CHARACTERIZATION OF DRUG- AND CONVULSION-NAIVE WITHDRAWAL SEIZURE PRONE AND WITHDRAWAL SEIZURE RESISTANT MICE USING ULTRASTRUCTURAL IMMUNOCYTOCHEMISTRY

by

Jennifer F. Buckman

A THESIS

Presented to the Department of Behavioral Neuroscience

and the Oregon Health Sciences University

School of Medicine

in partial fulfillment of

the requirements for the degree of

Master of Sciences

July 1996

APPROVED [redacted]

.....

(Professor in Charge of Thesis)

[redacted]

.....

(Associate Dean for Graduate Studies)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

This study was supported by the Department of Veterans Affairs Merit Review Program through a grant to Charles Meshul, National Institute of Alcohol Abuse and Alcoholism training grant T32AA07468 and a Tartar Trust fellowship to Jennifer F. Buckman.

A special thanks to Cindy Allen in the Electron Microscopy Facility for her invaluable assistance.

ABSTRACT

Withdrawal Seizure Prone (WSP) and Withdrawal Seizure Resistant (WSR) mice have been selectively bred for differential ethanol withdrawal handling induced convulsions (HICs). In addition, it has been observed that WSP mice exhibit drug-naive HICs. This latter finding suggests that WSP and WSR mice differ in their susceptibility to HICs. Alterations in the glutamatergic and GABAergic systems have been implicated in convulsive activity. These same neurotransmitter systems have been proposed to underlie the manifestation of ethanol withdrawal symptoms. It is therefore possible that WSP and WSR mice are genetically different in respect to their glutamatergic and/or GABA-ergic systems. To test this hypothesis, we have analyzed WSP and WSR mice that are both drug- and HIC-naive for differences in the density of glutamate and GABA immunoreactivity within the CA1 subfield of the hippocampus (CA1) and layer II of the somatosensory cortex (SSC). The major finding of this study is that drug- and HIC-naive WSP mice exhibit a significantly greater density of nerve terminal glutamate immunoreactivity in asymmetric synapses within the CA1 , but not in layer II of the SSC, when compared to WSR mice. The density of nerve terminal GABA immunoreactivity associated with symmetric synapses does not appear to differ between the selected lines in either brain region. Since prior drug exposure and HICs cannot account for the observed differences in these naive mice, it is suggested that the density of nerve terminal glutamate immunoreactivity within the CA1 is a reflection of inherent genetic differences between WSP and WSR mice. Furthermore, an elevated density of nerve terminal glutamate immunoreactivity may be an underlying neurochemical correlate to increased susceptibility to both drug-naive and ethanol withdrawal convulsions.

INTRODUCTION

Overall Aim:

To identify differences in the density of nerve terminal glutamate and GABA immunolabelling associated with drug- and convulsion-naive Withdrawal Seizure Prone (WSP) and Withdrawal Seizure Resistant (WSR) mice.

Selective breeding of WSP and WSR mice:

Withdrawal Seizure Prone (WSP) and Withdrawal Seizure Resistant (WSR) mice have been selectively bred for differential ethanol withdrawal susceptibility as measured by handling-induced convulsions (HICs). The breeding of these lines of mice (two replicate lines of both WSP and WSR mice) was initiated for use as a potential model for ethanol dependence. After being exposed to ethanol vapor for 72 hours, the mice were withdrawn and scored for HIC severity (see Table 1). Those mice that displayed the highest HIC scores were bred together to develop the WSP lines (WSP-1 and WSP-2) and those that displayed the lowest HIC scores were bred together for the WSR lines (WSR-1 and WSR-2) (Crabbe et al., 1985).

Selective breeding is used to develop lines of animals (i.e. the WSP and WSR lines) that markedly differ in a phenotypic response (i.e. sensitivity to HICs during ethanol withdrawal) and that genotypically differ at loci relevant to this phenotype. Frequently, other behavioral and neuro-chemical phenotypes co-segregate during selection and these traits are believed to be correlated to the same set (or a subset) of genes as the selection phenotype (Phillips et al., 1989; Crabbe et al., 1991b). Analysis of these correlated

Table 1: Scale for measuring severity of handling-induced convulsions (HICs)* modified from Crabbe et al., 1991b.

SCORE	SYMPTOM
0	No convulsion
1	Facial grimace only when lifted and spun
2	Tonic convulsion when lifted and spun
3	Tonic-clonic convulsion when lifted and spun
4	Tonic convulsion when lifted (no spin required)
5	Tonic-clonic convulsion when lifted
6	Severe tonic-clonic convulsion when lifted
7	Spontaneous tonic-clonic convulsion (prior to being lifted)

* HICs are elicited by gently picking a mouse up by the tail and lifting all four legs off the ground. If the mouse does not exhibit signs of a HIC, it is spun 180 degrees while being suspended in the air.

phenotypes can offer insight into the genes influenced by the selective breeding protocol.

Ethanol withdrawal in WSP and WSR mice:

Distinguishing these lines based upon ethanol withdrawal symptoms has a strong foundation in clinical research where it has been repeatedly shown that following chronic, long-term alcohol abuse, withdrawal from alcohol may be severe, possibly even resulting in death (Ballenger and Post, 1978). Delirium tremens and grand mal (generalized tonic-clonic) seizures have often been associated with long-term alcoholism (Freedland and McMicken, 1993; Ulrichsen et al., 1992) and have been shown to correlate strongly with the number of previous withdrawal episodes experienced by the alcohol abuser (Ballenger and Post, 1978; Lechtenberg and Worner, 1991). An increase in the severity of withdrawal symptoms, such as convulsions, has also been shown in animals repeatedly intoxicated and withdrawn from ethanol (Clemmesen and Hemmingsen, 1984; Ulrichsen et al., 1992; Becker, 1994).

There have been numerous reports establishing differences in ethanol withdrawal HICs in the WSP and WSR mouse lines (Crabbe et al., 1983, Crabbe et al., 1985). In addition, it has been reported that the WSP lines are also more prone to severe HICs induced by administration of or withdrawal from several drugs (see Table 2). The convulsant properties of these drugs have been studied using a variety of administration routes and doses (given either acutely or chronically). Each of these drugs is believed to act by altering the levels of excitation in the brain. Regardless of the drug's site of action (i.e.

Table 2: Correlated Phenotypes: drug exposures, other than chronic ethanol, that exacerbate HICs in WSP mice.

Drugs that lead to an increase in HIC severity in drug-naive WSP mice at subconvulsant doses	**References**
t-butylcyclophophorothionate (TBPS)	Crabbe et al., 1991a
pentylentetrazole (PTZ)	Feller et al., 1988; Crabbe et al., 1991a
strychnine	Crabbe et al., 1991a
nicotine	Crabbe et al., 1991a
N-methyl d-aspartate (NMDA)	Crabbe et al., 1991a
kainic acid	Crabbe et al., 1991a
picrotoxin	Feller et al., 1988; Crabbe et al., 1991a
bicuculline	Feller et al., 1988; Crabbe et al., 1991a
Ro 15-4513	Crabbe et al., 1991a
3-mercaptoproprionic acid (3-MPA)	Crabbe et al., 1991a
BayK 8644	Crabbe et al., 1991a

Drugs that lead to an increase in HIC severity in WSP mice during withdrawal following administration	**References**
acute ethanol	Kosobud and Crabbe, 1986; Crabbe et al., 1993
nitrous oxide	Belknap et al., 1987
diazepam	Belknap et al., 1989
acute acetylaldehyde	Crabbe et al., 1991b
acute tertiary butanol	Crabbe et al., 1991b
barbiturates	Belknap et al., 1988; Crabbe et al., 1991b

All drugs listed have significantly more influence on the WSP lines as compared to the WSR lines.

which neurotransmitter system it acts upon), WSP mice, as compared to WSR mice, have consistently higher HIC scores. Moreover, WSP mice frequently exhibit HICs in their drug-naive state whereas WSR mice do not (Crabbe et al., 1983; Kosobud and Crabbe, 1986). Therefore, it is possible that WSP and WSR mice inherently differ in their degree of excitatory (increased) and/or inhibitory (decreased) synaptic transmission (Crabbe et al., 1994a).

Convulsive activity in WSP and WSR mice:

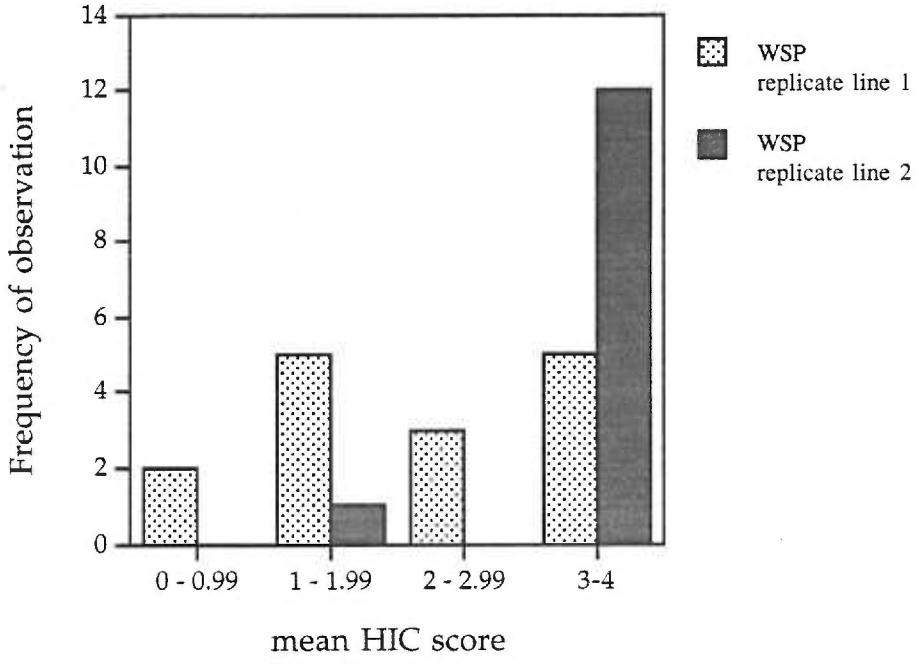
Handling-induced convulsive activity in WSP and WSR mice represents a sensitive behavioral measure that is thought to reflect the degree of CNS excitability (Crabbe et al., 1990a). Preliminary investigation of basal HICs in drug-naive WSP mice in this laboratory has revealed that these drug-naive mice do exhibit HICs and these HICs can be elicited simply by picking the mice up by the tail (see Figure 1, Table 1). This observation suggests that WSP mice may convulse during routine cage changing and when handled at the onset of experiments. Cage changing within 24 hours of testing in a place conditioning paradigm has been shown to decrease significantly the effects of an opioid antagonist (S. Dickinson, personal communication). In addition, drug-naive HICs have been observed to decrease mossy fiber zinc levels in WSP mice (D. Feller, personal communication). Therefore, routine animal handling, both prior to and during experiments, may also influence the activity of the glutamatergic and GABAergic systems.

History of seizure research:

In 1880, Sommer analyzed post-mortem brains and found a correlation

Figure 1 : Mean HIC scores of drug-naive WSP mice

Drug-naive WSP mice (28 male mice, approximately 60 days old) that had been specially handled (see Methods) were scored for severity of HICs based on a scale modified from Crabbe et al., 1991b (see Table 1). Two replicate lines of WSP mice were used in this analysis. Mice were scored ten times over a period of 90 minutes (handling occurred every ten minutes). The ten individual HIC scores were combined and the mean HIC score for each animal was calculated. Data are presented as the number of animals exhibiting a given mean HIC score.


14
12
10
8
6
4
2
0
Frequency of observation
0 - 0.99
1 - 1.99
2 - 2.99
3-4
mean HIC score
WSP
replicate line 1
WSP
replicate line 2

between hippocampal atrophy and epilepsy. Based on this finding, he concluded that hippocampal atrophy led to epilepsy. Spielmeyer (1927) and Scholz (1933), on the other hand, suggested that the opposite was true: that epilepsy led to hippocampal damage. This latter claim was supported by findings of hippocampal sensitivity to ischemia during seizures (Spielmeyer, 1927). It has been an ongoing endeavor to discover the direction of causality between seizures and neurological dysfunction.

One of the problems in directly addressing this issue is that there is a lack of screening tests for epilepsy. Diagnosis requires a detailed clinical evaluation of the patient and is only possible subsequent to a paroxysmal event (Annegers, 1994; Chadwick, 1994). In addition, it has been suggested that factors that predispose an individual to seizure activity may be distinct from factors that trigger a seizure (Anderson et al., 1986). Recent advances in the development of animal models offer researchers the ability to determine the direction of causality and the effects of multiple etiological factors. The selective breeding protocol used to create the WSP and WSR lines has resulted in mice that differ in their susceptibility to convulsions but have been bred from the same stock. Since WSP mice are susceptible to convulsions, investigation of these mice before they have convulsed will be useful in determining differences that make an animal at risk for a convulsion.

Inheritance of seizure-related disorders:

Genetic transmission of epilepsy has been well established with twin and family studies. It has been estimated that at least 20% of all epilepsies have a genetic etiology (McNamara, 1992). Unfortunately, due to the

heterogeneity of seizure-related disorders, it has been difficult to determine the exact nature of this genetic influence. While genes responsible for some types of seizures (i.e. benign familial neonatal convulsions (Leppert et al., 1989) have been mapped to specific chromosomal regions, it is likely that most seizure activity is polygenic in nature (Annegers, 1994).

A genetic basis for alcohol-related seizures has also been suggested. Thus far, research has been unsuccessful in correlating the likelihood of alcohol-induced seizures with alcohol related behaviors (such as the length of alcohol abuse or quantity of alcohol consumed) (Schaumann et al., 1994). The presence of alcohol-related seizures, though, has most commonly been associated with alcoholism (Freedland and McMicken, 1993) and susceptibility to alcoholism has been suggested to be partially genetically determined (Crabbe et al., 1983).

The role of glutamate in alcohol withdrawal and seizure activity:

The neurotoxic actions of glutamate, known as excitotoxicity, were first proposed by Olney et al. (1971, 1974) and are now well documented. Excitotoxicity is the result of excessive excitation due to an abnormal glutamatergic response that leads to an inability of a cell to maintain ionic homeostasis (Choi, 1987; Rothman and Olney, 1987; Siesjo and Bengtsson, 1989). Convulsive activity is commonly associated with excitotoxicity (Olney et al., 1974). Although it has been hypothesized that the NMDA receptor-associated calcium channel is of particular importance in the neuronal damage and death resulting from excitotoxicity (Choi, 1987; Siesjo and Bengtsson, 1989), the events that lead to hyperactivation of the glutamatergic system are

unknown. It is possible that susceptibility to excitotoxicity is associated with excess glutamate synthesis, release, uptake and/or receptor functioning. Furthermore, although it is unclear whether the glutamatergic dysfunction associated with excitotoxicity is of genetic or environmental origin, it is likely that both are involved.

