2002

Stress and Hippocampal Plasticity

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A thesis presented to the faculty of
The Rockefeller University
in partial fulfillment of the requirements
for the degree of
Doctor of Philosophy

March 25, 2002

For my parents,
with gratitude and love

ACKNOWLEDGEMENTS

I wish to thank my advisor, Bruce McEwen, for accepting me into his laboratory and for providing a nurturing environment for conducting graduate research. He has given me many freedoms in regard to developing this thesis and has thus allowed me to pursue my interests and to develop a sense of independence at this early stage of my training. At the same time, he can be very attentive when called upon, and I greatly appreciate his prompt responses to my numerous inquiries. He contributes an extensive knowledge of the literature in the field of Neuroendocrinology and has been a truly superlative mentor in many respects.

Members of the McEwen laboratory, past and present, have helped in various aspects of my training. I thank Ana Maria Magarinos for taking me under her wings during my early months in the lab and teaching me about animal handling techniques. Her research has also provided a foundation upon which this thesis was built, and in this regard, she and I have had stimulating discussions about our data. Constantine Pavlides spent a significant amount of time teaching me about electrophysiology and was generous enough to allow me to use his lab space and equipment. Juan Nacher contributed the bulk of the PSA-NCAM ideas and work, and he has been a very reliable collaborator. I have received useful advice from Karen Bulloch, Keith Akama, Dan Rosell, Larry Reagan, Susan Lee, and Gwendolyn Wood, on many topics dealing with science and graduate education. Anisha Patel and Elena Hoskin provided great social support, and I am grateful for their friendship. I was fortunate to have summer help from several undergraduate students, including Nishant Sekaran, Rupal Patel and Francois St-Pierre. Finally, Halina Korsun and Adelaide Acquava were wonderful with administrative matters while Maryse Aubourg was always helpful with general lab operations.

Many collaborators have aided me along the way. Dan Stettler, from the Gilbert lab, not only allowed me to use their sliding microtome on numerous occasions but has also been a good friend and neighbor. Karim Nader and Joseph LeDoux, from NYU, were crucial to the fear conditioning project. Brian Derrick, Desiree Villareal, and Viet Do, from UTSA, helped me perfect the art of sterile surgery. Pat Tanapat taught me BrdU immunohistochemistry at Princeton, and Patrick Hof trained me in the technique of unbiased stereology at Mount Sinai.

In addition to Bruce McEwen, my faculty advisory committee was composed of Paul Greengard, Mary Jeanne Kreek, and Elizabeth Gould. I thank Paul Greengard and Mary Jeanne Kreek for their advice over the past six years. In addition, I am grateful to have had opportunities to rotate through their laboratories during my first year in graduate school. They were supportive during the brief periods I spent in their labs and have been wonderful assets as committee members. I thank Elizabeth Gould of Princeton University for serving as the external committee member and for taking time to read the thesis and participate in the defense examination. She, more than anyone else, has a qualified background in the field of stress and hippocampal neurogenesis, and I am glad she could contribute to this thesis.

The past six years have sailed by quickly, most notably because I have so much enjoyed my experience in graduate school, and for this, I thank the Rockefeller University Dean's Office. The graduate program at Rockefeller is very special, in the sense that the university offers access to world-class research in a small, personable environment. The Dean's Office is the base for students, and the staff has been tremendously supportive of the student body. I would like to thank Marta Delgado and Kristen Cullen in particular, for they have been in the office throughout my tenure at Rockefeller. I also acknowledge the financial support of the National Institutes of Health (NRSA training grant GM07524).

I thank Kate Dredge, my classmate and roommate of five years. Sharing an apartment with Kate came with ease, and she continues to be a close friend. My sister, Vy, and I have rarely spent time on the same coast, or for that matter, in the same country. Despite this, she and I have enough in common to stimulate endless conversations, giving me an excuse, if ever I need one, to procrastinate just a little bit longer. I am entirely indebted to my parents for their love and the sacrifices they made to provide brighter futures for their children. Finally, I thank my husband, Morgan Huse. He encourages me when I am in doubt, makes me laugh when I am upset, and loves me unconditionally.

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ABBREVIATIONS

5HT	Serotonin
ADX	Adrenalectomy
ANOVA	Analysis of variance
AP	Anterior-posterior axis
BrdU	5-Bromo-2'-deoxyuridine
CE	Coefficient of error
CS	Conditioned stimulus
CV	Coefficient of variation
DAB	3,3'-diaminobenzidine
DV	Dorsal-ventral axis
EAA	Excitatory amino acid neurotransmitter
EPSP	Excitatory postsynaptic potential
GFAP	Glial fibrillary acidic protein
GR	Glucocorticoid receptor; type II receptor
HFS	High frequency stimulation
HPA	Hypothalamic-pituitary-adrenal axis
ISD	Immediate shock deficit
LTP	Long term potentiation
ML	Medial-lateral axis
MR	Mineralocorticoid receptor; type I receptor
NCAM	Neural cell adhesion molecule
NeuN	Neuronal Nuclei
NHS	Normal horse serum
NMDA	N-methyl-D-aspartate
NSE	Neuron specific enolase
PSA	Polysialic acid
PSA-NCAM	Polysialylated neural cell adhesion molecule
PST	Polysialyltransferase
SEM	Standard error of the mean
Shh	Sonic hedgehog
STX	Sialyltransferase-X
US	Unconditioned stimulus

ABSTRACT

The addition of new neurons continues in the dentate gyrus of the adult rat, and this process has been previously shown to be regulated by adrenal steroid hormones, N-methyl-D-aspartate (NMDA) receptor activity, psychosocial stress and learning and memory. Repeated restraint stress is known to induce neuroanatomical changes in the hippocampus, such as dendritic remodeling in CA3 neurons and mossy fiber terminal rearrangement, and these alterations are reversible, suggesting that the hippocampal system experiences a certain degree of plasticity in response to stress. This dissertation examines the effects of chronic restraint stress on several parameters of hippocampal plasticity, including dentate gyrus neurogenesis, polysialic acid neural cell adhesion molecule (PSA-NCAM) expression and neuronal excitability. In addition, the learning and memory process is dissected using a contextual fear conditioning paradigm in order to identify the component that can affect hippocampal cell proliferation.

The results presented demonstrate that repeated stress, which activates the hypothalamic-pituitary-adrenal (HPA) axis and leads to increased glucocorticoid and glutamate release, can inhibit hippocampal neurogenesis, reduce dentate gyrus cell numbers and volume, enhance the expression of PSA-NCAM and transiently increase the synaptic response in the dentate gyrus after perforant path stimulation. Contextual fear conditioning was also shown to temporarily decrease cell proliferation in the dentate gyrus. Taken together, the results suggest that the hippocampus is capable of responding to an animal's experiences with stress and learning tasks by inducing plasticity events that can revert to baseline, such as synaptic enhancement, dendritic remodeling, and PSA-NCAM elevation. However, when the stress was endured for extended periods, the morphological response became more extreme, exhibiting reductions in neurogenesis,

total granule neuron numbers and granule cell layer volume. At this level of stress, PSA-NCAM expression was no longer enhanced, implying that the dentate gyrus had acquired a more stable condition and one that is perhaps more vulnerable to permanent changes.

CHAPTER 1

INTRODUCTION

For most of the 20th century, it was widely accepted in the field of neurobiology that the generation of new nerve cells ceases before or shortly after birth. This belief lent critical support to the theory that the brain acquires structural stability very early in life and that the adult brain is hardwired and irreparable. Unlike skin, blood or intestinal cells, which self-renew constantly throughout life, the adult brain was deemed incapable of producing new neurons. Research in the recent decades has revealed that the brain is not as immutable as was once viewed, and in fact, it can be rather pliant. The modifications that take place in the brain include structural remodeling as well as the addition of newly generated neurons.

Postnatal development of granule neurons

Most neurons are born in proliferative regions termed the ventricular and subventricular zones during embryogenesis. From here, the cells migrate to their final destinations and extend axonal projections and dendrites to wire the brain with the appropriate connections. An exception to embryonic neurogenesis is the production of granule neurons in the cerebellum, olfactory bulb, and hippocampus. Granule cell neurogenesis in these regions occurs in the early postnatal period, usually within the first several weeks of a rodent's life (Altman and Das, 1966).

Cerebellar granule cells are the most abundant type of neurons in the brain, tallying in at around 10^8 cells in the mouse cerebellum (Wechsler-Reya and Scott, 2001). The generation of this large population of neurons begins with the propagation of early

precursor cells in the rhombic lip, a dorsal hindbrain structure (Alder et al., 1996). These cells then migrate from the rhombic lip during late embryogenesis to the surface of the cerebellum and compose the external germinal layer, visible in newborn rodents as a single layer of undifferentiated cells. It is here that the cells again proliferate, rather extensively during the first few postnatal days, and are thus termed granule cell precursors. As the pool of granule cell precursors expands, some cells begin to differentiate and migrate along radial astroglia inward past the Purkinje cell layer until they reach the internal granule layer, their final destination (Gasser and Hatten, 1990). In the mouse cerebellum, the processes of proliferation and differentiation take roughly two to three weeks, after which time the external germinal layer dwindles as the granule cell precursors mature into granule neurons.

The rostral extent of the lateral ventricle forms the olfactory ventricle in newly born rats. Cells in the ependymal layer and subependymal matrix of the olfactory ventricle proliferate rapidly during the first three weeks of a rat's life (Altman and Das, 1966). These cells then undergo long distance migration along the rostral migratory stream to generate some of the granule neurons in the olfactory bulb (Lois and Alvarez-Buylla, 1994). The olfactory bulb is composed of six layers: the external nerve layer, the glomerular layer, the external plexiform layer, the mitral layer, the inner plexiform layer, and the inner granular layer. While the granule cells of the mitral and inner granular layers originate from the olfactory ventricle as described, the granule cells of the other layers are derived from locally proliferating cells, and these processes occur primarily during the early postnatal period.

The hippocampus is composed of two primary neuronal cell layers: the pyramidal cells of Ammon's horn and the granule cells of the dentate gyrus. Although the pyramidal cells of Ammon's horn are formed prenatally, the majority of the dentate gyrus granule neurons are produced postnatally. In the neonate rat, the granule cells compose a layer

only two to three cells thick. The peak period of granule cell generation is immediately after birth, during the first two postnatal weeks (Schlessinger et al., 1975). New neurons added to the granule cell layer during this period originate from the subependymal wall of the lateral ventricle as well as from a local proliferative zone within the dentate gyrus itself. This proliferative region is the border between the granule cell layer and the hilus, termed the subgranular zone. The development of the dentate gyrus follows three morpho-cellular gradients: the dorsal blade cells are generated earlier than the ventral blade cells; the caudal portion is formed earlier than the rostral region; and younger cells are added at the hilar side of the granule cell layer, migrating outward toward the molecular layer as they mature (Schlessinger et al., 1975).

After neuronal precursors undergo cell division and become post-mitotic, they migrate into the granule cell layer and begin to extend neuronal processes (Hastings and Gould, 1999; Markakis and Gage, 1999). The hippocampal granule cells form dendrites that receive synaptic input from the entorhinal cortex via the perforant path, and they extend axonal projections, called mossy fibers, toward the CA3 pyramidal cells. As post-mitotic, immature neurons, they express cellular markers, such as Turned on after division-64kD protein (Minturn et al., 1995) and PSA-NCAM (Seki and Arai, 1993a). As they mature into granule cells, they begin to express markers of differentiated neurons, such as Neuronal Nuclei (Mullen et al., 1992), calbindin (Sloviter, 1989), and neuron-specific enolase (Cameron et al., 1993).

Adult hippocampal neurogenesis

Four decades ago, Joseph Altman (1962) reported the production of hippocampal granule neurons in adult mammals. While the early postnatal generation of neurons had been accepted as a developmental phenomenon, the finding that neurons could be born in adults went against the tide of conventional neurobiology. The adult brain was viewed as

a hardwired, stable organ, and thus, Altman's study was largely ignored after its publication. Fifteen years later, Michael Kaplan and James Hinds (1977) conducted an electron microscopic analysis of these putatively adult-generated neurons. Tritiated-thymidine was injected into adult rats, and after a one month survival period, the rats were sacrificed and their dentate gyri subjected to electron microscopy. Examining one-μm thick sections, Kaplan and Hinds were able to demonstrate that ^{3}H-thymidine-labeled cells in the dentate gyrus possessed ultrastructural features characteristic of neurons, such as dendrites and synapses. Further evidence supporting adult neurogenesis in the hippocampus came from Stanfield and Trice in 1988. Combining ^{3}H-thymidine autoradiography with retrograde fluorescent tracing, they demonstrated that the newly generated neurons in the dentate gyrus were capable of extending axons into the mossy fiber projection layer (Stanfield and Trice, 1988). Heather Cameron and colleagues substantiated the claim by showing that proliferating cells in the adult dentate gyrus can develop into both mature neurons and glia using antibodies directed against NSE and glial fibrillary acidic protein (GFAP) in immunohistochemical studies (Cameron et al., 1993). Recent studies have revealed that adult rats generate 6% of the total size of the granule cell population each month through the addition of new neurons (Cameron and McKay, 2001), and the neurons have been shown to be physiologically functional (van Praag et al., 2002). Today, there is widespread acceptance among the neuroscience community of adult generation of granule neurons not only in the dentate gyrus but also in the olfactory bulb, and in fact, hippocampal neurogenesis has been demonstrated to occur in a variety of adult species, including humans (Eriksson et al., 1998; Gould et al., 1999b; Kempermann et al., 1997a).

The existence of adult generation of new neurons in the dentate gyrus is particularly exciting because the hippocampus is a brain structure involved in spatial learning and memory processes, including declarative and episodic memory. This was

first observed with patient H.M., who received medial temporal lobe resection in 1953 to alleviate epileptic seizures and lost his ability to form new memories and to recall events that occurred shortly prior to the operation (Scoville and Milner, 1957). H.M. was, however, able to retrieve older memories. Studies with subsequent human patients and with laboratory animals confirmed the critical role of the hippocampus in spatial and associational learning and memory formation (Eichenbaum, 2000). Since memories formed long ago are unaffected by hippocampal damage, the hippocampus is not the seat of long term memory consolidation and thus is viewed as a relay station in which sensory input converges for processing and that will eventually transfer more permanent memories to cortical regions. Moreover, as a part of the limbic system, the hippocampus, together with the amygdala, is involved in emotional memory processing.

The dentate gyrus serves as a major gateway to the pyramidal neurons of the hippocampus. All inputs to the hippocampus travel through the entorhinal cortex, and the main synaptic contact from the entorhinal cortex is upon the granule cells of the dentate gyrus along the perforant pathway. According to the classic "trisynaptic" circuit of the hippocampus, the granule cells then extend mossy fibers to synapse with the CA3 pyramidal cells, which in turn connect with the CA1 region through the Schaffer collaterals. It is now evident that the circuitry of the hippocampus is far more complex. For example, the perforant path also extends directly from the entorhinal cortex to the CA3 cells, and the CA3 cells have recurrent collaterals that synapse back upon their own cell layer. Despite these and numerous other synaptic contacts throughout the hippocampal formation, the dentate gyrus remains the key relay point for convergence into the hippocampus. The addition of new granule neurons continues throughout the life of adult mammals and must play a pivotal role in the proper functioning of the hippocampus. Many studies have been conducted to identify regulatory and functional mechanisms of this process.

Regulation of hippocampal neurogenesis

The peak period of granule cell production is immediately after birth, during the first two postnatal weeks (Altman and Das, 1965). After this, the rate of cell proliferation sharply declines, continues at a low basal rate and steadily declines with age (Gould and Cameron, 1996; Kuhn et al., 1996). This pattern of proliferation rate happens to correlate inversely with the levels of circulating adrenal steroid hormones. Studies by Robert Sapolsky demonstrate that rats experience a stress hyporesponsive period during the first two postnatal weeks, at which time levels of endogenous adrenal hormones remain very low (Sapolsky et al., 1986). After this brief period, the adrenal hormone concentration begins to rise and is further elevated in aging animals. Thus, periods of low glucocorticoids correspond with periods of enhanced granule cell proliferation, and vice versa. These observations led scientists to postulate a role for glucocorticoid regulation of hippocampal neurogenesis both during development and throughout adult life.

Several reports show that administration of adrenal steroids during the stress hyporesponsive period significantly inhibits neurogenesis, and removal of endogenous adrenal hormones by bilateral adrenalectomy (ADX) stimulates cell division (Gould et al., 1991). It was then determined that adrenal steroids have similar effects in adulthood as in developmental stages. Using adult rats, Heather Cameron and Elizabeth Gould showed that acute corticosterone administration significantly suppressed neurogenesis and that adrenalectomy resulted in an increase of cell proliferation in the dentate gyrus (Cameron and Gould, 1994; Gould et al., 1992a). Interestingly, while mature neurons in the hippocampus robustly express receptors for glucocorticoids (McEwen et al., 1968), very few granule cell precursors undergoing mitosis are immunoreactive for adrenal hormone receptors (Gould et al., 1992b). This suggests that adrenal steroids do not act directly upon the granule cell progenitors and likely mediate their effects through some

other factor(s). The type I adrenal steroid receptor (mineralocorticoid receptor, MR) and type II adrenal steroid receptor (glucocorticoid receptor, GR) appear to play different roles in cell survival (Gould et al., 1997b). During early postnatal development, activation of GRs protects granule cells against naturally occurring cell death, whereas during adulthood, MR activation is required for granule cell survival.

In addition to adrenal steroids, pharmacological studies implicated a role for the NMDA subtype of glutamate receptors in regulation of neurogenesis (Cone and Cone, 1976). Indeed, pharmacological activation of the NMDA receptor by injection of NMDA resulted in a decrease of newly born cells (Cameron et al., 1995). Conversely, blockade of NMDA receptors by the antagonists MK-801, a noncompetitive antagonist, or CGP 37849, a competitive receptor antagonist, resulted in about a two-fold increase of neurogenesis. The relationship of NMDA receptors to proliferation of granule cell precursors implies that afferent input can modify rates of neurogenesis. While it has been shown that the granule cells of the dentate gyrus undergo extensive remodeling in temporal lobe epilepsy (Houser, 1992; Houser et al., 1992), it was only within the past five years that investigators linked neurogenesis to seizure models.

Jack Parent and coworkers used a model of temporal lobe epilepsy, induced either by pilocarpine administration or by perforant path stimulation, to examine the effects of seizures on dentate gyrus neurogenesis as well as on hippocampal network plasticity associated with epilepsy (Parent et al., 1997). They showed that an upregulation of cell proliferation took place in the dentate gyrus after seizure induction. Furthermore, they observed that these newly generated granule neurons appeared in ectopic locations in the hilus and inner molecular layer of the dentate gyrus and that the neurons formed aberrant projections to the CA3 region and the inner molecular layer. Subsequently, it was demonstrated that hippocampal kindling stimulation and kainic acid induced seizures independently contributed not only to proliferation of dentate gyrus neurons but also

increased apoptotic death of positively identified neurons in the dentate gyrus (Bengzon et al., 1997). Interestingly, amygdala kindling in the adult rat also enhanced genesis of dentate granule cells (Parent et al., 1998), suggesting an intimate link between the amygdala and hippocampal formation and the possibility that fear related experiences can alter hippocampal activity.

The entorhinal cortex provides the major excitatory input to granule cells of the dentate gyrus via NMDA receptors. Data on the relationship between neurogenesis and NMDA receptor activation implicated a role for the synaptic connection between the entorhinal cortex and the dentate gyrus in regulation of neural proliferation. As expected, based on the pharmacology studies, lesion of the entorhinal cortex, and thus removal of excitatory action on granule neurons, resulted in an increase of cell proliferation in the dentate gyrus (Cameron et al., 1995). In the realm of lesion studies, Elizabeth Gould and Patima Tanapat demonstrated that lesion of the granule cell layer both by mechanical and excitotoxic means led to an increase in neurogenesis, and this proliferative activity was positively correlated with cell death (Gould and Tanapat, 1997). These observations are consistent with ADX studies showing higher incidences of both granule cell death (Sloviter et al., 1989) and cell birth (Gould et al., 1992a).

Other factors have been identified in recent years that participate in the regulation of hippocampal neurogenesis. Brezun and Daszuta reported the role of serotonin in regulation of neurogenesis in the dentate gyrus (Brezun and Daszuta, 1999). They found that inhibition of serotonin synthesis and selective lesions of serotonergic neurons reduced the number of proliferating cells in the dentate gyrus. Furthermore, activation of serotonin-1A (5HT-1A) receptors in rats significantly stimulated the production of new granule neurons while blocking 5HT-1A receptors by administration of receptor antagonists decreased the proliferation of precursor cells (Gould, 1999; Jacobs et al., 1998). Like corticosterone, serotonin levels can be elevated by a stressful event.

However, it has been demonstrated that different types of stressors can produce different effects on serotonin levels in certain brain regions, such as the hippocampus (Kirby et al., 1997). These data indicate that serotonin must play a modulatory role in the regulation of neurogenesis, particularly in relation to stress and glucocorticoids.

Aside from serotonin, additional drugs associated with depression and anxiety have been linked to hippocampal neurogenesis. Haloperidol, used frequently to manage schizophrenia and mania, significantly stimulates granule cell proliferation (Dawirs et al., 1998). Hippocampal neurogenesis is also greatly enhanced by lithium treatment, a common remedy for manic-depressive illness (Chen et al., 2000). Finally, tianeptine, an antidepressant, has been used to successfully reverse the attenuation of neurogenesis associated with chronic psychosocial stress (Czeh et al., 2001). These results suggest possible mechanisms for how these drugs can reverse structural changes in the brain observed in patients with depressive disorders.

Patima Tanapat et al. (1999) compared the proliferation properties of hippocampal cells in male and female rats to determine if sex differences exist. They found that females exhibited a cyclic fluctuation in cell proliferation, producing more cells during proestrus, when estrogen levels are highest, compared to other stages of the estrus cycle. This suggested a role for estrogen regulation of cell proliferation. Indeed, this was confirmed with ovarian steroid manipulations. Removal of the ovaries significantly attenuated the number of proliferating cells in the dentate gyrus, and this was reversed with estrogen replacement. Although females produced more immature neurons than their male counterparts during periods of high estrogen, the net number or surviving, mature neurons is comparable between them. Thus, estrogen enhancement of cell proliferation produces a transient increase in immature neurons in females.

Adrenal hormone levels and NMDA receptor activity are involved in regulation of neurogenesis; accordingly, both glucocorticoids and glutamate are upregulated in

response to stress, thereby presenting a paradigm in which one could study neurogenesis in the context of environmental manipulations, such as stress. Exposure of rat pups to the odor of an unfamiliar adult male rat is a potent stressor that results in defensive behavior, such as freezing, or behavioral inhibition (Takahashi, 1995); this stressor can inhibit proliferation of granule cell precursors (Tanapat et al., 1998). Elizabeth Gould et al. (1997a) have shown that in the tree shrew, a primitive primate species, neurogenesis can be suppressed significantly by psychosocial stress. Moreover, these studies were extended to demonstrate that a resident-intruder model of stress can inhibit proliferation of neural precursors in the dentate gyrus of adult marmoset monkeys (Gould et al., 1998), thereby showing that regulation of neurogenesis by stressful events is common among several species and can probably be extended to most mammals and primates, including humans.

Neurogenesis in rats continues throughout adulthood but is drastically reduced in aged rats; this age-related decrease of neurogenesis is specifically attributable to the decrease in proliferation of granule cell precursors (Kuhn et al., 1996). However, precursor cells residing in the dentate gyrus of aged rats can be stimulated to proliferate by reducing corticosteroid levels (Cameron and McKay, 1999) or by blocking NMDA receptor activation (Nacher et al., 2001b); NMDA receptor blockade leads to delayed apoptosis after 14 days. Furthermore, allowing animals to live in enriched environments can elevate the rate of neurogenesis (Kempermann et al., 1998). These exciting results demonstrate that neurogenesis can be regulated by environmental influences throughout life, including senescence. Similarly, Kempermann has also found that adult mice living in enriched environments have more granule cell neurons and larger hippocampal granule cell layers in comparison to mice housed in standard laboratory cages (Kempermann et al., 1997b). When the various components of the enriched environment were separated for further analysis, it was discovered that the major contributor to the neurogenesis

increase was physical exercise in the form of running wheel usage (van Praag et al., 1999a; van Praag et al., 1999b). At a functional level, running was also shown to improve spatial learning ability and synaptic plasticity in the form of long term potentiation. The regulation of exercise-induced neurogenesis has been attributed to enhanced uptake of the neurotrophic factor insulin-like growth factor I (Trejo et al., 2001).

Functional significance of new dentate gyrus granule cells

Although it is well known that the hippocampus is crucial for learning and memory, little is known about the biological basis of this function. The production of new hippocampal neurons is also a well established phenomenon, but a functional link to learning and memory was not made until recently. In 1999, Elizabeth Gould and Tracey Shors reported that hippocampal-dependent learning enhances neurogenesis in the dentate gyrus (Gould et al., 1999a). When trained in trace eyeblink conditioning or the Morris water maze, both tasks requiring hippocampal participation, animals increased the number of hippocampal neurons added to the dentate gyrus. This increase in neurogenesis was due to the enhanced survival of recently generated cells.

Since a link between learning and neurogenesis was determined, Gould and Shors went on to conduct experiments to determine if the new neurons are involved in memory formation (Shors et al., 2001). An anti-mitotic drug, methylazoxymethanol acetate, was used to deplete the numbers of newly generated neurons in the dentate gyrus, resulting in the impairment in the ability of rats to learn a hippocampal-dependent task. This study demonstrates that the newly born neurons participate in the formation of hippocampal-dependent memory.

Structural plasticity of the hippocampus

Several factors that regulate hippocampal neurogenesis also participate in the induction of structural plasticity of the hippocampus. For example, excess glucocorticoids, which suppress neurogenesis, can bring about dendritic remodeling in CA3 pyramidal neurons (Woolley et al., 1990). The changes that occur include decreased numbers of apical dendritic branch points and decreased total apical dendritic length. Similar results may be obtained by subjecting animals to chronic restraint stress (Watanabe et al., 1992b) or psychosocial stress (Magarinos et al., 1996). In addition to glucocorticoids and stress, excitatory mechanisms involving NMDA receptors play a major role in driving the remodeling process (Magarinos and McEwen, 1995b; Magarinos et al., 1996). These structural changes require chronic exposure to stress and glucocorticoids and are reversible upon termination of the stress or glucocorticoid injections.

The CA3 pyramidal dendrites receive synaptic input from the dentate gyrus granule neurons via the mossy fiber pathway. Upon ultrastructural examination of mossy fiber terminals, it was observed that chronic stress also alters synaptic terminal structure (Magarinos et al., 1997). Compared to control animals, the mossy fiber terminals of stressed rats exhibited more densely packed synaptic vesicle clusters localized near active zones and more mitochondrial profiles present in the terminal area.

Repeated restraint is a well established stress system used to induce morphological plasticity throughout the dentate gyrus-CA3 hippocampal regions. It is likely that adult hippocampal neurogenesis, a process that can be regulated by adrenal steroid hormones, NMDA receptor activity and psychosocial stress, could also be altered by restraint stress. This dissertation examines the effects of chronic restraint stress on several parameters of hippocampal plasticity, including dentate gyrus neurogenesis, PSA-NCAM expression and neuronal excitability. In addition, the learning and memory

process is dissected using a contextual fear conditioning paradigm in order to identify the component that can affect hippocampal cell proliferation.

