The Effects of Surgical Stress on Hippocampal and Medial Prefrontal Dendritic Morphology.

by

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ABSTRACT

THE EFFECTS OF SURGICAL STRESS ON HIPPOCAMPAL AND MEDIAL PREFRONTAL DENDRITIC MORPHOLOGY IN ADULT MALE RATS

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Surgical stress can have profound effects on hippocampal and medial prefrontal cortex structure and function. Our laboratory demonstrated that sham-orchidectomy causes atrophy of CA3 apical dendrites while testosterone replacement partially restores CA3 apical dendrites. However, the lasting effects of surgery and the factors contributing to surgical stress remained unclear. This thesis demonstrates that the remodelling of hippocampal and mPFC apical dendrites following surgery persist for up to 2-months. While the anesthesia does not appear to be contributing to these effects, they may be in part due to glucocorticoid action as prolonged dexamethasone exposure produces similar dendritic remodelling as surgery. This indicates a potential contribution of sustained glucocorticoids during the recovery period. The use of a surgical approach with minimal post-operative complications did not produce the same atrophy of hippocampal and mPFC dendrites. These findings reveal some of the contributors to surgery and post-operative recovery on hippocampal and mPFC dendritic morphology.

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DECLARATION OF WORK PERFORMED

I declare that I have performed all of the work in this thesis with the exception of the tasks listed below.

Eric Lawton performed orchidectomy and sham surgeries and the imaging and neuronal tracings for the 1-month orchidectomy (with and without testosterone replacement) and sham animals in chapter 2. He also performed the isoflurane anesthesia for the rats in chapter 3 and assisted with the handling and dexamethasone treatment in chapter 4.

Dr. Ari Mendell assisted with the processing of hippocampal and medial prefrontal sections for some of the dexamethasone animals in chapter 3.

Dr. Neil MacLusky assisted with the abdominal sham-orchidectomies performed in chapter 5.

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17β-HSD	17β-hydroxysteroid dehydrogenase
3α-HSD	3α-hydroxysteroid dehydrogenase
3α-diol	5α-androstane-3α, 17β-diol; 3α-androstanediol
3β-HSD	3β-hydroxysteroid dehydrogenase
3β-diol	5α-androstane-3β, 17 β -diol; 3β-androstanediol
αCaMKII	alpha-Ca^{2+}/calmodulin-dependent protein kinase II
ACC	Anterior cingulate cortex
ACTH	Adrenocorticotropic hormone
AMPA	α-amino-3-hydroxyl-5-methyl-4-ixoxazolepropionic acid
AR	Androgen receptor
BDNF	Brain-derived neurotrophic factor
CA1	Cornu ammonis 1
CA3	Cornu ammonis 3
CBG	Corticosteroid-binding globulin
CREB	cAMP response element-binding protein
CRH	Corticotrophin-releasing hormone
CRHR1	Corticotrophin-releasing hormone receptor 1
CRHR2	Corticotrophin-releasing hormone receptor 2
CUMS	Chronic unpredictable mild stress
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DG	Dentate gyrus
EC	Entorhinal cortex
E-LTP	Early phase long-term potentiation
ERK1/2	Extracellular-signal-regulated kinases 1/2
FKBP5	FK506 binding protein 5
GABA	Gamma-aminobutyric acid
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
HPA	Hypothalamic-pituitary-adrenal axis

HPG	Hypothalamic-pituitary-gonadal axis
IL	Infralimbic cortex
IL-β	Interleukin-1β
IL-6	Interleukin-6
IL-18	Interleukin-18
LTD	Long-term depression
LTP	Long-term potentiation
L-LTP	Late phase long-term potentiation
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MC2-R	Melanocortin type 2 receptor
MF	Mossy fibers
mPFC	Medial prefrontal cortex
MR	Mineralocorticoid receptor
mRNA	messenger RNA
NAc	Nucleus accumbens
NLRP3	NOD-like receptor protein 3
NMDA	N-methyl-D-aspartic acid
ORCH	Orchidectomy
P450scc	P450 cholesterol side-chain cleavage enzyme
PFC	Prefrontal cortex
PKA	Protein kinase A
PKC	Protein kinase C
PL	Prelimbic cortex
POCD	Post-operative cognitive dysfunction
PTSD	Post-traumatic stress disorder
PVN	Paraventricular nucleus
SHAM	Sham-orchiectomized
SHBG	Sex hormone binding globulin
SGZ	Sub-granular zone
SVZ	Sub-ventricular zone

TNFα	Tumor necrosis factor α
TrkB	Tropomyosin receptor kinase B
VTA	Ventral tegmental area

1 General Introduction and Literature Review

1.1 Hippocampus

1.1.1 Introduction

The understanding of the functional role of the hippocampus in learning and memory has been developing since the first studies conducted by Scoville and Milner on patient H.M in 1957. Prior to these studies, the process of memory formation was thought to involve the entire brain rather than specific interconnected brain regions (Squire, 2009). H. M underwent a bilateral temporal lobe-resection, in an attempt to alleviate his severe epileptic condition (Milner & Scoville, 1957). Although his epileptic symptoms were cured and his intellect, short-term memory and many cognitive abilities remained intact, H.M developed major memory impairments specifically affecting his ability to convert short-term memories into long-term memories (Milner & Scoville, 1957). The procedure caused both anterograde amnesia causing the inability to form new memories and to a lesser extent retrograde amnesia resulting in the loss of previously formed memories (Milner & Scoville, 1957; Squire, 2009). The studies on H.M revealed the role of the medial temporal lobe in memory processes and sparked decades of research on identifying the distinct functions of the hippocampus, amygdala and perirhinal cortex in various forms of memory (Milner & Scoville, 1957; Squire, 2009).

1.1.2 Hippocampal Organization, Neuronal Morphology and Circuitry

The hippocampus is a key structure located in the medial temporal lobe and is known for its role in learning, spatial and emotional memory and the consolidation of short-term memories to long-term storage (Squire, Stark, & Clark, 2004). It is also part of the limbic system and this is involved in the regulation of mood state (Roxo, Franceschini, Zubaran, Kleber, & Sander, 2011). Due to its involvement in the limbic system, the hippocampus is affected in many neuropsychiatric disorders such as anxiety, depression and post-traumatic stress disorder (PTSD) (Bremner et al., 1995; Liu et al., 2017; Malivoire, Girard, Patel, & Monson, 2018; Malykhin & Coupland, 2015). The general anatomy and circuitry of the hippocampus are highly conserved across various species such as rats, non-human primates and humans (Naber, Lopes Da Silva, & Witter, 2001). The hippocampus is comprised of three main sections; the dentate gyrus (DG), Ammon's horn and the subiculum (Witter, 2010). The outer most layer of the dentate gyrus is known as the stratum moleculare which receives the majority of the inputs from the entorhinal cortex. The granular layer of the dentate gyrus contains granular cells, the primary neuron type of the dentate gyrus (Witter, 2010). The dentate gyrus also contains the subgranular zone (SGZ), a major site of neurogenesis where new neurons are created and undergo proliferation and differentiation into new hippocampal cells (Lieberwirth, Pan, Liu, Zhang, & Wang, 2016; Witter, 2010).

Interlocking with the dentate gyrus is Ammon's horn. Ammon's horn has four main sub-regions known as cornu ammonis (CA) 1 through 4 (Witter, 2012). Although

CA1, CA2, CA3 and CA4 are relatively distinct regions within the hippocampus, the two subfields that are primarily investigated for their role in synaptic plasticity are CA1 and CA3 (Sweatt, 2004). The primary cell type found within all the CA regions is pyramidal neurons that can be clearly identified by their pyramidal cell body with prominent apical dendrites projecting towards the dentate gyrus and shorter basal dendrites (Amaral, 1978; Sweatt, 2004; Witter, 2010). Although the cell type is consistent between CA1 and CA3, the morphology of the neuron within these regions is different (see Figure 1.1). CA3 neurons bifurcate closer to the cell body and display a more complex branching pattern while CA1 neurons are less arborized with a simpler branching pattern (Witter, 2010).

There are several distinct layers that receive projections from specific circuits within the hippocampus (see Figure 1.1). The stratum oriens (s. oriens) is the most superficial layer and contains the basal dendrites of the pyramidal neurons located within the CA regions (Witter, 2010). Next is the pyramidal layer or stratum pyramidale (s. pyramidale) where the cell body of pyramidal neurons are located (Witter, 2010). The apical dendrites of CA3 pyramidal neurons extend through the stratum lucidum (s. lucidum), stratum radiatum (s. radiatum) and the inner most layer known as the stratum lacunosum-moleculare (s. lacunosum-moleculare) while the CA1 sub-region contain only the s. oriens, s. radiatum and s. lacunosum-moleculare layers (Witter, 2012).

The final portion of the hippocampus is the subiculum. The subiculum is the most inferior portion of the hippocampus and is the main output region of the hippocampus

with projections extending out towards the cortex (O'Mara, Commins, Anderson, & Gigg, 2001).

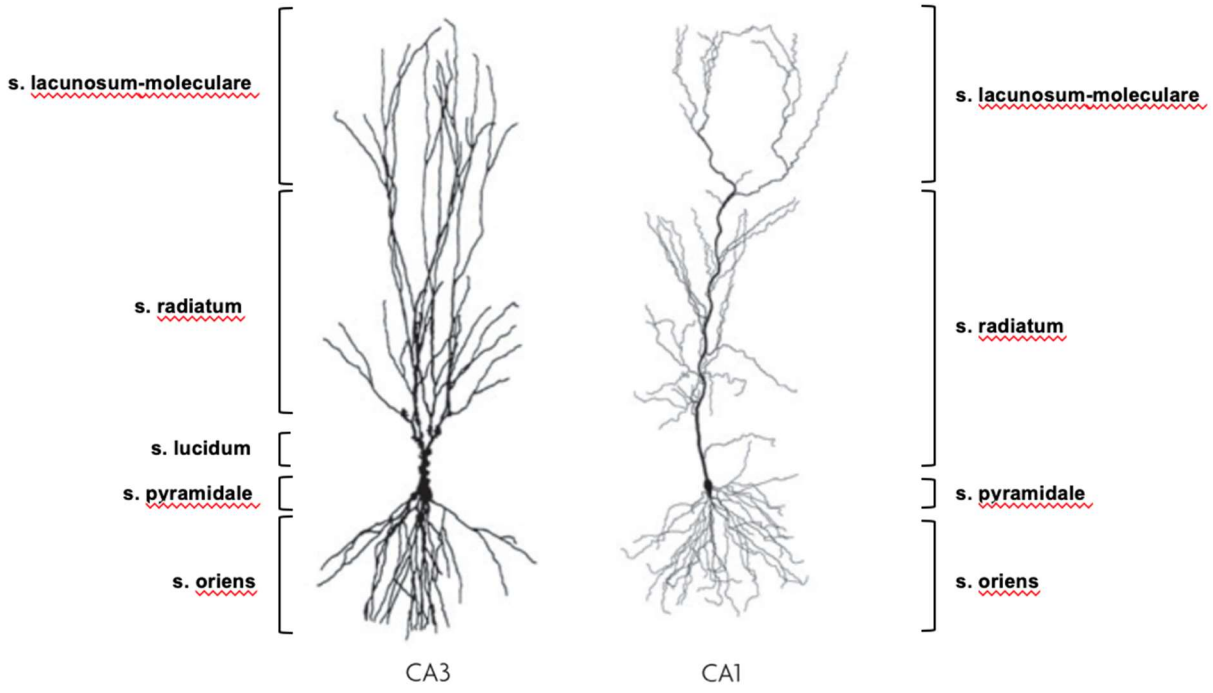

Figure 1.1: Comparison of the differences in morphology and layers of CA3 and CA1 hippocampal pyramidal neurons.

Representative CA3 and CA1 pyramidal neuron tracings displaying the differences in dendritic branching of neurons within these two regions. The dendritic branching patterns of CA3 neurons are more complex while CA1 neurons are less arborized with simpler branching patterns. The hippocampus contains several distinct layers that encase different regions of the dendritic tree. In both CA3 and CA1 neurons, the basal dendrites and cell body are found in the stratum oriens and stratum pyramidale layers respectively. The apical dendrites of CA3 neurons extend through the stratum lucidum, stratum radiatum and stratum lacunosum-moleculare. Within the CA1 sub-region there is only the stratum radiatum and stratum lacunosum-moleculare layers. (Diagram modified from (Spruston, 2008) Nature Reviews Neuroscience 9:206-221).

The connections between the dentate gyrus, CA3 and CA1 form the circuitry known as the trisynaptic loop (Anderson 1975). The trisynaptic loop consists of three main pathways; the perforant pathway, the mossy fibre system and the Schaffer Collateral pathway (Figure 1.2; Hjorth-Simonsen & Jeune, 1972; O'Mara et al., 2001). The perforant pathway sends information from neurons within layer II of the entorhinal cortex to the outer and middle molecular layer of the dentate gyrus (Hjorth-Simonsen & Jeune, 1972; Naber et al., 2001; Witter, Wouterlood, Naber, & Van Haeften, 2000). The axons of granule cells of the dentate gyrus then synapse onto the proximal apical dendrites of CA3 pyramidal neurons forming the mossy fibre system (Gaarskjaer, 1986; Witter, 2010). CA3 pyramidal neurons then innervate the apical dendrites of the CA1 pyramidal neurons via the Schaffer collateral pathway. CA1 pyramidal neurons then project to the entorhinal cortex (Collingridge, Kehl, & Mclennan, 1983) and other regions of the brain such as the medial prefrontal cortex (mPFC) (Jin & Maren, 2015; Swanson, 1981; Witter, 2010).

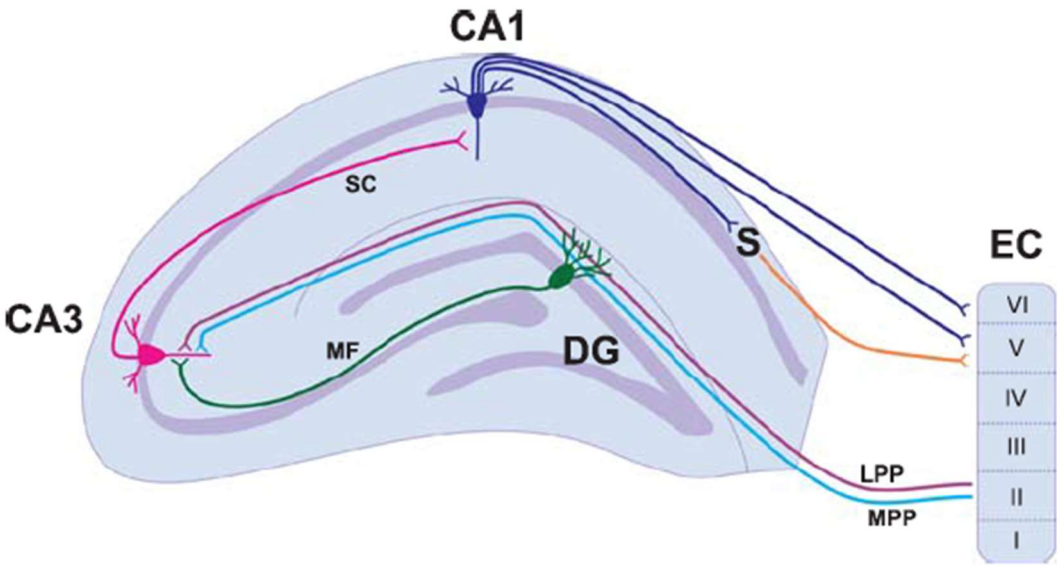

Figure 1.2: Hippocampal trisynaptic circuit indicating the three major pathways.

Simplified schematic displaying the major input into the hippocampus from the entorhinal cortex. These excitatory inputs originate from layer II of the entorhinal cortex and synapse onto granule cells of the dentate gyrus forming the perforant pathway (purple and light blue lines). Granule cell axons project to the CA3 sub-regions where they synapse on the apical dendrites of CA3 pyramidal neurons within the stratum pyramidale and lucidum layers forming the mossy fibre system (green line). Axons of CA3 pyramidal neurons then project and synapse onto apical dendrites of CA1 pyramidal neurons within the stratum radiatum and stratum lacunosum-moleculare layers which forms the Schaffer collateral pathway (pink line). CA1 pyramidal neurons then project to both the subiculum and the entorhinal cortex as well as other regions of the brain. The entorhinal cortex has direct projections to both CA3 and CA1 pyramidal neurons. (Diagram (Patten et al., 2016) from Brain Plasticity 1:97-127).

Connections between the hippocampus and the prefrontal cortex (PFC) are known to be important for working, episodic and contextual memory (Burwell & Amaral, 1998; Carmichael & Price, 1995; Jin & Maren, 2015). Particularly, the mPFC plays a key role in the retrieval and consolidation of memories and its connections with the hippocampus have been implicated in many neuropsychiatric disorders such as depression, anxiety and PTSD (Chen & Etkin, 2013; Liu et al., 2017). The hippocampus has both direct and indirect connections with the mPFC. The primary direct connection between the hippocampus and the mPFC are the excitatory glutamatergic projections from the ventral CA1 and subiculum that synapse onto pyramidal neurons of the prelimbic (PL) sub-region of the mPFC (Swanson, 1981). However, several indirect hippocampal connections project through intermediate structures that relay information to the mPFC. The ventral hippocampus contains dual-projecting neurons that send information to both the mPFC directly and via the basolateral amygdala which sends excitatory glutamatergic signals to the mPFC (Jin & Maren, 2015). While the dorsal hippocampus does not have directly innervate with the mPFC, CA1 pyramidal neurons from the dorsal hippocampus travel through the fimbria and fornix and then synapse onto pyramidal neurons within the infralimbic (IL) and PL sub-regions of the mPFC (Jin & Maren, 2015). The dorsal and ventral hippocampus also use the nucleus accumbens (NAc) and the ventral tegmental area (VTA) as intermediate regions which then project to the mPFC (Russo & Nestler, 2013). While glutamatergic CA1 ventral hippocampal neurons project to the shell of the NAc the glutamatergic CA1 neurons from the dorsal

hippocampus project to the core of the NAc (Bagot et al., 2015; Yang & Liang, 2014). Inhibitory GABAergic neurons from both the shell and the core of the NAc send information to the dopaminergic neurons within the VTA which then synapse onto the IL and PL regions of the mPFC (Goto & Grace, 2008). The VTA also has reciprocal dopaminergic connections with the CA1 sub-region of the hippocampus which are known to be important for memory consolidation from short-term memory in the hippocampus to long-term memory of the mPFC (Mahmoodi, Ahmadi, Pourmotabbed, Oryan, & Zarrindast, 2010; Nazari-Serenjeh, Rezayof, & Zarrindast, 2011). The hippocampus also projects to various nuclei of the thalamus which then synapse onto neurons within the mPFC (Wolff, Alcaraz, Marchand, & Coutureau, 2015). In particular, the hippocampus sends projections to the anterior medial nucleus of the thalamus and the mediodorsal thalamic nucleus of the thalamus sends afferent projections to the pyramidal neurons within later 2/3 of the mPFC. These hippocampal-mPFC circuits play a key role in the regulation of cognitive and emotional function (Jin & Maren, 2015). Dysregulation of these pathways has been implicated in many stress-related neuropsychiatric disorders such as PTSD, depression, anxiety and schizophrenia (Chen & Etkin, 2013; Sigurdsson, Stark, Karayiorgou, Gogos, & Gordon, 2010).

1.1.3 Synaptic and Structural Plasticity

Neurons within the hippocampus and mPFC are two highly plastic areas within the brain and can present drastic structural changes in response to a variety of stimuli. Synaptic plasticity within the hippocampus and mPFC has been shown to reflect

alterations in cognitive function due to behavioural changes such as learning or formation of new memories, physiological processes such as fluctuations in circulating levels of hormones as well as inflammatory process during recovery from physical injury (Foy, Stanton, Levine, & Thompson, 1987; Hajszan, MacLusky, Johansen, Jordan, & Leranth, 2007; Li et al., 2018; Shors & Dryver, 1994). The presence of synaptic plasticity can be identified by functional and structural indicators.

A key indicator of synaptic plasticity within the hippocampus and mPFC is the induction of long-term potentiation (LTP). LTP occurs when there is an influx of calcium ions (Ca^{2+}) through post-synaptic N-methyl-D-aspartate (NMDA) receptors (Bliss & Collingridge, 1993). For LTP to occur the post-synaptic neuron must receive excitatory glutamatergic stimulation that is strong enough to cause the cell to depolarize and for glutamate to bind to open the receptor (Bliss & Collingridge, 1993). Glutamate release from the pre-synaptic terminals activates the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors which cause an influx of calcium ions (Malinow, 2003). This depolarization results in the removal of the voltage-dependent magnesium block from the NMDA receptors (Bliss & Collingridge, 1993). While LTP has two distinct phases, early-phase LTP (E-LTP) and late-phase LTP (L-LTP), L-LTP is responsible for the lasting synaptic changes within the hippocampus and mPFC. L-LTP requires the activation of transcription factors and protein synthesis to produce a sustained response (Frey, Huang, & Kandel, 1993). The persistent response of L-LTP can alter the structure of dendrites and the density of dendritic spines within the hippocampus and mPFC that

are associated with changes in cognition (Bradshaw & Emptage, 2003). L-LTP relies on mechanisms involving alpha-Ca^{2+}/calmodulin-dependent protein kinase II (αCaMKII), protein kinase A (PKA), mitogen-activated protein kinases (MAPK), and cAMP response element-binding protein (CREB) (Frey et al., 1993; Pang & Lu, 2004; Yang, Wang, Frerking, & Zhou, 2008). Furthermore, brain-derived neurotrophic factor (BDNF), the primary neurotrophin in the brain, is well known for its role in strengthening synapses by acting on tropomyosin receptor kinase B (TrkB) (Korte et al., 1995). The induction of LTP can be regulated by various modulatory neurotransmitters such as serotonin, dopamine, norepinephrine and acetylcholine, as well as endogenous hormones such as glucocorticoids and androgens (Guo et al., 2017; Harley, Malsbury, Squires, & Brown, 2000; Izumi & Zorumski, 1999; Masuoka et al., 2019; Mlinar, Stocca, & Corradetti, 2015; Sousa, Cerqueira, Mailliet, Almeida, & Jay, 2007).

In response to LTP, the hippocampus and mPFC display an increase in dendritic spines (Yang et al., 2008). Dendritic spines are small protrusions on the surface of the apical and basal dendrites within the hippocampus and mPFC (Nimchinsky, Sabatini, & Svoboda, 2002). Dendritic spines receive inputs from presynaptic neurons and are the main area of post-synaptic excitatory synapses (Hering & Sheng, 2001). An increase in the number of spines is an indicator of an increase in the number of synapses and synaptic plasticity (Bourne & Harris, 2008; Nimchinsky et al., 2002). However, the number of spines present on the dendrites does not tell the full story of changes in synaptic plasticity as the different types of dendritic spines that express different levels

of plasticity (Bourne & Harris, 2008). Thin, stubby and filopodial spines (Figure 1.3) are considered to be immature as they are more transient and undergo rapid turnover and therefore are considered to be more plastic (Sorra & Harris, 2000). Mushroom spines (Figure 1.3) are the more mature and stable type of spine and are thought to represent a more permanent and less plastic synapse (Sorra & Harris, 2000). Dendritic spines have been shown to be regulated by physiological and environmental factors. The density of dendritic spines is known to respond to changes circulating hormones such as estrogens, androgens and glucocorticoids (Galea et al., 1997; Leranth, Petnehazy, & MacLusky, 2003; Phan et al., 2012). Environmental factors such as home cage enrichment can cause an increase in dendritic spine density associated with an increase in synapses on the dendrites of hippocampal neurons (Rojas et al., 2013). Changes in dendritic spine occur on a rapid time scale as changes are observed within seconds to minutes of the onset of the stimuli (Phan et al., 2012).

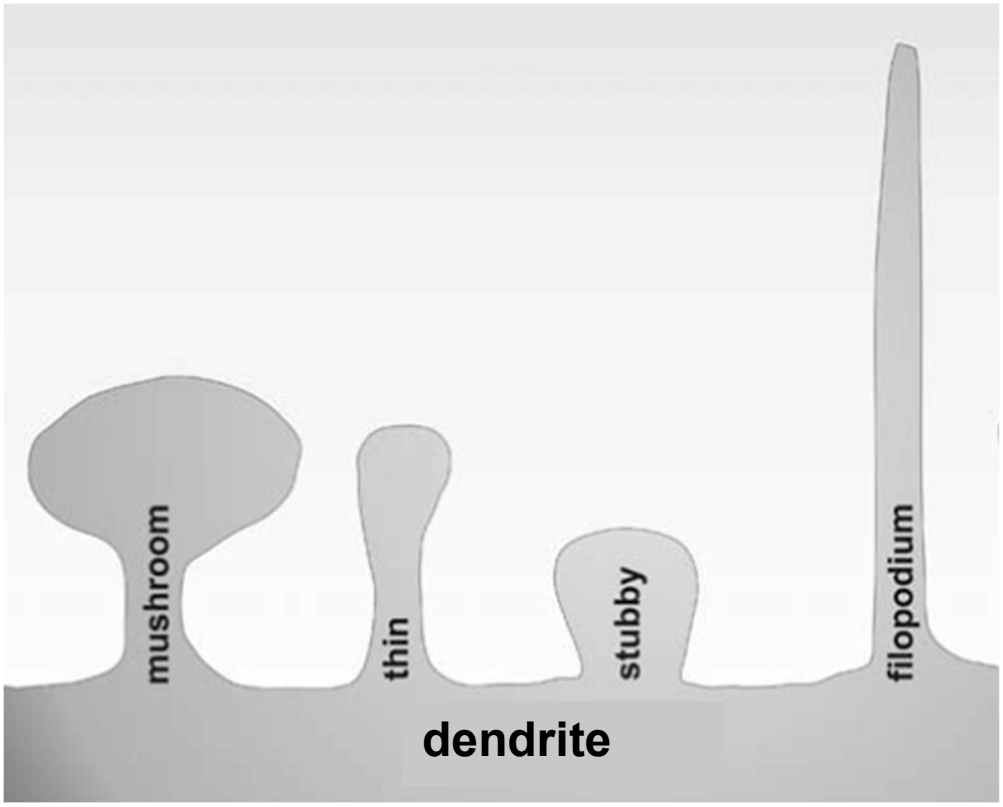

Figure 1.3: Morphology of the subtypes of dendritic spines.