Ethanol is a potent inhibitor of glutamatergic functioning and may act by blocking calcium influx (Lovinger et al., 1990; Hoffman et al., 1992). It has been suggested that during chronic ethanol exposure, compensatory changes lead to an up-regulation of glutamate receptors or NMDA-associated ion channels or an alteration in binding affinities or ionic sensitivities (Lancaster, 1992). During withdrawal, this compensatory modification has been hypothesized to lead to a hypersensitivity of the glutamatergic system that may be exhibited behaviorally as withdrawal symptoms such as seizures (Lancaster, 1992; Hoffman et al., 1992). Alcohol use is commonly associated with seizure activity and is considered one of the predominant causes of provoked seizures (i.e. with an identified environmental origin) (Freedland and McMicken, 1993; Chadwick, 1994). Excitotoxicity has been proposed to underlie the generation and/or the expression of withdrawal seizures (Lancaster, 1992; Lovinger, 1993).

Stress, on the other hand, acts indirectly to influence the glutamatergic system. The mechanisms by which stress can interfere with normal glutamatergic functioning have been reported (Sapolsky, 1993a, b). Briefly stated, it has been suggested that stress increases the concentration of circulating glucocorticoids (adrenal steroid hormones). Glucocorticoids have been proposed to exert a direct excitatory effect by actions at a subtype of intracellular steroid

receptors in the hippocampus and an indirect excitatory effect by impairing glucose transport into cells (Armanini et al., 1990). By interfering with cellular glucose utilization, glucocorticoids may alter the functioning of the glutamatergic system which is dependent on energy for proper transporter and receptor activity (Armanini et al., 1990). Thus, a stress-induced increase in glucocorticoids may decrease the availability of glucose at glutamatergic synapses thereby increasing excitatory activity both pre- and postsynaptically.

There are numerous accounts of increased plasma glucocorticoid levels associated with increased convulsion susceptibility. This observation has been made in humans and animals, in their drug-naive states as well as during drug withdrawal. Temkin and Davis (1984) reported a correlation between seizure frequency and subjectively-measured stress levels in human epileptics. In addition, Roberts et al. (1991, 1993) examined the hypothalamic-pituitary-adrenal axis in WSP and WSR mice and found evidence for a differential sensitivity to corticosterone (the principal glucocorticoid in rodents) between the selected lines. Acute and chronic corticosterone applications dose-dependently increased the severity of ethanol withdrawal HICs in WSP mice, but not in WSR mice (Roberts et al, 1993). Thus, since altering the concentration or activity of glucocorticoids endangers neurons, the differential sensitivity of WSP and WSR mice to HICs may represent a behavioral manifestation of a differential susceptibility to excitotoxicity.

Although it seems evident from the breeding protocol used to create the WSP and WSR mouse lines that genetic differences exist between the lines, the nature of these differences is unknown. There is strong evidence supporting the involvement of glutamate in convulsive activity (Olney et al.,

1974; Lallement et al., 1991; Carlson et al., 1992), but previous biochemical analysis of glutamatergic functioning in WSP and WSR mice has yielded conflicting results (Valverius et al., 1990; Carter et al., 1995). It is possible that the reported discrepancies are the result of confounding factors, such as basal, drug-naive HICs in WSP mice. Therefore, in this study WSP and WSR mice have been examined prior to both convulsive activity and drug exposure.

It is also possible that the contradictory reports result from the utilization of techniques that are not sufficiently sensitive to detect differences in synaptic glutamatergic activity. Both Valverius et al. (1990) and Carter et al. (1995) focus on changes occurring postsynaptically with the NMDA receptor, but excessive release of glutamate, due to an increased pre-synaptic accumulation, could lead to repetitive, and potentially toxic, post-synaptic activation (Sloviter, 1983). Ultrastructural immunocytochemistry allows quantification of presynaptic neurotransmitter densities. It may offer insight into whether differences exist in the density of nerve terminal glutamate immunolabelling in drug- and convulsion-naive WSP and WSR mice. It is plausible that WSP mice will exhibit elevated densities of glutamate immunoreactivity, as compared to the WSR mice. This finding would suggest that genetic alterations in the glutamate system result in WSP mice being more prone to an excitotoxic event.

The role of GABA in alcohol withdrawal and seizure activity:

Normal activity within the CNS is dependent on a precise balance of inhibitory and excitatory influences (Tabakoff and Hoffman, 1993). GABA is commonly acknowledged as the major inhibitory neurotransmitter and

much like glutamate, GABA has been implicated in the underlying pathologies associated with a variety of CNS disorders (see Table 3).

It has been hypothesized that both ethanol withdrawal symptoms (Crabbe et al., 1994a) and excitotoxicity (Lovinger, 1993) result from an imbalance in the levels of excitation and inhibition in the brain. The suggestion has been made that a decrease in GABAergic functioning, as opposed to or in conjunction with an increase in glutamatergic activity, is of critical importance (Ribak et al., 1982; Kamphius et al., 1987; Keir and Morrow, 1994). Researchers have repeatedly attempted to address the degree and nature of the GABAergic involvement in symptoms associated with ethanol dependence and withdrawal but the data, thus far, have been inconsistent (for review see Buck and Harris, 1991). The role of the GABAergic system in seizure activity is also unclear (for review see Glass and Dragunow, 1995).

With chronic ethanol exposure, GABA-mediated inhibitory neurotransmission appears enhanced, but desensitization of the $GABA_A$ receptor complex to such potentiation may develop (Glue and Nutt, 1990; Buck and Harris, 1991). Upon withdrawal, subsensitivity of $GABA_A$ receptors may lead to a decrease in GABAergic activity, which may result in a reduced inhibition of NMDA-mediated excitation (Glue and Nutt, 1990; Hoffman et al., 1992; Lovinger, 1993). This shift in excitatory and inhibitory activity may be responsible for withdrawal symptoms such as seizures (Glue and Nutt, 1990).

In a review of animal models of epilepsy, Horton (1991) examined evidence for GABA involvement in predisposing, precipitating and propagating seizure activity. Analysis of presynaptic GABA markers in inductive models of epilepsy (i.e. by pharmacological or electrical induction), using light

Table 3: Clinical and experimentally-induced neurological diseases and disorders associated with abnormalities in glutamatergic and/or GABAergic activity

Diseases and Disorders	References supporting glutamatergic involvement	References supporting GABAergic involvement
initiation & propagation of abnormal synchronous discharges	Choi, 1988*; Dingledine et al., 1990*	Horton, 1991*
seizure- and convulsion-induced brain damage	Olney et al., 1974; Rothman & Olney, 1987*; Choi, 1988*; Dingledine et al., 1990*; Lallement et al., 1991; Carlson et al., 1992	Ribak et al., 1982*; Kamphius et al., 1986, 1987, 1989; Horton, 1991*; Araki et al., 1994 ; Glass & Dragunow, 1995*; Rowley et al., 1995
Huntington's disease	Rothman & Olney, 1987*; Choi, 1988*; Meldrum & Garthwaite, 1990*	Kandel et al., 1991*
Guam amyotrophic lateral sclerosis/Parkinsonism-dementia	Choi, 1988*; Meldrum & Garthwaite, 1990*	
alcohol-related disorders	Glue & Nutt, 1990*; Lancaster, 1992*,;Lovinger, 1993*	Glue & Nutt, 1990*; Freedland & McMicken, 1993*; Lovinger, 1993*
Alzheimer's disease	Choi, 1988*; Meldrum & Garthwaite, 1990*	
hypoxia	Rothman & Olney, 1986*; Choi, 1988*; 1990*; Choi & Rothman, 1990*	
hypoglycemia	Rothman & Olney, 1987*; Choi, 1988**	
neuronal damage associated with insulin overdose	Choi,1988*	
neuronal damage associated with CNS trauma	Choi,1988*	
ischemia	Rothman & Olney, 1986*; Choi & Rothman, 1990*; Meldrum & Garthwaite, 1990*; Lekieffre et al., 1992	Lekieffre et al., 1992

* review articles

level microscopy, showed a decrease in presynaptic GABA marker levels preceding convulsive activity (Horton, 1991). Genetic animal models of epilepsy (i.e. Genetically Epilepsy Prone Rats), though, show evidence for an elevation in presynaptic GABA markers (Roberts et al., 1985a, b; Horton, 1991). Thus the role of GABA in inherited convulsion susceptibility remains in question.

Additional evidence for the involvement of GABA in both epileptic and withdrawal seizures comes from clinical research. $GABA_A$ receptor agonists have been reported to decrease withdrawal symptom severity (Ballenger and Post, 1978; Glue and Nutt, 1990) and are frequently used as treatment for epilepsy (Chadwick, 1994).

The GABAergic system in ethanol-naive WSP and WSR mice has been examined. No significant line differences in the GABA receptor or its associated chloride channel have been found (Feller et al., 1988; Buck et al., 1991b; also see review Phillips et al., 1989). No differences in binding densities or binding affinities of [^{35}S]TBPS (a cage convulsant believed to act at the picrotoxin binding site) were detected in the cortices, cerebella or hippocampi of ethanol-naive WSP and WSR mice (Feller et al., 1988). In addition, no differences between the WSP and WSR mice were reported in benzodiazepine receptor binding affinity when evaluated with [^{3}H]flunitrazepam (Feller et al., 1988). Interestingly, though, the ethanol-naive WSP mice exhibited lower levels of the α3 and α6 $GABA_A$ receptor subunit mRNA in whole brain (Buck et al., 1991a) and the α1, α6 and ß2 $GABA_A$ receptor subunit mRNA in the cerebellum, but not in the cortex, when compared to ethanol-naive WSR mice (Keir and Morrow, 1994).

These dendrites receive synaptic inputs from commissural fibers and from pyramidal cells in the CA3 which are typically responsible for spontaneous interictal bursts associated with epilepsy (Dingledine et al., 1990). It has been repeatedly shown that seizure activity leads to morphological changes in this limbic region (Olney et al., 1983; Sloviter, 1983; Cavazos et al., 1994). Ethanol also influences the morphology of the hippocampus, especially the CA1 subfield (McMullen et al., 1984; King et al., 1988). For these reasons, the CA1 subfield of the hippocampus in the WSP and WSR mice has been examined for inherent nerve terminal glutamate and GABA immunocytochemical differences.

The somatosensory cortex, on the other hand, appears to be relatively resistant to the effects of excitotoxicity. Animals subjected to amygdalar or olfactory bulb kindling exhibit a typical pattern of morphological change in the limbic system, with the decrease in neuronal density becoming more prominent as the number of kindled class V seizures increased (Cavazos et al., 1994). The somatosensory cortex, though, when examined morphologically at the light level, showed no alterations even following 150 kindled class V seizures (Cavazos et al., 1994). In addition, it has been established that seizure activity spreads through specific anatomical pathways (Goddard, 1983) to which the somatosensory cortex does not appear to be connected. Therefore, the somatosensory cortex will act as a control region in the current study and should show no immunocytochemical alterations since seizure disposition is associated with a specific pattern of selective vulnerability.

Rationale:

The overall aim of this thesis is to identify differences in the density of nerve terminal glutamate and GABA immunolabelling within asymmetric and symmetric synapses of the CA1 subfield of the hippocampus and layer II of the somatosensory cortex associated with drug- and convulsion-naive WSP and WSR mice. WSP and WSR mice have been bred to differentially respond to ethanol, as observed by their proneness or resistance to HICs during withdrawal (Crabbe et al., 1985). Such divergence in the phenotypic response suggests a strong genetic influence (Phillips et al, 1989).

It is often observed that other phenotypes, in addition to that for which the mice were selected, markedly differ following selection; this is known as genetic correlation (Crabbe et al., 1990b). Such correlations suggest that two traits found to co-segregate during selection are likely to act through a single mechanism (Crabbe et al., 1990b). One genetic correlate observable in WSP, but not in WSR mice, is a predisposition to drug-naive HICs. It is postulated that excitotoxicity is one mechanism that could result in both sensitivity to alcohol withdrawal symptoms and drug-naive convulsions.

Glutamate has been implicated in excitotoxicity (Olney et al., 1971,1974) and may underlie convulsions and ethanol withdrawal symptoms (see Table 3). Therefore, **the first aim of this thesis is to determine if differences in presynaptic glutamate immunoreactivity, as an index of glutamate content or concentration, exist between drug- and convulsion-naive WSP and WSR mice.** A role for GABA in excitotoxicity has also been suggested (Lovinger, 1993). Moreover, studies have suggested a GABAergic involvement in convulsions and alcohol withdrawal symptoms (see Table 3). Therefore, **the**

second aim of this thesis is to determine if differences in presynaptic GABA immunoreactivity, as an index of GABA content or concentration, exist between drug- and convulsion-naive WSP and WSR mice.

Intracellular glutamate and GABA concentrations can be assessed quantitatively using a technique that combines electron microscopy and immunocytochemistry (Ottersen et al., 1990; Phend et al., 1992; Meshul et al., 1994). This sensitive technique can be used to evaluate the neurochemistry at the synaptic level. In addition, by preventing the induction of basal, drug-naive HICs (see Methods), the genetic contribution to susceptibility for convulsion induced by handling and exacerbated by a variety of drugs can be more fully explored.

MATERIALS AND METHODS

Subjects:

Breeding pairs from replicate lines of Withdrawal Seizure Prone (WSP) and Withdrawal Seizure Resistant (WSR) mice from generation $S_{26}G_{57\text{-}58}$ were obtained from the Portland VA Medical Center (courtesy of Dr. John Crabbe). These mice were bred within each line and, 21 days after birth, male offspring were separated for use in the present experiments. The experiments were performed when the male WSP-1, WSP-2, WSR-1, WSR-2 mice were between 60 and 70 days old. All animals were given free access to standard lab chow and water and maintained on a 12:12 light-dark cycle. In addition, they were housed in groups of five and handled once per week for cage changing only. Handling was carried out exclusively by the experimenter.

Special handling procedures were implemented to ensure that no convulsive activity was induced by handling prior to the initiation of the experiments. The handling procedure was as follows: each mouse was gently scooped into a small cup, transferred and gently poured out of the cup onto fresh bedding. This technique allowed the mice to be moved between cages without removing their hindlimbs from the ground, which, in WSP mice, has been shown to induce convulsions. This convulsive activity may influence neurochemistry (D. Feller, personal communication).

Electron Microscopy:

Mice were overdosed with an anesthetic containing 5% ketamine, 2% xylazine and 1% acepromazine at a dose of 0.1 ml/kg. Once deeply

anesthetized (as determined by an animal's failure to respond to pinching with forceps), the chest cavity was opened and transcardial perfusion began immediately. Perfusions were carried out using a LKB Bromma 2115 Multoperpex perfusion pump at a flow rate of about 20 ml/min (H.J. Guldener, Zurich, Switzerland). Each animal was perfused with approximately 100 ml of fixative containing 2.5% glutaraldehyde, 0.5% paraformaldehyde, 0.1% picric acid in 0.1M HEPES, pH 7.3. Following fixation, whole brains were harvested and placed in cold fixative overnight.

