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**Effect of Chronic Mild Stress on the Neural Consequences of Permanent Bilateral
Common Carotid Artery Occlusion**

Submitted by

Lesley Ritchie

In partial fulfillment of the requirements for

The degree of Master of Science

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Lesley Ritchie

in Partial fulfillment of the requirements for
the degree of Master of Science

Chair
Thesis Supervisor

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Abstract

High levels of stress and, by association, glucocorticoids, are reported to increase neuronal susceptibility to various insults, including ischemia. The purpose of the current study was to examine the possibility that chronic mild stress (CMS) exacerbates the neural consequences of permanent bilateral occlusion of the common carotid arteries (2VO) in the adult rat. To this end, 8-month old male rats were assigned to 2VO and control conditions. At approximately 7 days post-surgery, control and 2VO animals were assigned to stress and non-stress conditions. Animals in the stress condition were exposed to a six-week chronic mild stress regime. At eight weeks post-surgery, 2VO rats exhibited significantly more CA1 pyramidal cell loss compared to sham animals, with Stress 2VO rats exhibiting the largest loss of CA1 pyramidal cells. Stress, by itself, did not result in significant CA1 cell loss. In contrast, stress promoted a significant reduction in glial fibrillary acidic protein (GFAP) expression in Stress 2VO and Stress Sham animals. No differences in brain-derived neurotrophic factor (BDNF) expression, the number of degenerating cells as quantified by Fluoro-Jade staining, or corticosterone levels were observed between groups. The results of this study suggest that chronic mild stress leaves hippocampal cells vulnerable to ischemic insult by altering their metabolic state. Since cerebral hypoperfusion is characteristic of mild cognitive impairment, probably the progenitor stage of Alzheimer's disease (AD), and since CA1 cell loss is an established feature of AD, exposure to CMS and higher levels of circulating glucocorticoids may potentiate the progression of neuropathology in AD.

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Introduction

High levels of stress and, by association, of glucocorticoids, are reported to increase neuronal susceptibility to various insults, including ischemia (Moghaddam et al, 1994; Sapolsky, 1985a, 1985b, 1986; Sapolsky & Pulsinelli, 1985, 1986). To the best of our knowledge little, if any, research has examined the neural consequences of chronic mild stress (CMS) in a model of the chronic ischemia that mimics the chronic hypoperfusion that occurs with aging. The purpose of the current study was to examine the possibility that CMS exacerbates the neural consequences of permanent bilateral occlusion of the common carotid arteries (2VO) in the adult rat.

Permanent Bilateral Artery Occlusion (2VO)

Ischemia and Local Cerebral Blood Flow

Over the past decade, a number of studies have examined the pathological consequences to the hippocampus following permanent bilateral occlusion of the common carotid arteries. Three main cerebral arteries maintain the brain's blood supply: the two carotid arteries and the basilar artery (Chang & Liu, 1928). Occlusion of the common carotid arteries reduces the local cerebral blood flow (LCBF) to various levels in differing brain structures. The percent reduction of LCBF in the rat was assessed by Tsuchiya et al (1992), following 2VO. Occlusion of the carotid arteries was observed to produce a decrease in LCBF to the extent of 60% in the caudate nucleus, 61-75% in the cortex, 48% in the hippocampus, and 15-55% in various other regions of the brain, as measured 2.5 hours after the onset of ischemia. At one week following the onset of

ischemia, blood flow rates were observed to increase, such that a 42% decrease in caudate nucleus LCBF, a 32-43% LCBF reduction in the cortex, and a 25% reduction in hippocampal LCBF were reported. All brain regions exhibited an increase in LCBF at the one-week time-point, with the exception of areas marked by neuronal damage (Tsuchiya et al, 1992).

Following bilateral cerebral artery occlusion, Tsuchiya et al (1992) found that 90% of the animals exhibited neural damage in the caudate nucleus, 23% in the cortex, and 30% in the hippocampus. The extent of neural damage, however, did not correlate with the reduced rate of LCBF. As a result of these findings, the researchers conducted a subsequent study examining changes in local cerebral glucose utilization (LCGU) following bilateral carotid artery occlusion. LCBF and LCGU were measured at 2.5 hours and one-week post-2VO. Consistent with earlier results (Tsuchiya et al, 1992), a significant reduction in LCBF was observed at the 2.5-hour time-point (25-87%), with a lesser decrease in LCBF by the one-week time-point (54-92%). In addition, a 23-28% decrease in LCGU was observed in the cerebral cortex and the nucleus accumbens of 2VO rats following 1 week of hypoperfusion, compared to control. LCGU and LCBF levels for both sham and ischemic rats were positively correlated. Thus, chronic ischemia causes persistent metabolic distress (Tsuchiya et al, 1993).

Neuropathological Consequences of 2VO

Permanent bilateral occlusion the common carotid arteries is reported to produce delayed CA1 hippocampal neurodegeneration (Bennett et al, 1998; Pappas et al, 1996; Ni et al, 1994). Pappas et al (1996) conducted a six-month longitudinal study examining the

pathological effects of permanent bilateral carotid artery occlusion, wherein they report no differences between control and 2VO rats in the total number of CA1 cells at 14 days post-surgery. However, at six months post-surgery, 2VO rats exhibited a significant reduction in CA1 pyramidal cells, as well as a significant increase in CA1 GFAP immunoreactivity. In a similar study, Ni et al (1994) observed a significant decline in CA1 pyramidal cells at four months, but not one month, following 2VO, confirming the occurrence of a delayed progressive CA1 cell loss following 2VO.

Based on the finding that bilateral occlusion of the common carotid arteries causes delayed CA1 cell loss, Bennett et al (1998) designed a study to investigate the mechanism of neurodegeneration following 2VO. Specifically, the researchers examined the viability of hippocampal cells at 2 and 27 weeks following 2VO in aged rats. 2VO rats exhibited apoptotic and necrotic cell degeneration at 2 weeks post-2VO in the CA1, CA3b, and CA3c regions of the hippocampus. Apoptotic nuclei were often clustered together and surrounded by glia. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) detection of DNA fragmentation, a stage in the apoptotic process, was also observed in the CA1 and CA3 hippocampal regions. By the 27-week time-point, a significant increase in pyramidal cell degeneration was observed in 2VO rats, compared to controls and the 2-week 2VO rats. However, apoptotic cells were no longer in clusters but were still surrounded by glia. The 27-week 2VO and control rats were tested on both the Morris water maze and the radial arm maze. Significant correlations were observed between the number of TUNEL positive neurons at 27 weeks and reference memory errors on the radial arm maze. Furthermore, a significant relationship was found between the number of CA1 apoptotic cells and radial arm maze

reference and working memory errors. The results of this study suggest that at least a portion of the hippocampal degeneration following 2VO is due to apoptosis and that the ensuing behavioral deficits may be the result of hippocampal cell loss (Bennett et al, 1998).

Behavioral Consequences of Permanent Bilateral Carotid Artery Occlusion (2VO)

Studies suggest that permanent occlusion of the 2 common carotid arteries has a negative effect on reference and working memory (Bennett et al, 1998). Ni et al (1994) examined the behavioral consequences of permanent bilateral carotid artery occlusion in rats, in order to determine whether the decrease in LCBF to regions associated with learning and memory, such as the cortex and the hippocampus, results in cognitive impairments typical of senile dementia and Alzheimer's type dementia. To this end, control and 2VO rats, both either pretrained or non-pretrained, were tested on the radial arm maze to assess retention and learning, respectively. Rats who had not been pretrained failed to learn the radial arm maze task and performance did not improve with repeated exposure, suggesting that learning is impaired under conditions of chronic ischemia. Pretrained rats, however, were able to perform at the level of controls beginning 3-7 days after surgery, suggesting that reference memory and working memory are intact at this time. Significant impairment, however, was detected among 2VO rats on the radial arm maze at the 4-month time-point, at which point a marked increase in CA1 cell loss was observed (Ni et al, 1994).

In contrast, pretrained 2VO rats were significantly impaired at the one-month time-point on a radial arm delay task wherein a 3-minute delay was interposed between

the fourth and fifth arms, which was indicative of an impaired short-term memory capacity. The significant impairment on the delayed-recall task, however, was not accompanied by morphological damage at the early time-point. The results of this study suggest that permanent bilateral carotid artery occlusion produces progressive cognitive impairments, accompanied by progressive neuronal degeneration (see above), similar to the cognitive impairments typical of senile dementia and dementia of the Alzheimer's type (Ni et al, 1994).

In a study investigating the long-term behavioral consequences of 2VO surgery in adult male rats, Pappas et al (1996) examined the Morris water maze and radial arm maze performance of chronic ischemic (2VO) rats at various time points. 2VO rats, as compared to controls, demonstrated Morris water maze impairment as early as one week following the onset of ischemia and persisting up to 175 days post-surgery. Radial arm maze impairment, however, was not evident until 21 days post-surgery, with 2VO rats committing significantly more unbaited arm re-entries (i.e. reference memory errors) on days 63, 105, and 168 post-surgery. 2VO rats were also found to execute more baited arm re-entries (working memory errors) on the radial arm maze task, apparent by day 21 post-surgery (Pappas et al, 1996). No relation was observed between performance on the Morris water maze and the radial arm maze or between Morris water maze and hippocampal CA1 degeneration (Pappas et al, 1996). Thus, as evidenced by Ni et al (1994), prolonged functional deficits do arise despite the lack of hippocampal degeneration in the early stages of permanent bilateral carotid artery occlusion.

Pappas et al (1996) proposed that the early detection of Morris water maze impairment in 2VO rats is due to the increased stress associated with the water-based task

and that the elevated stress has a damaging effect on the already degenerating neurons. Moreover, the researchers (Davidson & Pappas, unpublished) have observed elevated corticosterone levels in 2VO rats after Morris water maze. Since other evidence suggests that stress enhances neuronal vulnerability to various insults (Moghaddam et al, 1994; Sapolsky, 1985a; 1985b; 1986; Sapolsky & Pulsinelli, 1985; Stein-Behrens et al, 1994), there is reason to believe that stress could exacerbate the neural consequences of 2VO in rats.