A simplified diagram displaying the four main types of dendritic spines. The morphology of dendritic spines reflects their synaptic potential. Mushroom spines are larger and have a well-defined spine head making them optimal for forming stable synaptic connections. Thin, stubby and filopodium spines are highly plastic but less mature and stable. Changes in immature spines occur on a rapid time scale of seconds to minutes. (Diagram modified from (von Bohlen und Halbach, 2009) Annals of Anatomy 191:518-531).

In addition to structural changes at the synapse level, morphological changes to the apical and basal dendrites of hippocampal and mPFC pyramidal neurons can also occur in response to a variety of stimuli. In comparison to the rapid changes in dendritic spines, changes in dendritic branching take much longer to occur and develop over hours rather than minutes (Deitch & Rubel, 1984). Alterations in the morphology of the dendrites are much more dramatic as they can involve changes in the length, number of branches, diameter, volume and the overall complexity of the dendritic tree. Circulating hormones are one of the most characterized factors that can cause morphological changes in hippocampal and mPFC dendrites. Hippocampal and mPFC dendritic branching have shown to be regulated by gonadal hormones and stress hormones such as glucocorticoids as well as environmental factors such as enrichment (Bindu et al., 2007; Mendell et al., 2017; Woolley, Gould, & McEwen, 1990). These responses of dendritic morphology to physiological changes such as hormone levels are associated with altered hippocampal and mPFC dependent function (Aubele, Kaufman, Montalment, & Kritzer, 2008; Luine, Villegas, Martinez, & McEwen, 1994).

Hippocampal neurogenesis, or the production of new neurons, is another form of plasticity (Gage, 2002; Zhao, Deng, & Gage, 2008). Multipotent neural stem cells produce either neural stem cells or progenitor cells within the sub-granular zone of the dentate gyrus (Gage, 2000). Progenitor cells will then proliferate, differentiate and migrate into the CA regions of the hippocampus (Gage, 2000). These newly formed hippocampal neurons mature and become integrated into the circuitry. The process of

hippocampal neurogenesis can occur well into adulthood; however, it is known to decline with increased age (Gage, 2002). Much like other types of neuroplasticity, hippocampal neurogenesis can be affected by a variety of different factors including changes in gonadal hormones, stress responses, neurotrophic peptides and environmental stimuli (Galea, Spritzer, Barker, & Pawluski, 2006; Gould & Tanapat, 1999; Liu & Nusslock, 2018).

1.2 Medial Prefrontal Cortex

1.2.1 Introduction

The discovery and understanding of the prefrontal cortex (PFC) began with the identification of granular cortex located anteriorly to the motor cortices by Brodmann (1909). As the cortical cytoarchitecture of the PFC differs across humans, non-human primates and rats, the areas of the PFC can be identified by the reciprocal connections with the mediodorsal thalamic nucleus (Öngür & Price, 2000; Rose & Woolsey, 1948). The prefrontal cortex is known to play a crucial role in several cognitive and executive functions. While there are several sub-divisions of the PFC, the medial prefrontal cortex (mPFC) is known for its involvement in cognitive processes such as working memory (Goldman-Rakic, 1995), attention (Miller, 2007), decision making and emotional memory (Etkin, Egner, & Kalisch, 2011). In rats and mice, the mPFC is analogous to the dorsolateral prefrontal cortex in humans and non-human primates as they share structural and functional similarities (Heidbreder & Groenewegen, 2003; Öngür & Price, 2000; Seamans, Lapish, & Durstewitz, 2009). The mPFC is highly interconnected with

various regions of the brain such as the hippocampus and is, therefore, a key structure studied for its involvement in memory processes (Little & Carter, 2012).

1.2.2 Organization and Circuitry

The mPFC is located in the frontal cortex and can be separated into three sub-regions; the anterior cingulate cortex (ACC), prelimbic cortex (PL) and the infralimbic cortex (IL) (Heidbreder & Groenewegen, 2003; Ray & Price, 1992). In primates, the mPFC is separated into 6 distinct layers however, the mPFC in rats and mice is missing the fourth cortical layer (Öngür & Price, 2000). Glutamatergic pyramidal neurons are the primary cell type found within each layer of the mPFC collectively encompassing 80-90% of the neurons while the other 10-20% are GABAergic interneurons (Riga et al., 2014).

The cortical layers of the mPFC receive inputs from various areas of the brain making it a central area for information processing (Granon, Vidal, Thinus-Blanc, Changeux, & Poucet, 1994). This facilitates the various top-down cognitive processes that are regulated by the mPFC such as working memory and attention. The mPFC receives inputs from the basolateral amygdala, thalamus, hypothalamus, VTA, and hippocampus (Little & Carter, 2012). These afferent and efferent projections are distinct for each layer of the mPFC. Layer I of the mPFC receives cholinergic afferent inputs from the basal forebrain (Lysakowski, Wainer, Bruce, & Hersh, 1989). Additionally, the apical dendrites of the deeper layers of the mPFC can be found within layer 1 (Lysakowski et al., 1989). The basolateral amygdala and the mediodorsal thalamus

sends excitatory glutamatergic projections that synapse onto layer 2 and 3 of the mPFC (Jin & Maren, 2015; Little & Carter, 2012). Layer 2/3 also project back to the basolateral amygdala and these connections play a critical role in working and fear memory (Quirk, Likhtik, Pelletier, & Paré, 2018; Roozendaal, McReynolds, & McGaugh, 2004). Additionally, the VTA sends dopaminergic projections to the mPFC (Russo & Nestler, 2013). Layer 5 is the main output region of the mPFC and receives glutamatergic inputs from the thalamus and hippocampus (Vertes, 2006). Additionally, layer 5 pyramidal neurons synapse onto neurons within the basolateral amygdala, VTA and thalamus (Vertes, 2002; Zikopoulos & Barbas, 2006). Similarly, layer 6 also receives afferent projections from the thalamus and hippocampus and send efferent projections to regions such as the mediodorsal thalamus and hypothalamus (Vertes, 2002; Zikopoulos & Barbas, 2006). Dysregulation of these connections can lead to impairments in mPFC plasticity, function and has been associated with the development of neuropsychiatric disorders such as PTSD (Malivoire et al., 2018).

1.2.3 Synaptic and Structural Plasticity

The mPFC is a highly plastic region and displays both synaptic and structural plasticity markers. Neurons within the mPFC undergo changes in plasticity in response to a variety of stimuli including circulating steroid hormones (Hajszan, MacLusky, Johansen, Jordan, & Leranth, 2007b; Radley et al., 2008), social interaction (Burleson et al., 2017), and BDNF expression (Rosas-Vidal et al., 2018). Connections between the hippocampus and mPFC are known to be important for the proper formation of

memories and have been associated with many neuropsychiatric disorders (Yang & Liang, 2014). Previous studies have demonstrated the persistent induction of LTP in the mPFC following stimulation of the CA1 hippocampus (Taylor, Ohline, Moss, Ulrich, & Abraham, 2016). When male rats were given high-frequency stimulation within CA1 for 3 consecutive days there was a sustained induction of LTP within the mPFC (Taylor et al., 2016).

In addition to synaptic changes, neurons within the mPFC display changes in dendritic morphology in response to physiological and social changes. Changes in circulating steroid hormones are one of the most well-characterized factors known to induce structural changes within the mPFC (Radley et al., 2008). Stress has been well established to have detrimental effects on structural plasticity within the brain. Chronic stress can lead to the retraction of layer 2/3 mPFC apical dendrites with a corresponding reduction in dendritic spines (Radley et al., 2006; Wellman, 2001). Additionally, social interactions have been shown to lead to enhancements in mPFC dendritic morphology. Social isolation and the removal of social play can alter dendritic morphology within the mPFC (Burleson et al., 2017). Previous research has shown that when animals are deprived of social play during adolescence there is a significant loss of layer 2/3 apical dendritic branching in adulthood (Burleson et al., 2017). However, when given access to normal social interactions, animals display an increase in total dendritic length and the number of dendritic branch point within layer 2/3 of the mPFC

(Burleson et al., 2017). These alterations in mPFC dendritic morphology have been associated with functional changes in mPFC-dependent behaviour.

1.2.4 Medial Prefrontal Cortex Function

The mPFC is well known for its role in top-down cognitive processes such as working memory (Granon et al., 1994), attention, (Broersen & Uylings, 1999) and cognitive flexibility (de Bruin, Sànchez-Santed, Heinsbroek, Donker, & Postmes, 1994). Many of the studies that demonstrate the role of the mPFC in these cognitive processes have used the method of lesioning the mPFC to determine changes in behaviour. Lesions to the mPFC in male rats results in significant deficits in working memory (Granon et al., 1994). Furthermore, lesions to the mPFC have been shown to cause behavioural impairments in male rats when tested using several attention-based tasks. When the mPFC of male rats were lesioned they displayed impairments in the three-choice serial reaction time and the visual timing tasks (Broersen & Uylings, 1999). Additionally, male rats with either partial of full bilateral mPFC lesions displayed the same ability to learn and acquire the Morris water maze task (de Bruin et al., 1994). However, when they are tested using spatial reversal, animals with full bilateral mPFC lesioned displayed impairments in the ability to find the hidden platform compared to control and partially lesioned animals indicating impairments in cognitive flexibility (de Bruin et al., 1994).

Moreover, due to its connection with limbic structure, the mPFC is also involved in emotional memory. Lesions to the mPFC lead to longer delays in fear extinction in male

rats when compared to sham or control animals (Etkin et al., 2011). Furthermore, fear extinction may be dependent on the expression of BDNF within the mPFC. Inhibiting BDNF within the PL or IL regions of the mPFC impairs the recall of extinction avoidance (Rosas-Vidal et al., 2018). Additionally, increased expression of BDNF within the IL region of the mPFC facilitates fear extinction (Rosas-Vidal, Do-Monte, Sotres-Bayon, & Quirk, 2014). Deficits in these cognitive processes often parallel deficits in areas of the brain that project to the hippocampus and amygdala (Shin, Rauch, & Pitman, 2006). Impairments within the mPFC have been associated with the development in neuropsychiatric disorders such as PTSD (Shin et al., 2006).

1.3 Stress and Glucocorticoids

1.3.1 Hypothalamic Pituitary Adrenal (HPA) Axis

The physiological response to a stressful stimulus is regulated by the hypothalamic-pituitary-adrenal (HPA) axis (see Figure 1.4). When an individual is presented with a physical or psychological stressor, sensory information travels to the hypophysiotropic neurons within the paraventricular nucleus of the hypothalamus to stimulate the release of corticotrophin-releasing hormone (CRH) (Abou-Samra, Harwood, Catt, & Aguilera, 1987). CRH then travels through the hypophysial portal to the anterior pituitary where it acts on CRH type 1 receptors (CRHR1) located on pituitary corticotropes to initiate the release of adrenocorticotropic hormone (ACTH) into the circulation (Aguilera, 1994). ACTH then targets the melanocortin type 2 receptor (MC2-R) on the adrenal cortex to stimulate the synthesis and release of glucocorticoids

(cortisol in humans and corticosterone in rats and mice) into the circulation (Hadley & Haskell-Luevano, 2006; Simpson & Waterman, 1988). Glucocorticoids then travel to their target tissues and bind to the glucocorticoid (GR) and mineralocorticoid receptors (MR).

Under normal conditions, glucocorticoids feedback onto the anterior pituitary and hypothalamus to inhibit the release of CRH and ACTH thus stopping the stress response (Gómez, De Kloet, & Armario, 1998). However, under chronic stress conditions, the negative feedback mechanisms become dysregulated causing sustained activation of the HPA-axis and thus persistent elevation in glucocorticoids (Mizoguchi, Ishige, Aburada, & Tabira, 2003). Although the main driver of the effects of stress on the brain is thought to be through glucocorticoid action, CRH and ACTH can also feedback and act on stress-sensitive regions of the brain such as the hippocampus and mPFC (Herman et al., 2016).

Glucocorticoids synthesized from cholesterol and released into the circulation can act through MRs and GRs throughout the body and brain (Hanukoglu, 1992). Because MRs have a higher affinity for natural glucocorticoids, binding of glucocorticoids to MR occurs during basal conditions while GRs are only fully activated during negative feedback following the activation of a stress response (Mifsud & Reul, 2018; Reul & De Kloet, 1986). At rest, GRs are found mainly in the cytoplasm of the cell and are an integral part of many complexes such as chaperone proteins such as p23 and hsp90 as well as stress-related protein such as FK506 binding protein 5 (FKBP5;

Oakley & Cidlowski, 2013). When glucocorticoids circulate throughout the body during a stress response they are primarily bound to corticosteroid-binding globulin (CBG) in which form cortisol (or corticosterone in rats and mice) cannot bind to GRs (Herman et al., 2016; Oakley & Cidlowski, 2013). However, free cortisol can diffuse through the plasma membrane and bind to GRs (Oakley & Cidlowski, 2013). Once glucocorticoids are bound the GR changes conformation allowing it to translocate into the nucleus where it binds to glucocorticoid-responsive elements (GREs) to regulate subsequence gene expression (Oakley & Cidlowski, 2013).

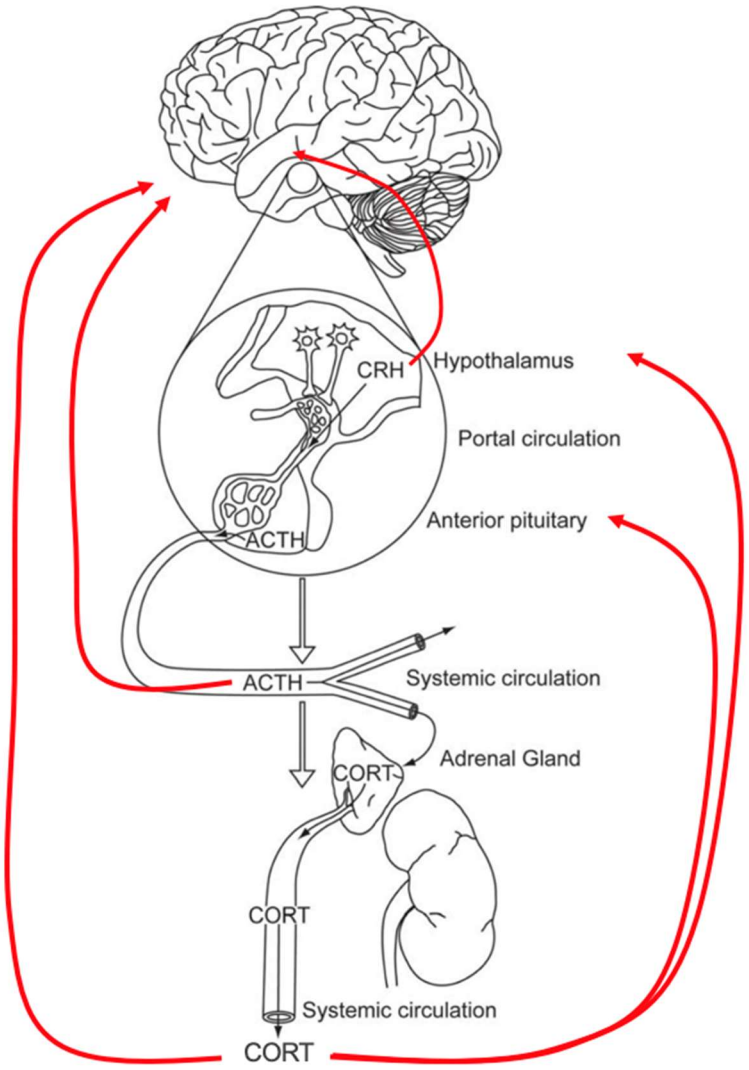

Figure 1.4: Organization of the hypothalamic-pituitary-adrenal (HPA) axis.

Stress causes the release of corticotrophin-releasing hormones (CRH) from the paraventricular nucleus of the hypothalamus. CRH travels through the hypophysial portal to the anterior pituitary to stimulate the release of adrenocorticotropic hormone (ACHT) into the circulation. ACTH then acts on the adrenal cortex causing the release of glucocorticoids into the circulation. Glucocorticoids then negatively feedback onto the anterior pituitary and hypothalamus to inhibit the release of CRH and ACTH thus stopping the stress response. Glucocorticoids, ACTH and CRH can also feedback and act on various regions of the brain such as the hippocampus and medial prefrontal cortex. (Diagram modified from (Herman et al., 2016) Comprehensive Physiology 6: 603-621.)

1.3.2 Types of Stress

Stress can have differential effects depending on the type of stressful stimulus. The type of stress is particularly important for stress-sensitive areas of the brain such as the hippocampus and mPFC that has an abundant expression of the GR and MR. The effects on the hippocampus and mPFC are often dependent on the magnitude and duration of the stressor typically producing an inverted-U shaped response curve (Kim, Pellman, & Kim, 2015; Kim & Yoon, 1998). Acute stress is known to result in enhancements in LTP, working memory and memory formation (Shors, 2001; Yuen et al., 2011). Although there is some evidence of the effects of acute stress, the majority of the literature focuses on the effects of chronic stress.

Chronic stress is caused by the dysregulation of the negative feedback mechanism of the HPA-axis leading to the prolonged elevation of glucocorticoids (Mizoguchi et al., 2003). Chronic stress can occur if an individual experiences persistent psychological or emotional stress that occurs in many neuropsychiatric disorders such as depression and anxiety (Qiao, An, Ren, & Ma, 2014; Qin, Xia, Huang, & Smith, 2011). Chronic stress cause suppressed immune function, suppression of reproductive function and exacerbate already existing disorders (Wang et al., 2017). Within the brain, chronic stress can lead to neuroinflammation, atrophy of dendrites and decreased dendritic spines and synapses (Chen, Dube, Rice, & Baram, 2008; Frank, Thompson, Watkins, & Maier, 2012; Watanabe, Gould, & McEwen, 1992).

Traumatic stress can be single or repeated exposures to an intense stressor. However, unlike acute stress, the severity is much higher, therefore, the effects on the brain and hippocampus are more extensive (Foa, Steketee, & Rothbaum, 1989). In cases such as PTSD, a traumatic stressor can have chronic effects on the individual and the hippocampus (Malivoire et al., 2018). This can cause dramatic impairments on the structure and function of neurons within the hippocampus and mPFC (Sapolsky, 2000).

While a larger portion of the literature focuses on the effects of psychological stress, physical stress or trauma can also lead to impairments in cognitive function. Surgery is a common physical stress event that can affect hippocampal and mPFC function. Patients have reported experiencing impairments in memory, attention, information processing and sleep following major surgery (Steinmetz et al., 2017). The presences of these symptoms are known as post-operative cognitive dysfunction (POCD) and while it can occur in patients of any age, it is most common in older individuals (Moller et al., 1998). The development of POCD can increase recovery time and the risk of post-operative complications (Steinmetz et al., 2017). Furthermore, previous research has shown that stress and inflammation play a critical role in the development of hippocampal memory impairments and POCD. Surgery and general anesthesia can cause the activation of stress responses that feedback onto the hippocampus and lead to cognitive impairments (Jacobsen, Kalliokoski, Teilmann, Hau, & Abelson, 2012; Rasmussen et al., 2003). Surgery can also stimulate inflammatory

responses causing an increase in proinflammatory cytokines can also lead to dysfunction in hippocampal-dependent memory (Cibelli et al., 2010; Skvarc et al., 2018).

1.3.3 Stress and Synaptic Plasticity

The effects of chronic stress on the hippocampus have been linked to a variety of alterations within the hippocampus including dendritic complexity, reduced neurogenesis, impairments in hippocampal LTP, deficits in spatial learning and memory and development of depressive- and anxiety-like behaviours (Foy et al., 1987; Luine et al., 1994; Watanabe et al., 1992). On a basic synaptic level, stress responses are known to affect the induction of LTP within the hippocampus (E. J. Kim et al., 2015). Stress has been shown to impair LTP within all the three major sub-regions of the hippocampus (CA1, CA3 and DG). The induction of LTP of the Schaffer collaterals within the CA1 sub-region is impaired in rats following 30-minutes of restraint stress alone or in combination with tail-shocks (Foy et al., 1987). Similar effects were seen in CA3 and the dentate gyrus 48-hours following chronic restraint stress or restraint stress + tail-shock (Pavlides, Nivón, & McEwen, 2002). The effects of stress on synaptic strength are supported by the evidence of stress on long-term depression (LTD) or the decrease in synaptic strength following a stimulus (Kim et al., 2015). When adult male rats were subjected to restraint stress + tail-shock that had been previously shown to impair LTP, there was a significant enhancement in LTD in CA1 (Kim, Foy, & Thompson, 1996).

Stress can also affect many molecular factors that are important for synaptic plasticity. One factor in the hippocampus that is regulated by stress is the expression of BDNF. Under normal conditions, BDNF is highly expressed in the hippocampus and essential for the maintenance and survival of neurons, induction of LTP and facilitation of adult neurogenesis (Suri & Vaidya, 2013). However, stress can negatively affect the expression of BDNF within the hippocampus and is associated with changes in synaptic and structural plasticity as well as chronic stress-related neuropsychiatric disorders (Lakshminarasimhan & Chattarji, 2012; Qiao, An, Xu, & Ma, 2017). These changes in BDNF expression following stress are also known to be sub-field specific within the hippocampus. When male rats were exposed to daily restraint stress for either 7-, 14- or 21-days there was reduced BDNF mRNA expression in CA3 and the dentate gyrus in all 3 groups compared to unstressed rats (Murakami, Imbe, Morikawa, Kubo, & Senba, 2005). The largest effect of chronic stress on BDNF expression was following 14-day of daily restraint stress that corresponded with the most dramatic increase in plasma corticosterone levels (Murakami et al., 2005). Moreover, acute and chronic stress have different effects on the expression of BDNF within the hippocampus. While chronic stress results in decreased expression, acute stress leads to a transient increase in expression (Shi, Shao, Yuan, Pan, & Li, 2010). Therefore, BDNF expression has been associated with enhancements in cognitive function following acute stress and the memory impairments following chronic stress (Shi et al., 2010; Suri & Vaidya, 2013).

BDNF also plays a role in the effects of stress on the function of the hippocampus. BDNF promotes the induction of LTP within the hippocampus through the strengthening of glutamatergic signalling (Figurov, Pozzo-Miller, Olafsson, Wang, & Lu, 1996). Applying corticosterone to hippocampal slices impairs the induction of LTP in CA1 pyramidal neurons with a corresponding decrease in BDNF expression within CA1 (Zhou, Zhang, & Zhang, 2000). However, when corticosterone and BDNF are administered simultaneously BDNF reverses the effect seen following corticosterone treatment alone (Zhou et al., 2000). These protective effects of BDNF following stress can also be observed in vivo. Rats that were given long-term infusions of BDNF and subjected to 7-days of daily restraint stress had improved spatial learning and memory compared to stressed rats without BDNF (Radecki, Brown, Martinez, & Teyler, 2005). Therefore, BDNF plays a critical role in the maintenance of synaptic plasticity and the regulation of hippocampal-dependent learning and memory following stress or glucocorticoids treatment (Radecki et al., 2005; Zhou et al., 2000).

1.3.4 Stress and Structural Plasticity

Changes in the structure of the dendrites of hippocampal and mPFC neurons have been associated with maladaptive behaviours and stress-related neuropsychiatric disorders such as anxiety, depression and PTSD (D'Aquila, Brain, & Willner, 1994; Foa et al., 1989). At the synaptic level, persistent exposure to stress can affect the density of dendritic spines located on the dendrites of neurons within the hippocampus and mPFC (Leuner & Shors, 2013; Qiao et al., 2016). Male rats subjected to 21-days of chronic

unpredictable mild stress (CUMS) display reduction in dendritic spines on CA3 pyramidal neurons (Conrad, Magarifios, Mcewen, & Ledoux, 1999; Qiao et al., 2014). Additionally, chronic restraint stress leads to an increase in stubby type spines while decreasing thin and mushroom type spines, therefore, affecting the ability of immature spines to develop into stable synapses (Magariños et al., 2011). Stress has also been shown to exert damaging effects on dendritic spines within the CA1 subfield. While chronic restraint stress causes a decrease in apical CA1 spines in males (Castañeda et al., 2015; Qiao et al., 2014), Additionally, 21-days of daily corticosterone injections cause a decrease in CA1 dendritic spines of male rats indicating that the effects of stress are glucocorticoid-mediated (Morales-Medina, Sanchez, Flores, Dumont, & Quirion, 2009). However, acute stress has an opposing effect on spine density and behaviour compared to chronic stress. Males exposed to acute stress have an increase in the density of CA1 dendritic spines while females display a reduction (Shors, Chua, & Falduto, 2001).

The effects of chronic stress on spine density in the mPFC often mirror the effects seen in the hippocampus. Adult male rats display a reduction in the density of dendritic spine of layer 2/3 pyramidal neurons of the mPFC following chronic restraint stress (Radley et al., 2006). The decrease in the density of dendritic spines in the mPFC following chronic stress has been shown to reflect a loss of mushroom type spines while the density of thin spines is increased (Bessa et al., 2009; Radley et al., 2008). Therefore, similar to the hippocampus, chronic stress is causing the reduction in the

pool of immature spines available to mature into stable excitatory inputs (Qiao et al., 2016; Radley et al., 2008).