Twenty to twenty-two hours after fixation, the CA1 subfield of the dorsal posterior hippocampus (CA1) and the most lateral region of the somatosensory cortex (SSC) were removed (Slotnick and Leonard, 1975). The dissected regions were then washed several times in 0.1M HEPES, pH 7.3 and incubated in 1% osmium tetroxide/1.5% potassium ferricyanide for one hour at room temperature. The samples were then washed in deionized water and stained *en block* with aqueous 0.5% uranyl acetate for 30 minutes at room temperature. The tissue was then dehydrated in ethanol, cleared in propylene oxide and embedded in Epon/Spurrs for 24 hours at 60°C. Thick sections (0.50 μm) were cut and stained with toluidine blue/azure II and viewed with the light microscope to ensure proper orientation and preservation. Poorly preserved tissue was discarded. Tissue was then thin sectioned (800 angstroms) and processed for immunocytochemistry as detailed below.

Sections were viewed and photographed using a JOEL 1200 EX TEMSCAN electron microscope. Ten photographs per grid were taken randomly in the neuropil following identification of the proper area. The

CA1 was identified by the organized layer of pyramidal cells with prominent apical and basal dendrites characteristic of this area. Photographs were taken of the neuropil in the region of the apical dendrites within 100 μm of the CA1 pyramidal cells. The molecular layer of the SSC was first identified and photographs were taken of the neuropil in the adjacent layer II, in the region surrounding the pyramidal cells. All photographing and analysis were done blind to the selection line.

The photomicrographs (initial magnification x25,000; final magnification x62,500) were analyzed for the density of immuno-gold labelling within nerve terminals. For glutamate immunocytochemistry, asymmetric synapses, presumed to be excitatory in nature (Ottersen et al., 1990) were analyzed. Asymmetric synapses were identified by an accumulation of synaptic vesicles within the presynaptic nerve terminal and a prominent postsynaptic density (PSD) (Becker, 1991). PSDs wider than the width of the synaptic cleft were considered associated with asymmetric synapses. For GABA labelling, symmetric synapses, presumed to be inhibitory by nature (Ottersen et al., 1990) were analyzed. Symmetric synapses were identified in the same manner as asymmetric ones, but with a PSD whose measurements did not extend beyond the width of the synaptic cleft. The area of each nerve terminal photographed was analyzed using a Zeiss Videoplan image analysis system.

Immunocytochemistry:

Post-embedding immuno-gold electron microscopy was carried out according to Phend et al. (1992), as modified by Meshul et al. (1994). Two separate immunocytochemical experiments (i.e. runs) were performed for

glutamate and GABA. The protocol for both experiments was the same except for the primary antibody used and the type of synapses analyzed (asymmetric versus symmetric). The basic procedure is as follows.

The thin sections (800-1000 angstroms) were cut on an RMC 6000 Ultramicrotome (RMC, Tucson, AZ), collected on 200 mesh nickel grids coated with a solution from a Coat-Quick "G" pen (Kiyota International; Elk Grove, IL) and air dried for at least three hours. Four grids containing thin sections were collected from each brain region (CA1 and SSC). Two of the grids were incubated with the glutamate antibody, while the other two were incubated with the GABA antibody.

The grids were washed in TRIS-buffered saline (0.05M TRIS with 0.9% NaCl) containing 0.1% Triton X-100 detergent, pH 7.6 (TBST7.6) and incubated in the primary antibody, either glutamate or GABA, overnight in a moist chamber at room temperature. The glutamate antibody (non-affinity purified, rabbit polyclonal; Arnel, Brooklyn, NY), previously characterized by Hepler et al. (1988), was diluted 1:50,000 in TBST7.6. Aspartate (1 mM) was added to the antibody solution to prevent cross-reactivity with aspartate. Specificity of glutamate immunoreactivity was established by incubating the antibody overnight with 3 mM glutamate according to Meshul et al. (1994). The GABA antibody (non-affinity purified, rabbit polyclonal; Arnel, Brooklyn, NY), previously characterized by Phend et al. (1992), was diluted 1:5000 in TBST7.6.

The following day, the samples were washed several times in TBST7.6 and TBST8.2 (TRIS-buffered saline containing 0.025% Triton X-100, pH 7.6 and 8.2) and incubated in goat anti-rabbit IgG (Amersham, Arlington Height, IL) conjugated to 10 nm gold (1:50 dilution in TBST8.2) for 90 minutes. The

samples were washed in TBST7.6 and deionized water, dried, counterstained with uranyl acetate and lead citrate (Meshul et al., 1994) and viewed and photographed on the electron microscope.

The number of 10 nm gold particles within a presynaptic terminal was counted and the area of the nerve terminal determined using a Zeiss Videoplan image analysis system. Counting did not include gold particles found within mitochondria since cross-reactivity with Kreb cycle intermediates may occur (Ottersen et al., 1990; Phend et al., 1992). For both glutamate and GABA, only synapses containing at least 5 gold particles were analyzed. This criteria ensured that only synapses that exhibited labelling above background were included in the analysis. Background immunolabelling density was defined as 10 gold particles/μm^2, based on labelling observed in glial processes (Meshul et al., 1994). Neurotransmitter densities were determined by dividing the number of 10 nm gold particles by the area of the presynaptic terminal (# particles/μm^2).

Statistical Analysis:

Two separate experiments were carried out. The first was designed to assess differences in the nerve terminal density of glutamate immunoreactivity (# of gold particles/μm^2) in the CA1 and layer II of the SSC of drug- and convulsion-naive WSP and WSR mice. The second was designed to examine differences in the nerve terminal density of GABA immunoreactivity in the CA1 and layer II of the SSC of the same WSP and WSR mice. Statistical analyses were performed separately for each experiment and the density of immunolabelling in the CA1 and layer II of the SSC were analyzed separately.

A variety of statistical procedures were implemented to assess group differences. First, it was necessary to obtain group means. Ten electron micrographs of synapses within the neuropil were taken from each brain region for each animal. These micrographs often contained more than one synapse that met the necessary criteria (i.e. for glutamate, criteria include an asymmetric postsynaptic density, an accumulation of synaptic vesicles near presynaptic active zone and at least 5 gold particles present in the nerve terminal). Approximately 30 asymmetric and 10 symmetric synapses were analyzed for each brain region from each animal. The number of immuno-gold particles, the area (in μm^2) and the density (# particles/μm^2) for each synapse was determined and the mean of each animal was recorded. A mean (of individual animal means) was then computed for each selected line (WSP-1, WSP-2, WSR-1, WSR-2) and brain region (CA1 and layer II of the SSC).

Due to the large number of tissue samples collected in these experiments, mice were randomly grouped into four immunocytochemical runs that were performed on four different days. Each immunocytochemical run was carried out in exactly the same manner, but significant differences in the density of immunolabelling between runs can arise due to unknown factors. It was therefore necessary to test for a potential confounding effect of run.

Ideally, a run effect should be analyzed separately for each line (both selected and replicate lines) and each brain region. The CA1 and layer II of the SSC were analyzed separately, but the extensive amount of time required for processing tissue samples resulted in a small number of animals (n=0-4) per line being tested on any given day. This prevented the otherwise appropriate

use of a three-way ANOVA (selected line x replicate line x run). In lieu of this analysis, we performed separate two-way ANOVAs (first collapsing over replicate line, then collapsing over selected line) in order to determine if there was a significant effect of run. This technique allowed us to determine whether the run differentially influenced the selected or replicate lines. ANOVAs that revealed statistical significance but no interactions suggested that run was a confounding factor. A correction factor was used to control for this run effect; these corrections are described in Appendix A. Briefly, the correction factor was calculated by determining the weighted mean immunolabelling density from individual means within each run ($\bar{x}_r$) and the grand weighted mean density of all individual means ($\bar{x}_g$). Each run mean was then subtracted from the grand mean ($\bar{x}_g - \bar{x}_r$) and the difference was added to each individual mean from that run.

After the data were corrected for run, it was necessary to determine if differences between selected and/or replicate lines existed. Using a two-way ANOVA, differences in mean nerve terminal area and nerve terminal immunolabelling densities in the CA1 and layer II of the SSC were compared between selected lines and replicate lines. If no significant differences were detected, replicate lines were combined. This is in accordance with previous publications (Crabbe et al., 1994a). 13 WSP and 11 WSR mice were used in each of the analyses. The number of nerve terminals analyzed from these animals for glutamate immunoreactivity were: 446 (WSP, CA1), 393 (WSR, CA1), 423 (WSP, SSC) and 325 (WSR, SSC). The number of nerve terminals analyzed for GABA immunoreactivity were: 135 (WSP, CA1), 105 (WSR, CA1), 157 (WSP, SSC) and 126 (WSR, SSC).

RESULTS

Glutamate immunoreactivity:

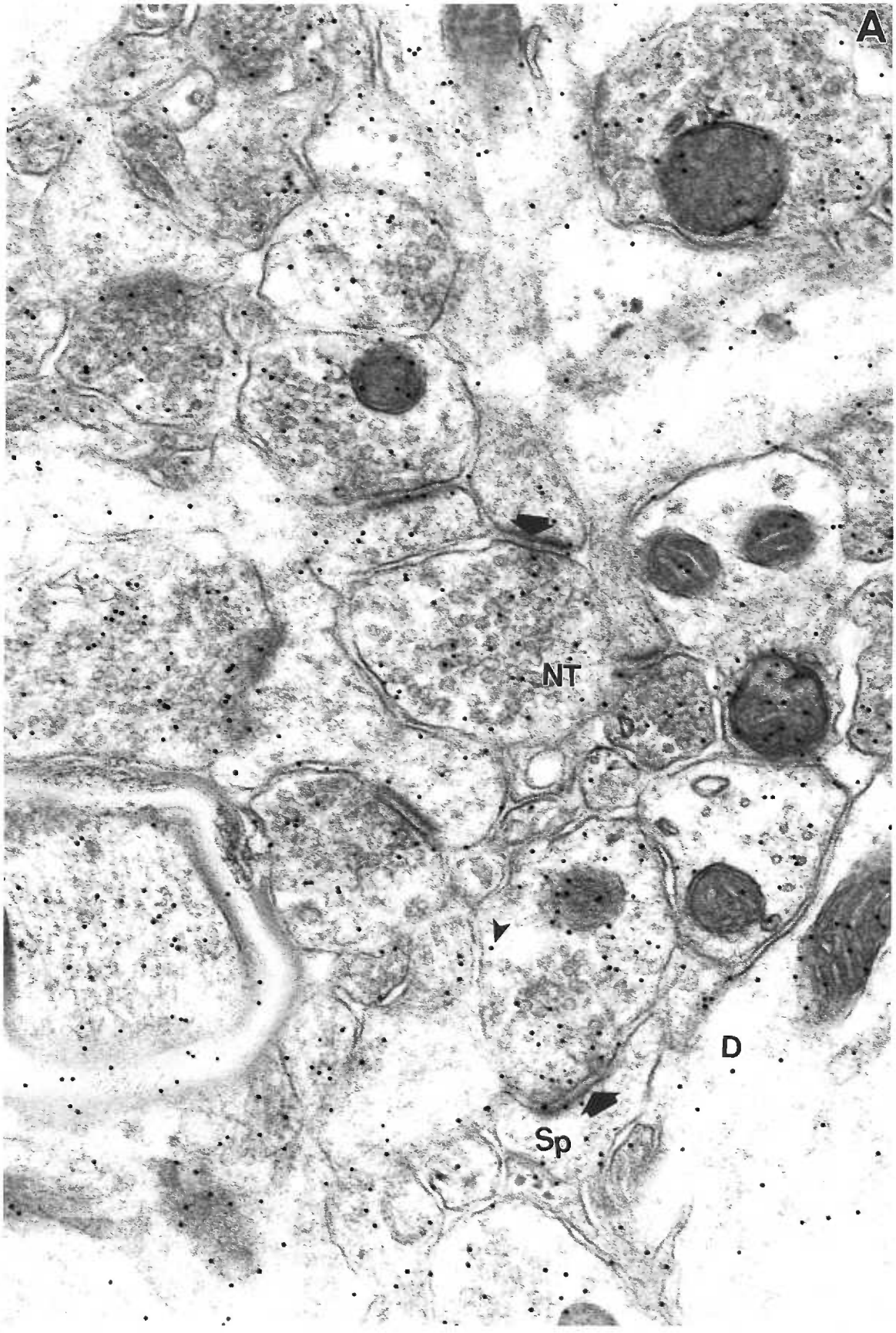
WSP (n=13) and WSR (n=11) mice were analyzed for differences in asymmetric nerve terminal area and the density of glutamate immunoreactivity. This experiment was carried out over four days (with four separate immunocytochemical runs), but statistical analyses revealed no significant effect of run (CA1: $F_{(3,20)} = 0.70$, $p = .5633$; SSC: $F_{(3,20)} = 2.493$, $p = .0894$). Therefore, data from all runs were combined.

Examples of glutamate immunolabelling from WSP and WSR mice are illustrated in Figures 2 (CA1) and 3 (layer II of the SSC). A two-way ANOVA (selected line x replicate line) revealed no significant differences in the density of glutamate immunoreactivity between the replicate lines (CA1: $F_{(1,20)} = 0.278$, $p = .6039$; SSC: $F_{(1,20)} = 1.045$, $p = .3189$) . In addition, no selected line by replicate line interaction was observed for either brain region. Therefore, data from replicate lines 1 and 2 were combined for both WSP mice and WSR mice. Significant differences between the selected lines were observed in the density of glutamate immunoreactivity in the CA1 ($F_{(1,20)} = 7.073$, $p = .0150$) (see Figure 4). Differences in the density of nerve terminal glutamate immunoreactivity in layer II of the SSC for the WSP and WSR mice approached, but did not reach, statistical significance ($F_{(1,20)} = 3.922$, $p = .0616$) (see Figure 4).

A two-way ANOVA (selected line x replicate line) of asymmetric nerve terminal areas in the CA1 and layer II of the SSC for WSP and WSR mice revealed no significant differences between replicate lines (CA1: $F_{(1,20)} = .168$,

p=.6863; SSC: $F_{(1,20)}$=.0003, p=.9863). Furthermore, no interactions were observed. Moreover, no significant differences in terminal areas between selected lines (CA1: $F_{(1,20)}$=0.014, p=.9075; SSC: $F_{(1,20)}$=0.730, p=.4030) were observed (see Figure 5).

