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Sex Differences in Stroke Recovery: Synaptic Proteins and the Growth Inhibitory Protein Nogo a

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SEX DIFFERENCES IN STROKE RECOVERY:

SYNAPTIC PROTEINS AND THE GROWTH INHIBITORY PROTEIN NOGO-A

IN THE AGED RAT

A DISSERTATION SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL IN

CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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BY

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LIST OF ABBREVIATIONS

11C7	Anti-Nogo-A IgG Antibody (anti-amino Nogo)
17beta-HSD	17beta-hydroxysteroid dehydrogenase
AAV	Adeno-Associated Virus
AKT/PKB	Protein Kinase B
ALS	Amyotrophic Lateral Sclerosis
AMP	Adenosine Monophosphate
AMPAR	Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid Receptor
BCL-2	B-cell Lymphoma 2
BDA	Biotinylated Dextran Amine
BDNF	Brain-Derived Neurotrophic Factor
BrdU	Bromodeoxyuridine
cAMP	Cyclic Adenosine Monophosphate
CCAO	Common Carotid Artery Occlusion
CNS	Central Nervous System
CREB	Cyclic-AMP Response Element Binding Protein
DAPI	4',6-Diamidino-2-Phenylindole
E2	Estradiol
EGFP	Expressed Green Fluorescent Protein
ELISA	Enzyme-linked Immunosorbent Assay
ER-alpha	Estrogen Receptor Alpha
ER-beta	Estrogen Receptor Beta
GAP-43	Growth-Associated Protein 43
GFP	Green Fluorescent Protein
GPER	G-Protein-coupled Estrogen Receptor

HRT	Hormone Replacement Therapy
ICV	Intracerebroventricular
IGF-1	Insulin-like Growth Factor 1
IHC	Immunohistochemistry
LTD	Long Term Depression
LTP	Long Term Potentiation
MAG	Myelin-Associated Glycoprotein
MCAO	Middle Cerebral Artery Occlusion
MLCP	Myosin Light Chain Phosphatase
mPR	Membrane Progesterone Receptor
NgR	Nogo-66 Receptor
NMDAR	N-Methyl-D-Aspartate Receptor
NMRI	Nuclear Magnetic Resonance Imaging
OMgp	Oligodendrocyte-Myelin Glycoprotein
OVX	Ovariectomy
PI3K	Phosphoinositide 3-Kinase
PirB	Paired Immunoglobulin-Like Receptor B
PKA	Protein Kinase A
PR	Progesterone Receptor
PSD95	Post-Synaptic Density 95
rCBF	Regional Cerebral Blood Flow
ROCK	Rho-associated, Coiled-coil containing protein Kinase
S1PR	Sphingosine-1-Phosphate Receptor
tPA	Tissue-type Plasminogen Activator
TIA	Transient Ischemic Attack
TrkB	Tropomyosin-related kinase B

TTC	2,3,5-Triphenyl-2H-Tetrazolium Chloride
VA/VL	Ventral Anterior/Ventral Lateral Nucleus of the Thalamus
VCD	4-Vinylcyclohexene Diepoxide
VGLUT	Vesicular Glutamate Transporter
WHI	Women's Health Initiative

ABSTRACT

Ischemic stroke is a major world-wide health problem, resulting in death and disability especially in the older population. A sex difference exists in functional recovery post-stroke, with post-menopausal women having worse functional outcome as compared to age-matched men. Although the mechanisms underlying this sex difference are not entirely clear, it is recommended that any potential therapy for stroke recovery be tested in pre-clinical models including both male and females in order to determine the efficacy of the proposed treatment on the entire population. We have reported a novel therapy to enhance brain plasticity and improve functional recovery after stroke in aged male rats by neutralizing the neurite inhibitory protein Nogo-A. We now propose to determine the efficacy of this treatment on synaptic proteins important for neural connectivity and functional recovery. We hypothesized that post-stroke sensorimotor recovery is worse in the aged female rat due to a decrease in synaptic proteins that are necessary for neuronal connectivity and this can be improved with anti-Nogo-A immunotherapy. We tested this hypothesis in the following specific aims. Specific Aim #1 determined sex differences in synaptic proteins in aged male and female rats post-stroke in the contralesional forelimb motor cortex using viral vectors to label cortical pyramidal neurons and high resolution confocal microscopy to examine synaptic proteins. Stroke was found to decrease excitatory synapse number, except in OVX females, who were already at a low number. Specific Aim #2 determined sex differences in aged male and female rats in synaptic plasticity that have been treated post-stroke with anti-Nogo-A immunotherapy and examined three weeks later for synaptic protein changes. The overall goal of this aim was to determine if anti-Nogo-A immunotherapy is useful in improving post-stroke synaptic changes in aged rats, thereby improving our

understanding and therapeutic approach to this devastating condition. It was shown that anti-Nogo-A immunotherapy increased excitatory number in males and intact females, but not OVX females. Specific Aim #3 determined sex differences in aged male and female rats post-stroke using behavioral tests of skilled sensorimotor function and examined dendritic profiles of pyramidal layer V motor neurons using Golgi-Cox stain. It was shown that while males learned the forelimb reaching task slower than both female groups, they had a better post-stroke recovery. Females had the largest decrease in dendritic complexity (pre-stroke they had the highest complexity). In conclusion, it has been shown that there is a sex difference in terms of excitatory synapse number following stroke and a sex difference with regard to post-stroke recovery. Anti-Nogo-A immunotherapy was shown to have a sex steroid-dependent effect and this could have far-reaching implications for post-menopausal women receiving the therapy.

CHAPTER ONE

OVERVIEW AND HYPOTHESIS

Stroke is a debilitating disorder that affects millions worldwide each year in both deaths and disability (Bushnell et al., 2014). The risk for stroke increases with age, as does the possibility of lasting neurologic damage (Bushnell et al., 2014). Stroke severity is also dependent on sex, as post-menopausal women have worse functional outcome post-stroke compared to age-matched men (Bushnell 2006; Bushnell 2008). With menopause being the turning point for functional recovery following stroke (Bailey et al., 2011; Bartoli et al., 2013), the mechanisms underlying stroke recovery (and its resulting sex differences) are paramount to uncover. Due to the large number of stroke victims, the increased risk of stroke with age, and the sex differences involved in stroke recovery, an in-depth study into those differences and underlying brain plasticity is warranted.

While treatments for stroke are rare, a novel therapeutic is anti-Nogo-A immunotherapy which is directed against Nogo-A, a myelin-associated growth inhibitor of both axons and dendrites (Schwab et al., 2014). It has been shown in our lab that anti-Nogo-A immunotherapy following focal cortical stroke resulted in improved performance in the skilled forelimb reaching task (Papadapoulos et al., 2002; Markus et al., 2005; Tsai et al., 2011). This research found a correlation between axonal and dendritic plasticity in the contralesional cortex with functional recovery following stroke. The contralesional cortex is considered an area of therapeutic value because new connections will be sent to the damaged hemisphere through the corpus collosum from there. While this past research has focused on male rats, experiments with female rats

are recommended now by the National Institutes of Health in order to better meet the appropriate experimental model.

Synaptic changes have also not been fully investigated, especially with regard to cortical connections to the area damaged by a middle cerebral artery occlusion (e.g. the forelimb motor cortex). These excitatory synaptic connections in the motor cortex play a role in motor movement for the entire body and as such are crucial to recovery of movement after stroke.

HYPOTHESIS

Post-stroke sensorimotor recovery is worse in the aged female rat due to a decrease in synaptic proteins in the contralesional motor cortex and this synaptic number can be improved with anti-Nogo-A immunotherapy

Specific Aim 1: To determine sex differences in synaptic proteins in aged male and female rats post-stroke in layer V pyramidal neurons in the contralesional forelimb sensorimotor cortex.

Rats will be divided into the following groups: aged males, aged intact females, and aged OVX females. At time of MCAO stroke, rats will be injected with AAV2/8-CMV-GFP-LDL-RCT in their contralesional motor cortex to label layer V pyramidal neurons. After 3 and 8 weeks, brains will be processed by immunohistochemistry for co-localization of NMDAR-NR1, VGLUT1/2 and pre-labeled viral AAV2/8-CMV-GFP-LDL-RCT neurons (on the dendrite), and synaptic counts will be quantified using confocal imaging.

Specific Aim 2: To determine sex differences in aged male and female rats in synaptic proteins in layer V pyramidal neurons in the contralesional forelimb motor cortex that have been treated post-stroke with anti-Nogo-A immunotherapy.
Rats will be divided into the following groups: aged males, aged intact females, and aged OVX females. At time of MCAO stroke, rats will be injected with AAV2/8-CMV-GFP-LDL-RCT in their contralesional motor cortex to label layer V pyramidal neurons. Rats will receive either anti-Nogo-A immunotherapy (11C7) or control antibody by intracerebroventricular (ICV) mini-osmotic pump into the lateral cerebral ventricle. Rats will be sacrificed at 3 weeks post-stroke for synaptic protein analysis, as in Aim 1.

Specific Aim 3: To determine sex differences in aged male and female rats post-stroke using behavioral tests of skilled sensorimotor function.
Pre-stroke, rats will be trained to perform the skilled forelimb reaching task and the skilled ladder rung walk task. They then will undergo middle cerebral artery occlusion (MCAO) on the side corresponding to the preferred forelimb and then will be tested for 8 weeks post-stroke on these skilled sensorimotor tasks.

CHAPTER TWO

REVIEW OF LITERATURE

STROKE

Stroke is a common and debilitating disorder resulting in 140 million US deaths and many more disabilities each year (Bushnell et al., 2014). A stroke can be one of several types. Ischemic is the most common (approximately 79%) and occurs when a blood vessel supplying an area of the brain is blocked or impeded, depriving that brain region of oxygenated blood (Egido and de Lecinana, 2007; Lang and McCullough, 2008). Hemorrhagic stroke is the less common of stroke variants. Hemorrhagic stroke occurs when the brain is deprived of blood through an occluded artery in which the occluded area becomes filled with blood from necrotic and degenerating vessels (Moore and Daley, 1999). A direct hemorrhage can also occur such as under the arachnoid [4] meningeal layer (subarachnoid hemorrhage) (Moore and Daley, 1999; Buma et al., 2013).

The middle cerebral artery (MCA) plays a crucial role in supplying the cerebrum (parietal, frontal and temporal lobes) (Moore and Daley, 1999) (Figure 1) with oxygenated blood. The MCA has a branch on both sides of the brain and arises from the [3] internal carotid and continues into the lateral sulcus (Moore and Daley, 1999; Buitrago et al., 2004). It is also connected to the anterior cerebral arteries and posterior communicating arteries while not directly being a part of the Circle of Willis (Moore and Daley, 1999). Occlusion of the MCA can have dramatic effects (depending on what part of the brain is occluded), such as weakness or paralysis of the contralateral face, arm, and leg, sensory loss of the contralateral face, arm, and leg, as well as aphasia, neglect

syndrome, and depression (Bradnam et al., 2013; Buma et al., 2013; Cheng et al., 2013). This loss of brain tissue has an impact on the outcome of the patient depending on what area of the brain was damaged, such as limb strength and sensation being affected by the opposite side middle cerebral artery occlusion (Kamal and Iadecola, 2012; Taylor and Sansing, 2013; Garcia-Bonilla et al., 2014).
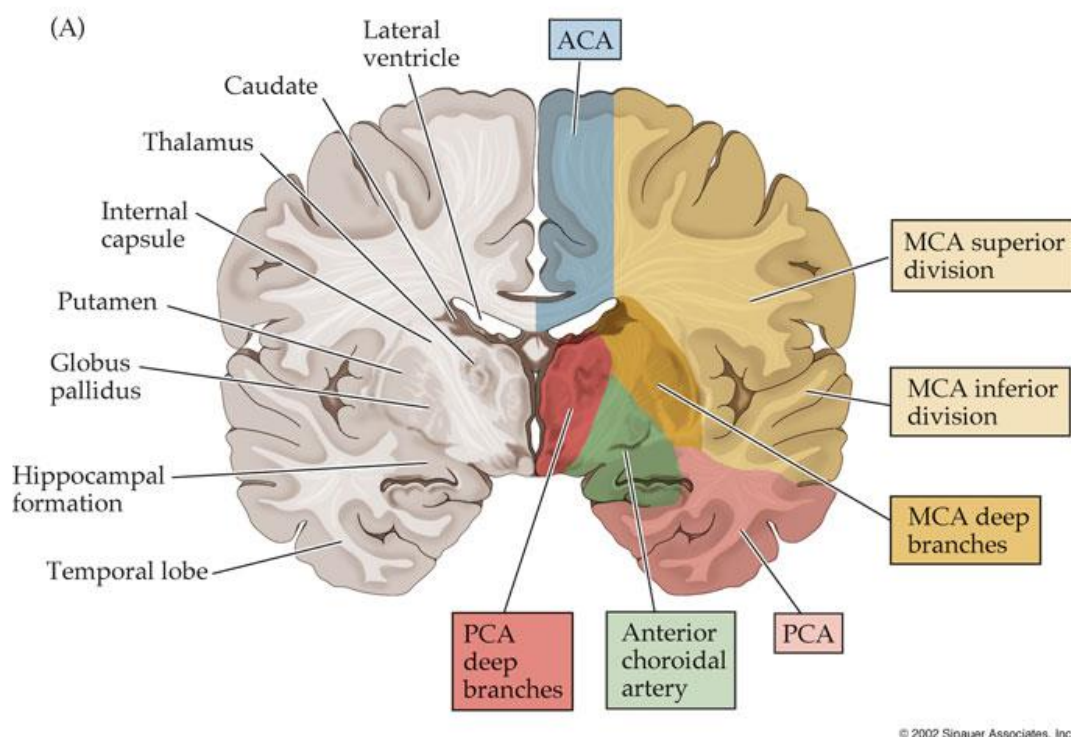
Figure 1. Brain Artery Perfusion Areas in the Human. Of note is the perfusion of the motor cortex by the middle cerebral artery, along with major fiber pathways. From Blumenfield Anatomy, 2002. ACA: Anterior Cerebral Artery; MCA: Middle Cerebral Artery; PCA: Posterior Cerebral Artery.

No matter the cause, a stroke results in damaged neural tissue due to toxin build-up and apoptosis and necrosis, along with oxidative stress and mitochondrial dysfunction (Kamal and Iadecola, 2012; Guesuete et al., 2014; Petrone et al., 2014) leading to cell death and immune responses. Apoptosis (Figure 2) in that area begins quickly as energy is depleted and wastes build up (Kamal and Iadecola, 2012; Guesuete et al., 2014). During a stroke the immune system also plays a role, with invasion by T cells and activated microglia (Brait et al., 2012; Xu et al., 2013) releasing inflammatory cytokines (Lambertsen et al., 2012; Shichita et al., 2012) and pro-apoptotic signals (Kamal and Iadecola, 2012; Taylor and Sansing, 2013; Garcia-Bonilla et al., 2014) with activation of microglia, and invasion of monocytes and leukocytes (Petrone et al., 2014).

Tissue plasminogen activator (tPA) is currently the only approved post-stroke pharmaceutical treatment, but must be administered in the early stage and carries risks of worsening of the stroke due to hemorrhaging (Moore and Daley, 1999). Many people are not eligible for this treatment since they will not be admitted to a hospital in that short time window and only seven percent of stroke patients receive it (Del Zoppo et al., 2009). Rehabilitation currently remains the main post-stroke treatment, with focuses on regaining lost ability and possibly stimulating new brain connections to either repair or compensate for damaged tissue (Wahl and Schwab; 2014; Wahl et al., 2014).

INTRINSIC APOPTOTIC PATHWAY
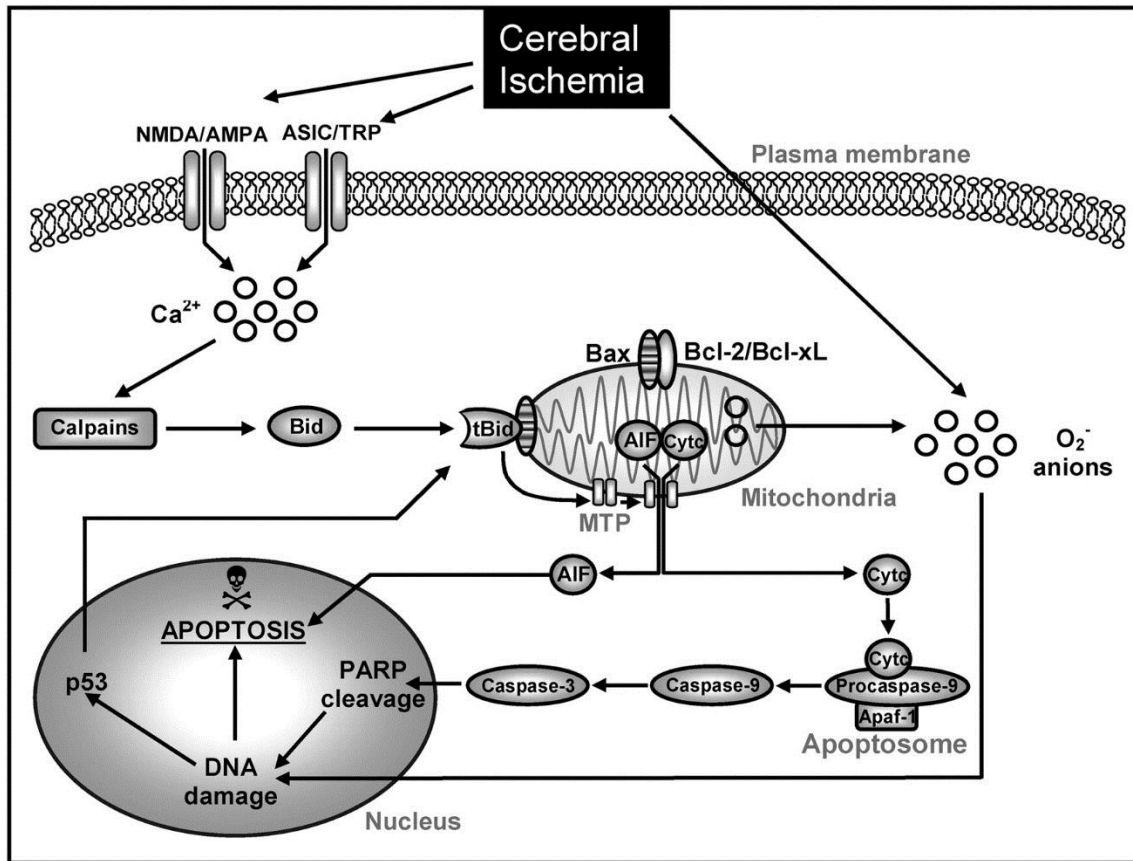
Figure 2. Apoptotic Pathways. Shown here are the cellular pathways starting at ischemic damage (lack of oxygen, build-up of toxins, excitotoxicity) and leading to apoptosis through oxidative damage and free radicals as well as apoptosome formation. Ischemia results in increased extracellular glutamate due to release and lack of reuptake, causing NMDA receptors to let in calcium. This increased intracellular calcium can activate calpains which in turn activate Bid. Bid binds to the mitochondrial membrane and allows Cytochrome C to exit (CytC) which activates caspases and apoptosis. From Broughton et al., 2009. PARP: Poly-ADP Ribose Polymerase.

Stroke has been shown to increase in both risk and mortality with increase in age (Brown et al., 2003; Merret et al., 2010), but the underlying mechanisms have yet to be elucidated. The increased risk in stroke due to age has been postulated to be due to vascular changes that deteriorate over time and are dependent on the health of the individual (Bayer and Hausman, 2011). Other factors such as increased vessel plaque, atherosclerosis, and increased risk for embolisms are also postulated. It has been shown in mice that age results in decreased white matter and increased damage following stroke (Suenaga et al., 2015). Of particular interest to this study is the change in sex hormones and receptors, seen both in female (Cai et al., 2014) and male (Wu and Gore, 2010) animal models that occur with aging. Aging increases stroke damage and affects the sexes differently (Liu and McCullough, 2012). An older (post-menopausal) woman affected by stroke is at an increased risk of death and disability following stroke (Bushnell, 2006; Bushnell, 2008).

NEUROLOGICAL SEX DIFFERENCES IN RODENTS AND HUMANS

There are many neurological sex differences, especially in the human brain. These differences could be used to explain the differences in stroke risk and recovery. Of particular note is the size, with males of both human and rat brains having a larger weight, although it is proportional to increased body weight of males in both species (Zaidi, 2010). Men have been found to have more gray matter, while women have more white matter (Zaidi, 2010). Of interest to this study is the corpus callosum, which is larger in women and does not change much with age (Ardekani et al., 2012). Astrocytes are in a higher number in females and react to damage differently (Chisholm and Sohrabji, 2015) with estrogen decreasing the astroglial scar (Chisholm and Sohrabji, 2015). Also of interest is that Broca's and Wernicke's Areas are significantly larger in women (Zaidi,

2010). While these anatomical differences have not been studied in detail in the rat brain, cellular mechanisms have been better studied.

There are sex differences in regard to cellular mechanisms. Males have higher brain estrogen receptor concentrations (Ritzel et al., 2013). The G-protein-coupled estrogen receptor (GPER) is upregulated in male rats more than females following ischemia (Broughton et al., 2013a, Broughton et al., 2013b). Females also have decreased inflammation compared to males (Manwani et al., 2013), especially in regards to ischemic inflammation (Ritzel et al., 2013; Zuo et al., 2013). Apoptosis has been shown to be decreased with increased estradiol concentrations (Lang and McCullough, 2008). Estradiol has also been shown to facilitate synthesis of progesterone in astrocytes (Wong et al., 2013) and astrocytes express the progesterone receptor (Wong et al., 2013). Estradiol and progesterone have been shown to decrease astrocyte proliferation following injury, even in aged animals (Wong et al., 2013). The circulating levels of progesterone that rise during the rat estrous cycle and the human menstrual cycle could also have neuroprotective effects similar to estradiol. While male rats and human men also have detectable progesterone levels (Amador et al., 1985), it is not as high a level as their female counterparts (MacBeth et al., 2007; Acosta et al., 2009). The sex differences in levels of estradiol, progesterone, and other sex hormones contributes to a different response to aging, injury and recovery. Mechanisms for these aforementioned hormones will be discussed in detail later.

Also of importance is that many psychological disorders have a sex bias to them, with women having increased risk for major depression, borderline personality disorder, and generalized anxiety disorder (Loke et al., 2015) all of which depend on brain dysfunction. Some psychological disorders have no sex bias to them, such as obsessive

compulsive disorder (Loke et al., 2015) while others have similar rates yet different timing (e.g. women typically develop schizophrenia earlier than men) (Loke et al., 2015).

SEX DIFFERENCES IN ISCHEMIC STROKE AND RECOVERY

Stroke is a leading cause of death and disability worldwide, especially in the aged population (Alkayed et al., 1999; Lewis et al., 2012), where mortality is different in males (5th leading cause of death) and females (3rd leading cause of death) (Bushnell et al., 2014). Incidence of stroke is also higher in aged females than males (Bushnell et al., 2014). Recovery post-stroke also differs between the sexes with post-menopausal women recovering worse than age-matched men (Bushnell 2008; Liu et al., 2009; Kim et al., 2010; Bushnell et al., 2014). Such trends will rise considering more people are expected to live longer, with women now living a larger portion of their lives post-menopausal (Manwani et al., 2013). Risk of death and worse recovery from stroke in females rises after menopause and may be related to the reduction in estrogens as they are thought to be neuroprotective (Miller et al., 2005; Dang et al., 2011; Liu et al., 2009; Soderstrom et al., 2009).

Based on these findings in humans, it has been proposed that female rats be used when studying recovery from stroke in addition to male animals, as it is believed that sex differences could exist. In fact, the National Institutes of Health (NIH) has strongly suggested that age-appropriate female animals be included when studying stroke recovery or therapeutic agents to improve recovery. Earlier research has shown that a sex difference does indeed exist when studying animal models of cerebrovascular disease (Wu et al., 2005; Zuo et al., 2013). Of particular interest to this study are the sex differences in sex hormone receptors, such as the G-protein-coupled estrogen receptor

(GPER). This receptor has been shown to have different effects in males and females (Broughton et al., 2013b). It is also more upregulated in male rats following stroke (Broughton et al., 2013a). The presence (and lack thereof following menopause) of estradiol has been shown to affect hippocampal aging (Han et al., 2013), infarct damage (McEwen and Alves, 1999; Garcia-Segura et al., 2001; Manwani and McCullough, 2012), inflammation (Petrone et al., 2014) and the insulin-like growth factor 1 (IGF-1) receptor and signaling cascade (Sohrabji, 2014). The last is of interest due to the interplay of estrogen's modulation of IGF-1's Akt up-regulation and decrease in apoptosis (Sohrabji, 2014). These examples illustrate the necessity of a female model to examine potential sex differences in stroke recovery and other studies.

There are psycho-social reasons why stroke recovery could be worse in females. Although not proven, it is suggested that of the reasons why aged women do not recover as well as aged men is that there exists decreased social links with aged women and social support (Bushnell, 2006; Bushnell, 2008). There is also an increased risk of needing longer hospital stay time and mental institutionalization, all of which have a bearing on, and are a barrier to, recovery (Bushnell, 2006; Bushnell, 2008; Bushnell et al., 2014). It is also proposed that women may live longer than their social links and outlive them, resulting in a diminished set of social links and support (Persky et al., 2010). Better recovery could occur with increased social support, such as through joining an exercise group (Persky et al., 2010; Bushnell et al., 2014). All these factors can contribute to the apparent sex difference in stroke recovery.

NEURONAL CONNECTIVITY, SYNAPTIC PROTEINS, AND SEX STEROIDS

Although many proteins are responsible for synaptic connectivity (Baudry et al., 2013; MacLusky, 2013; Kramer et al., 2013), of particular note are the NMDA receptors, as they are responsible for spine growth and signal transduction (Minano et al., 2008). NMDA receptors are heterotetramer channel proteins activated by glutamtate, D-serine, and glycine (Minano et al., 2008). Agonist binding along with membrane depolarization from AMPA receptors (which removes a magnesium plug) allows for NMDA receptors to open and let in sodium and calcium, depolarizing the post-synaptic cell (Minano et al., 2008). NMDA receptors are the post-synaptic components of most CNS excitatory signaling (Minano et al., 2008; Kaneko, 2013). The subunits of the heterotetrameric NMDA receptor include NR1, necessary to bind glutamate (Minano et al., 2008; Kaneko, 2013), along with NR2 and NR3. NR1 is included in every functional NMDA receptor due to the fact that glutamate does not bind without it (Minano et al., 2008).

Also of note is the post-synaptic density 95 (PSD95) protein, found in dendrites and spines which serves to anchor receptors to the post-synaptic membrane (Minano et al., 2008; Baudry et al., 2013) (Figure 3). Loss of these or other synaptic proteins leads to a decrease in neuronal connectivity and communication and can lead to loss or diminished function in associated brain regions. Estradiol has been shown to increase protein expression of the synaptic proteins PSD95 and NMDA-NR1 in young animal models (Norbury et al., 2003; Baudry et al., 2013), but has yet to be examined in aged female rats or after stroke in the aged female rat. Estradiol also increases expression of cytoskeletal proteins (Norbury et al., 2003; Baudry et al., 2013), which play a role in dendritic and spine formation (Hao et al., 2006; Sato et al. 2007). Estradiol has also been shown to promote long term potentiation and spine maturation by increasing the

number of AMPA receptors present on spines and by promoting actin polymerization

(Kramer et al., 2013).

The vesicular glutamate transporter proteins (VGLUT) are of importance in the

vesicular storage and reuptake of glutamate (through excitatory amino acid transporter),

as this is the main way of terminating its (usually) excitatory signaling (Baudry et al.,

2013; MacLusky, 2013; Kramer et al., 2013) (Figure 4). The subtypes of VGLUT include

VGLUT 1 and 2, which have been found to be localized to the pre-synaptic membranes

of cortico-cortical and thalamo-cortical connections, respectively (Kaneko, 2013). They

are also found in astrocytes. Other subtypes of VGLUT can be found in astrocytes and

other neurons (Kaneko, 2013), but are also located near the synapse.
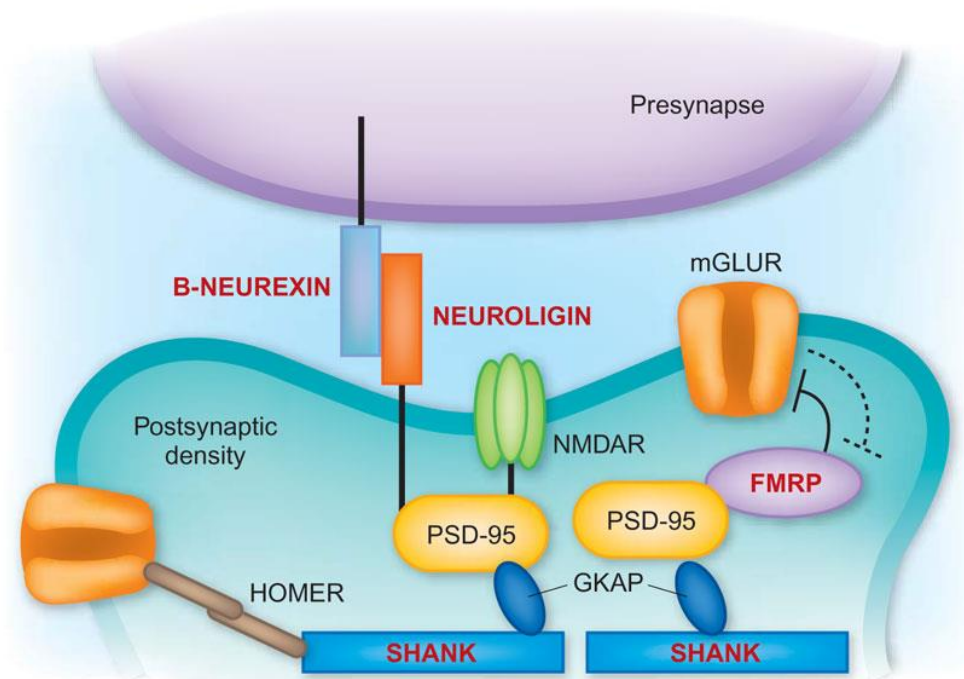
Figure 3. Post-synaptic Density 95 Anchors the NMDAR. PSD95 is shown here
anchoring the NMDAR to the post-synaptic membrane. mGLUR = metabotropic
glutamate receptor; GKAP = guanylate kinase-associated protein; FMRP = fragile X
mental retardation protein. From State, 2010.
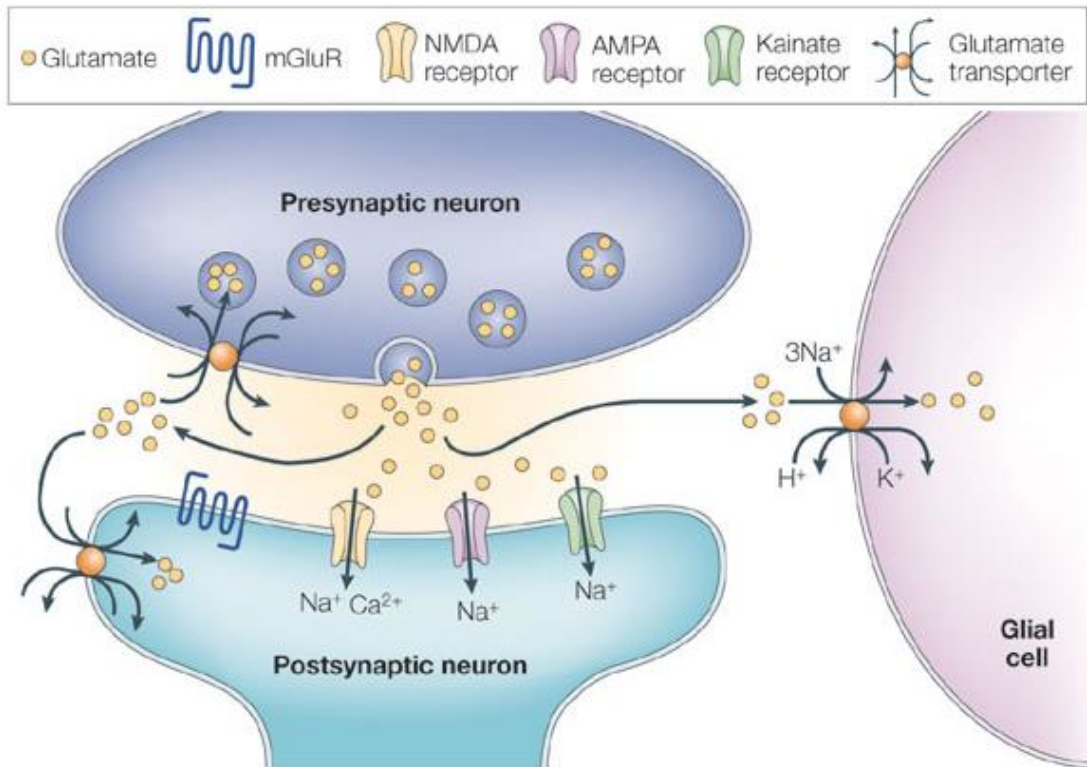
Figure 4. Glutamate in the Synapse. NMDAR as well as other glutamate receptors receive neurotransmitters released from the pre-synaptic cell while glutamate is taken back up by a glutamate transporter and is packaged into vesicles using vesicular glutamate transporter. From Atwell and Gibb, 2005.

