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**NEUROCHEMICAL AND NEUROCYTOLOGICAL ASPECTS OF THE
PORCINE STRESS SYNDROME**

Iowa State University

PH.D. 1983

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**Neurochemical and neurocytological aspects of the
porcine stress syndrome**

by

Lih-Fen Lue

**A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY**

**Department: Animal Science
Major: Meat Science**

Approved:

Members of the Committee:

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Ames, Iowa**

1983

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ABBREVIATIONS

ACh	acetylcholine
AN	all of the neurons
CA	catecholamine
CN	caudate nucleus
CPK	creatine phosphokinase
DA	dopamine
DAergic	dopaminergic
DP	dense projection
E-C	excitation-contraction
GABA	γ -aminobutyric acid
GABAergic	γ -aminobutyric acid-ergic
GABA-T	GABA- α -ketoglutarate transaminase
GAD	glutamic acid decarboxylase
GP	globus pallidus
HC	Huntington's chorea
HRP	horseradish peroxidase
IC	intracleft line
MH	malignant hyperthermia
NN	neurons stained with a nucleolus
PB	postsynaptic band
PD	Parkinson's disease
PLP	pyridoxal-5'-phosphate
PN	neurons stained with a process

PSE	pale, soft, exudative
PSS	porcine stress syndrome
SN	substantia nigra
SNC	pars compacta of substantia nigra
SNR	pars reticulata of substantia nigra
SR	stress-resistant
SS	stress-susceptible
SSADH	succinic semialdehyde dehydrogenase
STN	subthalamic nucleus

INTRODUCTION

The porcine stress syndrome (PSS) is a well-characterized, potentially fatal disease, particularly affecting pigs that demonstrate fast growth rates, high feed efficiency, and superior muscling (Campion and Topel, 1975). The clinical features of the PSS are muscle tremors, cyanosis, dyspnea, muscle rigidity, metabolic acidosis, and hyperthermia (Topel et al., 1968). Biochemical changes in blood plasma, such as elevation of creatine phosphokinase (CPK), lactic dehydrogenase, inorganic phosphate, potassium, calcium, magnesium, and catecholamines were observed (Brucker et al., 1973; Lister et al., 1977). The PSS can be induced by environmental factors, such as crowding, separation, exercise, fighting, weaning, mating, or slaughtering. The postmortem manifestation of the PSS is the formation of pale, soft, exudative (PSE) meat, which is commercially undesirable (Briskey, 1964; Cassens et al., 1975). The PSE condition is linked with rapid postmortem glycolysis (Bendall and Wismer-Pedersen, 1962; Cheah and Cheah, 1979), and denaturation of myofibrillar and sarcoplasmic proteins (Bendall and Wismer-Pedersen, 1962). This problem of poor-quality meat has seldom been observed in normal swine because the time required for chilling and processing of the animal is generally not slow enough, nor the metabolism sufficient

to produce the marked acidosis combined with high carcass temperature peculiar to the susceptible animals (Briskey, 1964; Nelson et al., 1974). There is a general belief that the intensive selection for increased muscling and fast-growing pigs has predisposed animals to undesirable characteristics both antemortem (stress-susceptibility) and postmortem (poor meat quality) (Christian, 1972; Cassens et al., 1975).

In 1966, Hall et al. reported that malignant hyperthermia (MH) could be induced by halothane and succinylcholine in stress-susceptible (SS) pigs. There is little doubt now that MH in swine is an anesthetic manifestation of a generalized susceptibility to stress, not only because of symptom similarities, but also because of the parallel biochemical processes described in PSS, PSE, and MH in swine (Briskey, 1964; Forrest et al., 1968; Nelson et al., 1974; Sybesma and Eikelenboom, 1978). Porcine MH has been suggested as a model for human MH (Jones et al., 1972), although some differences exist between these two cases. For instance: (1) histologic abnormalities are found in people, but seldom in swine; (2) MH can develop in swine in the absence of anesthetic drugs, but this occurs rarely in man; (3) total serum calcium increases in pigs, while it more often decreases in people (Gronert, 1980).

PSS and human MH are both genetic diseases. Their

genetic mechanisms, however, have not been well-determined. Porcine inheritance of MH susceptibility has been proposed, respectively, to be autosomal dominant (Allen et al., 1970; Jones et al., 1972; Williams and Lasley, 1977), recessive with variable penetrance (Christian, 1974; Minkema et al., 1976; Mabry et al., 1981), or multifactorial via two genes or alleles (Britt et al., 1969; Smith and Bamptom, 1977). Human MH inheritance may have a multifactorial inheritance with a range of susceptibility (Kalow et al., 1979; Ellis et al., 1977).

The etiology of the PSS is unknown, but considerable research has been done on skeletal muscle because the major metabolic defects and heat production have been found in this tissue (Sybesma and Eikelenboom, 1969; Berman et al., 1970; Gronert et al., 1977). The clinical features of the PSS are produced by an excess of Ca^{+2} in the myoplasm, which initiates the biochemical events and ultimately leads to severe acidosis through an increase in lactic acid formation (Moulds and Denborough, 1974; Gronert, 1980). The causes that trigger excess release of Ca^{+2} , however, are not well-determined. In resting skeletal muscles, the myoplasmic Ca^{+2} is stored within intracellular compartments (i.e., sarcoplasmic reticulum and mitochondria). But, a Ca^{+2} pool which is in equilibrium with extracellular Ca^{+2} also exists (Nelson and Chausmer, 1981). It is not clear

that the site and nature of the defects are linked with sarcoplasmic reticulum, mitochondria, or sarcolemma. Indeed, disorders in sarcoplasmic reticulum (Britt et al., 1973; Nelson, 1978), mitochondrial membranes (Britt et al., 1975; Cheah and Cheah, 1976, 1981; Heffron and Isaacs, 1976) and the sarcolemma (Britt et al., 1977; Willner et al., 1981) have been related to myoplasmic Ca^{+2} abnormality. There is another possibility that the lesion of the PSS may be associated with muscle excitation-contraction (E-C) coupling, because the mechanism by which halothane produces abnormal contracture of MH appears to be related to an extracellular Ca^{+2} -dependent pool involved in E-C coupling (Nelson and Chausmer, 1981). Furthermore, dantrolene, a unique muscle relaxant which directly acts on E-C coupling, has been used to prevent and reverse abnormal halothane contracture in MH pigs (Morgan and Bryant, 1977).

In addition to major clinical features in the skeletal muscle, subclinical symptoms of MH have been observed in the red blood cell. These include red blood cell fragility (Harrison and Verburg, 1973), increased Heinz body formation and reticulocytes (Sybesma and Zuidam, 1968), and methemoglobinemia. Recently, Schanus et al. (1981) reported that pigs suffering from MH have a deficiency in the glutathione peroxidase system of the red blood cell. They proposed that this deficiency was the molecular basis which allowed abnormal

oxidative damage in the red blood cell membrane of MH pigs. The membrane oxidative damage, thus, caused the failure of protective mechanisms in MH pigs. Similar deficiencies exist in the liver and muscle of halothane reactive pigs (Schanus et al., 1981). Therefore, it is possible that a generalized membrane defect which may cause changes in permeability may play a role in the cause of the PSS.

Other disorders, including hormonal (Cassens et al., 1975; Gronert, 1980) and neurological abnormalities (Gronert, 1980) have been reported. Thyroid function has been reported as both increased (Eighmy et al., 1978) and diminished (Briskey, 1964; Judge et al., 1968; Lister, 1973) in SS pigs. Circulating epinephrine and norepinephrine concentrations were all increased markedly during MH (Gronert, 1980; Davis, 1982). The sympathetic involvement was thought to be secondary (Gronert, 1980). The central nervous system contribution to the etiology of the PSS has been less documented. Recently, however, it has been shown that a catecholamine (CA) deficiency exists in the caudate nucleus (CN) and the substantia nigra (SN) of SS pigs (Altrogge et al., 1979; Hallberg et al., 1983). From these results, attention has been directed to similarities between the PSS and Parkinson's disease (PD) in humans (Hallberg et al., 1983). PD is a disease producing motor disturbances associated with basal ganglia dysfunction (Dray, 1980). Because

the PSS also shows muscle tremors and muscle rigidity during the syndrome, it is important to learn whether these disorders are associated with a basal ganglia dysfunction.

The purpose of this dissertation is to explore whether basal ganglia play a role in the mechanism of the PSS. Neurochemical and neurocytological studies were conducted to determine the role of basal ganglia in the PSS.

EXPLANATION OF DISSERTATION FORMAT

This dissertation is arranged as a series of three papers dealing with: (1) the activity of a neurotransmitter-synthesizing enzyme, glutamic acid decarboxylase (L-glutamic-1-carboxylase, E.C. 4.1.1.15; GAD); (2) the neuronal and synaptic morphometric characteristics of the porcine caudate nucleus; and (3) a Golgi analysis of the porcine caudate nucleus. Each paper is complete in itself, with an introduction, materials and methods, results, and discussion sections; all references have been combined into one list appearing at the end. A general literature review is included and placed before the three papers. Following the three individual papers is a summary of the entire work.

LITERATURE REVIEW

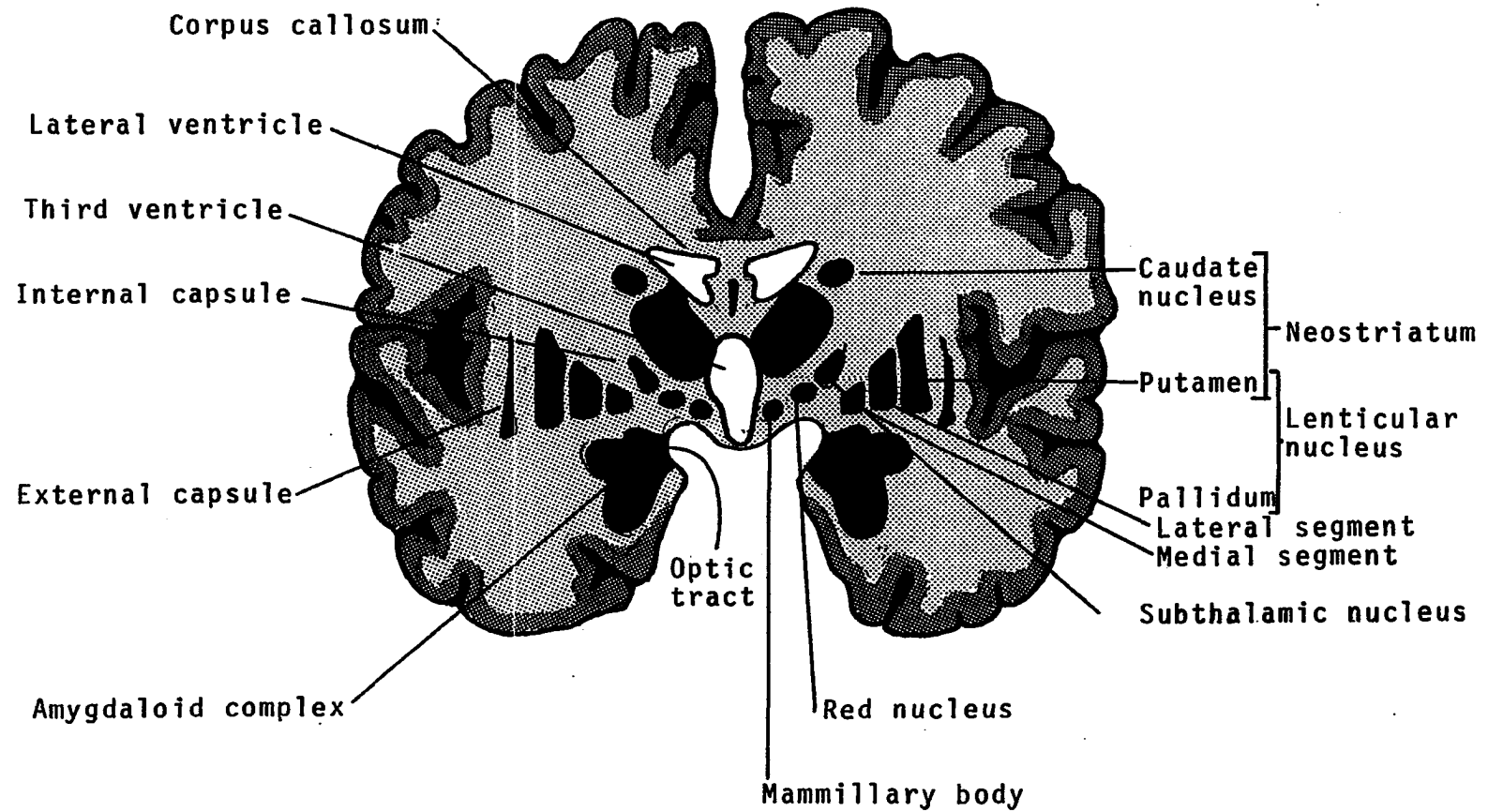
The Basal Ganglia

The components of the basal ganglia

The term basal ganglia has been used to describe certain large subcortical nuclear masses in the upper forebrain (Carpenter, 1976). Most commonly, the basal ganglia is limited to the caudate nucleus (CN), the putamen, the globus pallidus (GP), the subthalamic nucleus (STN), and the substantia nigra (SN). There are inconsistencies in the anatomical classification of the structures comprising the basal ganglia, so that the amygdaloid nuclear complex or other structurally and functionally related nuclei may be included (Carpenter, 1976; Dray, 1980). Figure 1 illustrates the major components of the basal ganglia.

The CN and the putamen, together referred to as the striatum, are separated by the internal capsule in the primate (Carpenter, 1976). They are considered as a single anatomical and functional entity, and constitute the largest component of the basal ganglia. Recently, Hassler (1978) suggested that the "fundus striati", which is situated basomedial to the CN or the putamen, should be included as one of the major parts of the striatum. Evidence to support this inclusion comes from the cytoarchitectonic and myeloarchitectonic similarities to the striatum, the

Figure 1. The basal ganglia and their relationship to other brain areas



developmental characteristics, and synaptic studies (Brockhaus, 1942; Swanson and Cowan, 1975; Hassler et al., 1978). The GP, located adjacent to the striatum, is the major recipient of the striatal outputs (Fox and Rafols, 1976; Carpenter, 1976). This nucleus is divided by a medial medullary lamina into medial and lateral pallidal segments. Both segments project to a small region called the STN (Nauta and Mehler, 1966; Fonnum et al., 1978). The STN is a small lens-shaped nucleus dorsal to the cerebral peduncles and internal capsule but rostral to the SN (Carpenter, 1976). The SN sits bilaterally as a band of cells between the cerebral peduncle and the tegmentum and is related functionally to the striatum by reciprocal connections (Dray, 1979).

The function of the basal ganglia

The basal ganglia occupy a large volume of the sub-cortical parts of the brain, but their functions are still not well-understood. Some clarification of function, however, has occurred during the past few years because of many clinical studies of human basal ganglia diseases. Pathologic lesions in the basal ganglia produce motor disturbances manifested by abnormal involuntary movements that include akinesia, chorea, athetosis, dystonia, ballism, and tremor (Marsden and Parkes, 1973; Marks, 1977). By

studying the cause of and therapy for the diseases, it has been shown that close correlation exists between the neurochemical and pathological profiles. For example, it is generally accepted that the loss of dopaminergic (DAergic) nigrostriatal neurons and dopamine (DA) deficiency in the striatum play an essential role in the pathophysiology of PD (Hornykiewicz, 1966; Berheimer et al., 1973). Other non-DAergic systems such as acetylcholine (ACh) and γ -aminobutyric acid (GABA) systems, may also be involved in the pathophysiology of PD (Lloyd and Hornykiewicz, 1973; Lloyd et al., 1975; McGeer and McGeer, 1976). PD is associated with the clinical features of muscle tremors, muscle rigidity, and restriction of movements. Huntington's chorea (HC), in contrast to those symptoms observed in PD, is characterized by choreiform movements. A primary lesion in HC is the considerable loss of striatonigral GABAergic neurons while the DA concentration is within the normal range, or at most, slightly decreased (McGeer and McGeer, 1976; Spokes, 1980). The exact functions of the basal ganglia, however, are still open to question. Several different points of view concerning their function have been cited in the literature. Martin (1967, cited by Dray, 1980), reported that the basal ganglia system, even though playing a cooperative role with other postural systems, has a separate identity and a large

measure of autonomy. Hassler (1978) proposed that the basal ganglia are involved in acts of "secondary automatisms", whereby, learned or acquired skills such as walking and running can be performed without intentional or conscious control. Conde et al. (1981) surveyed the literature and reported two lines of evidence on the function of the basal ganglia. The first aspect is from the study of Kornhuber (1971), who suggested that the basal ganglia act as a ramp generator for the command of "voluntary" goal-controlled movements, i.e., continuously regulated from the onset of the movement until the goal is reached. The second aspect is from the reports of Wise (1978), who emphasized a "cognitive" aspect of basal ganglia function. The term "cognitive" refers to those processes that add to the perception of the physical features of the stimuli which are known by the subject through its past experience. Comparisons of the neuropsychologic performance of Parkinson patients with age- and education-matched controls indicate several cognitive deficits, including impairment of memory, perception, and psychomotor speed (Reitan and Boll, 1971; Loranger et al., 1972; Portin and Rinne, 1980). Recently, Mortimer et al. (1982) was able to show significant correlation between motor and cognitive impairments in Parkinson patients, suggesting that cognitive impairment may result from the same subcortical lesions that cause motor symptoms.

Cools et al. (1981) stated that the basal ganglia seem to control the flexibility with which an organism copes with its environment. The striatum, in particular, seems to have the ability to control the ordering of behavioral units representing different levels of organization, i.e., behavioral strategies, individual behavioral acts, and motor patterns. Although the functions of the basal ganglia are not clearly understood, it is evident that functional heterogeneity is present in the basal ganglia.

The interconnections and neurotransmitters of the basal ganglia

There are numerous intra- and inter-connections in the basal ganglia. Also, several different chemical transmitters, such as GABA, DA, ACh, serotonin, substance P, and possibly enkephalin, function in the basal ganglia (Hökfelt et al., 1975; Hong et al., 1977; DiFiglia et al., 1982). These neuronal circuits and associated neurotransmitters will be reviewed with the focus on the connections between the striatum and the SN. These connections will be related to certain key pathways involving the rest of the basal ganglia.

Briefly, the neuronal circuitry of the basal ganglia include striatal afferents from the cortex, thalamus, SN, and dorsal raphe nucleus. The striatum projects to the SN and pallidum, and from the latter, outputs of the basal ganglia are sent to the thalamus, cortex, and mesencephalic

or pontine nuclei (Cheramy et al., 1981). Most of these pathways have been studied extensively. The existence of a striatonigral pathway arising from the striatum and terminating in the pars compacta (SNC) and pars reticulata (SNR) of the SN has been known for sometime (Grofova and Rinvick, 1970; Schwyyn and Fox, 1974). More recently, a descending pathway to the SN has been shown to arise from neurons in the pallidum (Grofova, 1975; Hattori et al., 1975; Kanazawa et al., 1976). Thus, both the striatonigral and pallidonigral pathways provide synaptic input to neurons in the SN. Results from biochemical studies of this brain region indicate that GABA and its synthesizing enzyme glutamic acid decarboxylase (GAD) are contained predominantly within axon terminals of the neurons that give rise to the striatonigral and pallidonigral pathways (Fonnum et al., 1974; McGeer et al., 1974; Brownstein et al., 1977; Gale et al., 1977). In addition, it has been shown by physiological and pharmacological studies that these two pathways monosynaptically inhibit neurons within the SN and that this postsynaptic inhibition is mediated by GABA (Feltz, 1971; Precht and Yosida, 1971; Ribak et al., 1980). The GABAergic terminals in the SN may terminate directly on dendrites of DA neurons and in addition, may form synaptic connections with interneurons, terminal afferents to the SN, and the nigral efferent projections (Cheramy et al., 1981).