Figure 2: **Immuno-gold electron microscopy using an antibody against glutamate showing glutamate immunoreactivity in the CA1. A:** An electron micrograph (initial magnification x25,000) from the CA1 of the hippocampus of a WSP mouse. **B:** An electron micrograph (initial magnification x25,000) from the CA1 of the hippocampus of a WSR mouse. The neuropil shows asymmetric synapses containing a number of gold particles within the presynaptic nerve terminals. (arrow head = gold particles, D = dendritic shaft, Sp = dendritic spine, NT = presynaptic nerve terminal, closed arrow = asymmetric synapses)


A
NT
D
Sp

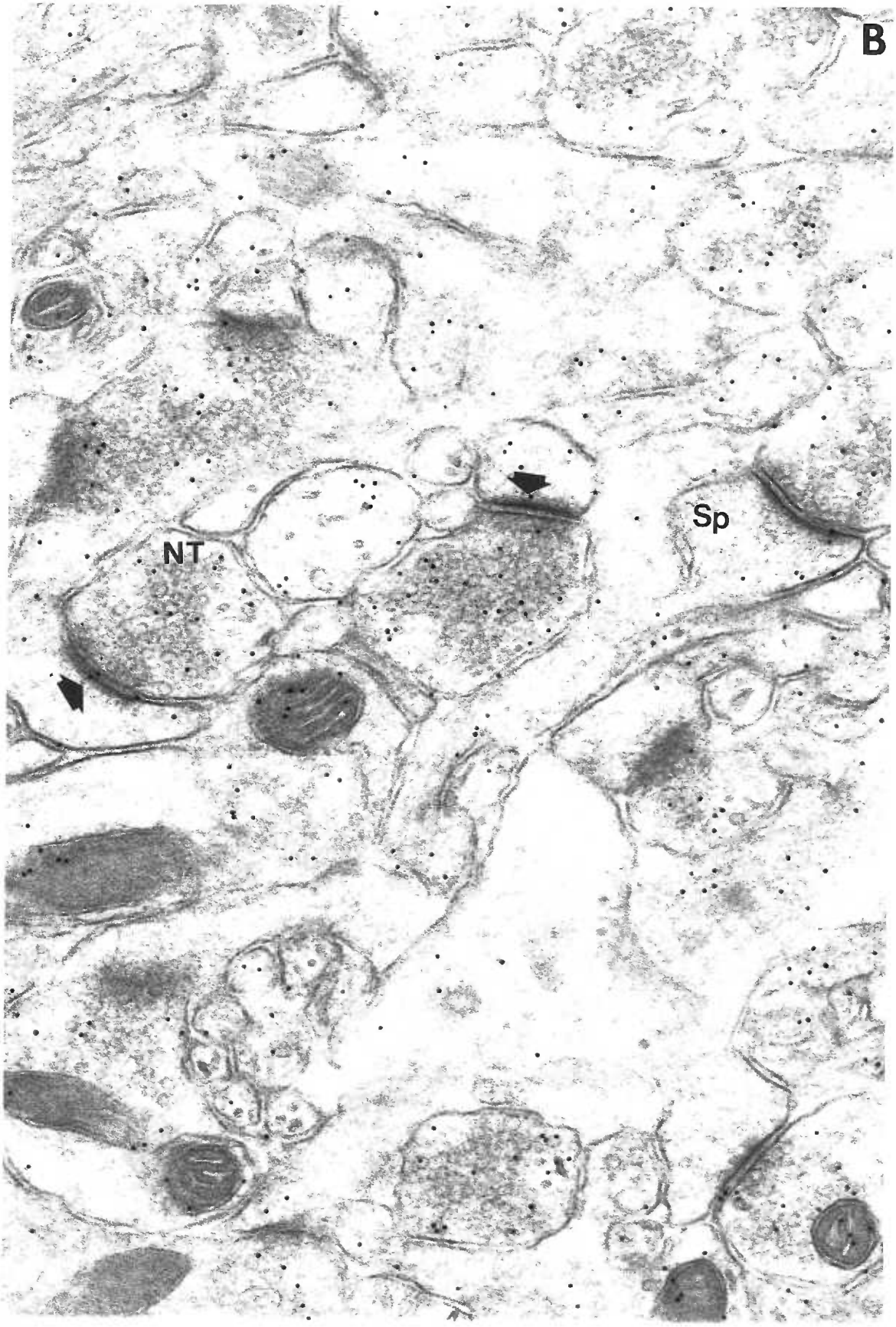
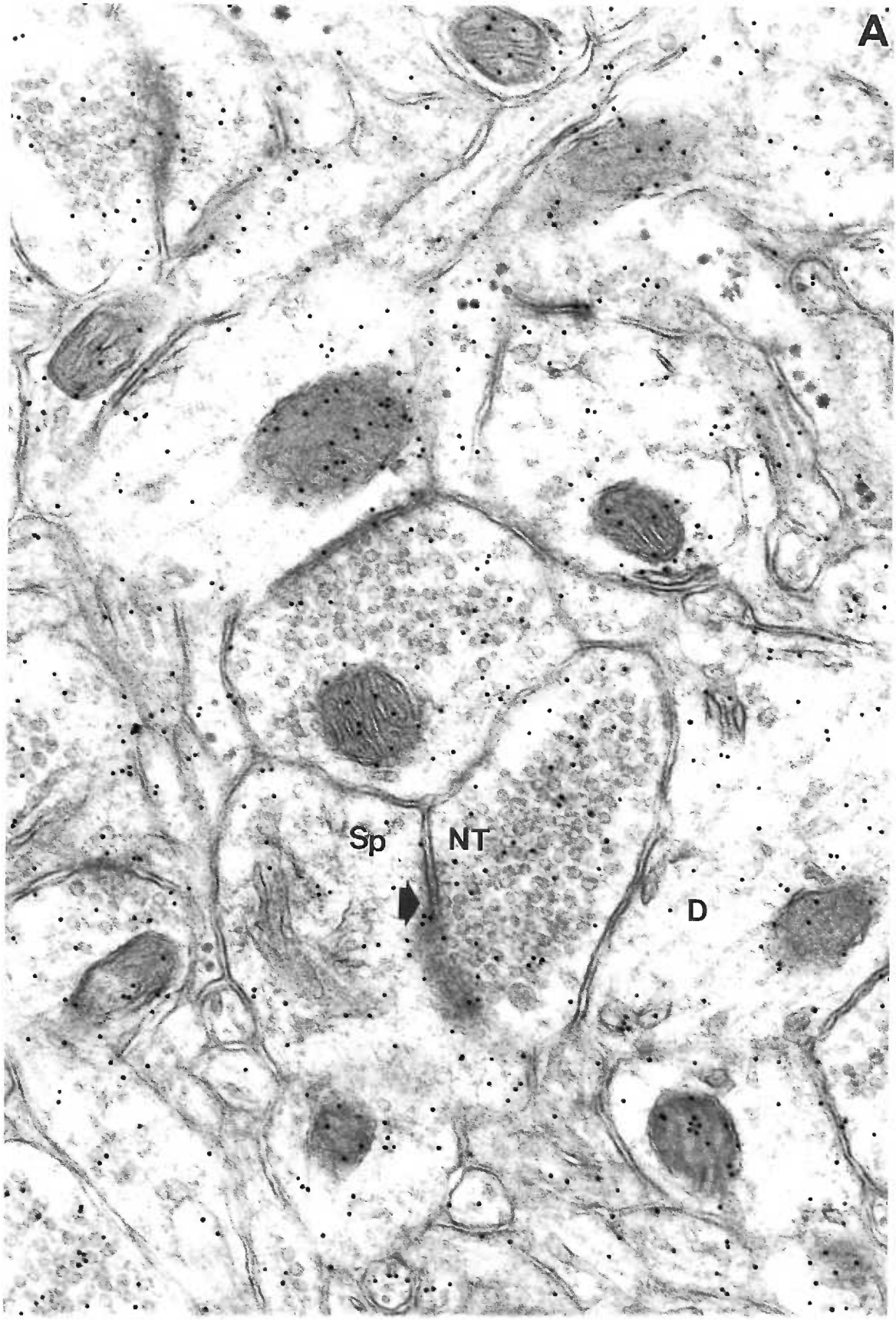

B
NT
Sp

Figure 3: Immuno-gold electron microscopy using an antibody against glutamate showing glutamate immunoreactivity in layer II of the SSC.
A: An electron micrograph (initial magnification x25,000) from layer II of the somatosensory cortex of a WSP mouse. **B:** An electron micrograph (initial magnification x25,000) from layer II of the somatosensory cortex of a WSR mouse. The neuropil shows asymmetric synapses containing a number of gold particles within the presynaptic nerve terminal. (D = dendritic shaft, Sp = dendritic spine, NT = presynaptic nerve terminal, closed arrow = asymmetric synapse)


A
Sp
NT
D

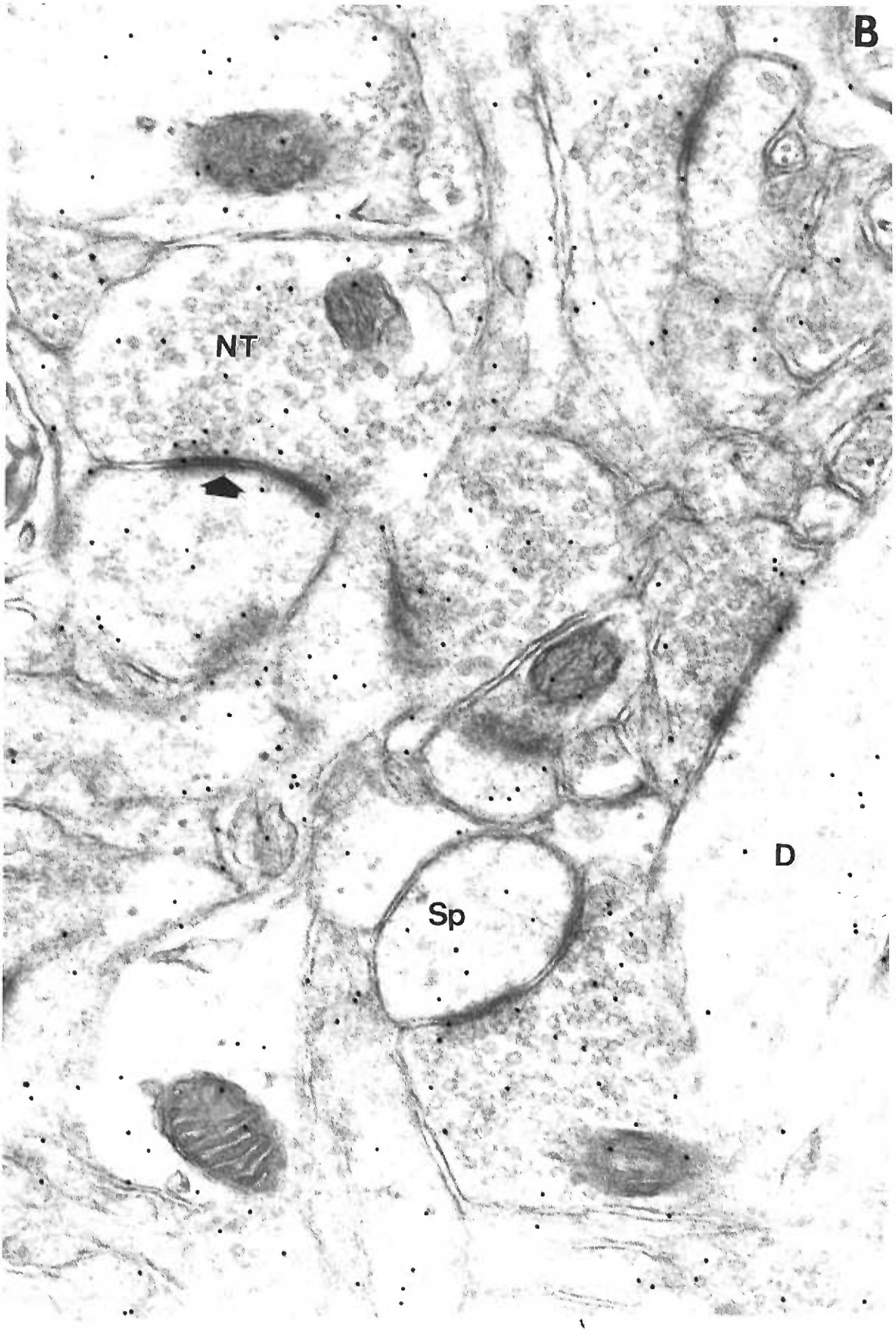
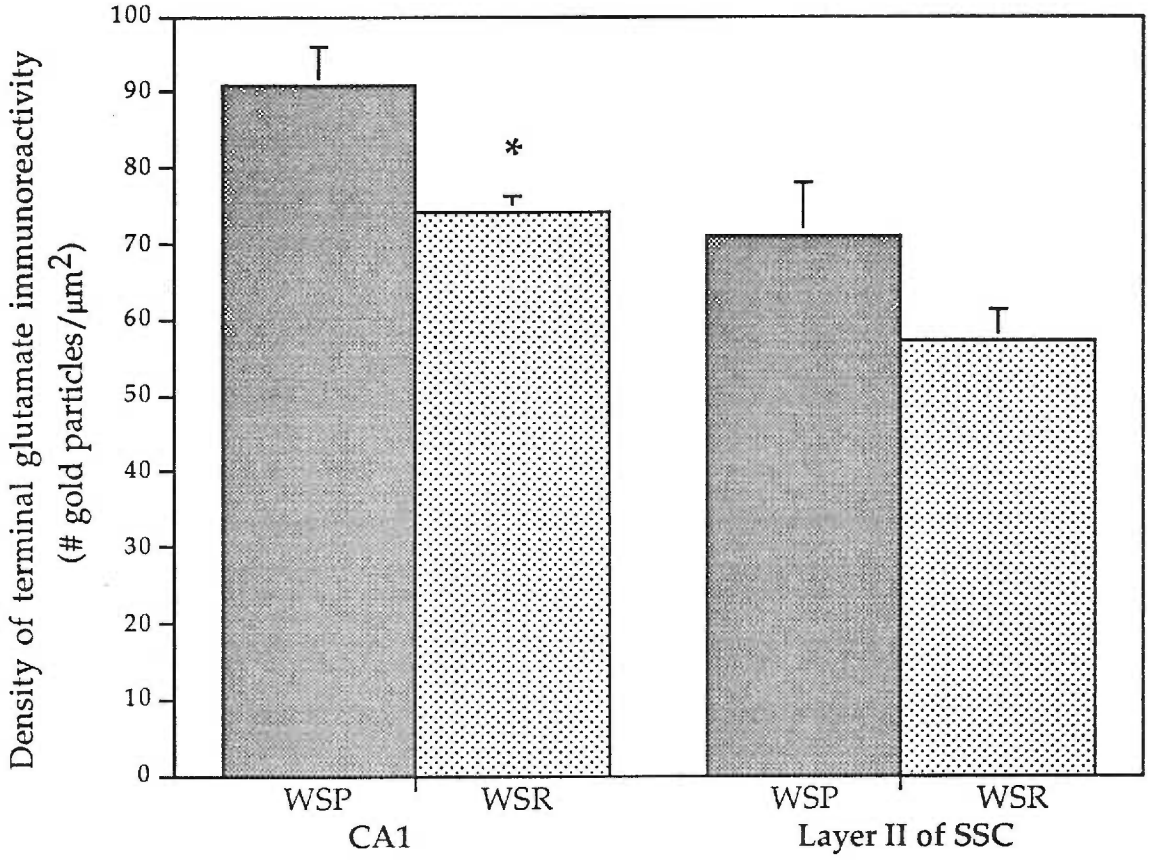
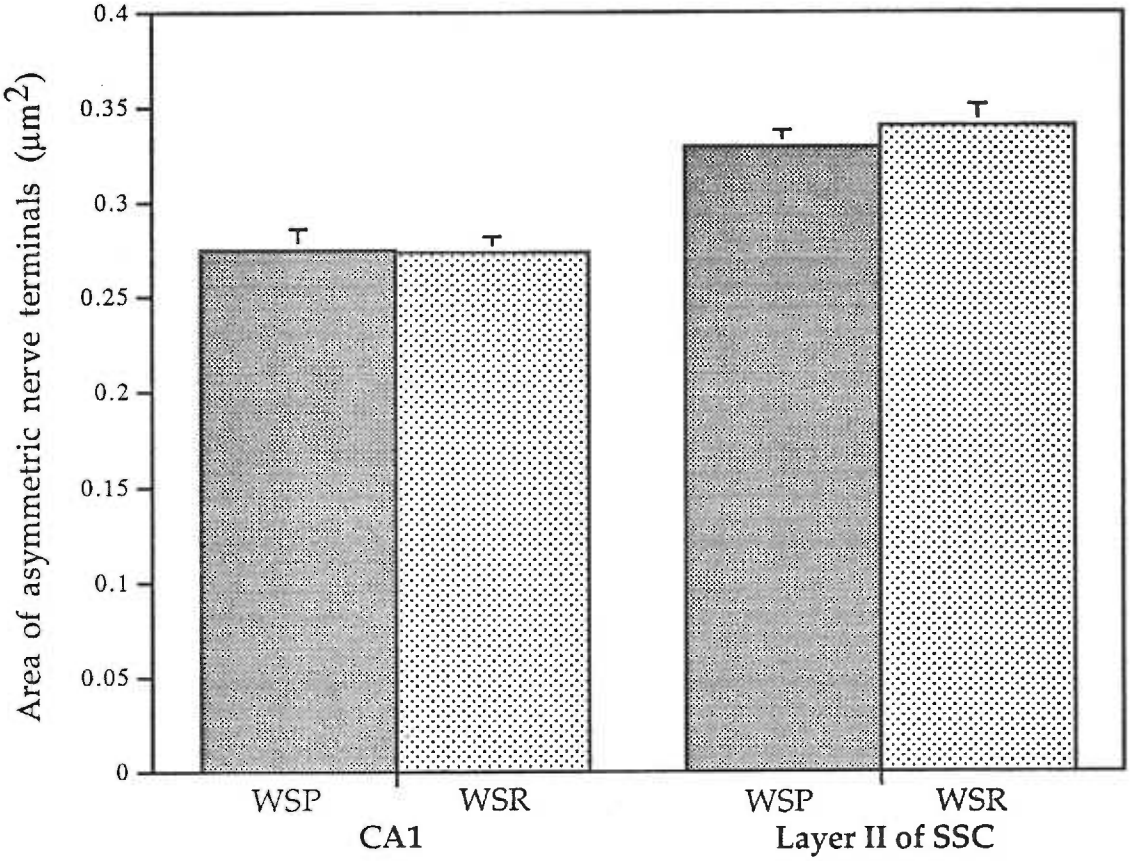