Spine and dendrite morphology have been shown to play a key role in learning and behavioral function (Phan et al., 2012; Brocca et al., 2013). Estradiol has been shown to increase spine number (Foy et al., 2008) as well as increase long-term potentiation and decrease long-term depression involved with those spines (Foy et al., 2008; Kramar et al., 2013). Estradiol has been shown to trigger actin polymerization in spines (Kramar et al., 2013). It is also of note that long-term depression occurs more in aged rodents, which is further exacerbated by stress (Foy et al., 2008). Estradiol shares its synapse-inducing effects along with progesterone (Rivera and Bethea, 2012). Several reports have also pointed to estradiol's ability to either induce or synergize synapse function and growth alongside neurotrophins (Schnell et al., 1994; Scharfman and MacLusky, 2006; Srivasta et al., 2013). Synapses are also sensitive to growth inhibitory factors (discussed in detail later) with Nogo-A as an identified antagonist (Xiao et al., 2012; Ritzel et al., 2013; Zemmar et al., 2014). There are numerous reports of decreases in spine density following ovariectomy, especially in the hippocampus and pre-frontal cortex (Velazquez-Zamora et al., 2012).

While spines have been shown to have the potential to be transient (Holtmaat et al., 2005) (Figure 5), there are many that are more permanent (Holtmaat et al., 2005). Spines are subject to pruning or strengthening based on experience and learning (Holtmaat et al., 2006; Holtmaat and Svoboda, 2009). Estrogen has been shown to be a modifier of spine growth and stability (Foy et al., 2008), especially due to the synaptic proteins that it up-regulates (Norbury et al., 2003; Baudry et al., 2013) as well as maintaining mature spines longer (Kramar et al., 2013).
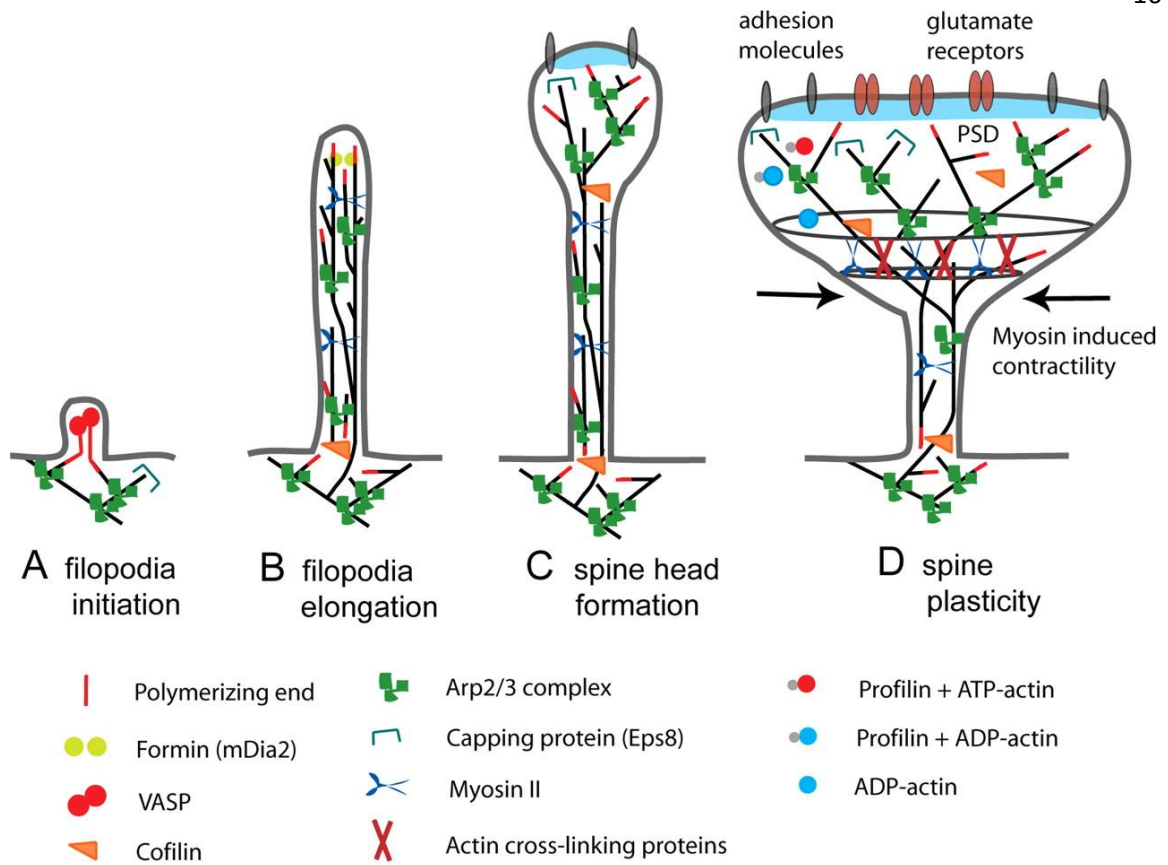
Figure 5. Dendritic Spine Plasticity. Shown here is the spine formation and actin dynamics required for it. From Hotulainen and Hoogenraad, 2010.

Estradiol is considered the most potent of the estrogens, being able to bind to the three main estrogen receptors with equal affinity (estrogen receptor alpha (ERA), estrogen receptor beta (ERB) (Morissette et al., 2008) and the G-protein-coupled estrogen receptor (GPER, formally known as GPR30) (McAllister et al., 2012; Schreihofer and Ma, 2013; Arevalo et al., 2014; Lamprecht and Morrison, 2014). It is also the main hormone released from the ovaries during the ovulatory cycle and has a large decrease in production during menopause (Wise and Dubal, 2000; Cheskis et al., 2007). In the rat model, menopause as seen in humans does not take place, but a type of 'reproductive senescence' occurs after approximately one year of age in which sex steroids no longer cycle but instead exist at a low basal level and fertilization is uncommon (Baeza et al., 2010). The other estrogens, estrone and estriol, do not bind as well as estradiol to the estrogen receptors and are present in higher concentrations during menopause (McClure et al., 2013).

Two mechanisms of estrogen signaling are the 'fast' versus 'slow' routes of action. Slow routes include activation of transcription or transcription (through binding directly to the DNA's estrogen response element (ERE)) of transcription factors. The GPER pathway calls for a faster mode of action, in which cellular events occur more rapidly than through transcription (Srivastava and Penzes, 2011; Srivastava et al., 2011). This utilizes the phosphoinositide 3-kinase activation by a membrane-bound estrogen receptor (Wu et al., 2005) that leads to activation of Bcl-2 and inhibition of apoptosis (Wu et al., 2005). Much of this research into a non-genomic route of action has focused on the newest of the estrogen receptors, GPER (formally GPR30 from its orphan receptor naming) (Hazell et al., 2009). GPER is activated by estradiol, causing activation of the Gαs subunit and activation of adenylyl cyclase with an increase in cAMP for quick

activation of protein kinase A (PKA) (Hazell et al., 2009). The general consensus seems to point to a combination of slow and fast mechanisms (Kramar et al., 2013), with a further postulation that estradiol may transactivate the TrkB receptor and promote spine growth through BDNF signaling (Kramar et al., 2013). BDNF also uses the Rho GTPase signaling pathway (Kramer et al., 2013) and depending on the cellular location this may cause an intersecting of estradiol, BDNF, and Nogo signaling (as described in more detail below). Estradiol has also been shown to upregulate aquaporin 4 levels leading to reduced edema following stroke (Shin et al., 2011). It has been shown that stroke itself does not affect measureable serum levels of estradiol (Shin et al., 2011) (Figure 8).

Although there is an increased risk for cancer (such as breast or ovary) with exogenous estradiol treatment (Petrone et al., 2014), the use of selective estrogen receptor modulators has grown. Some only activate ER-beta and are less likely to cause cancer (Petrone et al., 2014). Further clinical trials are needed, however, for their approved treatment usage. Timing is also an important issue in hormone treatments.

Of particular note is the ability of neurons and glia cells to produce sex steroids de novo from cholesterol, creating 'neurosteroids' (Raz et al., 2008; Wise et al., 2009; Rettberg et al., 2014). It has been shown that both neurons and astrocytes express aromatase as well as the estrogen receptors (Wise et al., 2009; Azcoitia et al., 2011; Brann et al., 2012) (Figure 6). ER alpha and beta are expressed by most cells, with ER beta having the higher expression in the motor cortex and hippocampus (Wise et al., 2009; Azcoitia et al., 2011; Brann et al., 2012), though ER alpha has been shown to be up-regulated immediately following ischemic stroke (Wise et al., 2009) (Figure 7). Currently being researched are the specific interactions estradiol has with its receptors, which are proposed to be used for neuroprotection or neural repair. ERB has been

proposed to be protective (Raval et al., 2013) while the effect is dependent on the dosage given (Strom et al., 2011), with too little or too much being considered detrimental (Strom et al., 2011). It has been shown in experimental studies that cerebral blood flow is not dependent on estradiol during ischemic stroke (Wang et al., 1998; Dang et al., 2011).

A crucial point of the Women's Health Initiative (WHI) study was the discovery of the 'window of opportunity' hypothesis, in which exogenous estradiol treatment must be begun within a very short window following natural or surgical menopause (Liu and Yang, 2013). While the timing is hypothesized to vary by individual, it has been suggested that hormone replacement therapy begin as soon as possible (Liu and Yang, 2013). Therapy beginning after the 'window' is believed to have caused the increased risk of stroke and cardiovascular disease seen in the WHI and elsewhere (Murphy et al., 2004; Nagasaki et al., 2013). Dosage, type of estrogen, timing, duration of treatment and age of participants were all determining factors in the WHI study (Murphy et al., 2004; Nagasaki et al., 2013). It has been shown that the type of estrogen is important, such as estradiol having more positive effects than estrone (McClure et al., 2013). Estrone is at a high level following menopause (McClure et al., 2013) but the ratio of each estrogen in the body can be altered by the estrogen interconversion enzymes (17beta-hydroxysteroid dehydrogenases) (McClure et al., 2013).

Of interest to this dissertation is the use of the ovariectomy model (OVX) which we have observed to cause a decrease in circulating estradiol concentrations. This mimics the loss of estradiol that occurs during human female menopause (Bushnell et al., 2014). This model, like all models, does not exactly mimic the human condition. A positive is that the surgery is a relatively simple procedure with quick recovery and

depletion of endogenous estradiol while a negative is that it does not perfectly model a

woman's slow decline of estradiol during menopause. Another method used to study

menopause in animal models is the use of the follicular toxin 4-Vinylcyclohexene

Diepoxide (VCD). This toxin is injection daily in order to induce death of the ovarian

follicles over time, resulting in their loss and the estradiol they produce (Hoyer et al.,

2001; Kappeler and Hoyer, 2012). This model more accurately represents the follicular

degeneration seen in women but is toxin-based, which is unlike human menopause and

could be toxic elsewhere in the body.
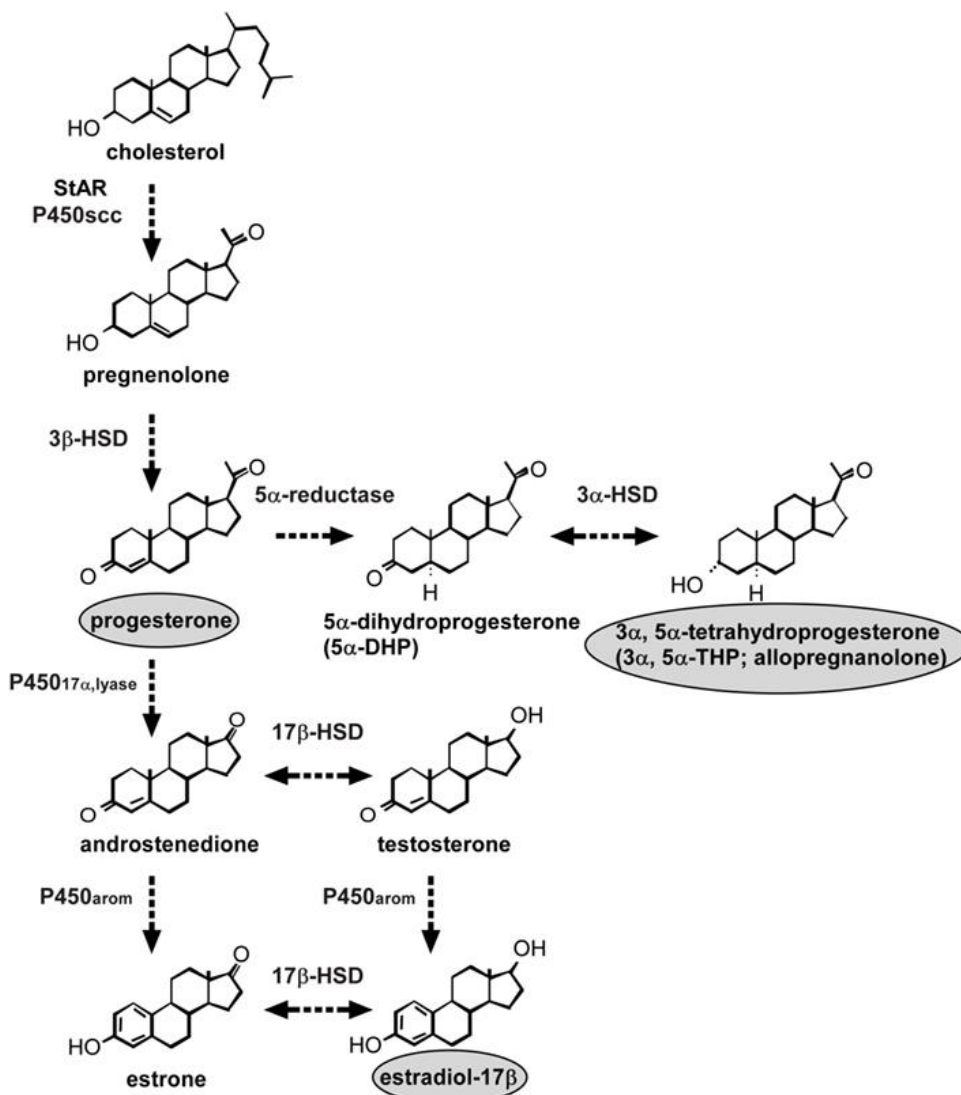
Figure 6. Steroid Synthesis. Shown here is the neurosteroids (and general synthesis) pathways of transforming cholesterol into progesterone, estradiol, and testosterone. Arom = aromatase, HSD = hydroxysteroid dehydrogenase, StAR = Steroidogenic acute regulatory protein. From Wang, 2011.
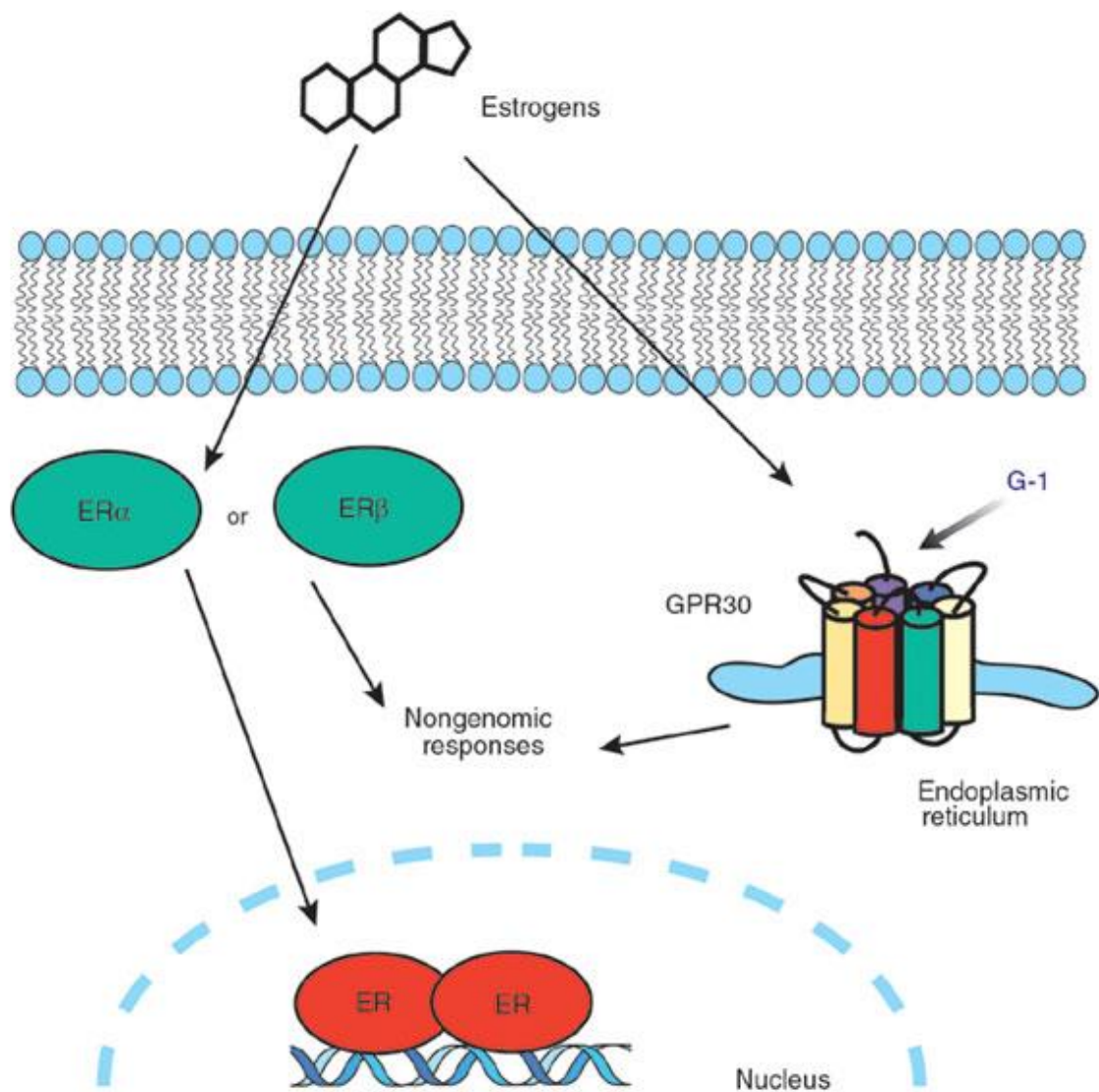
Figure 7. Estradiol Receptors. Shown here is the ability of estrogen to bind to ER-alpha, ER-beta, or the endoplasmic reticulum-located GPR30 (also known as GPER). From Weatherman, 2006.
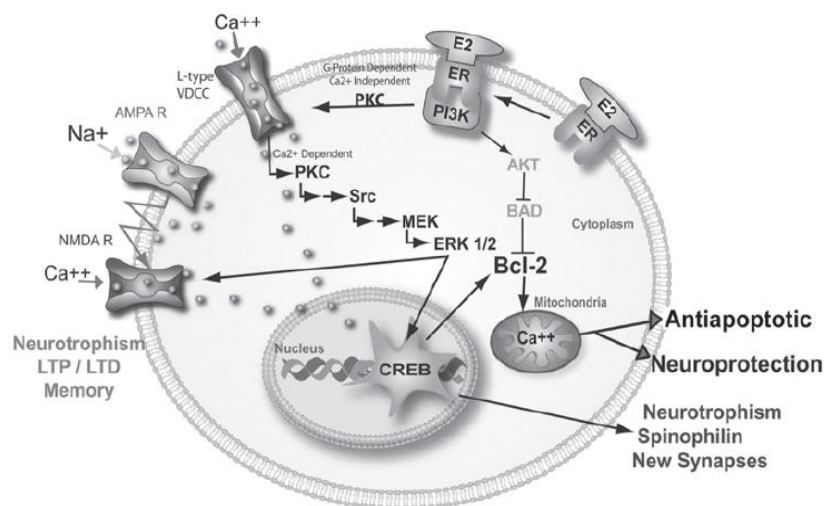
Figure 8. Estradiol Slow and Fast Routes. Shown here are the potential downstream pathways that estradiol (E2) along with excitatory neurotransmitter channels (AMPAR and NMDAR) and how anti-apoptotic and new synapse proteins can be transcribed (slow). There are also faster routes in which kinases are activated quickly to affect downstream proteins. Of note are the usage of calcium and the PI3K pathway. From Wu et al., 2005.

Along with estradiol (Gibson et al., 2006), progesterone is the other main

hormone produced by the ovaries and affected by menopause (Harsh et al., 2009; Liu et

al., 2014). Progesterone's action is directly affected by estradiol, as estradiol causes up-

regulation of some progesterone receptors (Sorwell and Urbanski, 2013; Liu et al.,

2014). Progesterone's affects in the brain are similar to estradiol's, such as increasing

dendritic spine number and increasing growth factor expression (Rehman and Masson,

2005; Hoffman et. al, 2006), with an on-going discussion as to its effects following

menopause. While some authors show a non-beneficial response to progesterone

therapy (usually given alongside estrogen), there are others that show positive or mixed

results depending on the report (Hoffman et. al, 2006; Lee et al., 2010; Liu and

McCullough, 2012; Liu et al., 2014) (Figure 9). Progesterone has been shown to

increase spine number, especially through PSD95 transcription (de Castilhos et al.,

2008; Rivera and Bethea, 2012; Sanchez et al., 2013; Rivera and Bethea, 2013). In terms of injury, progesterone has also been shown to decrease inflammation (Wong et al., 2013), decrease astrocyte proliferation following injury (Wong et al., 2013), and significantly decrease lesion volume (Wong et al., 2013). Progesterone has been shown to decrease Nogo-A and glial fibrillary acidic protein (a marker of glial cells) while increasing growth-associated protein 43 levels (Liu et al., 2014). It has been shown in the literature that progesterone levels are present in both aged rat sexes (Figure 10). Therefore, the literature has shown that progesterone has many neuroprotective qualities similar to estradiol.

Further under investigation are the effects of exogenous sex hormones given as treatment or preventative care. Examples include estrogens (in either estradiol form or conjugated equine estrogen (CEE) form) and progesterone, which may impact other neurological systems, such as mood (Backstrom et al., 2013). While still being investigated, this shows the wide range of targets and effects of sex hormones.

Following stroke, the levels of sex hormones could potentially change as brain anatomy is altered. This depends on the location of the stroke. A stroke infarct to the hypothalamus or pituitary could alter levels of sex hormone-releasing hormones (luteinizing hormone and follicle stimulating hormone). Middle cerebral artery occlusion has been shown to temporarily increase aromatase and estrogen receptor alpha (Alkayed et al., 1995). Other groups have shown that stroke decreases total serum testosterone and free serum testosterone, but not estradiol (Jeppesen et al., 1996). It has also been shown in both male and female rats that there is no change in estradiol following stroke (Shin et al., 2011).
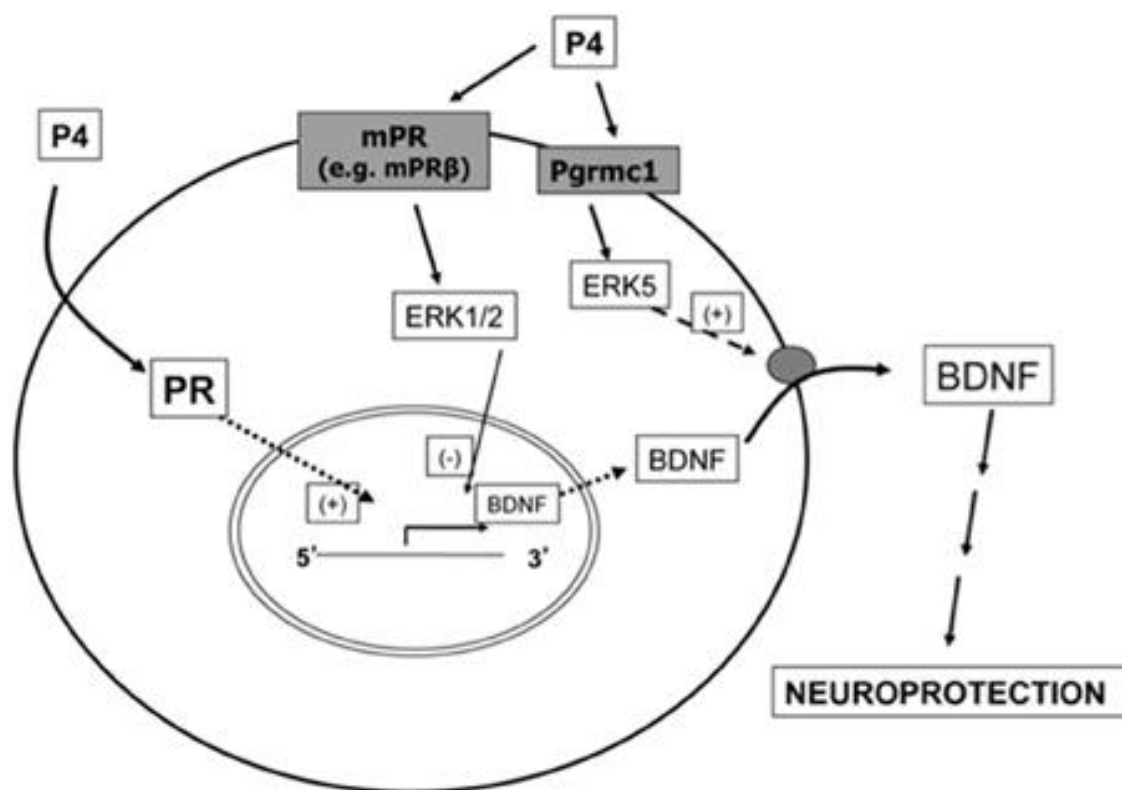
Figure 9. Progesterone Signaling. Shown here are the progesterone (P4) signaling pathways through the progesterone receptor (PR) (either cytosolic or membrane-bound (mPR)) and the possible mechanisms of neuroprotection through genomic and non-genomic brain-derived neurotrophic factor (BDNF). Suggested here is that progesterone can have differing effects if it binds to a cytosolic receptor (leading to increased BDNF transcription) or if it binds to a membrane receptor (which could decrease BDNF transcription) From Singh et al., 2013.

	18 m.o. male rat	18 m.o. female rat	18 m.o. OVX female rat
Estradiol	**3.9pg/mL** (Bimonte-Nelson et al., 2008)	**56.7 pg/mL** (Borkowski et al.) **10pg/mL** (Bimonte-Nelson et al., 2008)	**N/D** (Borkowski et al.) **5pg/mL** (Markowska and Savonenko, 2002)
Testosterone	**2.8ng/mL** (Amador et al., 1985) **1 ng/mL** (Bimonte-Nelson et al., 2008)	**13.29pg/mL** (MacBeth et al., 2007)	**N/D** (Acosta et al. 2009)
Progesterone	**0.5 ng/mL** (Amador et al., 1985)	**13.71pg/mL** (MacBeth et al., 2007)	**1pg/mL** (Acosta et al. 2009)

Figure 10. Hormone Levels. List of aged (18 month-old (m.o.)) rat levels of the three main sex hormones: estradiol, testosterone, and progesterone (N/D = Non-determinable or below assay detection level).

Testosterone is also a major sex hormone, especially in males. It can be aromatized to estradiol or bind to its own androgen receptors (Wu et al., 2005). Testosterone has been shown to be pro-growth in that in a similar fashion to estradiol it binds to its receptors which then act as transcription factors. This allows for the transcription of similar genes to estradiol (Chen et al., 2013). Testosterone has been shown to increase dendrites and spine number as well as be neuroprotective (Chen et al., 2013; Wu and Gore, 2010). Testosterone is also present in smaller concentrations in aged female rats (MacBeth et al., 2007). Female rats (as well as humans) are believed to have different concentrations and distributions of androgen receptors than males which may impact testosterone signaling.

NOGO-A AND GROWTH INHIBITORY FACTORS

Nogo-A is an inhibitory protein expressed on the surface of oligodendrocytes and in the myelin that they produce in the central nervous system, but can also be found in some neurons (Caroni & Schwab, 1988a; Caroni & Schwab, 1988b; Chen et al., 2000; Dodd et al., 2005; Papadopoulos et al., 2006; Cheatwood et al., 2008). Two extracellular regions of the Nogo-A protein have been attributed to axonal growth inhibition (see Figure 11). This is accomplished through disruption of neurite growth cones (Wang et al., 2002) and by limiting angiogenesis (Walchi et al., 2013). The extracellular regions contain the inhibitory domains Nogo-66 and delta-20, near the N-terminal portion. Nogo-66 is a 66-amino acid sequence that is common to all the isoforms of Nogo, while Nogo-A$^{544-725}$ is Nogo-A specific (Caroni & Schwab, 1988a; Caroni & Schwab, 1988b). Neutralization of Nogo-A's inhibitory domains by treatment with monoclonal antibodies (mAB) have been a long-term interest in our group to promote axonal outgrowth in the treatment of CNS injuries (Papadopoulos et al., 2002; Markus et al., 2005; Tsai et al., 2011).

The existence of Nogo and other growth inhibitory factors has been known for some time (McKerracher et al., 1994; Kawamata et al., 1997), but has only recently been better characterized (Kempf and Schwab, 2013). Along with Nogo-A, many other growth inhibitory factors exist, such as paired immunoglobin-like receptor B (PirB), oligodendrocyte-myelin glycoprotein (OMgp), and myelin associated glycoprotein (MAG), along with extracellular matrix proteins such as proteoglycans (Filbin, 2003; Buss et al., 2005; Cafferty et al., 2010). OMgp and MAG, along with Nogo-A, can be found on oligodendrocytes and act in a similar manner to Nogo-A by binding to and activating cellular receptors which can result in growth inhibition (Bongiorno and Petratos, 2010; Schwab et al., 2014). PirB acts in a similar manner to Nogo-A's receptors with similar downstream pathways (Filbin, 2003; Schwab et al., 2006).

GROWTH INHIBITION CELLULAR MECHANISMS

Nogo-A can bind to two different receptors, with different portions of the molecule binding. The delta-20 region has been shown to bind to the sphingosine-1-phosphate receptor 2 (S1PR2) (along with potential interactions with integrins) (Kempf et al., 2014). It is to this portion of Nogo-A (also called 'amino Nogo') that the antibody 11C7 binds (Tsai et al., 2007; Bongiorno and Petratos, 2010). The Nogo-66 region binds to the Nogo-66 Receptor (NgR1) as well as PirB (Chivatakarn et al., 2007; Schwab, 2010; Borrie et al., 2012). Binding to either of these receptors leads to a still poorly understood downstream mechanism, but which is believed to activate the Rho A GTPase which in turn activates Rho-associated, coiled-coil containing protein kinase (ROCK), a serine kinase (Kramar et al., 2013). This inhibits myosin light chain phosphatase (MLCP) causing a 'stabilization' of the growth cone actin, resulting in inhibited movement as actin cycling is arrested (Schwab, 2010; Fujita and Yamashita, 2014). This also has been

shown to inhibit the phosphorylation and deactivation of cofilin, destabilizing actin polymers to form monomers (Kramar et al., 2013). This process has also been shown to be dependent on protein synthesis (Manns et al., 2014) and several labs have shown other pathways induced by Nogo-A but ultimately resulting in RhoA activation (Schweigreiter et al., 2004; Yan et al., 2012) (Figure 12).