Although GABA receptors in the SN can influence nigrostriatal DA activity, this pathway is probably a feedback for adjusting the flow of information through the striatum, in order to keep pace with nigral (non-DA) efferent function. There is evidence that the striatonigral feedback loop consists of both excitatory and inhibitory components (Walters et al., 1975; Costa et al., 1978). Substance P is speculated to be the excitatory transmitter in this feedback circuit (Mroz et al., 1977; James and Starr, 1979). Both the striatonigral and pallidonigral pathways have been shown to contain the transmitter substance P (Brownstein et al., 1977; Gale et al., 1977; Kanazawa et al., 1977). In the complementary experiments, Glowinski et al. (1980) found that the striatonigral substance P neurons exert a tonic facilitary role on the nigrostriatal DA cells.

The projection of the SN to the striatum, first demonstrated with histochemical and fluorescence microscopic methods (Anden et al., 1964), and later analyzed with these and other anatomical techniques (Grofova, 1979), has been shown to arise largely, but probably not entirely, from DAergic neurons in the SNC, DAergic dendrites are distributed in the SNC and SNR (Bjorklund and Lindvall, 1975). DAergic fibers, in contrast to those of non-DAergic fibers of SNR, do not emit axon collaterals within the SN (Juraska et al., 1977). There is evidence that DA can be released,

synthesized, and stored in DAergic dendrites (Bjorklund and Lindvall, 1975; Geffen et al., 1976; Shepherd, 1978; Pickel et al., 1976). DA is thought to regulate the activity of adjacent DAergic neurons through its dendritic release by a local feedback mechanism (Bjorklund and Lindvall, 1975). Receptors for DA are located on both the cell bodies and striatonigral terminals, as well as on nigral interneurons, nigral non-DAergic efferent neurons, and nigral DAergic dendrites (Gale et al., 1977; Spano et al., 1977; Cheramy et al., 1981). Thus, DA, released from the dendrites of the nigrostriatal DA neurons, is involved in the self-regulation of the DAergic cells to control the release of neurotransmitters from nigral efferent fibers and to influence the activity of the nigral non-DAergic cells (Cheramy et al., 1981).

In the striatum, DA-containing terminals may form synapses with striatal interneurons (cholinergic and possibly GABAergic) as well as with afferent projections from the cerebral cortex. The neurons which contain DA receptors in the striatum impinge either directly or indirectly (via other striatal interneurons) on efferent pathways which project to the GP and the SN (Dray, 1979; Bartholini, 1980; Gale, 1980).

It is known that the axon bundles of the nigrostriatal pathway traverse the GP (Lindvall and Bjorklund, 1979).

Recently, a DAergic nigropallidal projection has also been found (Fallon and Moore, 1978; Lindvall and Bjorklund, 1979). Because the GP forms the principal efferent pathway of the basal ganglia to the thalamus, SN, and STN, the nigropallidal DAergic innervation, albeit very sparse in comparison with the innervation of the striatum, may be in a highly strategic position to exert a modulatory influence on striatal output systems (Lindvall and Bjorklund, 1979).

Evidence for the presence of the striatocortical projections was obtained recently (Jayaraman, 1980), by using the horseradish peroxidase (HRP) tracing method. Saline injected HRP was transported from the cortex to cells in different thalamic nuclei, GP, and striatum. In the striatum, large cells contained the HRP reaction product, suggesting reciprocal connections between the cortex and the striatum.

Other neuropathways may exist in the basal ganglia. For example, the striatofugal enkephalin pathway has been found. This pathway may directly influence the efferent systems originating in the GP and the SN (Pickel et al., 1980; DiFiglia et al., 1982).

From the interconnections mentioned above, intricate neuronal circuits in the basal ganglia are evident. Among them, the nigrostriatal DAergic neurons display prominent functional roles both in the striatum and in the SN. In the striatum, DA, released from the terminals of the nigrostriatal

afferents, contributed to the filtering of the messages delivered to the striatum by various other afferent fibers, including the cortico- and thalamo-striatal fibers. Striatal DA also modulates the transit of signals which converge on the GP and the SNR by influencing the activity of large populations of striatal cells. In the SN, DA, released from nigro-striatal dendrites, is involved in the processing of information passing through the SNR in the direction of the thalamus, the STN, and several mesencephalic or pontine nuclei. Therefore, it is feasible to say that the nigro-striatal DAergic system has a critical role in the coordination of sensory-motor processes.

The morphology of the striatum

Cell arrangements The CN and putamen are cytologically identical tissues, densely packed with cells. It has been shown by recent studies that regional arrangements of the cells and dendrites are present in at least cats and rodents (Kemp, 1968; Chronister et al., 1976; Mensah, 1977). Mensah (1977) observed clusters of 10 to 15 medium cells which may take the form of a clumped mass of cells in the mouse. Clumps may be composed of one large and several medium cells or of only medium cells. Around the axon fascicles of the internal capsule, cell clusters with their dendritic bundles are arranged into a ring-shape.

These three types of the cell clusters, i.e., clumped medium and large cells, clumped medium cells, and medium cells in ring-like arrangement occur through the anteroposterior and dorsoventral part of the CN.

Very similar cell arrangements were demonstrated by Golgi studies in the cat (Chronister et al., 1976). These authors showed that the dominant organization was the cell clusters and ring formation, although they did not comment on large cell participation in cell clusters. Fentress et al. (1981), however, while describing medium cell clusters in groups of 10 to 30, saw no evidence that large neurons were distributed within the central position of the striatum in rats and mice, as Mensah (1977) has suggested. They observed a more-or-less randomly distributed pattern of large neurons throughout the mediolateral and rostrocaudal region of the striatum.

There is increasing evidence for a heterogeneous organization of striatal connections and certain features of the intrinsic cytochemistry. Royce (1978) and Kalil (1978) demonstrated, respectively in cats and mice, a patch-like termination of the centromedian thalamostriatal projections in the CN and putamen. In addition, Goldman and Nauta (1977) studied autoradiographically the corticostriatal projections and found the grains were not uniformly distributed but rather were segregated into clusters in the CN. Individual clusters

of grains, circular or elliptical in shape, surround grain-free cores. They suggested that the CN is organized more as an anatomic and functional mosaic than as the homogeneously organized structure that it is commonly considered to be.

Goldman-Rakic (1982) examined the monkey CN in Nissl-stained material, in which the corticostriatal terminals had been labelled by anterograde transport of tritiated amino acids injected into prefrontal cortex. It was revealed by cytoarchitectonic analysis that there are two cellular compartments that can be distinguished on the basis of cell size, density, orientation, and tinctorial properties: (1) cell islands consisting of approximately 1500 to 15,000 densely packed neurons that form aggregates of variable shapes and sizes embedded and (2) a matrix compartment of slightly larger and more loosely packed neurons that comprise the remaining part of the CN. The corticostriatal connections project only to the matrix zone and not to the territory occupied by island cells. These compartment arrangements in the CN may be related to the histochemical and functional diversity of the striatum.

Cell types Cell types in the striatum have been studied by Nissl-stain and Golgi impregnation methods. From the Nissl-stained material, usually two to three types of

neurons have been recognized. Carpenter (1976) described small achromatic neurons and large multipolar neurons in the striatum. Mensah (1977) reported that the overwhelming majority of cells in the CN fall into one of two size categories: 10 to 14 μm or 15 to 20 μm in diameter. Both categories were considered as medium cells in that study and all have large, pale nuclei, surrounded by a thin rim of cytoplasm and very little Nissl substance. Another type of cell mentioned in the same study was the large cell with a diameter larger than 20 μm . These cells are multipolar or fusiform in appearance. They are very prominent in the population due to their size and abundant Nissl content.

Adinolf and Pappas (1968) showed that the majority of neurons in the cat CN are small neurons (diameter: 10 to 15 μm). Larger neurons (diameter: 18 to 20 μm) are also present but much less abundant. Bugiani et al. (1978) also reported neurons with a diameter between 10 and 15 μm as the major cell type in the human putamen. Large neurons, however, were classified as having a diameter larger than 30 μm .

Greater detail of neurons can be revealed in Golgi preparations (Scheibel and Scheibel, 1978), permitting morphologically distinct types of neurons to be identified. A variety of neuronal types with varied terminology have been disclosed by different investigators using different modifications of Golgi techniques on different species. For

example, Fox et al. (1971-1972a) found four types of neurons in the monkey; DiFiglia et al. (1976), Rafols and Fox (1979), and Eder et al. (1980) found six types in the monkey. Kemp and Powell (1971a) described six types of neurons in the cat, while Eder et al. (1980) described seven types in the cat. In the comparison of the Golgi neuronal architecture of the CN in rabbit, cat, monkey, and human, Eder et al. (1980) suggested that the number of types and subtypes of neurons show an increased order in the phylogenetic series.

The most prominent characteristic used to classify the Golgi neuronal types is the presence or absence of dendritic spines. In the striatum, spiny and aspiny neuronal types form two major groups, within which further subdivision can be made according to the somatic size, distribution pattern of the spines, arrangement of the dendrites and the axon characteristics (Fox et al., 1971-1972b; Kemp and Powell, 1971a; Pasik et al., 1976).

In the spiny category, the most frequently impregnated type of neuron is designated differently by various authors: spiny neurons (Fox et al., 1971-1972a), medium spiny cells (Kemp and Powell, 1971a), spiny I neurons (DiFiglia et al., 1976), medium neurons with dendritic spines (Rafols and Fox, 1979), and medium-size spiny neurons (Eder et al., 1980). These types of neurons comprise over 95% of the total detectable caudate neurons in Golgi preparations (Fox et al., 1971-1972a;

Kemp and Powell, 1971a; Kocsis et al., 1977). The cell body of this common type of neuron may be round, ovoid, fusiform, or triangular. The primary dendrites and initial portion of the secondary branches are usually spine-free, but the rest of the secondary branches and tertiary branches are studded with numerous pedunculated and sessile spines. Generally, the dendrites radiate in all directions for a distance of 150 to 200 μm and do not taper (DiFiglia et al., 1976; Rafols and Fox, 1979). The axons of medium-sized spiny neurons are longer than 1.0 mm (Wilson and Groves, 1980) and are thought to project to the GP and to the SN (Grofova, 1975; Kitai et al., 1976; Kocsis et al., 1977; Somogyi and Smith, 1979; Preston et al., 1980). These striatal spiny neurons have been shown to have an extensive local axon collateral system in cat and monkey (Fox et al., 1971-1972a; DiFiglia et al., 1976; Kocsis et al., 1977). Somogyi and Smith (1979) speculate that the striatal spiny neurons represent the GABA-containing neuronal projection to the SN and that local axon collaterals of these neurons might be involved in GABAergic interactions in the striatum.

Ribak et al. (1979), using immunocytochemical methods, showed the presence of GAD-positive reaction products in the somata and dendrites of striatal medium neurons. This result is in accord with the finding that medium-sized, spiny

neurons of the striatum give rise to a GABAergic striatonigral pathway. Reaction products of GAD were present within nerve terminals in the striatum, which indicated that the intrinsic local circuits in the striatum involve GABAergic mechanisms.

The medium-sized spiny neurons seem to be involved in utilizing other transmitters in addition to GABA. According to the more recent reports from DiFiglia et al. (1982) and Pickel et al. (1980), using immunoreactive methods, immunoreactive Leu-enkephalin was contained within medium-sized neurons.

In addition to medium spiny type I neurons, other spiny neuronal types also have been distinguished. For example, a spiny type II neuron can be differentiated from spiny type I neurons by its spine distribution pattern. The spiny type II neuron has spines on all portions of the cell, including the soma and primary dendrites (DiFiglia et al., 1976). The density of spines is only one-third the density observed in the spiny type I. Some of the spiny type II neurons are very large with the mean cross sectional area ranging from 500 to 1300 sq. μm . There is quantitative evidence from the work by Pasik et al. (1976), showing a relatively constant spine distribution along the dendrites of the spiny type II neurons in contrast to the presence of a peak distribution of spines at a distance of 48 to 72 μm from the soma in the spiny type II. The spiny type II neurons are less frequently

impregnated than type I neurons.

The aspiny category accounts for only 2 to 5% of the striatal cell population in Golgi preparations. These cells range in size from giant (40 to 50 μm in diameter) to small (10 to 12 μm in diameter) (Pasik et al., 1976; Eder et al., 1980). Three major types of aspiny neurons are noted in adult dogs (Tanaka, 1980): the medium aspiny type I cell with swirling varicose dendrites and short axon, the medium aspiny type III cells with straighter varicose dendrites, and the large aspiny type II cell, also with straight varicose dendrites. Type I had as many as six smooth and beaded dendrites radiating from it. The primary dendrites were often straighter and less varicose than the secondary or tertiary branches. The aspiny III cell differed from the aspiny I cell in that the dog lack the swirling dendritic pattern of the large aspiny cell described by DiFiglia et al. (1976) in the monkey. There are four types of aspiny neurons in the monkey. Aspiny I cells, being of medium-size, usually have 5-8 smooth-surfaced primary dendrites which emerge from somata. One of these dendrites is usually rather thick. The secondary branches are often thin and varicose and frequently curve about the soma, covering a relatively small dendritic field.

Aspiny type II are the largest neurons in the striatum. Ten or more smooth dendrites emerge from somata, branch many times and course in a swirling fashion around the neuronal body within a radius of about 250 μm . Most of the dendrites are highly varicose. Aspiny III type, being of small to medium size, have primary dendrites which branch rather sparsely within a dendritic field of about 150 μm radius. The dendritic surface appears irregular, and in some of these neurons, it exhibits an occasional spine-like process or a few varicosities. The fourth type is the so-called neurogliform neuron, in which at least 10 processes emerge from the somata, each branching several times. They give off many twigs and varicose appendages and cover a radius of 60 to 80 μm from the cell body.

The synapses of CN Numerous CN synapses can be observed readily with the electron microscope (Fox et al., 1971-1972a,b; Kemp and Powell, 1971a; Pasik et al., 1976; Hassler, 1978). Hassler and Chung (1976) and Chung et al. (1977) described nine distinct types of synapses in the striatum. The type I axo-spinous synapses (Bak et al., 1975) are characterized by small (less than 0.5 μm) axon terminals containing loosely arranged, small (40 nm), spherical, pale vesicles, terminating asymmetrically on a trapezoid spine of similar size which contains an irregular

spine apparatus. Type II synapses are "en passant" between a long axon and a large, often parallel running dendrite or a cell membrane. Only a few vesicles appear arranged in a row. Both type I and type II are from afferent terminals from the SN (Hassler et al., 1978). The type III synapse is an axo-spinous synapse with a narrow terminal bouton densely filled with small, spherical, electron-dense vesicles. There is always a strong subsynaptic thickening and the contact with a spine is short and straight. This type of synapse is of cortical origin (Hassler et al., 1978). One-third of all synapses in the CN are axo-spinous type IV synapse with a curved and divided asymmetric contact. Type IV synapses come from centro-median parafascicular thalamic inputs. Type V synapses are pleomorphic because of the large and pale vesicles, usually intermingled with some dense core vesicles. It forms contacts with large or medium-sized dendrites or with cell membranes without a postsynaptic thickening. Type VI has a dark symmetric contact with the nerve cell membrane and are still of unknown origin. Type VII synapses are characterized by slightly asymmetric contacts between relatively large boutons and the membrane of large dendrites. The boutons are filled with small, round densely arranged vesicles, intermingled with mitochondria. This type of synapse is of cortical and thalamic origin. Type VIII synapses show no specialization except that the sparsely

distributed round vesicles are accumulated near the symmetric contact. Sometimes this type of synapse shows a presynaptic thickening, perhaps resulting from the fusing of dense core vesicles. They are probably the dendritic terminal of Golgi type II cells. Type IX synapses represent contacts between the small intrinsic and the large efferent neurons. They are characterized by sparsely distributed, large spherical, pale vesicles (about 60 nm in diameter). The vesicles tend to accumulate towards the symmetrical contact with a large dendrite. It is the most frequently encountered axo-dendritic type of synapse in the CN (Hassler et al., 1978).

The correlation between the ultrastructural characteristics of these synapses and their chemical and pharmacological properties is not sufficiently understood. The striatum contains various transmitters. Much work has been done recently to clarify the neurochemical features of the specific synaptic types (Ribak et al., 1979; Kaiya and Namba, 1981; DiFiglia et al., 1982). GAD-positive terminals predominantly form symmetric synapses with somata, dendrites and spines, but a small number of them form asymmetric synapses with either dendrites or spines (Ribak et al., 1979). The monoaminergic synapses are divided into two types, according to their ultrastructural features. Type A is composed of a relatively large axon terminal and a dendritic spine with a

postsynaptic membrane thickening. The axon terminal of type B, that is a bouton en passant, is smaller than the postsynaptic profile, while the synaptic contact is symmetrical (Kaiya and Namba, 1981). Leu-enkephalin reactive terminals in the striatum have been identified to make symmetric contacts with unlabeled somata of spiny type I neurons and aspiny type I neurons and with the primary and distal branches of unlabeled spiny and aspiny dendrites. Few immunoreactive boutons synapse with unlabeled dendritic spines. Some immunoreactive Leu-enkephalin terminals form synapses with cell bodies and dendrites which also are positively labeled for Leu-enkephalin. The Leu-enkephalin reactive products are found within medium-size spiny neurons (spiny type I in the monkey), which are known to have axon collaterals intrinsic to the CN and to project to the GP and the SN (DiFiglia et al., 1982).

GABA and GAD in the Basal Ganglia

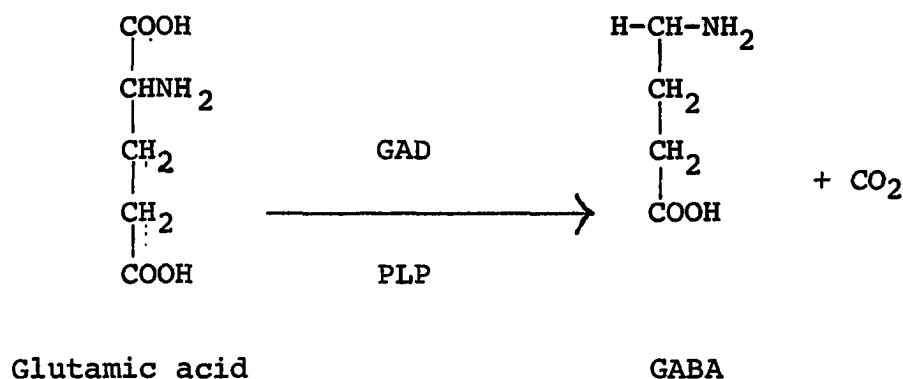
GABA is believed to be one of the major inhibitory neurotransmitters of the vertebrate central nervous system (Roberts, 1976). The brain content of GABA is 200 to 1,000-fold greater than that of other neurotransmitters such as DA, noradrenaline, ACh, and serotonin. Uptake studies with labeled GABA suggest that 25-45% of nerve endings, depending on the brain area, may contain this neurotransmitter (Iversen

and Schon, 1973). Some of the greatest GABA concentrations are found in the basal ganglia, particularly in the GP and the SN (Okada et al., 1971). Several neuropathways in the basal ganglia have been identified to be GABAergic and may participate in the extrapyramidal function (Kim et al., 1971; McGeer et al., 1971). There are pallidonigral (Hattori et al., 1973; McGeer et al., 1974; Ribak et al., 1980), striatonigral pathways (Gale et al., 1977; Ribak et al., 1980), and striatal interneurons (Bunney and Grace, 1978) that use GABA as the neurotransmitter. The GABA neurons, thus, interact with other neurosystems in the basal ganglia to form a complex network of neuronal circuits and play a prominent role in the function of the basal ganglia.