B
NT
D
Sp

Figure 4: **Density of nerve terminal glutamate immunoreactivity in drug- and convulsion-naive WSP and WSR mice.** The density of nerve terminal glutamate immunoreactivity is determined by counting the number of 10 nm gold particles within a given nerve terminal displaying an asymmetric postsynaptic density and analyzing the area of that nerve terminal (# of gold particles/μm^2) The density of glutamate immunoreactivity in the CA1 subfield of the hippocampus was significantly greater in WSP mice as compared to WSR mice ($F=7.073$, $df=1$,$p < 0.02$). No significant difference in glutamate immunoreactivity was observed in layer II of the somatosensory cortex of WSP and WSR mice ($F=1.181$, $df=1$, $p > 0.05$). Data are presented as means ± SEM.


Density of terminal glutamate immunoreactivity
(# gold particles/μm2)
100
90
80
70
60
50
40
30
20
10
0
*
WSP
WSR
WSP
WSR
CA1
Layer II of SSC

* $p < 0.02$

Figure 5: **Mean area (± SEM) of asymmetric nerve terminals analyzed for glutamate immunoreactivity in drug- and convulsion-naive WSP and WSR mice.** Glutamatergic nerve terminals were identified by: (a) labelling with 10nm gold particles; (b) an accumulation of synaptic vesicles near active zone; (c) an asymmetric postsynaptic density. The area (μm^2) of nerve terminals displaying all of these characteristics, as analyzed using a Zeiss Videoplan image analysis system, was determined. No significant differences were observed between WSP and WSR mice in either brain region tested.


Area of asymmetric nerve terminals (μm2)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
WSP
WSR
WSP
WSR
CA1
Layer II of SSC

GABA immunoreactivity:

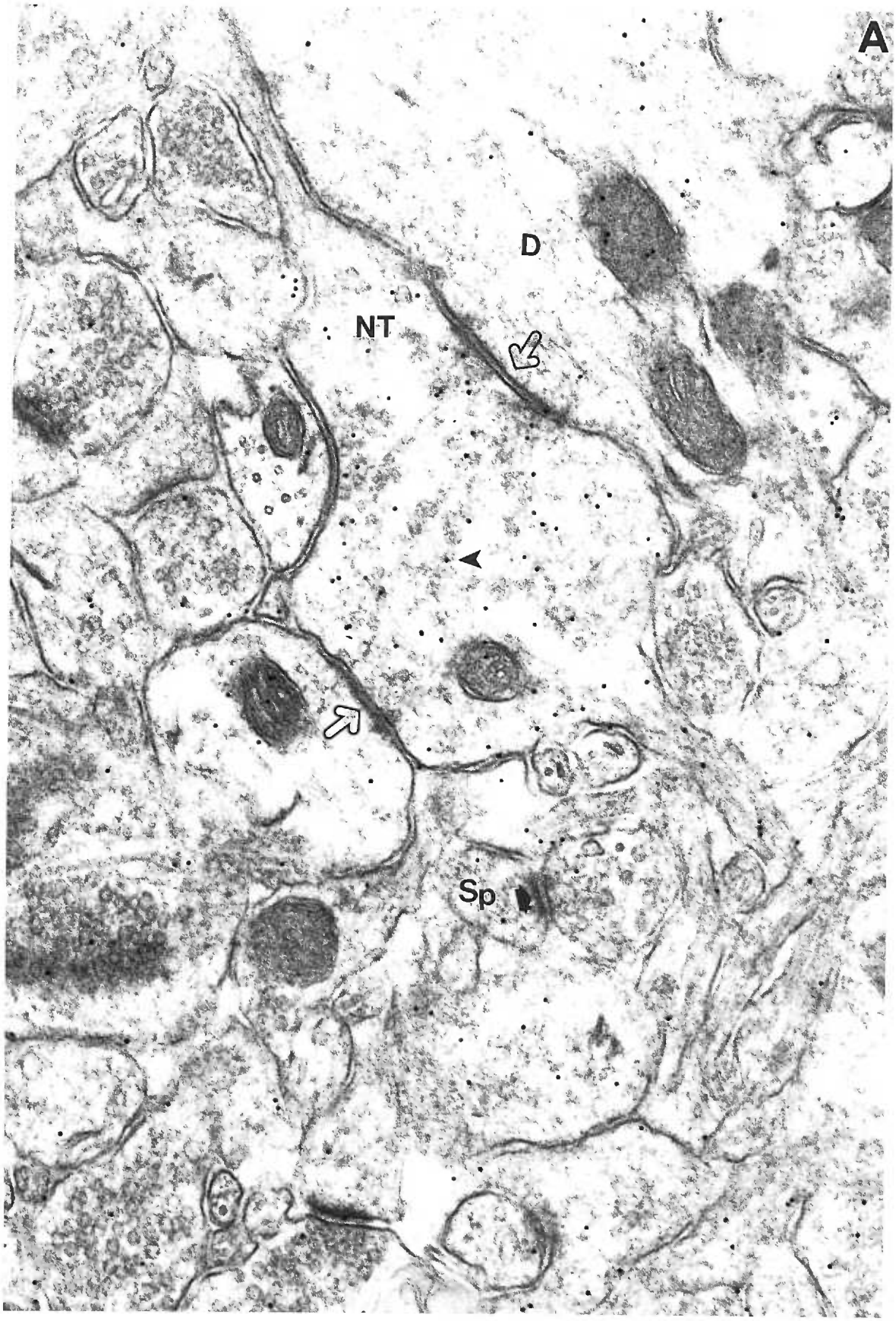
The density of nerve terminal GABA immunoreactivity was analyzed in a similar manner as that carried out for glutamate except symmetric nerve terminals were analyzed. Since this experiment was also run over four days, ANOVAs were performed to determine if there was an effect of run on the density of nerve terminal GABA immunoreactivity (see Appendix A). A highly significant run effect was observed for both the CA1 and layer II of the SSC (CA1: $F_{(3,20)} = 17.578$, $p = .0001$; SSC: $F_{(3,20)} = 9.833$, $p = .0003$). A correction factor was added (see Appendix A) and data from all days were grouped.

Examples of GABA immunolabelling from WSP and WSR mice are illustrated in Figures 6 (CA1) and 7 (layer II of the SSC). A two-way ANOVA (selected line x replicate line) for nerve terminal GABA immunoreactivity revealed no significant differences between the replicate lines (CA1: $F_{(1,20)}= 2.591$, $p = .1232$; SSC: $F_{(1,20)}= 0.059$, $p = .8108$) . In addition, there were no selected line by replicate line interactions in either brain region. Furthermore, no significant differences were observed between the selected lines in the density of presynaptic GABA immunoreactivity in the CA1 ($F_{(1,20)}= 0.723$, $p = .4052$) or layer II of the SSC ($F_{(1,20)}= 2.061$, $p = .1666$). The data from replicate lines 1 and 2 were combined for both WSP mice and WSR mice and are presented in Figure 8.

A two-way ANOVA (selected line x replicate line) of symmetric nerve terminal areas in the CA1 and layer II of the SSC for WSP and WSR mice revealed no significant differences between replicate lines (CA1: $F_{(1,20)}=0.687$, $p=.4168$; SSC: $F_{(1,20)}=0.484$, $p=.4947$). Furthermore, no interactions were observed. No significant differences between selected lines (CA1: $F_{(1,20)}=1.336$, $p=.2613$; SSC: $F_{(1,20)}=0.003$, $p=.9568$) were observed (see Figure 9).

Figure 6: Immuno-gold electron microscopy using an antibody against GABA showing GABA immunoreactivity in the CA1.

A: An electron micrograph (initial magnification x25,000) from the CA1 of the hippocampus of a WSP mouse. **B:** An electron micrograph (initial magnification x25,000) from the CA1 of the hippocampus of a WSR mouse. The neuropil shows specific immuno-gold labelling within the nerve terminals of symmetric synapses (arrow head = gold particle, D = dendritic shaft, Sp = dendritic spine, NT = presynaptic nerve terminal, closed arrow = asymmetric synapse, open arrow = symmetric synapse)


A
D
NT
Sp

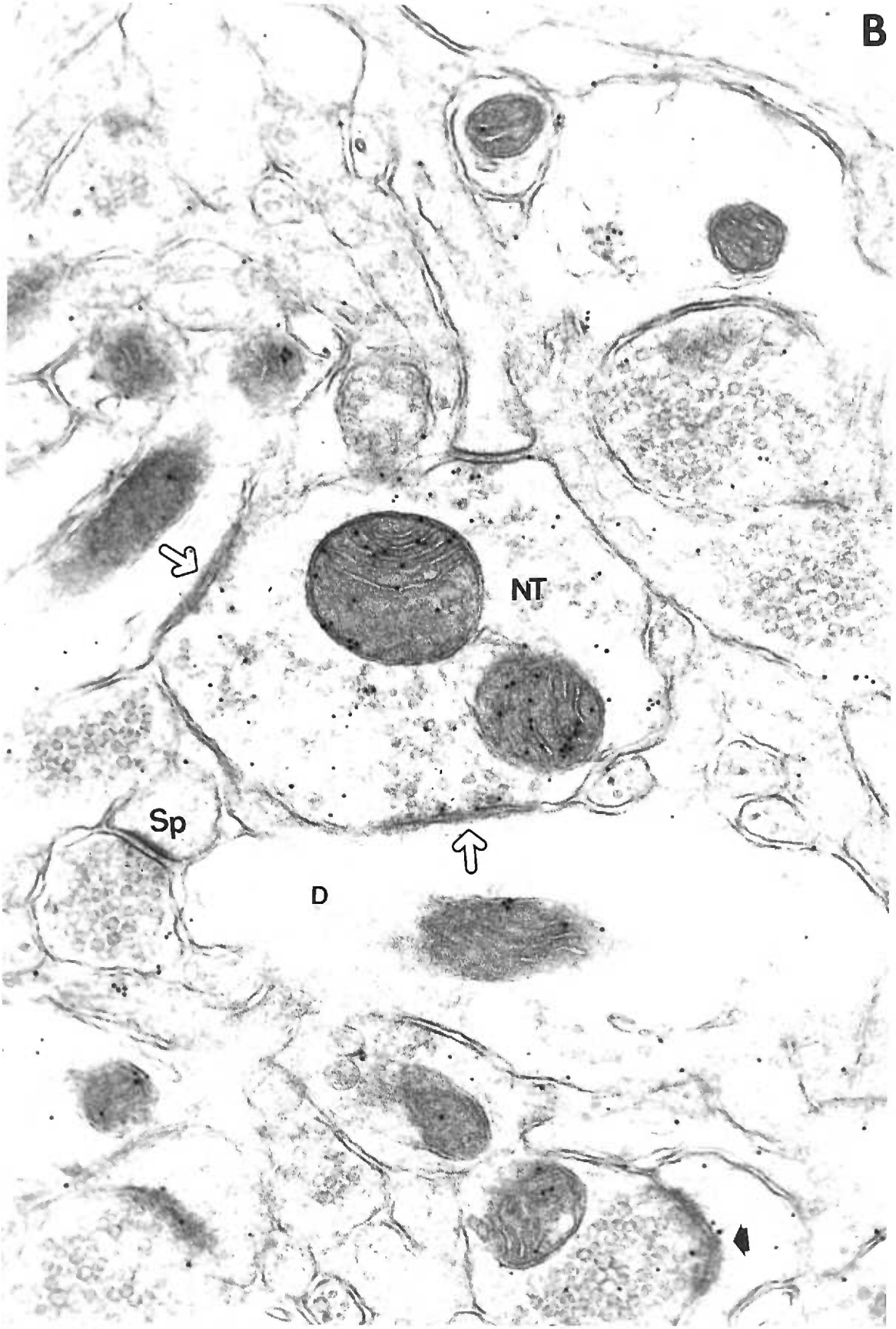
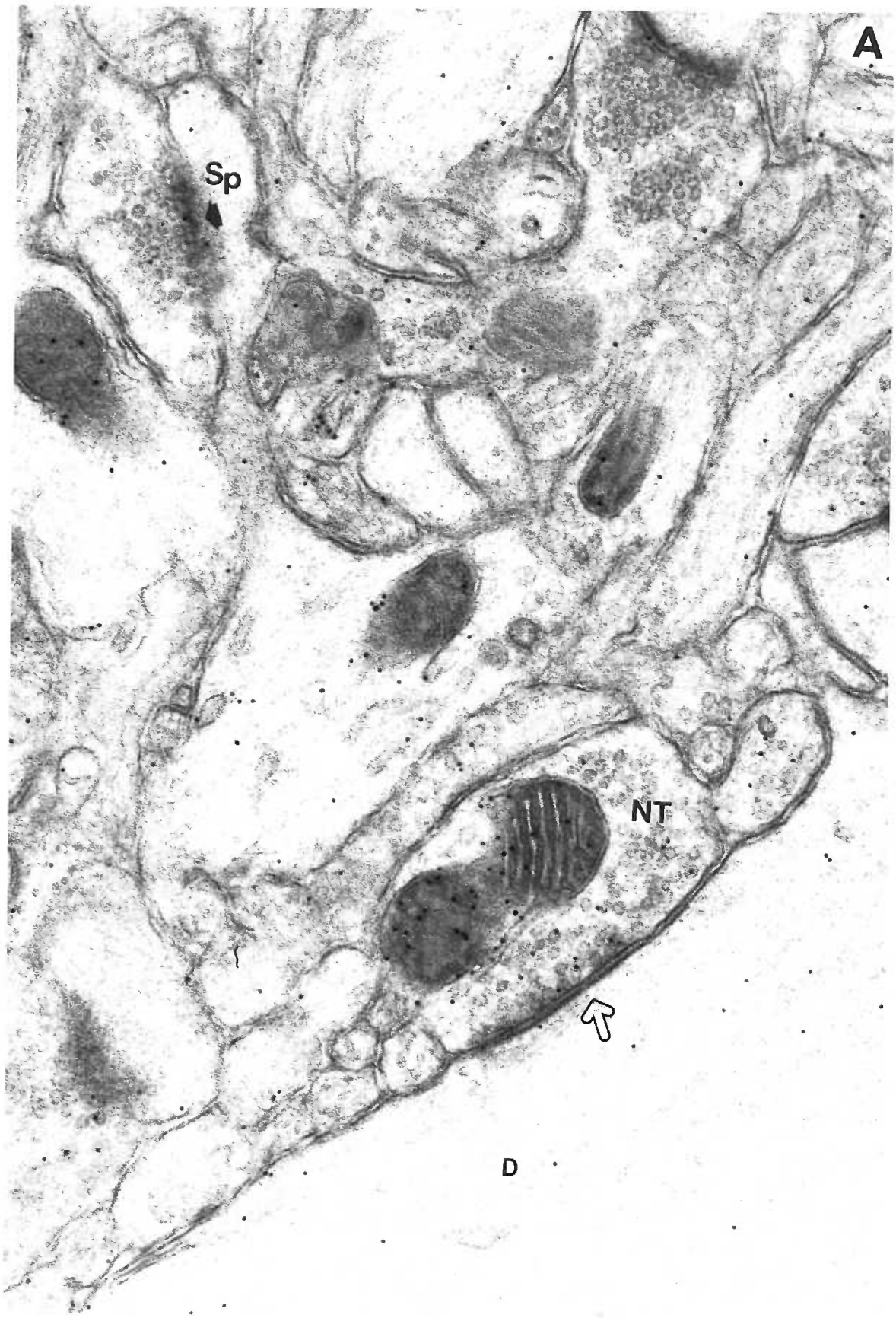