Of interest to this dissertation are the findings that growth inhibitory factors may have a sex difference associated with them. It has been shown that neuronal S1PR2 is expressed more in female brains (Cruz-Orengo et al., 2014). If this were the case then a higher expression may mean more growth inhibition in females. Also of interest is the fact that the RhoA pathway in which Nogo and its receptors use to halt the growth cone's growth (Schwab and Strittmatter, 2014) is also used by estradiol to cause actin polymerization from monomers (Kramar et al., 2013) as well as BDNF to stimulate spine growth (Kramar et al., 2013). Possible future directions may look at the complex intersection of these three pathways. The sex differences are especially highlighted when looking at data that suggests that estradiol activates the RhoA pathway as well (Kramar et al., 2013) but in a positive manner of inhibiting cofilin allowing actin monomers to form into filaments (Kramar et al., 2013).
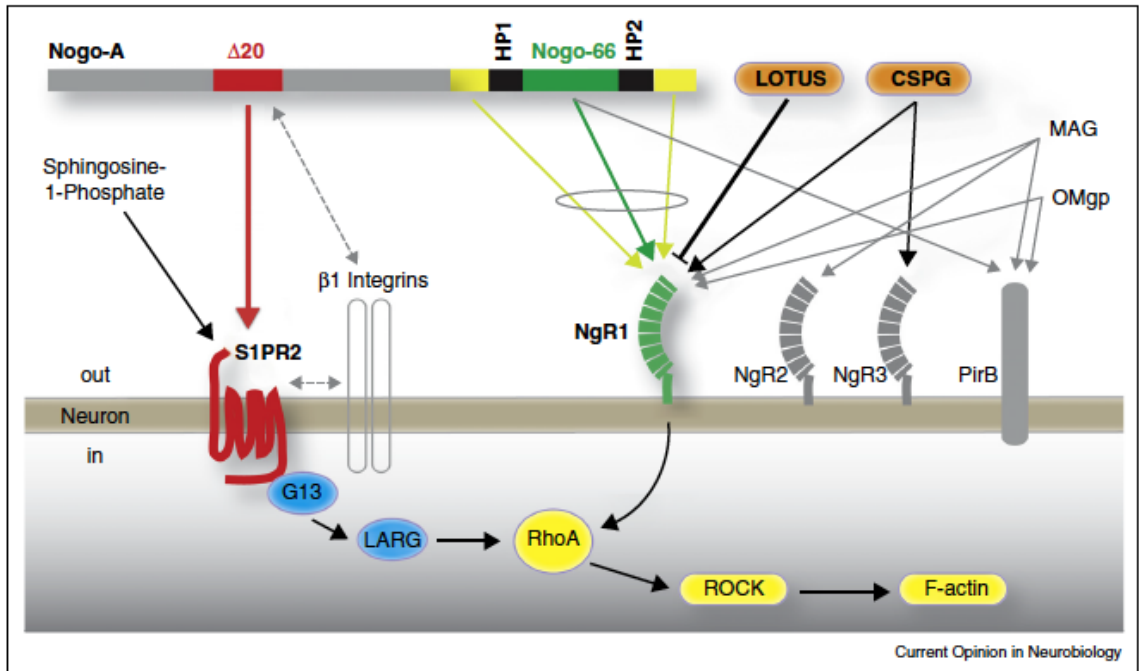
Figure 11. Nogo-A Signaling. Nogo-A is a transmembrane protein expressed primarily by oligodendrocytes. The amino portion of the protein, labeled here as "**Δ**20," is unique to Nogo-A, and is not found on either Nogo-B or Nogo-C. It is this portion of the protein bound by the specific antibodies used in these experiments. Using an antibody specific for **Δ**20 keeps Nogo-A from binding to its receptor, thereby allowing for axonal and dendritic plasticity after CNS injury. Shown here are the downstream proteins following receptor binding, starting with S1PR2's G-protein (G13) which activates RhoA to activate it. RhoA then activates ROCK (From Schwab and Strittmatter, 2014).
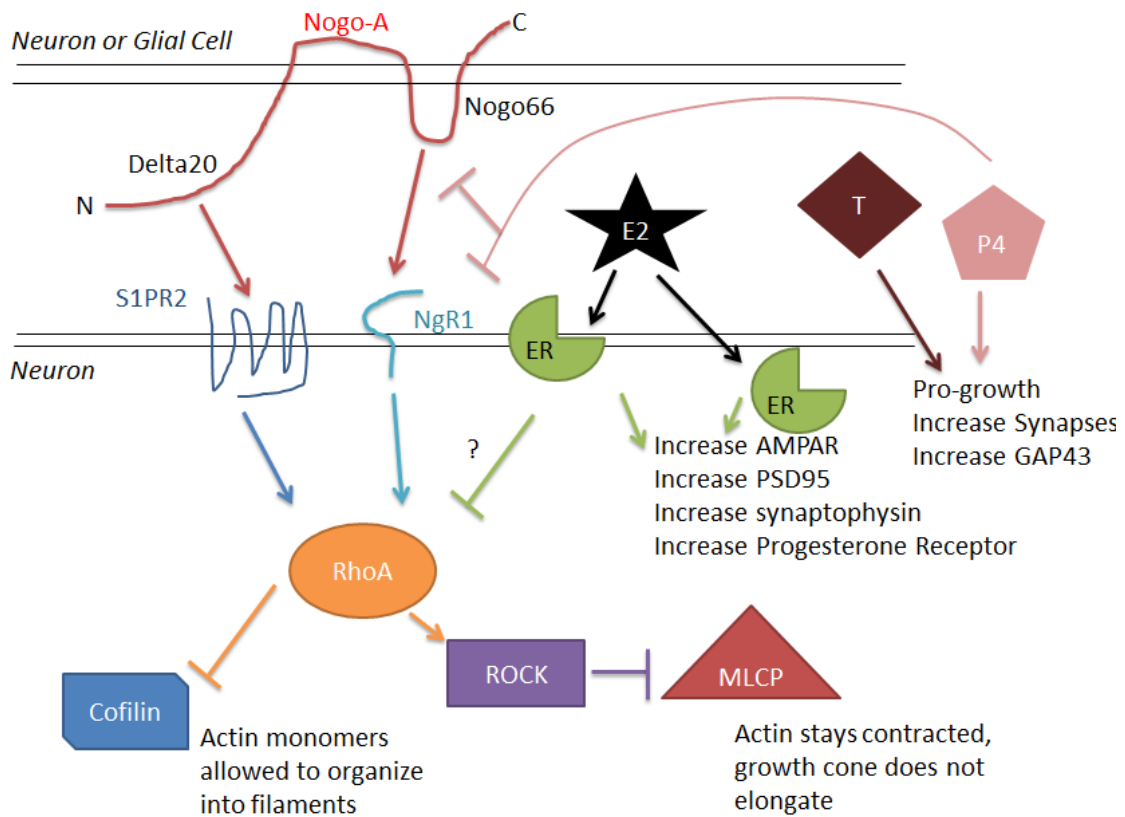
Figure 12. Nogo-A and Sex Hormones. Summary of the proposed mechanics of Nogo-A leading to growth cone stalling and collapse through S1PR2 and NgR1 to the RhoA pathway alongside possible interaction with estradiol (E2) membrane-bound and cytosolic receptors (Adapted from Wu et al., 2005; Kramer et al., 2013; Schwab and Strittmatter, 2014). P4: progesterone, T: testosterone, ROCK: Rho-associated coiled-coil kinase. MLCP: myosin light chain phosphatase.

ANTI-NOGO-A IMMUNOTHERAPY AND NEURONAL PLASTICITY FOLLOWING CNS INJURY

It was discovered in 1988 that antibody-mediated neutralization of the reticulon family proteins (of which Nogo-A is a member) could lead to increased growth of damaged and severed axons and neurites (Caroni and Schwab, 1988a; Caroni and Schwab, 1988b; Mukhopadhay et al., 1994). This was further expounded by Schwab and colleagues in which mature oligodendrocytes were inhibitive of this growth (Caroni and Schwab, 1988a). A monoclonal antibody was then raised against isolated membrane-bound proteins (Caroni and Schwab, 1988b; Schnell and Schwab, 1990). Later identification of this protein led to the title of Nogo-A (Chen et al., 2000).

The Nogo gene has three different splice variants (Nogo-A, -B, and –C) (Chen et al., 2000; Grandpre and Strittmatter, 2001) (Figure 13). In the Nogo-A protein there are two active sites of particular note for CNS recovery: Amino-Nogo, which binds to the Sphingosine-1-Phosphate Receptor (S1PR2) and the Nogo-66 region near the C-terminal domain which also induces growth cone collapse through binding to the Nogo-66 Receptor (NgR1) and PirB Receptor (Chen et al., 2000; Schwab, 2004; Schwab, 2010).

Our laboratory has shown that anti-Nogo-A immunotherapy enhanced new axonal growth from uninjured pathways that projected to areas denervated by a CNS lesion (Kartje et al., 1999; Wenk et al., 1999; Seymour et al., 2005). Furthermore, our lab has reported extensively on the administration of anti-Nogo-A antibodies which resulted in axonal plasticity of intact fiber tracts and brain reorganization (Kartje et al., 1999; Wenk et al., 1999; Emerick and Kartje, 2004; Emerick et al., 2005; Papadapoulos et al. 2006), and most significantly improved functional recovery of skilled forelimb movements in

adult and aged male rats post-stroke (Papadopoulos et al., 2002; Markus et al., 2005; Tsai et al., 2007; Gillani et al., 2010). The efficacy of anti-Nogo-A immunotherapy in stroke impaired aged female rats is an important area of investigation since females recover worse than males post-stroke and may respond differently to this therapeutic intervention.
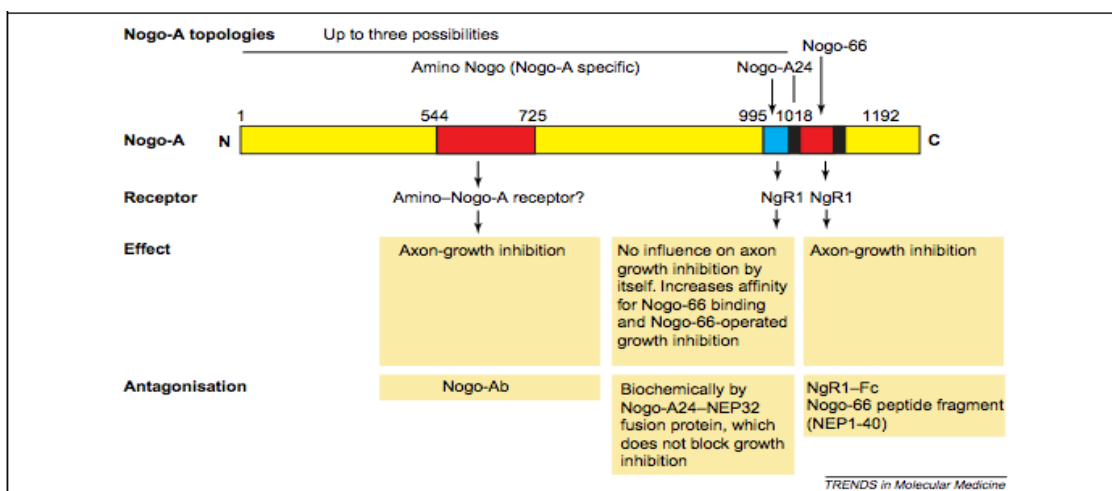
Figure 13. The Nogo-A Gene. This schematic shows the two active growth-inhibiting sites of Nogo-A and their targets (Amino Nogo and Nogo-66) with target receptors (Amino-Nogo-A receptor now reported to be the S1PR), effects and antibody binding locations. From Schwab et al., 2006.

NOGO-A IN CLINICAL TRIALS

The Nogo-A protein has been of potential clinical value for some time (Pernet and Schwab, 2012; Wang et al., 2012; Schwab and Strittmatter, 2014). Phase 1 clinical trials for ALS and multiple sclerosis are underway using the antibody named Ozanezumab through GlaxoSmithKline (Meinenger et al., 2014). Novartis earlier completed Phase 1 of a spinal cord injury trial using a similar antibody (Clinical Trials Identifier: NCT00406016) in 2011.

NOGO AND PSYCHIATRIC DISORDERS

Nogo-A levels have been seen altered in psychiatric disorders (Novak and Tallerico, 2006). Nogo-A has been shown to be implicated in several disorders, including rat models of schizophrenia (Willi and Schwab, 2013). Nogo-A's growth inhibition may play a role in impeding normal mental function as particular brain connections are reduced or inhibited. This theory has been put forward for many mental disorders including schizophrenia but also major depression, antisocial personality disorder, and autism (Willi and Schwab, 2013; Schmandke et al., 2014). While more research is needed on the subject, it is apparent Nogo-A has widespread effects not just on axonal and dendritic plasticity leading to sensorimotor recovery but also on higher-level functioning. Of particular note are the other roles Nogo has in normal animals and neural development, such as the reduced motivational behaviors seen in rats with reduced Nogo-A (Enkel et al., 2014). Transgenic rats with reduced Nogo-A showed deficits in cognition along with decreased anxiety and a change in circadian rhythms (Petrasek et al., 2014). This shows another reason why Nogo-A cannot be removed completely from the central nervous system.

BEHAVIORAL TASKS FOR LEARNING AND FUNCTIONAL RECOVERY

Measuring behavioral recovery from stroke has a long history in research and many tasks exist depending on which aspect of recovery is desired to be measured. Of interest to this dissertation is the skilled forelimb reaching task, developed by Castro and Whishaw (Castro, 1972) and the skilled ladder rung walking task, as developed by Metz (Metz and Whishaw, 2002). Usefulness of these tasks lies in their ability to be accurate, quantifiable, and translatable to human motor movements, such as in the fine grasping task of the skilled forelimb reaching and skilled ladder rung walking task. These tasks

have been used to measure cortical brain injuries and the extent of the behavioral deficit after cortical injuries (Papadapoulos et al., 2002; Papadapoulos et al., 2006; Tsai et al., 2007).

In addition, the use of behavioral tests to measure learning (particularly in the hippocampus) is widespread. Of interest to this dissertation are motor tasks for measuring age-related changes. This has been studied using nuclear magnetic resonance imaging (NMRI) studies in mice (with aged animals learning slower than younger animals) (Lamberty and Gower, 1992). These learning tasks also involve a change in the motor cortex possibly resulting from increased use and increased cortical connectivity (Kami et al., 1998) which is an area of research not yet fully investigated.

CORTICAL CONNECTIVITY

The neocortex is defined by its many layers and connections. Studying these connections can make it possible to study behavioral changes due to injury. There are six neocortical layers, with differing cell types in each. Layer I (the molecular layer) is mostly apical dendrites and glial cells. Layer II (the external granular layer) has small pyramidal neurons. Layer III (the external pyramidal layer) has small and medium pyramidal neurons. Layer IV (the internal granular layer) also contains pyramidal neurons. Layer V (the internal pyramidal layer) has large pyramidal cells and is the start of the corticospinal tract. The axons from these neurons form the large cortico-efferent descending pathways, including the cortico-spinal tract and the cortico-rubral tract. Layer VI (the multiform layer) has smaller pyramidal cells and multiform neurons and connects mainly to the thalamus (Markowska and Savonenko, 2002) (Figure 14). Of interest to this dissertation are the connections to and from the pyramidal layer V forelimb motor

cortex, as this is where voluntary motor movement is generated. Damage to this area results in forelimb reaching deficits (Markus et al., 2005; Tsai et al., 2007). Therefore, cortical connectivity is important to study as it dictates motor planning and movement and has a large impact on behavior.

One of the major connections to the layer V pyramidal neuron are the afferent signals from the thalamus, specifically the VA/VL nuclei of the thalamus used in motor movement (Yu et al., 2008; Kaneko, 2013). These are usually excitatory and release glutamate onto the post-synaptic receptors, such as AMPA receptors and NMDA receptors (Yu et al., 2008; Kaneko, 2013). The released glutamate is taken up by the vesicular glutamate 2 transporter (VGLUT2) (Kaneko, 2013). These connections are important because they communicate with part of the basal ganglia, in which motor movement is controlled and fine-tuned. Parkinson's Disease is an example of a deficit in this layer (Huang et al., 2015).

The other major connections to the layer V pyramidal neuron are from other layers, or even the same layer of the cortex itself (Kaneko, 2013). This can be from layers of the same hemisphere or from the contralateral hemisphere (traveling through the corpus callosum) (Yu et al., 2008; Huang et al., 2015). The excitatory signal is also glutamate onto the same receptors listed above (AMPA and NMDA receptors). After release, glutatmate is re-taken up by excitatory amino acid transporters and returned to vesicles via the vesicular glutamate 1 transporter (Hira et al., 2013; Kaneko, 2013). These signals can also be inhibitory using vasoactive intestinal peptide or gamma aminobutyric acid (Tanaka et al., 2011; Kaneko, 2013).

The motor cortex sends signals outwards to the contralateral motor cortex, striatum, globus pallidus, and spinal cord through the corticospinal tract and other structures (Yu et al., 2008; Huang et al., 2015). It is through these pathways that motor movement is initiated, inhibited, or dis-inhibited along with input from the cerebellum (Pi et al., 2013). Each hemisphere connects to each other through the corpus callosum (Yu et al., 2008) and this cross-talk is an important factor in motor recovery when one hemisphere is damaged (Papadapoulos et al., 2006).
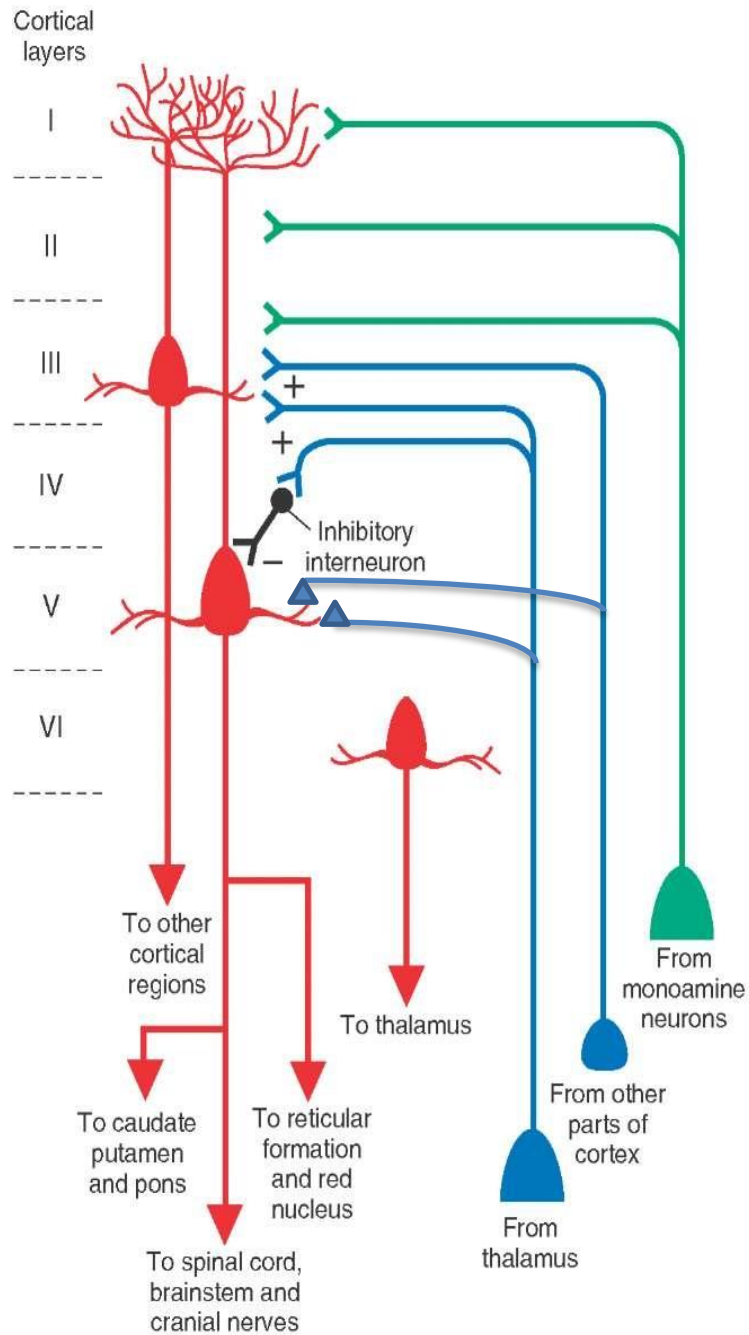
Figure 14. Neocortex Connectivity and Layers. The pyramidal cells can be seen in layers III, V and VI and their respective synapses, such as from the thalamus and from other neocortex areas. Adapted from The Thalamus and Cerebral Cortex (Integrative Systems) Part 1 Tutorial (What-When-How).

The focus of this dissertation is the repair of the cortex following an injury such as stroke and the resulting new synapses that are formed or re-formed. The rubro-spinal tract, for example, has been shown to be able to re-form cortical connections following a stroke (Takenobu et al., 2014) and it has been shown that there is a crossing of the fibers at the level of the red nucleus from the intact hemisphere to the de-enervated red nucleus following stroke and anti-Nogo-A immunotherapy (Papadapoulos et al., 2002; Papadapoulos et al., 2006). Our laboratory has also shown that there is a significant number of fibers crossing over from the uninjured cortex to innervate the de-enervated subcortical structures such as the striatum, spinal cord, pons and red nucleus (Papadapoulos et al., 2002; Markus et al., 2005; Papadapoulos et al., 2005; Tsai et al., 2007). This has shown that the uninjured contralesional cortex is a potential site for neural repair and functional recovery following injury.

Projections from the cortex were shown to be at least partially recoverable even in the absence of treatment (Carmichael, 2003; Jiang et al., 2013). It has been suggested that the large portions of the cortex reorganizes its connections following a stroke (Grefkes and Ward, 2014; Starkey and Schwab, 2014) which can be postulated to be the neurons working around the infarct and glial scar to recreate those previous connective pathways spontaneously, though to a limited effect (Wieloch and Nikolich, 2006). Aging itself results in neuronal changes (Heuninckx, et al., 2008), especially in cortical connections (Thouvarecq et al., 2007; Del Zoppo, 2012). The combination of aging and injury could result in a different reorganization that may not be effective (Nudo, 2013) and has been shown to affect dendrites and axons in different ways (Mizielinska et al., 2009). While the motor cortex has been shown to have defined areas

and connections (Neafsey et al., 1986) it has been proposed that the resulting brain

repair could result in a changed but effective model (Small et al., 2013).

CHAPTER THREE

SEX DIFFERENCES IN SYNAPSE NUMBER IN THE RAT FORELIMB MOTOR

CORTEX

ABSTRACT

Sex-based differences in recovery from stroke are an area of research not yet fully
explored. This is especially true with regard to aged rats' excitatory synapse number in
the motor cortex, which model human post-stroke functional recovery and synaptic
connections. In the human clinical population, post-menopausal females have been
shown to recover from stroke less successfully than age-matched males. Therefore,
studying sex differences in rat models of stroke recovery is clinically relevant. We set
out to determine if differences in excitatory synaptic number were evident in the
different sexes, which may influence recovery from stroke and connectivity relating to
motor movements and recovery. 18 month-old Fischer 344 male and female aged rats
were used for these studies. Rats were divided into three groups: ovariectomized (OVX)
females, intact females, and age-matched males. Half of each group received a stroke
and half did not. All rats received adeno-associated virus (AAV2/8-CMV-GFP-LDL-
RCT) injections to the motor cortex to pre-label cortico-efferent pyramidal neurons in
layer V. At the end of three or eight weeks, rats were sacrificed for
immunohistochemistry to quantify excitatory synapses in pyramidal layer V forelimb
motor cortex. Neurons that were GFP-positive with a typical pyramidal shape and good
dendritic labeling were identified via confocal microscopy. Using 630x oil magnification,
basilar dendrites were examined for presynaptic (vGlut1 or vGlut2) markers and co-
localization with the postsynaptic marker NMDAR-NR1. The number of co-localized

synapses was quantified using an orthogonal Z-stack (Z-step .89um, 16 Z-steps) on basilar dendrites for a 40um length starting at the cell soma. Our results showed that after 3 weeks, no differences were found between groups in the number of excitatory synapses on layer V pyramidal neurons. However, after 8 weeks, the number of excitatory synapses in the aged OVX female group was significantly less than that of the intact females and age-matched males. These results indicate a possible long-term effect of estradiol and related sex hormone depletion on neocortical synaptic number in aged females.

INTRODUCTION

Synaptic proteins are an integral part of the synaptic unit and are subject to many factors, including sex hormones, synaptic signal strength, neuronal health and the extra-synaptic environment (Foy et al., 2008). This synaptic integration is crucial for communication in the nervous system and allows for modification and inhibition of cell signaling (Phan et al., 2012; Brocca et al., 2013). Damage to the communication pathways in the brain has been shown to impair those signals and subsequently interrupt normal behavior or other physiologic transmission (Phan et al., 2012; Brocca et al., 2013). In this case, ischemic damage to the motor cortex results in a decrease in healthy brain tissue (resulting in an infarct, or damaged and dead tissue). Repair and subsequent functional recovery depends on these areas re-establishing proper communication to motor areas in order for functional motor behavior to ensue. Synaptic proteins are integral to forming these required synapses and are dependent on neuronal connectivity (Norbury et al., 2003; Minamo et al., 2008; Phan et al., 2012; Baudry et al., 2013; Brocca et al., 2013).

Potential sex differences in synaptic number in the motor cortex could be used to explain the behavioral differences seen in learning and post-stroke recovery. Estradiol has been shown to increase synaptic number (Wu et al., 2005; Foy et al., 2008; Kramer et al., 2013), but it is uncertain whether stroke reduces the number of synapse numbers in rats, especially at different time points of recovery. The motor cortex's connections to the thalamus and other parts of the cortex are used in relaying movement information (Kaneko, 2013). Proper cortical connectivity is essential in learning and recovery as this communication is necessary for the motor movement pathways (Brocca et al., 2013). Disruption of these pathways will negatively impact behavior, while eventual restoration of connectivity could allow for functional motor recovery. The focus of this study is the excitatory connections to the pyramidal layer V forelimb motor cortex via the pre-synaptic markers VGLUT1 (cortico-cortical connections) and VGLUT2 (thalamo-cortical connections), which are the glutamate transporters that fill the vesicles with glutamate (Kaneko, 2013).

METHODS

All experimenters were blinded to rat group and treatment.

Animal Subjects

All animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of the Hines Veterans Affairs Hospital in Hines, IL. A total of 36 aged male and female Fischer 344 albino rats (18 months of age at start of the study) were divided into three groups: normal males, normal (intact) females, and ovariectomized (OVX) females. Rats were housed two to a cage and were maintained in a 12 hour light/dark cycle with free access to water (Figure 15).

RAT GROUPS AND NUMBERS

	Stroke	No Stroke	
Male	6	6	
Female	6	6	
OVX Female	6	6	
Total	18	18	36

Power Analysis:

For this aim, we started with a power analysis and incorporated six experimental groups for comparison, setting alpha at 0.05. A sample size of 36 with 6 animals per group (IHC 3 and 8 week time-points) will be needed to achieve power of 0.8.
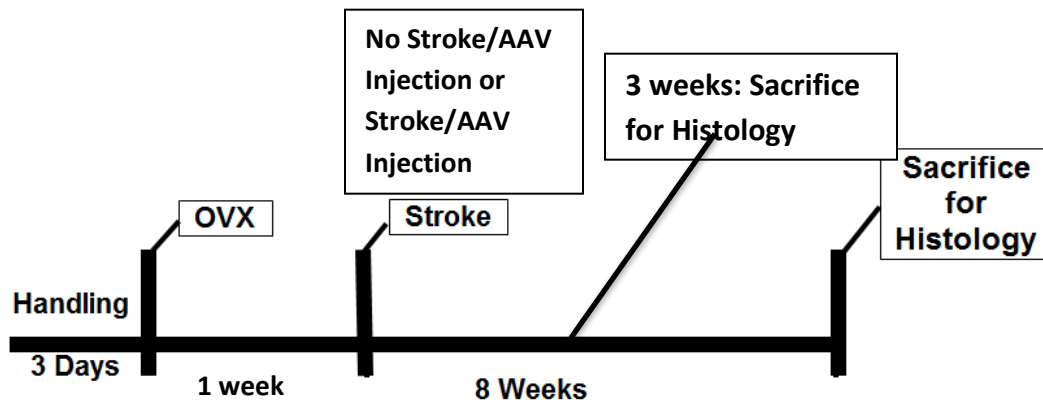
Figure 15. Experimental Timeline of Aim 1. Rats received a stroke to the right hemisphere and immediately after received an AAV injection to the contralesional cortex. Three weeks post-stroke, half of the rats were sacrificed for IHC and lesion analysis. The other half were sacrificed at 8 weeks post-stroke.

Ovariectomy (OVX)

Twelve females received an OVX in which both ovaries were removed. Rats were anesthetized using 2% isoflurane with oxygen and had both flanks shaved between rib cage and pelvic bones. A one centimeter incision was made in the skin followed by a one centimeter incision in the underlying muscle wall. Ovaries were found by pulling underlying fat tissue and isolating the ovary. Oviducts were tied off and cut right below the ovary body. The muscles and skin were then sutured.

Estradiol Serum Measurements Via Enzyme-linked Immunosorbent Assay (ELISA)

One week after ovariectomy all female rats had tail vein blood collected for ELISA estradiol serum level determination. Tail blood was spun down and the serum was collected and then processed using Cayman Chemical's Estradiol EIA Kit (Item 582251 Ann Arbor, MI). The kit's detectable level for estradiol was 19pg/mL.

Stroke Surgery

Middle Cerebral Artery Occlusion (MCAO) was performed as described in our previous work (Markus et al., 2005; Gillani et al., 2010). Animals were anesthetized (2-3% isoflurane in oxygen) and secured in a stereotaxic frame. An incision was made through the scalp and underlying muscle and the skull opened with a dental drill. The middle cerebral artery was then isolated and ligated by a 10-0 silk suture and transected with microscissors. Core body temperature was maintained at 37 degrees Celsius. The scalp was sutured closed and rats returned to their home cages.

Adeno-Associated Virus (AAV) Injection

AAV2/8-CMV-GFP-LDL-RCT (Adeno-associated Virus Serotype 2/8 – Cytomegalovirus plasmid – Green Fluorescent Protein – Directed to Plasma Membrane) was injected in the contralesional forelimb motor cortex to label layer V pyramidal neurons (2 injection sites (Coordinates: 3 mm lateral, bregma, 1.5 mm ventral; 2.5 mm lateral, 0.5 mm anterior, 1.5 mm ventral), 1 uL each injection site; 1.3x10E12 vector genomes/ml titer). This paradigm has been used in our previous publications (Pradhan et al, 2010) and results in excellent labeling of dendrites and spines as the myristoylated GFP targets the neuronal cell membrane (Kameda et al, 2008) (Figures 16 and 17).
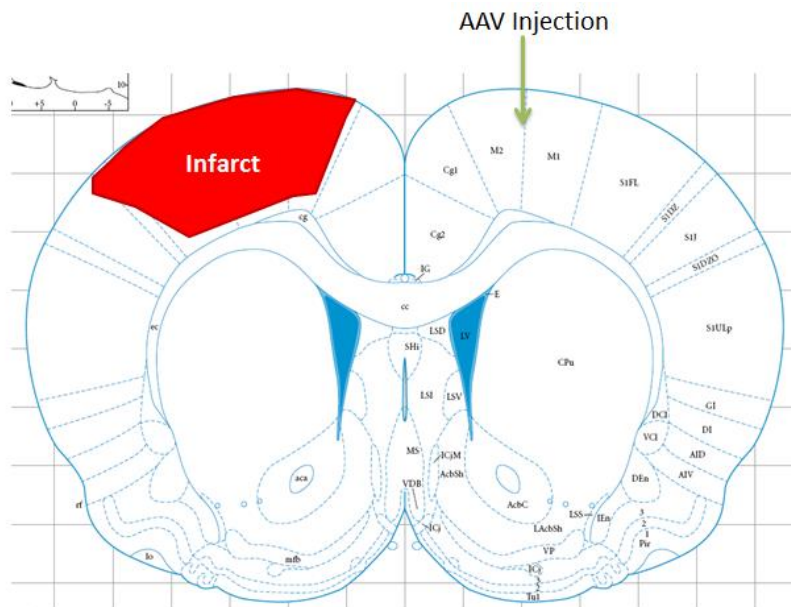
Figure 16. Infarct Location. Shown here is the typical infarct size and location of the rat motor cortex and the contralesional AAV injection that follows. No-stroke rats also received AAV injection in the left hemisphere.

Perfusion

Three and eight weeks after stroke, rats were sacrificed and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were allowed to sit overnight in 4% paraformaldehyde before being placed in 30% sucrose until sinking. They were then frozen in tissue medium (OCT) and stored at -80 degrees Celsius.

Immunohistochemistry and Analysis

Brains were cut at 50 um on a -20 degree Celsius cryostat before lipofuscin removal and antigen retrieval. Primary antibodies against NMDA Receptors (NR1 subunit) (mouse IgG2, Life Technologies 32-0500), vesicular glutamate transporter 1 and 2 (VGLUT1 and 2) (guinea pig IgG1, EMD AB5905) and anti-GFP (rabbit, Life Technologies A11122) were used. Sections from the same anatomical location from each brain were used for IHC (three from each brain) – 2nd, 8th, 14th sections starting

collecting at the beginning of when the hemispheres connect via the corpus callosum (Bregma +1.66mm) (Figure 17).

Free-floating sections were incubated for 30 minutes at room temperature in 50uM cupric sulfate in 100uM ammonium acetate for lipofuscin removal. Sections were then washed and incubated for 30 minutes at 80 degrees Celsius in pH 6 citrate buffer for antigen retrieval. Sections were washed and incubated at 4 degrees Celsius for 24 hours in one of the following parameters:

- Anti-VGlut1 (1:1000), Anti-GFP (1:1000), Anti-NMDAR (1:1000)

- Anti-VGlut2 (1:1000), Anti-GFP (1:1000), Anti-NMDAR (1:1000)

- Anti-Nogo-A (1:10,000), Anti-VGlut1 (1:1000), Anti-GFP (1:1000).