The metabolism of GABA

The immediate precursor of GABA is glutamate, which can be formed from either glutamine or α -ketoglutarate. Glucose, the main energy source of the brain, is probably the principal in vivo carbon source for glutamate synthesis; glutamate, in turn, is converted to GABA. The GABA shunt that has been proposed by Roberts (1956) contains three enzymes: GAD that converts glutamate to GABA, GABA- α -ketoglutarate transaminase (GABA-T) that converts GABA to succinic semialdehyde, and succinic semialdehyde dehydrogenase (SSADH) that returns the metabolic remnant to the Kreb's cycle as succinate.

GABA is biosynthesized by GAD in nervous tissue. GAD catalyzes the formation of GABA by the decarboxylation of glutamate, which requires the cofactor pyridoxal-5'-phosphate (PLP) (Roberts and Simonsen, 1963).



The cellular and subcellular localization of GAD is important because GAD is a specific marker for neurons that synthesize GABA and GAD activity may reflect sites of neuronal release of GABA (Gold and Roth, 1979; Tapia et al., 1975). The nerve endings of GABAergic neurons have been defined clearly by immunocytochemical studies of GAD (Ribak et al., 1977). It is known that GAD is concentrated mainly in the synaptosomes of the GABAergic neurons (Fonnum, 1968; Roberts, 1978), suggesting that the formation of GABA may occur in nerve endings in vivo. The activity of GAD is correlated positively with the distribution of GABA (Fonnum and Walberg, 1973; Tappaz et al., 1976; Yoneda and Kuriyama, 1978).

The GAD in brain tissues is particularly sensitive to in vitro (Roberts et al., 1964; Tapia et al., 1967) and in vivo (Tapia and Awapara, 1969) deficiencies of PLP. Furthermore, the dependence on PLP concentration in vivo has been demonstrated to occur in synaptic terminals (Perez de la Mora et al., 1973). Itoh and Uchimura (1981), who studied the regional distribution of GAD with and without PLP added in discrete brain regions, demonstrated uneven and lower activity (about 40-80%) in the absence of PLP. GAD activity, in the absence of exogenously added PLP, is thought to reflect the true neuronal rate of GABA formation, because GABA is produced exclusively by GAD-PLP holoenzyme. Modulation of cofactor binding to the GAD apoenzyme, thus, may constitute one means by which the rate of GABA synthesis is regulated.

Catabolism of GABA seems to be catalyzed exclusively by GABA-T. Great concentrations of this enzyme have been found in the basal ganglia, cerebral cortex, and cerebellar cortex (Salvador and Albers, 1959). The distribution of GABA-T in the brain differs somewhat from that of GABA (Albers and Brady, 1959). GABA-T was thought to be in the neurons postsynaptic to GABA neurons, or in the surrounding glial elements (Baxter, 1976). Vincent et al. (1980), however, demonstrated that neurons which receive GABA synapses do not necessarily

contain GABA-T. For example, GABA-T activity is lacking in the striatal AChergic and nigral DAergic neurons. Furthermore, they determined that all of the striatal GABA-T activity is neuronal, and probably associated with GABA neurons themselves. Likewise, nigral GABA-T activity is suggested to be contained in the perikarya of the GABAergic nigral efferents. There are two forms of GABA-T found in pig brain (Bloch-Tardy et al., 1980). Their biological significance is not yet understood. The ratio of activity of GABA-T to that of GAD is always greater than one, indicating that the activity of the GAD system is the rate-limiting reaction in determining brain GABA concentrations (Hall et al., 1970).

The distribution of GABA and GAD

Studies of GABA and GAD distribution in brain have been done in monkeys (Albers and Brady, 1959; Fahn and Côté, 1968; Jacobowitz et al., 1980), rats (Tappaz et al., 1976; Arregui and Barer, 1980), cats (Fonnum et al., 1974), and humans (Bird and Iversen, 1974; McGeer and McGeer, 1976). In the monkey, SN and GP were reported to have the greatest GAD activity and GABA concentrations, with the hypothalamus having the next highest activity. In addition to SN and GP, high concentrations of GABA and GAD were also found in the striatum in humans. Greatest GAD activity in cats and rats occurred in the SN and hypothalamus.

Activity of GAD is known to be contained preferentially in nerve terminals of striatonigral and pallidonigral efferents in the SN (Kim et al., 1971; Ribak et al., 1980). Nagy and Fibiger (1980), however, demonstrated that GP is not the source of the massive GABAergic innervation of the SN. A shell-like topographical arrangement of neurons in the striatum is proposed to be responsible for the intense GABAergic innervation.

Distribution of GAD activity differs between the two parts of the SN. The SNC has a lower GAD activity than does the SNR (Tappaz et al., 1976). Subdivisions within the SNC and SNR also have shown activity differences, at least in the cat. In the SNC, the greatest activity was found in the lateral part which merges with SNR, with lowest activity in the medial part. In contrast, the SNR exhibited greatest GAD activity medially and lowest laterally (Fonnum et al., 1974).

The role of GABA as a neurotransmitter has been studied less in the hypothalamus than in other brain tissues. Both GABA and GAD, however, have been reported to be concentrated in the hypothalamus (Albers and Brady, 1959; Fahn and Côté, 1968). There is evidence that the distribution of GAD is uneven between various hypothalamic nuclei (Tappaz, 1978). Tappaz and Brownstein (1977) indicated that most GAD-containing cells have their origin inside of the hypothalamus.

They are likely short interneurons providing intrahypothalamic connections.

The developmental changes of GAD

Activity of GAD is increased during growth and development, and the rate of production of GABA by GAD in vertebrates also increases as the animal develops (Roberts and Kuriyama, 1968). Blinderman et al. (1978) studied the developmental changes of GAD activity in rats. They found that the total GAD activity in the brain reaches its maximum during the second and the third postnatal weeks (from 10 to 26 days). The adult value for total GAD activity in the brain seemed to be reached after 30 days of age. The increase in GAD activity is because of a parallel increase of the amount of specific enzyme for the period studied. Changes in GAD activity also have been studied in the brain of the newborn and pubescent monkey (Jacobowitz et al., 1980). Increase in the pubescent/newborn ratios of GAD activity occur in approximately half of the brain areas studied in the pubescent monkey. The largest increase (2.3-fold), is observed in the SNR.

GABA and GAD in human neurological diseases

Neurotransmitters and their associated enzyme abnormalities have been observed in several neuropathological diseases in humans. One such disease is PD, characterized by a variety

of motor disturbances, including muscular rigidity, restriction of movement, and muscle tremors. The major neuropathological observation in Parkinsonism is a loss of the melanin-containing cell bodies in the SNC. This is paralleled by the disappearance of DA, its synthesizing enzymes and its metabolites in the SN, CN, and putamen (Hornykiewicz, 1966; Bernheimer et al., 1973). Involvement of GABA pathways in PD is indicated by the decrease in GABA concentration and GAD activity in extrapyramidal areas (McGeer et al., 1971; McGeer and McGeer, 1976), especially in the striatum, GP, and the SN. This GAD change is not likely related to cell loss, but to the reduced DA input to the striatum, as the GAD activity of patients chronically treated with L-DOPA is similar to that of control patients (Lloyd and Hornykiewicz, 1973). Reduced GABA concentration and GAD activity in PD are considered as one of the compensatory responses in the basal ganglia, although other compensatory mechanisms, such as decreased ACh in the striatum, have also been demonstrated (Lloyd, 1977). Huntington's chorea, characterized by severe choreiform movements and progressive dementia, involves severe neuronal losses in the striatum, pallidus, STN, and cortex (Lange et al., 1976). The major neurochemical finding in HC has been the decrease in GAD activity and GABA concentrations, especially in the striatum and the SN (Bird and

Iversen, 1974; Spokes, 1980). The GAD activity in chorea patients is about 20% of that in the normal controls, although the GABA concentration is reduced to a lesser degree. Because loss of striatal neurons is a prominent pathological feature in HC, neurochemical deficiencies are believed to reflect the degeneration of both striatal interneurons and output cells to the SN. Other neurochemical systems, such as the striatal cholinergic neurons, also appear to be involved in HC (McGeer and McGeer, 1976).

Functional role of GABA in the basal ganglia

In the striatum, several lines of evidence have been used to suggest that DAergic pathways are involved in the control of GABAergic processes in the basal ganglia. An inhibitory action by DA on the release of GABA in the rat striatum has been observed (Van der Heyden et al., 1979). In the SN, a mutual interaction of GABAergic and DAergic processes may occur. Descending GABAergic neurons regulate nigral activity of DAergic neurons and GABAergic nerve terminals localized in both SNC and SNR (Fonnum et al., 1974, 1978; Ribak et al., 1977). Most of these terminals originate in the striatum and GP, as revealed by lesion studies or retrograde tracing techniques (Kim et al., 1971; Grofova, 1975; Bunney and Aghjanian, 1976). Stimulation of CN produces release of GABA in the SN (Kondo and Iwatsubo, 1978). The GABA within the SN

is thought to be contained primarily in the terminals of feedback pathways projecting from the caudate-putamen to the SN which modulate the activity of DA-containing neurons located in the SNC (Kim et al., 1971; McGeer et al., 1974). The GABA in the SN increases the firing rate of the DA neurons (Waszczak et al., 1980). In the rostral part of the SN, there is contrasting evidence that GABA may inhibit the DA neurons (Scheel-Krüger et al., 1977). The regional SN difference in GABA function seems in agreement with the findings of a recent anatomical study in which it was suggested that the topographical organization of the striatonigral pathway does not support the hypothesis of a simple feedback system directed towards the DA cells (Tulloch et al., 1978).

It is true that the activity of nigral GABA receptors influence nigrostriatal DA function, and the activity of striatal DA receptors influence striatonigral GABA function. GABA receptors in the SN, however, function in a role more than just to regulate DA activity (Gale, 1980). Gale (1980) considered the striatonigral fibers as a major outflow route for the neural information which has been integrated in the striatum, and this outflow information is monitored by a DA pathway or possibly by a non-DA nigrostriatal pathway as well, either by modulating cortico-striatal afferents or by changing the responses of striatal interneurons, in order to

keep it coordinated with nigral outflow. Nigral activity in SN may be routed via direct synaptic connections between afferent and efferent pathways, and, additionally, by direct connections mediated by nigral interneurons. The outflow information through the relay in the SN are then sent out through the efferent projections to thalamus, reticular formation, and superior colliculus.

In conclusion, the intricate relationship between DA and GABA neurons in the basal ganglia may not be the only matter which should be emphasized, particularly because other transmitters may be utilized in the basal ganglia. It remains to be determined how substance P and enkephalin may participate in the function of the basal ganglia.

The Age-Related Changes in the Striatum

The morphology of the central nervous system changes with age (e.g., accumulation of lipofuscin, loss of Nissl substance, appearance of neurofibrillary tangles, loss of dendrites, and dendritic spines) and biochemistry (e.g., alterations in neurotransmitters) is also altered. These neuronal changes may be responsible for the accompanying decrements in neurological function and abnormalities of behavior or mental state (Timiras and Bignami, 1976). Age-related brain disorders include depression, PD, HC, and

various degrees of mental deterioration often associated with memory impairment (Samorajski, 1975).

Age-related reductions of CA concentrations and its metabolism in the basal ganglia, hypothalamus, and other brain regions were observed in humans (Carlsson and Winblad, 1976; Riederer and Waketich, 1976), mice (Finch, 1973; Jonec and Finch, 1975), and rats (Ponzio et al., 1978; Demarest et al., 1980). The DAergic system, in particular, seems to be more susceptible to the aging process. Decreased DA synthesis (Samorajski, 1975), decreased tyrosine hydroxylase (McGeer and McGeer, 1976), decreased DA-sensitive adenylyl cyclase activity (Walker and Boas-Walker, 1973; Schmidt and Thornberry, 1978), and decreased DA receptor numbers (Severson and Finch, 1980) are all age-related changes that have been observed in humans and rodents. Decreased DA concentrations have also been found in both the nigrostriatal and tuberoinfundibular DA systems (Demarest et al., 1980).

Decreased DA concentrations generally are believed to reflect DAergic neuronal losses (Bernheimer et al., 1973). Nigrostriatal denervation, however, cannot explain all of the aging changes that occur in the striatum. In young rodents, denervation of the nigrostriatal system resulted in increases in binding of DAergic agonists and antagonists (Muller and Seeman, 1978; Creese et al., 1977), in DA-

sensitive adenyl cyclase activity (Mishra et al., 1974), and in striatal turnover (Agid et al., 1973). This is in contrast to the finding of decreased neuroleptic receptor binding, and decreased DA-sensitive adenyl cyclase activity with age (Walker and Boas-Walker, 1973; Govani et al., 1977; Schmidt and Thornberry, 1978). Moreover, a postdenervation supersensitivity response has been shown in older humans by greater spiroperidol binding in the Parkinsonian striatum despite a severe loss of nigrostriatal neurons (Lee et al., 1978). In addition, DA receptor binding and DA-sensitive adenyl cyclase activity decrease at an earlier age and a greater extent than do the presynaptic markers of DAergic neurons (Randall, 1980). Severson and Finch (1980) suggested that reductions of striatal DA receptors with age may indicate a loss of striatal neurons on which reside a population of DAergic binding sites. There are other findings which are consistent with this hypothesis. Decreased choline acetyltransferase activity has been determined in the rat striatum (McGeer et al., 1971) and human putamen (McGeer and McGeer, 1976). Bugiani et al. (1978) reported a 15 to 30% striatal neuronal loss with age. Lost neurons were both large and small in size. In quantitative studies, Brizzee et al. (1981) demonstrated that older animals had a decrease in both number and volume of neuronal elements, and an increase

in number and volume of glial elements in the ventrolateral portion of the caudate-putamen.

Morphological changes of striatal neurons with age have been demonstrated clearly with Golgi studies (Scheibel, 1981). Medium-size, spiny neurons were thought to be most responsive to the aging process. Scheibel (1981), when comparing 23 and 90 year old patients, indicated that the spine complement decreased dramatically in number, often by 50 to 75% or more. Spine morphology showed greatly increased variability, ranging from scarcely recognizable lumps or nubbins, through long sinuous filaments, to large mushroom-like structures with almost parasol-like terminal enlargements. The total dendritic-spine surface area decreased greatly, because of massive spine loss, and also to thinning and shortening of the dendritic shafts.

SECTION I: GLUTAMIC ACID DECARBOXYLASE (GAD) ACTIVITY IN
DIFFERENT BRAIN REGIONS OF STRESS-SUSCEPTIBLE
AND STRESS-RESISTANT PIGS

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INTRODUCTION

γ -Aminobutyric acid (GABA), synthesized from glutamic acid by action of glutamic acid decarboxylase (GAD), serves as an inhibitory synaptic transmitter in the central nervous system (CNS) of mammals (Baxter, 1976; Roberts, 1972; 1974). The concentration of GABA and the associated activity of GAD in the substantia nigra (SN) are among the highest found in any region of the brain. Other regions of the brain, such as the globus pallidus (GP), caudate nucleus (CN), and putamen, contain moderate to high concentrations of GABA and GAD activity (Hattori et al., 1973; Fahn, 1976). The major portion of GABA content in SN is associated with nerve terminals of fibers, arising from cell bodies located in the striatum and GP (Kim et al., 1971; Fahn, 1976; Gale et al., 1977). The GABA and GAD in the striatum, however, seem to be localized mostly in interneurons (McGeer and McGeer, 1976), but also in cell somata of striatopallidal and striatonigral efferent fibers (Ribak et al., 1979).

GABAergic neurons interact with DAergic neurons in the nigrostriatonigral loop (Dray, 1979; Bartholini, 1980; Casey et al., 1980). The GABA input on DAergic cells in the SN is exerted by the striatonigral pathway. Striatal GABA interneurons or collaterals of the striatonigral

pathway innervate DAergic terminals in the CN. In addition, GABAergic efferents in the striatum affect the postsynaptic system of DAergic neurons (Bartholini, 1980; Scatton and Bartholini, 1980). These neuronal circuits play a key role in the motor functions of basal ganglia (Dray, 1980). Therefore, alterations in any of these systems may account for the symptoms of basal ganglia disorders, such as Parkinson's disease and Huntington's chorea (Dray, 1980).

Pigs that have the porcine stress syndrome (PSS) recently have been reported to have a DA deficiency in their SN and CN (Altrogge et al., 1979; Hallberg et al., 1983). Besides the DA deficiency, other clinical features of the PSS, including muscle tremor and muscle rigidity, are similar to the symptoms of Parkinson's disease in humans. Because of these similarities (Hallberg et al., 1983), a mechanism parallel to that of Parkinsonism may be involved in the PSS. To test this hypothesis, it is important to determine whether there are alterations of other neurotransmitters in the basal ganglia of stress-susceptible (SS) pigs. In the present study, GAD activity was measured in the SN, CN, putamen, hypothalamus, and cerebral cortex to determine whether GABA concentrations were abnormal in the PSS.

MATERIALS AND METHODS

Animals

Pigs used in this study were bred and raised at Iowa State University's Swine Breeding Farm. Pigs were exposed to halothane between eight to ten weeks of age to determine whether pigs were stress-susceptible (SS). Each pig was exposed to 6% halothane vapor in oxygen for 3 minutes or until muscle rigidity ensued. A stress-susceptible or positive reaction was indicated by pronounced skeletal muscle rigidity, sometimes accompanied by blotching of the skin and nervous physical resistance to halothane administration. A stress-resistant (SR) or negative reaction was indicated by loss of consciousness under the influence of halothane anesthesia without muscle rigidity (Christian, 1974). In addition to the halothane test, H blood typing (Rasmussen and Christian, 1976) and serum creatine phosphokinase activity (Christian, 1974) also were used to ensure the correct classification of SS and SR pigs. Each group of SS and SR pigs was divided into two age groups: 76-148 (ave. = 107) and 173-187 (ave. = 184) days.

Tissue Collection and Handling Procedures

Pig brains were removed as soon as possible after electrical stunning and exsanguination. The CN, SN, putamen, hypothalamus, and cerebral cortex of each brain then were

dissected from the brain and immediately frozen in liquid nitrogen. Frozen tissues were stored at -80°C until assayed.

GAD Analysis

Frozen tissues were homogenized in 99 volumes of 0.1 M potassium phosphate buffer ($\text{pH} = 6.4$) containing Triton X-100 (0.25%, v/v) and 2-aminoethylisothiuronium bromide hydrobromide (1 mM). Activity of GAD in the crude homogenate was measured by using carboxy-labeled $[1-^{14}\text{C}]\text{-L-glutamate}$, according to a modification of the method of Albers and Brady (1959), as described by Tappaz et al. (1976). To initiate the reaction, 100 μl of tissue homogenate were added to a 25-ml vial containing 100 μl of potassium phosphate buffer (0.1 M, $\text{pH} = 6.4$) with 1 μCi of $[1-^{14}\text{C}]\text{-L-glutamic acid}$ (5 mCi/mmol, Amersham Corp.), potassium glutamate, and pyridoxal phosphate and kept in an ice bath. The final concentrations of glutamate and pyridoxal phosphate were 10 mM and 0.1 mM, respectively. A piece of filter paper, impregnated with 0.1 M hyamine hydroxide in methanol, was held in a small well, hanging under the stopper of the incubation vial to trap released CO_2 . Before incubation, the reaction vial was flushed with a fine stream of nitrogen. Contents of reaction vials were incubated at 37°C for 60 minutes. The reaction was terminated by injecting 200 μl of 6 N HClO_4 into each vial. Diffusion and trapping of CO_2 were allowed

to proceed for an additional 60 minutes of incubation. The pieces of filter paper then were transferred to counting vials containing scintillation fluids (Beckman), and radioactivity of the samples was determined by a liquid scintillation counter (Beckman Instruments Co.). Controls were prepared by replacing the tissue homogenate with phosphate buffer.

Protein Analysis

Protein concentration of tissue homogenates was determined according to the method of Lowry et al. (1951).