CHAPTER 2

MATERIALS AND METHODS

Animals

All experiments were performed on adult male Sprague-Dawley rats (SD strain; Charles River, Wilmington, MA). At the start of each study, the rats ranged from two to four months of age; animals in each experiment were age matched. Animals had unlimited access to food and water except during experimental sessions and were maintained on a 12 hour:12 hour light:dark cycle with lights on from 7:00 a.m. to 7:00 p.m. All experiments were performed during the lights on period and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Rockefeller University Institutional Animal Care and Use Committee (protocol numbers 98141 and 01055). Animals were allowed one week of rest to recover from transportation prior to experimentation.

For studies involving restraint stress, rats were housed in groups of two or three per cage. Control rats were always kept in a room separate from stressed rats. For studies involving fear conditioning or electrophysiology, rats were individually housed in the same room but were moved to different rooms during experimentation.

Restraint stress

For acute restraint stress, animals were subjected to a single session of restraint for either two hours or six hours. Chronic restraint stress involved restraining the rats for six hours per day (10:00 a.m. to 4:00 p.m. each day) for at least 21 consecutive days. In one particular study, chronic stress was extended from 21 days to 42 days. Rats were

usually stressed inside cylindrical wire mesh restrainers that were clamped at both openings and were placed inside their home cages during the restraint sessions. For electrophysiological studies involving restraint stress, rats were stressed inside cylindrical plexiglass restrainers. Control rats were left undisturbed in their home cages.

Bromodeoxyuridine injections

5-Bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO) was prepared in saline to a dilution of 20 mg/ml BrdU and 0.007 N NaOH. The solution was dissolved by sonication and was always prepared the same day as it was used. Delivery was by intraperitoneal (i.p.) injection. To examine the proliferation of precursor cells, rats were given a single injection of BrdU (200 mg/kg) and a survival time of two hours; this is a sufficient amount of time for cells in S phase to incorporate BrdU but not to complete mitosis (Lewis, 1978; Packard et al., 1973). In order to trace the survival of recently born cells and to determine the developmental phenotypes of proliferating cells, rats received four daily injections of BrdU (100 mg/kg) and were allowed to survive for periods ranging from twelve days to three weeks.

Perfusion and tissue storage

Animals were given an overdose of Nembutal (sodium pentobarbital) and were transcardially perfused first with saline containing heparin (1 unit/ml) and subsequently with 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed from the skulls, postfixed overnight in 4% paraformaldehyde and transferred into 30% sucrose in 0.1 M PB. Serial 40 μm-thick sections throughout the entire dentate gyrus were cut on a sliding microtome. The sections were stored in cryoprotectant (25% ethylene glycol, 25% glycerol, 0.05 M PB, pH 7.4) at -20°C until ready for use.

BrdU immunohistochemistry

Every 12th section from each brain was processed for BrdU immunohistochemistry. Sections were heated for one minute in 0.1 M citrate buffer (pH 6.0) in a microwave oven, treated with 0.6% hydrogen peroxide in 0.1 M phosphate buffered saline (PBS, pH 7.4) for 20 minutes, and permeabilized with 0.1% trypsin and 0.1% $CaCl_2$ in 0.1 M Tris buffer (TB, pH 7.5) for ten minutes at room temperature. The sections were then incubated in 2 N HCl for 30 minutes at 37°C to separate double stranded DNA. After blocking in 3% normal horse serum (NHS) for 20 minutes, the sections were incubated overnight at 4°C in mouse anti-BrdU IgG (1:200 in PBS containing 0.5% Tween-20; Novocastra, Newcastle Upon Tyne, UK). The following day, the sections were rinsed in PBS, incubated in biotinylated horse anti-mouse secondary antibody (1:200 in PBS containing 1.5% NHS; Vector Laboratories, Burlingame, CA) for one hour at room temperature, rinsed in PBS, incubated for one hour in avidin-biotin-horseradish peroxidase (1:50 in PBS, ABC kit; Vector Laboratories), rinsed in PBS, and reacted with 3,3'-diaminobenzidine (DAB; Sigma). The sections were Nissl-stained with cresyl violet acetate, dehydrated in a series of ethanol, cleared in xylene, and coverslipped with DEPEX mounting medium (Electron Microscopy Sciences, Fort Washington, PA).

Immunofluorescence labeling

To determine the phenotypes of BrdU-labeled cells, every 24th section from each brain was labeled for BrdU, NeuN (a marker for mature neurons) and GFAP (a marker of astroglia). Sections were first pretreated by incubation in 2X SSC/50% formamide for two hours at 65°C, rinsed in 2X SSC, incubated in 2 N HCl for 30 minutes at 37°C and rinsed in borate buffer (0.1 M, pH 8.5) for ten minutes. After blocking for one hour in TBS (pH 7.5) containing 0.1% triton X-100 and 3% normal donkey serum (TBS-plus),

the sections were incubated overnight at 4°C in an antibody cocktail containing rat anti-BrdU monoclonal IgG (1:50; Accurate Chemical, Westbury, NY), mouse anti-NeuN monoclonal IgG (1:25; Chemicon, Temecula, CA), and goat anti-GFAP polyclonal IgG (1:250; Santa Cruz Biotechnology, Santa Cruz, CA) in TBS-plus. The following day, the sections were rinsed in TBS, blocked for ten minutes in TBS-plus and incubated in a secondary antibody cocktail for one hour at room temperature in the dark. The cocktail contained Cy3-conjugated donkey anti-rat IgG (1:200; Jackson Immunoresearch, West Grove, PA), Cy2-conjugated donkey anti-mouse F(ab')2 fragment (1:50; Jackson Immunoresearch), and Cy5-conjugated donkey anti-goat IgG (1:200; Jackson Immunoresearch) in TBS-plus. The sections were rinsed in TBS, coverslipped with Aquapolymount (Polysciences, Warrington, PA) and stored at 4°C in the dark.

PSA-NCAM immunohistochemistry

Sections were processed for PSA-NCAM immunohistochemistry using a mouse anti-Men B monoclonal IgM antibody at a dilution of 1:500 (gift of G. Rougon, Centre National de la Recherche Scientifique, Marseilles, France). This antibody has been shown to recognize specifically PSA-NCAM (Rougon et al., 1986). Antibody binding was visualized with a secondary anti-mouse IgM (1:200) and the ABC Vectastain kit (Vector Laboratories) using DAB (Sigma) as a chromogen.

Data analysis

All slides were coded prior to quantitative analysis, and the code was not broken until the quantification was completed. To determine the total number of proliferating cells in the dentate gyrus, a 1:12 systematic-random series of sections were viewed on a Nikon E600 microscope and analyzed by stereology using StereoInvestigator software (Microbrightfield, Maine). BrdU-labeled cells were identified and counted with a 100X

Nikon 1.4 numerical aperture (n.a.) objective and a 1.4 n.a. auxiliary condenser lens to achieve optimal optical sectioning of the tissue. Cells appearing in the upper focal plane were omitted to prevent counting cell caps. Labeled cells were counted in the entire extent of the dentate gyrus (granule cell layer and hilus combined). PSA-NCAM-containing cells were counted under a 40X objective using the same approach.

Total granule cell numbers were estimated in a 1:12 series of sections at 100X by the optical fractionator method (West et al., 1991) in which 1% of the area of the granule cell layer on each section was sampled through 5 μm of the section thickness using a stack of five 1 μm disectors and a 15 μm by 15 μm disector frame. The volume of the granule cell layer was measured in each animal on the same series of sections by the Cavalieri method using a counting grid with an area associated with the counting points of 2500 μm^2 at 10X magnification. Means were determined for each experimental group, and the data were subjected to analysis of variance (ANOVA) followed by Fisher's PLSD post hoc tests of significance using Statview software. Coefficients of variation (CV) and of error (CE) were determined as previously described (West et al., 1996).

For phenotypic analysis, sections were viewed on a confocal laser scanning microscope (Zeiss Axioplan2) with LSM 510 software. Eighty to 120 BrdU-labeled cells were identified per animal and colocalization with NeuN, GFAP or neither was determined from scans in single optical planes one μm-thick.

Stereotaxic surgery for chronic electrode implantation

Rats were injected with atropine sulfate (0.4 mg/kg) ten minutes prior to anesthesia with sodium pentobarbital (55 mg/kg, with supplemental injections as required throughout surgery) and placed in a stereotaxic apparatus (Kopf). Body temperature was maintained at 37°C with a monitored heating control unit (Harvard). The scalp was incised and retracted, and the head was adjusted to a skull flat position. Holes were

drilled for electrodes (teflon-coated stainless steel, 0.005" in diameter, beveled at the tip) and miniature screws (stainless steel, size #80) to support the ground and indifferent wires. Recording and stimulating electrodes were implanted following the stereotaxic coordinates for the medial perforant pathway (AP 7.9, ML 4.1, DV 2.2), dentate gyrus (AP 3.8, ML 2.1, DV 3.3), mossy fibers (AP 3.5, ML 2.0, DV 3.0), or CA3 layer (AP 2.8, ML 2.2, DV 3.2). All measurements are millimeters from bregma according to the adult rat brain atlas of Paxinos and Watson (1982). Electrode placement was monitored using electrophysiological criteria, and the final depths of the electrodes were determined by optimizing the amplitude of the population spike viewed on an oscilloscope and analyzed on-line with LabView software on an Apple computer. The electrodes were lowered at very slow rates (at least 15 minutes per electrode) in order to maximize the long term stability of the electrodes within the brain tissue. The electrodes, ground wire, and indifferent wire were led to a connector and cemented in place with dental acrylic on top of the skull. Animals were allowed seven to ten days to rest and recover from surgery. The positions of the electrodes were checked in each animal histologically after the end of the experiment, and only those animals with a correct positioning of the electrodes were included in further analyses.

In vivo electrophysiological recordings in freely behaving rats

Prior to obtaining recordings, animals were habituated to daily handling and brief exposure to the recording chamber, specially constructed from wooden panels to insulate the box from external electrical noise. Recordings took place inside the animal's home cage placed within the recording chamber, where the rat was connected by a flexible cable to a swivel/slip-ring commutator brush block assembly that allowed for 360° free rotational movement by the animal. Input-output curves were constructed for each rat by averaging three evoked responses at each of nine different single-pulse stimulus

intensities: 25, 50, 75, 100, 125, 150, 200, 250, and 300 μA. Stimulations were applied at a frequency of 0.033 Hz, or one pulse every 30 seconds. Electrical stimulation was provided by a Grass S88 stimulator passed through a Grass PSIU6 photoelectric stimulus isolation unit to provide constant current. Responses were amplified (100X gain on a homemade amplifier), bandpass filtered from 1 Hz to 3 kHz, and passed to the data acquisition system (Chart software, ADInstruments, Australia) for sampling (rate of 40 kHz), digitization, visualization, and storage for subsequent analysis. The animal's hippocampal EEG and awake state were constantly monitored during recording sessions in order to maintain consistency of behavioral state since it is known that the behavioral state can modulate neuronal responses in the hippocampus (Winson and Abzug, 1977; Winson and Abzug, 1978).

At the end of the chronic stress study, animals were subjected to high frequency stimulation (HFS) before sacrifice. Baseline evoked field potentials were recorded for ten minutes using a stimulation intensity that produced a population spike amplitude equal to 50% of the maximal response for each animal, and one pulse was delivered every 30 seconds. Immediately after baseline recording, HFS was applied (400 Hz, 50 msec, 5 times in 10 sec intervals for perforant path stimulations; 100 Hz, 1 sec, 2 times in 10 sec intervals for mossy fiber stimulations), and recording continued for 30 minutes to determine the effects of HFS.

Fear conditioning by immediate shock deficit

Animals were housed separately from the behavior room containing shocking chambers and were handled daily for several days prior to experimentation. Pre-exposure to the conditioned stimulus involved placing the rat inside the chamber for five minutes. The unconditioned stimulus was administered by delivering a single foot shock (1.5 mA for 1 second) along a metal grid floor immediately after placement into the chamber

(placement to shock interval was less than 15 seconds). Freezing response was scored for five minutes following the shock or during the recall test phase.

Corticosterone assay

Tail blood was taken from the rats, centrifuged and the serum utilized for corticosterone analysis using the Coat-A-Count rat corticosterone kit (Diagnostic Products Corporation).

CHAPTER 3

EFFECTS OF RESTRAINT STRESS ON ADULT HIPPOCAMPAL NEUROGENESIS

Introduction

Restraint stress is a form of psychological stress that can illicit activation of the HPA axis, resulting in the elevation of adrenal hormones when administered acutely. Over a period of weeks, chronic restraint stress subjected daily upon rats results in habituation of the HPA response (Magarinos and McEwen, 1995a). The baseline corticosterone level for rats is typically less than 5 μg/dl during the circadian period of the commencement of stress. Over the course of the first week of restraint stress, corticosterone levels rise within the first half hour to 50 μg/dl, a significant elevation above baseline, and slowly revert to baseline over the subsequent five hour period. By the second week of restraint stress, the peak in corticosterone circulation is reduced slightly from 50 μg/dl to 40 μg/dl, and within three hours of the onset of stress, corticosterone levels return to baseline values. By the third week of restraint, stressed rats exhibit a surge in corticosterone peaking at only 20 μg/dl and quickly reverting to baseline within three hours.

Despite the partial habituation of the physiological stress response occurring between the second and third weeks of restraint, this stressor induces a variety of anatomical and behavioral changes when given chronically. Three weeks of daily restraint stress results in atrophy of the CA3 apical dendrites, reducing both the number of dendritic branch points as well as the total dendritic length (Watanabe et al., 1992b). The remodeling that occurs, however, is not permanent, as the CA3 dendrites return to

prestress condition within ten days after the final stress session. Furthermore, the mossy fiber terminals that synapse upon the CA3 pyramidal neurons show a marked rearrangement of synaptic vesicle clustering near the active zones and increased numbers of mitochondrial profiles (Magarinos et al., 1997).

The hippocampus is critical for spatial learning in rats. Because chronic restraint stress induces neuroanatomical changes in the hippocampus, Cheryl Conrad and colleagues conducted experiments to determine whether these alterations translate into learning impairments (Conrad et al., 1996). Using the Y-maze, a spatial recognition memory task shown to be hippocampal-dependent, they showed that chronically restrained rats were significantly impaired in their spatial recall ability compared to control rats. In addition, this learning impairment can be prevented by tianeptine treatment, an antidepressant drug that increases serotonin uptake and blocks stress-dependent atrophy of CA3 neurons (Watanabe et al., 1992a). Together, the data provide evidence that chronic restraint stress can impact spatial memory ability, and this process is mediated by neuroanatomical changes induced by the stressor.

Restraint stress also affects other behavioral parameters. For example, Gwendolyn Wood et al. (2002) have shown that chronic stress enhances aggressive behavior in rats. Through videotape monitoring, she has observed that stressed rats display higher incidences of biting, boxing, pinning, chasing and physical displacing. The level of aggression has been positively correlated with circulating corticosterone. Additionally, these rats exhibit higher levels of anxiety, as evidenced by their decreased exploration in an open field test (Conrad et al., 1999).

In order to determine how restraint stress impacts hippocampal neurogenesis, adult rats were subjected to either acute or chronic restraint stress and BrdU injections. Proliferation, survival and phenotypic development of the recently born cells were determined using immunohistochemistry and unbiased stereology. The total granule cell

number and granule cell layer volume were determined for chronically stressed and control groups.

Experimental design

Experiment 1: Fifteen rats were divided into three groups containing five rats each: control, two hours restraint, or six hours restraint. The restrained rats received a single session of acute restraint stress. At the end of the stress period, all rats received a single injection of BrdU (200 mg/kg) and allowed a two hour survival period prior to perfusion. This experiment is designed to determine whether acute restraint stress affects proliferation of precursor cells in the dentate gyrus.

Experiment 2: Animals were restrained in wire mesh restrainers for six hours per day for 21 consecutive days. Control rats were left undisturbed. To examine the effects of chronic restraint stress on the proliferation of precursor cells, restrained and control rats (n = 6 for each group) were injected with BrdU (200 mg/kg) one day following the final stress session and allowed a survival time of two hours.

Experiment 3: To study the effects of chronic restraint stress on the survival of newly born cells and to determine the developmental phenotypes of proliferating cells, rats (n = 6 for each group) received four daily injections of BrdU (100 mg/kg) immediately prior to the three weeks of restraint and were sacrificed one day following the final stress session. This period of survival following the BrdU injections is sufficient for the BrdU-labeled cells to differentiate and express mature neuronal markers (Cameron et al., 1993).

Experiment 4: To study the long term effects of restraint stress on hippocampal neurogenesis, 16 rats were restrained daily for 21 days. These rats, along with eight

control rats, received four daily injections of BrdU (100 mg/kg) during days 18-21. Of the 16 rats that were restrained for 21 days, four rats were restrained for an additional 21 days (42 days in total), and six were allowed to recover for 21 days. At the end of the 42-day experiment, these ten rats along with four of the control rats were perfused. The other six rats that were restrained for the initial three weeks and the remaining four control rats were sacrificed on day 22, one day following the final stress session and BrdU injection.

Results

Neurogenesis can be traced by immunohistochemical detection of BrdU incorporated into the DNA of proliferating cells during the DNA synthesis (S) phase of the cell cycle. In the hippocampus, these cells generally reside in the subgranular zone at the border between the granule cell layer and the hilus, with occasional cells present in the hilus (Figure 1). While many of the BrdU-labeled cells were scattered individually throughout the rostrocaudal extent of the dentate gyrus, it was also common to find three or more cells clustered together, particularly at the crest of the dentate gyrus. The cells were typically oval or round with a nuclear diameter of approximately 10 μm, or they were smaller and more densely clustered.

Stereological estimates of the number of BrdU-labeled cells revealed no differences in neurogenesis between control and acutely restrained rats ($p > 0.94$; Figure 2). Control rats had 3050 $\pm$ 249 BrdU-positive cells in the dentate gyrus, whereas rats acutely restrained for two hours had 3070 $\pm$ 239 BrdU-positive cells, and rats acutely restrained for six hours had 3094 $\pm$ 506 BrdU-positive cells. After 21 days of repeated restraint stress, however, there was a statistically significant reduction of precursor cell proliferation by 24% in the stressed group compared to the control group ($p < 0.04$; Figures 3 and 4). The control group had 2888 $\pm$ 245 BrdU-immunoreactive cells in the dentate gyrus, and the chronically stressed animals had 2204 $\pm$ 149 BrdU-

immunoreactive cells. Proliferation was examined by sacrifice of the animals two hours after a single BrdU injection.

To compare the effects of chronic stress on the survival of newly born cells, animals received injections of BrdU prior to the three weeks of restraint. Quantitative analysis of the number of BrdU-positive cells that survived in animals after the stress period showed a slight decrease compared to matched controls, but the change was not statistically significant ($p > 0.65$; Table 1). Stereological estimates of the total number of granule neurons in the dentate gyrus and of the granule cell layer volume by the Cavalieri method did not reveal significant differences between the control and experimental groups ($p > 0.1$ and $p > 0.5$, respectively; Table 1), although there was a slight trend toward suppression in the chronically stressed group.

Coefficients of variation (CV) for the numbers of BrdU proliferating and surviving cells in the chronic stress paradigm were ranging between 0.17 and 0.38, revealing a certain degree of variability among animals. This was not related to a methodological problem because the coefficients of error (CE) were small (0.08 – 0.11) in these data sets. Similarly, in the acute stress paradigm, CVs ranged from 0.17 to 0.37 and the CEs from 0.08 to 0.13. A much lower variance was observed with respect to the total number of granule neurons, with CV values ranging from 0.12 to 0.18 and CEs ranging from 0.02 to 0.06. In both the control and repeatedly stressed groups, the total volume of the granule cell layer was highly comparable among animals (CV = 0.06 – 0.11 and CE of 0.03 in both groups).

For phenotypic identification, immunofluorescent triple labeling was carried out with antibodies against BrdU, NeuN, and GFAP. These tissue sections were examined on a confocal laser scanning microscope in order to verify that colocalization of fluorescent signals originated in the same optical plane and thus from the same cells (Figure 5). An unbiased sampling of BrdU-positive cells through the entire dentate gyrus indicated that

control and stressed animals had similar phenotypic expression patterns three weeks after the BrdU injections (Figure 6). Approximately 70% of the newly born cells differentiated and developed into mature granule neurons, expressing the marker for NeuN. Less than 10% of the BrdU-labeled cells displayed GFAP staining, an indication of astroglia development, and 20% of the cells became neither granule cells nor astroglia, showing neither immunoreactivity for NeuN or GFAP.

Because the only effect observed thus far was a suppression in cell proliferation after three weeks of chronic stress, the study was repeated and extended for an additional three weeks to trace the survival and development of the cells born after the initial three weeks of restraint. After the first 21 days of stress, we were able to reproduce our result showing that chronic restraint stress significantly suppressed cell proliferation. The control rats had 9992 $\pm$ 243 BrdU-positive cells in the dentate gyrus, whereas the stressed group had 6568 $\pm$ 1189 BrdU-positive cells (Figure 7). This represents a 34% suppression. The numbers of BrdU-labeled cells in this experiment were markedly higher than those in the earlier experiment because the rats received four daily injections of BrdU in this case. Thus, there were more opportunities for cells to be labeled, and the cells that incorporated BrdU had time to complete more than one cell cycle. In the first experiment involving chronic restraint stress, the rats received a single BrdU injection with a two hour survival period. It seems consistent then, that the first study produced a 24% suppression while the second study resulted in a 34% suppression. A lower rate of proliferation during this stage would seemingly become amplified as BrdU-incorporated cells pass through mitosis; that is, when fewer cells become labeled and fewer cells divide, each generational passage of cell division would produce larger differences between control and experimental groups.

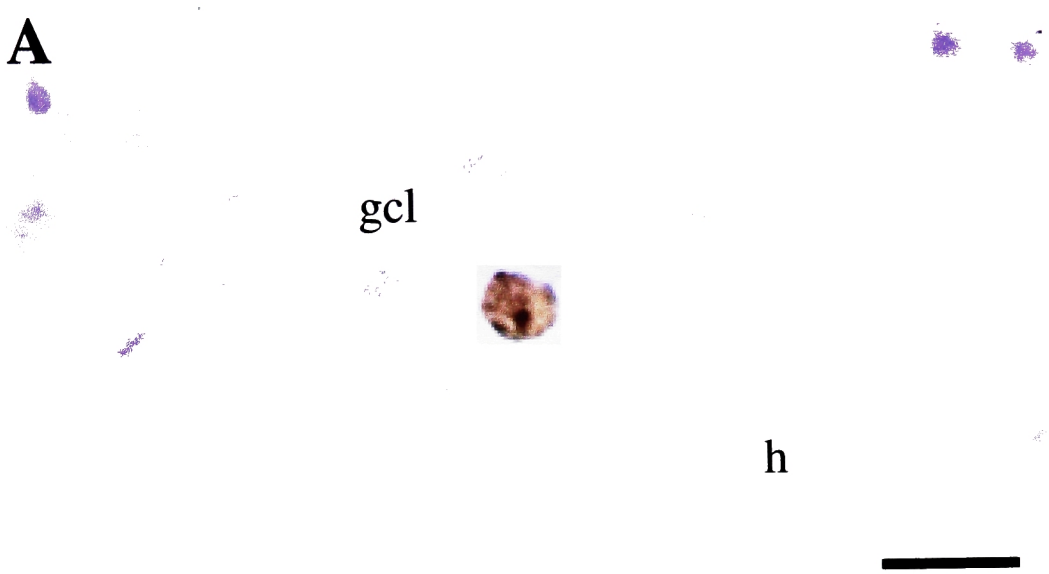
In the 42-day phase of the experiment, proliferating cells were pulsed with BrdU during days 18-21 and their survival determined three weeks later. Control rats had 4950

± 778 BrdU-labeled cells whereas rats stressed for three weeks and then allowed to recover for three weeks had 3488 ± 252 BrdU-labeled cells, and rats stressed continuously for six weeks had only 2472 ± 388 BrdU-labeled cells (Figure 8). No significant difference was observed between the control group and the stress and recovery group in BrdU-immunoreactivity ($p > 0.06$). However, there was a significant difference between the continuously stressed rats and the control rats ($p < 0.03$) as well as between the continuously stressed rats and the stress and recovery rats ($p < 0.05$). These data show that chronic restraint stress not only suppressed cell proliferation but also attenuated survival of the recently born cells after an extended time course, resulting in a reduction of granule cell neurogenesis in the dentate gyrus. When the number of BrdU-labeled cells detected after six weeks (surviving cells; Figure 8) is divided by the number of BrdU-labeled cells detected after three weeks (proliferating cells; Figure 7), the survival fraction is obtained for each group. Control rats had 50% survival; stressed and recovered rats had 53% survival, and continually stressed rats had 38% survival. Thus, termination of chronic restraint stress rescued the survival of recently born cells; however, the initial three weeks of restraint stress produced fewer proliferating cells, so the absolute value of BrdU-immunoreactive cells after six weeks in this group was lower than that of the control group, although the difference was not statistically significant.

Combined immunofluorescent labeling of these tissue using antibodies directed against BrdU, NeuN, and GFAP, revealed that among the populations of surviving BrdU-positive cells, similar proportions in all three groups matured into neurons (about 70%), astroglia (about 5%) or undetermined (about 25%; Figure 9). Thus, in the control group, it can be estimated that 3400 BrdU-labeled cells developed into granule neurons whereas the continuously stressed rats generated, on average, just 1800 cells immunoreactive for both BrdU and NeuN, representing a 47% suppression of neurogenesis in rats stressed for six weeks compared to naïve control rats.

Using unbiased stereology to estimate the total number of granule cells in the dentate gyrus and the volume of the granule cell layer, it was determined that six weeks of daily restraint significantly reduced both of these measures when compared to control treatment. (Table 2). Chronically stressed rats had a 13% reduction of absolute granule cell number and a 5% reduction in granule cell layer volume ($p < 0.03$ and 0.04, respectively). Although three weeks of restraint stress revealed a trend toward a decrease of these neuroanatomical parameters (Table 1), it required six weeks of stress to produce significant results (Table 2).

Figure 1: Light microscopic images of proliferating cells labeled by BrdU immunohistochemistry (brown cells) and counterstained with cresyl violet. The cells resided at the border of the granule cell layer and the hilus in the dentate gyrus. They were typically oval or round in shape and were about 10 μm in diameter. *gcl*, granule cell layer; *h*, hilus. Scale bars: *A*, 20 μm; *B*, 10 μm.