Sections were then washed and incubated in secondary antibody (goat anti-rabbit, IgG Life Technologies A-11070; goat anti-guinea pig, IgG VWR RL606-143-129; goat anti-mouse IgG Life Technologies A-11019) (1:500) for two hours at room temperature plus DAPI stain before washing and mounting on slides. Brain sections were mounted on slides and examined with a confocal microscope (Leica High Resolution Confocal TCS-SPE) and deconvolution software. GFP-labeled pyramidal neurons from the sensorimotor forelimb cortex were identified by morphology. For each rat, z-stacks of dendritic segments from 5 different neurons were imaged in the basilar arbors, and analyzed for density of the specific synaptic protein-positive puncta co-localized with EGFP with ImageJ software (NIH) (Z-step .89um, 16 Z-steps). After full z-stack photo was taken, the image was flipped orthogonally (Figure 18) and the chosen dendrite was measured 40um from the soma so as to confirm co-localization (Figure 19 and 20). The co-localized puncta were counted and categorized according to placement on the

dendritic shaft or spine within that 40um distance. Non-specific labeling was checked by

incubating one section per rat in a secondary antibody only, primary antibody only, and

cross-reactivity was checked by one section in each non-correct primary-secondary

antibody pairing (i.e., mouse primary antibody with anti-rabbit secondary antibody. No

non-specific binding or cross-reactivity was observed. Quantification was done by

confirming yellow co-localization within the 40 um distance from soma.
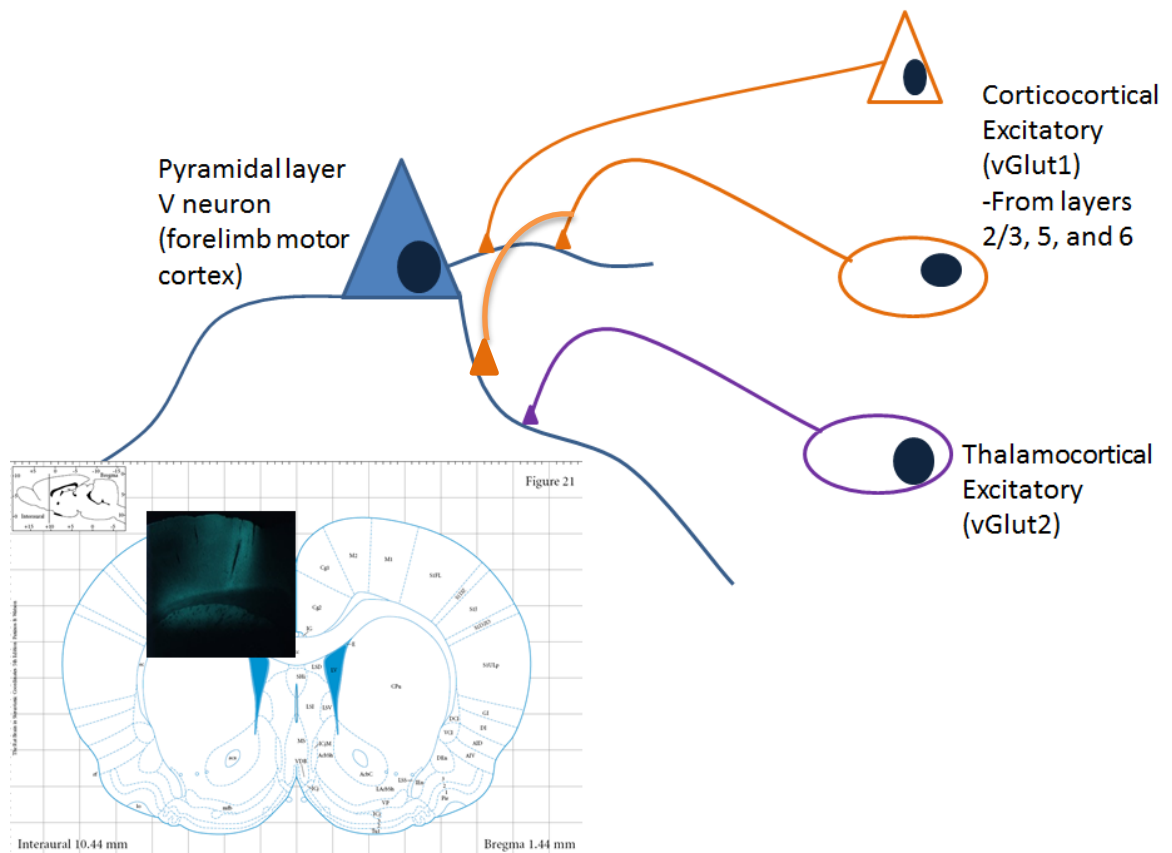
Figure 17. Cortical Connections and AAV Injection Location. Shown here are the pyramidal layer V neural connectivity with excitatory connections from the cortex (layers 2/3, 5 and 6 from both hemispheres) and from the VA/VL nuclei of the thalamus. Brain section diagram from Paxinos and Watson, 1998.
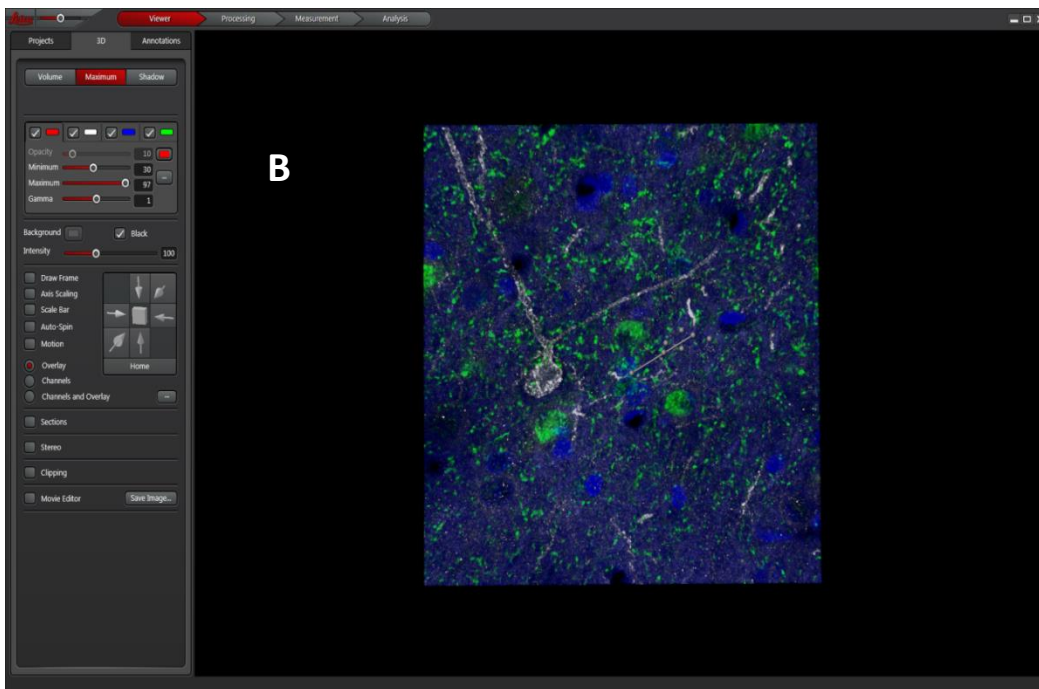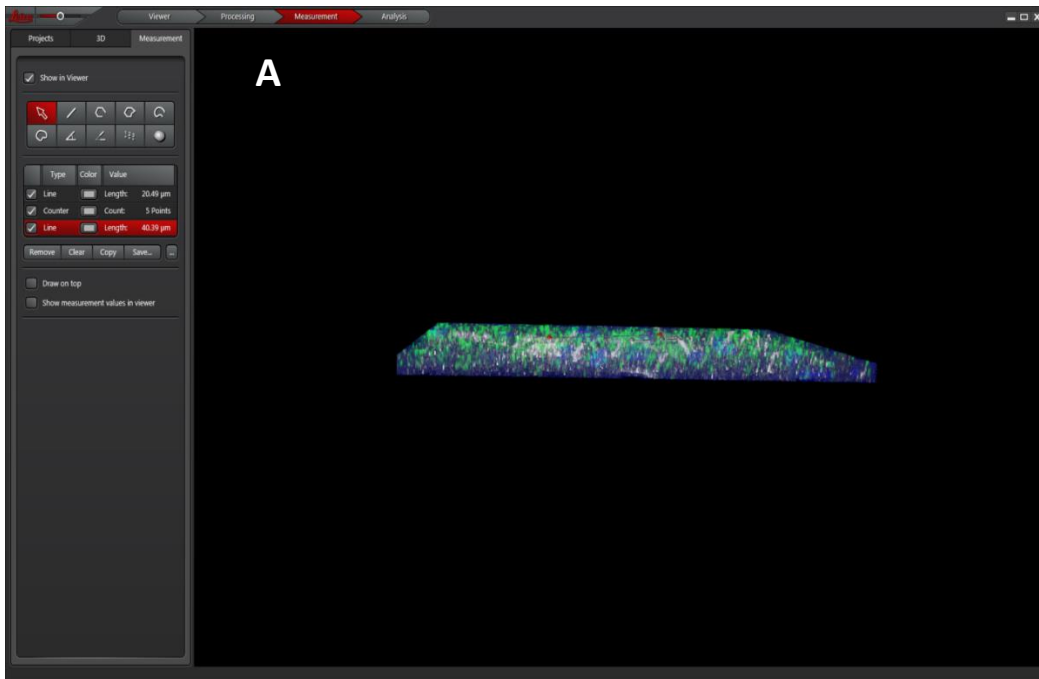
Figure 18. Orthogonal View of Z Stack. Panel A shows the Z-stack image after capturing on the confocal microscope, with panel B showing the same image flipped orthogonally to be certain of co-localization of pre- and post-synaptic fluorescence on the dendrite.
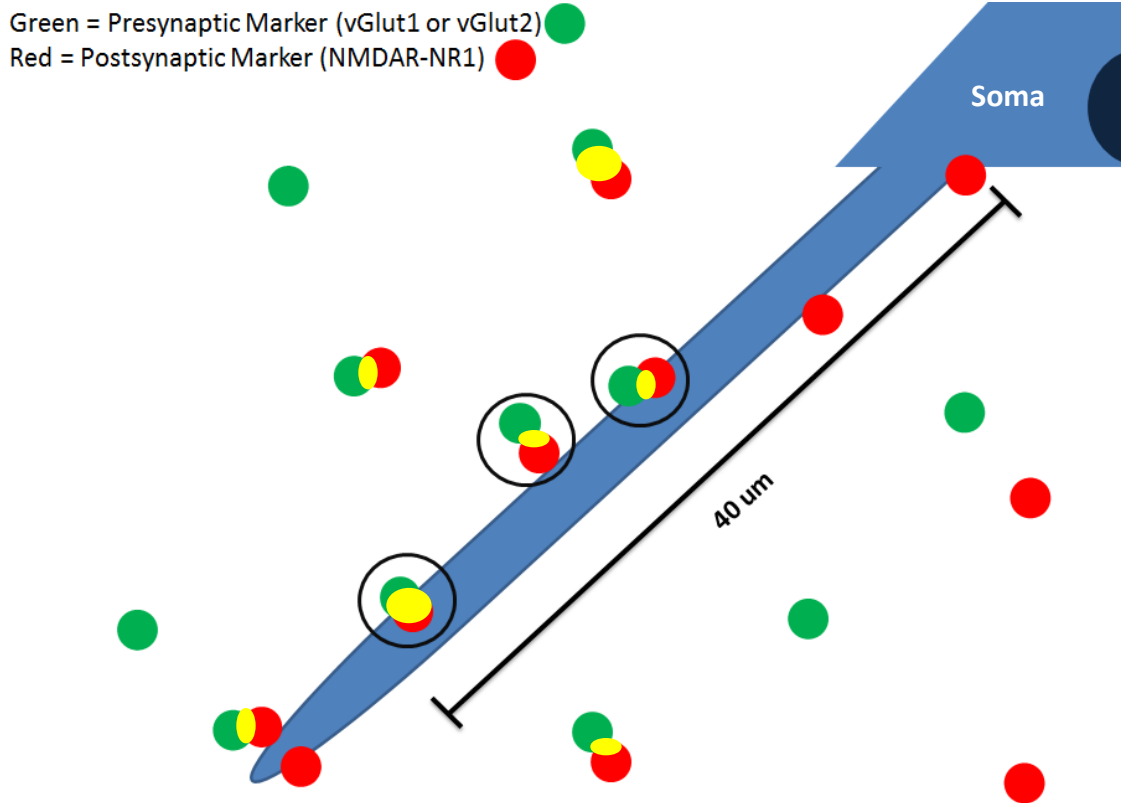
Figure 19. Quantification of Synapses. Quantification of green-labeled pre-synaptic markers (vGlut1 or vGlut2), red post-synaptic markers (NMDAR-NR1 subunit) and the GFP-labeled neuronal soma and dendrites. Quantification must be within the 40um distance from the soma, the post-synaptic marker must be making contact with the dendrite and there must be some overlap between the post- and pre-synaptic markers (yellow). Circles indicate a countable triple-labeling.
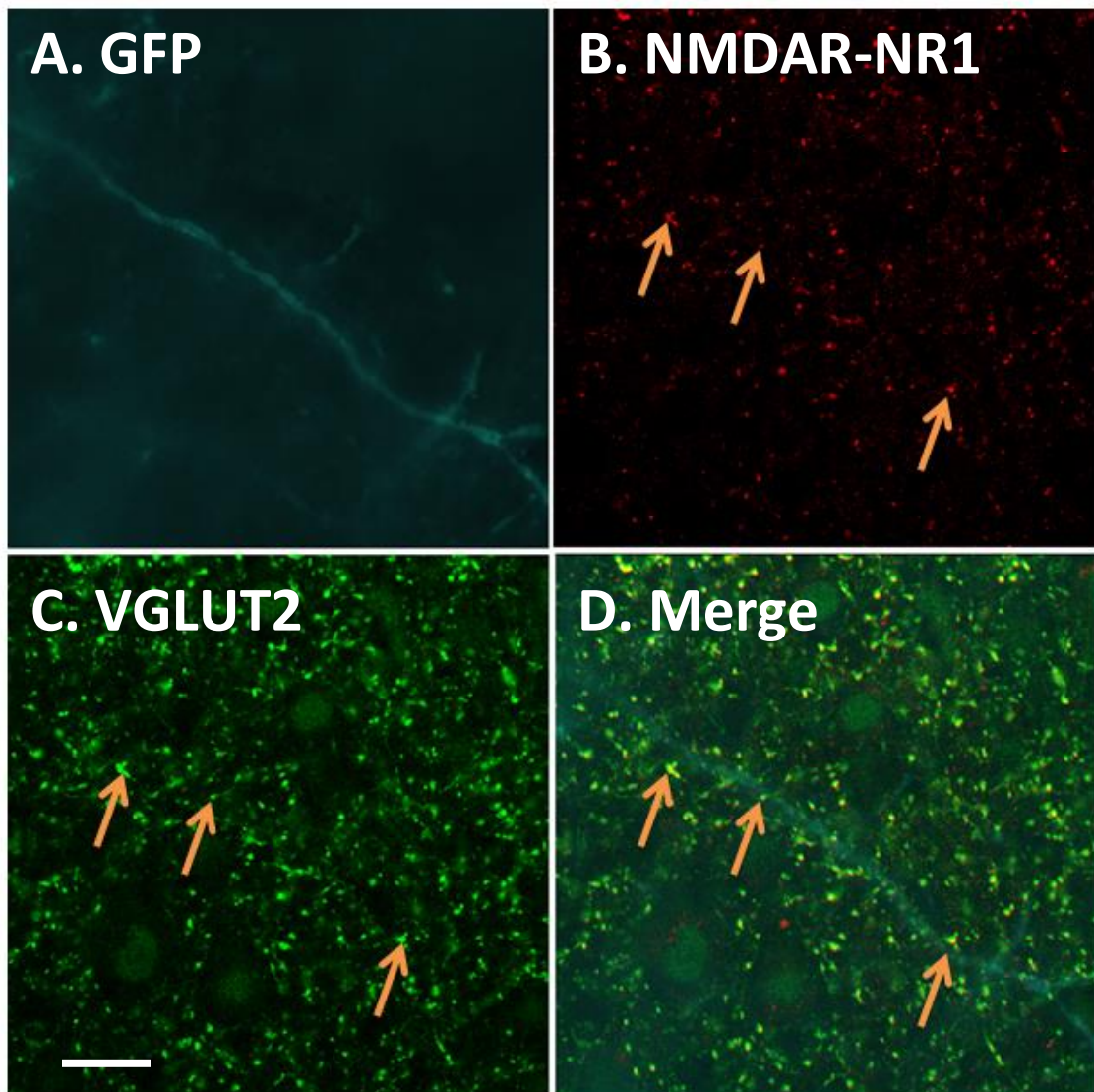
Figure 20. Example of Synapse Quantification. Sample photomicrographs obtained from an intact female rat shows (A) a GFP-labeled basilar dendrite, (B) the post-synaptic marker (NMDAR-NR1) in red, (C) the pre-synaptic marker (VGLUT2) in green (D) and the merge of all three images. Arrows indicate sample co-localizations (yellow). Scale bar = 5um.

Lesion Analysis

The lesion volume was analyzed using alternate Nissl stained sections. The area of the intact contralateral hemisphere was measured then subtracted from the area of the intact ipsilateral hemisphere. This was then multiplied by the distance between sections. Area of stroke was expressed as a percentage of the intact contralateral hemispheric volume.

Statistical Analysis

P values of less than 0.05 were considered significant.

All data analysis was performed with GraphPad Prism Version 6.0 software (La Jolla, CA USA) for repeated-measures ANOVA and one-way ANOVA with Tukey post-hoc tests or SPSS (IBM, Armonk, NY, USA) for F statistics.

F statistics were determined for this aim. A one-way ANOVA with Tukey's post hoc analysis was used to compare the mean of the IHC data and lesion analysis data after measuring F statistic. Correlative statistics were done for each rat on lesion size and synaptic number using the correlation coefficient (r). A *p* value less than or equal to 0.05 was be considered significant and a correlation coefficient of less than 0.5 was considered weakly correlating.

RESULTS

Estradiol Blood Serum Levels

The estradiol serum levels in the females were as follows: the average estradiol level in the intact females (n=9) was 53.7 pg/mL (± 10.36pg/mL) whereas the ovariectomized females (n=8) were all below the detectable limit of 19 pg/mL.

Lesion Analysis

Lesion analysis of the stroke brains revealed no significant difference in lesion size between groups (Figure 21).
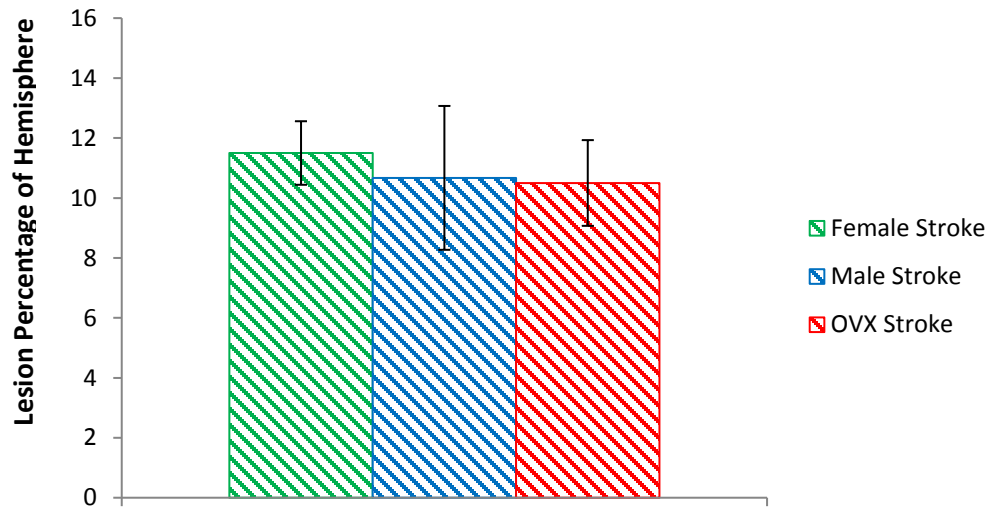
Figure 21. Lesion analysis in the aged rat after stroke. Lesion analysis showed no difference in stroke size between aged male, aged female, and aged female/ovariectomized (OVX) rats. (p>0.05). Error bars represent standard error of the mean.

VGLUT1 and VGLUT2 Co-Localization

When quantifying VGLUT1 synapses on Layer V pyramidal neurons with no cortical lesion (ie, no stroke), we found that all three groups had the same number of synapses with no differences between groups at the three week time point. Furthermore, at three weeks post-stroke/AAV injection there were still no differences between groups (Figure 22). Although there was a trend for decreased VGLUT1 synapses following stroke, this was not significantly different.

The F test conducted revealed significant group effects [$F_{(6, 35)} = 28.78$, $p < 0.05$]. When quantifying VGLUT1 synapses at eight weeks post-AAV injection, we found significant differences. AAV-only females had the highest counted co-localizations in this instance and post-hoc revealed were significant when compared to AAV-only OVX females ($p < 0.05$) and stroke intact females ($p < 0.05$). AAV-only males were significant when compared to stroke males ($p < 0.05$) (Figure 22). There was no difference between OVX groups with and without stroke.

When quantifying VGLUT2 synapses on Layer V pyramidal neurons with no cortical lesion (ie, no stroke), we found that all three groups had the same number of synapses with no differences between groups at the three week time point. Furthermore, at three weeks post-stroke/AAV injection there were still no differences between groups (Figure 23), although there was a trend for decreased VGLUT2 synapses following stroke, this was not significantly different.

The F test conducted revealed significant group effects [$F_{(6, 35)} = 31.11$, $p < 0.05$]. When quantifying VGLUT2 synapses at eight weeks post-AAV injection, we found significant differences. There was no decrease in synaptic number following stroke in either the intact female or OVX female groups. Post-hoc revealed AAV-only males were significant when compared to stroke males ($p < 0.05$) (Figure 23). Post-stroke, intact females had significantly higher synaptic counts than both stroke males ($p < 0.05$) and stroke OVX females ($p < 0.05$) (Figure 23). There was no difference between OVX groups with and without stroke. A representative image is used to illustrate the punctae in Figure 28.

There was no observed correlations between lesion size and synapse number for any

groups and either time point (Figures 23 through 27).
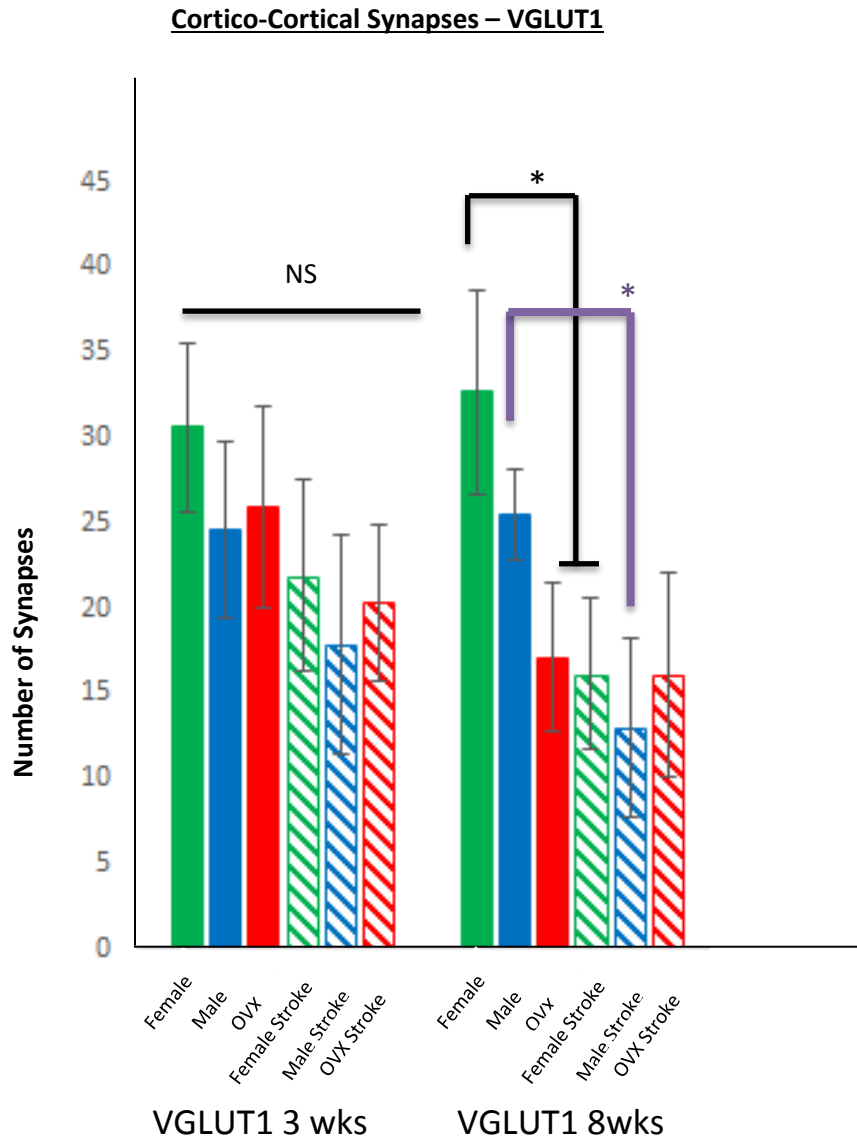
Figure 22. VGLUT1 Synapses. Above is VGLUT1 punctae co-localization with NMDAR and GFP-labeled dendrite quantification from both three weeks post-injection and eight weeks. * indicates $p < 0.05$. Error bars represent standard error of the mean. NS = Not significant.

Thalamo-Cortical Synapses – VGLUT2
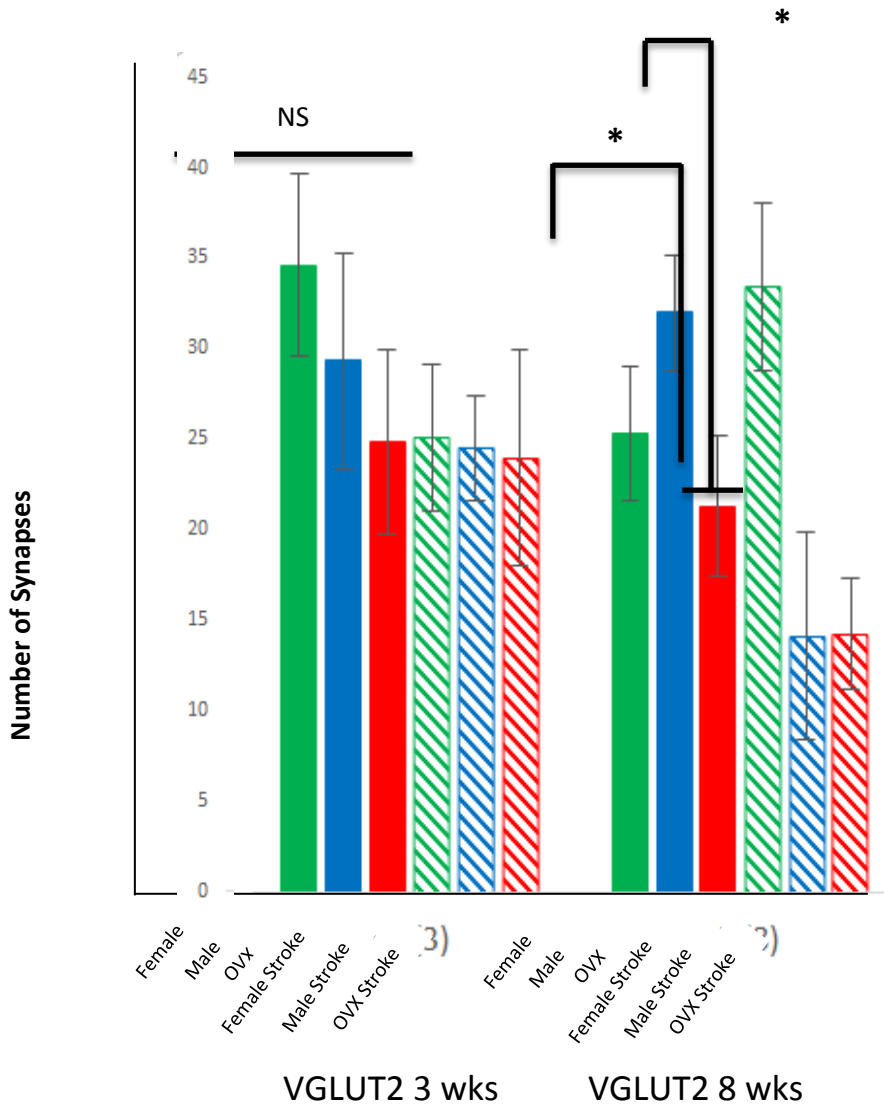
Figure 23. VGLUT2 Synapses. Above is VGLUT2 punctae co-localization with NMDAR and GFP-labeled dendrite quantification from both three weeks post-injection and eight weeks. * indicates p < 0.05. Error bars represent standard error of the mean. NS = Not Significant.
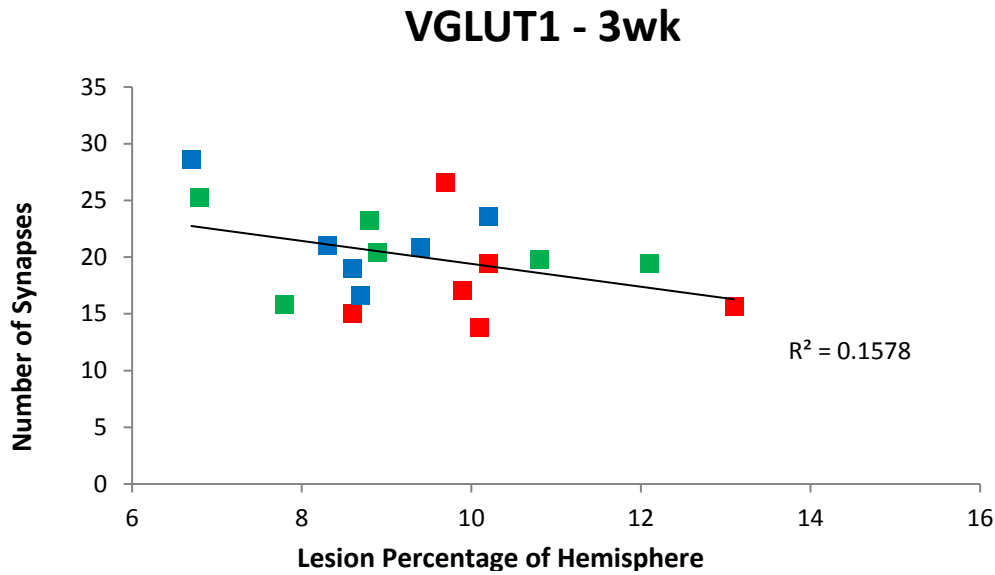
Figure 24. VGLUT1 3 week Correlation. VGLUT1 correlation showing weak correlation between lesion size and VGLUT1 punctae (green = intact female stroke, blue = male stroke, red = OVX stroke).
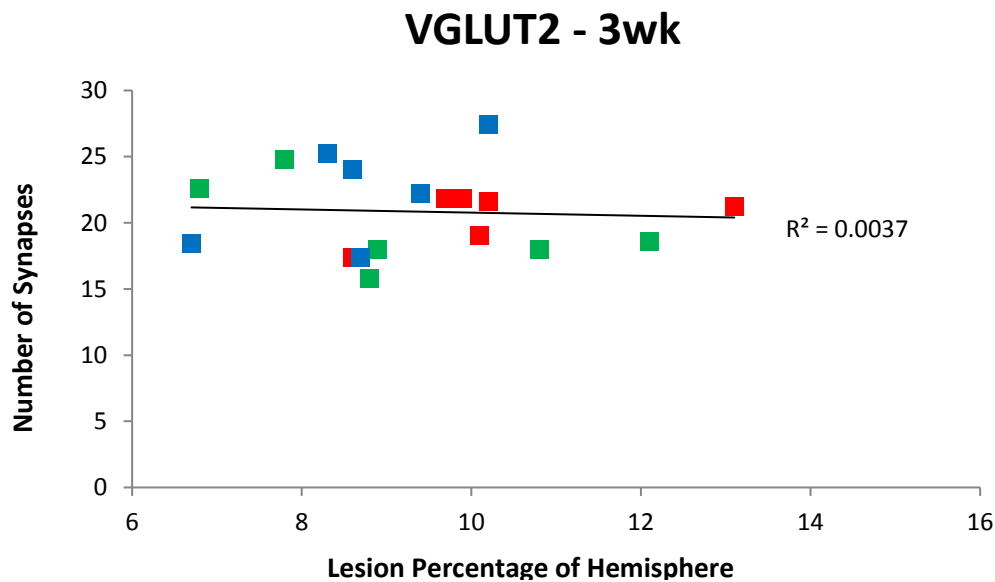
Figure 25. VGLUT2 week 3 Correlation. VGLUT2 correlation showing weak correlation between lesion size and VGLUT2 punctae (green = intact female stroke, blue = male stroke, red = OVX stroke).
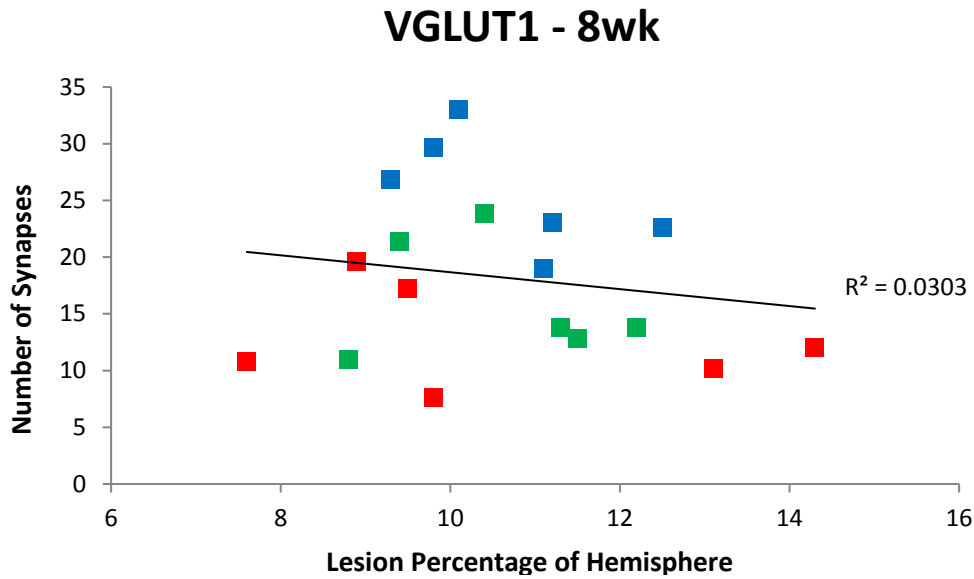
VGLUT1 - 8wk