Data Analysis

Activity of GAD was expressed as pmoles of glutamate decarboxylated/mg protein per hour. Measures of the statistical differences between treatments and age groups were made by a two-way analysis of variance.

RESULTS

Activity of GAD was measured in five regions of the brain in SS and SR pigs. Results are shown in Table 1. Two brain regions had statistically significant differences in GAD activity. The SN of SS pigs had significantly greater amounts (about 20%) of GAD activity than did the SN of SR pigs. The GAD activity in the hypothalamus of SS pigs, however, was significantly less (about 14%) than that in the hypothalamus of SR pigs. The GAD activity of other brain regions was similar in the two types of pigs.

In the brain regions which we analyzed, GAD activity was not evenly distributed. Highest GAD activity (mean 729.08 ± 30.10) was found in the hypothalamus of the SR pigs, while CN and putamen exhibited moderate activities (415.60 ± 35.93 and 354.97 ± 48.32 , respectively). Mean GAD activity in SN was only 279.75 ± 30.10 in SR pigs. The cerebral cortex mean GAD activity was 228.98 ± 17.29 .

Values representing the effects of age on GAD activity are presented in Figure 1. The CN, putamen, and SN had similar GAD activity changes with age in both types of pigs. Significantly greater GAD activity was associated with the older age group. In the cerebral cortex, a slight, but significant, reduction of GAD activity was found in the

Table 1. Glutamic acid decarboxylase (GAD) activity in different brain regions of stress-susceptible and stress-resistant pigs

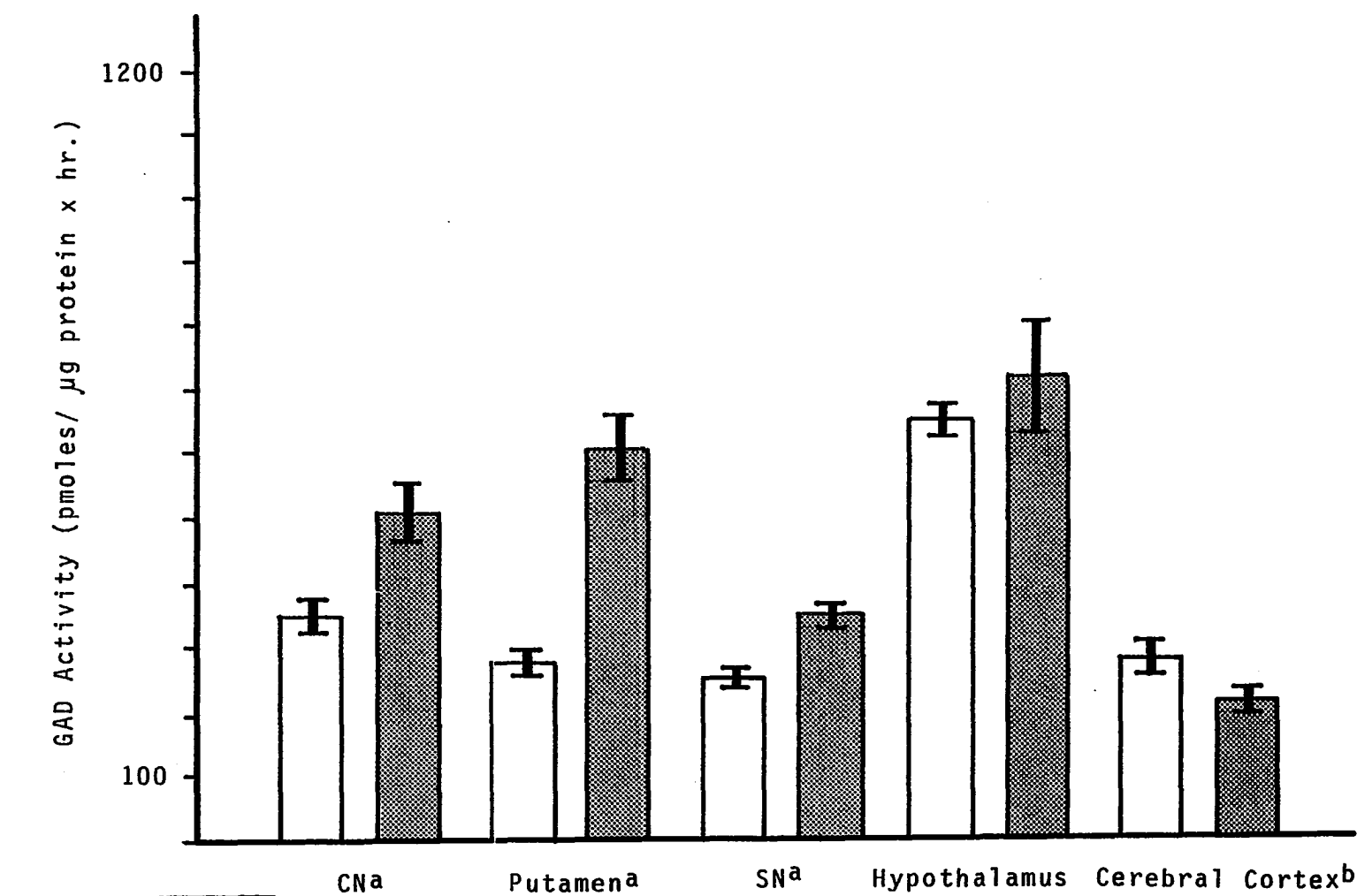
Brain regions	GAD activity (pmoles/ μ g protein x hr.)	
	Stress resistant	Stress susceptible
Caudate nucleus	415.60 \pm 35.93 (15) ^a	444.77 \pm 38.63 (14)
Putamen	354.97 \pm 48.32 (18)	408.83 \pm 35.15 (18)
Substantia nigra	279.75 \pm 16.78 ^b (21)	335.92 \pm 15.49 (12)
Hypothalamus	729.08 \pm 30.10 ^c (12)	626.08 \pm 32.39 (15)
Cerebral cortex	228.98 \pm 17.29 (9)	229.63 \pm 15.12 (9)

^aNumber of pigs in each group.

^bMeans for two types of pigs are different, $p < 0.01$.

^cMeans for two types of pigs are different, $p < 0.005$.

Figure 1. Activity of GAD in different regions of brain from pigs of different ages. Open bars are data from pigs that averaged 107 days of age and shaded bars are data from pigs that averaged 184 days of age



^{a,b}Indicates that means within a brain region are significantly greater at $p < 0.005$ and $p < 0.01$, respectively.

older age group. The hypothalamus, however, did not have any significant change in GAD activity with age.

DISCUSSION

The results showing that SS pigs had greater GAD activity in SN than SR pigs, but with similar activity in CN of both SS and SR pigs, are unlike the findings in human neurological diseases. In human HC and PD, considerably lower GAD activity has been observed in the SN and the striatum of affected patients (Dray, 1980). Especially in PD, where severe nigrostriatal dopamine deficiency exists, GAD activity reduction in the SN is about 78% of normal (McGeer and McGeer, 1976). In contrast, we found that GAD activity was about 20% greater in the SN of SS pigs, whereas, striatal GAD activity was unchanged.

There is evidence that CNS compensatory mechanisms occur in patients with PD (Lloyd, 1977) and in certain animal models (Melamed et al., 1982). Therefore, in PD, loss of DA neurons in SN and decreased DA release in the striatum reduces ACh synthesis and release which is necessary to maintain DA:ACh balance. The striatal GABA inputs to the SN decrease synthesis and release of GABA, allowing maximal firing of remaining DA neurons during the compensated stage. Acceleration of DA turnover, by surviving nigrostriatal neurons, and the development of striatal DA receptor supersensitivity in the rat animal model, may represent two compensatory mechanisms (Melamed et al., 1982). Although

SS pigs have a 30% DA deficiency (Hallberg et al., 1983) in the SN and CN, the finding of increased GAD activity in the SN of SS pigs in our study does not reflect similar compensatory mechanisms in the pig.

Increased GAD activity in the SN has not been reported in other studies. Nevertheless, higher concentration of GABA were found in the cerebrospinal fluid of patients with acute hypoxic encephalopathy than in the control subjects (Manyam et al., 1980). Anaerobic conditions, after decapitation, are also known to cause increases in GABA concentration in the rat brain (Minard and Mushahwar, 1966). In fact, Tappaz (1978) reported that, following decapitation and immediate freezing of tissues, postmortem increases in GABA levels are directly related to the GAD activity in the various hypothalamic and SN nuclei. It is not clear whether the GAD activity enhancement in the SS pigs reflects a severe anoxic state of the brain which may be induced by the immediate physiological response to slaughtering and decapitation.

GABA and GAD activity in the striatum are known to be present mainly in the interneuron population (McGeer and McGeer, 1975), although some may be present in the cell bodies of GABA-containing striatal output neurons whose terminals synapse in the GP and SN (Yoshida and Precht, 1971).

Because there was no apparent change of GAD activity in the CN of the SS pigs, this may represent an intact GABAergic interneuron population in the CN of the SS pigs.

There are many sources for the nigral GAD activity. If the differences of the GAD activity that we found in the SN of SS pigs do represent a pathophysiological alteration, then, it is difficult to determine whether the changes of GAD activity are of extrinsic or of intrinsic origin. The GAD activity in the SN may arise from nerve terminals of striatonigral (Roberts, 1976), pallidonigral (Hattori et al., 1973; Grofova, 1975), or possibly, of nucleus accumbens nigral tracts (Somogyi et al., 1981); the perikarya of nigral projections to tectum, thalamic nuclei (Vincent et al., 1978), and superior colliculus (DiChiara et al., 1979); and intrinsic nigral local neurons (Ribak et al., 1980). In addition, axons of the SNR projection neurons emit several intrinsic collaterals which arborize in both SNR and SNC (Karabelas and Purpora, 1980; Grofova and Fonnum, 1982). It is also not clear why GAD activity in the hypothalamus decreased in the SS pigs, although most of the GAD activity in this tissue is intrinsic (Tappaz and Brownstein, 1977).

The other major finding in this study was the increase in GAD activity with age in the SN, CN, and putamen, but

decreased GAD activity with age in the cerebral cortex and no change of the activity in the hypothalamus. The older age group animals in this study were pubescent. Our findings of increased GAD activity from age I to age II in SN, CN, and putamen are in agreement with studies in other species (Coyle and Enna, 1976; Brown and Brooksbank, 1979; Gottesfeld and Jacobowitz, 1978). Significantly higher GAD activity occurs during pubescent rather than the newborn period in the monkey (Jacobowitz et al., 1980). The latter authors reported that the ratio of pubescent to newborn GAD activity was 1.3-1.8 in the CN and putamen, and 2.3 in the SN of monkey. In our study with pigs, the GAD activity from age I to age II, however, increased at a greater magnitude in putamen than in the SN and CN. In pig cerebral cortex, the GAD activity decreased from age I to age II, which is in disagreement with findings from cerebral cortex of the monkey.

In our study, GAD activity was unevenly distributed in the different brain regions that we analyzed. GAD activity in the SN of both types of pigs was relatively low compared to the findings obtained from SN of other species (Tappaz et al., 1976; McGeer and McGeer, 1976; Jacobowitz et al., 1980). It is possible that GAD activity in the SN of pigs may have been less stable to the preparative

procedures before assay than in other brain regions.

In conclusion, there were differences in GAD activity in the SN and hypothalamus of SS and SR pigs, suggesting a possible GAD involvement along with a DA deficiency in the porcine stress syndrome.

SECTION II: AGE EFFECTS ON NEURONAL AND SYNAPTIC MORPHOMETRIC
CHARACTERISTICS OF THE PORCINE STRESS SYNDROME

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INTRODUCTION

Decrease of dopamine (DA) concentrations with age has been determined in the postmortem striatum of humans and laboratory rodents (Finch, 1973; Carlsson and Winblad, 1976; Ponzio et al., 1978; Osterburg et al., 1981). In humans, the progressive decrease of striatal DA concentration approaches 50% by 80 years of age (Adolfsson et al., 1979), whereas in rodents, it approaches 20% by 24-30 months (Osterburg et al., 1981).

Decreased DA concentration in the striatum is believed to reflect DAergic neuronal losses in the substantia nigra (SN), and subsequent nigrostriatal denervation of the striatum (Hornykiewicz, 1975). DAergic neuronal loss, however, may not be the only cause of age deficits in nigrostriatal function. There may be postsynaptic abnormalities such as decreased DA receptor binding sites or decreased DA-sensitive adenylyl cyclase, which may occur earlier and to a greater extent than the changes of nigrostriatal DA or tyrosine hydroxylase concentrations (Finch et al., 1981). Other evidence for age-related nigrostriatal postsynaptic abnormalities is the finding of a decrease in choline acetyltransferase activity with age in the rat striatum (McGeer et al., 1971; Meek et al., 1977) and human putamen (McGeer and McGeer, 1976). Thus, age-related loss of striatal

interneurons which use acetylcholine as a neurotransmitter has been proposed to be responsible for DA receptor loss in the striatum during aging (Severson and Finch, 1980).

Striatal neuronal loss during aging has been reported in the human putamen (Bugiani et al., 1978) and in C 57BL/6J mice (Mensah, 1979). The normal human striatum was estimated to have a neuronal loss up to 30-50%, affecting both large and small neurons (Bugiani et al., 1980). In mice, the neuronal loss was smaller. Losses of synaptic density, dendritic spines, and axon collaterals also have been observed with age in some brain regions (Machado-Salas et al., 1977; Huttenlocher, 1979). Direct evidence for the loss of striatal DAergic synaptic complexes during aging is lacking. In neostriatum, DA uptake by synaptosomes, however, is decreased in mice older than 18 months (Jones and Finch, 1975). Dense vesicles are fewer in number in old rat striatal synapses (Sun, 1976).

A possible involvement of the basal ganglia in the mechanism of the porcine stress syndrome (PSS) has been proposed recently (Altrogge et al., 1979; Hallberg et al., 1983) because a DA deficiency was found in the SN and caudate nucleus (CN) of stress-susceptible (SS) pigs. Age-related changes in striatal DA concentration have been demonstrated in both SS and stress-resistant (SR) pigs (Lue et al., 1982),

with a greater reduction of DA concentration occurring with age in the SS pigs. In order to further understand the age effects on the CN of SS and SR pigs, a morphometric analysis was carried out at light microscopic and ultrastructural levels. The caudate neuronal and synaptic densities, and characteristics in relation to age are reported here.

MATERIALS AND METHODS

The twenty-eight pigs used in this experiment were obtained from the Iowa State University Swine Breeding Farm. They were divided into three different age groups: twelve in group I (42-66 days of age), twelve in group II (148-237 days of age), and four in group III (751-1168 days of age). In each age group, there was an equal number of SS and SR pigs. The stress susceptibility of the animals was determined at 8-10 week age, by exposure to halothane anesthesia, serum CPK activities, and H blood typing (Rasmussen and Christian, 1976). The pigs were fed ad libitum a 16% protein corn-soy nonmedicated feed from the time of weaning until the day of slaughtering.

Pigs were killed by conventional slaughtering processes at the desired age. Pig brains were removed immediately after electrical stunning and bleeding. The head and body of the CN were dissected from the brain and cut into halves. The cranial half of the CN was immersed in 10% phosphate buffered neutral formalin for subsequent histological processing with the Nissl stain. The caudal half of CN was immersed immediately in 5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2-7.4) and processed for electron microscopic examination. Tissues were left in the respective fixatives for at least 4 months before further processing.

Neuronal Density Determinations

Formalin-fixed CN were embedded in paraffin. Seven μm thick cross sections were cut serially through the entire block. Three sections were selected from each block. These sections were obtained from the rostral, middle, and caudal one-third of the tissue and mounted on three different slides, and stained with cresyl echt violet. The three histological slides from each animal were then coded so that all subsequent morphometric analysis was performed without knowing the origin of the animal or the position of the section.

Each slide was examined through a 4x objective and 10x eyepiece on a Zeiss microscope. The center of the CN cross section on each slide was oriented to the center of the field. The objective was then increased to 40x and the tissue image projected through a closed-circuit colored video system onto a TV screen. The slide stage was adjusted to locate the center of the CN cross section at the center of the TV screen. The total magnification from the light microscope to the TV screen was $1.60 \times 10^3 \times$. All subsequent analyses were performed at this magnification.

Each section of CN tissue was examined with a grid system (Figure 1). This grid system was composed of seventeen grid units. The area of each grid unit on the TV

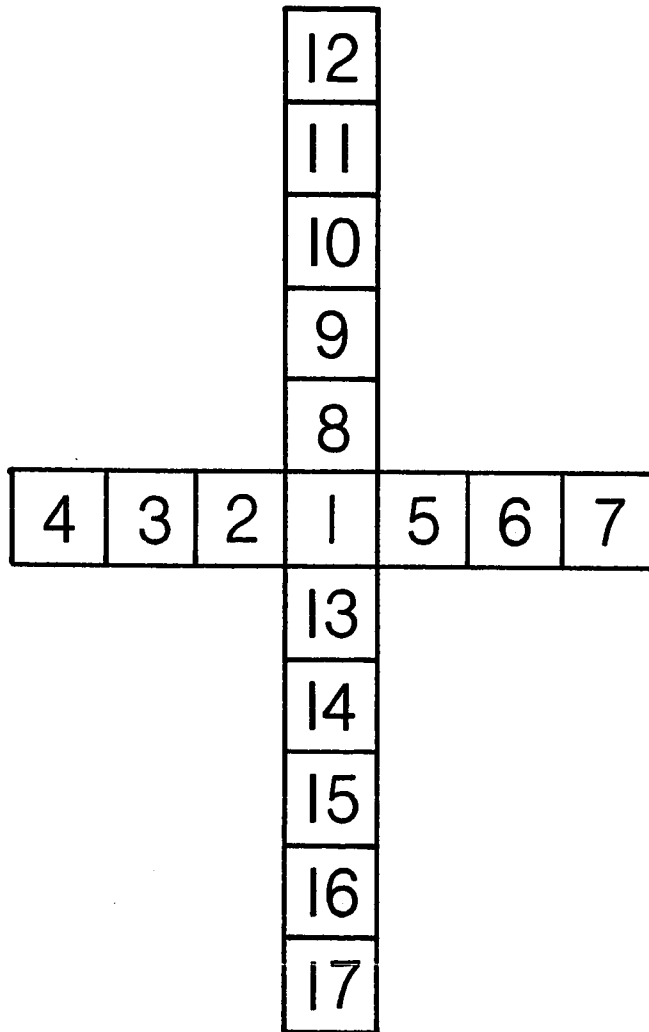


Figure 1. The counting grid system used in the morphometric study of the nerve cells in the caudate nucleus of pigs

screen was determined to be $65,858 \text{ mm}^2$ (equivalent to $26,000 \mu\text{m}^2$ of the tissue). This grid unit was tested and positioned to avoid any aberrations from the curvatures of the TV screen. The boundary of such a grid unit was marked directly on the screen so that it was easy to visualize and to use as a guide for the observers when a vinyl acetate sheet was superimposed on it. There were seventeen vinyl acetate sheets, one for each grid unit, for each histological slide. On the sheet, slide code number and grid unit number were recorded. Neurons present within the boundary of the grid unit were traced and numbered. Two independent observers were responsible for the identification and evaluation of all neurons on the seventeen grids of a slide. The first grid unit was located at the center of the CN cross section. After observation on the first grid unit, the microscopic stage was then moved along the short axis of the tissue to the second grid unit. There were three consecutive grid units at each side of the central grid unit. The stage was then moved perpendicular to the short axis and five grid units above and below the central grid unit were analyzed.

