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The Manipulation Of Apical Dendritic Plasticity And The Consequences For The Effects of Chronic Stress

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THE MANIPULATION OF APICAL DENDRITIC PLASTICITY AND THE CONSEQUENCES FOR THE EFFECTS OF CHRONIC STRESS

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Pyramidal cell apical dendrites in region CA3 of the hippocampus of male rats collapse in size and complexity following chronic stress. These dendrites return to control sizes with a recovery period. This suggests that dendritic remodeling can function as a protective response mechanism, protecting the region from exposure to the increased amounts of excitatory amino acid released in stress. If this is the case, then a situation in which remodeling is prevented during stress should be more damaging to the long-term survival of the organism. To examine this, animals were treated with endo N, an enzyme that cleaves PSA from NCAM for at least 30 days. PSA-NCAM is a cell adhesion molecule present during plasticity in development and also following chronic stress in the hippocampus. PSA-NCAM removal did not cause cell death on its own or following chronic stress by multiple measures. Phosphorylated-Tau is present in the hippocampus of severely damages brains, such as during Alzheimer's disease. Tau binds to microtubules and helps support the backbone structure making up dendrites and axons. Tau phosphorylates in response to chronic stress, and in animals without PSA-NCAM, this response was absent. This suggested that the neurons were not as plastic in response to stress. A Golgi stain analysis revealed that PSA-NCAM removal significantly blunted stress-induced dendritic remodeling. PSA-NCAM removal caused the apical dendrites in CA3 to be larger than control animals both with and without stress. To examine functional

consequences of this treatment, animals were put through multiple behavioral tests. PSA-NCAM removal increased the anxiety of rats following chronic stress. Animals also appeared more aggressive when they were housed two per cage. This indicates that without the normal level of dendritic remodeling, chronic stress has increased negative behavioral consequences. Various peptides and receptors were examined, including CART, NPY, and NMDA receptor subunits. PSA removal prevented stress-induced NR2B decreases and NPY increases. Although chronic immobilization stress was not enough to cause long-term damage, a future experiment presenting a insult to the hippocampus following chronic stress in animals with PSA-NCAM removal could show just how dangerous a lack of dendritic remodeling could be.

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CHAPTER 1: INTRODUCTION

1.1 Stress and Hippocampal Plasticity

Stress, an experience which disrupts body homeostasis, is a universal experience for living organisms (Selye, 1936). A stressful experience causes the release of corticosteroid hormones, which are regulated by hormone release in the hypothalamic-pituitary-adrenocortical (HPA) axis, over which the hippocampus (HC) has regulatory influence at the hypothalamus (Jacobson and Sapolsky, 1991; Jankord and Herman, 2008). This negative control works, in part, through binding of circulating corticosteroids directly to two types of adrenal steroid receptors in the hippocampus (McEwen et al., 1968; van Haarst et al., 1996). With an acute exposure to stress, the corticosteroid release works to keep all the body's various normal responses to stress in check, keeping the organism from major lasting harm (Munck et al., 1984). However, when the stress period is prolonged, whether through physical or psychological stimuli, protection fails, the HPA axis becomes disregulated, and there are long-term deleterious consequences (Chrousos and Gold, 1992; McEwen, 1998; Korte et al., 2005).

Chronic stress or chronic elevated levels of circulating glucocorticoids (GCs) are associated with several different types of damage and disease states. Depressed patients with the melancholic form of major depression show elevated circulating GCs and hippocampal atrophy (Sheline et al., 1996; Brown et al., 2004). Serotonin levels are highly responsive to stress (Linthorst and Reul, 2008). With increased exposure to circulating GCs, however, HC neurons become less responsive to serotonin (Karten et al., 1999). The hyperactivity of the HPA axis in depression and the development of

symptoms of depression that are directly related to hippocampal activity show just how directly chronic stress can influence mood in a deleterious fashion (Nemeroff, 1996; Raone et al., 2007).

In Cushing's syndrome, overproduction of GCs due to tumor presence in the hypothalamus, pituitary gland, adrenal glands, or pulmonary system causes changes similar to those seen in depression culminating in HC atrophy, with the amount of atrophy directly related to the level of GC hypersecretion (Starkman et al., 1992). Chronic stress has also been shown to increase the incidence of obesity and diabetes (Kyrou and Tsigos, 2007), and diabetics show HPA axis disregulation with increased circulating GCs (Golden, 2007; Reagan et al., 2008).

It is not in fact necessary for stress to be chronic to be damaging; if the stress is severe enough, damage will result. This is what is seen in posttraumatic stress disorder (PTSD). Combat veterans with PTSD show decreased hippocampal volumes by MRI (Bremner et al., 1995; Gurvits et al., 1996).

The binding of GCs to steroid receptors in the HC leads to glutamate release (Lowy et al., 1993). This excitatory amino acid (EAA) release leads to long term potentiation (LTP) in the hippocampus through N-methyl-D-aspartic acid (NMDA) receptor binding, a model for memory (Bliss and Collingridge, 1993). Memory enhancement would be beneficial for an organism operating under acute stress conditions, where it would be important to remember what caused the danger one would seek to avoid in the future (Sapolsky, 2003). The danger in having too much glutamate around in the HC is that a by-product of its release is free radical formation due to

elevated levels of cytoplasmic calcium (McCord, 1985). Free radical accumulation leads to lipid peroxidation DNA damage and cell death ((Reagan and McEwen, 1997; Keller and Mattson, 1998).

One of the major consequences of chronic stress and the increase in circulating GCs resulting from it is apical dendritic remodeling in the cornu ammonis 3 (CA3) region of HC. Three weeks of chronic restraint stress (CRS) in rats causes atrophy of CA3 pyramidal neuron apical dendrites as measured by dendritic length and number of dendritic branch points (Watanabe et al., 1992a). This phenomenon is specific to male rats; female rats have a different hormonal response to stress and do not show dendritic atrophy (Galea et al., 1997). Male mice also show dendritic retraction following CRS (Liu et al., 2006). Apical dendritic structural collapse is also seen with 10 days of chronic immobilization stress (CIS) in male rats (Vyas et al., 2002). In both of these paradigms, rats are placed in tight containers, either wire mesh (CRS) or plastic (CIS) that while not painful, prohibit movement. CRS is for 6 hours a day for 21 days, whereas CIS is for 2 hours a day for 10 days. The decrease in time in CIS to produce effects comparable to CRS is due to the increased severity of the stress. The plastic container is not as exposed to circulating air as wire mesh restrainers. Stress does not have to come through immobilization. Chronic multiple stress (a mix of shaking, restraint, and forced swim) also produces some dendritic retraction (Magarinos and McEwen, 1995b).

Activity stress, where rats are housed with a running wheel and food access is restricted to one hour a day, also produces CA3 apical dendritic retraction (Lambert et al., 1998). Rats housed together in a visible burrow

system, a housing condition that allows for dominance hierarchies to be established, show dendritic retraction at levels corresponding to their position in the social hierarchy and the levels of circulating GCs they have as a result (McKittrick et al., 2000). Tree shrews also show dendritic atrophy following psychosocial stress (Magarinos et al., 1996). This dendritic atrophy is severe, yet seven days after CRS termination, there is recovery, and apical pyramidal cell dendrites are no different than those from controls (Conrad et al., 1999). A better term to describe the apical dendritic changes is thus remodeling, due to the fact that there are two changes, first retraction and then recovery.

The same remodeling phenomenon produced by CRS is seen with chronic delivery of corticosterone, and blockage of steroid synthesis during CRS prevents dendritic retraction (Magarinos and McEwen, 1995a). Beyond the dendrites, chronic stress also alters the structure of mossy fiber terminals (MFTs) from dentate gyrus (DG) neurons that synapse onto the pyramidal cell dendrites. MFTs in stressed animals show more densely packed clusters of vesicles in the active zones of the synapses than controls, and the MFTs in stressed rats also have more mitochondrial support (Magarinos et al., 1997).

Also affected are neurotransmitter systems linked to depression. Chronic stress decreases the levels of the serotonin (5HT) transporter in the HC (McKittrick et al., 2000). Daily administration of phenytoin during CRS, an anti-epileptic drug that binds to the 5HT transporter, prevents dendritic remodeling (Watanabe et al., 1992b). Chronic lithium treatment also prevents dendritic retraction (Wood et al., 2004). The benzodiazepine agonist adinazolam also prevents retraction and shows that enhancing inhibitory

tone in the HC can work to counter the GC-glutamate enhancement of excitation that leads to remodeling (Magarinos et al., 1999).

This stress response is severe. The retraction of apical dendrites in the hippocampus leaves the animal less able to perform in tasks in which it is involved. Stressed animals show y-maze learning deficits, a test for spatial memory (Conrad et al., 1996). But the response, and presumably the behavioral deficits that accompany it, are not permanent. This begs the question of whether the remodeling changes seen with chronic stress are signs of damage or signs of an adaptive mechanism at work.

Allostasis describes adjustments made by the body in response to a change in circumstances in order to maintain homeostasis. Although baseline levels of hormones or the set points of various regulatory systems may change from those previous, the end result is to preserve normal functionality in an organism. Allostatic load refers to the consequences of a system remaining under allostasis for a long period of time (McEwen and Stellar, 1993; McEwen, 1998). Some results of the allostatic load of chronic stress, as mentioned above, are therefore depression, diabetes, and PTSD. It is harder to determine where allostasis ends and allostatic load begins. Dendritic remodeling in CA3 is, at least in part, the result of glutamate release and NMDA receptor binding, as NMDA receptor blockade prevents the response (de Kloet et al., 2005(Magarinos and McEwen, 1995a). But the remodeling response limits these same neurons not only to further input from the same source, but also limits the exposure to excess free radicals produced by extended EAA release (McEwen, 2001; de Kloet et al., 2005). Therefore, the interesting question is, is CA3 pyramidal cell apical dendritic remodeling

evidence of allostasis, or a casualty of allostatic load? Is it a protective homeostatic mechanism, or evidence of damage itself?

Adult dendritic plasticity does not occur only in response to stress. Ground squirrels in hibernation show HC pyramidal cell apical dendritic retraction that fully reverses to normal branching state within two hours of arousal from torpor (Popov et al., 1992). The apical dendritic synapses in ground squirrels also undergo a loss and recovery with torpor and arousal (von der Ohe et al., 2007). The dendritic retraction response is seen in hibernating European hamsters as well (Magarinos et al., 2006). Spine density in CA3 decreases in mice subjected to shorter photoperiods to mimic seasonality (Pyter et al., 2005). The fact that dendritic and synaptic remodeling occurs in studies of hibernation and simulated winter indicates that remodeling is not purely a damage response. Hibernation is not damage; it is an evolutionary adaptation to winter. Therefore dendritic remodeling in these instances is also adaptive.

Is dendritic remodeling in response to chronic stress also adaptive? Animals that receive an injection of ibotenic acid (IBO) to CA3 following CRS or chronic GC treatment show increased damage compared to non-stressed controls (Conrad et al., 2004; Conrad et al., 2007). This shows that the HC is more sensitive to insult following chronic stress. But is this damage sensitivity due to dendritic retraction? The only way to measure this is to perform chronic stress while at the same time preventing dendritic retraction. How the animal reacts to this altered stress state will then reveal whether remodeling is adaptive and an allostatic mechanism to protect from further harm.

1.2 PSA-NCAM and Plasticity

One of the major molecules involved in the formation and stabilization of synapses is neural cell adhesion molecule (NCAM) (Washbourne et al., 2004). This regulation of cell-cell interactions and the homophilic binding of NCAM is not regulated only through NCAM expression, but is also dependent on the presence of polysialic acid (PSA) (Rutishauser and Landmesser, 1996). PSA-NCAM disrupts cell-cell interaction because of both its negative charge and the fact that PSA itself has a large hydrated volume (Rutishauser et al., 1988; Yang et al., 1994). In fact, this volume-related steric inhibition of cell adhesion is disruptive enough that the presence of PSA-NCAM can inhibit non-NCAM cell-cell adhesion, interaction, and communication as well (Regan, 1991; Fujimoto et al., 2001). Polysialylation of NCAM is regulated by two polysialtransferases, STX and PST in a calcium-dependent manner (Bruses and Rutishauser, 1998; Angata and Fukuda, 2003). The addition of PSA to NCAM occurs in the Golgi compartment and is responsive to NMDA neural activity (Kiss and Rougon, 1997). PSA-NCAM is highly expressed during development in stage and tissue-specific patterns that correlate with the developmental timetable of the organism (Rutishauser, 1998). The expression creates a permissive environment for cell migration because the large PSA molecules keep neural cell surfaces from being sticky, giving them time to complete developmental migration (Monlezun et al., 2005; Schmid and Maness, 2008). PSA-NCAM mutant mice show ectopic synaptogenesis and excess mossy fiber innervations onto CA3 (Seki and Rutishauser, 1998). PSA-NCAM is also present in repair following injury. Global ischemia produces a

glutamate-dependent increase followed by a decrease of hippocampal PSA-NCAM (Conrad et al., 1999; Fox et al., 2001).

In contrast to development, PSA-NCAM is not highly expressed in the brain in adulthood. PSA-NCAM expression in the adult is limited to areas that undergo neurogenesis and plasticity (Bonfanti, 2006). Adult neurogenesis occurs in the olfactory bulb through the rostral migratory stream, and newborn neurons that possess PSA-NCAM are more likely to survive (Gascon et al., 2007). Cultured cortical neurons are also less likely to survive without PSA-NCAM, but this effect is reversed with brain-derived neurotrophic factor (BDNF) treatment (Vutskits et al., 2001).

One of the main regions of adult PSA-NCAM expression is the HC (Sandi, 2004). Most expression is in the granule cell layer (GCL) of the DG, but immunoreactivity is also present in the stratum oriens, lucidum, and radiatum of CA3 and CA1 (Nacher et al., 2002). PSA is present in small, immature-type boutons of mossy fiber bundles from the DG that synapse onto CA3 (Seki and Arai, 1999a). These immature mossy fibers are associated with glia also expressing PSA-NCAM (Seki and Arai, 1999b).

Through activity in these regions PSA-NCAM is involved in many of the main functions of the HC. Hippocampal slices from NCAM-deficient mice need PSA treatment to show normal LTP (Senkov et al., 2006). LTP involves the addition of alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors (Barria et al., 1997), and PSA has been shown to potentiate AMPA receptor current (Dityatev et al., 2004), so the LTP effect is probably due to that interaction. PSA-NCAM is only present in DG LTP, and is absent in LTP induced in CA3 (Schuster et al., 2001). PSA-NCAM deficient mice

show an enhanced seizure response to an AMPA-dependent anticonvulsant (Potschka et al., 2008).

Regulation of PSA-NCAM by neuraminidase, which cleaves sialic acids, can also affect excitability, in that PSA-NCAM cleavage by neuraminidase reduces high-potassium-induced seizure potential (Isaev et al., 2007). The spatial Morris water maze task results in an increase in PSA-NCAM in the DG, and the level of PSA-NCAM correlates with how well rats learn the task (Sandi et al., 2004). Animals that cannot synthesize PSA due to a lack of PST show impairment in learning the water maze (Markram et al., 2007). Passive avoidance training also increases hippocampal PSA-NCAM expression (Doyle and Regan, 1993). PSA-NCAM is first up-regulated and then down-regulated following contextual fear conditioning in a manner that follows the process of memory acquisition and consolidation (Merino et al., 2000; Lopez-Fernandez et al., 2007). Mouse models of Huntington's disease show decreased numbers of hippocampal and piriform cortex PSA-NCAM-positive cells, and this may relate to why Huntington's disease patients have impaired olfactory function and memory problems (van der Borght and Brundin, 2007).

PSA-NCAM plasticity in the hippocampus is directly related to corticosteroid-induced plasticity. PSA-NCAM expression is inhibited by GCs, and adrenalectomy increases PSA-NCAM expression (Rodriguez et al., 1998; Cremer et al., 2000). Adrenalectomized rats can have PSA-NCAM levels reduced to baseline with glucocorticoid receptor (GR) agonists, but not mineralocorticoid receptor (MR) agonists (Montaron et al., 2003). MR have a low affinity for corticosterone (CORT) and GR have a high CORT affinity;

therefore GR are only activated in extreme situations, such as stress (Reul et al., 1987).

Although chronic CORT decreases HC PSA-NCAM expression, chronic stress increases PSA-NCAM in the DG (Pham et al., 2003; Nacher et al., 2004a; Nacher et al., 2004b). This increase in DG PSA-NCAM following chronic stress is accompanied by a decrease in NCAM (Sandi et al., 2001). Antidepressant treatment of either fluoxetine or imipramine increases PSA-NCAM expression (Sairanen et al., 2007; Varea et al., 2007).

The fact that PSA-NCAM is expressed in the same areas that are involved with dendritic remodeling, taken with the fact that PSA-NCAM removal affects the hippocampus and the organism in ways similar to chronic stress, indicates that PSA-NCAM is a good target to examine stress effects. The addition of a large, sterically inhibitory and negatively charged molecule to a synapse would appear to be a good way to begin the retraction process. A synapse could be very well defined and established, but the arrival of PSA-NCAM could force the other cell adhesion molecules out of the way and prevent any tendency for sticking. This indicates that PSA-NCAM manipulation would be a great way of answering the allostasis versus allostatic load in retraction question. Using a PSA-NCAM or PST knockout mouse would not, however, be a good way to go about this, because it is important to have normal hippocampal connectivity and activity to start with. Fortunately, there is another experimental manipulation that appears to be a great way to answer the remodeling question.

The bacteriophage-derived enzyme endoneuraminidase (endo N) cleaves alpha-2, 8-linked polysialic acid in living cells without any toxic or non-

specific adhesion effects (Vimr et al., 1984; Rutishauser et al., 1985). It has been used in many experimental situations. Treatment of hippocampal cell cultures with endo N blocks the preferential formation of synapses onto NCAM-expressing cells and the increase in perforated spine synapses associated with NMDA receptor-dependent LTP (Dityatev et al., 2004). Intracerebroventricular endo N injection prevents the decrease in Morris water maze spatial memory caused by status epilepticus in rats (Pekcec et al., 2008). Neurons and glia in the hypothalamo-neurohypophysial system express high levels of PSA-NCAM throughout life, and the region displays high levels of plasticity (Theodosis et al., 1991). Endo N injection into the region prevents the normal response to lactation and dehydration, involving astrocytic process withdrawal (Theodosis et al., 1999).

Perhaps most relevant to the question of the purpose of chronic stress-induced dendritic retraction is a study examining chronic pain in the spinal cord (El Maarouf et al., 2005). Constriction of the sciatic nerve causes chronic pain, and one result of this chronic pain is a loss of nociceptive C fibers from their terminal region in spinal lamina II (Knyihar-Csillik and Torok, 1989). Along with this fiber loss there is expression of PSA-NCAM (Bonfanti et al., 1996). In this study, endo N was injected during a spinal nerve constriction surgery in mice. The result was an increase in the intensity of hyperalgesia, or sensitivity to pain. Here, remodeling of the neural pain pathway acts to blunt how much pain is experienced by the mouse. When remodeling is prevented, the pain experienced is more intense. The PSA-NCAM-dependent remodeling does not prevent pain; sciatic nerve constriction still hurts, but the animal is worse off without remodeling, as the endo N injection revealed.

This nociceptive C process retraction could serve an adaptive purpose, allowing the animal to still function and survive in the face of injury and pain that would otherwise be too intense to ignore.

The same phenomenon could be happening in region CA3 of the hippocampus. Chronic stress, and the increase in circulating GCs and glutamate that accompany it, might be too much of a load for an organism without CA3 dendritic retraction. Retraction may be a way for an animal to ride out the stress period in the same way the hippocampus reacts and changes during hibernation with minimal permanent damage. Even though this stress-induced retracted state is more susceptible to IBO injury (Conrad et al., 2004; Conrad et al., 2007), the possibility remains that stress may make the HC even more vulnerable in the absence of retraction. Treatment with endo N will allow us to begin to answer these questions. The first thing to determine is whether endo N injection is sufficient to prevent the dendritic retraction response. If this is the case and if the injection isn't harmful to the animal in and of itself, then we can begin to examine the function of dendritic remodeling and the consequences of its prevention.