Changes in the density of dendritic spines within the hippocampus and mPFC often match larger structural changes on dendritic branching. Chronic stress causes atrophy of CA3 apical dendrites in male rats compared to unstressed controls (Galea et al., 1997; Luine et al., 1994). CA3 apical dendritic trees of chronically stressed rats display a decrease in the number of apical dendritic branch points and the overall of total apical dendritic length (McLaughlin, Gomez, Baran, & Conrad, 2007). This response to chronic restraint stress can be reversed if the subjects are given a period without stress (Conrad, 2006). Additionally, when male rats were tested under less severe chronic stress conditions, the same atrophy in CA3 apical dendrites does not occur (Sousa, Lukoyanov, Madeira, Almeida, & Paula-Barbosa, 2000).

Similar to the comparable changes in dendritic spine density in the hippocampus and mPFC, changes in dendritic branching of pyramidal mPFC neurons often reflect the changes seen in the hippocampus. Following 21-days of daily restraint stress, there is a significant reduction in the number and length of the apical dendrites of layer 2/3 mPFC pyramidal neurons (Brown, Henning, & Wellman, 2005; Cook & Wellman, 2004b). The effects of stress on the brain are thought to be due to the elevated levels of circulating glucocorticoids that occur following a stress response (Wellman, 2014). When male rats are treated with chronic daily corticosterone injections to observe the effects of elevated glucocorticoids in isolation, there is a dramatic retraction of CA3 apical dendrites that is

consistent with the effects seen following chronic restraint stress as well as presents indicators of early stages of neuronal degeneration (Woolley et al., 1990). The mPFC display similar responses to corticosterone as 21-days of corticosterone injections causing the reorganization of the apical dendrites of 2/3 pyramidal apical dendrites with an increase in dendritic material in the proximal region while the distal region displayed a decrease in dendritic material (Wellman, 2014).

However, restraint stress causing the subsequent activation of the HPA-axis will not only result in the release of glucocorticoids but also CRH and ACTH that can both feedback onto the hippocampus and mPFC (Herman et al., 2016). While there is limited evidence of the effects of ACTH on hippocampal dendritic morphology, CRH has been shown to cause adverse outcomes on structural plasticity within the hippocampus. Both CRHR1 and CRHR2 are abundantly expressed throughout the hippocampus however CRHR1 located on dendritic spines of hippocampal pyramidal neurons is the main site of CRH action (Chen, Brunson, Muller, Cariaga, & Baram, 2000). CRH has been shown to contribute to the effects of stress on dendritic spine density in the CA3 hippocampus (Chen et al., 2008). When CRHR1 is blocked, the loss of CA3 dendritic spines seen following stress is prevented (Chen et al., 2008). Additionally, deletion of hippocampal CRHR1 lead to the expansion of CA1 and CA3 pyramidal neurons (Chen et al., 2004). Therefore, blocking the effects of CRH appears to help prevent the negative effects of stress on structural plasticity within the hippocampus. However, like glucocorticoids, CRH is just one portion of a stress response that involves a variety of different factors.

The effects of stress on the structure of neurons within the hippocampus and mPFC has been shown to be mediated by a variety of neurotransmitters and molecular factors. Glutamate is the most abundant excitatory neurotransmitter in the brain and is known to increase following stress (Gilad, Gilad, Wyatt, & Tizabi, 1990). When glutamate release is inhibited or NMDA receptors are blocked, glucocorticoid-induced hippocampal and mPFC dendritic atrophy is prevented (Martin & Wellman, 2011; McEwen & Magariños, 1995). Moreover, BDNF is also involved in the maintenance of neuronal structure in response to stress. Following 21-days of chronic restraint stress, BDNF mRNA expression is reduced in the CA3 hippocampus parallel with the retraction of CA3 apical dendrites (Lakshminarasimhan & Chattarji, 2012). BDNF returns to baseline levels when subjects are given a 21-day recovery period (Lakshminarasimhan & Chattarji, 2012). BDNF has also been shown to promote the formation of new spine synapses within the hippocampus (Ji, Pang, Feng, & Lu, 2005) and reverse the loss of dendritic spines following chronic stress (Qiao et al., 2017).

1.3.5 Stress and Cognitive Function

Changes in hippocampal synaptic plasticity following chronic or severe stress have been associated with subsequent changes in various types of hippocampal and mPFC dependent learning and memory. Male rats who were subjected to the same chronic restraint stress that leads to CA3 dendritic atrophy have significant impairments in spatial memory when tested in the radial arm maze task compared to unstressed controls (Luine et al., 1994). The same stressor also impairs male rats in hippocampal-

dependent tasks such as the Morris water maze, Y-maze object recognition task, and object placement task (Conrad, Ortiz, & Judd, 2017a). However, these impairments are often reversed with time. When rats who underwent chronic restraint stress were tested again with the radial arm maze performed the same as unstressed rats (Luine et al., 1994).

Chronic stress also leads to impairments in mPFC dependent memory. Following chronic stress, male rats display significant impairments in response inhibition and working memory when tested on the response withholding task and the radial arm water maze respectively (Mika et al., 2012). Chronic stress also impairs other prefrontal cortex dependent behaviours such as behavioural flexibility (Bondi, Rodriguez, Gould, Frazer, & Morilak, 2008; Cerqueira et al., 2005), and recall of fear extinction (Miracle, Brace, Huyck, Singler, & Wellman, 2006). Therefore, the changes in mPFC and hippocampal dendritic branching may be contributing to the impairments in cognitive function following stress.

1.3.6 Stress and Inflammation

Stress-induced peripheral and neuroinflammation has been investigated as a potential mechanism to help explain neuropsychiatric disorders such as schizophrenia, depression and PTSD (Körschenhausen, Hampel, Ackenheil, Penning, & Müller, 1996; Leonard, 2018; Rohleder, Wolf, & Wolf, 2010). While glucocorticoids are known for their anti-inflammatory properties, they can also exhibit pro-inflammatory action depending on the conditions of the stressor (De Bosscher, Vanden Berghe, & Haegeman, 2003).

For example, acute stress can lead to enhanced immune response, chronic stress can suppress the immune system while severe stress can lead to the over-activation of the immune system (Dhabhar, Miller, McEwen, & Spencer, 1996; Dhabhar & McEwen, 1997). Elevated glucocorticoids can activate the NOD-like receptor protein 3 (NLRP3) inflammasome leading to increased interleukin-1β (IL-1β), a pro-inflammatory cytokine, into the circulation (Ogikubo et al., 2004). Stress also primes the immune system causing greater inflammatory responses (Johnson et al., 2002). Injecting mice or rats with lipopolysaccharide (LPS) to cause the release of pro-inflammatory cytokines is often used as a method to study a controlled inflammatory response (Lieberman, Pithatt, Shint, & Shin, 1989; Ogikubo et al., 2004). Exposing rats to stress before an injection LPS will cause a greater inflammatory responses and higher levels of IL-1β within the hippocampus compared to unstressed controls (Johnson et al., 2002). This effect is also seen if animals are given injections of corticosterone before LPS, indicating that there is a link between glucocorticoid action and inflammatory responses (Frank, Miguel, Watkins, & Maier, 2010; Sorrells, Caso, Munhoz, & Sapolsky, 2009). The interaction between stress and inflammation has been shown to have a reciprocal relationship. Previous studies have shown that an increase in pro-inflammatory cytokines like IL-1β, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) can activate the release of ACTH from the anterior pituitary which in turn increases glucocorticoid release into the circulation (Liu, Wang, & Jiang, 2017).

The link between stress and neuroinflammation has been implicated in stress-related neuropsychiatric conditions such as depression and PTSD (Muhie et al., 2017; Peng et al., 2012). Previous research has shown that chronically stressed male rats have an increase in NLRP3 activation and pro-inflammatory markers IL-1β, IL-6, and interleukin-18 (IL-18) within the hippocampus (Wang et al., 2018). This upregulation in pro-inflammatory markers was also associated with increased anxiety- and depressive-like behaviours (Wang et al., 2018). Neuroinflammation is regulated by microglia and astrocytes. Microglia are responsible for *de-novo* synthesis and release of pro-inflammatory cytokines within the brain while communicating with astrocytes to regulate neuroinflammatory responses (Nayak, Roth, & McGavern, 2014; Xu et al., 2017). Therefore, in response to chronic stress, the function of microglia and astrocytes within the hippocampus and the prefrontal cortex can become dysregulated which can lead to exacerbated inflammatory responses (Almolda et al., 2015; de Pablos et al., 2006).

While stress and inflammation have been associated with neuropsychiatric disorders such as depression and PTSD, they have also been linked to common events such as surgical procedures (Hovens et al., 2014). Previous research has demonstrated that surgery can cause dysregulation of microglia and astrocyte function (Almolda et al., 2015; Feng et al., 2017; Xu et al., 2017). This leads to subsequent increase secretion of pro-inflammatory cytokines, suppress BDNF synthesis, inhibit hippocampal neurogenesis, and impair hippocampal function (Alam, Hana, Jin, Suen, & Ma, 2018; Fidalgo et al., 2011; Hovens et al., 2014; Li et al., 2017). Additionally, activation of the

immune system through LPS injection exacerbates the peripheral inflammatory and neuroinflammatory effects of surgery causing higher levels of IL-β in both the serum and hippocampus (Fidalgo et al., 2011). Furthermore, exposure to stress before surgery also exacerbates the neuroinflammatory effects within the hippocampus. This leads to a more robust neuroinflammatory response, a greater decrease in BDNF expression and larger impairments in cognitive function (Wang et al., 2016; Zhu, Shi, Wang, Wang, & Li, 2014). Therefore, the potentially lasting effects of the interaction between stress and inflammation following surgery is an important factor to consider when understanding the effects of surgery on both humans and in scientific research.

1.4 Androgens and Testosterone

1.4.1 Introduction

Androgens, the main gonadal steroid hormones in males, play critical roles during various stages of life and development. These include effects during early life development to promote masculinization, puberty and the development of secondary male sex characteristics such as promoting the enhancements in muscle mass, and during adulthood where they can exert effects on prostate weight and hair patterns and baldness (Hines, 2008; Matsumoto, 2003; Rogol, 2002). Circulating androgens are known to influence cognitive function and the loss or replacement of testosterone can alter the structure of neurons within androgen-sensitive regions such as the hippocampus (Genazzani, Pluchino, Freschi, Ninni, & Luisi, 2007). As men age, there

is a natural decline of circulating free testosterone due to the increase in sex hormone-binding globulin (SHBG) (Matsumoto, 2003; Stárka, Pospíšilová, & Hill, 2009).

1.4.2 Androgen Synthesis and Metabolism

The synthesis of androgens begins with the conversion of cholesterol to pregnenolone through P450 side-chain cleavage enzyme (P450scc; see Figure 1.5). Pregnenolone is then converted into progesterone and 17-α-hydroxypregnenolone by 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme and 17α-hydroxylase respectively. Progesterone is then converted to 17-α-hydroxyprogesterone by 17α-hydroxylase (Payne & Youngblood, 1995). Next, both 17-α-hydropregnenolone and 17-α-hydroxyprogesterone are converted to dehydroepiandrosterone (DHEA) and the testosterone precursor, androstenedione via 17, 20 lyase. DHEA can also be converted into androstenedione through 17β-hydroxysteroid dehydrogenase (17β-HSD). DHEA is also converted into the other testosterone precursor, androstenediol, via 17β-HSD. Androstenedione and androstenediol are both converted into testosterone through the action of 17β-HSD and 3β-HSD. The conversion of the precursors into testosterone occurs primarily in the Leydig cells of the testes (Payne & Youngblood, 1995), although some androgens are also synthesized in the adrenal (Rege et al., 2013). Testosterone is then converted into dihydrotestosterone (DHT) via 5α-reductase (Baulieu & Mauvais-Jarvis, 1964) in androgen target tissues. Finally, DHT is then converted into 5α-androstane-3α, 17β-diol (3α-diol) or 5α-androstane-3β, 17β-diol (3β-diol) via 3β-HSD (Baulieu & Mauvais-Jarvis, 1964). Additionally, while DHT and testosterone are the

main androgens that bind to the AR, 3α-diol can also exert effects in the brain as weak androgen with low affinity for the AR (Matias et al., 2000).

Androgens can also be converted into estrogens (Bagatell, Dahl, & Bremner, 1994). Androstenedione and testosterone and be converted into estrone and estradiol respectively through aromatase action (Bagatell et al., 1994). These estrogens can also have action on the brain while their effects are thought to be relatively limited in males (Leranth et al., 2003).

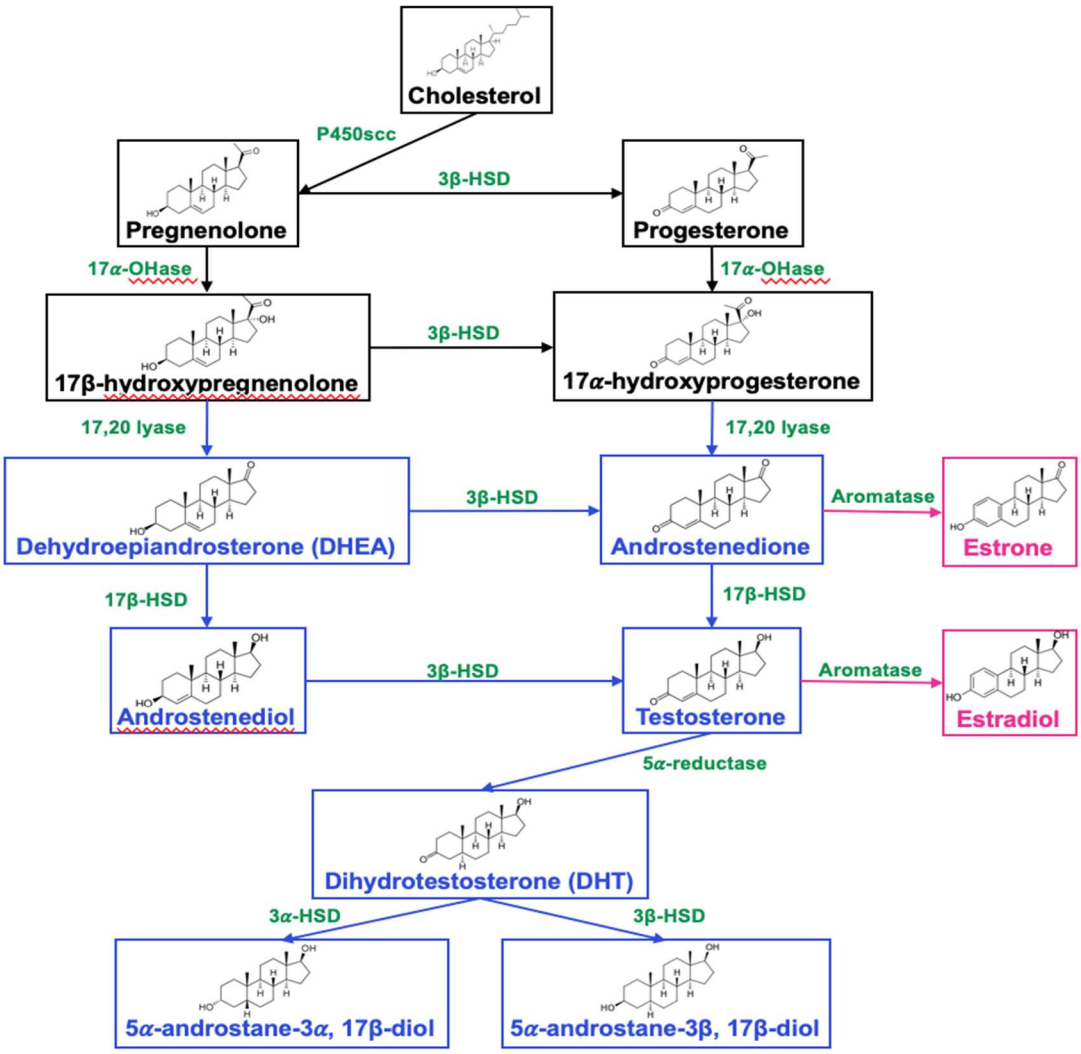

Figure 1.5: Androgen biosynthesis and metabolism.
Androgens are synthesized from cholesterol which if first metabolized to pregnenolone by P450scc. Pregnenolone is then metabolized to progesterone by 3β-HSD and 17β-hydroxypregnenolone by 17α-OHase. Progesterone is then converted into 17α-hydroxyprogesterone by 17αOHase while 17β-hydroxypregnenolone is converted to DHEA by 17,20 lyase. 17β-hydroxypregnenolone can also be converted into 17α-hydroxyprogesterone via 3β-HSD. 17α-hydroxyprogesterone is then converted into androstenedione by 17,20 lyase. DHEA and androstenedione are then converted to androstenediol and testosterone respectively by 17β-HSD. Androstenediol can also be converted to testosterone by 3β-HSD. Testosterone is then metabolized via 5α-reductase to DHT. DHT is then converted to 3α-diol and 3β-diol via 3α-HSD and 3β-HSD respectively. Androstenedione and testosterone can also undergo aromatization via aromatase into estrone and estradiol respectively.

1.4.3 Androgen Receptor and Signalling

The effects of androgens throughout the body are primarily exerted through the binding of DHT and testosterone to the androgen receptor (AR) (Liao et al., 2003). The AR can bind both testosterone and DHT, however, AR has a higher affinity for DHT (Liao et al., 2003; Matias et al., 2000). While testosterone and DHT freely circulated throughout the body, the majority is bound to binding proteins such as albumin or sex hormone-binding globulin (SHBG) (Burton & Westphal, 1972; Pugeat, Dunn, & Nisula, 1981). Once the bound testosterone and DHT reach their target tissues they are released from the binding proteins and diffuse across the membrane of the cell and bind to AR located in the cytoplasm of the cell (Pugeat et al., 1981; Rosner, Hryb, Khan, Nakhla, & Romas, 1991). Androgens that have bound to ARs undergo dimerization and form a complex that translocates into the nucleus (Liao et al., 2003). This complex can then bind to hormone response elements located on DNA and mediate subsequent gene expression. Although the genomic effects through the classical AR binding pathway is well characterized, androgens can also exert many effect through non-genomic signalling pathways (Bennett, Gardiner, Hooper, Johnson, & Gobe, 2010).

Androgens such as DHT and testosterone can also activate non-genomic cascades (Michels & Hoppe, 2008). This can subsequently activate various protein kinases pathways such as MAPK (ERK), protein kinase A (PKA), and protein kinase C (PKC), α-calcium-calmodulin-dependent protein kinase (CaMKs) and CREB (Michels & Hoppe, 2008). In in vivo cultured hippocampal neurons, administration of DHT and

testosterone leads to the rapid activation of the ERK/MAPK signalling pathway (Nguyen et al 2005). Additionally, DHT and testosterone can activate CREB through the PKC cascade (Nguyen et al. 2009).

1.4.4 Androgens and Synaptic Plasticity

Similar to stress, the hippocampus and mPFC are sensitive to the effects of androgens as these regions abundantly express the AR (Beyenburg et al., 2000; Nuñez, Huppenbauer, McAbee, Juraska, & DonCarlos, 2003). Although the beneficial effects of androgens and testosterone are less established compared to the effects of estrogens, the literature does show that androgens mediate structural plasticity and cognitive function (Genazzani et al., 2007; Leonard & Winsauer, 2011). The removal of the endogenous source of testosterone through orchidectomy (ORCH) has been shown to mediate the induction of LTP within the hippocampus. Following ORCH, male rats have increase facilitation of LTP within the CA1 hippocampal subfield (Atwi, McMahon, Scharfman, & MacLusky, 2016; Harley et al., 2000). Additionally, ORCH in male rats leads to an increase in mossy fibre transmission and LTP of CA3 pyramidal neurons (Skucas et al., 2013). Orchidectomy is also known to increased BDNF expression within the CA3 subfield in adult male rats therefore potentially explaining the enhancements in LTP (Skucas et al., 2013).

1.4.5 Androgens and Structural Plasticity

Androgens have been shown to have contradictory effects on dendritic spines and the morphology of dendritic branches within the hippocampus. The response of

dendritic spines to androgens seems to reflect their neuroprotective effects within the hippocampus. For example, pubertal male rats have an increase in the density of dendritic spines of apical dendrites of CA1 and CA3 pyramidal neurons due to the natural surge in serum testosterone during puberty (Meyer, Ferres-Torres, & Mas, 1978). However, the mechanisms that regulate the density of hippocampal dendritic spines in males does not appear to be dependent on the conversion of testosterone to estradiol (Leranth et al., 2003). When orchiectomized male rats, administration of estradiol has no effect on restoring the loss of CA1 dendritic spines (Leranth et al., 2003). Additionally, blocking the aromatization of testosterone to estradiol through the administration of letrozole, an aromatase inhibitor, did not affect the density of hippocampal dendritic spines of male rats (Fester et al., 2012). Therefore, previous literature has demonstrated that androgens appear to have beneficial effects on the density of hippocampal dendritic spines. When male rats are orchiectomized there is a dramatic loss of CA1 dendritic spines, while treatment with either DHT or testosterone helps restore this loss to levels of intact males (Hajszan, MacLusky, & Leranth, 2008; Hatanaka et al., 2015; Leranth et al., 2003). This effect of androgens on dendritic spines is also consistent in layer 2/3 of the mPFC. When male rats are orchiectomized there is a reduction in the number of spines of layer 2/3 pyramidal neurons compared to intact males, while treatment with DHT recovered the number of dendritic spines back to those of intact males (Hajszan et al., 2007b).

In addition to their effects on dendritic spines, androgens can also influence changes in dendritic branching of hippocampal and mPFC pyramidal neurons. However, while the effects of androgens on dendritic spines are more established, less is known about their effects on the dendritic structure. Previous research conducted from the MacLusky laboratory has worked to characterize the effects of orchidectomy on hippocampal and mPFC dendritic branching. When male rats were orchiectomized, there was an expansion of CA3 apical dendrites with a corresponding small decrease in dendritic spine density compared to sham-orchiectomized males 2-months following surgery (Mendell et al., 2017). This expansion in CA3 dendrites following orchidectomy is consistent with the evidence that orchidectomy facilitates the induction of LTP and mossy fibre transmission within the hippocampus (Skucas et al., 2013). Additionally, expansion of CA3 dendrites is also seen at shorter time-points following surgery. Male rats that have been orchiectomized show the same expansion of CA3 apical dendrites at 10-days following surgery that had been previously seen at 2-months by Mendell et al. (Lawton, 2018). Furthermore, replacing orchiectomized male rats with testosterone at the time of surgery caused CA3 apical dendritic branching to be comparable to patterns of both intact and orchiectomized males (Lawton, 2018). Therefore, androgens such as testosterone appear to be playing a role in changes in dendritic branching of neurons within androgen-sensitive regions.

1.4.6 Androgens and Cognitive Function

The neuroprotective effects of androgen have been observed in functional changes in cognitive function and hippocampal-dependent behaviours that often reflect changes seen on the cellular and molecular level. Previous literature has demonstrated the effects of androgens such as testosterone in both human and animal studies. In humans, there is a natural gradual decline in circulating free testosterone and DHEA while the increase in SHBG that is thought to contribute to some of the age-related cognitive decline and increased risk for developing age-related (Feldman et al., 2002; Stárka et al., 2009). Testosterone hormone replacement therapy has been shown to improve cognitive dysfunction and spatial memory in men (Atwi et al., 2016; Cherrier et al., 2001). Moreover, lower serum testosterone levels have also been related to increased inflammation in older men and have been associated with an increase of inflammatory cytokines such as TNF-α in men suffering from Alzheimer's disease (Butchart, Birch, Bassily, Wolfe, & Holmes, 2013).

In animal models, androgens such as testosterone and its metabolites, DHT and 3α-diol have been shown to affect various types of cognitive processes. Orchiectomized male rats and mice exhibit increased anxiety-like behaviours indicated by more time spent in the open arms of an elevated plus maze task compared to intact males. (Frye & Edinger, 2004; Frye, Park, Tanaka, Rosellini, & Svare, 2001). Additionally, orchiectomized males that receive testosterone replacement do not display anxiety-like behaviour and behave no different from intact males (Frye, Edinger, & Sumida, 2008).

Orchiectomized male rats also have impairments in hippocampal-dependent spatial memory (Aubele et al., 2008; Jacome et al., 2016) as well as mPFC dependent function such as non-spatial working memory (Hawley et al., 2013; Kritzer, McLaughlin, Smirlis, & Robinson, 2001). However, when these animals are given testosterone replacement, these impairments are completely abolished (Sandstrom, Kim, & Wasserman, 2006).

In addition to the effects of testosterone on hippocampal and mPFC function, testosterone metabolites such as DHT and 3α-diol also have effects on cognitive function (Frye, Edinger, Seliga, & Wawrzycki, 2004). DHT has a higher affinity for the AR, is not converted to estradiol through aromatization and has been demonstrated to have effects similar to those of testosterone (Frye et al., 2004). DHT supplementation in orchiectomized mice reverses the impairments in emotional memory when tested with an inhibitory avoidance task as well as working memory when tested using the Morris water maze task (Frye et al., 2004). DHT replacement in orchiectomized mice also decreases anxiety-like behaviour and enhances cognitive performance (Edinger & Frye, 2004). Additionally, replacing orchiectomized male mice with 3α-diol leads to similar effects as testosterone or DHT replacement. 3α-diol replaced mice have less anxiety-like and fear behaviours as well as increased cognitive performance in the inhibitory avoidance task (Edinger & Frye, 2004; Edinger, Lee, & Frye, 2004). Therefore, although many studies have focused on the effects of testosterone, its metabolites are also important to consider when looking at the overall effects of androgens on the brain.