A
gcl
h

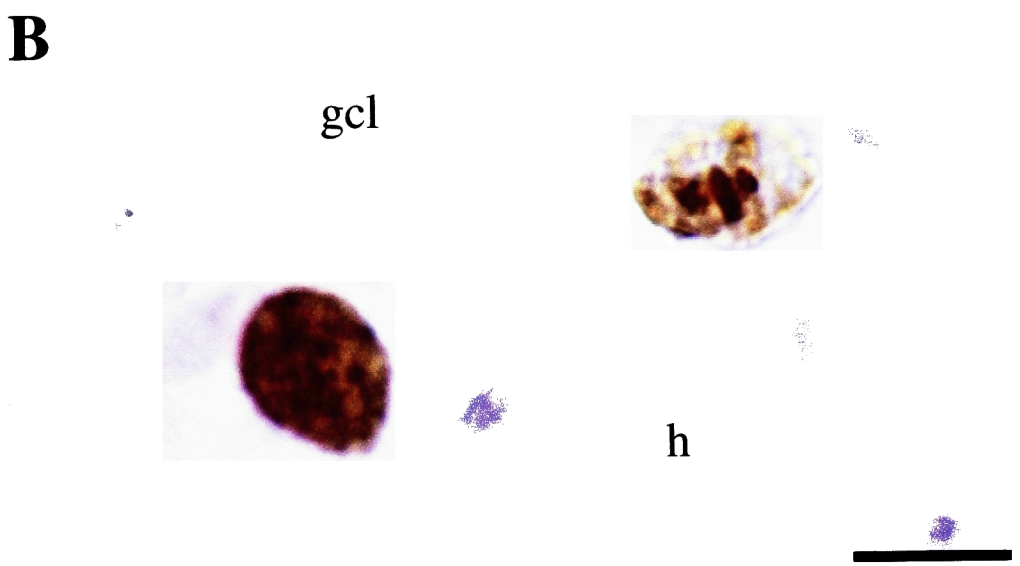

B
gcl
h

Figure 2: Acute restraint stress did not suppress cell proliferation in the dentate gyrus. Bar values are mean + S.E.M. Control rats had 3050 $\pm$ 249 BrdU-positive cells in the dentate gyrus, whereas rats acutely restrained for two hours had 3070 $\pm$ 239 BrdU-positive cells, and rats acutely restrained for six hours had 3094 $\pm$ 506 BrdU-positive cells. Analysis by one way ANOVA revealed no significant differences among the three groups ($p > 0.94$).

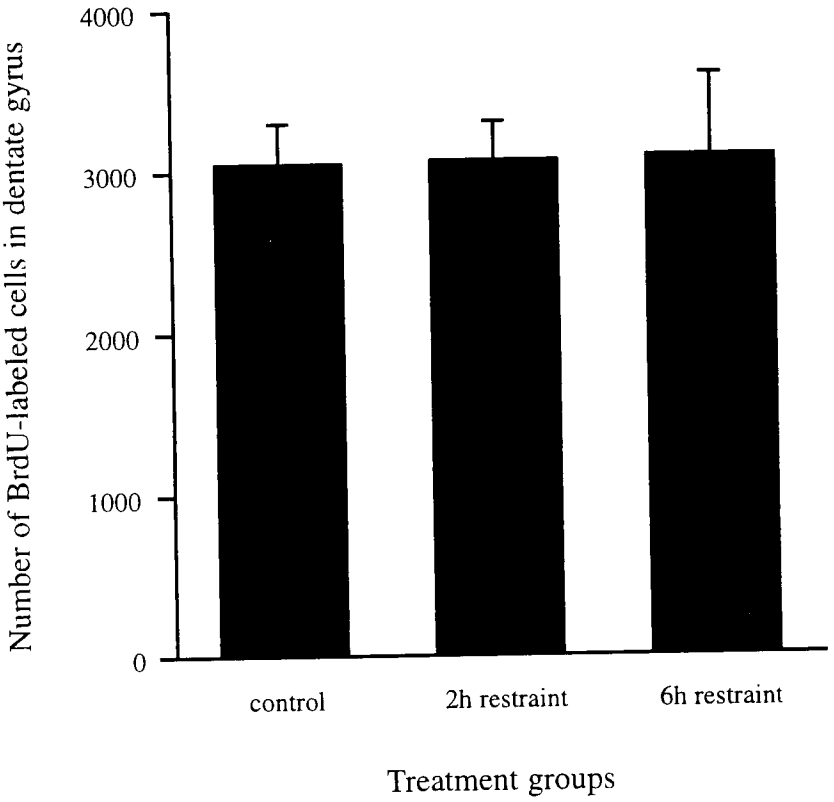

4000
3000
2000
1000
0
Number of BrdU-labeled cells in dentate gyrus
control
2h restraint
6h restraint
Treatment groups

Figure 3: Repeated restraint stress for 21 consecutive days reduced proliferation of precursor cells by 24%. Bar values are mean + S.E.M. The control group had 2888 $\pm$ 245 BrdU-immunoreactive cells in the dentate gyrus, and the chronically stressed animals had 2204 $\pm$ 149 BrdU-immunoreactive cells. Asterisk indicates a significant difference ($p < 0.04$) from control in Fisher's PLSD post hoc test following one way ANOVA.

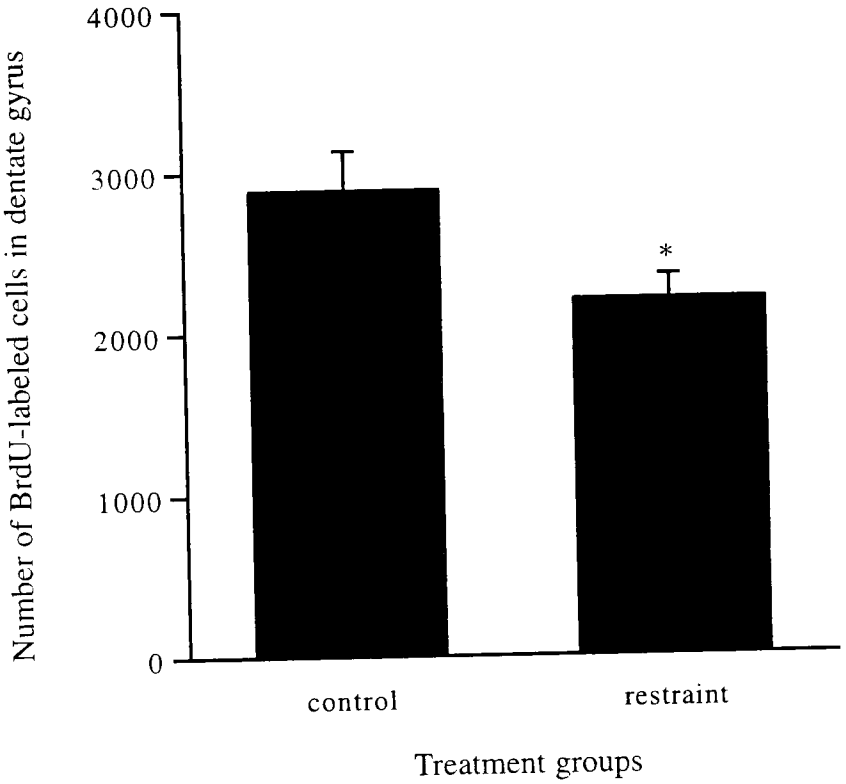

4000
3000
2000
1000
0
Number of BrdU-labeled cells in dentate gyrus
*
control
restraint
Treatment groups

Figure 4: Examples of Nissl-stained dentate gyrus of control (*A*) and chronically stressed (*B*) animals. Immunohistochemical labeling for BrdU incorporation showed proliferating cells (arrows) present mainly in the subgranular zone and occasionally in the hilus. *gcl*, granule cell layer; *h*, hilus. Scale bar: 100 μm (applies to *A,B*).

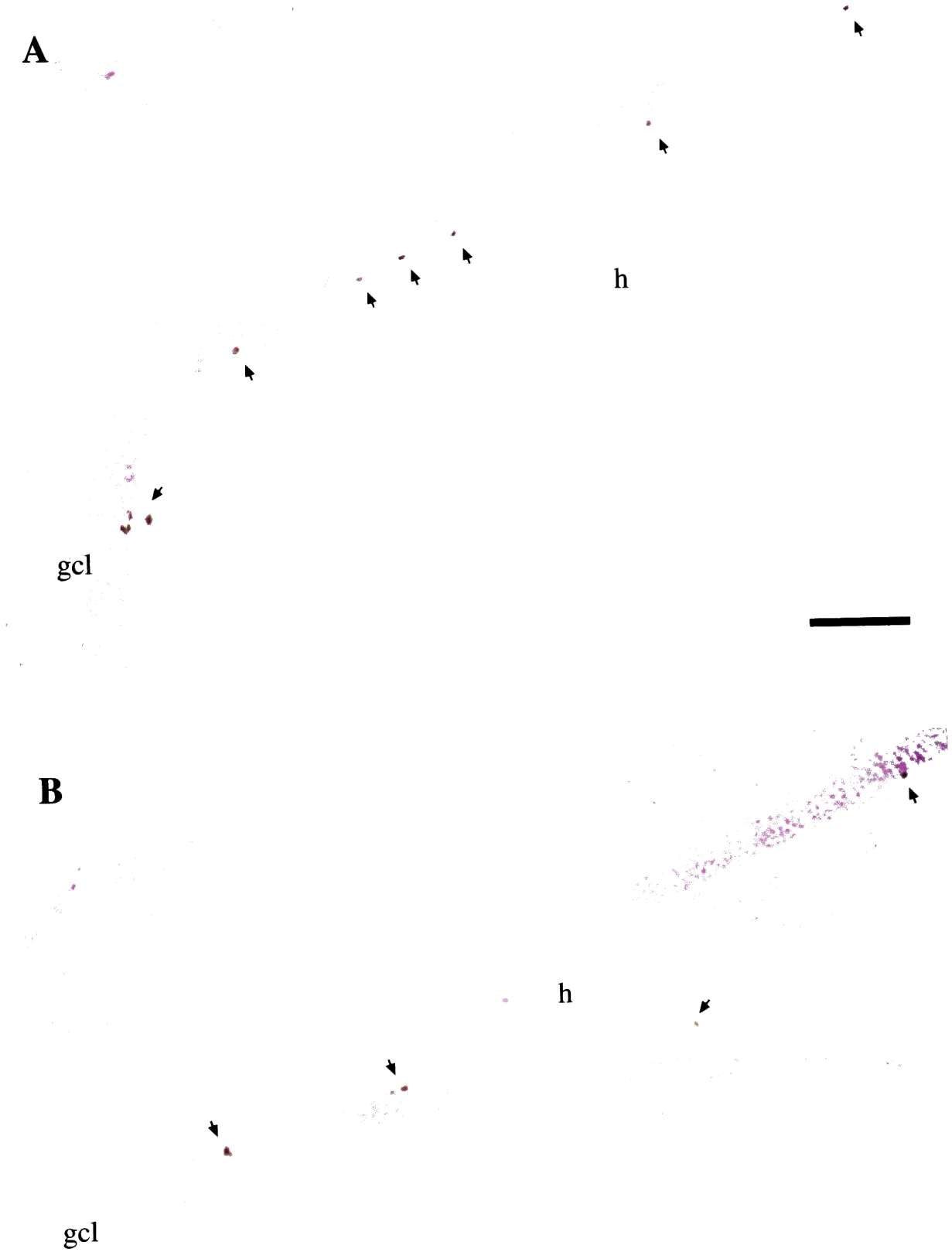

A
h
gcl
B
h
gcl

Figure 5: The majority of newly born cells developed into granule neurons. Confocal laser scanning microscopic images of the dentate gyrus showing green nuclear staining for NeuN (*A*), red nuclear staining for BrdU-containing cells (*B*), and blue GFAP staining for astroglia (*C*). *D*, Image of the three fields merged together, showing NeuN and BrdU colocalization in the granule cell layer. Images were acquired from line sequence scanning through an optical section one μm in the z-axis. Scale bar equals 10 μm and applies to *A*-*D*.

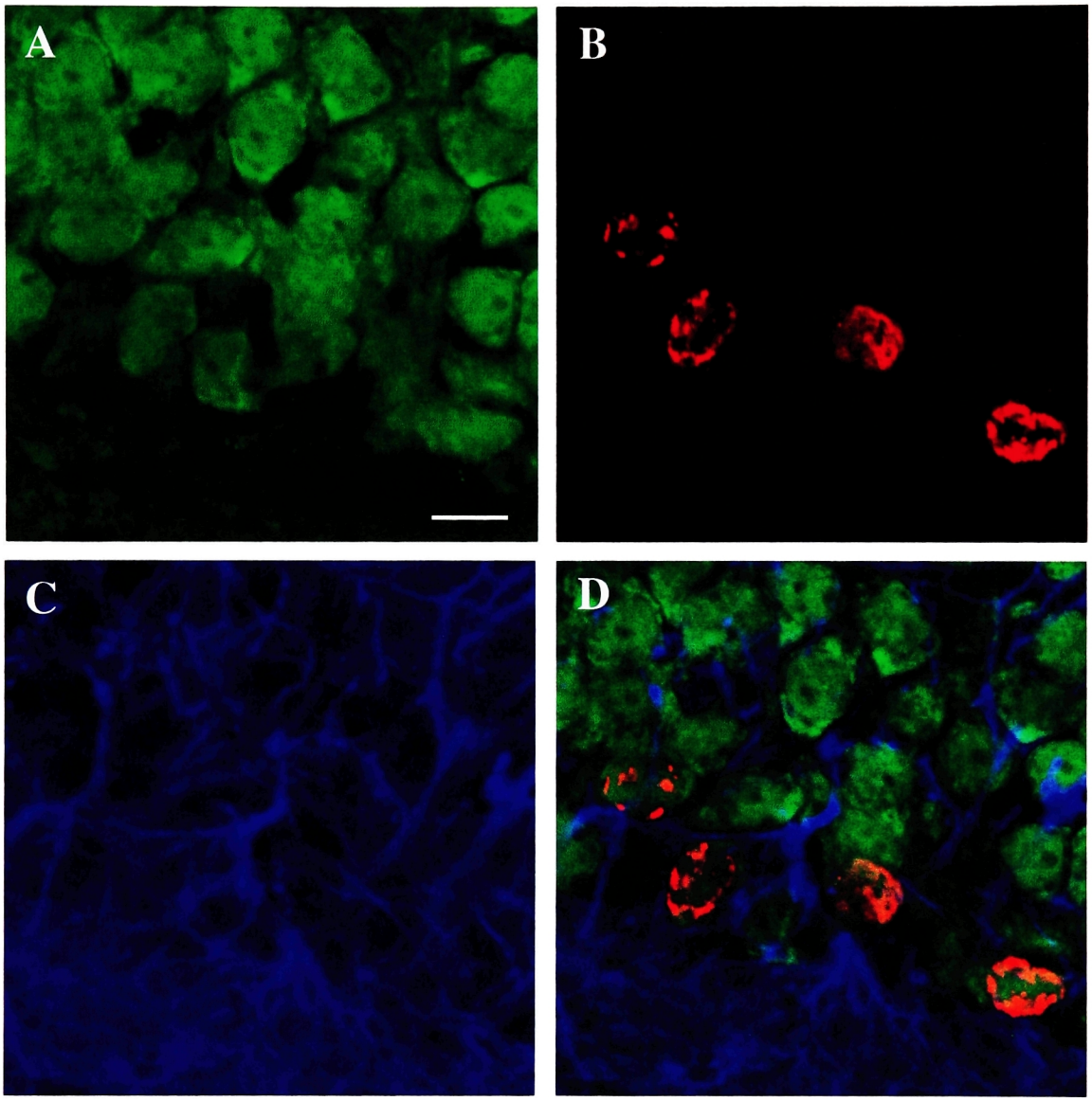

A
B
C
D

Figure 6: Three weeks after the BrdU injections, 70% of the BrdU cells matured into granule neurons and expressed NeuN. Six to eight percent of the BrdU cells developed into astroglia, and 20% remained undifferentiated, expressing neither NeuN nor GFAP. There were no differences in phenotypic expression patterns between control and stressed groups. Bar values are mean + S.E.M.

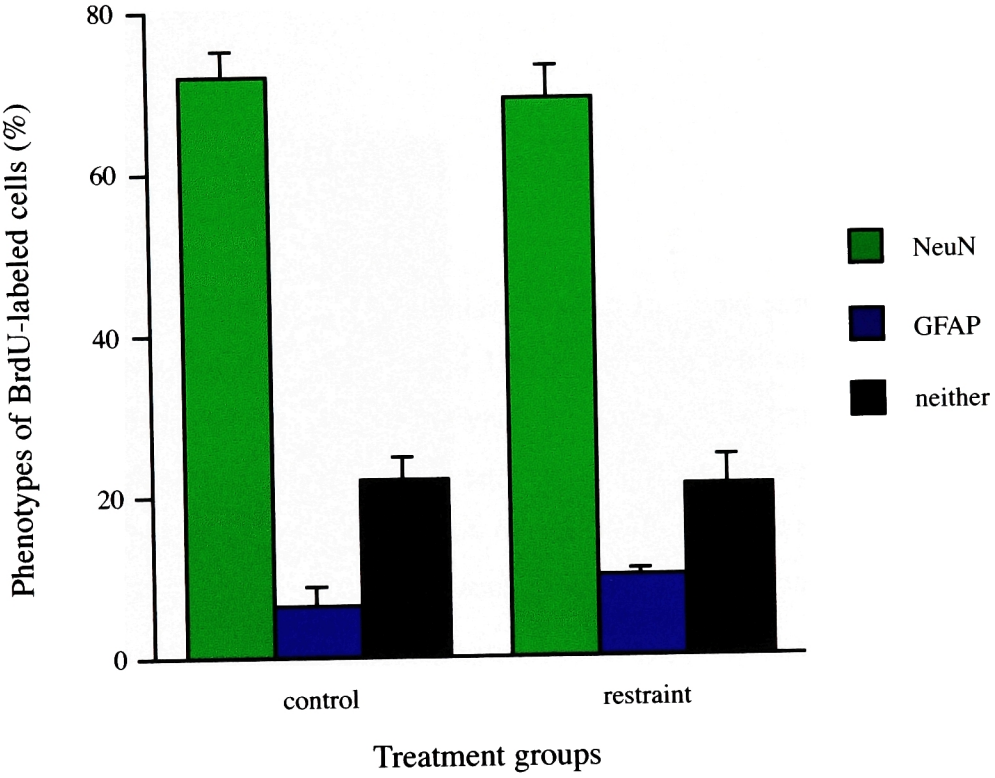

80
60
40
20
0
Phenotypes of BrdU-labeled cells (%)
control
restraint
Treatment groups
NeuN
GFAP
neither

Figure 7: Three weeks of daily restraint stress reduced cell proliferation in the dentate gyrus. All rats received four BrdU injections, one each during days 18-21 of the experiment, and were perfused on day 22. Control rats had 9992 $\pm$ 243 BrdU-positive cells in the dentate gyrus, whereas the stressed group had 6568 $\pm$ 1189 BrdU-positive cells, a 34% reduction compared to control. Bar values are mean + S.E.M., and the asterisk indicates a significant difference compared to control ($p < 0.05$).

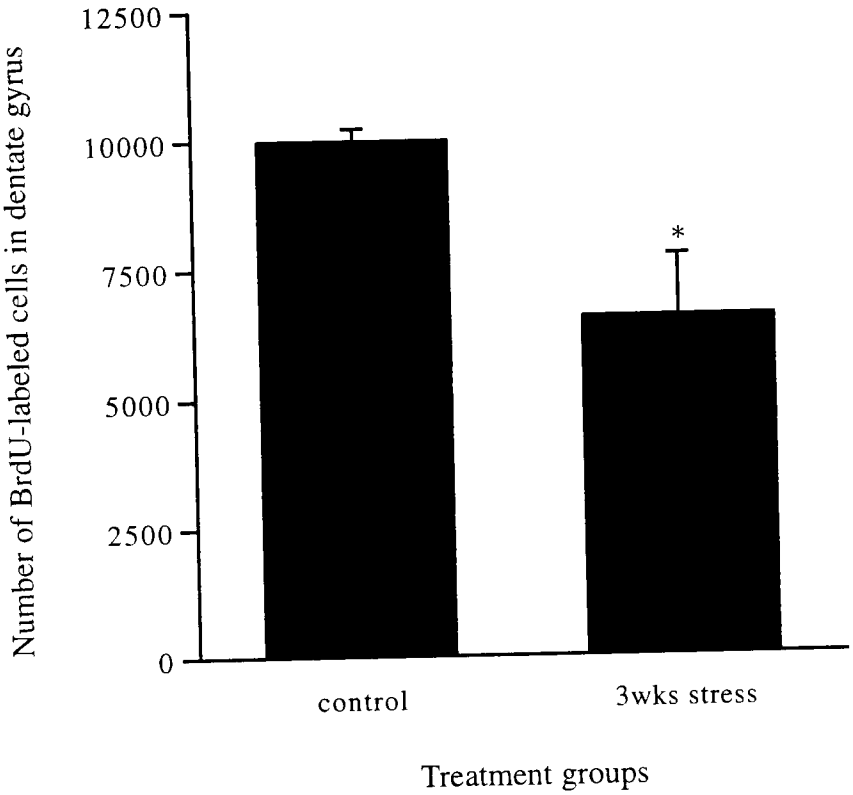

12500
10000
7500
5000
2500
0
Number of BrdU-labeled cells in dentate gyrus
*
control
3wks stress
Treatment groups

Figure 8: Extension of chronic restraint stress from three to six weeks reduced the survival of recently born cells in the dentate gyrus. Control rats had 4950 $\pm$ 778 surviving cells, representing 50% survival compared to control rats sacrificed on day 22 (refer to figure 7). Rats restrained for three weeks and then allowed to recover for the remaining three weeks (3wk S&R) had 3488 $\pm$ 252 BrdU-positive cells, a 53% survival rate. Rats stressed continuously for six weeks had only 38% of labeled cells detected at day 22 survive to day 43, showing 2472 $\pm$ 388 BrdU-positive cells. Bar values are mean + S.E.M., and the asterisk indicates a significant difference compared to control group ($p < 0.03$) and to 3wk S&R group ($p < 0.05$).

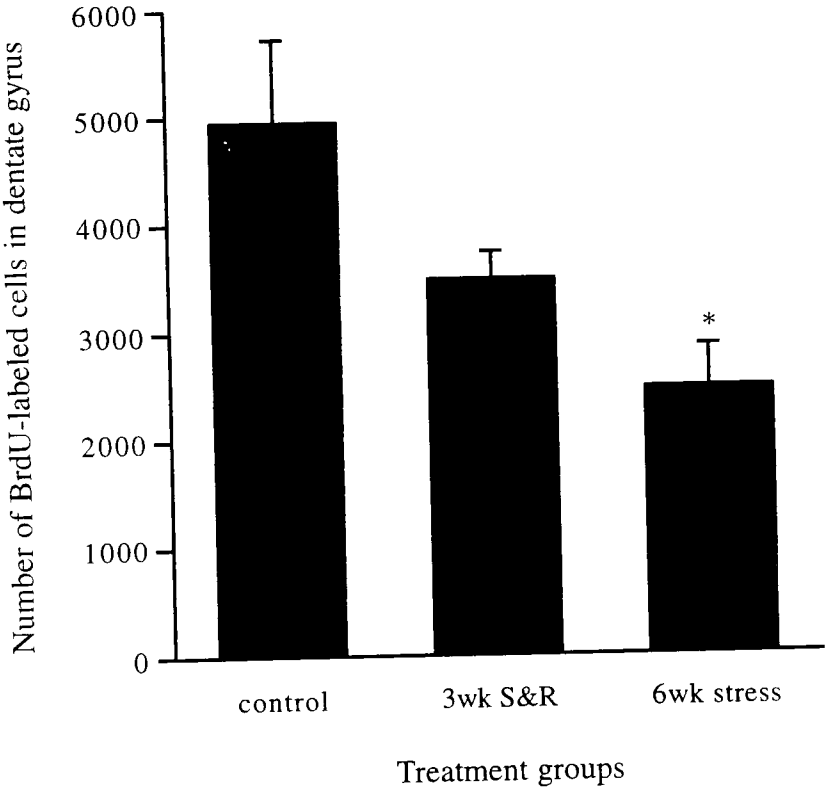

Number of BrdU-labeled cells in dentate gyrus
6000
5000
4000
3000
2000
1000
0
*
control
3wk S&R
6wk stress
Treatment groups

Figure 9: Combined immunofluorescent labeling revealed that among the populations of surviving BrdU-positive cells, similar proportions in all three groups matured into neurons (about 70%), astroglia (about 5%) or undetermined (about 25%). Bar values are mean + S.E.M.

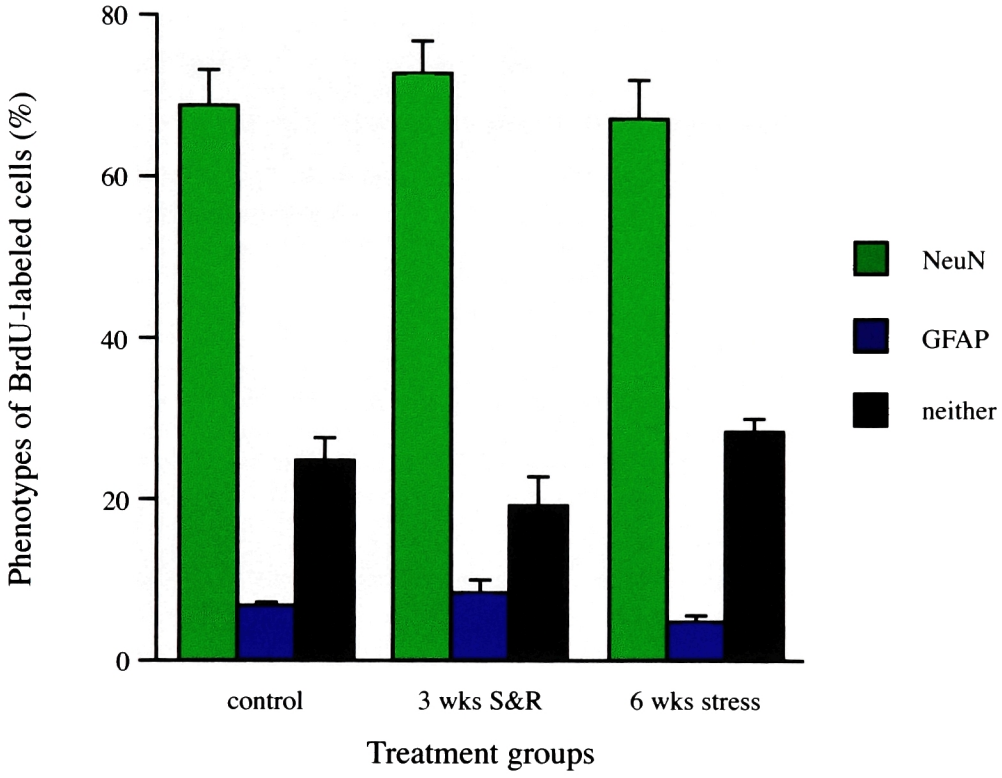

80
60
40
20
0
Phenotypes of BrdU-labeled cells (%)
control
3 wks S&R
6 wks stress
Treatment groups
NeuN
GFAP
neither

Table 1. Stereological data from the hippocampus of adult rats after three weeks of restraint stress

	Control	Chronically restrained
Number of surviving BrdU-labeled cells	2453 ± 307	2232 ± 346
Absolute number of granule cells	$2.69 \times 10^6 \pm 0.13 \times 10^6$	$2.37 \times 10^6 \pm 0.14 \times 10^6$
Volume of the granule cell layer (mm^3)	3.02 ± 0.08	2.93 ± 0.13

The numbers are mean ± S.E.M. No significant differences were obtained between the chronically stressed animals and matched controls, though there were trends toward reduction in all three measured variables.