1.3 Measuring the Consequences of PSA-NCAM Removal

1.3.1 Tau Phosphorylation

When ground squirrels undergo hibernation, along with the dendritic retraction there is an increase in phosphorylated tau (P-tau) in CA3 (Arendt et al., 2003). Levels of P-tau return to baseline within the same time frame it takes dendrites to recover. Tau is a microtubule-associated protein whose major function is to promote microtubule assembly and maintain microtubule

stability (Wang and Liu, 2008). When tau is phosphorylated, its location shifts from the microtubules in neuronal axons to the somatodendritic compartment (Mandell and Banker, 1996). Phosphorylation of tau to the hyperphosphorylated extent causes tau to self-aggregate and form neurofibrillary tangles (Alonso et al., 2001). This P-tau increase in hibernation appears to be related to the decrease in body temperature (Su et al., 2008). Cold water stress also causes a rapid reversible tau phosphorylation response in mice (Okawa et al., 2003; Feng et al., 2005). Anesthesia-induced hypothermia causes tau phosphorylation that results in disassociation of tau from microtubules, but that does not lead to overall tubulin depolymerization (Planel et al., 2007; Planel et al., 2008).

Tau is highly phosphorylated not only in Alzheimer's and other neurodegenerative diseases, but also in development (Burack and Halpain, 1996). A common site for phosphorylation to occur is Ser-202, which is located in the microtubule binding domain of the protein (Lee et al., 1989; Goedert et al., 1993). All of the above experiments show phosphorylation at this site.

Moreover, P-tau is also seen as a consequence of stress that is unrelated to temperature. Starvation of mice for two to three days progressively increases P-tau in the HC (Yanagisawa et al., 1999). The effect of stress on tau phosphorylation was examined in a mouse study (Rissman et al., 2007). In this study, acute stress increased P-tau in the hippocampus. This effect was not dependent on GCs, as evidenced by adrenalectomy, but was dependent on type-1 corticotropin releasing factor receptor. In this study, repeated stress not only increased tau phosphorylation but also led to the formation of

insoluble tau, which is normally only seen in Alzheimer's (Gustke et al., 1992). Although in this mouse study GCs did not influence tau phosphorylation, other studies have shown an increase in circulating GCs in mouse models of dementia (Green et al., 2006). The presence of insoluble tau in the above mouse study indicates that tau phosphorylation could be a damaging process, or at least evidence of damage. Insoluble tau is usually only seen in diseased brains. Since tau phosphorylation changes occur in tandem with dendritic remodeling, this could support the idea that remodeling is also evidence of damage, of allostatic load.

The questions we intended to ask are, first, whether CRS-induced remodeling increases P-tau and, second, whether prevention of remodeling also prevents the phosphorylation of tau protein. If the synapses are still stuck together, tau should stay in its normal, microtubule-associated role. Although the above study points to P-tau as if not an instigator of damage, at least evidence of damage having occurred, other experiments show a different phenomenon. Cultured neurons expressing phosphorylated tau are more resistant to apoptosis induced by N-methyl-D-aspartate (NMDA) (Lesort et al., 1997). If expression of P-tau is a protection response to excess excitatory stimulation, the prevention of plasticity with endo N could set up neurons to be very vulnerable to damage.

1.3.2 Synaptic Changes and pLIMK

LIM kinase (LIMK) proteins are involved in actin dynamics and synaptic plasticity. Their effects are exerted by the inactivation of the actin-depolymerizing factor (ADF)/cofilin (Arber et al., 1998). The protein kinase

ROCK phosphorylates LIMK, and then pLIMK binds to and phosphorylates cofilin, which prevents the cofilin-induced actin depolymerization and leads to actin reorganization (Yang et al., 1998; Maekawa et al., 1999). LIMK knockouts show abnormal synaptic structures and impaired fear conditioning and spatial learning (Meng et al., 2002; Meng et al., 2004). LIMK is also necessary for proper HC LTP (Meng et al., 2003). In rats, the age-related decline in CA1 synaptic plasticity is coupled with a decrease in pLIMK immunoreactivity (Yildirim et al., 2008). Preliminary results from the McEwen lab show an increase in pLIMK immunostaining with CRS but not with acute stress.

An animal that is prevented from making the dendritic remodeling response is in a unique, and possibly vulnerable position with regard to the effects chronic stress. All the changes that normally occur at the synapse could be prevented due just to the lack of PSA-NCAM, and the inability of the synapses to pop apart. If pLIMK is a marker of synaptic reorganization and plasticity, PSA-NCAM removal should prevent pLIMK increases following CIS.

1.3.3 Behavioral Deficits

Chronic stress produces many behavioral consequences that may be compromised with PSA-NCAM removal. Rats subjected to predator stress show an increase in anxiety as measured by elevated plus field behavior and startle response (Adamec et al., 2006). CIS and CRS also potentiate anxiety and fear conditioning (Wood et al., 2008). Acute restraint stress in rats significantly reduces in-cage aggression behaviors, but CRS produces a

significant increase in aggression (Wood et al., 2003). A rat model of PTSD shows that anxiety-related fear behavior and learning is potentiated by previous stress exposure (Rau et al., 2005). In addition to anxiety, chronic stress has also been shown to affect spatial memory. CRS rats show decreased spatial memory as measured by performance in a y-maze, and this deficit can by rescued by tianeptine treatment during the CRS period (Conrad et al., 1996). This treatment also prevents remodeling, as mentioned above.

This y-maze deficit is seen only in a purely spatial version of the task. Addition of in-maze cues removes the CRS-induced performance deficit (Wright and Conrad, 2005). Performance in a spatial task that includes reward for correct spatial memory, the eight-arm radial maze, is also impaired by CRS with performance recovery by stress-period treatment with either the antidepressant tianeptine or phenytoin (Luine et al., 1994).

The Morris water maze is another useful task to measure spatial memory, although it involves a longer training period than the y- or radial maze. Three months CORT treatment produces learning impairment in the water maze, as does six months of high social stress (Bodnoff et al., 1995). Examination of synapses following water maze training reveals that the training reverses the synaptic losses and retractions produced by CRS (Sousa et al., 2000; Stewart et al., 2005).

Animals that are chronically stressed show increased anxiety, as detailed by the above results. Anxiety is evidence of an increased general level of excitability in the brain, and therefore remodeling prevention may exacebate stress effects on anxiety. The same hypothesis could hold true for aggression, with an increase in aggressive behavior produced as a result of a more

connected and therefore active hippocampus. An increase in anxiety or aggression in animals that undergo remodeling prevention followed by chronic stress would indicate a protective, allostatic function of remodeling. Spatial memory is a more complicated behavior, but since CRS deficits are recovered by antidepressant treatment that also prevents dendritic remodeling, prevention of the remodeling response in the first place could prevent the stress-induced learning impairment. If this is the case, then this is evidence of remodeling not protecting the animal from harm, and therefore not an incidence of allostasis.

1.3.4 Neuropeptide Y

Neuropeptide Y (NPY) is a 36-amino acid peptide that is expressed in development and adulthood throughout the brain (Larhammar et al., 1987). It is released by stress and has an anxiolytic effect (Heilig et al., 1989; Broqua et al., 1995). In humans, lower haplotype-driven NPY expression predicts higher levels of emotionally-induced amygdala activation and a decreased resiliency to pain as measured by opioid activity (Zhou et al., 2008). Acute stress increases NPY expression in the DG (Conrad and McEwen, 2000), and chronic stress-induced effects on body weight, open field, and forced swim can be suppressed or blocked by an intra-hippocampal injection of NPY (Luo et al., 2008). Injection of NPY to either the DG or CA1 produces anxiolytic effects as measured by elevated plus maze activity (Smialowska et al., 2007). Infusion of NPY to the CA3 in learned helplessness rats has antidepressant-like effects, and the effects in this region are dependent on the NPY receptor Y1 but not on the Y2 receptor (Y2R) (Ishida et al., 2007). Mouse mutants for the Y2R

show decreased anxiety and depression behavior in response to immune stress in the open field, elevated plus maze, and tail suspension test (Painsipp et al., 2008a). The same effect is seen in NPY receptor Y4 mutants (Painsipp et al., 2008b).

The close relation between NPY and anxiety suggests that animals that undergo remodeling prevention will see an exaggerated increase in NPY. The increase in excitability engendered by the lack of remodeling and the availability of more synapses could produce a more excitable state. This could then produce a need for more NPY to counteract all the extra stimulation.

1.3.5 Cocaine-Amphetamine-Regulated Transcript

Cocaine-Amphetamine-Regulated Transcript (CART) peptides are found widely in the central nervous system where they work as neurotransmitters (Adams et al., 1999), and are known to be involved in feeding and reward function (Rogge et al., 2008). In the nucleus accumbens CART is regulated by dopamine through the D3 receptors (Hunter et al., 2006). CART is also regulated by GCs, with adrenalectomy producing a decrease in CART expression in the brain (Dominguez et al., 2004). CART expression is found throughout the limbic system, including the HC (Stanek, 2006).CART has been shown to be involved in anxiety. Intraperitoneal injections of CART decrease the time spent in the open arms of the elevated plus maze and decrease social interaction between mice (Chaki et al., 2003). Injection of CART into the cerebral ventricles produces the same plus maze deficits in exploratory behavior (Kask et al., 2000). Children with CART mutations show increased anxiety and depression (Miraglia del Giudice et al., 2006). Changes

have also been observed with stress in the HC. CRS upregulates CART mRNA in the DG whereas acute stress does not. Adrenalectomy reduces DG CART expression, and CORT replacement restores CART levels (Hunter et al., 2007).

Since CART expression is directly linked to anxiety, and it is likely that PSA-NCAM removal will increase anxiety through increased stimulation, it is possible that CART expression will also be increased in PSA-NCAM depleted animals. Further exacerbation of these animals by chronic stress could reveal even more CART expression. This would mean changes upstream from remodeling in the anatomical pathway of the hippocampus, as DG mossy fibers synapse onto CA3 pyramidal cell neurons, and the CART changes that occur in response to stress do so in the DG.

1.3.6 Glutamate Receptors

NMDA receptors have been linked to many of the consequences of stress and functions of the HC. Subtypes of the NMDAR include NR1, NR2A, and NR2B. Prepubertal adrenalectomized male rats with high-dose CORT replacement show increased NR2A expression in the DG and increased NR2B expression in CA3 (Lee et al., 2003). NR2A and NR2B in CA1 are both necessary for LTP, with NR2A also necessary for long term depression (LTD) (Bartlett et al., 2007). A single acute immobilization stress produces an increase in NR1 expression in CA1 and CA3 and an increase in NR2B but not NR2A in CA3 (Bartanusz et al., 1995). Chronic CORT treatment increases NR2A and NR2B but not NR1 expression throughout the hippocampus (Weiland et al., 1997). NMDAR blockage during CRS prevents dendritic

remodeling (Magarinos and McEwen, 1995a). Cell death induced by tau overexpression is mediated by extrasynaptic NR2B receptors, indicating an interaction between NMDARs and the microtubule backbone of cells (Amadoro et al., 2006). NR2B is also linked to PSA-NCAM, with glutamate-induced cell death in cultured hippocampal neurons inhibited by PSA-NCAM application in an NR2B dependent manner (Hammond et al., 2006).

The activity of NMDARs in stress and remodeling and the interaction between PSA-NCAM and NR2B indicate the NMDARs to be likely candidates for an altered response resulting from chronic stress in a PSA-NCAM depleted background. The increases in NMDARs following stress and the lack of remodeling seen with receptor blockage suggest that remodeling prevention might also prevent changes in NMDAR expression levels following chronic stress. Since PSA-NCAM works with NR2B to prevent cell death, the absence of PSA-NCAM could be very dangerous to the animal.

PSA-NCAM removal has experimental usefulness in that it allows us to examine stress-induced remodeling in a way that use of a knockout mouse cannot, by preserving normal development, and also in that it allows us to use the same model system in which much previous research has taken place. The fact the PSA-NCAM also has direct interactions with many molecules that experience fluctuation in response to chronic stress adds to the potential information to be gained in experiments where its formation is prevented.

CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Experiments were performed on adult male Sprague-Dawley rats (SD strain; Charles River Laboratories). Rats were obtained at two months of age, approximately 200-250 g. After arrival, rats were kept for one week 3 per cage to recover from the shipping process. Rats were then single-housed, and had unlimited access to food and water except during experimental manipulations. The cages were maintained on a 12-hour light/dark cycle, with lights on from 7:00 am to 7:00 pm. All experimental manipulations were performed during the light period. All experiments and procedures were conducted according to protocols approved by the Institutional Animal Care and Use Committee (IACUC).

2.2 EndoN Injections

2.2.1 Stereotaxic Surgery

Rats were anesthetized with xylazine (0.5 mg/kg plus additional supplements if necessary; i.p.) and ketamine (0.4 mg/kg plus additional supplements if necessary; i.m.) and placed in a Stereotaxic apparatus (Kopf). An incision was made on the scalp and the skin retracted to expose bregma on the surface of the skull. Once bregma was measured, a hole was drilled (1.00 mm, Stoelting) through the skull unilaterally on the right side P -0.38 mm L -0.15 mm. A 10 μl Flexifil Tapertip needle (World Precision Instruments, Inc.) attached to the swing arm of the stereotax was then lowered V -0.15 mm. All coordinates were determined using the rat brain atlas of Paxinos and Watson (1987) to obtain an injection site in the posterior

parietal associated area of the cortex. A volume of 2 μl was inserted steadily over a 2-minute period of either endo N (87 U/μl) or 0.9% saline. After injection, the needle was left in place for 5 minutes, and then slowly raised back out of the brain. The drill hole was sealed with bone wax (Lukens), and the head wound sutured closed with 3.0 metric Chromic Gut sutures (CP Medical). Animals were then moved to a clean home cage placed atop a heating pad while recovering from anesthesia. From surgery forward the animals were single-housed. Rats recovered in-cage undisturbed for one week following the surgery before the stress procedure began.

2.2.2 Preparation of Endo N

The enzyme endoneuraminidase N was a gift from the Rutishauser lab. It was expressed by IPTG induction of pREP4-repressed M15 cells containing a QE60 (Quiagen #33603) plasmid encoding endoN, which was originally cloned from PK1F phage (Vimr et al., 1984), plus a 6xHis-tag. The final endo N preparation was diluted with glycerol, dialyzed at 4˚C against PBS, pH 8.0 with 50% glycerol. The preparation was at a concentration of 87 U/μl, with one unit of activity equaling the amount of enzyme required to remove half of the PSA staining in 1 mg PSA-NCAM substrate obtained from embryonic chick brain. Endotoxin contamination was monitored using the E-Toxate assay (Sigma). Endo N was stored at -20˚C and kept on ice in the surgical room until the needle was filled immediately before the injection, as exposure to heat destroys the enzyme activity.

2.3 Chronic Stress Procedures

2.3.1 Chronic Immobilization Stress (CIS)

After a week's recovery from surgery, animals were subjected to CIS 2 hr a day for 10 days. The stress period was 10:00 am until 12:00 pm. Rats were placed in plastic bags resembling DecapiCones but made in the lab. Absorbent pads were affixed inside the bags to keep fluid in place, and the plastic cones were taped shut at the large opening to prevent movement. The narrow end of the cone was opened to allow unrestricted breathing. The animals were placed back inside their cages for the duration of the stress period. Control animals were kept in a separate room from stressed animals and handled 2 min/d for 10 d during the immobilization stress period to acclimate them for subsequent behavioral testing. All animals were weighed on the first and the last day of the stress experiment.

2.3.2 Chronic Restraint Stress (CRS)

A subset of male rats was stressed without first being subject to endo N injections. These animals underwent CRS 6 hr a day for 21 days. The stress period was 10:00 am until 4:00 pm. Rats were places in wire mesh restrainers clipped shut at either end. When not being stressed, the rats were housed 2 per cage, and throughout the experiment a group of control rats was housed in the same conditions undisturbed. The day after stress termination, animals were given an overdose of Nembutal (sodium pentobarbital). Brains were extracted from the rats and the hippocampi were quickly dissected from the brains before freezing on dry ice. All tissue was stored at -80˚C until use.

2.4 Tissue Collection and Storage

2.4.1 Perfusion

Rats were injected with an overdose of Nembutal and were then transcardially perfused first with 0.9% saline containing heparin (1 U/ml) added right before the start of perfusions until the liver was clear of blood, then with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.4) for 400 ml. Brains were then removed from skulls and post-fixed for one hour in 4% PFA before transfer to 30% sucrose in 0.1 M PB. The vibratome was used to cut the brain into 40 μm sections, which were stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB, pH 7.4) at -20˚C.

2.4.2 Fresh Frozen

For tissue that needed to be freshly frozen, rats were quickly decapitated. Brains were removed and immediately frozen in dry ice.

2.5 Golgi Staining and Analysis

Animals were injected with an overdose of Nembutal (sodium pentobarbital) and once unconscious, brains were rapidly removed and processed using an FD Rapid GolgiStain™ Kit (FD NeuroTechnologies) according to previously established methods (Glaser and Van der Loos, 1981) that give successful staining of hippocampal pyramidal cells (McLaughlin et al., 2007). Immediately after removal the brains were sliced in half coronally to allow for better impregnation. Brain tissue was left in impregnation solution for 2 weeks and solution C for 1 week. Brains were cut in 200 μm sections (Leica VT 1000S Vibratome) and mounted on Superfrost Plus slides (Fisher Scientific). Excess solution was absorbed onto blotting paper (Fisher

Scientific). Slides were then processed until stained according to Kit directions, and slides were coverslipped with DPX (Fluka).

To select cells for analysis, impregnated neurons had to fit the following characteristics. (1) full impregnation of the cell body and dendrites; (2) relative isolation from surrounding impregnated neurons; (3) located within the segment of CA3b excluding the curvature nearing CA2 and before the imaginary line connecting the dorsal and ventral blades of the dentate gyrus; (4) cell bodies located within the middle third of the tissue section to avoid false endings of dendrites at the edges of the section. Neurons were traced using a Nikon Eclipse E600 microscope using a 40X objective and Neurolucida 8 software (MBF Bioscience). There are three subtypes of pyramidal neurons in CA3: neurons with a single shaft that is either short (short shaft, SS), long (long shaft, LS), and neurons with more than one primary shaft (two-shaft, 2S) (Fitch et al., 1989). Each of the subtypes has a different degree of dendritic complexity, so care was taken to represent the subtypes equally across animals and across experimental groups. For each group, 18-22 neurons were selected. Analysis was conducted of total dendritic length and dendritic branch points for the apical dendrites. The Sholl method (Uylings et al., 1986) was also used to measure apical dendritic complexity in 30 μm intervals.

2.6 Immunohistochemistry and Analysis

2.6.1 Immunofluorescence Labeling

To determine if endo N treatment removed PSA-NCAM from the brain throughout the length of the experiment, every 24th section from each perfused brain was labeled for PSA-NCAM. Free-floating sections were rinsed in 0.1 M PBS with 0.2% Triton X-100, then blocked in 10% normal goat serum (NGS) for 30 minutes. Sections were then incubated overnight at room temperature in mouse monoclonal anti-PSA-NCAM IgM (1:700; Chemicon) in 10% NGS. The following day, sections were rinsed in 0.1 M PBS with 0.2% Triton X-100, then incubated in Alexa Fluor 488 goat anti-mouse IgM secondary antibody (1:200; Molecular Probes) in 10% NGS for 24 hr at 4˚C in the dark. Continuing in the dark, sections were rinsed in 0.1 M PBS with 0.2% Triton X-100 followed by rinses in 0.1 M PB. Sections were mounted and coverslipped with Permafluor (Thermo Scientific) and stored at 4˚C overnight. Slides were sealed with clear nail polish and analyzed on a Nikon Eclipse E600 microscope using StereoInvestigator software (MBF Bioscience).

2.6.2 Immunocytochemistry and Cell Counting

To perform accurate statistical analysis and ensure identical labeling conditions during immunostaining, sections from each treatment group were rinsed in PB and coded using hole-punches in the cortex before being pooled into color-coded mini-crucibles and carried through the staining procedure (Pierce et al., 1999). Tissue was washed in 0.1 M TBS with 0.1% Triton X-100, then incubated in 1% H_2O_2 in TBS for 30 min. Tissue was washed again in 0.1 M TBS with 0.1% Triton X-100 before blocking in 3% BSA in TBS/0.1% Triton X-100 for 1 hour. Tissue was then incubated overnight at 4˚C in rabbit

polyclonal IgG anti-cleaved caspase-3 (1:500; Cell Signaling) in 3%BSA/TBS/0.1% Triton X-100. The following day sections were rinsed in TBS/0.1% Triton X-100, and then incubated in biotinylated goat anti-rabbit IgG (1:600; Vector Laboratories) for 1 hr at room temperature. Sections were again washed in TBS/0.1% Triton X-100, then incubated in ABC for 1 hr at room temperature (Vectastain ABC Kits, Vector Laboratories) (Hsu et al., 1981). Sections were then washed in TBS/0.1% Triton X-100 and developed in DAB solution (Vector Laboratories). The DAB reaction was stopped with a wash in H_2O, then sections were rinsed in 0.1 M PB and mounted on Superfrost Plus slides (Fisher Scientific) and dried. Slides were dehydrated through an ethanol series and cleared with xylenes, then coverslipped with Permount (Fisher Scientific). Sections were analyzed on a Nikon Eclipse E600 microscope using StereoInvestigator software (MBF Bioscience).