The following characteristics of the neurons were scored independently and simultaneously by two observers: the presence or absence of a nucleolus (presence, 1; absence, 0); the presence or absence of a process (presence, 1;

absence, 0); staining intensity (light, 1; intermediate, 2; dark, 3); neuronal shape (round, 1; intermediate, 2; angular, 3). The area of each individual neuron was measured by a Zeiss graphic analyzer attached to a MOP III computer. The diameter of the neurons was obtained by the following calculation:

$$D = \sqrt{\text{Area} \times \frac{4}{\pi}}$$

All of the morphometric data from three slides of each animal were combined together for data analyses. Neuronal density was calculated by Equation 6 of Dubin (1970):

$$N = \frac{Nc \cdot T}{\frac{2}{\pi}(D-2K) + T}$$

where N = neuronal density, Nc = cell number counted, T = 7 μm , thickness of the tissue, D = corrected diameter, obtained by the Equation 10 of Dubin, K = a constant obtained by the Equation 2 of Dubin,

$$D = \frac{2\bar{D}}{\pi} - T + \sqrt{\left(T - \frac{2\bar{D}}{\pi}\right)^2 + 2T\bar{D}}$$

$$K = \frac{D - \sqrt{(D)^2 - (D_{\min})^2}}{2}$$

where \bar{D} = mean diameter of neurons, D_{\min} = minimum diameter of neurons.

An ANOVA statistical analysis of the morphometric data was done with computer assistance to determine treatment (SS and SR) and age (age I, II and III) effects.

Synaptic Density Determinations

The CN were cut into small pieces and transferred to fresh 5% glutaraldehyde in 0.1M phosphate buffer solutions (pH 7.2 to 7.4) for 24 hours. Tissues were then washed with 0.2 M phosphate buffer for 1 hour. Dehydration proceeded through 50%, 70%, 80%, 90%, 95%, and 100% ethanol for a total of 85 minutes. Tissues were then stained in three changes of 1.5% phosphotungstic acid in ethanol for 3.5 hours. Before infiltration, tissues were rinsed three times in propylene oxide for 25 minutes. Infiltration was done in different ratios of the propylene oxide and Spurr's resin mixture (1:1 for 6 hours; 1:3 for 12 hours). The final infiltration was carried out in the standard formula of the Spurr's resin in the desiccator for 6 hours. Tissues were embedded in fresh Spurr's resin and incubated in a 70°C vacuum oven for 24 hours.

Two blocks were selected randomly from the embedded tissues of each animal. They were trimmed with a pyramitome until the complete tissue surface was exposed. Blocks were then transferred to a Reichert OM UZ ultramicrotome and cut with a diamond knife. Sections were collected at

both the surface level and 50 μm deeper into the block, and mounted on 300 mesh copper grids. Tissues on the grids were viewed with a Hitachi HU-12A electron microscope at 50 KV. Specimen knobs were turned randomly until a grid window appeared in which a tissue section completely covered the grid window. Four corners of the selected grid window were then photographed at 10,000x. Subsequently, the specimen knobs were again rotated several times until another grid window, which was covered completely by tissue section, was found. Photographs were taken again from four corners of the grid windows. Because two grid windows from one block and two blocks from one animal were used, there were 32 micrographs obtained from each animal. The synapses were identified on prints having a total magnification of 17,000 with the assistance of a magnifying lamp. The synaptic profiles appearing in the electron micrographs were counted according to the diagrammatic representation of West et al. (1972). Two independent observers identified the synapses on each print and only synapses which were in agreement between two observers were used for further analysis. The length of the postsynaptic density and the area of the prints were measured with a Talos graphic digitizer interfaced with a PDP-11/23 computer. The synaptic density was then calculated according to Dubin (1970). Data analyses were done in a similar manner as in the neuronal density study.

RESULTS

Neuronal Density

Three Nissl-stained sections from three different positions of the rostral one-half of the head of the porcine CN were selected for morphometric studies at the light microscopic level. Neuronal density was expressed as the neuron number present in the 17 counting grid units (tissue area, 0.442 mm^2) from each animal. The neuronal density at different sampling positions was not significantly different. Therefore, the neuronal density was considered to be evenly present throughout the caudate tissues which were used.

The neuronal density tabulations of three neuronal populations, including all of the neurons (AN), neurons with nucleolus (NN), and neurons with process (PN), are presented in Table 1. The mean neuronal density of AN and NN were significantly decreased with advancing age. The mean neuronal density of PN, however, followed a different pattern of change with age. PN neuronal density increased at age II and then, decreased at age III. The neuronal density of AN, NN, and PN were not significantly different between treatments (SS vs. SR pigs). The magnitude of the neuronal density decline was significantly greater in the SS pigs. In AN, the density decline with age was about 58% in the SS pigs and 27% in the SR pigs. In NN, the magnitude of the density

Table 1. Numerical density of nerve cells in the caudate nucleus of the stress-susceptible and stress-resistant pigs in relation to age

		Stress-susceptible	Stress-resistant	Mean ^a
Cells (AN)	Age I	515	376	446**
	Age II	345	406	375**
	Age III	217	274	246**
	mean	359	352	
Cells with nucleolus (NN)	Age I	236	198	217**
	Age II	140	128	134
	Age III	111	131	121
	mean	178	159	
Cells with process (PN)	Age I	32	41	36
	Age II	55	64	60**
	Age III	16	34	25
	mean	39	50	

^aMeans expressed in the unit of cell number per grid unit volume ($3.10 \times 10^6 \mu\text{m}^3$).

**p < .01.

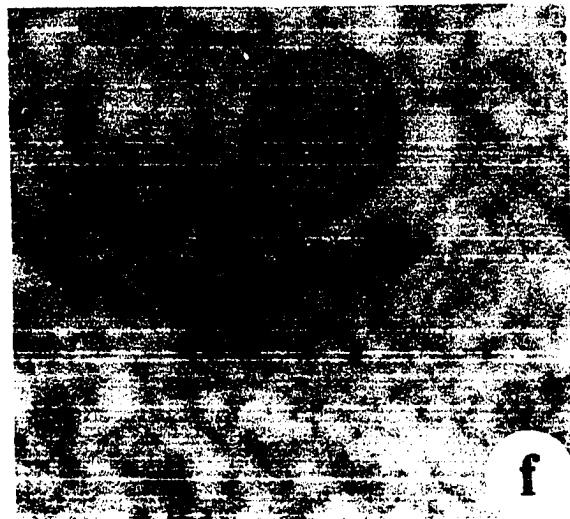
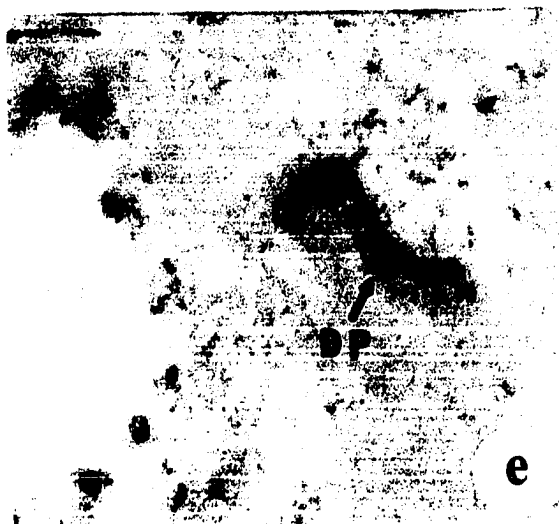
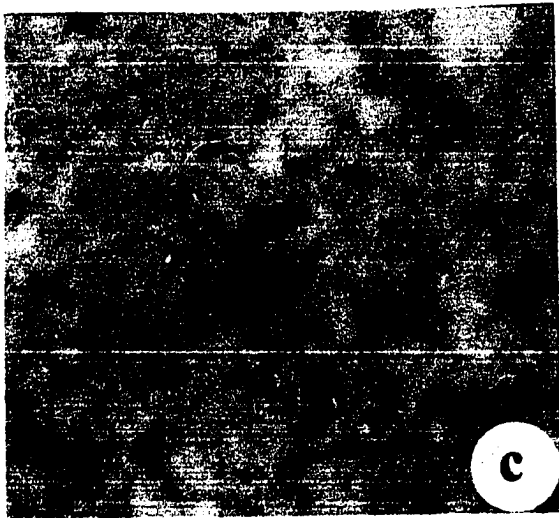
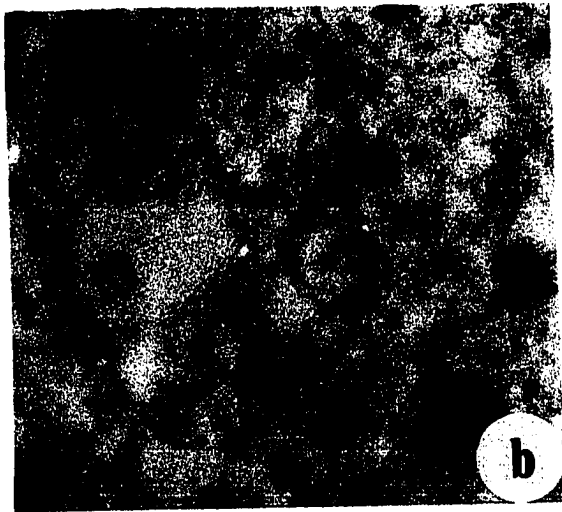
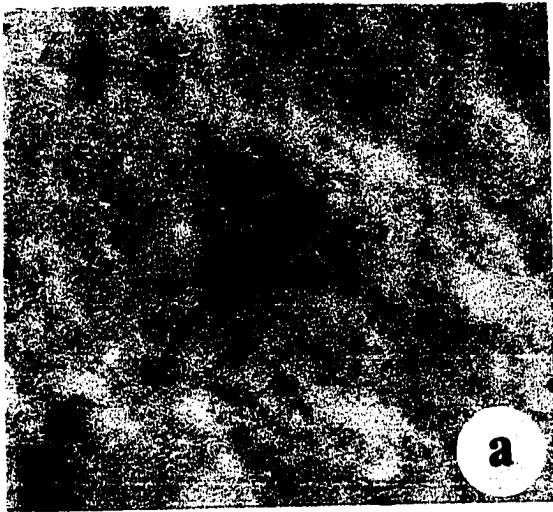
decline with age was 53% and 34% in the SS and SR pigs, respectively. Although there was a greater decline in neuronal density in the SS pigs with age, the neuronal density of AN and NN at age III were still not statistically different from that in the SR pigs.

Synaptic Density

Samples from the caudal one-half of the CN were used for counting synapses. The synaptic profiles were clearly exhibited in the E-PTA-stained postmortem porcine CN (Figure 2a). Both symmetrical and asymmetrical synaptic profiles (Figure 2b,c) occurred in the porcine CN. The synaptic contacts were straight, curved, or circled (Figure 2a,e,d); long, medium, or short (Figure 2e,b,c); and continuous or divided (Figure 2e,f). The synaptic density differed significantly between blocks within an animal, and between section levels within a block. Taking the variation of these sources into account, the synaptic density still declined significantly with age (Table 2). A similar decline of the synaptic density with age was found in both treatments. The mean synaptic density between treatments, however, was not statistically different. There was a linear relationship between age and the synaptic density (Figure 3). The magnitude of the synaptic density decline amounted to 15% from age I to age III.

Figure 2. Electron micrographs of synaptic profiles in porcine caudate nucleus (magnification x40,000)

- a. A synaptic profile is characterized by pre-synaptic dense projections (DP), an intracleft linear density (IC), and a postsynaptic band (PB). This particular synaptic contact has asymmetrical appearance at pre- and post-synaptic sides
- b. One asymmetrical synaptic profile (A) is present at upper left corner in this micrograph. There is a short synaptic contact having a nearly symmetrical appearance (S) with a continuous, irregular presynaptic band, at lower right corner
- c. This micrograph illustrates a short, straight asymmetrical synaptic profile with three well-identified presynaptic dense projections (DP)
- d. Here are a circled synaptic profile versus a relatively straight synaptic profile
- e. Long asymmetrical synaptic profile may have a curving and continuous appearance
- f. This is a set of synaptic profiles which look like a long synaptic profile which is in a divided appearance



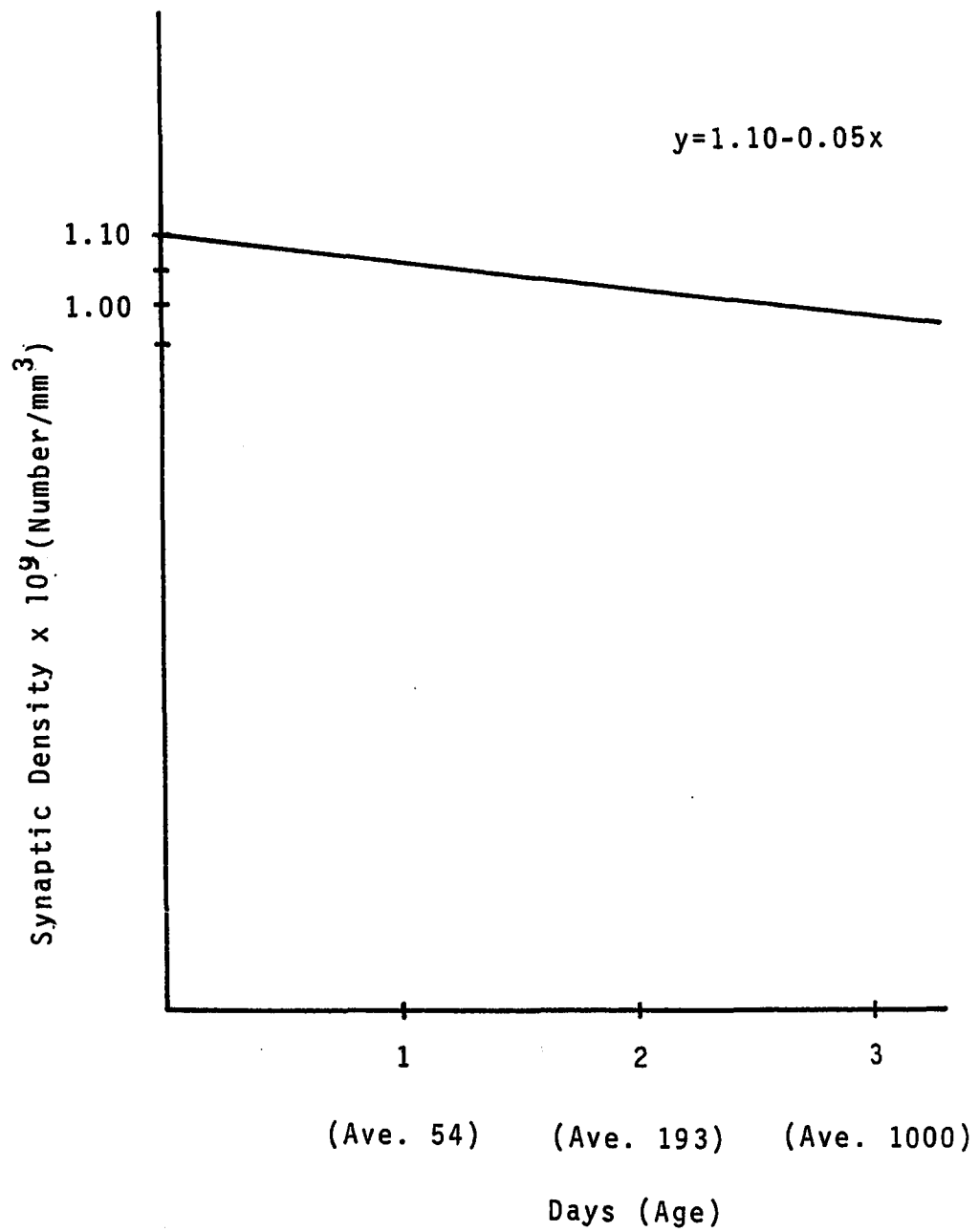


Figure 3. Relationship between synaptic density and age

Table 2. Synaptic density^a in the stress-susceptible and stress-resistant pigs in relation to age

	Stress-susceptible	Stress-resistant	Mean
Age I	1.07	0.96	1.02**
Age II	0.92	0.97	0.95**
Age III	0.92	0.79	0.87**
mean	0.98	0.94	

^aA synaptic density was expressed as the value in the unit of number per mm³.

** P < .01.

Characteristics of Neurons and Neuronal Populations

Approximately 10,837 neurons were analyzed from Nissl-stained CN of 28 pigs at three ages. The CN neurons were distinguished and characterized according to differences in cell diameter, cellular shape, cell staining intensity, the presence of a nucleolus, and the presence of a process. The frequency distribution of neurons classified by each of the characteristics is listed in Table 3.

The first classification of the neurons was by size. The diameter of AN ranged from 6.0 to 32.5 μ m. A frequency distribution of neuron diameters is illustrated in Figure 4.

Table 3. Frequency distribution of nerve cells categorized^a by various characteristics in stress-susceptible and stress-resistant pigs

Characteristics	Stress-susceptible ^b (%)	Stress-resistant ^b (%)
A. <u>By size</u>		
medium	24.2	24.3
small	75.4	75.3
B. <u>By nucleoli</u>		
present	43.4	45.3
C. <u>By process</u>		
present	12.9	10.5
D. <u>By stain</u> ^c		
light	55.5	58.9
dark	28.4	27.1
E. <u>By shape</u> ^d		
round	66.2	70.1
angular	24.2	20.7

^aFrequency distribution was only presented for two categories in a three-category characteristic and one category in a two-category characteristic. For example, intermediate-category frequency distribution in size, stain and shape characteristics can be calculated by subtracting the two values listed under each characteristic from 100%. The frequency distribution for each absence-category in nucleolus and process characteristics can also be known in a similar calculation.

^bNo significant difference between SS and SR groups in any of the categories.

^cLight stain: stain scores are less than or equal to 1.5; dark stain: stain scores are equal to or greater than 2.5.

^dRound shape: shape scores are less than or equal to 1.5; angular shape: shape scores equal to or greater than 2.5.

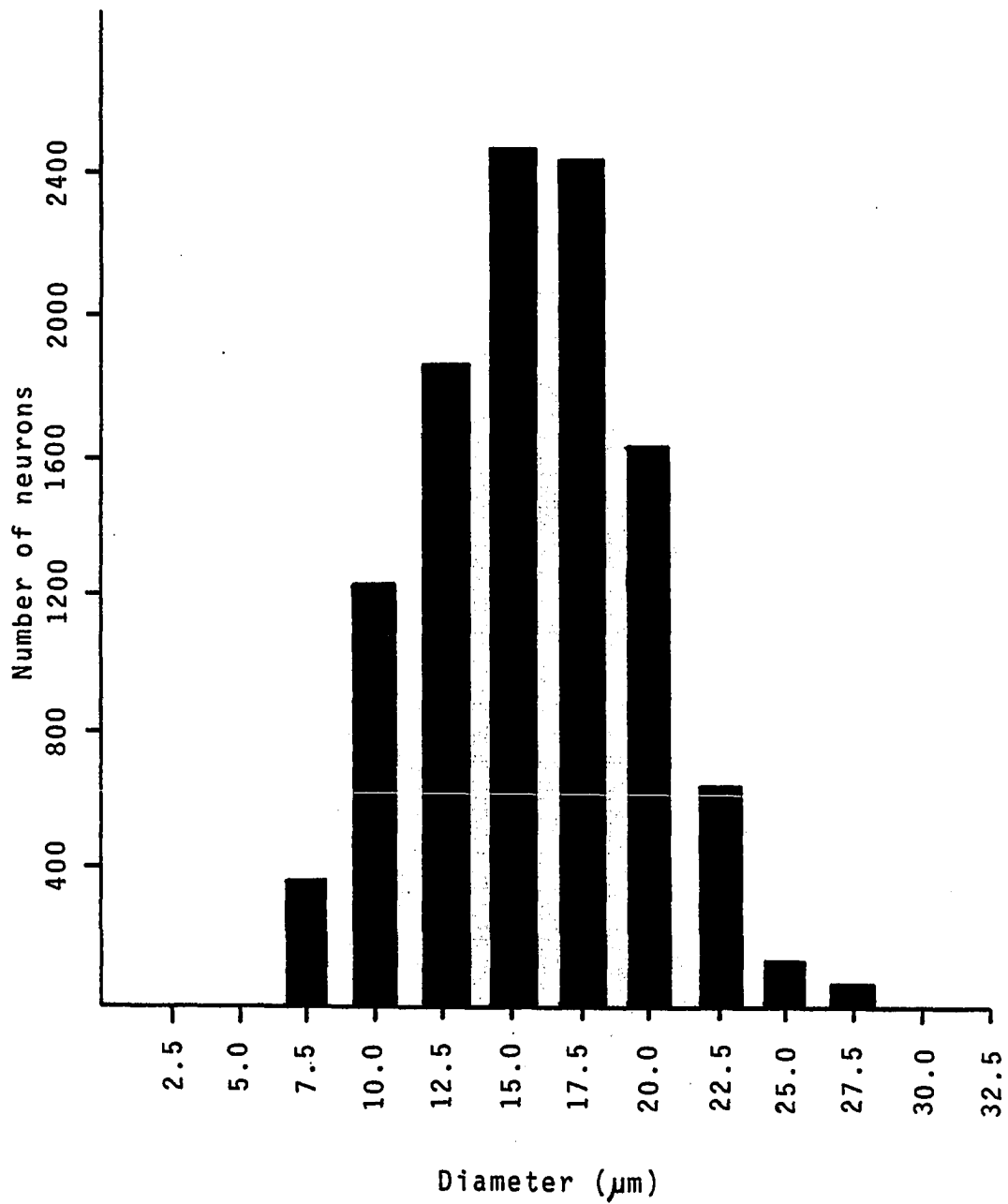


Figure 4. The frequency distribution of nerve cell sizes in the pig caudate nucleus

The highest frequency diameter occurred in the range of 15.0 to 17.0 μm . In this study, small neurons were defined as equal to or less than 18.5 μm in diameter. Medium neurons were between 18.5 and 26.0 μm in diameter. Large neurons, greater than 26.0 μm in diameter, were very few in number. It is obvious that small neurons comprised the majority (about 75%) of the neuronal populations in both the SS and SR pigs. Medium neurons were less abundant, contributing about 24% of the neuronal population. The second classification of the neurons was by the presence of the nucleolus in Nissl-stained material. About 43-45% of AN were observed to be NN. In a third classification, based on the presence or absence of a process, only 11-13% of AN were identified to be PN.