Stress

Stress and Glucocorticoid Secretion

A major component in the physiological response to stress is the increased secretion of glucocorticoid (GC) hormones. Glucocorticoids (cortisol in humans, corticosterone in rats) bind to intracellular mineralcorticoid (MR) and glucocorticoid (GR) receptors. The hippocampus is identified as having the largest concentration of MR and GR and is largely involved in regulating glucocorticoid secretion. The hippocampus regulates a negative feedback loop consisting of the hypothalamus, the pituitary, and the adrenal cortex. In the event of stress, hippocampal glucocorticoid binding serves to inhibit further hypothalamic release of corticotrophin-releasing factor (CRF) and vasopressin, decreases pituitary secretion of adrenal-corticotrophin hormone (ACTH), and ultimately, reduces glucocorticoid secretion from the adrenal cortex. Given the regulatory role it plays on the hypothalamic-pituitary-adrenal (HPA) axis, damage to the hippocampus results in increased glucocorticoid levels and, ultimately, increased neurodegeneration (reviewed in Seckl & Olsson, 1995)

Stress and Aging

Recent evidence suggests that chronic GC up-regulation, such as that observed as a result of stress, accelerates the aging process in the CNS (Kerr et al, 1991; Sapolsky et al, 1985c; Endo et al, 1999). To test the hypothesis that increased GC levels promote CNS aging, Sapolsky et al (1985c) investigated the potential for corticosterone-induced hippocampal neurodegeneration similar to that observed in the aged rat brain. To this end, adult male rats received acute (2 weeks) or chronic (3 months) corticosterone (CORT) injections. Prolonged CORT administration decreased the number of hippocampal corticosterone receptors (CORT-R), as determined by autoradiography. Both acute and chronically injected rats reached a maximal depletion of 55% of their CORT receptors. However, with a prolonged recovery time following CORT administration, acute rats returned to control level of CORT receptors, while chronic rats maintained a reduced level (Sapolsky et al, 1985c).

Chronic-CORT and aged rats showed similar neuropathology. Specifically, a similar pattern of CA3 cell loss was reported for aged and chronic rats with both groups exhibiting fewer large CA3 cells. Furthermore, the chronic group had an increase in smaller CA3 hippocampal cells with the morphological characteristics of glia, similar to what had been observed in aged rats. CORT treatment caused similar reductions in CORT receptors in the CA1 for acute and chronic rats. These results are suggestive of accelerated CNS aging due to increased GC circulation (Sapolsky et al, 1985c).

Further evidence in support of GC-potentiated CNS aging was gathered by Kerr et al (1991). These researchers tested the hypothesis that chronic stress, and by association,

elevated CORT, accelerates the electrophysiological and anatomical markers of aging by comparing the frequency potentiation (a measure of synaptic response), excitatory post-synaptic potential (EPSP) threshold, and EPSP amplitudes in young, middle-age, and old control and stressed rats. Stressed rats were trained for six months on a shuttle-escape paradigm with mild footshock.

Significant stress-induced acceleration of CA1 pyramidal cell loss was only observed in the aged rats compared to aged controls, and not in younger rats. In young and middle-aged groups, stress reduced population and EPSP thresholds to the level of aged controls. No significant differences were observed between the two aged groups. These results confirm the hypothesis that stress accelerates the electrophysiological and morphological changes characteristic of brain aging. This study has important implications with regard to early neurodegeneration among the middle-aged exposed to chronic stress, as well as elderly persons exhibiting AD features due to neuronal loss as a result of stress-induced CORT elevation (Kerr et al, 1991).

While the above studies provide evidence for a stress-induced acceleration of the aging process, they do not provide any evidence regarding the neurological mechanism. With this goal, Endo et al (1999) investigated the effect of chronic stress on local cerebral blood flow (LCBF). Adult male rats were assigned to stress or control groups. Stressed rats were immersed in 4°C water for 15 minutes daily for 12 weeks. LCBF was measured 3 months after the stress exposure by means of hydrogen clearance in free moving rats. LCBF was measured every hour for 24 hours. Significant variability was recorded in control rats over the 24-hour period, with higher LCBF recorded during the dark (active) cycle. However, no significant diurnal fluctuations in LCBF were reported for the

stressed rats. Furthermore, the absence of a significant increase in LCBF during the dark cycle coincided with the significant pathology in stressed rats. Specifically, stressed rats had fewer cells and an increase in shrunken cells in the CA2 and CA3 regions of the hippocampus. Three conclusions may be drawn from these results. First, stress inhibits LCBF increases during the dark cycle. Secondly, the stress-induced decrease in LCBF results in permanent hippocampal pathology leading to a decrease in neuronal activity, which perpetuates the observed reduction in LCBF. And finally, the researchers conclude that the decrease in LCBF in the dark cycle is due to an inhibited response to environmental cues as a result of hippocampal pathology (Endo et al, 1999). LCBF is reduced in the aging brain (Tsuchiya et al, 1992), therefore, exposure to chronic stress may accelerate CNS aging.

Further evidence regarding the effect of stress on the aging process comes from studies investigating the neurodegenerative mechanisms of Alzheimer's disease (AD). Stress has been identified as a risk factor in the progression of AD (Kropiunigg et al, 1999). For instance, higher cortisol levels have been reported in AD patients with definite cognitive impairment (Weiner et al, 1997). Recent evidence suggests that the hypothalamic-pituitary-adrenal (HPA) axis, a stress response system, may play a key role in the enhanced neurodegeneration associated with AD. Based on the hypothesis that exposure to an aversive stimulus would increase HPA activity in AD patients above and beyond that of elderly controls, Pascualy et al (2000) measured the endocrine changes of AD patients and elderly controls in response to the Cold Pressor Test (CPT). Prior to the CPT, two baseline plasma samples were collected. For the CPT, subjects placed one hand in 4°C slushy ice water for one minute. Plasma samples were again collected

following the CPT. While no differences were observed in the ACTH response to CPT, a significant increase in CORT following CPT was observed in AD patients, compared to controls. Furthermore, a significant negative correlation was reported between scores on the Mini-Mental State Exam (MMSE) and CORT levels suggestive of greater HPA suppression in patients in the more advanced stages of AD. Since patients in more advanced stages of AD have higher CORT levels, arguably this exacerbates neurodegeneration and lowers MMSE scores (Pascualy et al, 2000).

Stress, Glucocorticoids and Hippocampal Pathology

As previously described, a key feature of the stress response is the secretion of adrenal steroids or glucocorticoids. A large body of research investigating the neurodegenerative consequences of prolonged stress exposure focuses on the neuropathological significance of corticosterone administration in the rat. CORT injections are believed to produce comparable electrical and chemical neurological states to those observed following stress. Furthermore, GCs are believed to promote hippocampal vulnerability to various insults (Sapolsky, 1985a, 1985b).

As evidence for this, Sapolsky (1985a) examined GC-induced neuronal vulnerability in adrenalectomized (ADX) and control rats who were infused with the neurotoxin 3-acetylpyridine (3AP) which served to inhibit ATP. On its own, 3AP infusion caused significant hippocampal damage. However, 3AP infused rats with CORT replacement therapy exhibited a significant increase in total hippocampal pathology. Maximal neuropathology was reported in rats receiving CORT replacement both before and after 3AP infusion. In both instances, hippocampal damage was

restricted to the dentate gyrus. A trend toward a decrease in total hippocampal pathology was observed in ADX rats, thus implicating GCs in the enhanced hippocampal pathology. Based on the results of this study, Sapolsky (1985a) hypothesized that the GC-induced inhibition of glucose uptake leaves neurons vulnerable to various insults.

GCs have been found to inhibit glucose transport in hippocampal neurons and glia (Armanini et al, 1990; Horner et al, 1990; Virgin Jr. et al, 1991). For instance, in a study investigating whether GCs inhibit glucose transport in cultured hippocampal cells, Horner et al (1990) report a 33% reduction of glucose transport in mixed cultures (neurons and glia). Pure neuronal and glial cultures exhibited a 26% and 21% inhibition of glucose transport, respectively. Moreover, no inhibition was observed following treatment with other steroids (e.g. testosterone, estradiol) suggesting that the effect was specific to CORT receptors. Treatment with a Type II corticosterone receptor antagonist overturned the inhibition suggesting that the inhibition is mediated by Type II CORT receptors (Horner et al, 1990).

Subsequently, to test his hypothesis that GC-induced inhibition of glucose uptake leaves neurons vulnerable to various insults, Sapolsky (1985b) examined the effect of prolonged GC exposure on hippocampal vulnerability to excitotoxicity and neurotoxicity. To this end, adult male rats were assigned to ADX, intact, or CORT treatment groups. In the first experiment, rats were unilaterally infused with the excitotoxin kainate acid (KA) in the hippocampus, cerebellum, and hypothalamus. In the second experiment, rats were unilaterally infused with 3AP in the same regions. KA-induced hippocampal damage was limited to the hippocampus, with the majority of the pathology observed in the CA3 region. As in the previous study, ADX reduced hippocampal pathology, while CORT

treatment potentiated the damage in the CA3b region. 3AP infusion produced damage in the DG, which was potentiated by increased CORT levels. The results of this study suggest that the region of CORT-potentiated damage is dependent upon the toxin.

Sapolsky (1985b) concluded that CORT does not potentiate the toxin itself, as significant increases in neuropathology were not observed in the cerebellum or hypothalamus following the infusion of either toxin. Rather, the majority of KA-induced damage was observed in the CA3 region, an area with a large concentration of both KA and CORT receptors. As such, it is concluded that GCs act in a specific manner to potentiate the vulnerability of specific neuronal populations (i.e. those with CORT receptors) (Sapolsky, 1985b).

Given the results of the previous studies, Sapolsky (1986) investigated the hypothesis that GCs potentiate neuronal vulnerability to insult by reducing cellular energy metabolism. As in the previous two experiments, rats were unilaterally infused with the neurotoxin 3AP. In the first experiment, one group of rats received CORT injections 24 hours before and after 3AP infusion. Three other groups were co-administered CORT with glucose, mannose, or fructose. In the second experiment, rats were given daily CORT injections for 3 days after 3AP infusion. The final experiment differed from the first experiment only in that the rats were infused with KA, rather than 3AP, and they had also undergone prior ADX (Sapolsky, 1986).

Consistent with earlier studies (Sapolsky, 1985a, 1985b), 3AP and KA infusion produced damage in the dentate gyrus and the CA3 regions, respectively, which was potentiated by CORT. However, the damage was attenuated by sugar supplementation, with mannose producing the greatest decline in hippocampal damage, followed closely

by glucose. These results confirm the hypothesis that GC render hippocampal neurons vulnerable to insult by reducing their energy metabolism (Sapolsky, 1986).

Moghaddam et al (1994) confirmed Sapolsky's (1986) reports of GC-mediated metabolic alterations and subsequent increases in pathology in the hippocampus.

Moghaddam et al (1994) sought to determine the mechanism of GC-induced hippocampal pathology by examining the effect of GC on circulating glutamate concentration after twenty minutes of restraint stress in ADX rats. Microdialysis samples taken every twenty minutes over a period of 2 hours following stress exposure revealed a significant increase in extracellular glutamate levels in the hippocampus following stress in control and ADX + CORT rats. Removal of GCs altogether by ADX attenuated the increase in glutamate (Moghaddam et al, 1994). These results suggest that stress and, therefore, GCs, affect levels of glutamate in the hippocampus, and since glutamate can have excitotoxic effects at higher-than normal levels, this may explain how stress renders hippocampal neurons susceptible to damage by altering their metabolic state.