2.6.3 Immunocytochemistry and Densitometry

Immunohistochemistry was run multiple times for multiple proteins using perfused tissue using the same procedure. Tissue was divided into mini-crucibles as described in previous immunocytochemistry section. Sections were rinsed in 0.1 M TBS and blocked in 0.5% BSA in TBS for 30 min at room temperature. Sections were then incubated in either mouse monoclonal IgG anti-human PHF-Tau (1:15,000; Pierce Biotechnology) or rabbit polyclonal IgG anti-phosphoLIMK1 (1:500, Cell Signaling) in 0.25% Triton X-100 and 0.1%BSA/TBS for 24 hr at room temperature followed by 24 hr at 4˚C. Tissue was rinsed the following day in 0.1 M TBS, then incubated for 30 min in biotinylated horse anti-mouse or goat anti-rabbit secondary (1:400; Vector Laboratories) in 0.1% BSA/TBS. Tissue was then rinsed in 0.1 M TBS,

incubated in ABC for 30 min at room temperature (Vector Laboratories), washed in TBS and developed in DAB solution for 6 min (Vector Laboratories). The DAB reaction was stopped with a wash in H_2O, then sections were rinsed in TBS followed by 0.1 M PB and mounted on Superfrost Plus slides (Fisher Scientific) and dried. Slides were dehydrated through an ethanol series and cleared with xylenes, then coverslipped with Permount (Fisher Scientific).

Quantitative densitometry was then performed on the mounted sections. When sections were selected, care was taken in picking two sections from each animal that were the same in each animal, corresponding as closely as possibly to 1.46 mm and 2.18 mm caudal to bregma (Paxinos and Watson, 1987). Images of regions of interest (ROI) were captured using a Dage MTI CCD-72 camera and NIH Image 1.50 software on a Nikon Eclipse 80i microscope. The mean grey value (out of 256 grey levels) was calculated for each ROI. To compensate for background staining a background measurement from non-stained tissue on each section was taken and subtracted from the ROI value. Each individual mini-crucible contained tissue from multiple groups as another level of control. Net optical density values were converted to a percentage scale of the grey values using NIH Image.

2.7 In Situ *Hybridization and Analysis*

Brains were cut at 20 μm on a cryostat and placed on Fischer Superfrost Plus slides. The probes used were the following sequences: CART 5′-ATC GGA ATG CGT TTA CTC TTG AGC TTC TTC AGG-3′; NPY 5′-TGC CCG GAC CTG GCC CCT CTG CTC CGC CCC-3′, 5′-GCT GGC GCG TCC TCG CCC

GGA TTG TCC GGC TTG GAG GGG TA-3'; NR2A 5'-TCG GGA GTT CCC TTT GGA TTC AGT GCT GAC AGC-3'; NR2B 5'-CAT GTT CTT GGC CGT GCG GAG CAA GCG TAG GAT-3'. Two complement probe sequences for NPY were used as a cocktail to improve hybridization sensitivity. First a tailing reaction was performed to radioactively label the oligonucleotide probes with ^{33}P –dNTP at the 3' end using terminal deoxynucleotidyl transferase (Promega) to a specific activity of 5 x 10^9 cpm/μg. The probe was then purified with the QIA quick spin nucleotide removal kit (Quiagen). Slides were processed through a series of washes: 3.7% formaldehyde in PBS, PBS, ddH_2O, 0.1 M triethanolamine (Sigma) with 5 ml/l acetic anhydride, 2X SSC, and then ascending concentrations of ethanol (70%, 95%, and 100%), chloroform, and finally 100% ethanol. Slides were air-dried and then placed face up in a humidifying chamber containing kimwipes soaked in chamber solution (50% formamide (Sigma), 0.3 M NaCl, and 1x Tris-EDTA (TE; Ambion)). Sections were incubated for 1 hr in 175 μl prehybridization solution (50% deionized formamide (Sigma), 10% dextran sulfate (EMD), 0.3 M NaCl, 1X TE, 1x Denhardt's solution (Sigma), and 200 mM DTT (Sigma)) at 42˚C. Slides were then washed in a series of 2x SSC, 70% EtOH, and 95% EtOH before being placed back in the humidifying chamber and incubated with 150 μl hybridization solution (prehybridization solution, 500 mg/l sonicated salmon sperm DNA (Ambion)) at 42˚C overnight. The following day, sections were washed in a 3x in 1x SSC at 55˚C, then once in 1x SSC starting at 55˚C but removed from heat after the slides were placed in and subsequently brought to room temperature, a process that took about 2 hr. Slides were then washed in 50% ammonium acetate/EtOH, 85% ammonium

acetate/EtOH, and 100% EtOH before they were air-dried and exposed to Kodak MR autoradiography films for 2 weeks, developed, and analyzed for optical density using MCID (Imaging Research). Hippocampal measurements were taken bilaterally at 2.18 mm caudal to bregma (Paxinos and Watson, 1987).

2.8 Western Blotting

Frozen hippocampal tissue was homogenized using a procedure to enrich the sample for heat-stable proteins for analysis of levels of phosphorylated tau. To weighed tissue was added 10 volumes of buffer (10 mM sodium fluoride, 2 mM EGTA, 1mM activated sodium vanadate, 1 mM PMSF) in TBS. Sample was homogenized, then to it added beta-mercaptoethanol to 5% and NaCl to 2% (v/v). Sample was vortexed in the hood, and then incubated at 100˚C for 10 min. Sample was vortexed again and cooled on ice for 30 min, then spun in a microcentrifuge (Eppendorf) at 14,000 rpm at 4˚C for 15 minutes. The supernatant was pipetted off and frozen at -80˚C and the pellet discarded. Total protein in samples was determined by the Quick Start Bradford assay according to kit instructions (Bio-Rad). Protein samples were then run and separated by SDS-PAGE under reducing conditions with 4-12% acrylamide NuPage gels according to manufacturer's instructions (Invitrogen). Proteins were transferred from the gels to PDVF membranes (Invitrogen). After transfer, membranes were dipped in 0.1 M TBS with 0.1% Tween-20 (TBS-T), then blocked in 5% nonfat dry milk (Carnation) in TBS-T for 1 hr at room temperature. Membranes were washed with TBS-T, and then incubated with primary antibody in blocking solution overnight at 4˚C. All of the following

mouse monoclonal anti-tau antibodies were the gift of Peter Davies at Albert Einstein College of Medicine: CP3 IgM (1:10), CP9 IgM (1:20), CP13 IgG (1:500), MC6 IgG (1:10), PG5 IgG (1:10), PHF1 IgG (1:10), TG5 IgG (1:100,000). The next day membranes were washed with TBS-T and incubated with the appropriate anti-mouse (IgM or IgG) horseradish peroxidase-conjugated antibody (1:10,000; Pierce) in blocking solution for 1 hr at room temperature. Membranes were washed again with TBS-T, and then developed using SuperSignal West Pico substrate (Pierce). Membranes were exposed to X-ray film (X-OMAT AR, Kodak), and film was analyzed on the light box for optical density using MCID.

2.9 Behavioral Tests

2.9.1 Open Field Exploration

The day after the last stress treatment (day 11), rats were tested in an open-field apparatus to measure exploratory behavior and anxiety for one 5-min trial ($n = 9$/group). Rats were brought into the behavior room in groups of 3 and allowed to acclimate to the move for 15 minutes. Rats were then placed in a novel square arena (70 X 70 X 40 cm) made of black Plexiglas with a video camera mounted 1 m above the arena. The camera fed into a computer running Noldus Ethovision XT, a program that analyzes behavior in real-timed based on user-inputted parameters. A grid dividing the floor area into 25 equally sized blocks present on the floor of the maze was used to divide the maze on the program into perimeter and center areas. Perimeter included the single block increments around the edge of the maze, and center included the 9 inner blocks. The program identifies and tracks the animal itself as it

moves in the maze. The maze was thoroughly cleaned between subjects with 50% ethanol and water. Behavioral measures of activity, exploration, anxiety and fear included the total distance traveled, time spent in the center, time spent in the perimeter, rearing behavior, and the number of fecal boli.

2.9.2 Y-Maze

The y-maze was constructed of black Plexiglas with three identical arms (50 cm length X 16 cm width X 32 cm height) with visual cues located outside the maze and with a video camera mounted 1 m above the arena connected to a TV and VCR to record behavior for subsequent analysis. The floor of the maze was covered with cage bedding that was mixed between trials. Rats were brought into the behavior room in groups of 3 and allowed to acclimate to the move for 15 minutes. There were two trials during the light cycle on the day following CIS termination. For the first trial, access to one arm (novel arm) was blocked with black Plexiglas, and animals were allowed to freely explore the other two arms for 15 min. Animals were then removed from the maze for 2.5 hr. After this period, animals were reintroduced to the maze, this time with the Plexiglas panel removed and free access to the novel arm, and rats were allowed to explore for 10 min. Tapes of behavior were coded and then analyzed blind, with time spent in each area and total rears in the maze analyzed.

2.10 Corticosterone Assay

Blood was taken from rats during the perfusion procedure once the body cavity was opened but before beginning the pumping of fluids. Blood was

centrifuged and the serum was analyzed for corticosterone levels using the Coat-A-Count rat corticosterone kit (Diagnostic Products Corporation).

2.11 Fluoro Jade B Staining

Perfused sliced tissue was mounted and dried overnight. Slides were then stained with Fluoro-Jade B (FJB) as previously described (Schmued and Hopkins, 2000) to detect cells undergoing necrosis or apoptosis.

2.12 Data Analysis

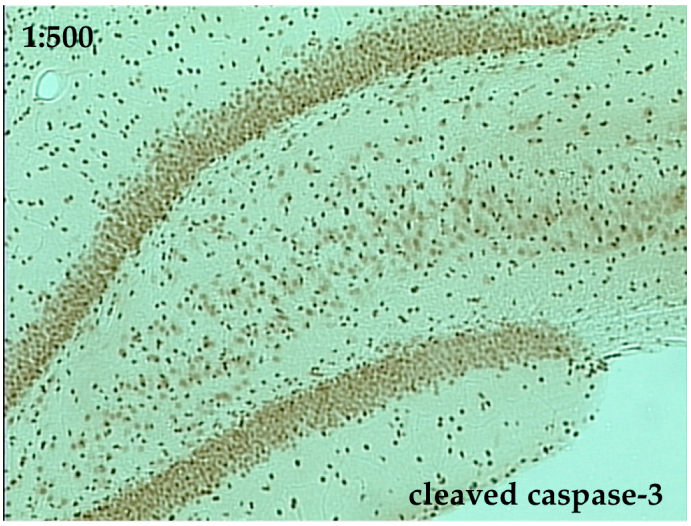
Data was analyzed using Prism 5 performing two-tailed t-tests or two-way ANOVA and Bonferroni post-tests. All data are presented as mean ± SEM. *P* values < 0.05 were considered significant.

CHAPTER 3: EFFECT OF ENDO N TREATMENT ON ANIMAL SURVIVAL AND HEALTH

The injection of a foreign substance directly into the brain of a subject is a complicated procedure that could possibly be harmful in multiple ways. The injection procedure itself could cause damage, the endo N could be directly harmful to the animal, and any PSA-NCAM removal caused by endo N injection could have detrimental effects before any further stress is added to the animal. All these possibilities were examined. It was also important to determine the physiological, system-wide effects of PSA-NCAM removal.

Animals were closely monitored after surgery and during the CIS period. Single-housed rats showed no sign of infection, and head wounds healed at a uniform speed and were not reinjured by CIS. Nissl stained tissue sections showed needle placement in some but not all brains, but no further damage beyond the width of the needle. Fluoro Jade B staining revealed no necrotic or apoptotic cells in any of the experimental groups, including animals subjected to CRS after PSA removal by endo N. However, as a positive control, mouse tissue from an ischemic study in the lab processed along with the rat tissue did in fact show apoptotic cells. ICC staining for activated cleaved caspase-3 revealed no significant difference between saline and endoN injected stressed groups, with sporadic staining throughout the brain but no significant pattern of staining in the DG or CA regions (Fig. 1).

A. representative staining



B. cleaved caspase-3 positive cells in HC

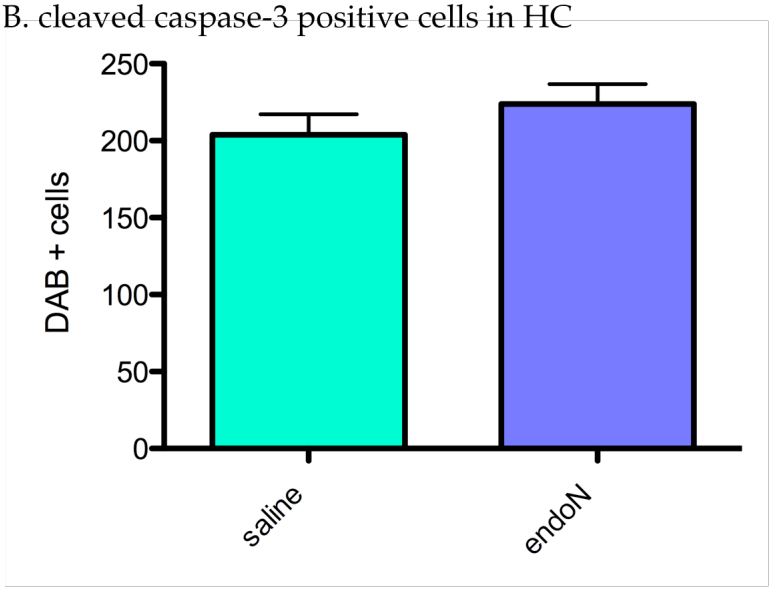


Figure 1. PSA-NCAM removal prior to chronic stress does not increase levels of activated caspase-3. The active form of caspase-3 is present in the brain of 10 d CIS rats, but not in a specific pattern that would indicate damage to the DG, CA3 or any other surrounding areas. Moreover, there was no evidence of cell death based on Fluoro Jade staining (see text). A. Representative picture of staining in the HC. B. Number of cells stained for cleaved-caspase 3 was counted throughout the HC. Quantification of DAB + cells, bound to cleaved caspase-3 antibody, indicates no significant difference between endo N-injected and saline-injected stressed rats by two-tailed t-test ($P > 0.05$; $n = 9$). Staining specificity was determined through the use of controls.

It also needed to be determined whether endo N injection was sufficient to remove PSA-NCAM for the duration of the experiment, throughout CIS and behavioral tests. Immunofluorescence staining revealed that while PSA-NCAM was present in DG in both stress and control animals, endo N injected had no PSA-NCAM (Figure 2). A control group of animals that did not undergo stereotaxic surgery but were still single-housed showed less variation between stress and control than animals that received a saline-injection, perhaps due to the stress of the surgical procedure itself.

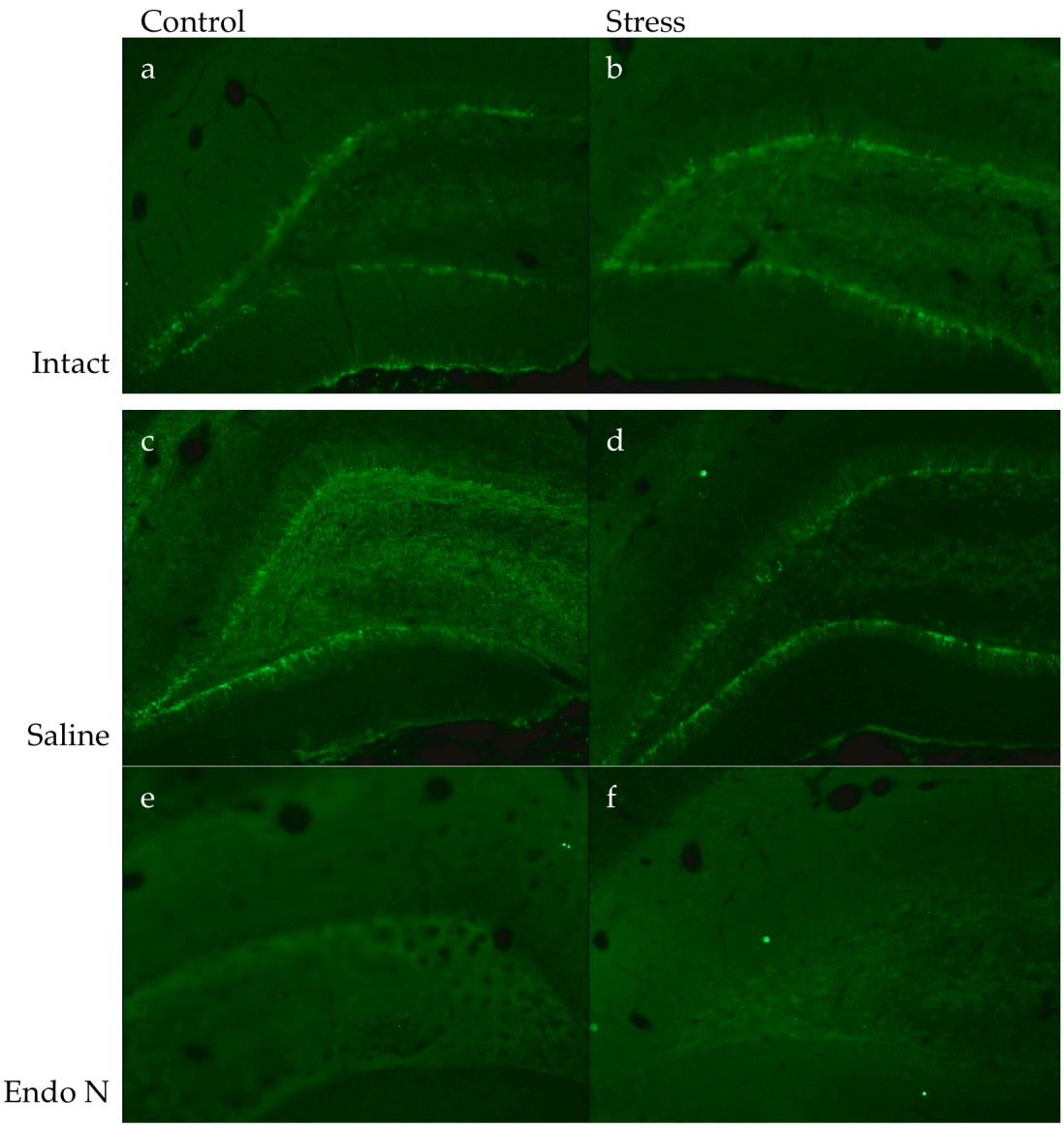


Figure 2. Endo N injection removes PSA-NCAM from the DG of the hippocampus. Male rats underwent 10d CIS. One week before CIS, some of the rats were injected with either endo N or saline. The day after stress termination, animals were sacrificed and their brains examined histologically for the presence of PSA-NCAM. A. Single-housed control rats showed some PSA-NCAM staining in the granule cell layer (GCL) of the dentate gyrus. B. Non-injected stressed rats showed more even PSA-NCAM expression following CIS. C&D. Saline injected rats, whether they underwent CIS or not, showed high levels of PSA-NCAM immunoreactivity. E&F. Endo N animals showed absolutely no PSA-NCAM immunoreactivity, regardless of CIS presence. ($n = 6$).

Radioimmunoassay of circulating serum corticosterone (CORT) was performed on animals on the day of sacrifice the day after the last CIS period. 10 d CIS had no effect on CORT levels in uninjected animals or in 10 d CIS groups that first received a saline injection. However, PSA-NCAM removal by endo N pretreatment caused a significant increase in stressed rats as compared to PSA-NCAM removed controls (Figure 3).