Other characteristics used for classification of CN neurons were staining intensity and neuronal shape. Light staining neurons (stain scores, 1.0-1.5) comprised 55% of AN, whereas, dark staining neurons (stain scores, 2.5-3.0) formed 27-28% of AN. The most frequently occurring neurons (about 66-70%), based on the neuronal shape, were round (shape scores, 1.0-1.5). Angular neurons (shape scores, 2.5-3.0) occurred less frequently and represented about 21-24% of AN.

From the above findings, it was estimated that small

neurons together with medium neurons comprised more than 99% of the neuronal population in both SS and SR pigs. The ratio of small neurons to medium neurons, as illustrated in Table 4, was not significantly different among age groups or between treatments. The interaction between age and treatments, however, was significant at $p < 0.09$. The ratio of small neurons to medium neurons in the SS pigs decreased with age whereas it increased with age in the SR pigs.

In addition to neuron diameter, staining intensity and neuronal shape were two prominent characteristics used to classify neuronal populations. The combination of these two attributes with neuron diameter provided further delineation of neurons, as shown in Table 5. The light staining and round neurons formed the main population (over 80% of the medium neurons). Both treatments possessed similar proportions at all three ages. In the population of small neurons, light staining and round neurons, however, were not as numerous and the proportion varied with age. There was a decrease in the percentage (32.7%) of small, light staining, and round neurons at age II, while at age III their percentage increased to 48.8% of the population. Small neurons had more variations in their relative proportion in the neuronal population.

Table 4. Small to medium cell ratios in relation to stress-susceptibility and age

	Stress-susceptible ^a	Stress-resistant	Mean
Age ^b I	9.6	3.2	6.4
Age II	3.2	3.5	3.3
Age III	1.5	9.5	5.5
Mean	5.7	4.2	

^{a,b}The interaction between age and treatments was significant at $P < .09$.

Table 5. The proportion of neurons classified as round and lightly-stained^a in the medium and small categories

Size categories	Age I		Age II		Age III	
	SS	SR	SS	SR	SS	SR
	(%)		(%)		(%)	
Medium	88.5	90.2	87.0	82.6	86.3	32.8
	mean 89.3		mean 84.8		mean 84.6	
Small	52.8	50.5	35.9	29.6	56.3	41.4
	mean 51.6 ^{b,c}		mean 32.7 ^{b,d}		mean 48.8 ^{d,c}	

^aRound and lightly-stained neurons have cellular shape and stain scores from 1.0 to 1.5.

^{b,c,d}Means in the same row with the same superscripts are significantly different ($P < .01$).

Comparisons of the morphometric data of NN and PN are presented in Table 6. The mean neuronal area of NN was 258 μm^2 which corresponds to a diameter of 18 μm . The mean staining and shape scores were 1.30 and 1.15, respectively. Thus, NN represented neurons characterized by the presence of a nucleolus, small to medium in size, light staining and round in shape. PN neurons had a mean area of 152 μm^2 , a mean staining score of 2.73, and a mean shape score of 2.72. The mean diameter of PN was smaller on the average, but ranged from small to medium. The PN were consistently dark staining and angular. Although NN and PN were two distinct neuronal populations, they exhibited similar positive correlations between staining intensity and shape attributes, as shown in Table 7. In NN alone, however, other significant relationships were seen. The neuronal area correlated negatively with staining scores and numerical density. Therefore, large area was related to low stain and shape scores, and low neuronal density. In both neuronal populations, higher stain scores were associated with an angular shape.

The effect of aging on various neuronal attributes in the populations of NN and PN was also of interest. The mean neuronal area of NN and PN according to age is compared in Table 8. Significantly greater neuronal area was found in

Table 6. Summary of cell characteristics of neurons with a nucleolus and neurons with process

	Area (μm^2)	Stain ^a	Shape ^a
<u>Neurons with a nucleolus</u>			
Mean	258	1.30	1.15
Std. dev.	34	0.11	0.09
Smallest	167	1.08	1.03
Largest	317	1.51	1.38
<u>Neurons with process</u>			
Mean	152	2.73	2.72
Std. dev.	32	0.15	0.13
Smallest	98	2.50	2.39
Largest	257	2.96	2.90

^aStain and shape scores were based on the scale 1, 2, and 3.

Table 7. Correlations between the morphometric parameters of porcine caudate nucleus neurons

Cell population	Stain	Shape	Numerical density
NN Area	-0.42*	-0.26	-0.66*
Stain		0.64*	0.37*
Shape			0.09
PN Area	0.01	0.06	-0.21
Stain		0.48*	0.24
Shape			0.01

*Significant at $p < 0.05$.

Table 8. Mean cellular areas of neurons with a nucleolus and neurons with process

Categories	Age I		Age II		Age III	
	SS (μm^2)	SR	SS (μm^2)	SR	SS (μm^2)	SR
Neurons with nucleolus (NN)	219 mean 236 ^{a,b,c}	253	277 mean 279 ^{a,b}	282	276 mean 260 ^{a,c}	244
Neurons with process (PN)	137 mean 137 ^{d,e,f}	136	152 mean 162 ^{d,e}	172	196 mean 170 ^{d,f}	143

^{a,d} Means in the same column with different superscripts are significantly different ($P < .01$).

^{b,c,e,f} Means in the same row with different superscripts are significantly different ($P < .01$).

NN than in PN, regardless of age or treatments. The neuronal area was significantly smaller at age I than at any age in both NN and PN populations. These findings were consistent in SS and SR pigs. Neuronal staining intensity was lighter in NN than in PN at all three ages and in both treatment groups, as illustrated in Table 9. The staining intensity of the neuronal populations, however, remained the same with advancing age. The neuronal shape scores in NN were significantly lower than in PN, the former being round and the latter angular. Again, age did not affect the neuronal shape scores in either NN or PN populations (Table 10).

In summary, there were at least four neuronal types

Table 9. Staining characteristics of neurons with a nucleolus and neurons with process

Categories	Age I		Age II		Age III	
	SS	SR	SS	SR	SS	SR
Neurons with nucleolus (NN)	1.34 mean	1.29 1.31 ^a	1.25 mean	1.32 1.29 ^a	1.19 mean	1.41 1.30 ^a
Neurons with process (PN)	2.64 mean	2.76 2.70 ^b	2.82 mean	2.78 2.80 ^b	2.76 mean	2.54 2.64 ^b

^{a,b} Means in the same column with different superscripts are significantly different ($P < .01$).

Table 10. Cellular shape characteristics of neurons with a nucleolus and neurons with process

Categories	Age I		Age II		Age III	
	SS	SR	SS	SR	SS	SR
Neurons with nucleolus (NN)	1.14 mean	1.14 1.14 ^{a,b}	1.11 mean	1.14 1.13 ^{a,c}	1.12 mean	1.36 1.24 ^{a,b,c}
Neurons with process (PN)	2.67 mean	2.67 2.67 ^d	2.74 mean	2.79 2.76 ^d	2.77 mean	2.69 2.73 ^d

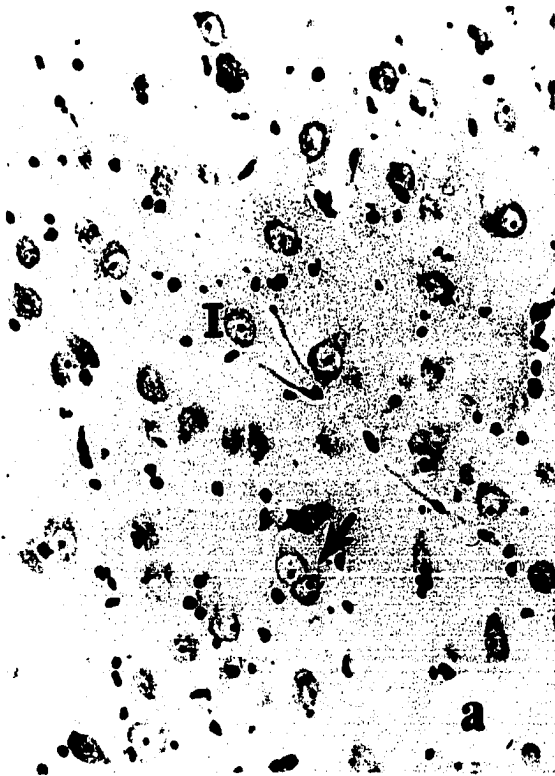
^{a,d} Means in the same column with the same superscripts are significantly different ($P < .01$).

^{b,c} Means in the same row with the same superscripts are significantly different ($P < .05$).

identified in Nissl preparations of the porcine CN. They are described as follows: (1) neurons with the presence of a nucleolus, small to medium in size, light staining and round in shape; (2) neurons with the presence of a process, medium to large in size, dark staining and angular in shape; (3) neurons with intermediate staining, and round to angular shape; (4) neurons with dark staining and round to intermediate shape (Figure 5a,b,c,d).

Figure 5. Four neuronal types identified in the Nissl preparation of porcine caudate nucleus

- a. Neuronal type I occurred most frequently in the caudate nucleus (arrow indicates pairing of type I neurons, 960X)
- b. Neuronal type II, III, and IV (2,400X)
- c. Neuronal type III (2,400X).
- d. Neuronal type II and III (2,400X)



DISCUSSION

Age changes were seen in the neuronal and synaptic densities of the porcine CN. The finding of a significant decrease in AN density with advancing age is in agreement with the values reported for the human putamen (Bugiani et al., 1978) and the mouse neostriatum (Mensah, 1979). Particularly, the magnitude of the mean neuronal density decline in 28 pigs is similar to the neuronal loss reported in the human putamen. Bugiani et al. (1978) demonstrated a 30-50% neuronal loss from age 1 (1 day to 3.3 years old) to age 3 (55-65 years old) in the human putamen. We found a 45% decline in the neuronal density in the porcine CN from age I (ave. 54 days old) to age III (ave. 1,000 days old). When examined separately, the neuronal density decline of SR pigs was about 27% and that of SS pigs was 58%. Thus, the normal porcine CN had a neuronal loss which approached the lower end of the neuronal loss in the human putamen. The reasons why SS pigs had a neuronal loss twice as great as in SR pigs during aging are not clear. It is possible that both pathological and aging mechanisms are operating in the SS pigs. In addition, the aging process may differ in the SS pigs. There was some evidence to suggest this. Specifically, there was an interaction between age and treatment in the ratio of small to medium neurons. That

is, SS pigs and SR pigs followed different trends in age-related changes of the ratio of small to medium neurons. The SS pigs had a decrease in the ratio of small to medium neurons from age I to age III. In contrast, SR pigs had an increase in this ratio from age I to age III. The SR pigs had a greater loss of medium neurons than small neurons, whereas, the SS pigs had a greater loss of small neurons than medium neurons with age. Therefore, age changes in the neuronal populations of the CN of SS and SR pigs may follow a different pattern. Further, the magnitude of the changes may differ between two treatment groups even though their mean neuronal densities were similar when all three ages were considered together.

There have been reports of neuronal losses in human Parkinson's disease and Huntington's chorea. In the putamen of Parkinson's patients, a moderate loss (up to 30% of normal) of the neurons was commonly seen in several kinds of cases, while in choreatic patients, more severe neuronal loss (about 30-50% or more severe) occurred in the caudate and putamen (Bernheimer et al., 1973). These findings seemed to be in concert with part of our data where SS pigs had a greater decline of neuronal density with age. Additionally, Lange et al. (1976) reported a more severe loss of small neurons in the striatum of choreatic patients

which changed the ratio of large to small neurons from 1:175 in the normal groups to 1:40 in the patients. In the Parkinson's disease, however, large neurons were affected more than small neurons in the striatum (Bugiani et al., 1980). Although direct comparison could not be made between SS pigs and human neurological diseases because of the different diameter range used for small neurons, the fact remains that the composition of the neuronal populations changed with age and diseases in both cases.

The synaptic density losses in the porcine CN were not of the same magnitude as the losses in neuronal density with age. Synaptic density losses may be independent of neuronal losses. There was a 15% decline in synaptic density in the porcine CN from age I to age III. A linear relationship existed between age and synaptic density. Evidence for age changes in human striatal synaptic density is lacking. However, there is no significant decline in synaptic density in human frontal cortex over 16-72 years (Cragg, 1975; Huttenlocher, 1979). Brain regions may differ in age changes in synaptic density. A 20% reduction of synaptosomal uptake of dopamine was found in mice over 18 months implying a possible loss of neostriatal dopaminergic synaptic complexes with age (Jonec and Finch, 1975). This type of nerve terminal change may exist in the pigs but was not

determined in our study.

The PN neuronal density followed a different pattern of aging change in SS than in SR pigs. The increase in neuronal density at age II in PN was somewhat surprising. It is generally accepted that neurons do not divide after differentiation and neurons lost are not replaced by division of the remaining cells (Vernadakis, 1975). Therefore, it is unlikely that at age II, CN would have more PN because of cell division. The PN only comprised 10-12% of the total population which reflects that some processes of the neurons are not stained by the Nissl preparation. Nissl substances are known to be composed mainly of rough endoplasmic reticulum, whose biochemical reaction compound is RNA when stained with cresyl echt violet. These substances can be present in the cytoplasm and dendrites but not axon and axon hillock (Copenhaver et al., 1971). The PN neurons appeared to have abundant Nissl substance packed in the cytoplasm and at least initial segments of the dendrites which gave the dark staining appearance to the neurons. It is possible that changes of RNA with age may influence the staining appearance of the neurons. Indeed, there are selective changes of RNA in striatum of mouse and rats (Chaconas and Finch, 1973; Shaskon, 1977), but not in other brain regions. At present, it still remains a question as

to why PN increased at age II.

Coinciding with the increase in proportion of PN at age II was the finding of a smaller proportion of small light staining round neurons. This change can be explained, in part, by the different rate of the neuronal loss in the various neuronal populations. For example, light staining and round neurons may decrease faster from age I to age II while dark staining and angular neurons may be lost at a relatively slower rate. Thus, dark staining and angular neurons constitute a relatively larger proportion of the small neuronal population.

Combinations of morphometric parameters were used in our study to classify neuronal types. Based on these combinations, four types of neurons can be identified readily. The first type was light staining and round, and usually associated with the presence of the nucleolus. This is the type which has been mentioned most frequently in other species (Adinolf and Pappas, 1968; Mensah, 1977). The neuron has a large and pale nucleus with a prominent nucleolus. The nucleus is surrounded by a thin rim of cytoplasm which contains sparse Nissl substance. In our study, this type of neuron was small to medium which is consistent with the work of Mensah (1977) in the mouse. The pig type I neurons are, however, larger than the values reported for

this cell type in the cat by Adinolf and Pappas (1968). The second type of neuron had a process and appeared dark staining and angular. Most of these neurons were medium size, nevertheless, some neurons in this type were large. The large type II neurons probably correspond to the neurons found scattered in the special neuronal clusters within the CN in other species (Mensah, 1977). Type III neurons had intermediate staining but did not always have a nucleolus. Our type IV neurons were dark staining but had no processes stained in Nissl preparations. They were more consistent in size, but the shape varied from round to intermediate. The difference between type III and type I is in the staining intensity of the nucleus. In type III neurons, the nucleus and the cytoplasm had similar staining intensities, as compared with the type I neuron which had a completely pale nucleus. The functional significance of this morphometric classification of neuronal types is not known. But, it is the first time that CN neuronal types were studied morphometrically in the pig. These classifications should provide a basis for further work on morphology and functions of the porcine CN.

SECTION III: A GOLGI ANALYSIS OF PORCINE CAUDATE NUCLEUS

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INTRODUCTION

Abnormalities of the basal ganglia may be involved in the mechanism of the porcine stress syndrome (Altrogge et al., 1979; Hallberg et al., 1983). One of the affected structures in the basal ganglia is the caudate nucleus (CN), which has a deficiency of the neurotransmitter dopamine in stress-susceptible (SS) pigs (Hallberg et al., 1983). Other studies in our laboratory are being done to confirm the role of the basal ganglia in the porcine stress syndrome (PSS). Because of the importance of the CN in the PSS, we did a Golgi analysis to determine the neuronal morphology and organization of the CN in pigs.

Golgi studies on the neostriatum of mammals have been of intense interest in recent years. Golgi material has been used to reveal neuronal types which vary in number and morphological characteristics in the rabbit (Eder et al., 1980), in the cat (Kemp and Powell, 1971a; Eder et al., 1980), in the rat (Mensah and Deadwyler, 1974; Lu and Brown, 1977; Dimova et al., 1980; Chang et al., 1982), in the dog (Eder et al., 1980; Tanaka, 1980), in the monkey (Fox et al., 1971-72a,b, 1974; DiFiglia et al., 1976; Rafols and Fox, 1979; Eder et al., 1980), and in the human (Eder et al., 1980). Spiny and aspiny neuronal types have been found in all species studied. The size range of the neurons was from

small to large. Among them, the medium densely spiny neurons are the most common type in the neuronal population of the CN, although the length of the axon may still be controversial (DiFiglia et al., 1976). In general, the neuronal types found in the porcine CN in this study are similar to those observed in other species. Three major aspiny and four major spiny types of neurons were identified in the porcine CN.

MATERIALS AND METHODS

Five 14 day old pigs were used in this study. The anesthetized pigs were perfused through the aorta with 1% paraformaldehyde and 1% glutaraldehyde in 0.1M phosphate buffer, pH 7.2. After perfusion, the CN were removed from the brain and stored in the same aldehyde mixtures for two weeks until further processing for Golgi impregnation. The reliable Golgi-Kopsch modification by Riley (1979) was used for Golgi impregnation procedures.