All of the above mentioned studies examine the effect of GC administration on hippocampal viability, rather than the direct effect of stress. In order to address the effect of stress itself on hippocampal pathology, as opposed to a pharmacologically-induced stress-like state, Stein-Behrens et al (1994) compared hippocampal damage in CORT injected rats with chronically stressed rats, all of whom received KA infusions. The chronic stress regime consisted of twelve hours in a 4°C room, a 30-minute tube restraint, 30 minutes on a cage on a rotator plate, and introducing unfamiliar rats into cages. Consistent with previous studies (Sapolsky, 1985b, 1986), increased CORT levels potentiated CA3 KA-induced damage. Interestingly, the most significant damage was

observed in the CA3 region of the stress-KA group, as assessed by counts of healthy cresyl-violet stained cells. Using the same method, 25% of the rats in the stress-KA group were found to have a reduced number of viable CA1 pyramidal cells suggesting that, as well as increasing CORT levels, stress endangers hippocampal neurons.

The finding that stress-KA rats also exhibited CA1 damage raises questions regarding corticosterone potentiation of hippocampal neurodegeneration (Stein-Behrens et al, 1994). Ischemia-induced neurodegeneration is largely present in the hippocampus, specifically in the CA1 field (Ni et al, 1994; Pappas et al, 1996; Bennett et al, 1998; de Butte et al, 2002). Moreover, CA1 hippocampal damage is also found in Alzheimer's disease (AD), a neurodegenerative disease associated with aging. Therefore, stress and the consequent increase in GC levels may potentiate the neural consequences of ischemia and may be pathogenic to AD (Stein-Behrens et al, 1994).

Stress and Ischemia

Sapolsky and Pulsinelli (1985) examined the effect of GCs on hippocampal neurons in an ischemic-reperfusion model. Following 20 minutes of four-vessel occlusion, rats were assigned to a CORT, ADX, or intact group. The CORT group received corticosterone injections resulting in CORT levels similar to those observed following major stress. Prolonged CORT elevation resulted in significant increases in hippocampal damage in the subiculum, CA3, CA4, and neocortex, as compared to control. In contrast, a significant reduction in hippocampal damage was reported in the subiculum, CA1, CA4, dentate gyrus, and striatum of ADX rats compared to control rats, suggesting that the removal of circulating GCs is neuroprotective in ischemia (Sapolsky

& Pulsinelli, 1985). It is noteworthy that GCs are used in stroke to reduce swelling caused by membrane impairment (Qizilbash et al, 2002) As well, GCs have been proposed as a pharmacological means by which to treat inflammatory mechanisms associated with Alzheimer's disease (Aisen, 1996). Clearly, these data of Sapolsky and Pulsinelli (1985) contraindicate this use of GCs.

Stress and Glial Fibrillary Acidic Protein (GFAP)

Astrocytes are involved in the uptake, storage, synthesis, and redistribution of glutamate (Feldman et al, 1997). Research suggests that glucocorticoids and stress inhibit the expression of glial fibrillary acidic protein (GFAP) (Maurel et al., 2000; O'Callaghan et al., 1991; O'Callaghan et al., 1989). GFAP is the primary protein of astrocytes in the mature CNS (Maurel et al, 2000). Alteration of GFAP expression may, therefore, have neural consequences given the supportive role that astrocytes play in the intact brain. In order to deduce the effect of glucocorticoids on GFAP expression, O'Callaghan et al (1989) examined the effects of exogenous corticosterone, adrenalectomy, and adrenalectomy with corticosterone replacement on GFAP expression in the adult rat. Rats were given daily corticosterone treatments for 12 consecutive days. Control rats were simply given the corticosterone vehicle, sesame oil. Rats were subsequently sacrificed on days 1, 7, 14, or 21 post-treatment. GFAP expression was determined by means of immunoassay. The results of the immunoassay suggest that increased corticosterone levels down-regulate GFAP expression. Rats sacrificed one day post-treatment had significantly lower GFAP levels in the hippocampus, frontal cortex, striatum, and cerebellum than did rats sacrificed on days seven through twenty-one.

GFAP levels in the hypothalamus did not return to normal levels until 14 days post-treatment.

To determine whether the removal of glucocorticoids increases GFAP expression, adult male rats were assigned to control, adrenalectomy (ADX), and adrenalectomy with corticosterone replacement (ADX + CORT) groups. ADX + CORT animals were given 12 days of corticosterone replacement post-surgery. All animals were sacrificed 3 weeks post-surgery. Compared to shams, ADX rats had an increase in GFAP expression in the hippocampus and the frontal cortex of 46% and 57%, respectively. ADX + CORT animals had a significant decline in GFAP expression in both the hippocampus and the frontal cortex at one day post-treatment, compared to shams (O'Callaghan et al., 1989).

In a follow-up study, O'Callaghan et al (1991) sought to characterize GFAP expression in sham and adrenalectomized rats, to assess whether other astrocytic proteins and structures are affected by glucocorticoids, and to examine whether glucocorticoid-mediated GFAP expression is further altered following brain injury. Rats underwent vehicle or corticosterone treatment for a duration ranging from one day to four months, and were assigned to the sham or ADX condition, as well as a sham or traumatic brain injury (TBI) condition. TBI was induced by one of two methods: stab wounds to the frontal cortex or injection of the hippocampal-lesioning neurotoxin trimethyltin (TMT). The results of the study show a 20% and 14% decrease in GFAP concentrations in intact rats in the hippocampus and frontal cortex, respectively, following 5 days of corticosterone treatment. Larger decreases in GFAP concentration were achieved by increasing both the duration and the quantity of corticosterone in both the hippocampus and the cortex. Maximal down-regulation of GFAP expression was observed following 3

weeks of CORT therapy at higher doses with decreases reaching up to 46% and 49% in the hippocampus and cortex, respectively.

ADX caused 20% and 27% increases in GFAP concentrations in the hippocampus and cortex, respectively, of ADX rats. Three weeks after ADX, a 184% increase in hippocampal GFAP was observed compared to shams. In contrast, a decrease in GFAP concentration was observed among ADX rats having received CORT replacement therapy.

O'Callaghan et al (1991) examined the effect of TBI, in conjunction with CORT treatment, on GFAP concentrations. As hypothesized, TBI caused significant increases in GFAP expression. The addition of corticosterone therapy, however, did not alter the GFAP results observed following TBI. This finding suggests that while TBI increases GFAP expression and corticosterone treatment decreases GFAP concentrations, they do so by different mechanisms (O'Callaghan et al, 1991).

Finally, due to the effect of corticosterone on GFAP expression, the researchers examined the effect of CORT on two other astrocytic proteins, S-100 and glutamine synthetase. While no differences were observed between groups for S-100, a small non-significant increase in glutamine synthetase was observed following CORT treatment. In addition, ADX decreased glutamine synthetase in the hippocampus and the cortex.

More recent research has confirmed O'Callaghan et al's (1989 & 1991) reports of glucocorticoid mediation of GFAP expression in the hippocampus. The inhibitory action of corticosterone on GFAP expression in the hippocampus has become so well established that it has been used as a control measure to which the effect of corticosterone on GFAP expression in the suprachiasmatic nucleus (SCN) of the hypothalamus has been

compared (Maurel et al, 2000). Maurel and his colleagues examined the effect of corticosterone treatment on GFAP expression in the hippocampus and SCN of sham and ADX rats. They report prominent staining the ventrolateral SCN, the floor of the third ventricle, and the paraventricular nucleus (PVN) compared to a down-regulation of GFAP staining in the hippocampus, particularly in the dentate gyrus and CA1-CA3 regions, following CORT replacement in ADX rats. In ADX rats with no CORT replacement a 28-34% decline in GFAP staining was reported in the SCN and PVN, compared to a 50-70% increase in GFAP expression in the dentate gyrus of the hippocampus. As a result, the researchers propose that the effect of glucocorticoids on GFAP expression may be regionally specific.

Brain-Derived Neurotrophic Factor (BDNF)

Ischemic and stress conditions have both been identified as neural antagonists. In addition to inducing hippocampal neurodegeneration, both conditions serve to alter the brain's protective mechanisms, including brain-derived neurotrophic factor. Brain-derived neurotrophic factor (BDNF), along with nerve growth factor (NGF), neurotrophin 3 (NT-3), and neurotrophin 4 (NT-4), belongs to a class of neurotrophic factors called neurotrophins. Neurotrophins are associated with a group of tyrosine kinase receptors (trkA, trkB, trkC receptors). BDNF interacts specifically with trkB. While each neurotrophin interacts with high affinity to at least one of the trk receptors, all neurotrophins interact with the low-affinity receptor p75. To date, the neural function of neurotrophins has been defined as one of differentiation and survival (Kandel, Schwartz, & Jessell, 2000).

Recent research investigating the localization of BDNF in the brain reveals its widespread distribution. Kawamoto et al (1996) conducted an immunohistochemical study to determine the role of BDNF in the adult rat brain. To this end, adult male rats were perfused and their brains removed, sectioned and processed for BDNF immunohistochemistry. Intense BDNF neuronal staining was observed in the cerebral cortex, the granule and pyramidal cells of the hippocampus, and the amygdaloid nucleus.

While previous studies have focused on the localization of BDNF mRNA, Yan et al (1997) conducted a study with the purpose of localizing BDNF protein and its cellular location in normal rats and in rats administered kainate acid (i.p.). Their brains were processed for immunohistochemistry 7 or 18 hours post-injection. BDNF immunohistochemistry was completed using an anti-BDNF antibody with less than 0.5% cross-reactivity with other neurotrophins. Similar to Kawamoto et al (1996), Yan et al (1997) found a widespread distribution of neural BDNF protein. Of particular interest is the localization of BDNF protein in the cerebral cortex, hippocampus, and amygdala. The hippocampus was observed to have the most intense staining, compared to the rest of the brain. Staining was observed in the dentate gyrus, hilus, CA2 and CA3 regions, but not in the CA1. However, CA1 BDNF protein staining was observed in the kainate-injected rats suggesting an up-regulation of BDNF protein in the CA1 following ischemic insult. Baseline CA1 BDNF protein levels, however, appear to be too low for immunohistochemical detection. The finding that CA1 BDNF protein levels were significantly increased following kainic acid treatment confirms the hypothesis the BDNF protein plays a key role in neuronal survival and plasticity (Yan et al, 1997). BDNF protein expression has been observed to differ according to the nature of a neural insult.

For instance, BDNF protein and mRNA levels are reported to significantly increase following an ischemic episode (Kirprianova et al, 1999; Kokaia et al, 1995; Zhao et al, 2001; Cheng & Mattson, 1994), whereas BDNF levels decrease in response to increased glucocorticoid levels, such as those associated with stressful experience (Smith et al, 1995; Nitta et al, 1997).