B
NT
Sp
D

Figure 7: Immuno-gold electron microscopy using an antibody against GABA showing GABA immunoreactivity in layer II of the SSC.

A: An electron micrograph (initial magnification x25,000) from layer II of the somatosensory cortex of a WSP mouse. **B:** An electron micrograph (initial magnification x25,000) from layer II of the somatosensory cortex of a WSR mouse. The neuropil shows specific immuno-gold labelling within symmetric synapses (D = dendritic shaft, Sp = dendritic spine, NT = presynaptic nerve terminal, closed arrow = asymmetric synapse, open arrow = symmetric synapse).


A
Sp
NT
D

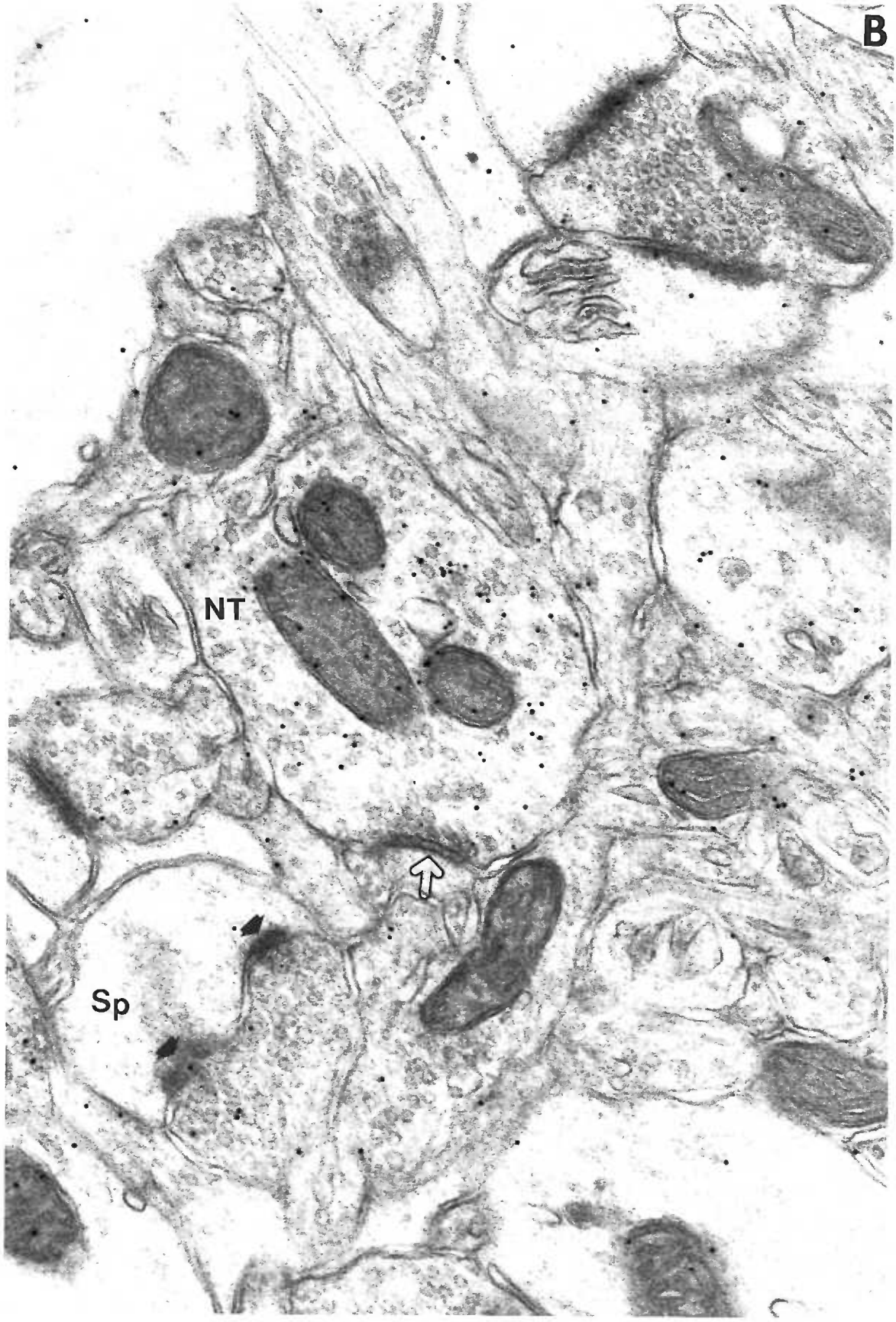

B
NT
Sp

Figure 8: Density of nerve terminal GABA immunoreactivity in drug- and convulsion-naive WSP and WSR mice. The density of GABA immunoreactivity is determined by counting the number of 10 nm gold particles within a given nerve terminal displaying a symmetric postsynaptic density and analyzing the area of that nerve terminal (# of gold particles/μm^2). No significant differences in GABA immunoreactivity was observed between WSP and WSR mice in either brain region tested. Data are presented as means ± SEM.

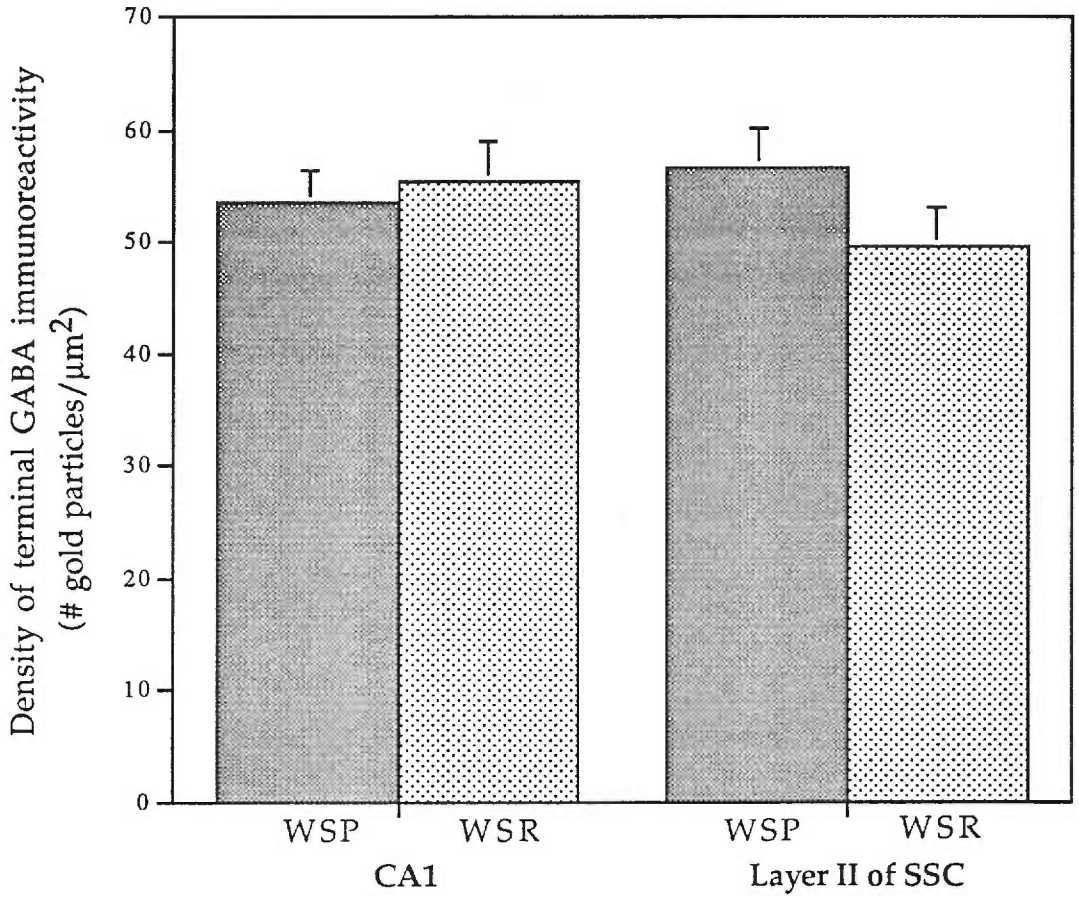
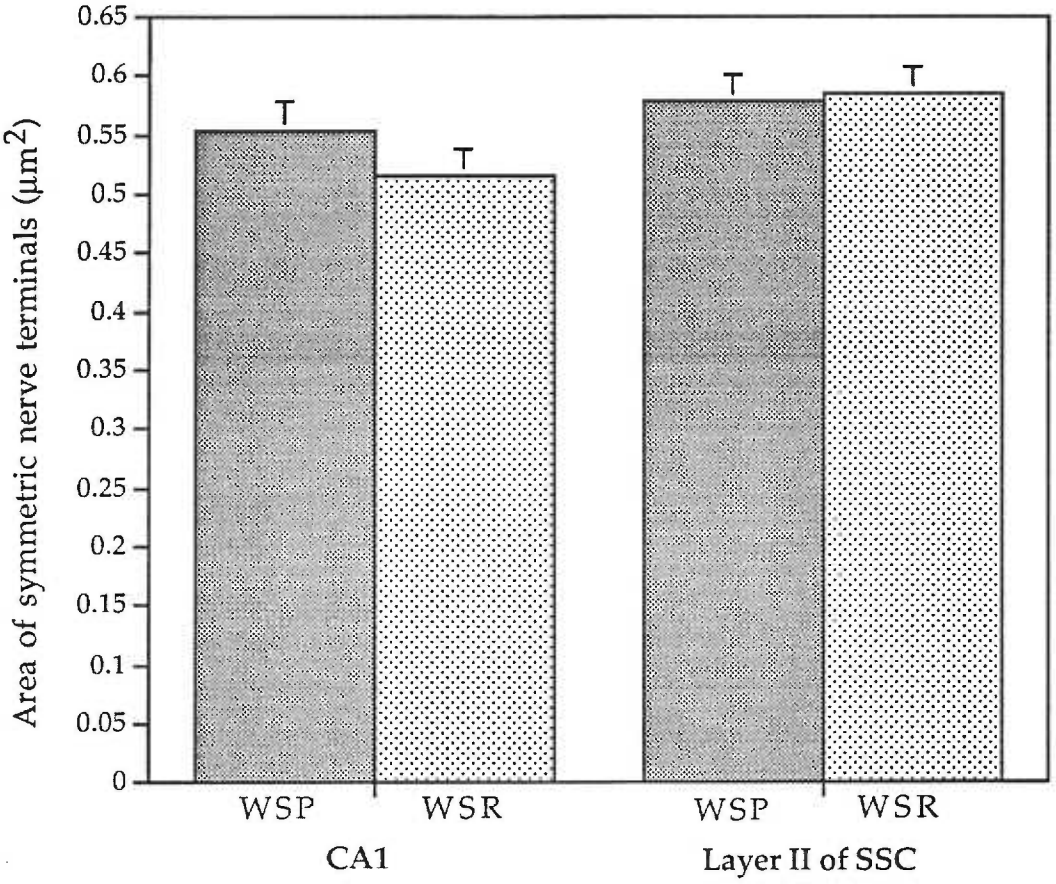

Density of terminal GABA immunoreactivity
(# gold particles/μm2)
70
60
50
40
30
20
10
0
WSP
WSR
WSP
WSR
CA1
Layer II of SSC

Figure 9: **Mean area (± SEM) of symmetric nerve terminals analyzed for GABA immunoreactivity in drug- and convulsion-naive WSP and WSR mice.** GABAergic nerve terminals were identified by: (a) labelling with 10 nm gold particles; (b) an accumulation of synaptic vesicles near active zone; (c) a symmetric postsynaptic density. The area (μm^2) of the nerve terminals displaying all of these characteristics, as analyzed using a Zeiss Videoplan image analysis system, was determined. No significant differences were observed between WSP and WSR mice in either brain region tested.


Area of symmetric nerve terminals (μm2)
0.65
0.6
0.55
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
WSP
WSR
WSP
WSR
CA1
Layer II of SSC

DISCUSSION

There are a variety of methods currently used to assess genetic influence on behavior. Behaviors are typically complex, polygenic traits that have proven exceedingly difficult to analyze genetically (Crabbe et al., 1994b). Genetic factors associated with a predisposition to alcohol related behaviors, such as dependence or tolerance, are currently being investigated. Selective breeding has successfully demonstrated that some alcohol related behaviors are heritable (for reviews see Crabbe, 1989, Crabbe et al., 1994b). The selection of WSP and WSR mice was based upon differential sensitivity to handling-induced convulsions (HICs) during ethanol withdrawal. Although there is considerable evidence suggesting that susceptibility to these withdrawal HICs is heritable, characterizing the genes involved in conferring this predisposition has proven difficult.