Table 2. Stereological data from the hippocampus of adult rats after six weeks of restraint stress

	Control 6 weeks	Stress 3 weeks/ Recover 3 weeks	Stress 6 weeks
Absolute number of granule cells	$2.70 \times 10^6 \pm 0.09 \times 10^6$	$2.59 \times 10^6 \pm 0.08 \times 10^6$	$2.35 \times 10^6 \pm 0.09 \times 10^6$*
Volume of the granule cell layer (mm^3)	3.02 ± 0.04	2.96 ± 0.04	2.85 ± 0.06**

The numbers are mean ± S.E.M. Asterisks indicate significant differences compared to six weeks control group (* $p < 0.03$; ** $p < 0.04$).

Discussion

The results of this study show that acute restraint stress did not change the baseline proliferative activity in the dentate gyrus, whereas chronic restraint stress significantly suppressed proliferation of dentate gyrus precursor cells. These changes occurred over a similar time frame in which repeated restraint stress has been shown to produce atrophy of CA3 apical dendrites and rearrangement of mossy fiber synaptic terminals (Magarinos et al., 1997; Watanabe et al., 1992b). Furthermore, restraining rats for an extended period of six weeks resulted in a reduction in the survival of recently born cells. Because both acute and chronic restraint stress induce corticosterone secretion via the HPA axis, the differential effects on cell proliferation must be explained by the duration of stress exposure and by the dynamic changes in other mediators that are activated by acute and chronic stress. Candidate mediators besides adrenal steroids include excitatory amino acid neurotransmitters (EAAs) and serotonin. For neurogenesis, EAAs have been shown to be inhibitory whereas serotonin has been shown to be stimulatory via 5HT-1A receptors.

The role of HPA response and excitatory amino acids in neurogenesis

Acute restraint stress has been shown to stimulate the HPA axis, inducing a release of corticosterone that peaks 20-30 minutes after the onset of the stressor (Magarinos and McEwen, 1995a). Corticosterone, as well as NMDA receptor activation, suppresses neurogenesis, with NMDA receptors acting downstream of corticosterone (Cameron et al., 1998). Restraint stress increases extracellular glutamate levels in the hippocampus by a process that is reduced by ADX (Lowy et al., 1993; Moghaddam et al., 1994). This suggests that acute restraint stress should suppress cell proliferation by means of glutamate acting on NMDA receptors, as well as by the synergistic actions of glucocorticoids on glutamatergic activity. However, our results indicate that acute

restraint stress did not reduce the number of proliferating cells. Nevertheless, acute psychosocial stress does inhibit hippocampal neurogenesis (Gould et al., 1997a; Gould et al., 1998). One possible explanation of the difference in efficacy between stressors is that acute restraint stress may be such a mild stressor that the activation of HPA activity or glutamate release is not sufficient to inhibit neurogenesis. However, another possible explanation involves the participation of other regulatory players, such as serotonin neurotransmission, that may act to counterbalance the inhibitory effects of EAAs and glucocorticoids.

Stress-induced regulation of the serotonergic pathway

The serotonergic system appears to enhance neurogenesis, as shown by recent reports that depletion of serotonin decreases the number of newly born cells in the dentate gyrus, and that activation of 5HT-1A receptors increases dentate gyrus neurogenesis (Brezun and Daszuta, 1999; Gould, 1999). Thus, enhanced serotonin neurotransmission during acute restraint stress might compensate for decreases in neurogenesis caused by glutamate release and HPA activation. In this connection, adrenal steroids have been shown to facilitate serotonin synthesis via increased tryptophan uptake and elevated tryptophan hydroxylase activity (Azmitia and McEwen, 1974). Moreover, acute restraint stress has been shown to increase hippocampal 5HT-1A receptor binding, particularly in the hilus and in the dentate gyrus (Mendelson and McEwen, 1991). Kaoru Fujino and colleagues recently published results showing that various types of acute stress caused an immediate, significant and reversible increase in extracellular serotonin in the hippocampus, suggesting that the serotonergic system is involved in the response to acute stress (Fujino et al., 2002). In order to determine if serotonin neurotransmission is counterbalancing the suppressive effects of glucocorticoids during acute restraint stress, the study should be repeated using a blocker of serotonin synthesis or an antagonist of the

5HT-1A receptor. If the hypothesis is correct, one would predict that interference of serotonin neurotransmission would result in a reduction of hippocampal neurogenesis in response to an acute session of restraint stress. This experiment is currently being conducted by Helen Bateup in the lab.

Chronic restraint stress-induced decrease in neurogenesis

Although acute restraint did not alter neurogenesis, repeated daily application of this stressor for three weeks significantly reduced the number of proliferating cells in the dentate gyrus, and survival and neurogenesis were decreased after six weeks of stress. This occurred in spite of the partial habituation of the HPA response over the chronic period of restraint stress (Magarinos and McEwen, 1995a). Thus, the suppression of neurogenesis may be related to changes in the balance between EAA suppression of neurogenesis and the enhancing effects of serotonin. Chronic glucocorticoid treatment increases NMDA receptor subunit expression in the hippocampus (Weiland et al., 1997), while at the same time it reduces electrophysiological responses to serotonin in the rat hippocampus (Karten et al., 1999). Thus, compensatory action of the serotonergic pathway in acute restraint stress, which was suggested above, may be curtailed during chronic stress, leading to the observed suppression of neurogenesis by the enhanced activity of the EAA pathway. This notion is further supported by studies conducted by Lopez and colleagues showing that chronic stress causes a decrease in 5HT-1A receptor mRNA and binding in the hippocampus (Lopez et al., 1998).

In summary, chronic restraint stress suppressed cell proliferation in the dentate gyrus and survival of the recently born cells, resulting in a significant attenuation of hippocampal neurogenesis in adult rats. Other morphological alterations included reductions in the total granule cell number and granule cell layer volume. These events

may be mediated, at least in part, by adrenal steroids acting in concert with EAAs and serotonin, and they may be involved in the wider structural plasticity of the dentate gyrus-CA3 region, which also involves atrophy of apical dendrites of CA3 neurons and reorganization of synaptic vesicles in mossy fiber terminals.

CHAPTER 4

EFFECTS OF RESTRAINT STRESS ON PSA-NCAM EXPRESSION

Introduction

The developmental processes governing the maturation of neurons often involve cell migration and neurite extension. Cell adhesion molecules, particularly the neural cell adhesion molecule (NCAM), mediate the adhesion of cells to other cells as well as to components of the extracellular matrix and are thus critical for nervous system development. NCAM is a member of the immunoglobulin superfamily, and several isoforms are generated via alternative splicing of a single copy gene. NCAM can be further modified at the post-translational level by the addition of polysialic acid (PSA), a large, negatively charged sugar moiety. PSA is composed of long, linear homopolymers of α-2,8-linked sialic acid residues and is carried exclusively on NCAM in the vertebrate brain (Rougon et al., 1986). The attachment of PSA to NCAM has been shown to be catalyzed by one of two enzymes: polysialyltransferase (PST) and sialyltransferase-X (STX; Kiss and Rougon, 1997). The glycosylation of NCAM attenuates the adhesion properties of NCAM by interfering with homophilic binding. Thus, cells expressing PSA-NCAM are believed to be more "slippery," allowing them to participate in developmental and plasticity associated events, such as migration, neurite extension and synaptogenesis.

PSA-NCAM is widely expressed throughout the embryonic and early postnatal brain but is more restricted in its expression in the adult brain. Interestingly, the regions of the adult brain that continue to display PSA-NCAM expression are those which generate new neurons throughout an animal's life span, such as the olfactory system and

the hippocampus (Seki and Arai, 1993a). In fact, PSA-NCAM has been shown to be expressed by newly generated granule cells in the dentate gyrus of the adult rat and is important for morphological plasticity in the hippocampus (Seki and Arai, 1993b). Conditions, like repeated stress, which alter hippocampal neurogenesis and cause dendritic reorganization are likely to be accompanied by changes in PSA-NCAM expression. In order to examine the effects of restraint stress on PSA-NCAM expression, we performed immunohistochemical studies on tissue from adult rats subjected to various treatments of restraint stress.

Experimental design

PSA-NCAM immunohistochemical detection and quantitative analyses were completed as described in the methods section (Chapter 2) using tissue generated from experiments involving acute, chronic (21 days), and extended (42 days) restraint stress (experiments 1, 2, and 4, respectively) listed in Chapter 3.

Results

PSA-NCAM immunohistochemistry revealed a subpopulation of cells located in the innermost portion of the granule cell layer (Figure 10). These cells displayed the characteristic morphology of granule neurons, with apical dendritic trees expanding into the molecular layer. Moreover, many of these neurons exhibited basal dendrites comparable to those described by Spigelman et al. (1998) after temporal lobe epilepsy. The mossy fibers were also labeled in the hilus and the stratum lucidum and stratum radiatum of CA3. Quantitative analysis revealed that chronic stress induced a significant mean increase of 40% in the number of PSA-NCAM-immunoreactive cells in the subgranular zone of the dentate gyrus with respect to the control group ($p < 0.04$; Figure 11). Control animals had 43,637 $\pm$ 3620 PSA-NCAM-positive cells in the dentate gyrus

while chronically stressed animals had 60,998 ± 5638 PSA-NCAM-positive cells. There were no differences in the number of PSA-NCAM-immunoreactive granule neurons between control and acutely restrained rats (Figure 12).

In experiment 4, when restraint stress was extended to six weeks, we were able to reproduce our result showing a significant elevation of PSA-NCAM expression at the midpoint (after three weeks) of daily stress ($p < 0.05$; Figure 13). Control rats had 37,536 ± 3098 PSA-NCAM-labeled cells in the dentate gyrus while the stressed rats displayed a 51% increase over control, showing 56,817 ± 5424 PSA-NCAM-labeled cells. The level of PSA-NCAM expression reverted to control value when rats were allowed to recover for three weeks after three weeks of restraint stress (Figure 14). The control group had 39,024 ± 4859 PSA-NCAM-labeled cells, and the stress and recovery group had 39,640 ± 1749 PSA-NCAM-labeled cells. Interestingly, the rats continuously restrained for six weeks showed no significant difference in PSA-NCAM staining compared to control rats (Figure 14). The six weeks stress group had 37,452 ± 3521 PSA-NCAM-immunoreactive cells.

Figure 10: *A*, Photomicrograph of PSA-NCAM-immunoreactive cell in the dentate gyrus. Light microscopic images of the dentate gyrus of a control (*B*) and chronically stressed (*C*) rat, showing higher expression of PSA-NCAM in stressed rats in a population of neurons residing in the subgranular zone. *gcl*, granule cell layer; *h*, hilus. Scale bars: *A*, 25 μm; *B*, 200 μm (applies to *B,C*).

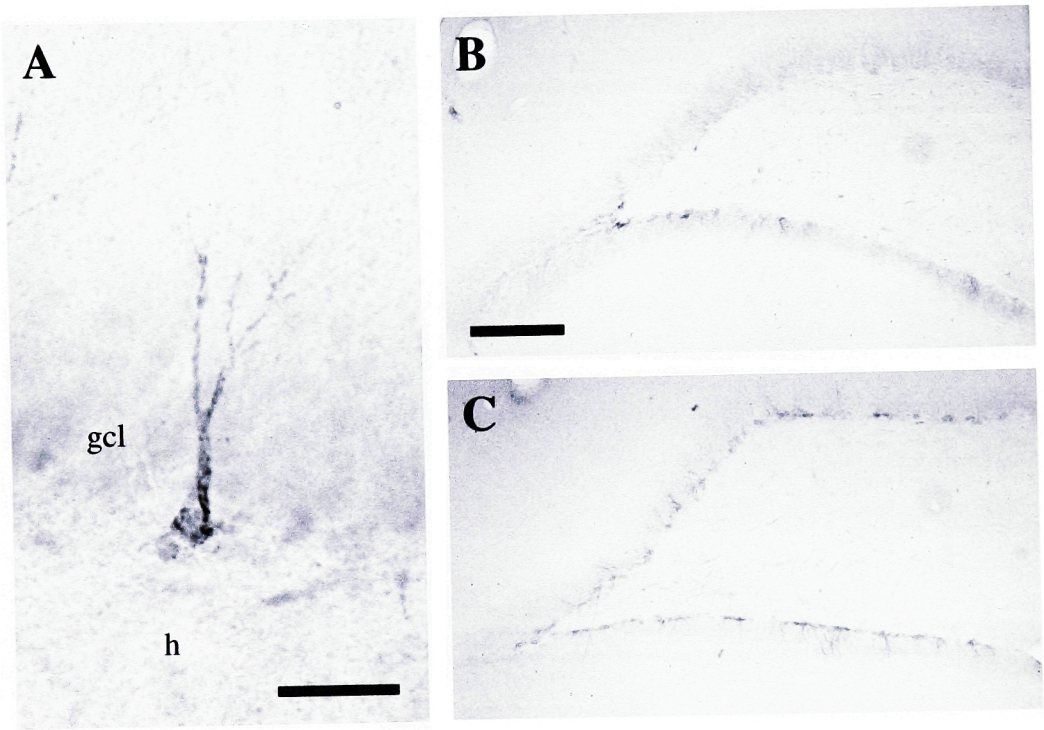

A
gcl
h
B
C

Figure 11: Three weeks of chronic restraint stress increased PSA-NCAM expression in the dentate gyrus of adult rats. ANOVA followed by Fisher's PLSD post hoc test showed 40% higher levels of PSA-NCAM-containing cells in chronically stressed animals compared to matched controls. Bar values are mean + S.E.M. Asterisk indicates a significant difference.

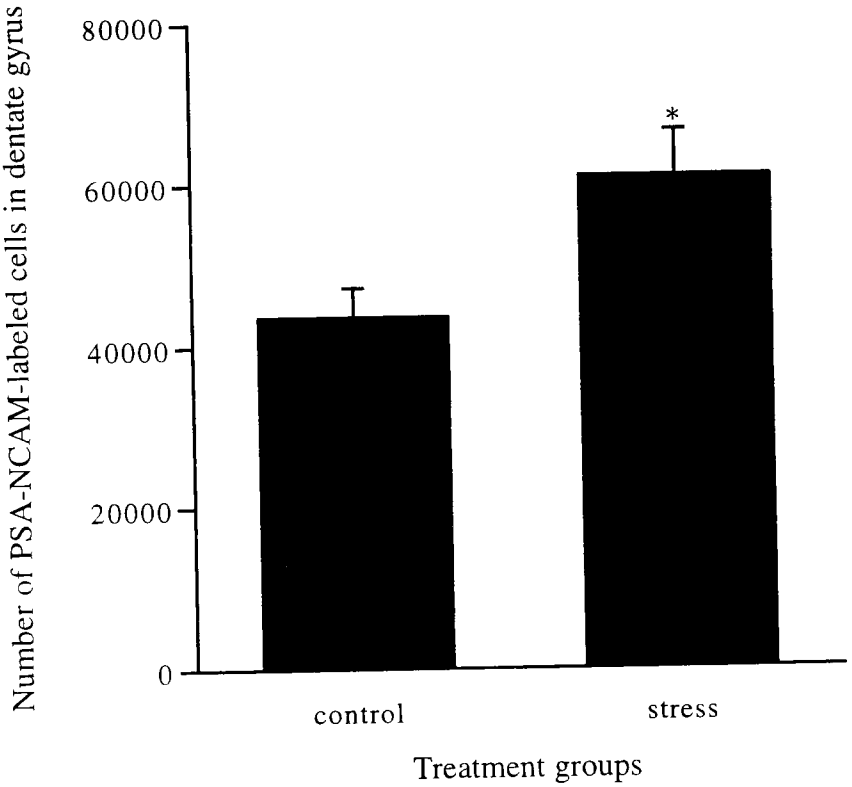

Number of PSA-NCAM-labeled cells in dentate gyrus
80000
60000
40000
20000
0
*
control
stress
Treatment groups

Figure 12: Unlike 21 days of daily restraint stress, a single session of acute restraint stress did not alter the expression of PSA-NCAM expression in the dentate gyrus. Rats restrained for two hours had very similar numbers of PSA-NCAM-labeled cells as the control rats, and rats restrained for six hours displayed a trend toward an increase in PSA-NCAM expression, but it was not significantly different from the control group.

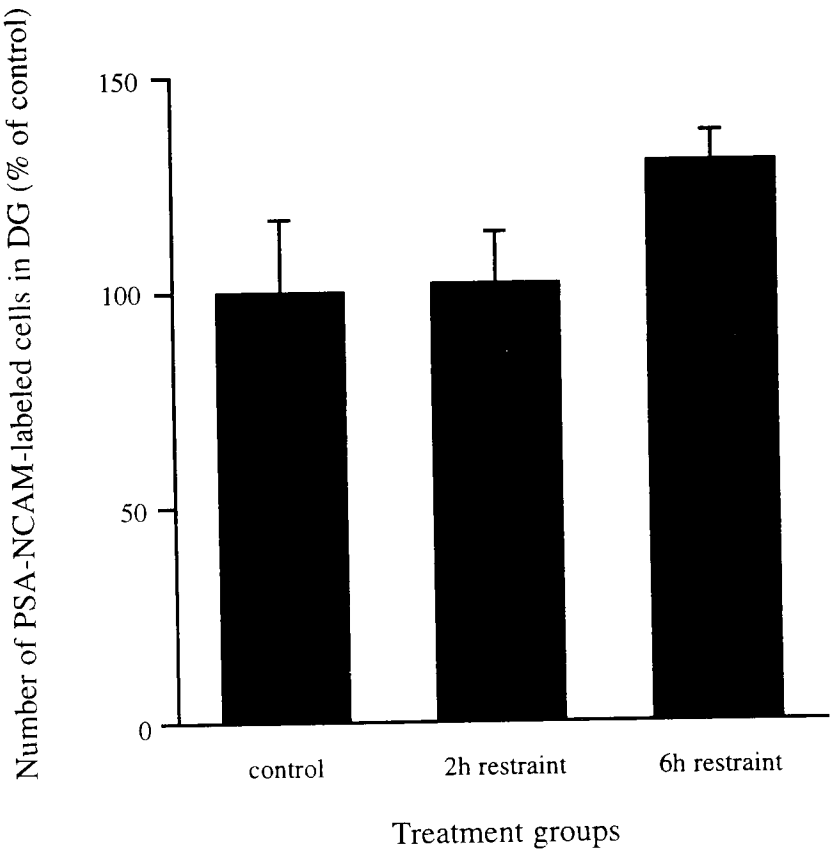

Number of PSA-NCAM-labeled cells in DG (% of control)
150
100
50
0
control
2h restraint
6h restraint
Treatment groups

Figure 13: A repeat study showing that three weeks of restraint stress increased PSA-NCAM expression in the dentate gyrus by 51%. The control group had 37,536 $\pm$ 3098 PSA-NCAM-labeled cells whereas the stressed group had 56,817 $\pm$ 5424 PSA-NCAM-labeled cells. Bar values are mean + S.E.M. Asterisk indicates a significant difference.

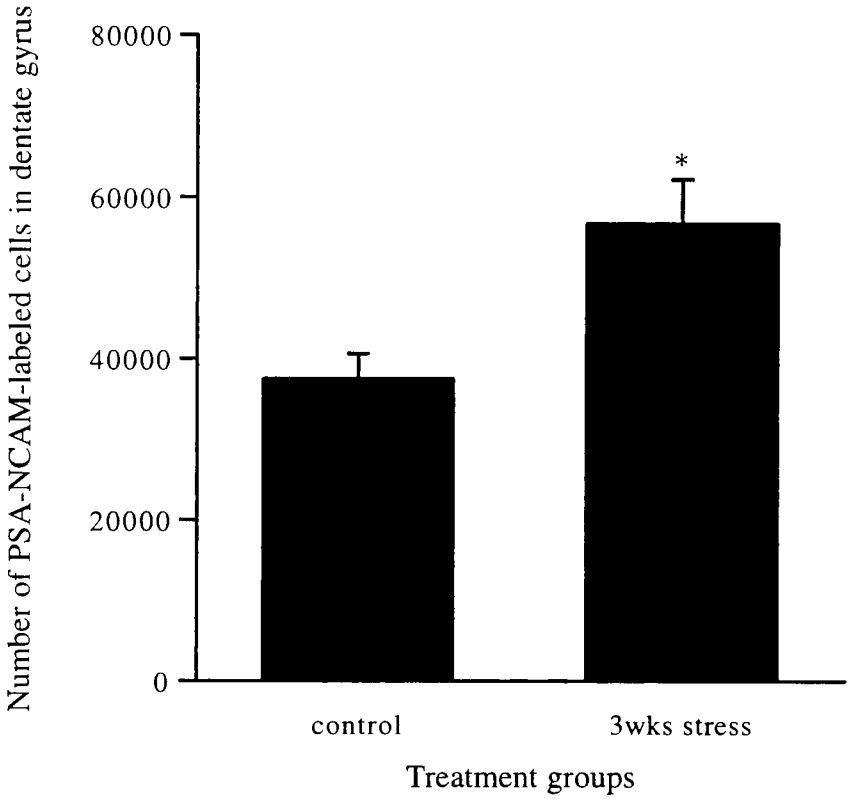
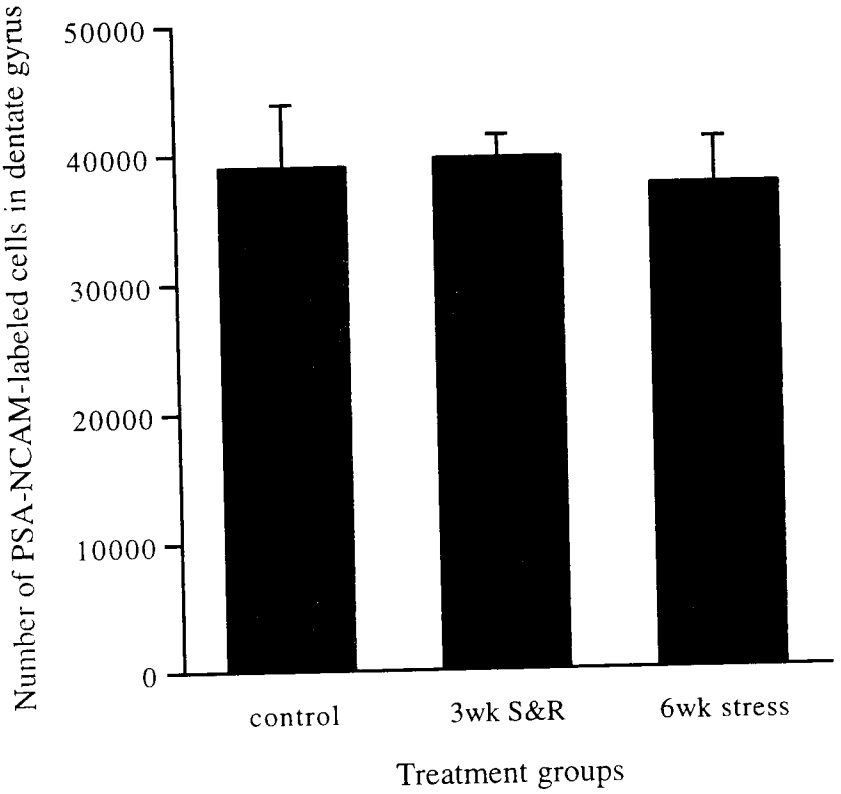

Number of PSA-NCAM-labeled cells in dentate gyrus
80000
60000
40000
20000
0
*
control
3wks stress
Treatment groups

Figure 14: Recovery from chronic restraint stress and 42 days of daily stress produced similar PSA-NCAM expression as the control group. Control rats had had 39,024 $\pm$ 4859 PSA-NCAM-labeled cells, the three weeks stress and three weeks recovery rats had 39,640 $\pm$ 1749 PSA-NCAM-labeled cells, and the six weeks stress rats had 37,452 $\pm$ 3521 PSA-NCAM-labeled cells. There were no significant differences among any groups ($p > 0.63$). Bar values are mean + S.E.M.


Number of PSA-NCAM-labeled cells in dentate gyrus
50000
40000
30000
20000
10000
0
control
3wk S&R
6wk stress
Treatment groups

Discussion

The results of this study show that daily restraint stress for 21 days caused a significant increase in PSA-NCAM expression in the dentate gyrus whereas extension of this stressor for an additional 21 days reversed the elevation. Acute restraint stress did not alter PSA-NCAM expression. The findings we observed in PSA-NCAM expression, acutely and at three weeks, are consistent with the reported morphological reorganization after chronic stress that takes place throughout the interconnected dentate gyrus–CA3 pyramidal neuron system. There is evidence that PSA-NCAM is regulated by adrenal steroids and EAAs and is important for structural plasticity in the hippocampus and hypothalamus.

A role for PSA-NCAM in structural plasticity

Whereas neurogenesis was decreased after three weeks of chronic restraint stress, PSA-NCAM expression was increased. This may be explained, at least in part, by effects of adrenal steroids and EAA activity on the polysialylation of NCAM, resulting in a form of NCAM that allows for greater movement and plasticity of cells. The principal site of hippocampal PSA-NCAM expression is in a subpopulation of neurons located in the innermost region of the dentate granular layer (Seki and Arai, 1991). Many of these neurons have been recently generated and express PSA-NCAM transiently as they differentiate and migrate to their final location in the granule cell layer (Seki and Arai, 1993b).

Chronic stress (21 days) reduced cell proliferation in the adult dentate gyrus but did not alter the total number or survival of previously generated granule neurons. Therefore, the increase in the number of PSA-NCAM labeled granule neurons after chronic restraint stress is likely due to an increase in the expression of NCAM and/or to an increase in the amount of PSA attached to this molecule in preexisting granule

neurons. There is also PSA-NCAM on the mossy fiber terminals in the stratum lucidum of the CA3 region, and increases in PSA attachment to NCAM may have consequences for the reported structural plasticity of the dentate gyrus and CA3 region. PSA-NCAM appears to be an important player in morphological plasticity in the nervous system, participating in cell migration, neurite outgrowth and axonal fasciculation (Rutishauser and Landmesser, 1996; Yoshida et al., 1999). For example, removal of PSA from NCAM in the hypothalamic magnocellular nuclei prevented the withdrawal of astrocytic processes and the increase in synaptic contacts normally induced by lactation and dehydration (Theodosis et al., 1999).

Thus, the PSA-NCAM on granule neurons and mossy fiber boutons in the adult hippocampus could participate in the processes of bouton formation and remodeling that accompany synaptic formation between granule neurons and CA3 pyramidal neurons (Seki and Arai, 1999). The increased expression of PSA-NCAM that we have found in the granule neurons after chronic stress could reflect some morphological changes in mossy fiber boutons induced by apical dendritic atrophy of CA3 pyramidal cells. Previous studies in our laboratory have shown that chronic stress altered the ultrastructure of mossy fibers (Magarinos et al., 1997) and induced atrophy of apical dendrites of CA3 neurons (Watanabe et al., 1992b). Chronic stress could affect immature mossy fibers (those from recently generated cells) or also could promote the sprouting of preexisting mossy fibers as has been demonstrated after epilepsy by Jack Parent et al. (1999) in irradiated brains.