BDNF and Ischemia

Kokaia et al (1996) conducted a study on BDNF and ischemia with 3 objectives in mind. The first was to quantify basal levels of hippocampal and cortical BDNF protein levels in comparison with BDNF mRNA levels. The second objective was to assess whether changes in BDNF mRNA levels produce a corresponding change in BDNF protein levels following ischemia. Finally, the researchers sought to assess changes in BDNF protein levels in hippocampal and cortical regions that typically exhibit ischemic damage.

To this end, cerebral ischemia was induced for 10 minutes by occluding the common carotid arteries in combination with hypotension. BDNF mRNA was quantified by in situ hybridization in rats sacrificed 1, 2, 4, and 24 hours after recirculation and BDNF protein levels were quantified by means of immunoassay in rats sacrificed after 6, 12, 24 hours and one week of recovery. Under basal conditions, hippocampal BDNF mRNA levels were the highest in the dentate gyrus and CA3 pyramidal cells and lower in CA1 and cortical regions. Following 10 minutes of transient ischemia, significant increases in BDNF mRNA levels were observed in the dentate gyrus and CA3 hippocampal areas with a peak in levels at 2 hours post-ischemia. No significant

differences were observed in CA1 or cortical regions. Similar regional differences were observed for BDNF protein with the dentate gyrus expressing the greatest amount of protein, followed by the CA3. There was a significant increase in DG BDNF protein at 6 hours post-ischemia, with a return to basal levels thereafter. Significant increases in CA3 protein levels were also noted at one week post-ischemia. In contrast, significant decreases in BDNF protein levels were observed in CA1 and cortex regions, reaching a peak at 24 hours post-ischemia and a return to basal levels by the one-week time point.

The results of this study suggest that BDNF is neuroprotective against ischemia. The neocortex, and more specifically, CA1 hippocampal regions are more susceptible to ischemic damage than are the CA3 and DG hippocampal regions. Lower levels of BDNF protein are observed in CA1 and neocortical regions following transient ischemia (Kokaia et al, 1996).

To explicitly assess the neuroprotective properties of BDNF in ischemia, Kirprianova et al (1999) examined the effect of intraventricular administration of BDNF after global ischemia and reperfusion. Global ischemia was induced by four-vessel occlusion wherein the vertebral arteries were coagulated and the common carotid arteries were occluded for 15 minutes before reperfusion. In BDNF-treated 4-VO rats, BDNF was diluted in artificial cerebrospinal fluid and administered over the course of 7 days by an osmotic pump. Animals were sacrificed at 24, 48, 96 hours, and 7 days post-ischemia. Global ischemia produced delayed neurodegeneration primarily in the CA1 region, with minimal cell loss observed in CA3 and CA4 areas. Degeneration was first observed 48 hours post-ischemia and reached a peak of 95.8% CA1 cell loss by 7 days post-ischemia, compared to controls. By the 48 hours time point, GFAP up-regulation was evident in

the CA1 region. Of particular interest, however, is the finding that BDNF administration inhibited cell loss in all hippocampal regions, as well as GFAP hypertrophy. Taken together, these results support the hypothesis that the neurotrophin BDNF plays a large role in hippocampal neuron survival following an ischemic event.

The neuroprotective properties BDNF and may be due to its ability to stabilize intracellular calcium (Ca^{2+}) concentrations (Cheng and Mattson, 1994). Cheng and Mattson (1994) applied glucose deprivation to six to fifteen day old hippocampal, septal, and cortical cell cultures derived from 18-day-old rat fetuses. BDNF, NT-3, and NGF were added to some cultures 24 hours before and after the onset of hypoglycemia. Neuronal survival was also assessed 24 hours before and after the onset of hypoglycemia. The effect of neurotrophins on glutamate neurotoxicity was also investigated by exposing the cell cultures to increased levels of glutamate. Intracellular free calcium levels were determined by means of a ratio imaging of calcium indicator dye. Finally, immunocytochemistry was used to determine trk B and trk C receptor sites.

The study revealed a 90-95% reduction in cell viability in the glucose-free cultures. Furthermore, NT-3 and BDNF pretreatment significantly increased cell survival by 60% and 70-80%, respectively in the hypoglycemic cultures. The percent neuronal loss was dependent on neurotrophin concentration. Similar results of BDNF and NT-3 neuroprotection were observed in glia-free cultures suggesting that the neuroprotective effect is not mediated by glia (Cheng & Mattson, 1994). BDNF and NT-3 also attenuated glutamate-induced neuronal damage.

In support of the initial hypothesis that calcium influx largely mediates excitotoxic injury, hypoglycemic cell cultures maintained in a stable calcium-deficient

medium had less damage compared to controls (regular calcium medium). BDNF and NT-3 attenuated the resulting 3-5 times increase in intracellular calcium concentrations in hippocampal and septal neurons. Neurotrophin pre-treated cultures also had decreased Ca^{2+} levels following glutamate exposure (Cheng & Mattson, 1994).

BDNF and Stress

As reviewed earlier, stress and glucocorticoids have been found to induce hippocampal pathology (Sapolsky et al, 1985a, 1985b, 1985c, 1986; Kerr et al, 1991; Moghaddam et al, 1994; Stein-Behrens, 1994). In an attempt to deduce the manner in which chronic stress and glucocorticoids induce hippocampal pathology, Smith et al (1995) examined the effect of chronic restraint stress and glucocorticoids on neurotrophic factors and their receptors in the hippocampus. Immobilization stress was employed for two hours per day for one or seven consecutive days. Animals were sacrificed immediately or 24 hours following the stress procedure. As well, some animals received a CORT or saline injection for one or seven consecutive days to examine the effect of exogenous glucocorticoids on neurotrophin expression. In situ hybridization revealed large amounts of BDNF mRNA in the CA2, CA3, CA4, and, in particular, the dentate gyrus of non-stressed controls, with lower levels observed in the CA1. In contrast, stressed rats were reported to have a 75% decrease in BDNF mRNA in the dentate gyrus, with no differences between rats receiving one or seven days of restraint stress. Rats sacrificed 24 hours following the stress regime were observed to have BDNF levels falling between those of stress and control rats, suggesting that BDNF levels begin to return to normal once endogenous corticosterone levels return to baseline.

In contrast to BDNF expression, significant increases in NT-3 expression were observed following 7 days, but not one day, of restraint stress in the CA1 and CA2 hippocampal regions. Corticosterone injections resulted in a 65-75% decrease in BDNF mRNA in the DG, compared to controls. Again, significant increases in NT-3 were observed in the DG and hippocampus only following seven days of CORT injections, compared to controls. ADX did not produce any significant changes in BDNF mRNA, but did result in a significant decrease in NT-3 in the DG and, especially in the CA2 and CA3. Interestingly, ADX, combined with immobilization stress, produced a decrease in BDNF mRNA. However, ADX, in combination with restraint stress and CORT replacement produced significantly larger decrease in BDNF mRNA in the hippocampus. The results of this study indicate that chronic stress, but more specifically, corticosterone, inhibits BDNF mRNA expression in the hippocampus and supports the idea that stress-induced decrease of BDNF plays a role in hippocampal pathology (Smith et al, 1995).

Current Study

No previous studies, to our knowledge, have examined the effect of chronic mild stress on the delayed progressive neurodegeneration observed following permanent bilateral carotid artery occlusion. As previously mentioned, the 2VO model produces progressive behavioral and neural deficits. Recent studies have established that cerebral hypoperfusion characterizes mild cognitive impairment (MCI), which is considered to be the pre-clinical phase of AD (Huang et al, 2002). Similar to AD, the 2VO model produces capillary degeneration, decreased glucose utilization, and reduced energy metabolism (de la Torre, 2002).

Studies suggest that chronic stress and increased glucocorticoid levels are involved in accelerating the neural aging process (Sapolsky et al, 1985c; Kerr et al, 1991; Endo et al, 1999). Additionally, patients in the advanced stages of AD have higher concentrations of circulating GCs (Pascualy et al, 2000), suggesting that stress may exacerbate hippocampal neurodegeneration induced by chronic cerebral hypoperfusion. Consequently, it is important that the neurobehavioral effects of stress be examined in an animal model that mimics the early stage of AD. The current study was conducted in order to determine the neural consequences of exposure to chronic mild stress in a model of chronic hypoperfusion in the rat. It was predicted that chronic mild stress would exacerbate the neural consequences of bilateral carotid artery occlusion.

Chronic Mild Stress

The chronic mild stress model (CMS) was initially developed to serve as an animal model of depression. It has since been used to test the effectiveness of new and traditional forms of antidepressants and to facilitate further investigation of the pathogenesis of depression (Willner, 1997). To date, variations on the CMS model have been used to investigate the effect of stress on CNS aging (Kerr et al, 1991), local cerebral blood flow (Endo et al, 1999), hippocampal cell loss (Stein-Behrens et al, 1994), hippocampal CA3 dendrite atrophy (Magarinos & McEwen, 1995), and immune response (Shanks et al, 1994), to name a few.

In some of the aforementioned studies, the chronic stress model was composed of a single stressor. For instance, Endo et al (1999) report a reduction in LCBF 3 months after 12 weeks of daily immersion in 4°C water for 15 minutes. In contrast, the effects of

a variety of stressors over only 3 days on hippocampal neuronal vulnerability were examined by Stein-Behrens et al (1994). These stressors included 12 hours in a 4°C room, tube restraint, ether exposure, placement of cage on a rotator plate, and exposure to novel animals.

According to Willner (1997), there are two essential elements to a valid model of chronic mild stress. Essentially, the stressors must be mild and not severe and repeated exposure to random stressors must not result in habituation. The development of a stress regime first involves the selection of the stressors. There are three types of stressors. The first type, psychogenic stressors, are stressors of a psychological nature (e.g. predator odor, novel environment) and do not involve any physical manipulation to the subject. In contrast, neurogenic stressors consist of physically aversive stimuli (e.g. footshock, tail shock). The third type of stressor is a systemic stressor, which involves the manipulation of endogenous functions (e.g. hormone replacement, drug therapy), (Anisman et al, 1997).

In order to be able to extrapolate our results from rats to humans, we have selected primarily psychogenic stressors, given the fact that psychogenic stressors are those stressors most typically chronically experienced by humans. While these stressors do involve some physical manipulation, they are considered to be psychogenic in that they are not pain inflicting (Anisman, et al, 1997).

Methods

Animals

48 male rats, aged 8 months, were obtained from Harlan-Spague-Dawley (Indianapolis, IN). Upon arrival, the rats were individually housed in a 12-hour light/dark cycle. Food and water were available ad libitum. Following a one-week habituation period under quarantine, the animals were moved to a separate housing room. All animals were handled for 5 minutes for 2 days prior to surgery.

