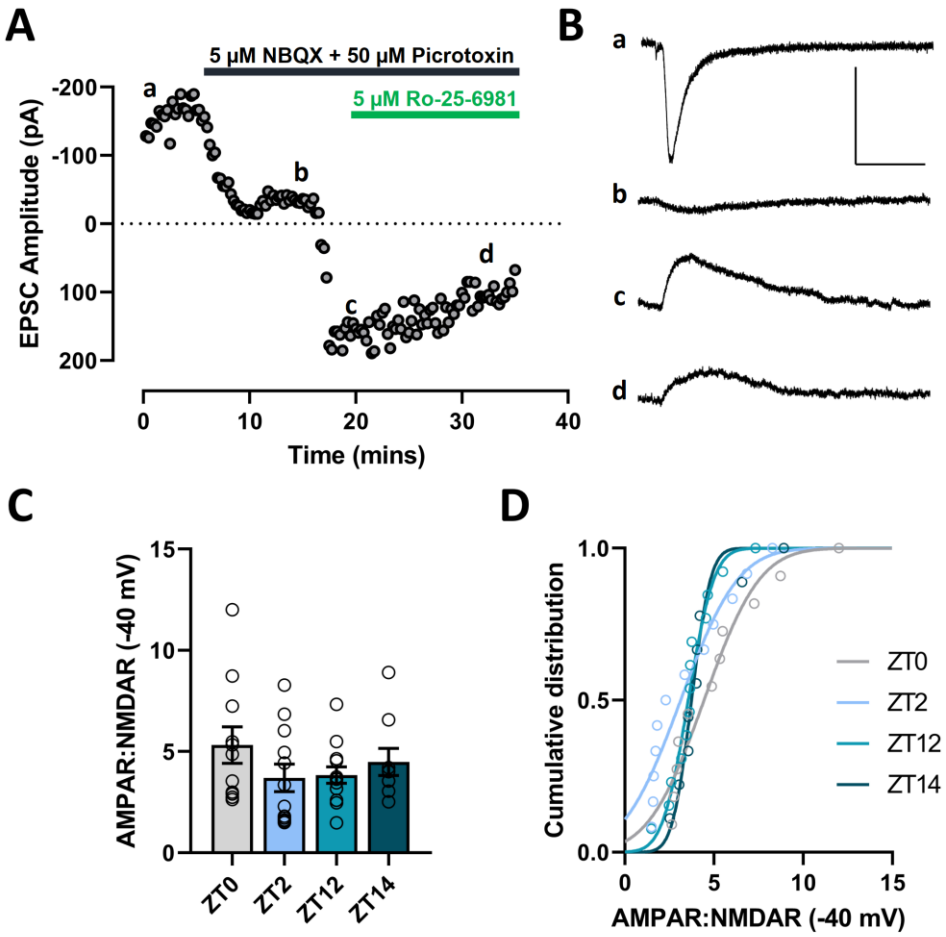

Figure 4.7. Time of day does not affect AMPAR:NMDAR ratio in perirhinal principal cells. ***A)*** *A single representative experiment showing how AMPAR:NMDAR ratios were obtained. AMPAR mediated currents were recorded at -70 mV in the presence of 50 µM picrotoxin. 5 µM NBQX was then added to the perfusion and cells held at -40 mV to relieve the voltage dependent* Mg^{2+} *block. NMDAR mediated EPSCs were recorded at -40 mV and +40 mV before the addition of 5 µM Ro-25-6981 to the perfusion to isolate the GluN2A mediated NMDAR EPSC component.* ***B)*** *Representative traces for AMPAR-mediated EPSCs at -70 mV (a), NMDAR-mediated EPSCs at -40 mV (b), NMDAR-mediated EPSCs at +40 mV (c), and NMDAR-mediated EPSCs after 5 µM Ro-25-6981 (d). Scale bar represents x = 10 ms and y = 50 pA.* ***C)*** *AMPAR:NMDAR ratio recorded at -40 mV was not different across any time point measured in control animals (n = 11, 8; 12, 8; 13, 7; 9, 6) (slices, animals; left to right). Brown-Forsythe ANOVA and Dunnett's T3 multiple comparisons tests were used to test for statistical significance. Data are mean ± SEM.* ***D)*** *Cumulative distributions of AMPAR:NMDAR ratios for each time point.*

4.3.5. AMPAR:NMDAR ratio at ZT12 is increased by MPL treatment

Data from a NOR task showed that chronic MPL treatment led to a deficit in memory consolidation, and these behavioural data were acquired during the rats' active phase. Consistent with this finding, I showed that chronic MPL treatment also resulted in deficits in NMDAR dependent LTD in PRH slices prepared at the onset of the active phase. Consequently, it might be that rhythmic expression of NMDA receptor subunits is compromised by steroid treatment, resulting in alterations to their function at the synapse at a time crucial to learning, during the active phase. I therefore investigated whether chronic MPL treatment induced changes in AMPAR:NMDAR ratio at ZT12 (the onset of the active phase).

AMPAR:NMDAR ratio measured at a holding potential of -40 mV was significantly increased in perirhinal principal neurons from rats treated chronically with MPL (5.408 ± 0.5232, n = 13, 7 (slices, animals)) compared to controls (3.836 ± 0.4062, n = 13, 7) (t_{12} = 2.312, P < 0.05, nested t-test, Fig 4.8B). The same was true when cells were held at +40 mV (1.583 ± 0.1202 vs. 1.177 ± 0.0961, n = 13, 7; 13, 7) (t_{24} = 2.635, P < 0.05, nested t-test, Fig 4.8C).

Because GluN2B-containing NMDARs have been shown to be important in PRH LTD, as well as in mediating the effects of MPL on hippocampal function, it is plausible that the ratio of GluN2A- to GluN2B-containing NMDAR mediated current might be affected by MPL. However, GluN2A:2B ratio was not significantly affected by MPL treatment (2.929 ± 0.2874, n =11, 6) compared to controls (2.157 ± 0.2505, n = 11, 6) (t_{10} = 1.436, P = 0.1816, nested t-test, Fig 4.8D), though there is a non-significant trend for a decrease in the GluN2B mediated component (Fig 4.8G).

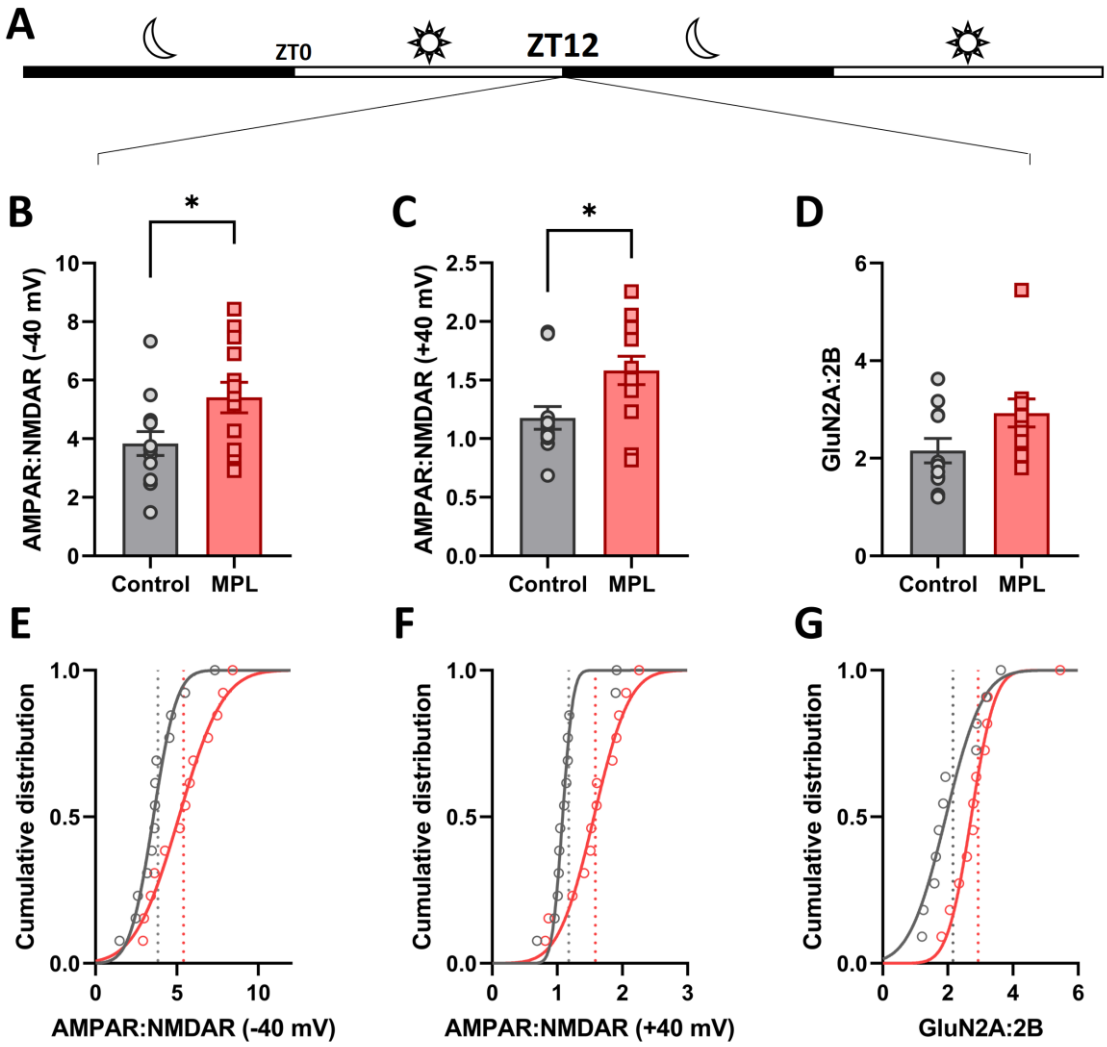

Figure 4.8. Chronic MPL treatment increases AMPAR:NMDAR ratios in PRH principal neurons at ZT12. ***A)*** *Schematic of the light/dark cycle illustrating that these recordings were made at the start of the dark/active period of the rat (ZT12).* ***B)*** *Bar graph showing AMPAR:NMDAR ratio recorded at -40 mV is raised in MPL treated rats (n = 13, 7) (slices, animals) compared to controls (n = 13, 7).* ***C)*** *Bar graph showing that AMPAR:NMDAR ratio recorded at +40 mV is raised in rats treated with MPL (n = 13, 7), compared to controls (n = 13, 7).* ***D)*** *Bar graph representing GluN2A:GluN2B ratios from control and MPL groups.* ***E-G)*** *Cumulative distributions of AMPAR:NMDAR ratios at -40 mV (**E**) and +40 mV (**F**), and GluN2A:2B ratios (**G**). Data in bar graphs are plotted as mean ± SEM. Dotted lines in cumulative distributions represent mean values. Nested t-tests were performed to check for statistical significance, which is indicated at P < 0.05 (*).*

4.3.6. AMPAR:NMDAR ratio in PRH principal neurons at ZT0 is unaffected by MPL treatment

Although no modulation by time was found for AMPAR:NMDAR ratio (Fig 4.7), the disruption of glucocorticoid driven rhythmic expression of target genes in the PRH by MPL treatment indicates that there may be interplay between time of day, glucocorticoid mediated effects, and learning and memory.

AMPAR:NMDAR ratio measured at a holding potential of -40 mV was not significantly different in control (4.755 ± 0.8448, n = 13, 5 (slices, animals)) or MPL (5.307 ± 0.5899, n = 11, 5) groups (t_{22} = 0.5168, P = 0.6105, nested t-test, Fig 4.9B). When measured at a +40 mV holding potential, there was again no difference between control (1.172 ± 0.1397, n = 10, 5) or MPL (1.543 ± 0.133, n = 11, 5) groups (t_{20} = 0.8843, P = 0.3871, nested t-test, Fig 4.9C). GluN2A:2B ratio was not different between control (2.514 ± 0.2997, n = 5, 4) and MPL (2.830 ± 0.1987, n = 6, 5) groups (t_9 = 0.9068, P = 0.3881, nested t-test, Fig 4.9D). These results indicate that unlike at ZT12, MPL did not significantly alter the distribution (and possibly expression) of synaptic AMPARs or NMDARs at this time, suggesting that disruptions to NMDAR-dependent processes may be specific to certain epochs. For instance, the disruption of glucocorticoid directed expression of target genes at ZT12 when GR activation is naturally raised may result in deficits that aren't apparent during the inactive phase, when GR activation is already reduced.

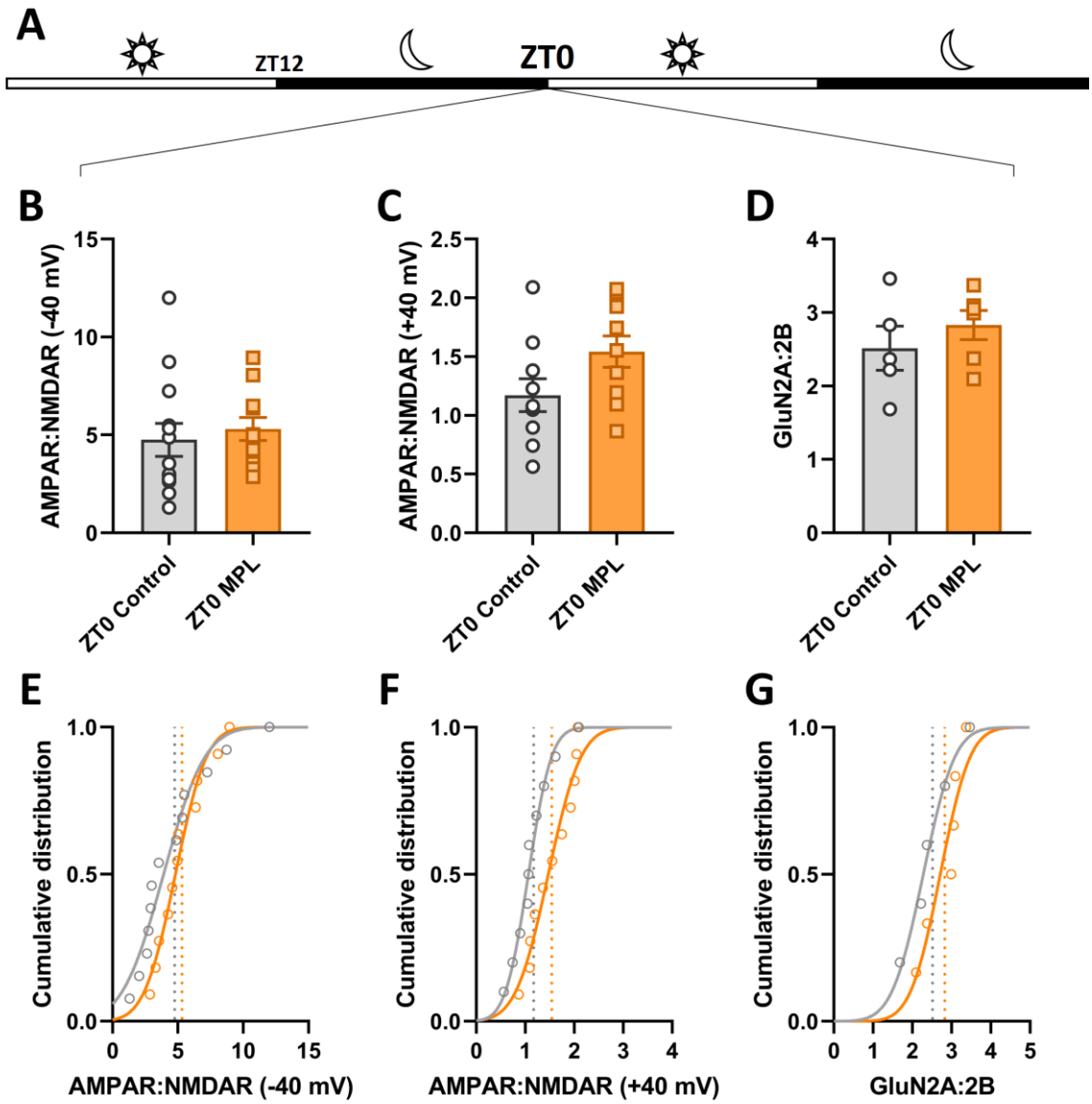

Figure 4.9. MPL does not alter AMPAR:NMDAR ratios in PRH principal neurons at ZT0. ***A)*** *Schematic of the light/dark cycle illustrating that these recordings were made at the start of the light/inactive period of the rat (ZT0).* ***B)*** *Bar graph showing AMPAR:NMDAR ratio recorded at -40 mV is the same in MPL treated rats (n = 11, 5) (slices, animals) compared to controls (n = 13, 5).* ***C)*** *Bar graph showing that AMPAR:NMDAR ratio recorded at +40 mV is not different in rats treated with MPL (n = 11, 5), compared to controls (n = 10, 5).* ***D)*** *Bar graph representing GluN2A:GluN2B ratios from control (n = 5, 4) and MPL (n = 6, 5) groups.* ***E-G)*** *Cumulative distributions of AMPAR:NMDAR ratios at -40 mV (**E**) and +40 mV (**F**), and GluN2A:2B ratios (**G**). Data in bar graphs are plotted as mean ± SEM. Dotted lines in cumulative distributions represent mean values. Experimental n is indicated as (n = slices, animals). Nested t-tests were performed to test for statistical significance.*

4.3.7. AMPAR:NMDAR ratio is altered by treatment, but not time

When data from both time points are collated, there was no effect of treatment ($F_{1,46}$ = 2.961, P = 0.092), time ($F_{1,46}$ = 0.4378, P = 0.5115), or interaction ($F_{1,46}$ = 0.6830, P = 0.4128, Two-way ANOVA, Fig 4.10A) for AMPAR:NMDAR ratios with the NMDAR mediated current recorded at a -40 mV holding potential. On the other hand, when the NMDAR currents were recorded at +40 mV, a significant effect of treatment ($F_{1,43}$ = 10.17, P < 0.01) was found, but not for time ($F_{1,43}$ = 0.0341, P = 0.8544) or interaction between time and treatment ($F_{1,43}$ = 0.0202, P = 0.8876, Two-way ANOVA, Fig 4.10B). No effect of treatment ($F_{1,29}$ = 2.022, P = 0.1657), time ($F_{1,29}$ = 0.014, P = 0.9067), or interaction ($F_{1,29}$ = 0.1841, P = 0.0.671, Two-way ANOVA, Fig 4.10C) was found for GluN2A:2B ratios. Tukey's multiple comparisons test did not report any statistical significance between the means of any groups in any of the conditions. It must be noted, though, that in the whole-cell patch clamp experiments assessing the AMPAR:NMDAR ratios in perirhinal neurons, glycine was added to the perfusate in order to ensure the presence of co-agonist for NMDAR activation, whereas in field electrophysiology experiments assessing synaptic plasticity no glycine was added to the perfusate. This makes the interpretation of any comparison between the field and whole-cell data somewhat difficult, as there is a possibility that the presence or absence of glycine from either condition could affect the experimental outcome.

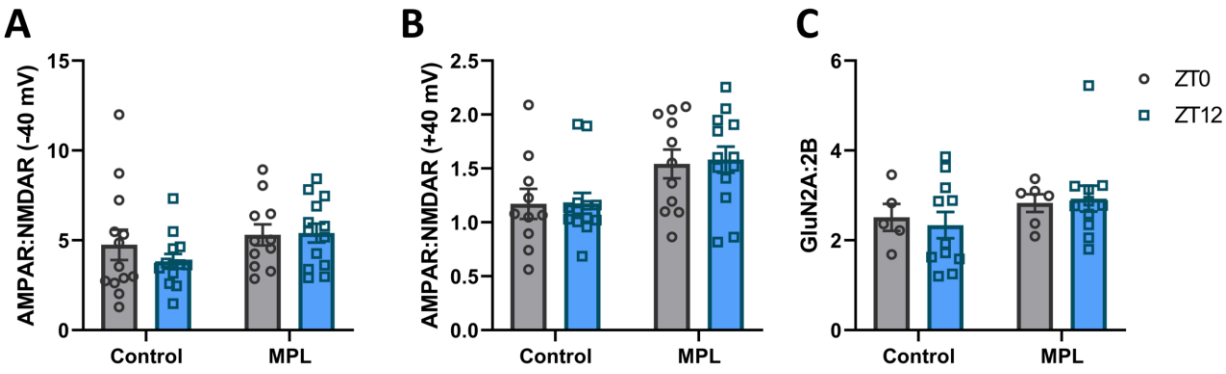

Figure 4.10. AMPAR:NMDAR ratios across treatment and time. ***A)*** *AMPAR:NMDAR ratios recorded at -40 mV in control and MPL groups at ZT0 and ZT12 (n = 13, 5; 13, 7; 13, 5; and 13, 7 (slices, animals; left to right)).* ***B)*** *AMPAR:NMDAR ratios recorded at +40 mV in control and MPL groups at ZT0 and ZT12 (n = 10, 5; 13, 7; 11, 5 and 13, 7 (left to right)).* ***C)*** *GluN2A:2B ratios in control and MPL groups recorded at ZT0 and ZT12 (n = 5, 4; 11, 5; 6, 5; and 11, 5 (left to right)). Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance. Data are presented as mean ± SEM.*

4.3.8. MPL induces circadian changes in mEPSC frequency

Relatively little is known about the functional impact of spontaneous synaptic events, though evidence has accumulated demonstrating the importance of this distinct form of neurotransmission in the modulation of dendritic protein synthesis and homeostatic plasticity (Sutton et al. 2007; Sutton et al. 2006). In the previous chapter I reported disruption to the natural rhythms and balance of mEPSC and mIPSC events in the hippocampus by the chronic action of MPL, therefore, similar aberrations may occur in the PRH.