Figure 26. VGLUT1 8 week Correlation. VGLUT1 correlation showing weak correlation between lesion size and VGLUT1 punctae (green = intact female stroke, blue = male stroke, red = OVX stroke).
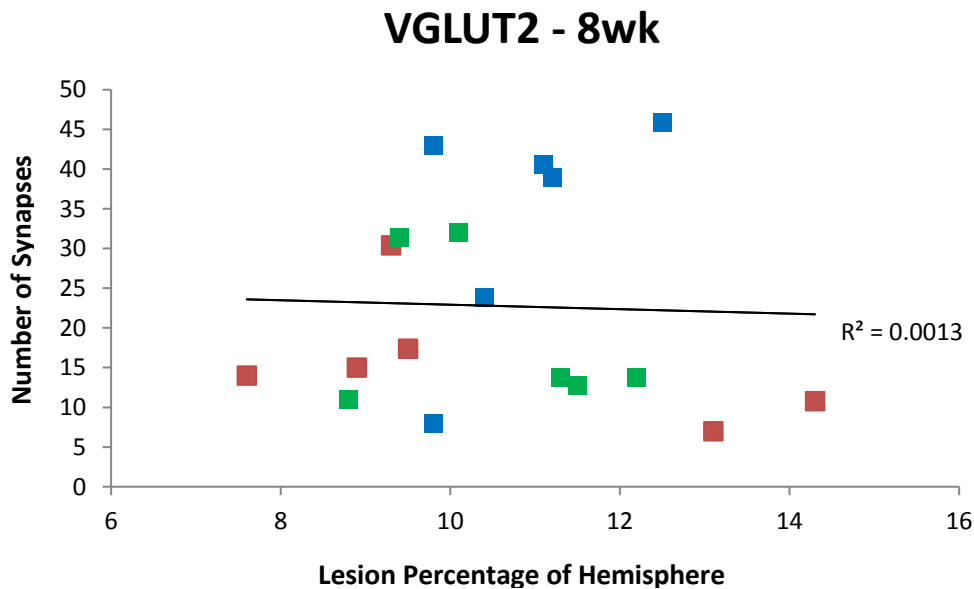
VGLUT2 - 8wk

Figure 27. VGLUT2 8 week Correlation. VGLUT2 correlation showing weak correlation between lesion size and VGLUT2 punctae (green = intact female stroke, blue = male stroke, red = OVX stroke).
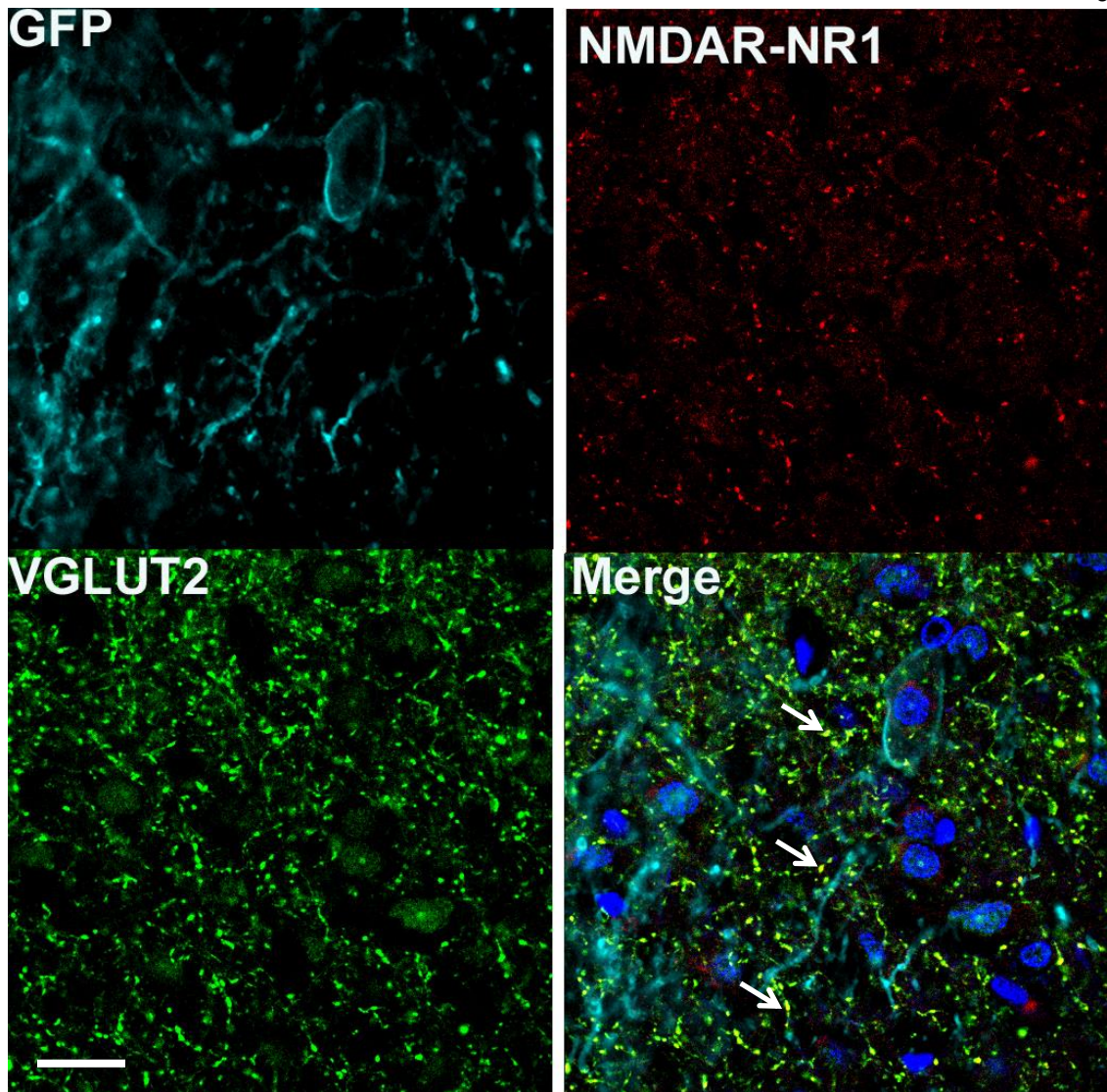
Figure 28. Intact Female Sample Image. Sample photomicrographs obtained from an intact female rat shows a very high density of NMDAR co-localizing (yellow) with a pre-synaptic marker on cyan dendrites (Blue = DAPI) in an intact female. Scale bar = 5um. Arrows denote three examples of yellow co-localization.

DISCUSSION

The results from this study showed that the number of excitatory synapses on layer V pyramidal neurons in the forelimb motor cortex are similar between aged males and female rats. However, this number is influenced by factors such as estradiol and related sex hormone levels and cortical injury. Specifically, when examining VGLUT1 synapses, which are indicative of cortico-cortical connections, there was a decrease in number by nine weeks post-OVX. VGLUT2 synapse number, which primarily arises from thalamo-cortical connections, did not show this decrease, supporting the idea that estradiol and related sex hormones may be more important for maintaining cortico-cortical connectivity.

When examining synapse number in the contralesional cortex post-stroke, both aged males and females showed a decrease in VGLUT1 synapse number by eight weeks post-stroke. This is consistent with others who have reported a decrease in dendritic complexity (Papadopoulos et al., 2002; Papadopoulos et al., 2006; Gillani et al., 2010). This decrease in expected due to the effects of the stroke lesion which would essentially eliminate and de-enervate any cortico-cortical connections to the homotopic forelimb motor cortex. In our study we did not observe a further decrease in VGLUT1 synapse number in the OVX group, suggesting that the effect of the stroke was not 'strong' enough to deplete this already low synapse number further than it had already dropped. This shows a potential time effect post-stroke of a loss of excitatory synapses in the pyramidal layer V motor cortex.

When examining VGLUT2 synapse number post-stroke, we found that only the aged male group showed a significant decrease in this synapse number. In fact, aged

females had significantly more VGLUT2 synapses than aged males or OVX females. Therefore, VGLUT2 synapses, which are a reflection of thalamo-cortical connectivity, were differentially affected by the cortical ischemic infarction, with aged males being more susceptible to this injury. This also shows a potential time effect post-stroke of a loss of excitatory synapses in the pyramidal layer V motor cortex.

These results also show that there was no correlation between lesion size and synapse counts for any protein or time point examined (Figures 24 through 27). This is possibly due to the consistent focal stroke size across all groups and both time points. Stroke size has been shown by others to be reduced by estradiol presence (Dang et al., 2011) but those levels used in that report were high (and exogenous) and not the low basal levels seen in our intact females (or possibly in our males through aromatization of testosterone). With a larger n there may be a stronger correlation.

Estradiol has been shown to play a role in upregulating dendrites, spines, and synapse proteins (Norbury et al., 2003; Minamo et al., 2008; Baudry et al., 2013). Estradiol has been shown to be neuroprotective in some studies (Dang et al., 2011; Kao et al., 2013) but the mechanism is still under investigation. It is proposed that adipose tissue could release stored sex hormone during stroke, which may have been seen at three weeks but not eight. It has been proposed to activate Akt (Spencer et al., 2008; Kao et al., 2013) and also proposed to upregulate cyclic-AMP response element binding protein (CREB) which can increase transcription of anti-apoptotic genes such as Bcl-2 (Lokuge et al., 2010) as well as activating both Akt and Bcl-2 (Li et al., 2013). Others have argued that estradiol's neuroprotection depends on the age of the organism (Leon et al., 2011) with an older animal not responding as well to estradiol administration as stroke treatment. Not yet investigated widely is the effect of testosterone on synapses,

but it is possible to aromatize it to estradiol via aromatase and have a similar effect as long as the receptors are present (Dubal and Wise, 2001; Etgen et al., 2011; Chen et al., 2013).

Some have speculated that estrogen and progesterone may act through other mediators, such as secondary response systems to enhance recovery following stroke. Stem cells are proposed to be activated by estradiol (Li et al., 2011; McClure et al., 2013) and estradiol has been proposed to even have effects in the peripheral nervous system (Nowacek and Sengelaub, 2006). Furthermore, progesterone has been shown to have neuroprotective and pro-growth abilities (Stein, 2001; Toung et al., 2004).

The neuronal synapse has much astrocytic involvement, especially in terms of neurotransmitter reuptake (Chisholm and Sohrabji, 2015) and plays a large part in aging, stroke, and potentially post-stroke repair (Chisholm and Sohrabji, 2015), with others proposing a sex difference as well (namely females have more astrocytic involvement on synapses) (Brandt et al., 2013).

In conclusion, the results from this study show that the number of excitatory synapses is affected by sex hormones and cortical lesion, and are dependent on the time point examined following stroke. Synaptic numbers are signifiers of axonal pathways and are important in measuring recovery. The infarct from a stroke (or similar lesion, such as from a traumatic brain injury) results in a death of neurons and decrease in synaptic connections. The contralesional hemisphere is believed to play a significant role in taking over some of the function lost by the damaged hemisphere. The differences observed in this aim show that synaptic differences do exist between groups and that time changes the numbers of these synapses. Inhibitory synapses is another area to

study as only excitatory synapses were studied here, and present another realm of altered synapses following injury, along with the fact that only the pyramidal cells of the layer V motor cortex were examined. Other areas may yield different results, such as another cortical layer or another brain region (thalamus or red nucleus for example). Different cell types may also yield other results, such as the inhibitory cells of the cortex or the non-pyramidal cells of layer V. The dendrites studied were also only basilar dendrites, and apical dendrites are another area of study.

CHAPTER FOUR

EFFECTS OF ANTI-NOGO-A IMMUNOTHERAPY ON SYNAPTIC NUMBER IN THE RAT FORELIMB MOTOR CORTEX AFTER STROKE

ABSTRACT

We have shown previously that anti-Nogo-A immunotherapy post-stroke increased functional recovery and axonal and dendritic complexity in the male rat (Papadopoulos et al., 2002; Papadopoulos et al., 2006; Markus et al., 2005; Seymour et al., 2005). Dendritic plasticity following stroke has been previously measured (Papadopoulos et al., 2002; Papadopoulos et al., 2006; Gillani et al., 2010), but which synapses these dendrites are making has not yet been investigated. Male, intact female, and OVX females received a stroke to the right hemisphere (because no behavioral analysis to determine limb preference was done) one week following OVX. Immediately following stroke a miniosmotic pump was inserted into the contralesional lateral ventricle with either 11C7 or control antibody (anti-BrdU) and AAV pre-labeling was injected into the contralesional forelimb motor cortex. Two weeks later pumps were removed. Rats were sacrificed 3 weeks after stroke for IHC. Results showed that anti-Nogo-A immunotherapy significantly increased excitatory synapse number for both VGLUT1 and VGLUT2 in male and intact female groups. OVX females did not have a significant increase following immunotherapy. Our results show that anti-Nogo-A immunotherapy is effective in increasing excitatory synapse number in the aged rat, but lack of estradiol or other sex hormones could impact its usefulness.

INTRODUCTION

The disabilities left by a stroke can be very damaging, with rehabilitation being the mainstay of alleviating those disabilities. Patients are often left with permanent deficits (Alaverdashvili and Whishaw, 2010; Wahl and Schwab; 2014; Wahl et al., 2014). The novel therapeutic Anti-Nogo-A immunotherapy has been shown in the past to reduce post-stroke motor deficits and increase functional recovery (Papadapoulos et al., 2002; Markus et al., 2005; Tsai et al., 2007). Most research into stroke recovery, and specifically Anti-Nogo-A immunotherapy, has focused on young, male rats. This makes an aged female investigation particularly noteworthy and necessary due to the apparent sex differences observed in the human population. This is true not only in stroke recovery, but also in neuronal connectivity (Bushnell, 2005) and the how synapses can contribute to motor recovery.

Of interest is the mechanism of how anti-Nogo-A immunotherapy increases functional recovery. While it has been known that both the Nogo receptors and estradiol act through the RhoA pathway (Kramar et al., 2013; Schwab and Strittmatter, 2014), it is unclear if there is any interaction between the two and the resulting synaptic changes that occur. Earlier work has shown that intrathecal infusion of the anti-Nogo-A antibody over two and four weeks increased the amount of VGLUT1 in the hippocampus in young male rats (Craveiro et al., 2013).

Much research into anti-Nogo-A immunotherapy has focused on behavioral and morphological changes (Papadapoulos et al., 2002; Papadapoulos et al., 2006; Gillani et al., 2010). The previous work in our lab has correlated improved functional outcome with both dendritic plasticity (Papadopoulos et al., 2006) and axonal plasticity (Padapolous et

al., 2002; Seymour et al., 2007; Tsai et al., 2007; Tsai et al., 2011) emanating from the

contralesional motor cortex after treatment with anti-Nogo-A immunotherapy post-stroke.

Specifically in these studies the dendritic and axonal plasticity targeted large pyramidal

neurons in layer V of the contralesional cortex. There neurons constitute the main

efferent output to important descending motor pathways controlling motor movements.

The novelty of this study then is the usage of confocal microscopy to determine 'triple

labeling' of a pre-synaptic marker (VGLUT), a post-synaptic marker (NMDAR), with the

NMDAR on the GFP expressed by pre-label layer V pyramidal dendrites. How much

anti-Nogo-A changes these excitatory cortical connections can also reveal sex

differences and if the behavioral effects of anti-Nogo-A could be mechanistically

explained by excitatory synaptic changes.

Therefore, the purpose of this study was to investigate the effect of anti-Nogo-A

immunotherapy on excitatory synaptic proteins in the contralesional motor cortex post-

stroke.

METHODS

All experimenters were blinded to rat group and treatment.

Animal Subjects

All animal experiments and protocols were approved by the Institutional Animal Care

and Use Committee of the Hines Veterans Affairs Hospital in Hines, IL. A total of 29

aged male and female Fischer 344 albino rats (18 months of age at start of the study)

were divided into three groups: normal males, normal (intact) females, and

ovariectomized (OVX) females (Figure 29). Rats were housed two to a cage and were

maintained in a 12 hour light/dark cycle with free access to water. Rats were given food and water ad libitum.

ANIMAL GROUPS AND NUMBERS

	Stroke + 11C7 (IHC)	Stroke + control Ab (IHC)	
Male	4	5	
Female	5	5	
OVX Female	5	5	
Total	14	15	29

Power analysis:

For this aim, we started with a power analysis and incorporated six experimental groups for comparison, setting alpha at 0.05. A sample size of 29 and 4-5 animals per group will be needed to achieve power of 0.8.

EXPERIMENTAL TIMELINE
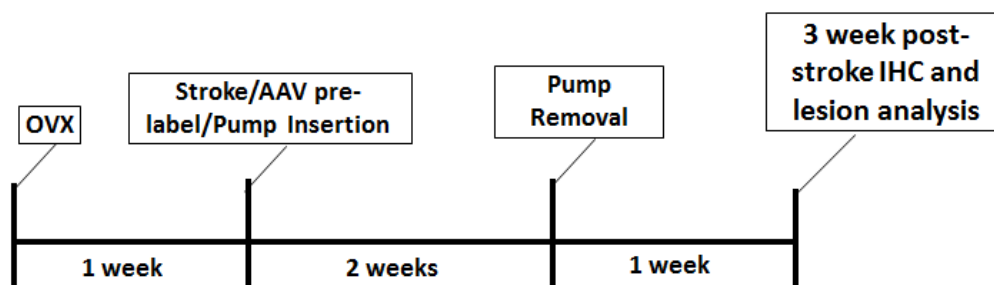
Figure 29: Experimental Timeline of Aim 2. Rats will receive a stroke to the right hemisphere and immediately after receive an AAV injection to the contralesional cortex followed by antibody pump placement into the contralesional lateral cerebral ventricle. Two weeks post-stroke pumps will be removed. Three weeks post-stroke, the rats will be sacrificed for IHC and lesion analysis.

Ovariectomy (OVX)

Ten females received an OVX in which both ovaries were removed. Rats were anesthetized using 2% isoflurane with oxygen and had both flanks shaved between rib cage and pelvic bones. A one centimeter incision was made in the skin followed by a one centimeter incision in the underlying muscle wall. Ovaries were found by pulling underlying fat tissue and isolating the ovary. Oviducts were tied off and cut right below the ovary body. The muscles and skin were then sutured.

Estradiol Serum Measurements Via Enzyme-linked Immunosorbent Assay (ELISA)

One week after ovariectomy all female rats had tail vein blood collected for ELISA estradiol serum level determination. Tail blood was spun down and the serum was collected then processed using Cayman Chemical's Estradiol EIA Kit (Item 582251 Ann Arbor, MI). The kit's detectable level for estradiol was 19pg/mL.

Stroke Surgery

MCAO was performed as described in our previous work (Markus et al., 2005; Gillani et al., 2010). Animals were anesthetized (2-3% isoflurane in oxygen) and secured in a stereotaxic frame. An incision was made through the scalp and underlying muscle and the skull was opened with a dental drill. The middle cerebral artery was then isolated and ligated by a 10-0 silk suture and transected with microscissors. Core body temperature was maintained at 37 degrees Celsius. The scalp was sutured closed and rats returned to their home cages.

Adeno-Associated Virus (AAV) Injection

AAV2/8-CMV-GFP-LDL-RCT was injected in the contralesional forelimb motor cortex to label layer 5 pyramidal neurons (2 injection sites (Coordinates: 3 mm lateral, bregma,

1.5 mm ventral; 2.5 mm lateral, 0.5 mm anterior, 1.5 mm ventral), 1 uL each injection

site; 1.3x10E12 vector genomes/ml titer). This paradigm has been used in our previous

publications (Pradhan et al, 2010) and results in excellent labeling of dendrites and

spines as the myristoylated GFP targets the neuronal cell membrane (Kameda et al,

2008) (Figure 30).

Antibody Infusion Via Osmotic Mini-pumps

Immediately following stroke and AAV injection, mini-osmotic pumps were placed for ICV

delivery of either purified mouse monoclonal anti-Nogo-A antibody (11C7, IgG1; 2.5

mg/mL concentration) or control antibody (anti-bromodeoxyuridine (BrdU), IgG1; 2.5

mg/mL concentration) infused at a rate of 12.5 micrograms/hour for two weeks as

described in our previous publications in the contralesional lateral ventricle

(Papadapoulos et al., 2002; Gillani et al., 2010). At the end of two weeks, pumps were

removed and rats were re-sutured. Pumps were confirmed to work by measuring amount

of antibody solution left over after pumps were removed. Any pump with over half the

amount of the starting volume (2 milliliters) was determined to be not working and the rat

was removed from the study.
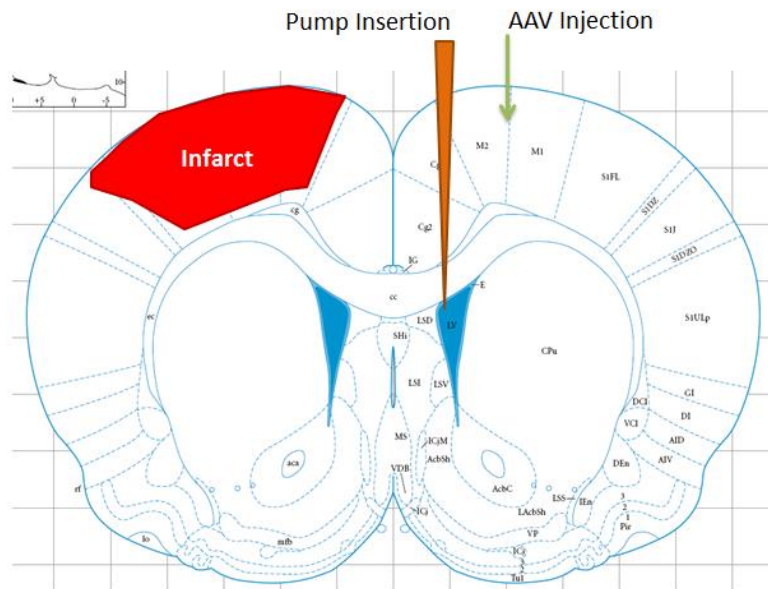
Figure 30. Injection and Pump Insertion sites. Shown here is the typical infarct size and location in the rat motor cortex, the AAV inject site, and the location of the mini-osmotic pump as it is inserted into the lateral ventricle.

Perfusion

Three weeks after stroke, rats were sacrificed and perfused with 0.9% saline followed by 4% paraformaldehyde. Brains were allowed to sit overnight in 4% paraformaldehyde before being placed in 30% sucrose until sinking. They were then frozen in tissue medium (OCT) and stored at -80 degrees Celsius.

Immunohistochemistry and Analysis

Brains were cut at 50 um on a -20 degree Celsius cryostat before lipofuscin removal and antigen retrieval. Primary antibodies against NMDA Receptors (NR1 subunit) (mouse IgG2, Life Technologies 32-0500), vesicular glutamate transporter 1 and 2 (VGLUT1 and 2) (guinea pig IgG1, EMD AB5905) and anti-GFP (rabbit, Life Technologies A11122) were used. The same sections from each brain were used for IHC (three from each brain) – 2nd, 8th, 14th sections starting collecting at the beginning of when the hemispheres connect (Bregma +1.66mm).

Sections were incubated for 30 minutes at room temperature in 50uM cupric sulfate in 100uM ammonium acetate for lipofuscin removal. Sections were then washed and incubated for 30 minutes at 80 degrees Celsius in pH 6 citrate buffer for antigen retrieval. Sections were washed and incubated at 4 degrees Celsius for 24 hours in one of the following parameters:

- Anti-VGlut1 (1:1000), Anti-GFP (1:1000), Anti-NMDAR (1:1000)

- Anti-VGlut2 (1:1000), Anti-GFP (1:1000), Anti-NMDAR (1:1000)

Sections were then washed and incubated in secondary antibody (goat anti-rabbit, IgG Life Technologies A-11070; goat anti-guinea pig, IgG VWR RL606-143-129; goat anti-mouse IgG Life Technologies A-11019) (1:500) for two hours at room temperature plus DAPI stain before washing and mounting on slides. Brain sections were mounted on slides and examined with a confocal microscope (Leica High Resolution Confocal TCS-SPE) and deconvolution software. EGFP-labeled pyramidal neurons from the sensorimotor forelimb cortex were identified by morphology. For each rat, z-stacks of dendritic segments from 5 different neurons were imaged in the basilar arbors, and analyzed for density of the specific synaptic protein-positive puncta co-localized with EGFP with ImageJ software (NIH) (Z-step .89um, 16 Z-steps). After full z-stack photo was taken, the image was flipped orthogonally (Figure 17) and the chosen dendrite was measured 40um from the soma so as to confirm co-localization (Figure 18 and 19). The co-localized puncta were counted and categorized according to placement on the dendritic shaft or spine within that 40um distance. Non-specific labeling was checked by incubating one section per rat in a secondary antibody only, primary antibody only, and cross-reactivity was checked by one section in each non-correct primary-secondary antibody pairing (ie, mouse primary antibody with anti-rabbit secondary antibody. No non-specific binding or cross-reactivity was observed.

Lesion Analysis

The lesion volume was analyzed on alternate Nissl stained sections. The area of the intact contralateral hemisphere minus the area of the intact ipsilateral hemisphere was multiplied by the distance between sections. Area of stroke was expressed as a percentage of the intact contralateral hemispheric volume.

Statistical Analysis

All data analysis was performed with GraphPad Prism Version 6.0 software (La Jolla, CA USA) for repeated-measures ANOVA and one-way ANOVA with Tukey post-hoc tests or SPSS (IBM, Armonk, NY, USA) for F statistics. A one-way ANOVA with Tukey's post hoc analysis was used to compare the mean of the IHC data and lesion analysis data after measuring F statistic. Aim 1 rat values for the stroke-only groups were used for synaptic number comparison. Correlative statistics was done for each rat on lesion size and synaptic plasticity using the correlation coefficient (r). A *p* value less than or equal to 0.05 will be considered significant and a correlation coefficient (r squared) of less than .50 will be considered weakly correlating.

RESULTS

Lesion analysis revealed that there were no group differences in lesion size, nor was there a lesion size difference due to antibody treatment (Figures 31 and 32). Previously, our lab has shown that anti-Nogo-A immunotherapy does not alter lesion size (Seymour et al., 2005; Tsai et al., 2007; Gillani et al., 2011).

The F test conducted revealed significant group differences [$F_{(3, 28)} = 19.74$, $p < 0.05$]. For VGLUT1 post-hoc, there was a significant increase seen in the number of synapses following anti-Nogo-A immunotherapy for intact females and males (Figure 37) ($p < 0.05$). There was no significant increase for OVX females ($p = .68$). The control antibody did not significantly increase synaptic counts for any group.

The ANOVA conducted revealed significant group effects [$F_{(3, 28)} = 22.65$, $p < 0.05$]. For VGLUT2 post-hoc, there was a significant increase seen in the number of synapses

following anti-Nogo-A immunotherapy for intact females and males (Figure 38) (p <

0.05). There was no significant increase for OVX females (p = .70). The control antibody

did not significantly increase synaptic counts for any group. Representative images are

shown in figure 33. A sample imaged zoomed to show synapses on the dendritic spines

is shown in figure 34.

It was also shown that there was no correlation between lesion size and co-localized punctae counts (Figures 37 through 40).
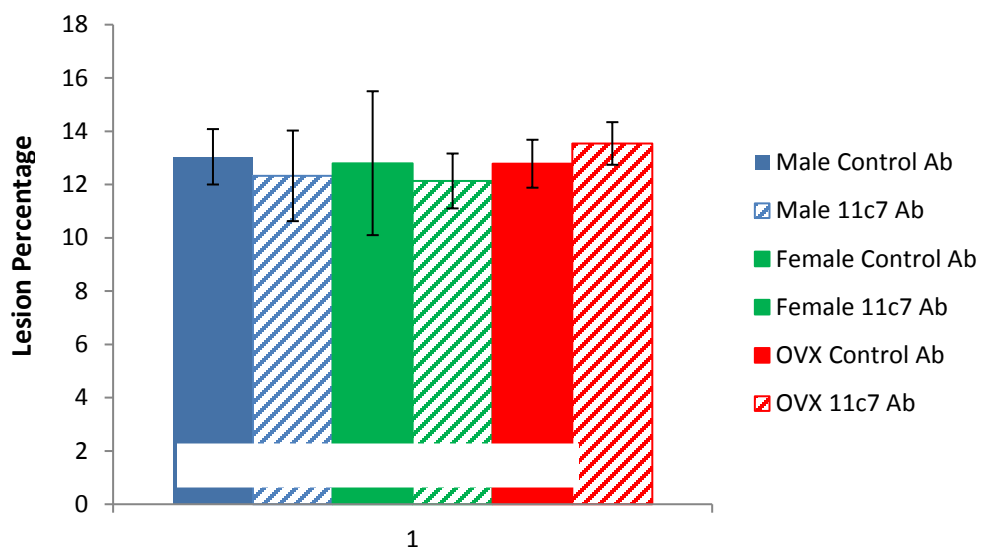
Figure 31. Aim 2 Lesion Analysis. There was no observed significant difference between groups or antibody treatment. Error bars represent standard error of the mean. Figure 32 shows a sample lesion size for the antibody treatment.

Figure 32: Representative pictures of typical lesion sizes and locations.
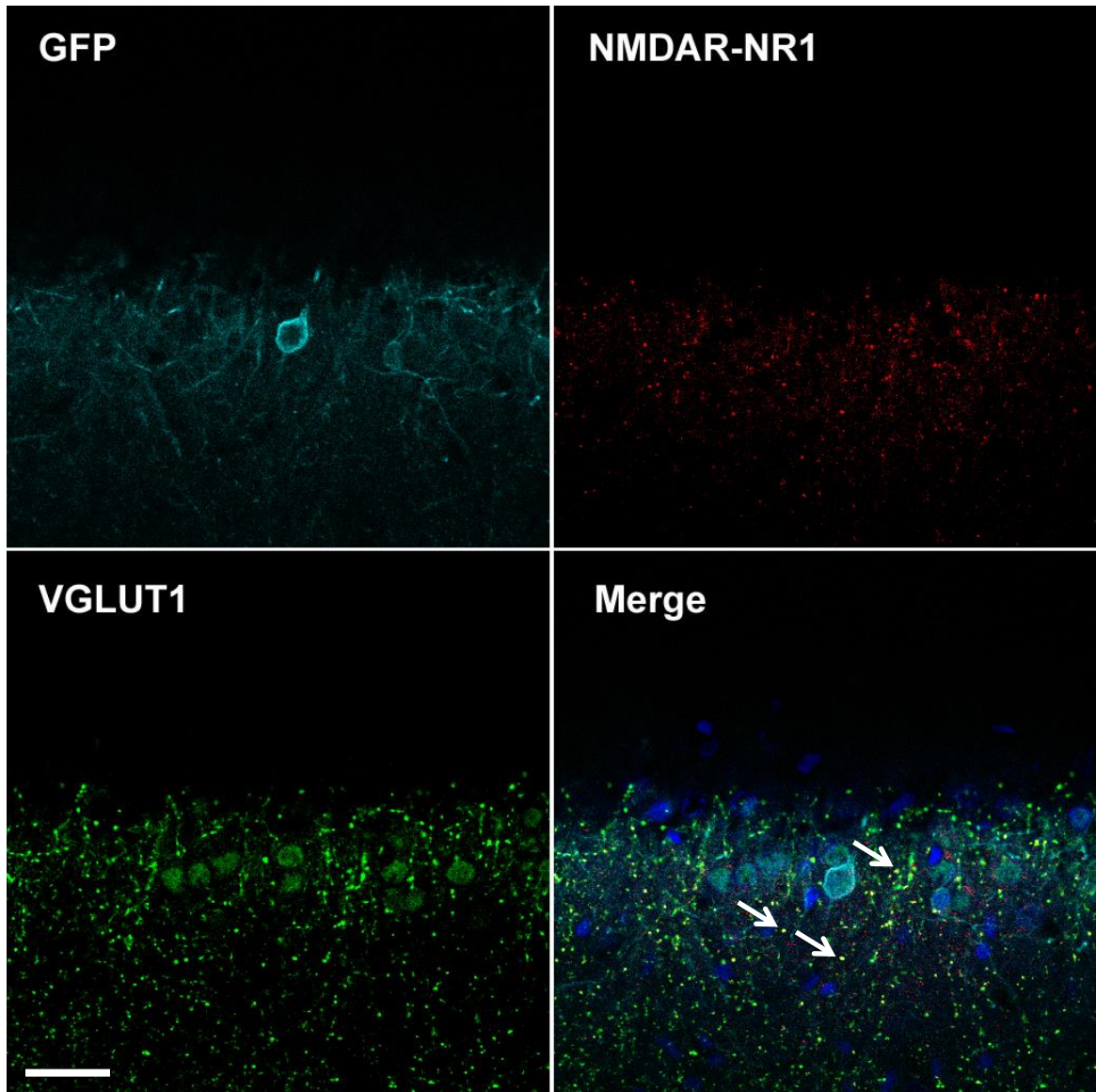
Figure 33. MCAO Intact Female Control Antibody Image. This sample photomicrograph is a representative image of a very high density of NMDAR co-localizing (yellow) with a pre-synaptic marker on cyan dendrites (Blue = DAPI) in a control antibody MCAO intact female. Scale bar 5um. Arrows denote three examples of yellow co-localization.