Complex regulation of PSA-NCAM by glucocorticoids and EAAs

As to the mechanism for increased PSA attachment to NCAM in chronic stress, PSA is attached to NCAM by either PST or STX, and glucocorticoids may play a role in facilitating this process. Dexamethasone increases total sialyltransferase enzymatic

activity in serum and also an increase specifically in the activity of alpha 2,3 sialyltransferase in a neural cell line (Coughlan and Breen, 1998; Maguire et al., 1998). Moreover, ADX results in a decrease in neural sialyltransferase activity (Coughlan et al., 1996). Since NCAM expression does not change with chronic corticosterone treatment (Sandi and Loscertales, 1999), the increase in PSA-NCAM we observed in the granule cells after chronic stress may be due only to an elevation of the attachment of PSA to this molecule and not to an increase in NCAM. However, regulation of PSA-NCAM expression in the hippocampus by glucocorticoids has been demonstrated to be quite complex, since ADX, which increases the production of granule neurons (Cameron and Gould, 1994), can also enhance PSA-NCAM (Rodriguez et al., 1998). Such treatment did not alter NCAM expression, indicating that the observed changes were attributable to greater polysialylation of NCAM molecules, results seemingly at odds with Coughlan et al. Furthermore, the generation of new granule neurons and PSA-NCAM expression can be dissociable; very few BrdU-positive cells coexpressed PSA-NCAM after ADX. Thus, enhancement of PSA-NCAM immunoreactivity likely occurs in pre-existing granule cells. Our results show that a reduction in cell proliferation was accompanied by an enhancement in PSA-NCAM expression, suggesting that upregulation of PSA-NCAM took place in previously generated granule cells. Rodriguez and colleagues also demonstrated that cell proliferation and PSA-NCAM expression are not identically influenced by glucocorticoids. After ADX, neurogenesis was normalized by diurnal or nocturnal corticosterone replacement, whereas normalization of PSA-NCAM expression occurred only after simulation of the complete circadian cycle.

Another possible mediator of stress-induced PSA-NCAM expression are EAAs. Administration of MK-801, an NMDA receptor antagonist, in the developing striatum abolishes PSA-NCAM immunoreactivity and leads to a loss of synapses (Butler et al., 1998), suggesting that EAAs could regulate PSA-NCAM expression via NMDA

receptors. It is interesting to note that the apical dendritic atrophy of CA3 neurons also involves the participation of EAAs, which are elevated after chronic stress, and this effect appears to be mediated by the NMDA type of glutamate receptors (Magarinos and McEwen, 1995b).

Correlation of PSA-NCAM enhancement with reversible plasticity

The neuroanatomical remodeling occurring after three weeks of restraint stress has been shown to be reversible within ten days of termination of stress. Our results indicated that PSA-NCAM expression in the dentate gyrus also reverted to control levels within three weeks of recovery from stress. Acute restraint stress, which does not induce dendritic atrophy on CA3 pyramidal neurons, also did not produce changes in PSA-NCAM expression, suggesting that elevation of PSA-NCAM expression is positively correlated with periods of structural remodeling in the hippocampus.

While three weeks of restraint stress induces morphological alterations that are reversible, Robert Sapolsky has demonstrated that severe, prolonged stress can lead to permanent changes in the hippocampus of rodents and primates, such as neuronal degeneration and depletion of glucocorticoid receptors (Sapolsky et al., 1985; Uno et al., 1989). Our examination of PSA-NCAM expression in response to restraint stress revealed a significant enhancement after three weeks but no change compared to control after six weeks. It is possible that six weeks of stress is a period long enough to induce permanent hippocampal damage, and it is widely believed that downregulation of PSA accompanies a change in NCAM function from a plasticity-promoting to a stability-promoting molecule. Morphological characterization of the hippocampus after six weeks of stress has not been demonstrated, although we did show a significant reduction of neurogenesis in the dentate gyrus after this treatment. It would be interesting to repeat the

study, allowing the rats to recover after six weeks of daily stress, to determine whether these hippocampal changes are reversible or permanent.

In summary, PSA-NCAM expression was significantly elevated in the dentate gyrus of rats restrained for three weeks but remained unchanged in acutely restrained rats and in those restrained for six weeks. The increase in PSA-NCAM expression occurred during a period when morphological remodeling of the dentate gyrus-CA3 regions is known to take place, and it is likely that PSA-NCAM is involved in these plasticity events. After six weeks, restraint stress no longer induced differences in PSA-NCAM expression between control and experimental groups, suggesting that this prolonged period of stress may have shifted the hippocampal response from plasticity mediated events to more permanently damaging events. It would be interesting to determine how chronic restraint stress could affect other markers of structural plasticity, such as the collapsin response-mediated protein 4 (rCRMP-4), a protein involved in axonal growth (Nacher et al., 2000), or doublecortin (DCX), a protein required for normal neuronal migration (Nacher et al., 2001a).

CHAPTER 5

EFFECTS OF RESTRAINT STRESS ON NEURONAL EXCITABILITY IN THE HIPPOCAMPUS

Introduction

It has long been observed that behavioral performance in learning tasks could be modulated by arousal state or stress and that the correlation between the two is nonlinear (Stennett, 1957). One approach toward understanding the role of hormones in learning and memory is to study neuroendocrine modulation of a physiological model of memory. Common models of memory formation are long term potentiation (LTP), a long lasting enhancement of synaptic efficacy induced by high frequency stimulation (HFS) of afferent fibers, and primed burst potentiation (PBP), a low threshold form of conventional LTP. One decade ago, David Diamond and colleagues (1992) reported an inverted-U relationship between corticosterone levels and the magnitude of hippocampal PBP; that is, moderate doses of serum corticosterone (10-20 μg/dl) produced the maximal level of potentiation while lower and higher doses were not as efficacious, and extremely high doses of corticosterone (greater than 60 μg/dl) resulted in long term depression (LTD). Since then, a series of studies conducted by Constantine Pavlides revealed a biphasic effect on synaptic plasticity by glucocorticoids (Pavlides and McEwen, 1999; Pavlides et al., 1996; Pavlides et al., 1995). Activation of type I adrenal steroid receptors produced an enhancement of LTP whereas activation of type II receptors suppressed LTP.

Chronic restraint stress activates the HPA axis and induces morphological changes in the CA3 apical dendrites and in the mossy fiber terminals that make contact with the CA3 neurons (Magarinos et al., 1997; Watanabe et al., 1992b). These changes

do not occur with acute restraint stress and are reversible upon the termination of stress. Since the hippocampus contains high levels of glucocorticoid receptors and responds to restraint stress by altering synaptic processes, it is likely that the neurophysiology of the hippocampal system undergoes changes throughout a chronic stress paradigm. In order to examine the neurophysiological properties of the hippocampus during stress, we implanted chronic electrodes in various regions of the hippocampus and recorded evoked field potentials daily from rats throughout a chronic stress study while the animals were awake and freely behaving.

The primary projection into the hippocampus from the entorhinal cortex is the perforant path to the dentate gyrus. In the classical "trisynaptic" circuit of the hippocampus, the perforant path input to the dentate gyrus is the first synaptic connection, followed by the mossy fiber projection from the dentate gyrus to the CA3 pyramidal neurons, and subsequently, CA3 neurons project to CA1 neurons via Schaffer collaterals. This simplified description of hippocampal circuitry suggests that CA3 neurons receive afferent input only from granule neurons, but in fact, the connections in the CA3 cell layer are more complex than the "trisynaptic" model proposes. CA3 pyramidal neurons have extensive dendritic arborizations and receive several sources of afferent projections. Mossy fibers from the dentate gyrus granule neurons synapse upon the most proximal apical dendrites in the stratum lucidum while perforant path fibers from the entorhinal cortex synapse upon the most distal apical dendrites in the stratum lacunosum-moleculare (Berzhanskaya et al., 1998; Steward, 1976; Yeckel and Berger, 1990). Additionally, CA3 neurons have recurrent collaterals, composed of the commissural/associational fibers from both ipsilateral and contralateral CA3 neurons, which synapse upon themselves throughout the basal dendrites and the mid-apical dendrites in the stratum oriens and stratum radiatum (Li et al., 1994). The dendrites that undergo atrophy due to restraint stress are the distal apical dendrites. Since this region

receives direct, monosynaptic input from the entorhinal cortex, we examined this connection by implanting an extracellular stimulating electrode in the medial perforant path and an extracellular recording electrode in the CA3 cell layer. We also investigated the entorhinal cortex projection to the dentate gyrus by implanting rats with an extracellular stimulating electrode in the medial perforant path and an extracellular recording electrode in the dentate gyrus, and additionally, we investigated the mossy fiber projection to CA3 (Figure 15).

It was recently demonstrated that immediately after chronic restraint stress, there is suppression of LTP in the medial perforant input to the dentate gyrus and the commissural/associational input to CA3, but not in the mossy fiber input to CA3 (Pavlides et al., 2002). Since cessation of stress for at least ten days can reverse the effects of dendritic atrophy (Conrad et al., 1999), we wished to determine whether LTP impairment could be rescued after termination of stress. Therefore, at the end of three weeks of daily restraint, rats were allowed two weeks to recover. We then applied HFS to study LTP induction in the rats.

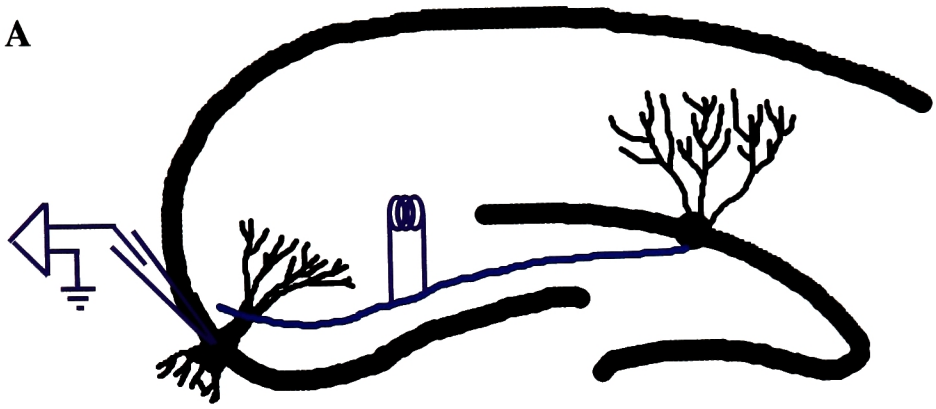
Experimental design

Adult rats were implanted with chronic electrodes in the following pathways: stimulate the medial perforant path while recording the dentate gyrus (n=8), stimulate the medial perforant path while recording CA3 (n=8), and stimulate the mossy fibers while recording CA3 (n=6; Figure 15). After sufficient recovery from surgery and habituation to the recording chamber, baseline recordings were obtained from all rats for three days to ensure stability of electrode placement. Stressed rats were recorded from each day in the morning before 10:00 a.m. and in the afternoon after 4:00 p.m.; they were restrained for 21 consecutive days between 10:00 a.m. and 4:00 p.m. Control rats were recorded from each day in the morning before 12:00 p.m. while circadian levels of glucocorticoids

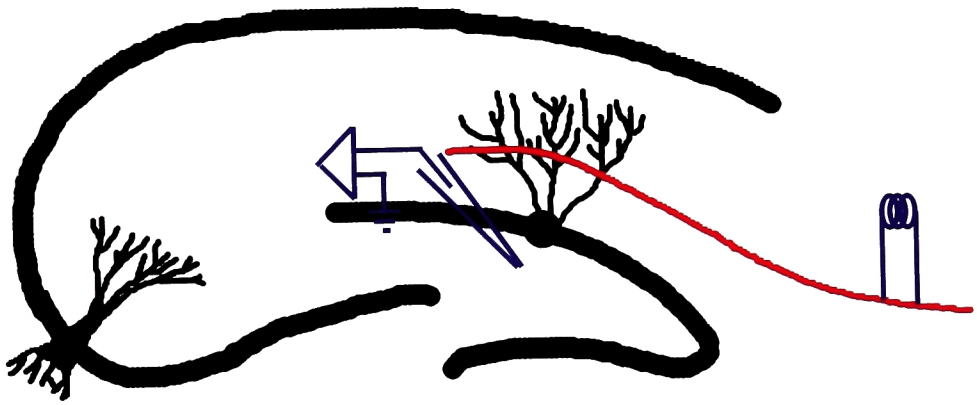
were low. At the end of the experiment, all rats were subjected to HFS and then perfused for histological confirmation of electrode positioning.

Figure 15: Diagrams of the hippocampus showing electrode positionings in the mossy fiber to CA3 pathway (*A*, blue line), perforant path to dentate gyrus (*B*, red line), and perforant path directly to CA3 (*C*, green line). Ammon's horn and the dentate gyrus are shown in black line form, with representative neurons drawn in the dentate gyrus and CA3 fields. Notice that the apical dendrites of CA3 neurons receive mossy fiber input proximal to the cell bodies while the more distally located apical dendrites receive direct connections from the perforant path.

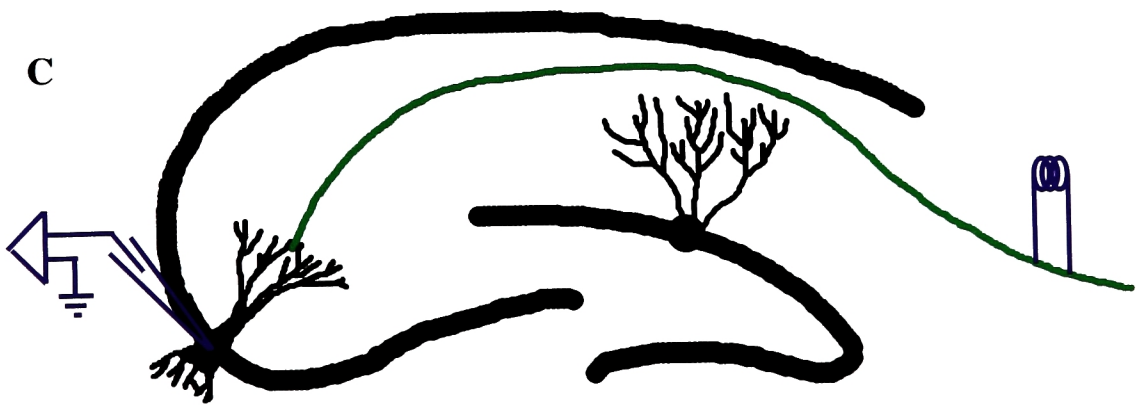
A



B



C



Results

Throughout the course of *in vivo* recordings, it became apparent that the mossy fiber electrodes were unstable in their positioning, as the field potential recordings were inconsistent and small. At a thickness of only 100 μm, the mossy fiber bundle is difficult to impale chronically, as even slight (less than 0.01 mm) shifts in the dorsal-ventral axis will cause a lost response. One alternative to this challenge would be to use electrodes controlled by a microdrive, such that the positioning of the electrode within the mossy fiber bundle could be tuned each day for recordings. Unfortunately, the data obtained from the mossy fibers in this study were unreliable and could not be further analyzed. Histological examination of the remaining electrodes confirmed the proper positioning in the medial perforant path, dentate gyrus and CA3 cell layer (Figure 16) with the exception of one CA3 electrode that was placed too medially and ended up in the dentate gyrus. This animal was removed from the perforant path to CA3 group and placed in the perforant path to dentate gyrus group.

Evoked field potentials were recorded from all rats each day using a range of stimulus intensities (Figure 17), and the responses were analyzed by measuring the slope of the field excitatory postsynaptic potential (EPSP) and the height of the population spike amplitude (Figure 18A). The data obtained were plotted against current intensity to generate input-output curves (Figure 18B-C). The current intensity that produced the half-maximal response was selected for subsequent analyses; data recorded during the 21 days of experimentation were expressed as percentages of baseline value for each rat, and the results for all rats within one group were pooled for statistical analyses.

No differences between the control and stress groups were observed in the medial perforant path to CA3 field (Figure 19). The EPSP slope remained stable, hovering near 95% of baseline, throughout the three weeks for both groups. The height of the population spike amplitude, however, fluctuated up and down during the course of the

study and ranged from 65-100% of baseline value. The trend was parallel for the two groups, such that control and stressed rats exhibited somewhat synchronous oscillations in population spike height. This effect may be due to global variations on behavioral state. While every effort was made to prevent rats from falling asleep during electrophysiological recordings, the awake state can range from active exploration to passive alertness. It is known that the vigilance state of an animal can profoundly alter neural transmission in the hippocampus (Winson and Abzug, 1977; Winson and Abzug, 1978). Global effects on behavioral state could be attributable to environmental disturbances, such as noisy construction near the laboratory or a change of cage during that day.

In the medial perforant path stimulations of the dentate gyrus, there was significant elevation ($p < 0.05$) in the EPSP slope of the restrained rats compared to control during the period roughly corresponding with the second week of the study (days 6-12; Figure 20A). Control rats had slope measurements ranging from 76-103% of baseline whereas stressed rats displayed 103-142% of baseline slope values over the same time period. There were no differences between the two groups in the first five days and after day 12, and in fact, during the last six days of the experiment, the slope values for both groups were close to 100% of baseline. Figure 21 illustrates the differences in the recording traces acquired during either day 7 (Figure 21A) or day 21 (Figure 21B) compared to the same baseline trace, and figure 22 shows input-output curves generated on day 7, revealing an upward shift for the EPSP slope (Figure 22A) but not for the height of the population spike amplitude (Figure 22B). The population spike height measurements for medial perforant path to dentate gyrus were similar to those obtained for medial perforant path to CA3; values in the dentate gyrus ranged from 70-109% of baseline but did not differ between the control and stressed groups (Figure 20B).

At the end of the three weeks of stress, rats were maintained for two additional weeks without stress and were then subjected to HFS to determine the effect on LTP after recovery from chronic stress. LTP was induced in both the perforant path to dentate gyrus and perforant path to CA3 fields, and the degree of potentiation was comparable for the control and stressed groups (Figure 23). Thus, while chronic stress can induce dendritic atrophy (Watanabe et al., 1992b) and suppress dentate gyrus LTP (Pavlides et al., 2002), termination of stress for two weeks, a period sufficient for reversal of dendritic atrophy, resulted in similar potentiation in the dentate gyrus and CA3 of both groups. HFS applied to the mossy fibers resulted in electrical discharges indicative of seizure activity in five out of six rats; this is a common observation with mossy fiber to CA3 LTP induction in awake animals, and perhaps the degree of HFS in the mossy fibers should be reduced when working with non-anesthetized rats.

Figure 16: Representative photomicrographs showing electrode positioning in the CA3 cell layer (*A*), mid-range between the dorsal and ventral blades of the dentate gyrus (*B*), and the medial perforant path (*C*). Electrode placement was verified in all rats.

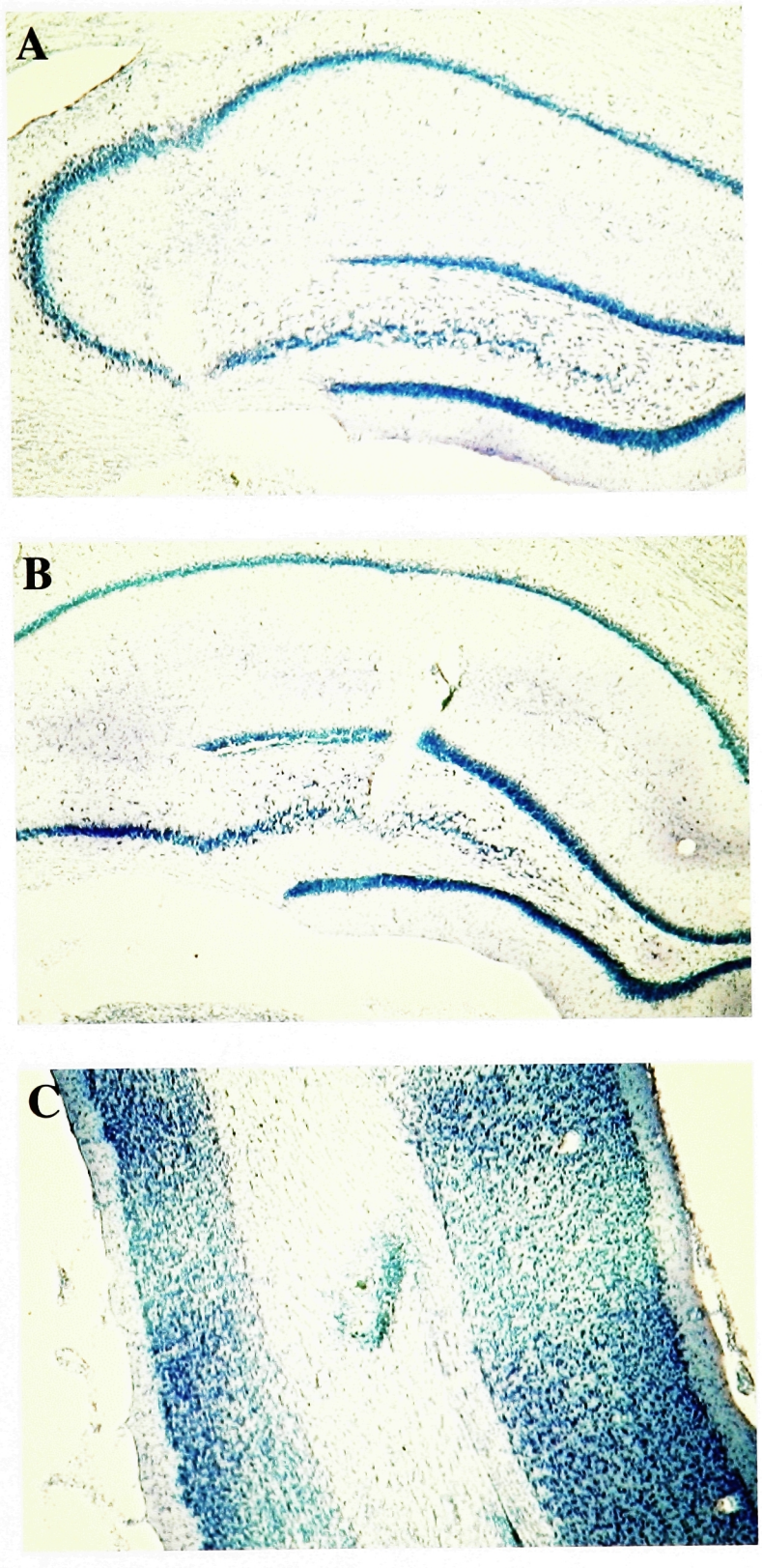

A
B
C

Figure 17: Stimulus-response relationship of dentate gyrus field potentials evoked by medial perforant path stimulation. Single pulse stimulation was applied at current levels shown on the left of each trace.

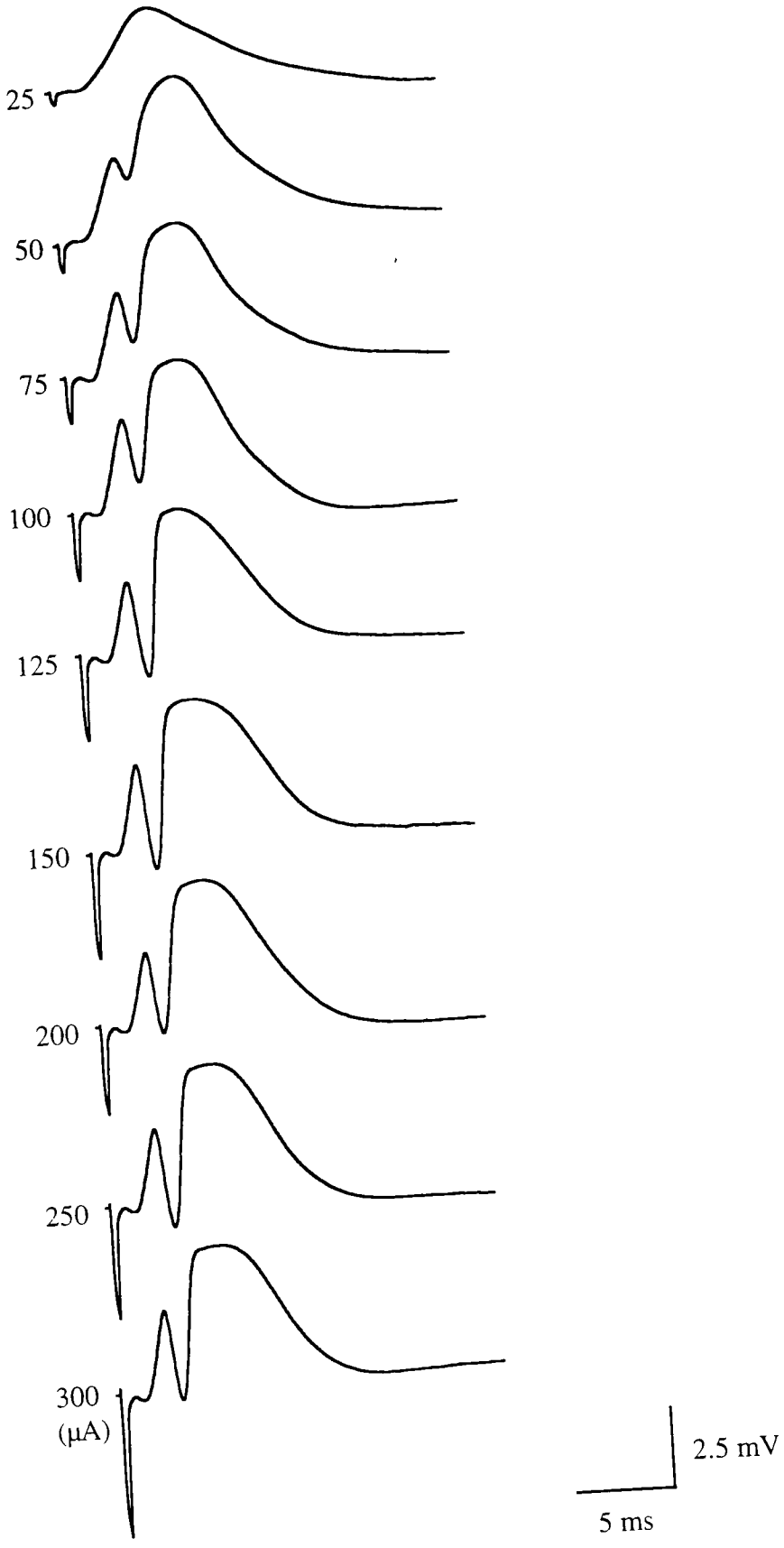
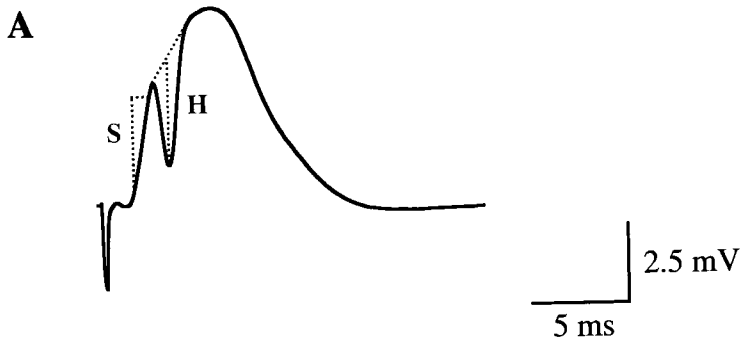
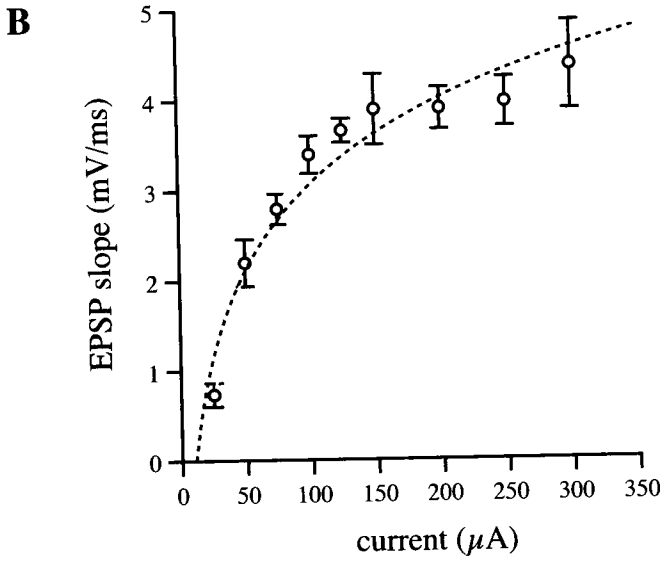

25
50
75
100
125
150
200
250
300
(μA)
2.5 mV
5 ms

Figure 18: *A*, Field potentials were analyzed by measurement of the EPSP slope (S) and the height (H), or amplitude, of the population spike. Input-output curves were generated to plot EPSP slope (*B*) or population spike height (*C*) versus increasing stimulus intensities.