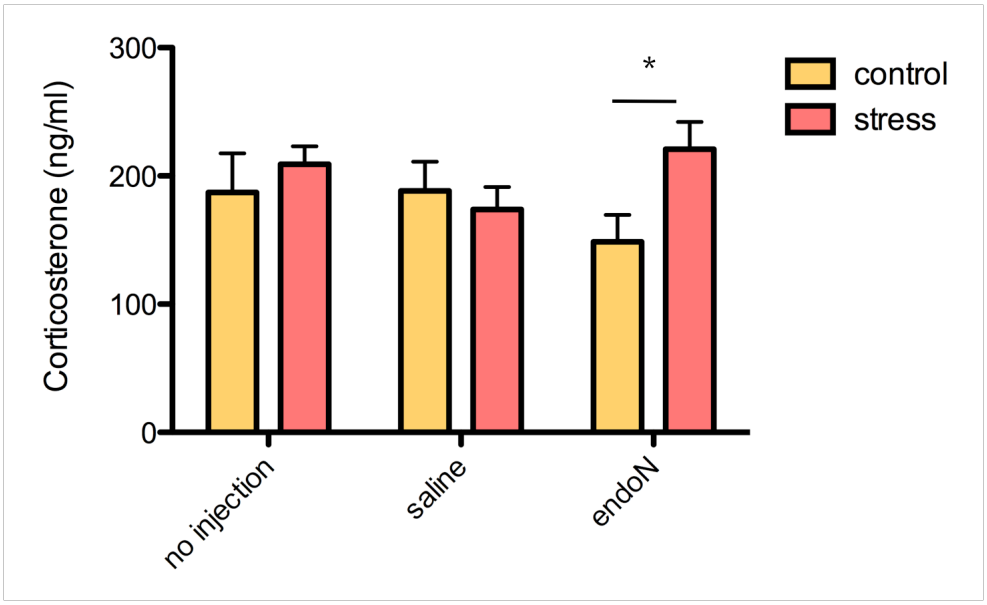


Figure 3. PSA-NCAM removed animals show an increase in CORT in response to chronic stress. Single-housed male rats do not show an increase in circulating CORT in response to 10 d CIS. The same is true in animals that are stereotaxically microinjected with saline 1 week before 10 d CIS. However, intracerebral microinjection of endo N to remove PSA-NCAM 1 week before 10 d CIS causes animals to show an increase in circulating CORT, taken from the abdominal cavity shortly before the beginning of perfusion on the day after stress termination by two-tailed t-test ($P < 0.05$; $n = 6$).

Total amount of weight lost was quantitated for every run of the experiment. 10 d CIS caused animals to gain significantly less weight over the course of the experiment than control rats (Figure 4). Animals with PSA-NCAM removed also showed a stress-induced decrease in weight gain. Endo N injection alone did not contribute to this decreased weight gain, as PSA-NCAM removed animals that did not experience stress were no different from control saline-injected animals. In these experiments PSA-NCAM removed stressed rats showed the same weight gain difference as saline-injected stressed rats, albeit with a significantly larger variance.

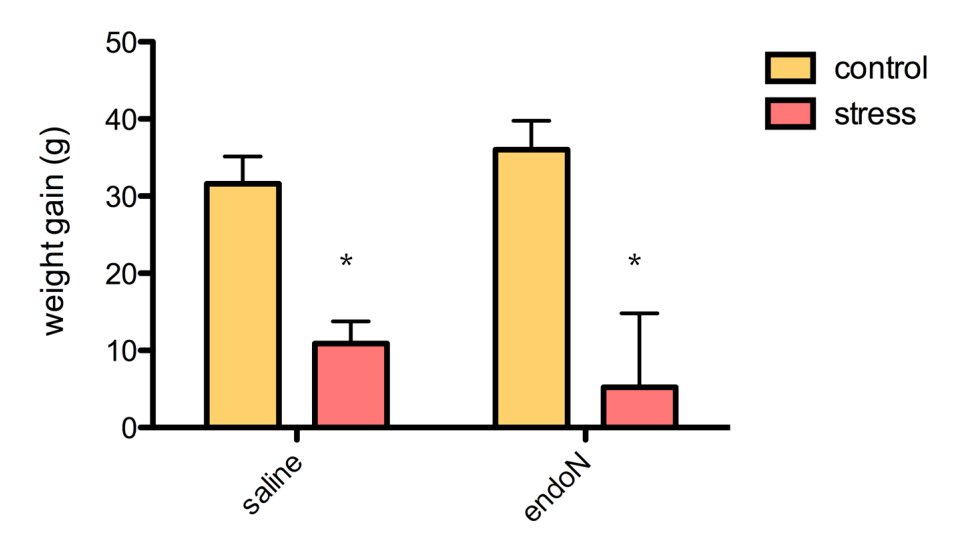


Figure 4. Chronic stress causes decreased weight gain regardless of PSA-NCAM presence. 10 d CIS causes a decreased level of weight gain compared to non-stressed rats ($P < 0.0001$). PSA-NCAM removal did not affect weight gain of non-stressed rats, and although it did not modify the stress effect on weight loss, PSA-NCAM deficient rats showed significantly more variance in the amount of weight gained compared to saline-injected stressed rats by two-way ANOVA ($P < 0.005$, $n = 9$).

In the final run of the endoN + 10 d CIS experiment, a different pattern of weight gain data emerged. 10 d CIS caused a decrease in weight gain over the course of the experiment, and PSA-NCAM removal had no effect on weight gain in non-stressed animals, but in stressed animals PSA-NCAM had such a severe effect on weight gain that overall the PSA-NCAM removed group lost weight over the course of the experiment (Figure 5). Overall weight loss had been seen in PSA-NCAM removed stressed group in previous experimental runs, but there were always large variances in the data that masked this in the final statistics. The greater effect in this run is most likely due to increased success with the endoN injection technique, having at this point performed it on over 100 rats, to the point were the surgery was successful enough repeatedly to remove the extra variance.

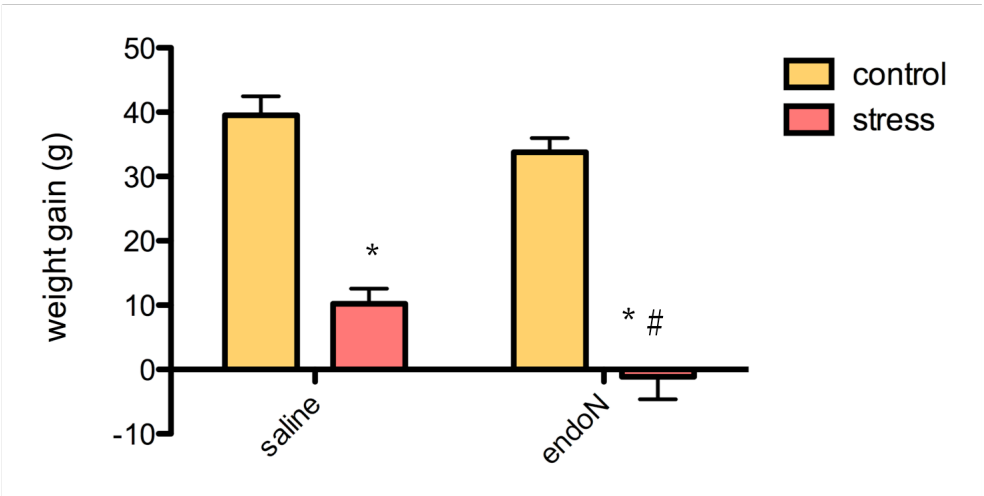
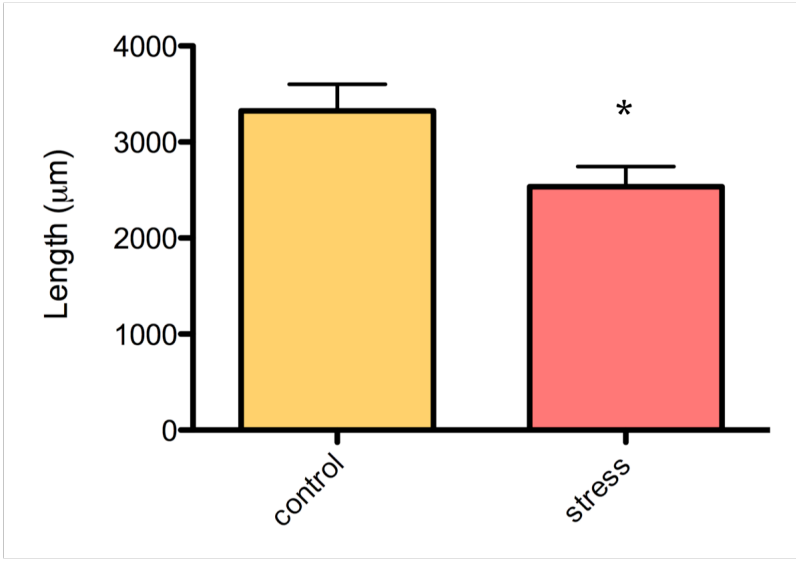


Figure 5. Chronic stress causes decreased weight gain and stress-induced decrease is exacerbated by lack of PSA-NCAM. 10 d CIS decreases level of weight game compared to control rats (* $P < 0.0001$). PSA-NCAM removal did not affect weight gain of non-stressed rats, but in the presence of CIS, PSA-NCAM deficient rats lose weight over the stress period, a significant difference from saline-injected chronically stressed rats as measured by two-way ANOVA (# $P < 0.005$; $n = 10$).

CHAPTER 4: EFFECT OF RESTRAINT STRESS IN PSA-NCAM REMOVED ANIMALS ON DENDRITIC REMODELING

Three weeks of chronic restraint stress produces dendritic retraction in the apical dendrites of region CA3 of the HC in male rats (Watanabe et al., 1992a). The stress procedure in these experiments was 10 days of chronic immobilization stress (CIS). To examine the effects of PSA removal on dendritic remodeling, it is helpful to first look at the effects of the stress paradigm in the animals injected with the saline control. Ten days of CIS caused a significant decrease in the total length of apical dendrites and the total number of branch points in region CA3 of the HC in animals that had previously received an intracerebral saline injection (Figure 6). This result is similar to that seen in standard three week chronic restraint stress experiments and shows that the 10-day bag stress produces chronic stress-related changes, and that these dendritic remodeling changes are present in the animals that were microinjected with saline. Sholl analysis of the stress-induced remodeling showed that the dendritic complexity differences occurred between 120 – 210 μm from the cell soma (Figure 7).

A. Total dendritic material



B. Number of branch points

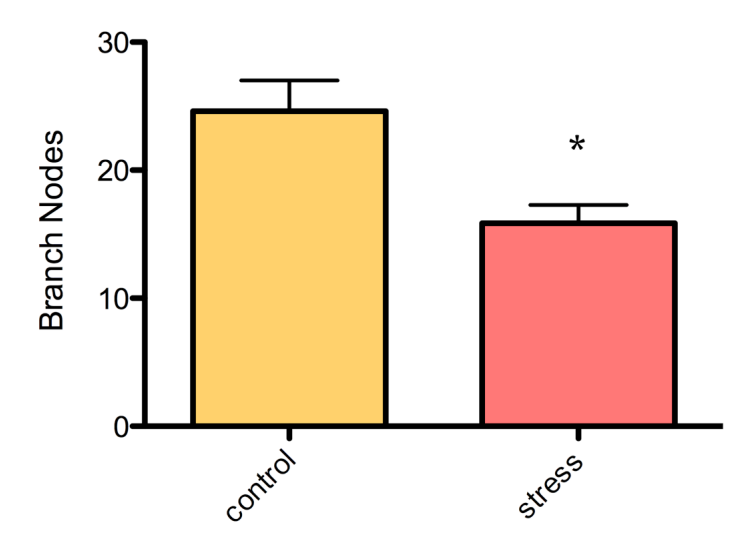


Figure 6. Chronic stress decreases apical dendritic length and branch points in saline-injected rats. Adult male rats received a unilateral stereotaxic microinjection of 2 μl saline into the cortex. After one week of recovery, animals were subjected to 10 days of CIS. After the final day of stress, rats were sacrificed and the brains removed for processing according to RapidQuick Golgi protocol. Analysis of Neurolucida tracing showed a decrease in total apical dendritic material (A) and number of apical branch points (B) in chronically stressed rats by two-tailed t-test ($P < 0.05$, $n = 20$).

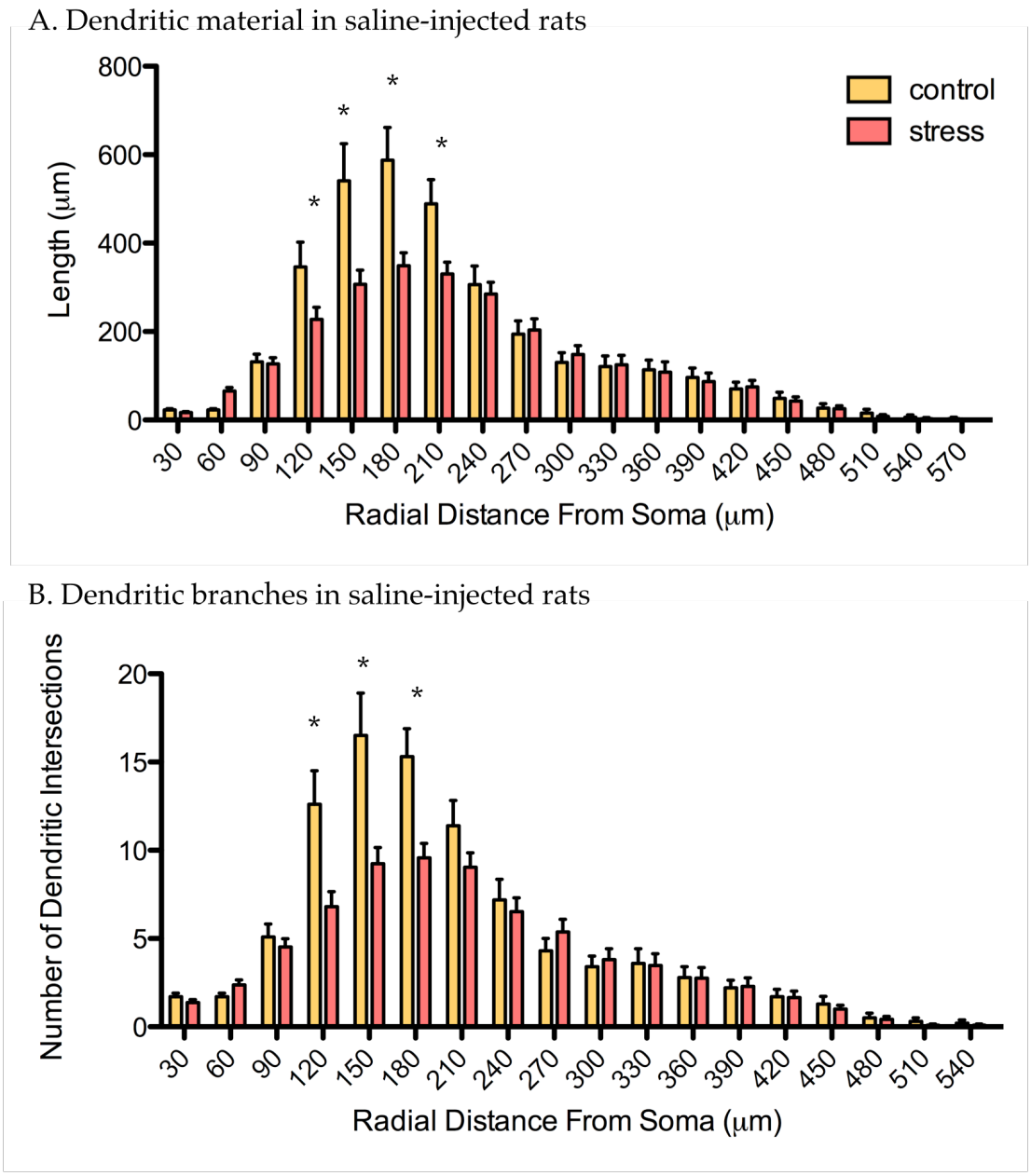


Figure 7. CIS-induced reductions in dendritic complexity occur 120 – 210 μm from cell soma. Sholl analysis of Golgi-stained tissue from saline-injected CIS rats revealed a decrease in dendritic material starting at the 120 μm distance from the cell body and continuing until 210 μm by two-way ANOVA with Bonferroni posttests ($P < 0.05$, $n = 20$).

After achieving confirmation of stress-induced remodeling, PSA-NCAM depleted animals were added into the analysis. PSA-NCAM removal prevents chronic stress from causing total dendritic remodeling. Both control and 10 d CIS rats pretreated with endo N show significantly larger and more complex apical dendritic trees than the saline-injected control and 10 d CIS rats (Fig. 8). The PSA-NCAM depleted animals also showed some significant differences when comparing their control and stress conditions. PSA-NCAM depleted stressed rats had significantly less apical branch nodes than PSA-NCAM depleted controls (Fig. 8b), but the total apical dendritic length between PSA-NCAM depleted stressed and control rats was not significantly different. The differences between PSA-NCAM depleted neurons and saline-injected neurons can be seen by examining Neurolucida tracings of representative neurons from each group (Fig. 9). The PSA-NCAM depleted neurons are much more complex to the naked eye.

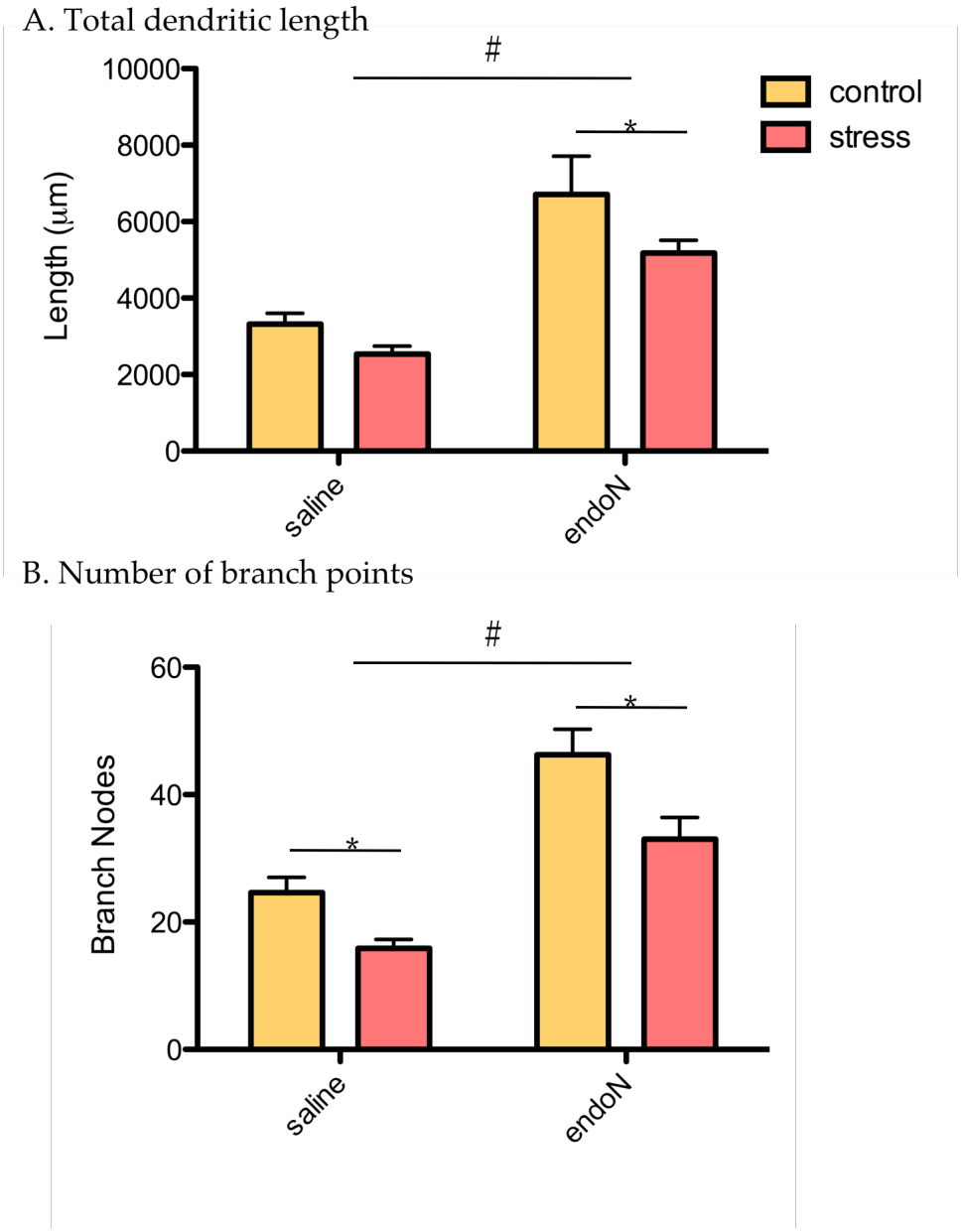
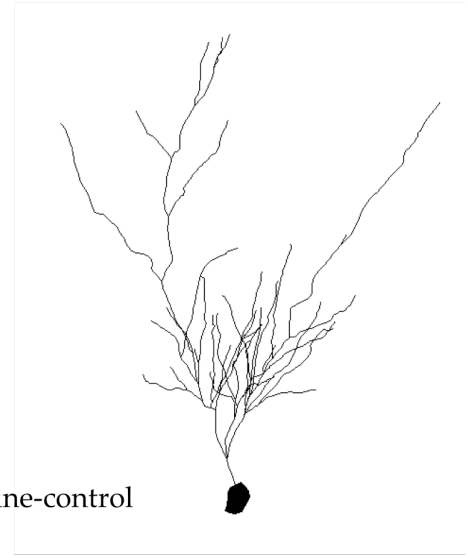


Figure 8. PSA-NCAM depleted neurons are significantly more complex than saline-injected neurons while still retaining some measure of plasticity. Animals that received an intracranial injection of endo N to remove PSA-NCAM have much larger and more complex neurons as measured by total apical dendritic length (A) and number of apical branch points (B) (# $P < 0.0001$). Saline-injected animals showed the normal plasticity response following 10 d CIS (* $P < 0.05$), and PSA-NCAM depleted animals also showed some plasticity. Total apical dendritic length was not affected by 10 d CIS, but CIS did significantly decrease apical dendritic branch points in PSA-NCAM depleted stressed rats as compared to PSA-NCAM depleted controls. Analysis by two-way ANOVA with Bonferroni posttests (* $P < 0.05$; $n = 20$).