A significant effect of time ($F_{1, 83} = 8.417$, $P < 0.01$), but not treatment ($F_{1, 83} = 0.1052$, $P = 0.7465$) was found for mEPSC frequency. There was also a significant interaction between the two factors ($F_{1, 83} = 10.48$, $P < 0.01$, Two-way ANOVA, Fig 4.11A). Tukey's multiple comparisons test further revealed a significant difference between the means of the ZT12 control and ZT12 MPL groups ($P < 0.05$) and between the ZT0 MPL and ZT12 MPL groups ($P < 0.001$). In the hippocampus I reported an inversion of the daily rhythm of mEPSC frequency, however in PRH neurons under control conditions, no difference was found between ZT0 and ZT12. Interestingly, following MPL treatment, mEPSC frequency was suppressed at ZT12 and increased at ZT0.

For mEPSC amplitude, a significant effect of time ($F_{1, 83} = 8.579$, $P < 0.01$), but not treatment ($F_{1, 83} = 0.9375$, $P = 0.3357$) or interaction ($F_{1, 83} = 0.3618$, $P = 0.5491$, Two-way ANOVA, Fig 4.11C) was found. Tukey's multiple comparisons revealed a significant difference between the ZT0 control and ZT12 MPL groups ($P < 0.05$). Similar to the results from the hippocampus, it is the frequency, rather than the amplitude, of spontaneous events that changes with time of day and chronic MPL treatment.

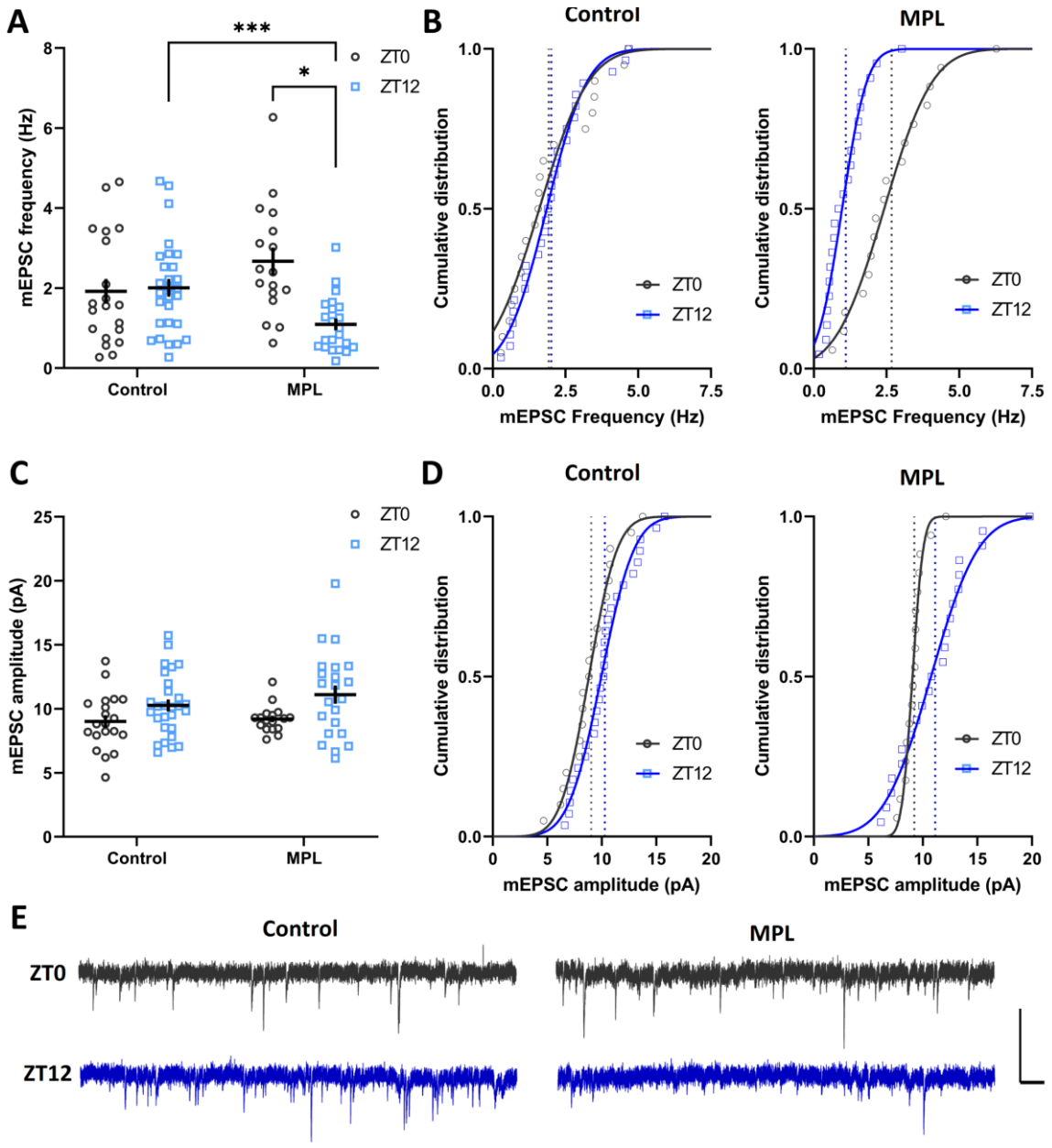

Figure 4.11. mEPSC frequency not amplitude is affected by MPL treatment. ***A)*** *mEPSC frequency at ZT0 (n = 20, 8, 8) (cells, slices, animals) and ZT12 (n = 28, 9, 8) in control, and at ZT0 (n = 17, 10, 8) and ZT12 in MPL, groups (n = 22, 9, 8).* ***B)*** *Cumulative distributions of mEPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***C)*** *mEPSC amplitude at ZT0 (n = 20, 8, 8) and ZT12 (n = 28, 9, 8) in control, and at ZT0 (n = 17, 10, 8) and ZT12 (n= 22, 9, 8) in MPL, groups.* ***D)*** *Cumulative distributions of mEPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***E)*** *Representative traces from each condition, scale bar represents x = 200 ms and y = 20 pA. Data in* ***A*** *and* ***C*** *are plotted as mean ± SEM. Dotted lines in* ***B*** *and* ***D*** *represent means. Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*) and P < 0.001 (***).*

4.3.9. MPL inverts the circadian rhythm of sEPSC frequency in PRH principal cells

sEPSCs are recorded in the absence of TTX (a voltage-gated sodium channel blocker), therefore action potential induced events are preserved. Thusly, in this recording environment, a relative change in the local network activity (within the slice preparation) can be inferred.

A significant effect of treatment ($F_{1, 34} = 6.947$, $P < 0.05$), but not time ($F_{1, 34} = 0.7088$, $P = 0.4057$) was found for sEPSC frequency. A significant interaction between the two factors was also reported ($F_{1, 34} = 20.49$, $P < 0.0001$, Two-way ANOVA, Fig 4.12A). Tukey's multiple comparisons test further showed a significant difference between the ZT0 control (1.269 ± 0.4268 Hz, n = 10) and ZT12 control groups (3.025 ± 0.4667 Hz, n = 11) ($P < 0.05$), indicating a modulation by time of sEPSC frequency in control rats. Tukey's also showed a significant difference between the ZT0 MPL (4.682 ± 0.5096 Hz, n = 10) and ZT12 MPL groups (2.124 ± 0.4221 Hz, n = 7) ($P < 0.01$), but crucially, this pattern was the reverse of that observed in control animals, indicating a phase-shift of the temporal modulation of sEPSC frequency induced by MPL. A significant increase at ZT0 in MPL compared to control ($P < 0.0001$) groups was largely responsible for this effect, showing that PRH network activity is greatly increased in slices from MPL animals during the inactive phase.

For sEPSC amplitude, a significant effect of neither time ($F_{1, 34} = 0.1124$, $P = 0.7395$) nor treatment ($F_{1, 34} = 3.790$, $P = 0.0599$) could be found. However, an interaction between the two factors was reported ($F_{1, 34} = 6.828$, $P < 0.05$, Two-way ANOVA, Fig 4.12A). Tukey's multiple comparisons test also showed a significant increase in sEPSC amplitude in MPL (12.3 ± 0.6322 pA, n = 10) compared to control (9.278 ± 0.182 pA, n = 10) ($P < 0.05$) groups at ZT0, indicating that both the frequency and amplitude of sEPSC events were increased at the start of the inactive phase in the PRH of MPL treated rats, compared to time-matched controls.

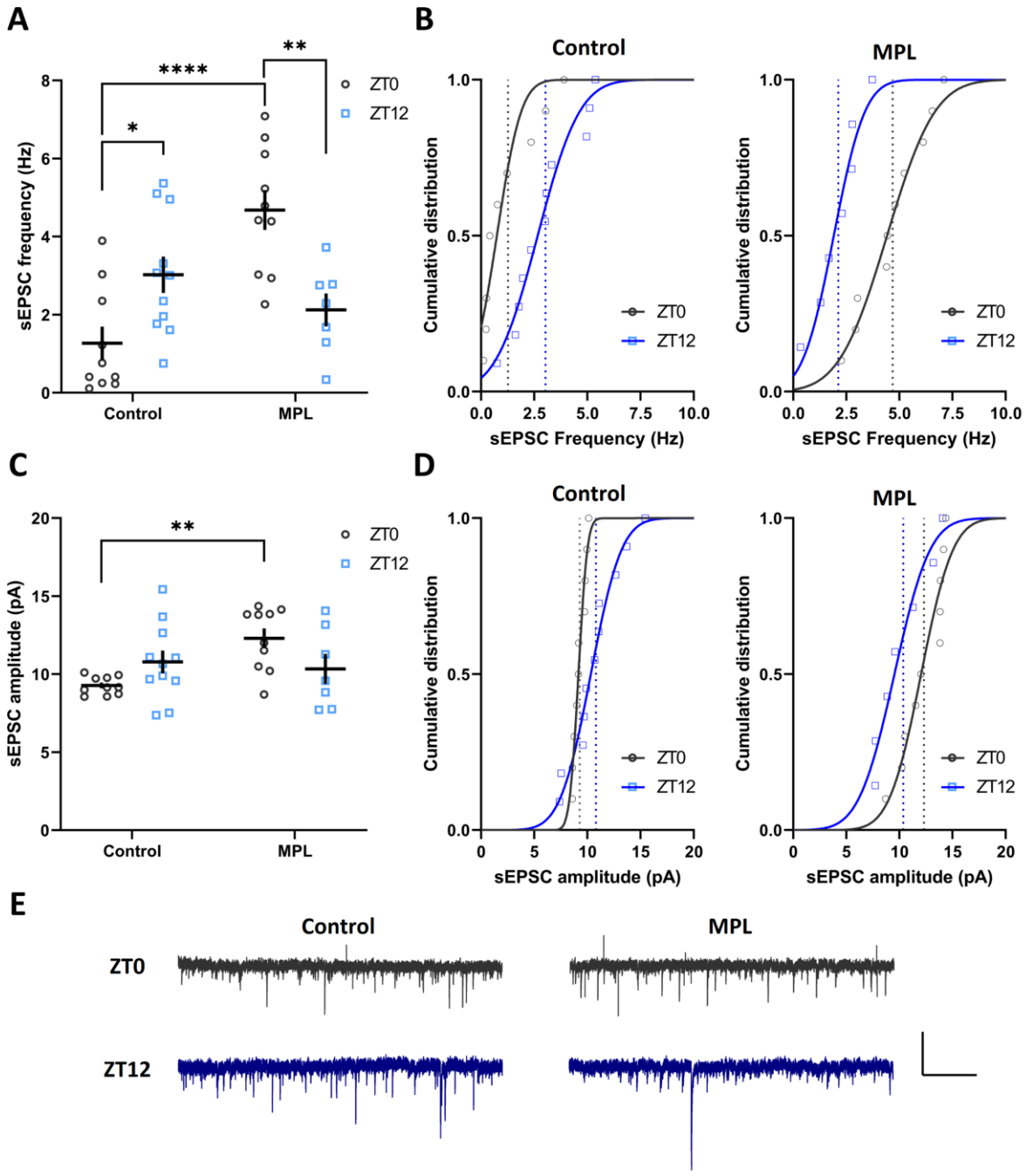

Figure 4.12. MPL inverts the circadian fluctuation in sEPSC frequency in perirhinal cortex. ***A)*** *sEPSC frequency at ZT0 (n = 10, 10, 8) (cells, slices, animals) and ZT12 (n = 11, 11, 8) in control, and at ZT0 (n = 10, 10, 8) and ZT12 (n = 7, 7, 7) in MPL, groups.* ***B)*** *Cumulative distributions of sEPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***C)*** *sEPSC amplitude at ZT0 (n = 10, 10, 8) (cells, slices, animals) and ZT12 (n = 11, 11, 8) in control, and at ZT0 (n = 10, 10, 8) and ZT12 (n = 7, 7, 7) in MPL, groups.* ***D)*** *Cumulative distributions of sEPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***E)*** *Representative traces from each condition, scale bar represents x = 1 s and y = 20 pA. Data in* ***A*** *and* ***C*** *are plotted as mean ± SEM.*

*Dotted lines in **B** and **D** represent means. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparisons tests were used to test for statistical significance, which is indicated at P < 0.05 (*), P < 0.01 (**), and P < 0.0001 (****).*

4.3.10. MPL alters mIPSC amplitude, not frequency

Prolonged activation of glutamate receptors can lead to a cascade of neurotoxicity (excitotoxicity). These abnormalities include mitochondrial dysfunction, reactive oxygen species overproduction, and the release of pro-apoptotic factors, which can all result in neuronal damage, and excitotoxicity has been associated with the pathogenesis of neurodegenerative disease (Armada-Moreira et al. 2020). The balance between excitatory and inhibitory neurotransmission is thought to be crucial in maintaining the brain in a stable state and preventing excitotoxic damage that can lead to neurodegeneration. Because MPL caused shifts in the frequency of spontaneous and miniature EPSCs, it was important to assess whether compensatory changes to inhibitory EPSCs had occurred, or whether alterations to the delicate balance between excitation and inhibition in the PRH had been caused by chronic MPL treatment.

Two-way ANOVA revealed no significant effect on mIPSC frequency by time ($F_{1, 79}$ = 0.5546, P = 0.4587), treatment ($F_{1, 79}$ = 0.6386, P = 0.4266), or interaction ($F_{1, 79}$ = 0.2567, P = 0.6138, Two-way ANOVA) and Tukey's multiple comparisons test demonstrated no significant differences between the means of any group (Fig 4.13A).

On the other hand, two-way ANOVA showed a significant main effect of time on mIPSC amplitude ($F_{1, 79}$ = 10.93, P < 0.01). No main effect, though, could be found for treatment ($F_{1, 79}$ = 2.196, P = 0.1424) or interaction ($F_{1,79}$ = 3.398, P = 0.069, Two-way ANOVA) and Tukey's multiple comparisons test revealed a significant increase at ZT0 in the MPL group (11.53 ± 0.4230 pA, n = 20) compared to ZT12 MPL (9.696 ± 0.2970 pA, n = 13) (P < 0.01) and compared to ZT12 control (9.825 ± 0.2368 pA, n = 28) (P < 0.01) (Fig 3.13C).

The small increase in mIPSC amplitude observed at ZT0 in the MPL group may not be sufficient to offset the relatively larger changes to mEPSC frequency observed at ZT0 and ZT12 in the MPL group, and this augmented excitatory drive may underlie some of the neuronal degradation that can be seen following bouts of chronic stress (Vyas et al. 2002).

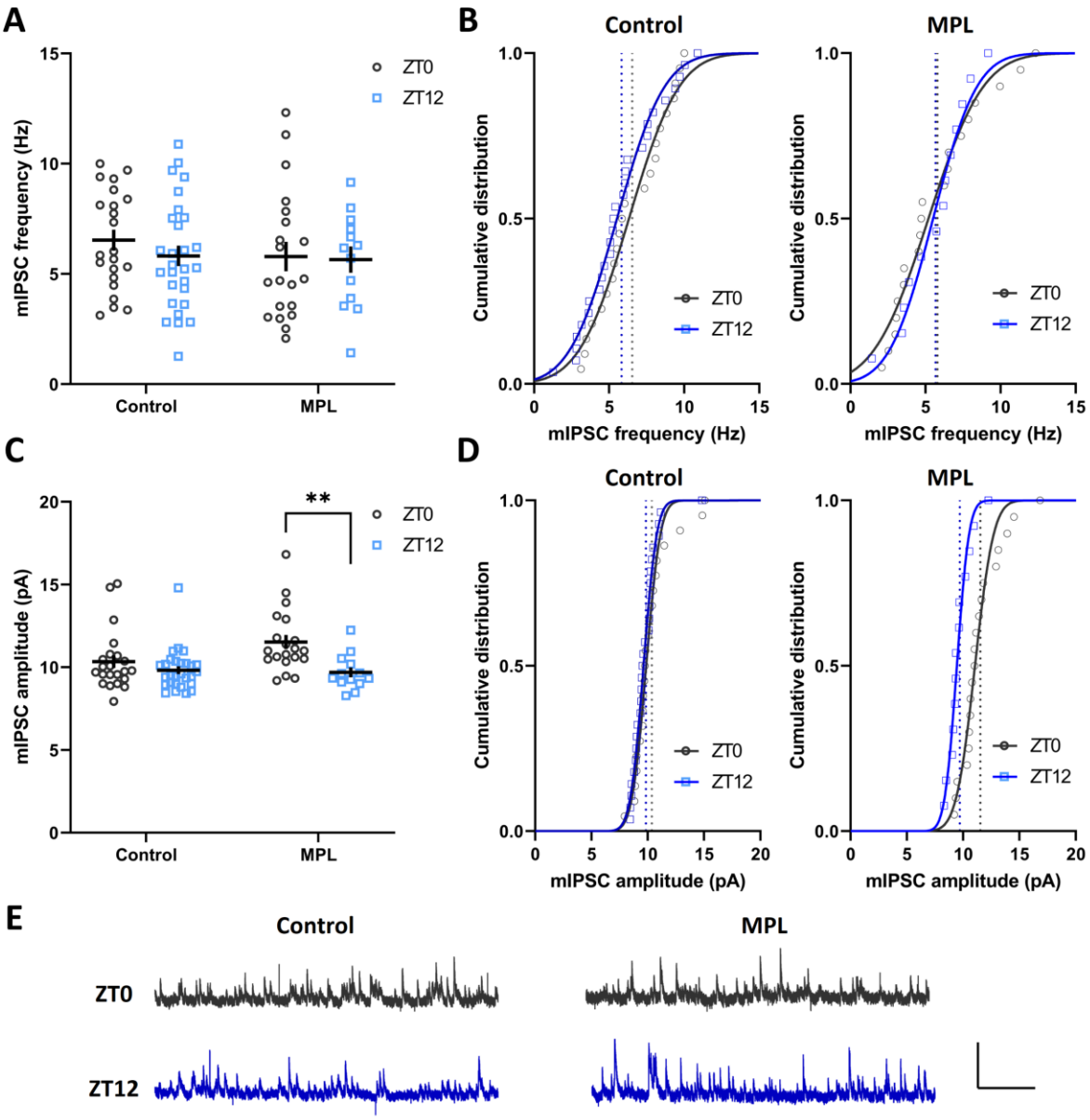

Figure 4.13. MPL treatment effects on mIPSC frequency and amplitude. ***A)*** *mIPSC frequency at ZT0 (n = 22, 7, 8) (cells, slices, animals) and ZT12 (n = 28, 10, 8) in control, and at ZT0 (n = 20, 8, 7) and ZT12 in MPL, groups (n = 13, 4, 4).* ***B)*** *Cumulative distributions of mIPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***C)*** *mIPSC amplitude at ZT0 (n = 22, 8, 8) (cells, slices, animals) and ZT12 (n = 28, 10, 8) in control, and at ZT0 (n = 20, 8, 7) and ZT12 in MPL, groups (n = 13, 4, 4).* ***D)*** *Cumulative distributions of mIPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***E)*** *Representative traces from each condition, scale bar represents x = 1 s and y = 20 pA. Data in* ***A*** *and* ***C*** *are plotted as mean ± SEM. Dotted lines in* ***B*** *and* ***D*** *represent means. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance, which is indicated at P < 0.01 (**).*

4.3.11. MPL does not affect sIPSC properties

Unlike the effects reported on sEPSC properties, no significant main effects were found on sIPSC frequency by either time ($F_{1, 29} = 1.591$, $P = 0.2172$), treatment ($F_{1, 29} = 0.7953$, $P = 0.3799$), or interaction ($F_{1, 29} = 0.2246$, $P = 0.6391$, Two-way ANOVA, Fig 4.14A).