A
S
H
2.5 mV
5 ms


B
EPSP slope (mV/ms)
0
1
2
3
4
5
0
50
100
150
200
250
300
350
current (μA)

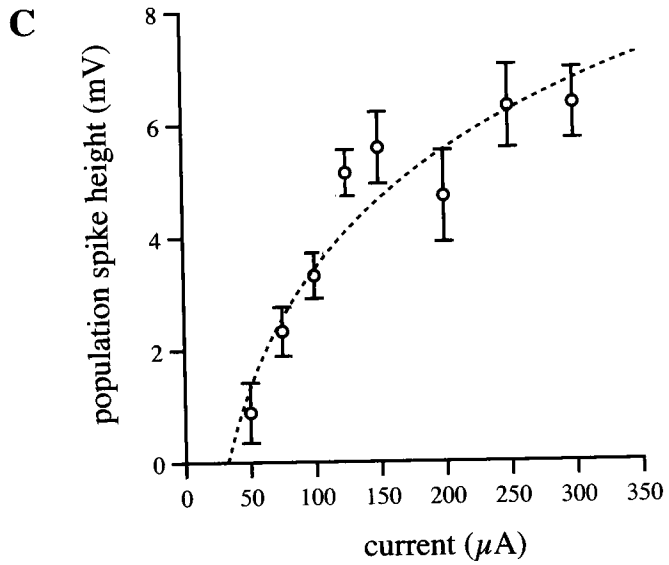
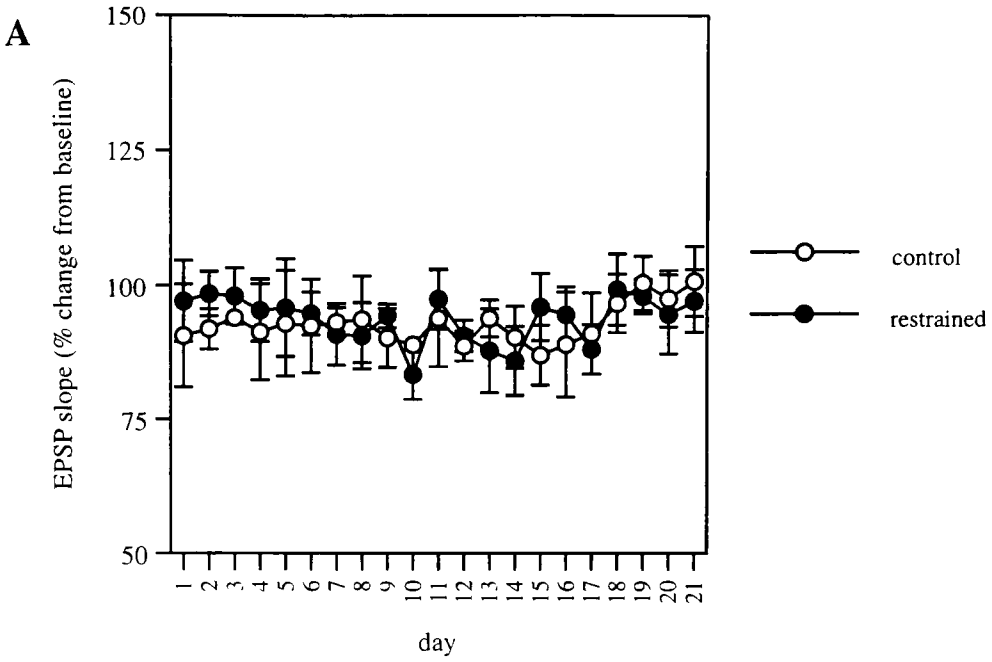

C
population spike height (mV)
0
2
4
6
8
0
50
100
150
200
250
300
350
current (μA)

Figure 19: *A*, Measurements of EPSP slope in the CA3 field evoked by perforant path stimulation throughout the 21 day study revealed that the control and stressed groups did not differ from each other statistically, and the recordings for both groups ranged from 90-100% of baseline value, suggesting that the electrode placements remained stable. *B*, The population spike height varied considerably from day to day, ranging from 65-100% of baseline value. The control and stressed groups fluctuated in parallel and were not significantly distinguishable.


A
EPSP slope (% change from baseline)
150
125
100
75
50
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day
control
restrained

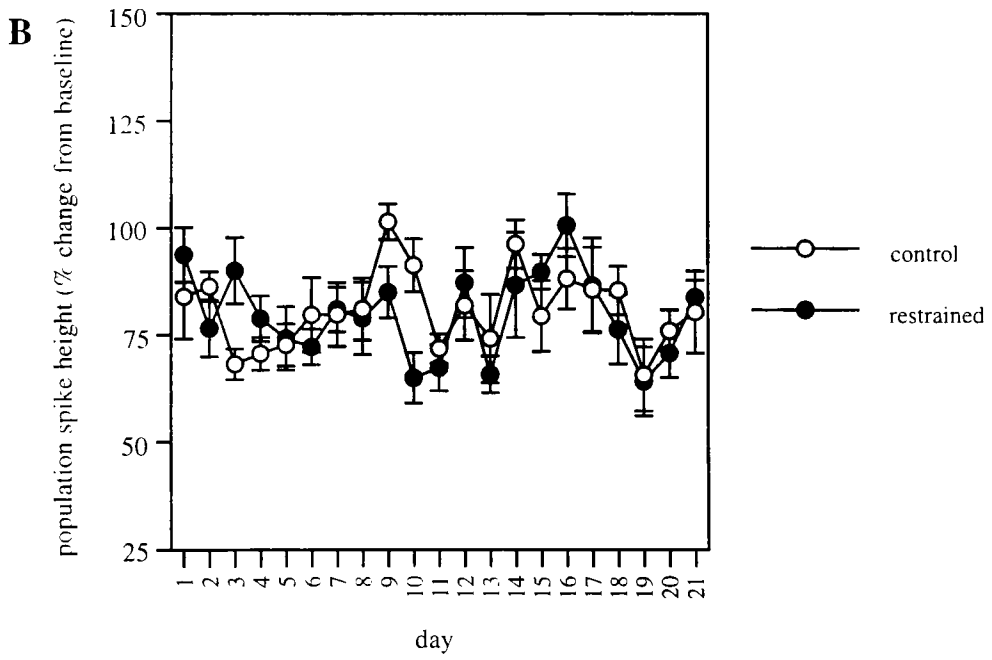
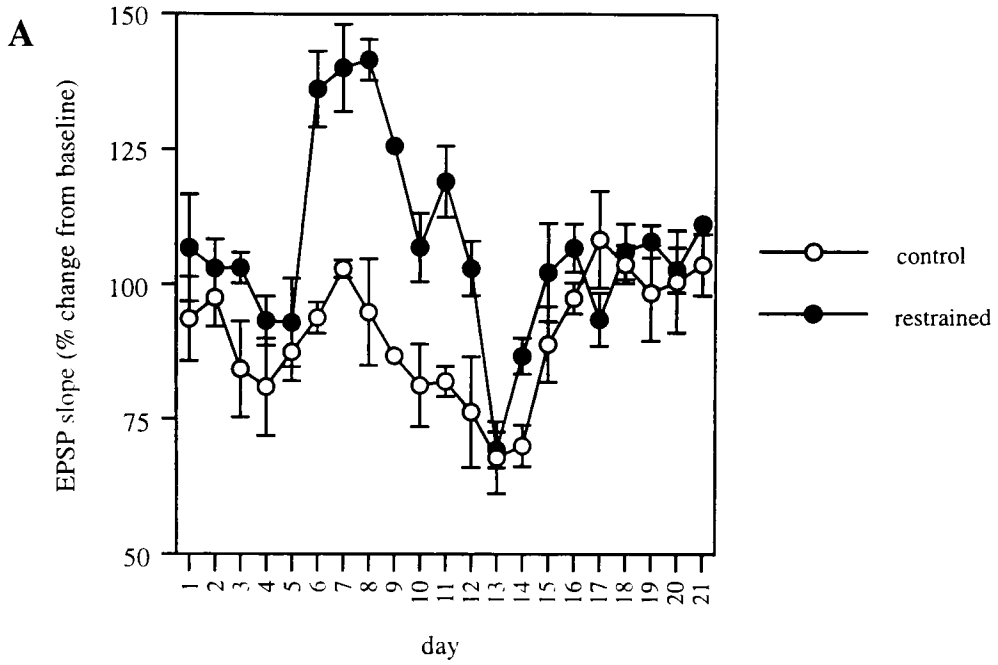

B
population spike height (% change from baseline)
150
125
100
75
50
25
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day
control
restrained

Figure 20: *A*, Field potentials recorded in the dentate gyrus evoked by perforant path stimulation showed significantly increased EPSP slope in stressed rats compared to controls during the second week of stress, from days 6 to 12; control rats had slope measurements ranging from 76-103% of baseline whereas stressed rats displayed 103-142% of baseline slope values over the same time period. In the first five days of the experiment, both groups exhibited a trend toward declining slope measurements, but by the third week, the slope returned to roughly 100% of baseline for both groups. *B*, Values for the population spike height in the dentate gyrus ranged from 70-109% of baseline but did not differ between the control and stressed groups.


A
150
125
100
75
50
EPSP slope (% change from baseline)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day
control
restrained

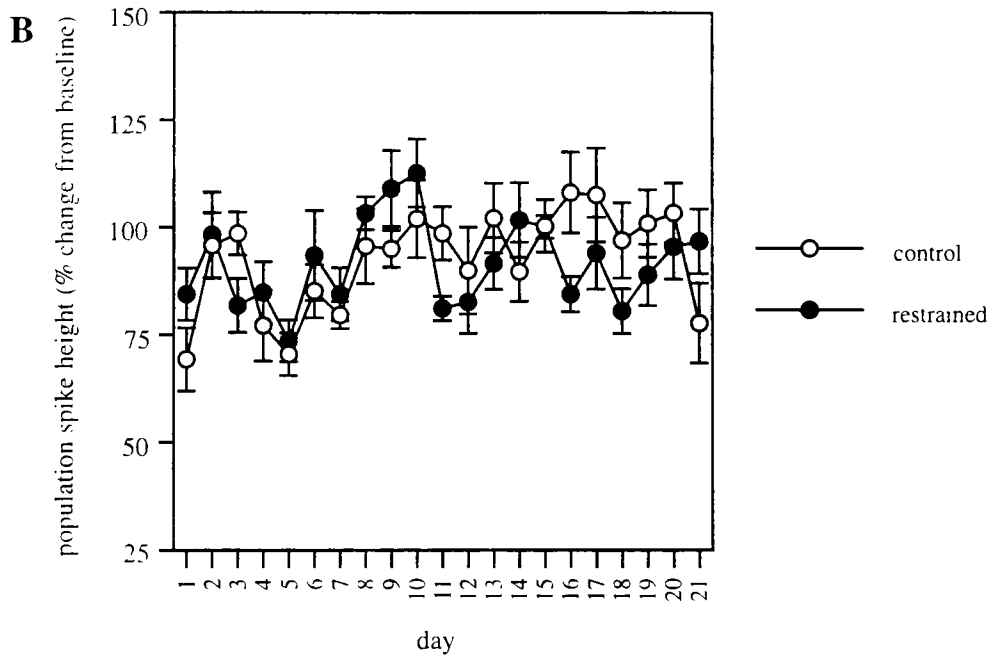

B
150
125
100
75
50
25
population spike height (% change from baseline)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
day
control
restrained

Figure 21: *A*, Traces of perforant path-stimulated dentate gyrus responses from recordings obtained during baseline and day 7 of a stressed rat. The traces are overlaid, demonstrating that the slope from day 7 was steeper than the baseline slope. The population spike amplitude, however, remained fairly consistent despite the elevated slope. *B*, Recording from the same pathway in the same rat on day 21 revealed that the slope had reversed to baseline value. The baseline traces in *A* and *B* are the same.

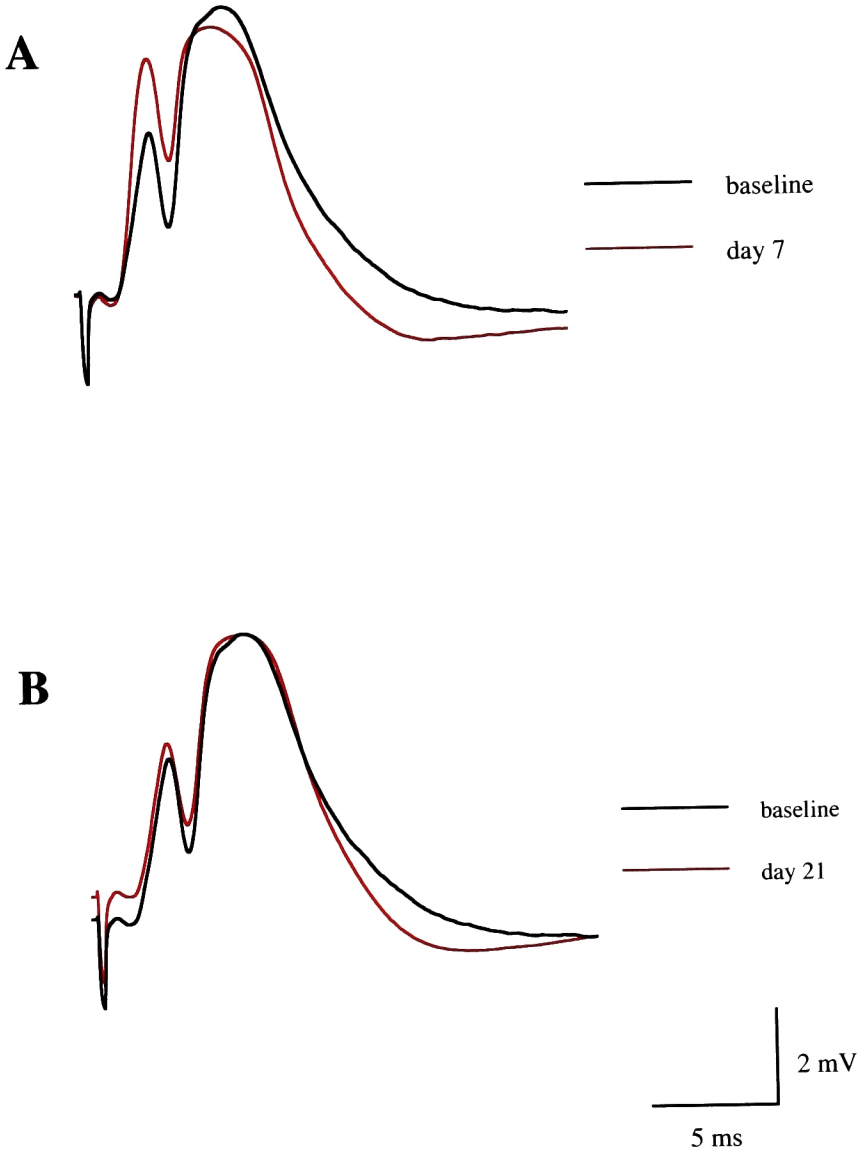

A
baseline
day 7
B
baseline
day 21
2 mV
5 ms

Figure 22: Input-output curves generated from day 7 recordings (red line) plotted with baseline recordings (black line) showing EPSP slope (*A*) or population spike height (*B*) versus stimulus intensity. The day 7 curve shifted upward relative to the baseline curve in the slope measurements, indicating that the same current input could produce a higher slope output on day 7 compared to baseline. The population spike amplitude remained unaltered on day 7 relative to baseline.

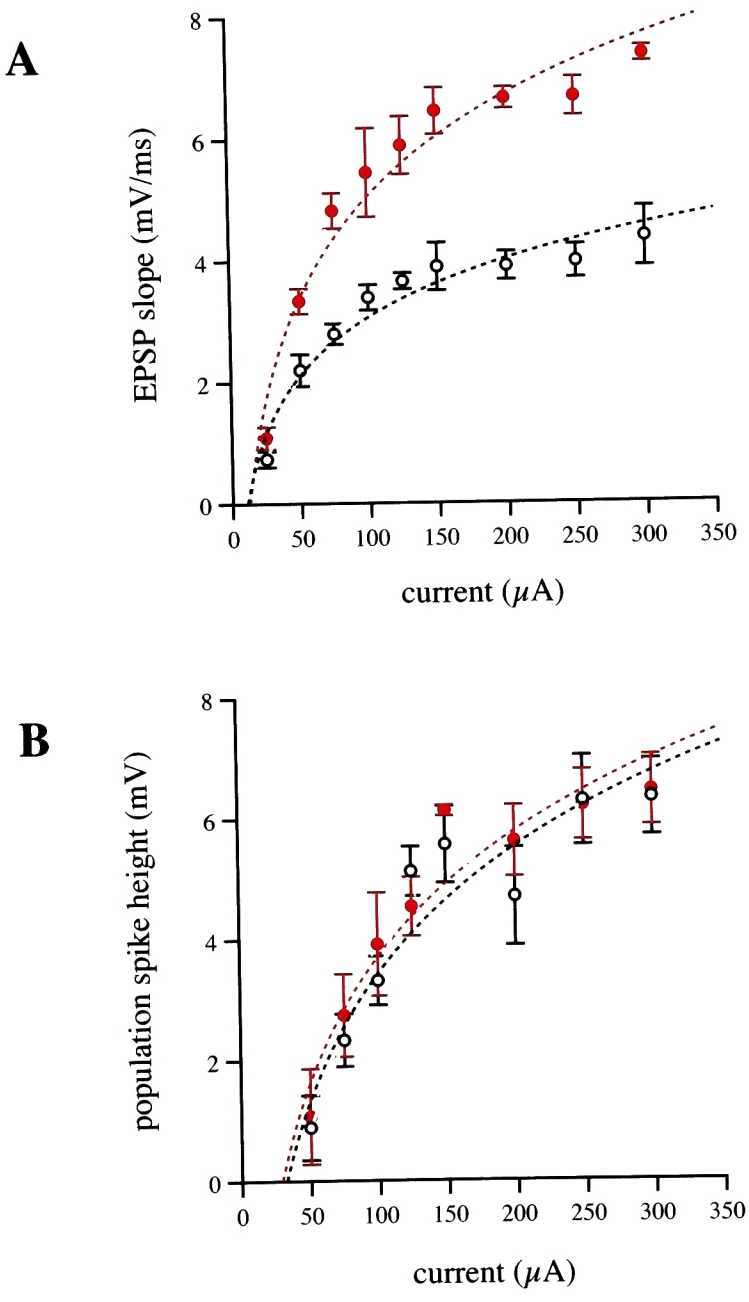
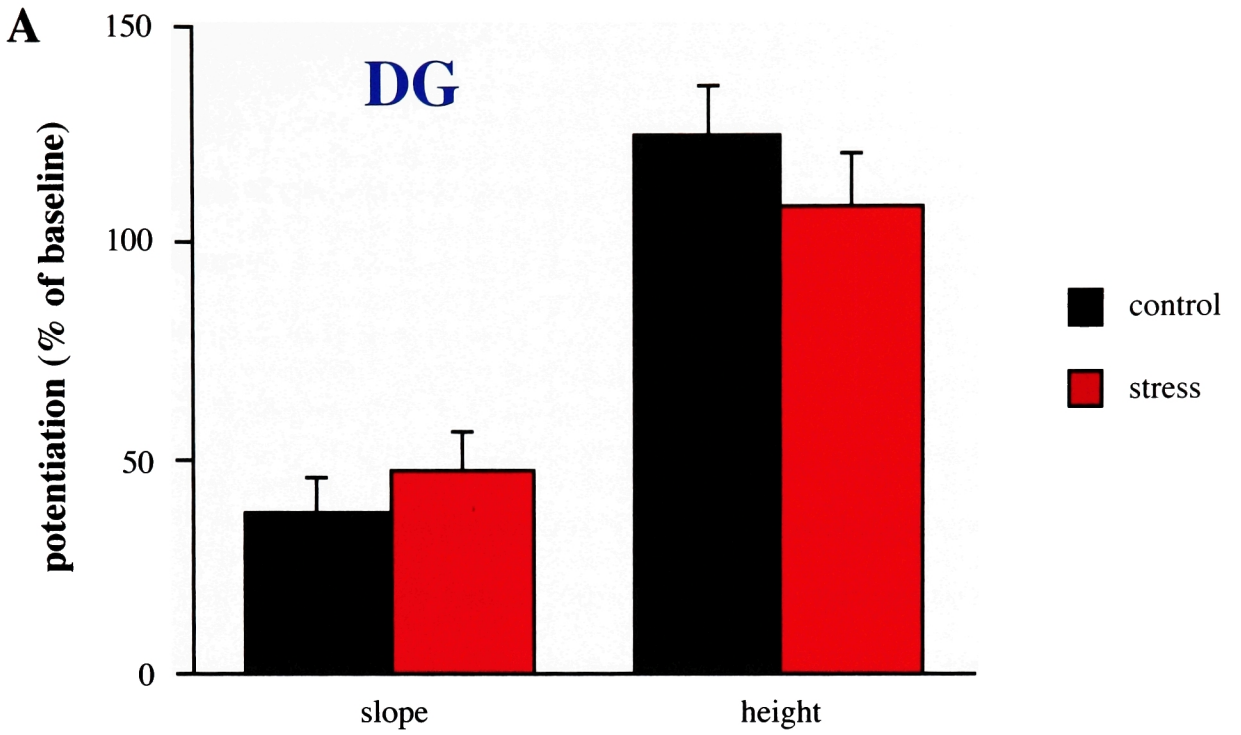
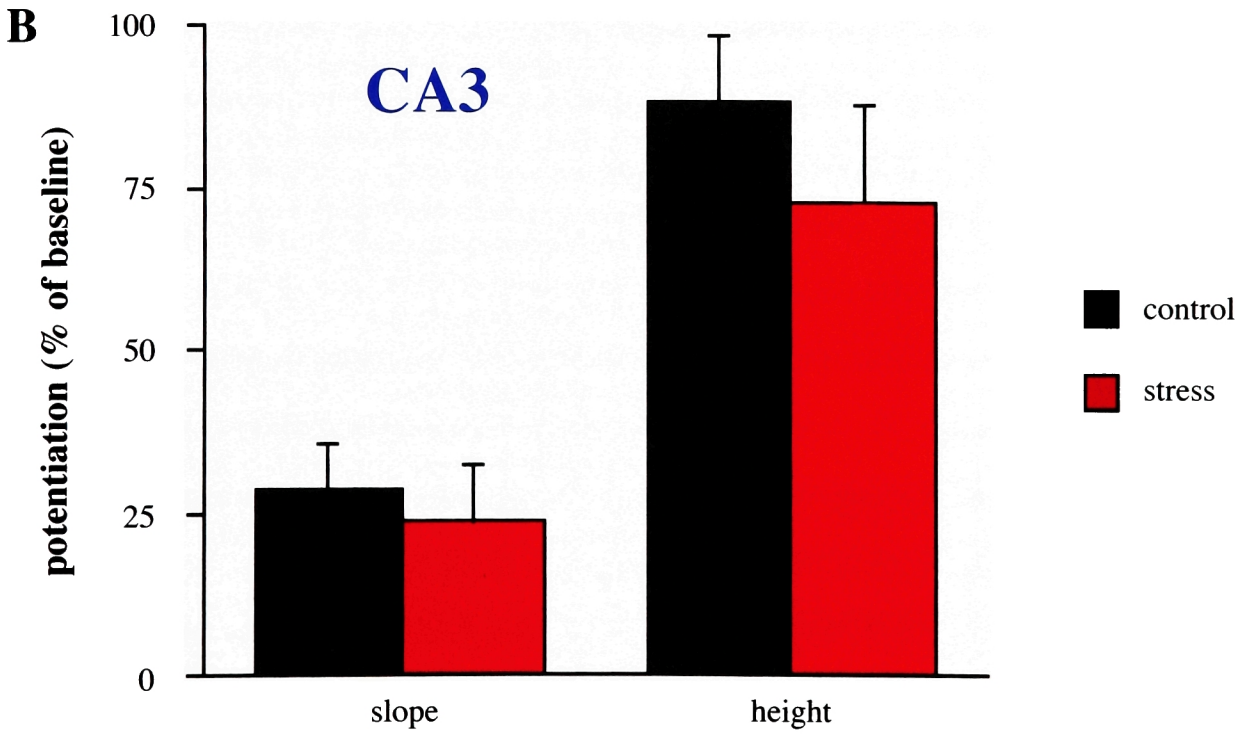

A
EPSP slope (mV/ms)
0
2
4
6
8
0
50
100
150
200
250
300
350
current (μA)
B
population spike height (mV)
0
2
4
6
8
0
50
100
150
200
250
300
350
current (μA)

Figure 23: After two weeks recovery from chronic stress, LTP in both the dentate gyrus (*A*) and the CA3 field (*B*) were similar for control and stressed rats. Magnitude of potentiation relative to baseline is shown here. There were no distinguishable differences between the two groups.


A
DG
potentiation (% of baseline)
150
100
50
0
slope
height
control
stress


B
CA3
potentiation (% of baseline)
100
75
50
25
0
slope
height
control
stress

Discussion

The results of this study show that there was a one week transient period in which the field EPSP slope in restrained rats was significantly elevated in the dentate gyrus in response to medial perforant path stimulation; this occurred during the second week of a three week stress study. Other properties of the evoked field responses, such as the height of the population spike amplitude in both the dentate gyrus and CA3 fields and the EPSP slope in CA3, did not differ between the experimental and control groups. Furthermore, LTP responses were similar for both groups after animals were given recovery time sufficient to reverse stress-induced atrophy. These observations suggest that adaptational events take place in the hippocampus during chronic stress that may serve to revert the system to a baseline level of operation.