Permanent Bilateral Common Carotid Artery Occlusion (2VO)

All animals were randomly assigned to one of two treatment groups: sham (N=24) and 2VO (N=24). All rats were anaesthetized with 2% halothane. The 2VO surgery consisted of a ventral midline incision to bilaterally expose the carotid arteries. The carotid arteries were then carefully dissected away from the vagus nerve and surrounding tissues. The carotid arteries of the 2VO animals were permanently doubly ligated with 5-0 silk. Animals in the sham treatment group underwent the same surgical procedure with the exception of the carotid artery ligation. Prior to the commencement of the chronic mild stress regimen, all animals were given 7 days of recovery.

Chronic Mild Stress Regimen

By 7 days of post-operative recovery, the total number of rats decreased from 48 to 38, due to surgical complications. At this time, the 2VO and sham animals were randomly assigned to one of four treatment groups: non-stress sham (N = 9), non-stress 2VO (N = 9), stress sham (N = 10), and stress 2VO (N = 10). All animals in both stress

treatment groups were housed separately from the non-stressed animals, under the same conditions.

Stress sham (SS) and stress 2VO (S2VO) animals underwent a six-week chronic mild stress regimen. During the six-week period, stress exposure was limited to six days a week. On each given day, all animals were exposed to one of eight possible stressors. Stress conditions were randomly assigned such that each stressor was experienced once within an eight-day span. The stress model consisted of:

Physical restraint: the rats were placed in a semicircular plastic bag, typically used for decapitations, which serves to inhibit physical movement. A hole in the small end of the bag permitted the protrusion of the rat's nose. Once inside the bag, the larger end was firmly secured and, in order to prevent injury, the base of the rat's tail was taped down to the counter. Animals underwent this procedure for 15 minutes in groups of 5, after which they were returned to their housing room.

Saline injection: the rats were injected with 0.2 ml of 9% saline solution (i.p.) once in the morning and once in the afternoon. The animals were returned to their home room between injections.

Open field: Animals were placed in the corner of an 80 X 80 X 60.5 cm large gray Plexiglas open field box for a period of 10 minutes, during which, they were free to roam about the novel environment..

Fox odor: In order to create a stressful experience in a familiar environment, animals were exposed to predator odor (fox urine) in their home cages. Fox urine was placed on a Q-tip and, using a 50ml syringe, was administered to the animals

by means of air puff once every minute for 5 minutes in a testing room. Animals were exposed to this stressor in groups of 5, once in the morning and once in the afternoon. Animals were returned to their home room between stressors.

Cat odor: Animals were transferred to the testing room in their home cages. They were then placed in individual empty cages containing cat feces in the corner for a period of one hour. Animals were exposed to this stressor twice on a given day, once in the morning and once in the afternoon, in groups of 10. Animals were returned to their home cages and home room between stress exposures.

Plus Maze: Rats were placed in the center of a raised platform (50 cm) consisting of four arms (52 X 12 cm). Forty-centimeter high opaque white polyvinyl chloride walls enclosed two of the arms, whereas the remaining two arms were open. For the purposes of this experiment, entry into the 2 enclosed arms was prevented by means of 2 clear Plexiglas sheets. This prevented animals from retreating into an enclosed arm, thereby ensuring the same experience for all rats. The animals were free to roam about for the duration of 10 minutes.

Food Deprivation (water ad libitum): Animals were completely deprived of food for 16 hours (6 a.m. to 10 p.m.).

Forced Swim: This stressor involved the placement of the animal in a large white polypropylene pool (1.6 meters in diameter) filled 15 cm from the top with tepid water for 3 minutes. This was repeated 3 times at one minute intervals.

Startle: This stressor was conducted on the second last day of the stress regimen. The startle stressor involved the placement of the rat in a closed plastic (10.1 X 19.0 cm) cylinder, which was, in turn, attached to a startle platform and enclosed

in a 30 X 55 X 50 cm sound proof box. Loud, random bursts of sound were projected into the box to elicit a startle response whose resulting pressure change on the startle platform is ultimately recorded using Startle Reflex Software (MED Associates). Baseline startle responses were recorded during the first of two blocks of testing wherein 5 baseline startle stimuli (115 dB) with an intertrial interval of 50ms were presented. Additionally, 45dB of continuous background white noise was projected into the startle boxes to eliminate any exterior stimuli. The second block of testing was composed of 32 trials. The 32 trials, with an intertrial interval of 30ms, consisting of 4 tones ranging from 85-115 dB, which were randomly, presented 8 times each. The entire startle stress condition had a duration of 20 minutes. Animals were then returned to their home cages.

In addition to the above, on the final day of the stress regimen, SS and S2VO animals received 2 injections (50mg/kg) of bromo-deoxyuridine (Sigma) with a 4 hour delay between injections. Animals were sacrificed 24 hours following the first injection. This was for another project, the results of which are not reported here.

Histological Procedures

On the day of sacrifice, all animals were deeply anaesthetized with somnotol. Rats were transcardially perfused with 75mls of heparinized saline followed by 500 mls of 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Upon removal, the brains were cut coronally to remove the cerebellum and the frontal lobes. Secondly, the brains were cut down the midline to separate the 2 hemispheres. Both halves of the brains were

postfixed in the fixative for 24 hours at 4°C. Following postfixation, the right hemisphere was rinsed 0.1 M PB + azide + 10% sucrose, pH 7.4 at 4°C, while the left hemisphere was rinsed in 0.1 M PB + azide, pH 7.4 at 4°C. The first wash was for 4 hours and the second wash was overnight. The right hemisphere was then placed in another change of 0.1 M PB + azide, pH 7.4, this time with 20% sucrose, while the left hemisphere was again rinsed in 0.1 M PB + azide, pH 7.4. All tissues remained in their final rinse at 4°C and were stored until processed.

Tissue Sectioning

1- Paraffin Sections

The left hemisphere was dehydrated in graded alcohols, cleared in clearene and paraffin embedded. Forty micron sagittal sections consisting of the hippocampus were cut using a sliding microtome. In order to view hippocampal pyramidal cells, forty micron sections were stained with Gill's hematoxylin (Surgipath). Briefly, sections were cleared in clearene, rehydrated in graded alcohols, rinsed in distilled water, and incubated for 30 minutes in a 25% Gill's hematoxylin solution (50mls hematoxylin to 150 mls distilled water). The sections were then rinsed in lukewarm water for 30 minutes before being dehydrated, cleared, and coverslipped.

In order to determine astrocyte density, forty micron paraffin sections were also stained immunohistochemically with a glial fibrillary acidic protein (GFAP) monoclonal antibody (Sigma). 3 representative tissue sections from each animal were selected for GFAP immunohistochemistry. These 3 sections were made up of a medial, medial-lateral, and lateral section. Briefly, the sections were cleared, rehydrated in graded

alcohols, and rinsed in 10 mM PBS, pH 7.2 (azide) for 5 minutes. Sections were then incubated in a humidifying plastic box with a monoclonal antibody to GFAP (1:600; Sigma) in a blocking buffer overnight. The next day, following a 15 minute rinse in 10 mM PBS, pH 7.2 (azide), the sections were incubated with a biotinylated anti-mouse Ig secondary (1:100; Amersham) for 2 hours in the humidifying box. Sections were again rinsed in 10 mM PBS, pH 7.2 (no azide) and incubated in a Streptavidin biotinylated horseradish peroxidase complex (1:100; Amersham) 2 hours. A DAB (3,3 diaminobenzidine, Sigma) reaction was performed to visualize the antibody complex. Sections were placed in 0.2% DAB, 0.6% ammonium nickel sulphate, and 0.76 trizma pre-set crystals (pH 7.6) for 5 minutes. Following the DAB presoak, 40 µl of 6% hydrogen peroxide was added to the DAB solution. The tissue sections remained in this solution for 7 minutes after which another 40 µl of hydrogen peroxide was added. After rinsing, the sections were counterstained with 0.085% pyronin Y in a 0.05N acetate buffer, pH 4.8. Slides were then dehydrated, cleared and coverslipped. GFAP density was quantified with a light microscope at a 20X magnification using the MCID Imaging program

In order to quantify degenerating cells, 2 representative paraffin sections (medial & medial-lateral) were selected for Fluoro-Jade staining. Briefly, sections were cleared and placed in 2 rinses of 100% absolute ethanol (10 & 5 minutes). The slides were then placed in 70% ETOH, 30% ETOH, and distilled water for 1 minute each. Sections were then incubated at room temperature for 15 minutes in a 0.06% potassium permanganate solution, followed by a brief rinse in distilled water. Slides were placed in the Fluoro-Jade B solution (0.001% Fluoro-Jade B in 0.1% acetic acid) for 30 minutes. Finally, the

sections were rinsed in distilled water, dehydrated in graded alcohols, cleared in clearene and coverslipped. Positive fluoro-jade staining was quantified by means of a manual counting technique, wherein fluorescent cells were manually counted using an Olympus BX51 light microscope fitted with an FITC filter. Fluorescing cells were counted under a blue fluorescent light at 40X magnification.

2- Floated Frozen Sections

The right hemisphere of the brain was reserved for frozen sections. The tissue was flash frozen in a cryostat with Histo-freeze (Fisher) for 10-15 seconds just prior to cutting. Coronal sections were cut at 80µm to compensate for shrinkage. Cut sections were placed in 20% sucrose in 0.1 M phosphate buffer, pH 7.4. Sections were then transferred into cryoprotectant and left overnight at 4°C and finally transferred to -20°C for long-term storage.

BDNF Immunohistochemistry

In order to assess brain-derived neurotrophic factor immunoreactivity, 2 representative tissue sections (medial & medial-lateral) from each animal were selected for BDNF immunohistochemistry. On the day before staining, selected sections were removed from the freezer and transferred into 20% sucrose in 0.1 M PB, pH 7.4 in cell culture well plates, and placed in the fridge overnight. On the day of staining, sections were rinsed 2 X 30 minutes in 0.1 M PB, 7.4. Sections were then rinsed 2 X 5 minutes in 10 mM PBS, pH 7.2. After the initial rinses, the sections were incubated for 30 minutes in 3% H₂O₂ to remove endogenous peroxidase. After rinsing in 10 mM PBS, pH 7.2, the

sections were incubated overnight at 4°C with a polyclonal antibody to BDNF (1:250; Chemicon) in a blocking buffer. The following day, the sections were rinsed and incubated with a biotinylated anti-rabbit Ig secondary (1:100; Amersham) for 2 hours. The sections were then rinsed and incubated in a Streptavidin biotinylated horseradish peroxidase complex (1:100; Amersham) 2 hours. A DAB reaction was performed to visualize the antibody complex. Sections were placed in a well plate containing 0.02% 3,3 diaminobenzidine, 0.6% ammonium nickel sulphate, and 0.76 trizma pre-set crystals (pH 7.4) for 5 minutes. Following the DAB presoak, 16 µl of 6% hydrogen peroxide was added to each well of DAB solution. The tissue sections remained in this solution for 7 minutes after which another 16 µl of hydrogen peroxide was added to each well for 7 minutes. After washing, the sections were mounted on gelatinized slides and allowed to air dry overnight. On the third day, the slides were then dehydrated, cleared and coverslipped. BDNF density was quantified for CA1, dentate gyrus, and cortex-amygdala transition zone with a light microscope at a 40X magnification using the MCID Imaging program (St. Catherine's, Ont.). BDNF density in layer 2 of the entorhinal cortex was quantified at a 20X magnification using the same program.