1.5 Rationale

Androgens and stress steroid hormones play critical roles in synaptic and structural plasticity within the hippocampus and mPFC. Glucocorticoids and testosterone can both profoundly alter neuronal structure within these two regions which can have implications for the connectivity and function of these two areas. Chronic stress and glucocorticoids are known to cause atrophy of CA3 hippocampal and layer 2/3 mPFC apical dendrites (Galea et al., 1997; Wellman, 2001; Woolley et al., 1990) that is associated with impairments in cognitive function (Luine et al., 1994). However, previous work conducted in our laboratory has demonstrated the same atrophy of CA3 hippocampal and layer 2/3 mPFC apical dendrites 10-days following sham-orchidectomy surgery, therefore, suggesting that there is a major effect of the stress of the surgical procedure and post-operative recovery (Lawton, 2018). Furthermore, previous research has demonstrated that surgery can have effects on hippocampal BDNF as well as inducing both stress and peripheral and neuroinflammatory responses.

Testosterone appears to attenuate the effects of surgery. Previous work from our laboratory demonstrated that testosterone replacement helps to partially restore CA3 apical dendritic branching or orchiectomized rats to levels of unoperated males 10-days following surgery. However, what remained unknown from this study was how long the effects of surgery persist and how testosterone contributes to the long-term recovery of hippocampal and mPFC dendritic morphology. Additionally, it was unclear how the surgery, elevated glucocorticoids, anesthesia and post-operative recovery contribute to

the effect seen 10-days following surgery. The work performed in this thesis aimed to determine what factors contribute to the effects of surgery on hippocampal and medial prefrontal dendritic morphology.

1.5.1 Hypothesis

Surgical stress and post-operative recovery alters hippocampal and medial prefrontal dendritic structure.

1.5.2 Objectives

1. Determine the time course of post-operative changes of hippocampal and medial prefrontal dendrites.
2. Evaluate the contribution of anesthesia to the morphological changes following surgery.
3. Determine the specific effects of elevated glucocorticoids in the absence of surgical stress
4. Determine how pre-surgical treatment and post-operative recovery can minimize the effects of surgery.

2 Orchidectomy and sham-surgery causes remodelling of hippocampal and medial prefrontal apical dendrites.

2.1 Introduction

Circulating steroid hormones can induce profound changes on the structure and function of neurons within the hippocampus and mPFC which may underlie the development of neuropsychiatric disorders such as depression, PTSD and schizophrenia (Kokras & Dalla, 2014; MacLusky, Hajszan, Prange-Kiel, & Leranth, 2006; McEwen, 2016). Androgens such as testosterone have been shown to alter hippocampal dendritic morphology and regulate normal cognitive function (Atwi et al., 2016). When male rats are orchiectomized, they display impairments in spatial and working memory compared to intact males (Aubele et al., 2008). Orchiectomized males also display memory impairments and an increase in anxiety- and depressive-like behaviours (Aubele et al., 2008; Edinger & Frye, 2004). However, when orchiectomized males are replaced with testosterone, they no longer display memory impairments or anxiety-like behaviours (Aubele et al., 2008; Edinger & Frye, 2004; Jacome et al., 2016).

Testosterone can also regulate the structure and function of neurons within the hippocampus and mPFC. The removal of endogenous testosterone through orchidectomy facilitates mossy fibre transmission and LTP within the hippocampus (Skucas et al., 2013). Furthermore, dendritic spine density within the CA1 hippocampus

is reduced following orchidectomy (Leranth et al., 2003; Leranth, Prange-Kiel, Frick, & Horvath, 2004) but is restored if orchiectomized animals are replaced with either testosterone or DHT (Hatanaka et al., 2015; Jacome et al., 2016; Leranth et al., 2003). Additionally, testosterone also mediates changes in apical dendritic branching of CA3 neurons. CA3 apical dendritic branching increases after orchidectomy at both short and long-term time-points following surgery (Lawton, 2018; Mendell et al., 2017). Previous research from our laboratory has shown that CA3 apical dendrites increase 10-days and 2-months following orchidectomy surgery (Lawton, 2018; Mendell et al., 2017). Additionally, when orchiectomized males are replaced with testosterone at the time of surgery, CA3 apical dendrites were partially restored to levels resembling intact unoperated male (Lawton, 2018). However, the process of surgery and post-operative recovery can also have effects on the hippocampus and mPFC that may be mediated by androgens and glucocorticoids. At 10-days, males rats that received a sham-orchidectomy, therefore keeping their endogenous source of testosterone intact, had decreased CA3 and layer 2/3 mPFC apical dendritic branching compared to unoperated males (Lawton, 2018). This suggests that there are potential factors associated with the surgical procedure and recovery period that lead to the atrophy of hippocampal and mPFC apical dendrites following sham-orchidectomy. However, what was still unknown from this study was how long the effects of surgery last and how testosterone plays a role in the long-term post-operative recovery of hippocampal and mPFC dendritic morphology.

Therefore, in the present experiment, we hypothesize that surgery results in the lasting remodelling of hippocampal and mPFC dendrites while testosterone attenuates the effects of the surgical procedure. The results from this experiment suggest that the combination of orchidectomy surgery and the post-operative recovery period leads to a lasting restructuring of hippocampal and layer 2/3 mPFC apical dendrites. These changes in dendritic structure have significant implications for the connectivity of the hippocampus and mPFC.

2.2 Materials and Methods

2.2.1 Animals

Thirty three young adult male Sprague Dawley rats (60-70 days old, ~250g) were acquired from Charles River Laboratories (Saint-Constant, Quebec, Canada). Rats were housed in groups of 2-3 in standard large plastic cages under 12-hour light/dark (light between 0700 - 1900 hour) with food (Purina 5001, WF Fisher, Somerville, NJ, USA) and water ad libitum. Animals were left to acclimatize for one week following shipping prior to conducting any procedures. Animals were cared for as per the Canadian Council on Animal Care and all experimental procedures conducted were approved by the Animal Care and Use Committees at the University of Guelph.

2.2.2 Surgical Procedures

Male rats underwent either an orchidectomy with testosterone replacement (ORCH+T), or cholesterol replacement (ORCH+C), sham orchidectomy (SHAM) or remained surgically naïve. Carprofen (5 mg/kg) was injected subcutaneously 2-hours

prior to surgery to allow for a long acting anti-inflammatory and a 2mg/kg lidocaine/bupivacaine combination was injected subcutaneously at the main incision site. All rats were anesthetized under 4-5% isoflurane anesthesia and then maintained on 1-2% isoflurane while the surgery was performed.

First, a small incision was made on the back between the shoulder blades where crystalline testosterone-filled (Galenova, Saint-Hyacinthe, QC, Canada) or cholesterol (Sigma-Aldrich) filled Silastic capsule was implanted for the ORCH+T and ORCH+C groups respectively. Silastic capsules had a 10mm functional length and provide a slow release at levels that mimic low-normal physiological levels in adult male rats. An incision was made laterally across the scrotal sac allowing the testes to be exposed. The tunic was sutured and the testes, seminiferous tubules, epididymis, sperm duct and seminal vesicles were removed. This process was repeated for the other teste. The incision was then stapled shut and the rat was placed in the recovery chamber and given another 2mg/kg lidocaine/bupivacaine and 5 mg/kg of saline subcutaneously. Once the rat was conscious and moving around normally, they were placed back in clean home cages with their cage mates and monitored for post-operative complications. For SHAM, the same procedure was performed however the testes were only exposed and placed back into the cavity and the incision was closed. The intact surgically naïve rats were left in their home cages for their respective timepoints. Each group (n=8-9) was split in half and sacrificed 1-month or 2-months (n=4-5) following their surgical procedures.

It is important to note that in the days following surgery, nine orchiectomized animals received enrofloxacin, a broad spectrum anti-biotic, carprofen and the incisions were flushed due to post-operative infections. Additionally, six of orchiectomized animals with testosterone replacement were monitored as they displayed swelling at the incision site.

2.2.3 Brain and Blood Collection

At the respective time-points following orchidectomy, rats were sacrificed by inducing a deep CO_2 anesthetic state followed by decapitation. Their brains were then extracted and dissected in half sagittally down the midline. Alternating left and right half brains were either placed in Golgi Cox solution for morphological analysis and the other half was flash frozen on dry ice and then stored at -80°C. Trunk blood was collected at the time of sacrifice. Whole blood was centrifuged at 3000rpm for 10 minutes to separate the serum. Serum was extracted and aliquoted and stored at -80°C for subsequent hormone analysis.

2.2.4 Golgi Staining and Tissue Processing

Golgi Cox solution [1% potassium dichromate (Fisher Scientific, Ottawa, ON, Canada), 0.8% potassium chromate (Sigma-Aldrich, Oakville, ON, Canada), 1% mercuric chloride (Sigma-Aldrich) was prepared and filtered (Whatman grade 1 filter paper; Fisher Scientific) and stored at room temperature and was protected from light exposure. Following sacrifice, half of the brain was immediately placed in 20mL scintillation vials containing the prepared Golgi solution and stored for 26-days at room

temperature in the dark. They were then transferred to 30% sucrose solution in 0.1M phosphate buffer (PB) for 48-hours at 4°C. Half brains were then sliced using a vibratome (Leica) into 300µm and 400µm sections for the mPFC and hippocampus respectively and placed in 6% sucrose solution and stored at 4°C for 24-hours. Sections were then fixed in 2% paraformaldehyde for 15-minutes, 2.7% NH4OH for 15-minutes, and 10% Kodak rapid fixative A for 25-minutes with 5-minute Milli-Q water wash steps in between each step. Sections were then mounted onto microscope slides and left to air dry for approximately 45 minutes and were protected from light exposure. Sections were then dehydrated by submerging the slide in 50%, 70%, 85% and 95% ethanol for 2-minutes each followed by two separate 100% ethanol and xylene steps for 5-minutes each. Slides were then removed from the final xylene step and cover-slipped using Permount mounting medium (Sigma-Aldrich). Slides were left overnight and then sealed with clear nail polish to prevent oxidization.

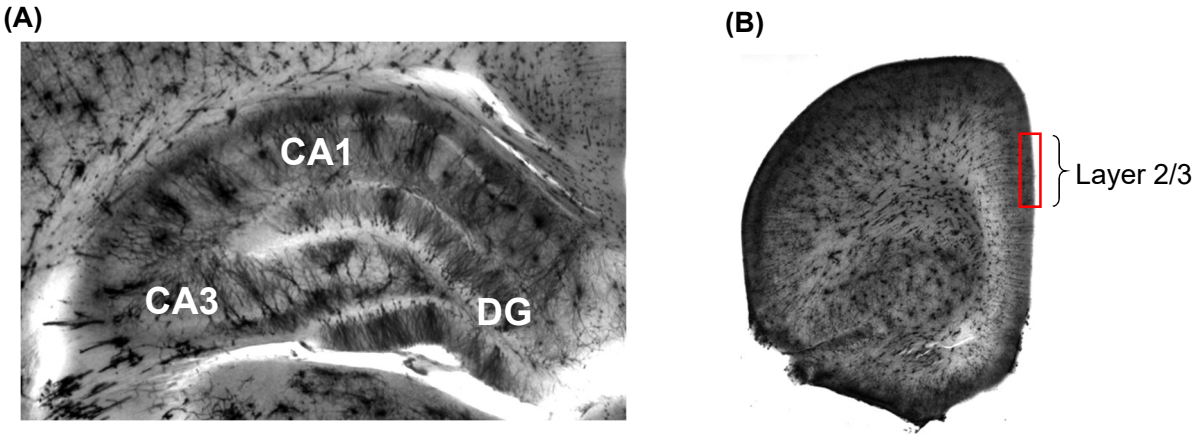

Figure 2.1: Golgi stained dorsal hippocampus and prefrontal cortex sections. **(A)** Golgi-Cox stained dorsal rat hippocampus indicating the 3 major sub-regions; CA1, CA3 and the dentate gyrus (DG). Neurons from CA1 and CA3 are imaged for morphological analysis. **(B)** Golgi-Cox stained rat prefrontal cortex section indicating layer 2/3 of the mPFC.

2.2.5 Dendritic Structure and Sholl Analysis

Neurons within the CA1 and CA3 of the dorsal hippocampus (see figure 2.1) and mPFC layer 2/3 regions were selected based on the following criteria; (1) neurons must be fully impregnated by the Golgi Cox staining; (2) relatively isolated from surrounding neurons; (3) neurons must be fully contained within the specific hippocampal or mPFC region (4) basal and apical dendrites are not broken or damaged. Selected neurons were then imaged (Nikon 90i/80i microscope, QImaging camera) every 2μm through the brain section producing three-dimensional image stacks. Using the Neurolucida Software (Version 10, MBF Bioscience, Williston, VT, USA) image stacks were used to trace the neurons to produce a three-dimensional representation. An average of 4 to 5 neurons were taken for each of CA1, CA3 and mPFC layer 2/3 for each animal.

Once all of the neurons had been traced, Sholl analysis was used to determine changes in dendritic structure (Sholl, 1953). Sholl analysis was conducted using the Neurolucida Explorer software (MBF Bioscience, Williston, VT, USA) which places increasing concentric spheres every 20μm starting from the cell body and extending the entire length of the basal and apical dendritic trees. Sholl analysis data for the number of intersections and dendritic length were analyzed.

2.2.6 Testosterone Assay

Using serum samples collected from each animal at the time of sacrifice, a Mouse/Rat Testosterone enzyme-linked immunosorbent assay (ELISA; Calbiotech Inc, El Cajon, California, USA) was used to determine changes in circulating levels of

testosterone in each treatment group. Samples and standard curve were run based on manufactures instructions and absorbance was read at 450nm. Mean absorbance values were calculated and testosterone levels in ng/mL based on the standard curve.

2.2.7 Statistical Analysis

Two-way ANOVA was used for the analysis of apical and basal dendritic length followed by a Tukey-Kramer post hoc. Sample sizes for the 1-month groups are as followed; ORCH+T n=4, ORCH+C n=4, SHAM n=4, Intact n=5. Sample sizes for the 2-month groups are as followed; ORCH+T n=4, ORCH+C n=4, SHAM n=4, Intact n=4.

To determine changes in serum testosterone levels, raw data was transformed using the natural logarithm (ln) and analyzed using a two-way ANOVA followed by Tukey-Kramer post hoc test was conducted. Significance for all statistical analysis was determined as $p<0.05$.

2.3 Results

2.3.1 Orchidectomy and sham surgery results in the remodelling of CA3 apical dendrites 1- and 2-months following surgery.

At 1-month following surgery, two-way ANOVA indicated both a significant effect of treatment (Treatment; $F(3, 480) = 39$, $p<0.0001$) and a significant interaction between treatment and Sholl radius (Treatment*Sholl Radius; $F(117, 480) = 1.565$, $p=0.0006$). The interaction effect indicated specific areas across the dendritic tree where dendritic branching was significantly altered. ORCH+C rats have increased dendritic branching compared to SHAM males however this is restricted to 140-200μm

from the cell body. Additionally, similar to the ORCH+C group, the ORCH+T have increased apical dendritic branching compared to SHAM animals in the proximal region between the 120-200μm. ORCH+C, ORCH+T and SHAM male rats have significantly decreased dendritic branching compared to unoperated males. While there was a slight decrease in dendritic branching of the surgical groups in the medial region, the main effect of surgery is seen in the loss of the distal dendritic branches between 480-580μm from the cell body (see Figure 2.2A).

By 2-months, there was still lasting effects of the surgical procedure on CA3 apical dendritic structure. Two-way ANOVA revealed a significant effect of treatment (Treatment; $F (3, 480) = 20.39$, $p<0.0001$) and an interaction effect between treatment and distance from the soma (Treatment*Sholl radius; $F (39, 480) = 120.2$, $p<0.0001$). While apical dendritic branching of SHAM male rats had comparable dendritic branching compared to ORCH+T and ORCH+C animals, there were significant changes to dendritic structure present in all surgical groups compared to unoperated intact males (see Figure 2.2B). All surgical groups had an increase in dendritic branching in the proximal region of the dendritic tree compared to the unoperated intact males. Additionally, similar to 1-month following surgery, all the surgical groups displayed less dendritic branching farther away from the cell body compared to unoperated male rats.

Based on the results of the loss of the distal dendrites in CA3, the length of the apical dendrites between each Sholl radius was analyzed to better understand the overall changes in dendritic structure following surgery. Two-way ANOVA revealed a

significant effect of treatment (Treatment; $F (3, 2480) = 516.7$, $p<0.0001$) as well as a significant interaction between treatment and distance from the soma (Treatment*Sholl radius; $F (117, 2480) = 7.688$, $p<0.0001$). While there was a small increase in apical dendritic length between 140-180μm in the ORCH+T animals compared to SHAM animals, there was a dramatic effect of the surgical procedures on the length of the apical dendrites. All surgical groups, regardless of if it was an orchidectomy with or without testosterone replacement or sham procedure, had a dramatic decrease in apical dendritic length compared to unoperated males. SHAM display decreased apical dendritic length between 60-620μm while both ORCH+T and ORCH+C have reduced apical dendritic length between 100-620μm (see Figure 2.2C). See figure 2.2E for representative 1-month CA3 tracings.

Moreover, the surgical procedure and post-operative recovery period lead to persistent changes on CA3 apical dendritic length 2-months following surgery. There was both a significant effect of treatment (Treatment; $F (3, 2400) = 145.9$, $p<0.0001$) on dendritic branching and a significant interaction between treatment and Sholl radii (Treatment*Sholl radius; $F (117, 2400) = 4.482$, $p<0.0001$). Similar to 1-month following surgery there was a slight increase in the apical dendritic length of ORCH+T compared to SHAM male rats between 240-300μm however the most noticeable effect is seen when comparing all surgical groups to the unoperated intact group. While the apical dendritic length of the ORCH+T, ORCH+C and SHAM groups appeared to begin to recover up to the unoperated group, there was still a dramatic reduction in apical

dendritic length of in all group that received any form of surgical procedure. This reduction was seen in dendrites within the 200-620μm of the apical dendritic trees of ORCH+T, ORCH+C and SHAM groups (see Figure 2.2D). See figure 2.2E for representative 2-month CA3 tracings.

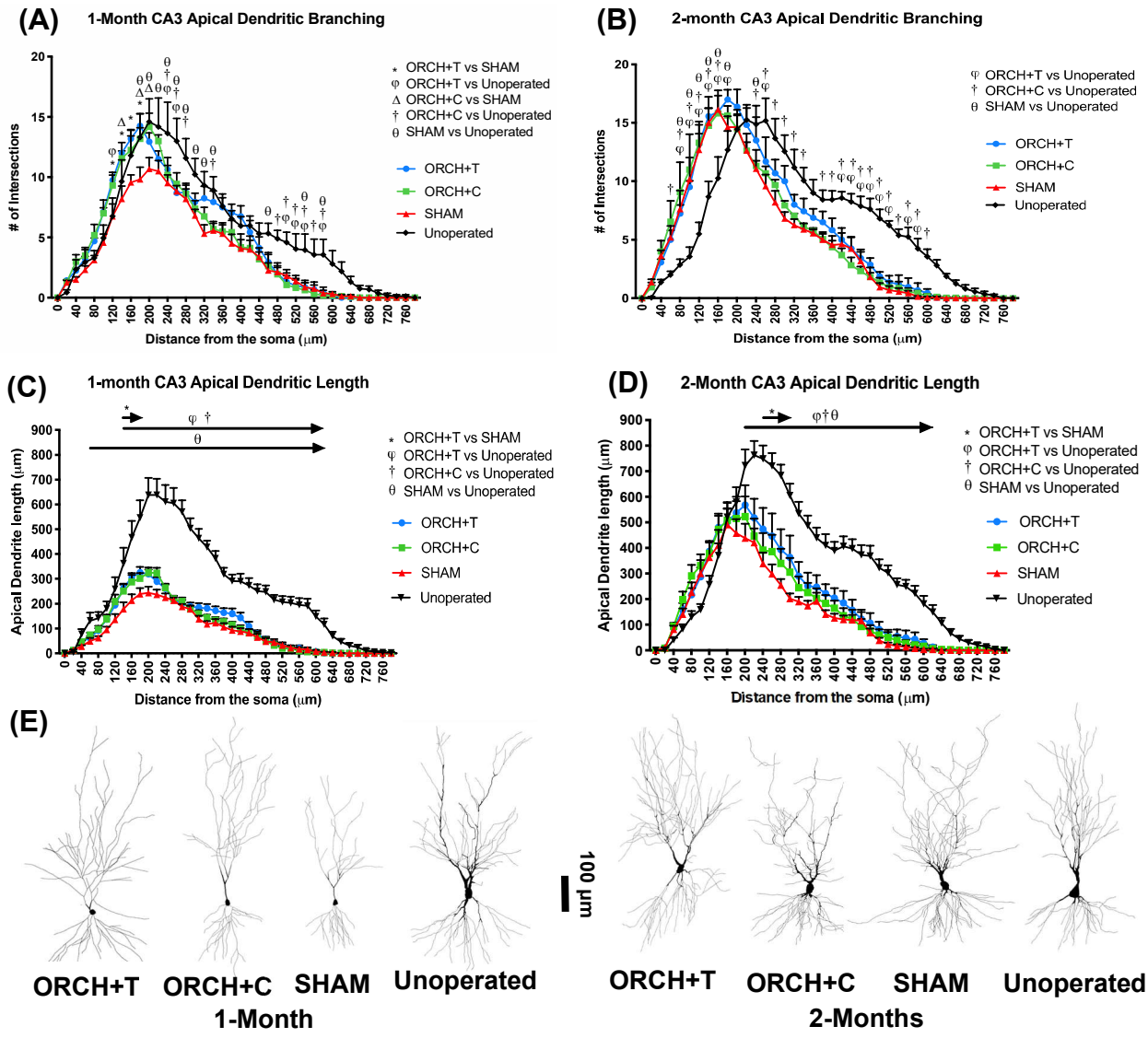

Figure 2.2: Apical dendritic branching and dendritic length for the CA3 sub-region.

(A) SHAM rats have reduced CA3 dendritic branching compared to ORCH+T, ORCH+C and unoperated rats at 1-month. ORCH+T, ORCH+C and SHAM rats have less dendritic branching distal to the cell body compared to unoperated rats. **(B)** At 2-months, ORCH+T, ORCH+C and SHAM rats have an increased number of dendrites close to the cell body and reduced dendritic branching in the distal region **(C)** All surgical groups have reduced apical dendritic length compared to unoperated rats 1-month **(D)** and 2-months after surgery. **(E)** Representative 1- and 2-month tracings of ORCH+T, ORCH+C, SHAM and unoperated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

2.3.2 Surgery and post-operative recovery results in the remodelling of CA1 apical dendrites 1-month following surgery.

In the CA1 hippocampal subfield, there appeared to be a significant remodelling of the apical dendrites associated with surgery and post-operative recovery. Sholl analysis revealed a significant treatment effect (Treatment; $F (3, 533) = 61.34$, $p<0.0001$) and an interaction effect between treatment and distance from the soma (Treatment*Sholl radius; $F (120, 533) = 1.346$, $p=0.0149$). The process of any surgical procedure, ORCH+T, ORCH+C or SHAM, lead to a decrease in dendritic branching compared to the unoperated rats. This reduction in dendritic branching was significant between the following Sholl radii; ORCH+T: 120-440μm; ORCH+C: 140-420μm; SHAM: 140-400μm (see Figure 2.3A). Apical dendritic length of CA1 dendrites was significantly reduced following any form of surgical procedure. ORCH+T, ORCH+C, and SHAM all displayed a significant decrease in apical dendritic length (Treatment; $F (3, 2583) = 564.4$, $p<0.0001$. Treatment*Sholl radius; $F (120, 2583) = 10.29$, $p<0.0001$). This reduction in apical dendritic length extended between 60μm-520μm for ORCH+C and SHAM animals and 540μm for ORCH+T animals (see Figure 2.3C).

By 2-months following surgery, CA1 apical dendrites did not display the same atrophy seen at 1-month following surgery. While there is an effect of treatment (Treatment; $F (3, 528) = 6.495$, $p=0.0003$) between SHAM and unoperated animals and ORCH+T and unoperated animals there was no significant interaction (Treatment*Sholl radius; $F (129, 528) = 0.3447$, $p>0.9999$) between treatment and distance from the

soma (see Figure 2.3B). This effect was consistent with the length of the apical dendrites there was no interaction (Treatment*Sholl radius; $F(129, 2640) = 0.6749$, $p=0.9979$) between treatment and Sholl radius however there was a treatment effect (Treatment; $F(3, 2640) = 8.803$, $p<0.0001$) between ORCH+T, ORCH+C and sham-orchiectomized animals compared to unoperated intact animals (see Figure 2.3D). Figure 2.3E displays representative 1- and 2-month CA1 neuronal tracings.