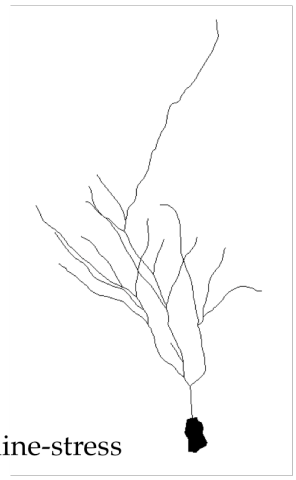
Figure 9. CA3 Neurolucida tracings of representative neurons from each experimental group show stress-induced dendritic retraction and endo N-induced dendritic hypertrophy. A. Single-housed saline-injected control rat. B. Single-housed saline-injected CIS rat. C. Single-housed PSA-NCAM removed control rat. D. Single-housed PSA-NCAM removed CIS rat. After analysis of Neurolucida Golgi-stain tracings was finished, the mean value for each experimental group was determined. The neurons closest to that value were selected for the figure above. LS neurons were selected for all groups except D, for which only a 2S neuron was close to the mean. The difference in levels of complexity can be clearly seen when comparing saline-injected A and B to PSA-NCAM removed C and D. In neurons A and B, the complexity differences occurring between 120 – 210 μm from the cell soma are evident in the sparseness of the branch pattern of neuron B. It can be seen from tracings C and D that dendritic complexity extends well beyond 120 – 210 μm range, where there are very few dendrites in the saline-injected neurons. It is also evident that neuron D, the PSA-NCAM removed stressed brain, still has complexity much closer to the PSA-NCAM removed control neuron than either of the saline-injected neuronal tracings. LS: long shaft neuron. 2S: two main shafts neuron.

A



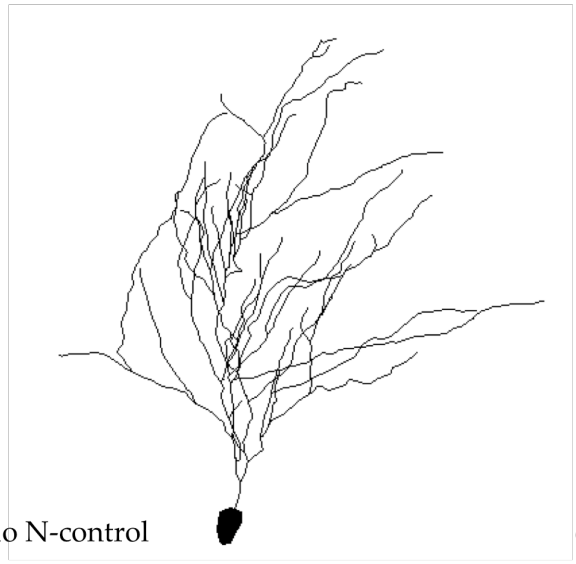
saline-control

B



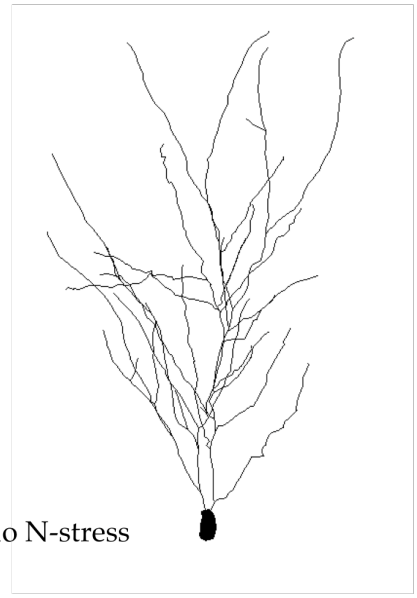
saline-stress

C



endo N-control

D

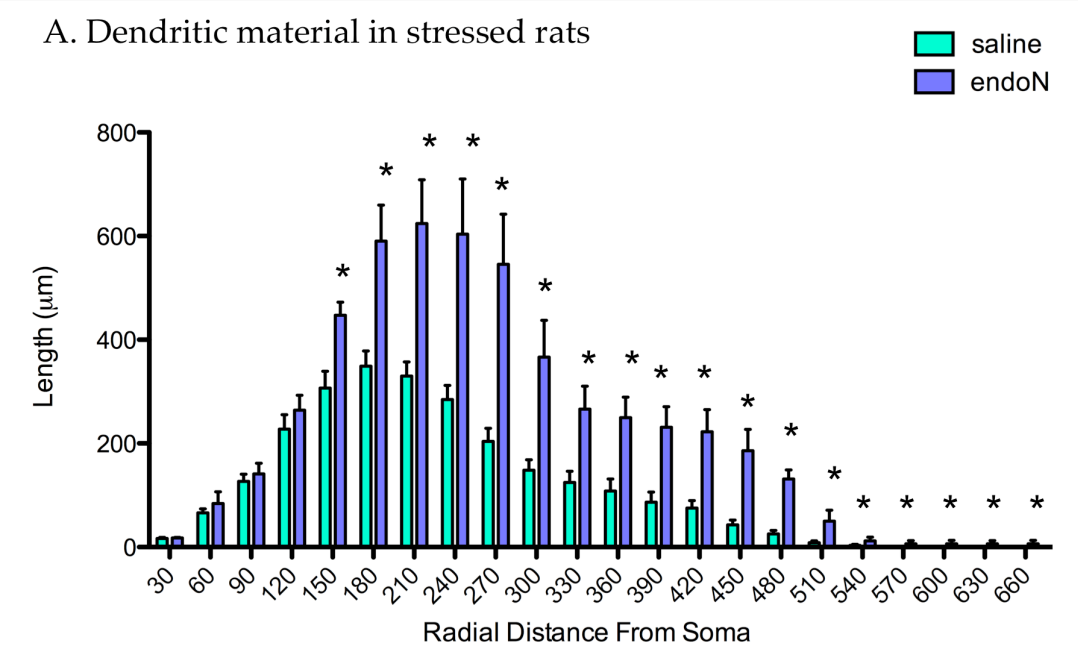


endo N-stress

Sholl Analysis of PSA-NCAM depleted animals in comparison to saline-injected animals further illustrates the large complexity difference. PSA-NCAM depleted CIS neurons have significantly more dendritic material and dendritic line crossings per 30 μm radial area than saline-injected CIS neurons (Fig. 10). Apical dendrites from PSA-NCAM depleted controls were also significantly larger and more complex than those from saline-injected control rats (Fig. 11). In addition, unlike the stress effect in saline-injected animals, PSA-NCAM depleted neurons have significantly more dendritic material at distances distal to the cell soma.

Figure 10. PSA-NCAM depleted CIS animals have significantly more complex apical dendritic arbors than saline-injected CIS animals. Animals received an intracerebral microinjection of endo N to permanently remove PSA-NCAM from the brain. 10 d CIS yielded significantly more complex neurons over the whole length of the dendritic tree as measured by Sholl analysis as compared to saline-injected 10 d CIS rats. Complexity was measured by (A) amount of dendritic material at a given radial distance, and (B) number of dendrites crossing the radial intersection. The increased complexity in PSA-NCAM depleted animals was seen throughout the length of the apical dendritic arbor, as opposed to the stress effect in saline-injected animals, which was only present from 120-210 µm from the cell soma. The PSA-NCAM depleted CIS neurons also reach farther distances, with dendritic material present up to 660 µm from the cell soma, as opposed to saline-injected CIS neurons with a maximum reach of 570 µm. Analysis with two-way ANOVA and Bonferroni posttests ($P < 0.0001$, $n = 20$).

A. Dendritic material in stressed rats



B. Dendritic branches in stressed rats

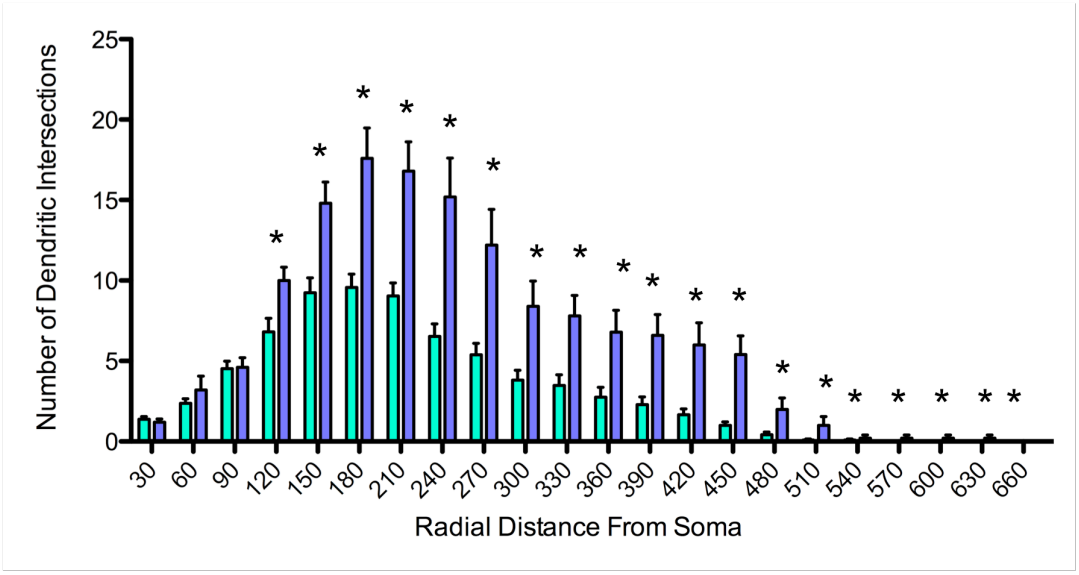
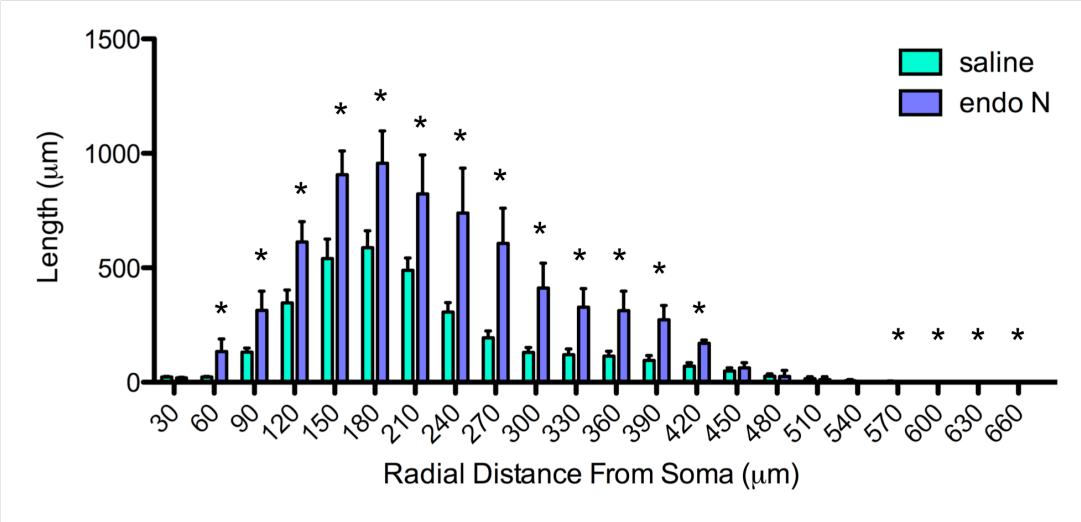
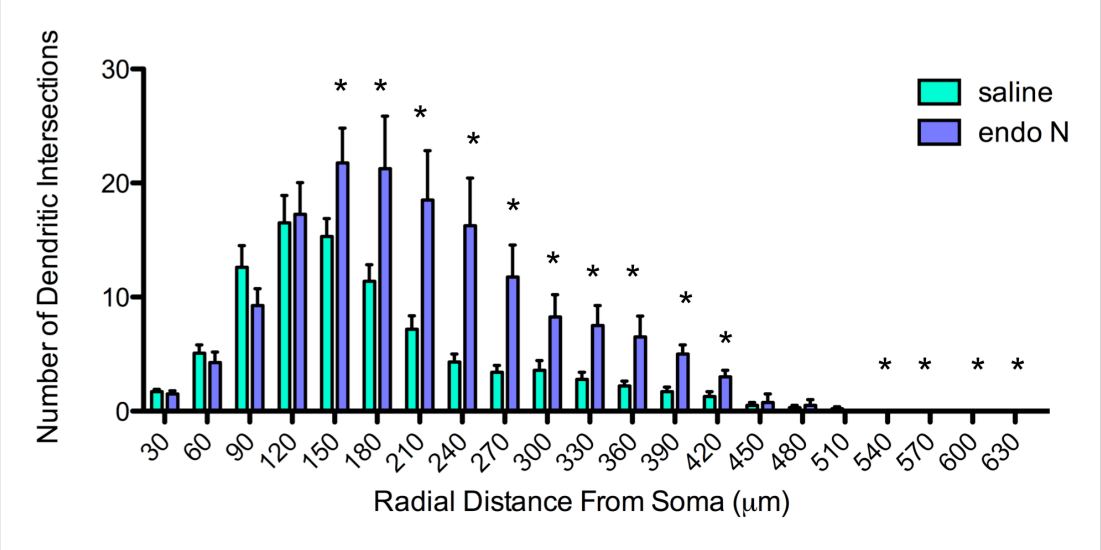


Figure 11. PSA-NCAM depleted control animals have significantly more complex apical dendritic arbors than saline-injected controls animals. Animals received an intracerebral microinjection of endo N to permanently remove PSA-NCAM from the brain, and then remained in housed single-caged while CIS was performed on a separate group of animals. Apical dendrites in PSA-NCAM removed animals were significantly more complex over the whole length of the dendritic tree as measured by Sholl analysis as compared to saline-injected control rats. Complexity was measured by (A) amount of dendritic material at a given radial distance, and (B) number of dendrites crossing the radial intersection. The increased complexity in PSA-NCAM depleted animals was seen throughout the length of the apical dendritic arbor. Analysis with two-way ANOVA and Bonferroni posttests ($P < 0.0001$, $n = 20$).

A. Dendritic material in control rats

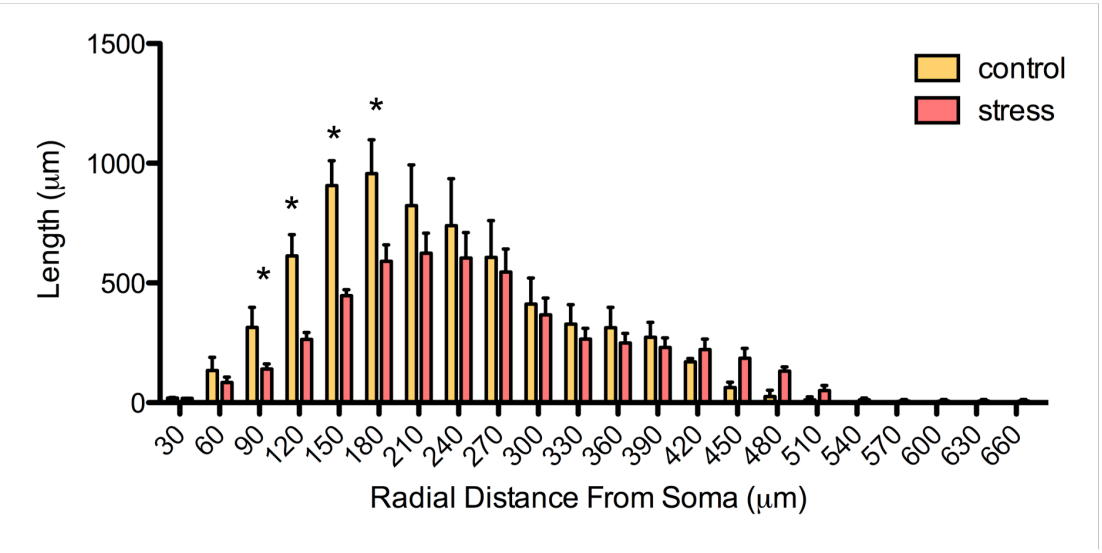


B. Dendritic branches in control rats



Sholl analysis further examined how much plasticity is present in PSA-NCAM removed stressed rats. CIS in PSA-NCAM depleted rats yielded CA3 apical dendritic trees with significantly less total dendritic length than PSA-NCAM removed controls (Fig. 12a), but Sholl analysis of radial dendritic intersections revealed no significant effect of CIS (Fig. 12b). Some plasticity, therefore, occurs following 10 d CIS in PSA-NCAM removed rats, but it is not as complete a phenomenon as seen in the absence of endo N injection. The plasticity that does occur in PSA-NCAM depleted animals with 10 d CIS results in apical dendritic trees that are still significantly larger than those of saline-injected control rats (Fig. 13). Each experimental condition resulted in a unique apical dendritic profile, and the PSA-NCAM removal relationship and the saline-injection relationship of relative size is seen when Sholl anaylsis was performed for all the groups together (Fig. 14).

A. Dendritic material in endo N injected rats



B. Dendritic branches in endo N injected rats

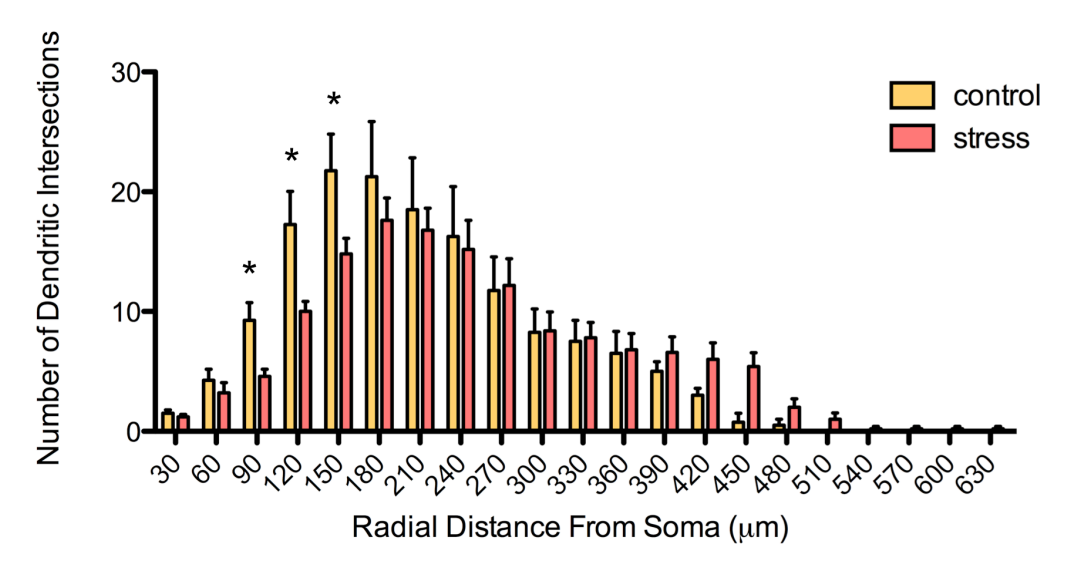
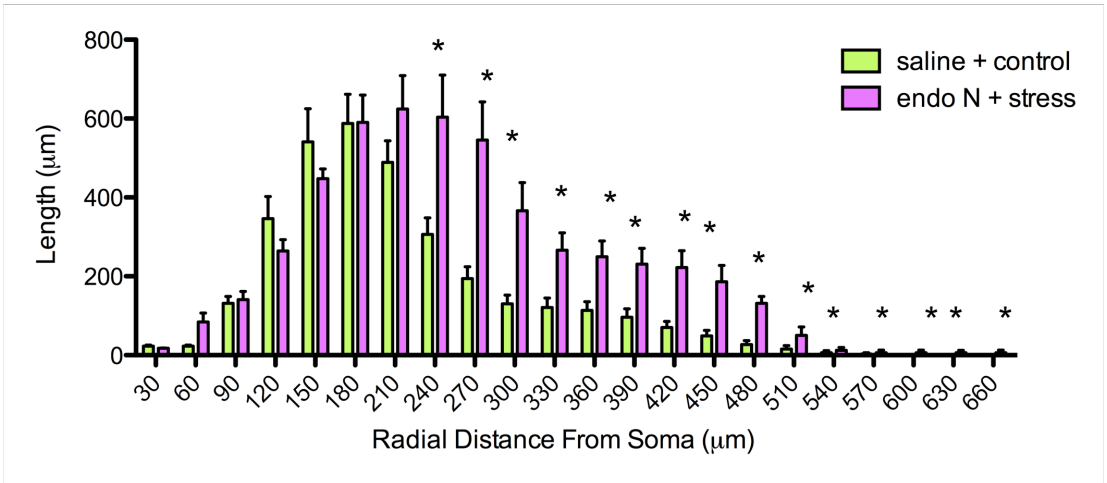


Figure 12. 10 d CIS in PSA-NCAM depleted rats decreased dendritic material per radial unit but did not significantly affect the number of dendritic intersections per unit. A. 10 d CIS significantly decreased dendritic material per radial distance block from the cell soma in PSA-NCAM removed rats ($P < 0.05$). B. 10 d CIS did not significantly decrease the number of dendritic intersections of the radial divisions in PSA-NCAM removed rats. Analysis with two-way ANOVA and Bonferroni posttests ($n = 20$).