Corticosterone Assay

Plasma samples were collected from each animal at the time of perfusion. Samples were centrifuged at 3.0 RPM and stored at -20°C until processed. Corticosterone quantification was accomplished by means of a rat corticosterone ³H radioimmunoassay kit (ICN Diagnostics). Briefly, sample preparation involved a 1:500 dilution of rat serum by adding 2.5 mls of steroid diluent to 5µl of rat serum. To begin

the assay procedure, 0.6 ml of steroid diluent was added to 10 X 75 mm tubes 1 and 2. 0.5ml of steroid diluent was added to tubes 3 and 4. Corticosterone standards, ranging from 0.025-1.0 ng/0.5 ml, were added in duplicate to tubes 5-16. Diluted serum samples were added to the remaining tubes. All tubes were incubated at 98°C for 10 minutes to denature the corticosterone binding proteins. Once the tubes had returned to room temperature, 0.1 ml of anti-corticosterone was added to all tubes with the exception of tubes 1 and 2. Following the addition of 0.1ml of corticosterone-³H tracer to all tubes, the tubes were mixed and incubated overnight at 4°C. The next day, 0.2ml of cold charcoal-dextran solution was added to all tubes for 20 minutes. Finally, all tubes were centrifuged at 2,500 rpm for 15 minutes, the supernatant extracted and placed into scintillation fluid, and counted in a beta counter for 1 minute.

Stereological and Densitometric Quantification

Quantification of hippocampal CA1 pyramidal cells was accomplished by means of standard stereological techniques (West, Slomianka, & Gundersen, 1991). Using an unbiased systematic sampling technique, every consecutive sixth section containing the hippocampus was selected from a random starting point. Because stereology does not make any assumptions regarding cell size, shape, or orientation, it is a very precise and unbiased method of obtaining highly accurate estimates of total cell number. As a result, changes in section thickness (i.e. shrinkage) due to tissue processing do not affect the estimates of total cell number. Stereology involves the use of a three-dimensional probe, the optical dissector, enabling cell quantification within a given volume. Stereological determination of CA1 estimates of total cell number were carried out using the

StereoInvestigator Software package (Microbrightfield; Chelchester, VT) and a light microscope (Olympus BH-2) at 100X magnification

Densitometry was used to measure hippocampal GFAP and BDNF expression in the hippocampus, amygdala, and entorhinal cortex. Densitometry involved the use of a light microscope (Olympus BH-2) and imaging analysis software (Imaging Research, Inc., St. Catherine's, Ont.) to determine the proportion of area displaying positive immunoreactivity. A threshold was established to detect positive immunoreactivity and was used to determine optical density. All stereological estimates of total cell number and all densitometry measures were carried out by an investigator who was blind to group membership.

Results

Hippocampal Measures

CA1 Pathology

The results are shown in Figures 1 and 2 and Photomicrograph 1. A 2 X 2 statistical analysis of variance (ANOVA) with Stress (Non-stress vs. Stress) and 2VO (Sham vs. 2VO) as between group variables revealed a significant 2VO effect ($F(1,31) = 27.212$; $p > .01$), as well as significant Stress X 2VO interaction effect ($F(1,31) = 5.367$; $p < .05$). Stress 2VO rats had the lowest total CA1 cell numbers (83.3% of controls), followed by Non-stress 2VO (92.8% of controls). A one-way ANOVA with Group (SS, S2VO, NSS, NS2VO) as the independent variable and CA1 cell counts as the dependent variable and subsequent post-hoc analysis (Tukey) revealed a significant

Figure 1. Estimated mean ($\pm$ SEM) hippocampal CA1 cell counts for the four groups. A significant Stress X Surgery interaction was found ($F(1,31) = 5.367$; $p < .05$), with significant reductions in Stress 2VO CA1 cell number compared to control.

CA1 Pyramidal Cell Number

Figure 2. Box-plot representation of the estimated hippocampal CA1 cell counts. The boundaries of the box represent the 25th and the 75th percentiles, the line represents the median, and the whiskers represent the 5th and 95th percentiles.

CA1 Pyramidal Cell Number

Photomicrograph 1: Photomicrograph of CA1 hippocampal pyramidal cell layer at 20X magnification in Non-stress Sham (A), Non-stress 2VO (B), Stress Sham (C), and Stress 2VO rats.

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Figure 3. **Top Panel:** Mean ($\pm$ SEM) percentage of GFAP staining in the CA1 oriens layer for the four groups. A significant Stress effect was found ($F(1, 37) = 10.647$; $p < 0.01$). **Bottom Panel:** Mean ($\pm$ SEM) percentage of GFAP staining in the CA3 oriens layer for the four groups. Again, a significant Stress effect was found ($F(1, 37) = 5.464$; $p < 0.05$). * represents significant differences between stress and non-stress animals.

CA1 Percent Area GFAP Staining

CA3 Percent Area GFAP Staining

Figure 4. **Top Panel:** Mean ($\pm$ SEM) percentage of GFAP staining in the hilus. A significant Stress effect was found ($F(1, 37) = 7.824; p < 0.01$). **Bottom Panel:** Mean ($\pm$ SEM) percentage of GFAP staining in the medial parietal cortex. No significant differences were found. * represents significant differences between stress and non-stress animals.

Photomicrograph 2: Photomicrograph of GFAP immunoreactivity in the CA1 hippocampal pyramidal cell layer at 20X magnification in Non-stress Sham (A), Non-stress 2VO (B), Stress Sham (C), and Stress 2VO rats. Significantly lower densities of GFAP immunoreactivity were observed in stressed animals compared to non-stressed animals in CA1, CA3, and the hilus gyrus regions of the hippocampus.

Table 2. Descriptive statistics for GFAP cell counts.

TABLE 1

Descriptive Statistics for GFAP Cell Counts

	Non-Stress Sham	Stress Sham
First Section	437	195
Second Section	415	94
Third Section	592	319
Sum	1,444	608
Mean	481	203

is clear that the GFAP down-regulation among stressed animals was due to a reduction in the number of cells expressing GFAP, rather than reduced GFAP expression within the cells (cell size).

BDNF Immunohistochemistry

The results are shown in Figures 5-7, photomicrographs 3-4, and appendices A.4 – A.5. A 2 X 2 ANOVA with Stress (Non-stress vs. Stress) and 2VO (Sham vs. 2VO) as between groups variables did not reveal any significant differences between groups for the CA1/CA2 hippocampal region, cortex-amygdala transition zone, and layer 2 of the entorhinal cortex. Densitometry revealed a significant effect of 2VO in the dentate gyrus ($F(1,37) = 4.545, p < .05$). Stereological assessment of the dentate gyrus did not reveal any significant group differences in dentate gyrus BDNF-IR.

Fluoro-Jade Staining

A 2 X 2 ANOVA with Stress (Non-stress vs. Stress) and 2VO (Sham vs. 2VO) as between groups variables did not reveal any significant differences (results shown in Figures 8-9 and Appendix A.6) in Fluoro-Jade staining between groups for any of the three brain regions (CA1, CA3, and the dentate gyrus).

Corticosterone Measurement

The results of the corticosterone quantification are shown in Figure 10 and Appendix A.6. A 2 X 2 ANOVA with Stress (Non-stress vs. Stress) and 2VO (Sham vs. 2VO) as between groups variables did not reveal any significant differences in

Figure 5. **Top Panel:** Mean ($\pm$ SEM) percentage of BDNF staining in the CA1. **Bottom Panel:** Mean ($\pm$ SEM) percentage of BDNF staining in the cortex-amygdala transition zone. No significant differences were found between groups in either region.

CA1 Percent Area BDNF Staining

**Cortex-Amygdala Transition Zone
Percent Area BDNF Staining**

Figure 6. Mean ($\pm$ SEM) percent area BDNF staining in layer 2 of the entorhinal cortex.

No significant group differences were found.

**Layer 2 Entorhinal Cortex
Percent Area BDNF Staining**

Figure 7. **Top Panel:** Mean ($\pm$ SEM) percent area of BDNF staining in the dentate gyrus. A significant 2VO effect was found ($F(1,37) = 4.545, p < .05$). **Bottom Panel:** Estimated dentate gyrus BDNF cell number. No significant group differences were found.

DG Percent Area BDNF Staining

Mean DG BDNF Cell Counts

Photomicrograph 3: Photomicrograph of BDNF immunoreactivity in the CA1 hippocampal pyramidal cell layer (A & B) and the dentate gyrus (C & D) of a control rat. No significant differences in BDNF immunoreactivity were observed between groups in the CA1. A significant 2VO effect was observed for BDNF-IR in the dentate gyrus. Photos are taken at 40X (A & C) and 100X (B & D) magnification.

Photomicrograph 4: Photomicrograph of BDNF immunoreactivity in the cortex-amygdala transition zone (A & B), and layer 2 of the entorhinal cortex (C & D) of a control rat. No significant differences in BDNF immunoreactivity were observed between groups in the cortex-amygdala transition zone or the entorhinal cortex. Photos are taken at 40X (A & C) and 100X (B & D) magnification.

Figure 8. **Top Panel:** Estimated hippocampal CA1 Fluoro-Jade cell counts. **Bottom Panel:** Estimated hippocampal CA3 cell counts. No significant differences between groups were observed in either hippocampal region.

CA1 Fluoro-Jade Cell Number

CA3 Fluoro-Jade Cell Number

Figure 9. Estimated hippocampal dentate gyrus Fluoro-Jade cell counts. No significant differences between groups were found.

DG Fluoro-Jade Cell Number

Figure 10. Estimated plasma corticosterone levels (ng/dl) at sacrifice. No significant group differences were found.

Plasma CORT Levels

corticosterone levels between groups. Furthermore, no significant Stress X 2VO interaction effect was observed.