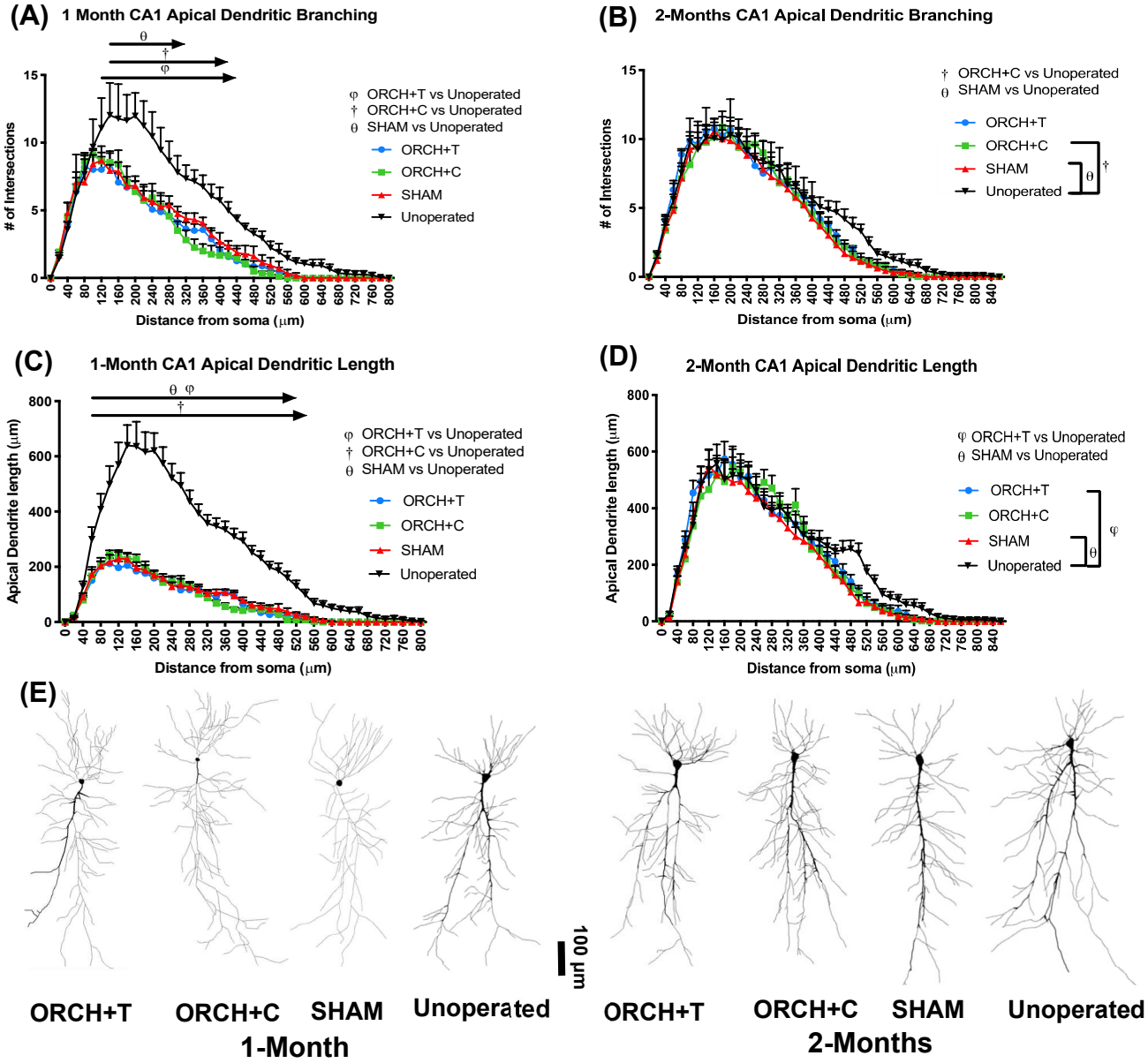

Figure 2.3: Apical dendritic branching and length for the CA1 sub-region.
(A) All surgical groups have decreased dendritic branching compared to unoperated rats 1-month following surgery. **(B)** By 2-months, there were minimal effects on CA1 apical dendrites. **(C)** Apical dendritic length of all surgical groups was reduced compared to unoperated rats at 1-months. **(D)** Minimal effects of surgery on CA1 apical dendritic length at 2-months. **(E)** Representative CA1 tracings of ORCH+T, ORCH+C, SHAM and unoperated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

2.3.3 Surgery alters apical dendritic length of layer 2/3 medial prefrontal cortex neurons 1-month following surgery.

While the effects of the surgical procedure were also be observed on pyramidal neurons of layer 2/3 of the mPFC, they were only seen 1-month following surgery. There was no interaction effect (Treatment*Sholl radius; $F (60, 252) = 0.8732$ $p=0.7308$) observed for apical dendritic branching 1-month following surgery in the mPFC. However, there was an effect of treatment in the ORCH+T and SHAM compared to the unoperated intact males with ORCH+T and SHAM animals displaying a decrease in dendritic branching compared to unoperated animals (see Figure 2.4A).

Layer 2/3 mPFC apical dendritic branching also shows no interaction between treatment and Sholl radius (Treatment*Sholl radius; $F (66, 207) = 0.6097$, $p=0.9900$) however, there was a significant overall treatment effect (Treatment; $F (3, 207) = 3.773$, $p=0.0115$) 2-months following surgery (see Figure 2.4B). Apical dendritic branching of ORCH+T was significantly higher than ORCH+C. Additionally, there was a significant increase in apical dendritic branching of SHAM rats compared to ORCH+C while dendritic branching of ORCH+C was significantly decreased compared to unoperated intact males.

Consistent with the effects of surgery observed in CA3 and CA1, the apical dendritic length of layer 2/3 mPFC neurons was significantly reduced compared to unoperated intact male rats. Two-way ANOVA revealed both an overall effect of treatment (Treatment; $F (3, 1260) = 182.5$, $p<0.0001$) and an interaction between

treatment and distance from the soma (Treatment*Sholl radius; $F (60, 1260) = 6.676$, $p<0.0001$). The reduction in apical dendritic length extended throughout the majority of the dendritic tree and was statistically significant between the following Sholl radii ORCH+C vs. Unoperated: 60-260μm; ORCH+T vs Unoperated and SHAM vs Unoperated: 40-260μm (see Figure 2.4C).

In contrast to the effects of surgery on apical dendritic length in the mPFC at 1-month following surgery, by 2-months, there was no significant difference between the apical dendritic length across any treatment groups at any of the Sholl radii (Treatment; $F (2, 984) = 1.63$, $p=0.1964$; Treatment*Sholl radius; $F (46, 984) = 0.8829$, $p=0.6940$; see Figure 2.4D). Figure 2.4E shows representative tracings of 1- and 2-month layer 2/3 mPFC neuronal tracings.

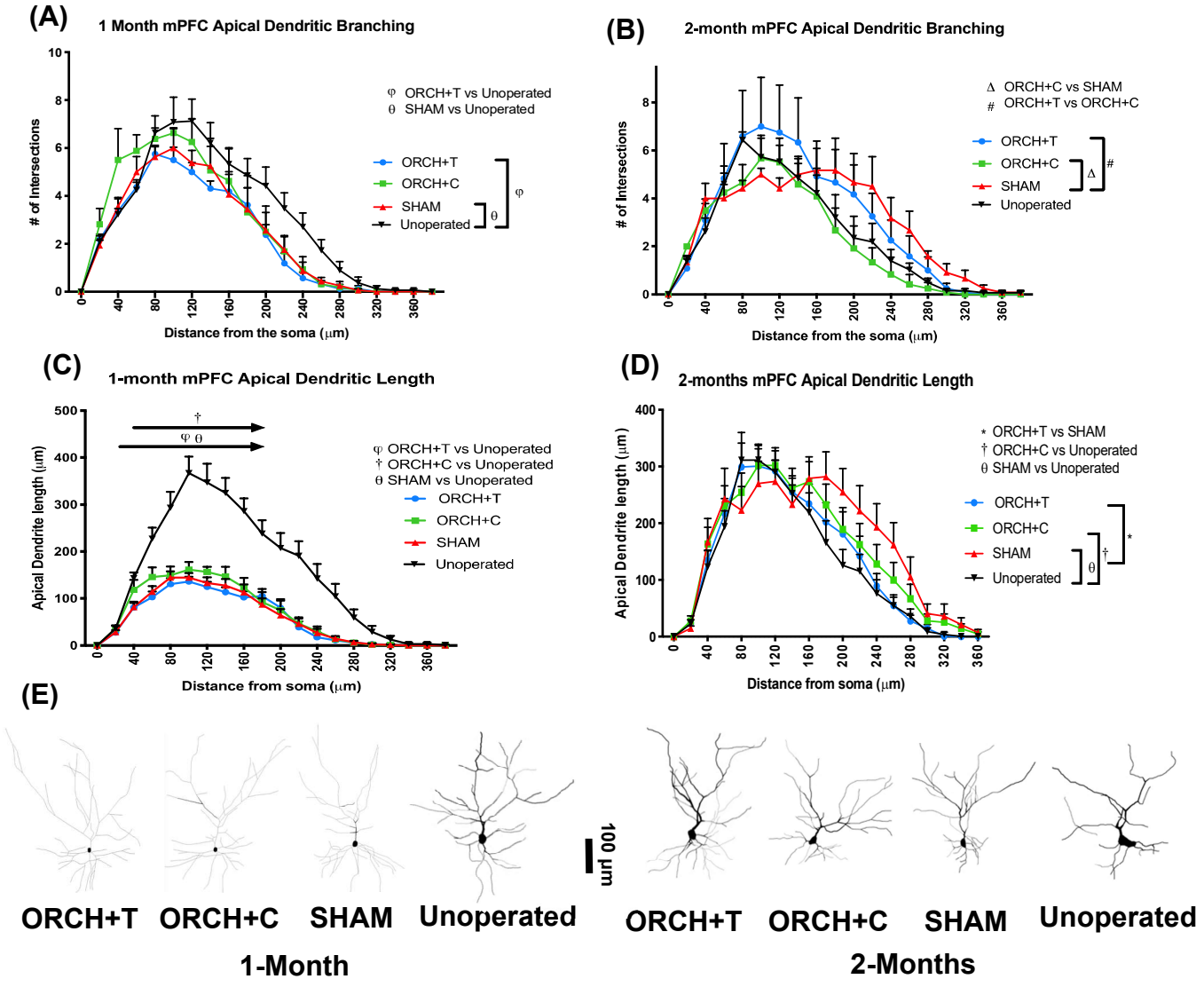

Figure 2.4: Apical dendritic branching and dendritic length for layer 2/3 of the mPFC.

(A) SHAM and ORCH+T displayed decreased dendritic branching compared to unoperated rats at 1-month. **(B)** SHAM rats had a slight increase in dendritic branching compared to ORCH+C rats at 2-months. ORCH+T display an increase in dendritic branching compared to SHAM rats. **(C)** All surgical groups had reduced apical dendritic length compared to unoperated rats at 1-month. **(D)** No significant differences in apical dendritic length at 2-months. **(E)** Representative 1- and 2-month mPFC tracings of ORCH+T, ORCH+C, SHAM and unoperated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

2.3.4 Testosterone levels

Two-way ANOVA on ln transformed data revealed a significant effect of treatment (Treatment; $F(3,25) = 118.718$, $p<0.001$) but not time (Time; $F(1,25) = 1.309$, $p=0.2635$) between the 1-month and 2-month ORCH+T, ORCH+C, SHAM and unoperated testosterone levels. Tukey-Kramer post hoc test revealed a significant treatment effect across all comparisons. While ORCH+T had significantly lower testosterone levels compared to unoperated intact males, ORCH+C testosterone levels were significantly lower compared to both ORCH+T and unoperated intact males. Furthermore, SHAM testosterone levels were significantly higher compared to ORCH+T, ORCH+C and unoperated animals (see Figure 2.5).

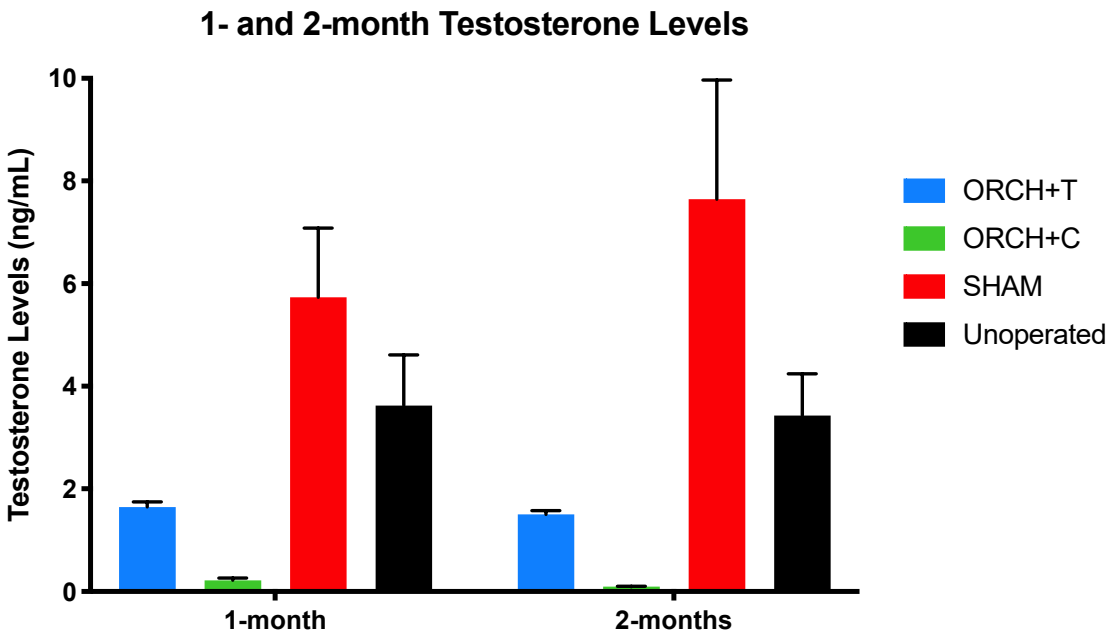

Figure 2.5: Serum testosterone levels of 1- and 2-month ORCH+T, ORCH+C, SHAM and unoperated adult male rats.

While the two-way ANOVA of ln transformed data indicated no significant effect of time, there was a significant treatment effect between all groups. ORCH+T rats have reduced testosterone compared to SHAM and unoperated males at both time-points. ORCH+C display a significant reduction in testosterone compared to both ORCH+T, SHAM and unoperated rats. While testosterone levels of SHAM rats were significantly increased compared to unoperated rats at both 1- and 2-months. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

2.4 Discussion

The findings from this study demonstrated that surgery and the post-operative recovery period can have lasting effects on the structure of apical dendritic within the hippocampus and layer 2/3 of the mPFC. While the effects of dendritic branching based on the number of intersections are relatively consistent with the previous results obtained at 10-days following surgery, it appears that the overall effects of undergoing a surgical procedure affect various aspects of the apical dendritic tree. At 1-month following surgery, ORCH+T and ORCH+C had comparable apical dendritic branching to unoperated intact males however, sham-orchiectomized males had decreased CA3 dendritic branching compared to the unoperated males indicating a significant effect of the surgical procedure. However, when compared to unoperated male rats, all surgical groups regardless of the procedure displayed fewer CA3 branches farther away from the cell body. This loss of the long apical dendrites is critical as they project information to other regions of the brain (Nazari-Serenjeh et al., 2011; Witter, 2010).

At 2-month following surgery, the CA3 apical dendritic branching of sham-orchiectomized males have recovered back to levels of ORCH+T animals however there still appeared to be an effect of surgery and post-operative recovery compared to unoperated males. The number of apical dendritic branching closer to the cell body is significantly increased in all surgical groups whether it was a full orchidectomy or simply a sham-operation. Additionally, consistent with CA3 branching at 1-month following surgery, there was a significant loss of the long apical dendrites in all animals who had

undergone a surgical procedure. An increase in the number of intersection closer to the cell body within the CA3 sub-field may indicate an increase in the interconnectivity with other areas of the hippocampus (Witter, 2010). However, the loss of dendrites farther away from the cell body suggests that there could be impairments in the ability of the hippocampus to make connections with other regions of brain such as the mPFC (Anne-Marie, Yves, Eric, & Jacques, 2000; Laroche, Davis, Jay, Suzuki, & Clayton, 2000; Witter, 2010). These connections are critical as they play a role in maintaining normal cognitive function and proper memory formation, therefore a loss of these connections could lead to significant impairments in the function of these two areas (Laroche et al., 2000).

However, analyzing dendritic structure based on the number of intersections alone will determine the complexity of the dendritic tree but it may not tell the full story of all of the morphological changes that are occurring (Sholl, 1953). Therefore, looking at the apical dendritic length between each of the Sholl radii provides a better understanding of the amount of dendritic material throughout the dendritic tree. These results suggested that any form of surgery leads to the dramatic reduction in apical dendritic length across the majority of the dendritic at both 1- and 2-months following surgery.

However, the effects of surgery were not restricted to the CA3 subfield of the hippocampus. Apical dendritic branching and length of CA1 hippocampal neurons were both significantly reduced following surgery compared to unoperated male, however,

these effects were much more pronounced 1-month after the surgical procedures. Moreover, in layer 2/3 of the mPFC, although the effects of surgery were still present, they were less extensive than what is seen in the hippocampus. The main effect seen in the mPFC is the reduction in apical dendritic length 1-month following surgery, while dendritic branching at 1- and 2-months and dendritic length at 2-months appeared to be relatively unaffected. This suggests that the process of undergoing a surgery, regardless of testosterone levels, caused persistent changes on the structure of apical dendritic tree throughout the hippocampus and in a critical area of the mPFC and therefore could be implicated in altered cognitive function.

Several potential mechanisms that may help explain why surgery produces lasting effects in the hippocampus and mPFC. One possible explanation for these results is that the stress of the surgical procedures causes atrophy of apical dendrites of the hippocampus and mPFC. At both 10-days and 1-month following surgery, there was a decrease in CA3 apical dendritic branching of SHAM rats compared to unoperated males. Because male rats who undergo 21-days of chronic stress displayed similar atrophy to what is seen in the present experiment following surgery the effects may be due to a response to elevated levels of circulating glucocorticoids (Galea et al., 1997; Wellman, 2014).

One common factor between the prior study conducted at 10-days following surgery and the present experiment is the development of post-operative complications that could be contributing to the response of hippocampal and mPFC apical dendrites.

The animals in both the 10-day and present experiment were operated on using the scrotal approach for orchidectomy which is known to have an increased risk for developing post-operative infections (Guilmette, Langlois, Hélie, & Warrak, 2015; Jenkins, 2000). Following all of these surgeries, several of the orchiectomized and sham-operated animals were placed on antibiotic treatment as they had developed post-operative infections. Therefore, a potential explanation for the lasting changes seen following these surgeries is the combination of surgical stress and the development of infections during recovery. Post-operative infections may place animals in a state of chronic inflammation leading to profound changes on apical dendrites within the hippocampus and mPFC. Chronic inflammation causes the prolonged secretion of pro-inflammatory cytokines such as IL-1β and TNF-α which can act on the hippocampus resulting in impairments in cognitive function (Liu et al., 2017; Peng et al., 2012). Surgery has also been shown to cause neuroinflammation within the hippocampus due to the dysregulation of microglia which leads to the local secretion of pro-inflammatory cytokines (Alam et al., 2018; Feng et al., 2017; Z. Li et al., 2018). Additionally, inflammation and stress have a reciprocal relationship. For example, elevated levels of circulating pro-inflammatory cytokines can activate the HPA-axis leading to persistent exposure to glucocorticoids while prolonged elevation of glucocorticoids increases the release of pro-inflammatory cytokines (Peng et al., 2012; Sorrells, Munhoz, Manley, Yen, & Sapolsky, 2014). Both glucocorticoids and pro-inflammatory cytokines reduce the expression of hippocampal BDNF (Suri & Vaidya,

2013; Wang et al., 2017). BDNF helps to maintain normal structure and function of neurons throughout the brain, therefore a reduction in BDNF due to chronic elevation in glucocorticoid and pro-inflammation cytokines may be one of the underlying mechanisms contributing to the surgery-induced atrophy of apical dendrites.

However, although the effects of surgery on hippocampal and mPFC apical dendrites 1- and 2-months following surgery can be hypothesized to be due to a combination of elevated glucocorticoids and chronic inflammation, it is difficult to accurately determine what factors are contributing to the effects seen following surgery. Additionally, when looking at the results of this experiment it is unclear how elevation in glucocorticoids and the post-operative recovery period are contributing to the dendritic remodelling. However, this study does provide some insight into the post-operative changes of hippocampal and mPFC dendritic morphology following surgery and how post-operative complications may exacerbate these effects. It also fills in some of the gaps in understanding long-term time-course of the effects of surgery and post-operative recovery.

3 Isoflurane exposure results in minor effects on hippocampal and medial prefrontal apical dendrites.

3.1 Introduction

Undergoing a major surgical procedure can activate a variety of physiological processes that are known to affect areas of the brain such as the hippocampus (Krenk, Rasmussen, & Kehlet, 2010). In humans, major surgery is known to cause periods of short-term memory loss and cognitive dysfunction however the reasons behind this are poorly understood as there are several components of surgery that could be playing a role (Alam et al., 2018; Krenk et al., 2010; Skvarc et al., 2018). While many studies evaluate the total effects of the surgical procedure, the effects of the anesthesia are often not considered. Isoflurane, ketamine/xylazine and pentobarbital are the most commonly used anesthetics for surgery although isoflurane appears to be the most popular option in research. While all these forms of anesthesia have shown to induce changes in hippocampal LTP, post-operative behaviour and duration of post-operative recovery ketamine/xylazine and pentobarbital appear to exert more damaging effects on the hippocampus compared to isoflurane (Freo & Ori, 2004; Janocko & Mycek, 1986; Merrill, Lee, Neigh, Bekhbat, & Kelly, 2016; Ribeiro, Tomé, Silva, Cunha, & Antunes, 2014; Wu et al., 2012).

In the experiments conducted in this thesis, isoflurane was used to anesthetize the rats prior to and during surgery. However, isoflurane has been shown to increase

plasma corticosterone levels which can have significant effects on hippocampal and mPFC dendritic structure and function (Bekhbat, Merrill, Kelly, Lee, & Gretchen, 2016; Jacobsen et al., 2012). Isoflurane has also been shown to activate neuroinflammatory processes causing increased secretion of IL-β within the hippocampus and causes significant impairments spatial memory that persists for 7-days (Cao et al., 2018; Li et al., 2016; Wu et al., 2012).

This experiment aims to determine how isoflurane is contributing to the effects seen 1- and 2-months following orchidectomy in chapter 2. Therefore, based on previous literature suggesting that isoflurane cause an increase in circulating glucocorticoids (Jacobsen et al., 2012) we hypothesize that isoflurane is contributing to the remodelling of hippocampal and mPFC apical dendrites. The results from this experiment indicate that isoflurane alone has very little effect on hippocampal and mPFC dendritic structure.

3.2 Materials and Methods

3.2.1 Animals

Eight young adult male Sprague-Dawley rats (~60-70 days old, ~250g) from Charles River Laboratories were housed under the same conditions as previously describes. All experimental protocols conducted were revised and approved from the Animal Utilization Protocol (AUP) in accordance with the Animal Care and Use Committees at the University of Guelph.

3.2.2 Isoflurane Treatment

The male rats treated with isoflurane were anesthetized with 4-5% isoflurane as they normally would prior to performing the surgery. Once the animals were fully anesthetized, they were placed in the recovery chamber until conscious and exhibited normal behaviour. They were then placed in clean home cages. The untreated animals remained in their home cages for the duration of the experiment. The isoflurane treated and untreated rats were sacrificed at 10-days after treatment.

3.2.3 Brain and Blood Collection

Isoflurane and untreated animals were sacrificed, and brain samples were collected in the same manner as previously described in chapter 2.

3.2.4 Golgi Staining and Tissue Processing

Golgi staining and tissue process were conducted as previously state in chapter 2.

3.2.5 Dendritic Structure and Sholl Analysis

Analysis of dendritic branching and dendritic length for apical dendrites was conducted in the same manner as chapter 2

3.2.6 Statistical Analysis

Two-way ANOVA followed by a Sidak's multiple comparisons post hoc test was used to analyze changes in dendritic branching and dendritic length of the basal and apical dendrites. The sample sizes for each group was as followed; isoflurane treated n=4, untreated n=4. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$

3.3 Results

3.3.1 Isoflurane has minor effects of CA3 apical dendrites.

While a two-way ANOVA revealed that there was no interaction between treatment or Sholl radius on CA3 apical dendritic branching (Treatment*Sholl radius; $F(43, 1144) = 0.621$, $p=0.9743$) or apical dendritic length (Treatment*Sholl radius; $F(43, 1144) = 0.804$, $p=0.8135$), there was a statistically significant effect of treatment on both CA3 apical dendritic branching (Treatment; $F(1, 1144) = 48.73$, $p<0.0001$) and length (Treatment; $F(1, 1144) = 53.25$, $p<0.0001$) compared to untreated intact males (see Figure 3.1A and Figure 3.1B). See Figure 3.1C for representative CA3 neuronal tracing from isoflurane treated and untreated male rats.