A. Dendritic material



B. Dendritic branches

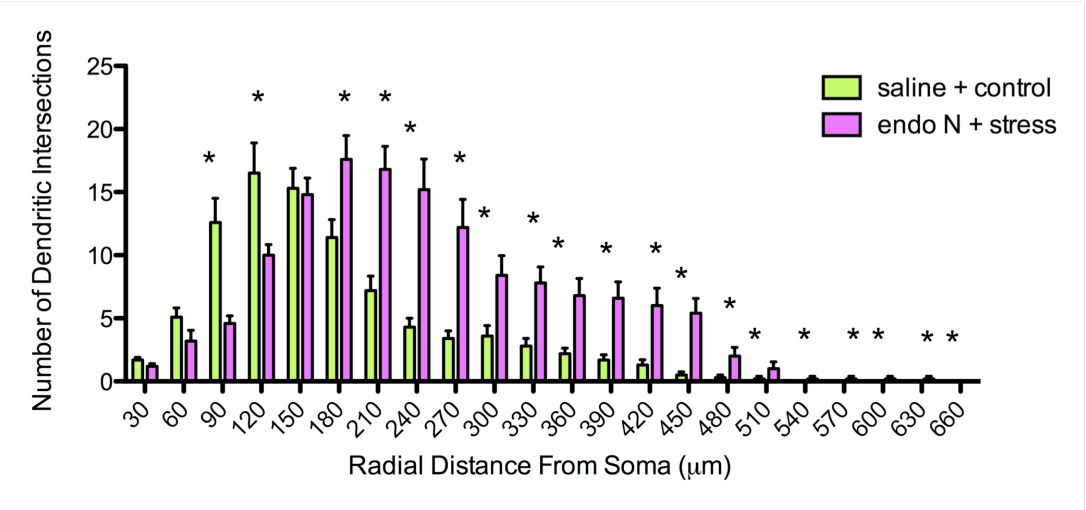


Figure 13. PSA-NCAM removed 10 d CIS rats had significantly more complex apical dendritic trees than saline-injected controls. PSA-NCAM removal by endoN injection produced CA3 apical dendritic trees that were significantly more complex than those seen in saline-injected control rats as measured by Sholl analysis of (A) dendritic material per radial unit, and (B) number of individual dendrites crossing each radial plane. Analysis with two-way ANOVA and Bonferroni posttests ($P < 0.0001$, $n = 20$).

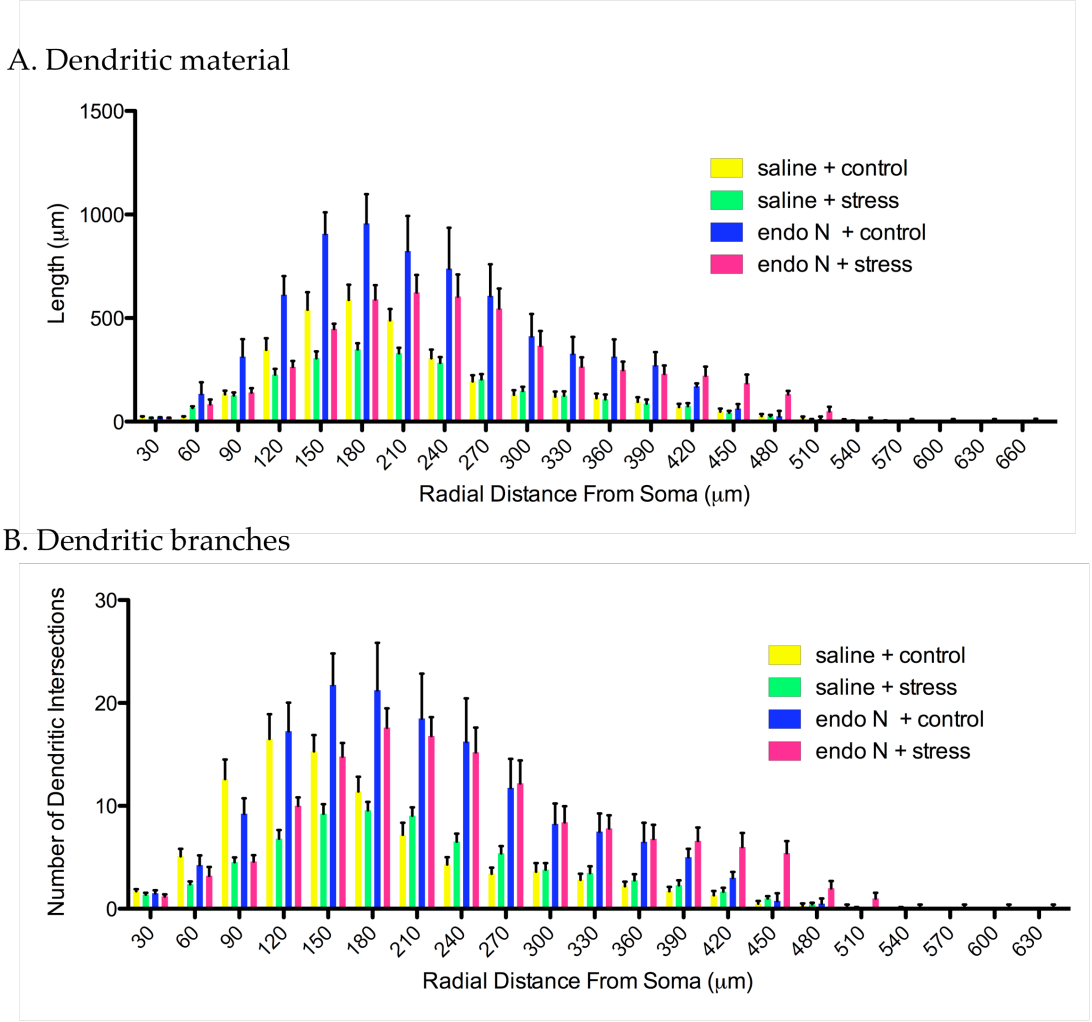


Fig 14. Both CIS and PSA-NCAM removal significantly affect the size and complexity of CA3 apical dendrites. Sholl analysis of Golgi stained brains shows 10 d CIS decreases apical dendritic complexity and PSA-NCAM removal increases dendritic complexity as measured by (A) amount of dendritic material per radial distance from cell soma, and by (B) number of dendritic crossings of the radial boundary line. Analysis with two-way ANOVA and Bonferroni posttests ($P < 0.001$, $n = 20$).

CHAPTER 5: EFFECT OF RESTRAINT STRESS IN PSA-NCAM REMOVED ANIMALS ON STRESS-MODULATED BEHAVIORS

It was very important to determine if PSA-NCAM removal had any functional consequences for the animal. After previously determining that CIS after PSA-NCAM removal did not lead to an increase in cell death or cause death to the animal itself (Chapter 3), we looked for effects of PSA-NCAM removal on behaviors affected by chronic stress.

5.1 Anxiety Behavior

The day following the final day of CIS, animals were subjected to an open field maze to measure stress effects on anxiety. After an acclimation period to adjust to movement to the behavioral testing room, rats were placed in the open field for five minutes and recorded. Stress greatly increased the amount of fecal boli released in the maze (Fig. 15a). Stress also had a significant effect on the total amount of movement in the maze during the 5 minute period (Fig. 15b). The control, non-stressed rats traveled more in the maze than the CIS rats. Saline-injected rats showed no effect of CIS stress on the amount of time spent in the center of the open field and the amount of time spent in the perimeter (Fig. 15c,d). PSA-NCAM depletion had a significant effect in CIS. These rats spent significantly more time in the perimeter and less time in the center of the open field. This effect was not present in non-stressed PSA-NCAM depleted rats (Fig. 15c,d).

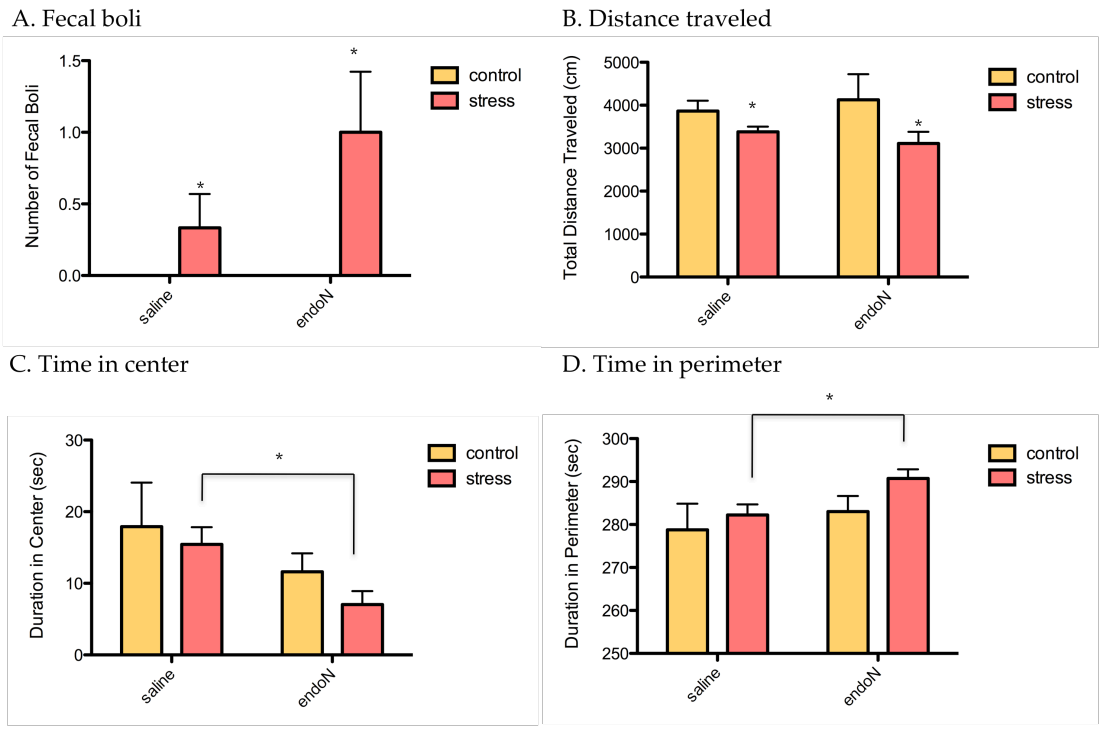


Fig 15. CIS has limited effects on increased anxiety that are enhanced with PSA-NCAM depletion. A and B. 10 d CIS in single-housed animals increased anxiety as measured by amount of fecal boli released in the open field maze and total distance traveled in the maze by two-way ANOVA with Bonferroni posttests ($P < 0.05$). PSA-NCAM depletion by endo N injection did not affect these two behaviors on top of the stress effect. C and D. Saline-injected single-housed rats showed no effect of stress on anxiety as measured by amount of time spent in the center and in the perimeter of the open field maze. PSA-NCAM removal before CIS significantly decreased the amount of time rats spent in the center of the maze and increased the time spent in the perimeter measured by two-way ANOVA and two-tailed t-test ($P < 0.05$). This change was not seen in control, non-stressed PSA-NCAM depleted animals. ($n = 9$).

5.2 Aggression

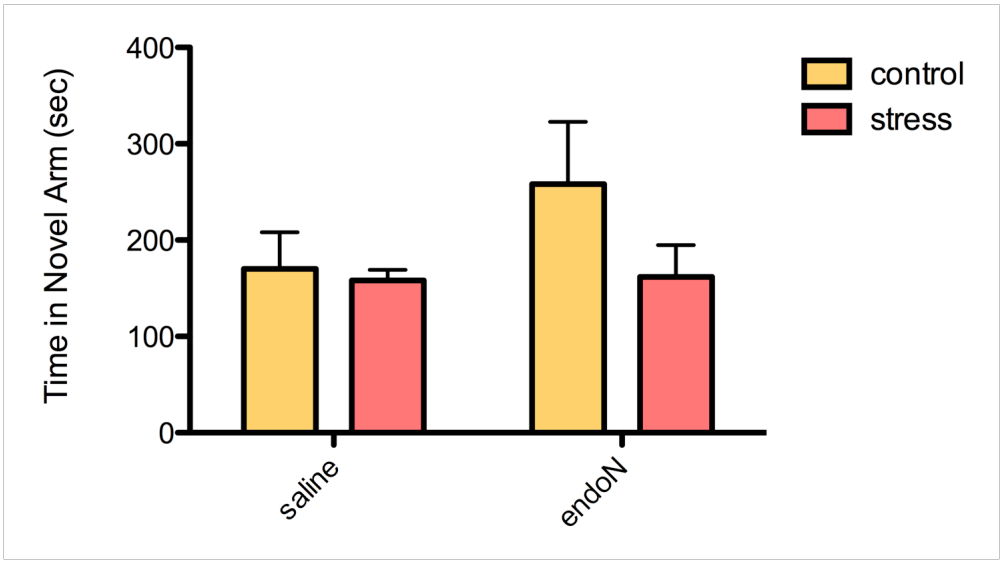
The single-housing conditions made it impossible to measure effects of CIS and PSA-NCAM removal on aggression. However, single-housing the animals was chosen based on the aggressiveness of the first trial run of the experiment, so anecdotally, some information can be contributed. The test run was performed to ensure that endo N injection and 10 d CIS did not physically hurt the animals or cause death. For the test run of the endo N injection experiment, animals were housed three per cage, as the plan was to record in-cage aggressive behavior during the actual experiment. This housing strategy was abandoned because the endo N injected rats would not stop hurting each other. After release from immobilization, they would attack each other very aggressively, resulting in re-injury of the surgical head would, visible wounds elsewhere on the flesh, and in some cases serious infection on the head and elsewhere in the body. One animal had to be put down due to a serious foot infection. After this, animals were single-housed in subsequent experiments and none of these problems were ever seen again because animals were no longer co-housed. CIS has been performed in animals housed three to a cage before, and although there is an increase in aggression (Wood et al., 2008), nothing on this scale is observed. Therefore, it is obvious that PSA-NCAM increased aggression in these animals, although quantitative analysis of this effect does not exist.

5.3 Spatial Memory

There was no effect of CIS on y-maze behavior in saline-injected animals. Although there was a trend for an increase in time spent in the novel arm in PSA-NCAM depleted controls, the effect was not significant (Fig. 16a). CIS significantly increased the amount of rears animals made in the maze (Fig. 16b).

There was further evidence of an anxiety increase in PSA-NCAM removed rats. They made significantly less rears onto their hind limbs in order to look out of the maze than saline-injected rats, and this indicates a higher state of fearfulness and anxiety (Fig. 16b).

A. Time spent in novel arm of y-maze



B. Total rears in y-maze

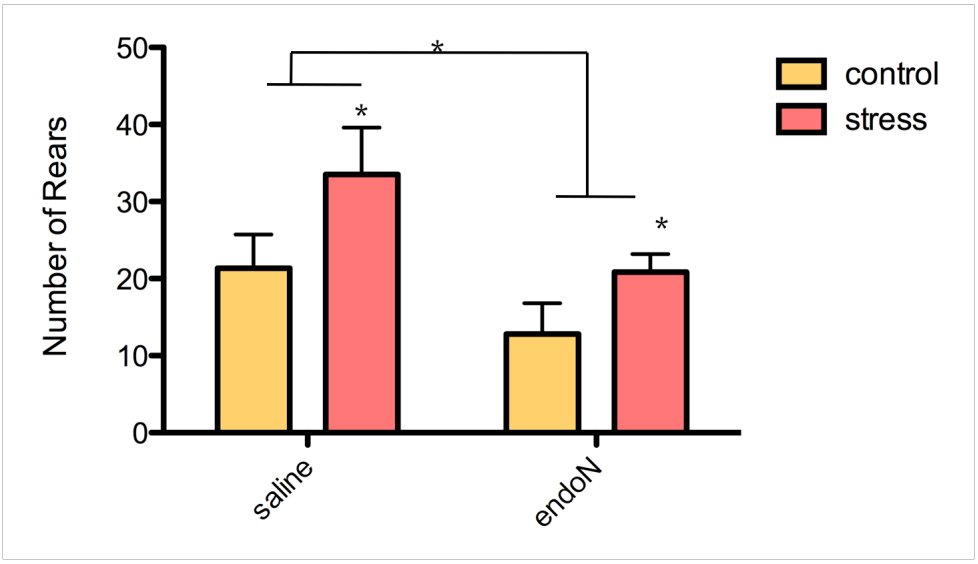


Fig 16. CIS and PSA-NCAM removal did not significantly affect spatial memory, but PSA-NCAM removal increased anxiety-like behavior in the y-maze. Single housed male rats were injected with either saline or endo N, and then submitted to 10 d CIS. Animals then were tested in the y-maze, where their natural desire to seek out novel situations is dependent on remembering where that novelty exists spatially. A. CIS did not affect time spent in novel arm of y-maze. A trend for endo N injected controls to spend more time in the novel arm of the y-maze was insignificant. B. CIS increased the amount of rears rats made in the maze**.** PSA-NCAM removal decreased the amount of rears, another measure of an increase in anxiety. Analyzed by two-way ANOVA with Bonferroni posttests (* $P < 0.05$; $n = 6$).

CHAPTER 6: EFFECT OF RESTRAINT STRESS AND PSA-NCAM REMOVAL ON EXPRESSION OF PLASTICITY AND AGGRESSION-RELATED PROTEINS

A host of molecular changes occur in response to chronic stress, and it is through these changes that dendritic remodeling and behavior alterations are produced. The removal of PSA-NCAM was expected to affect many of these changes, both because of the absence of remodeling and the lack of a direct interaction with PSA-NCAM itself. We looked for both evidence of dendritic remodeling-associated protein changes and changes in the levels of molecules affecting anxiety and general excitation in the HC.

6.1 Tau Phosphorylation

Western blot analysis was first performed on whole hippocampal dissections of chronically stressed rats to examine phosphorylation of tau at various residues (Fig 17). Levels of total tau protein were measured as a control for the phosphorylation differences (TG5 antibody), with no significant difference between stress and control rats. Chronic stress caused tau phosphorylation at Ser-205 as examined with the MC6 antibody. Hippocampal homogenates showed tau phosphorylation at Thr-231 (CP9 antibody) and Ser-202 (CP13 antibody), but levels were not affected by chronic stress. PKA-dependant phosphorylation at Ser-214 and 409 was not seen in stress or in control rats. PKA phosphorylation leads to insoluble tau like that seen in Alzheimer's disease (Jicha et al., 1999).

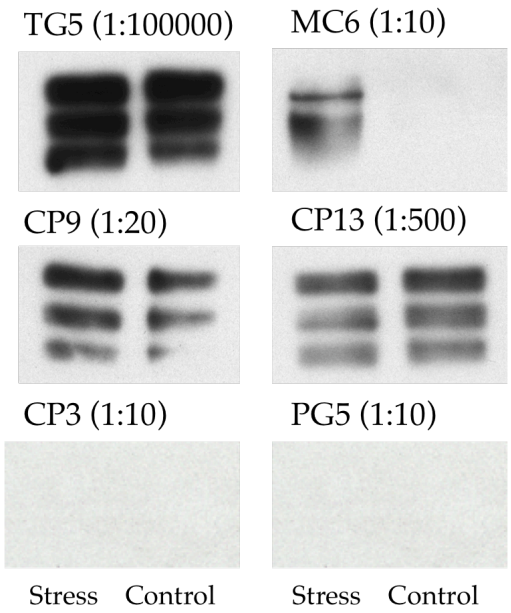


Figure 17. Levels of tau phosphorylation at various residues in response to stress. Rats were subjected to 21 d CRS. Animals were sacrificed, brains removed and the hippocampus quickly dissected and frozen. Western blot analysis revealed CRS causes phosphorylation of tau at Ser-205 (MC6) and that this phosphorylation is not present in control animals. The hippocampal protein samples revealed no stress-induced differences in total tau (TG5), Ser-202 tau phosphorylation (CP13), or Thr-231 phosphorylation (CP9). PKA-dependant tau phosphorylation at Ser-214 and 409 was not seen in stress or control rats (CP3, PG5). All staining showed 51 kDa bands.