Glutamate immunoreactivity:

The present data demonstrate that intracellular, nerve terminal glutamate immunoreactivity in the CA1 is significantly higher in both WSP replicate lines, as compared to both WSR replicate lines (Figure 4). There were no significant differences observed in mean asymmetric nerve terminal areas between these selected lines. These observations suggest that differences in the density of nerve terminal glutamate immunolabelling exist as a result of an increased number of gold particles (implying an increase in intraterminal glutamate) and not from alterations in asymmetric nerve terminal size (i.e. from swelling or degeneration). Furthermore, this difference is present prior

to the induction of HICs suggesting that the greater concentration of intracellular, presynaptic glutamate immunoreactivity observed in the CA1 of WSP mice, may be associated with a genetic propensity for HICs. The density of terminal glutamate immunoreactivity in layer II of the SSC approached, but did not reach, statistical significance (Figure 4). Closer inspection of these latter data revealed that this trend was most likely the result of two outlying data points. To test for this possibility, the data were re-analyzed using a non-parametric Mann-Whitney U test. Using this analysis, the trend toward significance was no longer observable (p=.2129).

The anatomical specificity of the increase in glutamate immuno-reactivity in WSP mice lends additional support to the suggestion that this finding is associated with convulsive activity. The somatosensory cortex has been shown to be resistant to morphological changes induced by 150 kindled seizures (Cavazos et al., 1994). In addition, there is evidence that seizure propagation follows a specific anatomical pathway (Goddard, 1983). In the hippocampus, the CA3 subfield is suspected to be responsible for spontaneous interictal bursts associated with epilepsy (Dingledine et al., 1990). This region sends excitatory projections to the CA1 subfield (Dingledine et al., 1990). Feller et al. (1991) reported that drug-naive WSP mice had a significantly lower density of mossy fiber zinc in the CA3 subfield of the hippocampus as compared to drug-naive WSR mice. This suggests that there may be greater activity of the Schaffer collaterals which make excitatory synaptic connections within the CA1. Therefore, the findings presented in this paper are consistent with previous studies and suggest that susceptibility of WSP mice to HICs is associated with a greater presynaptic glutamate concentration within the CA1

subfield of the hippocampus when compared to WSR mice.

Since the genetic differences between these lines are believed to be correlated with HICs (Crabbe et al., 1986), the differences observed in the density of presynaptic glutamate immunoreactivity may represent a neurochemical correlate of convulsion susceptibility. Therefore, it is of interest to determine which genes influence presynaptic glutamate accumulation and assess whether WSP and WSR mice express allelic differences at these loci.

There are several potential genes that may be involved in the accumulation of glutamate presynaptically. The most straightforward suggestion is that genes influencing glutamate synthesis are involved. Alternatively, presynaptic glutamate concentrations may be regulated by genes associated with the functioning of proteins related to exocytosis, the neuronal and glial transporters, the vesicular transporter and/or proteins involved in glutamate degradation. Research investigating the role of these genes in regulating presynaptic glutamate concentrations in WSP and WSR mice should be undertaken.

One theory that ties the present data together with previously published findings is excitotoxicity, which suggests that the brain requires a very tight balance between the level of excitation and inhibition to be maintained. Although most individuals are susceptible to some degree of imbalance during stress, such as during alcohol withdrawal, the likelihood that this imbalance will reach the theoretical "threshold", where excitotoxicity and convulsions can be observed, may be genetically determined. If withdrawal from ethanol results in an up-regulation of the NMDA receptor complex of

most individuals (for review see Hoffman et al., 1992), then this up-regulation alone cannot explain why some individuals are prone to convulsions and others are not. Perhaps, it is the increased concentration of intracellular presynaptic glutamate that predisposes an individual for convulsive behavior. In other words, it may be the combination of increased release (from increased presynaptic vesicular availability) and NMDA receptor up-regulation that results in a pathological excitatory imbalance, observed behaviorally as convulsions and neurochemically as excitotoxicity.

It should be noted that interpretation of the data presented in the current study is based on the following assumptions: (a) The WSP mice tested in this experiment were naive to all convulsive activity. Although a special handling procedure was used in the present study, spontaneous convulsive activity cannot be completely ruled out; (b) HICs observed in the WSP mice are a behavioral reflection of paroxysmal events. To date, no electrophysiological experiments have been conducted to determine if HICs exhibit the typical abnormal neuronal discharges observed with seizures; (c) It is a relative increase in terminal glutamate immunoreactivity that enhances the propensity of WSP mice to convulsive activity. It is also possible that it is a relative decrease in glutamate immunoreactivity that protects the WSR mice from exhibiting HICs.

GABA immunoreactivity:

In the present study, no significant differences in GABA immunolabelling are observed between any of the four selected lines tested (see Figure 8). This finding correlates with other neurochemical studies which have

suggested that GABAergic dysfunction is not essential to the underlying phenotype associated with the selection of WSP and WSR mice (Crabbe et al., 1991a; Buck et al., 1991b). It is therefore likely that the genes associated with the GABAergic system are not altered by the selection of WSP and WSR mice (Crabbe et al., 1991b; Buck et al., 1991b).

It is interesting to note that molecular biological studies have reported decreased levels of $GABA_A$ receptor subunit mRNA in drug-naive WSP mice, as compared to drug-naive WSR mice (Buck et al., 1991a; Keir and Morrow, 1994) In addition, GABA is believed to be involved in maintaining normal neuronal firing and excitatory balance (Tabakoff and Hoffman, 1993) and alcohol withdrawal may involve alterations in GABAergic function (for reviews see Glue and Nutt, 1990; Lovinger, 1993). The experiments reported here attempted to address whether intracellular presynaptic GABA immunoreactivity was associated with the differential proclivity towards convulsive activity observed in the WSP and WSR mice. The results suggest that presynaptic GABA immunoreactivity within the CA1 subfield of the hippocampus and layer II of the SSC is not involved in conferring susceptibility to convulsive activity or excitotoxicity. However, this experiment cannot rule out the possibilities that GABAergic alterations occur postsynaptically, in other brain regions (i.e. cerebellum) or <u>following</u> the onset of convulsive activity.

SUMMARY AND CONCLUSIONS

The present findings suggest that the genetic selection process used in creating the WSP and WSR mouse lines selected for genes responsible for the synthesis, storage, release and/or reuptake of the excitatory amino acid, glutamate. The selection process does not appear to have influenced pre-synaptic GABAergic immunoreactivity, but GABA may act in a compensatory manner or at a postsynaptic site and therefore line differences may not be detectable in the present experiment. In conclusion, the increase in hippocampal nerve terminal glutamate immunoreactivity observed in WSP mice, as compared to the WSR mice, cannot be attributed to drug withdrawal or convulsive activity, but may reflect an increased susceptibility of WSP mice to these events.

REFERENCES

Anderson, VE, Hauser, WA and Rich, SS (1986) Genetic heterogeneity in the epilepsies. Advances in Neurology 44: 59-73.

Annegers, JF (1994) Epidemiology and genetics of epilepsy. Neurologic Clinics 12(1): 15-29.

Araki, T, Kato, M and Goto, I (1994) Altered GABAergic effects on kainic acid-induced seizures in the presence of hippocampal sclerosis in rats. Brain Research 653: 237-242.

Armanini, MP, Hutchins, M, Stein, BA and Sapolsky, RM (1990) Glucocorticoid endangerment of hippocampal neurons is NMDA-receptor dependent. Brain Research 532: 7-12.

Ballenger, JC and Post, RM (1978) Kindling as a model for alcohol withdrawal syndromes. British Journal of Psychiatry 133: 1-14.

Becker, HC (1994) Positive relationship between the number of prior ethanol withdrawal episodes and the severity of subsequent withdrawal seizures. Psychopharmacology 116: 26-32.

Becker, LE (1991) Synaptic dysgenesis. Canadian Journal of Neurological Science 18: 170-180.

Belknap, JK, Laursen, SE and Crabbe, JC (1987) Ethanol and nitrous oxide produce withdrawal-induced convulsions by similar mechanisms in mice. Life Sciences 41: 2033-2040.

Belknap, JK, Danielson, PW, Lame, M and Crabbe, JC (1988) Ethanol and barbiturate withdrawal convulsions are extensively codetermined in mice. Alcohol 5(2): 161-171.

Belknap, JK, Crabbe, JC and Laursen, SE (1989) Ethanol and diazepam withdrawal convulsions are extensively codetermined in WSP and WSR mice. Life Sciences 44: 2075-2080.

Buck, KJ and Harris, RA (1991) Neuroadaptive responses to chronic ethanol. Alcoholism: Clinical and Experimental Research 15(3): 460-470.

Buck, KJ, Hahner, L, Sikela, J and Harris, RA (1991a) Chronic ethanol treatment alters brain levels of γ-aminobutyric acid$_A$ receptor subunit mRNAs: Relationship to genetic differences in ethanol withdrawal seizure severity. Journal of Neurochemistry 57: 1452-1455.

Buck, KJ, McQuilkin, SJ and Harris, RA (1991b) Modulation of γ-aminobutyric acid$_A$ receptor-operated chloride channels by benzodiazepine inverse agonists is related to genetic differences in ethanol withdrawal seizure severity. Journal of Neurochemistry 57: 2100-2105.

Carlson, H, Ronne-Engström, E, Ungerstedt, U and Hillered, L (1992) Seizure related elevations of extracellular amino acids in human focal epilepsy. Neuroscience Letters 140: 30-32.

Carter, LA, Belknap, JK, Crabbe, JC, and Janowsky, A (1995) Allosteric regulation of the N-methyl-D-aspartate receptor-linked ion channel complex and effects of ethanol in ethanol-withdrawal seizure-prone and -resistant mice. Journal of Neurochemistry 64(1): 213-219.

Cavazos, JE, Das, I and Sutula, TP (1994) Neuronal loss induced in limbic pathways by kindling: Evidence for induction of hippocampal sclerosis by repeated brief seizures. Journal of Neuroscience 14(5): 3106-3121.

Chadwick, D (1994) Epilepsy. Journal of Neurology, Neurosurgery, and Psychiatry 57: 264-277.

Choi, DW (1987) Ionic dependence of glutamate neurotoxicity. Journal of Neuroscience 7(2): 369-379.

Choi, DW (1988) Glutamate neurotoxicity and diseases of the nervous system. Neuron 1: 623-634.

Choi, DW (1990) Cerebral hypoxia: Some new approaches and unanswered questions. Journal of Neuroscience 10(8): 2493-2501.

Choi, DW and Rothman, SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. Annual Rev in Neuroscience 13: 171-182.

Clemmesen, L and Hemmingsen, R (1984) Physical dependence on ethanol during multiple intoxication and withdrawal episodes in the rat: Evidence of a potentiation. Acta Pharmacologica et Toxicological 55: 345-350.

Crabbe, JC, Kosobud, A and Young, ER (1983) Genetic selection for ethanol withdrawal severity: differences in replicate mouse lines. Life Science 33(10): 955-962.

Crabbe, JC, Kosobud, A, Young, ER, Tam, BR and McSwigan, JD (1985) Bidirectional selection for susceptibility to ethanol withdrawal seizures in *Mus musculus*. Behavior Genetics 15(6): 521- 536.

Crabbe, JC, Young, ER, Tam, B, Kosobud, A, Belknap, JK and Laursen, SE (1986) Genetic differences in anticonvulsant sensitivity in mouse lines selectively bred for ethanol withdrawal severity. Journal of Pharmacology and Experimental Therapeutics 239(1): 154-159.

Crabbe, JC (1989) Genetic animal models in the study of alcoholism. Alcoholism: Clinical and Experimental Research 13(1): 120-127.

Crabbe, JC, Feller, DJ, Terdal, ES and Merrill, CM (1990a) Genetic components of ethanol responses. Alcohol 7: 245-248.

Crabbe, JC, Phillips, TJ, Kosobud, A and Belknap, JK (1990b) Estimation of genetic correlation: Interpretation of experiments using selectively bred and inbred animals. Alcoholism: Clinical and Experimental Research 14(2): 141-151.

Crabbe, JC, Merrill, CM and Belknap, JK (1991a) Effects of convulsants on handling-induced convulsions in mice selected for ethanol withdrawal severity. Brain Research 550: 1-6.

Crabbe, JC, Merrill, CM and Belknap, JK (1991b) Acute dependence on depressant drugs is determined by common genes in mice. Journal of Pharmacology and Experimental Therapeutics 257(2): 663-667.

Crabbe, JC, Merrill, CM and Belknap, JK (1993) Effect of acute alcohol withdrawal on sensitivity to pro- and anticonvulsant treatments in WSP mice. Alcoholism: Clinical and Experimental Research 17(6): 1233-1239.

Crabbe, JC, Young, ER and Dorow, J (1994a) Effects of Dizocilpine in Withdrawal Seizure-Prone (WSP) and Withdrawal Seizure-Resistant (WSR) mice. Pharmacology, Biochemistry and Behavior 47(3): 443-450.

Crabbe, JC, Belknap, JK and Buck, KJ (1994b) Genetic animal models of alcohol and drug abuse. Science 264: 1715-1723.

Dingledine, R, McBain, CJ and McNamara, JO (1990) Excitatory amino acid receptors in epilepsy. Trends in Pharmacological Sciences 11: 334-338.

Feller, DJ, Harris, RA and Crabbe, JC (1988) Differences in GABA activity between ethanol withdrawal seizure prone and resistant mice. European Journal of Pharmacology 157: 147-154.

Feller, DJ, Tso-Olivas, DY and Savage, DD (1991) Hippocampal mossy fiber zinc deficit in mice genetically selected for ethanol withdrawal seizure susceptibility. Brain Research 545: 73-79.

Freedland, ES and McMicken, DB (1993) Alcohol-related seizures, Part I: Pathophysiology, differential diagnosis, and evaluation. Journal of Emergency Medicine 11: 463-473.

Glass, M and Dragunow, M (1995) Neurochemical and morphological changes associated with human epilepsy. Brain Research Reviews 21: 29-41.

Glue, P and Nutt, D (1990) Overexcitement and disinhibition: Dynamic neurotransmitter interactions in alcohol withdrawal. British Journal of Psychiatry 157: 491-499.

Goddard, GV (1983) The kindling model of epilepsy. Trends in Neurosciences 6: 275-279.

Greenamyre,JT, Young,AB and Penney,JB (1983) Quantitative autoradiography of L-[^{3}H]glutamate binding to rat brain. Neuroscience Letters 37: 155-160.

Halpain, D, Parsons, B and Rainbow, TC (1983) Tritium-film autoradiography of sodium-independent glutamate binding sites in rat brain. European Journal of Pharmacology 86: 313-314.

Hepler, JR, Toomim, CS, McCarthy, KD, Conti, F, Battaglia, G, Rustioni, A and Petrusz, P (1988) Characterization of antisera to glutamate and aspartate. Journal of Histochemistry and Cytochemistry 36: 13-22.