Examining the properties of an evoked field response is a means to determine whether the neurophysiological state of a population of cells changes in response to particular manipulations. The EPSP slope measures the inflow of current through synapses while the population spike amplitude is a reflection on the number of action potentials. Changes in the EPSP slope may accompany parallel changes in population spike height; this theoretically occurs when an elevation in the slope represents more current flow through synapses, leading to enhanced membrane depolarization, more action potentials firing, and a larger population spike height. However, it has been suggested that different mechanisms underlie the two components of the response, such that potentiation in both the slope and the spike is achieved by an increase in synaptically evoked excitation as well as a reduction in tonic synaptic inhibition through GABA or glycine channels (Chavez-Noriega et al., 1990). An increase in the height of the population spike amplitude can be interpreted as an enhancement in neuronal excitability for that particular population of cells.

The distal apical dendrites of CA3 pyramidal neurons undergo reversible atrophy after chronic restraint stress. These dendrites receive direct, monosynaptic input from the perforant path, and yet, our observations indicate that CA3 field potentials evoked by medial perforant path stimulation were not altered throughout a chronic stress paradigm. It is known that the excitatory component of perforant path to CA3 synaptic junctions is mediated by glutamate acting upon both NMDA receptors as well as on AMPA receptors (Berzhanskaya et al., 1998). Pharmacological characterization of CA3 neurons has revealed that stimulation of the perforant path also results in disynaptic inhibition, mediated by $GABA_A$ and $GABA_B$ receptors. Inhibitory input could change neuronal responses and reduce excitation by afferent summation, and this may account for the net lack of change in CA3 response to perforant path stimulation throughout chronic stress.

Maintenance of perforant path physiology during restraint stress

Studies involving either chronic restraint stress or chronic corticosterone administration have shown altered morphology in the mossy fiber terminals and the distal region of the CA3 apical dendrites while anatomical properties of the granule neurons remained unaffected. Here, we show that at the end of three weeks of daily stress, neural transmission in both the CA3 field and the dentate gyrus did not significantly change relative to control rats and baseline recordings. Thus, these findings do not support a loss in functional properties of hippocampal neurons due to chronic stress. Rather, the changes that occur during chronic stress seem to be adaptational. The observed increase in the EPSP slope in the dentate gyrus may be indicative of synaptic plasticity events that take place during the second week before the hippocampal system counteracts to adjust to the chronic stress. The rats used in this study were young adults about four months of age, and it has been reported that aged rats suffer more learning deficits after chronic corticosterone treatment compared to young rats (Bodnoff et al., 1995). This may signify

that adaptational events could serve to counterbalance changes induced by chronic stress in young rats, an ability that may disappear when animals age.

CHAPTER 6

EFFECTS OF CONTEXTUAL FEAR LEARNING ON HIPPOCAMPAL CELL PROLIFERATION

Introduction

Adult rats generate 9,000 new granule cells each day, or 6% of the total size of the granule cell population per month (Cameron and McKay, 2001). The creation of such a large number of new neurons suggests that these granule cells participate in hippocampal function. Recently, the production of new neurons in the adult dentate gyrus has been shown to contribute to hippocampal-dependent learning (Shors et al., 2001). By depleting the population of proliferating cells in the hippocampus, Tracey Shors and colleagues demonstrated the requirement of adult neurogenesis for the acquisition of trace eyeblink conditioning, a learning task that is dependent on hippocampal performance.

We asked what effect contextual fear conditioning, another hippocampal-dependent learning task, would have on dentate gyrus cell proliferation and what is the critical process impacting the change. Unlike eyeblink conditioning, which can require 800 learning trials administered over a period of several days, contextual fear conditioning is a powerful learning task that can be accomplished with just a single training session.

Contextual fear conditioning involves the association between the stimuli constituting an animal's environment or context (the conditioned stimulus; CS) and an aversive experience such as administration of a footshock (the unconditioned stimulus; US). In this paradigm, the role of the hippocampus is thought to be the creation of a rich sensory representation of the context, which is then sent to the amygdala where that

contextual representation is associated with the US and is stored (Anagnostaras et al., 2001).

In studies that have demonstrated an effect of learning and memory on hippocampal neurogenesis, it is not clear which aspect of the task is causal to the change in neurogenesis. For example, the change could be due to the learning of the task, the performance of the task, or some other component, such as the experience of a US. Thus, we asked what specific component of hippocampal-dependent learning and memory formation could be responsible for alterations in neurogenesis. Toward this end, we used the Immediate Shock Deficit paradigm (ISD; Fanselow, 1986). Rats given a footshock immediately after being placed in a new environment do not form a context-shock association. Pre-exposure to that environment, however, rescues this learning deficit. The advantage of this paradigm is that by varying the combinations of context pre-exposure and shock administration, learning and memory can be deconstructed into its components, such as the CS, the US, the expression of an association, and the formation of the association. Using ISD in combination with BrdU immunohistochemistry, we examined how each of the various components causes a change in cell proliferation in the dentate gyrus.

Experimental design

Experiment 1 (Figure 24A): In order to determine the effect of contextual fear learning on cell proliferation, rats (n=6) were pre-exposed to the CS 24 hours prior to receiving the US, an immediate footshock. Control rats (n=6) were pre-exposed to the shocking chamber but were not shocked the following day. BrdU (200 mg/kg) was injected after the five minute behavioral observation period and the animals given a two hour survival time.

Experiment 2 (Figure 25A): In order to ascertain whether pre-exposure to the CS alone has any effect on cell proliferation, rats (n=6 per group) were separated into pre-exposed or non pre-exposed groups. Pre-exposed rats were individually placed into the shocking chamber for five minutes while non pre-exposed rats remained in their home cages. The following day, all rats were placed into the shocking chamber, and freezing responses were observed for five minutes. None of the rats received a footshock. After the five minute behavioral observation period during which freezing response was scored, the rats received a single i.p. injection of BrdU (200 mg/kg) and were perfused two hours later.

Experiment 3 (Figure 26A): In order to see whether experience of the US alone could affect cell proliferation, one group of rats (n=6) received an immediate shock after placement into the chamber while the control group (n=6) received no shock. The rats were not previously exposed to the shocking chamber. Their freezing responses were scored, BrdU (200 mg/kg) was injected, and they were perfused after two hours.

Experiment 4 (Figure 27A): In order to determine whether the expression of fear (the act of freezing) contributes to alterations in cell proliferation, animals were pre-exposed to the CS on the first day of the study, split into two groups (n=6 per group) of shock or non-shock controls the following day, and then placed back into the chamber on the third day to test memory retention. After five minutes of freezing observation, the rats received BrdU injections (200 mg/kg) and were perfused after two hours.

Experiment 5 (Figure 28A): In order to learn whether contextual fear learning could affect the survival or phenotypic differentiation of recently born cells, we conducted a study in which rats (n=5 per group) were pre-injected with BrdU (200 mg/kg) and

subjected to the training trial on day 10 (pre-exposure) and day 11 (shock or no shock treatment). On day 12, the rats were perfused.

Experiment 6 (Figure 29A): In order to determine the involvement of glucocorticoids on cell proliferation, serum corticosterone levels were measured in several groups of animals (n=5-8 per group). On the first day, rats were divided into pre-exposed and non pre-exposed groups, and on the second day the rats in each group were further sub-divided into shock and no shock groups. A fifth group consisted of naïve rats for the purpose of obtaining baseline corticosterone levels. Tail blood was taken from each rat 15 minutes after receiving shock or no shock and the sera used for corticosterone analysis.

Results

In contextual fear conditioning, learning success can be measured by an animal's freezing response; immobility is a naturally occurring defensive fear response that rats display to cues associated with an aversive event (Fanselow, 1989). The amount of time a rat spends in a frozen position correlates with his learning index. All of the animals that received paired training, including pre-exposure to the CS and administration of the US, learned to make the CS-US association, as evidenced by their robust freezing behavior (78 $\pm$ 3.7% of time under observation). In contrast, rats that did not receive an aversive stimulus froze only 2.3 $\pm$ 0.7%. At the same time, the shocked rats had 2216 $\pm$ 292 BrdU-positive cells, 33% fewer proliferating cells compared to the non-shock controls, which had 3300 $\pm$ 216 BrdU-positive cells. Thus, contextual fear learning induced significant freezing response and reduced hippocampal cell proliferation (Figure 24B).

Contextual fear conditioning is a complex behavioral learning task consisting of multiple components, including exposure to a novel environment, experience of an aversive footshock, learning to make an association between the context and the shock,

and expression of fear by physical immobility. It is unclear whether the suppression of cell proliferation is due to one or more of these components. In order to address this question, we tested the contribution of each component separately.

To determine whether the CS alters the proliferation of cells in the dentate gyrus, rats either remained in their home cages or received CS pre-exposure. The following day, all animals were placed inside the conditioning chamber without administration of the US. Not surprisingly, all rats exhibited practically no freezing response. Pre-exposed rats froze only 2.3 $\pm$ 0.7% of the time they were under observation while non pre-exposed rats froze 1 $\pm$ 0.3%. The two groups also had similar numbers of BrdU-labeled cells, with the pre-exposed group showing 3300 $\pm$ 216 cells and the non pre-exposed group showing 2900 $\pm$ 215 labeled cells. No significant differences were detected between the two groups in both behavior and cell proliferation, indicating that CS pre-exposure alone did not contribute to changes in cell proliferation (Figure 25B).

Experience of the US in the absence of CS pre-exposure also produced minimal freezing and no alteration in cell proliferation. Rats given a shock immediately after being placed in a novel environment did not form a CS-US association, indicated by the lack of robust freezing behavior (9.3 $\pm$ 2.7%). They had 2812 $\pm$ 308 BrdU-positive cells in the dentate gyrus, similar to the non-shocked controls that had 2900 $\pm$ 216 labeled cells. The control rats that received neither the CS nor the US froze for only 1 $\pm$ 0.3% of the time under observation. Thus, the experience of a single, aversive shock alone did not induce changes in cell proliferation (Figure 26B).

The freezing behavior during contextual fear learning reflects both the CS-US formation and the expression of conditioned fear, the physical act of freezing. Since physical activity has been shown to enhance hippocampal neurogenesis (van Praag et al., 1999b), we sought to determine how freezing alone affects cell proliferation. To do this, we repeated the paired training but extended the study for an additional day to test

whether the expression of freezing was sufficient to cause a change in cell proliferation. In theory, the rats that learned to make the CS-US association should remember that the CS is linked to the US and freeze upon re-exposure to the CS alone. This was in fact the case, as those rats froze 48.7 ± 5.3% of the observed time, a significant enhancement over the control rats (1 ± 0.3%). Both groups, however, had similar numbers of proliferating cells in the dentate gyrus, with 2957 ± 408 positive cells for the control group and 2688 ± 171 positive cells for the paired group, thereby showing that freezing alone did not contribute to the decrease in cell proliferation observed in rats during the training day (Figure 27B). Furthermore, the suppression in cell proliferation associated with fear learning was transient, as 24 hours later, during the recall test, this effect was no longer detected.

The results presented thus far show that animals forming an association between a CS and US experienced a transient suppression of cell proliferation in the dentate gyrus. In order to determine whether the same conditioned learning task could affect either the survival or the phenotypic differentiation of recently generated hippocampal cells, we injected rats with BrdU and subjected them to training 10-11 days after the labeling, a period known to be critical for cell survival decisions (Cameron et al., 1993). Rats that experienced hippocampal-dependent learning displayed significant freezing response (80.1 ± 3.3%) whereas rats exposed to the CS in the absence of the US froze only 1 ± 0.5%. The numbers of BrdU-labeled cells that survived were similar for both groups, 3360 ± 279 cells for the pre-exposed and shocked rats and 3558 ± 182 for the pre-exposed, non-shocked control rats. These results indicate that contextual fear learning suppressed cell proliferation but not cell survival in the dentate gyrus of adult rats (Figure 28B).

We conducted immunofluorescent staining to determine the phenotypic development of the recently born cells in the dentate gyrus. Rats that underwent paired

CS-US training had similar proportions of neural and glial development as the control rats that were exposed to the CS without the US (Figure 29). For the paired group, 43.2 ± 1.8% of the BrdU-positive cells colocalized with NeuN, a marker for mature neurons; 21.5 ± 2.0% of the cells co-expressed GFAP, indicative of astroglia phenotype, and 35.4 ± 2.8 % remained undifferentiated or developed into a phenotype for which we did not label. Likewise, the non-shock control rats had 46.0 ± 2.2 % NeuN cells, 19.2 ± 2.0% GFAP cells, and 34.8 ± 3.3% neither. Thus, phenotypic development of neurons and glia remained unaltered after contextual fear learning.

Adrenal steroids have been shown to suppress cell proliferation (Cameron and Gould, 1994). We obtained serum corticosterone levels in rats subjected to contextual fear conditioning in order to determine whether glucocorticoid enhancement accompanied a decrease in cell proliferation. Baseline corticosterone level of naïve rats was 4.4 ± 1.2 μg/dl. The only group in which we observed an attenuation in cell proliferation (the pre-exposed and shocked rats) displayed a significant activation of the hypothalamus-pituitary-adrenal cortex axis, with corticosterone levels at 35 ± 4.2 μg/dl. However, all other treatments (whether pre-exposed or not and whether shocked or not) also resulted in significant elevation of serum corticosterone (all above 30 μg/dl; Figure 30). While glucocorticoids can play an important role in the regulation of hippocampal neurogenesis, we must conclude that they are not the sole mediators.

Figure 24: The formation and expression of a contextual fear association caused a 33% decrease in proliferating cell numbers. Rats that were pre-exposed and shocked froze 78 $\pm$ 3.7% and had 2216 $\pm$ 292 BrdU-positive cells. In comparison, pre-exposed, non shocked rats froze only 2.3 $\pm$ 0.7% and had 3300 $\pm$ 216 labeled cells. Fisher's PLSD post hoc tests revealed significant differences in freezing behavior ($p<0.0001$*) and cell proliferation ($p=0.01$**). Values are mean + S.E.M. The blue bars represent freezing behavior and correspond to the left side axis while the red bars represent BrdU cell counts and correspond to the right side axis.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B

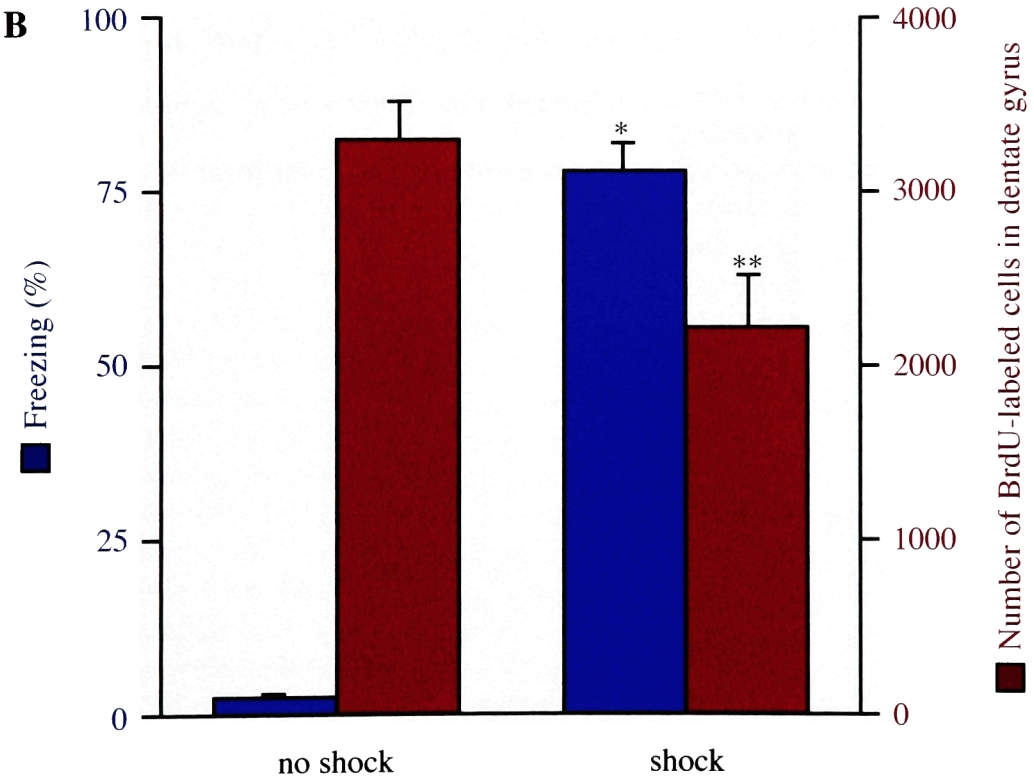


Figure 25: Forming a representation of the contextual CS had no effect on behavior and cell proliferation. Rats pre-exposed to the shocking chamber froze 2.3 $\pm$ 0.7% and had 3300 $\pm$ 216 BrdU-positive cells in the dentate gyrus. The control, non pre-exposed rats froze 1 $\pm$ 0.3% and had 2900 $\pm$ 215 labeled cells. There were no significant differences in behavior and cell number between the two groups. Values are mean + S.E.M.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B

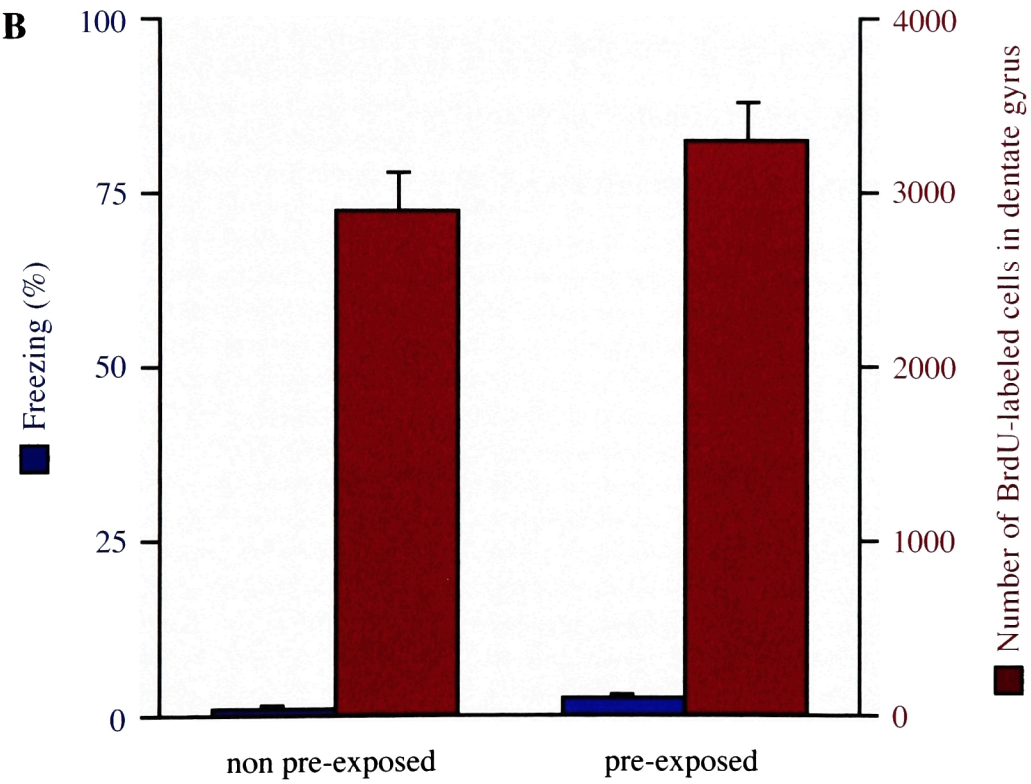


Figure 26: The shock alone in the absence of learning had no effect on behavior and cell proliferation. Rats given a shock immediately after being placed in a new environment did not form a context-shock (CS-US) association, as shown by their minimal freezing response to the US, and had similar numbers of proliferating cells in comparison to their non shocked controls. Values are mean + S.E.M.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B

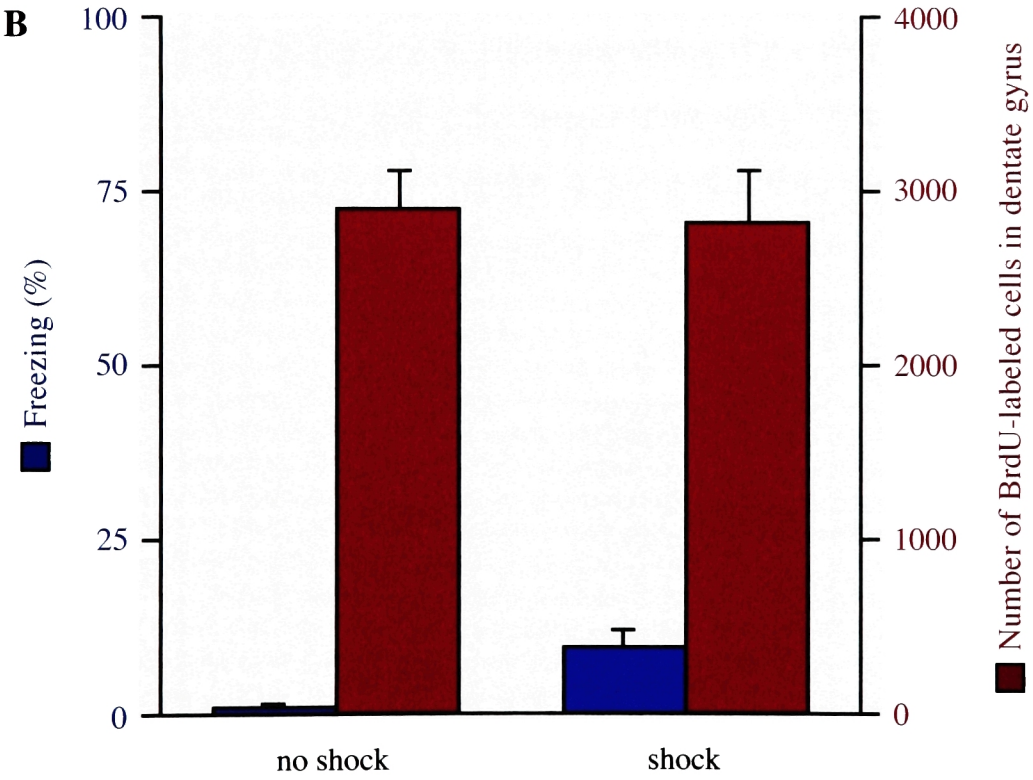


Figure 27: Upon re-exposure to the shocking chamber one day following fear conditioning, rats that were previously shocked displayed a freezing response (48.7 $\pm$ 5.3%) and had 2688 $\pm$ 171 proliferating cells. The non-shocked controls froze only 1 $\pm$ 0.3% and had 2957 $\pm$ 408 BrdU-labeled cells. There was a significant difference in freezing behavior ($p<0.0001$*) but no difference in proliferating cells, showing that expression of fear did not affect cell proliferation. Values are mean + S.E.M.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock
Day 3			Test	Test

B

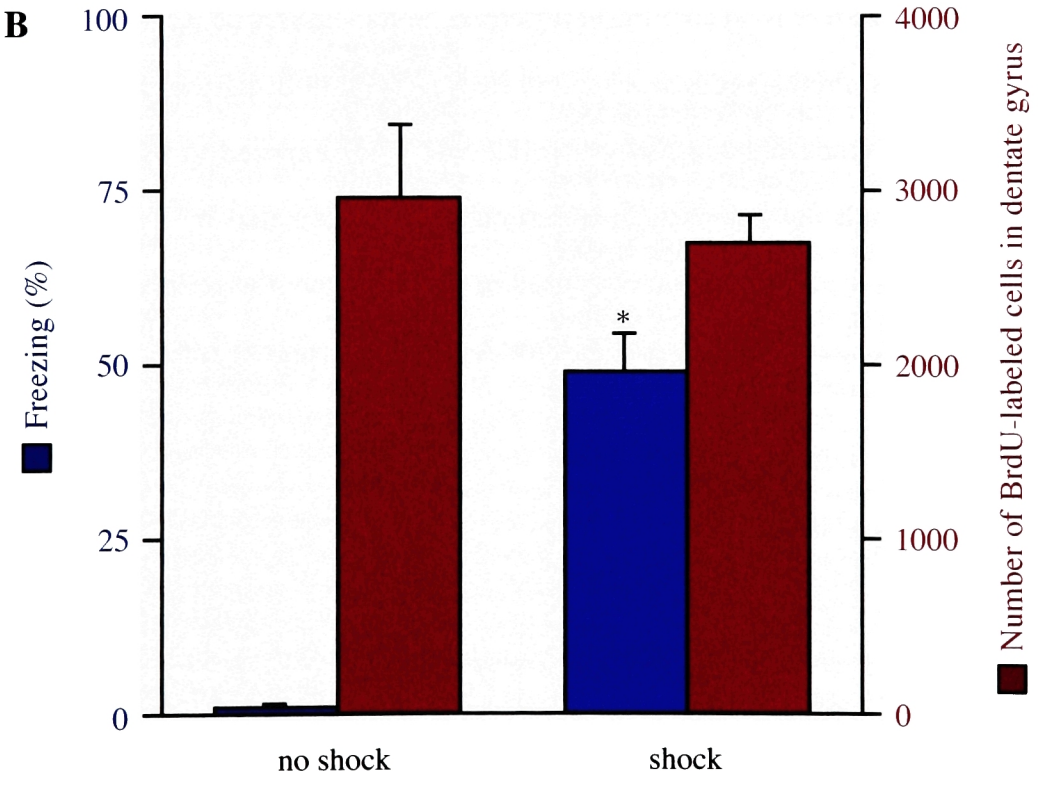


Figure 28: Contextual fear learning did not affect cell survival. All rats received BrdU injections ten days prior to training, and quantitation of BrdU-labeled cells after the task revealed that there was no significant difference in the number of surviving cells between the two groups. Pre-exposed, shocked rats had 3360 $\pm$ 279 BrdU-positive cells while pre-exposed, non shocked rats had 3558 $\pm$ 182 cells. Rats exposed to both the CS and US learned to make the context-shock association, as indicated by the robust freezing response (80.1 $\pm$ 3.3%). Rats pre-exposed to the CS without experience of the US froze only 1 $\pm$ 0.5%. Values are mean + S.E.M. Asterisk indicates significant difference from control.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B