Examination of whole hippocampal homogenates did not show stress-induced changes in tau phosphorylated at Ser-202. To see if there was any difference in Ser-202 phosphorylated tau in the CA3 alone, immunocytochemical analysis was performed on fixed tissue. PSA-NCAM depleted rats had significantly less Ser-202 phosphorylated tau in CA3 than saline-injected stressed rats (Fig. 18). This resembles the effect seen in ground squirrels undergoing torpor with retracted CA3 apical dendrites (Popov et al., 1992; Su et al., 2008).

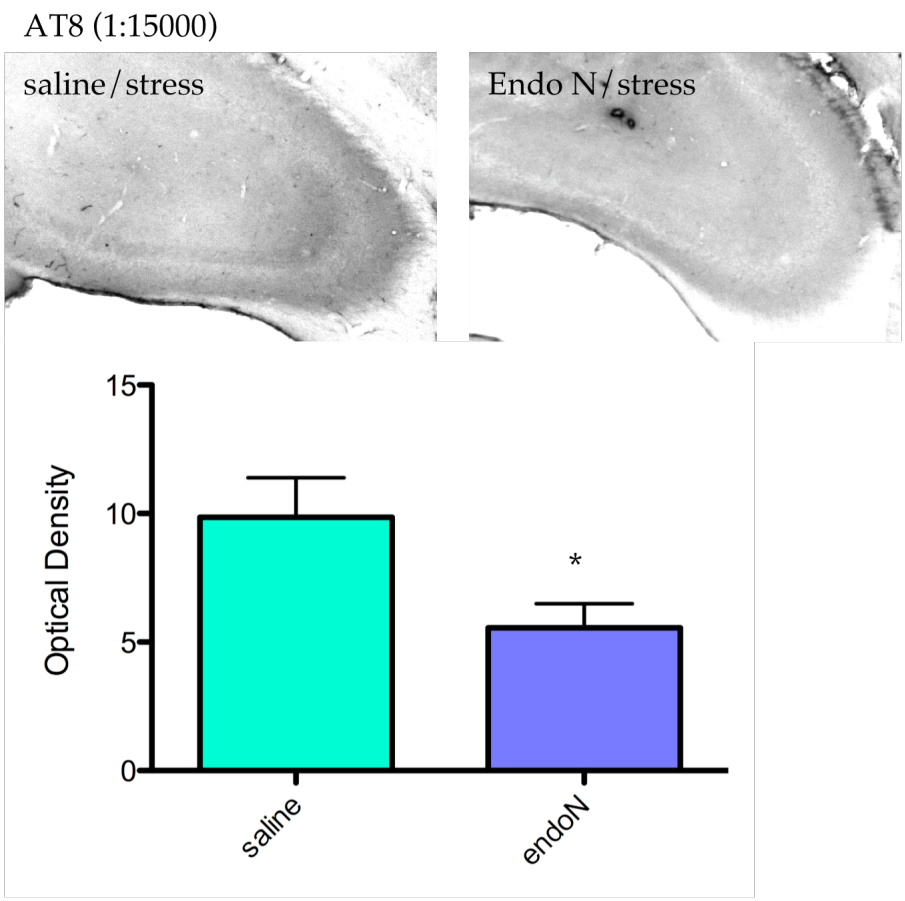


Figure 18. PSA-NCAM removal decreased Ser-202 tau phosphorylation in CA3 of stressed rats. Rats underwent 10 d CIS. Animals were perfused, brains were sectioned and immunocytochemistry was performed for Ser-202 phosphorylated tau. PSA-NCAM removal with endo N significantly reduced the amount of tau phosphorylation in CA3 in stressed rats analyzed by two-tailed t-test ($P < 0.05$; $n = 15$).

6.2 Phospho-LIM Kinase

Phospho-LIMK was examined to quantitate changes in synaptic organization and shape. Immunohistochemistry and densitometry was performed on perfused, sectioned 10 d CIS rat tissue. There was a trend for CIS to increase the amount of pLIMK protein staining in CA3, but it did not reach significance (P = 0.06). There was no trend toward a stress-induced increase in endo N-injected PSA-NCAM depleted rats (Fig 19).

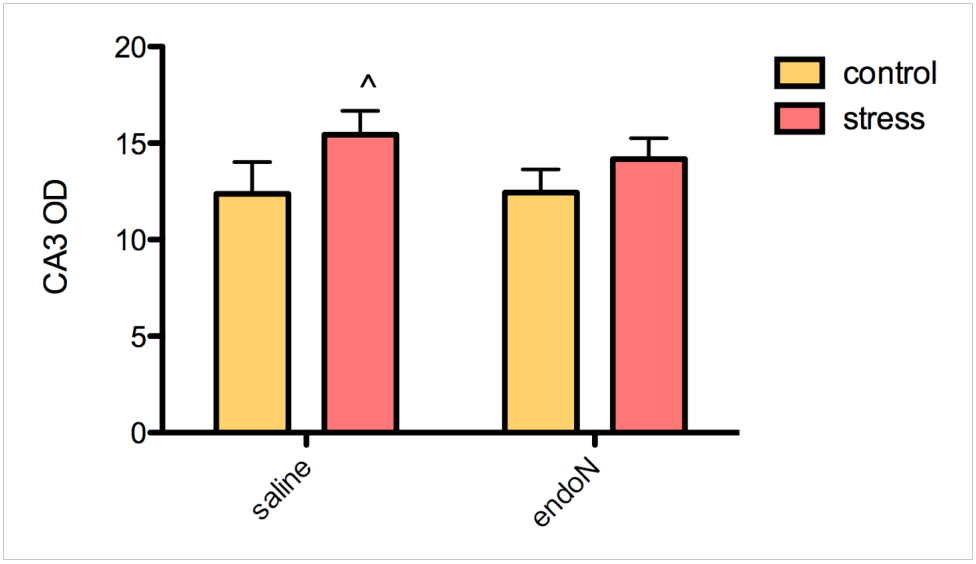


Figure 19. 10 d CIS did not significantly increase pLIMK protein levels in single-housed male rats. 10 d CIS in single-housed saline-injected rats trended to increase pLIMK levels in region CA3 of the hippocampus, but the effect was not significant (^ $P = 0.06$). There was no stress-effect trend in PSA-NCAM depleted animals. Analyzed by two-way ANOVA ($n = 12$).

6.3 Neuropeptide Y

NPY is an anxiolytic molecule that has been shown to increase in the DG in response to chronic stress (Conrad and McEwen, 2000). The effects of stress and PSA-NCAM removal on NPY mRNA expression in the hippocampus were seen when the hippocampus was divided up into sub-regions. Chronic stress increased NPY expression in the lower blade of the dentate gyrus and in region CA3b. CA3b refers to the horizontal region of CA3 cells, whereas CA3a refers to the cells in the curved vertical region that connects to CA2. In region CA3b, the same region where PSA-NCAM removal prevents dendritic remodeling, PSA-NCAM removal prevented the stress-induced increase in NPY. PSA-NCAM removal did not affect CA3b NPY expression in non-stressed controls (Fig 20).

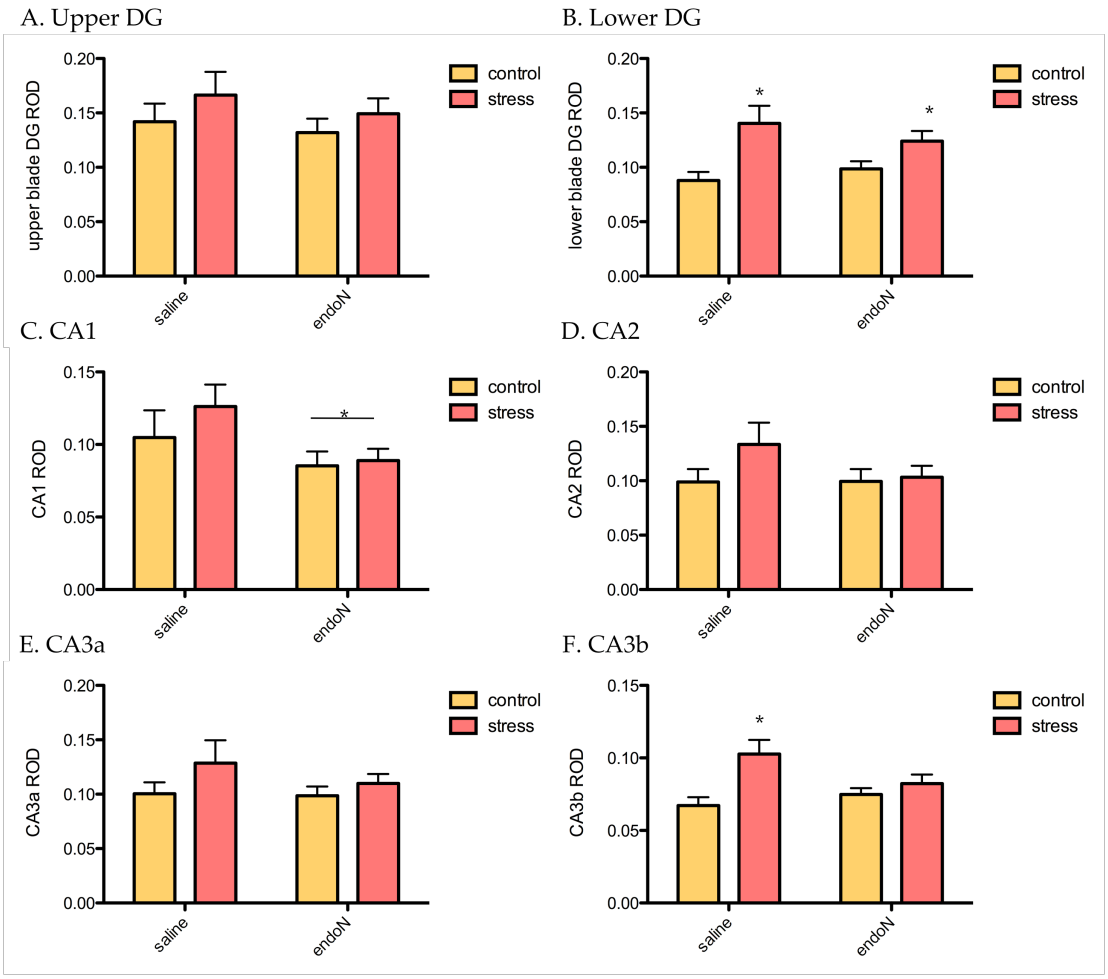


Figure 20. Increased CIS-induced hippocampal NPY expression is PSA-NCAM dependent in CA3b, but not in the lower blade of the DG. *In situ* hybridization was performed on fresh-frozen tissue from 10 CIS rats. CIS increased NPY mRNA expression in the lower blade of the dentate gyrus (B) and CA3b, but not in the upper blade of the dentate gyrus (A), CA1 (C), CA2 (D), or CA3a (E) based on two-way ANOVA. PSA-NCAM removal prevented the stress-induced NPY expression increase in CA3b (F). Endo N animals show lower levels of NPY mRNA expression in CA1 (C). Analyzed using two-way ANOVA with Bonferroni posttests and two-tailed t-tests ($P < 0.05$; $n = 10$).

6.4 CART

CART is an anxiety-related peptide that has been shown to increase in expression in the DG in response to CS (Hunter et al., 2009). *In Situ* autoradiography for CART mRNA in the dentate gyrus of the hippocampus revealed no significant effect of stress or PSA-NCAM removal (Fig 21). However, there was a lot of variability that could contribute to the lack of significant changes.

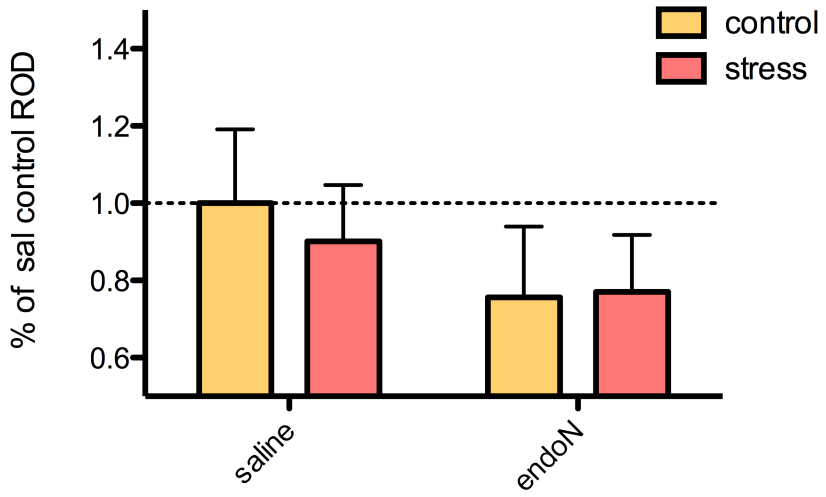


Figure 21. 10 d CIS and PSA-NCAM removal had no significant effect on CART mRNA expression in the DG. mRNA levels of CART were not affected by 10d CIS or PSA-NCAM removal in the upper blade of the dentate gyrus. *In situ* autoradiography produced a lot of variability even when data were examined as a percent of the control values. Analyzed by two-way ANOVA ($n = 10$).

6.5 NMDA Glutamate Receptors

Chronic stress increases NMDA receptors in the HC and blockage of these receptors prevents dendritic remodeling (Magarinos and McEwen, 1995a). These receptors are likely targets for expression changes in the PSA-NCAM removal experimental condition both for this reason and also because PSA-NCAM interacts with the NR2B receptor itself (Dityatev et al., 2004).

In situ autoradiography for NR2A mRNA in the DG of the hippocampus revealed no effect of stress or injection (Fig. 22).

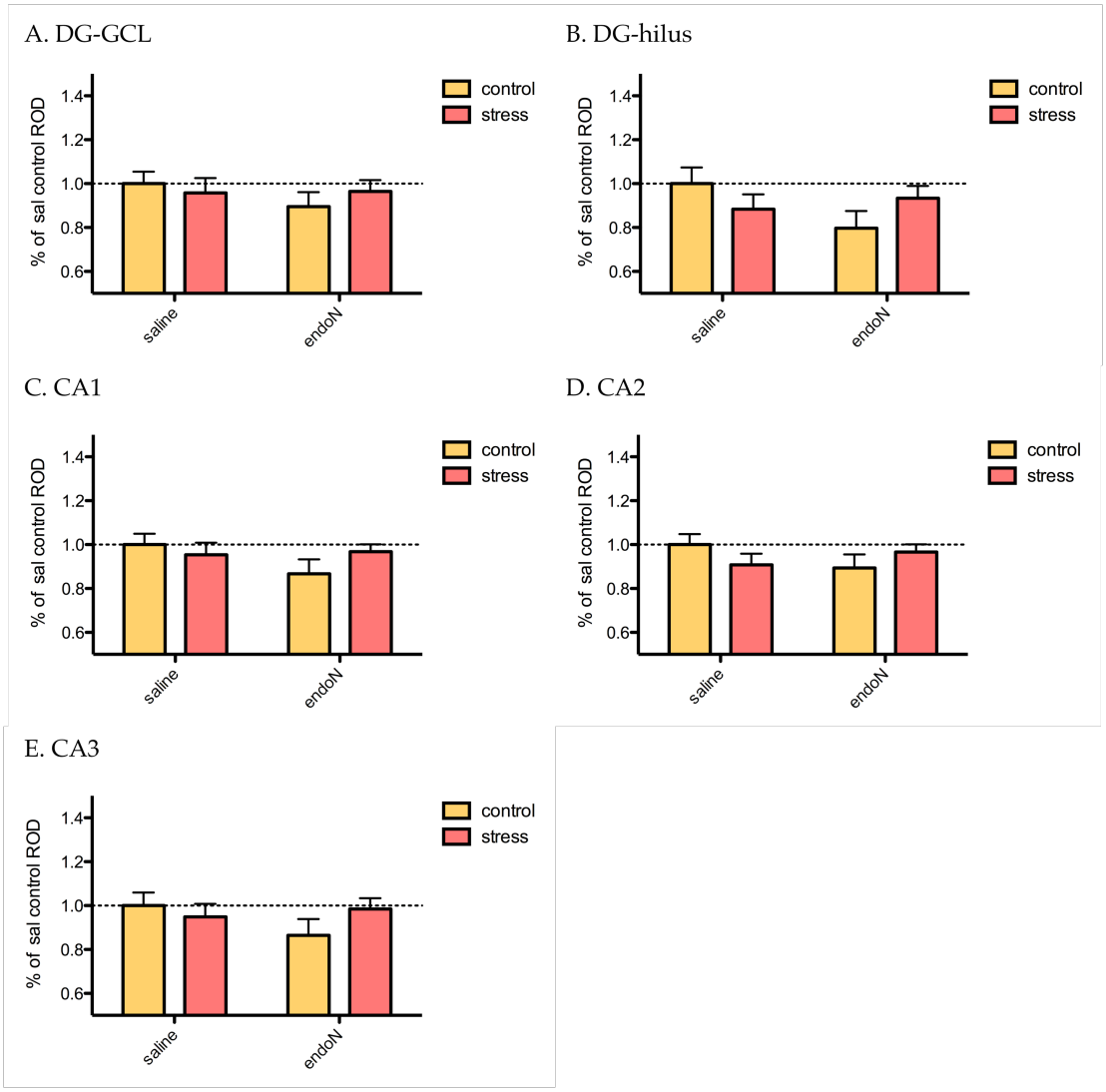


Figure 22. CIS and PSA-NCAM removal had no effect on expression levels of NR2A mRNA. *In situ* autoradiography revealed no significant CIS or PSA-NCAM removal effect on NR2A mRNA levels in the (A) granule cell layer of the DG, (B) hilus region of the DG, (C) CA1, (D) CA2, or (E) CA3. Values are shown based on % of the saline-injected control rats mRNA levels. Analyzed using two-way ANOVA ($n = 10$).

In situ autoradiography for NR2B mRNA in the DG of the hippocampus revealed a significant decrease in NR2B mRNA in saline-injected CIS rats relative to controls. PSA-NCAM removal abolished this decrease in stressed rats (Fig 23). There was no effect of stress or endo N injection in CA1, CA2, or CA3.

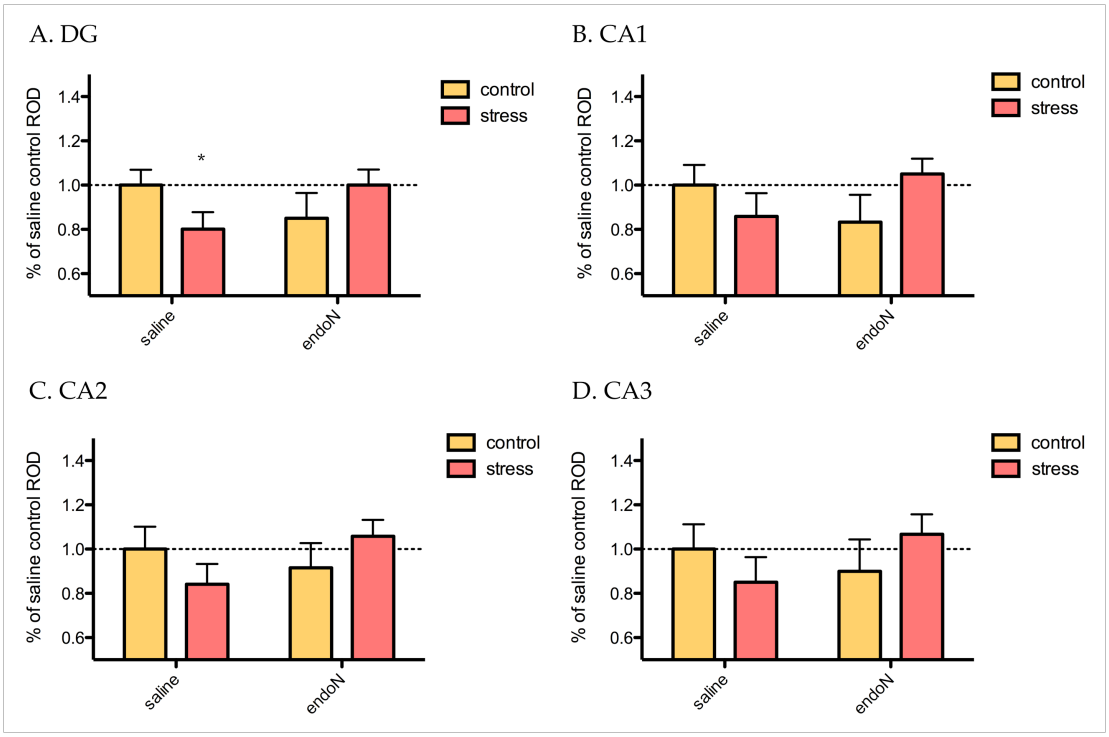


Figure 23. The CIS-induced decrease in NR2B mRNA expression in the DG was eliminated by PSA-NCAM removal. Fresh frozen tissue was used for *in situ* autoradiography of NMDA receptor NR2B. A. 10 d CIS significantly decreased NR2B mRNA expression in the dentate gyrus of saline-injected single-housed male rats ($P < 0.05$). This decrease did not occur in PSA-NCAM depleted CIS rats. B-D. 10 d CIS and PSA-NCAM removal had no significant effect on NR2B mRNA expression in CA1, CA2, and CA3. Analyzed using two-way ANOVA and Bonferroni posttests ($n = 10$).