Correlational analysis of hippocampal cell number and GFAP-IR

The results are shown in Table 2. Correlational analyses of CA1 cell number and hippocampal GFAP staining proved to be non-significant for all animals. No relationship was observed between CA1 total cell number and any of CA1, CA3, or hilus GFAP density. However, significant positive relationships between CA1 and CA3 GFAP densities ($r = .837, p < .01$), CA1 and hilus GFAP densities ($r = .688, p < .01$), and CA3 and hilus GFAP densities ($r = .676, p < .01$) were observed across groups. GFAP densities in the medial fronto-parietal cortex were also found to be positively correlated with GFAP densities in CA1 ($r = .597, p < .01$), CA3 ($r = .520, p < .01$), and hilus ($r = .757, p < .01$) regions. Interestingly, no significant correlation was observed between CA1 pyramidal cell number and GFAP densities for any region. However, significant positive relationships were observed (Table 3) between CA1 and CA3 GFAP densities ($r = .645, p < .05$), and CA1 and hilus densities ($r = .643, p > .05$) were observed. Finally, no significant relationship was observed between dentate gyrus BDNF densities and any of the following variables: CA1 cell number, CA1 GFAP, CA3 GFAP, hilus GFAP.

Table 2: Intercorrelations (Pearson Product Moment) between the estimated total number of CA1 pyramidal cells and the percent area of GFAP immunoreactivity in the CA1, CA3, hilar, and cortical regions across groups

	CA1 Total Cell Number	CA1 GFAP	CA3 GFAP	Hilus GFAP	Cortex GFAP
CA1 Total Cell Number	----	.120	.129	.140	.129
CA1 GFAP	.120	----	.837**	.688**	.597**
CA3 GFAP	.129	.837**	----	.676**	.520**
Hilus GFAP	.140	.688**	.676**	----	.757**
Cortex	.129	.597**	.520**	.757**	----

** Correlation is significant at the 0.01 level

Table 3: Intercorrelations (Pearson Product Moment) between the estimated total number of CA1 pyramidal cells and the percent area of GFAP immunoreactivity in the CA1, CA3, hilar, and cortical regions in Stress 2VO rats

	CA1 Total Cell Number	CA1 GFAP	CA3 GFAP	Hilus GFAP	Cortex GFAP
CA1 Total Cell Number	----	.138	.330	-.200	-.404
CA1 GFAP	.138	----	.645**	.643**	.562
CA3 GFAP	.330	.645**	----	.549	.004
Hilus GFAP	-.200	.643**	.549	----	.416
Cortex	-.404	.562**	.004	.416	----

** Correlation is significant at the 0.05 level

Discussion

CA1 Pathology

Following 6 weeks of a chronic mild stress regime, stereological cell counting identified significant CA1 pyramidal cell loss in stressed 2VO rats. Compared to sham rats, stressed showed a 16.7% loss. Non-stressed 2VO rats showed a non-significant 7.2% loss of cells, compared to sham controls. It is clear that the stress condition potentiated the CA1 pyramidal cell loss resulting from the bilateral occlusion of the common carotid arteries.

The reduction in total CA1 pyramidal cell number in 2VO rats is consistent with previous reports of hippocampal cell degeneration in chronically hypoperfused rats (Bennett et al, 1998; Pappas et al, 1996; Ni et al, 1994). In earlier studies, Pappas et al (1996) report no differences between control and 2VO rats in the total number of CA1 cells at 14 days post-surgery. However, at six months post-surgery, there was a significant reduction (8.8%) in CA1 pyramidal cells, thus providing evidence for delayed hippocampal pathology following 2VO. Similarly, while Ni et al (1994) found no significant differences between control and 2VO rats at one month post-surgery, significant CA1 pyramidal cell loss (32.3%) is reported at the four-month time-point. Moreover, while not statistically significant, de Butte et al (2002) report a 10% loss in CA1 pyramidal cells at 4 months post-2VO.

It should be noted that Bennett et al (1998) identified apoptotic and necrotic cell degeneration 2 weeks post-2VO. Thus, it seems that CA1 cells may be unhealthy soon after 2VO but there is significant loss only after accumulated death. With the exception

of de Butte's (2002) study, these previous studies did not employ stereology and systematic sampling of the entire hippocampus, as was done here. Hence, it is difficult to compare the results of these studies. As well, it should be noted that the estimates of cell number in the current study and those of de Butte et al (2002) are more accurate and reliable. Thus, it is noteworthy that the loss of CA1 cells after 2VO found here (7.2% loss at 7 weeks post-surgery) and the de Butte (2002) study (10% loss at 122 days post-surgery) are very similar.

GFAP Immunohistochemistry

Positive GFAP immunoreactivity was observed in all animals. Densitometric analysis indicated significant group differences in GFAP expression between stressed and non-stressed rats. The stressed sham and stressed 2VO animals had significantly lower GFAP densities than both the non-stressed sham and non-stressed 2VO animals. Down-regulation of GFAP expression was observed in the CA1 (25% reduction), CA3 (17.4% reduction), and hilar (27% reduction) regions of the hippocampus in stressed animals, whereas no significant difference in GFAP expression was observed in the medial parietal cortex, suggesting that suppression of GFAP expression in stressed animals may be unique to the hippocampus. GFAP down-regulation was present throughout the entire hippocampus, due to a reduction in GFAP-IR cell number and the level of GFAP-IR in any region correlated with the levels in other regions, in all rats

The results of the current study confirm earlier reports by O'Callaghan et al (1989) and Maurel et al (2000) of glucocorticoid-mediated GFAP expression in the hippocampus. O'Callaghan et al (1989) investigated the effect of exogenous

corticosterone on GFAP expression in the adult male rat. They report that increased corticosterone level down-regulates GFAP expression in the hippocampus. Furthermore, removal of endogenous corticosterone by means of adrenalectomy resulted in significant increases in GFAP immunoreactivity in the hippocampus and the frontal cortex.

Maurel et al (2000) found a down-regulation of GFAP staining in the hippocampus of adrenalectomized (ADX) rats given corticosterone replacement therapy following the removal of their adrenal glands. In addition, they report a 50-70% increase in GFAP immunoreactivity in the dentate gyrus of the hippocampus of adrenalectomized rats with no corticosterone replacement therapy.

The current study indicated no change in GFAP expression following approximately 8 weeks of 2VO. These results are consistent with previous research. Pappas et al (1996) reported an up-regulation of GFAP expression following 190 days of chronic ischemia. However, no evidence of increased GFAP expression was reported at the 14-day time-point. Thus, it seems that upregulation of GFAP after 2VO occurs with a long latency (at least 50 days).

Brain-Derived Neurotrophic Factor Expression

Densitometric analysis of BDNF protein immunoreactivity did not reveal any significant group differences in the CA1/CA2 hippocampal region, the cortex-amygdala transition zone, or layer 2 of the entorhinal cortex. In contrast to the results reported here, Smith et al (1995) report a 75% decrease in BDNF mRNA expression in the DG following 2 hours of immobilization stress. Moreover, BDNF levels are reduced in animals models of chronic stress-induced depression (Shirayama et al, 2002).

Nevertheless, the absence here of significant differences between stressed and non-stressed animals is consistent with previous reports that suggest that BDNF expression begins to return to normal levels once endogenous corticosterone levels begin to normalize (Smith et al, 1995). In the current study, animals were sacrificed 24 hours following the completion of the stress regime at which time CORT levels were found to have been at the control level.

It should also be noted that decreased BDNF immunoreactivity has been reported when freshly perfused brains are left in post-fix (4% paraformaldehyde) for periods longer than 2 hours (Yan et al, 1997). In the current study, the brains were postfixed for 24 hours. This may have obscured differences in BDNF immunoreactivity between groups.

Significantly lower levels of BDNF immunoreactivity were observed in the dentate gyrus of 2VO animals, as assessed by densitometry. In order to confirm these results, stereological counts were conducted on the three BDNF sections for each brain. Interestingly, no significant differences between groups were found. Given that stereology is a more accurate method of cell quantification, these results are believed to reflect the true level of BDNF staining in the dentate gyrus.

Fluoro-Jade Staining

In the current study, Fluoro-Jade B was used to detect degenerating cells in the CA1, CA3, and dentate gyrus hippocampal regions. No significant group differences in Fluoro-Jade B staining were observed for any of the regions. In fact, for all groups only minimal amounts of positive staining were observed. Thus, while a decrease in CA1

pyramidal cell number was observed in Stress 2VO and Non-stress 2VO rats, very little hippocampal neurodegeneration was actually occurring at the time of sacrifice (8 weeks post-surgery).

Previous studies report significant CA1 Fluoro-Jade staining forty-eight hours and one week following 30 minutes of global forebrain ischemia. However, at 4 weeks post-ischemia, CA1 Fluoro-Jade staining is significantly reduced. This progressive reduction in Fluoro-Jade staining corresponds with the pattern of CA1 pyramidal cell loss associated with transient global forebrain ischemia. Significant progressive cell loss is reported at forty-eight hours and one week, but is attenuated by four weeks post-surgery. Moreover, no significant CA3 and hilus cell loss was detected, corresponding with minimal Fluoro-Jade staining in either region at all 3 time-points (Larsson et al, 2001).

The current results of the Fluoro-Jade B staining reinforce previous reports identifying the importance of the time-point of Fluoro-Jade staining within a study (Krinke et al, 2001). In a study examining the ability of Fluoro-Jade to detect necrotic neurons following neurotoxin injection, Krinke et al (2001) report marked neuronal staining 3 days after injection, but little positive staining at the 15-day time-point. Given the results of the Krinke et al study, and the significant CA1 pyramidal cell loss observed in the current study, it is likely that an up-regulation of Fluoro-Jade staining would be observed in S2VO and NS2VO rats at an earlier time-point. Moreover, it is possible that active cell loss only occurred during conjoint 2VO and stress. It is unlikely, but possible that the delay of 24 hours imposed between the final stressor and sacrifice contributed to the minimal Fluoro-Jade staining. Future studies should examine Fluoro-Jade staining immediately following stress exposure and at an earlier time-point post-surgery.

Plasma Corticosterone

No significant differences in plasma CORT levels were observed between groups at the time of sacrifice indicating an absence of persisting post-stress CORT elevation in both of the chronic stress groups. It may be concluded that chronic mild stress does not induce chronic elevations of plasma CORT concentrations. Previous studies suggest that maximal CORT elevation occurs 10-15 minutes post-stress (Anisman et al, 1998; 2001). The absence of differences in plasma CORT between groups in the present study is, therefore, not surprising, given that the blood samples were collected 24 hours after the final stress exposure. It would be instructive to measure CORT at intervals during CMS and to relate this to the emergence of CA1 cell loss.

Why does Chronic Mild Stress Potentiate CA1 Cell Loss in 2VO Rats?