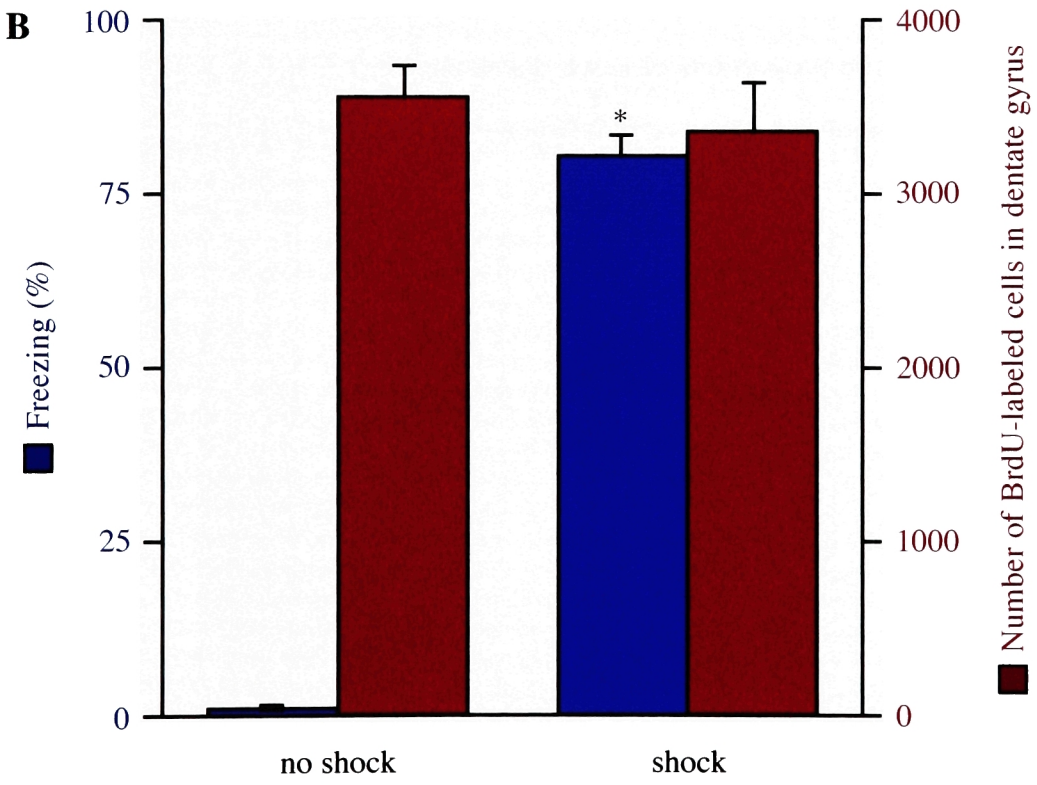


Figure 29: Contextual fear learning does not alter phenotypic differentiation of recently born cells in the dentate gyrus. Rats were injected with BrdU 10 days prior to conditioning. The proportions of newly generated cells that developed into neurons and astroglia were similar for both conditioned and unconditioned groups.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B

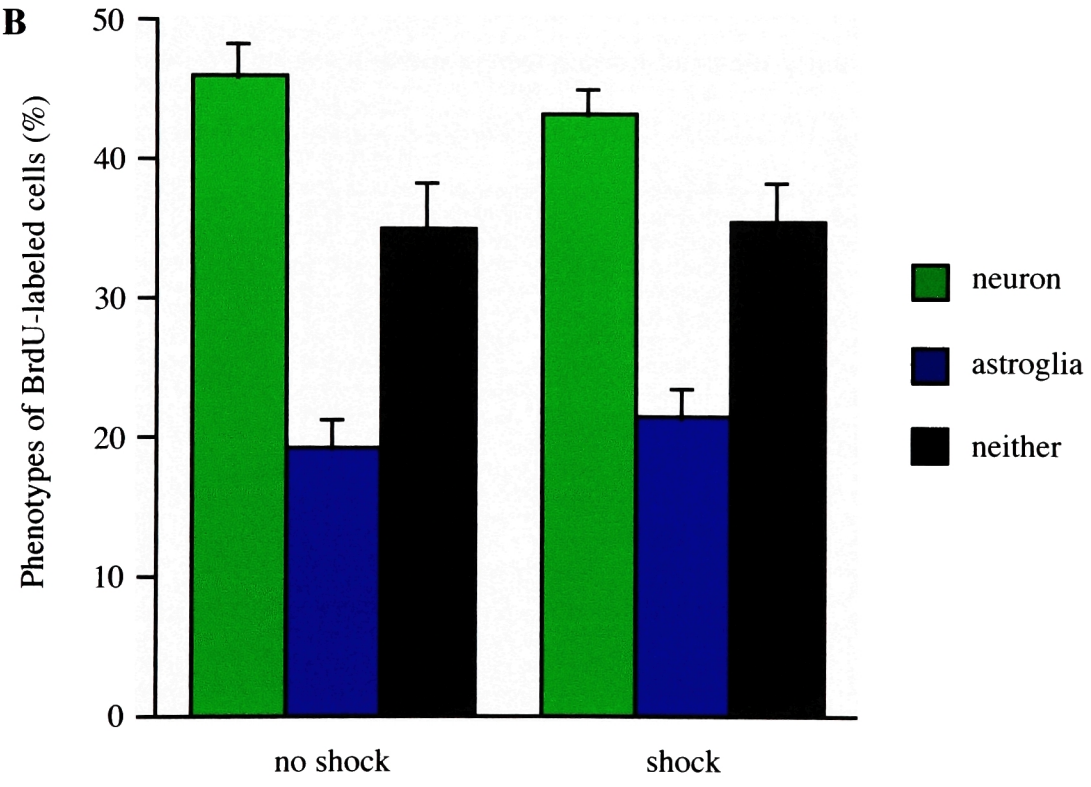
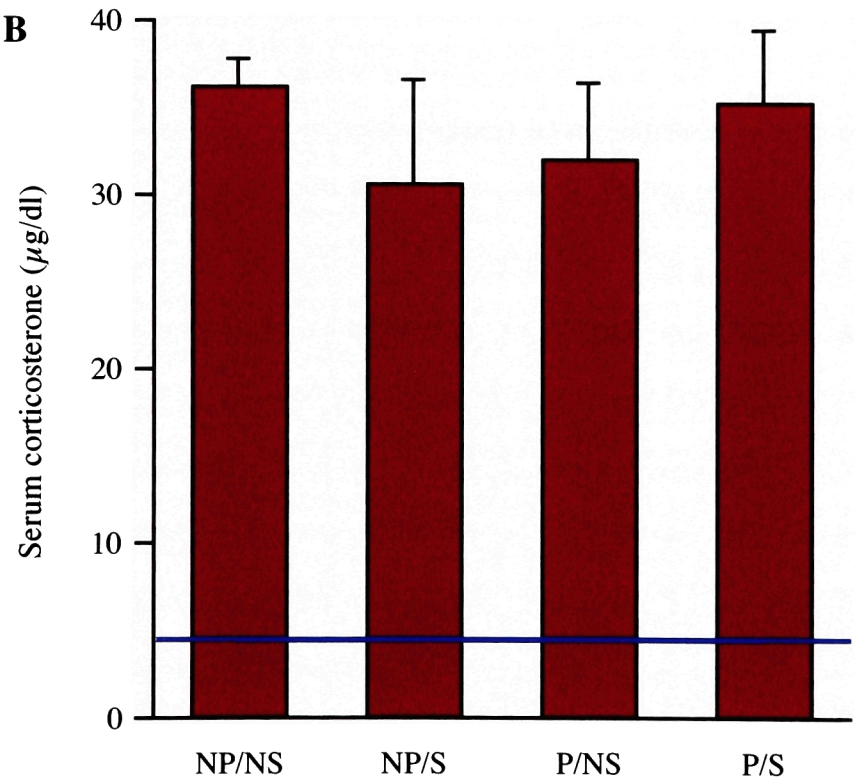


Figure 30: Tail blood was taken from the rats 15 minutes after receiving shock or no shock to assess corticosterone levels. All rats, whether pre-exposed or not and shocked or not, had significantly elevated corticosterone secretion compared to baseline controls (blue line; 4.4 $\pm$ 1.2 μg/dl). Only the P/S group, however, had suppression of cell proliferation, indicating that corticosterone was not solely responsible for regulation of cell proliferation. Abbreviations: *NP*, not pre-exposed; *P*, pre-exposed; *NS*, not shocked; *S*, shocked. Values are mean + S.E.M.

A

Day 1	No pre-exposure		Pre-exposure	
Day 2	No shock	Shock	No shock	Shock

B



Discussion

The results of these experiments show that the formation of an association between a contextual CS and a footshock US temporarily decreased proliferation of neural precursor cells in the dentate gyrus. Exposure to the CS alone or to the US alone did not change proliferation rates, and expression of fear in a recall test did not contribute to the suppression of dentate gyrus cells. The effect was specific to cell proliferation, as fear learning did not affect the survival or differentiation of recently born cells, and was not mediated solely by glucocorticoids. Since contextual fear conditioning is a learning task that is dependent on both the hippocampus and the amygdala (Antoniadis and McDonald, 2000; Phillips and LeDoux, 1992), changes in hippocampal neurogenesis during this process may be regulated by functional connections between the two structures.

Reduction in cell proliferation via excitatory hippocampal-amygdala connections

With sufficient exposure to contextual cues, the hippocampus forms a representation of the context. Once the US is experienced in the presence of the CS, communication flows from the CA1 hippocampal field and subiculum to the lateral, basal and accessory basal nuclei of the amygdala (Canteras and Swanson, 1992). In these structures, contextual representations relayed from the hippocampus can interact with incoming US information to form an association. In turn, these areas project to the central amygdala, which controls the expression of fear responses by way of output pathways to brainstem areas, such as the hypothalamus (for review see LeDoux, 2000). The amygdala then projects to the dentate gyrus of the hippocampus by way of the entorhinal cortex, and the perforant path input to the dentate gyrus can be facilitated by stimulation of the amygdala (Thomas et al., 1984). Similarly, LTP can be induced in the amygdala by stimulation of the hippocampus (Maren and Fanselow, 1995). It is then likely that during

contextual fear learning, the CS and US inputs converge upon the amygdala to mediate synaptic plasticity, and these changes are communicated back to the hippocampus via the entorhinal cortex. Perforant path activation of NMDA receptors in the dentate gyrus has been previously shown to reduce cell proliferation (Cameron et al., 1995).

Adrenal steroids can suppress adult hippocampal neurogenesis (Cameron and Gould, 1994). Here we show that experiencing the various components of contextual fear conditioning in the absence of learning results in stress levels of glucocorticoid secretion without alterations in cell proliferation. Pre-exposure to the CS alone was not accompanied by an attenuation in cell proliferation despite an elevation in corticosterone. Similarly, the experience of a shock in a novel environment resulted in increased corticosterone and basal levels of proliferating cells in the dentate gyrus. These results may be attributable to the observance that corticosterone regulation of neurogenesis occurs in the same pathway as and can be outweighed by NMDA receptor activity (Cameron et al., 1998). Alterations in hippocampal neurogenesis by manipulation of adrenal steroids can be overridden by modulation of NMDA receptor activity. Since acquisition of fear learning requires communication between the amygdala and the hippocampus, it is probable that glutamate activation of NMDA receptors during learning directly inhibits cell proliferation and that glucocorticoid influence is quelled in this learning paradigm. NMDA receptor activity has been shown to be required for contextual fear conditioning; intracerebroventricular administration of an NMDA receptor antagonist caused a dose-dependent suppression of fear acquisition for contextual cues associated with shock, and this appears to be mediated through NMDA receptors in the hippocampus (Fanselow et al., 1994). Furthermore, the requirement for NMDA receptors has been demonstrated specifically for acquisition of fear learning and not for expression of fear (Rodrigues et al., 2001). Thus, adrenal steroid modulation of hippocampal

neurogenesis could be averted in contextual fear conditioning by the participation of NMDA receptors in this learning task.

The role of task difficulty in hippocampal neurogenesis

Elizabeth Gould and Tracey Shors reported that hippocampal-dependent learning could increase hippocampal neurogenesis specifically by enhancing cell survival but not cell proliferation (Gould et al., 1999a). Their results contradict the ones presented here; we show that hippocampal-dependent learning decreased cell proliferation and had no effect on cell survival. The inconsistent results may be partially attributable to the degree of difficulty of the learning tasks used. It appears that the hippocampus may become more engaged in the learning task when the task is more difficult to learn (Beylin et al., 2001). Contextual fear conditioning requires just one training trial for memory formation, and extinction of the memory takes an extraordinarily long period of time. It is an evolutionarily advantageous defensive skill, having been observed in a variety of species, such as flies, snails, fish and most mammals, including humans (LeDoux, 2000). It can be inferred, then, that fear conditioning through contextual cues is a relatively easy task, and while it is dependent upon hippocampal function, it may not encumber the hippocampus greatly. Gould and Shors, on the other hand, used a trace eyeblink conditioning paradigm, which required 800 training trials administered over four days. This is arguably a much more difficult learning task and one that likely places more demand on hippocampal participation. It may be that the enhancement of cell survival in the trace eyeblink paradigm is reflective of the technical difficulty associated with learning that task, and it is therefore necessary to engage newly born cells during training and thus increase their chances for survival. As for the opposing results on cell proliferation, we showed a suppression that was ephemeral; the difference was no longer detected 24 hours after the initial training when learning occurred. In the trace eyeblink conditioning study,

proliferation was examined at the end of 800 trials; however, significant acquisition of the skill appeared often by 300-400 trials, after which the percentage of conditioned responses seemingly reached a plateau. By determining the number of proliferating cells after the fourth day of training, it is possible that the investigators missed the brief period in which learning could reduce the number of proliferating cells, which probably transpired during the second day of training. Another difference between the two learning tasks is the requirement for the amygdala during fear conditioning which is not a requisite for trace eyeblink conditioning. The circuits linking the amygdala and the hippocampus may influence hippocampal neurogenesis in a manner that is more specified for learning tasks with emotional components.

CHAPTER 7

CONCLUSION

The hippocampus contains an abundance of adrenal steroid hormone receptors (McEwen et al., 1968), and glucocorticoid influence on neuronal plasticity is quite complex (McEwen et al., 1993). The results presented in this dissertation demonstrate that repeated stress, which activates the HPA axis and leads to increased glucocorticoid and glutamate release, can inhibit hippocampal neurogenesis, reduce dentate gyrus cell numbers and volume, enhance the expression of PSA-NCAM and transiently increase the synaptic response in the dentate gyrus after perforant path stimulation (Figure 31). Contextual fear conditioning was also shown to temporarily decrease cell proliferation in the dentate gyrus. Taken together, the results suggest that the hippocampus is capable of responding to an animal's experiences with stress and learning tasks by inducing plasticity events that can revert to baseline, such as synaptic enhancement, dendritic remodeling, and PSA-NCAM elevation. However, when the stress was endured for extended periods, the morphological response became more extreme, exhibiting reductions in neurogenesis, total granule neuron numbers and granule cell layer volume. At this level of stress, PSA-NCAM expression was no longer enhanced, implying that the dentate gyrus had acquired a more stable condition and one that is perhaps reflective of permanent changes.

Humans and other animals exposed to chronic elevations of stress hormones often exhibit neuronal loss and reduced hippocampal volume (Bremner et al., 2000; Horn et al., 1996; Sapolsky, 2000). Conditions that display similar manifestations, such as aging and certain depressive illnesses, are often accompanied by a decline in cognitive skill and an

increase in anxiety. It will be necessary to determine whether the results obtained during this dissertation project are indeed permanent or reversible and to assess the functional implications of such changes. Understanding the mechanisms that induce these alterations can lead to the prevention or alleviation of undesired clinical symptoms.

A correlation has been noted between reduced hippocampal volume and certain diseases that affect mental ability, like major depression and Alzheimer's disease (Bremner et al., 2000; Horn et al., 1996). Shrinkage of the hippocampus can occur by several mechanisms, including loss of neurons or glia, retraction of synaptic processes, or suppressed neurogenesis. Chronic stress can promote atrophy of CA3 apical dendrites (Magarinos et al., 1996; Watanabe et al., 1992b), but this event is reversible, resulting in no long term or permanent change in the morphology of the hippocampus. Here we demonstrate that between three and six weeks of daily restraint stress, we can induce significant suppression of hippocampal neurogenesis as well as granule cell layer volume. These changes are accompanied by a normalization of PSA-NCAM expression after an enhancement was detected following three weeks of stress. Since PSA-NCAM is a molecule that confers plasticity upon neurons, we hypothesize that reversion of the molecule to baseline levels of expression after six weeks of stress represents a shift in hippocampal response from an adaptive response to a permanently altered state. In order to verify this, experiments should be conducted to measure the anatomical changes induced by six weeks of stress. The parameters to investigate could include dendritic arborization, synaptic contact, total cell number, and volumetric measurements of the various hippocampal subfields and of the whole hippocampus. In addition, cognitive skill and emotional state could be assessed using various tests, like the Morris water maze, open field test, and elevated platform test.

If it can be demonstrated that six weeks of restraint stress leads to widespread neuroanatomical alterations in the hippocampus and to increased cognitive deficits and

emotionality, then the next task would involve showing a causal relationship between anatomy and behavior. Are the behavioral changes a consequence of morphological changes, and if so, what is the contribution of each anatomical modification? To do so would require subjecting animals to chronic stress while interfering with specific modifiable processes. For example, tianeptine administration concurrent with restraint stress can rescue CA3 neurons from dendritic atrophy (Watanabe et al., 1992a). Hippocampal neurogenesis is attenuated in chronically stressed rats, but running enhances neurogenesis (van Praag et al., 1999a; van Praag et al., 1999b). By restraining rats during the day and housing them in cages with running wheels at other times, one may be able to counter the reduction in neurogenesis due to stress with an increased effect due to physical exercise. These experiments are designed to determine whether a direct link, and not just a correlative one, exists between hippocampal volume reduction and behavioral deficits.

Another important criterion to resolve is the reversibility of these modifications. Are the consequences of chronic stress permanent, or can they revert to baseline after a period of recovery, and what amount of time would be sufficient if a return to normalcy were possible? In order to address these questions, one would need to subject animals to stress for the length of time required for anatomical and behavioral changes to arise and then conduct a time course study for recovery effect.

An important variable in the study of stress research is the selection of a stressor. Restraint stress, while it is sufficient for inducing a physiological stress response, does result in partial habituation throughout a chronic experiment. We have observed that rats become accustomed to the routine of restraint and crawl eagerly inside the restrainer after experiencing several days of restraint. There were exceptions, and few rats have been known to resist restraint everyday even after enduring several weeks of stress. It would be intriguing to make notes of these exceptions to determine if a correlation should exist

between behavioral habituation and physiological habituation. Perhaps the use of a more severe stressor could produce more profound neuroanatomical changes in the same time frame or similar alterations within a shorter period. This appears to be the case in regard to dentate gyrus neurogenesis. Neurogenesis is significantly impaired after prolonged restraint stress but not after acute restraint stress. However, certain forms of psychosocial stress can suppress neurogenesis just after a single stress session (Gould et al., 1997a; Gould et al., 1998). In examining hippocampal response to stress, one must consider the effects of various stressors on other neuronal systems. For example, serotonin neurotransmission is differentially affected by a variety of stressors (Kirby et al., 1997). Thus, the specific attributes of individual stressors will need to be considered in the context of experimental purpose.

In the realm of neurogenesis detection, BrdU immunohistochemistry is widely used for quantitative analysis. Since incorporation of BrdU is dependent upon the quality, preparation, and dose of BrdU as well as on success of the injection, internal controls should be used in future studies to judge the uniform standard of BrdU availability and uptake. One such control could be to count the number of BrdU-labeled cells present in the subventricular zone. It has been shown that the number of proliferating cells in the subventricular zone remains stable under conditions that can manipulate hippocampal neurogenesis. Thus, examining subventricular zone cells could indicate whether differences detected in the hippocampus were due to real changes or simply due to variations in BrdU availability. Another control assay is to detect and quantitate an endogenous marker of the cell cycle. One such candidate is the phosphorylated histone-3, a protein expressed in nearly all stages of cells undergoing mitosis (Balmain et al., 1977).

While various factors have been identified in the regulation of dendritic atrophy and hippocampal neurogenesis, little is known about the intracellular events mediating these processes. Which factors act directly upon responsive cells, and how are the signals

translated into actions? Do modifications occur on existing proteins, such as by phosphorylation or ubiquination, or do genes get transcribed to produce new proteins? These questions are more easily addressed, at least initially, using *in vitro* systems.

Neuronal precursors to dentate gyrus granule cells can be isolated and cultured (Gage et al., 1995), and given the proper conditions, they can develop into mature neurons and glia. Serotonin can upregulate proliferation in this population of cells (Brezun and Daszuta, 1999) while glucocorticoids downregulate this process (Cameron and Gould, 1994). A possible experiment to conduct is one that involves culturing these precursor cells under various conditions to either stimulate or suppress proliferation rates and then compare the gene expression profiles of the different populations. While the technology for gene chip studies continues to evolve quickly, producing cleaner results and better software for analysis, these experiments can generate an overwhelming volume of data. The advantage of expression profiling is the potential to detect novel genes and proteins involved in neurogenesis regulation.

Another approach toward identifying potential regulators is to apply what is known in comparable systems to the hippocampal system of adult neurogenesis. For example, sonic hedgehog (Shh) induces proliferation of granule cell precursors in the cerebellum (Wechsler-Reya and Scott, 1999). In order to assess the role of Shh in the proliferation of granule cells of the hippocampus, one could culture hippocampal precursors with Shh and observe the outcome. *In vitro* experiments can be combined with *in vivo* studies to further confirm results. If, or example, Shh significantly enhances cell proliferation, it may be possible to culture granule cell precursors and infect them with a retrovirus encoding Shh and a marker, such as green fluorescent protein. Replacing these retrovirally infected cells back into the dentate gyrus would allow one to monitor the effects of Shh on cell proliferation within the context of the cell's natural environment.

Transgenic and knock out mice can also be utilized to study genes of interest. However, it should be noted that compensatory mechanisms could produce mice lacking an obvious phenotype, or interference with essential genes could result in non-viable mice. A more specific approach involving this technology is the use of genes under the guidance of spatially or temporally controlled promoters. Unfortunately, a promoter specific for granule cell precursors in the hippocampus has not yet been identified.

Given the crucial role the hippocampus holds in memory formation and the links that have been made between reduced hippocampal volume and conditions that affect mental clarity, understanding the basis of hippocampal shrinkage may allow for better insight into the roots of mental disorders. Stress is a common experience in our human existence, from the underprivileged youths of our society living in conditions of poverty to the highest executives of business corporations working under profit pressures. Stressors can include both physical and psychological challenges. In our post-September 11^{th} world, we have become increasingly prone to stress of the psychological kind, where recurring feelings of anticipatory anxiety and worry can contribute to chronic stress and its accompanying physiological manifestations. Studies on how stress can affect the brain, whether at a plasticity level or at a more permanently damaging level, will help us understand how our day to day experiences and behavioral responses may alter our long term mental well being.

While stress often connotes negative associations, it is important to recall that acute stress and the mounting of a proper response serve to protect organisms in the short term. The brain is ultimately responsible for the perception of stress, initiating the release of stress hormones, including glucocorticoids and catecholamines. These hormones alter the function of a variety of cells and tissues throughout the body and aid organisms in their immediate responses to the challenge at hand. For example, in a "fight or flight" situation, cardiovascular tone and blood pressure are elevated, reproductive and digestive

activities are temporarily suppressed, energy mobilization is enhanced, and immune cells are redistributed to sites of potential damage (For review see McEwen, 1998). All of these responses are intended to assist organisms in their ability to successfully overcome the stressful experience, and in normal organisms, restoration of the systems to basal operation quickly follows the stress onset, returning the body to homeostasis. From an evolutionary standpoint, the presence of receptors for stress hormones throughout the hippocampus signifies the importance of learning and memory formation associated with stressful events in the promotion of survival of the species. In other words, if a foraging rabbit suddenly encounters a pack of hungry wolves and manages to escape harm, the rabbit would certainly benefit from memory formation of the event, thereby dissuading it from returning to that particular locale in the future and minimizing its risk for a similar attack.

Problems arise when systems that respond to stress become chronically activated. This can be achieved by several means, including the inability to shut off a stress response during recovery, the experience of frequent stress, or the lack of adaptation to repeated stress of the same kind. These situations place chronic burden on the cardiovascular, metabolic, and immune systems, to name only a few, and eventually promote damage to the body. For example, repeated episodes of blood pressure elevation over sustained periods can stimulate atherosclerosis and increase myocardial infarction. Furthermore, immunological response becomes inhibited after chronic stress. Thus, overexposure to stress hormones can accelerate pathophysiology.

The objective of this thesis was to determine how stress, namely restraint stress or conditioned fear learning, could impact hippocampal structural and functional plasticity, and the results obtained contribute to the foundation of what we know currently about restraint stress effects upon the brain. The good news is that the brain appears to be fairly resilient in the face of stressful challenges. Acute stress produces few significant changes

in the hippocampus, and moderate duration stress, such as three weeks of daily restraint, induces a variety of alterations, which are for the most part reversible. These changes include reduced cell proliferation, dendritic remodeling, mossy fiber terminal rearrangement, and dentate gyrus LTP suppression, and occur during a time frame accompanied by an elevation of PSA-NCAM expression, a molecule important for structural plasticity in neurons. We demonstrate that prolonging restraint stress from three weeks to six weeks produces suppressed neurogenesis and resulted in reductions in both the numbers of granule cells and the granule cell layer volume. This is the first evidence of cell reduction due to stress in a laboratory setting, and it remains to be determined whether these changes are permanent or reversible. The fact that PSA-NCAM enhancement can no longer be detected after six weeks of stress suggests that the hippocampus may be transitioning from a state of adaptive plasticity to one that is more vulnerable to permanent changes. Determining the molecular and cellular mediators underlying these changes in future studies can help us understand processes that could lead to hippocampal shrinkage and provide mechanistic insight into diseases that are correlated with atrophy of the hippocampus.

Figure 31: A diagram showing the temporal effects of chronic restraint stress and a model suggesting that repeated stress experienced for brief periods leads to neuronal adaptations that are reversible while stress under extremely long periods may result in hippocampal damage or loss of plasticity. Abbreviations: *PP*, perforant path; *DG*, dentate gyrus; *gcl*, granule cell layer.

Stress timeline

2 wks

↑ PP to DG EPSP slope

3 wks

↓ cell proliferation

↑ PSA-NCAM expression

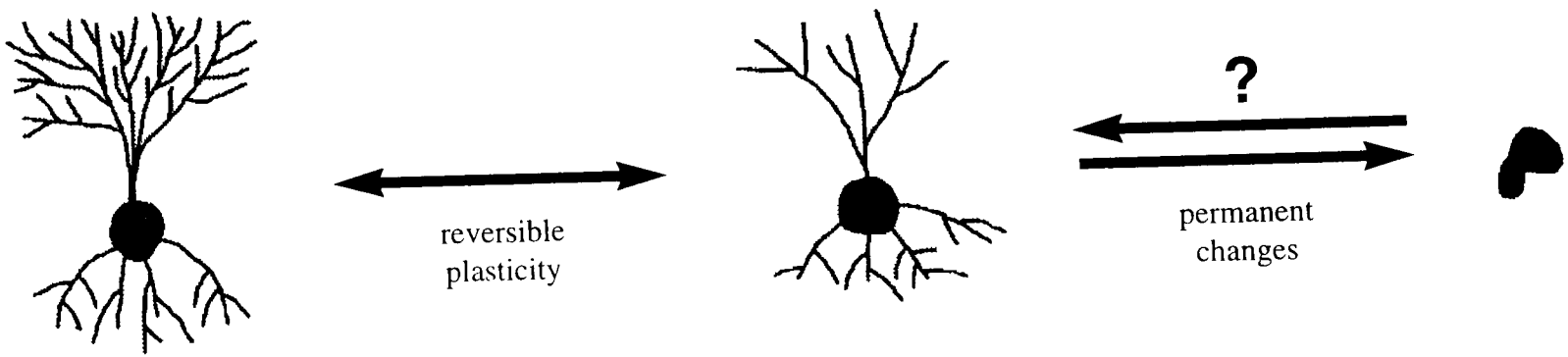
6 wks

↓ cell survival

↓ neurogenesis

↓ granule cells

↓ gcl volume



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