Although no main effect of time ($F_{1, 29} = 3.622$, $P = 0.067$) or treatment ($F_{1, 29} = 1.633$, $P = 0.2114$) could be found, a significant interaction between the two factors was reported ($F_{1, 29} = 4.753$, $P < 0.05$, Two-way ANOVA). Tukey's multiple comparisons test revealed no significant difference between the means of any group (Fig 4.14C). This indicates a large shift in the excitatory network activity in the PRH induced by MPL, which is not mirrored by any changes to inhibitory network activity, therefore potentially leading to excitotoxicity, a phenomenon linked with many neurological diseases (Beal 1992).

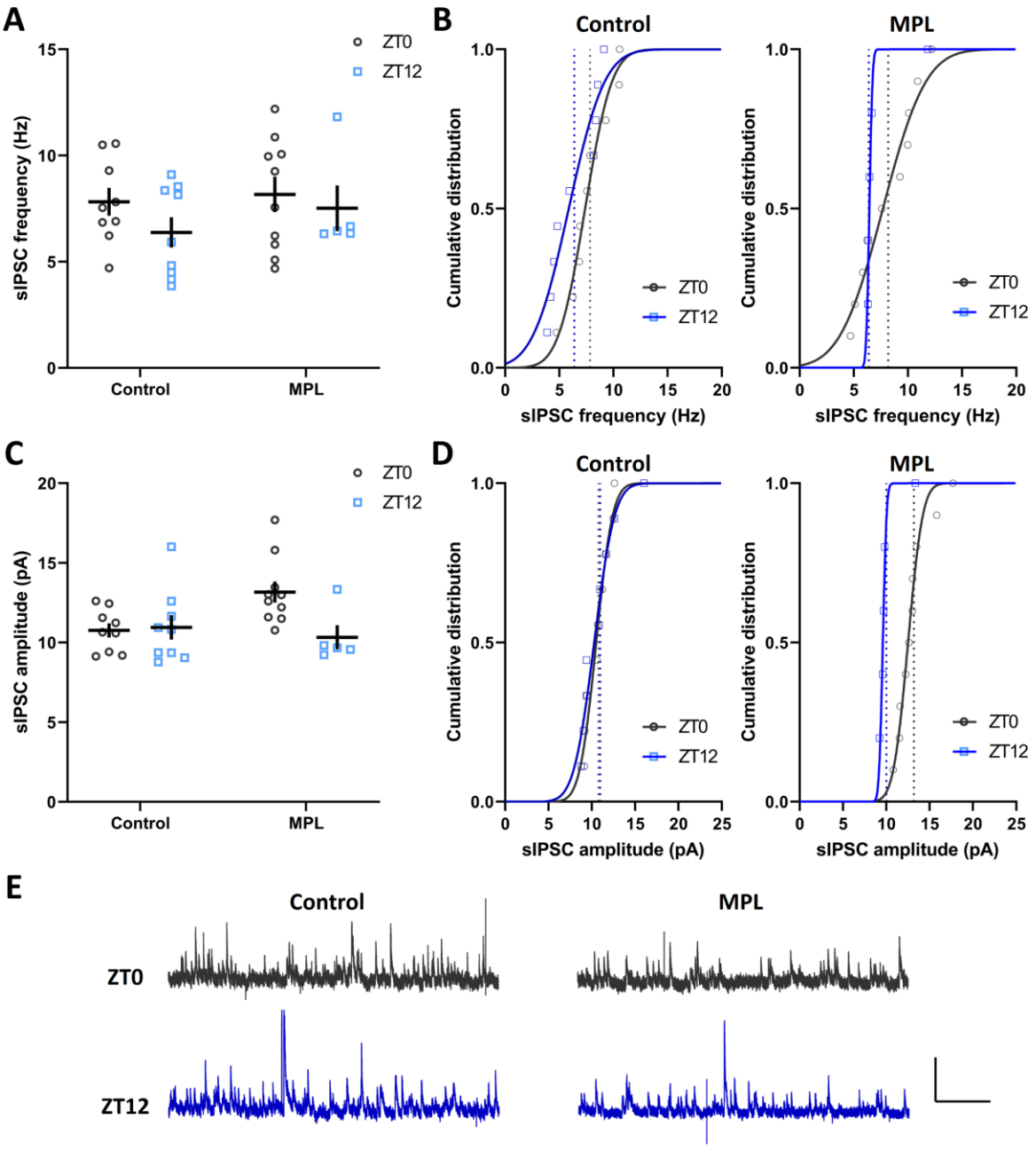

Figure 4.14. MPL does not alter sIPSC frequency or amplitude. ***A)*** *sIPSC frequency at ZT0 (n = 9, 9, 8) (cells, slices, animals) and ZT12 (n = 9, 9, 8) in control, and at ZT0 (n = 10, 10, 7) and ZT12 in MPL, groups (n = 5, 5, 4).* ***B)*** *Cumulative distributions of sIPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***C)*** *sIPSC amplitude at ZT0 (n = 9, 9, 8) (cells, slices, animals) and ZT12 (n = 9, 9, 8) in control, and at ZT0 (n = 10, 10, 7) and ZT12 in MPL, groups (n = 5, 5, 4).* ***D)*** *Cumulative distributions of sIPSC frequencies at ZT0 and ZT12 in controls (left) and MPL (right).* ***E)*** *Representative traces from each condition, scale bar represents x = 1 s and y = 20 pA. Dotted lines in* ***B*** *and* ***D*** *represent means. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance.*

4.4. Discussion

Chronic treatment with methylprednisolone, a mixed MR/GR agonist, causes impairments in a novel object recognition (NOR) memory task following a delay after acquisition of 6 h or more, but not following a 1 h delay. This form of recognition memory is known to depend heavily upon the neurons of the PRH, and qPCR experiments revealed that the same treatment disrupted the circadian profile of gene expression in this region (Rozwaha 2017).

Since deficits in NOR performance are present at 6 h, but not 1 h, post-learning, it is likely that MPL treatment is affecting the consolidation phase of memory. This is the process by which a labile short-term memory is converted into a more stable long-term memory, and has been reported to involve gene expression and protein synthesis (Kandel 2001). Early evidence from adrenalectomized rats revealed that GR activation is a fundamental modulator of this process (Roozendaal, Portillo-Marquez, and McGaugh 1996; Oitzl and De Kloet 1992). Chronic steroid treatment is known to result in memory problems in both animal and human studies (Keenan et al. 1996; Coluccia et al. 2008; McLay, Freeman, and Zadina 1998). The effects of acute steroid exposure, and of chronic stress on the hippocampus and amygdala, brain regions crucial for different types of memory processing, are well documented (Roozendaal, McEwen, and Chattarji 2009b; McEwen 2001; Roozendaal 2003). However, detailed studies of chronic steroid exposure, particularly in other brain regions important in memory, like PRH, are sparse. Moreover, although many studies have reported the various effects of chronic stress in the hippocampus, the importance of the GR and molecular clock driven rhythms in the transcriptome, and their impacts on memory/synaptic physiology are novel findings.

The expression of LTD induced by 10 minutes of 5 Hz stimulation was completely abolished in PRH slices taken from animals chronically treated with MPL (Fig 4.4). This form of PRH LTD has been shown to be crucial in successful encoding of NOR memory (Winters and Bussey 2005; Griffiths et al. 2008). The blockade of NMDA receptors using the competitive inhibitor D-AP5 inhibits the expression of the same form of LTD at PRH layer I/II synapses that is abolished by MPL treatment (Fig 4.5), indicating that chronic steroid exposure may incur alterations to NMDA receptor signalling. NMDA receptors are known to play a critical role in many common forms of synaptic plasticity in the brain (Malenka and Nicoll 1993), primarily by facilitating the modification and movement of ligand-gated ion channels in the post-synaptic neuron. Given that GR and MR are ligand-activated transcription factors (Escriva, Delaunay, and Laudet 2000), it is plausible that altered transcription of NMDA receptor subunits could account for the deficits reported in synaptic plasticity, although confirmation of this with molecular techniques is needed.

The results described in this chapter demonstrate that, similar to the effects of chronic stress on neurons of the hippocampus (McEwen 2001), chronic exposure to corticosteroids results in deficits to synaptic plasticity in PRH neurons. Synaptic plasticity has been demonstrated to be a crucial correlate of learning and underlies the successful encoding of memory in several brain regions (Neves, Cooke, and Bliss 2008; Bear 1996; Maren 2003), and so this impairment could account for the reported deficits in the NOR task following MPL treatment (Birnie 2018). However, the use of one dataset of controls for comparison against multiple other data sets in this chapter makes the interpretation of results from the plasticity studies more challenging. Indeed, in using an interleaved experimental design, the conclusions drawn from this data would be significantly more robust.

The importance of the perirhinal cortex in memory was first established following lesion studies (Aggleton, Kyd, and Bilkey 2004), but there is now considerable evidence from many avenues supporting its role in recognition memory in rodents (Brown and Aggleton 2001), as well as a more nuanced role in associative recognition memory in monkeys and humans (Miyashita 2019). There has been little research, however, into the impact that chronic steroid exposure has on this brain region. Though this chapter focuses on single item recognition in rodents, a highly PRH specific task (Aggleton, Kyd, and Bilkey 2004), the research may be translatable to a more complex set of cognitive deficits reported by patients on long-term steroid treatment (Brown and Chandler 2001; Keenan et al. 1996; Naber, Sand, and Heigl 1996). Studies in primates have revealed that, besides its role in single item recognition, the PRH also serves complex roles in associative memory (Naya, Yoshida, and Miyashita 2003), indicating that chronic steroid exposure could have further reaching implications on memory besides the reported deficits in a NOR task through the disruption of perirhinal rhythms and plasticity.

GluN2B containing NMDA receptors have been shown to be key in mediating the impacts of corticosteroids (Mikasova et al. 2017) and chronic stress on neuronal function in the hippocampus (Kiselycznyk et al. 2011) and amygdala (Lehner et al. 2017), and in this chapter I have shown that the form of PRH LTD affected by chronic MPL treatment can be altered by blockade of GluN2B containing NMDA receptors by application of the highly selective antagonist Ro 25-6981. *Grin2b* is associated with GR binding sites (Barik et al. 2010), and its transcription is directly regulated by glucocorticoids (Mifsud et al. 2021), suggesting that disruption to the expression profile of GluN2B NMDA receptor subunits by MPL is a plausible mechanism resulting in impaired LTD. However, there are many forms of synaptic plasticity and many molecular mechanisms that underlie these events, and the chronic activation of MR/GR may result in much wider reaching effects on transcription, epigenetics and post translational modification that could also impact the changes in synaptic efficacy that support learning and

memory. Indeed, in the hippocampus, the rhythmic expression of many other proteins associated with plasticity (like CaMKII) is disrupted (Birnie 2018), however, further studies using RNAseq would be needed to confirm whether the same transcriptomic disruptions are taking place in the PRH. Moreover, the effects of MPL on plasticity mediated by NMDARs of differing subunit composition remains to be investigated. Nevertheless, the important role that GluN2B containing NMDA receptors seem to play in mediating the impacts of steroid use on learning and memory highlights them as potential therapeutic target in reducing side effects associated with steroid treatment.

Synthetic steroids like MPL have a significantly higher binding affinity and half-life than endogenous corticosteroids (Stavreva et al. 2009), meaning that chronic use results in significant deviations from the natural pulsatile nature of the HPA axis. The impact that the ultradian and circadian nature of glucocorticoid action has on cognition, and how disruption to these physiological release patterns might disrupt mnemonic processes has not been adequately addressed. Only more recently have studies aimed to investigate this. Several pathophysiologies have now been associated with chronic disruption to glucocorticoid rhythms, but the mechanisms of their interactions remain unclear (Keller et al. 2006; Yehuda et al. 1996).

Deviations from the ultradian/circadian glucocorticoid release induced by chronic MPL treatment are reflected by aberrations to the natural expression profile of clock genes in PRH (Rozwaha 2017). It has been recently demonstrated that the pulsatile nature of the HPA axis is crucial for normal cognitive function in humans (Kalafatakis et al. 2018), and electrophysiogical investigations of amygdala and hippocampus have shown that corticosterone pulses are necessary to maintain the plasticity of neural connections (den Boon et al. 2019; Sarabdjitsingh et al. 2016b; Sarabdjitsingh et al. 2014; Karst et al. 2010). Spontaneous neurotransmission is also thought to play a pivotal role in maintaining certain forms of neural plasticity, and this distinct form of neurotransmission is responsive to glucocorticoid pulses (Sarabdjitsingh et al. 2014), providing a potential mechanism by which GCs may maintain homeostasis and plasticity. Clock genes, particularly *Per1,* are known to be associated with learning and memory processes and disruption to their natural expression profiles may present another route by which chronic steroid exposure disrupts healthy cognitive processing. This could result in memory loss and perhaps neuropathologies like depression. Indeed, mice lacking *Per1* have been shown to exhibit significantly altered time-dependent memory performance, accompanied by loss of regulation of the phosphorylation of CREB in the hippocampus (Rawashdeh et al. 2014), which is known to be pivotal in memory processing (Mizuno et al. 2002). *Per1* knockout mice also exhibit dysregulation of the co-ordinated expression of other clock genes (*Per2, Cry1, Cry2, Clock, Bmal1*) (Jilg et al. 2010), similar to the effect induced by chronic MPL treatment. Targeted

knockout of *Bmal1*, another essential timing gene, from excitatory forebrain neurons also induces cognitive deficits (Snider et al. 2016), indicating that the circadian regulation of cognition by clock genes is important in other brain regions. Since *Per1* knockout mice exhibited altered hippocampal LTP *in vivo* as well as circadian modulation of histone modifications (Rawashdeh et al. 2014), it is possible that perturbations to the molecular clock mechanisms of PRH neurons could underlie the deficits in synaptic plasticity and recognition memory that are reported in this chapter.

It is likely that chronic MPL exposure leads to not only the desynchronization of the local molecular clock, which is driven by GCs, but also disruption to the dynamic transcription of many other GC regulated target genes, as RNAseq data has demonstrated in the hippocampus. MPL induced disruption to physiological glucocorticoid rhythms also interferes with spontaneous neurotransmission in the PRH. I have shown that mEPSC frequency is suppressed at ZT12 and increased at ZT0 in MPL treated rats, compared to controls, and that these changes are not compensated for by similar alterations to mIPSC properties.

Recently, mutations to *SNAP25*, a key component of the synaptic vesicle fusion machinery, have been shown to central in the onset of developmental and epileptic encephalopathies (DEEs) by altering spontaneous neurotransmitter release and impairing evoked release. Crucially, the specific dysregulation of spontaneous neurotransmission was sufficient to trigger DEEs (Alten et al. 2021). The augmentation of spontaneous glutamate release has been implicated in endoplasmic reticulum stress induced neurodegeneration (Chanaday et al. 2021) and has been proposed to be a central component of the deficits in neurotransmission found in a mouse model of Huntington's disease (Mackay et al. 2020). Taken together, these studies and data from this chapter provide evidence the that long-term use of glucocorticoids may be sufficient to trigger the onset of neurological disease by altering the delicate balance of spontaneous neurotransmission in key brain regions.

Moreover, the blockade of spontaneous NMDAR activity by ketamine is vital in mediating its antidepressant effects, by deactivation of eukaryotic elongation factor 2 kinase (also known as CaMKIII), ultimately resulting in the translation of BDNF (Autry et al. 2011). This further illustrates the importance of physiological spontaneous neurotransmission and that alterations to the frequency or amplitude of these events may have implications for mood disorders.

The data from previous studies (Birnie 2018; Rozwaha 2017) combined with the data presented in this thesis show that chronic treatment with the synthetic glucocorticoid MPL disrupts the pulsatile nature of endogenous GCs in the PRH, leading to alterations to the rhythmic nature of the PRH transcriptome. In turn, synaptic plasticity and glutamate receptor ratios are perturbed

during the active phase, when novel situations are experienced and learned from. NMDA receptor dependent processes seem to be particularly sensitive to these GC induced changes, potentially via disruption to the timing of transcription of NMDAR subunits, with GluN2B seeming to play a critical role. Further to this, spontaneous neurotransmission is disrupted, skewing towards an augmented excitatory balance, providing a further route for the disruption of plasticity and the development of several neurological issues. Ultimately, these changes may explain the observed deficits in a PRH dependent NOR memory task, though with long term MPL treatment, there are avenues for more severe side effects to take hold.

What remains to investigated are further components of the circuit regulating learning and memory processing. Therefore, the following chapter will explore the effects of the synthetic glucocorticoid MPL, and it's interaction with circadian processes in the medial prefrontal cortex, a region highly interconnected with the hippocampal network that is also crucially involved in many aspects of memory and mood.

Chapter V

Effects of methylprednisolone in medial prefrontal cortex

5.1. Introduction

The mPFC is a brain region known to be important in executive control (Yuan, Raz, and Reviews 2014), exerting top-down influence over lower order circuits within the CNS. It is a region that has among the highest baseline metabolic rates during rest, which is liable to change during goal-directed behaviours. Evidence suggests that the mPFC is critical in several facets of complex thought and behaviour, including the neural representation of "self" (Gusnard et al. 2001), social cognition (Grossmann 2013; Yang and Raine 2009), language, working memory (Gabrieli, Poldrack, and Desmond 1998), and attention (Baldauf and Desimone 2014).

The rodent mPFC is further divided in to three regions, the prelimbic cortex (PrL), the infralimbic cortex (IL) and the anterior cingulate cortex (ACC). These regions were differentiated by their cytoarchitecture and functional connectivity, and the human brain analogues are thought to be Brodmann areas 32, 25, and 24b, respectively (Uylings, Groenewegen, and Kolb 2003; Gabbott et al. 2005). Crucially, these regions share many reciprocal connections with other GC-sensitive regions like the hippocampus and amygdala. Moreover, the functional connectivity between these areas is vital in many forms of memory processing. The modulation of certain forms of memory processing by glucocorticoids are known to depend on action in mPFC cell populations, with functional connections to the BLA being of particular importance (Roozendaal et al. 2009).

5.1.1. Effects of acute glucocorticoid exposure in mPFC

Compared to the hippocampus and amygdala, though, very little is known about the effects of stress and glucocorticoids in the mPFC, but by and large, the acute action of glucocorticoids seems to enhance glutamatergic output. Synaptosome preparations from mPFC neurons dissociated from the brains of adult rats subjected to acute foot shock stress exhibit increased glutamate release, via a GR dependent mechanism resulting in the increased accumulation of presynaptic SNARE complexes. Acute stress was also found to increase the amplitude, as well as rise and decay times, of EPSCs in layer III dorsal mPFC pyramidal neurons (Musazzi et al. 2010b).

In adolescent rats, acute stress or acute corticosterone exposure was shown to augment AMPAR and NMDAR mediated EPSCs in layer V pyramidal neurons due to SGK and Rab4 dependent upregulation of the glutamate receptors, ultimately resulting in facilitation of working memory (Yuen et al. 2009; Yuen et al. 2011b). Acute GC-induced augmentation of PFC glutamate transmission, which is thought to be crucial in working memory (Lisman, Fellous, and Wang 1998; Goldman-Rakic 1995), provides some mechanistic insight in to the memory enhancing effects of acute stress.

Considerably less is known about how acute GC exposure alters inhibitory neurotransmission in mPFC, though one study reported a decreased mIPSC frequency in layer V PrL neurons in response to bath perfusion of 100 nM corticosterone, which was dependent on endocannabinoid signalling (Hill et al. 2011b).

5.1.2. Effects of chronic glucocorticoid exposure in mPFC

The effects of chronic glucocorticoid exposure on synaptic physiology in mPFC are not fully understood, however, several chronic stress paradigms (Radley et al. 2006; Liston et al. 2006; Goldwater et al. 2009; Cook and Wellman 2004) and chronic corticosterone exposure (Wellman 2001; Cerqueira et al. 2007) have been shown to result in decreases in the complexity of apical dendrites of pyramidal neurons in this region. Two weeks of chronic corticosterone exposure resulted in structural alterations to PrL pyramidal neurons that persisted following a three week washout period in which HPA axis rhythmicity was restored (Anderson et al. 2016). Conversely, chronic restraint stress increased dendritic arborization in inhibitory interneurons and resulted in increased transcription of GABA receptor subunit *GABAAα1* (Gilabert-Juan et al. 2013). These changes to dendritic structure in mPFC were accompanied by alterations to perceptual attention and cognitive flexibility in behavioural tasks (Cerqueira et al. 2007; Liston et al. 2006).