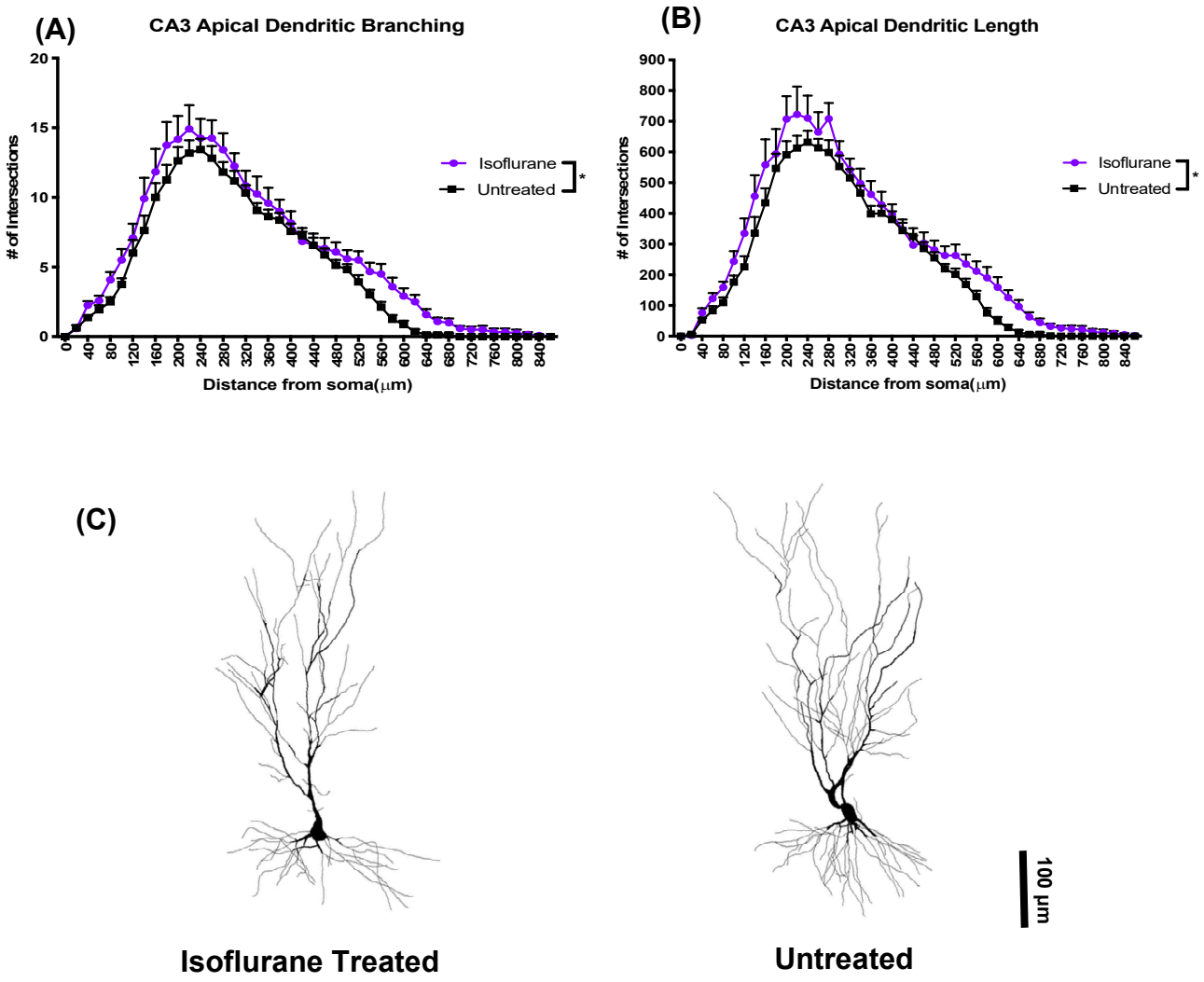

Figure 3.1: CA3 apical dendritic branching and dendritic length of male rats anesthetized with isoflurane compared to untreated rats.

(A) While there was a slight significant effect of treatment on the distal region, isoflurane resulted in minimal effects on CA3 apical dendritic branching **(B)** and apical dendritic length 10-days following anesthetization. **(C)** Representative CA3 tracings from isoflurane treated and untreated male rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

3.3.2 Isoflurane has minimal effects on CA1 apical dendritic branching.

Similarly, in the CA1 sub-region of the hippocampus, there was no significant interaction treatment and distance from the soma on apical dendritic branching (Treatment*Sholl radius; $F (39, 1200) = 0.9321$, $p=0.5909$) or length (Treatment*Sholl radius; $F (39, 1200) = 1.125$, $p=0.2770$) compared to untreated males. However, two-way ANOVA indicated a significant effect of treatment on both apical dendritic branching (Treatment; $F (1, 1200) = 7.778$, $p=0.0054$; Figure 3.2A) and apical dendritic length (Treatment; $F (1, 1200) = 5.695$, $p=0.0172$; Figure 3.2B). Figure 3.2C displays representative CA1 tracings from isoflurane treated or untreated rats.

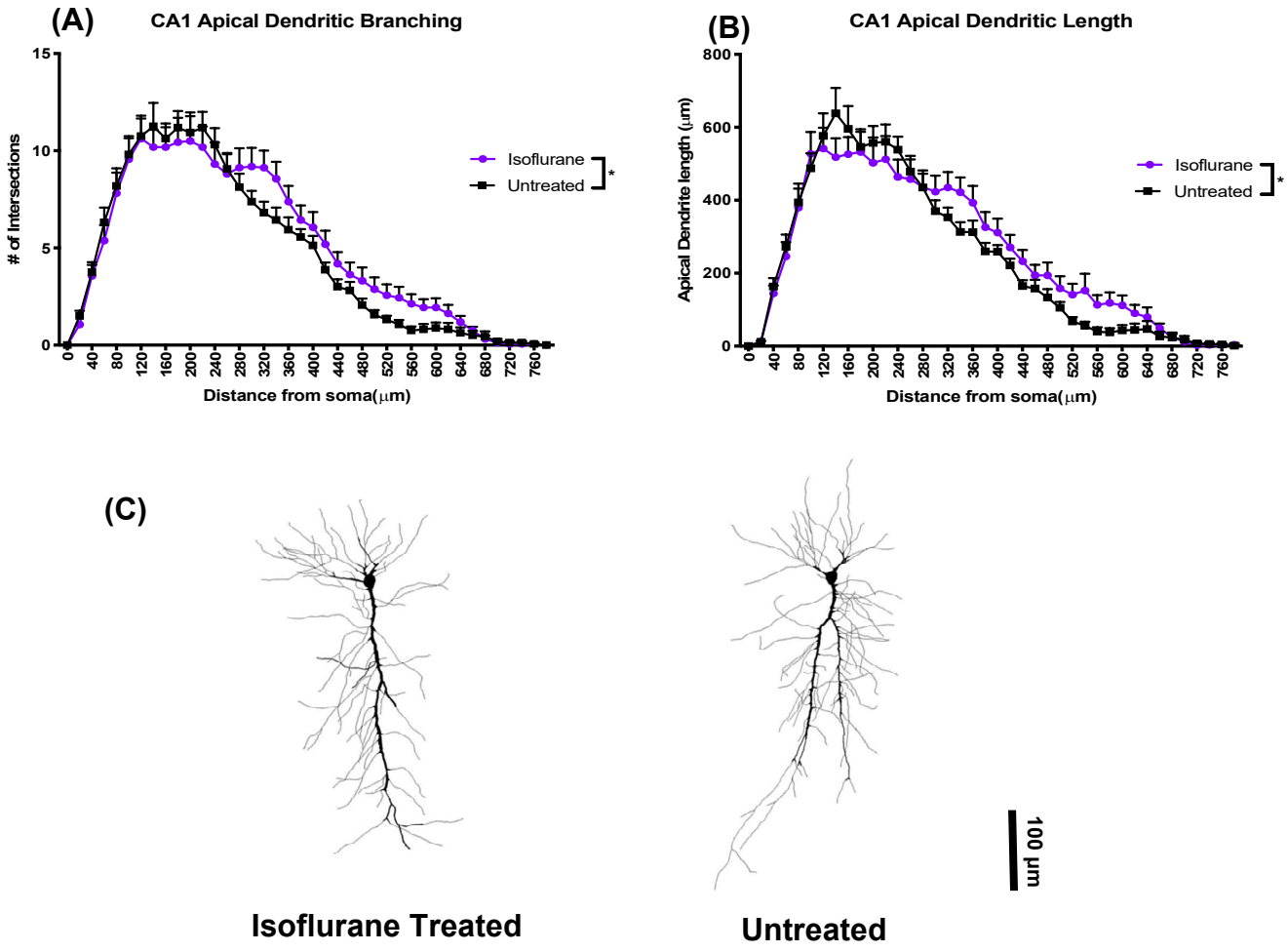

Figure 3.2: CA1 apical dendritic branching and dendritic length of male rats anesthetized with isoflurane compared to untreated rats.

(A) While there was a slight significant effect of treatment, isoflurane resulted in minimal effects on CA1 apical dendritic branching **(B)** and apical dendritic length 10-days following anesthetization. **(C)** Representative CA1 tracings from isoflurane treated and untreated male rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

3.3.3 Isoflurane results in minor effects on layer 2/3 mPFC apical dendrites.

In layer 2/3 of the mPFC the two-way ANOVA revealed no significant overall treatment effect on apical dendritic branching (Treatment; $F (1, 580) = 0.6512$, $p=0.4200$) or length (Treatment; $F (1, 520) = 0.4507$, $p=0.5023$) of isoflurane treated compared to untreated male rats. However, there was an interaction between treatment and distance from the soma for apical dendritic branching (Treatment*Sholl radius; $F (19, 580) = 2.201$, $p=0.0024$; Figure 3.3A) and length (Treatment*Sholl radius; $F (19, 520) = 3.015$, $p<0.0001$; Figure 3.3B). See Figure 3.3C for representative neuronal tracings

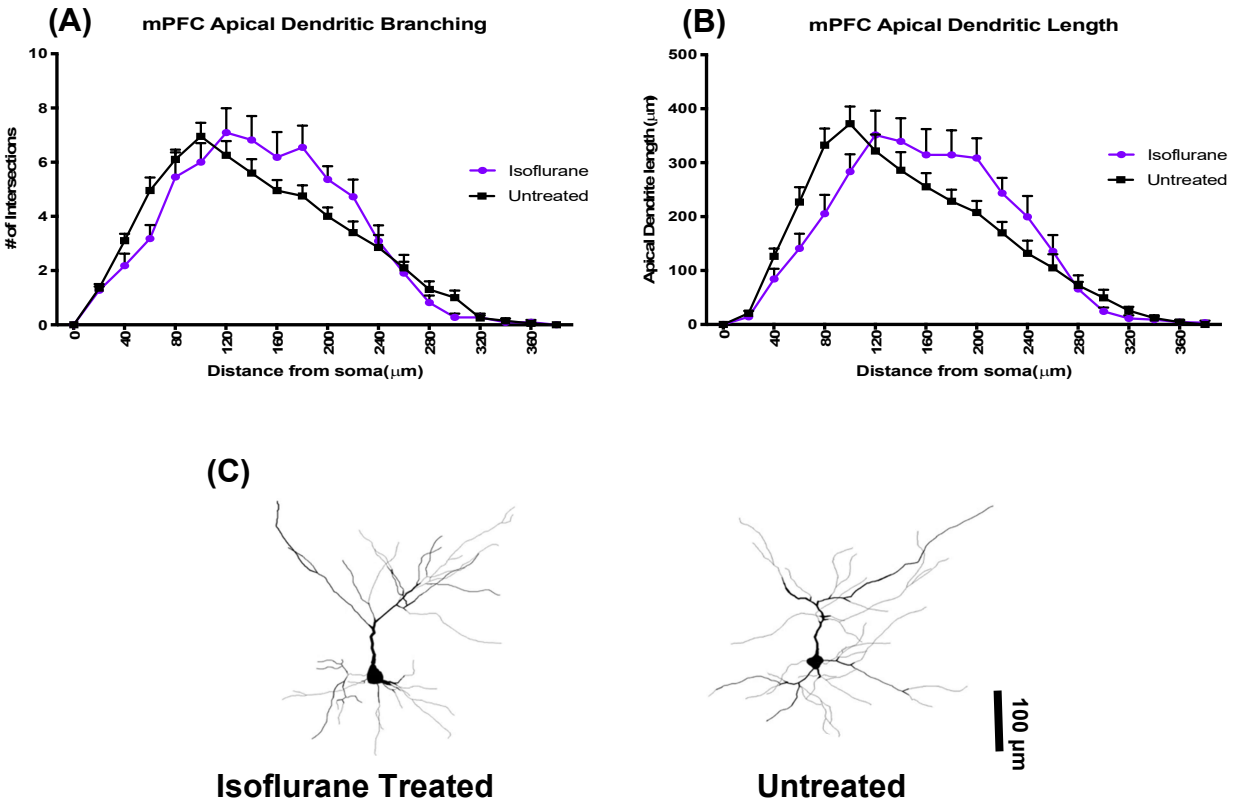

Figure 3.3: mPFC apical dendritic branching and dendritic length of male rats anesthetized with isoflurane compared to untreated rats.

(A) Isoflurane produced minor effects on mPFC apical dendritic branching **(B)** and apical dendritic length 10-days following anesthetization. **(C)** Representative mPFC tracings from isoflurane treated and untreated male rats. All data is represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

3.4 Discussion

This experiment was conducted to determine the role of anesthesia in the response of hippocampal and mPFC following surgery. Surgery has been shown to impair hippocampal-dependent memory, induce peripheral inflammation and hippocampal neuroinflammation (Fidalgo et al., 2011; Hovens et al., 2014). However, in studies that look at the effects of surgery as a whole, it is difficult to determine if there is any effect of the anesthesia used that are contributing to the effects after surgery. Isoflurane can increase circulating levels of glucocorticoids and increased local production of pro-inflammatory cytokines within the hippocampus (Bekhbat et al., 2016; Jacobsen et al., 2012; Li et al., 2013). To determine if isoflurane anesthesia is contributing to the effects of surgery previously seen at 10-days, 1-month and 2-months following surgery, male rats were anesthetized as they normally would be prior to performing a surgical procedure and then were left to recover for 10-days. The results indicated that isoflurane alone causes minor increases in apical dendritic branching and length within CA3, CA1 and layer 2/3 of the mPFC, but was not sufficient to cause the same atrophy seen following surgery at 1- and 2-months.

While the literature suggests that isoflurane increases corticosterone levels, these appear to be short-term effects. Corticosterone levels of male mice anesthetized with isoflurane without undergoing a surgical procedure peaks 5 minutes following the end of the isoflurane exposure and return to baseline by 4-hours (Jacobsen et al., 2012). Thus, the effects seen in chapter 2 are potentially due to more sustained levels of

glucocorticoids. Additionally, in humans, major surgery has been associated with the development of POCD in which the patient experiences a period of short-term memory loss and cognitive impairment following their surgery. However, it appears that the anesthetic used during surgery is not a major contributing factor to POCD. A randomized clinical trial using an electroencephalograph (EEG) guided method to administer the lowest amount of anesthetic necessary to the patient found that reducing the amount of general anesthetic used does not significantly decrease the incidence of POCD (Wildes et al., 2019).

These results indicate that isoflurane does not appear to be a major contributor to the effects that were seen 1- and 2-months following surgery. Therefore, there may be a combination of other factors from the surgery or post-operative recovery that are driving the atrophy of hippocampal and mPFC apical dendrites. However, this experiment helps the understanding of how isoflurane contributes to the effects of surgery on hippocampal and mPFC neuronal structure.

4 Glucocorticoids cause atrophy of hippocampal and medial prefrontal apical dendrites in unstressed male rats.

4.1 Introduction

The activation of stress responses through the activation of the HPA-axis leads to a rise in circulating levels of stress hormones such as glucocorticoids that can affect stress-sensitive regions of the brain such as the hippocampus and mPFC (Herman et al., 2003, 2016; McEwen, 2002). Under chronic or severe stress conditions the prolonged exposure to elevated glucocorticoids has been implicated in many stress-related neuropsychiatric disorders such as anxiety, depression and PTSD (Malivoire et al., 2018; Pooley et al., 2018). Previous research has suggested that underlying the development of stress-related neuropsychiatric disorders may reflect changes in the structure and function of neurons within the hippocampus and mPFC (Kokras & Dalla, 2014).

Stress and glucocorticoids can alter the structure of dendrites within the hippocampus and mPFC (McEwen, 2016; Sousa et al., 2007). In male rats, chronic restraint or unpredictable stress can lead to atrophy of CA3 and layer 2/3 mPFC apical dendrites (Cook & Wellman, 2004a; Galea et al., 1997; Wellman, 2001). Furthermore, this retraction of hippocampal and mPFC apical dendrites is associated with stress-induced impairments in spatial and working memory (Conrad, Ortiz, & Judd, 2017b; Luine et al., 1994). However, the response of hippocampal and mPFC apical dendrites to stress can be reversed if animals are given a sufficient recovery period where they

are not exposed to any form of stress (Luine et al., 1994). Although other stress hormones such as CRH have been shown to affect hippocampal dendritic morphology (Chen et al., 2008) the majority of the literature suggests that glucocorticoids are the driving factor responsible to the effects of stress on dendritic morphology (Woolley et al., 1990). For example, chronic corticosterone injections lead to the same dendritic atrophy of CA3 and layer 2/3 mPFC apical dendrites that are seen following chronic restraint stress (Wellman, 2001; Woolley et al., 1990).

Based on this previous research that prolonged exposure to glucocorticoids leads to atrophy of hippocampal and mPFC apical dendrites we believe that elevated levels of glucocorticoids are playing a role in the effects seen following surgery. However, although our previous experiments demonstrated that surgery results in the dramatic remodelling of hippocampal and mPFC apical dendrites at 10-days, 1-month and 2-months following surgery what was still unknown is where exactly this effect was coming from. Surgery induces a stress response in the rats however in the previous experiments (Frank et al., 2012), this stress response was combined with post-operative complications and potentially chronic inflammatory responses. Therefore, in this experiment, we aimed to determine the effects of elevated glucocorticoids on hippocampal and mPFC dendritic morphology in the absence of surgical stress. The results from this experiment suggest that prolonged exposure to glucocorticoids leads to the remodelling of hippocampal and mPFC apical dendrites that partially reflects the changes previously seen following surgery.

4.2 Materials and Methods

4.2.1 Animals

Nineteen young adult male Sprague-Dawley rats (60-70 days old, ~250g) from Charles River Laboratories were housed under the same conditions as previously described. Animals were cared for as per the Canadian Council on Animal Care and all experimental protocols conducted were approved from the Animal Utilization Protocol (AUP) in accordance with the Animal Care and Use Committees at the University of Guelph.

4.2.2 Habituation

Rats were handled daily at 10:00 am for one-week before the start of the experiment for the animals to become familiar with being handled at any point in the experiment and to eliminate inducing a stress response when picked up during the study.

4.2.3 Dexamethasone Treatment

Following habituation, the regular drinking water in the cages was replaced with water containing 1.5µg/mL of dexamethasone (Sigma-Aldrich, Oakville, ON, Canada) for 16-hours ad libitum. Following the 16-hours, the rats were switched back onto regular water for the remainder of the experiment. This treatment method had previously been used to mimic the GR occupation seen following acute restraint stress (A. H. Miller, Spencer, Stein, & McEwen, 1990). The approximate amount of water that each rat drank was calculated by subtracting weights of the water bottles after treatment

to the weight before treatment and divided by the number of rats in the cage. The approximate dose per rat was calculated based on the concentration of dexamethasone in the water. Animals were sacrificed 1-day, 3-days and 10-days after then end of the dexamethasone treatment. Untreated rats remained on regular drinking water for the entire experiment and were sacrificed at the 10-day time-point.

4.2.4 Brain and Blood Collection

Following their respective time points dexamethasone-treated and untreated animals were sacrificed, and brain and blood samples were collected in the same manner as previously described.

4.2.5 Golgi Staining and Tissue Processing

Golgi staining and tissue processing were conducted as described in chapter 2.

4.2.6 Dendritic Structure and Sholl Analysis

Analysis of dendritic branching and dendritic length for apical dendrites was conducted in the same manner as chapter 2

4.2.7 Testosterone Assay

Testosterone ELISA was conducted with the same Calbiotech kit using manufactures instructions and absorbance was read at 450nm as previously described.

4.2.8 Statistical Analysis

Two-way ANOVA followed by Tukey-Kramer post hoc tests was used to analyze changes in dendritic branching and dendritic length. The sample sizes for each group was as followed; 1-day dexamethasone n=5, 3-day dexamethasone n=5, 10-day

dexamethasone n=5, untreated n=4. All dendritic structure data are expressed as mean ± SEM with the level of significance set at $p<0.05$.

To determine changes in serum testosterone levels, raw data was [ln] transformed and analyzed using a one-way ANOVA followed by Tukey-Kramer post hoc test was conducted. Significance for all statistical analysis was determined as $p<0.05$.

4.3 Results

4.3.1 Dexamethasone treatment in unstressed male rats leads to similar remodelling of CA3 apical dendrites as surgery.

CA3 apical dendrites displayed a significant effect of both treatment (Treatment; $F(3, 2807) = 26.73$, $p<0.0001$) and an interaction between treatment and distance from the soma (Treatment*Sholl radius; $F(114, 2807) = 2.484$, $p<0.0001$). While the 1-day and 10-day dexamethasone treated groups had comparable CA3 apical dendritic branching, the 3-day dexamethasone treated animals displayed a decrease in dendritic branching compared to the 1-day and 10-day animals between 120 to 220μm and 140 to 200μm respectively. Moreover, apical dendrites of animals sacrificed 1-, 3- and 10-days following dexamethasone treatment were significantly altered compared to untreated male rats (see Figure 4.1A). The apical dendritic trees of animals sacrificed 1- and 10-days following dexamethasone displayed an increased number of dendritic branches in the proximal region and decreased in dendrites in the medial and distal regions of the dendritic tree compared to untreated males. This increase in dendritic branching between animals 1-day and untreated animals was seen from 100-160μm

while dendritic branching was decreased between 300-320μm and 500-520μm. Similarly, animals sacrificed 10-days following dexamethasone treatment had an increase in dendritic branching between 120-140μm but decreased from 240-280μm. Furthermore, rats sacrificed 3-days following dexamethasone treatment displayed a significant decrease in dendritic branching between 200-460μm.

The 16-hour dexamethasone treatment resulted in a significant reduction in CA3 apical dendritic length compared regardless of the number of days following treatment when compared to untreated rats (see Figure 4.1B). Two-way ANOVA showed both an overall treatment effect (Treatment; $F(3, 2808) = 175.1$, $p<0.0001$) and an interaction between treatment and distance from the soma (Treatment*Sholl radius; $F(114, 2808) = 4.954$, $p<0.0001$). Apical dendritic length of animals sacrificed 1-, 3- and 10-days following treatment was significantly reduced compared to untreated males between the following Sholl radii; 1-day vs untreated; 160-560μm, 3-days vs untreated; 140-540μm, 10-days vs untreated; 180-540μm. See Figure 4.1C for representative CA3 neuronal tracings.

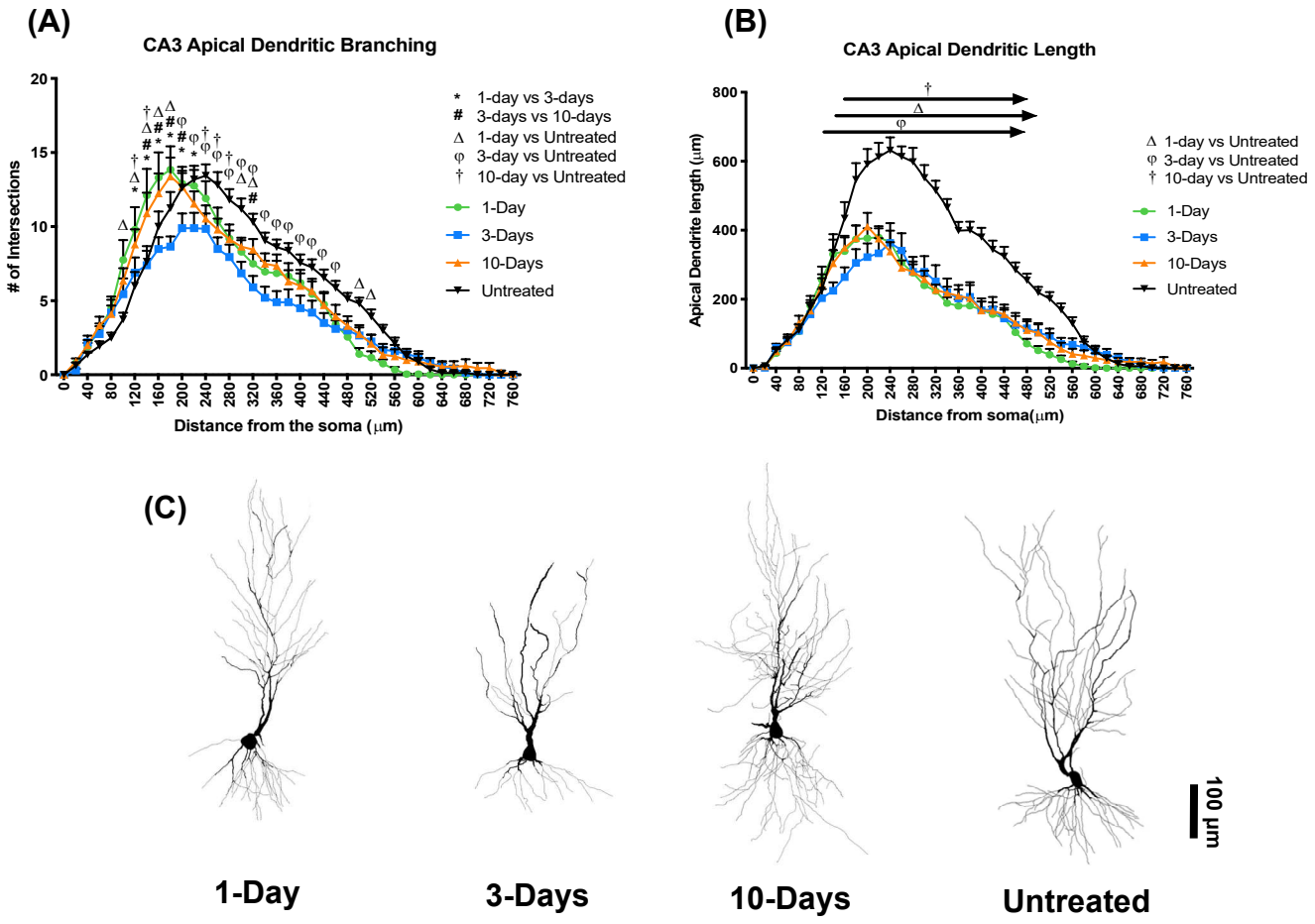

Figure 4.1: CA3 apical dendritic branching and dendritic length of dexamethasone treated male rats.