Blocks which were embedded in cellodin were cut by a sliding microtome. The 120 μ m thick sections were collected serially in 95% ethanol. Sections were dehydrated in two changes of absolute ethanol. Celloidin in the section was removed by a 1:1 mixture of absolute ethanol and anhydrous ether.

The neuronal morphological characteristics were observed under a Zeiss light microscope with 25x and 40x objectives. The representative neuronal types were drawn using the camera lucida attached to the light microscope. Photographs were taken for the purpose of analysis and illustration. The morphometric data such as area of somata cross-section, length of the dendrites, thickness of the dendritic trunks, length of the long and short axis of the dendrites and radius of the dendritic field, were

measured from the drawing tube tracing with the aid of a graphic analyzer attached to a MOP-III computer.

RESULTS

Neurons in the porcine CN were classified into three major aspiny and four major spiny neuronal types according to their somatic cross-sectional area and their somatic and dendritic morphological characteristics. The neurons were examined for the presence or absence of the dendritic spines, the presence or absence of the dendritic varicosities, the emerging pattern of the dendrites, the dendritic branching pattern, the dendritic length, and the features of the axon. In this study, the large neurons had cross-sectional somatic areas larger than $520 \mu\text{m}^2$, the medium neurons had somatic areas between $200 \mu\text{m}^2$ and $520 \mu\text{m}^2$, and the small neurons had somatic areas less than $200 \mu\text{m}^2$.

Large Spiny Neurons

This cell type (Figure 1a,b) was few in number but easily found in most of the sections because of its large somatic area, which ranged from 550 to $976 \mu\text{m}^2$ in this study. The shapes of the somata were variously oval, triangular, and polygonal. Four to eight primary dendrites emerged from the soma and usually were 5-9 μm thick. The primary dendrites were either branched or unbranched but they all were directed away from the soma for a distance which was more than 200 μm . Long, thin, and sometimes hairy-like

spines were found on the primary dendrites and their branches. A variable number of spines were also visible on the soma. The spine density varied among different segments of the dendrites, with higher density occurring at the secondary dendrites (about 5 spines per 10 μm length of the dendrite).

Large Aspiny Neurons

This is a rarely impregnated neuronal type in the porcine CN. The smooth soma with a cross-sectional area of $586 \mu\text{m}^2$ was spherical (Figure 2). Six thick primary dendrites with no spines emerged from the soma and all branched twice and briefly. The dendritic field was smaller than the large spiny type. The dendritic length from the soma was less than 200 μm . The axon came out from the stem of the dendritic trunk.

Medium Densely Spiny Neurons

These neurons were the most frequently impregnated type in the Golgi material from pigs. The morphological characteristics were identical to the medium spiny or spiny I neurons described in other species. The shapes of the somata were variously spherical, ovoid, fusiforms and triangular (Figure 3). The range of the somatic cross-

Figure 1. Two large spiny neurons (a,b) (arrows point to the dendritic and somatic spines, 1600X)

Figure 2. Large aspiny neuron (1600X)

Figure 3. Medium densely spiny neuron (1600X)



sectional area was 256 to 350 μm^2 while the measurements of the long and short axes were 19 to 23 μm and 14 to 15 μm , respectively. Usually, four or five primary dendrites emerged from the soma and branched several times. This type of neuron consistently had no spines on the soma, primary dendrites, and initial portion of the secondary dendrites. The rest of the dendrites, however, were studded densely with various shapes of spines. The dendritic length from the soma were often within 200 μm but longer dendritic lengths were also present. The first segment of the axon was clearly seen in most of this type of neuron, and arose either directly from the soma or from the proximal portion of the primary dendrites. The axons were impregnated for a limited distance within 200 μm . They, however, gave collaterals at various intervals which ranged in length from 32 to 58 μm . Some axons which were impregnated up to 100 μm showed no signs of collaterization. In this latter case, the axon was frequently seen to approach its own dendritic spines or the neighboring densely spiny neurons. The axon from this densely spiny neuron was also observed to reach the sparsely laden thin spines on the other type of neuron.

Medium to Large Neurons, Moderately to
Sparsely Spined

This neuronal type differed from the densely spiny neurons in both the somato-dendritic characteristics and the distribution of the spines. They had great variation and were divided into five subtypes.

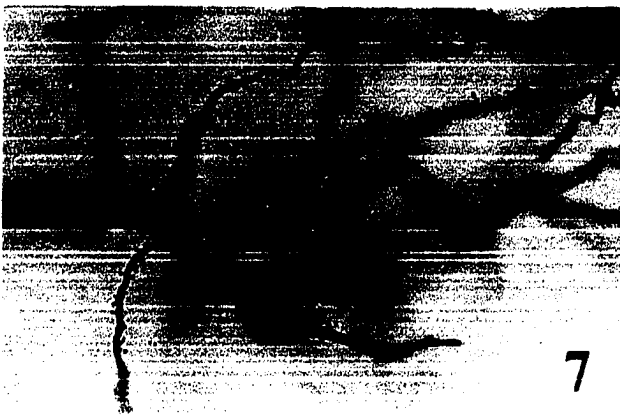
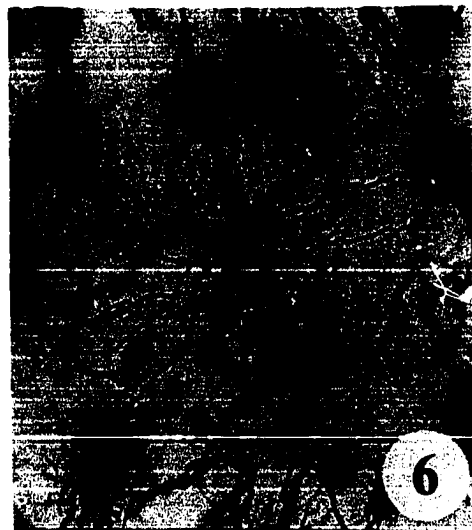
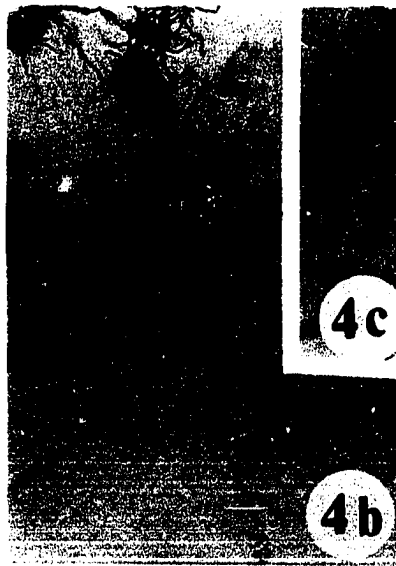
Subtype I: These were spindle- or pyramidal-shaped neurons with bipolar dendritic trunks. Neurons within this subtype had a somatic cross-sectional area of 203 to 762 μm^2 , which covered the range of the medium and large sizes. The somatic shapes of this subtype were almost spindle- or pyramidal-shaped. The longest axis was 48 μm and the shortest axis was 13 μm . The most important feature was the dendritic emerging pattern. In the spindle-shaped neurons (Figure 4a), there were always two major dendritic trunks emerging bipolarly from the opposing poles of the soma. The thickest portion of the dendritic trunk was 9 μm . In addition to these two main trunks, one or two dendrites might arise from the stem of one trunk or from the central portion of the soma. In the pyramidal-shaped neurons (Figure 4b), however, one thicker dendritic trunk often emerged from the narrow end of the pyramid, and other dendrites occupied other corners of the pyramids. Primary dendrites also emerged from the cell body. The dendrites in both spindle-

and pyramidal-shaped neurons were ramified sparsely. Spines (Figure 4c) were distributed on the primary, and dendritic branches moderately or sparsely. The soma was usually devoid of spines. A variety of dendritic lengths were observed. Some were over 300 μm . The axon came out near the stem of the main dendritic trunk or from the soma. There was one neuron having a similar dendritic emerging pattern which showed moderately studded spines on the soma (Figure 4d).

Subtype II: These were triangular-shaped neurons with few dendrites. This neuronal type had three dendrites emerging from three tips of the triangle and appeared to be a plane-like projection (Figure 5). They bifurcated into secondary dendrites and extended away from the soma. The length of the dendrites from the soma reached over 450 μm . One or two dendrites were also seen to emerge from the stem of those three main dendrites. Spines were distributed sparsely on the dendrites but not the soma. The initial segment of the axon was clearly impregnated at the stem of the main dendrites.

Subtype III: These cells were triangular- or polygonal-shaped neurons with profusely branched dendrites (Figure 6). Four to five primary dendrites emerged from the soma and radiated in all directions. The dendrites ramified

- Figure 4. Medium sparsely spiny neurons, subtype I
(a. Spindle neuron (640X), b. Pyramidal neuron (640X), c. Sparsely studded spines on dendrites of pyramidal neurons (1600X), d. Spindle neuron with spine-studded soma and dendrites (1600X))
- Figure 5. Subtype II. Few dendrites emerged from the soma, and spines were distributed on all dendrites (1600X)
- Figure 6. Subtype III. Profusely-branched dendrites were sparsely studded with few spines (640X)
- Figure 7. Subtype IV. Axon (ax) came out from the base of the primary dendrites (1600X)
- Figure 8. Subtype V. Dendrites were swirling over the dendritic field (640X)



immediately into fine branches and formed abundant dendritic trees. The dendrites were laden sparsely with spines. The somata, however, were less studded with spines. The dendritic lengths were within 200 μm .

Subtype IV: Spherical- or oval-shaped neurons with few dendrites. The shapes of this type of neuron were spherical or ovoid (Figure 7). The somatic cross-sectional area ranged from 270 to 430 μm^2 . The measurements of the long and short axes were 20-23 and 15-20 μm , respectively. Three to seven dendrites emerged from the soma. Among them, there were always two to three main dendrites with thicker diameters, 4-5 μm instead of the 2-3 μm diameter seen in the others. The projecting angles of these main dendrites were approximately 120° between either two. The primary dendrites branched infrequently. Spines were distributed on the dendrites sparsely and also occasionally on the somata.

Subtype V: Spindle-shaped neurons with profusely swirling dendrites were also seen. The most common shape of the soma was spindle. The cross-sectional area ranged from 418 to 490 μm^2 . The measurements of the long and short axes were 34-40 μm and 14-18 μm , respectively. Usually, six thick dendrites emerged from the portions near two poles of the spindle (Figure 8). The dendrites curving

and swirling around the soma branched profusely and formed a complex dendritic field. The length of the dendrites extended to more than 200 μm . Spines studded all of the dendrites moderately, but, sometimes also the soma.

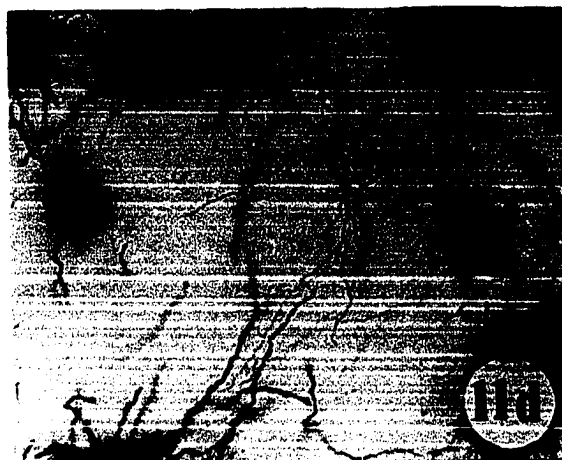
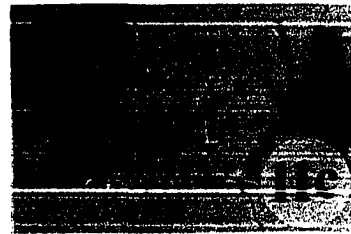
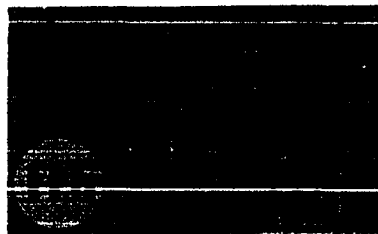
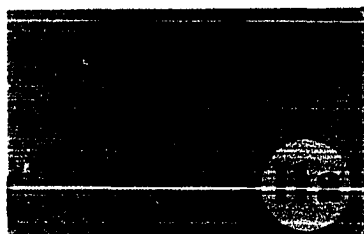
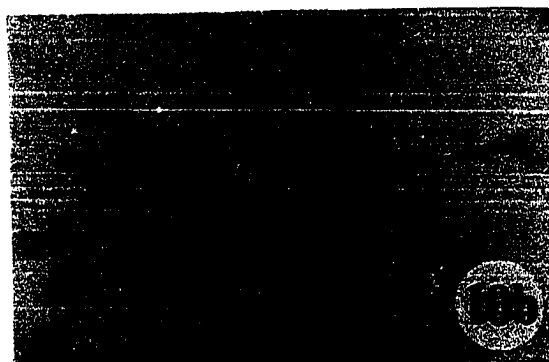
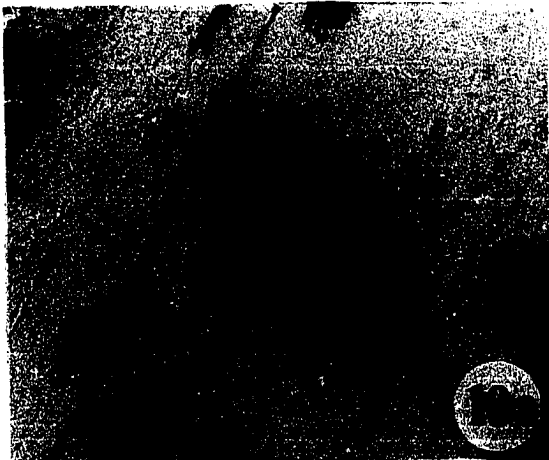
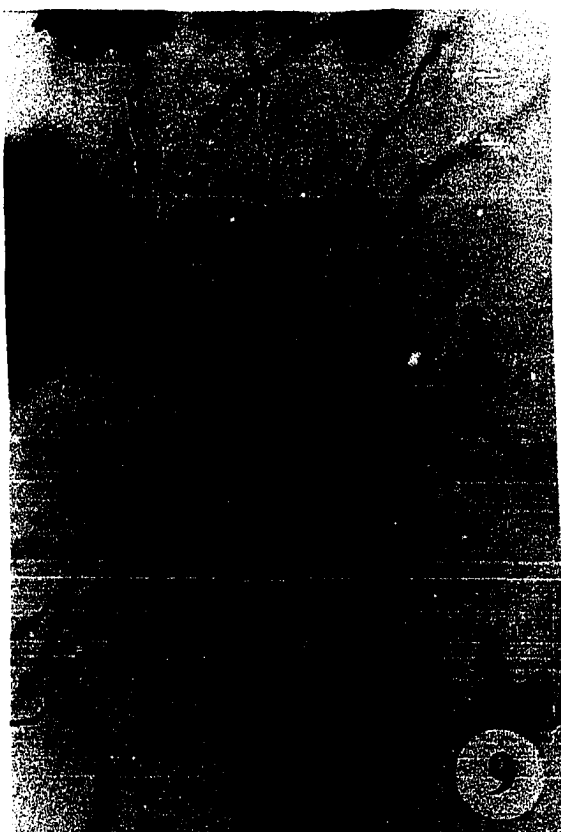
Medium Aspiny Neurons

There were many somatic shapes such as ovoid, spherical, triangular, pyramidal, and stomach-like (Figure 9). They normally did not bear any spines on the somata and the dendrites. Usually, four to six primary dendrites emerged from the soma and among them, two or three were apparently thicker. The dendrites branched near the somata, and were oriented in either one direction or all directions. The dendritic branches usually extended more than 200 μm from the soma. The dendritic field was complicated with an overlapping and folding appearance. The surfaces of the dendrites were irregular with numerous varicosities on the secondary dendrites and their branches (Figure 9). The axon was often impregnated on the initial segment and arose from either the soma or near the stem of the primary dendrites.

Figure 9. Medium aspiny neurons with irregular-surfaced dendrites (1600X)

Figure 10. Neurons (a, Small spiny neurons (640X), b, Spines were studded sparsely on the dendrites (1600X))

Figure 11. Four small aspiny neurons (a,b,c, neurons had short dendrites which usually branches near the somata (640X), b, small aspiny neuron had long and varicosed dendrites (640X), e, varicosities on the dendrites (1600X))



Small Spiny Neurons

This neuronal type was rarely found. They were spherical, triangular, and polygonal in somatic shapes (Figure 10). The cross-sectional area of the somata ranged from 114 to 167 μm^2 . The long and short axes were 14-20 μm and 9-11 μm . Three to five dendrites emerged from the soma and were sparsely to moderately covered with spines. The dendrites branched infrequently but the dendritic length only attained 150 μm . The axon which arose from the initial segment of the dendrite had constantly beaded collaterals.

Small Aspiny Neurons

There were some variations in this type. The common feature of this type of neuron was the smooth somata, but dendrites with varicosities. The somatic cross-sectional area was 130-191 μm^2 . The shape of the soma was oval, spherical, triangular, or polygonal. This neuronal type was further subdivided into two subtypes according to their dendritic fields. Subtype I had a dendritic field within 150 μm of the soma. The dendrites branched abundantly near the soma (Figure 11a,b,c). Subtype II had a dendritic field larger than subtype I and could reach to 200 μm from the soma. The dendrites were less branched and directed away

from the somata. These neurons all exhibited many varicosities on the surface of the dendrites (Figure 11d,e). In addition to micrographs, some of the neuronal types which have been mentioned above are illustrated in Figures 12 and 13 by camera lucida drawing in order to show better overall neuronal structures.