Hoffman, PL, Grant, KA, Snell, LD, Reinlib, L, Iorio, K and Tabakoff, B (1992) NMDA Receptors: Role in ethanol withdrawal seizures. Annals of the New York Academy of Science 654: 52-60.

Horton, RW (1991) GABA dysfunction in animal models of epilepsy. In: *GABA Mechanisms in Epilepsy*, Wiley-Liss: 121-147.

Kamphius, W, Wadman, WJ, Buijs, RM and Lopes da Silva, FH (1986) Decrease in number of hippocampal gamma-aminobutyric acid (GABA) immunoreactive cells in the rat kindling model of epilepsy. Experimental Brain Research 64: 491-495.

Kamphius, W, Wadman, WJ, Buijs, RM and Lopes da Silva, FH (1987) The development of changes in hippocampal GABA immunoreactivity in the rat kindling model of epilepsy: a light microscopic study with GABA antibodies. Neuroscience 23(2): 433-446.

Kamphius, W, Huisman, E, Wadman, WJ and Lopes da Silva, FH (1989) Decrease in GABA immunoreactivity and alterations of GABA metabolism after kindling in the rat hippocampus. Experimental Brain Research 74: 375-386.

Kandel, ER, Schwartz, JH and Jessell, TM (Eds.). *Principles of Neural Science.* Appleton & Lange, Connecticut, 1991, pp.654-658.

Keir, WJ and Morrow, AL (1994) Differential expression of $GABA_A$ receptor subunit mRNAs in ethanol-naive withdrawal seizure resistant (WSR) vs. withdrawal seizure prone (WSP) mouse brain. Molecular Brain Research 25: 200-208.

King, MA, Hunter, BE and Walker, DW (1988) Alterations and recovery of dendritic spine density in rat hippocampus following long-term ethanol ingestion. Brain Research 459: 381-385.

Kosobud, A and Crabbe, JC (1986) Ethanol withdrawal in mice bred to be genetically prone and resistant to ethanol withdrawal seizures. Journal of Pharmacology and Experimental Therapeutics 238(1): 170-177.

Lallement, G, Carpentier, P, Collet, A, Pernot-Marino, I, Baubichon, D and Blanchet, G (1991) Effects of soman-induced seizures on different extracellular amino acid levels and on glutamate uptake in rat hippocampus. Brain Research 563: 234-240.

Lancaster, FE (1992) Alcohol, nitric oxide, and neurotoxicity: Is there a connection? - a review. Alcoholism: Clinical and Experimental Research 16(3): 539-541.

Lechtenberg, R and Worner, TM (1991) Relative kindling effect of detoxification and non-detoxification admissions in alcoholics. Alcohol and Alcoholism 26(2): 221-225.

Lekieffre, D, Callebert, J, Plotkine, M and Boulu, RG (1992) Concomitant increases in the extracellular concentrations of excitatory and inhibitory amino acids in the rat hippocampus during forebrain ischemia. Neuroscience Letters 137: 78-82.

Leppert, M, Anderson, VE, Quattlebaum, T, Stauffer, D, O'Connell, P, Nakamura, Y, Lalouel, J and White, R (1989) Benign familial neonatal convulsions linked to genetic markers on chromosome 20. Nature 337: 647-648.

Lovinger, DM, White, G and Weight, FF (1990) Ethanol inhibition of neuronal glutamate receptor function. Annals of Medicine 22: 247-252.

Lovinger, DM (1993) Excitotoxicity and alcohol-related brain damage. Alcoholism: Clinical and Experimental Research 17(1): 19-27.

McMullen, PA, Saint-Cyr, JA and Carlen, PL (1984) Morphological alterations in rat CA1 hippocampal pyramidal cell dendrites resulting from chronic ethanol consumption and withdrawal. Journal of Comparative Neurology 225: 111-118.

McNamara, JO (1992) The neurobiological basis of epilepsy. Trends in Neuroscience 15(10): 357-359.

Meldrum, B and Garthwaite, J (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. Trends in Pharmacological Sciences 11: 379-387.

Meshul, CK, Stallbaumer, RK, Taylor, B and Janowsky, A (1994) Halperidol-induced morphological changes in striatum are associated with glutamate synapses. Brain Research 648: 181-195.

Olney, JW, Ho, OL and Rhee, V (1971) Cytotoxic effects of acidic and sulphur containing amino acids on the infant mouse central nervous system. Experimental Brain Research 14: 61-76.

Olney, JW, Rhee, V and Ho, OL (1974) Kainic acid: a powerful neurotoxic analogue of glutamate. Brain Research 77: 507-512.

Olney, JW, DeGubareff, T and Sloviter, RS (1983) "Epileptic" brain damage in rats induced by sustained electrical stimulation of the perforant path. II. Ultrastructural analysis of acute hippocampal pathology. Brain Research Bulletin 10: 699-712.

Ottersen, OP, Storm-Mathisen, J, Bramham, C, Torp, R, Laake, J and Gundersen, V (1990) A quantitative electron microscopic immunocytochemical study of the distribution and synaptic handling of glutamate in rat hippocampus. Progress in Brain Research 83: 99-114.

Phend, KD, Weinberg, RJ and Rustioni, A (1992) Techniques to optimize post-embedding single and double staining for amino acid neurotransmitters. Journal of Histochemistry and Cytochemistry 40(7): 1011-1020.

Phillips, TJ, Feller, DJ and Crabbe, JC (1989) Selected mouse lines, alcohol and behavior. Experientia 45: 805-827.

Ribak, CE, Bradburne, RM and Harris, AB (1982) A preferential loss of GABAergic, symmetric synapses in epileptic foci: A quantitative ultrastructural analysis of monkey neocortex. Journal of Neuroscience 2(12): 1725-1735.

Roberts, AJ, Chu, HP, Crabbe, JC and Keith, LD (1991) Differential modulation by the stress axis of ethanol withdrawal seizure expression in WSP and WSR mice. Alcoholism: Clinical and Experimental Research 15(3): 412-417.

Roberts, AJ, Crabbe, JC and Keith, LD (1993) Type I corticosteroid receptors modulate PTZ-induced convulsions of withdrawal seizure prone mice. Brain Research 626: 143-148.

Roberts, RC, Ribak, CE and Oertel, WH (1985a) Increased numbers of GABAergic neurons occur in the inferior colliculus of an audiogenic model of genetic epilepsy. Brain Research 361: 324-338.

Roberts, RC, Kim, HL and Ribak, CE (1985b) Increased numbers of neurons occur in the inferior colliculus of the young genetically epilepsy-prone rat. Developmental Brain Research 23: 277-281.

Rothman, SM and Olney, JW (1986) Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Annals of Neurology 19: 105-111.

Rothman, SM and Olney, JW (1987) Excitotoxicity and the NMDA receptor. Trends in Neuroscience 10: 299-302.

Rowley, HL, Martin, KF and Marsden, CA (1995) Decreased GABA release following tonic-clonic seizures is associated with an increase in extracellular glutamate in rat hippocampus *in vivo*. Neuroscience 68(2): 415-422.

Sapolsky, RM (1993a) Potential behavioral modification of glucocorticoid damage to the hippocampus. Behavioural Brain Research 57: 175-182.

Sapolsky, RM (1993b) The physiological relevance of glucocorticoid endangerment of the hippocampus. Annals of the New York Academy of Science 746: 294-304.Schaumann, BA, Annegers, JF, Johnson, SB, Moore, KJ, Lubozynski, MF and Salinsky, MC (1994) Family history of seizures in posttraumatic and alcohol-associated seizure disorders. Epilepsia 35(1): 48-52.

Scholz, W (1933) Uber die entstehung des hirnbefundes bei der epilepsie. Z Neurol Psychiatr 145: 471-515.

Siesjo, BK and Bengtsson, F (1989) Calcium fluxes, calcium antagonists, and calcium-related pathology in brain ischemia, hypoglycemia, and spreading depression: A unifying hypothesis. Journal of Cerebral Blood Flow and Metabolism 9: 127-140.

Slotnick, BM and Leonard, CM (1975) A Stereotaxic Atlas of the Albino Mouse Forebrain. US Dept. of Health, Education and Welfare. Rockville, MD.

Sloviter, RS (1983) "Epileptic" brain damage in rats induced by sustained electrical stimulation of the perforant path. I. Acute electrophysiological and light microscopic studies. Brain Research Bulletin 10: 675-697.

Sommer, W (1880) Erkrandkung des ammonshorns als aetiologisches moment der epilepsie. Arch Psychiatr Nervenkr 10: 631-675.

Spielmeyer, W (1927) Die pathogenese des epileptischen krampfes. Histopathologischer Teil. Z Neurol Psychiatr 109: 501-520.

Tabakoff, B and Hoffman, PL (1993) Ethanol, sedative hypnotics, and glutamate receptor function in brain and cultured cells. Behavior Genetics 23(2): 231-236.

Temkin, NR and Davis, GR (1984) Stress as a risk factor for seizures among adults with epilepsy. Epilepsia 25(4): 450-456.

Ulrichsen, J, Clemmesen, L and Hemmingsen, R (1992) Convulsive behaviour during alcohol dependence: discrimination between the role of intoxication and withdrawal. Psychopharmacology 107: 97-102.

Valverius, P, Crabbe, JC, Hoffman, PL and Tabakoff, B (1990) NMDA receptors in mice bred to be prone and resistant to ethanol withdrawal seizures. European Journal of Pharmacology 184: 185-189.

APPENDIX A:
Method for correcting for effect of run

Ultrastructural immunocytochemistry is a time consuming and labor-intensive procedure. It was therefore necessary to carry out the immunolabelling experiments presented here over a course of four days. Each tissue sample was randomly assigned into one of the four runs (immunocytochemical run). An identical protocol (as detailed in the Methods section) was used for each run, but slight differences in procedure, temperature and chemical factors can result in significant differences in the density of immunolabelling between runs. It was therefore necessary to test for the potential confounding influence of run.

Tissue samples from each run were grouped together to obtain a mean of individual means ($\bar{x}_r$). Two separate two-way ANOVA were then performed to test whether significant differences in density of immunoreactivity existed between runs. The glutamate immunocytochemical experiment did not reveal any significant differences. Therefore, tissue samples from all runs were combined. Both two-way ANOVAs revealed a significant effect of run for the GABA immunocytochemical experiment. Post-hoc analyses revealed that this statistical significance was not the result of a single run, but was a trend across all runs. Therefore, a correction factor was calculated, as shown below.

For the CA1 subfield of the hippocampus (CA1), this correction factor was easily calculated. The two-way ANOVAs performed revealed a significant effect of run with no interactions. This suggested that the run did not differentially effect the selected lines or the replicate lines. Thus, all samples

from each run were combined and a weighted mean (of individual animal means) for each run ($\bar{x}_r$) was calculated. An overall, grand weighted mean (across runs) was also calculated ($\bar{x}_g$). The correction factor was then determined by subtracting each run mean from the grand mean ($\bar{x}_g - \bar{x}_r$). The correction factor then was added to each of the individual (raw) densities. This method is detailed below.

Analysis of the data from layer II of the somatosensory cortex (SSC) was more complicated. The two-way ANOVAs revealed a significant effect of run, but an interaction between selected line and run was observed (see page 54). This finding was difficult to interpret because these samples were tested in the same runs as the CA1 samples and that ANOVA revealed no significant interaction between selected line and run (p=.7329). Furthermore, the effect of run was much greater than the interaction. Therefore, it was decided that a single correction factor would be added to all lines (both selected and replicate lines). The mean density (± S.E.M.) of GABA immunoreactivity in layer II of the SSC of WSP mice was corrected from 56.13 (± 4.7) to 56.65 (± 3.6). For WSR mice, the density was corrected from 53.50 (± 6.1) to 49.59 (± 3.6).

As an additional note, there were no SSC tissue samples from WSR animals tested in run 1. Therefore, this analysis was done using an estimated value for WSR mice in this run. To assure that this estimate was reasonable, the two-way ANOVA was repeated, excluding data from run 1. This analysis revealed nearly the same pattern of results (selected line: $F_{(1,15)}=2.449$, p=.1385; run: $F_{(2,15)}=20.294$, p=.0001; selected line x run: $F_{(2,15)}=6.160$, p=.0111).

Method for correcting for effect of run
GABA immunoreactivity in the CA1

ANOVA Table for density of immunoreactivity, collapsed over replicate line

	df	F-Value	P-Value
selected line	1	.145	.7087
run	3	12.594	.0002
selected line x run	3	.432	.7329
Residual	16		

ANOVA Table for density of immunoreactivity, collapsed over selected line

	df	F-Value	P-Value
replicate line	1	3.622	.0752
run	3	13.679	.0001
replicate line x run	3	1.674	.2124
Residual	16		

ANOVA Table for overall density of immunoreactivity in the CA1 (all lines)

	df	F-Value	P-Value
run	3	17.578	.0001
Residual	20		

Run Mean ($\bar{x}_r$): Mean of individual mean densities of immunoreactivity grouped by run

run	# of animals	# synapses (n)	mean density
1	4	46	82.17
2	5	51	70.45
3	8	76	42.31
4	6	65	41.27

Grand Mean ($\bar{x}_g$): Mean of all individual means in CA1

N_g	SS_g	$\bar{X}_g$
238	13271.08	55.76

Correction Factor for GABA immunoreactivity in the CA1

run	n	sum of squares	$\bar{x}_g$	$\bar{x}_r$	Correction Factor	Corrected Mean Density
1	46	3779.86	55.76	82.17	**-26.41**	54.29
2	51	3592.96	55.76	70.45	**-14.69**	55.77
3	76	3215.87	55.76	42.31	**+13.45**	55.43
4	65	2682.39	55.76	41.27	**+14.49**	56.15

Correction Factor for GABA immunoreactivity in the SSC

ANOVA Table for density of immunoreactivity, collapsed over replicate line

	df	F-Value	P-Value
selected line	1	2.655	.1216
run	3	16.583	.0001
selected line x run	2	6.679	.0072
Residual	17		

ANOVA Table for density of immunoreactivity, collapsed over selected line

	df	F-Value	P-Value
replicate line	1	.052	.8221
run	3	8.831	.0011
replicate line x run	3	.886	.4693
Residual	16		

ANOVA Table for overall density of immunoreactivity in the SSC (all lines)

	df	F-Value	P-Value
run	3	9.833	.0003
Residual	20		

Run Mean ($\bar{x}_r$): Mean of individual mean densities of immunoreactivity grouped by run

run	# of animals	# synapses (n)	mean density
1	3	39	71.10
2	6	78	73.25
3	9	103	42.37
4	5	63	39.23

Grand Mean ($\bar{x}_g$): Mean of all individual means in SSC

N_g	SS_g	$\bar{X}_g$
283	15322.51	54.14

Correction Factor for GABA immunoreactivity in the SSC

run	n	sum of squares	$\bar{x}_g$	$\bar{x}_r$	Correction Factor	Corrected Mean Density
1	39	2772.88	54.14	71.10	**-16.96**	55.01
2	78	5713.78	54.14	73.25	**-19.11**	54.28
3	103	4364.37	54.14	42.37	**+11.77**	53.26
4	63	2471.48	54.14	39.23	**+14.91**	53.64