(A) 3-days following dexamethasone treatment, male rats had significantly decreased CA3 apical dendritic branching compared to 1- and 10-days following dexamethasone treatment and untreated rats. **(B)** All dexamethasone treated rats had significantly reduced apical dendritic length compared to untreated rats regardless of the time-point following treatment. **(C)** Representative CA3 tracings of male rats sacrificed 1-, 3- or 10-days following dexamethasone water treatment and untreated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

4.3.2 Dexamethasone treatment decreases CA1 apical dendritic branching and length.

Apical dendrites of CA1 pyramidal neurons displayed a significant effect of dexamethasone treatment compared to untreated male regardless of the timepoint following the treatment (See Figure 4.2A). Two-way ANOVA revealed a significant overall effect of treatment (Treatment; $F(3, 2880) = 48.75$, $p<0.0001$) on CA1 apical dendritic branching and an interaction between treatment and distance from the soma (Treatment*Sholl radius; $F(117, 2880) = 1.656$ $p<0.0001$). Tukey post-hoc test revealed that animals sacrificed 1-day, 3-days and 10-days following dexamethasone treatment had a significant decrease in apical dendritic branching compared to untreated males between the following distances from the soma; 1-day vs untreated: 100-260μm, 320-360μm; 3-days vs untreated; 200-280μm, 320-400μm; 10-days vs untreated; 140-300μm, 340-360μm.

Furthermore, dexamethasone treatment also caused CA1 apical dendritic length to be significantly reduced compared to untreated males however this was even more exacerbated than the effect seen on apical dendritic branching (See Figure 4.2B). The two-way ANOVA revealed a significant overall treatment effect (Treatment; $F(3, 2880) = 240.9$, $p<0.0001$) as well as an interaction between treatment with specific regions of the dendritic tree (Treatment*Sholl radius; $F(117, 2880) = 5.243$. $p<0.0001$). Dexamethasone caused a significant decrease in apical dendritic length compared to untreated males between the following distances from the soma; 1-day vs untreated:

80-460μm; 3-days vs untreated: 80-420μm; 10-days vs untreated: 60-460μm. See Figure 4.2C for representative CA1 neuronal tracings.

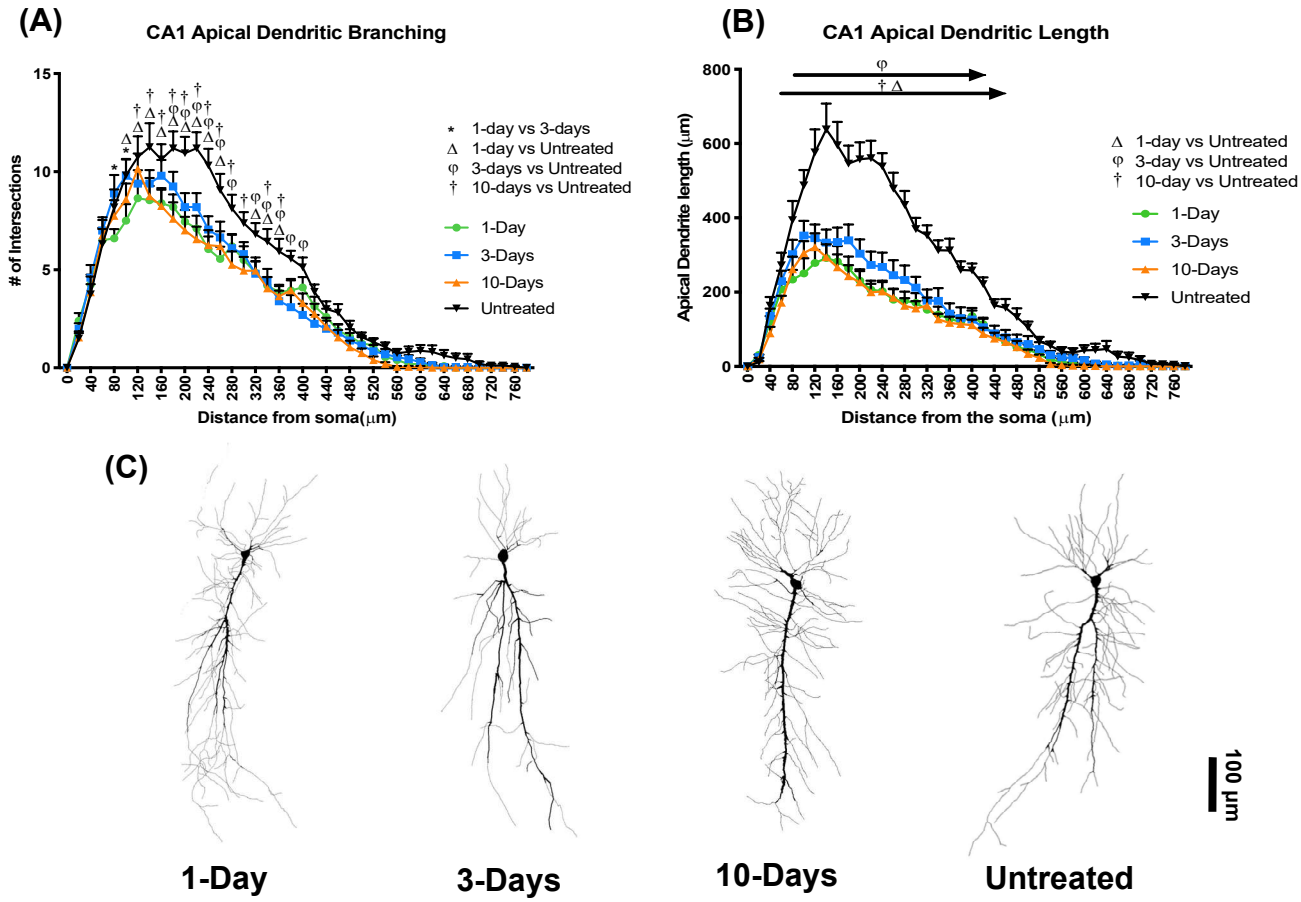

Figure 4.2: CA1 apical dendritic branching and dendritic length of dexamethasone treated male rats.

(A) All dexamethasone treated rats at all time-points following treatment had significantly reduced CA1 apical dendritic branching **(B)** and apical dendritic length compared to untreated rats. **(C)** Representative CA1 tracings of male rats sacrificed 1-, 3- or 10-days following dexamethasone water treatment and untreated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

4.3.3 Dexamethasone treatment causes the remodelling of layer 2/3 medial prefrontal apical dendrites.

While the two-way ANOVA revealed both a significant treatment (Treatment; $F(3, 1083) = 9.373$, $p<0.0001$) and interaction effect (Treatment*Sholl radius; $F(54, 1083) = 1.507$, $p=0.0115$) on layer 2/3 mPFC apical dendritic branching (Figure 4.3A), the results were similar to the hippocampus but had a larger variance of the individual measurements. There was a significant decrease in apical branching 1-day and 10-days following dexamethasone treatment compared to untreated males (1-day vs untreated:100μm, 200-240μm; 10-days vs untreated; 100-120μm). Additionally, there was a significant reduction in apical branching 1-day after dexamethasone compared to 3-days between 200-240μm.

There was a significant treatment (Treatment; $F(3, 1100) = 7.953$, $p<0.0001$) and interaction effect (Treatment*Sholl radius; $F(57, 1100) = 1.75$, $p=0.0006$) on mPFC apical dendritic length (Figure 4.3B) however the effects of dexamethasone were less clear than the hippocampus. Tukey's multiple comparisons test revealed that 1-day following dexamethasone treatment there was a significant decreased dendritic length compared to untreated males between 100-140μm. Additionally, 3-days following dexamethasone treatment apical dendritic length was significantly decreased compared to both 10-days following treatment (40-100μm) and untreated animals (60-100μm). Figure 4.3C displays representative mPFC neuronal tracings.

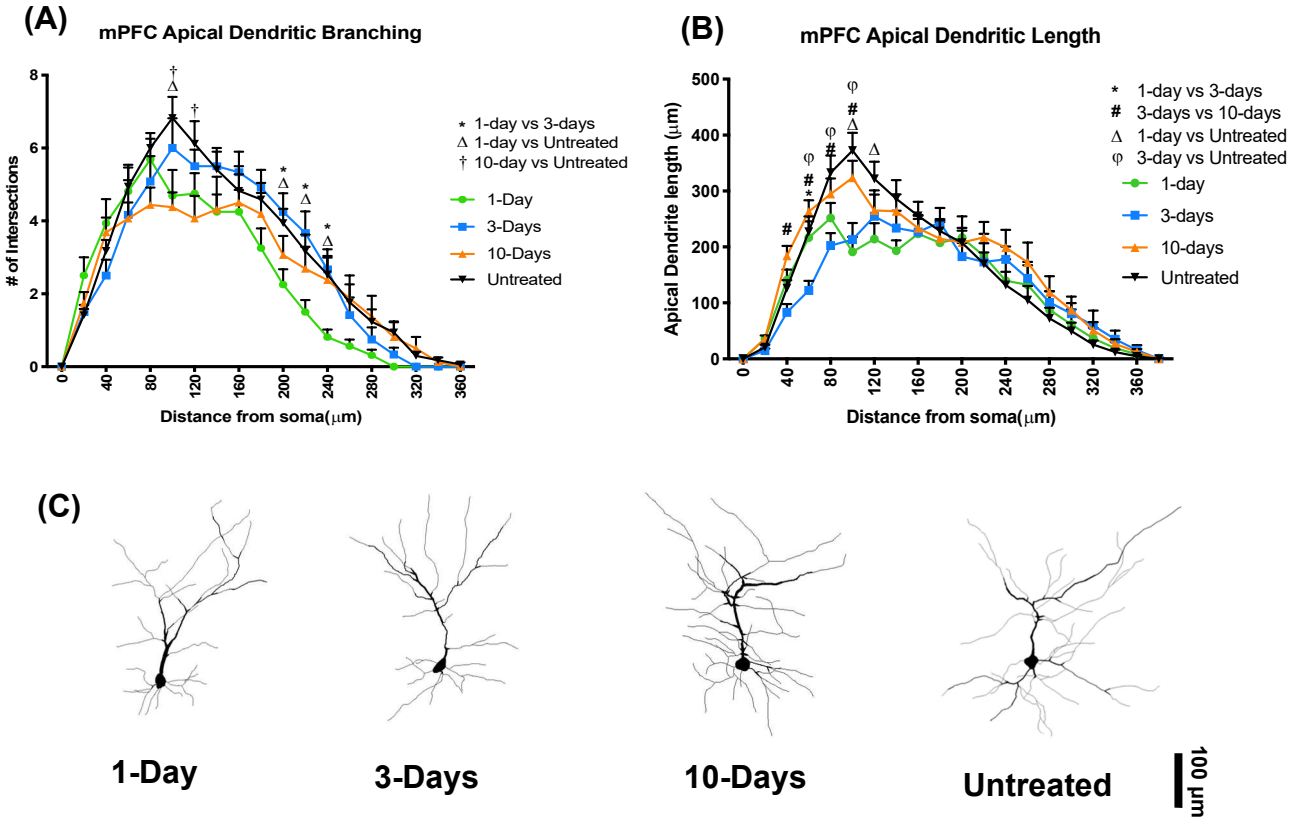

Figure 4.3: mPFC apical dendritic branching and dendritic length of dexamethasone treated male rats.

(A) mPFC apical dendrites displayed a significant decrease dendritic branching 1- and 10-days following dexamethasone treatment compared to untreated rats. Rats sacrificed 1-day following dexamethasone treatment displayed a significant decrease in apical dendritic branching compared to 3-days. **(B)** mPFC apical dendritic length was significantly reduced 1- and 3-days following dexamethasone treatment compared to untreated rats. Apical dendritic length was also reduced in 3-days compared to 10-days following dexamethasone treatment. (**C)** Representative mPFC tracings of male rats sacrificed 1-, 3- or 10-days following dexamethasone water treatment and untreated rats. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

4.3.4 Dexamethasone treatment decreases serum testosterone levels in adult male rats.

The results of the testosterone ELISA revealed that placing dexamethasone in the drinking water reduced serum testosterone levels compared to untreated males (Figure 4.4). One-way ANOVA and Tukey's Kramer post-hoc test of ln transformed data revealed that testosterone levels 1-day, 3-days and 10-days following dexamethasone treatment were significantly decreased compared to untreated adult male rats (ANOVA; $F(3,14) = 6.942$, $p = 0.0043$).

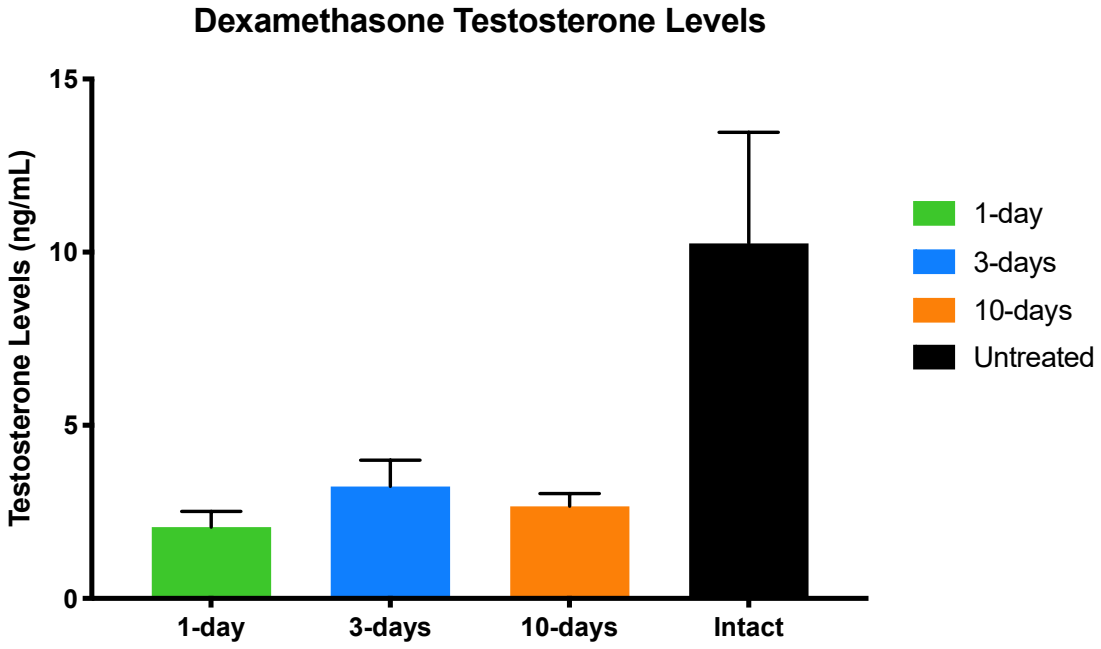

Figure 4.4: Serum testosterone levels of dexamethasone treated adult male rats. Male rats treated with dexamethasone had significantly decreased serum testosterone compared to untreated rats regardless of the time-point following treatment. All data are represented as mean +/- SEM (n=4-5 rats/group). Significant data points represent $p<0.05$.

4.4 Discussion

To determine the effects of elevated glucocorticoids in the absence of surgical stress, the normal drinking water was replaced with water containing dexamethasone at a concentration of 1.5ng/mL for 16 hours (Miller et al., 1990). These specific treatment conditions have been previously shown to cause similar GR occupation as acute restraint stress (Miller et al., 1990). Therefore, it was chosen to mimic the effects of glucocorticoids following surgery without inducing an overall stress response. Moreover, serum testosterone levels were reduced in all animals treated with dexamethasone compared to untreated males. As glucocorticoids are known to inhibit testosterone secretion, this reduction in testosterone levels may be a contributing factor to the effects of surgical stress on hippocampal and mPFC dendritic structure (Brownlee, Moore, & Hackney, 2005)

The results of this experiment suggest that glucocorticoids alone are sufficient to induce rapid dendritic remodelling of hippocampal and mPFC that is similar to what is seen 1- and 2-months following surgery and therefore may be contributing to the lasting effects following surgical stress. Dexamethasone resulted in the remodelling of both hippocampal and mPFC however these effects were more pronounced in CA3 and CA1 apical dendrites. In CA3, dexamethasone altered apical dendritic branching however, the reduction in apical dendritic length was strikingly similar to the response of CA3 dendrites 1- and 2-months following surgery. Additionally, there a noticeable similarity between CA1 apical dendritic branching and length of dexamethasone treated to CA1

dendrites 1-month following orchidectomy. This indicated that glucocorticoid exposure may be a common factor between these two experiments and may be a significant factor contributing to the changes in apical dendritic morphology following surgery.

Although the treatment of dexamethasone used is known to produce similar GR occupation seen following acute restraint stress (Miller et al., 1990), it does not exactly mimic the normal physiological response of glucocorticoids following stress. Under normal conditions, circulating levels of glucocorticoids will rapidly increase when presented with a stressor and will return to baseline within 2-hours of the removal of the stressful stimuli (Herman et al., 2016). By placing dexamethasone in the drinking water for 16-hours the effects of glucocorticoids can be isolated from a stress response however due to the half-life of dexamethasone in an adult male rat it will most likely remain in the circulation for at least 12-hours following the removal of the water (Wiedemann & Holsboer, 1987). This will result in stress-like levels of glucocorticoids that remain in the circulation for approximately 24-hours causing the duration of GR occupation will be longer than what would normally occur during a physiological stress response. However, due to the similarity in dendritic structure 1- and 2-months following surgery and 1-, 3- or 10-days following dexamethasone treatment it suggests that these effects seen following surgery are, at least in part, due to prolonged exposure to glucocorticoids.

Because the dexamethasone treatment is not the same as surgical stress it remains unknown if there are other factors of the surgery and recovery period are

contributing to the remodelling of hippocampal and mPFC apical dendrites. However, this experiment helps to better understand how the persistent elevation of glucocorticoids alone contributes to the effects of an overall stress response following surgery and the post-operative recovery period.

5 Sham-orchidectomy using an abdominal surgical approach produces minimal effects on hippocampal and medial prefrontal apical dendrites.

5.1 Introduction

Undergoing a major surgical procedure can have profound effects on the hippocampus (Krenk et al., 2010). In humans, major surgery can cause impairments in hippocampal-dependent memory as well as induce an increase in IL-β secretion (Alam et al., 2018; Cibelli et al., 2010; Krenk et al., 2010; Skvarc et al., 2018). Animal studies have demonstrated that surgical procedures alter BDNF expression, stress and inflammatory responses as well as neuroinflammation, which can all have effects on the structure and function of neurons within the hippocampus (Frank et al., 2012; Hovens et al., 2014; Wang et al., 2017). Surgery has shown to reduce BDNF expression within the hippocampus that can persist for a week after the procedure (Fan, Li, Zheng, Hua, & Zuo, 2016; Hovens et al., 2014). The effects of surgery on BDNF expression have been associated with the dysregulation of microglia leading to increased secretion of pro-inflammatory cytokines within the hippocampus as well as an increase in circulating glucocorticoids (Wang et al., 2017).

Stress and inflammatory processes can feed off each other leading to more extensive effects. Stress prior to surgery leads to a more exacerbated

neuroinflammatory response in the hippocampus, a larger reduction in BDNF and increased memory impairments (Wang et al., 2017; Wang et al., 2016).

Additionally, the scrotal approach for orchidectomy has an increased risk of post-operative complications and the development of infections which can exacerbate the effects of surgery (Guilmette et al., 2015; Jenkins, 2000). The development of post-operative infections will activate the immune system in the animals which will lead to peripheral inflammatory responses (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). While there is little evidence of the effects of inflammation on the dendritic structure of hippocampal and mPFC neurons, an increase in pro-inflammatory cytokines leads to the development of depressive-like behaviour in male rats (Alam et al., 2018; Liu et al., 2017).

Therefore, based on the results obtained in the previous chapters of this thesis it appears that surgery and elevated levels of glucocorticoids a profound effect on hippocampal and mPFC apical dendrites particularly the apical dendritic length. However, it was unclear if the effects are due to the surgery for if the post-operative inflammation during recovery were exacerbating the effects on hippocampal and mPFC apical dendrites. Therefore, this experiment aims to determine how we can minimize the effects of surgery on the hippocampus and mPFC through the use of handling and an alternate surgical approach with less risk of infections helps to prevent the remodelling of hippocampal and mPFC dendrites following surgery. The results obtained from this experiment indicate that the use of handling to reduce the stress of the animals and a

more careful surgical approach to reduce the duration of the post-operative recovery prevents the dramatic remodelling of hippocampal and mPFC apical dendritic morphology due to surgery.

5.2 Materials and Methods

5.2.1 Animals

Sixteen young adult male Sprague-Dawley rats (60-70 days old, ~250g) from Charles River Laboratories were housed under the same conditions as previously describes. All experimental protocols conducted were approved from the Animal Utilization Protocol (AUP) in accordance with the Animal Care and Use Committees at the University of Guelph.

5.2.2 Habituation

Rats were handled daily at 10:00 am for one-week in order for the animals to become familiar with being handled at any point in the experiment and to eliminate inducing a stress response when picked up during the study.

5.2.3 Surgical Procedures

Male rats underwent either a sham-orchidectomy using an abdominal surgical approach or remained surgically naïve. Carprofen (5 mg/kg) was injected subcutaneously 2-hours prior to surgery to allow for a long-acting anti-inflammatory. Sham-operated rats were anesthetized under 4-5% isoflurane and then maintained under 1-2% isoflurane during the surgery. A lidocaine/bupivacaine combination (2mg/kg) was injected subcutaneously at the incision site. An incision was made on the

midline of the abdomen. Subcutaneous fat and muscle tissue were gently separated to expose the abdominal cavity. The fat pad onto of the testes was located and pulled up to expose the teste and then placed back in the abdominal cavity. This procedure was repeated for the other teste. The abdominal muscle was sutured, and the skin was closed using a single surgical staple. The rats were given 5 mg/kg of saline and 2mg/kg lidocaine/bupivacaine subcutaneous then placed in the recovery chamber. Once the rat was conscious and moving around normally, they were placed back in clean home cages and monitored for post-operative complications. Sham-operated animals were sacrificed 1-day, 3-days, or 10-days following their procedures. The unoperated animals remained in their home cages for the duration of the experiment and sacrificed at the 10-day time-point.

5.2.4 Brain and Blood Collection

Following their respective time-points sham-orchiectomized, isoflurane and unoperated animals were sacrificed and brain and blood samples were collected in the same manner as previously described in chapter 2.

5.2.5 Golgi Staining and Tissue Processing

Golgi staining and tissue process were conducted the exact same as previously state in chapter 2.

5.2.6 Dendritic Structure and Sholl Analysis

Golgi staining and tissue process were conducted the exact same as previously state in chapter 2.

5.2.7 Testosterone Assay

Testosterone ELISA was conducted with the same Calbiotech kit using manufactures instructions and absorbance was read at 450nm as previously described in chapter 2.

5.2.8 Statistical Analysis

Two-way ANOVA followed by a Tukey-Kramer post hoc test was used to analyze changes in dendritic branching and dendritic length of the basal and apical dendrites. The sample sizes for each group was as followed; 1-day SHAM n=4, 3-day SHAM n=4, 10-day SHAM n=4, surgically naïve n=4. One-way ANOVA followed by a Tukey-Kramer post hoc test of ln transformed data was conducted to analyze changes in serum testosterone levels of sham-operated and unoperated adult male rats. Significance for all statistical analysis was determined as $p<0.05$.

5.3 Results

5.3.1 Abdominal sham-orchidectomy has minimal effects on CA3 apical dendrites.

Overall, CA3 apical dendritic branching and length of animals 1-day, 3-day and 10-days sacrificed following SHAM using an abdominal surgical approach were similar to those of unoperated males (Figure 5.1A). The two-way ANOVA revealed significant treatment and interactions effect on both apical dendritic branching (Treatment; $F (3, 2460) = 10.42$, $p<0.0001$, Treatment*Sholl radius; $F (120, 2460) = 1.515$, $p=0.0004$) and apical dendritic length (Treatment; $F (3, 2460) = 9.128$, $p<0.0001$Treatment*Sholl

radius; F (120, 2460) = 1.551, p=0.0002). CA3 apical dendritic branching 1- and 3-days following abdominal SHAM was significantly decreased between the following Sholl radii compared to the 10-day and unoperated groups; 1-day vs 10-days: 140-180μm, 300-340μm; 3-days vs. 10-days: 300-360μm; 3-day vs unoperated: 300-420μm. Similarly, CA3 apical dendritic length 1-day and 3-days following abdominal SHAM was significantly reduced compared to 10-day and unoperated males at the following distances from the soma; 1-day vs 3-days: 140-160μm; 1-day vs 10-days: 140-160μm, 320-340μm; 3-days vs 10-days: 220, 320-360μm; 3-days vs unoperated: 320-440μm (Figure 5.1B). See Figure 5.1C for representative CA3 neuronal tracings.