Figure 12. Camera lucida drawing of large and medium spiny neurons seen in the young pigs. (a. Large spiny neuron, b. Medium densely spiny neuron, c. Medium sparsely spiny neuron-subtype I, d. Medium sparsely spiny neuron-subtype IV, e. Medium sparsely spiny neuron-subtype III) (the axon (ax) was impregnated only in some types, scale bar = 50 μ m)

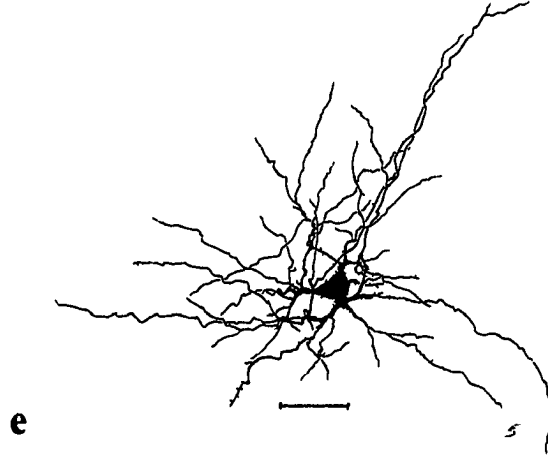
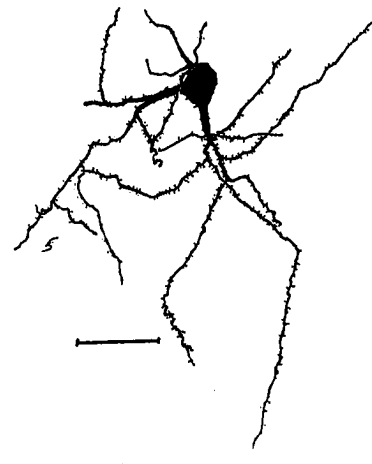
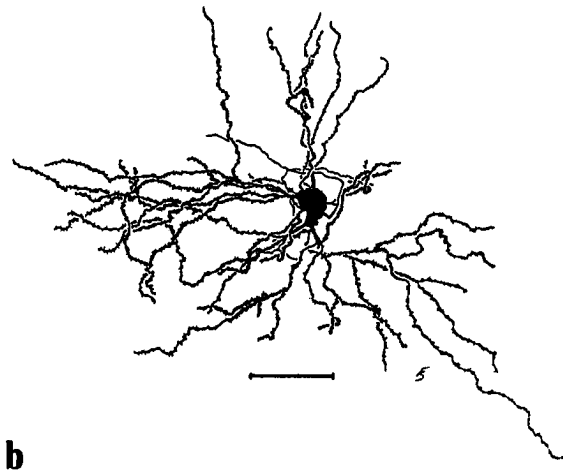
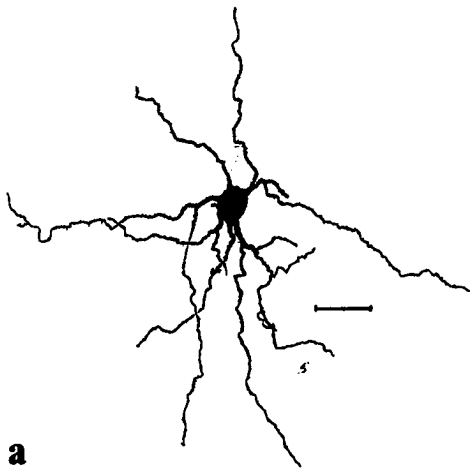
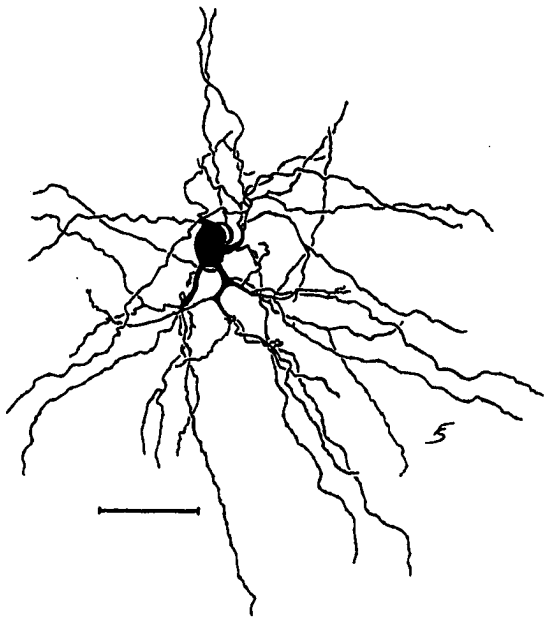
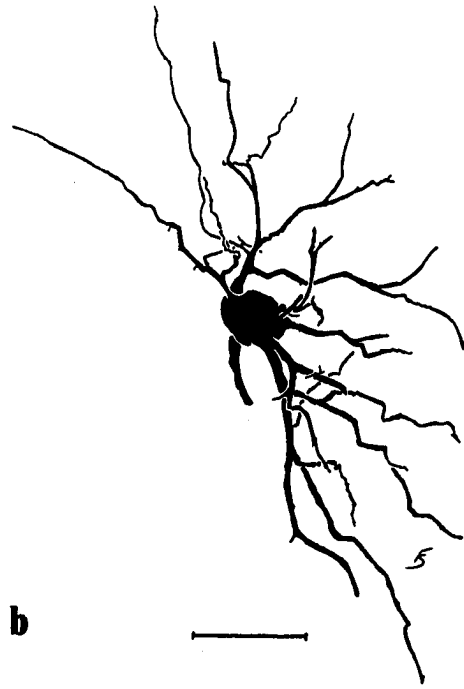
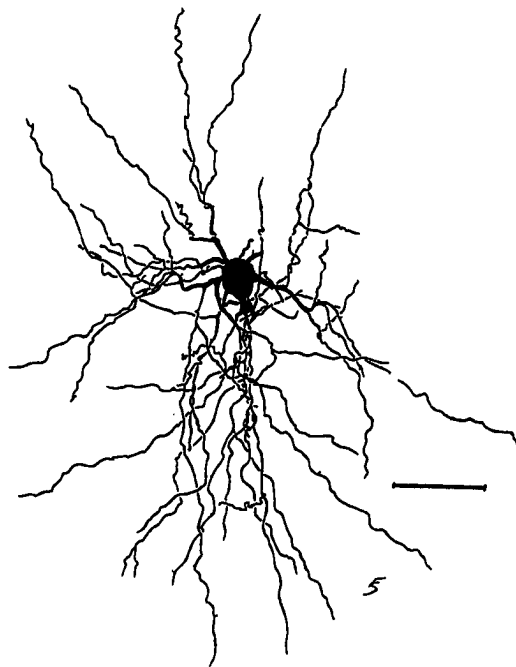


Figure 13. Camera lucida drawing of large and medium aspiny neurons seen in the caudate nucleus of the young pigs (a. Medium aspiny neurons with varicosed dendrites, b. Large aspiny neurons, c. Medium aspiny neurons with irregular-surfaced dendrites, scale bar = 50 μ m)

**a****b****c**

DISCUSSION

It is known that neuronal appearance changes postnatally (Chronister et al., 1976; Adinolf, 1977; Lu and Brown, 1977; Tanaka, 1980). The regular spine configurations are not discernible until day 16, while development is completed by one month of age by rodents (Chronister et al., 1976; Lu and Brown, 1977). Tanaka (1980) reported that aspiny I neurons which were immature at birth, were completely developed at 30 days of age. The spiny I neurons, however, show less spine density and dendritic lengths than those seen in the adult dogs. Therefore, developmental changes of neuronal characteristics may depend upon species and neuronal types. Although the postnatal maturation of the neurons has been studied for specific neuronal types, little is known of the distinct neuronal types in young animals. The Golgi material used in our study are taken from young pigs at 14 days of age, and may account for the variation of the neuronal types that were distinguished. A comparison of the neuronal types were made with those found in the adult animals in other species.

Large Neurons

Large neurons have been described in the neostriatum in several species (Kemp and Powell, 1971a; DiFiglia et al., 1976; Rafols and Fox, 1979; Tanaka, 1980; Chang et al., 1982). The morphological features and somatic sizes of large neurons, however, varied among these studies. In our study, the large neuron had a somatic cross-sectional area of about $995 \mu\text{m}^2$, and was found in the large spiny category. DiFiglia et al. (1976) described giant neurons of the aspiny neuronal type in monkeys, which was similar to the findings as did Dimova et al. (1980) in rats. Large aspiny neurons rarely occurred in our porcine material. The dendritic patterns of the large spiny neurons seemed to be like the large neurons with many dendrites observed by Rafols and Fox (1979) in monkeys and the large neurons in Figure 7 of Lu and Brown (1977) in rats. Therefore, the common feature was, among several primary dendrites, a few thick and fairly straight dendrites which may or may not be studded with two or three spines. Other large aspiny neuronal types, such as aspiny II of DiFiglia et al., (1976), and Tanaka (1980), and aspiny I of Chang et al. (1982), were not impregnated in the porcine CN.

The large spiny neurons have been reported in the dog (Tanaka, 1980), monkey (DiFiglia et al., 1976) and rat

(Chang et al., 1982). The morphology of our large spiny neurons was more like those found in the dog and the monkey. The spiny dendrites were very thick and rather straight, and branched sparsely, extending for a long distance. The large neuron which was described as spiny type II by DiFiglia et al. (1976) has characteristics similar to our large spiny neurons. Nevertheless, the medium sized spiny type II of DiFiglia et al. (1976) possessed similar features to our subtype I of medium sparsely to moderately spiny neurons.

Medium Densely Spiny Neurons

This is the neuronal type which occurs consistently in many species. Its morphology has been well-studied in monkey (DiFiglia et al., 1976; Rafols and Fox, 1979), dogs (Tanaka, 1980), and rats (Chang et al., 1982). In our material, the general features of the spine distribution were similar to those found in other adult species, although it is not known whether the dendritic length and spine density reached the maturation level of the adult pigs or not. The axons of these neurons were not well-impregnated in preparatives. The axon length and degree of arborization have been reported to be increased during maturation of this type of neuron (Tanaka, 1980). This may explain the

relatively short appearance of the axon in our material. It has been shown by recent studies combining Golgi and retrograde tracing methods or intracellular labeling techniques (Somogyi and Smith, 1979; Preston et al., 1980; Chang et al., 1981), that medium spiny neurons are projection neurons.

Medium, Moderate, to Sparsely Spiny Neurons

In young pigs, in addition to the densely spiny neurons, a variety of medium spiny neurons were impregnated. According to somatodendritic traits, they were further subdivided into five subtypes. Whether these subtypes exist in the adult pigs is unknown. The subtype I neurons were similar to the neurons shown in Figure 2 of Rafols and Fox's work (1979) and the spiny type II of DiFiglia et al. (1976). A postnatal decrease in spine density on dendrites of medium spiny II neurons has been found in monkeys (DiFiglia et al., 1979). The spine density of subtype I dendrites varied from moderate to sparse. This variation may represent a differentiation process of the neuronal types. The subtype II cells in our material were differentiated from subtype I cells by having less branching and longer dendrites. It is like the large spiny II neurons in monkeys (DiFiglia et al., 1976), except is smaller in size. The subtype III

cells were characterized by a fine and profusely branching dendritic tree and by a decrease in spine distribution from the proximal to the distal end of the dendrite. The spines were present at irregular distances from one another and were sparse in number. No comparable type was found in the CN of adults of other species. It is not known whether this type is under transition type or not. The subtype IV neurons were similar to the type II medium neurons of Chang et al. (1982). The subtype V neurons in our material were specialized by their swirling and curving dendritic branches around the somata. Although this feature of the dendritic pattern had also been reported in the monkey (DiFiglia et al., 1976), as aspiny type II, the impregnated neurons with this dendritic pattern were moderately spiny in appearance in our sections.

Medium Aspiny Neurons

Based on the absence of dendritic and somatic spines, many medium aspiny neurons have been described in rats (Dimova et al., 1980; Chang et al., 1982), monkeys (DiFiglia et al., 1976; Rafols and Fox, 1979), and dogs (Tanaka, 1980). The medium aspiny neurons were often found in every part of the tissue. Although they showed many somatic shapes, the overall dendritic pattern was not apparently

different. The degree of dendritic branching may be varied, in our medium aspiny neurons, but no clear separation can be made according to the dendritic tips which has been used as one of the criteria for identification of medium neurons in rats (Chang et al., 1982).

Small Spiny Neurons

The small spiny neurons found in the porcine CN had areas ranging from 114 to 167 μm^2 . The spines were either moderately or sparsely studded on the dendritic processes. There were few reports about small spiny neurons. Chang et al. (1982) showed several small neurons with spiny dendrites. Some of them were similar to our small spiny neurons. The difficulties in finding this type of the neuron may be partly because of their small somata, thin processes, and rare occurrence.

Small Aspiny Neurons

The small aspiny neurons were impregnated more frequently than small spiny neurons. Two subtypes were described in this study, and can be differentiated mainly by the radius of the dendritic field (150 vs. 200 μm). Similar cells have been observed in other adult species (Dimova et al., 1980; DiFiglia et al., 1976; Chang et al., 1982).

Developmental changes in the morphology of neurons take place in different ways in spiny and aspiny types. Aspiny neurons in the CN of dogs mature faster than spiny neurons and the absence of the spines or filopodia are shown even at birth. The spiny neurons, however, show varicosities, filopodia, and thick dendritic stumps at birth, which then decrease during maturation and are replaced by increasing growth of the spines (Tanaka, 1980), but pigs at this age do possess morphological characteristics which can be readily distinguished. Generally, the porcine CN has a similar variety of neuronal types with other animals, and may follow similar morphological changes during development.

SUMMARY

To determine whether another neurotransmitter, γ -aminobutyric acid (GABA), in addition to dopamine, is involved in the etiology of the porcine stress syndrome, we measured the activity of glutamic acid decarboxylase (GAD), the biosynthetic enzyme of GABA. Five brain regions, substantia nigra (SN), caudate nucleus (CN), putamen, hypothalamus, and cerebral cortex, were collected and assayed for GAD activity. Significantly greater GAD activity was found in the SN of the stress-susceptible (SS) pigs than in the SN of stress-resistant (SR) pigs, whereas, striatal GAD activity in both types of pigs was similar. The reason for the increase of GAD activity in the SN of SS pigs is unknown. The hypothalamus of SS pigs contained lower GAD activity than did the hypothalamus of SR pigs. Two age groups (group I: ave. = 107 days; group II: ave. = 184 days) of SS and SR pigs were used to examine GAD changes with age. Age did exert significant effects on brain GAD activity. Greater activity was present in the SN, CN, and putamen of the older pigs. The cerebral cortex of the older pigs, however, had lower GAD activity. Uneven distribution of GAD activity throughout the brain was observed. The hypothalamus had the greatest amount of GAD activity. The SN, which has greatest activity in other species, showed surprising low activity in this

study. Possible reasons have been discussed. It is still unclear how the alteration in GAD activity of the SN and the hypothalamus of SS pigs correlates to dopamine deficiency and the etiology of the porcine stress syndrome.

The age effects on neuronal and synaptic morphometric characteristics of the porcine CN were also studied. Several attributes such as the presence or absence of the nucleolus, the presence or the absence of a process, the staining intensity, and the neuronal shape were used to identify the neuronal populations in the caudate nucleus. Significant age differences in both SS and SR pigs were found in three neuronal populations which included all of the neurons (AN), neurons with a nucleolus (NN), and neurons with a process (PN). The neuronal density of AN and NN were decreased with advancing age, whereas, that of PN increased from age I (42-66 days of age) to age II (148-237 days of age), and then decreased at age III (751-1168 days). The neuronal density of AN, NN, and PN were not significantly different between treatments (SS vs. SR). The magnitude of the density decline with age, however, was significantly greater in the AN and NN of the SS pigs. The synaptic density on CN neurons also showed a significant decline with age in both SS and SR pigs, although the magnitude of the synaptic density decline was smaller than the magnitude of decline of the neuronal density. There

was a linear relationship between age and synaptic density.

The porcine CN prepared by Nissl stain showed neuronal populations mainly characterized by small (diameter $< 18.5 \mu\text{m}$) and medium ($18.5 < \text{diameter} < 26.0 \mu\text{m}$) neurons, which comprised 75% and 24% of the population, respectively. The ratio of small neurons to medium neurons was not significantly different among age groups or between treatments. The interaction between age and treatments was, however, significant at $p < 0.09$. This neuronal ratio was decreased with age in SS pigs, but increased with age in SR pigs. Therefore, age seems to exert different effects on different neuronal populations in SS and SR pigs.

Other important findings include the characterization of the neuronal types by the combination of several neuronal attributes. At least four neuronal types could be identified in this study. They were: (1) neurons with the presence of a nucleolus, small to medium in size, light staining and round in shape; (2) neurons with the presence of a process, medium to large in size, dark staining, and angular shape; (3) neurons with intermediate staining, and round to angular shape; and (4) neurons with dark staining and round to intermediate shape.

From the above finding, the greater magnitude of the neuronal density decline associated with SS pigs represents an

abnormal neuronal alteration which occurs with age in the CN of the SS pigs. The reversed trend of the small to medium neuron ratio in SS pigs also suggests a qualitative neuronal loss in the CN of the SS pigs.

The neuronal types in the porcine caudate nucleus were further studied by Golgi techniques according to their somatodendritic characteristics and the soma size. Four major spiny neuronal types and three major aspiny neuronal types were described in our study. The large aspiny neurons were rarely impregnated in the CN of the young pigs. The large spiny neurons were impregnated more frequently than the large aspiny neurons. They showed a relatively simple dendritic pattern with several fairly straight, thick, long dendrites which branched infrequently. Medium neurons can be divided into three main categories by the distribution and density of the spines. The densely spiny neurons were identical to the most frequently impregnated medium spiny neurons found in other species. Moderately to sparsely spiny neurons had a variety of somatodendritic features and could be further subdivided into five subtypes. Medium aspiny neurons also were impregnated often in this study. Variation in the somatodendritic features was not apparent in this neuronal type so no further subdivision was made. There were spiny and aspiny small neurons observed. The small spiny neurons were

hard to identify probably because of their small soma size, very fine processes, and also rare impregnation. The aspiny small neurons could be distinguished into two subtypes by the radius of their dendritic fields. Generally, the neuronal types in the porcine caudate nucleus were similar to those in the CN in other species.

In conclusion, it would seem that there are both neurochemical and neurocytological alterations in pigs affected with the PSS. In addition, the synthesizing enzyme, GAD, of the neurotransmitter GABA, may participate in a causal role, as well as dopamine, in the PSS. The greater magnitude of the decline in neuronal density in SS pigs represented a quantitative difference in neuronal loss. Furthermore, a different trend of the neuronal loss may cause the alteration of the small to medium neuron ratio in SS pigs. These results are suggestive for the PSS being an age-related disease.

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To Him be the glory forever. Amen.

Rom. 11:33-36

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APPENDIX A

Method I. Procedure for Tissue Preparation for
Neuronal Density Determination

The cranial one-half of the CN was immersed in 10% phosphate buffered neutral formalin (pH=7.0) and fixed for at least 4 months at room temperature. Tissues were dehydrated in a series of alcohols (70%, 95%, 95%, 95%, 100%, and 100%) for two hours at each concentration. After dehydration with alcohols, the tissues were cleared by two changes of xylene in two hours. The tissues were infiltrated through three changes of "Paraplast Plus" paraffin (Lancer) for a total of 6 hours. The final infiltration was done in fresh paraffin in a vacuum oven and left over night. The next morning, tissues were embedded in fresh "Paraplast Plus" paraffin. After the paraffin was hardened, the blocks were hand-trimmed and mounted on the microtome for further trimming until the tissue started to be cut. Seven μ m thick cross-sections were collected serially through the entire tissue. Three sections were obtained, one from the rostral, middle, and caudal one-thirds of the tissue and mounted on three different slides. The slides with tissue sections were transferred to a series of solutions: 5 minutes for each change of xylene (two changes), 2 minutes for each of the following solutions: 100%, 95%, 95%, 70% alcohols, and distilled water. Tissues were

stained in cresyl echt violet (Chroma-Gesellschaft) solution (5 grams of cresyl echt violet dissolved in 1000 ml distilled water with 3.0 ml of 10% acetic acid) for 5 minutes and washed in distilled water three times. The slides with tissues were then placed in acetic formalin solution, which was made up by adding 4.0 ml of formaldehyde, 0.2 ml of glacial acetic acid, to 100 ml of distilled water, for 3 minutes, and then washed twice in distilled water. The dehydration was done through three trays of 95% alcohol, each for 2 minutes, and n-butyl alcohol for 2 minutes. Dehydrated tissue sections were then cleared in cedar wood oil for 8 minutes and four changes of xylene, each for 2 minutes. The slides were then covered with drops of "Permunt" (Fischer-Scientific Co.) and cover-slipped.

APPENDIX B

Method II. Procedure for Tissue Preparation for Synaptic Density Determination

The caudal one-half of the CN was fixed in 5% glutaraldehyde in 0.1 M phosphate buffer solution (pH = 7.2-7.4) for at least 4 months at 4°C. Tissues then were cut into small pieces and transferred to freshly prepared 5% glutaraldehyde in 0.1 M phosphate buffer solution at the same pH for 24 hours at 4°C. Tissues were washed with 0.2 M phosphate buffer solution for 1 hour at room temperature. Dehydration was done in 50% alcohol for 10 minutes and 15 minutes for each of the following concentrations of alcohols: 70%, 80%, 95%, 95%, and 100%. After dehydration, tissues were stained with three changes of 1.5% phosphotungstic acid (Mallinckrodt) in 100% alcohol for a total of 3.5 hours. Before resin infiltration, tissues were rinsed three times in 100% propylene oxide for a total of 25 minutes. Infiltration with resin was done in different ratios of the propylene oxide and Spurr's resin mixture (1:1 for 6 hours; 1:3 for 12 hours). The final infiltration was carried out using the standard formula of Spurr's firm resin (Spurr, 1969) in the desiccator for 6 hours. Tissues were embedded in fresh Spurr's resin and incubated in a 70°C vacuum oven for 24 hours.

¹Reference: Spurr, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-42.