The most obvious explanation pertains to the finding that stressed rats exhibited a significant reduction in GFAP-IR. Glial fibrillary acidic protein is the dominant protein of astrocytes in the mature CNS (Maurel et al, 2000). Astrocytes have a supportive role in neuron viability. For instance, they are actively involved in glutamate buffering (Siesjö, 1988) and the provision of neurotrophins (Kandel et al, 2000). Stress sham rats also exhibited a down-regulation of GFAP-IR but it was not associated with increased CA1 pyramidal cell loss, undoubtedly because of the absence of additional trauma caused by hypoperfusion. Thus, chronic mild stress, by itself, is insufficient to induce CA1 pyramidal cell degeneration. However, stress is more damaging in 2VO rats, whose CA1 cells are vulnerable to injury. Ischemia is reported to increase extracellular concentrations and reduce re-uptake of circulating concentrations of the excitatory amino

acid glutamate. Prolonged exposure to glutamate results in increased neural pathology, presumably due to increased intracellular calcium concentrations as a result of glutamate activation of NMDA receptors. Astrocytes reduce extracellular glutamate concentrations as they convert glutamate to glutamine for storage (Siesjö, 1988). Increased levels of circulating GCs compromise astrocyte glutamate re-uptake. Specifically, CORT reduces GFAP expression in the hippocampus (O'Callaghan et al, 1989). Stress also decreases GFAP expression and glutamate re-uptake in the hippocampus, given the rise in CORT associated with stress (Moghaddam et al, 1994). It is important to note, however, that GFAP is not the only protein exhibited by astrocytes. Astrocytes also express S-100 whose expression is not altered following CORT exposure (O'Callaghan et al, 1991). Therefore, the reduction in GFAP expression observed here may not necessarily reflect a reduction in the number of astrocytes.

Earlier reports indicate that stress and, by association, glucocorticoids leave cells vulnerable to a variety of insults seemingly by altering the neuronal metabolic state (Moghaddam et al, 1994; Sapolsky, 1985a, 1985b, 1986; Sapolsky & Pulsinelli, 1986). Specifically, GCs have been found to cause a 25% reduction in neuronal transportation of glucose (Horner et al, 1990). This energy reduction attenuates the removal of excess glutamate by astrocytes at the synapse resulting in hippocampal cell loss (Virgin Jr. et al, 1991). The glucocorticoid-potentiated hippocampal pathology is attenuated with the addition of energy supplements, such as glucose and mannose (Sapolsky, 1986). Of additional importance to this study is the fact that ischemia also reduces glucose availability, thereby reducing the energy state of the cells (Tsuchiya et al, 1993). The

combined metabolic insults of the chronic mild stress and 2VO conditions may, therefore, account for the increased CA1 pathology observed in the Stress 2VO rats.

Clinical Implications

Our findings suggest that exposure to chronic mild stress and, by association, glucocorticoids exacerbates the neural consequences of chronic cerebral hypoperfusion. As such, this study has clinical implications, especially with respect to aging, mild cognitive impairment (MCI) and Alzheimer's disease (AD). MCI has been identified as the early stage of AD and the progression rate from MCI to AD is reported to be 13.7% per year. Similar to AD, cerebral hypoperfusion is a common feature of MCI, specifically in the posterior cingulate cortex, a region important for memory (Huang et al, 2002). AD is a progressive neurodegenerative disease associated with aging, which shares many risk factors and pathological features with vascular dementia (de la Torre, 2002). Previous studies suggest that glucocorticoids affect the progression of AD. Pascualy et al (2000) found increased cortisol response to the Cold Pressor Test in patients in the more advanced stages of AD (lower MMSE scores). Similarly, Weiner et al (1997) report higher cortisol levels with increased cognitive impairment as assessed by a modified Alzheimer's disease Assessment Scale in AD patients. Increased levels of GCs, therefore, may potentiate the neurodegeneration associated with AD.

Our results suggest that stress potentiated the neural consequences of 2VO by minimizing the cellular metabolic state. Specifically, chronic mild stress may potentiate an ischemia-induced decrease in glucose metabolism. Research suggests that glucose metabolism is already compromised in AD, especially in the temporal and parietal lobes

(Benson et al, 1983; Friedland et al, 1989). Exposure to chronic mild stress in individuals with AD may increase the progression of the disease by exacerbating a known feature of AD: a reduction in glucose metabolism. Maintaining a relatively stress-free environment is, therefore, crucial for AD patients.

Conclusions

In conclusion, the results of this study confirm the original hypothesis that chronic mild stress exposure exacerbates the neural consequences of bilateral occlusion of the common carotid arteries. Stress 2VO rats exhibited substantial CA1 pyramidal cell loss, whereas non-stressed 2VO rats showed only modest loss. The down-regulation of GFAP expression in stressed animals suggests that stress potentiates neurodegeneration following 2VO by enhancing “weak excitotoxicity.” It is likely that stress-induced reduction in GFAP expression, and a corresponding inability to buffer glutamate compromises hippocampal cell viability. While we cannot make any direct causal statement regarding the mechanism of stress-potentiated neurodegeneration, it is clear that exposure to CMS further perpetuates the effects of 2VO and that these findings have clinical implications for the evolution of Alzheimer’s disease.

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APPNDIX A.1

ANOVA Summary Table for the Estimated Total of Hippocampal CA1 Cells

CA1:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	1.119E+9	1	1.119E+9	1.794	.191
2VO	1.697E+10	1	1.697E+10	27.212	.000
Stress X 2VO	3.516E+9	1	3.516E+9	5.637	.025
Error	1.746E+10	28	6.237E+9		

APPENDIX A.2

ANOVA Summary Tables for GFAP Density in the CA1, CA3, & Hilar Hippocampal
Regions and the Fronto-parietal Medial Cortex

CA1:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	12.045	1	12.045	10.647	.003
2VO	.203	1	.203	.179	.675
Stress X 2VO	.138	1	.138	.122	.729
Error	38.465	34	1.131		

CA3:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	5.833	1	5.833	5.464	.025
2VO	.190	1	.190	.178	.676
Stress X 2VO	1.954	1	1.954	1.830	.185
Error	36.298	34	1.068		

Hilus:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	11.684	1	11.684	7.824	.008
2VO	.420	1	.420	.281	.599
Stress X 2VO	.107	1	.107	.071	.791
Error	50.775	34	1.493		

Fronto-parietal Medial Cortex:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	.126	1	.126	2.053	.163
2VO	2.24 E-03	1	2.24 E-03	.036	.850
Stress X 2VO	1.22 E-03	1	1.22 E-03	.020	.889
Error	1.721	34	6.15 E-02		

APPENDIX A.3

ANOVA Summary Tables for BDNF Density in the CA1, Amygdala-Cortex Transition
Zone, and Entorhinal Cortex

CA1:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	2.094	1	2.094	1.380	.249
2VO	1.541	1	1.541	1.015	.321
Stress X 2VO	.161	1	.161	.106	.746
Error	50.095	33	1.518		

Amygdala-Cortex Transition Zone:

<i>Source</i>	<i>SS</i>	<i>Df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	.560	1	.560	.624	.434
2VO	4.209	1	4.209	4.071	.052
Stress X 2VO	2.824	1	2.824	2.731	.108
Error	33.090	32	1.034		

Entorhinal Cortex:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	.381	1	.381	.185	.670
2VO	.585	1	.585	.284	.598
Stress X 2VO	2.839	1	2.839	1.379	.249
Error	65.867	32	2.058		

APPENDIX A.4

ANOVA Summary Tables for Percent Area BDNF Staining and Estimated Total BDNF
Immuno-positive Cells in the Dentate Gyrus

Dentate Gyrus BDNF Densitometry:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	3.387E-04	1	3.387E-04	.000	.990
2VO	9.755	1	9.755	4.545	.041
Stress X 2VO	1.738	1	1.738	.810	.375
Error	70.836	33	2.147		

Dentate Gyrus BDNF Stereology:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	216.097	1	216.097	.077	.784
2VO	220.086	1	220.086	.078	.782
Stress X 2VO	656.742	1	656.742	.233	.632
Error	92961.191	33	2817.006		

APPENDIX A.5

ANOVA Summary Tables for the Estimated Total Number of Fluoro-Jade Cells in the
CA1, CA3, & Dentate Gyrus Regions of the Hippocampus

CA1:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	92.024	1	92.024	.301	.587
2VO	105.614	1	105.614	.345	.561
Stress X 2VO	674.667	1	674.667	2.205	.147
Error	10404.678	34	306.020		

CA3:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	582.966	1	582.966	1.661	.206
2VO	38.742	1	38.742	.110	.742
Stress X 2VO	62.952	1	62.952	.179	.675
Error	11933.556	34	350.987		

Hilus:

<i>Source</i>	<i>SS</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
Stress	267.233	1	267.233	1.791	.190
2VO	47.253	1	47.253	.317	.577
Stress X 2VO	62.411	1	62.411	.418	.522
Error	5072.778	34	149.199		

APPENDIX A.6

ANOVA Summary Table for Estimated Plasma Corticosterone Levels (ng/dl)**Corticosterone Levels:**

<i>Source</i>	SS	Df	MS	F	P
Stress	.550	1	.550	.004	.952
2VO	11.731	1	11.731	.078	.752
Stress X 2VO	111.638	1	111.638	.742	.395
Error	5112.721	34	150.374		

APPENDIX B

List of Abbreviations

2VO:	Two-vessel occlusion
3AP:	3-acetylpyridine
ACTH:	Adrenal-corticotrophin hormone
AD:	Alzheimer's disease
ADX:	Adrenalectomized
BDNF:	Brain-derived neurotrophic factor
BDNF-IR:	Brain-derived neurotrophic factor immunoreactivity
CMS:	Chronic mild stress
CNS:	Central nervous system
CORT:	Corticosterone
CORT-R:	Corticosterone receptors
CPT:	Cold Pressor Test
CRF:	Corticotrophin-releasing factor
DAB:	3,3 diaminobenzidine
DG:	Dentate gyrus
EPSP:	Excitatory post-synaptic potential
GC:	Glucocorticoid
GFAP:	Glial fibrillary acidic protein
GFAP-IR:	Glial fibrillary acidic protein immunoreactivity
GR:	Glucocorticoid receptor

HPA axis:	Hypothalamic-pituitary-adrenal axis
KA:	Kainate acid
LCBF:	Local cerebral blood flow
LCGU:	Local cerebral glucose utilization
MCI:	Mild cognitive impairment
MMSE:	Mini-Mental State Exam
MR:	Mineralcorticoid receptor
MWM:	Morris water maze
NGF:	Nerve growth factor
NT-3:	Neurotrophin 3
NT-4:	Neurotrophin 4
NS2VO:	Non-stress 2VO
NSS:	Non-stress sham
PVN:	Paraventricular nucleus
SCN:	Suprachiasmatic nucleus
SS:	Stress sham
S2VO:	Stress 2VO
TBI:	Traumatic brain injury
TdT:	Terminal deoxynucleotidyl transferase
TMT:	Trimethyltin
Trk:	Tyrosine kinase receptors
TUNEL:	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling