NEONATAL IBOTENIC ACID LESIONS OF THE VENTRAL HIPPOCAMPUS: THE EFFECTS OF STRESS ON GENE EXPRESSION AND APOPTOSIS

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Paula C. Ashe

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PAULA C. ASHE

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ABSTRACT

Recently, it has been suggested that neurodevelopmental abnormalities underlie schizophrenia. However, it has also been suggested that schizophrenia is a neurodegenerative disease as evidenced by a progressive worsening of symptoms over time. Neurodevelopmental abnormalities may, therefore, create a functionally compromised system that is more susceptible to neuronal atrophy and/or death caused by environmental factors such as stress (a known precipitant of acute psychotic episodes and exacerbant of schizophrenia). This hypothesis was tested using the putative neurodevelopmental animal model of schizophrenia described by Lipska *et al.* (1993). This model, which parallels a number of aspects of schizophrenia, involves bilateral ibotenic acid lesions in postnatal day 7 male rats. The effects of neonatal hippocampal lesions on BDNF mRNA and NMDAR1 mRNA, factors involved in development, cell survival and cell communication, were investigated in adult rats following exposure to a physiological stressor. Apoptosis levels were also investigated in these rats to determine if neurodegeneration was present.

Results demonstrate that BDNF mRNA was reduced in the prefrontal cortex and hippocampus of lesioned as compared to sham rats. Increased BNDF mRNA resulted from swim stress in both groups, but the increase in lesioned animals was more pronounced than controls. NMDAR1 mRNA was also reduced in the prefrontal cortex and CA3 and CA1 regions of the hippocampus in lesioned versus sham rats. There was an increase, however, in the dentate gyrus of lesioned versus sham rats. Swim stress increased NMDAR1 mRNA in the prefrontal cortex and decreased it in the hippocampus. There was also an increase in apoptosis in lesioned versus sham rats, with no significant increase in response to stress.

Reductions in BDNF mRNA in lesioned versus control animals support the hypothesis that neurodevelopmental lesions may result in a system more susceptible to stressors. Reductions in NMDAR1 mRNA are in accordance with the NMDA glutamate receptor hypofunction theory of schizophrenia. It is possible that reductions in glutamate function can remove the inhibitory effect of GABA, thereby resulting in overexcitation of the system and a potential for neurodegeneration. Increased apoptosis supports the presence of neurodegeneration as an ongoing phenomenon, however, it appears that these animals are resistant to increased cell death induced by acute stressors. The question remains whether chronic stress may have more serious consequences in these lesioned animals.

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To my father,

who gave me the strength and determination to face challenges.

Thank you.

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LIST OF ABBREVIATIONS

ACSF	artificial cerebrospinal fluid
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
cDNA	complementary deoxyribonucleic acid
CNS	central nervous system
CSF	cerebrospinal fluid
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylene diaminetetraacetic acid
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
H&E	haematoxylin and eosin
L-DOPA	L-3,4-dihydroxyphenylalanine
mRNA	messenger ribonucleic acid
NADPH-d	nicotinamide adenine dinucleotide phosphate-diaphorase
NCAM	neural cell adhesion molecule
NMDA	N-methyl-D-aspartic acid
PBS	phosphate buffered saline
PCP	phencyclidine
PND	postnatal day
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SOD	superoxide dismutase
SSC	sodium chloride-sodium citrate

STE	sodium chloride-tris-EDTA
TACS	Trevigen Apoptotic Cell System
TNFα	tumor necrosis factor alpha
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling

1. INTRODUCTION

1.1 Characteristics of Schizophrenia

Schizophrenia is a biological brain disease that affects approximately 1% of the world's population (Sartorius *et al.*, 1972). The term schizophrenia, first coined by Bleuler (1950), refers to a break from reality caused by disorganization of mental functions such that thoughts and emotions cease to function normally together. Schizophrenia generally onsets between the ages of 17 and 30 in men and 20 to 40 in women (Carpenter & Buchanan, 1994). It is characterized by two major classes of symptoms, positive symptoms (the presence of something that should be absent) and negative symptoms (the absence of something that should be present) (Andreasen, 1995). Positive symptoms include hallucinations, delusions, disorganized speech, disorganized behavior and thought disorders. This category of symptoms has been subdivided into two classes, psychotic symptoms comprised of hallucinations and delusions, and cognitive symptoms comprised of thought disorders and disorganization of behavior and speech. These symptoms are generally the first recognizable symptoms of the disorder and are relatively treatable. Negative symptoms include blunted affect (decreased emotional expression), social withdrawal, avolition (loss of motivational drive), anhedonia (loss of ability to experience pleasure) and restricted speech. These symptoms are particularly disabling for persons with schizophrenia, as they are persistent, very difficult to treat and often prevent people from leading productive lives.

Despite the prevalence and devastating effects of schizophrenia, very little is known about the underlying pathology. There has been shown to be a

genetic component as evidenced by twin, family and adoption studies, but the results of these studies indicate that genetics is not the only factor involved in the expression of schizophrenia. Family studies indicate that relationship to an affected individual results in an exponential increase in an individual's risk of developing schizophrenia (Karayiorgou & Gogos, 1997). Twin studies demonstrate a 50% concordance rate between monozygotic twins and a 4% to 14% concordance rate between dyzygotic twins (Farmer *et al.*, 1987, McGuffin *et al.*, 1995). Adoption studies also show that schizophrenia is more prevalent in biological relatives than in adoptive relatives (Karayiorgou & Gogos, 1997). The prevalence rates predicted by Mendelian genetics, however, are significantly higher than those seen in the population, therefore suggesting that other factors are integral to development of schizophrenia. It has been proposed that schizophrenia is associated with low penetrance variations in alleles that confer susceptibility to schizophrenia, but are not sufficient to cause the disease (Karayiorgou & Gogos, 1997). With the failure of genetics to completely account for schizophrenia, a number of theories based on neurochemical, neuropharmacological and neuropathological findings have attempted to explain the process behind schizophrenia, but still no one theory explains all facets of the disorder.

1.2 Neurotransmitter Theories of Schizophrenia

1.2.1 The Dopamine Hypothesis

One of the first theories attempting to explain schizophrenia was a relative hyperactivity of central dopaminergic neurons (Matthyse, 1973, Snyder, 1976, Stevens, 1973) projecting from the ventral tegmental area to limbic and cortical regions. Evidence for disruption of this system was inferred from pharmacological information. It was discovered that pharmacological agents used to treat schizophrenia were dopamine D_2 receptor antagonists and that the efficacy of these drugs was directly correlated with D_2 receptor blockade

(Creese *et al.*, 1976, Seeman *et al.*, 1976). Further evidence was obtained from the demonstration that dopamine agonists such as amphetamine, L-DOPA, bromocriptine and lisuride induce acute psychotic reactions (Carlsson, 1988, Connell, 1958, Griffith *et al.*, 1972). Pharmacological evidence led researchers to search for direct evidence of dopaminergic involvement in schizophrenia. The first direct evidence was the finding of increased striatal D_2 dopamine receptors in postmortem tissue from schizophrenic patients (Owen *et al.*, 1978). Controversy surrounds this observation, however, as the increase in D_2 dopamine receptors may be due to neuroleptic treatment. In support of this, Farde *et al.* (1987) found no alterations in dopamine D_2 receptor density between drug-naïve schizophrenics and controls. The discovery of additional members of the D_2-like dopamine receptor family and their affinity for some neuroleptics, led researchers to investigate if these receptors were altered in schizophrenia. It was discovered that the D_4 dopamine receptor was elevated in the striatum of postmortem tissue from schizophrenic brains (Murray *et al.*, 1995). However, the alteration of this receptor type can also not be conclusively proven independent of neuroleptic treatment.

The inconsistency of reports documenting the involvement of dopamine, combined with increased understanding of pharmacological treatment and knowledge of dopaminergic pathways in the brain led to a reformulation of the dopamine hypothesis of schizophrenia. The modified hypothesis states that a hyperdopaminergic state, resulting in positive symptoms, exists in the mesolimbic dopamine system and a hypodopaminergic state, resulting in negative symptoms, exists in the mesocortical dopamine system (Duncan *et al.*, 1999, Weinberger, 1987). Support for this hypothesis comes from earlier studies demonstrating that depletion of dopamine in the prefrontal cortex of monkeys results in cognitive deficits similar to those seen in schizophrenia (Brozoski *et al.*, 1979). Reductions in D_1 dopamine receptor binding in the prefrontal cortex of drug naïve schizophrenic patients also provide evidence for

mesocortical dopaminergic hypofunction (Okubo *et al.*, 1997), however, this finding has not been replicated.

Inconclusive findings in support of the role of dopamine in schizophrenia in conjunction with the inability of neuroleptics to effectively treat negative symptoms, leads to the conclusion that disruptions in the dopaminergic system cannot fully account for the plethora of symptoms and pathophysiological findings. Although dopamine is involved in schizophrenia it appears that it may be a resultant factor rather than a pathogenic factor.

1.2.2 The Glutamate Hypothesis

With the inability of dopamine to account for all of the various findings in schizophrenia, came the emergence of candidates previously overshadowed by dopamine. Glutamate, the major excitatory neurotransmitter in the central nervous system (Cotman & Iversen, 1987), became a major candidate primarily due to parallels between phencyclidine (PCP) psychosis and schizophrenia (for review see Javitt and Zukin, 1991). Phencyclidine was originally proposed as a model of schizophrenia in 1962 (Luby *et al.*, 1962), although at this time its neurochemical action was not fully understood. It is now known that phencyclidine is a non-competitive NMDA glutamate antagonist (Anis *et al.*, 1983). In its initial indication as an anaesthetic, phencyclidine produced a behavioral state characteristic of a dissociation from the environment although consciousness was retained (Collins *et al.*, 1960). For this reason, phencyclidine and its derivative, ketamine, were termed "dissociative anesthetics" (Corssen & Domino, 1966). In up to 50% of people treated with phencyclidine, psychotic reactions including bizarre behavior, excitation, hallucinations and paranoia resulted and persisted for several hours following anesthesia (Collins *et al.*, 1960). The administration of phencyclidine or ketamine to patients with schizophrenia led to exacerbation of symptoms (Lahti

et al., 1995b) and in stabilized patients led to the re-presentation of symptoms (Heresco-Levy *et al.*, 1993). These effects persisted for days to weeks and were reported by patients to be characteristic of their non-drug induced symptoms (Lahti *et al.*, 1995a). Ketamine has also been demonstrated to impair performance in psychological tests of prefrontal cortical function similar to what is observed in schizophrenia (Ghoneim *et al.*, 1985, Oye *et al.*, 1992). Further disruption of cognitive performance in schizophrenia is demonstrated by impairments in prepulse inhibition of startle, a paradigm for assessing deficits in information processing (Swerdlow *et al.*, 1994). In this paradigm, a weak stimulus (the prepulse) is presented immediately before a startling stimulus (the pulse) and under normal circumstances, results in a reduction in the magnitude of response to the pulse (Hoffman & Ison, 1980). NMDA receptor antagonists have been demonstrated to mimic schizophrenia with respect to reductions in prepulse inhibition of startle (Geyer *et al.*, 1990, Mansbach, 1991). Possibly one of the most relevant parallels between schizophrenia and NMDA receptor antagonist administration is the age dependency relationship. Phencyclidine and ketamine are psychoactive in adults but not in children, therefore, similar to schizophrenia, there appears to be an age dependent vulnerability to psychosis (White *et al.*, 1982).

In addition to the convincing evidence of glutamate's involvement in schizophrenia as demonstrated by phencyclidine and ketamine, Kim *et al.* (1980) also proposed a role for glutamate in schizophrenia. Based on reductions in cerebrospinal fluid (CSF) glutamate levels in schizophrenic patients, it was hypothesized that a deficiency in glutamatergic function is associated with schizophrenia. This finding, in combination with the information regarding phencyclidine and schizophrenia prompted other researchers to investigate the role of glutamate in schizophrenia. The demonstration of alterations of glutamate in the CSF could not be replicated (Korpi *et al.*, 1987, Perry, 1982), however, Tsai *et al.* (1995) found reductions

of glutamate in the hippocampus and prefrontal cortex of postmortem schizophrenic brains. Decreases in stimulated release of glutamate in the frontal and temporal lobes of postmortem schizophrenic brains were also found (Sherman *et al.*, 1991a). Further analysis using synaptosomes prepared from schizophrenic brains demonstrated that NMDA stimulated release was specifically disrupted thus supporting a deficiency in glutamate transmission mediated by the NMDA receptor (Sherman *et al.*, 1991b).

A third line of evidence for the involvement of glutamate in schizophrenia comes from binding studies and mRNA analysis. In 1983, Nishikawa *et al.* reported increased ^{3}H-kainate binding in the prefrontal cortex of patients with schizophrenia. In contrast to prefrontal cortex binding, ^{3}H-kainate binding in the hippocampus and parahippocampal gyrus of schizophrenic patients was found to be significantly reduced (Kerwin *et al.*, 1990). AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor (a non-NMDA ionotropic glutamate receptor) binding in the hippocampus of schizophrenic patients was also decreased in this investigation. In support of reduced binding, mRNA levels for kainate receptors and AMPA receptors were also found to be reduced in the hippocampus of schizophrenic patients (Breese *et al.*, 1995, Eastwood *et al.*, 1995b, Harrison *et al.*, 1991). The finding by Breese *et al.* (1995), however, was no longer significant when alcohol abusers were removed from the control group. NMDA receptor binding in the hippocampus was reported to be unaltered (Kerwin *et al.*, 1990, Kornhuber *et al.*, 1989), whereas, both increases and no change have been reported in various cortical brain regions investigated (Ishimaru *et al.*, 1994, Simpson *et al.*, 1992). The possibility exists that controversial reports are, at least in part, a result of the schizophrenic population examined. Humphries *et al.* (1996) reported a reduction in mRNA of the NR-1 subunit of the NMDA receptor in the temporal cortex in a cohort of patients with cognitive impairment. There was a direct correlation between NMDA receptor subunit expression and cognitive performance. Patients with no

cognitive impairment did not show this alteration. A slight decrease in NMDA receptor subunit expression was also found in the frontal lobe (Hirsch *et al.*, 1997). A screen of mRNA expression of the various subunits of the NMDA receptor in schizophrenic brains revealed no generalized abnormalities, however, abnormal increases in the relative levels of the NR2D subunit mRNA were found (Akbarian *et al.*, 1996b). The NR2D subunit is a subunit of the NMDA receptor that has a lower threshold of activation and a prolonged rate of ionic current decay (Monyer *et al.*, 1994). Relative increases in this subunit in schizophrenia suggest that compensatory mechanisms for glutamatergic hypofunction may be in place (Akbarian *et al.*, 1996b).

The ability of phencyclidine and ketamine to mimic or exacerbate fundamental characteristics of schizophrenia in association with alterations in binding and mRNA levels provide strong evidence for glutamatergic involvement in schizophrenia. The glutamatergic hypothesis is strengthened further by its ability to explain alterations in dopaminergic neurotransmission in schizophrenia. Glutamatergic efferents project from the cortex to subcortical dopaminergic cell bodies (Björklund & Lindvall, 1984, Sesack *et al.*, 1989) and the prefrontal cortex receives reciprocal dopaminergic inputs (Björklund & Lindvall, 1984, Thierry *et al.*, 1973). In accordance with the hypothesized role of dopamine in schizophrenia, acute NMDA antagonist administration has been demonstrated to increase dopamine turnover and release in mesolimbic and mesocortical dopamine systems (Bubser *et al.*, 1992). Subchronic NMDA receptor antagonist administration, possibly more relevant to schizophrenia, reduces dopamine in the prefrontal cortex (Jentsch *et al.*, 1997). Acute ketamine produces dopamine dependent impairments in prefrontal cortex-sensitive cognitive tasks that can be blocked by non-NMDA receptor antagonists and dopamine antagonists (Moghaddam *et al.*, 1997, Verma & Moghaddam, 1996). It has been demonstrated, however, that NMDA antagonists can produce their effects independently of dopaminergic activity (Carlsson &

Carlsson, 1989), but under normal conditions dopamine neurotransmission contributes to the drug effects (Bubser *et al.*, 1992). These findings support a situation of dopamine dysfunction occurring downstream from glutamatergic dysfunction.

1.3 Neurodevelopmental versus Neurodegenerative Theories

Other neurotransmitters have been implicated in the pathophysiology of schizophrenia as well, but strict neurotransmitter theories do not account for pathological reports. In addition, the limitations in symptom treatment by neuroleptics suggest neurotransmitters are not the primary disruption. The emergence of neuropathological data (see section 1.3.1) and consequent theories produced a paradigm shift in views on schizophrenia. In 1987, Weinberger (1987) first proposed a neurodevelopmental hypothesis of schizophrenia. This hypothesis states that schizophrenia is a primary brain disease resulting from a fixed structural defect occurring early in life and interacting with maturational events. These maturational events include neuronal and glial proliferation and migration, axonal and dendritic proliferation, myelination of axons, programmed cell death and synaptic pruning (Lieberman, 1999). A widely accepted model suggests that a neurodevelopmental insult results in altered morphology and cytoarchitecture and, therefore, a deficiency in the modulatory capacity of neurons (Duncan *et al.*, 1999). During adolescence or early adulthood, this deficiency, in conjunction with environmental triggers, such as stress, leads to observable symptoms. This emergence is proposed to occur due to the elimination of synaptic connections that previously compensated for the deficiency. It has been demonstrated that obstetric complications, low birth weight (Cannon *et al.*, 1989) and perinatal infections (Mednick *et al.*, 1988) are positively correlated with the occurrence of schizophrenia. Minor physical abnormalities also lend support to a developmental correlate of schizophrenia (Waddington *et*

al., 1990) as do the presence of childhood behavioral precursors such as withdrawn behavior and attentional problems (Baum & Walker, 1995, Done *et al.*, 1994, Jones *et al.*, 1994, Walker & Lewine, 1990). Previous reports also suggest that structural defects are present at the onset of illness (DeLisi *et al.*, 1991, Weinberger *et al.*, 1982), are static (Illowsky *et al.*, 1988, Lieberman *et al.*, 1992, Marsh *et al.*, 1994) and are not associated with gliosis, a marker of neurodegeneration (Roberts *et al.*, 1986, Stevens *et al.*, 1988).

In contrast to these reports, others have suggested that schizophrenia is an ongoing degenerative process as evidenced by progression of symptoms (Fenton & McGlashan, 1994, Loebel *et al.*, 1992, Wyatt, 1991), structural defects (DeLisi *et al.*, 1997, DeLisi *et al.*, 1995, Jacobsen *et al.*, 1998, Nair *et al.*, 1997) and increased gliosis (Stevens, 1982). These studies report that the length of time of symptom presentation prior to pharmacological treatment is a significant predictor of treatment response, relapse and long term outcome. Indications of disease progression led to the proposal that the biological defect in schizophrenia is a continual lifetime process of alterations in cell growth and repair most likely resulting from a genetic defect(s) (DeLisi, 1997). Aberrant neurodevelopment would lead to disorganization and dysconnectivity of neurons and subsequent cell loss (See Figure 1.1).

In addition to clinical evidence for neurodegeneration, the involvement of glutamate, as outlined above, also supports a role for neurodegeneration. NMDA receptor antagonists, such as those used to model schizophrenia, have been shown to be neurotoxic (Olney *et al.*, 1989). The extent of cell death is dependent on age as neurotoxic effects are only evident at the onset of puberty and become maximal in early adulthood (Farber *et al.*, 1995). Repeated NMDA receptor antagonist treatment has been demonstrated to result in subtle but permanent changes in neocortical and limbic structures that are reminiscent of

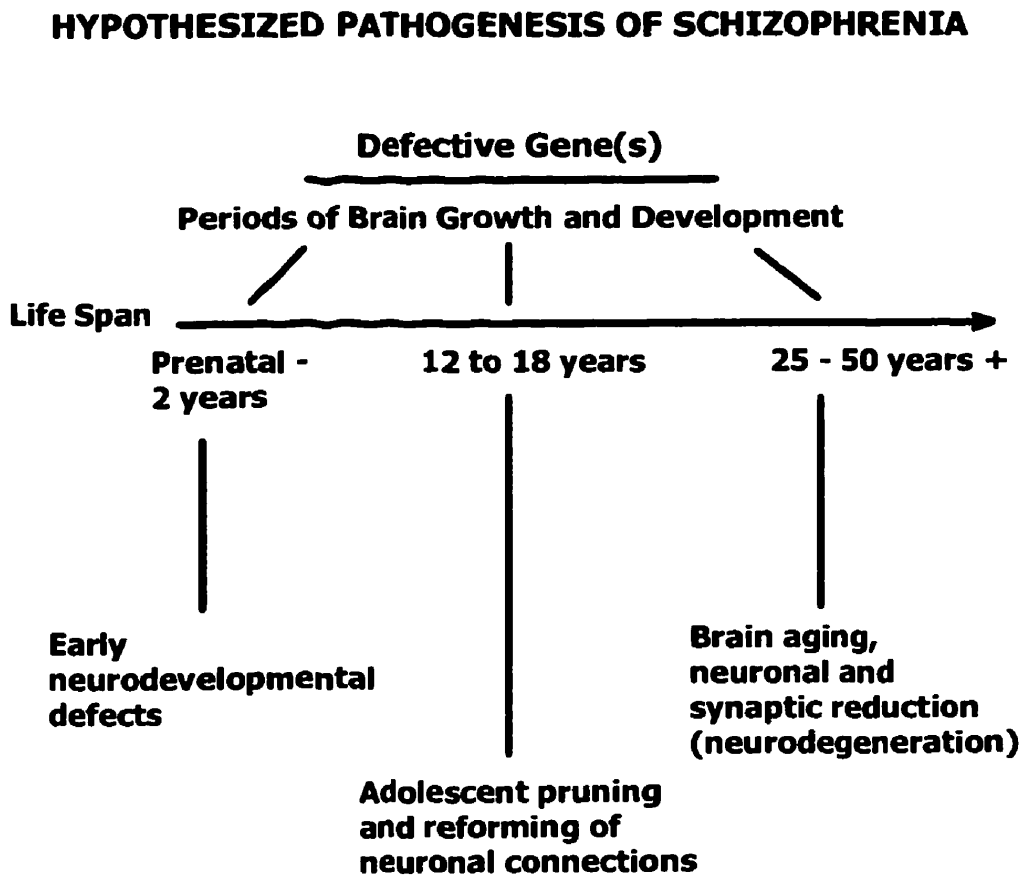


Figure 1.1. Hypothesis of the pathogenesis of schizophrenia suggesting schizophrenia is a lifetime disorder with a genetic defect resulting in neurodevelopmental and neurodegenerative components.
Adapted from DeLisi, 1997.

those observed in schizophrenia (Olney & Farber, 1995). These authors suggest that NMDA receptor hypofunction, as seen by NMDA receptor antagonist administration and as proposed in schizophrenia, exerts effects via the GABAergic system. NMDA receptor hypofunction may result in disinhibition of excitatory pathways normally tonically inhibited by GABAergic neurons under the control of NMDA receptors. This could result in increased excitatory neurotransmission and, therefore, excitotoxic damage to neurons. This situation could result from either a defect in the glutamatergic system or by a defect in the GABAergic system.

In sum, evidence exists for both neurodevelopmental and neurodegenerative components in the pathophysiology of schizophrenia. These processes are not necessarily mutually exclusive but rather, it is possible that both components co-exist to produce the pathophysiology of schizophrenia. In addition, they are both attractive hypotheses in terms of their ability to accommodate neurotransmitter theories. Alterations in neurodevelopment or neurodegeneration would not likely occur without consequent alterations in neurotransmission.

1.3.1 Neuropathological Findings in Schizophrenia

1.3.1.1 Gross Morphological Abnormalities

The first structural abnormality in schizophrenia, that of enlarged ventricles, was reported by Jacobi and Winkler (1927). The advent of computerized tomography (CT) allowed the confirmation of enlarged ventricles in schizophrenia (Johnstone *et al.*, 1976, Weinberger *et al.*, 1979). Johnstone *et al.* (1976) also demonstrated that enlarged ventricles were significantly correlated with impairments in cognitive performance. These findings provided the impetus to search for further abnormalities that would provide a more complete understanding of the pathogenesis of schizophrenia.

Gross morphological examination has revealed widespread alterations in brain structure in schizophrenic patients (See Table 1.1). These alterations include decreases in brain weight (Brown *et al.*, 1986, Pakkenberg, 1987), decreases in cortical volume (Pakkenberg, 1987, Zipursky *et al.*, 1992), alterations in subcortical structures (Bogerts *et al.*, 1985, Jeste & Lohr, 1989, Pakkenberg, 1990) and abnormalities in sulcogyral parameters (Jakob & Beckmann, 1986, Kulynych *et al.*, 1997, Pfefferbaum *et al.*, 1988). These findings lend support to the hypothesis that the primary defect in schizophrenia is a neurodevelopmentally derived structural abnormality that appears to affect multiple brain regions. In accordance with morphological abnormalities resulting in symptom presentation, alterations in cortical gray matter are positively correlated with cognitive impairment in schizophrenia (Andreasen *et al.*, 1986). Further, there is evidence that frontal gray matter reductions are correlated more strongly with negative symptoms than are temporal gray matter reductions (Zipursky *et al.*, 1992). Support for this possibility comes from a study demonstrating no significant difference in prefrontal gray matter in a cohort of schizophrenics with predominantly positive symptoms (Wible *et al.*, 1995). It is possible that although subtle abnormalities are seemingly universal in schizophrenia, morphological abnormalities are as heterogeneous as the symptoms expressed (Weinberger & Lipska, 1995). This heterogeneity and subtlety of morphological abnormalities may account for the lack of morphological alterations reported by a number of researchers.

BRAIN REGION/PARAMETER	ABNORMALITY	POSITIVE REPORTS	NEGATIVE REPORTS
Brain Weight	Decreased	Brown *et al.*, 1986 Pakkenberg, 1987	Heckers *et al.*, 1991b
Cortical Volume (Cerebral Gray Matter)	Decreased	Pakkenberg, 1987 Zipursky *et al.*, 1992 Gur *et al.*, 1999	Heckers *et al.*, 1991b
Lateral Ventricle	Increased volume	Johnstone *et al.*, 1976 Weinberger *et al.*, 1979 Brown *et al.*, 1986 Pakkenberg, 1987 Crow *et al.*, 1989 DeLisi *et al.*, 1995	
Temporal Lobe	Reduced volume	Suddath *et al.*, 1989 Menon *et al.*, 1995 Barta *et al.*, 1997	Brown *et al.*, 1986
Hippocampus	Reduced volume/area	Bogerts *et al.*, 1985 Jeste & Lohr, 1989 Barta *et al.*, 1997	Altshuler *et al.*, 1990
Parahippocampal Region	Decreased volume/area	Bogerts *et al.*, 1985 Brown *et al.*, 1986 Colter *et al.*, 1987 Falkai *et al.*, 1988 Altshuler *et al.*, 1990	
Striatum	Increased size	Heckers *et al.*, 1991b Lauer & Beckmann, 1997	Bogerts *et al.*, 1985 Brown *et al.*, 1986
Thalamus	Decreased size	Pakkenberg, 1990 Andreasen, 1994	
Sylvian Fissure	Shortened (left)	Crow *et al.*, 1992 Falkai *et al.*, 1992	
Sulcogyral Patterns	Abnormal	Jakob & Beckmann, 1986	
Cortical Folding	Reduced	Kulynych *et al.*, 1997	
Sulcal Width	Increased	Pfefferbaum *et al.*, 1988	

Table 1.1. Gross morphological alterations demonstrated in schizophrenic brains.

1.3.1.2 Cytoarchitectonic Abnormalities

The presence of macroscopic alterations in the brains of schizophrenic patients leads to the assumption that microscopic alterations co-exist and underlie these gross abnormalities. Numerous investigators (See Table 1.2) have reported widespread neuronal disruptions. There appears to be a general decrease in neuronal size in the hippocampus (Arnold *et al.*, 1995), and various cortical regions (Arnold *et al.*, 1995, Benes *et al.*, 1986, Rajkowska *et al.*, 1998). A general decrease in neuron density is also reported to exist in various brain regions (Arnold *et al.*, 1995, Benes *et al.*, 1986, Benes *et al.*, 1991, Falkai *et al.*, 1988, Holinger *et al.*, 1995, Jeste & Lohr, 1989). The prefrontal cortex, however, appears not to follow this pattern, as reports suggest increased pyramidal cell density in this region (Benes *et al.*, 1991, Selemon *et al.*, 1998, Selemon *et al.*, 1995). No overall alterations in cell number are evident in the disorder (Pakkenberg, 1993), however, it is suggested that decreases in cell numbers exist in discrete brain regions, thus accounting for decreased cell density. Benes *et al.* (1991) reported a specific loss of interneurons in the prefrontal and cingulate cortices of schizophrenic patients. These decreases, however, are not dramatic enough to account for the significant reduction in cortical volume as seen in schizophrenic brains. This supports the concept that increases in cell density in the prefrontal cortex may involve cell loss but, this may co-exist with a reduction in neuropil, interneuronal material consisting of dendritic trees and axons (Selemon & Goldman-Rakic, 1999). In support of this hypothesis is the demonstration of reduced dendritic spines, sites of excitatory synapses on pyramidal neurons, in layer III pyramidal cells in the cortex of schizophrenic patients (Garey *et al.*, 1998, Glantz & Lewis, 1995). This indicates a reduction in the number of synapses formed in the prefrontal cortex. In addition to abnormal numbers and densities of neurons in the brains of schizophrenic patients, a number of alterations in neuron distribution have also been reported. Akbarian *et al.*

(1993a, 1996a, 1993b) reported alterations in nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) containing neurons in the temporal and frontal lobes of schizophrenic patients. NADPH-d is a neuron-specific enzyme present in both gray and white matter cell populations. In the white matter, NADPH-d containing cells remain from the subplate, a transient structure in neurodevelopment. Interstitial neurons of the adult neocortical white matter, remaining from the subplate, are proposed to be involved in the establishment, maintenance and function of cortical circuitry (Chun & Shatz, 1989). The decrease in NADPH-d positive neurons in gray matter and associated increase in subcortical white matter suggests a failure of neurons to appropriately migrate during neural development or a failure of appropriate programmed cell death (Akbarian *et al.*, 1993a, 1996a, 1993b). Further support for aberrant neuronal migration is demonstrated by displacement of single groups of neurons in the entorhinal cortex of schizophrenic patients (Jakob & Beckmann, 1986). Hippocampal pyramidal cells also appear to be disorganized in schizophrenic patients (Scheibel & Kovelman, 1981), however, attempts to replicate this finding showed no significant differences overall, but specific cases with more severe symptoms did show pyramidal cell disorganization (Altshuler *et al.*, 1987). In accordance with neurodevelopmental abnormalities as a pathogenic factor in schizophrenia, hippocampal neuronal disarray was more pronounced at the interface between hippocampal regions which are known to migrate at distinct developmental times.

Neuropathological findings demonstrate that schizophrenia is a disease characterized by a large number of morphological abnormalities that appear to have their origins in neurodevelopmental abnormalities. Neurodevelopmentally derived alterations in neuronal structure and organization suggest that alterations in the expression of factors involved in neuronal migration, connectivity and viability may also be evident.

BRAIN REGION/PARAMETER	ABNORMALITY	POSITIVE REPORTS	NEGATIVE REPORTS
Superior Temporal Gyrus Neuron Density	Decreased	Holinger *et al.*, 1995	
Hippocampal Neuron Density	Decreased	Jeste & Lohr, 1989	Heckers *et al.*, 1991a
Hippocampal Neuron Size	Decreased	Arnold *et al.*, 1995	
Entorhinal Cortex Neuron Density	Decreased	Falkai *et al.*, 1988 Krimer *et al.*, 1995	Heckers *et al.*, 1991a Arnold *et al.*, 1995
Entorhinal Cortex Neuron Size	Decreased	Arnold *et al.*, 1995	
Prefrontal Cortex Neuron Density	Decreased in pyramidal cell layers	Benes *et al.*, 1986	Akbarian *et al.*, 1995a
	Increased in pyramidal cell layers	Benes *et al.*, 1991 Selemon *et al.*, 1995 Selemon *et al.*, 1998	
Prefrontal Cortex Neuron Size	Decreased	Rajkowska *et al.*, 1998	
Cingulate Cortex Neuron Density and Size	Decreased in pyramidal cell layers	Benes *et al.*, 1986 Benes *et al.*, 1991	
Striatal Neuron Counts	Increased	Beckmann & Lauer, 1997)	Pakkenberg, 1990
Mediodorsal Thalamic Neuron Counts	Decreased	Pakkenberg, 1990	

Table 1.2. Cytoarchitectonic abnormalities reported in schizophrenia.

1.3.1.3 Biochemical Abnormalities in Schizophrenia

Recent evidence indicates that alterations involved in neurodevelopment, neuronal connectivity and viability do exist in schizophrenia. A 115 kDa isoform of neural cell adhesion molecule (NCAM) is increased in the CSF (Poltorak *et al.*, 1995), hippocampus and prefrontal cortex of schizophrenic patients (Vawter *et al.*, 1998). NCAMs are members of the immunoglobulin superfamily that are involved in axon guidance, cell migration and synapse stabilization, however, the 115 kDa isoform does not effectively mediate these actions. Rather, this isoform is a putative secreted protein that is potentially neurotoxic. Its increase in schizophrenia could be due to abnormal glial and/or neuronal processing or by structural abnormalities which would induce changes via cell loss where these molecules are localized (Vawter *et al.*, 1998). Increases in this soluble NCAM may have significant effects on synaptic plasticity and learning. Further evidence for alterations in NCAM was reported by Barbeau *et al.*, (1995) who demonstrated a reduction in polysialation of NCAM in the hilus region of the hippocampus. Polysialic acid (PSA) residues on NCAM increase interactions between multiple NCAMs, enhance the neurite growth promoting characteristics of NCAM and prevent aberrant neuronal connections during neurodevelopment. In adulthood, polysialated NCAM is present in areas that possess high degrees of plasticity such as the hippocampus (Miller *et al.*, 1993). Reductions in PSA-NCAM in schizophrenia may reflect abnormal neuronal connections between hippocampal neurons and may also have significant implications for hippocampal connectivity to cortical regions (Barbeau *et al.*, 1995). The question remains whether alterations in PSA-NCAM exist throughout neurodevelopment and are a primary cause of the disorder or whether these alterations result from other abnormalities in schizophrenia.

Other neurodevelopmental factors altered in schizophrenia include ß-catenin and γ-catenin (Cotter *et al.*, 1998). The catenins are members of the

Wnt signaling pathway, which are involved in cell adhesion, proliferation, migration and gene transcription. The Wnt family is a family of genes involved in normal neurodevelopmental processes such as neuronal migration (Dickinson *et al.*, 1995) and neural crest expansion (Ikeya *et al.*, 1997). Reductions in ß-catenin were found in the CA3 and CA4 region of the hippocampus and reductions in γ-catenin were localized to the CA3 region of the hippocampus. These changes could be associated with alterations in neurodevelopment or with alterations in synaptogenesis or transcription.

Neurodevelopmental abnormalities are also implicated in schizophrenia by the demonstration of reduced reelin mRNA and protein in schizophrenic cortical regions (Impagnatiello *et al.*, 1998). Reelin is a protein involved in the regulation of cortical pyramidal cell, interneuron and Purkinje cell positioning and/or trophic support during neurodevelopment (Curran & D'Arcangelo, 1998). Heterozygote reelin knockout mice show a similar reduction in reelin expression to that seen in schizophrenia. These mice show a number of neuroanatomical abnormalities reminiscent of those seen in schizophrenia (Impagnatiello *et al.*, 1998). The abnormalities appear to be symptomatically silent with the exception of reduced prepulse inhibition of startle, a deficit also present in schizophrenia. A consequence of the neurodevelopmental abnormalities in the reelin deficient mouse appears to be an enhanced vulnerability to excitotoxicity. This may have implications for a neurodegenerative component in schizophrenia.

Tumor necrosis factor alpha (TNF_α), a cytokine involved in neuron growth and differentiation, also appears to be affected in schizophrenia. Increased plasma levels of TNF_α were demonstrated in neuroleptic free schizophrenics as compared to controls (Monteleone *et al.*, 1997). This increase is specific to schizophrenia, as non-schizophrenic psychiatric patients showed no alterations in TNF_α. Also, this increase was reduced by chronic

clozapine treatment (Monteleone *et al.*, 1997). TNF_{α} has also been demonstrated to have neurotoxic properties (Grell *et al.*, 1994, Heller *et al.*, 1992, Zheng *et al.*, 1995) and, therefore, its increase in schizophrenia may have neurodegenerative as well as neurodevelopmental consequences.

In accordance with neurodevelopmentally or neurodegeneratively induced alterations in interneuron number (Benes *et al.*, 1991), alterations in glutamic acid decarboxylase (GAD) are also found (Akbarian *et al.*, 1995a). GAD is the enzyme responsible for the synthesis of GABA from its precursor, glutamate. A decrease in GAD mRNA was demonstrated in the prefrontal cortex of schizophrenic patients thus suggesting a decrease in inhibitory neurotransmission in schizophrenia. The cortical layers most dramatically affected are those involved in corticocortical associative information processing which has significant implications for the negative symptoms of schizophrenia. Increased $GABA_A$ receptor binding in schizophrenia has also been reported (Benes *et al.*, 1992). This could represent a compensatory upregulation thereby supporting reduced GABAergic neurotransmission in schizophrenia. Alterations in GABAergic neurotransmission are hypothesized to result in relative increases in dopaminergic input to remaining GABAergic neurons in areas where GABAergic interneurons are lost (Benes *et al.*, 1997). Evidence for this hypothesis is demonstrated by an apparent shift in the distribution of tyrosine hydroxylase (a marker of dopaminergic terminals) immunoreactive varicosities from pyramidal neurons to interneurons in the anterior cingulate cortex. This effect is selective to layer II of the cortex which is the major layer of interneuron loss (Benes *et al.*, 1991). This shift suggests a "miswiring" of dopaminergic inputs from the ventral tegmental area which could result in a relative, but not absolute, hyperdopaminergic state in relation to the impaired GABAergic interneuron population.

Further evidence for disruptions in neuronal connectivity and transmission comes from the demonstration of altered synaptophysin in schizophrenic patients (Eastwood *et al.*, 1995a, Glantz & Lewis, 1997). Synaptophysin is a protein located in the majority of cortical synaptic terminals (Jahn *et al.*, 1985) and is considered to be a reliable marker of synaptic density (Masliah *et al.*, 1990). Eastwood *et al.*, (1995a) reported a reduction in synaptophysin mRNA in the hippocampus of schizophrenic patients. There was no change in hippocampal synaptophysin immunoreactivity, therefore, it was suggested that the loss of synapses is in extra-hippocampal sites that receive projections from neurons with reduced synaptophysin mRNA. In accordance with this, subsequent reports demonstrated reduced synaptophysin immunoreactivity in the prefrontal cortex, but not the visual cortex, of patients with schizophrenia (Glantz & Lewis, 1997). This reduction was not evident in non-schizophrenic psychiatric patients, nor in subjects with alcohol dependence, therefore, it appears to be relatively specific to schizophrenia. Reductions in synaptophysin in the prefrontal cortex suggest that either fewer synapses are present or that fewer vesicles are present per synaptic terminal. Regardless of the reason for the reduction, impairments in neurotransmission in the prefrontal cortex would be a significant consequence. Reductions in synaptophysin are also consistent with the hypothesis of reduced neuropil and increased neuronal density in the prefrontal cortex (Selemon & Goldman-Rakic, 1999, Selemon *et al.*, 1995). The reduction in synaptophysin is found across all cortical layers, therefore, it was proposed that GABAergic terminals, found in all cortical layers and responsible for corticocortical connections, may be those specifically affected (Glantz & Lewis, 1997). This suggestion is in accordance with alterations of the GABAergic system in schizophrenia as described above.

Disruptions in neurotransmission are also evidenced by the demonstration of altered RNA editing of the GluR-2 subunit of the AMPA-type glutamate receptor in schizophrenia (Akbarian *et al.*, 1995b). The GluR-2

subunit is a component of the AMPA receptor important for regulating calcium conduction. The edited form of the GluR-2 subunit originally contains a glutamine codon that is posttranscriptionally edited to an arginine codon (Sommer *et al.*, 1991). This edited form normally constitutes more than 99% of the total GluR-2 expressed and is responsible for restricting calcium permeability (Hollmann *et al.*, 1991, Verdoon *et al.*, 1991). In the prefrontal cortex of schizophrenics a significant increase in the ratio of unedited to edited GluR-2 molecules was found thus suggesting increases in calcium permeability (Akbarian *et al.*, 1995b). Increased calcium conductance has significant implications for neurotoxicity and in support of this, known neurodegenerative diseases such as Alzheimer's and Huntington's also show altered editing. The editing in Alzheimer's disease is decreased in the prefrontal cortex and in Huntington's disease, the effect is specific to the striatum. The effects in these diseases are also much greater than that seen in schizophrenia. It is not known if the alterations in editing are general or if they are specific to a subpopulation of neurons, thereby making a specific population more vulnerable to neurotoxicity. Recently, in the hippocampus, it has been demonstrated that GluR-2 subunits are less abundant on GABAergic interneurons than on pyramidal cells thereby resulting in GABAergic interneurons having higher calcium permeability and potentially greater vulnerability to neurotoxicity (He *et al.*, 1998). If this pattern were also seen in the prefrontal cortex in combination with alterations in editing of GluR-2 subunits, greater support would be found for GABAergic cell loss and neurodegeneration in schizophrenia.

Increasing evidence, other than imaging and clinical data, does suggest a role for neurodegeneration in schizophrenia. Recently it was demonstrated that schizophrenics have increased plasma levels of S-100b protein (Weismann *et al.*, 1999). The concentration of S-100b protein, a calcium-binding protein found in the CNS, has been shown to increase in CSF and blood in proportion to

CNS damage and therefore, is considered a marker of damage in patients with neurological disorders. This finding indicates ongoing neural damage in patients with schizophrenia. In accordance with this are findings of increased superoxide anion production (Melamed *et al.*, 1998) and reduced scavenging capabilities, thereby enhancing vulnerability to free radical damage. Reduced superoxide dismutase (SOD) activity has been demonstrated in neuroleptic naïve, first episode patients (Mukherjee *et al.*, 1994) and reduced SOD mRNA in postmortem tissue of schizophrenics has also been reported (Lau *et al.*, 1999). Glutathione has also been reported to be reduced in schizophrenic patients (Do *et al.*, 1999) as has glutathione peroxidase (Reddy & Yao, 1996). A deficit in glutathione is proposed to result in free radical induced neurodegeneration. Glutathione also potentiates the effect of glutamate at the NMDA receptor, therefore, its reduction could also contribute to NMDA receptor hypofunction. Increased lipid peroxidation, indicating free radical damage, has also been demonstrated in the blood of schizophrenic patients (Murthy *et al.*, 1989). These findings, generally associated with poorer outcome, lead to the conclusion that oxidative stress leads to membrane abnormalities and neuronal dysfunction (Reddy & Yao, 1996). Further evidence for membrane abnormalities stems from the demonstration of decreased phosphomonoesterases, precursors of membrane phospholipids, and increased phosphodiesterases, breakdown products of membrane phospholipids (Pettegrew *et al.*, 1990). This proposed decrease in synthesis and increase in breakdown of membranes in schizophrenia could be indicative of neuronal loss or loss of connections.

In an attempt to further elucidate the potential role of neurodegeneration, mitochondrial function in schizophrenia was addressed. Mitochondria are responsible for energy production within cells and, therefore, play an important role in cell viability. Cavelier *et al.* (1995) reported a reduction in cytochrome c oxidase, a mitochondrial respiration enzyme and

marker of mitochondrial function, activity in the frontal cortex and caudate nucleus of schizophrenics. It was proposed that this reduction reflected a general decrease in mitochondrial oxidative phosphorylation, and therefore, ATP production. Subsequently, another group investigated the number of mitochondria in the striatum from schizophrenic and control postmortem tissue. It was reported that the striatal neuropil of schizophrenic patients contained significantly fewer mitochondria than did controls (Kung & Roberts, 1999). This suggests either decreased energy demands or a reduced capacity to meet the energy requirements of structures with fewer mitochondria. The majority of striatal terminals arise from cortical neurons, therefore, the suggestion was made that a decrease in energy exists in corticostriatal circuitry in schizophrenia. It remains to be determined if specific populations are effected and whether the mitochondrial reduction is present in other brain structures. Reduced energy supply, in combination with the above alterations, has significant implications for cell connectivity, neurotransmission and cell viability.

Regardless of what the primary pathogenic factor is, it appears that neurodevelopment plays an important role, but many other factors are related to the expression of the disorder. No one theory on its own can explain the pathophysiology and heterogeneity of schizophrenia, but each explains various components. More recently, a holistic theory explaining how many of the abnormalities and hypotheses described interrelate to create the disease of schizophrenia has evolved (Weinberger & Lipska, 1995).

1.3.2 The Dysconnectivity Hypothesis

The dysconnectivity hypothesis originally proposed that symptoms of schizophrenia result from aberrant connections or from abnormal integration of information (Bunney *et al.*, 1995, Friston & Frith, 1995, Weinberger & Lipska, 1995). Historically, the name schizophrenia ("split mind") as coined by Bleuler,

implied a cognitive dysconnectivity or a splitting of the mind from reality (Bleuler, 1950). The more recent dysconnectivity theory, however, is an anatomical theory with consequent cognitive dysfunction rather than a cognitive theory per se (Bunney *et al.*, 1995, Friston & Frith, 1995, Weinberger & Lipska, 1995). Reports of reduced spine density (Glantz & Lewis, 1995), reduced neuropil (Selemon & Goldman-Rakic, 1999), reduced synaptophysin (Eastwood *et al.*, 1995a, Glantz & Lewis, 1997) as well as abnormalities in cytoarchitecture and neurotransmission all suggest abnormalities in neuronal connectivity.

Functional abnormalities also lend strong support to the dysconnectivity hypothesis of schizophrenia. Negative symptoms are thought to have their origin in the prefrontal cortex, a region of association cortex involved in motivation, planning and other higher cognitive functions. The prefrontal cortex also has reciprocal connections with other brain regions implicated in the pathophysiology of schizophrenia (Carr & Sesack, 1996, Sesack *et al.*, 1989). Imaging studies demonstrate compromised functionality of the prefrontal cortex in schizophrenia as evidenced by reduced glucose metabolism in the prefrontal cortex of schizophrenics (Buchsbaum *et al.*, 1990, Cohen *et al.*, 1987). Reduced regional cerebral blood flow in the prefrontal cortex, with concomitant increases in blood flow in the occipito-temporal cortex, is also seen in schizophrenia (Ingvar & Franzen, 1974, Paulman *et al.*, 1990). Additional investigators (Berman *et al.*, 1988, Weinberger *et al.*, 1986) also reported reduced prefrontal blood flow in schizophrenic patients versus controls however, this reduction was only seen during the Wisconsin Card Sorting Test, a cognitive test of dorsolateral prefrontal function requiring interactions between the prefrontal and temporolimbic cortices. Findings of regional specificity in combination with task dependency suggest functional dysconnectivity of circuitry relevant to schizophrenia (Weinberger & Lipska, 1995). More recently, investigation of functional connectivity resulted in

significantly different patterns between schizophrenics and controls again suggesting dysconnectivity between the frontal and temporal lobes in schizophrenia (Berman *et al.*, 1999).

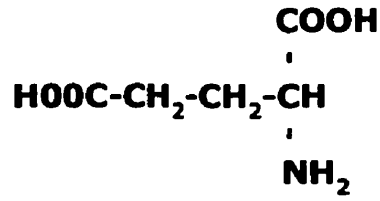
Further evidence for dysconnectivity stems from a neurological model of schizophrenia. Metachromatic leukodystrophy is a genetic disorder of sulfatide metabolism resulting in abnormal development of intracortical connectivity (Polten *et al.*, 1991). Demyelination results in disturbed but intact neuronal connectivity. Although the pathology is different in schizophrenia, many of the symptoms seen in schizophrenia are also seen in metachromatic leukodystrophy. At the onset of psychosis in metachromatic leukodystrophy, the demyelination is primarily in the prefrontal region, thus indicating that psychosis in this illness is related to a functional impairment in prefrontal connectivity (Hyde *et al.*, 1991). Although this disorder does not address many characteristics of schizophrenia, it does support the concept that alterations in connectivity of neurons can result in schizophrenic symptoms.

1.4 A Neurodevelopmental Animal Model of Schizophrenia

1.4.1 Characteristics of the Model

The widespread acceptance of the neurodevelopmental hypothesis of schizophrenia led to the establishment of a neurodevelopmental animal model of schizophrenia (Lipska *et al.*, 1993). This model involves the use of ibotenic acid to bilaterally lesion the ventral hippocampus at postnatal day 7 (PND 7) in male Sprague-Dawley rats. Ibotenic acid is a glutamate analogue extracted from the mushroom *Amanita muscaria* (Eugster *et al.*, 1965) (See Figure 1.2). It has been shown to produce axon sparing lesions in the hippocampus with toxic effects to GABAergic and glutamatergic intrinsic and projection neurons (Steiner *et al.*, 1984). It causes relatively little damage as compared to surgical or electrolytic ablation which have been shown to damage adjacent areas,

A.



B.

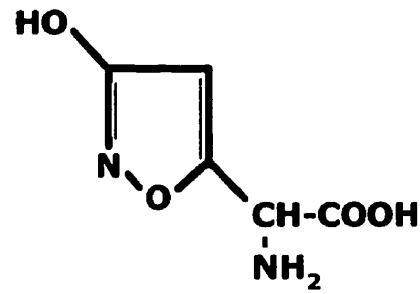


Figure 1.2. Chemical structure of glutamate (A) and its excitotoxic analog, ibotenic acid (B).

fibers passing through the hippocampus and vasculature (Jarrard, 1989). It is also a useful neurotoxin for use in neurodevelopmental models as it is able to induce lesions in immature brain structures. Kainic acid, another example of a glutamate analogue capable of producing excitotoxic lesions, is not toxic to neonatal animals, however, it has been demonstrate to result in apoptosis in the ventral hippocampus of adult animals (Contestabile *et al.*, 1998). It is proposed that ibotenic acid exerts its effects through the NMDA receptor and is, therefore, not age dependent as NMDA receptors are expressed throughout development (for review see McDonald and Johnston, 1990). In fact, hippocampal NMDA receptor binding has been demonstrated to be significantly higher from PND 4 to PND 9 than that seen in the adult hippocampus (Baudry *et al.*, 1981). Kainic acid is proposed to act through non-NMDA ionotropic receptors which are not expressed at PND 7 (Steiner *et al.*, 1984). For these reasons, ibotenic acid appears to be an ideal toxin for use in the development of this neonatal animal model.

Post natal day 7 is relevant for a neurodevelopmental animal model of schizophrenia as the first week of post natal development in the rat corresponds to the second trimester of gestation in the human (Bayer, 1980). During the second trimester, neurons migrate and settle into their appropriate cortical sites (Rakic, 1988). Apparent neurodevelopmental abnormalities in neuronal migration during this period (see section 1.3.1.2) are proposed to result in the structural abnormalities seen in adulthood (Kerwin & Murray, 1992). The second trimester is also the period of neurodevelopment coincident with maternal infections that correlate with the expression of schizophrenia (Mednick *et al.*, 1988). These factors indicate that rough parallels to the morphological features in schizophrenia may be able to be produced in a neurodevelopmental animal model.

The ventral hippocampus, the region lesioned in the model, was chosen as it was deemed the region to most frequently show abnormalities in schizophrenia by Lipska and colleagues (1993). Evidence to support this conclusion comes from multiple sources (Altshuler *et al.*, 1990, Altshuler *et al.*, 1987, Arnold *et al.*, 1995, Arnold *et al.*, 1991, Bogerts *et al.*, 1985, Jakob & Beckmann, 1986, Jeste & Lohr, 1989). The ventral hippocampus is also relevant to schizophrenia as it has been shown to be responsible for hippocampal regulation of the mesolimbic dopamine system (Springer & Isaacson, 1982) as well as having connections with the prefrontal cortex (Ferino *et al.*, 1987, Goldman-Rakic *et al.*, 1984), the region implicated in negative symptom expression. Specifically, monosynaptic excitatory afferents project from the CA1 region of the hippocampus onto pyramidal neurons within the prefrontal cortex (Ferino *et al.*, 1987, Laroche *et al.*, 1990). In terms of neurodevelopment, the synaptic formation of the hippocampus is completed much earlier in development than cortical regions (Kostovic *et al.*, 1989). This implies that the hippocampus may demonstrate increased vulnerability to early stage developmental abnormalities (Kerwin & Murray, 1992).

These factors discussed above all interact to produce a model of schizophrenia that is essentially a neurodevelopmental model of dysconnectivity. In effect, the ibotenic acid, during a period of critical neurodevelopment, results in deprivation of cortical and subcortical structures of their normal hippocampal connectivity (Weinberger & Lipska, 1995). This model, therefore, is in accordance with both the neurodevelopmental and the dysconnectivity hypotheses of schizophrenia. Whether consequent alterations in cell viability occur in adulthood, in support of the neurodegenerative hypothesis, has not been addressed.

1.4.2 Parallels to Schizophrenia

In an attempt to characterize the model, a number of parameters relevant to schizophrenia were originally investigated: dysregulation of dopaminergic systems, developmental/maturational dependency, frontal cortical dysfunction, vulnerability to stress, sensory gating (prepulse inhibition of startle), responsiveness to neuroleptic administration and genetic predisposition (Lillrank *et al.*, 1995).

Excitotoxic lesions in the ventral hippocampus of adult rats have been shown to result in enhanced spontaneous exploration with concurrent decreases in dopamine release/transmission in the medial prefrontal cortex and the limbic system (Lipska *et al.*, 1992), areas innervated by the hippocampus (Ferino *et al.*, 1987, Goldman-Rakic *et al.*, 1984). Animals lesioned as neonates show similar increases in spontaneous exploration (Lipska *et al.*, 1993), however, *in vivo* microdialysis indicates no alterations in basal dopamine levels in the prefrontal cortex or the nucleus accumbens (Lillrank *et al.*, 1996b). When rats with neonatal lesions are exposed to stress, however, contrary to sham animals, no increase is seen in extracellular dopamine. In addition, neonatally lesioned rats demonstrate an attenuated increase in dopamine levels in response to amphetamine challenge compared to sham animals. This finding was not replicated by Wan *et al.* (1996), however, they did find an enhanced behavioral response to amphetamine challenge compared to sham animals. Further exploration to identify the subtype of receptor involved demonstrated a role for increased sensitivity of the dopamine D_2 receptor subtype (Wan & Corbett, 1997), although specific isoforms of this receptor were not investigated. Lesioned animals demonstrate hyperresponsivity to quinpirole (a dopamine D_2-like receptor agonist) as compared to sham animals, attenuation of amphetamine induced locomotion by raclopride (a dopamine D_2 receptor antagonist), and show no differential effects with SKF38393 (a dopamine D_1

receptor agonist). These findings suggest that animals with neonatal ventral hippocampal lesions have hyperresponsive/ hypersensitive dopaminergic systems, especially with respect to dopamine D_2 receptors. This is especially relevant to schizophrenia as neuroleptics are thought to exert their effects, at least in part, through antagonism of D_2 receptors (Creese *et al.*, 1976, Seeman *et al.*, 1976). In addition, similar to schizophrenia, no overt alterations in the dopaminergic system have been identified but alterations are inferred based on behavioral and pharmacological responses (Lipska *et al.*, 1993).

Of further relevance to schizophrenia is the demonstration that dopaminergic hypersensitivity only becomes evident after puberty. Rats tested at PND 35 (prepubertal) demonstrate normal behaviors and dopamine responsiveness whereas rats tested on PND 56 (postpubertal) demonstrate hyperresponsiveness (Lipska *et al.*, 1993). The reason for the postpubertal onset is uncertain, however, it is not hormone dependent as animals castrated prior to puberty still demonstrate behavioral abnormalities (Lipska & Weinberger, 1994a). The timing of the neonatal lesion is critical to the delayed onset, as rats lesioned at PND 14, beyond the period of cell migration and settling, do not show the temporal onset of symptoms reminiscent of schizophrenia (Wood *et al.*, 1997).

In addition to the inferred abnormalities in the dopaminergic system, further evidence for dysregulation of neurotransmission, comes from *c-fos* mapping studies. *C-fos* is an immediate early gene expressed in most neurons at low levels, and therefore, is a useful tool for identifying transynaptically activated neurons in various brain regions (Morgan & Curran, 1991). Amphetamine challenge, to which lesioned animals are behaviorally hyperresponsive as compared to sham (Lipska *et al.*, 1993), results in a decreased expression of *c-fos* in lesioned rats as compared to sham in the prefrontal cortex and the striatum (Lillrank *et al.*, 1996a). This effect is seen at

30 minutes post-challenge, but levels of *c-fos* are the same 90 minutes post-challenge. This suggests a delayed response although animals do have the ability to express *c-fos* at normal levels. It is proposed that this delayed response could negatively affect the expression of late-response genes that regulate neuronal activity. The *c-fos* response to amphetamine could conceivably be via activation of dopamine D_1 receptors or inactivation of dopamine D_2 receptors. This does not seem likely, however, as to obtain the observed *c-fos* response, receptor numbers would most likely be altered and previous binding studies demonstrated no alterations in dopamine D_1 or D_2 receptors (Knable *et al.*, 1994). It is also possible that the ability of amphetamine to promote dopamine release is impaired in lesioned animals (Lillrank *et al.*, 1996a). Another possibility for the observed *c-fos* response is a decrease in glutamatergic neurotransmission. The destruction of glutamatergic projection fibers in the ventral hippocampus may result in reduced glutamate release in innervated regions such as the prefrontal cortex. Consistent with reduced glutamatergic neurotransmission is the demonstration that rats with neonatal lesions are hypersensitive, in adulthood, to the effects of MK-801, an NMDA antagonist (Al-Amin *et al.*, 1996). This observation is especially relevant as it demonstrates that other systems may be altered in the model and that dysconnectivity is almost certainly present and significant to impaired function. In addition, this observation brings the model one step closer to schizophrenia as these animals demonstrate hyperresponsivity to NMDA receptor antagonists. This is in accordance with the glutamatergic (NMDA receptor) hypofunction hypothesis of schizophrenia.

Animals with neonatal lesions of the ventral hippocampus, unlike animals lesioned in adulthood, also demonstrate a hypersensitivity to stress (Lipska *et al.*, 1993). Interestingly, animals with adult lesions in the medial prefrontal cortex show similar hyperresponsivity to stress (Jaskiw & Weinberger, 1992). The medial prefrontal cortex is known to play a role in stress and in the

regulation of subcortical dopamine systems (Deutch *et al.*, 1990). This leads to the conclusion that a neonatal lesion in the ventral hippocampus results in a loss of hippocampal – prefrontal connectivity and that this connectivity is essential for normal maturation of the prefrontal cortex (Lipska *et al.*, 1993). There is evidence, in support of this conclusion, that the synaptic connectivity of the prefrontal cortex does not fully develop into adulthood (Kalsbeek *et al.*, 1988) (for review see Lewis, 1997). This conclusion supports the dysconnectivity hypothesis of schizophrenia and an important role for the postpubertal development of the prefrontal cortex in the emergence and presentation of symptoms.

Various other behavioral abnormalities reminiscent of schizophrenia are also observed in animals with neonatal lesions of the ventral hippocampus. One such example is deficits in prepulse inhibition of startle as compared to sham animals, although the amplitude of the startle response in not different (Lipska *et al.*, 1995). This effect is also dependent on development, as differences are seen in PND 56 animals but not in PND 35 animals. Lesioned animals treated with apomorphine, a dopamine agonist, also show an exaggerated reduction in prepulse inhibition as compared to controls. This provides further evidence for dopaminergic dysregulation in the model. A second example of behavioral abnormalities associated with schizophrenia and the model is social withdrawal (Sams Dodd *et al.*, 1997). Deficits in social interaction are classified as negative symptoms in schizophrenia, therefore, the social withdrawal in rats with neonatal lesions indicates that aspects of both positive and negative symptoms can be reproduced in the model.

In addition to showing characteristics similar to schizophrenia, these animals with neonatal hippocampal lesions are also responsive to neuroleptic treatment (Lipska & Weinberger, 1994b). Low doses of both haloperidol and clozapine suppress spontaneous locomotor activity in lesioned animals, whereas

only haloperidol suppresses locomotor activity in sham rats. Haloperidol, at a dose lower than that seen to cause immobility, rigidity or catalepsy was proposed to continuously block dopamine D_2 receptors thereby inhibiting locomotor activity in both lesion and sham animals. It is suggested that clozapine may exert its actions in lesioned animals through receptors other than D_2. Alternatively, its effects may be subthreshold in intact animals but the dysfunctional system set up by the neurodevelopmental lesion may be more susceptible to its effects.

The final factor originally investigated in the model, genetic predisposition, also demonstrates parallels between the model and schizophrenia. A rat strain (Fisher 344) characterized by hyperresponsivity of dopamine systems to stress required a much smaller lesion to demonstrate abnormalities equivalent to Sprague-Dawley rats, the typical strain used (Lipska & Weinberger, 1995). In addition, a lesion of similar size to that seen in the Sprague-Dawley rats resulted in a much earlier onset of abnormalities. Contrary to this, rats (Lewis rats) with hyporesponsivity of dopamine show no abnormalities with typical lesions. This demonstrates that genetics can set up a predisposition for vulnerability to insults and can regulate the severity and expression of the defect. This suggests that genetics may play a significant role in the heterogeneous phenotypic expression of schizophrenia.

1.4.3 Implications and Limitations of the Model

This model demonstrates that a wide variety of factors associated with schizophrenia can be reproduced in an animal model, including dopamine dysregulation, prefrontal dysfunction, vulnerability to stress, behavioral and sensorimotor gating defects, responsiveness to dopamine antagonists and a genetic predisposition. It also demonstrates a potential dysregulation of other systems, such as the glutamatergic system, which is also relevant to

observations in schizophrenia. Perhaps most importantly, however, it proves that the neurodevelopmental theory of schizophrenia is biologically possible, i.e., that a neurodevelopmental insult can result in consequences that manifest only after puberty (Lillrank *et al.*, 1995).

There are a number of limitations associated with this model. Of primary concern is the fact that schizophrenia is a human disease affecting cognition, perception, behavior and emotion, therefore, the human condition is much more complex than could ever be seen in a rat model. The effects seen in the model, although proposed to result from neuropathology similar to schizophrenia, cannot be identical to symptoms seen in schizophrenia in humans. A second limitation is that the limited lesion produced in the rat model is still far greater than the subtle neuropathology seen in schizophrenia. Third, although genetic factors can be demonstrated to play a role, the specific causative factors that lead to aberrant neurodevelopment are not addressed in the model. Despite the limitations, this model allows for the exploration of a number of elements that would not be feasibly or ethically possible in a human patient. This includes elements such as mechanisms of neuroleptic action, drug testing, genetic – environmental interactions as well as time point studies of gene expression patterns. If extrapolations to the human condition of schizophrenia can be made, this model also has the advantage of being able to control more factors than is possible with a human population.

1.5 Potential Factors Involved in Schizophrenia

1.5.1 Stress

A physiological stressor is defined as any external perturbation, or anticipation thereof, that results in a disruption of homeostasis (Sapolsky, 1992). Exposure to stress results in a characteristic response of neural and endocrine alterations in an attempt to reestablish homeostasis. These

responses include mobilization of energy, increase in cardiovascular tone to facilitate oxygen and glucose delivery to active tissues, suppression of digestion, growth, reproduction, and inflammatory and pain responses. In addition, there is an alteration in cognition such that a sharpening of senses is demonstrated. Central to the stress response is the secretion of glucocorticoids, cortisol in the human and corticosterone in the rat. In general, adaptation to acute stress with minimal consequences is seen, but chronic stress can result in a disease process as the responses to stress become damaging over time (See Table 1.3). In particular, chronic or excessive exposure to glucocorticoids is especially damaging to hippocampal neurons (Sapolsky *et al.*, 1984, Watanabe *et al.*, 1992, Woolley *et al.*, 1990), an effect proposed to be mediated by glutamate via NMDA receptors (Armanini *et al.*, 1990). Chronic exposure to stress can result in dendritic atrophy as well as cell death. Lui *et al.* (1996) also demonstrated that stress results in lipid peroxidation, protein oxidation and nuclear DNA damage in the cerebral cortex as well as the hippocampus. This suggests oxidative stress and subsequent neurodegeneration can be induced by physiological stress, at least in extreme conditions (8 hours of immobilization stress). In addition, dysregulation or reduction of glucocorticoid secretion can also result in damage due to an inability to appropriately adapt to stress (Sapolsky, 1992). The regulation of glucocorticoid secretion is under the control of the dorsal hippocampus (Feldman & Conforti, 1980) via a multisynaptic projection from the hippocampus to the paraventricular nucleus of the hypothalamus (Sapolsky, 1992). This input inhibits the adrenocortical axis resulting in the downstream inhibition of glucocorticoid secretion (for review see Sapolsky, 1992). The hippocampus also mediates feedback inhibition, therefore, loss of corticosteriod receptors, due to compensatory downregulation, cell loss or aging, results in a hypersecretion of glucocorticoids and a potentially neurotoxic cascade.

STRESS RESPONSE	STRESS RELATED DISORDER
Mobilization of energy	Myopathy, fatigue, steroid diabetes
Increased cardiovascular tone	Stress-induced hypertension
Suppression of digestion	Ulceration
Suppression of growth	Psychogenic dwarfism
Suppression of reproduction	Amenorrhea, impotency, loss of libido
Suppression of immune system	Increased disease risk
Sharpening of cognition	Neuron death

Table 1.3. The stress response and consequent disorders from chronic exposure. Adapted from Sapolsky, 1992.

As described, stress can result in serious consequences in a normal animal or human (for reviews see Sapolsky, 1992 and Selye, 1976), however, the ramifications are even greater in a person with schizophrenia. In addition to neurotoxic consequences of chronic stress, stress has been shown to exacerbate the symptoms of schizophrenia, as well as precipitate the onset and relapse of schizophrenia (Brown & Birley, 1968, Dohrenwend & Egri, 1981, Mari & Steiner, 1994). Dopamine and glutamate, neurotransmitters proposed to be involved in schizophrenia, are increased in the prefrontal cortex in response to stress (Abercrombie *et al.*, 1989, Moghaddam, 1993, Thierry *et al.*, 1976). The increase in dopamine is proposed to be due to the action of glutamate on prefrontal AMPA receptors as dopamine release is blocked by AMPA antagonists but not by NMDA antagonists in this region (Takahata & Moghaddam, 1998). In the ventral tegmental area, the origin of dopaminergic afferents to the prefrontal cortex, glutamate acts on both AMPA and NMDA receptors, to regulate dopamine release in the prefrontal cortex. This suggests that dysregulation of glutamate neurotransmission is upstream from dopamine dysregulation, and may result in the proposed abnormalities in dopaminergic neurotransmission in schizophrenia. These findings serve to integrate the role of stress in schizophrenia with the glutamate hypothesis as well as the dopamine hypothesis of schizophrenia. In addition, it cannot be ignored that dysconnectivity, rather than strict dysregulation of glutamate, may result in enhanced responsivity to stress.

1.5.2 Brain-Derived Neurotrophic Factor

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, was first isolated from pig brain in 1982 (Barde *et al.*, 1982) and was subsequently cloned and characterized in the rat and the human (Leibrock *et al.*, 1989, Maisonpierre *et al.*, 1991). BDNF is expressed in neurons throughout the brain with the highest levels found in the hippocampus (Ernfors *et al.*,

1990, Hofer *et al.*, 1990, Wetmore *et al.*, 1990). More recently, however, it has been demonstrated that BDNF protein is not confined to neuronal cell bodies, but it is also found in neuropil, in both nerve terminals and preterminal axons (Conner *et al.*, 1997). The absence of BDNF mRNA in protein rich neuropil suggests that BDNF protein is anterogradely transported by axons to terminal regions.

From the BDNF gene, multiple mRNA transcripts are derived through the use of four different promoter sequences in combination with alternative splicing of exons within the 5' untranslated region of the gene (Timmusk *et al.*, 1993). Alternative promoters are responsible for developmental stage, tissue type and cell type specific expression. Exons I and II are specific to brain, exon III is found in brain and heart, and exon IV is abundant in heart and lungs with low levels in brain. The expression of BDNF is also dependent on the balance between the activity of the glutamatergic and GABAergic systems (Zafra *et al.*, 1990, 1991). Increased glutamate neurotransmission and reduced GABAergic neurotransmission are associated with increased BDNF expression. $GABA_A$ receptor antagonism results in a specific activation of promoters associated with exons I and III predominantly within the dentate gyrus of the hippocampus (Metsis *et al.*, 1993). In the neocortex, BDNF expression of exon I, II and III transcripts is blocked by NMDA and AMPA/kainate receptor antagonists suggesting these receptors are involved in BDNF regulation in cortical regions. In the hippocampus, however, with the exception of the exon III transcript in the CA1 region, NMDA receptor antagonism does not block BDNF expression. In fact, pretreatment with NMDA receptor antagonists potentiates the expression of BDNF exon I and II transcripts in the CA3 region and the dentate gyrus of the hippocampus. In addition, NMDA receptor antagonism has been shown to increase BDNF expression in the entorhinal, retrosplenial and cingulate cortices although transcript variants were not examined (Castren *et al.*, 1993). These results demonstrate that each transcript shows regional

variation in its expression pattern and regulation. This results in a complicated system of which the functional consequences are not yet known.

In the hippocampus, cortex and *in vitro*, BDNF may regulate the survival, differentiation, morphology and synaptic remodeling of neurons (Alderson *et al.*, 1990, Ghosh *et al.*, 1994, Jones *et al.*, 1994, Korte *et al.*, 1995, McCallister *et al.*, 1995, Thoenen, 1995). It has also been demonstrated that BDNF may modulate neurotransmitter synthesis, metabolism and release, postsynaptic ion channel fluxes, neuronal activity and long term potentiation (Altar *et al.*, 1997, Croll *et al.*, 1994, Kang & Schuman, 1995, Thoenen, 1995). The association of BDNF with neurodevelopment, cell viability and synaptic strength make it an attractive candidate for involvement in schizophrenia. Support for a role for BDNF also stems from the demonstration that it is decreased by factors correlated with first episode onset such as stress (Smith *et al.*, 1995a, 1995b) and estrogen withdrawal (Singh *et al.*, 1995). In addition, stress induced decreases in BDNF are blocked by 5-HT_2 receptor antagonists, a receptor binding property of many neuroleptics (Vaidya *et al.*, 1997). Electroconvulsive treatment (ECT), effective in treatment-resistant schizophrenia in combination with neuroleptics (Sajatovic & Meltzer, 1993), also upregulates the expression of BDNF (Lindefors *et al.*, 1995, Nibuya *et al.*, 1995). These findings lead to the inference that BDNF may be altered in schizophrenia. Direct investigations of BDNF in schizophrenia also suggest that it may be altered. Findings indicate that hippocampal BDNF mRNA is reduced (Brouha *et al.*, 1996) as is serum BDNF in schizophrenic patients (Toyooka *et al.*, 1999). An allele variant of the BDNF gene has also been identified in a population of schizophrenic patients (Vicente *et al.*, 1996). These factors suggest that disruptions of BDNF may play a role in the etiology of this disorder by compromising neuroplasticity or altering neurotransmission.

1.5.3 N-Methyl-D-Aspartate Glutamate Receptor

Excitatory amino acids, primarily L-glutamate and L-aspartate, are the major excitatory neurotransmitters in the brain, and as such, the most abundant neurotransmitter class in the mammalian brain (Cotman & Iversen, 1987). These neurotransmitters exert their actions through two major classes of receptors: ionotropic (ion-specific transmembrane channels) and metabotropic (G-protein coupled) receptors. The ionotropic receptors can be further subdivided into NMDA, AMPA and kainate receptors (for review see (Hollmann & Heinemann, 1994, Nakanishi, 1992, Seeburg, 1993). NMDA receptors have been shown to play an important role in neurodevelopment (McDonald & Johnston, 1990), synaptogenesis (Constantine-Paton *et al.*, 1990), synaptic plasticity (Bliss & Collingridge, 1993), and excitotoxicity (Rothman & Olney, 1987). They have also been shown to have neurotrophic effects (Balazs *et al.*, 1988). The functions of NMDA receptors are mediated by characteristic receptor properties. NMDA receptors are characterized by high permeability to calcium, sodium and potassium. These receptors require glycine for activation (Johnson & Ascher, 1987), they are activated by polyamines (Durand *et al.*, 1992), and are inhibited by zinc (Nakanishi, 1992). In addition, NMDA receptor channels are blocked in a voltage-dependent manner by magnesium (Mori *et al.*, 1992). NMDA receptors are multimeric complexes comprised of NMDAR1 and NMDAR2 subunits, each with four transmembrane segments, that assemble in various combinations to produce various electrophysiological receptor properties (Nakanishi, 1992). There are four different NMDAR2 subunits, responsible for modulation of NMDA receptor activity, encoded on four distinct genes. The NMDAR1 subunit is essential to the activity of the receptor and its mRNA is expressed in almost all neurons throughout the central nervous system with especially high levels in the hippocampus, cerebral cortex, cerebellum and olfactory bulb (Moriyoshi *et al.*, 1991). Various isoforms of the NMDAR1 receptor, generated by alternative splicing of a single gene, also exist

(Nakanishi *et al.*, 1992, Nakanishi, 1992); so far, nine have been identified (McBain & Mayer, 1994). These splice variants show differential spatial and temporal expression (Laurie & Seeburg, 1994), and pharmacological properties (Durand *et al.*, 1992). To date, however, little is known about the expression profile and regulatory mechanisms of these splice variants. However, the multiple forms of NMDAR1 and NMDAR2 and various possible combinations, lead to distinct receptor properties and the subsequent ability to differentially regulate various functions and microenvironments.

The extensive expression and function of NMDA receptors, in combination with reported alterations in glutamate neurotransmission and NMDA receptor antagonist sensitivity, make them likely candidates for abnormalities in schizophrenia. They influence neurodevelopment and neuronal viability and, therefore, alterations in neurodevelopment may result in the morphological and cytoarchitectural abnormalities observed in schizophrenia. If NMDA receptors are not a primary pathogenic factor, but rather are dysregulated as a consequence of other abnormalities, they still have significant implications for progression of the disease state. This conclusion is supported by the demonstration of neuroleptic regulation of various NMDA receptor subunits (Fitzgerald *et al.*, 1995, Riva *et al.*, 1997) and the fact that glutamatergic projections are the main projections connecting prefrontal and limbic structures (Cotman *et al.*, 1987, Fagg & Foster, 1983), primary regions implicated in the pathophysiology of schizophrenia.

1.5.4 Apoptosis

Apoptosis is a tightly regulated genetic program for active cell death (Majno & Joris, 1995, Savill, 1994). It is engaged in a precise manner to achieve physiological cell clearance, such as that seen in neural pruning (Oppenheim, 1991). It is also becoming increasingly apparent that apoptosis is

a factor in neurodegenerative diseases such as Alzheimer's disease (Stefanis *et al.*, 1997). Abnormalities in the program of apoptosis, resulting in inappropriate time, place or quantity of cell death are proposed to be the mediating factor in neurodegenerative disorders (Margolis *et al.*, 1994). Apoptosis is characterized by a morphological and biochemical profile distinct from that of necrosis, a form of cell death not tightly regulated (See Table 1.4) (for reviews see Majno and Joris, 1995, Wyllie *et al.*, 1980, Sastry & Rao, 2000). Apoptosis is characterized by cell shrinkage, chromatin condensation, membrane blebbing and fragmentation of the cell into apoptotic bodies. Perhaps most characteristic, they also show a DNA fragmentation pattern of internucleosomal cleavage. Apoptosis can be induced by a wide variety of insults including ischemia (MacManus *et al.*, 1993), glutamate excitotoxicity (Kure *et al.*, 1991), NMDA antagonist administration (Johnson *et al.*, 1998, Zhang *et al.*, 1996), trophic factor withdrawal (Deckwerth & Johnson, 1993), oxidative stress and disruption of calcium homeostasis (Boobis *et al.*, 1989). For a comprehensive review of apoptosis refer to McConkey *et al.* (1996).

There has been no direct evidence of apoptosis in schizophrenia, but the suggestion of symptoms and morphological abnormalities progressively deteriorating over the course of the disease has led to the proposal that neurodegeneration, via apoptotic mechanisms occurs in schizophrenia (Coyle, 1996, DeLisi, 1997, Margolis *et al.*, 1994). This proposal is also supported by the demonstrated reduction in neurons in the prefrontal cortex (Benes *et al.*, 1991), the hippocampus (Jeste & Lohr, 1989) and in the thalamus (Pakkenberg, 1990) of schizophrenics and is in accordance with the lack of gliosis in schizophrenics. The detection of apoptosis is not technically possible at this time in the brain of a living person, nor is postmortem analysis sufficient to identify if apoptosis is an ongoing phenomenon in schizophrenia. Therefore, until technology can overcome this obstacle, one is left to only speculate on a role for apoptosis in schizophrenia.

CHARACTERISTICS	APOPTOSIS	NECROSIS
Morphology	Reduced volume, chromatin condensation, organelles intact	Increased volume, early membrane rupture, damaged organelles
Macromolecule Synthesis	Typically essential; increased early	Unnecessary, rapidly shut down
DNA Cleavage	Internucleosomal pattern	No detectable pattern
Mechanism	Cascade of genetically controlled events	Loss of water and electrolyte balance
Calcium	Moderate influx	Massive influx
Typical Causes	Normal development, hormones, loss of trophic support	Hypoxia, hypothermia, complement attack, toxins
Immune Response	Phagocytosis	Inflammatory response

Table 1.4. Comparison of apoptosis and necrosis as mechanisms of cell death. Adapted from Margolis *et al.*, 1994.

1.6 Objectives of the Project

Many questions regarding the pathophysiology of schizophrenia remain unanswered and the limitations of investigations in human patients lead one to conclude that definite answers are still some distance away. The development of animal models, such as that developed by Lipska and Weinberger (Lipska *et al.*, 1993), therefore, offer a great advantage as they provide an avenue for investigations not possible in humans. Although great care must be taken in making conclusions about schizophrenia from such models, significant advances can still be made. With the acceptance of the neurodevelopmental hypothesis of schizophrenia and consequent dysconnectivity comes the need to investigate factors that may be involved in neurodevelopment, neuronal and synaptic viability and neurotransmission, factors all proposed to be compromised in schizophrenia. The objectives of this research project were to investigate the expression of BDNF mRNA and NMDAR1 mRNA, both factors involved in neurodevelopment, cell viability and neurotransmission, in animals with neurodevelopmental lesions of the ventral hippocampus. It was also of interest with respect to schizophrenia to investigate if these factors were differentially regulated in response to stress in lesioned versus control animals. A second major objective of this project was to determine if animals with developmental lesions modeling schizophrenia demonstrate increased apoptosis in the prefrontal cortex as compared to control animals. A second part of this question was whether or not animals with neurodevelopmental lesions show increased vulnerability to neurodegeneration induced by stress.

The hypothesis tested was that neurodevelopmental abnormalities modeling schizophrenia create a functionally compromised system with alterations in factors necessary for maintaining neuron viability and neuronal communication. The overall result would be a system more susceptible to neuronal atrophy and/or death caused by environmental factors such as stress.

2. MATERIALS AND METHODS

2.1 Chemicals

All chemicals used were either reagent or molecular biology grade. All solutions were made with nanopure water, i.e. water purified by reverse osmosis, ion exchange and organic component removal (Barnstead RO pure and nanopure II with organic cartridge; Sybron Corp., Boston, Mass., U.S.A.). Nanopure water used for in situ hybridization procedures was treated overnight with 0.1% diethylpyrocarbonate (DEPC) then autoclaved for 30 minutes.

2.2 Animals

Timed pregnant Sprague-Dawley rats were obtained from Charles River Canada (Montreal, Quebec, Canada) at 14 days gestation. All animals were housed in clear plastic cages at a room temperature of 19-21°C on a 12-hour light/dark cycle. Animals were allowed access to food and water ad libitum. All procedures involving animals were done in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Care Committee.

2.2.1 Surgery

Post-natal day (PND) 7 male rats were randomized to lesion or sham status and anaesthetized by hypothermia. Specifically, the pups were placed on wet ice for 5 minutes following which they were covered, with the exception of their head, with approximately 2 cm of ice and left for an additional 10 minutes. This induced a state of anesthesia lasting approximately 30 minutes

with no associated mortality. Xylocaine was applied to the head and an incision was made to expose the skull. The pups were placed in a modified neonatal stereotaxic apparatus (Cunningham & McKay, 1993). Bilateral injections of 1.5 µg (±)-ibotenic acid (RBI, Natick, MA, U.S.A.) in 0.3 µl of artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 5 mM KCl, 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgCl_2$, 1mM NaH_2PO_4, 24 mM $NaHCO_3$ and 11 mM D-glucose) (lesion) or ACSF alone (sham) were infused into the ventral hippocampus (AP –3.0, ML ±3.5, VD –5.0 relative to bregma) at a flow rate of 0.15 µl/minute. The delivery system consisted of a 10 µl removable needle Hamilton syringe (Chromatographic Specialties, Brockville, Ontario, Canada) attached to fused silica tubing (0.4 mm outer diameter, 0.2 mm inner diameter; Chromatographic Specialties, Brockville, Ontario, Canada). A microinfusion pump (MA 100, Medicin, Stockholm, Sweden) controlled delivery. The tubing was left in the brain for 4 minutes following completion of the delivery to allow for diffusion. Simple interrupted sutures were used to close the incision and the pups were allowed to recover in a 37°C humid chamber. Following recovery they were returned to the mother. Rats were weaned at PND 25 and housed in groups of 4 with 2 lesion and 2 sham per cage.

2.2.2 Behavioral Testing

At PND 35 and PND 56 rats underwent open field locomotor activity testing. Rats were placed in a 36 cm X 36 cm X 23 cm clear Plexiglas test box positioned inside a Columbus Instruments infra-red photobeam recording device consisting of a 12 X 12 photobeam grid. The photobeam device was interfaced with an Apple II+ computer that recorded photobeam breaks as a measure of locomotor activity over time. At each age, the locomotor activity of each animal was recorded for 60 minutes. Test chambers were cleaned with 70% ethanol before each trial.

2.2.3 Stress Paradigm

At PND 75 lesion and sham animals, in groups of 4, were exposed to either a stress or a non-stress condition. The stress condition was a forced swim test consisting of 15 minutes in 30 cm of 25°C water. The control environment was identical to the swim environment with the exception that bedding material was placed in the bottom of the cage instead of water. Test cages were made of opaque plastic measuring 55 cm x 42 cm x 42 cm. Following the forced swim test, open field locomotor activity (as described above) was monitored for 90 minutes.

2.2.4 Perfusion

Twenty-four hours post-stress, animals were transcardially perfused with 200 ml 0.1 M phosphate buffered saline (PBS) at pH 7.4 followed by 250 ml 4% paraformaldehyde (PFA) in 0.1 M (PBS). Brains were removed, cut into two blocks (one containing the prefrontal cortex and the other containing the hippocampus), post-fixed in 4% PFA in 0.1 M PBS for 3 hours then cryoprotected in 30% sucrose in 0.1 M PBS at 4°C. Following cryoprotection, brains were flash frozen in isopentane/dry ice at –50°C and stored at –70°C.

2.3 Tissue Processing

2.3.1 Poly-L-lysine Coating Procedure

Poly-L-lysine (Sigma Chemical Co., St. Louis, MO, U.S.A.) was diluted to a 0.1% solution (1:10 v/v) in diethylpyrocarbonate (DEPC) treated water. Precleaned J Melvin Freed microscope slides (VWR, Mississauga, Ontario, Canada) were dipped for 5 minutes in 0.1% poly-L-lysine, allowed to dry at 60°C, dipped a second time for 5 minutes and allowed to dry at 60°C again. Slides were stored 4°C.

2.3.2 Sectioning

At least one hour prior to sectioning, poly-L-lysine coated slides and brain blocks were equilibrated in a Shandon cryostat set at –22°C. Brain blocks were mounted with OCT embedding compound (VWR, Mississauga, Ontario, Canada) and 10 µm sections through the prefrontal cortex and the hippocampus were generated. Sections were thaw mounted onto poly-L-lysine coated slides in a rotating order such that each slide had one section from each of the four conditions and on sequential slides each of the four conditions were represented in each of the positions available on the slides. Sections through the hippocampus designated for histological staining were collected at 100 µm intervals and thaw mounted onto poly-L-lysine coated slides.

2.3.3 Histology

Sections through the ventral hippocampus were stained with haematoxylin and eosin (H&E). Sections were rehydrated through descending ethanols, stained 5 minutes in Harris' haematoxylin (Sigma Chemical Co., St. Louis, MO, U.S.A.), washed in tap water, differentiated in acid alcohol (95% ethanol containing 1% v/v HCl) then rinsed. Sections were counterstained in eosin solution containing 1 part stock (0.8% w/v erythrosin, 0.1% w/v eosin Y, 0.1% w/v eosin B and 0.2% v/v glacial acetic acid) and 3 parts 80% ethanol, rinsed, dehydrated through ascending ethanols, cleared and mounted with D.P.X. Neutral Mounting Medium (Aldrich Chemical Co Inc., Milwaukee, WI, U.S.A.)

Sections through the ventral hippocampus were evaluated for animal inclusion or exclusion based of the following exclusion criteria: damage in ACSF treated animals, damage outside the hippocampus in ibotenic acid treated animals, unilateral damage in ibotenic acid treated animals or lack of damage in

ibotenic acid treated animals. Animals were also excluded if they demonstrated convulsive behavior.

2.3.4 BDNF and NMDAR1 Probe Preparation

2.3.4.1 BDNF and NMDAR1 cDNA

BDNF cDNA in pBluescript SK-, designated pSK-rB(C1), was obtained from Dr. Ron Lindsay (Regeneron Pharmaceuticals, Tarrytown, NY, U.S.A.). Rat NMDAR1 cDNA in pBluescript SK-, designated pNMDAR1-1a, was obtained from Dr. Jim Boulter (The Salk Institute, San Diego, CA). pSK-rB(C1) contained an 1127 base pair sequence of rat BDNF cDNA encoding prepro rat BDNF and mature rat BDNF, useful for the detection of all alternative mRNA transcripts of the BDNF gene. This sequence was cloned into the *Eco*R1 polylinker site of pBluescript SK-. pNMDAR1-1a contained a 4183 base pair sequence containing the coding region of the rat glutamate receptor subunit gene NMDAR1-1a. This sequence was inserted between the *Eco*R1 (5') and the *Xho*I (3') sites of the polylinker region. Both of the designated clones contained the gene for ampicillin resistance as well as promoter sites for T7 and T3 RNA Polymerase enzymes (See Figure 2.1).

2.3.4.2 Preparation and Transformation of Competent Bacteria

LB (Luria-Bertani) medium (1% w/v bacto-tryptone, 0.5% w/v bacto-yeast extract, 0.1% w/v NaCl, pH 7.4) containing 1.5% w/v agar was poured into tissue culture plates and following solidification, plates were inoculated with *E. coli* JM109 and incubated at 37°C until distinct bacterial colonies could be identified. A distinct colony was removed from the plate and transferred to 40 ml of sterile LB medium and placed in a shaking water bath at 37°C. The optical density of the bacterial suspension was monitored at 600 nm until 0.4 O.D. was obtained. At this time, the bacteria were collected by centrifugation

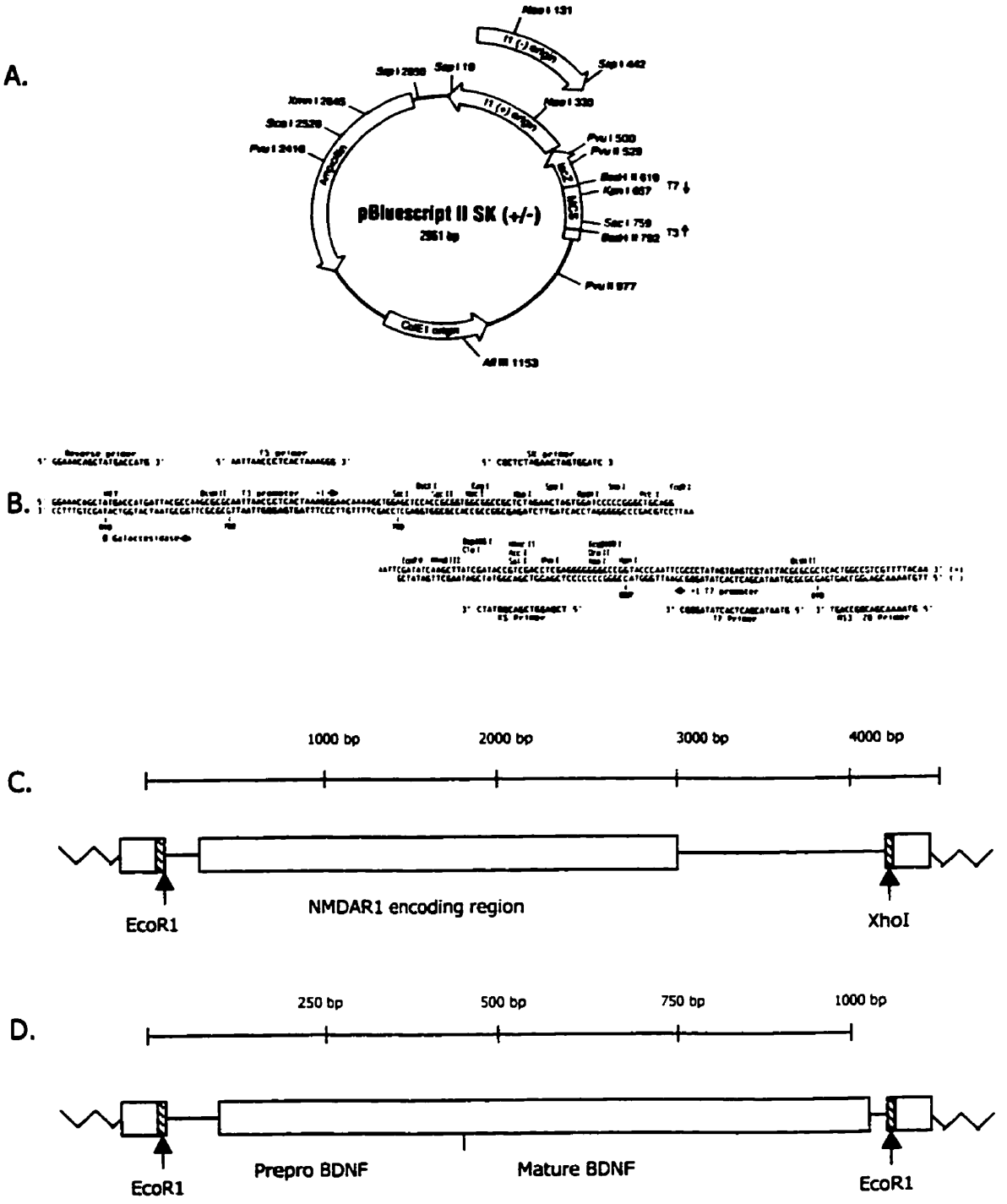


Figure 2.1. Structure of the pBluescript-SK plasmid (A) with the multicloning site (MCS) expanded to show the restriction digest sites surrounding the RNA Polymerase T7 and T3 promotor sites (B). C and D depict the NMDAR1-1a cDNA and BDNF cDNA inserts, respectively.

at 2500 x g at room temperature and resuspended in 4 ml of 50 mM $CaCl_2$. The volume of the suspension was brought to 20 ml with $CaCl_2$ solution and the suspension was incubated on ice for 30 minutes to produce competent bacteria. Competent bacteria were collected by centrifugation at 2500 x g for 5 minutes at 4°C then resuspended in 4 ml ice cold $CaCl_2$ solution. Ten nanograms of either pSK-rB(C1) or pNMDAR1-1a were incubated for 20 minutes on ice with 200 µl of competent JM109. The bacteria were heat shocked at 42°C for 2 minutes then incubated in 1 ml of LB medium for 45 minutes at 37°C to allow for expression of antibiotic resistance. The resulting suspension was plated onto LB agar plates containing 0.05 mg/ml ampicillin for selection of transfected JM109. Untransformed JM 109 were plated separately on ampicillin plates as a negative control. Plates were incubated overnight at 37°C to allow colonies to form. A single colony was transferred to 100 ml of LB medium and allowed to grow in a shaking water bath at 37°C overnight for amplification of the plasmid and bacteria.

2.3.4.3 Amplification and Isolation of pSK-rB(C1) and pNMDAR1-1a

Transformed bacterial cells were collected by centrifugation, resuspended and lysed in 0.2M NaOH containing 1% (w/v) SDS. Cell debris was removed and the plasmid DNA was precipitated with isopropanol. Proteins and lipids were removed by SS-phenol (salt-saturated phenol) and (1:24) isoamyl alcohol:chloroform extraction. Plasmid DNA was ethanol precipitated, washed, air dried and resuspended in water.

2.3.4.4 Purification of the Plasmid DNA

The plasmid DNA was combined 1:1 with DNA loading buffer and ran on a 1% agarose gel containing ethidium bromide. Plasmid DNA was excised from

the agarose gel and purified using a Geneclean II kit (BIO 101 Inc., LaJolla, CA, U.S.A.). Approximately 0.3 g aliquots of agarose were transferred to eppendorf tubes and 3 volumes of NaI solution were added. Tubes were placed in a 50°C waterbath to dissolve the agarose. Glassmilk (an insoluble silica matrix) was added to adsorb the DNA. The mixture was placed on ice for 5 minutes with periodic vortexing. The Glassmilk was collected by pulse centrifugation and the supernatant was removed. Plasmid DNA was eluted with water and its concentration was determined by measuring the absorbance at 260 nm and using the following equation:

$$\text{DNA } (\mu g/\mu l) = A_{260} \times EC \times DF$$

where: A_{260} is the absorbance at 260 nm, EC is the extinction coefficient of DNA (0.05 µg/µl) and DF is the dilution factor.

2.3.4.5 Restriction Digestion of pSK-rB(C1) and pNMDAR1-1a

Mini-digests were performed on 1 µg of plasmid DNA to map the plasmids and test the efficacy of the restriction enzymes. pNMDAR1-1a was mapped using *Bam*HI and *Apa*I. pSK-rB(C1) was mapped using *Bam*HI and *Xho*I. Once the identities of the plasmids were verified, large scale restriction digests were carried out to produce probe templates. pNMDAR1-1a was linearized with *Bam*HI to produce an antisense probe template and with *Apa*I to produce a sense probe template. pSK-rB(C1) was linearized with *Bam*HI to produce an antisense probe template and with *Xho*I to produce a sense probe template. All reactions were incubated overnight at 37°C, the enzymes were heat inactivated and completion of linearization was confirmed by 1% agarose gel electrophoresis with ethidium bromide. Concentrations of the templates were determined by measuring the absorbance at 260 nm, and dilutions were carried out to make the final concentration approximately 0.5 µg/4 µl. Four µl aliquots of the probe templates were stored at –20°C.

2.3.4.6 Radiolabeling RNA Probes

Radiolabeled RNA probes were transcribed from cDNA templates using T7 RNA Polymerase (Pharmacia Biotech, Uppsala, Sweden) (antisense) or T3 RNA Polymerase (Pharmacia Biotech, Uppsala, Sweden) (sense). Prior to labeling, T7 and T3 RNA Polymerase enzymes were diluted to a concentration of 10 units/µl in 20 mM K_2HPO_4/KH_2PO_4, pH 7.5, 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 100 µg/ml BSA and 5% v/v glycerol. The labeling reaction was performed using 0.5 µg template DNA, 0.5 mM ATP, GTP and UTP (Pharmacia Biotech, Uppsala, Sweden), 1X transcription buffer (400 mM Tris·HCl, pH 8.0, 60 mM $MgCl_2$, 100 mM DTT and 20 mM spermidine), 2 units/µl RNAguard (Pharmacia Biotech, Uppsala, Sweden), 0.5 units/µl T7 or T3 RNA Polymerase, 8 µM CTP and 50 µCi $^{35}S\alpha$-CTP (Dupont NEN, Boston, MA, U.S.A.). The reaction was incubated at 37°C for 2 hours then heat inactivated (65°C for 15 minutes). Ten units of DNase I was added to the reaction mixture and incubated for 45 minutes at 37°C. Following template degradation, 10 µl of 0.05 M EDTA and 65 µl of STE buffer (NaCl-Tris-EDTA) The probe was purified on a Bio-Spin Chromatography Column (Bio-Rad Laboratories, Mississauga, ON) containing a Bio-Gel P-6 matrix then the activity was measured in an LS5000TD Liquid Scintillation Counter (Beckman Instruments, Fullerton, CA, U.S.A.).

2.3.4.7 Preparation of Probes for In Situ Hybridization

Hybridization buffer (0.6 M NaCl, 0.08 M Tris, pH 7.5, 0.004 M EDTA, 0.1% w/v sodium pyrophosphate, 10% w/v dextran sulfate, 0.2% w/v SDS, 0.02% w/v heparin sodium salt, 50% v/v ultraformamide) was added to the probe to produce a final activity of ~2 X 10^6 cpm/40 µl. The mixture was boiled for 5 minutes, DTT was added to a final concentration of 100 mM and the probe was placed on ice.

2.3.5 Detection of mRNA by In Situ Hybridization

2.3.5.1 Theory of In Situ Hybridization

In situ hybridization is a technique used to detect specific RNA or DNA species in tissue sections through the use of RNA (or DNA) probes complementary to the species of interest. This technique has the advantage of investigating discrete brain regions that are difficult to dissect out or do not contain sufficient RNA for other detection methods (Wilcox, 1993). A second advantage is the ability to investigate mRNA expression at the cellular level. This allows for differentiation of cell populations expressing the species of interest. In situ hybridization is also compatible with immunohistochemical techniques thus allowing for the specific identification of cell types expressing the gene of interest. In addition, multiple detection methods including radioisotopes, non-isotopic fluorochromes or enzymatic conjugates allow for the simultaneous detection of multiple species (Bresser & Evinger-Hodges, 1987). Despite the many advantages of in situ hybridization over other nucleic acid hybridization techniques, there are also a number of limitations associated with this technique. Limitations include the inability to assess transcription rates or stability of transcripts. The technique is also limited by the artificial, static conditions within the tissue as the sections are fixed and processed to ensure optimum hybridization conditions. The choice of fixative can dramatically alter the sensitivity of the technique and optimization of fixation should be performed for each cell or tissue type (Bresser & Evinger-Hodges, 1987). The procedure is sensitive, but is dependent on investigator technique as the sections must be morphologically intact following multiple processes including perfusion, brain removal, freezing, sectioning, thaw mounting, prehybridizing, hybridizing and washing. In situ hybridization is also time consuming and labor intensive especially when considering the multiple controls necessary to establish a sensitive, specific and reproducible protocol (Bresser & Evinger-Hodges, 1987).

Hybridization is a reversible process that occurs through hydrogen bonding between complementary nucleotide bases of single stranded RNA or DNA molecules. Three possible hybrids are formed: RNA:RNA, DNA:RNA and DNA:DNA. RNA:RNA hybrids are the most stable whereas DNA:DNA hybrids are the least stable. In addition to the type of hybrid, the stability of the duplex formed is determined by the length of the probe, the degree of complementarity to the sequence of interest, the composition of the sequences, the temperature of hybridization, and the pH and the ionic strength of the hybridization buffer (Emson, 1993). Long sequences with high degrees of complementarity are more stable than shorter sequences or those containing a number of nucleotide mismatches. Duplexes containing higher incidences of G-C base pairs are more stable than are those containing more A-T (U) base pairs. This is because base pairing between guanine and cytosine is stabilized by three hydrogen bonds whereas adenine – thymine (uracil) base pairings are stabilized by only two hydrogen bonds. High temperature, low salt concentration and alkaline pH are considered high stringency conditions (Tecott *et al.*, 1994). Stringency is a measure of the ability of single stranded molecules to appropriately associate with molecules of high complementarity. High stringency conditions favor the formation of specific interactions between the probe and the species of interest. This results in a high signal to noise ratio. High salt concentrations interfere with the electrostatic repulsion forces between negatively charged nucleic acid molecules, therefore, allowing non-specific associations. Low salt concentrations do not interfere with electrostatic forces so only those sequences with high complementarity can overcome the electrostatic repulsion to form stable hybrids. pH influences stringency by altering ionization states of molecules. An acidic pH neutralizes phosphates which are negatively charged at near neutral pH. This allows for association of non-complementary strands. An alkaline pH increases ionization, thereby promoting electrostatic repulsion and the association of sequences with high degrees of complementarity. Formamide can also be used to increase

stringency as it reduces the temperature necessary for disassociation of non-complementary hybrids by destabilizing hydrogen bonds.

2.3.5.2 Prehybridization

Sections were rehydrated in 0.1 M PBS (pH 7.4), acetylated for 10 minutes in 0.1 M triethanolamine containing 0.25% acetic anhydride to prevent electrostatic interactions between the probe and the proteins, washed in 2X SSC, dehydrated in ascending ethanols, delipidated 2 X 5 minutes in chloroform, transferred to 100% ethanol, 95% ethanol, then allowed to air dry. Slides were stored at –20°C prior to hybridization. Prehybridization treatments were designed to increase penetrance of the probe into the tissue and to reduce nonspecific binding of the probe to cellular constituents (Bresser & Evinger-Hodges, 1987).

2.3.5.3 In Situ Hybridization

Two million counts per minute (cpm) of radiolabeled probe in 40 µl of hybridization buffer were applied to each slide and incubated overnight at 54°C in a humid chamber. Sense probes were included as a control for binding specificity. Coverslips were floated off in 2X SSC and slides were treated with 5 µg/ml RNase A in RNase digestion buffer (0.5 M NaCl, 10 mM Tris (pH 8.0) and 1 mM EDTA) for 30 minutes. Slides were then transferred to RNase buffer for 30 minutes, 2X SSC for 4 minutes, 2X SSC/50% formamide at 50°C for 20 minutes then 0.1X SSC at 50°C for 15 minutes. Slides were dehydrated in ascending ethanols containing 0.3 M ammonium actetate then air dried. Slides were dipped in NTB2 photographic emulsion (Eastman Kodak Co., Rochester, NY, U.S.A.) diluted 1:1 with nanopure water then left to expose at 4°C. When adequate exposure time was confirmed through test slides, samples slides were developed in D-19 Kodak developer (Eastman Kodak Co., Rochester, NY, U.S.A.) and fixed with Kodak general fixer (Eastman Kodak Co., Rochester, NY,

U.S.A.). Slides were lightly counterstained with cresyl violet, dehydrated, cleared and mounted with DPX Neutral Mounting Medium (Aldrich, Chemical Co. Inc., Milwaukee, WI, U.S.A.).

2.3.5.4 Determination of Probe Specificity

To confirm the specificity of the transcribed probes three control procedures were performed: 1) Sense transcripts were used in place of antisense transcripts in in situ hybridization experiments. No signal was detected in these sections. 2) RNase pretreatment of tissue to degrade mRNA and test for binding of the antisense probe to other cell components also resulted in no signal. 3) The final control was a competition assay to demonstrate that radiolabeled probe binding could be effectively competed out by non-radiolabeled probe. Various concentrations of unlabeled probe were added to a constant amount of radiolabeled probe. Results demonstrated consistently decreasing amounts of signal with increasing non-labeled probe concentration. All steps in these control procedures were carried out identical to the in situ hybridization procedure (see section 2.3.5.3)

2.3.5.5 Quantification

mRNA was quantified using an Axioskop microscope (Carl Zeiss, Inc., Thornwood, NY, U.S.A.) interfaced to a PC computer using Northern Eclipse Image Analysis Software (Empix Imaging Inc., Mississauga, Ontario, Canada). Percent area covered by silver grains in individual cells in the prefrontal cortex was measured in minimum of 100 cells per animal over three sections per animal per experiment. Percent area covered by silver grains in the dentate gyrus, CA3 and CA1 regions of the hippocampus were measured over the entire area in three sections per animal per experiment. Averaged percent area values were converted to percent control values and averaged across two

experiments. All statistical analyses were performed on percent control values. Various numbers of animals in each group were analyzed due to availability of sections, preservation of regions, and surgery groups included in the hybridization experiments.

2.3.6 Apoptosis Detection

Terminal deoxynucleotidyl Transferase (TdT) mediated dUTP-biotin nick end labeling (TUNEL) is a procedure used to detect fragmented DNA. During the process of apoptosis, internucleosomal fragmentation of DNA occurs. The generated DNA fragments contain double stranded ends that are accessible for the enzymatic incorporation of biotinylated nucleotides. TdT binds to exposed 3′ hydroxyl ends of DNA and synthesizes a labeled polydeoxynucleotide molecule (Ben-Sasson *et al.*, 1995).

TUNEL was performed using a TACS2 TdT (Fluor) kit (Trevigen Inc., Gaithersburg, MD, U.S.A.). Specifically, sections were rehydrated in 0.1 M PBS then treated with proteinase K (0.02 µg/µl) for 15 minutes to permeablize the tissue. Slides were equilibrated in labeling buffer then incubated for 1 hour at 37°C with 50 µl of TdT enzyme (15 units per slide) in labeling buffer in the presence of 0.25 mM biotinylated dNTP and 1 mM Co^{+2}. The reaction was stopped in 0.01 M EDTA (pH 8.0) and sections were washed in 0.1 M PBS. In the dark, 50 µl of streptavidin-fluorescein (0.005 µg/µl) was added to each slide and incubated for 20 minutes at room temperature. Sections were washed in 0.1 M PBS in the dark then mounted with fluorescent mounting medium (DAKO Corporation, Carpinteria, CA, U.S.A.) containing 5 µg/ml bisbenzamide (Hoechst 33258). Bisbenzamide is a DNA stain that detects apoptosis on the basis of nuclear condensation and cytoplasmic compaction. Apoptotic cells were visualized under a fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY,

U.S.A.) using a 495 nm filter for fluorescein detection and a 365 nm filter for bisbenzamide.

Neurodegeneration was also investigated using Fluoro-Jade B (received from Dr. L.C. Schmued, National Center for Toxicological Research, Jefferson, AR, U.S.A.). Fluoro-Jade B is an anionic fluorescein derivative that selectively stains degenerating neurons (Schmued *et al.*, 1997). It has been demonstrated to stain cell bodies, dendrites, axons and axon terminals of degenerating neurons, but not healthy neurons, neuropil, myelin or vascular elements. The mechanism by which this occurs is unknown, but it is speculated that degenerating neurons express a basic molecule to which Fluoro-Jade, a strongly acidic molecule, has a high affinity. Briefly, sections were immersed in 1% potassium hydroxide in 80% ethanol for 5 minutes, followed by 2 minutes in 70% ethanol, and 2 minutes in nanopure water. To stabilize the Fluoro-Jade B fluorescence and reduce background staining slides were immersed in 0.06% potassium permanganate for 10 minutes with gentle shaking. Sections were washed 2 minutes in nanopure water then stained with 0.0004% Fluoro-Jade B solution (made from a 0.01% (w/v) stock diluted in 0.1% (v/v) acetic acid) for 20 minutes. Sections were washed 3 X 1 minute in nanopure water then quickly dried at 50°C on a slide warmer. Sections were cleared in xylene, mounted with DPX Neutral Mounting Medium (Aldrich, Chemical Co. Inc., Milwaukee, WI, U.S.A.). Fluorescence indicative of neurodegeneration was viewed under a fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY, U.S.A.) using a 495 nm filter.

2.3.6.1 Quantification

Four tissue sections from the prefrontal cortex of each animal were processed for detection of apoptosis in 2 separate experiments. Apoptotic cells were expressed as a percent of the total number of cells counted. A minimum

of 800 cells per animal per experiment were counted and averaged to obtain percent apoptosis values. Total cell number and apoptotic cell number per field of view were counted using a 365 nm filter to visualize bisbenzamide fluorescence. Cells were confirmed as apoptotic using a 495 nm fluorescein filter to visualize TACS positive nuclei in sections double labeled with bisbenzamide and TUNEL.

2.4 Statistics

Two way analysis of variance (ANOVA) (SPSS for Windows, SPSS Inc., Chicago, IL, U.S.A.) was used for all statistical analyses. Newman-Keuls post-hoc testing was used to explore all significant interactions. In the present study, a two way analysis of variance resolved whether differences in means were due to lesion status (ACSF versus ibotenic acid), treatment (swim stress versus control environment) or due to an interaction between status and treatment. The first test in the two way ANOVA investigated a main effect of status by averaging scores across treatment conditions (control environment versus swim stress) to eliminate that source of variability. A significant main effect of status indicated that differences existed between ACSF and ibotenic acid treated animals irrespective of treatment condition. The second test in the two way ANOVA investigated a main effect of treatment by averaging scores across lesion status (ACSF versus ibotenic acid). A significant main effect of treatment indicated that differences existed between stressed and non-stressed animals irrespective of lesion status. The third test in the two way ANOVA was a test of interaction, i.e., whether ACSF and ibotenic acid treated animals responded differentially to the treatment conditions. When an interaction occurred between two independent variables such as lesion status and treatment, post hoc tests were performed to determine which components of the interaction were significant.

2.4.1 Behavioral Testing

Locomotor activity at PND 35 and PND 56 was analyzed by two-way ANOVA with repeated measures. Status (ACSF versus ibotenic acid) was a between subjects independent variable and age was a within subjects independent variable. Photocell beam breaks were the dependent variable. The specific question addressed was "do lesion animals exhibit different locomotor activity behavior than sham animals at PND 35 and/or PND 56?"

2.4.2 Stress Paradigm

Two way ANOVA with status (ACSF versus ibotenic acid) and treatment (control or swim) as independent variables and photocell beam breaks as the dependent variable was performed to investigate the effect of lesion status and stress on locomotor activity. The specific questions addressed were: "do lesion and sham rats exhibit different locomotor activity behavior at PND 75 and do lesion animals show increased responsivity to stress as compared to sham animals?"

2.4.3 Detection of mRNA by In Situ Hybridization

Two way ANOVA with status (ACSF versus ibotenic acid) and treatment (control or swim) as independent variables and percent control mRNA as a dependent variable was performed to assess the effect of stress on BDNF and NMDAR1 mRNA in lesion and sham animals. Specific questions addressed were: "does baseline expression differ between lesion and sham animals, does stress affect the expression of BDNF and/or NMDAR1 mRNA, and do lesion and sham animals respond differently to stress?"

2.4.4 Apoptosis Detection

Two way ANOVA with status (ACSF versus ibotenic acid) and treatment (control or swim) as independent variables and percent apoptosis as a dependent variable was performed to assess the effect of stress on cell death in lesion and sham rats. Specific questions addressed were: "does baseline apoptosis differ between lesion and sham animals, does stress affect the amount of apoptosis in lesion and/or sham animals, and do lesion and sham animals respond differently to stress?"

3. RESULTS

3.1 Behavioral Testing

This experiment was performed to evaluate the present animal model with respect to the criteria established by Lipska and Weinberger in the original model (Lipska *et al.*, 1993). Animals with postnatal day 7 ibotenic acid lesions of the ventral hippocampus have been shown to display postpubertal emergence of hyperresponsivity to dopamine mediated behaviors. The test used to investigate this effect was open field locomotor activity in response to novelty. All animals were tested at both PND 35 (prepubertal) and PND 56 (postpubertal) with n=45 in the ACSF group and n=24 in the ibotenic acid group. There was no significant effect of age ($F_{(1,67)}$=3.56, p=.06), no significant effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,67)}$=2.50, p=.12) and no age by lesion status interaction ($F_{(1,67)}$=0.50, p=.48). Overall, lesion and sham animals demonstrated similar locomotor behavior following exposure to a novel environment. This effect was not influenced by the age of the animals (see Figure 3.1).

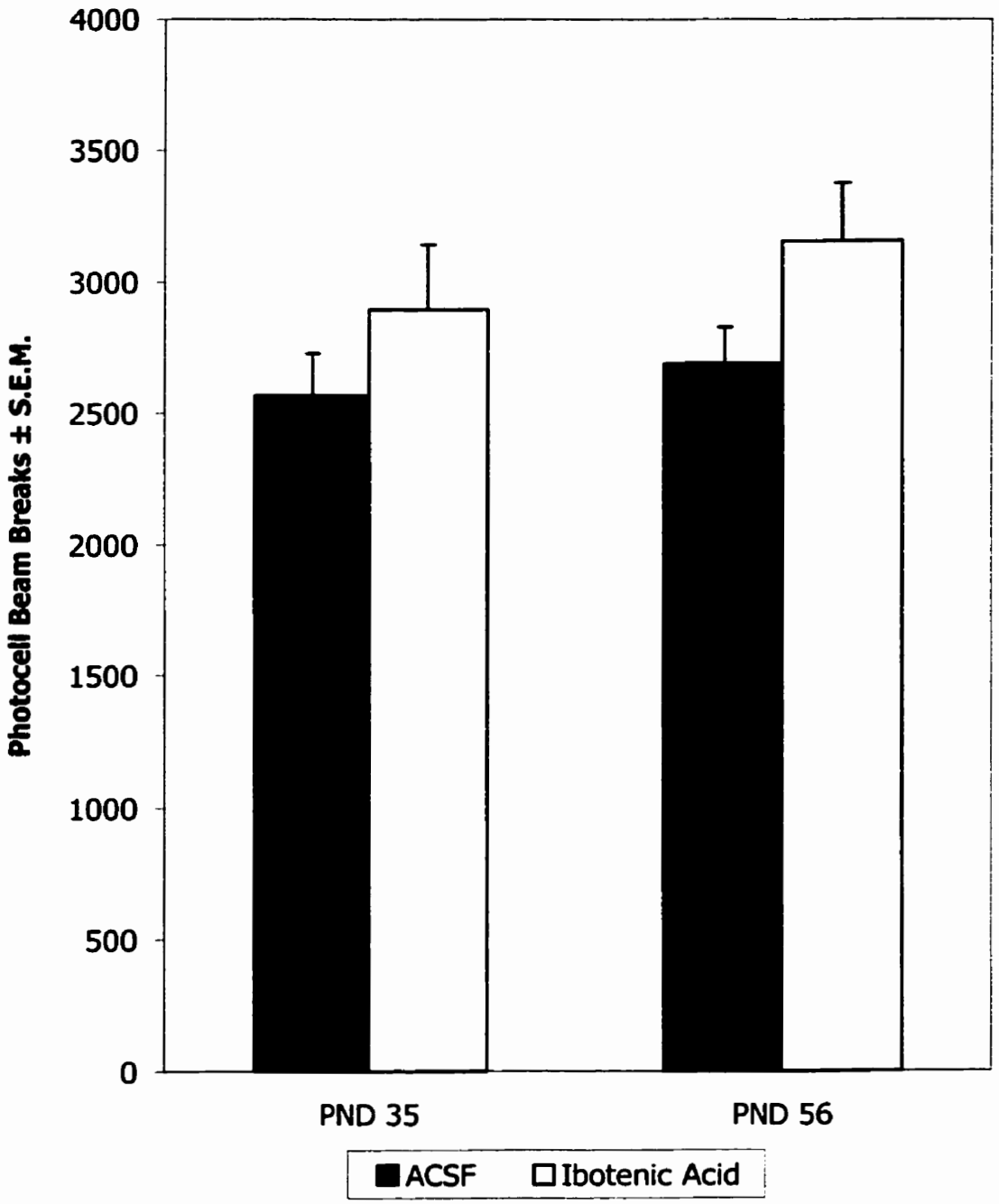


Figure 3.1. Total locomotor activity following exposure to a novel environment in an open field (the novel environment) paradigm in lesion and sham rats. ANOVA resulted in no significant differences between lesion and sham animals at PND 35 or PND 56.

3.2 Stress Paradigm

Previous characterization of the model (Lipska *et al.*, 1993) demonstrated a tendency for hyperresponsivity to stress as evidenced by greater increases in locomotor activity in lesion versus sham animals following exposure to physiological stress. This experiment was performed to replicate the paradigm used by Lipska *et al.* (1993) and to determine if lesioned animals were hyperresponsive to stress as compared to sham animals in the present study. In addition to allowing comparison to the model established by Lipska and Weinberger, the physiological stressor was used to investigate alterations in factors potentially involved in schizophrenia. Results were obtained from a total of 23 animals in the ACSF – control group, 23 in the ACSF – swim stress group, 11 in the ibotenic acid – control environment group and 13 in the ibotenic acid – swim stress group. Only behavioral values from animals meeting the histological criteria were included in the analysis. Two way ANOVA on total photocell beam break values revealed a significant main effect of lesion status (ibotenic acid versus ACSF) ($F_{(1,66)}=34.97$, $p<.001$) and of treatment (swim stress versus control environment) ($F_{(1,66)}=26.19$, $p<.001$). No interaction was found between lesion status and treatment ($F_{(1,66)}=0.01$, $p=.93$) (see Figure 3.2). Cumulative data in 15 minute intervals, assessed by two way ANOVA, revealed a significant within subjects main effect of time ($F_{(1,66)}=740.87$, $p<.001$) as well as a within subjects time by treatment (swim stress versus control environment) interaction ($F_{(1,66)}=80.54$, $p<.001$) and a within subjects time by lesion status (ACSF versus ibotenic acid) interaction ($F_{(1,66)}=26.83$, $p<.001$) (see figure 3.3). There was no time by treatment by lesion status interaction ($F_{(1,66)}=0.58$, $p=.75$). Exploration of the time by lesion status interaction revealed significant differences in locomotor activity between ACSF and ibotenic acid treated animals at 30 minutes through to 90 minutes ($p<.001$). Exploration of the time by treatment interaction revealed significant

differences in locomotor activity between animals exposed to swim stress and control environment at 15, 45, 60, 75 and 90 minutes ($p<.001$).

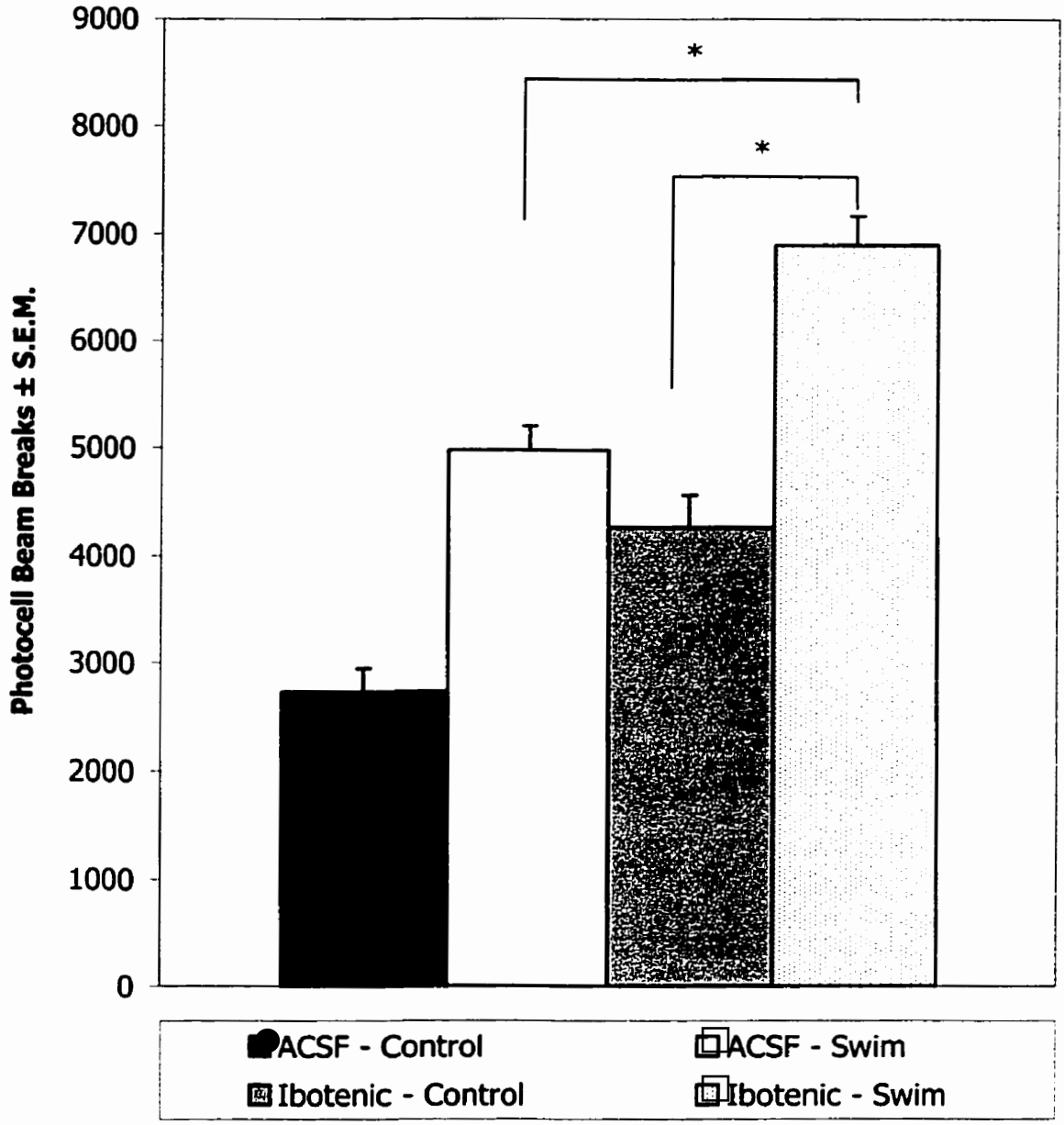


Figure 3.2. Total locomotor activity in response to swim stress or a control environment in lesion and sham animals at PND 75. ANOVA revealed a significant main effect of status (ACSF versus ibotenic acid) ($F=_{(1,66)}34.97$, $p<.001$) and a significant effect of treatment (swim stress versus a control environment) ($F_{(1,66)}=26.19$, $p<.001$). There was no interaction between status and treatment. * indicates significant main effects at $p<.001$.

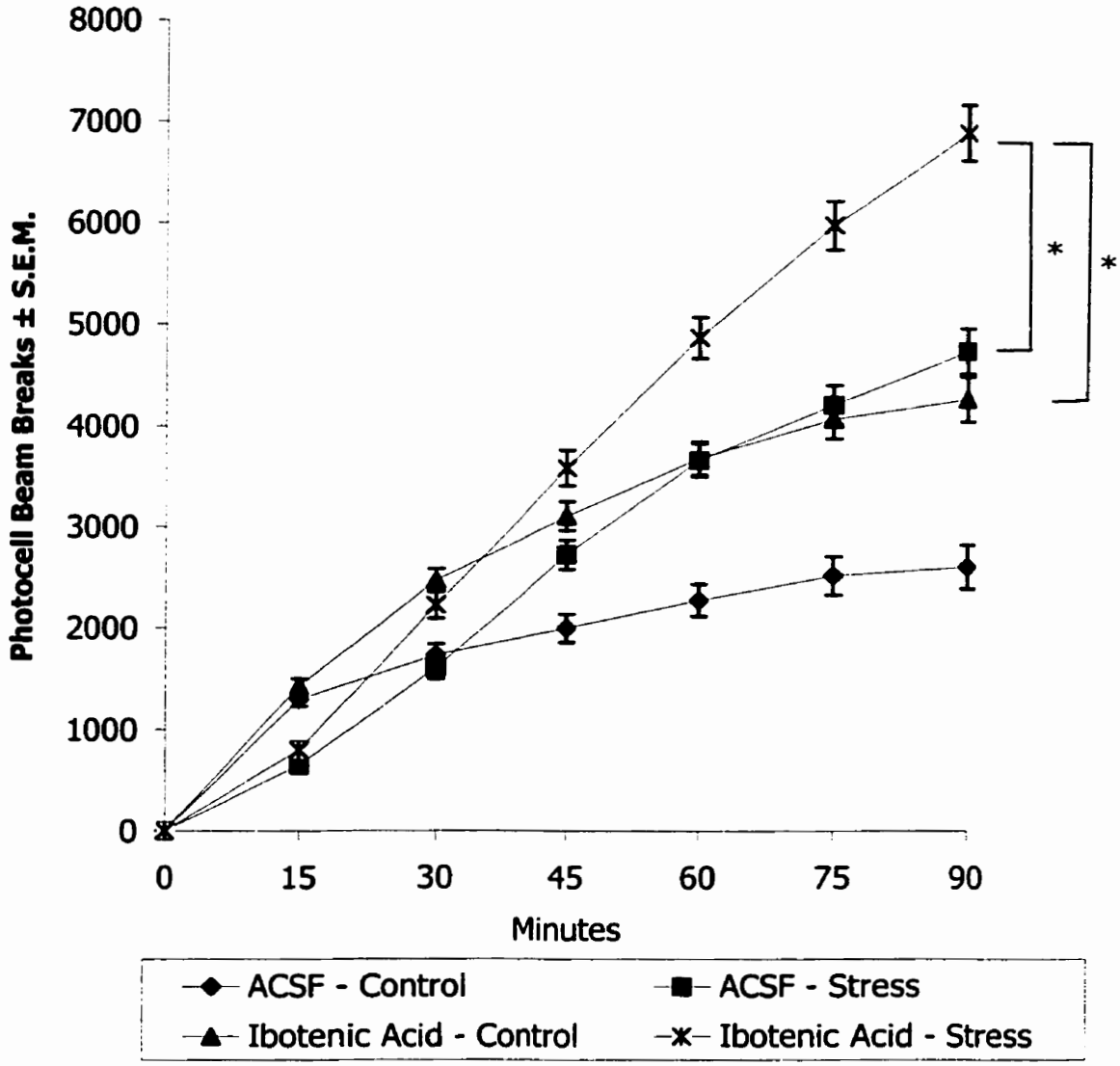


Figure 3.3. Cumulative open field locomotor activity in lesion and sham rats following exposure to swim stress or a control environment. ANOVA revealed a significant effect of status (ACSF versus ibotenic acid) ($F_{(1,66)}$=34.97, p<.001), a significant effect of treatment (swim stress versus control environment) ($F_{(1,66)}$=26.19, p<.001) and a significant effect of time ($F_{(1,66)}$=740.87, p<.001). There was a significant interaction between time and treatment (swim stress versus control environment) ($F_{(1,66)}$=80.54, p<.001) and a significant interaction between time and status ($F_{(1,66)}$=26.83, p<.001). There was no time by treatment by status interaction. * indicates significant main effects at p<.001.

3.3 Histology

Sections through the ventral hippocampus of lesion and sham rats, stained with haematoxylin and eosin were assessed for animal inclusion or exclusion. Rats infused with ACSF that were included in the study demonstrated no observable cell loss in or around the ventral hippocampus (see Figure 3.4). Of the total number of animals infused with ACSF, approximately 70% were included in further experiments. Animals excluded generally showed small rims of unilateral or bilateral cell loss in the CA3 pyramidal region of the ventral hippocampus; less than 300 μm anterior-posteriorly and ventro-dorsally. Animals infused with ibotenic acid showed varying degrees of cell loss in the ventral hippocampus. The dorsal hippocampus showed little to no cell loss in included animals, but excluded animals treated with ibotenic acid often demonstrated dorsal hippocampal damage or ablation, often in conjunction with thalamic damage. This was evident in approximately 25% of ibotenic acid treated animals. A small percentage of included animals showed narrowing of the polymorphic layer of the dentate gyrus resulting from the suprapyramidal and infrapyramidal blades of the dentate gyrus approaching each other. There was, however, no apparent granule cell loss. Animals in the ibotenic acid treated group were also excluded on the basis of unilateral damage only (~10%), limited extent of damage not meeting the criteria (~15%), or behavioral abnormalities (seizures) (~10%). Included ibotenic acid treated animals showed significant cell loss in the CA3 and CA1 pyramidal layers of the ventral hippocampus. This region of damage averaged a distance of 1 mm anterior – posterior through the ventral hippocampus and was most dramatic in the CA3 region. The ventricular region surrounding the hippocampus was greatly enlarged in the majority of animals. In total, approximately 45% of animals infused with ibotenic acid were included in subsequent in situ hybridization experiments.

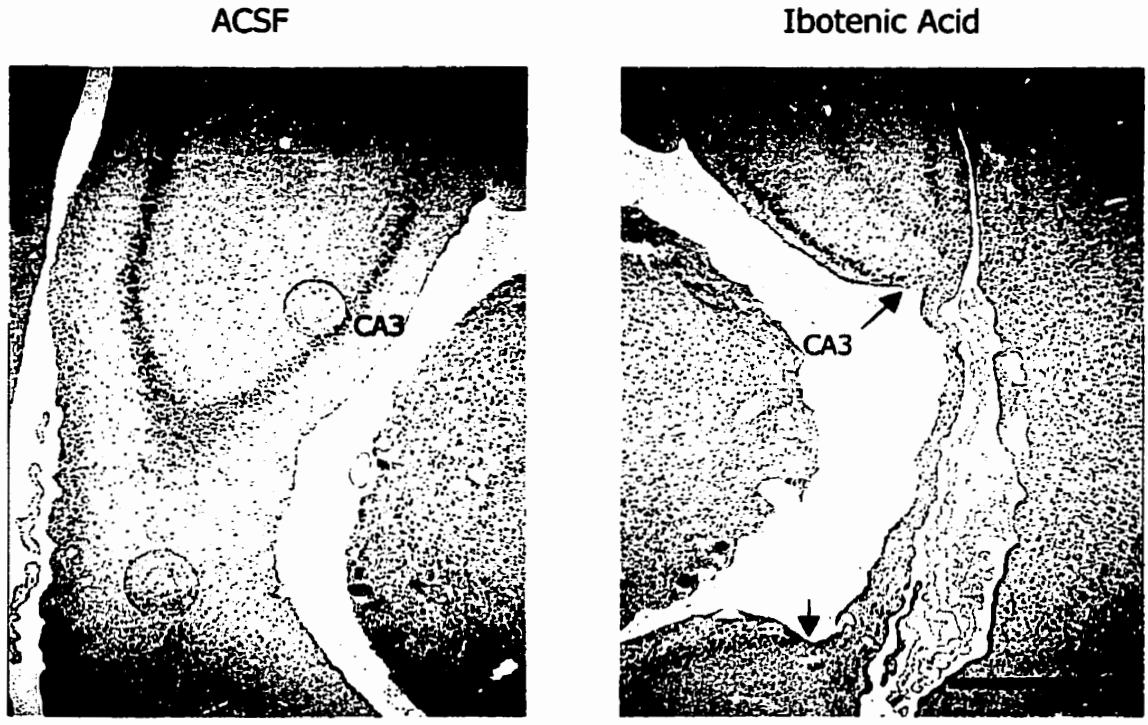


Figure 3.4. Haematoxylin and eosin stained sections through the ventral hippocampus of ACSF and ibotenic acid treated animals. Arrows point to areas of major cell loss. Scale bar = 500 μm.

3.4 Detection of mRNA by In Situ Hybridization

3.4.1 BDNF mRNA in the Prefrontal Cortex

This experiment was performed to determine if lesioned animals demonstrate alterations in baseline BDNF mRNA in the prefrontal cortex and to determine if appropriate regulation of BDNF mRNA is maintained. Sections from a total of 19 animals in the ACSF – control environment group, 17 in the ACSF – swim stress group, 11 from the ibotenic acid – control environment group and 11 from the ibotenic acid – swim stress group were analyzed. Two way ANOVA revealed a significant main effect of treatment (swim stress versus control environment) ($F_{(1,54)}=88.43$, $p<.001$) and a significant interaction between lesion status (ACSF versus ibotenic acid) and treatment ($F_{(1,54)}=13.03$, $p<.01$). There was no main effect for lesion status ($F_{(1,54)}=0.70$, $p=.41$). Exploration of the interaction revealed a significant change in baseline BDNF mRNA in lesion versus sham animals ($p<.05$) and a significantly different response to stress in lesion animals as compared to sham ($p<.01$). Overall, BDNF mRNA was reduced in ibotenic acid treated animals to 88% of control values (see Figure 3.5) and was increased in both lesion (139%) and sham (122%) animals in response to stress. Significantly different responses to stress were seen in lesion versus sham animals such that a relative 51% increase in BDNF mRNA was demonstrated in ibotenic acid treated animals, whereas only a 22% relative increase was demonstrated in sham animals. Bright field photomicrographs demonstrating silver grains indicative of BDNF mRNA in coronal sections through the prefrontal cortex of lesion and sham rats exposed to swim stress and a control environment are seen in Figure 3.6. In response to stress, although no cell counts were performed, it appears that BDNF mRNA in the prefrontal cortex is upregulated in individual cells in addition to detectable levels being observed in a greater number of cells as compared to baseline conditions. Ibotenic acid treatment, under control conditions, appears to decrease the expression of BDNF mRNA within individual cells.

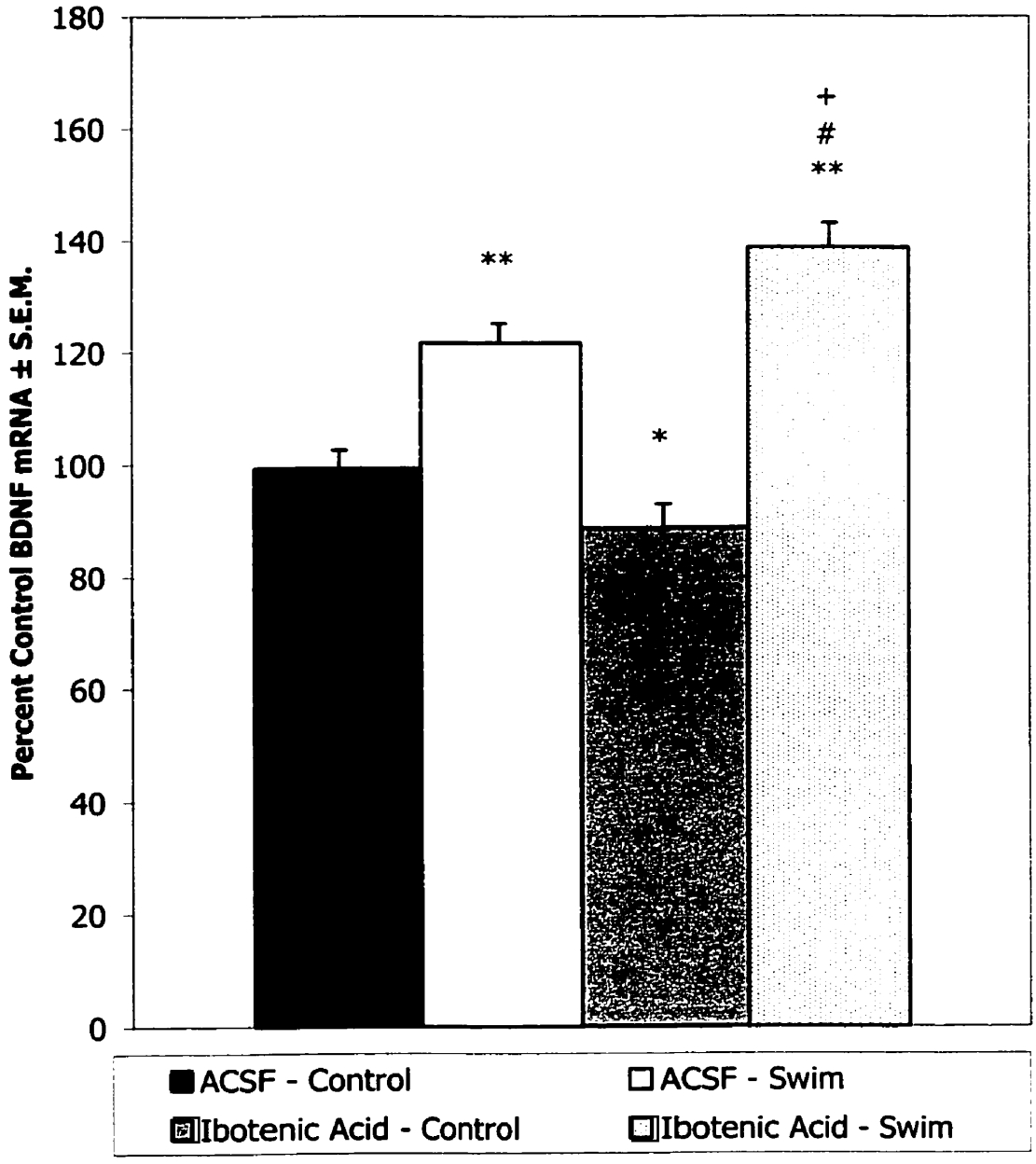


Figure 3.5. BDNF mRNA in the prefrontal cortex. Two way ANOVA revealed a significant effect of treatment (control environment vs. forced swim) ($F_{(1,54)}$=88.43, p<.001) and a significant interaction between treatment and lesion status (ACSF vs. ibotenic acid) ($F_{(1,54)}$=13.03, p<.01). * significant with respect to ACSF-Control, p<.05; ** significant with respect to ACSF-Control, p<.01; # significant with respect to Ibotenic Acid-Control, p<.01; + significant with respect to ACSF-Swim, p<.05.

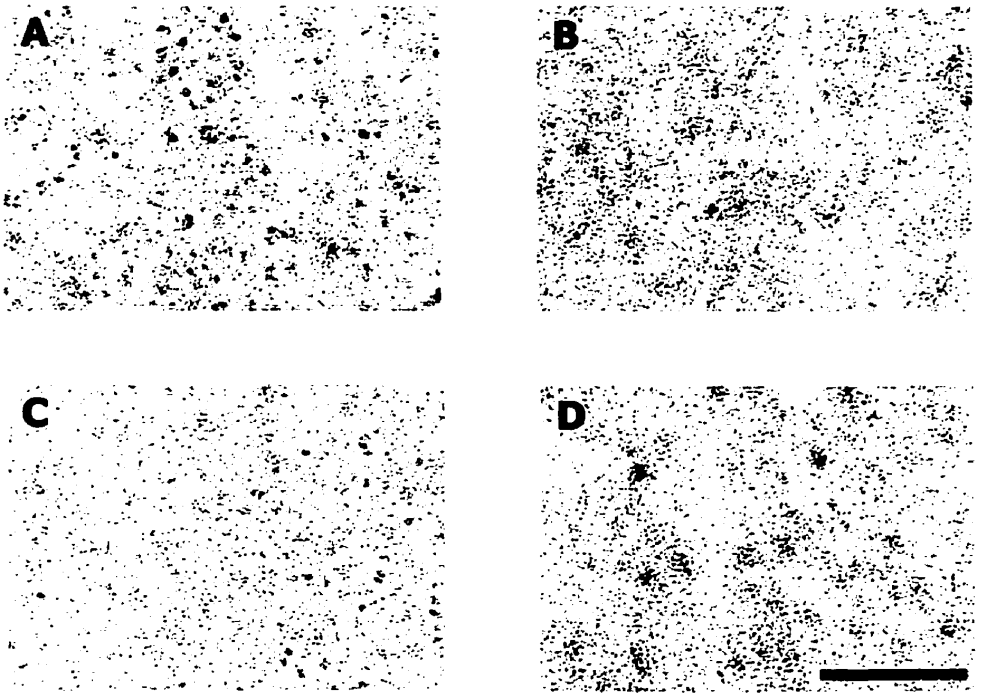


Figure 3.6. In situ hybridization of BDNF mRNA in the prefrontal cortex of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.2 BDNF mRNA in the Dentate Gyrus of the Hippocampus

This experiment was performed to determine if lesioned animals demonstrate alterations in baseline BDNF mRNA in the dentate gyrus of the hippocampus and to determine if appropriate regulation of BDNF mRNA is maintained during exposure to stress. Sections from a total of 12 animals in the ACSF – control environment group, 7 in the ACSF – swim stress group, 6 from the ibotenic acid – control environment group and 7 from the ibotenic acid – swim stress group were analyzed. Two way ANOVA revealed a significant main effect of treatment (swim stress versus control environment) ($F_{(1,28)}$=33.27, $p<.001$) and a significant interaction between lesion status (ACSF versus ibotenic acid) and treatment ($F_{(1,28)}$=4.30, $p<.05$). There was no main effect of status ($F_{(1,28)}$=2.96, $p=.10$). Exploration of the interaction revealed a significant change in baseline BDNF mRNA in lesion versus sham animals ($p<.05$). Overall, BDNF mRNA in ibotenic acid treated animals was reduced to 75% of control values (see Figure 3.7) and was increased to similar overall levels following stress in lesion (127%) and sham (124%) animals as compared to control levels. Relative increases in BDNF mRNA following exposure to stress, however, were not equivalent as a 52% increase in ibotenic acid treated animals was demonstrated whereas only a 24% increase in sham animals was seen. Bright field photomicrographs showing silver grains in coronal sections through the dentate gyrus of the hippocampus are seen in Figure 3.8. Silver grains indicative of BDNF mRNA are distributed over granule cells throughout the granule layer of the dentate gyrus. Following stress, the induction of BDNF mRNA appears to be distributed throughout the granular region of the dentate gyrus rather than an induction within a specific subset of cells.

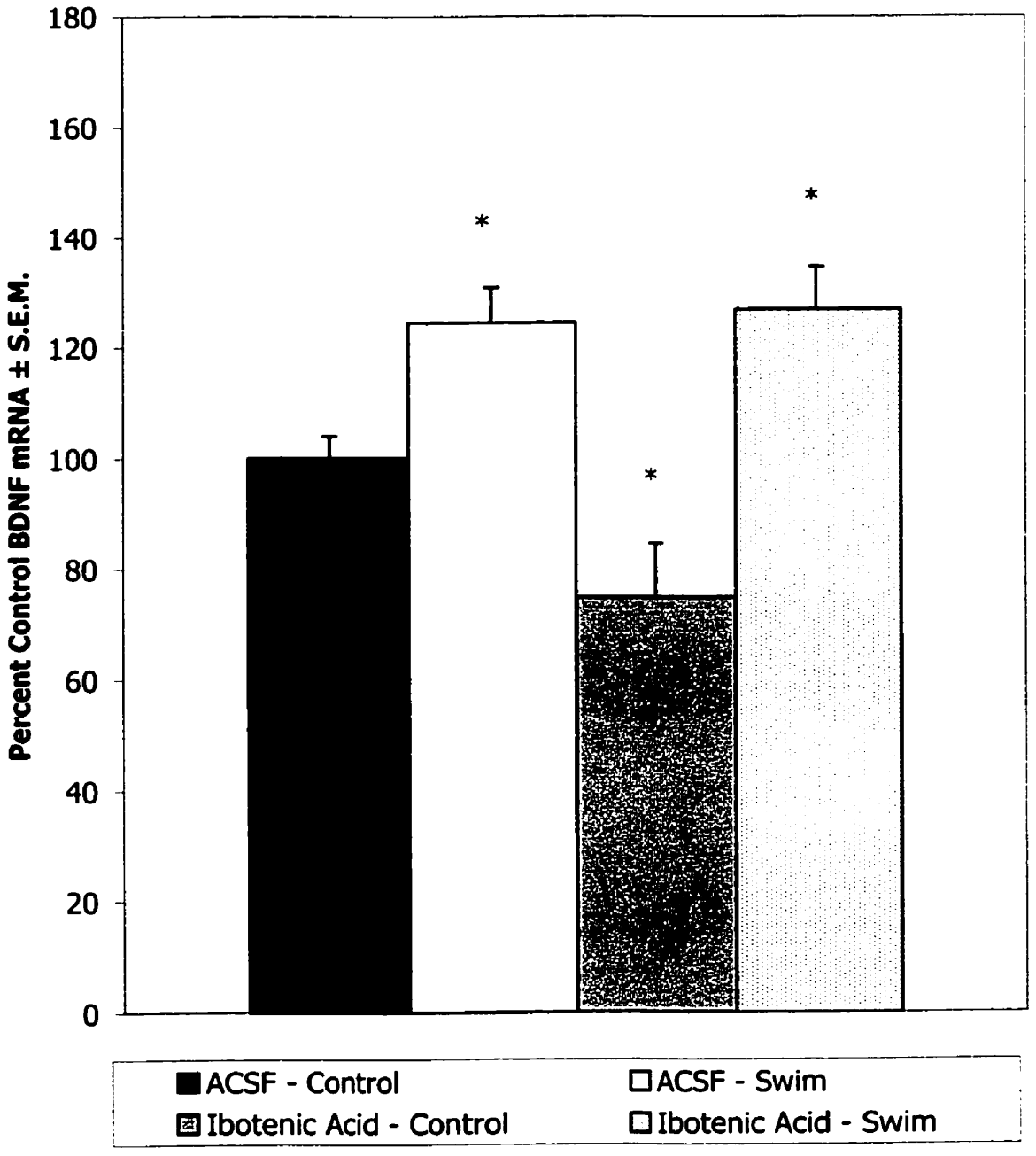


Figure 3.7. BDNF mRNA in the dentate gyrus of the hippocampus. Two way ANOVA revealed a significant effect of treatment (control environment vs. forced swim) ($F_{(1,28)}$=33.27, $p<.001$) and a significant interaction between treatment and status (ACSF vs. ibotenic acid) ($F_{(1,28)}$=4.30, $p<.05$). * significant with respect to ACSF - Control ($p<.05$).

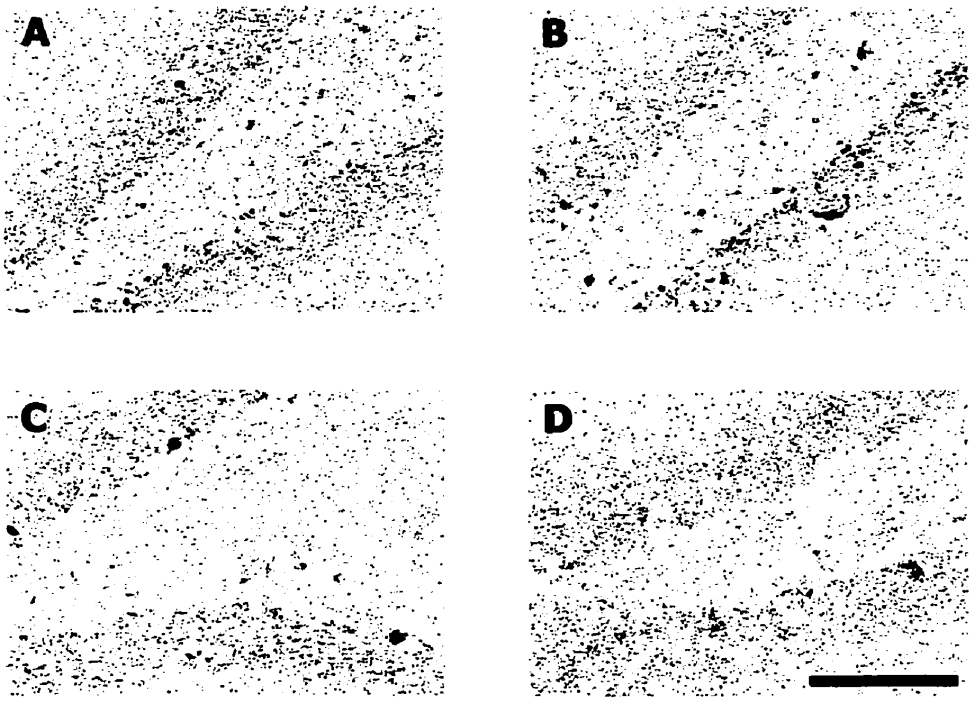


Figure 3.8. In situ hybridization of BDNF mRNA in the dentate gyrus of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.3 BDNF mRNA in the CA3 Region of the Hippocampus

This experiment was performed to determine if lesioned animals demonstrate alterations in baseline BDNF mRNA in the CA3 region of the hippocampus and to determine if appropriate regulation of BDNF mRNA is maintained during exposure to stress. Sections from a total of 9 animals in the ACSF – control environment group, 7 in the ACSF – swim stress group, 7 from the ibotenic acid – control environment group and 6 from the ibotenic acid – swim stress group were analyzed. Two way ANOVA revealed a significant main effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,25)}=11.46$, $p<.01$) and a significant main effect of treatment (swim stress versus control environment) ($F_{(1,25)}=44.91$, $p<.001$). There was no lesion status by treatment interaction ($F_{(1,25)}=0.70$, $p=.41$). Overall, neonatal ibotenic acid lesions resulted in reduced levels of BDNF mRNA in the CA3 region of the hippocampus as compared to sham animals (see Figure 3.9). There was a reduction of BDNF mRNA to 70% of control levels under baseline conditions. Further contributing to the main effect of lesion status, BDNF mRNA was induced in lesioned animals to 123% of control following exposure to stress, whereas, sham animals showed an induction to 141% of control. Despite the overall lower levels of BDNF mRNA in lesioned animals as compared to controls, however, similar relative increases in BDNF mRNA following stress were seen; a 53% increase from baseline in lesioned animals and a 41% increase in sham animals. Figure 3.10 depicts silver grains observed in coronal sections through the CA3 region of the hippocampus in lesion and sham animals exposed to swim stress and a control environment. Following exposure to stress, a large number of cells in both lesion and sham animals showed a strong induction of BDNF mRNA as evidenced by the large number of silver grains over individual cells. Under basal conditions, the majority of cells throughout the CA3 region of the hippocampus showed relatively low levels of BDNF mRNA expression.

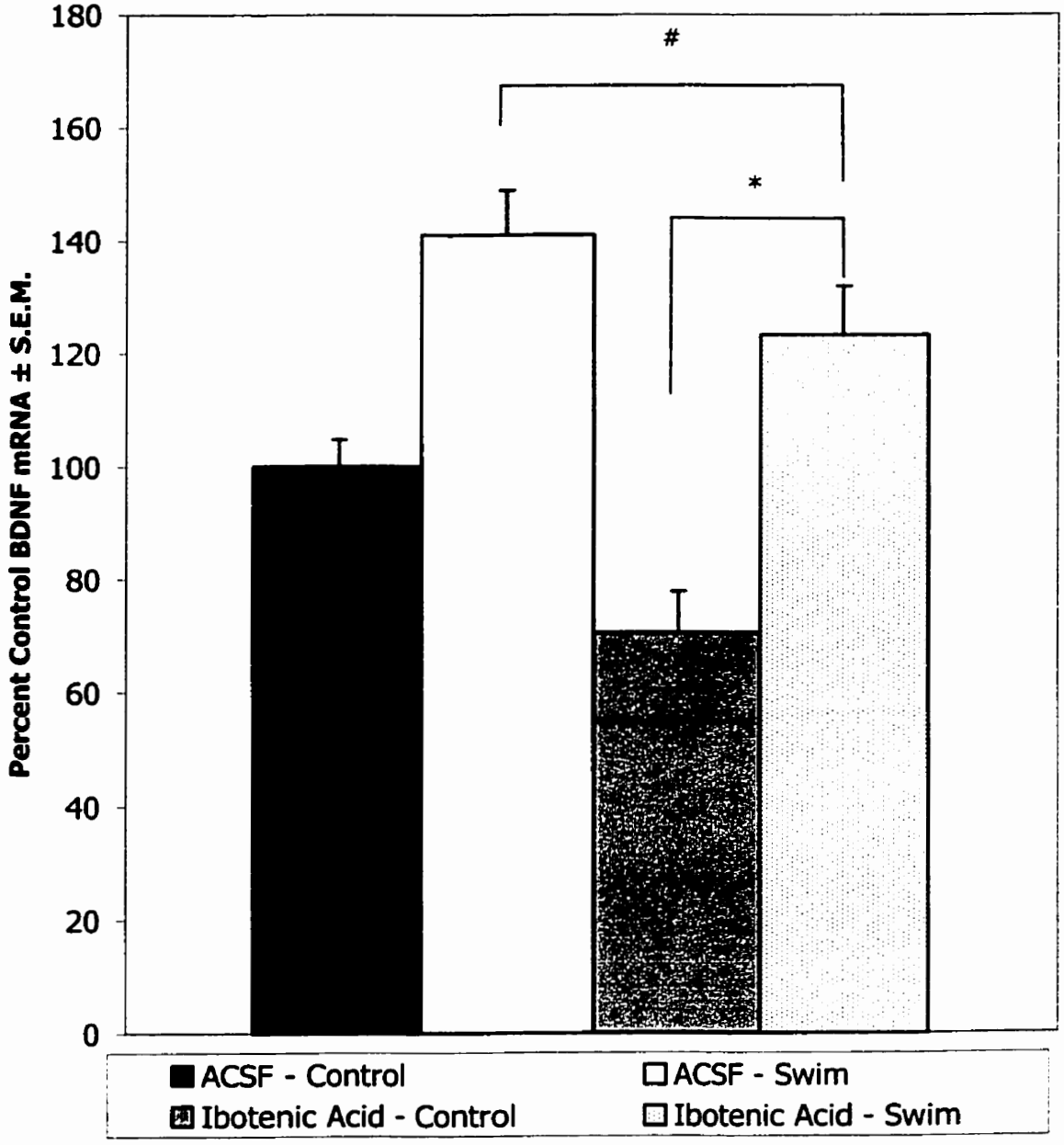


Figure 3.9. BDNF mRNA in the CA3 region of the hippocampus. Two way ANOVA revealed a significant effect of lesion status (ACSF vs Ibotenic Acid) ($F_{(1,25)}$=11.46, p<.01) and a significant effect of treatment (control environment vs swim stress) ($F_{(1,25)}$=44.91, p<.001). There was no interaction between lesion status and treatment. * indicates a significant main effect at p<.001, # indicates a main effect significant at p<.01.

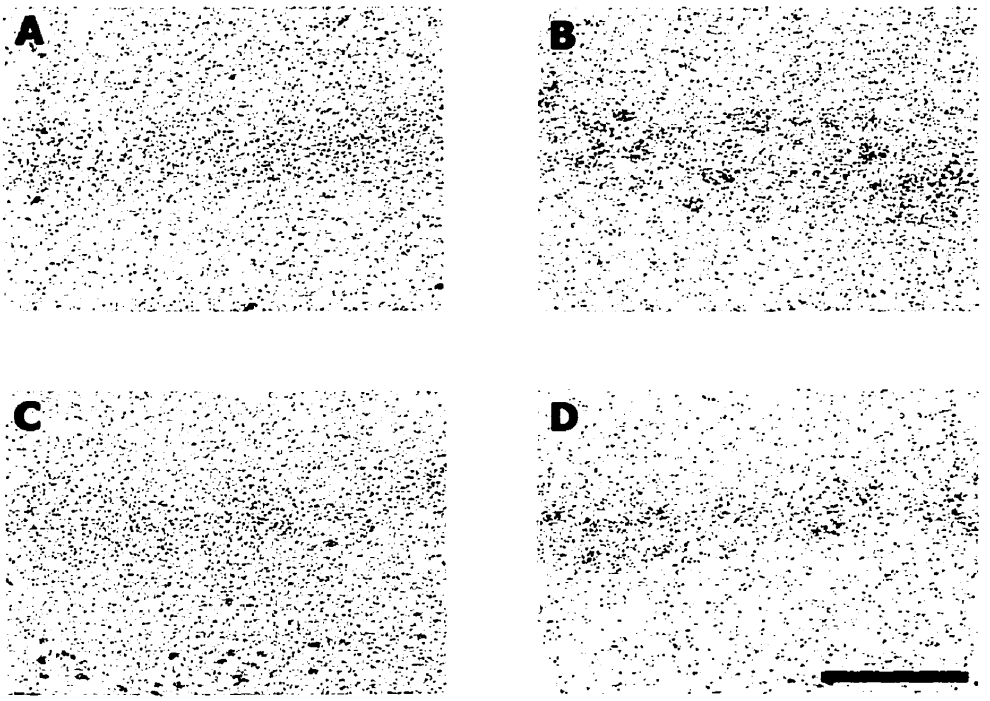


Figure 3.10. In situ hybridization of BDNF mRNA in the CA3 region of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.4 BDNF mRNA in the CA1 Region of the Hippocampus

This experiment was performed to determine if lesioned animals demonstrate alterations in baseline BDNF mRNA in the CA1 region of the hippocampus and to determine if appropriate regulation of BDNF mRNA is maintained during exposure to stress. Sections from a total of 8 animals in the ACSF – control environment group, 7 in the ACSF – swim stress group, 5 from the ibotenic acid – control environment group and 6 from the ibotenic acid – swim stress group were analyzed. Two way ANOVA revealed a significant main effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,22)}=5.67$, $p<.05$) and a significant main effect of treatment (swim stress versus control environment) ($F_{(1,22)}=21.87$, $p<.001$). There was no lesion status by treatment interaction ($F_{(1,22)}=0.12$, $p=.73$). Overall, neonatal ibotenic acid lesions appeared to decrease levels of BDNF mRNA in the CA1 region of the hippocampus as compared to sham animals (see Figure 3.11). Baseline BDNF mRNA levels in ibotenic acid treated animals was reduced to 74% of control levels. Further contributing to the main effect of lesion status was the lesser induction of BDNF mRNA in lesion (122%) versus sham (141%) animals following exposure to stress. There was, however, no significant difference in the relative degree of the upregulation in lesion (48%) and sham (41%) animals. Figure 3.12 depicts silver grains indicative of BDNF mRNA in the CA1 region of the hippocampus in lesion and sham animals exposed to swim stress and a control environment. The increase in BDNF mRNA in response to stress appears to result from a general increase in BDNF mRNA expression throughout the CA1 region of the hippocampus rather than specific induction in a subset of cells. The reduction in BDNF mRNA in ibotenic acid treated animals also appears to be throughout cells of the CA1 region rather than a specific downregulation in a subset of cells.

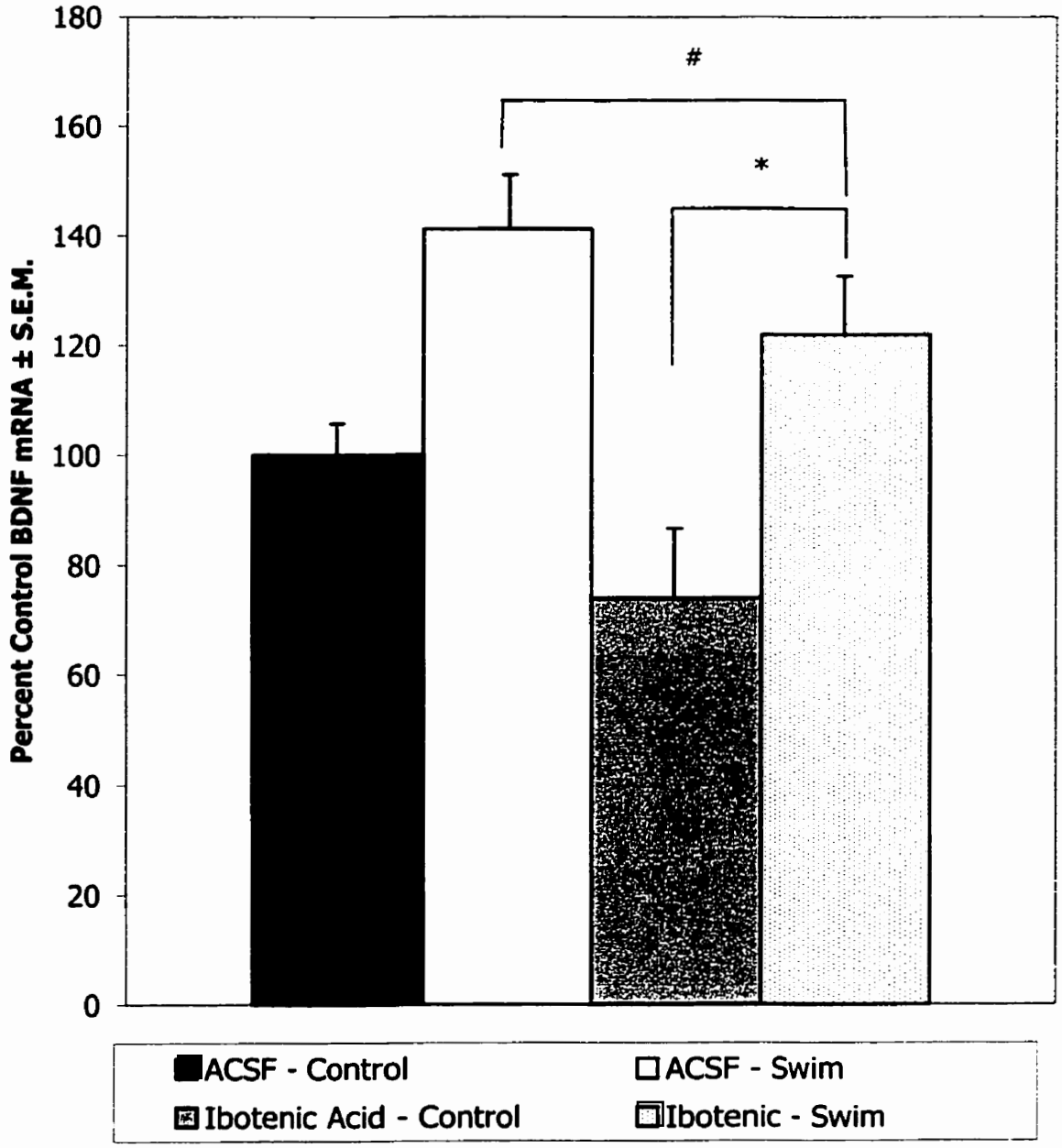


Figure 3.11. BDNF mRNA in the CA1 region of the hippocampus. Two way ANOVA revealed a significant effect of lesion status (ACSF vs ibotenic acid) ($F_{(1,22)}$=5.67, $p<.05$) and a significant effect of treatment (control environment vs. swim stress) ($F_{(1,22)}$=21.87, $p<.001$). There was no interaction between lesion status and treatment. # indicates a main effect significant at $p<.05$, * indicates a main effect significant at $p<.001$.

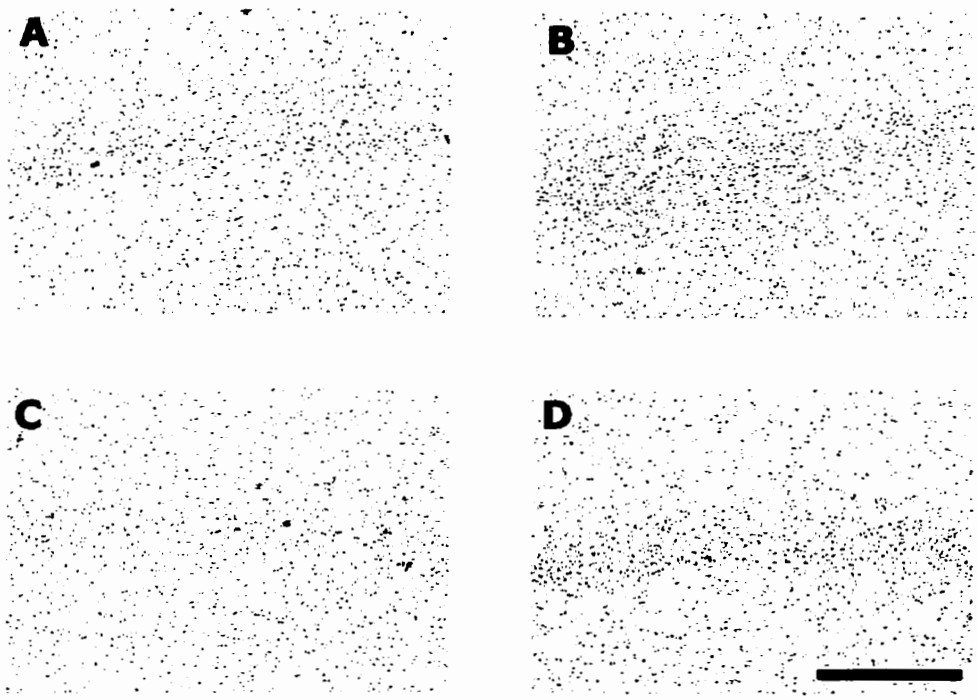


Figure 3.12. In situ hybridization of BDNF mRNA in the CA1 region of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.5 BDNF mRNA and Locomotor Activity

Increases in BDNF mRNA have been correlated with increased physical activity. For this reason, it was necessary to determine if the increases in BDNF mRNA in the animals exposed to swim stress was due to increased physical activity. Animals exposed to swim stress were more active during the 15 minutes of swimming and displayed increased locomotor activity for a minimum of 90 minutes following exposure to swim stress. Sham animals appeared to be approaching a plateau in activity after 90 minutes (see Figure 3.3, pg. 68) whereas, lesioned animals appeared to continue exhibiting locomotor behavior. The increase in activity, both amount and duration, may have directly resulted in the increase in BDNF mRNA due to swim stress. The possibility that a correlation existed was explored in the prefrontal cortex. Results indicated no direct relationship between the amount of physical activity and the level of BDNF mRNA expressed in lesion or sham animals (see Figure 3.13).

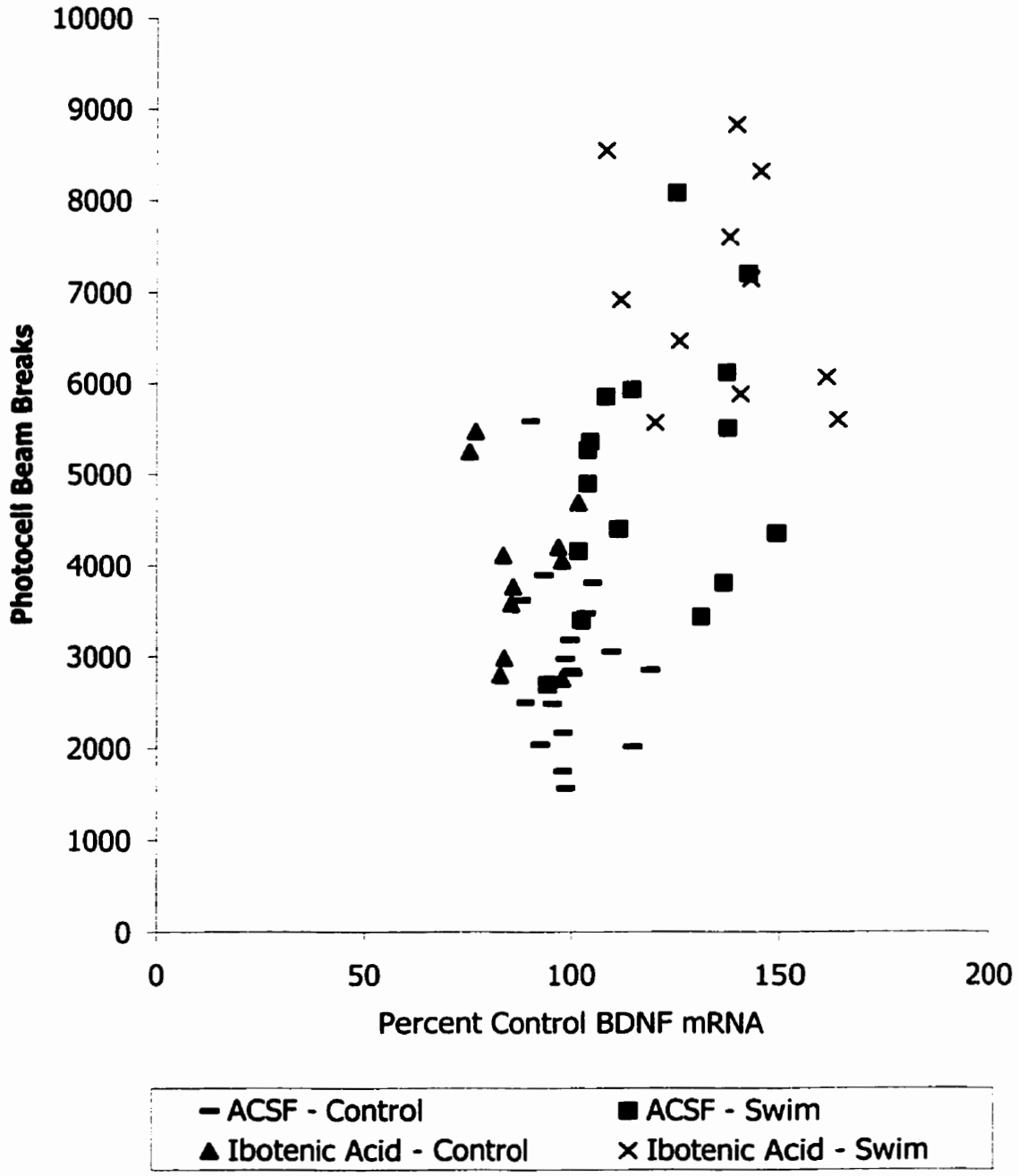


Figure 3.13. Relationship between locomotor activity and BDNF mRNA in the PFC of lesion and sham rats. No correlation between physical activity, as measured by photocell beam breaks, and expression of BDNF mRNA was found.

3.4.6 NMDAR1 mRNA in the Prefrontal Cortex

This experiment was performed to determine if neonatal ibotenic acid lesions of the ventral hippocampus influence the expression of NMDAR1 mRNA in the prefrontal cortex of adult rats. The goal was to investigate if basal expression was altered and if stress influenced the expression of this subunit. If stress affected the expression, it was also important to determine whether differential regulation was seen between lesion and sham rats. Sections from a total of 12 animals from the ACSF – control environment group, 7 from the ACSF – swim stress group, 5 from the ibotenic acid – control environment group and 5 from the ibotenic acid – swim stress group were analyzed. Results from the two way ANOVA indicated a significant main effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,25)}=9.08$, $p<.01$) and a significant main effect of treatment (control environment versus swim stress) ($F_{(1,25)}=8.64$, $p<.01$). There was no interaction between lesion status and treatment ($F_{(1,25)}=1.30$, $p=.27$). Overall, the results demonstrated a significant reduction in NMDAR1 mRNA expression in the prefrontal cortex of rats with neonatal ibotenic acid lesions of the ventral hippocampus (see Figure 3.14). Under baseline conditions, a 20% reduction compared to control was seen in lesioned animals. Swim stress resulted in an increase in the expression of NMDAR1 mRNA in both lesion (100%) and sham (108%) animals as compared to baseline levels. The relative increase in NMDAR1 mRNA in response to stress was not significantly different between lesion and sham animals with 20% and 8% increases, respectively. Figure 3.15 depicts silver grains indicative of NMDAR1 mRNA in the prefrontal cortex in lesion and sham animals exposed to swim stress and a control environment. It appears that individual cells show alterations in NMDAR1 mRNA levels and, although cell counts were not performed, that the number of cells expressing NMDAR1 mRNA is not altered in response to stress in lesion or sham animals nor in ibotenic acid treated animals under baseline conditions.

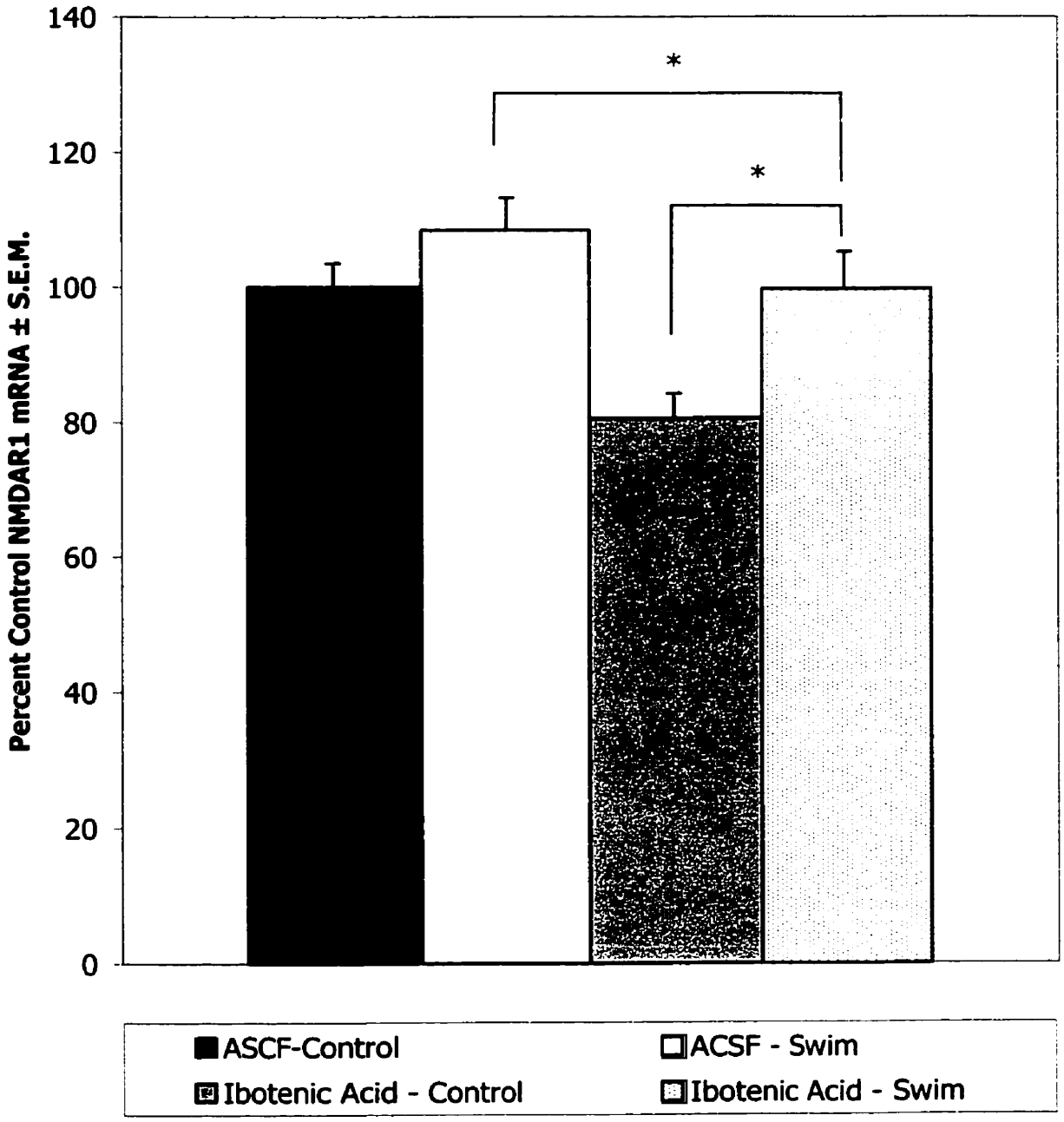


Figure 3.14. NMDAR1 mRNA in the prefrontal cortex. Two way ANOVA revealed a significant effect of lesion status (ACSF vs. ibotenic acid) ($F_{(1,25)}$=9.08, $p<.01$) and a significant effect of treatment (control environment vs. swim stress) ($F_{(1,25)}$=8.64, $p<.01$). There was no interaction between lesion status and treatment. * indicates a main effect significant at $p<.01$.

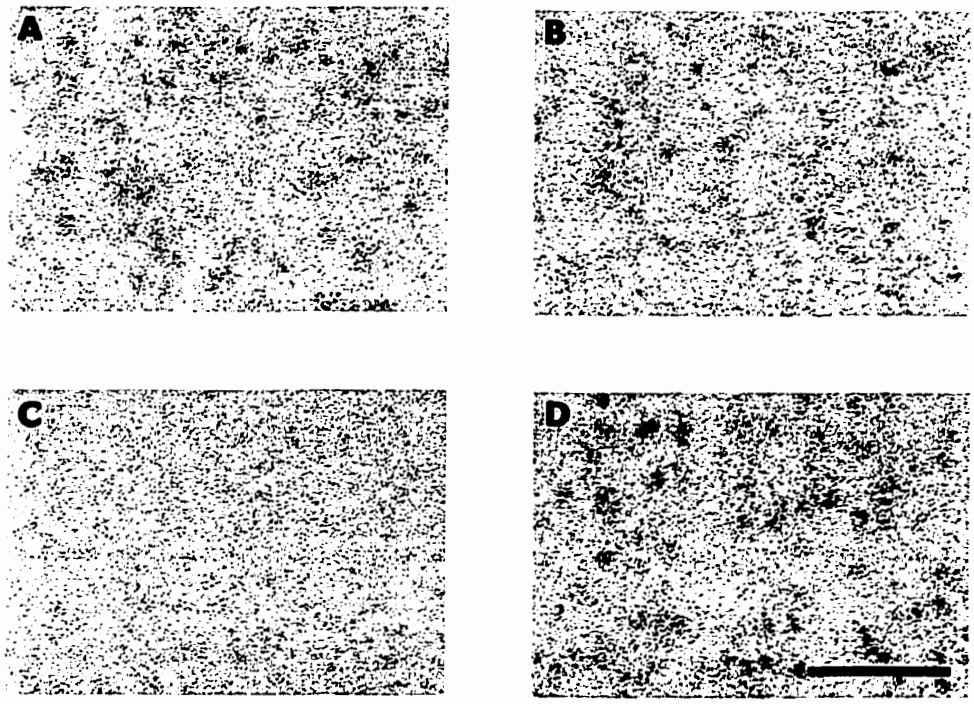


Figure 3.15. In situ hybridization of NMDAR1 mRNA in the prefrontal cortex of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control, B) ACSF – swim stress, C) Ibotenic acid – control, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.7 NMDAR1 mRNA in the Dentate Gyrus of the Hippocampus

This experiment was performed to determine if neonatal ibotenic acid lesions of the ventral hippocampus influence the expression of NMDAR1 mRNA in the dentate gyrus of the hippocampus of adult rats. The goal was to investigate if basal expression was altered and if stress influenced the expression of this subunit. If stress affected the expression, it was also important to determine whether differential regulation was seen between lesion and sham rats. Sections from a total of 7 animals from the ACSF – control environment group, 7 from the ACSF – swim stress group, 4 from the ibotenic acid – control environment group and 3 from the ibotenic acid – swim stress group were analyzed. Results indicated a significant main effect of treatment (swim stress versus control environment) ($F_{(1,17)}=14.42$, $p<.01$) and a significant interaction between treatment and status (ACSF versus ibotenic acid) ($F_{(1,17)}=7.43$, $p<.05$). There was no main effect of status ($F_{(1,17)}=1.93$, $p=.18$). Overall, the results indicated a decrease in the expression of NMDAR1 mRNA following stress in both lesion (33%) and sham (15%) animals as compared to non-stressed sham animals (see Figure 3.16). Exploration of the interaction revealed a significant increase in the expression of NMDAR1 mRNA in non-stressed ibotenic acid treated animals (157%) as compared to non-stressed sham animals. The relative decreases seen in lesion and sham animals in response to stress were 90% and 15% respectively. These decreases appear to be distributed throughout the granule cell layer of the dentate gyrus as are the increases in NMDAR1 mRNA expression in ibotenic acid treated animals under non-stress conditions. Bright field photomicrographs of silver grains indicative of NMDAR1 mRNA in the dentate gyrus of the hippocampus under the above conditions are shown in Figure 3.17.

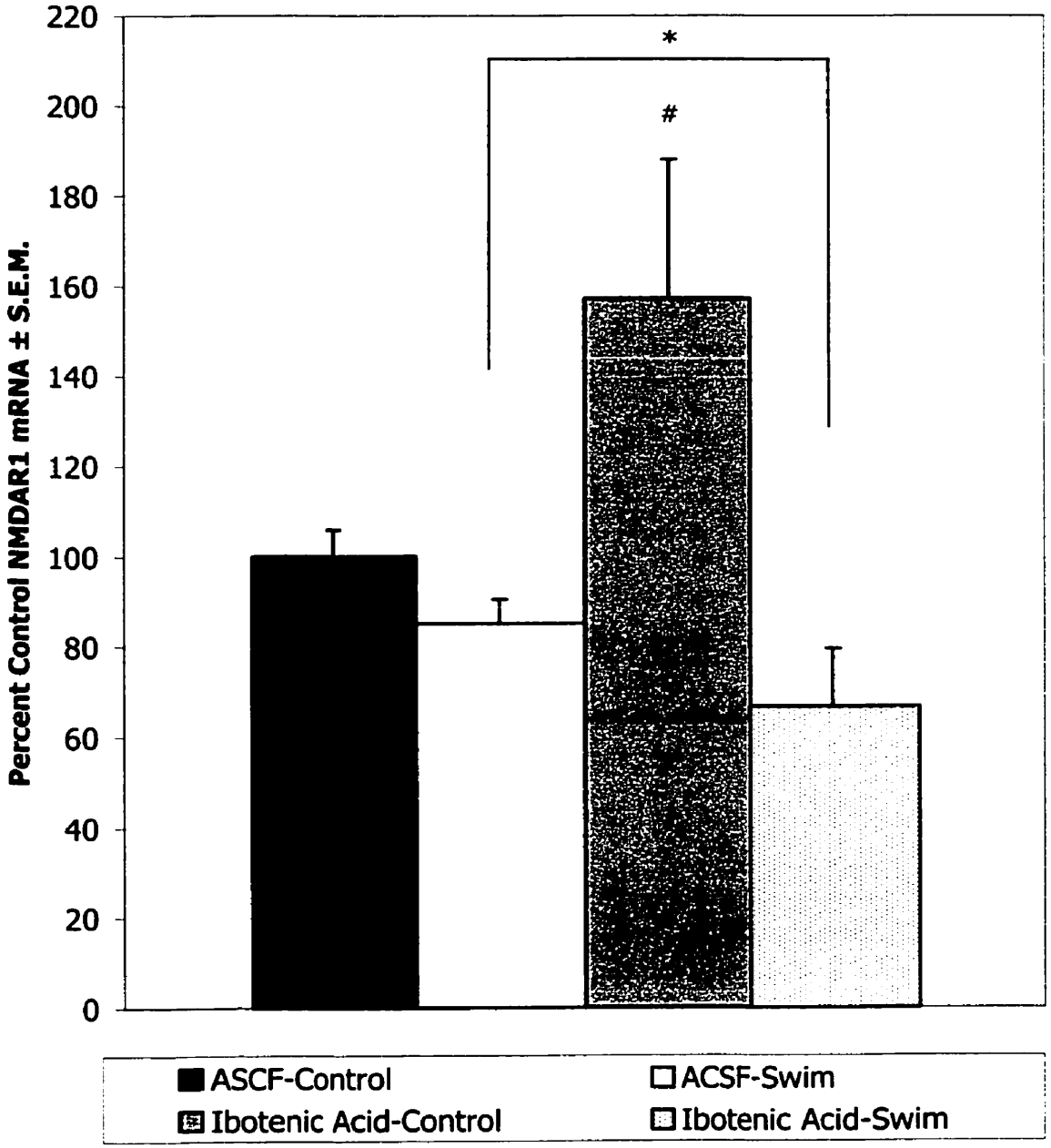


Figure 3.16. NMDAR1 mRNA in the Dentate Gyrus of the Hippocampus. ANOVA revealed a significant effect of treatment (swim stress vs. control environment) ($F_{(1,17)}$=14.42, $p<.01$) and a significant interaction between status (ACSF vs ibotenic acid) and treatment ($F_{(1,17)}$=7.43, $p<.05$). * indicates a main effect significant at $p<.01$ and # indicates significant with respect to ACSF-control at $p<.01$.

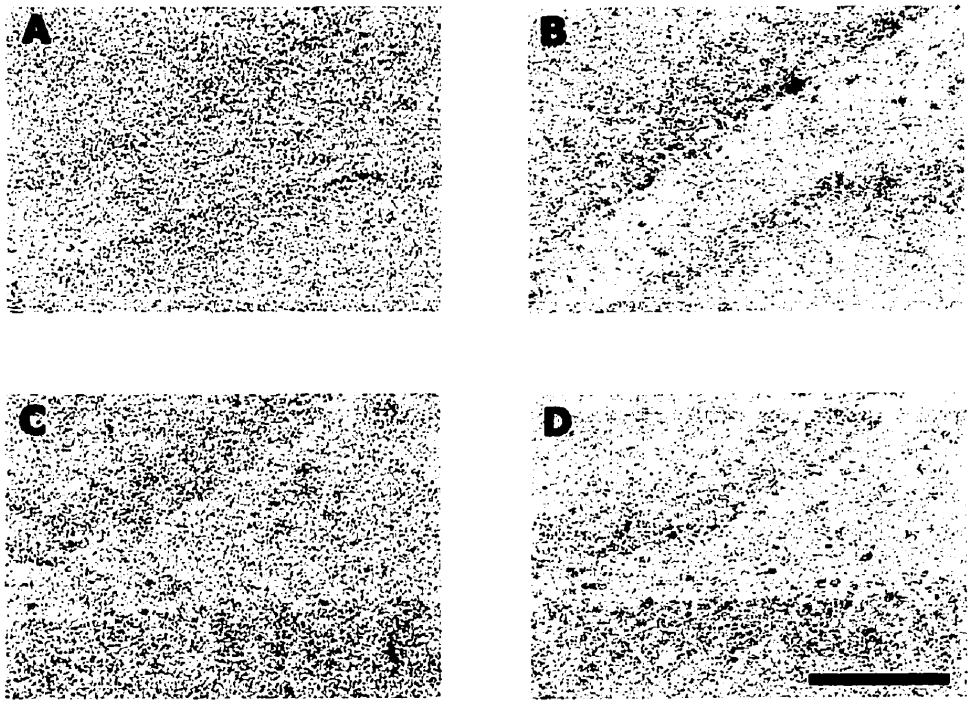


Figure 3.17. In situ hybridization of NMDAR1 mRNA in the dentate gyrus of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.8 NMDAR1 mRNA in the CA3 Region of the Hippocampus

This experiment was performed to determine if neonatal ibotenic acid lesions of the ventral hippocampus influence the expression of NMDAR1 mRNA in the CA3 region of the hippocampus of adult rats. The goal was to investigate if basal expression was altered and if stress influenced the expression of this subunit. If stress affected the expression, it was also important to determine whether differential regulation was seen between lesion and sham rats. Sections from a total of 10 animals from the ACSF – control environment group, 9 from the ACSF – swim stress group, 6 from the ibotenic acid – control environment group and 5 from the ibotenic acid – swim stress group were analyzed. Results from the two way ANOVA indicated a significant main effect of treatment (swim stress versus control environment) ($F_{(1,26)}=10.88$, $p<.01$) but no significant main effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,26)}=2.03$, $p=.17$). There was also no interaction between lesion status and treatment ($F_{(1,26)}=0.05$, $p=.82$). Overall, the results indicated a similar relative reduction in NMDAR1 mRNA expression in the CA3 region of the hippocampus in lesion (34%) and sham (30%) animals (see Figure 3.18). The approximate 10% reduction in NMDAR1 mRNA in lesion animals under both baseline and stress conditions as compared to sham animals under the same conditions was not significantly different. The reduction in NMDAR1 mRNA in response to stress appears to result from a general downregulation in individual cells rather than a decrease in the number of cells expressing detectable levels of NMDAR1 mRNA. Bright field photomicrographs of silver grains indicative of NMDAR1 mRNA in the CA3 region of the hippocampus of lesion and sham rats exposed to swim stress or a control environment are shown in Figure 3.19.

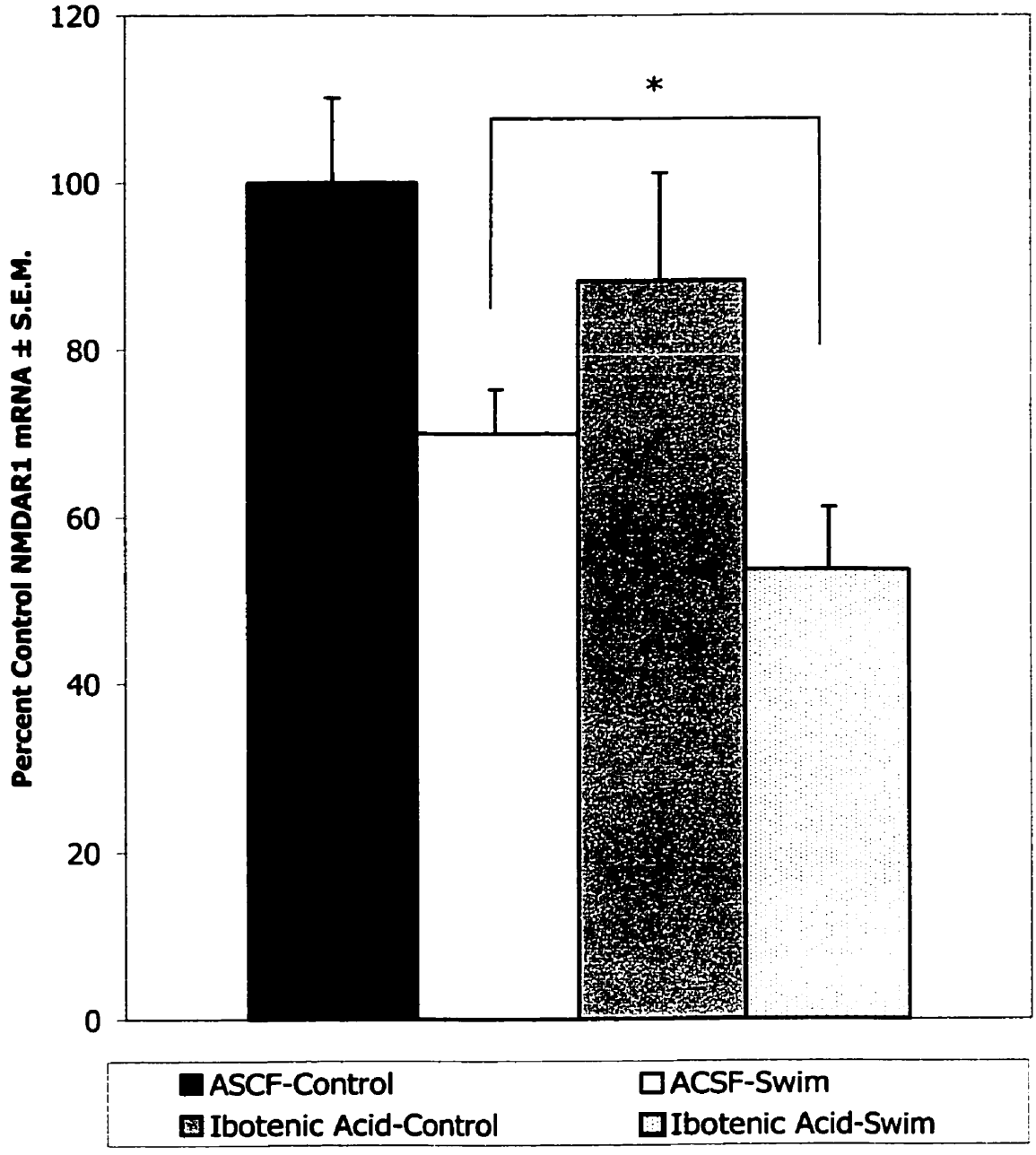


Figure 3.18. NMDAR1 mRNA in the CA3 region of the hippocampus. Two way ANOVA revealed a significant main effect of treatment (swim stress vs control environment ($F_{(1,26)}$=10.88, p<.01). There was no effect of lesion status (ACSF vs ibotenic acid) and no interaction between lesion status and treatment. * indicates a main effect significant at p<.01.

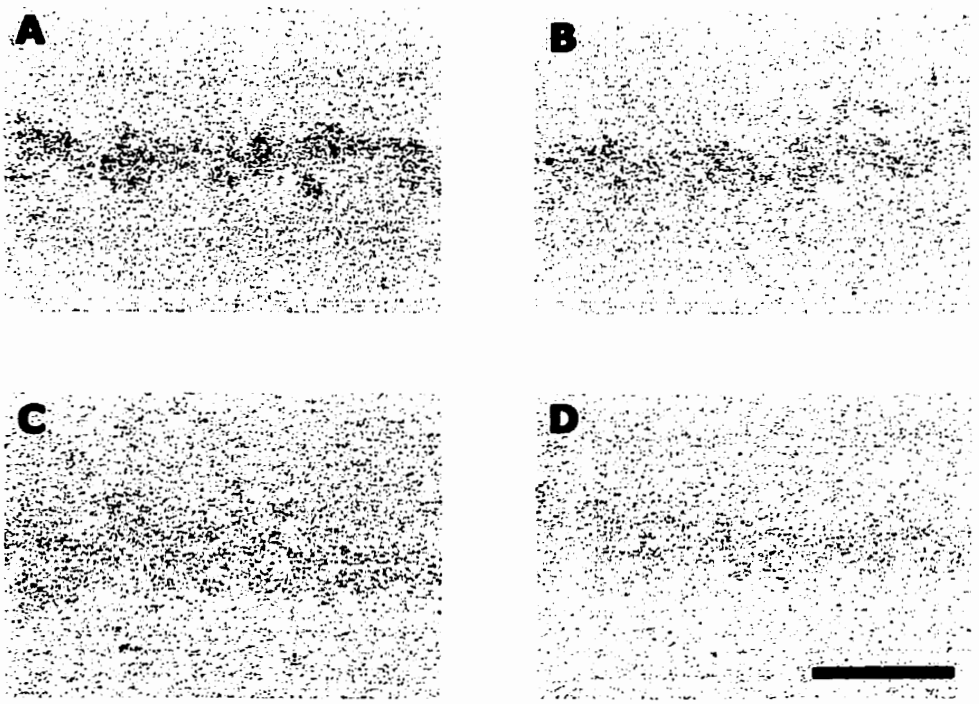


Figure 3.19. In situ hybridization of NMDAR1 mRNA in the CA3 region of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.4.9 NMDAR1 mRNA in the CA1 Region of the Hippocampus

This experiment was performed to determine if neonatal ibotenic acid lesions of the ventral hippocampus influence the expression of NMDAR1 mRNA in the CA1 region of the hippocampus of adult rats. The goal was to investigate if basal expression was altered and if stress influenced the expression of this subunit. If stress affected the expression, it was also important to determine whether differential regulation was seen between lesion and sham rats. Sections from a total of 8 animals from the ACSF – control environment group, 10 from the ACSF – swim stress group, 5 from the ibotenic acid – control environment group and 4 from the ibotenic acid – swim stress group were analyzed. Results indicated a significant main effect of treatment (swim stress versus control environment) ($F_{(1,23)}=12.85$, $p<.01$). There was no significant main effect of status (ACSF versus ibotenic acid) ($F_{(1,23)}=2.54$, $p=.13$), nor was there a significant interaction between lesion status and treatment ($F_{(1,23)}=0.22$, $p=.64$). Overall, the results indicated a similar relative reduction in NMDAR1 mRNA expression in the CA1 region of the hippocampus in lesion (36%) and sham (28%) animals in response to stress (see Figure 3.20). The approximate 10% and 20% reductions in NMDAR1 mRNA in lesioned animals under baseline and stress conditions respectively, were collectively not significantly reduced compared to sham animals under the same conditions. The reduction in NMDAR1 mRNA in response to stress appeared to result from a general downregulation in individual cells rather than a decrease in the number of cells expressing detectable levels of NMDAR1 mRNA. Bright field photomicrographs of silver grains indicative of NMDAR1 mRNA in the CA1 region of the hippocampus of lesion and sham animals exposed to swim stress or a control environment are shown in Figure 3.21.

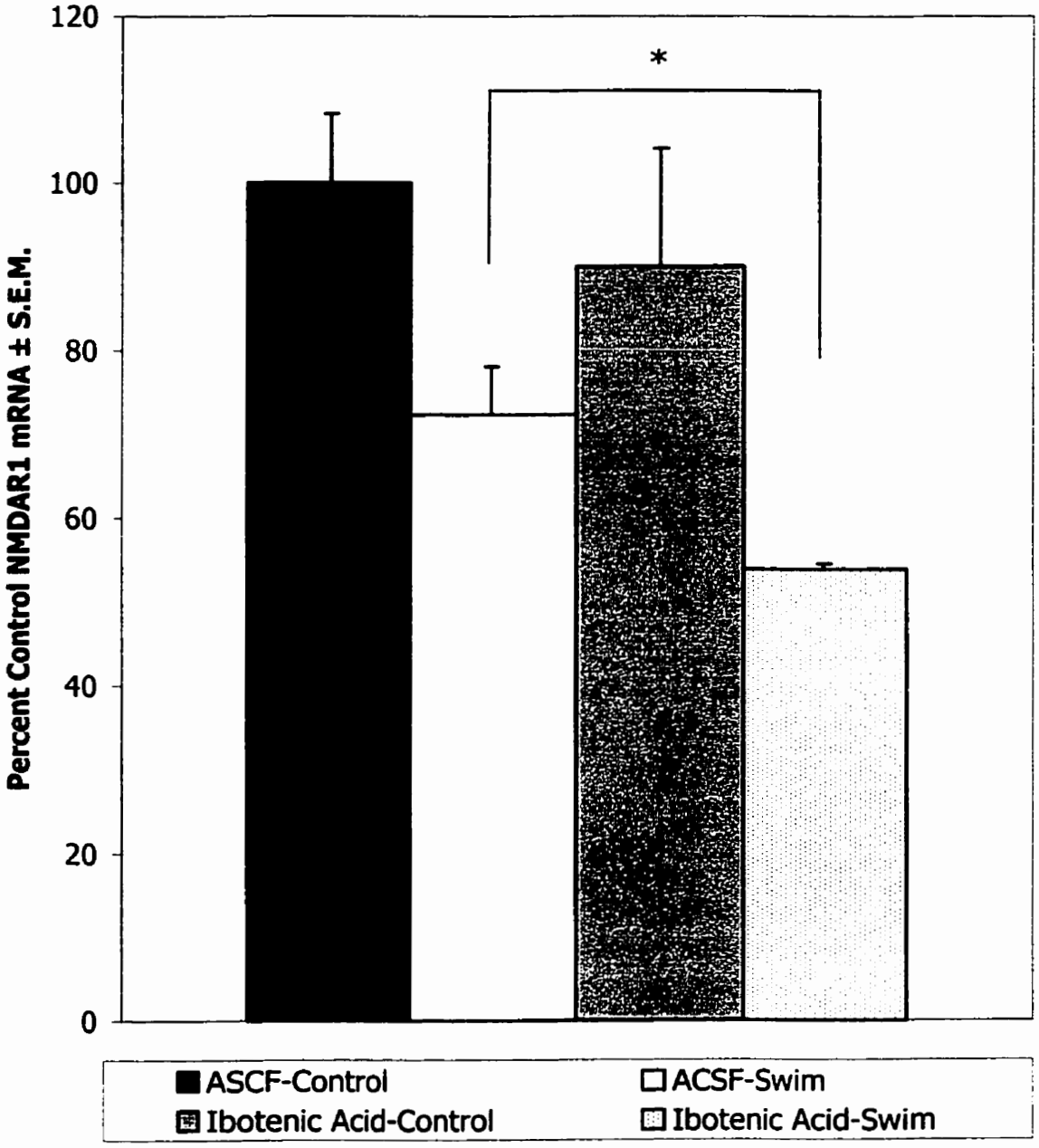


Figure 3.20. NMDAR1 mRNA in the CA1 region of the hippocampus. Two way ANOVA revealed a significant main effect of treatment (swim stress vs control environment) ($F_{(1,23)}$=12.85, $p<.01$). There was no main effect of lesion status (ACSF vs ibotenic acid) and there was no interaction. * indicates a main effect significant at $p<.01$.

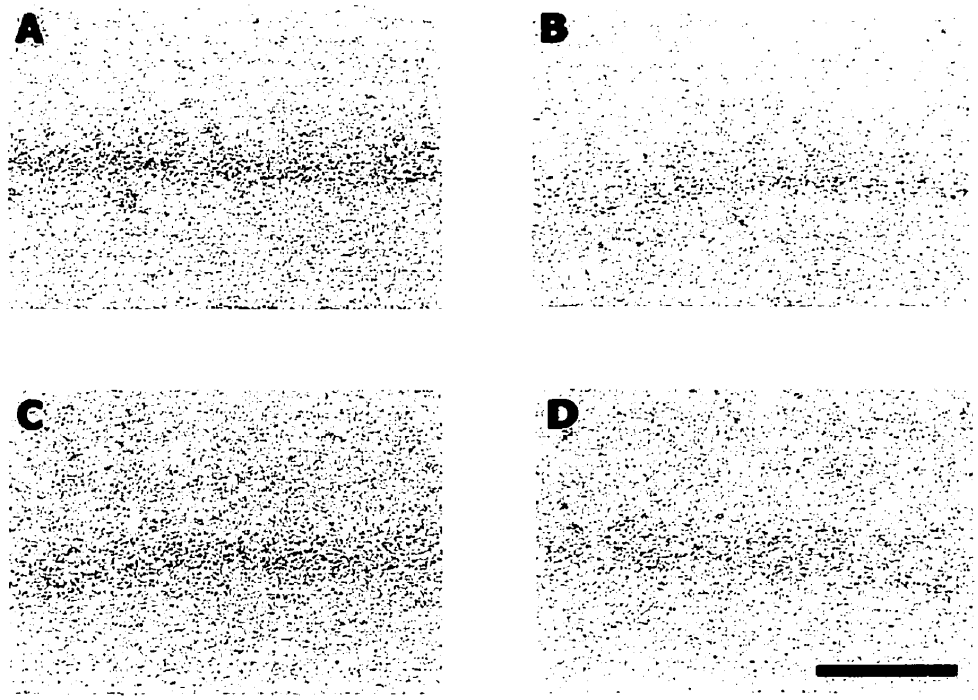


Figure 3.21. In situ hybridization of NMDAR1 mRNA in the CA1 region of the hippocampus of lesion and sham animals exposed to a control environment or swim stress. A) ACSF – control environment, B) ACSF – swim stress, C) Ibotenic acid – control environment, D) Ibotenic acid – swim stress. Scale bar = 100 μm.

3.5 Apoptosis in the Prefrontal Cortex

Apoptosis was investigated to determine whether animals with functionally compromised circuitry, neonatal ibotenic acid lesions, displayed increased levels of apoptosis compared to normal animals. The link between cell death and stress and schizophrenia and stress also led to the investigation of whether animals with symptoms reminiscent of schizophrenia displayed increased apoptosis in response to stress. Sections from a total of 4 animals from the ACSF – control environment group, 5 from the ACSF – swim stress group, 4 from the ibotenic acid – control environment group and 4 from the ibotenic acid – swim stress group were analyzed. Analysis of bisbenzamide counts, confirmed by TUNEL staining, by two way ANOVA indicated a significant main effect of lesion status (ACSF versus ibotenic acid) ($F_{(1,13)}=55.30$, $p<.001$). There was no significant main effect of treatment (swim stress versus control environment ($F_{(1,13)}=3.40$, $p=.09$) nor was there a significant interaction between lesion status and treatment ($F_{(1,13)}=3.66$, $p=.08$). Overall, the results indicated that animals with neonatal ibotenic acid lesions of the ventral hippocampus had greater baseline levels of apoptosis (see Figure 3.22). Lesion animals demonstrated 12.1% and 14.0% apoptosis under baseline and swim stress conditions, respectively, whereas, sham animals demonstrated 9.3% and 9.2% apoptosis under baseline and swim stress conditions, respectively. Apoptosis levels were not significantly altered by acute stress (14.0% in lesioned animals versus 9.2% in sham animals). Figure 3.23 depicts bisbenzamide staining of cells in the prefrontal cortex of lesion and sham animals exposed to a control environment or swim stress. Apoptotic cells are characterized by more intense bisbenzamide fluorescence than healthy cells. In addition, apoptotic cells demonstrate condensation of DNA resulting in reduced nuclear size compared to healthy cells. Figure 3.24 demonstrates the reliability of bisbenzamide staining to detect apoptosis by depicting double-stained TUNEL positive neurons and neurons appearing apoptotic by bisbenzamide staining.

There is overlap in cells demonstrating intense bisbenzamide fluorescence and TUNEL positive nuclei. Increased neurodegeneration in animals with neonatal ibotenic acid lesions of the ventral hippocampus as compared to sham animals was also apparent in Fluoro-Jade stained sections (see Figure 3.25) although no quantification was performed. Regions of intense fluorescence indicate regions of neuronal degeneration within the prefrontal cortex.

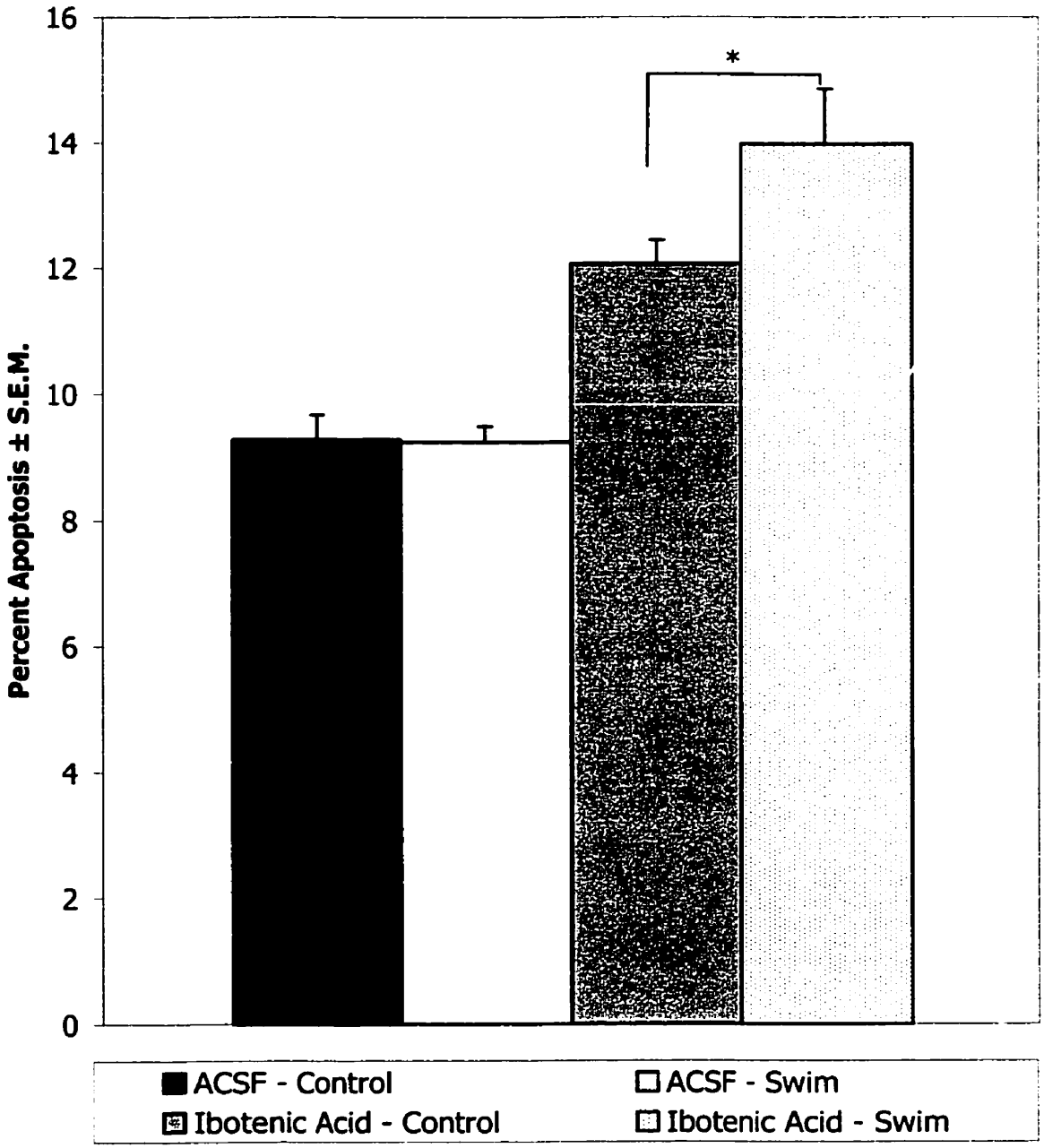


Figure 3.22. Percent apoptosis in the prefrontal cortex. Apoptotic cells were identified by bisbenzamide staining and confirmed apoptic by TUNEL labeling. Two way ANOVA revealed a significant effect of lesion status (ACSF vs ibotenic acid) ($F_{(1,13)}$=55.3, p<.001). There was no interaction. * indicates a main effect significant at p<.001.

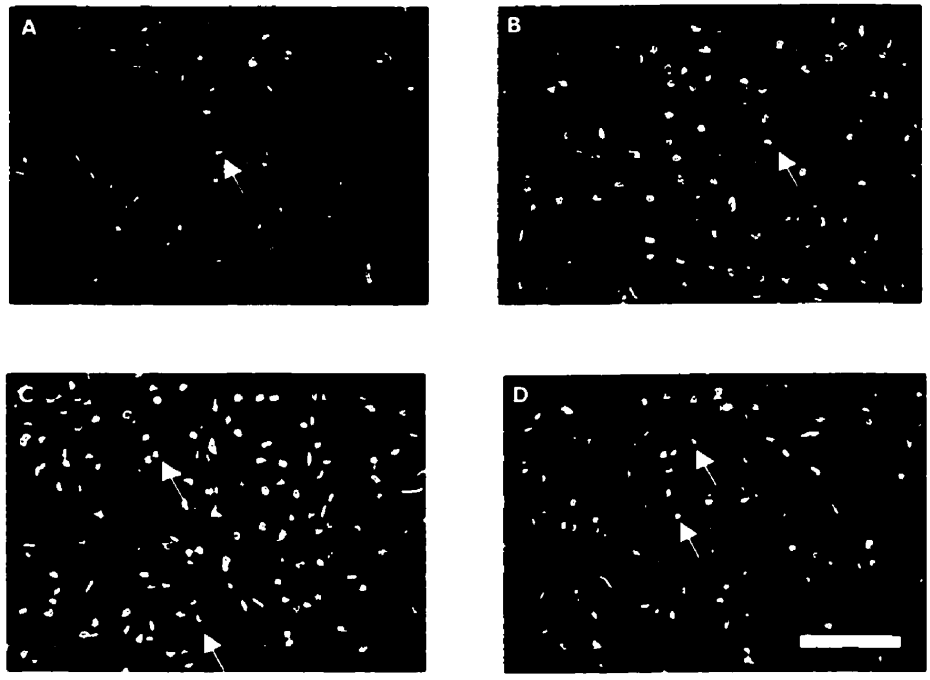


Figure 3.23. Bisbenzamide staining of apoptotic neurons in the prefrontal cortex. A) ACSF – control, B) ACSF – swim stress, C) Ibotenic acid – control, D) Ibotenic acid – swim stress. Scale bar = 500 μm.

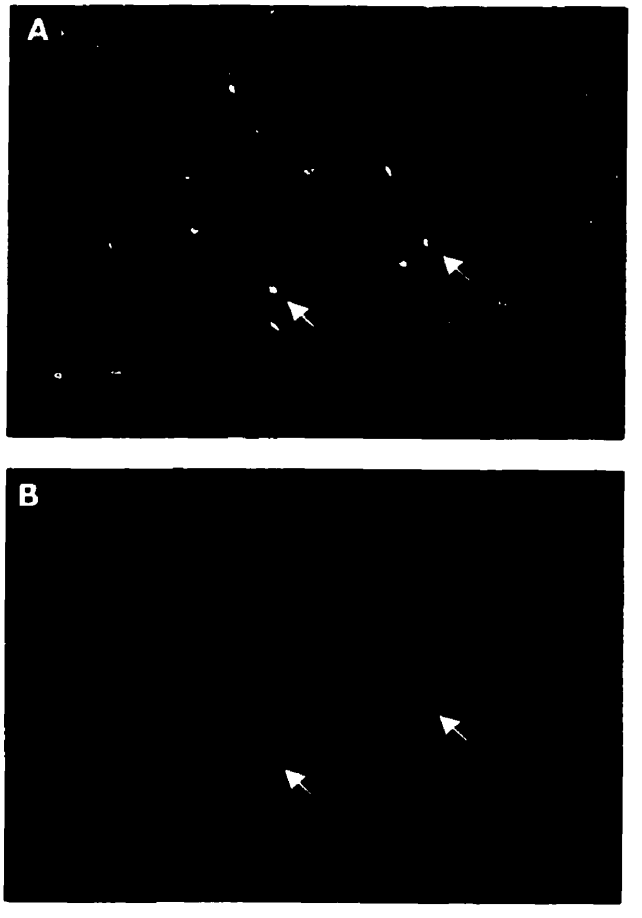


Figure 3.24. Comparison of apoptosis detection by bisbenzamide staining (A) and TUNEL staining (B) in identical areas of the prefrontal cortex. This example is taken from an animal with bilateral neonatal ibotenic acid lesions of the ventral hippocampus. Scale bar = 100 µm.

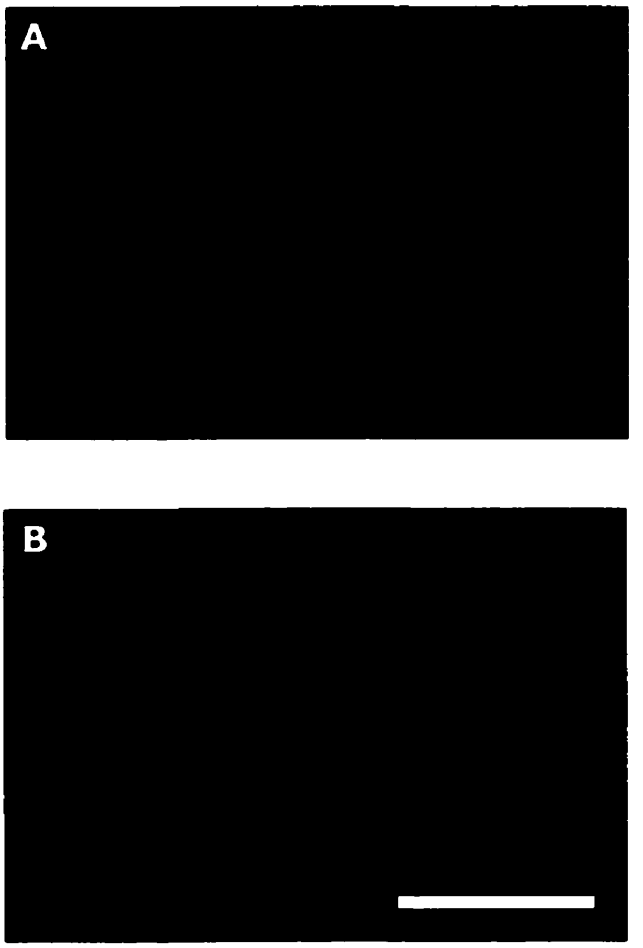


Figure 3.25. Fluoro-Jade B staining indiciating neurodegeneration in the prefrontal cortex of ACSF treated animals (A) and ibotenic acid treated animals (B) under control environment conditions. Scale bar = 100 μm.

4. DISCUSSION

4.1 Behavioral Testing

Animals with neonatal ibotenic acid lesions of the ventral hippocampus, a model of schizophrenia, have previously been shown to be hyperactive in locomotor activity paradigms in adulthood, but not prior to adulthood (Lipska *et al.*, 1993). In an attempt to replicate this model of schizophrenia-like dysconnectivity, locomotor activity in response to a novel environment was investigated in prepubertal (PND 35) and postpubertal (PND 56) lesion and sham rats. Lesion and sham rats demonstrated no differences in locomotor activity at either of these time points. This suggests that these animals are not hyperresponsive to dopamine-mediated behaviors at PND 56 as previously reported (Lipska *et al.*, 1993). Alternatively, the possibility exists that the delay in emergence of hyperresponsivity is increased. Previous reports demonstrate that the extent of the lesion is related to the emergence of behavioral hyperresponsivity (Lipska & Weinberger, 1995). These authors reported that Sprague-Dawley rats with small ventral hippocampal lesions demonstrated no emergence of hyperresponsivity at PND 56, whereas rats with large lesions did. No other lesion sizes were investigated in Sprague-Dawley rats, however, small and large lesions in other rats strains resulted in varying emergence times of behavioral hyperresponsivity. This supports the conclusion that a lesion of lesser extent than the original model could result in a further delay in the emergence of hyperresponsivity. Further support for the claim of a lesser lesion extent is the exclusion of any animals with thalamic damage in the present study compared to the 25% cohort with a minor rim of thalamic damage in the original study (Lipska *et al.*, 1993). In the present study,

thalamic damage was associated with a greater degree of hippocampal damage as compared to included animals. Locomotor activity of animals with this degree of damage was not examined, however, as the number of animals was not sufficient when those with associated dorsal hippocampal ablation, cortical damage, and seizures were removed from the cohort.

Relevant to the suggestion of an increased delay in the emergence of behavioral hyperresponsivity is the demonstration of a main effect of lesion status at PND 75 in the stress paradigm. This means that collapsed across treatment groups, ibotenic acid treated animals are significantly more active than ACSF treated animals. Although no direct comparison of ACSF and ibotenic acid treated animals exposed to a control environment is acceptable from a statistical standpoint, the overall results suggest an emergence of hyperresponsivity to dopamine-mediated behaviors at this time point. This supports the conclusion that the extent of the lesion is less than that of the original model, and also leads to the conclusion that facets of the original model have been replicated.

The mechanism underlying the delayed emergence of dopamine-mediated behaviors is unknown, but it has been suggested that the modulation of the mesolimbic dopamine system by the hippocampus may not be significant until later maturational stages (Lipska *et al.*, 1993). Alternatively, Lipska *et al.*, (1993) suggest that secondary neurodegenerative processes resulting in alterations in behavioral expression may occur over time. This leads to the suggestion that this neurodevelopmental model is a more comprehensive model of factors associated with schizophrenia than first described. The accuracy of this suggestion would implicate neurodevelopment, dysconnectivity and neurodegeneration in the model, all factors hypothesized to be relevant to schizophrenia. This alternative hypothesis also makes plausible the suggestion of a further delay in emergence of dopamine hyperresponsivity. It is possible

that a threshold of neurodegeneration must necessarily be reached before behavioral changes manifest. The lesion would not necessarily impact the age at which functional hippocampal modulation of the dopaminergic system occurs, but rather, secondary neurodegenerative changes could result in a diminished functional capacity of this system later in life.

4.2 Stress Paradigm

As discussed above, neonatal lesions of the ventral hippocampus appear to result in a delayed emergence of dopamine-mediated behaviors, with the present study demonstrating an even later emergence than previous reports. In addition to this difference between the original and the present model, Lipska *et al.,* (1993) demonstrated a trend for hyperresponsivity to stress in adult animals, a finding not replicated in the present study. A significant increase in locomotor activity is demonstrated in response to stress, but the relative magnitude of the increase is virtually identical in lesion and sham animals. The reason for the difference between the original model and the present findings is unknown, but one could speculate that the assumed reduction in lesion size in the present study may result in a relative sparing of neurons projecting to the prefrontal cortex. As described earlier, the prefrontal cortex is known to play a role in stress (Deutch *et al.*, 1990) and lesions of this region in adult animals result in hyperresponsivity to stress (Jaskiw & Weinberger, 1992). The demonstration that neonatal, but not adult induced, ibotenic acid lesions of the ventral hippocampus can result in hyperresponsivity to stress indicates that prefrontal cortex function and/or connectivity is affected. A reduction in the extent of damage to the hippocampus may result in a lesser downstream impairment in the prefrontal cortex, thereby resulting in animals better able to respond appropriately to stress than animals in the original model. Contrary to this suggestion, however, is the demonstration of hyperresponsivity to stress with respect to BDNF mRNA expression in the

prefrontal cortex (to be discussed later). In addition, incremental locomotor activity data (Figure 3.3) suggests that a longer time period of behavioral monitoring may reveal differences in the response to stress in lesion and sham animals. Ninety minutes parallels the monitoring time in the original investigation, however, laboratory variances such as specific testing environment (light conditions, time of day) and activity measurement units may necessitate a longer monitoring time. At the 75 and 90 minute intervals, the lesion and sham animals exposed to a control environment show similar increases in locomotor activity. Ibotenic acid treated animals exposed to swim stress, however, show 1.5 to 2 times the number of photocell beam breaks as ACSF treated animals exposed to swim stress. The ibotenic acid treated animals exposed to swim stress, also do not appear to be habituating as quickly as the other groups. It is, therefore, possible that hyperresponsivity to stress would be revealed following extended monitoring periods.

The emergence of behavioral hyperresponsivity to stress in postpubertal rats is hypothesized to be due to developmental maturation of the prefrontal cortex (Lipska *et al.*, 1993). During adolescence the synaptic density of the prefrontal cortex declines (Huttenlocher, 1979) with synapses on dendritic spines of pyramidal neurons accounting for the majority of the decline (Bourgeois *et al.*, 1994). This reduction in synapses on dendritic spines suggests a reduction in excitatory input to these neurons as spines are the major location of excitatory synapses on pyramidal neurons (Colonnier, 1968, Mates & Lund, 1983). Dopaminergic afferents to the prefrontal cortex also undergo maturational changes. These afferents form synapses with dendritic spines and shafts of pyramidal neurons (Goldman-Rakic *et al.*, 1989) as well as with GABAergic interneurons (Sesack *et al.*, 1995). There is a rise in the density of dopaminergic synapses prior to the decline of synapses on pyramidal neuron spines (Lewis, 1997). The pattern of neuronal connectivity and of change suggests that dopamine may influence the maturation of pyramidal

neuron connectivity in the prefrontal cortex. The link between the prefrontal cortex, dopamine, glutamate and stress makes it possible that maturational changes affecting these variables, in an already compromised system, may result in dysregulation, an inability to compensate, and, therefore, emergence of hyperresponsivity to stress. Prior to this maturational point, it is assumed that functional compensation accounted for the absence of behavioral abnormalities (Lipska *et al.*, 1993). A dysregulation of maturational processes resulting from the neurodevelopmental lesion, however, would result in inappropriate pruning and consequent behavioral and biochemical abnormalities.

4.3 BDNF mRNA Detection

4.3.1 BDNF mRNA in the Prefrontal Cortex

The results of the present study demonstrate that basal BDNF mRNA levels are reduced in the prefrontal cortex of rats with neonatal ibotenic acid lesions of the ventral hippocampus. Reductions in baseline BDNF mRNA expression could result in alterations in neuronal morphology, specifically dendritic atrophy (McCallister *et al.*, 1995), as well as reduced synaptic plasticity (Thoenen, 1995) and reduced neuronal survival (Ghosh *et al.*, 1994). The inter-relationship between BDNF and the neurotransmitters GABA and glutamate also suggests that reductions in basal BDNF mRNA expression could relate to alterations in inhibitory and excitatory neurotransmission in the prefrontal cortex. Specifically, it has been demonstrated that BDNF expression is positively correlated with glutamatergic neurotransmission and negatively correlated with GABAergic neurotransmission (Zafra *et al.*, 1990, 1991). A bi-directional regulatory relationship exists, however, between these systems. This implies that altered BDNF expression can directly influence glutamatergic neurotransmission just as glutamate can directly regulate BDNF expression (Kang & Schuman, 1995, Lebmann *et al.*, 1994, Levine *et al.*, 1995). As will be

discussed later, this inter-relationship and the reduction in baseline BDNF levels can have significant consequences with respect to brain function, synaptic connectivity, neurotransmission, and neuronal viability and/or vulnerability.

Previous investigations using restraint stress demonstrate that stress results in a decrease in BDNF expression (Smith *et al.*, 1995a, 1995b), however, the present study demonstrates a significant upregulation of BDNF mRNA following swim stress. In addition, animals with neonatal ibotenic acid lesions demonstrate significantly greater increases in BDNF mRNA expression than do sham animals exposed to stress. This finding is contrary to what was expected according to previous reports and the hypothesis tested in this study. It was hypothesized that animals with lesions modeling schizophrenia would show reductions in baseline BDNF, as was demonstrated, and also show significantly reduced BDNF mRNA levels as compared to sham animals exposed to swim stress. It is well documented that stress increases glutamate release in the prefrontal cortex (Moghaddam, 1993) and that glutamate signal transduction can directly increase BDNF expression (Zafra *et al.*, 1990, 1991). It is possible that the increased glutamate release following stress has a direct effect of increasing BDNF mRNA, although the results by Smith *et al.* (1995a, 1995b) are not in accordance with this conclusion. It is unknown what accounts for the discrepancy between the previous reports and the present study, however, the possibility of exon-specific probes can be negated. All of the probes used were directed against the region of the rat BDNF gene encoding the mature protein; a region conserved in all mRNA transcripts. Recently, a corroborating report of increased BDNF mRNA in the prefrontal cortex following restraint stress was published (Molteni *et al.*, 1999), however no comment was made regarding the demonstration of increased BDNF following stress compared to the previous reports of its reduction following stress.

The demonstration that lesioned animals exhibit significantly increased BDNF mRNA expression compared to sham animals following exposure to stress suggests that these animals are hyperresponsive to the effects of stress. In addition to this, one can conclude that the ability to regulate BDNF expression is not impaired. The alternative to this conclusion would be that the stability of the mRNA species may be differentially effected between treatments. In the event that BDNF mRNA stability is not altered there are three possible explanations for the relative overexpression of BDNF mRNA. First, a relative hyper-release of glutamate resulting in a proportional increase in BDNF mRNA may occur. Second, a compensatory mechanism in response to increased demands on an already compromised system may result in an increase in neuroprotective factors. Third, there may be a dysregulation in the expression of BDNF mRNA. This could result from differential promoter usage or from altered regulation of splice variants thereby resulting in an inappropriate response. The glutamatergic system has been proposed to play a role in schizophrenia (see section 1.2.2). This system is also suggested to be hypofunctional in the animal model of schizophrenia (Lillrank *et al.*, 1996a). Al-Amin *et al.*, (1996) demonstrated that rats with neonatal ibotenic acid lesions are hypersensitive to NMDA antagonists, suggesting a possible dysregulation of the glutamatergic system. It was concluded that the destruction of glutamatergic projection fibers results in reduced glutamate release in the prefrontal cortex. Investigation of stimulus-induced amino acid release from prefrontal cortical and hippocampal slices prepared from adult rats lesioned as neonates revealed a reduction in release (Schroeder *et al.*, 1999), thereby supporting this conclusion. These findings suggest that increased glutamate release is not responsible for the increase in BDNF expression, however, stimulus-induced alterations in glutamate have not been investigated *in vivo*. In addition, different stimuli may result in different glutamate release, therefore, one cannot conclude definitively that glutamate release is reduced. The role that BDNF plays in neuronal protection suggests that its expression

may be upregulated in response to a threat to the system as is the situation with ischemia (Lindvall *et al.*, 1992) and kainic acid toxicity (Zafra *et al.*, 1990). The functionally compromised system set up by the neonatal lesion may demonstrate increased vulnerability to stress, therefore, protection from atrophy and/or death may require a greater upregulation of BDNF to overcome the threat. Alternatively, dysregulation of BDNF expression may result from the neonatal lesion. Whether this effect is due to specific promoter usage or to altered expression of individual splice variants could be investigated through the use of probes specific for these variants. Regardless of whether an individual BDNF mRNA splice variant is altered or whether the effect is more global, the possibility exists that unnecessary overexpression of BDNF mRNA may put further stress on the system. It has been reported that overexpression of BDNF can potentiate NMDA-induced necrotic cell death, at least under cell culture conditions (Koh *et al.*, 1995). It is questionable whether necrotic cell death would occur in this system, however, the conditions under which this would occur could be present as there is a potential for increased glutamate release following stress and a marked increase in BDNF mRNA was demonstrated in the present study.

4.3.2 BDNF mRNA in the Hippocampus

The hippocampal formation is a heterogeneous structure consisting of six cytoarchitechtonically distinct regions: the dentate gyrus, the hippocampus proper (CA3, CA2 and CA1 fields), the subiculum, the presubiculum, the parasubiculum, and the entorhinal cortex (Amaral & Witter, 1995). The major input to the dentate gyrus arises from the entorhinal cortex via the unidirectional perforant pathway. The dentate gyrus, in turn projects unidirectionally to the CA3 field of the hippocampus via mossy fibers. The CA3 field projects unidirectionally to the CA1 field of the hippocampus via the Schaffer collaterals. This intrinsic "tri-synaptic" circuit is the major route of

information transfer within the hippocampal formation, however, other connections have also been identified that involve direct communication between various hippocampal fields (Amaral & Witter, 1995). The perforant pathway projecting to the dentate gyrus is suggested to be a glutamatergic pathway (Fonnum, 1970), however, more recent studies indicate other cell types including GABAergic neurons also project to the hippocampus via the perforant pathway (Germroth *et al.*, 1989). The mossy fiber projections and the Schaffer collaterals use glutamate as their primary neurotransmitter as do the CA1 pyramidal cells (Storm-Mathisen & Fonnum, 1972). It is the CA1 pyramidal cells that are responsible for the majority of extra-hippocampal projections including both cortical and subcortical regions (Van Groen & Wyss, 1990).

The present study demonstrates a significant reduction in baseline BDNF mRNA in the dentate gyrus of the hippocampus in animals with neonatal ibotenic acid lesions. An overall reduction in BDNF mRNA expression is also seen in both the CA3 and the CA1 fields of the hippocampus following the neonatal lesion. In response to stress, however, differences exist between the various hippocampal regions. Although stress consistently increases the expression of BDNF mRNA in all regions, only the dentate gyrus demonstrates a hyperresponsivity. The relative response to stress in the CA3 and CA1 regions are comparable in lesion and sham animals, whereas the dentate gyrus of lesioned animals demonstrates a significant upregulation of BDNF mRNA following exposure to stress as compared to sham animals.

As discussed above, reductions in BDNF expression can have serious consequences on neurotransmission as well as neuronal plasticity and viability. The "tri-synaptic" connectivity of the hippocampus, with the dentate gyrus being the first point of information communication, may be significantly compromised by alterations in BDNF expression. The system may be able to

function with relative normality under basal conditions, but the hyperresponsivity to stress in the dentate gyrus may lead to downstream abnormalities in neurotransmission and neuronal viability. As discussed previously, BDNF can regulate glutamatergic neurotransmission (Kang & Schuman, 1995, Lebmann *et al.*, 1994, Levine *et al.*, 1995), therefore, hyperresponsive expression of BDNF in lesioned animals may result in increased glutamatergic neurotransmission throughout the hippocampus. The results of stimulus-induced amino acid release in hippocampal slices from neonatally lesioned animals, however, do not support this conclusion (Schroeder *et al.*, 1999). In the event that glutamate release is not altered by BDNF expression, it remains possible that the basal reduction seen in lesioned animals may produce neuronal atrophy and consequent compromised neurotransmission. Under conditions of stress, the increase in BDNF in the dentate gyrus of lesioned versus sham rats may be an attempt for additional compensation on an already vulnerable system. One may also suggest that differential input to the dentate gyrus may account for its hyperresponsivity as compared to the CA3 and CA1 fields. As described above, the predominant inputs to the CA3 and CA1 fields are glutamatergic whereas the dentate receives both glutamatergic and GABAergic input (Fonnum, 1970, Germroth *et al.*, 1989). It is also known that BDNF expression is dependent on the balance of glutamatergic and GABAergic input (Zafra *et al.*, 1990, 1991). The possibility exists, therefore, that alterations in GABAergic neurotransmission following exposure to stress in lesioned animals account for the differential regulation of BDNF mRNA in the dentate gyrus.

In the hippocampus, as in the prefrontal cortex, the demonstration of increased BDNF mRNA expression is contrary to previous reports of the effects of stress on BDNF mRNA (Smith *et al.*, 1995a, 1995b). The increase in glutamate neurotransmission following exposure to stress would be expected to increase BDNF mRNA, and Smith *et al.* (1995a, 1995b) report that their results

were unexpected based on glutamate increase as well as the effect of other system stressors, such as ischemia, on BDNF mRNA expression.

4.3.3 BDNF mRNA and Locomotor Activity

The previous reports of reduced BDNF mRNA expression following stress (Smith *et al.*, 1995a, 1995b), in addition to the demonstration of increased BDNF mRNA expression following physical activity (Neeper *et al.*, 1996) led to the investigation of the effect of swim stress and locomotor activity on BDNF mRNA expression. Animals lesioned as neonates did appear to exhibit more basal activity than their sham counterparts at PND 75, however, they demonstrated reduced BDNF mRNA expression. This suggests that basal levels of activity did not have a stimulating effect on BDNF mRNA expression. The swim stress, however, resulted in a dramatic increase in physical activity as well as an increase in BDNF mRNA in both lesion and sham animals. The 15 minutes of swimming activity, in addition to the enhanced locomotor activity for at least 90 minutes following exposure to stress may have been sufficient to enhance BDNF mRNA expression. In addition, the significant increase in BDNF mRNA in lesion versus sham rats exposed to stress could also be explained by the suggested increase in physical activity. As discussed above, the lesioned animals exposed to stress were 1.5 to 2 times more active than their sham-stressed counterparts at later time points in the locomotor activity paradigm. The conclusion could be made that these animals also continued to be more active once placed back in the home cage, therefore exhibiting an overall greater level of physical activity. The assessment of whether locomotor activity directly correlated with BDNF mRNA expression demonstrated no direct relationship between physical activity and BDNF mRNA expression. This leads to the conclusion that the effect of stress on BDNF mRNA is opposite to that previously reported (Smith *et al.*, 1995a, 1995b) and that the hyperresponsivity

seen in the present study is a result of the lesion, not a result of enhanced physical activity.

4.3.4 Significance of Alterations in BDNF mRNA Expression

As discussed in section 1.5.2, BDNF is an attractive candidate molecule in schizophrenia due to its regulation by factors used to treat schizophrenia such as ECT and 5-HT_2 antagonists (Lindefores *et al.*, 1995; Nibuya *et al.*, 1997; Vaidya *et al.*, 1997). The potential role of BDNF is also supported by its reduction by factors associated with first onset such as stress and estrogen withdrawal (Smith *et al.*, 1995a, 1995b; Singh *et al.*, 1995). In addition, serum BDNF is reported to be reduced in schizophrenia (Toyooka *et al.*, 1999), as is hippocampal BDNF mRNA (Brouha *et al.*, 1996). The present study demonstrates a reduction in basal BDNF mRNA levels in the prefrontal cortex and the hippocampal formation of animals with neonatal ibotenic acid lesions of the hippocampus. This finding parallels those in schizophrenia and, therefore, adds validity to the model. Increased validity allows for a better basis for extrapolation of information gained from the model to the human condition of schizophrenia. Reductions in basal BDNF levels could result in alterations in neuronal connectivity by producing a situation of increased neuronal atrophy and fewer synapses. This situation is supported by the demonstration of altered synaptophysin immunoreactivity in the prefrontal cortex of schizophrenic patients (Eastwood *et al.*, 1995a, Glantz & Lewis, 1997). Alterations in synaptic density have not been investigated in the model, however, the reduction in BDNF mRNA supports this possibility. Glutamatergic neurotransmission can also be directly affected by alterations in BDNF (Kang & Schuman, 1995, Lebmann *et al.*, 1994, Levine *et al.*, 1995). The relative hypoglutamatergic state proposed in schizophrenia is, therefore, in accordance with reduced basal BDNF. As stated in the hypothesis, reductions in BDNF may also increase the vulnerability of neurons to stress. Hyperresponsivity to stress

is demonstrated by increases in BDNF mRNA in the prefrontal cortex and the dentate gyrus. This could be a direct attempt to compensate for the increased vulnerability of the system induced by the neonatal lesion.

4.4 NMDAR1 mRNA Detection

4.4.1 NMDAR1 mRNA in the Prefrontal Cortex

The results of the present study demonstrate a reduction in the level of NMDAR1 mRNA expression in the prefrontal cortex of lesion versus sham animals. Three possible explanations for this reduced expression are proposed. First, a general reduction in glutamate neurotransmission via a lesion-induced downregulation of receptor expression may occur. Second, a compensatory downregulation resulting from increased glutamate release may result in reduced NMDAR1 mRNA expression. Third, an alteration in channel properties may result from altered glutamate neurotransmission such that an alternative splice variant is expressed in place of NMDAR1a. Various splice variant probes are available, but to date, these variants have not been investigated in the model, so this remains a possibility that cannot be ruled out. The second suggestion of a compensatory downregulation is not supported by the previous demonstration of reduced glutamate release from prefrontal cortical and hippocampal slices from lesioned animals (Schroeder *et al.*, 1999). In addition, the suggested lesion-induced reduction in glutamatergic projections from the hippocampus to the prefrontal cortex also supports an overall reduction in glutamate release in the prefrontal cortex of lesioned animals (Lillrank *et al.*, 1996a). The demonstration of reduced BDNF levels in the prefrontal cortex of lesioned animals also supports the conclusion that glutamate release is not increased as increased glutamate release would result in increased BDNF expression. The suggestion that the lesion induces a reduction in glutamatergic neurotransmission via receptor downregulation remains a possibility. Reductions in NMDAR1 subunit expression have been reported in schizophrenia

in both the temporal lobe and the prefrontal cortex (Hirsch *et al.*, 1997, Humphries *et al.*, 1996). This reduction was correlated with the severity of symptom presentation, but the conclusion could be made that a lesion as seen in the model would produce a symptom severity as great or greater than that seen in severe schizophrenia. Reduced glutamate receptor subunit expression is also in accordance with the NMDA receptor hypofunction theory of schizophrenia (Olney & Farber, 1995). The demonstration of reduced NMDAR1 mRNA expression, therefore, supports the hypothesis that the neonatal lesion sets up a functionally compromised system. Reductions in glutamate neurotransmission via NMDA receptors would result in improper communication between neurons and the potential for cell death. Olney and Farber (1995) propose that reduced glutamate neurotransmission through the NMDA receptor results in downstream disinhibition of excitatory neurotransmission. This, in turn, could result in excitotoxic damage to neurons. As previously stated, reduced glutamate release was detected in slices from lesioned animals thus supporting glutamate hypofunction (Schroeder *et al.*, 1999). The second part of the hypothesis requiring downstream excitotoxicity, however, is difficult to reconcile with the overall demonstration of reduced release. In accordance with glutamate receptor hypofunction and excitotoxicity in this model, and perhaps in schizophrenia, however is the demonstration BDNF can protect against NMDA excitotoxicity (Tremblay *et al.*, 1999). Reductions in BDNF would reduce the ability of a system to protect itself from excitotoxic insults, therefore resulting in increased cell death and further detriment to the system. This would support a progressive course of the disorder. Despite the lack of a demonstrable increase in glutamate release, excitotoxicity could still result from relatively low levels of glutamate due to the lack of protective elements in place and a potentially hypersensitive system.

In addition to a reduction in overall expression of NMDAR1 mRNA in the animals lesioned as neonates, there appears to be an increase in NMDAR1

mRNA in response to stress. This increase is demonstrated in both lesion and sham animals to a similar degree. One can, therefore, conclude that although lesioned animals demonstrate reduced basal NMDAR1 mRNA expression, they maintain normal regulatory capacity. The reason for the upregulation is not known and appears contrary to the expectations based on increased glutamate release following stress. It is possible that other factors influence the expression of NMDAR1, however, very little is known about the interaction between stress and NMDA receptors. If the increase in NMDAR1 mRNA expression results in a functional increase in NMDA receptors, it is possible that this could contribute to stress induced neuronal death. Liu *et al.* (1996) demonstrated a stress-induces increase in lipid peroxidation, protein oxidation and nuclear DNA damage. Despite the fact that the relative NMDAR1 mRNA expression is similar following exposure to stress, it is possible that the effect on cell death could be more dramatic due to the presence of a functionally compromised system in lesion versus sham animals.

4.4.2 NMDAR1 mRNA in the Hippocampus

Investigation of NMDAR1 mRNA expression in the hippocampus reveals that there is a significant upregulation of this gene in the dentate gyrus. This result is not paralleled in the CA3 or the CA1 region of the hippocampus. The reason for the dramatic upregulation is not known, however, one could speculate that it is a compensation for reduced glutamate transmission from the entorhinal cortex. Glutamate release from this region has not been investigated in the model, but reduced release has been demonstrated in prefrontal cortical slices and hippocampal slices obtained from rats with neonatal ibotenic acid lesions (Schroeder *et al.*, 1999). If this is a compensatory mechanism, the question remains why no compensatory increase is demonstrated in the prefrontal cortex. It is possible that GABAergic input to the dentate gyrus also plays a role in regulating the expression of NMDAR1

mRNA, therefore, resulting in differential responses in the prefrontal cortex and the dentate gyrus. It is also possible that the downstream regulatory effects are different than those seen directly within the lesion site.

In response to stress, all three regions of the hippocampus investigated demonstrated a downregulation of NMDAR1 mRNA. This would be expected as a compensatory mechanism following increased circulating levels of glucocorticoids. Glucocorticoids are increased in response to stress and can result in extensive damage to hippocampal neurons (Sapolsky *et al.*, 1984, Watanabe *et al.*, 1992, Woolley *et al.*, 1990). This effect is proposed to be mediated by glutamate acting through NMDA receptors (Armanini *et al.*, 1990). The conclusion can be made that despite the neonatal lesion, these animals retain the ability to appropriately regulate NMDAR1 mRNA expression in response to stress. Although very little research has been done regarding NMDA receptors and stress one report of increased NMDAR1 mRNA following exposure to stress exists (Bartanusz *et al.*, 1995). This is contrary to the present findings, however, apparent differences do exist. The stress paradigm was restraint stress as opposed to swim stress, and the probe used in the in situ hybridization analysis detected all splice variants of the NMDAR1 subunit not only the NMDAR-1a variant as in the present study. It is possible that splice variants are differentially regulated under conditions of stress to effectively reduce glutamatergic neurotransmission and subsequent neuronal atrophy or death. Splice variant regulation would allow maintained expression of NMDA receptors with alterations in electrophysiological properties accounting for the effective reduction in neurotransmission.

4.4.3 Significance of Alterations in NMDAR1 mRNA Expression

The demonstrated reduction of NMDAR1 mRNA in the prefrontal cortex suggests that NMDA receptor hypofunction may be a significant factor

contributing to the behavioral and biochemical changes seen in the model. This finding is also in accordance with the demonstration of reduced BDNF mRNA expression. These results indicate that the hypothesis has been satisfied as they suggest the presence of a functionally compromised system with alterations in neurotransmission as well as neuronal viability and vulnerability. The results also demonstrate that the ability to regulate NMDAR1 mRNA is maintained and that the effects are regionally specific, not global as evidenced by results from the CA3 and CA1 hippocampal fields. The effects of stress on NMDA receptor subunit expression are not well understood, however, similarities to control indicate that the response is not specific to the lesioned system. Although the regulation is similar to control, it can not be concluded that the lesioned system demonstrates identical susceptibility to stress induced damage.

4.5 Apoptosis in the Prefrontal Cortex

TUNEL, in combination with bisbenzamide staining and Fluoro-Jade B staining, indicates that there is increased apoptosis in animals with neonatal lesions of the ventral hippocampus. In the present study, stress did not appear to result in increased apoptosis in sham animals as was previously reported (Liu *et al.*, 1996), however, the stress in this study was not as severe as that used by these authors. In addition, relatively high levels of baseline apoptosis were demonstrated in lesioned and sham animals in the absence of stress. This may have masked any effect of stress in sham lesioned animals. The inclusion of unoperated controls may have served to establish a lower baseline as would be expected under control conditions. It is possible that sham lesioned animals did exhibit minor damage not readily detectable in the histological assessment which subsequently resulted in increased susceptibility to cell death. As stated, there was a significant increase in overall levels of apoptosis in lesioned animals and this level was not significantly affected by stress. One can conclude that

increased apoptosis does occur in a functionally compromised system and may serve to further reduce the functionality of the system. In addition, increased apoptosis was not evident in lesioned animals exposed to acute swim stress ($p=.08$), but one cannot definitively conclude that alternate stress paradigms or chronic stress would not result in increased cell death. It is also possible that long term cumulative effects of stress may result in increased apoptosis and this effect may be more relevant to the proposed slow progression of schizophrenia (Fenton & McGlashan, 1994, Illowsky *et al.*, 1988, Lieberman *et al.*, 1992, Loebel *et al.*, 1992, Marsh *et al.*, 1994, Wyatt, 1991). If extrapolations can be drawn, marginal increases in apoptosis would be difficult to detect and in addition would not result in dramatic and relatively rapid loss of function as is evident in characteristic neurodegenerative diseases such as Alzheimer's and Huntington's (Lieberman, 1999). Relatively small increases in neuronal loss in combination with human neuron number variability may also account for the inability to detect overall changes in neuron number in the cortex of schizophrenic patients (Pakkenberg, 1993).

In addition to supporting a progression of the course of schizophrenia, the demonstration of increased apoptosis in animals with neonatal ibotenic acid lesions also has relevance to gender differences in schizophrenia. As previously stated, women show a later peak onset time of schizophrenia than men. In addition, women show a second peak following the onset of menopause when estrogen levels are known to decrease (Häfner *et al.*, 1993; Hambrecht *et al.*, 1992). Symptom severity is also known to fluctuate with estrogen levels as evidenced by premenstrual exacerbation of symptoms, and symptom improvement during pregnancy (Chang and Renshaw, 1986, Gerada and Reveley, 1988, for review see Seeman, 1996). The demonstration that estrogen is neuroprotective (Behl *et al.*, 1995, Singer *et al.*, 1996, Toung *et al.*, 1998) and that increased estrogen improves the symptoms of schizophrenia supports a role for neurodegeneration in this disorder. The demonstration of

increased apoptosis also supports a role for neurodegeneration in schizophrenia. As only male rats were used in the model, it would be of interest to determine if female rats demonstrate comparable results following neonatal ibotenic acid lesions of the hippocampus and if ovariectomy would influence susceptibility.

The demonstration of increased apoptosis is in accordance with the demonstration of reduced BDNF mRNA and reduced NMDAR1 mRNA. Many other factors also play important roles in apoptosis and it can not be conclusively stated that increases in this model are solely due to BDNF and/or NMDAR1 alterations. The conclusion can be drawn, however, that the neonatal lesion does produce a functionally compromised system that demonstrates alterations in neuron viability.

5. CONCLUSIONS

The present results support the hypothesis of a neurodevelopmentally-compromised system resulting in significant consequences in adulthood, including alterations in neuronal viability and neuronal communication. Based on the investigation of BDNF, NMDAR1 and apoptosis, the results all suggest the presence of a functionally compromised system. In addition, BDNF and NMDAR1 mRNA results suggest that the system is more susceptible to damage induced by stressors. As discussed above, the demonstration of reduced BDNF mRNA and NMDAR1 mRNA parallel previous reports in schizophrenic patients. These factors in association with increased apoptosis have significant functional consequences that can be related to the symptoms present in schizophrenia. It is known that the prefrontal cortex plays an important role in the organization of behavior as well as cognitive functions and adaptive processes (Kolb, 1984, Rosenkilde, 1983). The negative symptoms expressed in schizophrenia are proposed to be deficits in these prefrontal functions and a tentative extrapolation from the present results suggests that neurodegenerative factors may play a role in their emergence.

In the model, Lipska *et al.* (1993) suggested that secondary neurodegenerative changes could result in the postpubertal emergence of behavioral abnormalities. The present results suggest that neurodegenerative changes do exist following neonatal ibotenic acid lesions. The question not answered by this investigation was whether increased apoptosis is evident early in post lesion development. To account for the emergence of abnormal behaviors, and to reach the hypothesized threshold of dysfunction for emergence, the neurodegenerative changes would necessarily begin early in

post-lesion development. The same situation holds true for alterations in BDNF and NMDAR1 mRNA expression. It remains undetermined whether they are altered due to changes in adulthood or whether they are a direct result of the lesion and occur early in post-lesion development. The question of whether the changes demonstrated in the present study are causative or resultant needs to be addressed. Regardless of the answer, it appears that these alterations result in significant functional consequences. If accurate parallels to schizophrenia can be drawn, the results indicate the importance of early intervention in treatment of schizophrenia to prevent or slow the disease progression. A second important consideration derived from this study is the mechanism of action of pharmacological agents used in the treatment of schizophrenia. While symptoms can be partially controlled with current neuroleptics, improvement of patient prognosis could occur through the use of drugs specifically targeted to block cell death or to enhance neuronal viability.

Questions that remain to be answered from this research project include whether protein changes parallel the changes in mRNA expression of BDNF and NMDAR1 and what is the specific time course of events leading to these changes. Investigation of the BDNF receptor (TrkB) to determine the extent of alterations in neurotrophic support would also be of interest. In addition, the investigation of apoptosis related genes may provide further insight into the pathophysiology of schizophrenia. With respect to early treatment intervention in schizophrenia, it would be of interest to determine whether neuroleptics or current neuroprotective agents could prevent the emergence of behavioral and biochemical changes seen in the model.

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