CHAPTER 7: DISCUSSION

7.1 Experimental Hypothesis

Chronic stress causes reversible dendritic remodeling in region CA3 of the hippocampus, a region of this important brain structure that is key to the formation of memories in place and time and yet also vulnerable to excitotoxic damage (Watanabe et al., 1992a; Conrad et al., 1999). These changes occur together with a host of behavioral and molecular changes, all of which are influenced in some way or another by the increased circulating GCs and their interaction with steroid receptors in the brain, along with a key role for excitatory amino acids and several other transmitter systems (McEwen, 1999). Whether the remodeling seen in response to chronic stress is evidence of damage or of a susceptible but protective state is a major question that determines how we think about the purpose of the HC changes in the stress response. Evidence from hibernation studies show that remodeling occurs outside of damage situations, and evidence from spinal cord pain studies shows a protective effect of PSA-NCAM-dependent remodeling on experienced pain (Popov et al., 1992; El Maarouf et al., 2005). If the stress-induced remodeling seen in CA3 is a protective mechanism as opposed to evidence of damage, this affects the treatment strategies not just for chronic stress, but also for depression and anxiety disorders. In this study we attempted to prevent dendritic remodeling, and from there start to determine the consequences of stress in a non-plastic environment.

7.2 The Effect of PSA-NCAM Removal on Dendritic Plasticity

Ten days of chronic restraint stress produced dendritic retraction in the pyramidal cell apical dendrites in region CA3 of the hippocampus of saline-injected rats compared to saline-injected controls. These stressed neurons showed reduced complexity by multiple measures. Overall, they had less dendritic material, and the dendrites that did exist were simpler than controls, with fewer branch points. Most of the differences between stress and control rats existed between 120 and 210 μm from the cell body. The differences did not begin closer to the cell body because at closer distances, there is usually just a single dendrite, and when branching begins it takes space for the branch order to rise and the complexity to build up. At further distances from the cell body, there is also a lack of difference between stress and control dendrites. This is due to the fact that at these distances there are very few dendrites at all. Most of the dendritic branching stops at about an equal distance from the cell body, with just a single process here or there continuing out.

PSA-NCAM removal through endo N injection significantly and powerfully affected the size and shape of the pyramidal cell apical dendritic trees. Animals that were depleted of all PSA-NCAM expression showed significantly larger and more complex dendritic arborizations compared to saline-injected animals. What was really interesting about PSA-NCAM removal is that it resulted in differences at multiple levels of complexity. In comparing saline-injected CIS rats to PSA-NCAM-depleted control rats, it was seen that at the distances from the cell body where CIS produces its retraction effects, namely, 120 to 210 μm, PSA-NCAM depleted control

animals showed significantly more dendritic material than saline-injected controls. This complexity increase then continued on for the rest of the length of the dendritic tree, with apical dendrites from PSA-NCAM removed control rats significantly more complex than saline-injected controls even at the farther reaches of the dendritic tree. The longest dendritic processes in PSA-NCAM removed rats were about 100 μm longer than the longest processes in control saline-injected rats. This shows that PSA-NCAM removal produces significantly more complex neurons than those seen with saline-injected single housed controls.

Other interesting differences can be noted by comparing saline-injected control rat apical dendrites to PSA-NCAM depleted CIS rat apical dendrites. There was a significant difference in dendritic complexity between these two groups, as measured by total dendritic length and by number of branch points. But upon examination by Sholl analysis, it was seen that this difference is not present 120 to 210 μm from the cell body, where the stress effect in saline-injected animals is seen. Rather, saline-injected controls and PSA-NCAM removed CIS rats showed similar dendritic complexity at these earlier distances from the cell soma. Differences were manifested at around the same distance where the stress effect in saline-injected animals disappeared, indicating that at around the level where saline-injected rat neurons began to terminate, there were still much dendritic material in PSA-NCAM removed stressed rats.

A final comparison of dendritic complexity between the PSA-NCAM depleted experimental groups, control and CIS, revealed a surprising relationship. In these groups, CIS still had some effects on dendritic

complexity. After PSA removal, CIS decreased dendritic complexity as measured by dendritic length, the total dendritic material in each radial unit of the Sholl analysis, but not as measured by branch points, the number of dendrites crossing each radial plane. What is interesting is that these effects also occurred around 120 to 210 μm from the cell body. The CIS-induced decrease in PSA-NCAM removed animals, as mentioned above, brought the dendritic measurements down to about level with those from saline-injected controls. The result is then that PSA-NCAM removal results in a general amplification of the apical dendritic trees, while still maintaining some of the relationship between stress and control neurons. Another similarity between the PSA-NCAM removed and saline-injected stress and controls was that at further distances the dendrites in stress and control PSA-NCAM removed animals were of similar complexity. However, in this situation this was not due to a general lack of dendritic material as there were still many dendrites present at distal distances in both groups.

There are several different interpretations and implications of these results. First, PSA-NCAM removal did not prevent all neuronal plasticity in response to stress. This could complicate interpretations of further experiments. However, it is likely that the absolute size of the dendritic arbor is what is most important. PSA-NCAM removal is still a good tool for looking at the effects of stress in an altered neuronal environment. The region where CIS causes retraction is also a site of plasticity in PSA-NCAM removed rats. However, the plasticity response in endo N injected stress rats in comparison to endo N injected controls is not completely similar to that seen in saline-injected animals, as at distal distances the dendrites of PSA-NCAM removed

rats are more complex than saline-injected controls. This could once again be due to the space requirement in the building up of complexity. Since a dendritic tree starts from one main branch, even the most complex neuron will not be complex at close distances from the cell body. Second, there may be a ceiling effect on just how complex these neurons can get. The PSA-NCAM-removed control neuron could represent the maximal state of dendritic branching, and the PSA-NCAM-removed CIS neurons a step down, showing a truncated remodeling response to CIS in the region where it normally occurs, while showing no decrease in complexity at distal regions not normally involved in stress-induced dendritic remodeling. Overall, what PSA-NCAM removal produces is a more complex neuron, even in response to CIS, and with this brain background we can begin to analyze the consequences of chronic stress without a normal dendritic retraction response.

7.3 Tau Phosphorylation

Dendritic retraction has been accompanied by tau phosphorylation in various experimental situations. P-tau does not bind and support the microtubule backbone of the cell as well as non-phosphorylated tau, and therefore its presence indicates an abundance of disassociated microtubules. Various chronic stress paradigms have produced an increase in phosphorylated tau, and preliminary immunohistochemical data from the McEwen lab also showed this to be the case with CRS in CA3. Western blot analysis of HC from CRS rats showed an increase in tau phosphorylation at only one site, Ser-205. There was no phosphorylation difference between

stress and control at Ser-202, the site examined in most of the previous studies. However, there was phosphorylation at the site, and there were different amounts of Ser-202 P-tau protein in different animals, so it is possible that either the hippocampal dissection was not uniform across the animals, or that increases in P-tau in some regions of the HC, such as CA3, is accompanied by decreases in other subregions that give the net effect of no change.

There was no PKA-dependent P-tau in CRS rats or in controls. This is important because the tau that is phosphorylated by PKA is what ends up insoluble and forming neurofibrillary tangles (Jicha et al., 1999). A study by another group did show insoluble tau in response to 14 days of 30 min restraint in mice (Rissman et al., 2007), but 21 days of chronic restraint in rats is not sufficient to produce disease state tau phosphorylations. This lack of insoluble tau lends support to the hypothesis that dendritic remodeling is not damage, but rather an allostatic adaptation to non-ideal situations.

PSA-NCAM injected CIS stressed rats showed significantly less Ser-202 P-tau than saline-injected stressed rats in region CA3 of the HC. This indicates that there is less microtubule disorganization in these neurons. This is interesting and complimentary to the morphology data described above, but is a much more indirect way of answering the question of what the HC looks like without PSA-NCAM than the Golgi stain data, and so it is less useful for analysis.

7.4 pLIMK and Synaptic Plasticity

pLIMK analysis of region CA3 of the HC of PSA-NCAM removed and stressed rats is a way to look at the effects of a lack of PSA at the synapse. When LIMK is phosphorylated, it leads to actin cytoskeleton reorganization at the synapse. Chronic stress has been shown to increase pLIMK in the HC. In this study, 10 days of CIS trended to increase pLIMK immunostaining in the HC of saline-injected rats, although this did not quite reach significance. PSA-NCAM depleted animals, however, showed no such trend. This lack of change in PSA-NCAM injected animals is expected both because the loss of PSA prevents a major avenue for plasticity initiation, and because the Golgi analysis revealed such highly differentiated neurons.

It is assumed but not known for sure that the PSA-NCAM depleted neurons have an increase in active synapses to accompany their increase in dendritic branching. But this has not been proven. An important future direction for this study will be to do electron microscopic analysis of the pyramidal cell apical dendrites following CIS. Although there are immunohistochemical ways to quantitate synaptic material and activity, the more information one can get from the brains the better. The response to PSA-NCAM removal in CA3 with stress is complex, as the Golgi analysis proved, and anything less will not fully answer the question of how these neurons are affected.

7.5 The Effect of Retraction Attenuation on Hormones and Behavior

When examining the functional consequences of PSA-NCAM removal and chronic stress, one phenomenon was seen repeatedly, namely, that while CIS alone did not produce changes, PSA-NCAM removal before chronic stress enhanced the stress experience to produce or enhance some effects of chronic stress. With CRS, there is habituation in the CORT response to restraint, and after 21 days CORT elevations no longer occur (Magarinos and McEwen, 1995a). The 10 day CIS procedure also results in habituation of the stress response. However, PSA-NCAM depleted CIS animals did show elevated CORT levels at the time of sacrifice, with PSA-NCAM removed control rats not showing an elevation. Taken together, this indicates a lack of endocrine adjustment to chronic stress in these animals, and as such is evidence of a lack of allostasis in PSA-NCAM removed stressed rats.

Another instance of poor allostatic adjustment in PSA-NCAM removed stressed rats is in the effects of chronic stress on body weight gain. CIS slowed the increase in weight relative to controls over the course of the experiment. In the most recent endo N injection experiment, where the procedure had been perfected, weight gain stopped altogether in PSA-NCAM depleted stressed rats, and there was even a net loss of body weight over the course of the CIS duration, whereas saline-injected controls still gained some weight, and PSA-NCAM injected controls gained as much weight as saline-injected controls. Once again, it appears that PSA-NCAM removal is exacerbating the stress experience without an effect in non-stressed animals.

PSA-NCAM depletion enhanced anxiety in stressed rats as measured by multiple behavioral tests. Although CIS increased the number of exploratory

rears rats made in the y-maze, PSA-NCAM depletion significantly reduced the number of rears, which can be interpreted as a higher level of anxiety in PSA-NCAM removed animals.

This anxiety was better seen in the open field test, where CIS increased anxiety in the open field only as measured by the amount of fecal boli and the total movement in the maze. These two measures are secondary to the main analysis of open field behavior, namely, time spent in the center of the open field versus time spent in the perimeter. More anxious animals will spend more time in the darker, more protected perimeter area then they spend in the wide-open and bright center. CIS in saline-injected animals had no effect on the amount of time spent in the center or the perimeter. PSA-NCAM depletion in CIS rats, however, caused the rats to spend significantly more time in the periphery and less time in the center. Yet again, an effect of repeated stress is revealed and shown to be exacerbated by the attenuation of dendritic remodeling in response to stress.

As mentioned in the results, PSA-NCAM injection caused an increase in aggression in both stressed and control rats that was extreme enough to lead to injury and illness and which prevented further statistical analysis of aggression behavior.

CIS revealed no deficit in spatial memory as measured by y-maze testing. There was a trend for PSA-NCAM injected controls to show better spatial memory than the rest of the groups, but this was not significant. Saline-injected animals and PSA-NCAM depleted controls showed equally poor spatial memory.

7.6 The Effect of Single Housing and Surgery on Experienced Stress

One of the major complications of these studies is the lack of a stress effect in saline-injected animals. In analyzing most of the behavioral data, there was no difference between control and stress saline-injected rats, even though the literature states that chronic stress does indeed produce deficits. This is most likely due to the saline-injected controls also experiencing a significant amount of stress. The first source of stress would be the microinjection surgery. Although rats were fully recovered physically with the one week rest period before beginning CIS, then may have still been emotionally disturbed. The second source of stress is the single housing. As mentioned above, it was necessary to single-house the rats to keep them from aggressively attacking each other. But single housing is not ideal for chronic stress experiments. Single-housing decreases DG neurogenesis and y-maze performance (Lu et al., 2003). Single housing also decreases object location and recognition memory (Beck and Luine, 2002; Douglas et al., 2003). Single-housed rats show poorer responses to aggressive behavior (Von Frijtag et al., 2002). In general, male rats housed singly show deficits that mirror those seen as a result of chronic stress. Maybe most interesting to the PSA-NCAM removal studies, however, is the fact that single-housing male rats causes an increase in circulating CORT in response to stress that does not adapt in response to continuation of the stress protocol as it does in pair housed animals (Giralt and Armario, 1989; Dronjak et al., 2004; Weiss et al., 2004; Lucas et al., 2007). The fact that PSA-NCAM depleted rats showed elevated CORT when the saline-injected CIS animals were living in an elevated CORT environment speaks to the importance of the normal dendritic remodeling

response in the regulation of the HPA axis. But for many of the experimental measures attempted in these studies, the surgery plus single housing conditions may have been enough to give the saline-injected control rats more in common with the stress rats than we preferred. For example, this may have accounted for the lack of a significant effect on spatial memory, even though CRS has been reported to impair spatial memory in male rats (Luine et al., 1994; Conrad et al., 1996).

7.7 The Effect of Retraction Attenuation on Anxiety-Related Molecules

NPY is an anxiolytic peptide that is released in response to chronic stress. In these studies we saw a CIS-induced increase in NPY expression in both the lower blade of the DG and region CA3b. CA3b is the same region where dendritic remodeling occurs in response to chronic stress, and where the retraction response is attenuated by PSA-NCAM removal. Although PSA-NCAM removal did not affect NPY mRNA expression in the lower blade of the DG, where a stress-induced increase is still seen, PSA-removal wiped out the increase in NPY in region CA3b. PSA-NCAM removal had no effect on NPY mRNA expression in control rats.

NPY release in general is evidence of allostasis, i.e., an active adaptive response that seeks to promote homeostasis. In chronic stress, there is an increase in anxiety. The release of NPY is therefore an attempt of the organism to normalize its behavior. PSA-NCAM removal and CIS produce an anxiety increase bigger than CIS alone, and the fact that there is not an increased NPY release in response indicates that allostasis is disrupted in

PSA-NCAM depleted animals, and supports the hypothesis that dendritic retraction is part of the allostatic response.

CRS is known to increase CART expression in the DG (Hunter et al., 2007). In these studies, CIS did not significantly affect CART expression, and neither did PSA-NCAM removal. There is a possible explanation for this. The stress of surgery and single housing may have been enough to eliminate differences between saline-injected stress and control, just as it was enough to eliminate differences in anxiety. Since CART expression is directly related to anxiety, this is likely.

Thus, in the present studies, NPY expression appears to be a better indicator of PSA-NCAM depletion effects on stress and anxiety.

7.8 The Effect of Retraction Attenuation on Neuronal Excitability

NR2A and NR2B expression are modulated by chronic stress and chronic CORT, and show increased expression as a result of their application (Fitzgerald et al., 1996; Schwendt and Jezova, 2000; Lee et al., 2003; Owen and Matthews, 2007; Gourley et al., 2009). In these studies, however, CIS produced no change in NR2A expression and a decrease in DG NR2B expression. This NR2B decrease was absent in PSA-NCAM depleted CIS rats. This lack of decrease after PSA removal could be directly due to the lack of PSA-NCAM, as application of PSA-NCAM to cultured HC neurons has been reported to directly inhibit NR2B as a competitive antagonist (Hammond et al., 2006). The significance of a decrease following CIS as opposed to the reported increase in NR2B is unclear, but the fact that PSA-NCAM removed CIS rats show no NMDAR adjustments shows yet again that the plasticity

response is disturbed. It is also possible that single-housing the rats has effects in NR2A and NR2B expression on its own.

7.9 The Evidence for Allostasis and Allostatic Load

Hippocampal dendritic retraction and recovery is a phenomenon that occurs across species and in response to varied physiological stimuli. Remodeling after stress or in conditions like hibernation or jet lag is seen in rats, mice, ground squirrels, tree shrews (Popov et al., 1992; Watanabe et al., 1992a; Magarinos et al., 1996; Izquierdo et al., 2006; Czeh and Lucassen, 2007) and inferred to occur in humans (Cho, 2001; Gianaros et al., 2007). Retraction is seen in both adaptive situations and damage states. The question is whether this remodeling is itself damage. Just the fact that the retraction can recover doesn't prove that it did not increase vulnerability to permanent damage. The PSA-NCAM removal studies detailed here attempted to directly address this question, with varying degrees of success.

Manipulation of the hippocampus to prevent dendritic remodeling was arguably successful. Although PSA-NCAM depleted rats still showed some pyramidal cell apical dendritic plasticity, the resulting retraction left the brain with neurons still larger than those in saline-injected control rats. This result then allowed us to interpret any subsequent findings as a possible consequence of the lack of hippocampal plasticity.

Several behavioral effects emerged: PSA-NCAM depletion in chronically stressed rats increased circulating CORT levels, decreased weight gain, and increased anxiety. These phenomena are all classic consequences of chronic stress. That they can be increased by PSA-NCAM removal and retraction

attenuation supports the hypothesis that dendritic remodeling is a protective, homeostatic mechanism, and that dendritic remodeling is part of the allostasis process (i.e., active process of maintaining homeostasis) rather than a consequence of damage.

There are, however, stress-induced changes that are prevented by the prior removal of PSA-NCAM. These include NPY and NR2B mRNA expression. It could be argued that expression levels fail to change because the attenuation of dendritic retraction is preventing damage, which could be what normally prompts their expression changes. This logic is convoluted, however. The observed increase in anxiety may be partly explained by the decrease in NPY, and this would indicate that the normal increase in NPY following chronic stress is also an allostatic response. As for the meaning behind the lack of NR2B expression changes, further experiments are necessary come to a full understanding.

7.10 Future Directions – Remodeling as Allostasis

We propose that the increase in circulating CORT, decrease in weight gain, increase in anxiety, and decrease in CA3 NPY expression with chronic stress in animals with plasticity attenuation as a result of PSA-NCAM removal are evidence of the protective nature of the dendritic remodeling response. To directly test this hypothesis, the best tactic is to replicate the experiment that shows chronic stress increases the damage caused by ibotenic acid (IBO) injection. Such an experiment supported the hypothesis that dendritic retraction indicates a compromised HC (Conrad et al., 2004; Conrad et al., 2007). As a result, one could assume preventing dendritic retraction

would be useful in stress and mood disorders and would reduce damage caused by a moderate level of excitotoxicity.

However our data indicates that this may not be the outcome. An experiment in which animals are injected with endo N to remove PSA-NCAM, followed by chronic stress, and then followed by IBO injection to CA3 would address the question. The alternative prediction, based upon the results in this thesis, would be that the consequences of IBO injection would be much worse for animals with PSA-NCAM depletion and retraction attenuation. If damage is also increased in non-stress PSA-NCAM removed animals, this would lend even more support to the hypothesis that remodeling is an allostatic response critical for protection in chronic stress situations involving excitotoxicity.

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