In adolescent rats, chronic stress impairs temporal order recognition memory (Yuen et al. 2012), which is known to depend, at least in part, on the mPFC (Barker et al. 2007b). These deficits were accompanied by diminished AMPAR- and NMDAR-mediated evoked EPSCs as well as reductions in the frequency and amplitude of mEPSC events. GR-mediated degradation of GluN1, GluN2A, GluN2B, GluR1, and GluR2 glutamate receptor subunits was found to underlie the changes to glutamatergic currents in mPFC (Yuen et al. 2012). It must be noted that adolescence is a period in which prefrontal synapses undergo pruning (Cressman et al. 2010), therefore the degree to which glutamate receptor degradation is due to chronic stress, adolescence, or their interaction, is yet to be resolved. Indeed, a recent study has highlighted the interaction between developmental stage and the HPA axis in mPFC, with GR antagonism abolishing LTP in adults, but not juveniles, on a control diet. Conversely, GR antagonism could reverse a high fat diet induced loss of LTP in juvenile, but not adult, rats (Shrivastava et al. 2021).

The effects of chronic stress on the dendritic morphology of prefrontal neurons have been shown to be accompanied by the development of persistent depressive behaviour (He et al. 2021; Checkley 1996), with the helix-loop-helix transcription factor TWIST1 playing a central role (He et al. 2021). Chronic corticosterone alone leads to deficits in several mPFC dependent memory tasks, reduced neuronal activity (Cabeza et al. 2021a), and in adult rats, reduced

GluN2B, GluR2 and GluR3 expression in IL and PrL (Gourley et al. 2009b). Chronic exposure to corticosterone also resulted in the downregulation of the GR and in HPA axis dysregulation (Cabeza et al. 2021b), although forebrain-specific knockout of GR does not seem to alter the HPA axis hyperactivity and facilitation caused by chronic variable stress (Furay, Bruestle, and Herman 2008).

Recent evidence has shown that mice subjected to 2 hours of restraint stress for 14 days exhibited impairments in a social interaction, sucrose preference, and tail suspension test. These behavioural changes were accompanied by differential expression of a multitude of genes in PrL, including *CaMKIIα*, *ERK1* and 2, and *Nr3c1*. However, 5 minutes of restraint stress for 14 days reversed these chronic stress-induced alterations to behaviour and gene expression. The rescue effects of 5-minute restraint stress could be mimicked by repeated injection of 0.1 or 0.5 mg kg^{-1} of corticosterone. Knockdown of CaMKIIα in PrL was sufficient to rescue GR expression levels and restore behavioural deficits, suggesting that mPFC CamKII and GR are critical components in the expression of depression (Lee et al. 2021). Interestingly, PrL GRs have been shown to be vital in mediating stress-induced inhibition of pain contagion in male mice (Lidhar et al. 2021), suggesting that GC regulation of this mPFC subregion is crucial in the modulation of pain and social behaviours.

Research into the modulation of mPFC neurophysiology by GCs has favoured studying changes in excitatory transmission, meaning that relatively little is known about how these steroids alter inhibitory neurotransmission. Long-term changes in glutamatergic signalling in prefrontal populations could have severe effects, particularly if these alterations are not compensated for by coincidental shifts in inhibitory transmission. Indeed, abnormal glutamate receptor activation, particularly of NMDA receptors, in the mPFC is considered to be a central component of neuropsychiatric disorders such as schizophrenia (Moghaddam 2003; Frankle, Lerma, and Laruelle 2003; Tsai, Coyle, and toxicology 2002).

On the other hand, concomitant treatment with the synthetic GC dexamethasone and the antidepressants sertraline or fluoxetine (Dinan et al. 1997), or a single injection of cortisol (DeBattista et al. 2000; Goodwin et al. 1992) have been shown to improve depressive symptoms. The role of glucocorticoids in mood disorders and their interaction with other systems in PFC, however, remains unresolved. It could be the case that consideration of a factor so simple as time of day, such that other interventions coincide with certain phases of the endogenous glucocorticoid cycle, could provide improved results in the reappraisal of existing stress or negative bias in memory.

5.2. Methods

5.2.1. Evoked EPSCs

Whole-cell voltage clamp recordings were made in layer V pyramidal neurons from either the IL or PrL. EPSCs were evoked by a concentric bipolar stimulating electrode placed in layer I of the slice that was set to stimulate at a frequency of 0.067 Hz. Regions were distinguished based gross anatomy and the position of the slice on the rostro-caudal axis. AMPAR mediated EPSCs were recorded at a holding potential of -70 mV in the presence of 50 µM picrotoxin and NMDAR mediated EPSCs were recorded at a holding potential of +40 mV in the presence of 50 µM picrotoxin and 5 µM NBQX.

EPSCs were analysed offline in the WinLTP software in which EPSCs were low pass filtered at 1 KHz before being analysed. Briefly, the rise time was calculated from 20-80% of the EPSC peak amplitude during the rising phase, and decay was calculated by the time it took for the EPSC amplitude to reduce from 80% to 20%. The EPSC slope was calculated between 25% and 75% of the rising phase of the EPSC. AMPAR:NMDAR ratios were calculated by comparing the peak amplitudes of the AMPAR and NMDAR mediated EPSCs from the same cell.

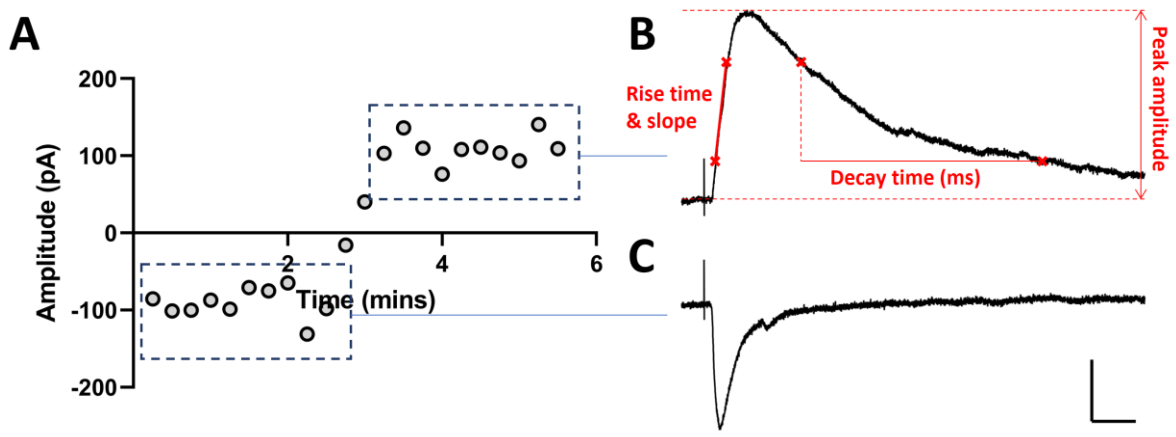

Figure 5.1. Example evoked EPSC experiment with evoked EPSC analysis graph. ***A)*** *Example experiment investigating EPSC properties and AMPAR:NMDAR ratios in mPFC neurons. AMPAR-mediated currents were recorded at -70 mV and NMDAR-mediated currents at +40 mV holding potential.* ***B)*** *A representative NMDAR-mediated EPSC annotated with analysis protocols used to calculate rise time, slope, decay time and peak amplitude.* ***C)*** *Representative AMPAR-mediated current recorded at -70 mV. Scale bar represents x = 20 ms and y = 50 pA.*

5.2.2. Spontaneous and miniature PSCs

For sEPSC recordings, 100 µM D-AP5 and 50 µM Picrotoxin were added to the perfusion to block NMDAR and $GABA_A$ mediated currents, respectively. For mEPSC recordings 1 µM TTX was

also added to the perfusion to block action-potential induced neurotransmission. In both instances the holding potential was maintained at -70 mV.

For spontaneous and miniature IPSCs, cells were instead held at +10 mV, and 100 μM D-AP5 and 5 μM NBQX were added to the perfusion to block NMDAR and AMPAR mediated currents, respectively. For mIPSC recordings, 1 μM TTX was included in the perfusion.

5.3. Results

5.3.1. AMPAR:NMDAR ratio is altered by MPL treatment in PrL, but not IL, neurons

Previous evidence has shown that glutamate currents are altered by chronic corticosterone in IL and PrL mPFC in juvenile mice (Cabeza et al. 2021a) and by chronic stress in juvenile rats (Yuen et al. 2012), therefore I expected to observe changes in AMPAR- and NMDAR-mediated EPSCs induced by MPL treatment in both subregions. Little research has been done on the effect of the circadian cycle and the interaction with chronic glucocorticoid exposure on glutamate transmission in the mPFC, though.

Indeed, a significant effect of treatment ($F_{1, 35} = 5.346$, $P < 0.05$), but not time ($F_{1,35} = 1.738$, $P = 0.1959$) or interaction ($F_{1, 35} = 0.0121$, $P = 0.9130$, Two-way ANOVA, Fig 5.2C) was found for AMPAR:NMDAR ratios in PrL L5 neurons. However, in IL L5 neurons, no significant effect of treatment ($F_{1,27} = 1.143$, $P = 0.2945$), time ($F_{1,27} = 0.0442$, $P = 0.835$), or an interaction ($F_{1, 27} = 3.792$, $P = 0.062$, Two-way ANOVA, Fig 5.2D) could be found, suggesting that long-term glucocorticoid use might preferentially disrupt PrL glutamate signalling.

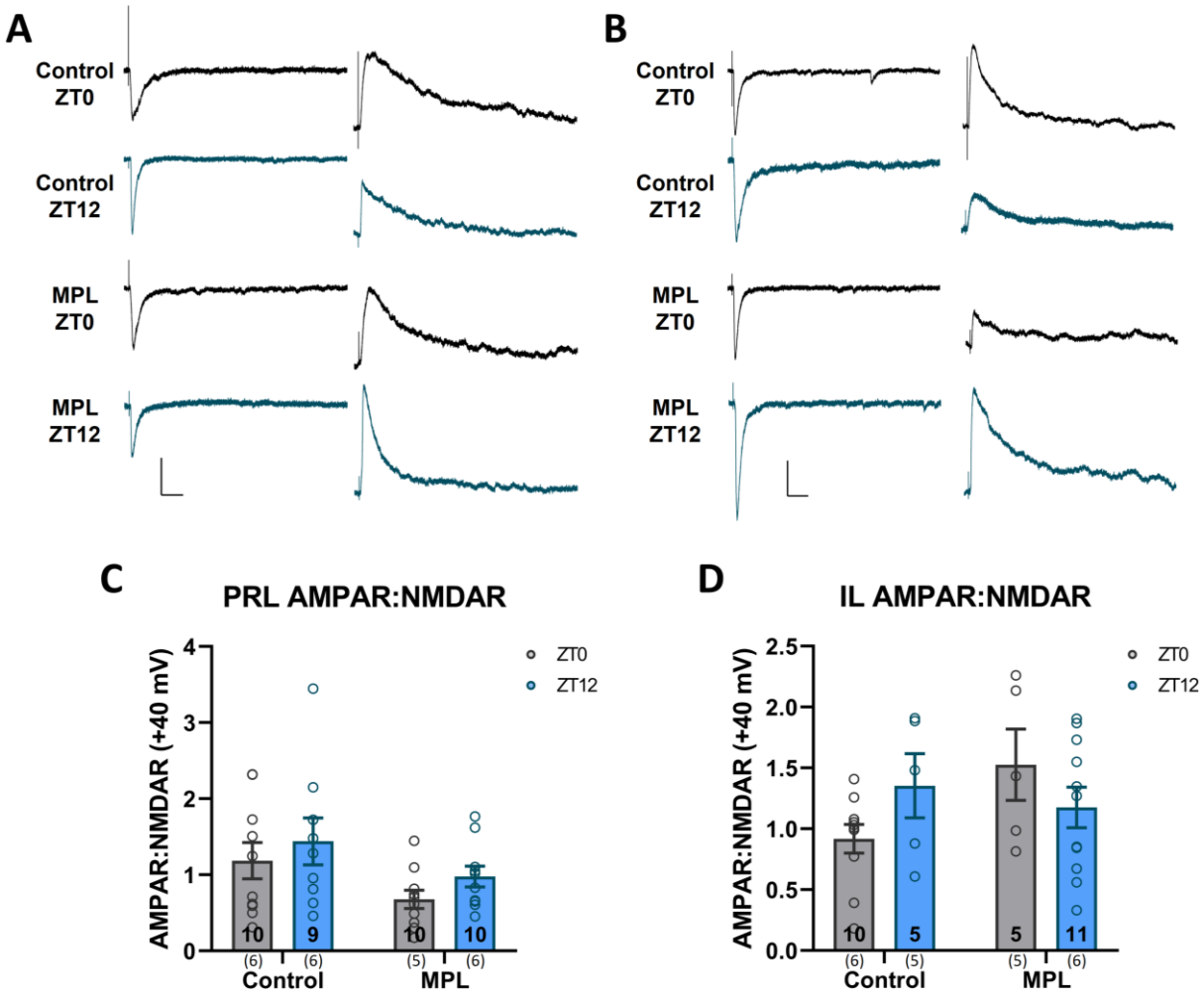

Figure 5.2. AMPAR:NMDAR ratios are reduced by MPL treatment in PrL, but not IL, neurons. ***A)*** *Representative AMPAR- and NMDAR-mediated EPSC traces from layer V pyramidal neurons in the PrL.* ***B)*** *Representative AMPAR- and NMDAR-mediated EPSC traces from layer V pyramidal neurons in the IL. Scale bars represent x = 50 ms and y = 50 pA.* ***C)*** *AMPAR:NMDAR ratios calculated from neurons in the PrL of slices taken at ZT0 or ZT12 from control and MPL treated rats.* ***D)*** *AMPAR:NMDAR ratios calculated from neurons in the IL of slices taken at ZT0 or ZT12 from control and MPL treated rats. Data are plotted as mean ± SEM. n is indicated at the base of the bar (number of slices within bar, number of animals below bar). Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance.*

5.3.2. AMPAR-mediated EPSC properties of prelimbic neurons

To further investigate the changes to glutamatergic signalling in mPFC I analysed the properties of the AMPAR-mediated EPSCs from each condition. Two-way ANOVA showed a significant main effect of time ($F_{1, 36}$ = 4.623, P = 0.0383), but not treatment ($F_{1, 36}$ = 1.176, P = 0.2854) or interaction ($F_{1. 36}$ = 1.479, P = 0.2319, Fig 5.3A), on AMPAR EPSC peak amplitude.

No significant changes could be found in rise time or slope (Fig 5.3B + D), though a significant effect of time ($F_{1, 35} = 11.69$, P = 0.0016), and an interaction between time and treatment ($F_{1, 35} = 4.967$, P = 0.0323, Two-way ANOVA, Fig 5.3C) was reported for the decay of AMPAR-mediated currents. Tukey's multiple comparisons test revealed a significant increase in the decay time in MPL treated animals at ZT0 (36.044 ± 4.440 ms) compared to ZT12 (18.943 ± 2.111 ms) (P < 0.01).

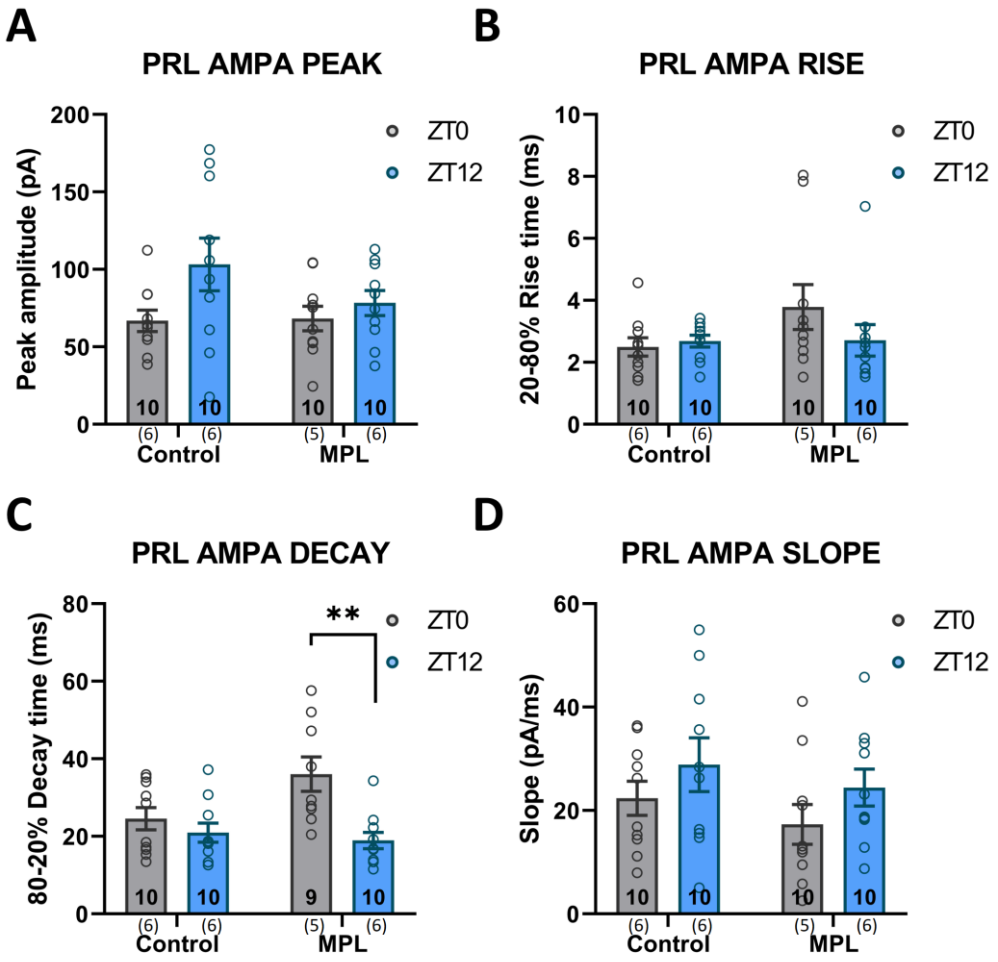

Figure 5.3. AMPAR-mediated EPSC properties of PrL L5 pyramidal neurons. *Peak amplitude **(A)**, rise time **(B)**, decay time **(C)**, and slope **(D)** of AMPAR mediated EPSCs from neurons in the PrL of slices taken at ZT0 or ZT12 from control and MPL treated rats. n is indicated at the base of the bar (number of slices within bar, number of animals below bar). Data are plotted as mean ± SEM. Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance, which is indicated at P < 0.01 (**).*

5.3.3. AMPAR-mediated EPSC properties in infralimbic neurons

Unlike prelimbic neurons, no significant alterations to AMPAR-mediated EPSC properties were observed in infralimbic cells (Fig 5.4). This is consistent with the data in figure 5.2 showing that AMPAR:NMDAR ratios were not affected by treatment or time in infralimbic neurons. Previous investigation has shown that AMPAR phosphorylation is significantly decreased in mPFC tissue homogenate containing PrL and IL cortices.

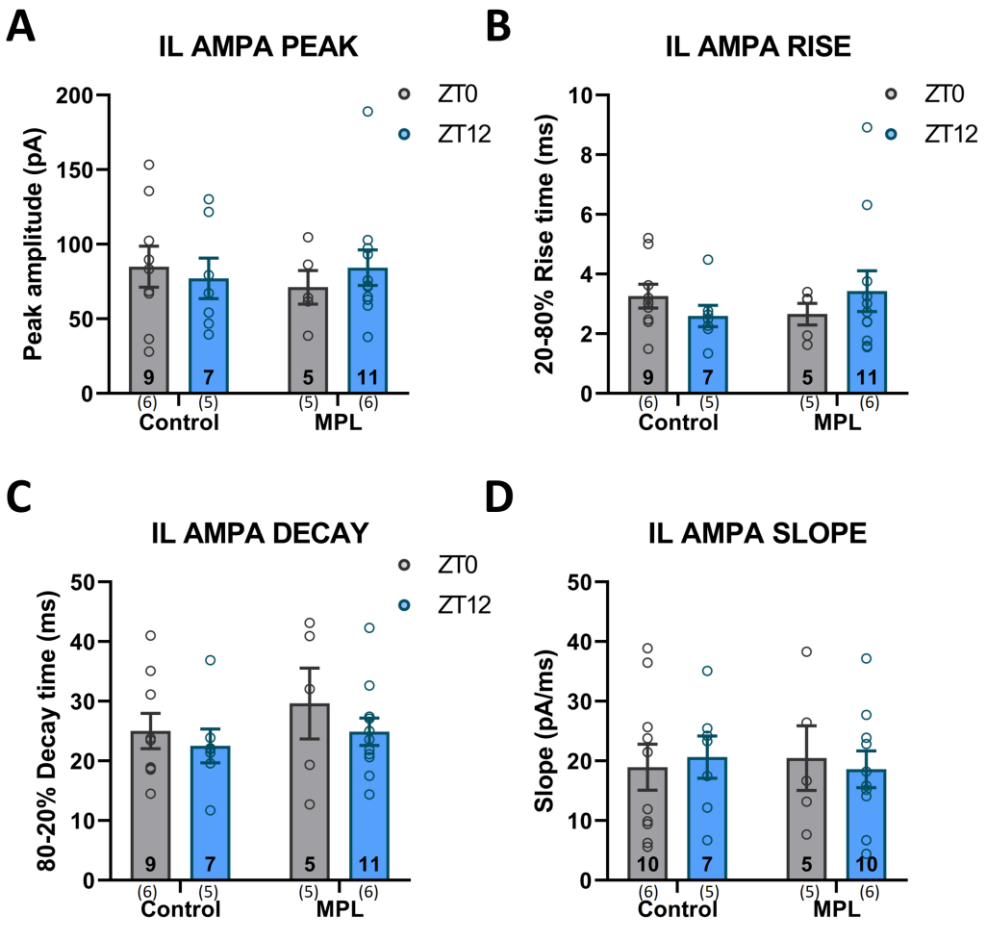

Figure 5.4. AMPAR-mediated EPSC properties of IL L5 pyramidal neurons. *Peak amplitude **(A)**, rise time **(B)**, decay time **(C)**, and slope **(D)** of AMPAR mediated EPSCs from neurons in the IL of slices taken at ZT0 or ZT12 from control and MPL treated rats. n is indicated at the base of the bar (number of slices within bar, number of animals below bar). Data are plotted as mean ± SEM. Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance.*

5.3.4. Properties of NMDAR-mediated EPSCs in prelimbic neurons

To further investigate any changes to NMDAR-dependent transmission in mPFC, I analysed the properties of NMDAR-mediated evoked EPSCs recorded at +40 mV holding potential. In prelimbic neurons, NMDAR-mediated peak current, rise time, and slope were all unaffected by treatment and time. However, a significant effect of treatment was found on NMDAR EPSC decay time ($F_{1, 35}$ = 6.333, P = 0.0166, Two-way ANOVA, Fig 5.5C). This could be consistent with an alteration to the subunit composition of surface NMDARs in prelimbic neurons, caused by chronic MPL treatment, as different subunits compositions confer different kinetic properties to the NMDAR conductance. GluN1-GluN2B diheteromers have a larger decay time constant than GluN2C containing diheteromers, and a considerably larger decay time than GluN2A diheteromeric channels (Cull-Candy, Brickley, and Farrant 2001). Previous evidence has also shown that GluN2B subunits are downregulated in mPFC following chronic stress (Yuen et al. 2012), so it could be that the decrease in NMDAR-EPSC decay time in treated animals is due to a change in NMDA receptor subunit composition, with fewer GluN2B subunits functioning at the synapse.

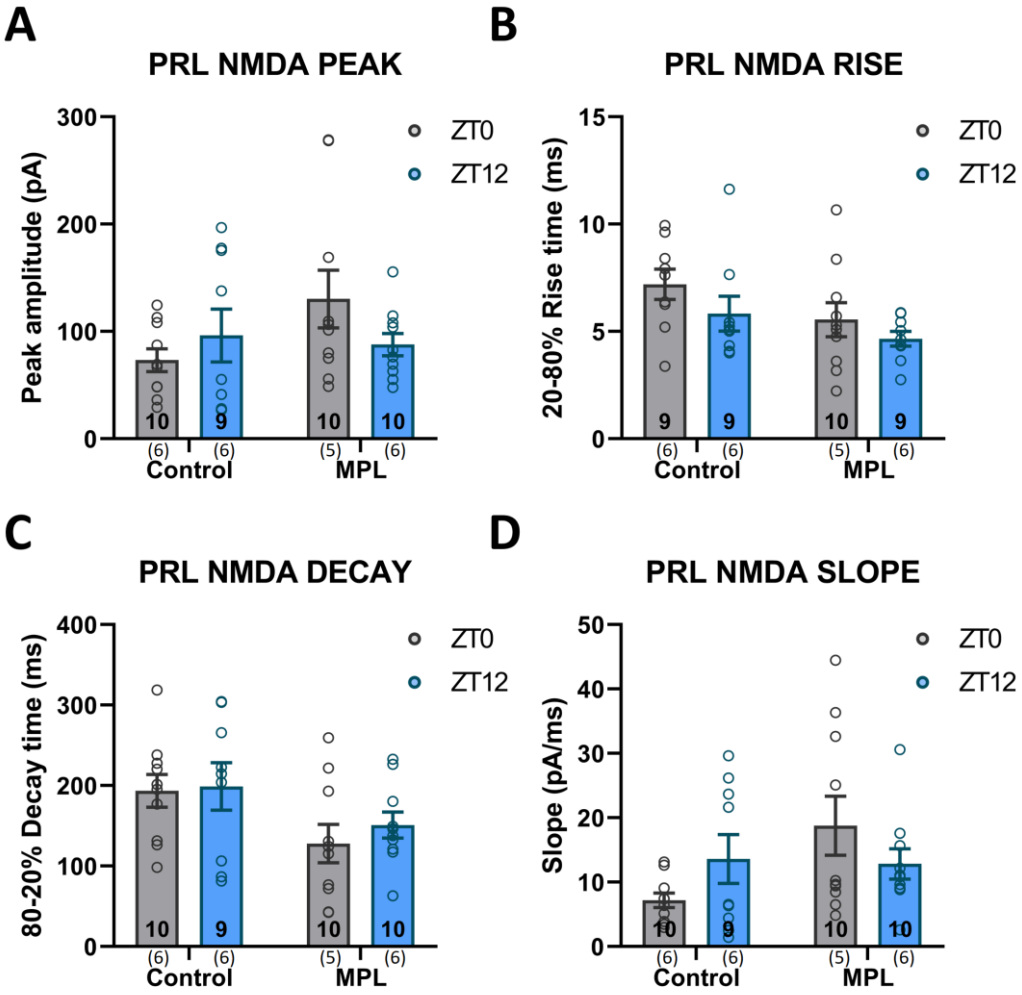

Figure 5.5. NMDAR-mediated EPSC properties of PrL L5 pyramidal neurons. *Peak amplitude **(A)**, rise time **(B)**, decay time **(C)**, and slope **(D)** of NMDAR mediated EPSCs from neurons in the PrL of slices taken at ZT0 or ZT12 from control and MPL treated rats. n is indicated at the base of the bar (number of slices within bar, number of animals below bar). Data are plotted as mean ± SEM. Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance.*

5.3.5. Properties of NMDAR-mediated EPSCs in infralimbic neurons

Similar to what was observed in the AMPAR-EPSC analysis, the NMDAR-mediated current properties were not affected by either treatment or time in infralimbic neurons (Fig 5.6). This reinforces the notion that chronic glucocorticoid treatment preferentially alters glutamatergic transmission in the prelimbic region.

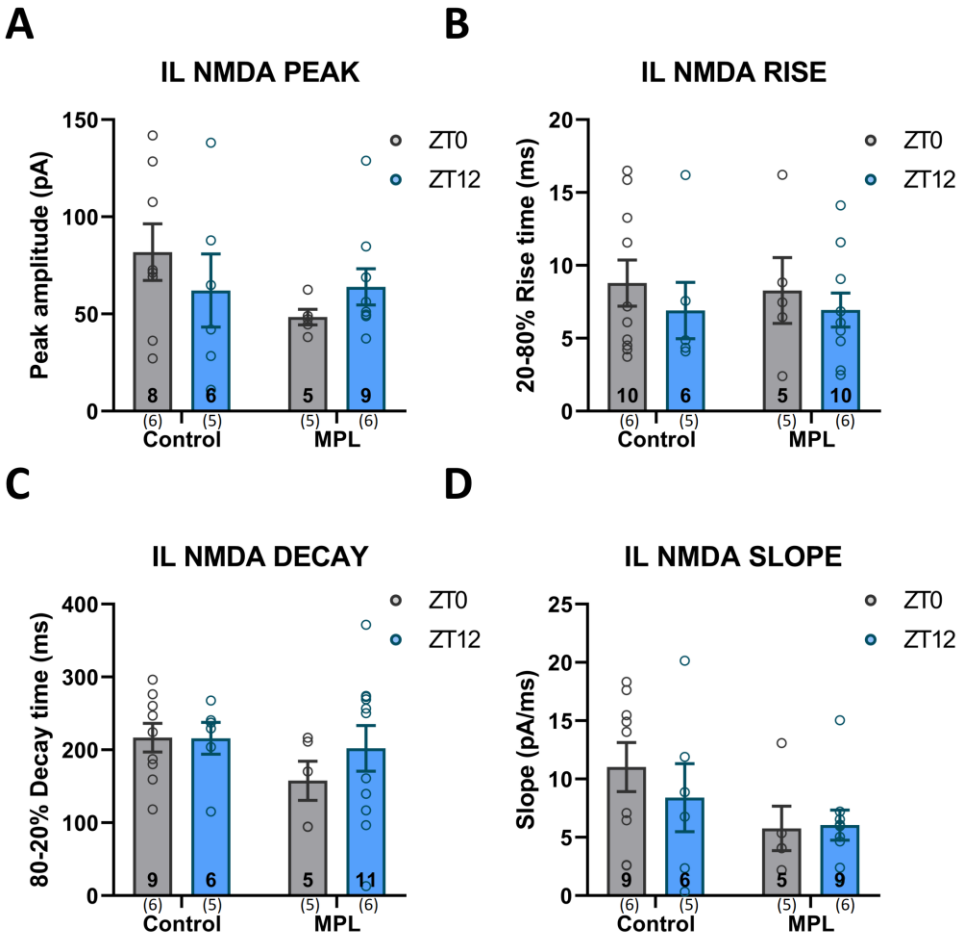

Figure 5.6. NMDAR-mediated EPSC properties of IL L5 pyramidal neurons. *Peak amplitude **(A)**, rise time **(B)**, decay time **(C)**, and slope **(D)** of NMDAR mediated EPSCs from neurons in the IL of slices taken at ZT0 or ZT12 from control and MPL treated rats. n is indicated at the base of the bar (number of slices within bar, number of animals below bar). Data are plotted as mean ± SEM. Two-way ANOVA and Tukey's multiple comparisons tests were used to check for statistical significance.*

5.3.6. Miniature EPSC amplitude in prelimbic neurons is suppressed by MPL

As well as investigating the effects of time of day and MPL treatment on evoked synaptic transmission, I also conducted experiments analysing the effects on spontaneous excitatory and inhibitory neurotransmission in mPFC. It has been demonstrated that chronic stress decreases the amplitude and frequency of mEPSCs in mPFC slices from juvenile rats, and seven-day corticosterone incubation decreases the amplitude and frequency of mEPSCs in cultured PFC neurons (Yuen et al. 2012).

Unexpectedly, no significant effects were found on the frequency of mEPSCs in prelimbic neurons, however, a significant effect of treatment ($F_{1, 94}$ = 12.98, P = 0.0005, Two-way ANOVA, Fig 5.7D) was found for mEPSC amplitude. Tukey's post-hoc analysis revealed a significant suppression of mEPSC amplitude at ZT0 in MPL treated (11.321 ± 0.689 pA) vs. control (14.428 ± 0.687 pA) animals (P < 0.01). Similarly, mEPSC amplitude was reduced at ZT12 in MPL treated animals (11.505 ± 0.702 pA) compared to controls (13.330 ± 0.546 pA) (P < 0.05).

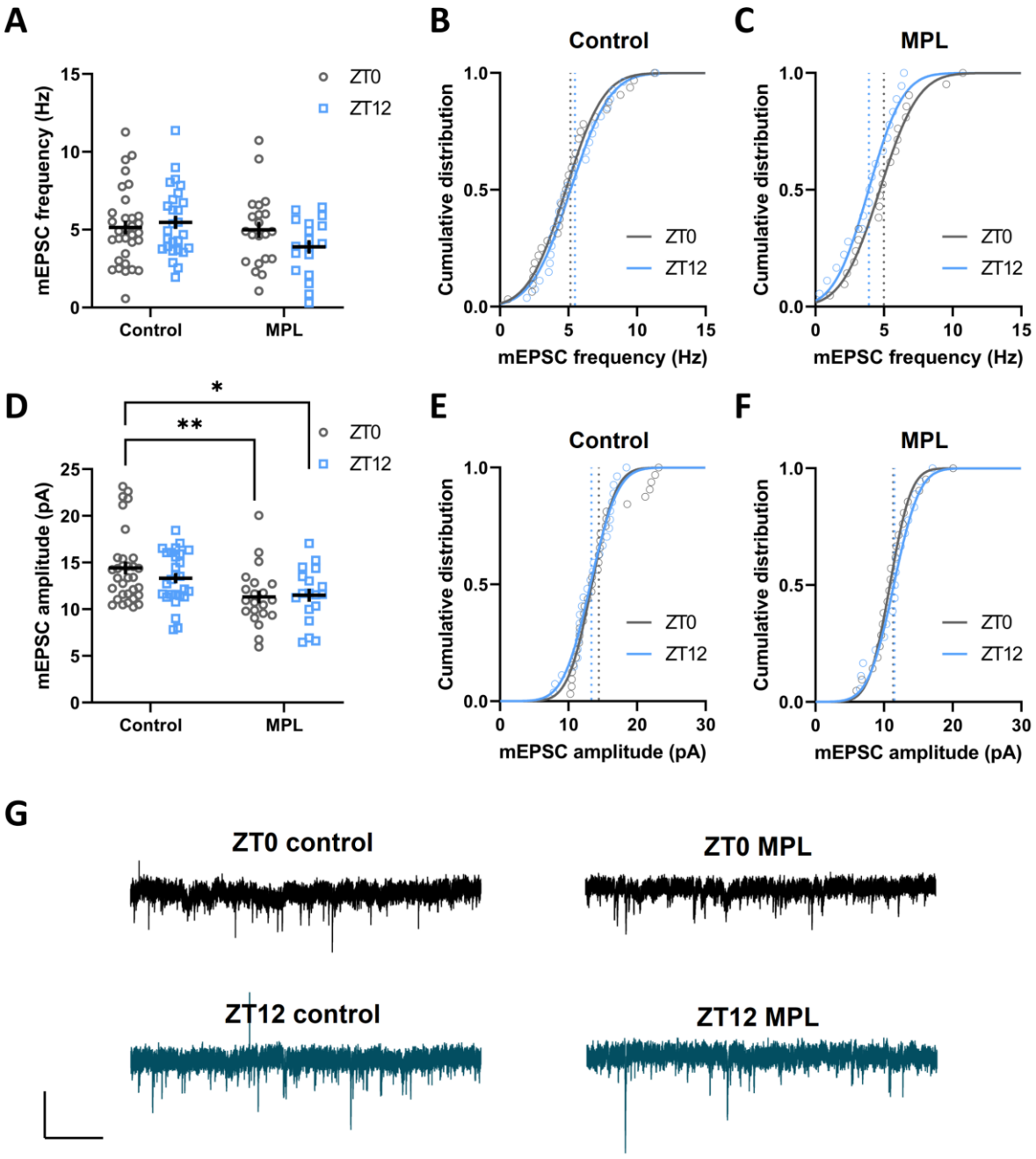

Figure 5.7. mEPSC amplitude, but not frequency, in PrL neurons is altered by MPL treatment. ***A)*** *mEPSC frequency at ZT0 (n = 32, 15, 10) (cells, slices, animals) and ZT12 (n = 27,*

12, 10) in control, and at ZT0 (n = 21, 9, 8) and ZT12 in MPL, groups (n = 18, 8, 8). ***B)*** *Cumulative distributions of mEPSC frequencies from ZT0 and ZT12 controls.* ***C)*** *Cumulative distributions of mEPSC frequencies from ZT0 and ZT12 MPL groups.* ***D)*** *mEPSC amplitude at ZT0 (n = 32, 15, 10) and ZT12 (n = 27, 15, 10) in control, and at ZT0 (n = 21, 10, 8) and ZT12 in MPL, groups (n = 18, 8, 8).* ***E)*** *Cumulative distributions of mEPSC amplitudes of ZT0 and ZT12 controls.* ***F)*** *Cumulative distributions of mEPSC amplitudes from ZT0 and ZT12 MPL groups.* ***G)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 10 pA. Data in* ***A*** *and* ***D*** *are plotted as mean ± SEM. Dotted lines in* ***B***, ***C***, ***E***, *and* ***F*** *represent means. Experimental n is indicated as (cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*) and P < 0.01 (**).*

5.3.7. Miniature EPSC amplitude is suppressed by MPL in infralimbic neurons

Although no significant effect was uncovered for time or treatment on mEPSC frequency in infralimbic L5 neurons, a significant effect for their interaction was revealed ($F_{1, 99} = 4.73$, P = 0.032, Two-way ANOVA, Fig 5.8A). Tukey's multiple comparisons revealed a significant increase at ZT0 in MPL treated (5.469 ± 0.533 Hz) compared to control (3.822 ± 0.356 Hz) animals (P < 0.05).

Similar to what was observed in prelimbic cells, amplitude was significantly affected by treatment ($F_{1, 99} = 38.95$, P < 0.0001, Two-way ANOVA, Fig 5.8D), but not by time, and no interaction was reported. Tukey's post-hoc analysis revealed a significant reduction in the amplitude of events in MPL treated animals compared to controls at both ZT0 (10.959 ± 0.453 vs. 15.853 ± 0.945 pA, P < 0.0001) and ZT12 (12.727 ± 0.445 vs. 16.807 ± 0.837 pA, P < 0.001). The data in Fig 5.7 and Fig 5.8 suggest that miniature AMPAR-mediated currents are suppressed at both the circadian peak and nadir by chronic MPL treatment, indicating that steroid treatment may cause a consistent depression of mPFC excitatory neurotransmission.

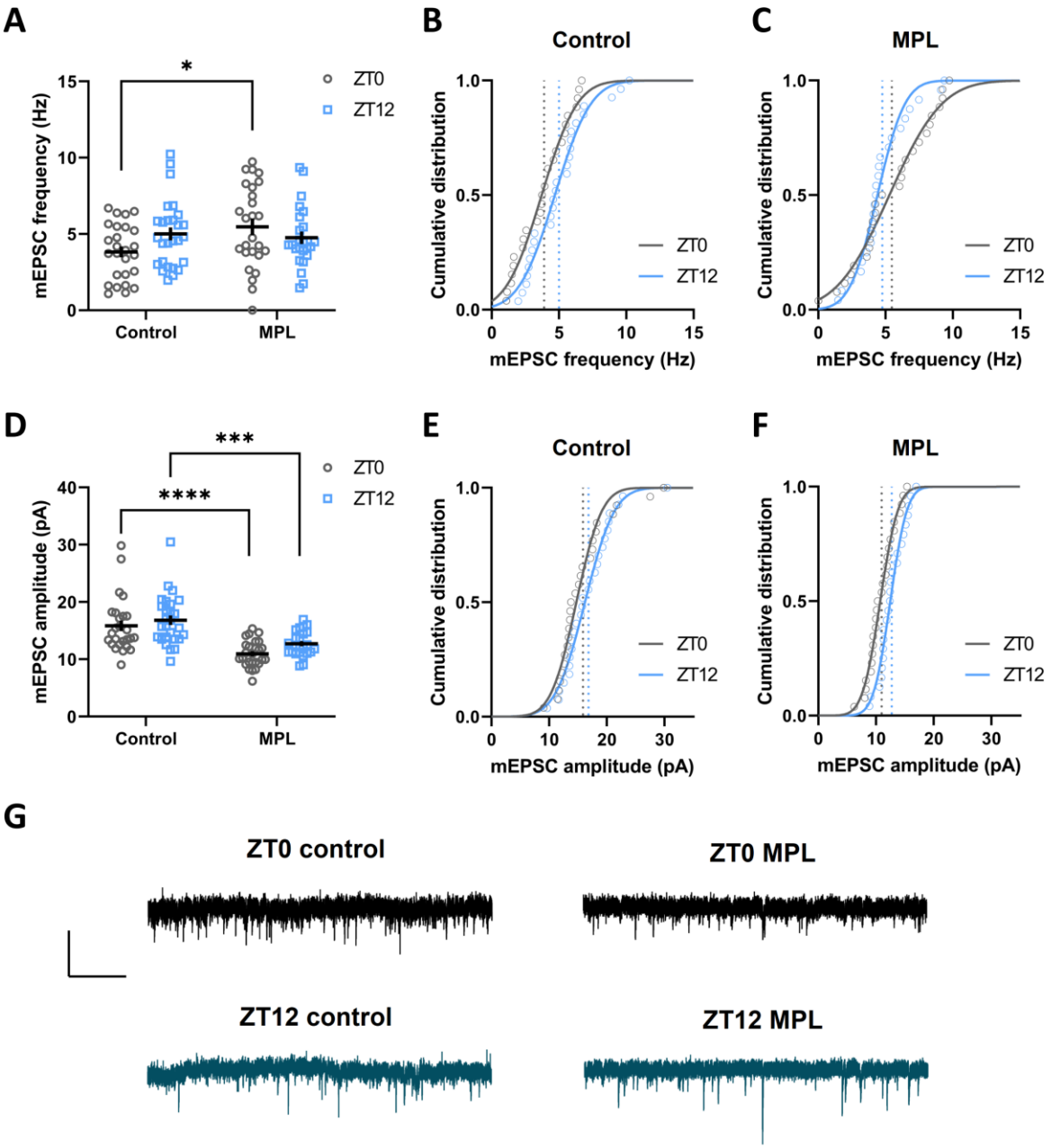

Figure 5.8. mEPSC properties are altered in IL neurons by MPL treatment. ***A)*** *mEPSC frequency at ZT0 (n = 26, 12, 10) (cells, slices, animals) and ZT12 (n = 27, 12, 10) in control, and at ZT0 (n = 26, 12, 8) and ZT12 in MPL, groups (n = 24, 11, 8).* ***B)*** *Cumulative distributions of mEPSC frequencies from ZT0 and ZT12 controls.* ***C)*** *Cumulative distributions of mEPSC frequencies from ZT0 and ZT12 MPL groups.* ***D)*** *mEPSC amplitude at ZT0 (n = 26, 12, 10) and ZT12 (n = 27, 12, 10) in control, and at ZT0 (n = 26, 12, 8) and ZT12 in MPL, groups (n = 24, 11, 8).* ***E)*** *Cumulative distributions of mEPSC amplitudes of ZT0 and ZT12 controls.* ***F)*** *Cumulative distributions of mEPSC amplitudes from ZT0 and ZT12 MPL groups.* ***G)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 10 pA. Data in* ***A*** *and* ***D*** *are plotted as mean ± SEM. Dotted lines in* ***B****,*

C, ***E***, *and* ***F*** *represent means. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*), P < 0.001 (***), and P < 0.0001 (****).*

5.3.8. Spontaneous EPSC properties of prelimbic neurons are affected by treatment, not time

Both the frequency ($F_{1, 37}$ = 5.334, P = 0.0266, Fig 5.9A) and amplitude ($F_{1, 37}$ = 6.535, P = 0.0148, Fig 5.9B) of sEPSCs in prelimbic neurons were found to be significantly regulated by MPL treatment. Amplitude of these events was also found to be significantly regulated by an interaction between treatment and time ($F_{1, 37}$ = 5.072, P = 0.303, Two-way ANOVA). Tukey's multiple comparisons test further revealed a significant reduction in sEPSC amplitude in MPL treated animals at ZT0 compared to the controls at the same ZT (10.371 ± 1.072 vs. 16.179 ± 1.085 pA, P < 0.05).

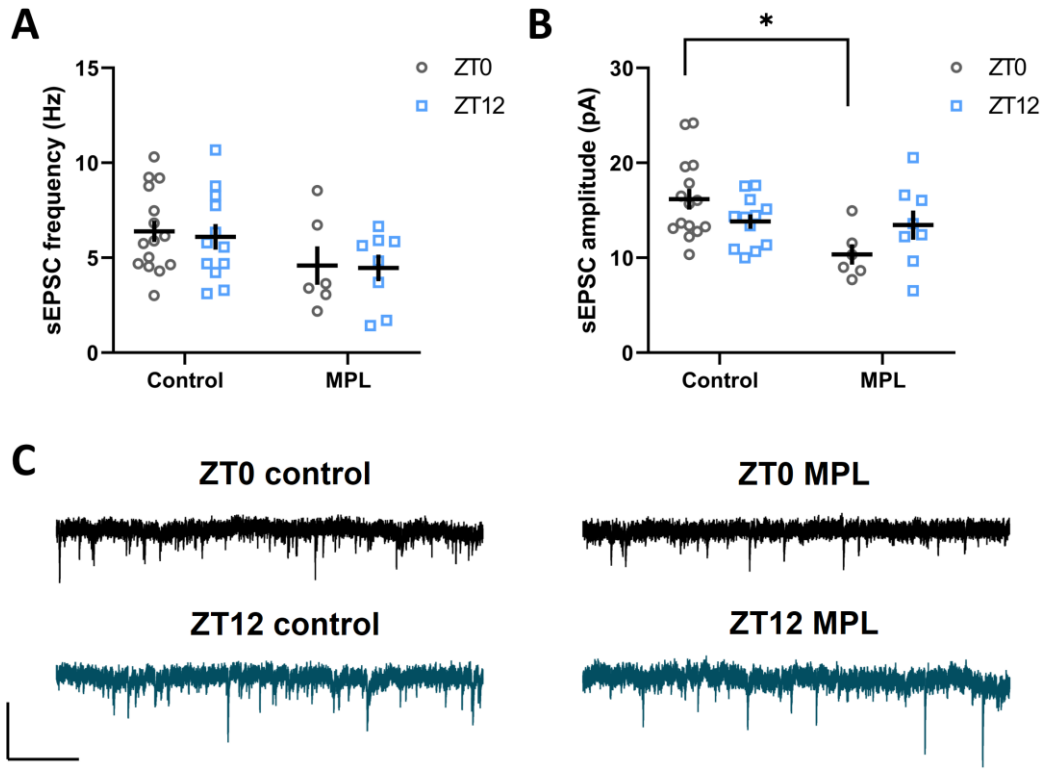

Figure 5.9. sEPSC properties of prelimbic neurons are altered by MPL treatment, but not time. ***A)*** *sEPSC frequency at ZT0 (n = 15, 15, 10) (cells, slices, animals) and ZT12 (n = 12, 12, 10) in control, and at ZT0 (n = 6, 6, 6) and ZT12 in MPL, groups (n = 8, 8, 8).* ***B)*** *sEPSC amplitude at ZT0 (n = 15, 15, 10) and ZT12 (n = 12, 12, 10) in control, and at ZT0 (n = 6, 6, 6) and ZT12 in MPL, groups (n = 8, 8, 8).* ***C)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 10*

*pA. Data in **A** and **B** are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*).*

5.3.9. sEPSC properties of infralimbic neurons are altered by treatment, not time

MPL treatment also had a significant effect on the amplitude of sEPSCs in infralimbic neurons ($F_{1, 39}$ = 16.17, P = 0.0003, Fig 5.10B) although no effect of treatment, time or interaction was apparent on sEPSC frequency in this subregion (Fig 5.10A). The interaction between treatment and time was also found to have a significant effect on sEPSC amplitude ($F_{1, 39}$ = 5.279, P = 0.027, Two-way ANOVA). Tukey's post-hoc analysis confirmed a significant reduction in the amplitude of events in MPL treated rats at ZT0 (9.609 ± 0.959 pA, P < 0.001) and ZT12 (13.152 ± 0.802 pA, P < 0.05) compared to controls at ZT0 (20.405 ± 2.447 pA). These data are consistent with the aforementioned reduction in mEPSC amplitudes in mPFC neurons caused by MPL treatment.

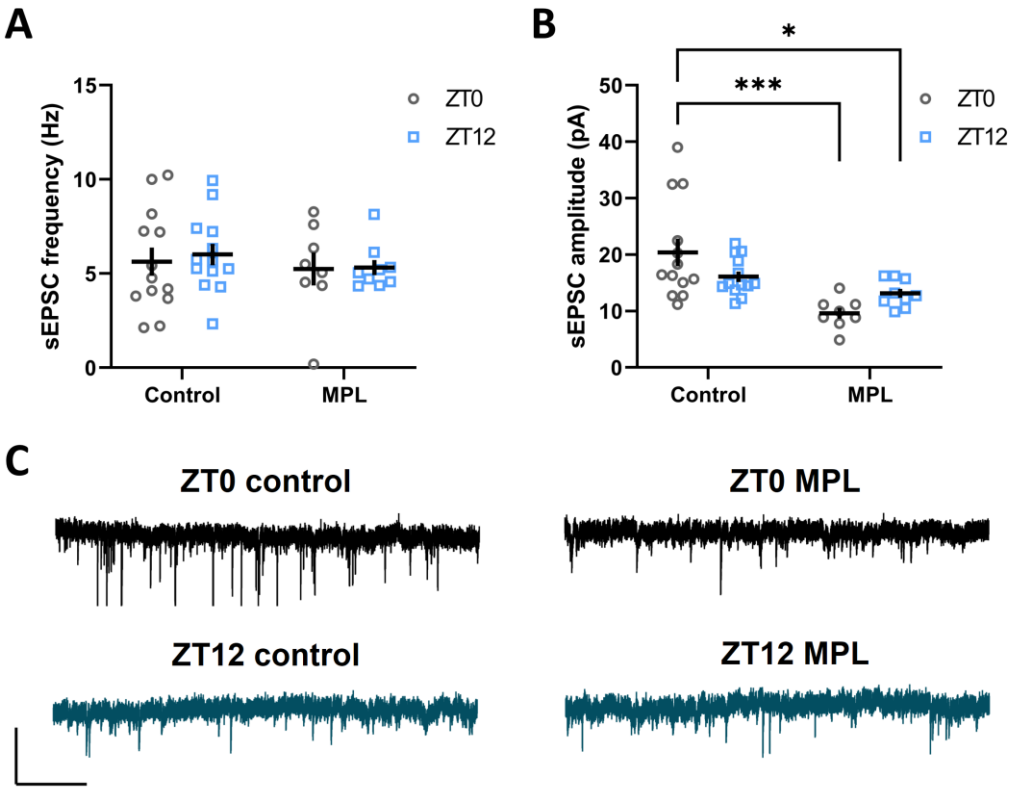

Figure 5.10. sEPSC properties of IL neurons are altered by MPL treatment, but not time. ***A)*** *sEPSC frequency at ZT0 (n = 13, 12, 10) (cells, slices, animals) and ZT12 (n = 13, 12, 10) in control, and at ZT0 (n = 8, 8, 8) and ZT12 in MPL, groups (n = 9, 9, 8).* ***B)*** *sEPSC amplitude at ZT0 (n = 13, 12, 10) (cells, slices, animals) and ZT12 (n = 13, 12, 10) in control, and at ZT0 (n = 8, 8, 8) and*

ZT12 in MPL, groups (n = 9, 9, 8). ***C)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 10 pA. Data in* ***A*** *and* ***B*** *are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*) and P < 0.001 (***).*

5.3.10. Miniature IPSC properties of prelimbic neurons

Relatively little is known about the effects of chronic steroid exposure or time of day on inhibitory neurotransmission in the mPFC. Given the alterations to glutamatergic transmission incurred by MPL, I expected that I might see compensatory changes to spontaneous inhibitory transmission, in order to preserve the balance between excitation and inhibition in this region. However, the effects on spontaneous inhibitory neurotransmission in the prefrontal cortex are somewhat different and less profound than the effects on excitation. Two-way ANOVA revealed no significant effects of on mIPSC frequency in prelimbic neurons (Fig 5.11A), but there was a significant main effect of time ($F_{1, 75} = 8.582$, P = 0.0045, Two-way ANOVA, Fig 5.11D) in this population. Tukey's multiple comparisons further revealed a significant increase in the amplitude of inhibitory events in MPL treated rats at ZT12 (22.702 ± 0.877 pA) compared to ZT0 in both controls (18.081 ± 1.014 pA, P < 0.05) and MPL treated rats (18.235 ± 1.216 pA, P < 0.05).

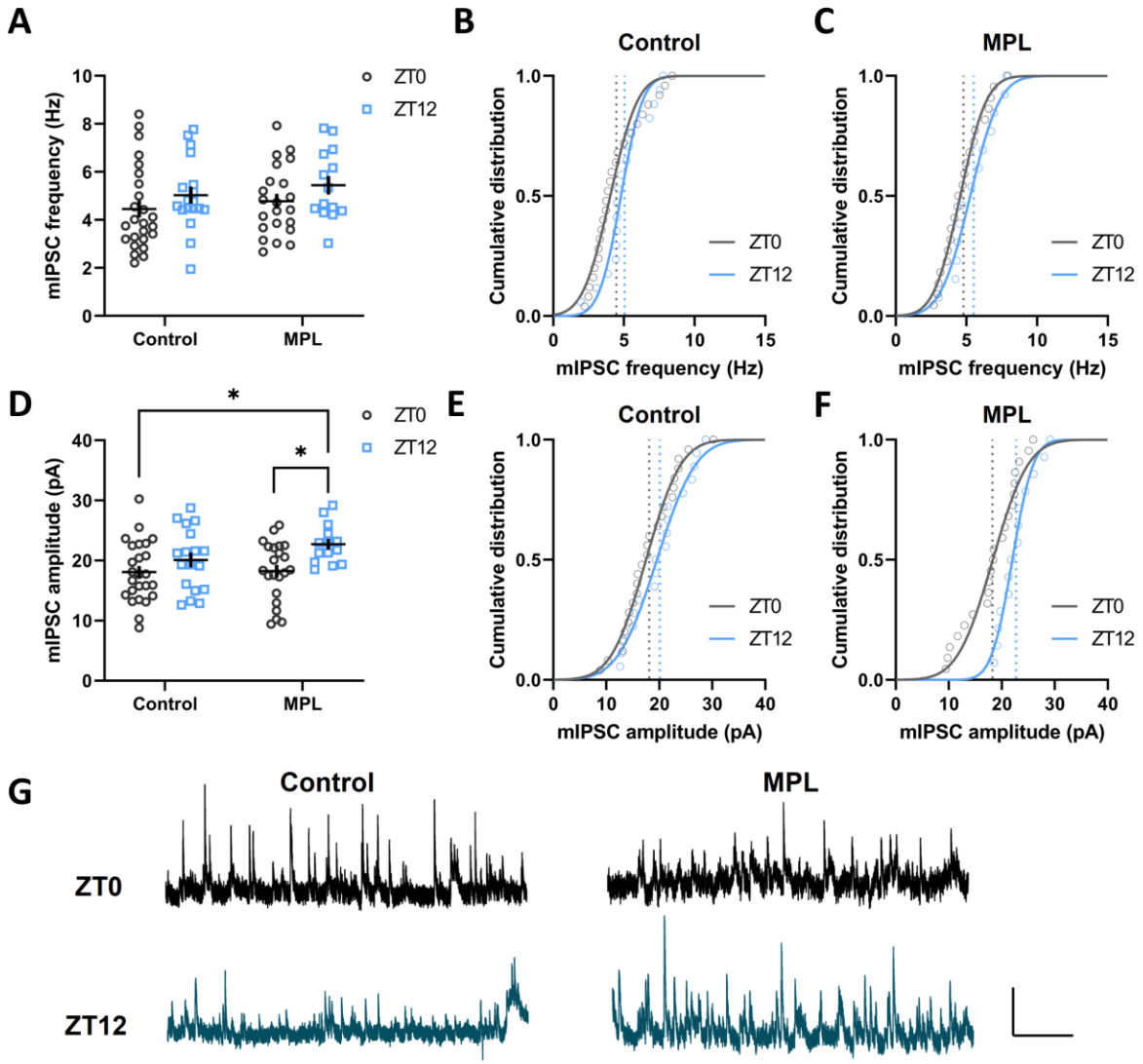

Figure 5.11. mIPSC amplitude, but not frequency, in PrL neurons is altered by MPL treatment. ***A)*** *mIPSC frequency at ZT0 (n = 25, 12, 10) (cells, slices, animals) and ZT12 (n = 18, 10, 10) in control, and at ZT0 (n = 22, 8, 8) and ZT12 in MPL, groups (n = 14, 8, 6).* ***B)*** *Cumulative distributions of mIPSC frequencies from ZT0 and ZT12 controls.* ***C)*** *Cumulative distributions of mIPSC frequencies from ZT0 and ZT12 MPL groups.* ***D)*** *mIPSC amplitude at ZT0 (n = 25, 12, 10) and ZT12 (n = 18, 10, 10) in control, and at ZT0 (n = 22, 10, 8) and ZT12 in MPL, groups (n = 14, 8, 6)* ***E)*** *Cumulative distributions of mIPSC amplitudes of ZT0 and ZT12 controls.* ***F)*** *Cumulative distributions of mIPSC amplitudes from ZT0 and ZT12 MPL groups.* ***G)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 20 pA. Data in* ***A*** *and* ***D*** *are plotted as mean ± SEM. Dotted lines in* ***B***, ***C***, ***E***, *and* ***F*** *represent means. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*).*

5.3.11. Miniature IPSC properties in infralimbic neurons

In infralimbic neurons, a different trend was observed to that seen in the prelimbic region. A significant main effect of time ($F_{1, 81}$ = 8.818, P = 0.0039, Two-way ANOVA, Fig 5.12A) was observed on mIPSC frequency, and no significant effects could be found on mIPSC amplitude (Fig 5.12D). Tukey's post-hoc analysis revealed a significant increase in mIPSC frequency at ZT12 in MPL treated rats (4.372 ± 0.432 Hz) compared to ZT0 in both controls (3.172 ± 0.226 Hz, P < 0.05) and MPL treated animals (3.231 ± 0.228 Hz, P < 0.05).

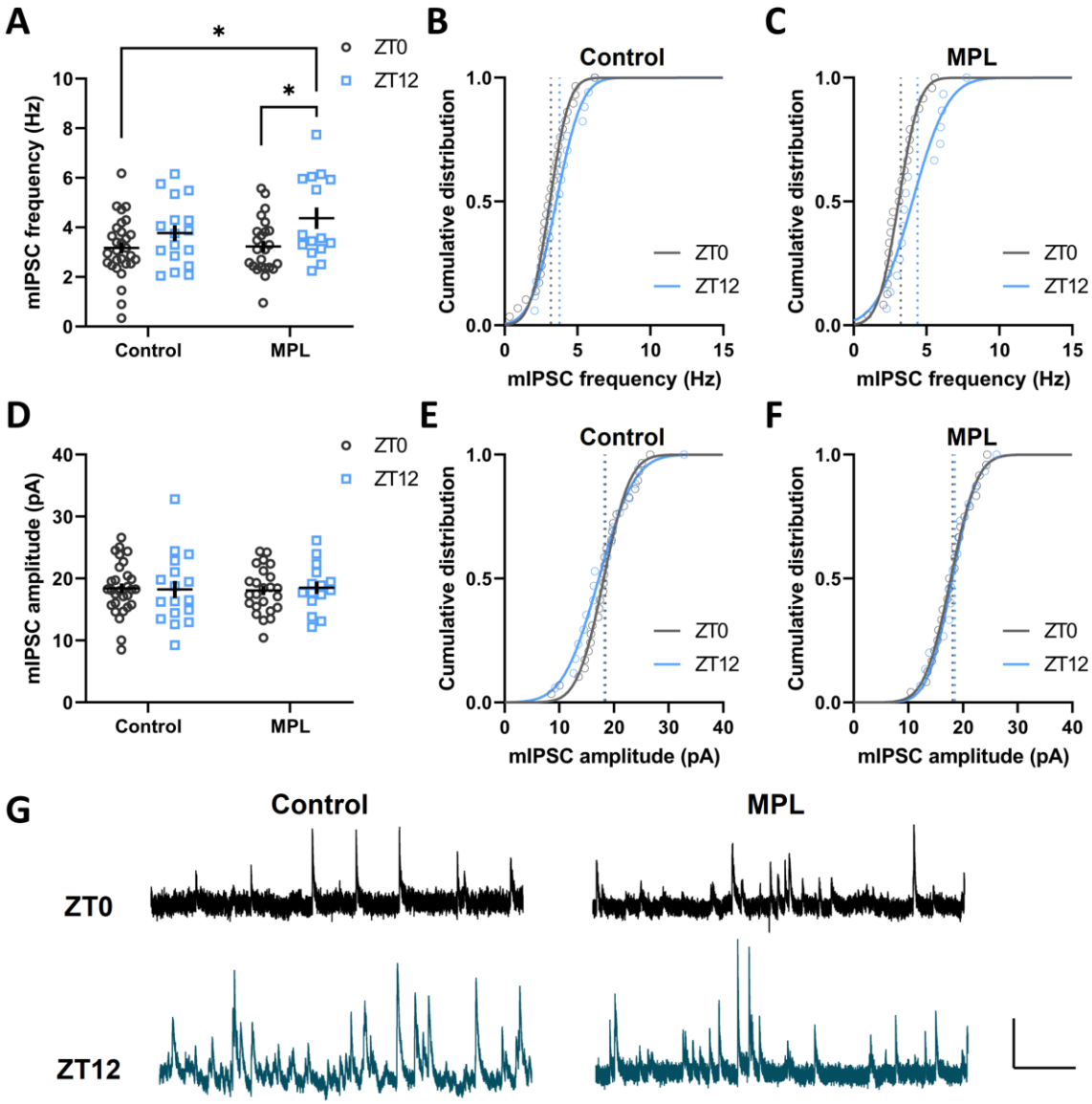

Figure 5.12. mIPSC frequency, but not amplitude, in IL neurons is altered by MPL treatment. ***A)*** *mIPSC frequency at ZT0 (n = 29, 12, 10) (cells, slices, animals) and ZT12 (n = 17, 10, 10) in control, and at ZT0 (n = 24, 10, 8) and ZT12 in MPL, groups (n = 15, 7, 6).* ***B)*** *Cumulative distributions of mIPSC frequencies from ZT0 and ZT12 controls.* ***C)*** *Cumulative distributions of*

mIPSC frequencies from ZT0 and ZT12 MPL groups. ***D)*** *mIPSC amplitude at ZT0 (n = 29, 12, 10) and ZT12 (n = 17, 10, 10) in control, and at ZT0 (n = 24, 10, 8) and ZT12 in MPL, groups (n = 15, 7, 6).* ***E)*** *Cumulative distributions of mIPSC amplitudes of ZT0 and ZT12 controls.* ***F)*** *Cumulative distributions of mIPSC amplitudes from ZT0 and ZT12 MPL groups.* ***G)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 20 pA. Data in* ***A*** *and* ***D*** *are plotted as mean ± SEM. Dotted lines in* ***B***, ***C***, ***E***, *and* ***F*** *represent means. Experimental n is indicated at (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance, which is indicated at P < 0.05 (*).*

5.3.12. Spontaneous IPSC properties in prelimbic neurons

No significant effects of time or treatment could be found on sIPSC frequency or amplitude; however a significant effect of their interaction was observed on both frequency ($F_{1, 20} = 4.520$, P = 0.0461, Fig 5.13A) and amplitude ($F_{1, 20} = 4.514$, P = 0.0463, Two-way ANOVA, Fig 5.13B).

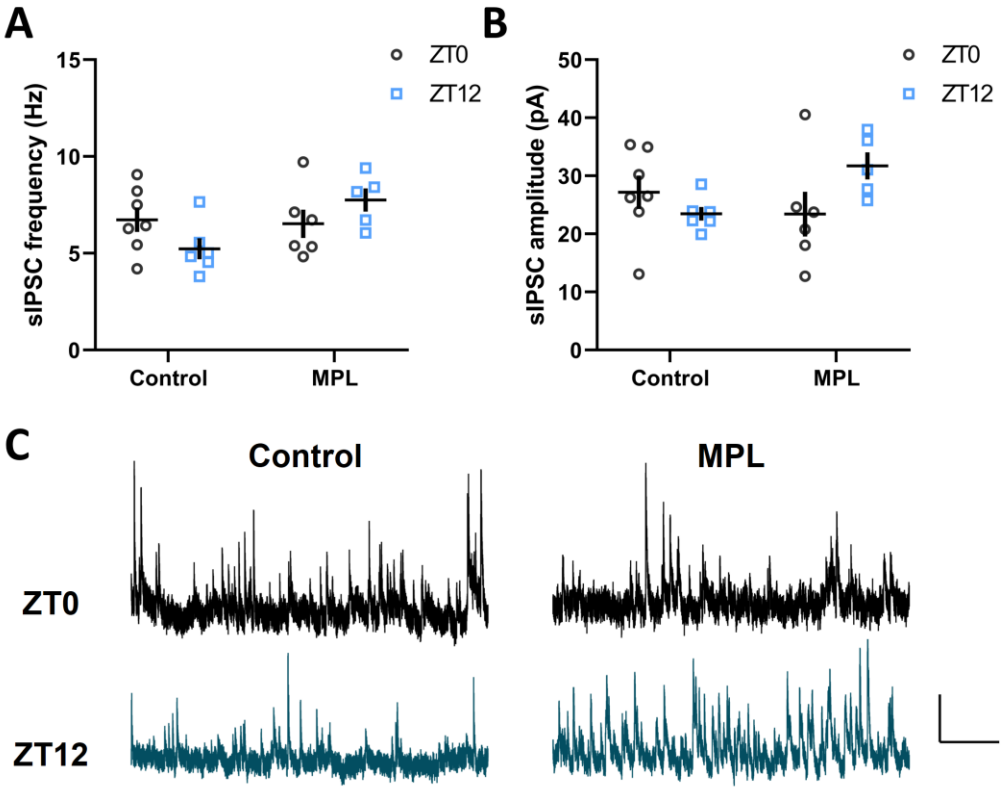

Figure 5.13. sIPSC properties of PrL neurons. ***A)*** *sIPSC frequency at ZT0 (n = 7, 7, 6) (cells, slices, animals) and ZT12 (n = 6, 6, 6) in control, and at ZT0 (n = 6, 6, 5) and ZT12 in MPL, groups (n = 5, 5, 4).* ***B)*** *sIPSC amplitude at (n = 7, 7, 6) and ZT12 (n = 6, 6, 6) in control, and at ZT0 (n = 6, 6, 5) and ZT12 in MPL, groups (n = 5, 5, 4).* ***C)*** *Representative traces from each condition. Scale bar*

*represents x = 1 s and y = 20 pA. Data in **A** and **B** are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance.*

5.3.13. Spontaneous IPSC properties in infralimbic neurons

In infralimbic neurons, no effects of time, treatment, or their interaction were uncovered, though this is potentially due to the low n number reducing the power of the statistical test.

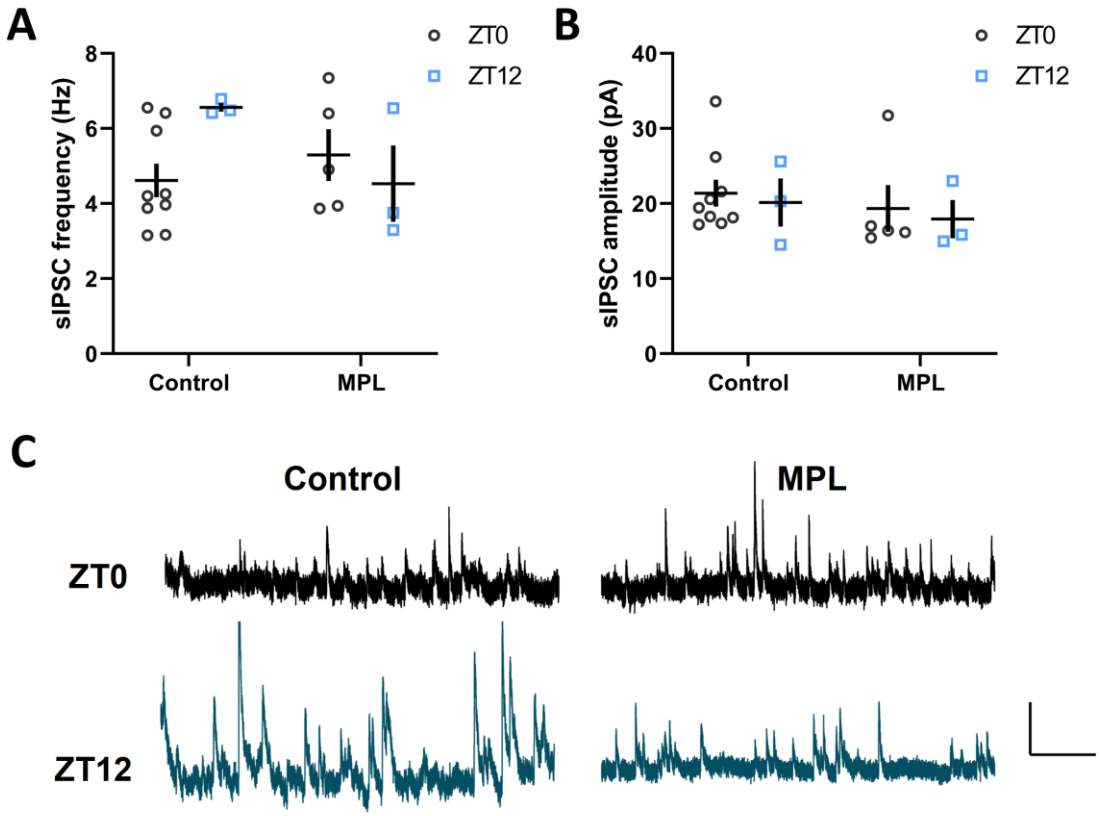

Figure 5.14. sIPSC properties of IL neurons. ***A)*** *sIPSC frequency at ZT0 (n = 9, 9, 7) (cells, slices, animals) and ZT12 (n = 3, 3, 3) in control, and at ZT0 (n = 5, 5, 5) and ZT12 in MPL, groups (n = 3, 3, 3).* ***B)*** *sIPSC amplitude at ZT0 (n = 9, 9, 7) and ZT12 (n = 3, 3, 3) in control, and at ZT0 (n = 5, 5, 5) and ZT12 in MPL, groups (n = 3, 3, 3).* ***C)*** *Representative traces from each condition. Scale bar represents x = 1 s and y = 20 pA. Data in **A** and **B** are plotted as mean ± SEM. Experimental n is indicated as (n = cells, slices, animals). Two-way ANOVA and Tukey's multiple comparison tests were used to test for statistical significance.*

5.4. Discussion

In this chapter I have demonstrated that chronic treatment with the synthetic glucocorticoid MPL, results in reductions in AMPAR:NMDAR ratios in neurons in PrL, but not IL, at both the circadian peak and nadir. This was somewhat surprising given that IL is known to be involved in the augmentation of the HPA axis and emotional responses (Shirazi et al. 2015), however, prelimbic neurons are known to be involved in glucocorticoid modulation of recognition memory (Barsegyan et al. 2019), and glucocorticoid induced alterations to glutamate receptor expression have been shown in both PrL and IL (Gourley et al. 2009a).

The roles of the prelimbic and infralimbic cortices are somewhat defined, with the more dorsal prelimbic and anterior cingulate regions playing a greater role in decision making and action and the more ventral infralimbic cortex being more involved in the regulation of motivation and emotion (Anastasiades and Carter 2021). Specifically, IL has been implicated in stress, mood and fear memory, via strong projections to the lateral septum, hypothalamus, amygdala, BNST, and to a lesser extent the hippocampus (Wood et al. 2019). Recent evidence shows that PrL and IL populations operate in a complex and opposing manner (Capuzzo and Floresco 2020), which depends on their reciprocal connections (Mukherjee and Caroni 2018). Although some sites of innervation are common between PrL and IL, many of their outputs are quite distinct. PrL primarily innervates the nucleus reuniens of the thalamus, the capsular part of the central amygdala and the raphe nuclei (Radtke-Schuller 2018).

Recent evidence has further shown the laminar distribution of neurons projecting to distinct sites in the PrL in mice, and distinct inhibitory and excitatory microcircuits within the mPFC are positioned to control the outputs to different regions, allowing local microcircuit control of behavioural outcomes and positioning the mPFC to support sustained activity during short-term memory tasks (Anastasiades and Carter 2021). It is quite likely, therefore, that MPL may be altering gene expression distinctly in certain cell types and consequently modulating separate circuits in a different way, depending on factors like chromatin accessibility and the differential expression of GR and MR. However, cell-type specific dissection of glucocorticoid actions in mPFC was beyond the scope of this chapter, though single cell RNA-seq combined with circuit specific manipulations would provide great insight into the actions of chronic glucocorticoid exposure and physiological rhythms on mPFC neurophysiology, and ultimately behaviour.

Interestingly, the changes in AMPAR:NMDAR ratio in PrL were accompanied by a reduction in the decay time of NMDAR-mediated EPSCs caused by MPL treatment. Given that GluN2B subunits confer a greater decay time to the NMDAR compared to GluN2A and GluN2C subunits (Cull-Candy, Brickley, and Farrant 2001), this data indicates that NMDAR subunit composition

in this subregion may underlie these changes in NMDAR-EPSC kinetics. In particular, a downregulation of GluN2B may be the cause of the synaptic and mnemonic deficits caused by chronic steroid treatment, which would be consistent with data from the first two chapters in this thesis, and data showing downregulation of mPFC GluN2B following chronic stress (Yuen et al. 2012).

Intriguingly, although alterations to AMPAR:NMDAR ratio and NMDAR-mediated EPSC kinetics were observed only in PrL, suppression of mEPSC amplitude by MPL was found in both PrL and IL neurons. These data suggest that AMPAR function is compromised by chronic steroid treatment in both regions. A potential explanation for this discrepancy is that while AMPAR subunit expression is disrupted in both subregions, NMDAR subunit expression is altered only in PrL. Further experiments utilising either immunofluorescent histochemistry co-localised to synaptic markers, and/or western blotting of proteins from synaptic fractionation methodologies would be necessary to determine whether changes in receptor protein levels are responsible for these alterations in spontaneous neurotransmission. Furthermore, RNA-sequencing of mPFC subregions, like that described in the hippocampus in Chapter IV, would provide valuable insight into potential disruption to the transcriptome incurred by MPL. Single cell RNA-sequencing of mPFC subregions would provide even more detailed information at the cellular level in this heterogenous tissue. Nevertheless, importantly, the electrophysiological data presented in this chapter characterizing changes to excitatory neurotransmission following chronic MPL treatment provide robust evidence that is consistent with reports demonstrating altered expression of AMPAR and NMDAR subunits in mPFC following chronic stress (Yuen et al. 2012) and chronic corticosterone exposure (Gourley et al. 2009a).

AMPAR phosphorylation can confer changes to the electrophysiological properties of the receptor, for example phosphorylation of serine 845 increases channel open probability and peak current amplitude, and phosphorylation at serine 831 increases the single-channel conductance.

The mPFC has been implicated in diurnal rhythms in the mesolimbic system (Baltazar, Coolen, and Webb 2013), which are thought to be important in the circadian modulation of reward and drug-seeking behaviour (Webb et al. 2009). Time of day effects on evoked and spontaneous neurotransmission were largely absent from the mPFC slice preparation, unlike the PRH and hippocampus, although some non-significant trends for circadian rhythmicity were observed for the frequency of spontaneous and miniature excitatory and inhibitory events. Interestingly, those trends were reversed by MPL in the excitatory, but not inhibitory, system.

Chapter VI

General Discussion

6.1. Summary of findings

In this thesis, I have demonstrated for the first time that chronic treatment with the synthetic glucocorticoid methylprednisolone disrupts the natural circadian oscillations in hippocampal neurophysiology and plasticity. I have presented evidence that suggest disruption to plasticity and spontaneous neurotransmission are physiological consequences of the dysregulation of hippocampal clock oscillations that ultimately lead to deficits in learning and memory. This data demonstrates the importance of the maintenance of natural glucocorticoid rhythms and reveals mechanisms by which adverse side effects of synthetic glucocorticoid treatment manifest themselves. Further to deficits in hippocampal function, circadian oscillations in spontaneous neurotransmission in the perirhinal cortex were inverted by the disruption to physiological pulsing of glucocorticoid receptor activation caused by chronic MPL treatment. These changes also led to deficits in perirhinal plasticity during the active phase, which I hypothesise to underlie deficits in an object recognition task. I have shown that changes to glutamate receptor ratios and NMDA receptor mediated currents in prelimbic mPFC are also incurred by chronic MP treatment, as well as suppression of the amplitude of spontaneous excitatory transmission in mPFC neurons.

6.2. Circadian and glucocorticoid control of synaptic plasticity

The glucocorticoid receptors GR and MR are expressed in many brain regions including hippocampus, amygdala, PFC, lateral septum, and brainstem (Diorio, Viau, and Meaney 1993; Mitra, Ferguson, and Sapolsky 2009; Reul and Kloet 1985; Senft, Meddle, and Baugh 2016). The hippocampus, which is enriched in the expression of GR and MR, has been extensively studied for its role in memory processing, and how this can be modulated by stress.

Circadian rhythms in cognitive performance have been described in many species and in a variety of tasks (Hoffmann and Balschun 1992; Hauber and Bareiss 2001; Valentinuzzi, Menna-Barreto, and Xavier 2004; Chaudhury and Colwell 2002). Generally speaking, learning and memory processes are enhanced during the organisms' active phase, presumably by the facilitation of learning-related neural activity. Synaptic plasticity is known to be crucial in the successful encoding of memory (Nabavi et al. 2014), and here I have shown that hippocampal LTP is subject to circadian control. In particular, I have demonstrated that LTP could be induced in slices taken at the onset of the active period, whereas LTP could not be induced in slices taken at the start of the inactive period. This provides a candidate mechanism for the aforementioned circadian fluctuations in cognition. I have also shown that at the circadian nadir, LTP induction can be facilitated by the application of the synthetic glucocorticoid methylprednisolone, highlighting the importance of glucocorticoid signalling in mediating these circadian effects on synaptic plasticity in the hippocampus.

Endogenous glucocorticoids bind rapidly to GR, causing brief epochs of activation, in the order of minutes. MPL, on the other hand, has a prolonged half-life *in vivo* (Al-Habet and Rogers 1989; Uhl et al. 2002), and consequently maintains very low levels of endogenous glucocorticoids via negative feedback on the HPA axis. All the while, the levels of GR and MR activation by the synthetic steroid remain chronically elevated (Kalafatakis et al. 2018).

The chronic activation of GR, which is normally pulsatile, facilitates the increased expression of the clock gene *Per1*, thereby driving the dysregulation of downstream clock gene products, as well as a number of genes associated with synaptic function. *Per1* gene expression in the hippocampus has been shown to follow a ~4-hour phase-shift as a consequence of MPL treatment (Birnie 2018), meaning that the circadian regulation of synaptic function is liable to disruption by steroid treatment. This phase shift is due to the suppression of the endogenous glucocorticoid rhythms by chronic MPL treatment. The circadian peak is anticipatory of the active phase, whereas animals must wake and start drinking in order to begin activating glucocorticoid receptors with the exogenous synthetic glucocorticoid. This is analogous to the suppression of endogenous rhythms in patients who must then wake before taking their prescribed medication.

Furthermore, behavioural data show that chronic treatment with MPL results in deficits in a hippocampus dependent NOL task, with poor performance occurring at a 6 hour, but not 1 hour, delay post-training (Birnie 2018). This led to the theory that it was the consolidation of long-term memory that is affected by MPL treatment, a process that is known to be mediated, at least in part, by synaptic plasticity.

In this thesis, I show that chronic treatment with the synthetic glucocorticoid methylprednisolone ablates the circadian regulation of hippocampal LTP, demonstrating that GR regulation of the molecular clock is a key driver of circadian neurophysiology in the hippocampus. The disruption of the hippocampal clock by MPL results in altered expression of a number of synaptic gene products, including NMDA receptor subunits and CaMKII (Birnie 2018), and I hypothesise that hippocampal plasticity is impaired during the active phase by the disruption of the association between GluN2B NMDAR subunits and CaMKII during the active phase, at a time critical to learning. Indeed, both of these genes are known to modulated by GR activation (Birnie 2018; Mifsud et al. 2021) and the blockade of hippocampal LTP by Ro-25-6981, a selective GluN2B antagonist, further supports this notion. Moreover, it has been shown previously that the rhythmic phosphorylation of CREB is regulated by *Per1,* and clock gene knockout models have resulted in impaired hippocampal plasticity (Kwapis et al. 2018; Rawashdeh et al. 2016; Rawashdeh et al. 2014; Garcia et al. 2000; Wardlaw et al. 2014; Snider et

al. 2016), supporting the notion that clock-driven rhythms in the hippocampus are crucial for synchronizing learning-related synaptic pathways.

Interestingly, a strong induction protocol (spaced theta burst stimulation, sTBS) was still able to produce late LTP of Schaffer collateral fibres in CA1 following MPL treatment. sTBS provokes calcium entry to the cell via extra-NMDAR routes, for example via VDCCs. Analysis of GO pathways predicted a specific loss of the rhythmic regulation of NMDARs in whole hippocampus extracts from MPL treated animals, while other synaptic pathways and proteins (including VDCCs) remained intact. These data provide further evidence for the dysregulation of NMDAR expression being a vital mediator of the negative side effects of chronic steroid treatment. Recent evidence demonstrating circadian- and stress-induced changes in GluN2B expression lend further credence to the idea that GluN2B-containing NMDARs are central to this mechanism.

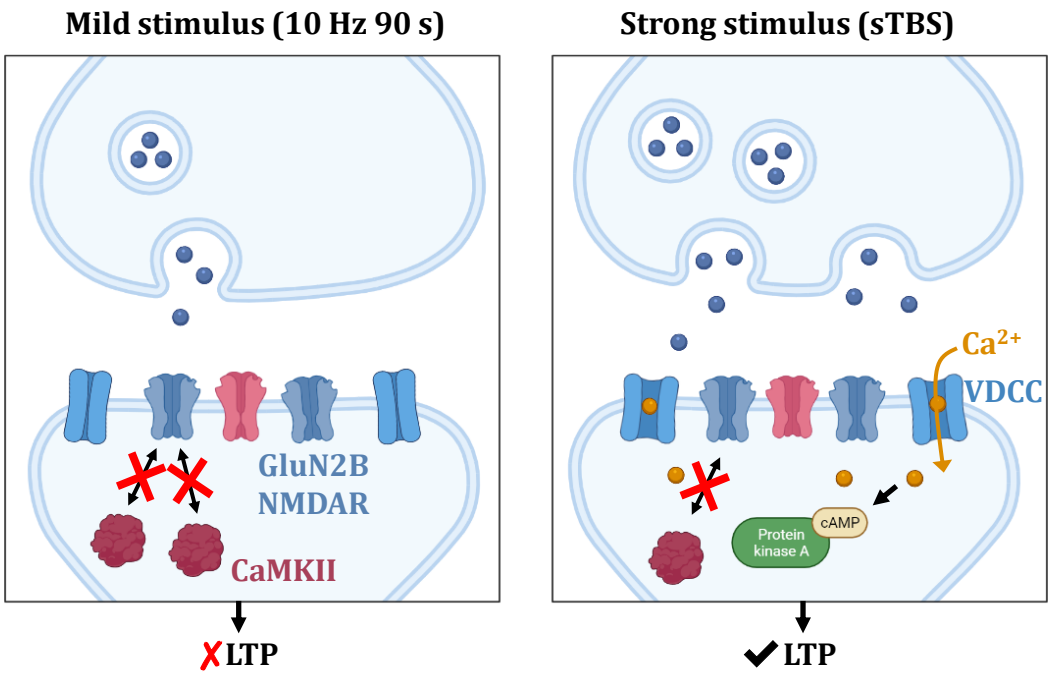

Figure 6.1. MPL treatment induces selective deficits in NMDAR-dependent plasticity. *Schematic diagram illustrating how MPL treatment disrupts the association of GluN2B-containing NMDARs and CaMKIIα, thereby reducing the expression of LTP in response to a mild stimulus, at a time critical to learning (i.e. during the active phase) (left panel). When a strong stimulus is evoked (right panel), calcium enters the post-synaptic cell from extra-NMDAR routes, including VDCCs, activating other plasticity pathways, for instance PKA, therefore LTP is still elicited despite the disrupted GluN2B-CaMKII association.*

As well as inducing deficits in a NOL task, chronic MPL treatment was shown to negatively affect performance in a NOR task, at 6, but not 1, hours post-training (Birnie 2018). The NOR task is known to depend on perirhinal cortex (Aggleton, Kyd, and Bilkey 2004; Brown and Aggleton 2001), thus, as well as causing deficits in hippocampal plasticity, I theorized that MPL would alter the expression of perirhinal LTD, which is known to be central in the formation of single-item recognition memory (Griffiths et al. 2008).

Indeed, I have shown for the first time that chronic treatment with methylprednisolone resulted in the abolition of LTD in the perirhinal cortex in slices taken at the onset of the active phase. In line with data from the hippocampus, the MPL-affected protocol is dependent on NMDAR activation. It is worth noting, though, that although full blockade of LTD was achieved in all experiments by the NMDAR antagonist D-AP5, some expression of LTD was still observed in 50% of experiments using the selective GluN2B antagonist, Ro. Contribution from NMDARs consisting of a different subunit composition could account for this, or perhaps only partial blockade of GluN2B-containing receptors was achieved at the onset of low frequency stimulation. Nonetheless, it seems likely that MPL is acting via a similar mechanism in PRH to HPC, given the similar dysregulation of clock genes reported in both regions by chronic steroid treatment (Birnie 2018; Rozwaha 2017). Again, the use of molecular techniques to ascertain protein and mRNA levels of NMDAR and AMPAR subunits at the synapse following MPL treatment would provide key insight into the precise mechanisms of synaptic dysfunction.

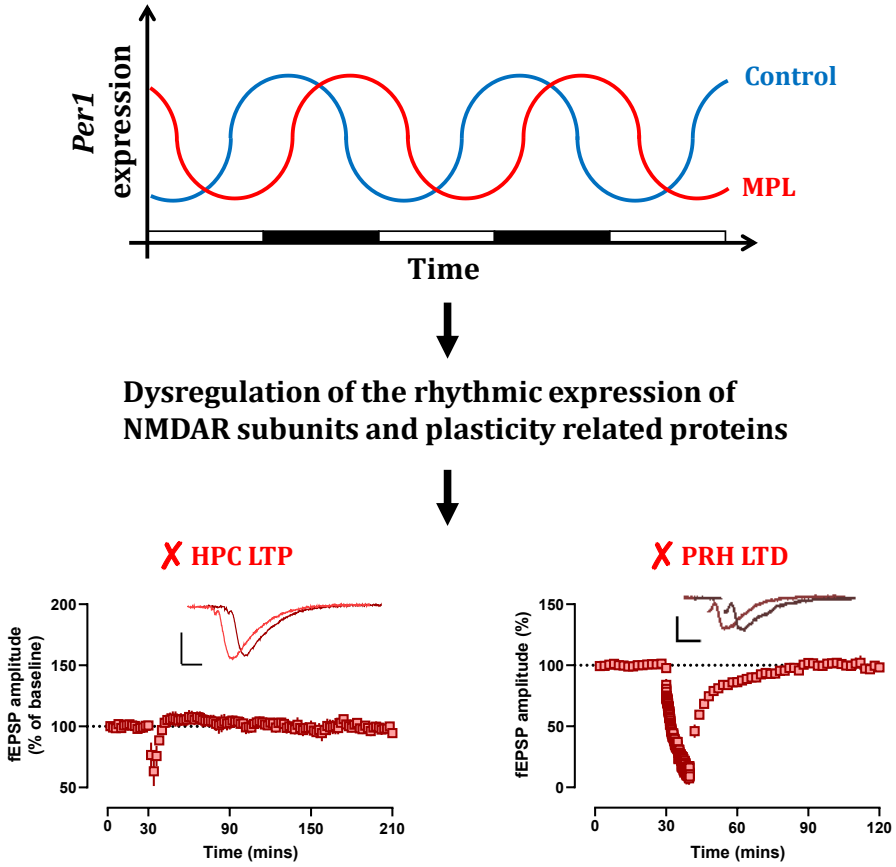

Figure 6.2. Phase misalignment could underlie deficits in synaptic plasticity. *Schematic diagram showing how in MPL treated animals the expression of clock genes in HPC and PRH becomes misaligned with external circadian cues (in this case, the light/dark cycle). This also results in the altered expression of synaptic gene targets, altering their transcription in a manner that impedes the association of plasticity-related proteins (i.e. GluN2B and CaMKIIα) at a time critical to learning. This results in deficits in LTP and LTD that ultimately lead to impairments in learning and memory.*

Nevertheless, the GluN2B-containing NMDAR appears to play a critical role in mediating the impacts of long-term steroid use on mnemonic processing and is a potential therapeutic target in reducing side effects associated with treatment.

Synaptic plasticity has been shown to be a crucial correlate of learning and memory, and a causal link between synaptic plasticity and the successful formation of memory has been demonstrated (Nabavi et al. 2014). Therefore, the deficits in synaptic plasticity induced by chronic MPL treatment that I have described in this thesis provide a physiological substrate for the memory deficits reported following chronic steroid treatment. An enhanced understanding of the mechanisms by which chronic steroid treatment can affect learning and memory will aid

in the prevention of unwanted side effects, the development of safer drugs, and improved delivery of the drugs currently available, leading to better outcomes.

6.3. Chronic synthetic glucocorticoid use alters synaptic receptor ratios and potentially subunit composition

In the hippocampus, I demonstrated that the AMPAR:NMDAR ratio in CA1 neurons is reduced by chronic treatment with methylprednisolone, in slices taken at the onset of the active phase. This could either reflect a decrease in the number of functional AMPAR receptors at the surface, a decrease in AMPAR receptor function at the surface, or potentially an increase in NMDAR receptor function/number at the synapse. As mentioned previously, GO pathway analysis of RNA-seq data predicted a specific effect on the NMDAR, so altered regulation of AMPAR subunits is somewhat less likely. Indeed, MPL treatment did not induce any changes to the amplitude of mEPSC events, indicating that post-synaptic AMPAR content was not reduced. However, the plasticity data presented in this thesis are indicative of a loss of function of NMDARs in the hippocampus, and an increase in NMDAR-dependent currents seems contradictory. It is possible, though, that the subunit-composition of NMDARs at hippocampal synapses is altered by chronic steroid treatment, and that a reduction in GluN2B-containing receptors, or a loss in their functionality, is responsible for the deficits in plasticity, rather than a reduction in overall synaptic content of NMDARs. Indeed, the data I have shown here are consistent with reports of a reduction in the AMPAR:NMDAR ratio of fEPSPs in temeroammonic-CA1 synapses, though in this thesis, Schaffer collateral-CA1 synapses were investigated (Kvarta et al. 2015). There was a trend towards a reduction in GluN2A:GluN2B ratio in MPL treated hippocampal slices, but due to the low n number for the control group, no conclusions can be drawn from this data, and further experiments are needed. Additional supporting molecular evidence, for example, using western blotting to determine the relative ratio of GluN2A:GluN2B in synaptic fractions would also provide valuable insight into how MPL alters synaptic receptor content.

RNA-seq data of the hippocampus from control and MPL treated animals, combined with the analysis of plasticity and spontaneous neurotransmission, suggests that it is the precisely co-ordinated timing of transcription of certain synaptic genes that is crucial for the expression of plasticity, and thus learning, at the appropriate time of day.

Very little research has been published on the effects of glucocorticoids in the perirhinal cortex, however, in this thesis I have demonstrated that AMPAR:NMDAR ratios of layer II/III pyramidal neurons were affected by MPL treatment, and not time. Specifically, at ZT12, the onset of the active phase, there was a significant increase in AMPAR:NMDAR ratio in slices from treated rats.

Moreover, when all time points were analysed, a significant main effect of treatment was found. Although this may seem at odds with what I observed in the hippocampus, it was an expected effect, given that it could indicate a reduction in the expression of NMDARs in PRH, thus explaining the deficits in NMDAR-dependent synaptic plasticity. Moreover, given the complex nature of glucocorticoid action in the CNS, region specific effects are to be expected. There was also a non-significant trend toward a reduction in the NMDAR EPSC component mediated by GluN2B, indicating a possible reduction in the function of GluN2B-containing receptors during the active phase, however, further experiments utilising western blotting of proteins from synaptic fractionation methodologies, and/or immunofluorescent histochemistry co-localised to synaptic markers would be needed to resolve this.

Interestingly, in the medial prefrontal cortex, MPL treatment was found to have a significant effect on AMPAR:NMDAR ratio in prelimbic, but not infralimbic, pyramidal neurons. This represented a decrease in AMPAR:NMDAR ratio in treated animals at both ZT0 and ZT12, although post-hoc analysis did not reveal a significant difference. This data shows that the MPL-induced effects in prelimbic cortex may be more akin to those observed in the hippocampus, rather than the PRH. However, with no molecular analysis of mRNA or protein levels in mPFC, it is difficult to posit the exact cause with much certainty, though these data are in line with reports of degradation of AMPAR subunits in mPFC following chronic stress (Yuen et al. 2012). Taken together, the data presented in this thesis underscore the importance of considering the region-specific effects of glucocorticoid action, likely mediated by many factors including relative receptor expression, chromatin accessibility, and the expression of co-factors/modulatory elements.

The prelimbic cortex is thought to be important in several aspects of cognition, including strategy set shifting and the modulation of attention in the face of changing experimental conditions (Balleine and Dickinson 1998; Sharpe and Killcross 2014). Interestingly, glucocorticoids have also been implicated in the re-allocation of cognitive resources to achieve balance between salience and executive control networks (Hermans et al. 2014). Indeed, blockade of MR prevented the adaptive switch between a spatial and stimulus-response strategy in a spatial task (Vogel et al. 2016; Schwabe et al. 2013). Therefore, it is possible that chronic steroid treatment may affect cognitive performance by impeding the access to flexible and reflective processing, by causing errant reorganization of brain networks. This could favour shifts towards salience networks and away from executive control networks, thereby impeding the shifting of strategies and attention.

Analysis of NMDAR-mediated EPSC kinetics in prelimbic neurons revealed a significant decrease in decay time following MPL treatment. I hypothesize that this could be due to the

downregulation of GluN2B subunits in these neurons, as the GluN2B subunit confers a longer open time, and therefore a greater decay time (Cull-Candy, Brickley, and Farrant 2001). This would be consistent with previous data demonstrating the downregulation of GluN2B subunits in mPFC following chronic stress (Yuen et al. 2012). Given the importance of GluN2B-containing NMDARs in mediating the negative effects of MPL in the HPC and PRH, it appears to be a good candidate for alterations to synaptic receptor content in prelimbic mPFC, and also a potential therapeutic target for alleviating the unwanted side-effects of chronic steroid treatment, chronic stress, and circadian disturbances.

6.4. Circadian and glucocorticoid control of spontaneous neurotransmission

I have also demonstrated that the frequency of miniature EPSCs in the hippocampus is elevated during the circadian peak, and reduced at the nadir, potentially facilitating the induction of LTP during the active phase, and the consolidation of synaptic changes during the inactive phase.

As well as disrupting the circadian regulation of synaptic plasticity in area CA1, I have demonstrated that chronic MPL treatment resulted in the dysregulation of circadian changes in mEPSC and mIPSC frequency in CA1 pyramidal neurons. Interestingly, the circadian pattern of mEPSC frequency was inverted in MPL treated animals, compared to controls, reflecting the phase-shifting of circadian clock gene expression that is evident following chronic steroid treatment (Birnie 2018). This reinforces the hypothesis that desynchronization of the hippocampal (and other extra-SCN) clocks from external cues results in brain activity that is not appropriate for the time of day, or current environmental challenges, thus hampering learning and memory. Crucially, at ZT12, when chronic MPL treatment resulted in blockade of NMDAR-dependent LTP, mEPSC frequency was suppressed in treated animals, compared to controls. This suppression of spontaneous excitatory activity could contribute to the deficits in plasticity of hippocampal neurons during the active phase. Indeed, evidence from *Drosophila* indicate that enhanced spontaneous glutamate release is required for activity-dependent synaptic growth (Cho et al. 2015) and whole-cell recordings from rat hippocampal slices indicate that spontaneous glutamate neurotransmission is critical in setting the threshold for LTP (Bonansco et al. 2011).

Further to this, the changes to mEPSC and mIPSC frequency in MPL treated animals were not equivalent, thereby offsetting the delicate balance between excitation and inhibition. Many studies have now demonstrated that alterations to the E-I ratio in the forebrain are central in mouse models of several psychiatric disorders including autism, schizophrenia, Angelman and Rett syndromes, and Alzheimer's disease (Lee, Lee, and Kim 2017; Antoine et al. 2019; Calfa et al. 2015; Judson et al. 2016; Busche and Konnerth 2016; Busche et al. 2015). Generally, chronic increases in excitation that are not compensated for by increases in inhibition are thought to

result in pathogenesis, and although these observations are often made in sensory and visual cortical areas, altered E-I balance in the hippocampus and mPFC has been implicated in Rett and Angelman syndromes, respectively (Calfa et al. 2015; Rotaru et al. 2018). In addition to being dysregulated in disease, it is thought that the E-I balance is maintained within a target range by homeostatic mechanisms in physiological conditions, which act primarily to adjust the strength of inhibition in response to changes in excitation, in order to promote learning and cortical remodelling (D'amour and Froemke 2015; Froemke 2015; Nanou and Catterall 2018). Recently, early-life stress has been shown to accelerate developmental shifts in the E-I balance (Karst et al. 2020), emphasizing the role of glucocorticoids in the regulation of this phenomenon.

In the hippocampus and perirhinal cortex, methylprednisolone treatment leads to the dysregulation of the molecular clock (Rozwaha 2017), and in the hippocampus RNA-seq revealed that chronic MPL treatment also resulted in the disruption of the dynamic transcriptional rhythms of many synaptic genes, including NMDAR subunits and CaMKII (Birnie 2018). It is likely, therefore, that similar dysregulation of the transcriptional profiles of synaptic gene targets occurs in perirhinal and prefrontal cortices. Similar to the hippocampus, I have shown for the first time that mEPSC frequency in PRH neurons is decreased at ZT12, and increased at ZT0, by MPL treatment, and these alterations are not balanced by similar alterations to mIPSC properties. Further to this, MPL treatment induced a striking inversion of the circadian regulation of sEPSC frequency in PRH neurons.

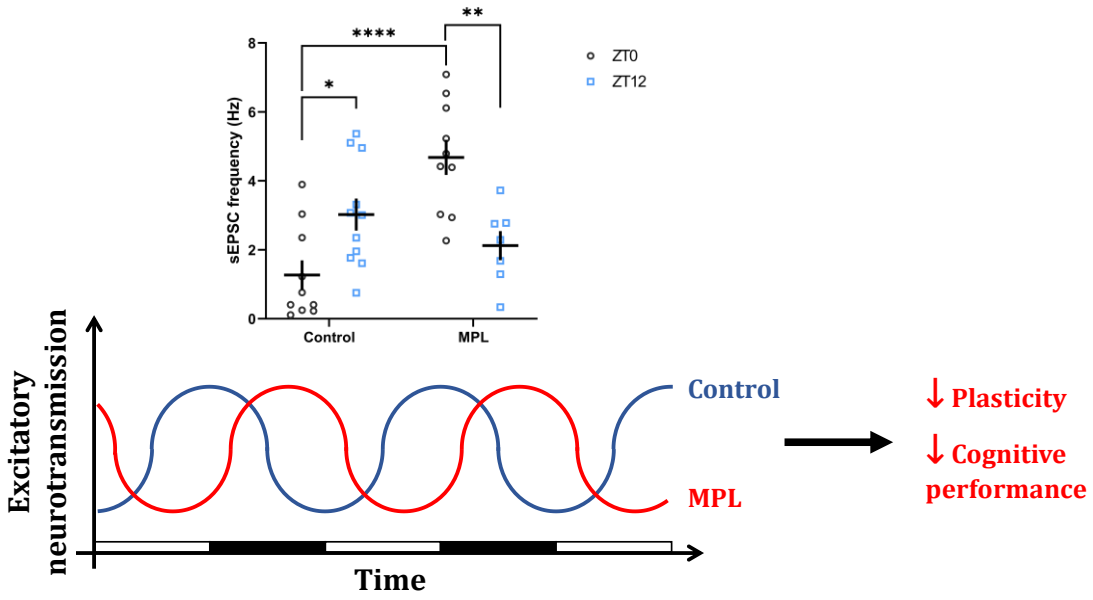

Figure 6.3. Phase inversions in spontaneous excitatory neurotransmission may cause cognitive impairments. *Chronic treatment with MPL resulted in the inversion of the circadian rhythms of sEPSC frequency and mEPSC frequency, in the PRH and HPC, respectively. Recently, spontaneous glutamate release has been shown to be pivotal in the synaptic plasticity, and so these changes could underlie the cognitive deficits associated with chronic steroid treatment.*

Recent evidence has shown that dysregulation of spontaneous neurotransmission was crucial in the onset of developmental and epileptic encephalopathies (Alten et al. 2021). Moreover, increases in spontaneous glutamatergic transmission were associated with endoplasmic reticulum stress induced neurodegeneration (Chanaday et al. 2021) and found to be central to deficits in neurotransmission in a mouse model of Huntington's disease (Mackay et al. 2020). These studies, combined with the data presented in this thesis, provide concerning evidence that long-term synthetic glucocorticoid causes alterations to spontaneous neurotransmission in brain regions key for learning and memory, and that these alterations may trigger or accelerate the onset of neurological disease.

Intriguingly, the MPL and circadian effects on spontaneous neurotransmission in mPFC were quite different to those observed in the hippocampus and PRH. Although alterations to AMPAR:NMDAR ratio were only observed in prelimbic neurons, mEPSC amplitude was suppressed by MPL treatment in both PrL and IL. These data suggest that AMPAR function at the synapse is reduced in both subregions by chronic steroid treatment, and that NMDAR function and/or subunit composition is altered primarily in the PrL. These data are consistent with reports that have shown the altered expression of AMPAR and NMDAR subunits in mPFC

neurons following chronic stress and chronic corticosterone exposure (Yuen et al. 2012; Gourley et al. 2009a). Further electrophysiological experiments would be desirable to analyse the subunit composition of NMDARs in PrL, and molecular techniques could be utilized to investigate the potential reduction in AMPAR subunit mRNA and proteins in mPFC synapses. It is possible that a reduction in the excitatory drive in the mPFC, coupled with alterations to NMDAR function, could induce reductions in cognition that are associated with chronic steroid treatment. These alterations may lead to deficits in cognitive flexibility, attention, and working memory.

6.5. Conclusions and limitations

These data provide a novel insight into the action of synthetic glucocorticoids in the brain, and how they interact with the circadian cycle, however, there are some limitations of the studies. Firstly, the method of delivery of methylprednisolone, via ad libitum treatment in drinking water, limits the known exact dose that each individual received during each experiment. Moreover, due to the metabolic effects of MPL, treatment was limited to a maximum of seven days, which limits the assessment of longer-term steroid exposure. Some patients can be on glucocorticoid treatment for many months at a time, and in this thesis, I report several neuropathic effects occurring after only seven days treatment.

Synthetic glucocorticoids are very widely used today in medical practice, with almost 2% of the adult UK population using oral glucocorticoids at any given time (Van Staa et al. 2000; Overman, Yeh, and Deal 2013). Despite their clinical usefulness, long-term treatment with synthetic glucocorticoids has been associated with cognitive deficits, depression, and even an increased risk of suicide (Brown and Chandler 2001; Judd et al. 2014; Fardet, Petersen, and Nazareth 2012). In this thesis, I believe I have elucidated several novel aspects of synthetic glucocorticoid action in the brain, and the interaction of glucocorticoid treatment with circadian rhythms. These neural deficits provide a physiological substrate for the cognitive impairments associated with glucocorticoid treatment and provide better understanding of the side-effects incurred by chronic glucocorticoid exposure.

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