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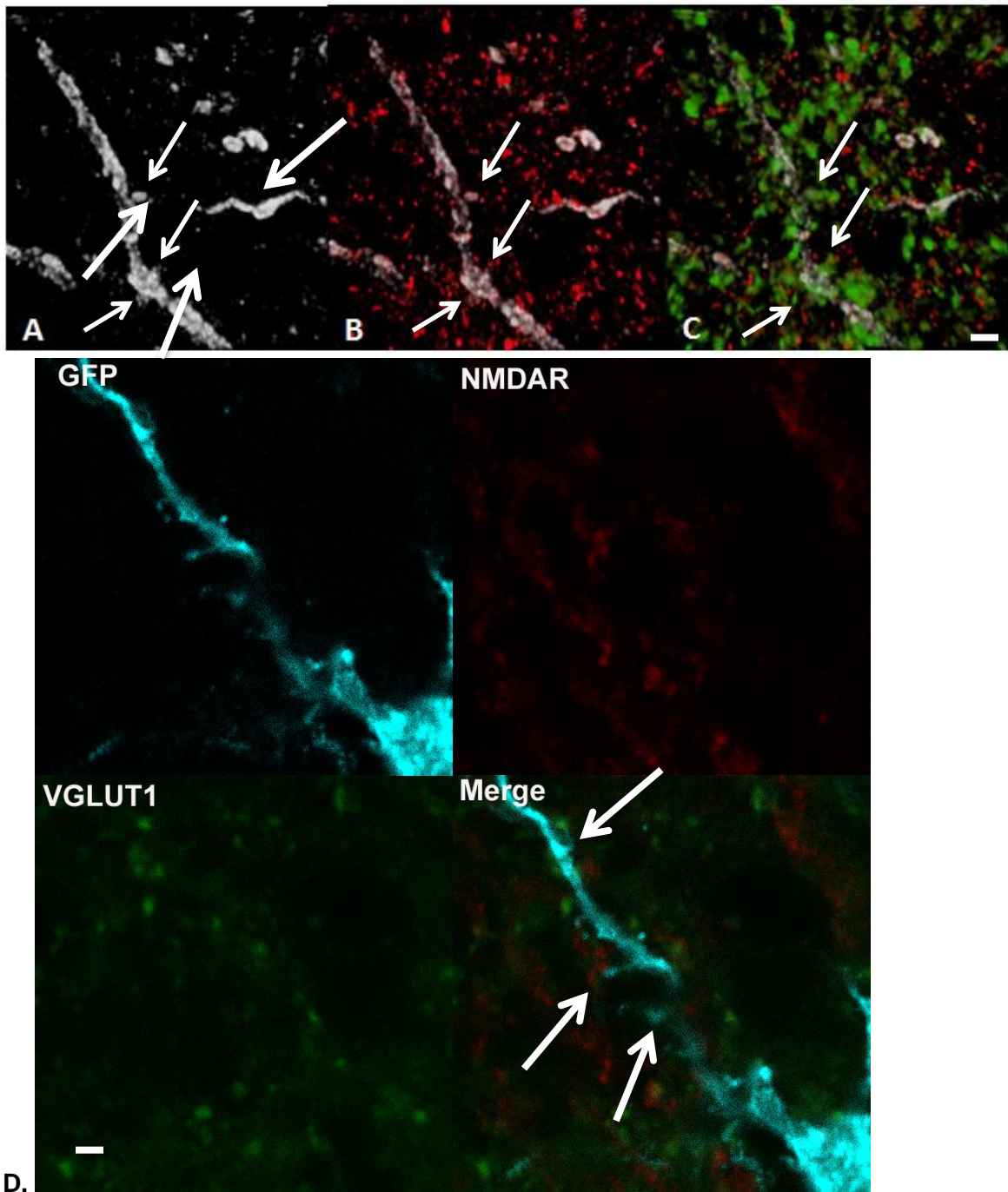
Figure 34. Dendritic Spines. With 400X magnification, some dendritic spines (arrows) can be seen in grey (A), along with NMDAR marking the post-synapse (red) (B), and VGLUT2 marking the pre-synapse (green) (C, merge). Figure D shows the green and red punctae on a dendritic spine connected to a soma (also 400X magnification) using VGLUT1. Scale bar 1um.
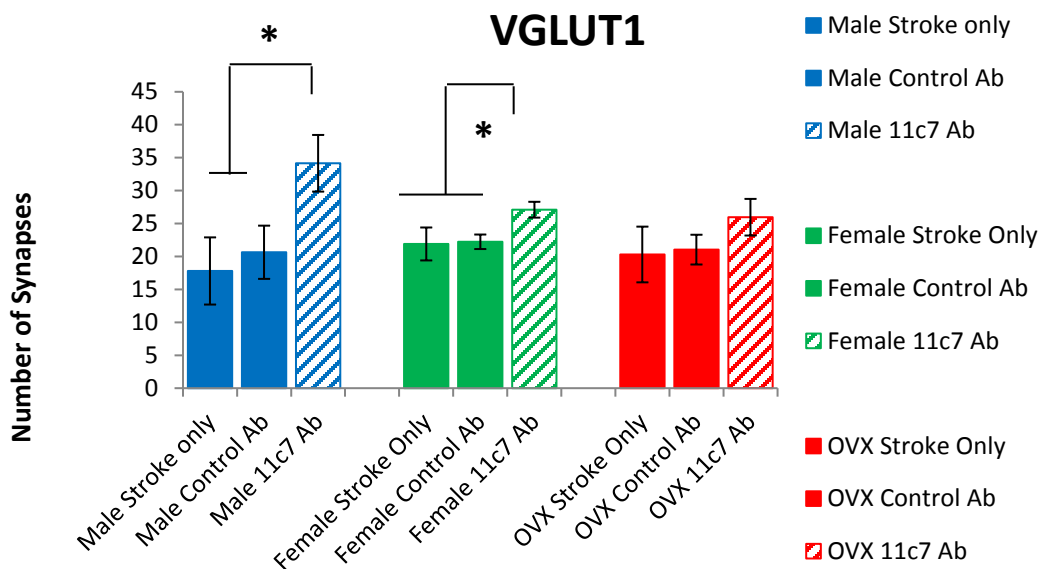
Figure 35. VGLUT1 Synapses After Immunotherapy. Above is a bar graph of VGLUT1 punctae co-localization with NMDAR and GFP-labeled dendrite quantification from three weeks post-injection in control antibody and 11C7-infused rats. Included from Aim 1 are the stroke-only group values. * indicates $p < 0.05$. Error bars represent standard error of the mean.
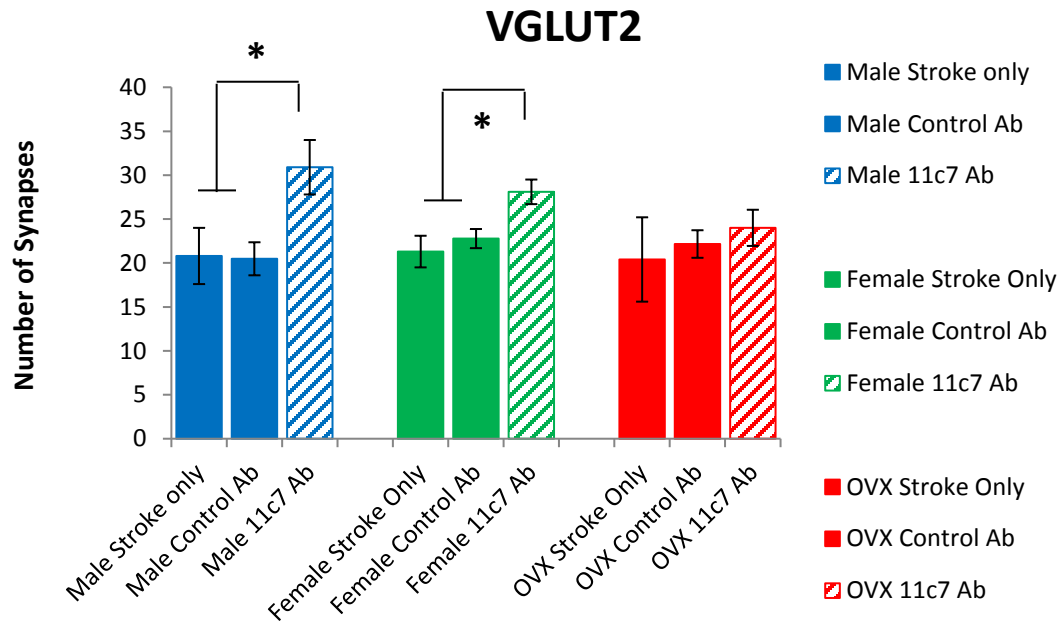
Figure 36. VGLUT2 Synapses after Immunotherapy. Above is a bar graph of VGLUT2 punctae co-localization with NMDAR and GFP-labeled dendrite quantification from three weeks post-injection in control antibody and 11C7-infused rats. Included from Aim 1 are the stroke-only group values. * indicates p < 0.05. Error bars represent standard error of the mean.
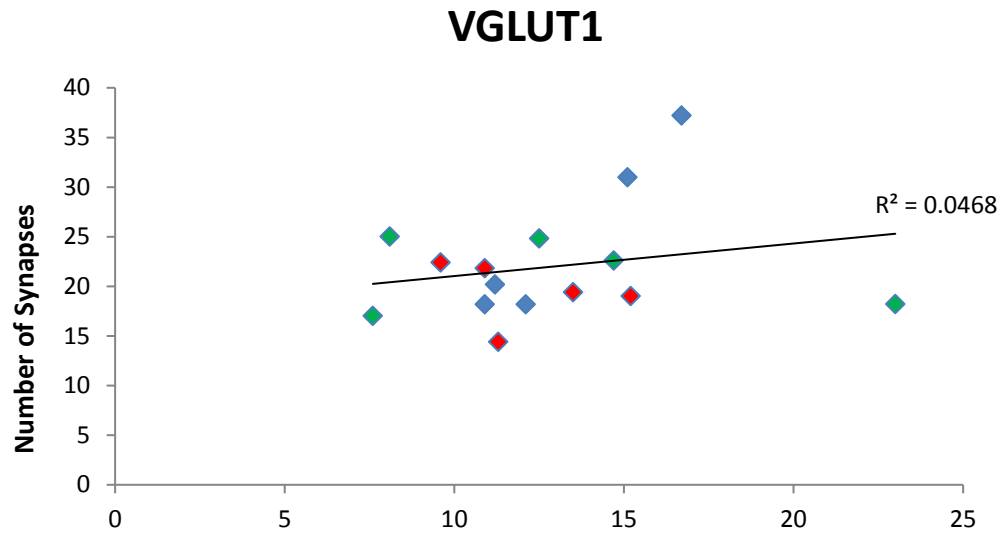
Figure 37. VGLUT1 with Control Antibody Correlations. VGLUT1 correlation showing weak correlation between lesion size and VGLUT1 punctae (blue = stroke males, green = stroke intact females, red = stroke OVX females) in control antibody-treated rats.
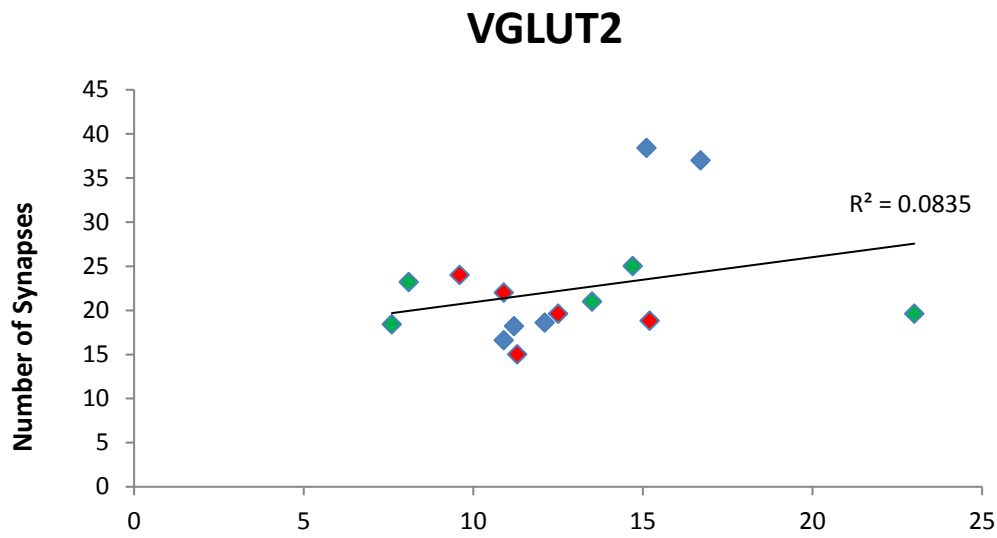
Figure 38. VGLUT2 with Control Antibody Correlations. VGLUT2 correlation showing weak correlation between lesion size and VGLUT2 punctae (blue = stroke males, green = stroke intact females, red = stroke OVX females) in control antibody-treated rats.
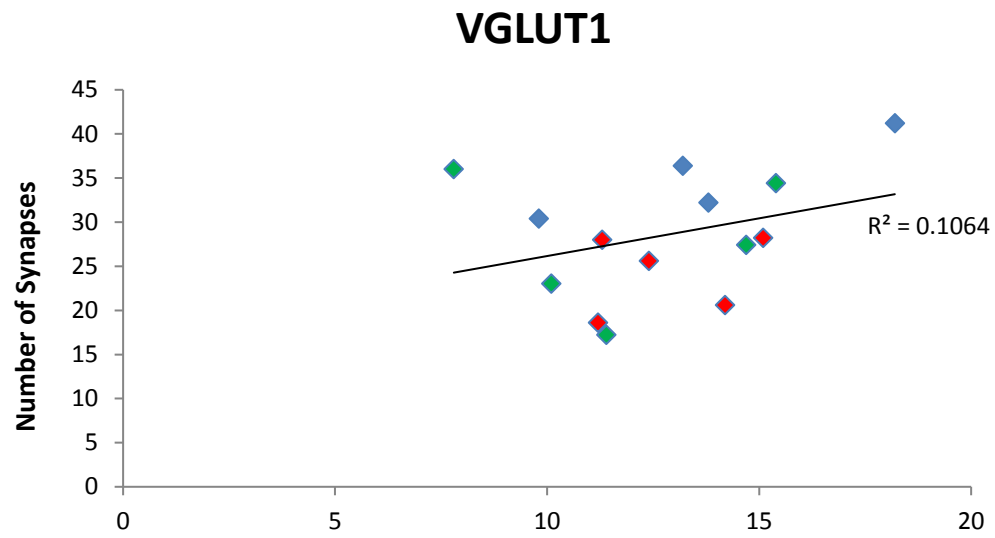
VGLUT1

Figure 39. VGLUT1 with 11C7 Antibody Correlations. VGLUT1 correlation showing weak correlation between lesion size and VGLUT1 punctae (blue = MCAO males, green = MCAO intact females, red = MCAO OVX females) in 11C7-treated rats.
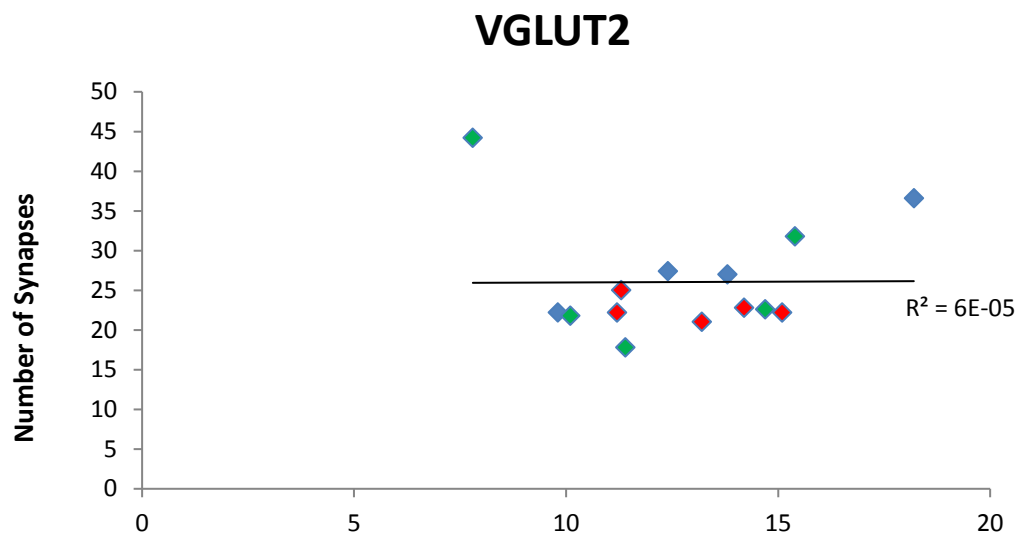
Figure 40. VGLUT2 with 11C7 Antibody Correlations. VGLUT2 correlation showing weak correlation between lesion size and VGLUT2 punctae (blue = MCAO males, green = MCAO intact females, red = MCAO OVX females) in 11C7-treated rats.

DISCUSSION

The results from this study show that synaptic increases were seen following 11C7 immunotherapy in male and intact female groups in both synaptic proteins examined (Figure 31). Males and intact females had a significant increase in VGLUT1 (Figure 35) and VGLUT2 (Figure 36) synapses on contralesional layer V neurons following stroke and anti-Nogo-A immunotherapy. OVX females were not shown to increase synapse number following immunotherapy for either protein.

The synaptic proteins we examined reflects part of the sensorimotor functional connectivity. VGLUT1 has been shown to be specific for cortico-cortical connections while VGLUT2 has been shown to be specific for thalamo-cortical connections (Kaneko, 2013). Based on previous work, the cortico-cortical connections could be to and from the damaged hemisphere, as it has been shown that axon fibers cross the midline to innervate the damaged infarct side (Papadapoulos et al., 2002; Papadapoulos et al., 2006). Using fMRI, our lab has shown that the thalamus is activated following a stroke and anti-Nogo-A immunotherapy in aged male rats (Markus et al., 2005), which may explain the VGLUT2 (thalamo-cortical) synaptic increase that we report with aged males and intact females.

The lack of increase in the OVX female VGLUT synapses following 11C7 treatment could be explained by the lack of estradiol and related sex hormones. Estrogen increases dendritic and synaptic number (Schnell et al., 1994; Scharfman and MacLusky, 2006; Srivasta et al., 2013). Estradiol signaling has been shown to lead to increased levels of CREB (Wu et al., 2005), which as a transcription factor transcribes

many pro-growth genes (Wu et al., 2005). Nogo-A signaling has been shown to decrease or dephosphorylate CREB, decreasing its activity (Schwab, 2010).

Progesterone has been shown to reduce neurite growth inhibitors following ischemia (Espinosa-Garcia et al., 2014) and the presence of this and estradiol was expected to decrease Nogo-A inhibition. We have observed that there was no significant difference in VGLUT1 and VGLUT2 co-localization (in either antibody group) due to low estradiol (which could result in low progesterone) in the OVX group. Intact females, with a small amount of estradiol, did have an increase in synapse number following 11C7 treatment. Furthermore, an earlier study reported that 11C7 infusion increased VGLUT1 expression in young adult Long-Evans male rats in the hippocampus following intrathecal infusion (Craveiro et al., 2013) using Western blot analysis. We have found in terms of our triple labeling that this is also shown for the aged males and intact females, but not aged OVX females.

Lesion analysis showed no significant group differences. This can be expected based on consistent lesion sizes with this model. This was also seen in our earlier studies using anti-Nogo-A immunotherapy (Markus et al., 2005; Seymour et al., 2005; Gillani et al. 2010). Estradiol and other sex hormones had no noticeable neuroprotective effect, and this is believed to be due to the old age of the intact females. It has been shown in young adult male and OVX rats that estradiol can reduce infarct size (Dang et al., 2011). In terms of correlation between lesion size and synaptic protein punctae, there was no correlation found. This was expected because lesion size is not believed to have an impact on synaptic protein concentration. If such a correlation exists, it was not detected

here. The study of other proteins involved in synaptic connectivity (such as AMPA receptors, PSD95, or inhibitory synapse markers) may be of use in the future.

It has been shown here that following stroke, aged males and intact females responded better to 11C7 antibody treatment in terms of increased VGLUT1 and VGLUT2 synapses on pyramidal layer V basilar dendrites. The OVX females were shown to have no significant difference between control or 11C7 antibody infusion. This could correlate with the human clinical data that suggests aged women recover less than men following a similar stroke (Bushnell, 2006; Bushnell, 2008; Bushnell et al., 2014). This would be due to their low estrogen levels. An underlying mechanism has been postulated that estradiol plays a role in actin stabilization through a similar mechanism of Nogo-A via RhoA (Wu et al., 2005; Kramer et al., 2013). While estradiol has been shown to have many positive effects on dendritic and spine growth and increases NMDAR levels (Schnell et al., 1994; Scharfman and MacLusky, 2006; Srivasta et al., 2013) it is postulated that estradiol may stabilize actin in this way. In the present study, different results were seen in both the intact (with a low level of estradiol) and OVX (no detectable serum estradiol) making the level of estradiol present an important factor. The difference in estrogen receptor type and concentration between male and female rats may also be a resulting difference here. Estradiol, even at a low level in the intact female group, would have pro-growth properties for the dendrites and resulting synaptic connections, along with inhibiting apoptosis (Wu et al., 2005). Sex hormones were thus shown to play a positive role in this study, as evidenced by the lower synaptic counts in the OVX female group.

In conclusion, it has been shown here that anti-Nogo-A immunotherapy increased the number of excitatory synapses in the forelimb motor cortex at three weeks post-stroke for aged females and males, however OVX females were shown to have no significant increase in synapse number over the control antibody. The low level of estradiol as seen in intact aged females may play a role in increasing their responsiveness to anti-Nogo-A immunotherapy in terms of excitatory synapse number.

CHAPTER FIVE

SEX DIFFERENCES IN LEARNING SKILLED MOTOR TASKS AND POST-STROKE
RECOVERY IN THE AGED RAT

ABSTRACT

Sex-based differences in learning and recovery from stroke are an area of research not yet fully explored, especially with regard to aged rats performing sensorimotor tasks. In the human clinical population, post-menopausal females have been shown to recover from stroke less successfully than age-matched males, and therefore studying sex differences in rat models of stroke is clinically relevant. Our work has shown that aged female rats learn the sensorimotor tasks faster than age-matched males, but following a stroke, age-matched males recovered better. We then sought to correlate these behavioral results with dendritic plasticity.18 month-old Fischer 344 male and female aged rats were divided into four groups: ovariectomized (OVX) females, intact females, sham OVX surgery males, and intact males. Pre-stroke, animals were trained in the skilled forelimb reaching task. The rats then underwent focal ischemic stroke via middle cerebral artery occlusion (MCAO) to affect the sensorimotor cortex associated with the preferred forelimb. Rats were then tested on the behavioral tests for eight weeks to assess post-stroke recovery, and then sacrificed for Golgi-Cox staining and dendritic characterization of contralateral pyramidal layer V motor neurons for complexity, length, number and the density of dendritic spines. Analysis of dendritic arborization showed that pre-stroke, intact females had the greatest number of dendrites

in the higher complexity levels whereas the OVX females had the lowest in the earlier complexity levels. Intact females also had the greatest length and number of dendrites whereas the OVX females had the lowest. OVX females had the lowest density of spines compared to all other groups. Post-stroke, all groups showed a decrease in complexity with the largest decrease seen in the intact female group. In conclusion, our results show that removing ovary-produced estradiol and related sex hormones results in less dendritic complexity, number, length and spine density in the pyramidal layer V motor cortex neurons in aged female rats.

INTRODUCTION

Ischemic stroke is a prevalent cause of death and disability worldwide, with an increase in death and disability as age increases (Alkayed et al., 1999; Lewis et al., 2012). Each year more than 795,000 people in the US have a stroke, with about 130,000 resulting in death. (Bushnell et al., 2014). Recovery following stroke also shows a sex difference, with a pre-menopausal woman recovering better than an age-matched man, while following menopause the opposite is true (Bushnell et al., 2014). There have been many hypotheses for why this occurs, but the major cause seems to be the decrease in estradiol (and its related hormones, such as progesterone) that occurs during menopause (Bushnell, 2006; Bushnell, 2008). Estradiol has been found to not only be neuroprotective (Dang et al., 2011) but also useful in reducing apoptosis (Wu et al., 2005), which the loss of estradiol potentially disrupting both of these mechanisms.

Of particular interest are the differences in stroke recovery observed between the sexes and the resulting difference in functional recovery observed. The Women's Health Initiative (WHI) has set guidelines for use of female research animals to better

understand women's health, which until now has been mainly focused on male animals (Bushnell et al., 2014). Thus a relevant animal model is necessary to evaluate post-stroke recovery and any potential brain changes.

Based on these reported sex differences we examined the potential motor learning speed differences apparent in the aged rat, along with their resulting post-stroke functional recovery and the morphological changes in their pyramidal layer V forelimb motor cortex dendrites. Stroke has been shown before to decrease contralateral total dendritic length and complexity (Papadapoulos et al., 2002; Papadapoulos et al., 2006; Gillani et al., 2010) but has since only been looked at in male rats. Golgi stain was also used to show that estradiol increased spine density and dendrite length in the hippocampus (Brocca et al., 2013). An aged male rat study showed a motor recovery following stroke using anti-Nogo-A (Markus et al., 2005) but did not include females. Measurement of the speed of learning the skilled forelimb reaching task is novel to this study, along with the inclusion of intact female and OVX female groups. The inclusion of these new groups will allow for a clinically relevant study of aged stroke functional recovery and resulting dendritic morphology differences.

METHODS

All experimenters were blinded to rat group and treatment.

Animal Subjects

All animal experiments and protocols were approved by the Institutional Animal Care and Use Committee of the Hines Veterans Affairs Hospital in Hines, IL. A total of 73 aged male and female Fischer 344 albino rats (18 months of age at start of the study)

were divided into four groups: normal males, sham ovariectomy (OVX) surgery males

(anesthetized, flanks shaved, and skin/muscle walls cut before suturing together),

normal (intact) females, and ovariectomized (OVX) females (Figure 43). Rats were

housed two to a cage and were maintained in a 12 hour light/dark cycle with free access

to water. Rats were food deprived to 95% of their body weight in order to encourage

compliance with behavioral testing (Figure 41).

RAT GROUPS AND NUMBERS

	No Stroke	Stroke	
Male	6	6	
Sham OVX Males	6	6	
Female	6	7	
OVX Female	6	6	
Total	36	37	73

Power analysis:

For this aim, we started with a power analysis and incorporated eight experimental

groups for comparison, setting alpha at 0.05. A sample size of 72 (behavioral tests) and

6 animals per group will be needed to achieve power of 0.8.
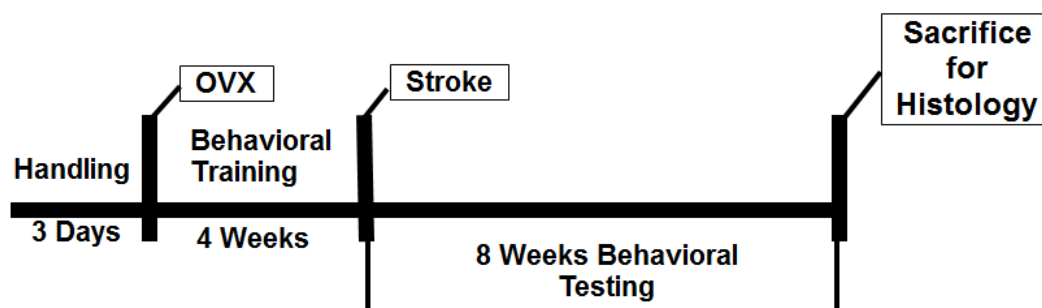
Figure 41. Experimental Timeline of Aim 3. Rats will receive four weeks of behavioral training on the skilled forelimb reaching task and skilled ladder rung walking task before receiving a stroke to the hemisphere used for reaching. Post-stroke, rats will be tested for eight weeks on both behavioral tests before being sacrificed for Golgi-Cox histology.

Ovariectomy (OVX) and Sham OVX Males

Twelve females received an OVX in which both ovaries were removed. Rats were

anesthetized using 2% isoflurane with oxygen and had both flanks shaved between the

rib cage and pelvic bones. A one centimeter incision was made in the skin followed by a

one centimeter incision in the underlying muscle wall. Ovaries were found by pulling

underlying fat tissue and isolating the ovary. Oviducts were tied off and cut right below

the ovary body. The muscles and skin were then sutured. Some of the males received a

'sham OVX' in which the flanks were shaved and the skin and muscle incisions made as

above before being sutured.

Estradiol Serum Measurements Via Enzyme-linked Immunosorbent Assay (ELISA)

One week after ovariectomy all female rats had their tail vein blood collected for ELISA

estradiol serum level determination. Tail blood was spun down and the serum was

collected then processed using Cayman Chemical's Estradiol EIA Kit (Item 582251 Ann Arbor, MI). The kit's detectable level for estradiol was 19pg/mL.

Skilled Forelimb Reaching Task

The skilled forelimb reaching task, as described in our previous studies (Papadopoulos et al., 2002; Markus et al., 2005; Tsai et al., 2007), was performed by all rats in a pre-stroke training period (until baseline of 14 out of 20 pellet successes for three consecutive days) and a post-stroke testing period (for eight weeks following stroke). Rats were placed in a Plexiglas chamber (30 x 36 x 30 cm) with a rectangular opening (1.5 x 3 cm) with a Plexiglas shelf attached outside the opening. Rats then learned to reach through the opening to grasp small sucrose pellets place on the shelf. The first week of testing involved two rats to a box at once then continue from then on as one rat per session. A success was defined as the rat reaching through the opening, grasping the pellet, and bringing it to its mouth. Rats were tested starting two days post-stroke. Rats were filmed at baseline and 1, 3 and 8 weeks post-stroke.

Skilled Horizontal Ladder Rung Walking Task

The rats' accurate forelimb placement was examined by assessing the ability to cross a one-meter long runway of small bars with randomly assigned gaps as described by Metz (Metz & Whishaw, 2002). Errors in foot placement of the stroke-impaired forelimb were analyzed (total number of errors/total number of steps) and the time to cross the grid was recorded. Latency was measured as the time it took rats to walk completely across the ladder. Rats were filmed at baseline and 1, 3 and 8 weeks post-stroke.

Stroke Surgery

MCAO was performed as described in our previous work (Markus et al., 2005; Gillani et al., 2010). Animals were anesthetized (2-3% isoflurane in oxygen) and secured in a stereotaxic frame. An incision was made through the scalp and underlying muscle and the skull opened with a dental drill. The middle cerebral artery was then isolated and ligated by a 10-0 silk suture and transected with microscissors. Core body temperature was maintained at 37 degrees Celsius. The scalp was sutured closed and rats returned to their home cages.

Perfusion for Golgi-Cox Staining and Reacting

8 weeks after behavioral testing, rats were sacrificed and perfused with 0.9% saline. After extraction, brains sat in Golgi-Cox stain for two weeks (allowing for staining of a random group of neurons in their entirety) then were submerged in 30% sucrose until sinking. After sinking the brains were cut into 200um thick sections, placed on slides, and reacted by the modified Golgi-Cox method (Gibb and Kolb, 1998). Sections were placed in ammonium hydroxide for 30 minutes before being transferred to Kodak Fix Solution for 30 minutes. Sections were then dehydrated in increasing concentrations of ethanol before being placed in absolute ethanol for 15 minutes. Sections were then placed in a mixture of HemoDe, chloroform, and absolute ethanol before being placed in HemoDe. Sections were then coverslipped with permount and allowed to dry. Sections were examined for lesion size analysis and dendritic profile tracing in the contralesional forelimb motor cortex using Neurolucida.

Lesion Analysis

The lesion volume was analyzed on alternate Golgi stained sections. The area of the intact contralateral hemisphere minus the area of the intact ipsilateral hemisphere was multiplied by the distance between sections. Area of stroke was expressed as a percentage of the intact contralateral hemispheric volume.

Histological and Dendritic Analysis

Basilar dendrites were traced at 630X magnification in the pyramidal layer V forelimb motor cortex and visual cortex layer II pyramidal neurons with the aid of Neurolucida software (Figure 42). Criteria for inclusion in the analyses is that the neuron has to be well impregnated with stain, unobstructed by other dendrites, blood vessels or glial cells, and the dendritic arborizations are intact and visible in the plane of section. For all analyses the slides were coded and investigators were blind to the treatment group.

Twelve neurons from each animal were traced. Dendritic trees were analyzed for total dendritic length and total number of branch order. Branch order was determined by considering that branches emanating directly from the cell body are first order; once bifurcation occurs, two secondary dendrites are formed and so on. Corresponding procedures were performed in the occipital cortex as a control measurement. Dendritic spine densities were analyzed by counting visible spines along a measured 50um section of dendrite (starting at the soma). Spine density was expressed as the number of spines per 10 µm.
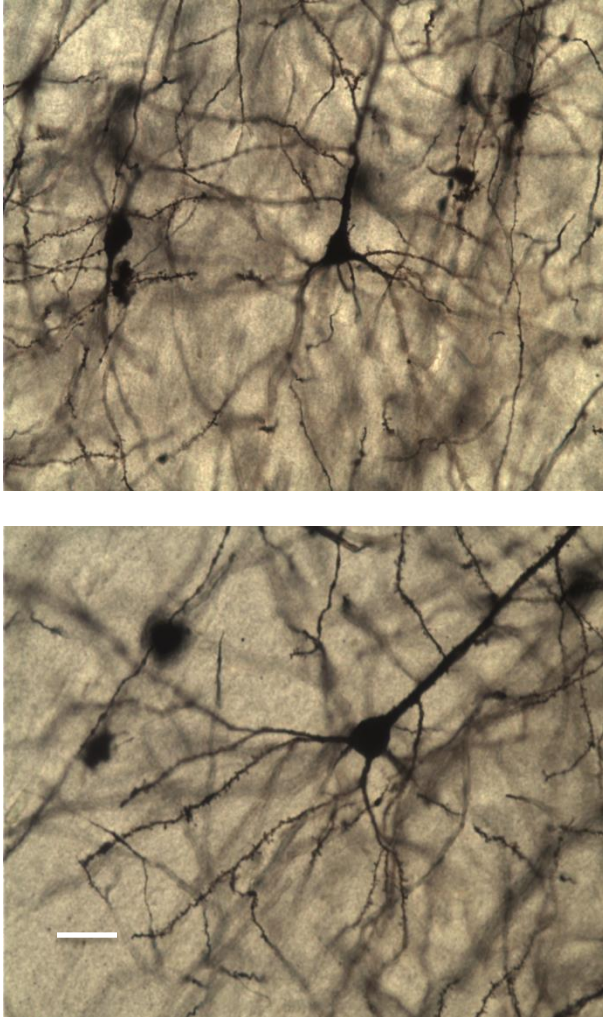
Figure 42. Golgi-Cox Stain. Representative photomicrographs of neurons stained by Golgi-Cox in the contralesional forelimb motor cortex. Note the defined dendrites and spines visualized by the stain, which through a heavy metal makes the neuron dark. Scale bar 10 um.

Statistical Analysis

All data analysis was performed with GraphPad Prism Version 6.0 software (La Jolla, CA USA) for repeated-measures ANOVA and one-way ANOVA with Tukey post-hoc tests or SPSS (IBM, Armonk, NY, USA) for F statistics.

The main effect of group, main effect of week, and Group x Week (reported as F value) were used. Afterwards, a repeated-measures ANOVA was used to compare the rate of improvement and learning on the behavioral tasks and a one-way ANOVA with Tukey's post hoc analysis was used to compare the mean of success scores, the Golgi-Cox data and lesion analysis data. A p value less than or equal to 0.05 was considered significant.

RESULTS

Behavioral Data

Skilled Forelimb Reaching Task

Pre-stroke, there was a significant sex difference in learning the task over four weeks, with males learning the task significantly slower than both female groups (Figure 43). A group difference [$F_{(3, 66)}$ = 31.22, p < 0.05], week difference [$F_{(2, 22)}$ = 17.49, p < 0.05] and Group x Week difference [$F_{(6, 66)}$ = 50.03, p < 0.05] were seen. There was no significant difference between the intact and OVX female. Both male groups were significantly slower than both female groups at weeks two and three. Both male groups also had significantly lower successful reach percentages at weeks two and three. However, all groups were able to attain the same number of pellets by four weeks of training, with no significant difference between groups at that point (Figure 44). There was a slight majority of left-handed rats over right-handed, but this was equally spread amongst all groups.

The F test conducted revealed significant differences by group [$F_{(3, 66)} = 66.42$, $p < 0.05$], week [$F_{(2, 22)} = 13.58$, $p < 0.05$], and Group x Week [$F_{(6, 66)} = 52.17$, $p < 0.05$] for post-stroke recovery. Post-stroke, there was also a sex difference in functional recovery of pellet success scores, with OVX females performing the worst over 8 weeks following stroke while intact females were slightly and significantly better (Figure 43). A significant difference of week for OVX females at weeks 5, 6, 7 and 8 was revealed. Both male groups reached successfully significantly more at all time points, with intact females reaching significantly better than OVX females at weeks five through eight.

Both female groups had significantly lower successful reach percentages at weeks two, three, and four. There was no significant difference between groups from week five onwards for group, where males continued to reach successfully at around 50% while both female groups improved their reach percentages. Although both female groups improved their reach percentages, the total successful reaches remained significantly lower than both male groups (Figure 45).
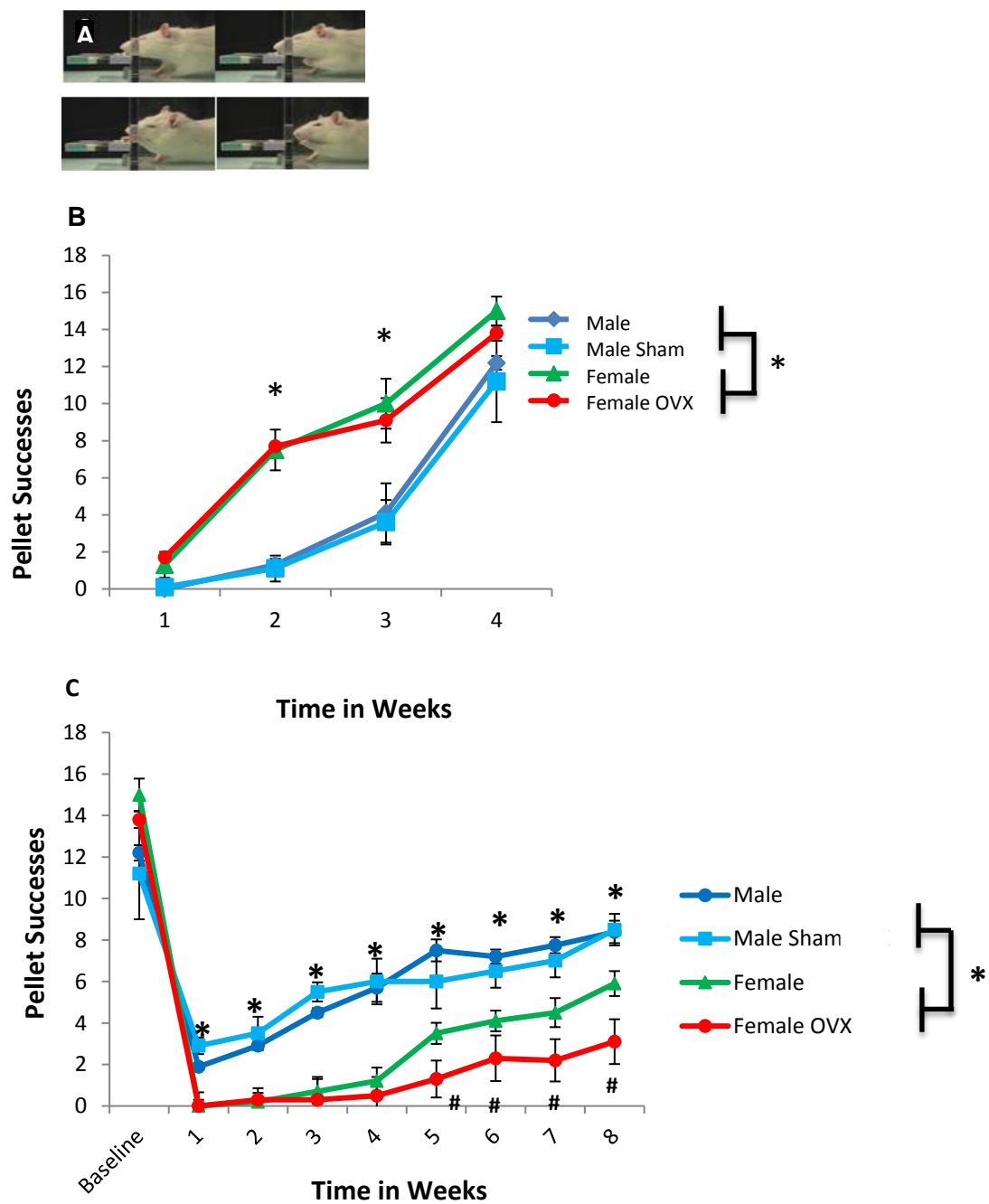
Figure 43. Skilled forelimb reaching task in the aged rat. (A) Rat reaching for pellet. (B) Successes pre-stroke and (C) post-stroke. Intact females and OVX females learn faster than males pre-stroke but have slower recovery post-stroke. * = p < 0.05 (Repeated Measures ANOVA with Tukey Post-Hoc) comparing both male groups against both female groups. # = p < 0.05 (Repeated Measures ANOVA with Tukey Post-Hoc) comparing OVX females against all groups. Error bars: standard error of the mean.
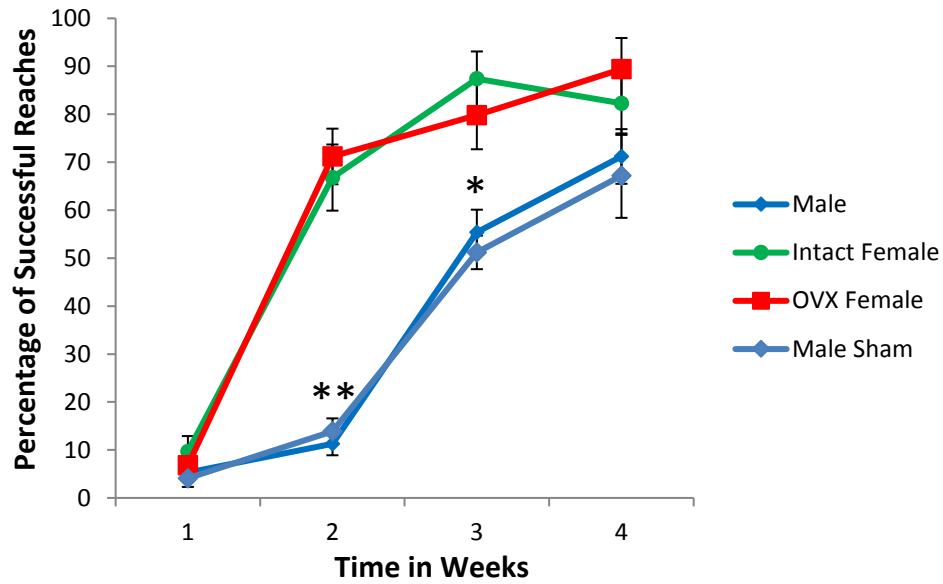
Figure 44. Reaching Percentages Pre-stroke. Percentages of successful reaches during the pre-stroke learning phase. Males had significantly lower success percentages than females during weeks two and three. Error bars represent standard error of the mean. * signifies $p < 0.05$, ** signifies $p < 0.01$ (One-way ANOVA with Bonferroni post hoc test) compared with female groups.
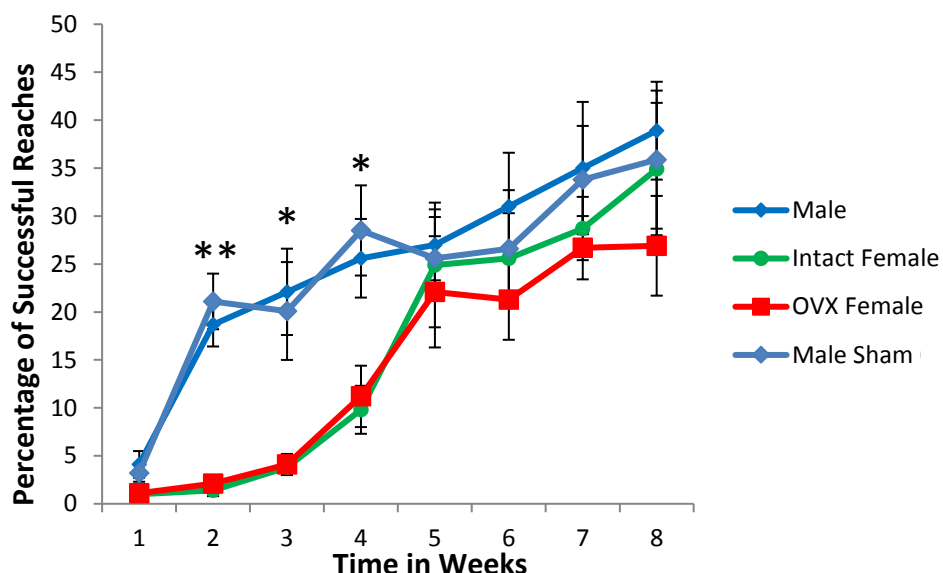
Figure 45. Reaching Percentages Post-stroke. Percentages of successful reaches during the post-stroke phase. Males had significantly higher success percentages than females during weeks one through four. Error bars represent standard error of the mean. * signifies $p < 0.05$, ** signifies $p < 0.01$ (One-way ANOVA with Bonferroni post hoc test).

Skilled Ladder Rung Walking Task

Pre-stroke, there was a group difference in learning the task over four weeks, with OVX females performing the worst (most number of forelimb slips) compared to both male groups and the intact females (Figure 46). This was seen in group differences [$F(3, 66) = 60.89$, $p < 0.05$]), and week differences [$F(2,22) = 17.22$, $p < 0.05$], and Group x Week [$F(6,66) = 44.65$, $p < 0.05$]. At weeks one, two, and three a significant difference was seen between female OVX rats and all other groups. However, by four weeks of training, all groups had achieved the same number of slips, with no significant difference between groups. There were no significant differences between groups for latency (time it took to cross the ladder) in speed for crossing the barwalk (Figure 47) for group [$F(3, 66) = 15.67$, $p = .77$] or week [$F(2, 22) = 10.33$, $p = .62$].

Post-stroke, there was a sex difference between the males and female groups, with the males performing better (lower number of forelimb slips) than both female groups (no significant difference between the female groups) (Figure 46). The F test conducted revealed significant group differences [$F_{(3, 66)} = 62.54$, $p < 0.05$], week differences [$F_{(2, 22)} = 14.88$, $p < 0.05$], and Group x Week differences [$F_{(6, 66)} = 52.31$, $p < 0.05$]. There was a significant difference at all time points between the male groups and the female groups. There were no significant differences between groups for latency in speed for crossing the barwalk at each time period (Figure 48) for group [$F_{(3, 66)} = 18.89$, $p = .56$] or week [$F_{(2, 22)} = 10.13$, $p = .68$].

Lesion Analysis

Lesion analysis of the stroke brains revealed no significant difference in lesion size between groups (Figure 50).

Weight

There was a difference in weights between males and females, however no significant weight loss was seen (Figure 49).

Golgi-Cox Dendritic Profiles

The dendritic profiles revealed that pre-stroke, intact females had the highest complexity, total branch number and lengths (Figures 51 through 53). OVX females had the lowest complexity, total branch number and lengths, and also had the lowest spine density (Figure 56). There were no significant group differences in the primary visual cortex for complexity, branch length, and spine density (Figures 55 and 56).

Post-stroke, males had the highest dendritic complexity for the primary and secondary dendrites (Figure 57). At the third dendrite complexity onwards, there were no significant

differences between groups. Males had the longest lengths (Figure 58) and highest

spine density (Figure 59).

A

B
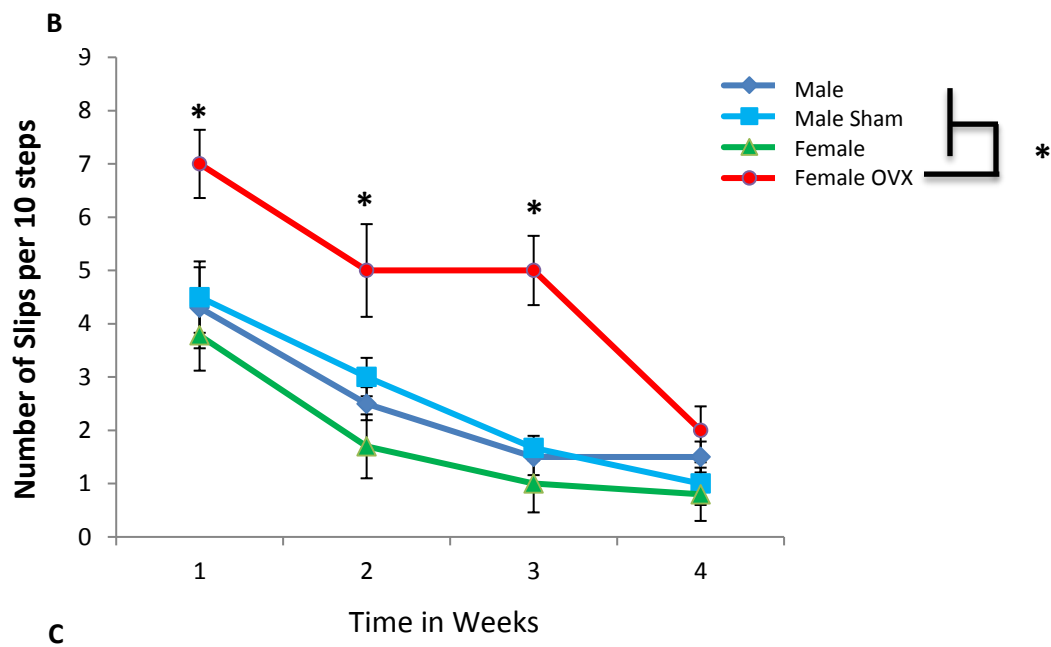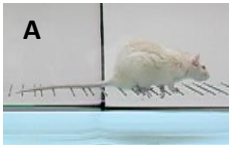
C
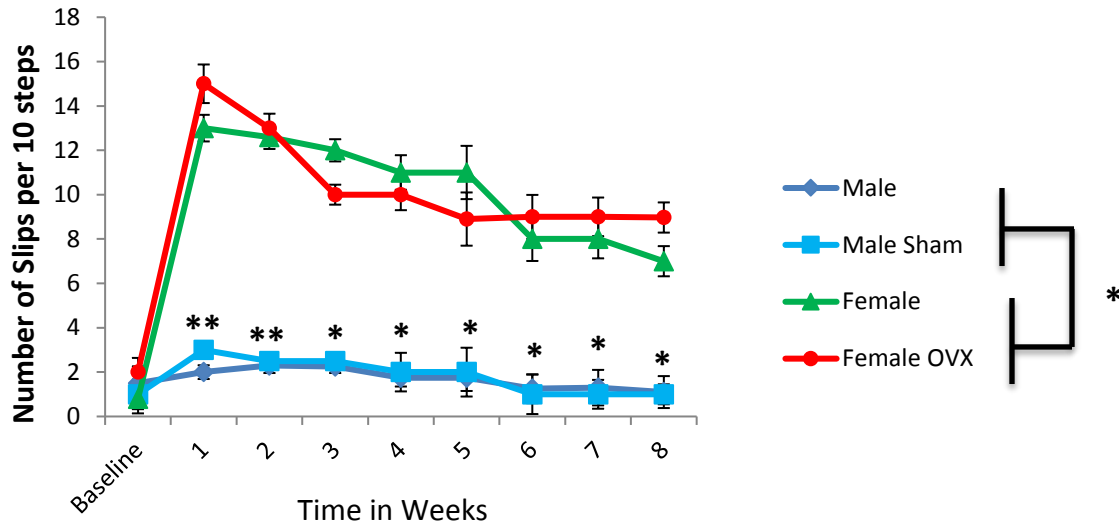
Figure 46. Walking task. Rat performing task (A), Pre-stroke average number of weekly falls per 10 steps (B), Post-stroke average number of weekly falls per 10 steps (C). * p < 0.05, ** p < 0.01. (Repeated Measures ANOVA, Tukey Post-Hoc Test) compared to all groups (A) and to both female groups (B). Error bars: standard error of the mean.
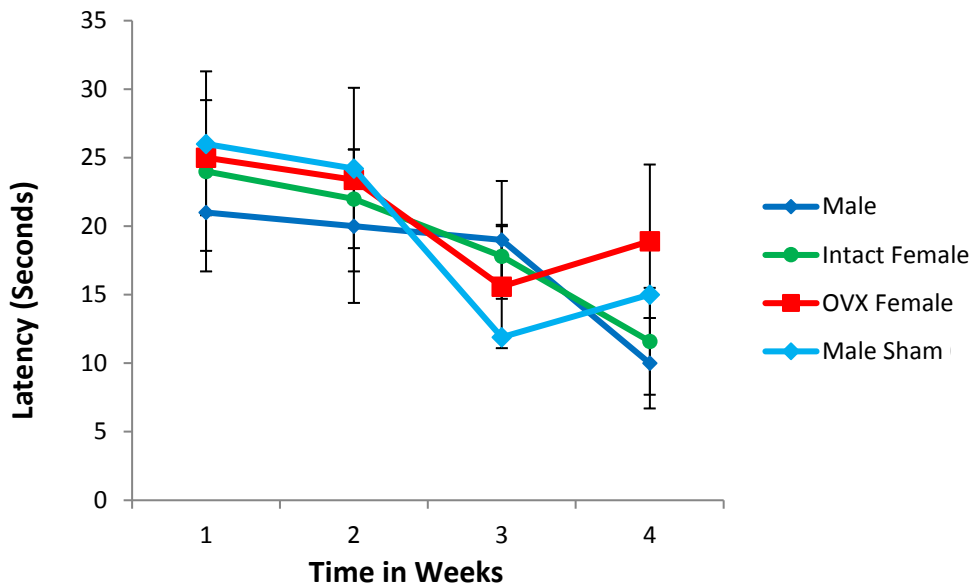
Figure 47. Pre-stroke Latency. Latency in seconds for the pre-stroke learning portion of the skilled ladder rung walking task. There was no significant time difference between groups. Error bars represent standard error of the mean.
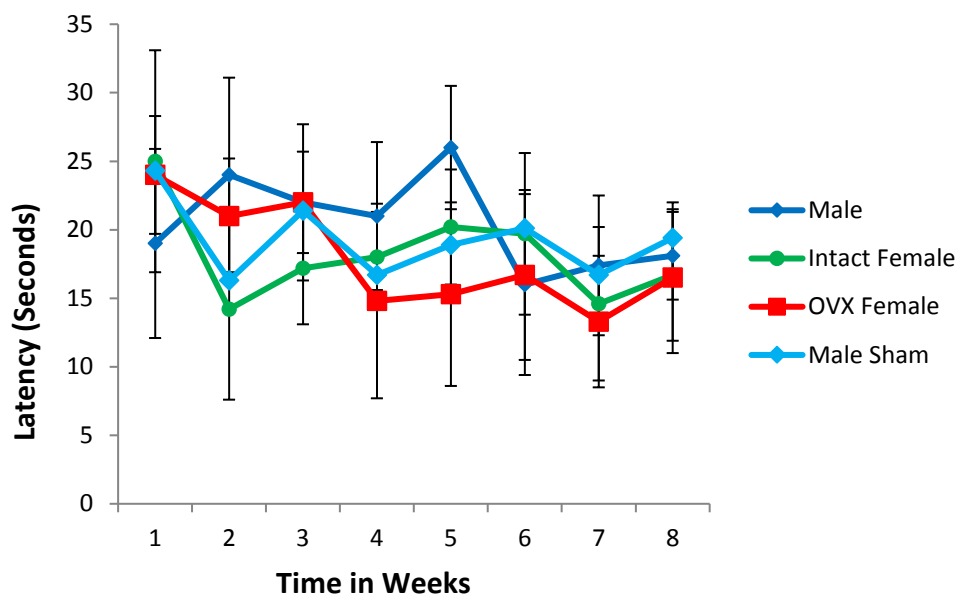
Figure 48. Post-stroke latency. Latency in seconds for the skilled ladder rung walking task during the post-stroke phase. There was no significant time difference between groups. Error bars represent standard error of the mean.

Table 1. Animal Body weight (Mean ± SEM)

	Male	Sham Male	Intact Female	OVX Female
Week 0	461 ± 40	458 ± 37	256 ± 12	260 ± 13
Week 4	450 ± 42	452 ± 50	251 ± 11	252 ± 12

Figure 49. Weight Changes in Aim 3. Weight changes (in grams) over the four week learning period are not significantly different between weeks 0 and 4, although males weighed significantly more than females.
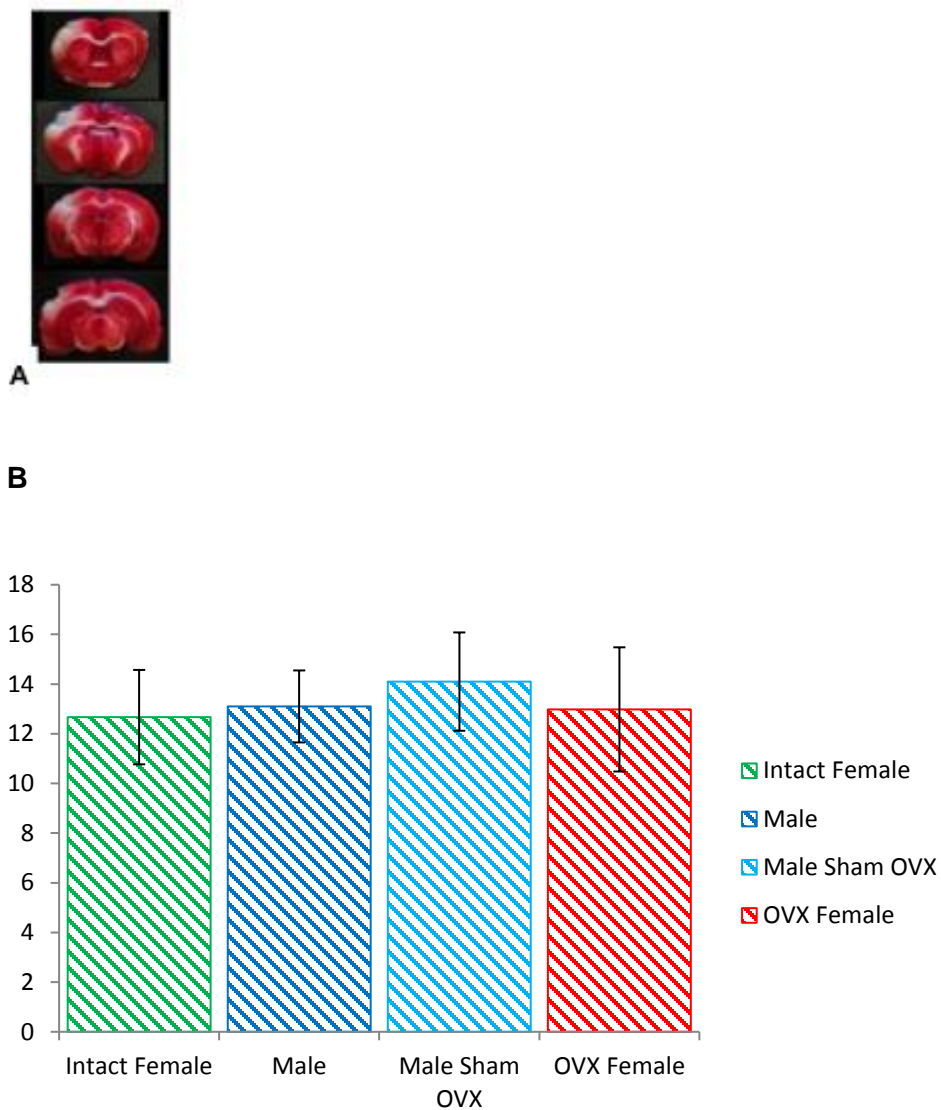
Figure 50. Lesion analysis (Aim 3). (A) TTC (2,3,5-triphenyl-2H-tetrazolium chloride)-stained coronal brain sections of an aged male rat at 2 days after MCAO demonstrates a representative stroke size and location in the ispilateral sensorimotor cortex. Viable tissue appears red and the area of the ischemic infarction appears white. (B) Lesion analysis showed no difference in stroke size between aged male, aged female, and aged female/ovariectomized (OVX) rats. (p>0.05). TTC photo courtesy of DJ Shepherd. Error bars represent standard deviation.
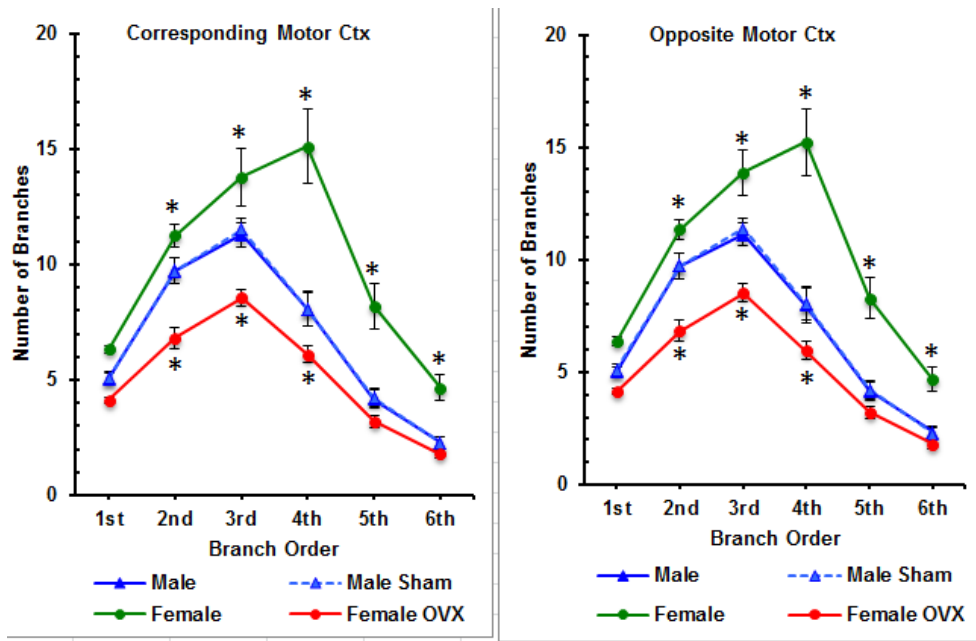
Figure 51. Pre-stroke neuronal dendritic complexity showing branch order of forelimb sensorimotor cortex corresponding to the preferred forelimb (A) and the opposite motor cortex (B). (* p < 0.05). Error bars represent standard error of the mean.
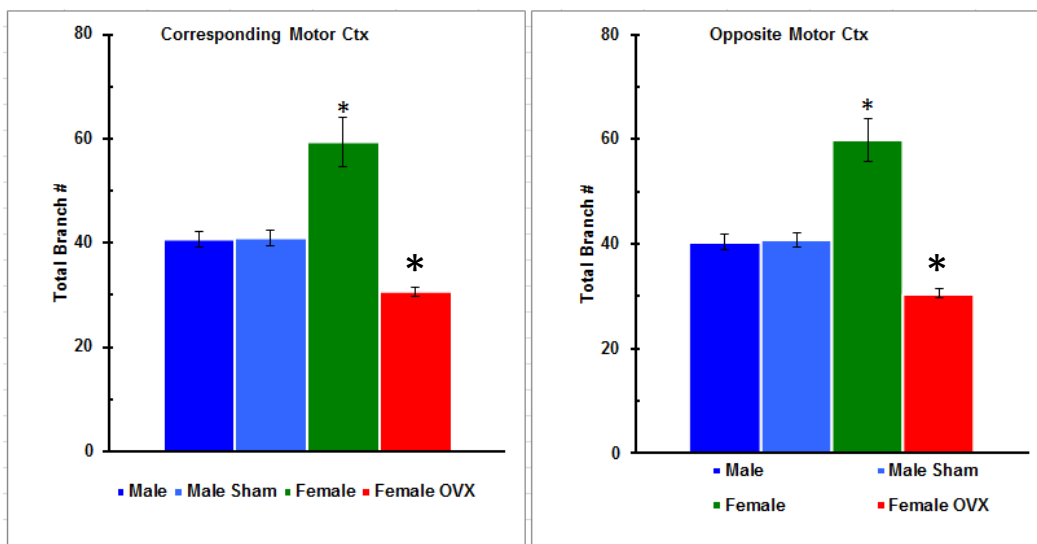
Figure 52. Pre-stroke neuronal dendritic total branch number (* p < 0.05). Error bars represent standard error of the mean.
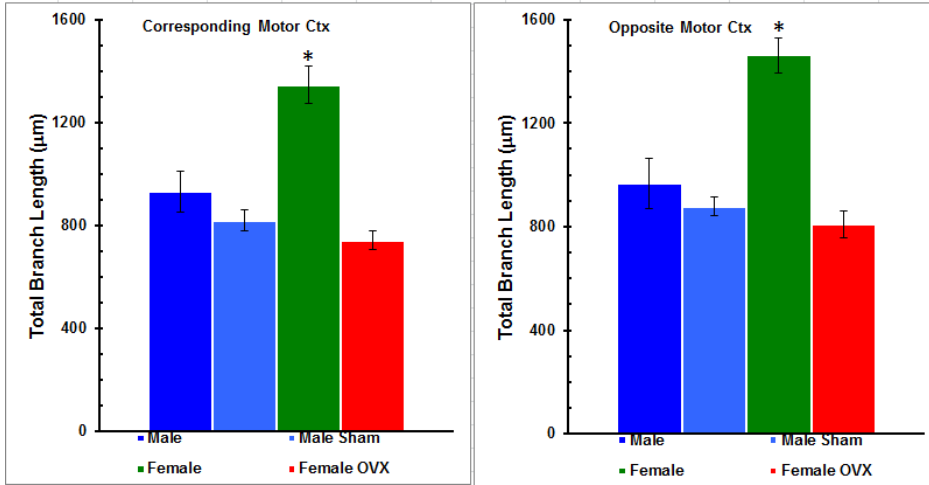
Figure 53. Pre-stroke neuronal dendritic total branch length (* $p < 0.05$). Error bars represent standard error of the mean.
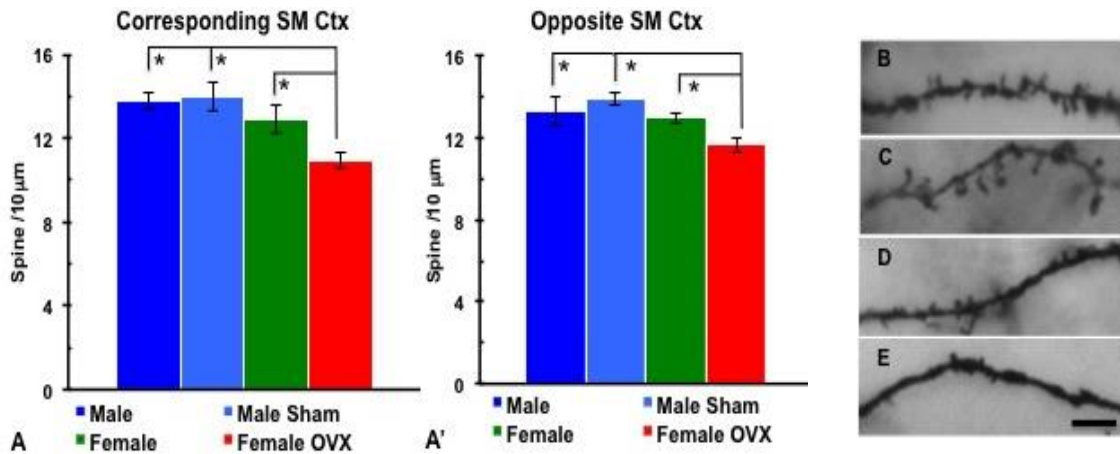
Figure 54. Dendritic spine density of neurons pre-stroke (n=12 neurons per animal) in the forelimb sensorimotor cortex corresponding to the preferred forelimb (A) and opposite cortex (A'). Representative Golgi-Cox stained images of spines in the different groups: male (B), male sham (C), intact female (D), and OVX female (E). * $p < 0.05$. Error bars represent +/- SEM. (One-way ANOVA, Bonferroni post hoc). Bar indicates 1um. Error bars represent standard error of the mean.
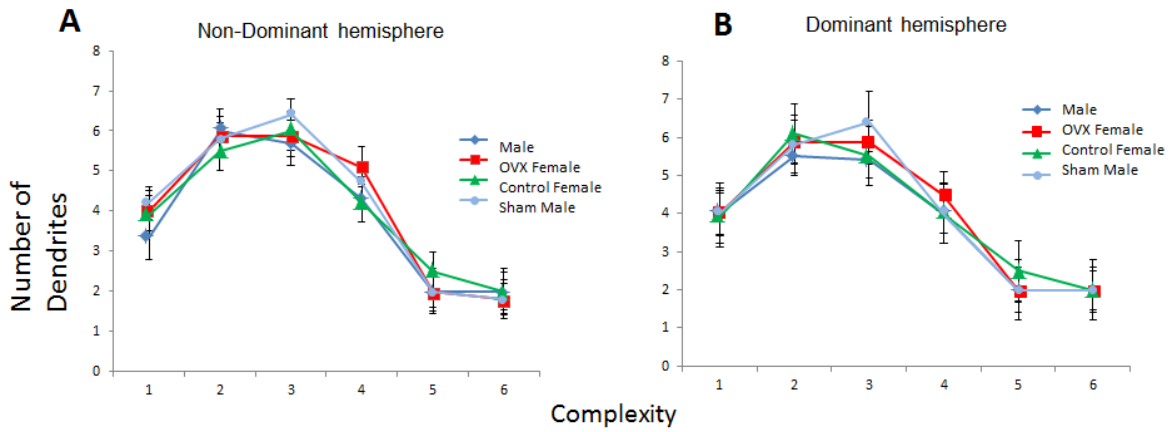
Figure 55: Pre-stroke neuronal dendritic complexity (visual cortex). Branch order of primary visual cortex corresponding to the preferred forelimb (A) and the opposite motor cortex (B). No significance was detected. Error bars represent standard error of the mean.
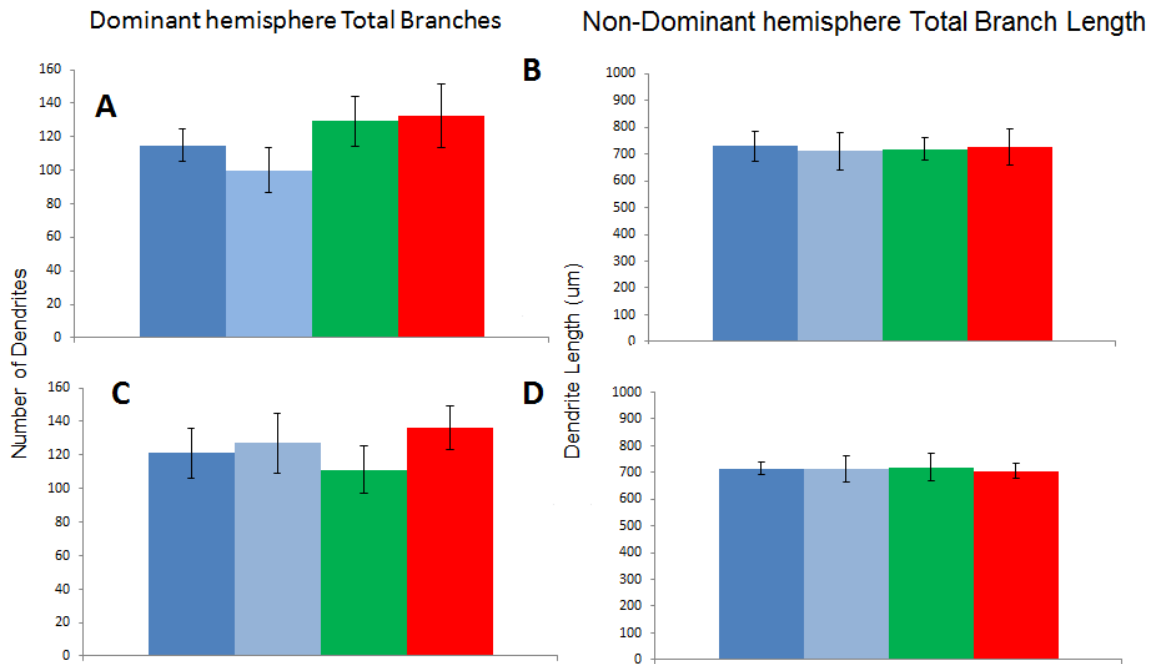
Figure 56. Pre-stroke number of dendrites and length in the primary visual cortex corresponding to the preferred forelimb (A,C) and the opposite motor cortex (B,D). No significance was detected. Error bars represent standard error of the mean.
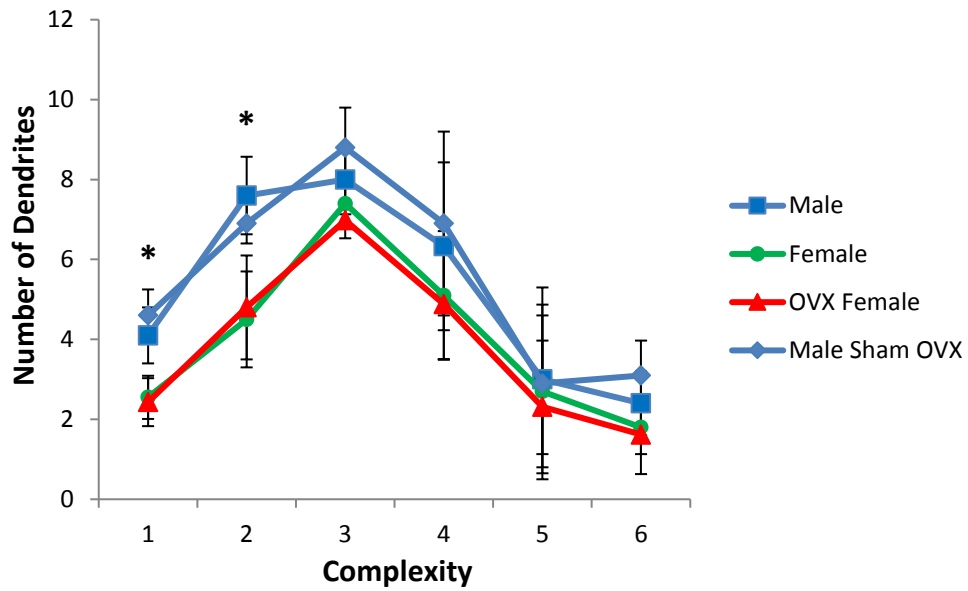
Figure 57. Post-stroke neuronal dendritic complexity showing branch order of contralesional forelimb sensorimotor cortex (* p < 0.05). Error bars represent standard error of the mean.
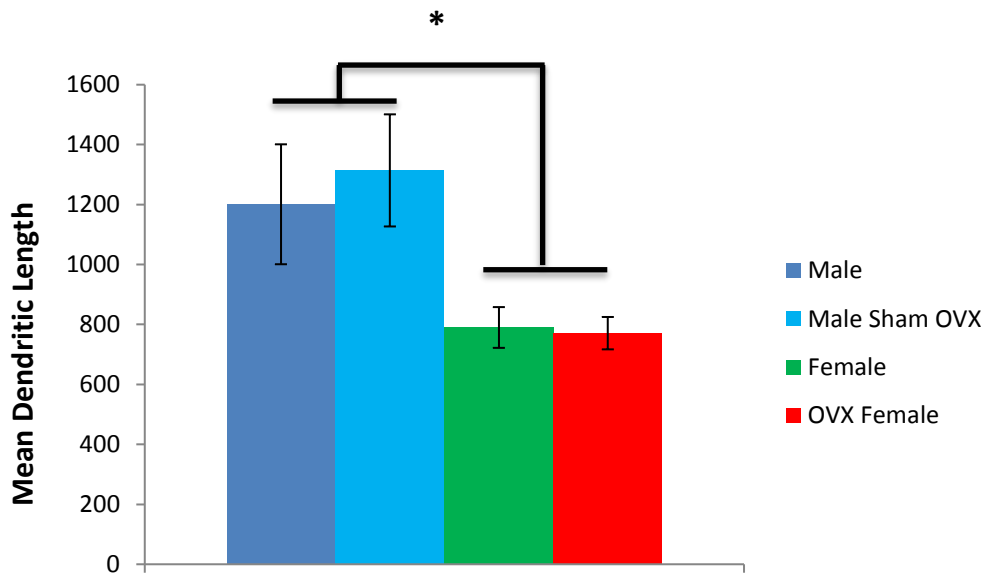
Figure 58. Post-stroke neuronal dendritic length of forelimb sensorimotor cortex contralesional sensorimotor cortex (* p < 0.05). Error bars represent standard error of the mean.
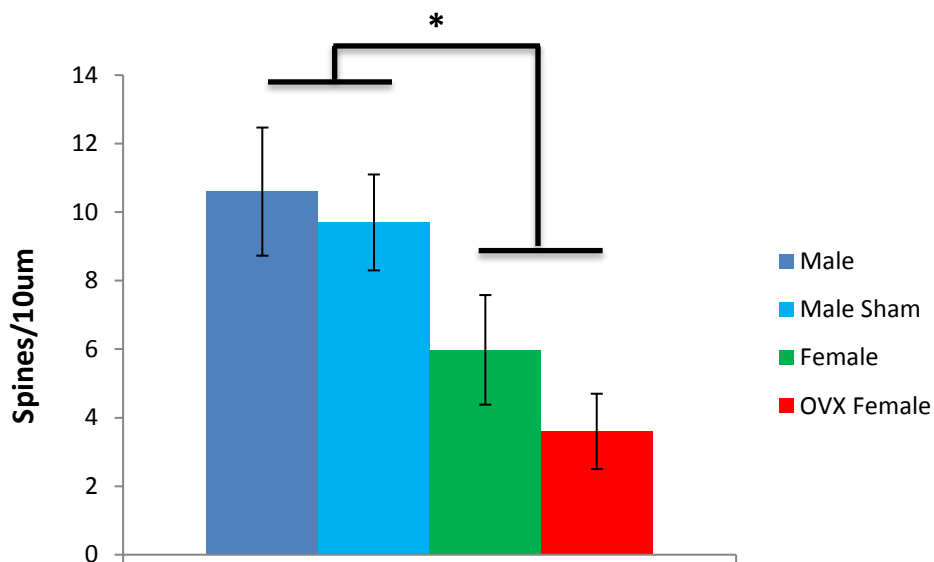
Figure 59. Post-stroke dendritic spine density of neurons in the contralesional forelimb sensorimotor cortex (* p < 0.05). Error bars represent standard error of the mean.

DISCUSSION

The results from this study show that sex differences exist in aged rats when examining both the training period to learn a task, as well as the post-stroke recovery period for that task. Specifically, for the forelimb reaching task, both groups of females learned skilled reaching faster than the males. For the skilled walking task, OVX females took the longest time to learn the test. Interestingly, post-stroke recovery was worse for both female groups as compared to males for both tasks. Golgi analysis from pyramidal neurons in the contralesional cortex correlated with these post-stroke behavioral results, with both females groups having less dendritic complexity and spine density (Figure 54) when compared to males post-stroke.

These results show that there exists a sex difference in learning skilled motor tasks, one involving skilled reaching and the other skilled walking. Various reasons have been put forth to explain the underlying mechanism for such sex differences, including differences in encoding learning engrams (behavioral patterns useful to the animal) (Monfils et al., 2005; Wallace et al., 2012) or differences in body positioning during the task (Field and Whishaw, 2005). The damage to this engram during brain injury could be believed to impact post-stroke recovery as the engram needs to be reformed or formed elsewhere in the brain (Whishaw, 2000; Wallace et al., 2012). Learning differences may be due to the significant difference in body weight between males and females (Figure 51).

Of interesting note is the fast learning speed in both tasks by the intact females, while OVX females were not as fast to learn the skilled ladder rung walking task. Males were slowest to learn the skilled forelimb reaching task yet were fast to learn the skilled ladder rung walking task. These tasks are very different in what they measure and the

differences described above could be attributed to that. The skilled forelimb reaching task is considered a very fine task of upper extremity function, while the skilled ladder rung walking task involves far more body movements. Both require the rat to balance and make fine forelimb movements, which serve to accurately measure post-stroke functional recovery. Different brain pathways are responsible, yet both require the forelimb motor cortex in order to function properly (Whishaw, 2000; Metz and Whishaw, 2002; Metz and Whishaw, 2009; Alaverdashvili and Whishaw, 2010). The body weights of the rats could also be a factor (Figure 51), as the males may have a better ability to balance with a larger body. Males may also have a longer stride than females based on this larger size.

Post-stroke sex differences in functional motor recovery are dependent on relating motor cortex damage to the behavior in question along with any possible repair or plasticity (Whishaw et al., 2008). The sex differences observed in post-stroke behavioral analysis correlate to the human post-stroke clinical data in which older women do not recover from stroke as well as men (Bushnell et al., 2014). There are several proposed reasons for why this is the case. One theory is that estradiol has been neuroprotective throughout the entire reproductive life of the female and its removal during menopause (or lowering to a low constant level in rats) may render the neurons who have since relied upon it very 'fragile' and susceptible to cell death or dysfunction. Men (although not male rats) undergo 'Andropause' in which testosterone is secreted at lower levels than normal, but a detectable level still remains and may act as a neuro-protectant either as testosterone or aromatized to estradiol. It is speculated that testosterone may have a strong neuroprotective effect in male rats as their post-stroke recovery was higher than both female groups. This could corroborate with literature in which testosterone is seen to be

very pro-growth and positively modulates dendrites and spines (Chen et al., 2013; Wu and Gore, 2010).

The lack of significant difference in lesion size is believed to be due to consistent technique as well as lack of neuroprotection by neurosteroids at this old age. At young adult age (8 weeks), exogenous estradiol was shown to reduce infarct sizes in both male and OVX female rats (Dang et al., 2011).

Sex differences in regard to Golgi-Cox dendritic analysis yielded results somewhat consistent with the behavioral results. Pre-stroke, intact females were found to have the highest dendritic complexity, males the next highest, with OVX females having the lowest complexity. While for intact females this correlates to the skilled forelimb reaching and skilled ladder rung walking tasks, it does not explain why OVX females learned the skilled forelimb reaching task quickly but not the skilled ladder rung walking task. It also does not correlate with the male behavioral task performance. Males were shown to still have high testosterone levels, and this pro-growth hormone may have a beneficial effect in recovery and excitatory synapse number, but not in learning.

The pre-stroke spine data corroborates earlier literature in which estradiol increases spine number (Foy et al., 2008) but does not correlate to the different group performances in learning pre-stroke. It is also believed that sex hormone levels will not change drastically over this testing period, which could have impacted recovery.

The secondary visual cortex data is similar to what was hypothesized and no group differences are seen in dendritic complexity, length, or dendrite number. This area has

previously acted as an internal control for examining dendritic differences

(Papadapoulos et al., 2002; Gillani et al., 2010).

Post-stroke Golgi-Cox profiles do tend to correlate well with the post-stroke behavioral

data. Intact and OVX females had the lowest dendritic complexity post-stroke and this

correlates with the skilled forelimb reaching task and skilled ladder rung walking task.

Intact females had the largest drop in complexity following stroke while males did not. All

groups had a resulting drop in complexity and spine density following stroke. OVX

females remained the lowest spine density following stroke, again following previous

reports in which estradiol has been shown to increase spine number (Foy et al., 2008).

In instances in which the dendritic profile data does not correlate with the observed

behavioral data, it is proposed that other factors may play a role than simply the number

of dendrites or spines. We only looked at a single region responsible for motor

movements, despite that region being the area that is affected by middle cerebral artery

occlusion stroke and its resulting behavioral deficits. In other brain regions

(supplementary motor cortex or cerebellum) a difference may be seen. This reasoning is

further examined in the next chapter which seeks to better characterize neural

communication and show a more detailed and molecular approach to determining cortex

connectivity.

In conclusion, it has been shown here that while both female groups learned faster (in

the skilled reaching task), males had better post-stroke recovery (in both tasks). There

were correlates in the Golgi-Cox data, in which pre-stroke females had the greatest

dendritic complexity and length, but OVX females had the lowest density of dendritic

spines. Post-stroke males had the highest dendritic complexity and dendritic length. It

has been shown here that ovariectomy reduces dendritic complexity, length, and spine density.

CHAPTER SIX

GENERAL DISCUSSION

The results from this dissertation show that there exists a sex difference in motor
learning (aged females learned the skilled forelimb reaching task faster than males) and
post-stroke motor recovery (both female groups had lower functional recovery in both
behavioral tasks), and that morphological differences exist in terms of dendrite length,
number and spine number (pre-stroke, intact females have the most complexity, length,
and spine density, but post-stroke males have the most of all of these parameters).
There are group differences with regard to excitatory synapse numbers (OVX females
have the lowest amount of synapses at eight weeks). Furthermore, stroke reduced
synapse number at eight weeks in both intact females and males but not OVX females.
It has also been shown that anti-Nogo-A immunotherapy given post-stroke increases the
synaptic numbers in aged male and intact female groups, but not OVX female group.

Aim 1 sought to look at the number of excitatory connections synapsing on pyramidal
neurons in the forelimb motor cortex. Through specific markers (VGLUT1 for cortico-
cortical excitatory connections and VGLUT 2 for thalamo-cortical excitatory connections)
the numbers of synapses were quantified at 3 and 8 weeks in stroke and non-stroke
rats. This revealed group differences especially at the eight week time point, with
decreases seen following stroke. This is expected due to the fact that the synapses will
be lost that communicated with parts of the infarct hemisphere. Intact females did not
see a significant drop following stroke (VGLUT2) and this is postulated to be due to
continued strong thalamic communication with the forelimb motor cortex.

Aim 2 further examined the synaptic proteins looked at in Aim 1 with the added treatment of 11C7 immediately after stroke (anti-Nogo-A immunotherapy) and examining the synaptic changes at 3 weeks. It was found that males and intact females had increases in both VGLUT1 and VGLUT2 number following anti-Nogo-A immunotherapy and this was not seen in OVX females. This showed a sex hormone difference in response to 11C7, with only intact females and males having a significant increase in synapse number.

Aim 3 examined behavioral learning pre-stroke and functional recovery post-stroke, along with Golgi-Cox dendritic analysis. It was shown that while males were the slowest group to reach baseline in the skilled forelimb reaching task, they had the best functional recovery post-stroke. While intact females had the highest dendritic complexity pre-stroke, this was reduced below males post-stroke. OVX females had the worst dendritic profile pre-stroke and this continued on into post-stroke, including the lowest spine density.

Our lab has a long history of studying functional recovery after stroke and possible therapeutic interventions that may aid recovery. There is, however, an important gap in our knowledge concerning therapeutic interventions and their effect on aged females. Aged OVX females are a suitable model for studying post-menopausal women, as shown by the undetectable estradiol levels and the poor functional recovery following stroke. Behaviorally we have shown that following stroke, OVX females have significantly lower functional recovery than all other groups in the skilled forelimb reaching task and lower functional recovery than the male groups in the skilled ladder rung walking task. In this model we also saw significantly lower pre-stroke dendritic

complexity, dendritic length, and spine density. These low values were also seen post-stroke.

The behavioral effects seen here could be attributed to the sex of the investigator, which has been shown to affect rat behavior and stress levels. It is believed that these results should be followed up with an investigator of the opposite sex to corroborate.

Strain is also a potential variant in interpretation of these results. The Fischer 344 strain is a common aged rat model and as such was chosen for this study. Other rat strains are available, such as Long-Evanns (black-hooded) rats. Strains could have differences in cognition, behavioral results, and dendritic morphology.

The complex interactions between sex hormones, synaptic proteins, and growth inhibitory molecules like Nogo-A form a complicated pathway that requires more research to understand. We have found that anti-Nogo-A immunotherapy does increase excitatory synaptic number in aged male and intact female rats, but not OVX rats. This could mean that an almost zero level of estradiol (as in the OVX females) is not sufficient to increase synapse number, even in the absence of a major growth inhibitory molecule. It is not suggested that estradiol is required for increased excitatory synaptic number following 11C7, although it may play a large role in increasing connections after immunotherapy. Male rats have significant levels of testosterone, which has been shown to be pro-dendritic and pro-growth (Dubal and Wise, 2001; Etgen et al., 2011; Chen et al., 2013) and can be aromatized to estradiol.

Presented here is a link between forelimb motor cortex dendritic morphology (in terms of length, complexity, and spine number) and behavioral learning and post-stroke motor

recovery. Pre-stroke, intact females had longer dendrites and this correlates well to their faster learning speed. OVX females had a decreased number of spines, yet still had a fast learning speed compared to males (who were slow to learn and had a dendritic complexity less than intact females but greater than the OVX females).

Males had the best motor recovery following stroke as compared to intact females and OVX females. This did correlate with the dendritic complexity data in which males post-stroke had a very small drop while intact females and OVX females had a large decrease in complexity. These results may be useful as a clinical model as this correlates with the outcome for functional recovery in the aged population following stroke. This morphological data can be compared to Aim 1 in which the specific synaptic protein changes and locations could be examined in the forelimb motor cortex. Aim 1showed that males had a large drop in excitatory synapses following stroke at eight weeks, seen also with VGLUT1 and intact females. This does not correlate with the males' better functional recovery while it did correlate with the intact females (poor post-stroke behavioral recovery). The low number of post-stroke excitatory synapses in OVX females does correlate with their poor post-stroke recovery, but not with their fast pre-stroke learning. While some of the parts of the above aims correlate, there are parts that do not correlate. The parts that do not correlate could be explained by looking at a different brain region or cortex layer (as the examine layer V pyramidal cells did not show a difference). There may also be a change seen on the apical dendrites instead, whereas only the basilar were examined here.

There was some data that did not fit as neatly, however. When comparing the Aim 3 dendritic complexity of OVX females, it did not correlate to the learning speed of the

skilled reaching task (they have less dendritic complexity but learned the task quickly), but did seem to correlate to the skilled ladder rung walking task (in which they did poorly post-stroke). The VGLUT2 synapse numbers of intact females showed no significant decrease, and yet intact females did not recover well for both post-stroke behavioral tasks and had a decrease in dendritic complexity (it did correlate for OVX females, however). These differences in expected correlations can be explained by the scope of the study. Only a specific brain region was studied, and as such it is believed that there may be a change in the brain elsewhere that can explain these behavioral changes. Different cell types could also be studied in these regions to explain these differences.

The signaling mechanisms used by Nogo-A to exert its growth inhibition have shown that a downstream target is CREB, which through ROCK it causes to be dephosphorylated (and lowers its activity) (Schmandke et al., 2014). CREB is also a target of estradiol signaling (Wu et al., 2005), which it phosphorylates through protein kinase A. This potential interaction could explain the results of Aim 2, in which OVX females did not respond to anti-Nogo-A immunotherapy. Even the low level of endogenous sex hormones in the intact female group could have a positive benefit to the animal through CREB phosphorylation. CREB acts as a transcription factor to upregulate expression of many synaptic proteins, including the NMDA receptor (Wu et al., 2005). As this was the post-synaptic protein studied in Aims 1 and 2, this could explain why excitatory synapses were found to be low in OVX females, both pre- and post-stroke (Figure 61).

The above show the impact of anti-Nogo-A immunotherapy on synaptic number and its possible mechanistic pathways alongside estradiol signaling. Taken together, a cohesive picture of sex differences in the aged rat model behavior (learning and recovery)

alongside dendritic morphology and synaptic protein differences emerges. Treatment with anti-Nogo-A also showed changes in synaptic proteins, shown by Aim 3. Overall, OVX females were found to have lower dendritic number, spines, dendritic complexity and a lack of increase in VGLUT following 11C7. The removal of estradiol and related sex hormones via ovariectomy seems to pose a large problem at the cellular neuron level as well as behaviorally in terms of functional recovery following stroke (Aim 1). Removal of estradiol has been shown here to have many negative effects at the neuronal level which also translate to behavior (Aim 1). Sex differences are also very apparent here, with the apparent benefits of having estradiol and its related sex hormones (Summarized in figure 62).

	Pre-stroke	Post-stroke	Post-stroke with Anti-Nogo-A
Males	Learning: Slowest of all groups (reaching) Golgi: Between Intact Females (most complex) and OVX Females (least complex) Synapses: Higher than in post-stroke (8 weeks, both VGLUT1 and 2)	Functional Recovery: Best Golgi: Highest complexity Synapses: Lower than pre-stroke (8 weeks, both VGLUT1 and 2)	Synapses: Significant increase
Intact Females	Learning: Fastest, with OVX Females (reaching) Golgi: Highest complexity Synapses: Higher than in post-stroke (8 weeks, VGLUT1)	Functional Recovery: Poor, along with OVX Females (better than OVX at end) Golgi: Low complexity, near OVX Females Synapses: Higher than males and OVX females (VGLUT2)	Synapses: Significant increase
OVX Females	Learning: Fastest, with Intact Females (reaching) Golgi: Lowest complexity and spine density Synapses: Lower than intact females (8 weeks, VGLUT1)	Functional Recovery: Worst Golgi: Low complexity, near Intact Females Synapses: Lower than intact females (8 weeks, VGLUT2)	Synapses: No increase

Figure 60. Summary of all results. Pre-, post- and post-stroke with anti-Nogo-A effects chart.
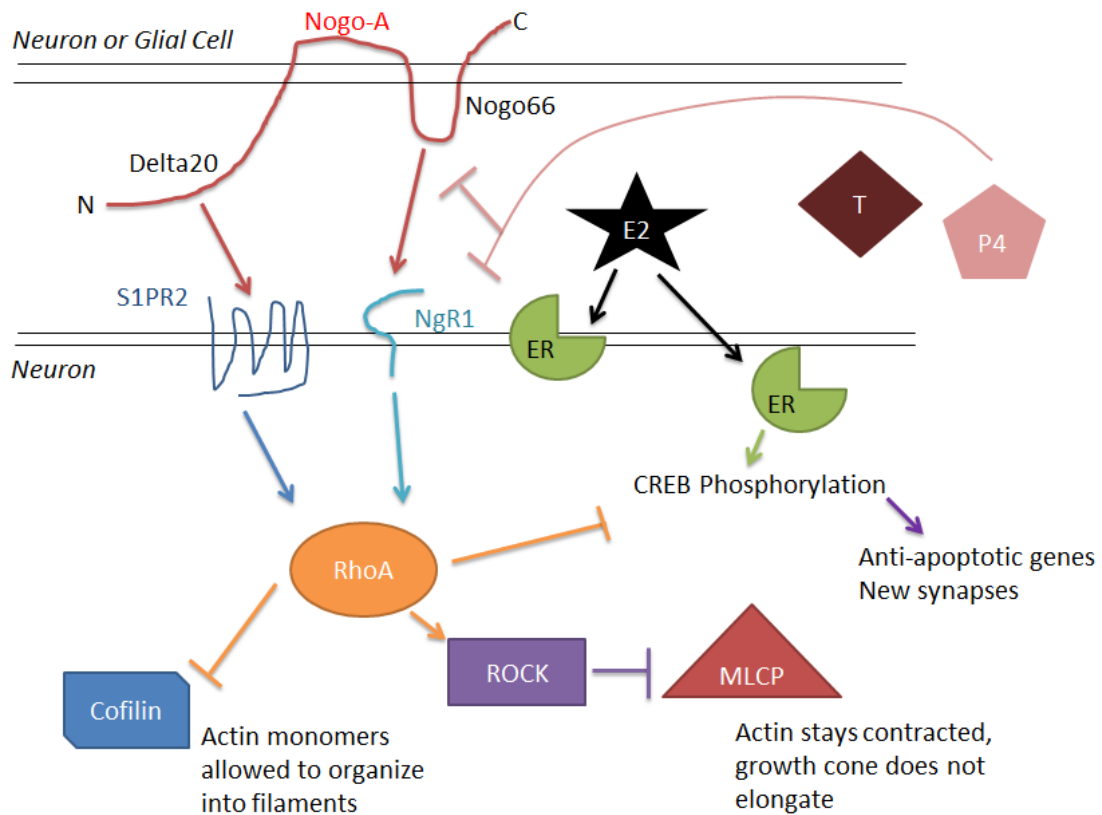
Figure 61. Summary of Sex Hormone and Nogo-A interaction. The intersection of sex hormone signaling such as estradiol with Nogo-A's growth inhibitory cascade is believed to be at CREB. Estradiol increases CREB phosphorylation (Wu et al., 2005) while Nogo-A dephosphorylates it (Schmandke et al., 2013). CREB, when phosphorylated, is an active transcription factor responsible for increasing expression of anti-apoptotic genes such as Bcl-2 and proteins used in new synapses.

FUTURE DIRECTIONS

It is proposed for future directions that this work be continued in a manner as to ascertain the underlying mechanisms of sex differences. One proposal is the use of hormone replacement therapy (HRT) translated into the rat model in which exogenous estradiol is placed in a capsule form subcutaneously to mimic the HRT that women may receive. This is believed to be an acceptable replacement for the estrogen loss from OVX. The literature is filled with reports on hormone therapy (Nelson et al., 2012; Wise, 2015) and a large debate continues over dosage, timing, usage of estrogen, progesterone (Paris et al., 2011), or both as well as potential benefits or health risks (Wassertheil-Smoller, 2010; Wise, 2015), as complications can arise from estrogen or other sex hormone therapy, such as risks for cancer and cardiovascular effects (Goodrow et al., 2005). An even further direction can be to use the aged rat stroke model to examine the different HRT types, such as conjugated equine estrogens and others. Gonadectomized male rats are also proposed to see the effects of removing testosterone on post-stroke behavior and synaptic changes.

Another proposal is for the quantification of Nogo-A and other growth inhibitory proteins in the aged rat brain (as well as specifically the forelimb motor cortex) in order to determine if sex differences exist in the levels of Nogo-A, Nogo-A receptors, OMgp, MAG, and PirB. This could add a mechanistic approach to determining differences in pre-stroke motor learning as well as post-stroke functional recovery. Western blotting using either whole brain homogenates or dissected homogenized motor cortices is proposed for this, along with protein quantification of the proteins of interest mentioned in this study: VGLUT1 and 2, NMDAR, AMPAR, BDNF, the estrogen receptors, and

other synaptic proteins. Localization of the estrogen receptors, while already looked at, has not yet been looked at in aged or OVX rats.

A further proposal is the use of an enriched environment and rat therapy and rehabilitation to measure potential changes in functional motor recovery and dendritic plasticity and synaptic connectivity. An enriched environment has been shown to be helpful for motor recovery (Janssen et al., 2010). Combination with the above and HRT is also proposed, along with combination with 11C7 treatment.

Further exploration of synaptic connectivity proteins is another possibility. Explored in depth here were excitatory connections, but inhibitory connections remains a possibility, as well as further classification of cortical connectivity to areas of interest such as the sensorimotor cortex and its individual layers, the basal ganglia, the red nucleus, and the spinal cord.

For a mechanistic future direction, it is proposed that the relevant pathway for Nogo signaling (RhoA) could be better understood, along with the interaction that this same pathway has with estradiol and BDNF/TrkB signaling (Kramar et al., 2013). Manipulation of these three important pathways and their relevance to neuroplasticity are currently not well understood and the effects of the three ligands on the same pathway are proposed to be better studied in the future.

Finally, it is proposed that altering the post-stroke treatment and observation times may shed more light on functional recovery and effects of timing of the immunotherapy. In the past delayed treatment has been used (such as a six month delay for beginning immunotherapy). Proposed here is a longer immunotherapy session (three weeks or

more) as well as carrying out post-stroke behavioral testing until 15 weeks or more

before sacrifice. This could reveal any further potential functional recovery if the rats

have not completely plateaued and provide a more accurate clinical model in which long

delayed recovery could occur. Behavioral analysis of post-stroke aged intact and OVX

females with antibody treatment is also a future direction, as well as usage of

gonadectomized males.

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VITA

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