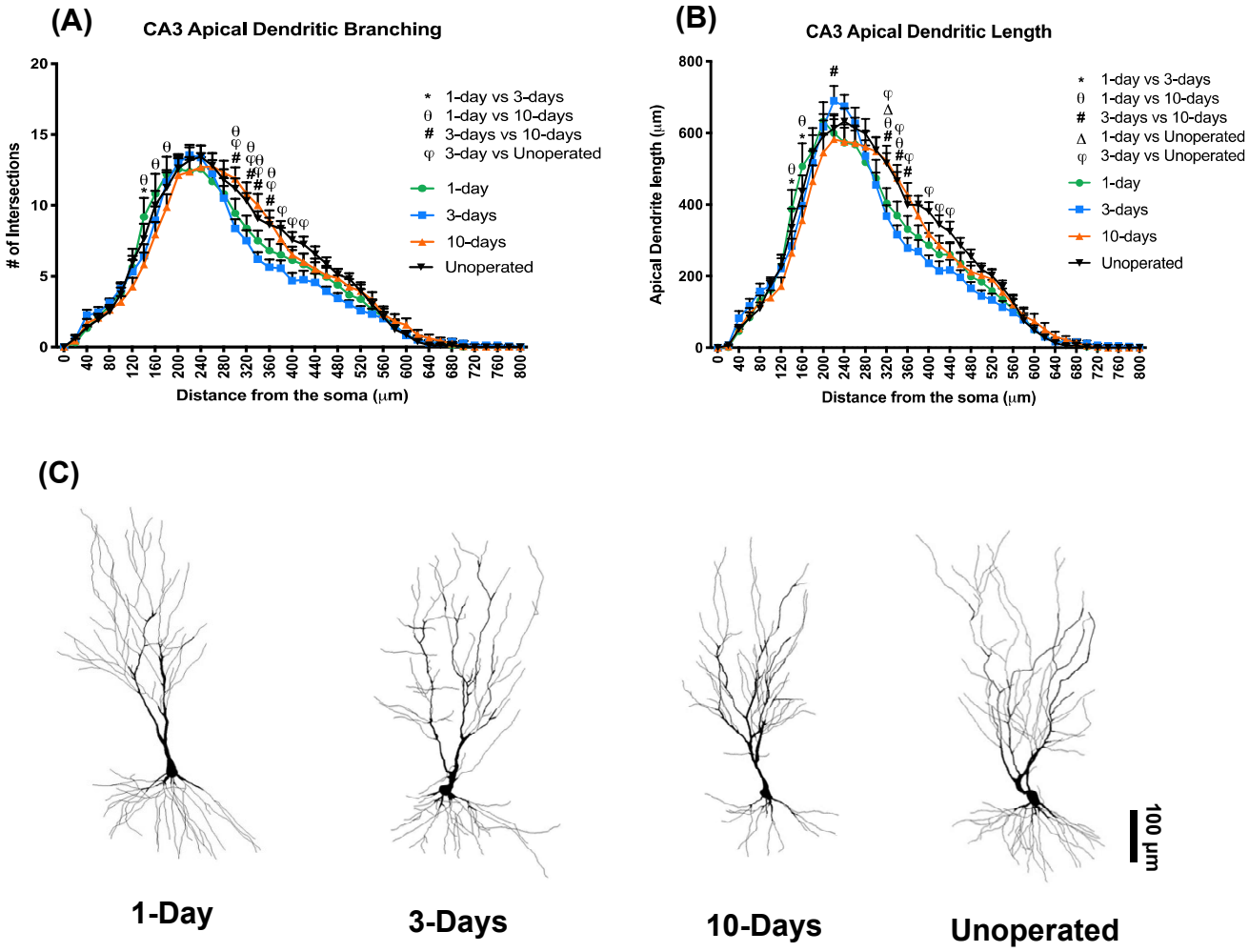

Figure 5.1: CA3 apical dendritic branching and dendritic length of sham-orchiectomized male rats using the abdominal surgical approach.

(A) Abdominal SHAM slightly decreased CA3 apical dendritic branching 1- and 3-days following surgery compared to unoperated rats. 10-days following surgery, apical dendritic branching was comparable to unoperated rats. **(B)** SHAM using the abdominal surgical approach caused minor decreases in apical dendritic length compared to 10-days following surgery and unoperated rats. **(C)** Representative CA3 tracings from abdominal SHAM 1-, 3- and 10-days following surgery and unoperated rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

5.3.2 Sham-orchidectomy using the abdominal approach little to no effects on CA1 apical dendrites.

Two-way ANOVA of CA1 apical dendritic dendrites revealed no overall treatment effect on apical branching (Treatment; $F(3, 2400) = 1.756$, $p=0.1536$; Figure 5.2A) but there is a treatment effect on apical dendritic length (Treatment; $F(3, 2400) = 4.534$, $p=0.0036$; Figure 5.2B) between 1-day vs 10-day SHAM animals ($p= 0.0388$) and 1-day vs unoperated animals ($p= 0.0023$). There was no interaction effect between treatment and distance from the soma on apical dendritic branching (Treatment*Sholl radius; $F(117, 2400) = 0.6235$, $p=0.9994$) or length (Treatment*Sholl radius; $F(117, 2400) = 0.7133$, $p=0.9908$). Figure 5.2C shows representative CA1 tracings from 1-, 3- and 10-days following abdominal SHAM and unoperated male rats.

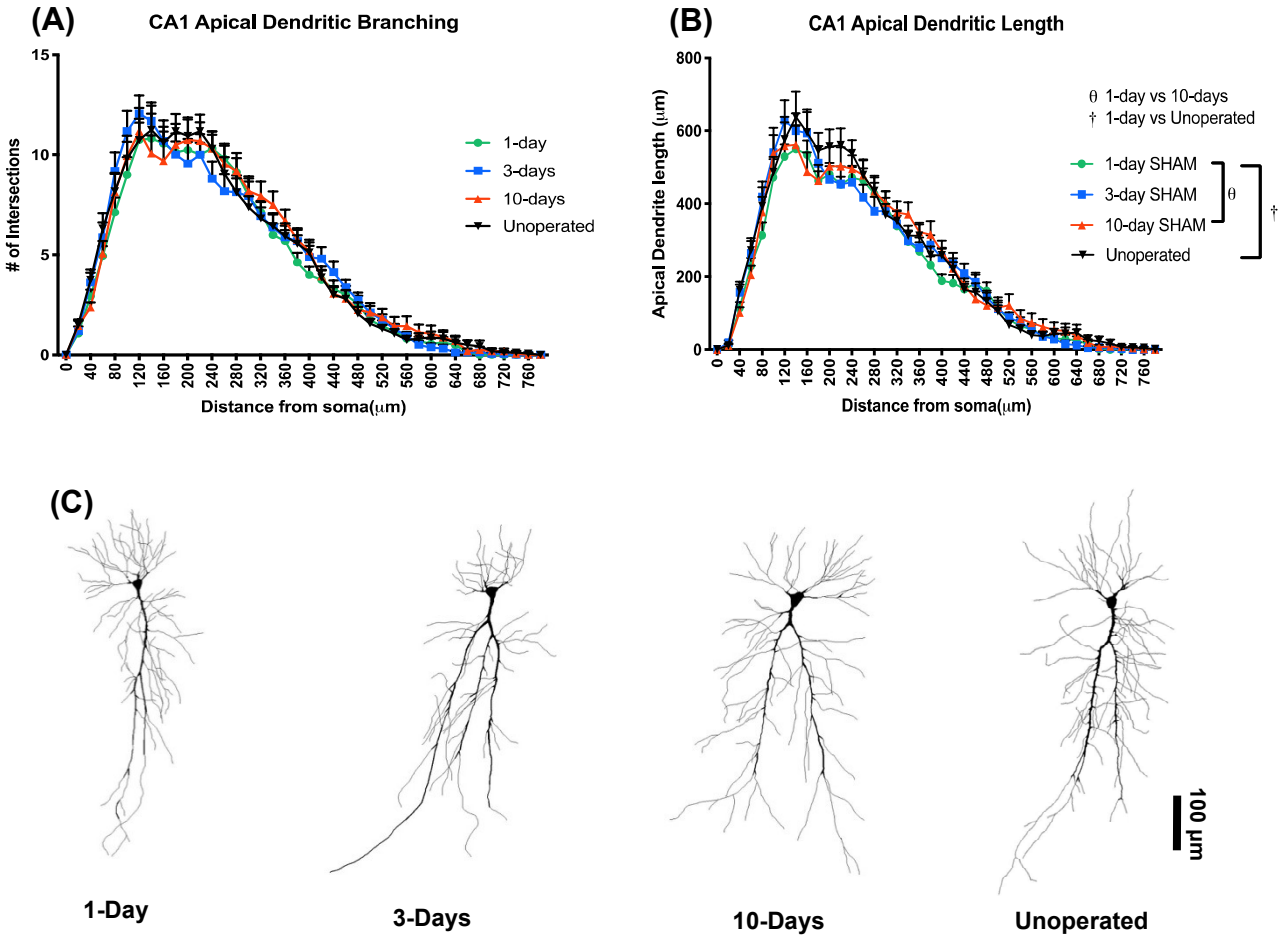

Figure 5.2: CA1 apical dendritic branching and dendritic length of sham-orchiectomized male rats using the abdominal surgical approach.

(A) No significant differences in CA1 apical dendritic branching 1-, 3- or 10-days following abdominal sham-orchiectomy compared to unoperated rats. **(B)** SHAM using the abdominal surgical approach caused minor treatment effects between 1-day and 10-days following surgery and 1-day and unoperated rats. **(C)** Representative CA1 tracings from abdominal SHAM 1-, 3- and 10-days following surgery and unoperated rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

5.3.3 Abdominal sham-orchidectomy has minimal effects on mPFC apical dendrites.

Similar to the effect of abdominal SHAM in the hippocampus, this surgical approach produced some effects on layer 2/3 apical dendrites however the effects appeared to be relatively minor compared to unoperated animals (See Figure 5.3A-C). Two-way ANOVA revealed a significant treatment (Treatment; $F (3, 1140) = 4.631$, $p=0.0032$) between 1-day and 10-day SHAM animals ($p=0.0170$) and 10-day and unoperated animals ($p=0.0218$). No significant interaction effect of apical dendritic branching. Apical dendritic length also had a significant effect of treatment (Treatment; $F (3, 1140) = 5.474$, $p=0.0010$) between 1-day and 10-day following SHAM animals ($p= 0.0189$), 3-days following SHAM and unoperated males ($p= 0.0310$), and 10-days following SHAM and unoperated males ($p= 0.0071$). No interaction effect was seen on apical dendritic length (Treatment*Sholl radius; $F (54, 1140) = 1.248$, $p=0.1107$).

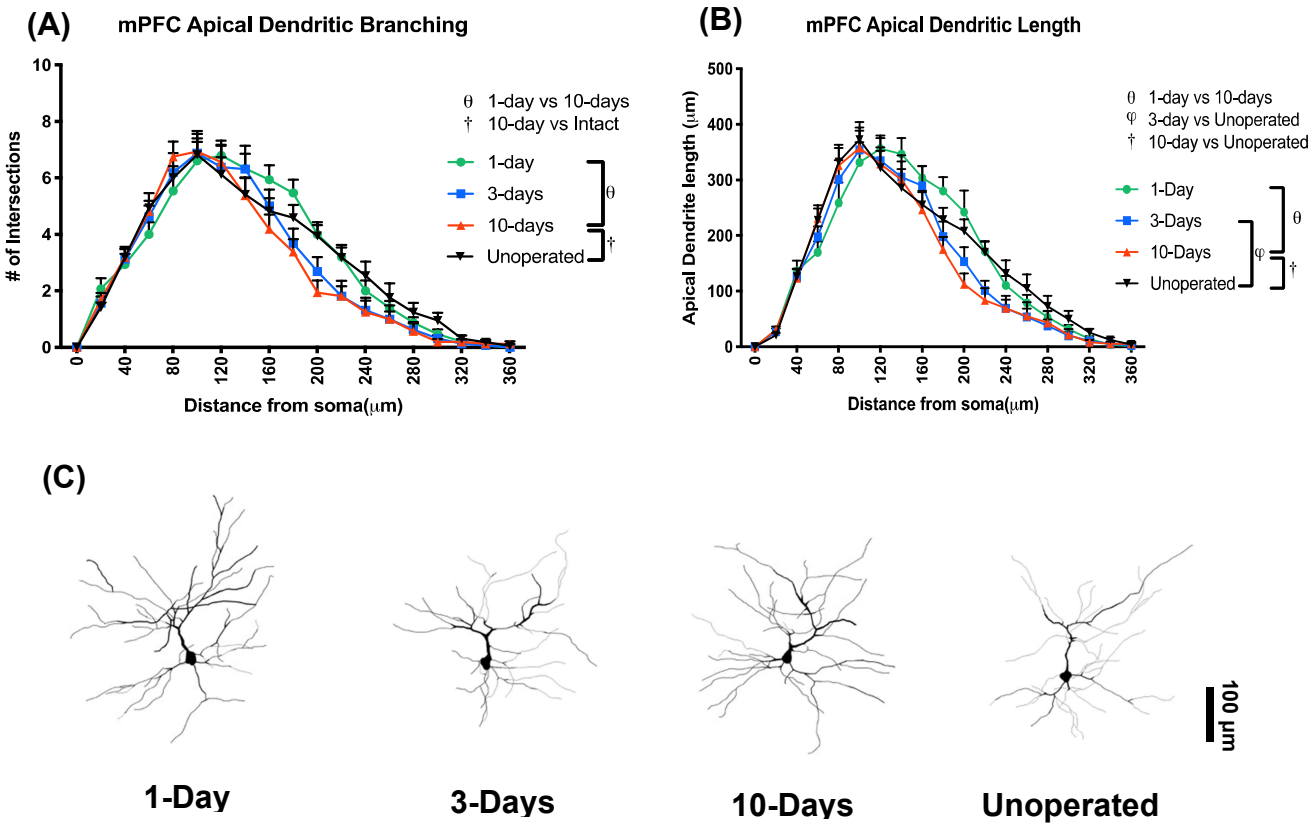

Figure 5.3: mPFC apical dendritic branching and dendritic length of sham-orchiectomized male rats using the abdominal surgical approach.

(A)SHAM rats 10-days following surgery displayed a decrease in apical dendritic branching **(B)** and apical dendritic length compared to unoperated rats and rats sacrificed 1-day following surgery. Rats sacrificed 3-days following surgery had a significant reduction in apical dendritic length compared to unoperated rats. **(C)** Representative CA1 tracings from abdominal SHAM 1-, 3- and 10-days following surgery and unoperated rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

5.3.4 Sham-orchidectomy through an abdominal approach decreases serum testosterone levels 1-day following surgery.

One-way ANOVA on ln transformed data revealed a significant treatment effect (Treatment; $F(3,12) = 4.335$, $p=0.075$). Tukey Kramer post-hoc test revealed that testosterone levels of animals 1-day following abdominal SHAM had a significant decreased in serum testosterone levels compared to unoperated adult male rats. No significant difference between 3- and 10-days following surgery compared to unoperated rats (see Figure 5.4).

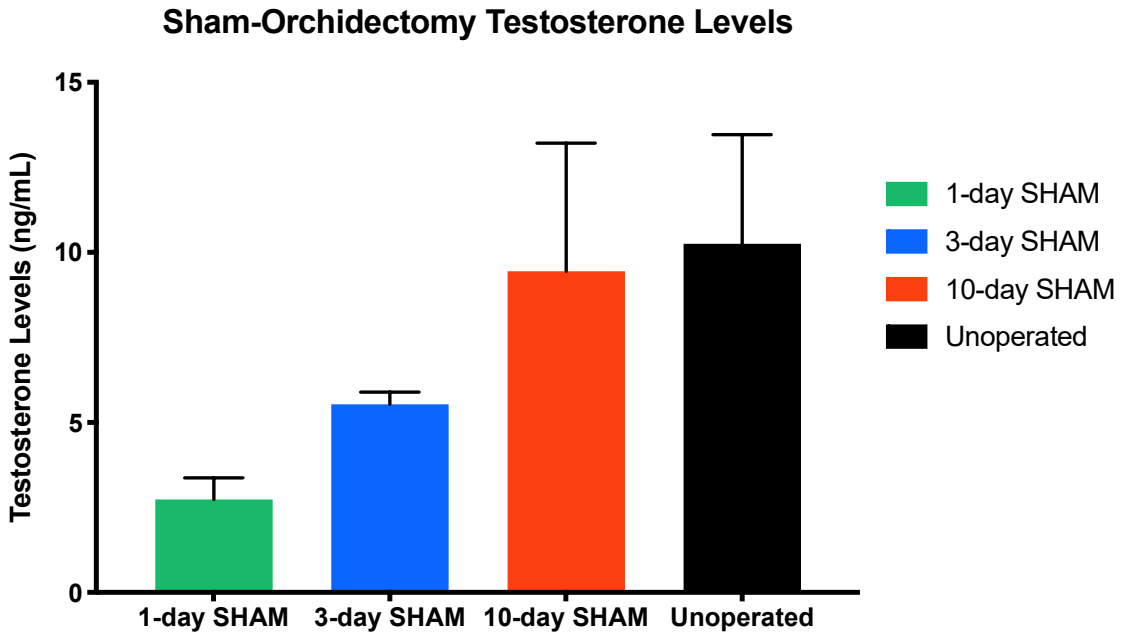

Figure 5.4: Serum testosterone levels of sham-orchiectomized male rats using the abdominal surgical approach.

Abdominal sham-orchidectomy significantly decreased serum testosterone levels 1-day following surgery. Testosterone levels gradually increased back up to levels of unoperated males as there was no significant difference between testosterone levels of rats sacrificed 3- and 10-days following surgery compared to unoperated rats. All data are represented as mean +/- SEM (n=4 rats/group). Significant data points represent $p<0.05$.

5.4 Discussion

The results obtained in the first three chapters of this thesis indicate that the effects of surgery and sustained elevated glucocorticoids leads to the dramatic remodelling of hippocampal and mPFC apical dendrites. However, the animals in chapter 2 were not handled prior to the surgery and experienced post-operative complications, therefore, several other factors that could have contributed to these effects. The environment and experience before surgery or stress can affect how the animals respond to the stressor. Stress prior to undergoing a surgical procedure has been shown to exacerbate the stress and inflammatory effects of surgery (Wang et al., 2017; Wang et al., 2016). However, handling animals prior to stress has shown to reduce anxiety-like behaviour and decreased HPA-axis reactivity (Nuñez et al., 2003). As the animals in the present experiment were handled for a week prior to surgery, it could explain why there were only minor effects of the abdominal SHAM on hippocampal and mPFC apical dendrites.

Post-operative complications also play a critical role in the rate of recovery following surgery. In humans, surgery can lead to post-operative pain, nausea and cognitive impairments that can increase recovery time (Kehlet & Dahl, 2003). Additionally, the standard way of performing an orchidectomy on rats is through a scrotal incision to expose the testes (Brown, 2008; Jenkins, 2000). However, due to the location of the incision, there is a greater risk of post-operative complications and infection as the incision is more likely to come in contact with bedding of the animal's cage (Brown, 2008). It has also been previously reported that the scrotal approach leads to a 33% risk

for post-operative complications compared to 0% when using the abdominal approach (Guilmette et al., 2015). This was seen in our experiments conducted at 10-days, 1-month and 2-months following surgery. All of the surgery performed on the animals in these experiments were using the scrotal approach which leads to many post-operative complications at each of these time-points. Many animals were placed on antibiotic treatment due to post-operative infections while others were monitored for swelling of the incision. The development of post-operative infections causing the activation of inflammatory responses may have detrimental effects on hippocampal and mPFC structural plasticity (Dantzer et al., 2008; Guilmette et al., 2015). Surgery-induced inflammation increases pro-inflammatory cytokines which can lead to decreased hippocampal BDNF and memory impairments (Hovens et al., 2014; Skvarc et al., 2018). Because the rats the 1- and 2-month experiment in chapter 2 displayed lasting changes to their hippocampal and mPFC apical dendritic structure particularly in the length of the apical dendrites post-operative inflammation may be playing a role in the dramatic remodelling of hippocampal and mPFC dendrites.

To determine how post-operative recovery plays a role in the response an abdominal surgical approach was used for performing sham-orchidectomy to reduce the duration of the recovery period following surgery. This method uses a small superficial incision in the abdominal skin and the abdominal muscle is simply spread without cutting to expose the testes (Guilmette et al., 2015; Jenkins, 2000). The abdominal approach for orchidectomy resulted in no post-operative complications or development

of infections. Additionally, the abdominal approach had minimal effects on hippocampal and mPFC apical dendritic structure. While there were slight changes in CA3, CA1 and mPFC apical dendritic branching and length, it did not cause the same atrophy and decrease in dendritic length that was observed 1- and 2- months following surgery.

These results indicate that handling the animals prior to surgery to reduce stress in combination with a good recovery period with no post-operative complications is critical for preventing the remodelling of hippocampal and mPFC apical dendrites. Therefore, the relationship between chronic inflammation, infections and prolonged elevation of glucocorticoids may lead to the dramatic remodelling of hippocampal and mPFC apical dendrites following surgery. Although the potential contribution of inflammation and the mechanisms of this hypothesis remain unclear, the results of this study indicate that improving the pre-operative treatment and a more careful surgical approach to reduce the duration of the post-operative recovery and risk for complications prevents major structural changes within the hippocampus and mPFC.

6 Conclusions and Future Directions

6.1 Summary of Findings

The results obtained in this thesis indicate that when an orchidectomy surgery using the scrotal approach is combined with the prolonged elevated glucocorticoids and post-operative complications there is a dramatic remodelling of hippocampal and layer 2/3 apical dendrites. When orchidectomy surgeries are conducted using the scrotal approach, there is a significant loss of the long CA3 apical dendrites and apical dendritic length throughout the dendritic tree of CA3, CA1 and mPFC neurons at both 1- and 2-months following surgery. However, it does not appear that the anesthesia used is producing a significant effect on the hippocampus and mPFC at 1- and 2-months following surgery as when animals anesthetized with isoflurane but did not undergo a surgical procedure, there were very minimal effects on apical dendritic structure in the hippocampus and mPFC.

Additionally, 16-hour dexamethasone treatment in unstressed male rats produced similar atrophy of hippocampal and mPFC apical dendrites to dendritic branching patterns observed 1- and 2-months following surgery. However, due to the half-life of dexamethasone in adult male rats, it most likely remains in the circulation for much longer than 16-hours. Therefore, because these effects of glucocorticoids were isolated from an overall stress response, we can conclude that the remodelling of apical dendrites seen 1-month and 2-months following surgery may be due to prolonged exposure to elevated glucocorticoids.

Moreover, the animals operated with the scrotal approach displayed post-operative complications that may have contributed to the remodelling of hippocampal and mPFC apical dendrites. Therefore, we reduced stress by handling the animals for a week before surgery and used an alternate surgical approach was used, therefore, reducing the risk of post-operative complications. This resulted in very little effect on CA3, CA1 and layer 2/3 apical dendrites. The abdominal orchidectomy approach eliminated the development of post-operative complications and prevent both the loss of distal CA3 apical dendrites as well as the loss of apical dendritic length in CA3, CA1 and layer 2/3. This suggests that reducing stress prior to surgery and eliminating post-operative complications, maximized the rate of recovery following surgery producing very minimal effects on the hippocampus and mPFC.

6.2 Potential Implications

The restructuring of hippocampal and mPFC dendrites often reflects changes in the connectivity of these two regions. The loss of the long and apical dendritic length of CA3 and CA1 apical dendrites indicates that the connections between hippocampus and mPFC may be impaired following surgery. The connections within the hippocampus and between the hippocampus and mPFC are crucial for maintaining normal cognitive function and proper memory formation (Naber et al., 2001; Nazari-Serenjeh et al., 2011). Therefore, impairments in the connectivity of these two areas may result in significant behavioural deficits.

Changes in the structure of hippocampal and mPFC apical dendrites may also have several clinical implications. Humans, particularly adult, undergoing a major surgical procedure are at risk for developing POCD (Skvarc et al., 2018). A potential mechanism underlying this condition could be the loss of apical dendritic branching and length and the connectivity within the hippocampus and mPFC. Furthermore, if the effects of surgery seen on hippocampal and mPFC dendrites at 1- and 2-month are due to a combination of prolonged elevated glucocorticoids and chronic inflammation during the recovery period. The combination of these factors may have implications for neuropsychiatric disorders. Inflammation has recently been implicated in many stress-related neuropsychiatric disorders such as depression, anxiety and PTSD (Calcia et al., 2016; Leonard, 2018; Liu et al., 2017). Therefore, it is possible that if chronic inflammation or infections and chronic stress are present in these disorders that the same remodelling of hippocampal and mPFC apical dendrites may occur. Therefore, the results from this thesis contribute to the understanding of the underlying mechanism of cognitive dysfunction and stress-related disorders.

6.3 Future Directions

Although the results from the series of studies in this thesis provide insight into the effects of surgery and post-operative recovery on the structure of neurons within the hippocampus and mPFC, it leads to many new unresolved questions that require further investigation. The experiment in chapter 3 revealed that dexamethasone results in the similar remodelling of hippocampal and mPFC as orchidectomy using a scrotal

approach. However, because the rats only had access to the dexamethasone water for 16-hours this may be underestimating the duration of elevated glucocorticoids during the recovery period following surgery. Therefore, conducting a similar dexamethasone experiment with a longer treatment time may provide more understanding of the effects of persistent glucocorticoid exposure of hippocampal and mPFC dendrites.

Additionally, the results of the two surgical approaches indicate that there is some part of the post-operative recovery period that leads to the dramatic remodelling seen following surgery using the scrotal approach. As previously mentioned, this may be due to chronic inflammation during post-surgical recovery. Therefore, to determine if inflammation is exacerbating the effects of surgery, sham-orchidectomy performed using the abdominal surgical method that does not cause post-operative infections in combination with an LPS injection to produce a controlled inflammatory response. This will provide critical information on the contribution of inflammation to the effects of post-operative recovery on hippocampal and mPFC dendritic morphology.

Therefore, although the results obtained in this thesis open up several questions that remain unknown, it does provide novel information on the response of hippocampal and mPFC dendritic morphology following surgery and what potential factors of the surgical procedure and post-operative recovery period contribute to the changes in the hippocampus and mPFC.

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