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An Immunocytochemical And Morphological Examination Of The Cerebellum Of Mutant Dystonic (Dt) Rats And Rats With 3-Acetylpyridine Lesions.

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AN IMMUNOCYTOCHEMICAL AND MORPHOLOGICAL EXAMINATION OF THE CEREBELLUM OF MUTANT DYSTONIC (*dt*) RATS AND RATS WITH 3-ACETYLPYRIDINE LESIONS

by

JACQUELINE LUTES GOLDSTEIN

A DISSERTATION

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ABSTRACT OF DISSERTATION
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Title An Immunocytochemical and Morphological Examination of the Cerebellum of Mutant Dystonic (*dt*) Rats and Rats with 3-Acetylpyridine Lesions

The morphology of cerebellar elements was examined in the mutant rat, *dt*, and in rats with lesions to the climbing fiber system as a result of administration of the neurotoxin, 3-acetylpyridine (3-AP). Both of these animals display a motor syndrome characterized by ataxia and axial twisting and both are insensitive to the tremorgenic effects of harmaline, a drug whose action is initiated in climbing fibers arising in the inferior olive and projecting to the cerebellum. Abnormal firing rates in cerebellar Purkinje cells and cells of the deep cerebellar nuclei (DCN) have been recorded in both groups. In addition, glutamate decarboxylase (GAD) activity, as well as metabolic activity, are increased in the DCN of both groups. In the studies presented here, in agreement with previously measured increases in DCN GAD activity, gamma-aminobutyric acid (GABA) levels in the cerebellum of animals with 3-AP lesions were elevated 24 hours after administration of the neurotoxin, and, one day later, there was an increase in the size of GAD+ puncta in the DCN. These puncta represent terminals of GABAergic cells. In addition, animals with 3-AP lesions showed an increase in the size of cells in the interpositus division of the DCN. Dystonic rats, in spite of increased GAD activity in the DCN, showed no change in GABA levels or GAD+ puncta size at this site. In addition, neither Purkinje cell number, DCN cell size, nor DCN volume were altered in this mutant as they are in many cerebellar mouse mutants. Therefore, increased firing rates in cells of the

DCN, previously measured in these animals, are not due to deafferentation. GAD+ puncta density was reduced in the DCN of the *dt* rat and may be a reflection of decreased Purkinje cell firing rates, which have previously been measured in these animals. It is suggested that different molecular forms of GAD may be responsible for paradoxical GAD activity findings in both animals. In addition, similarities between the *dt* rat and rats with long survival times following a 3-AP lesion are discussed. Findings from these experiments provide further evidence to support a hypothesis of altered Purkinje cell function in the genetically dystonic rat.

Abstract Approved by: Committee Chairman _____

Program Director _____

Date 4/15/91 Dean of Graduate School _____

PS-5744

DEDICATION

This entire project, the work which produced it, and the results produced by it, is dedicated to the two most important people in my life, my beloved children, Jennifer and Jonathan. While they must, at times, have felt themselves to be neglected, they uttered few complaints, were constantly encouraging, and moved forward confidently in their own lives, gathering personal success along the way. Their youthful enthusiasm and cheerful attitude have often sustained me in times of discouragement and have surely helped me to keep a perspective on what is and is not important.

ACKNOWLEDGMENTS

I would like to thank the members of my committee, Drs. Joan F. Lorden, Edward J. Rickert, Thomas T. Norton, Barry J. Davis and Rosalyn E. Weller, for their time, assistance, and encouragement.

Special thanks go to my advisor and mentor, Joan Lorden. She has been, for me, a model for the meaning of professional thoroughness, honesty, and generosity. Her intelligent and knowledgable guidance and persistent prodding are the foundation of the entire project. Thanks are due Barry Davis, for teaching me the art of immunocytochemistry; Roz Weller, for expert advice on photomicroscopy; Ed Rickert, for statistical expertise; Dr. Daniel Jones, of University of Alabama at Birmingham's Biology Department, for allowing me the use of their life-saving Bioscan image analysis system; and Dr. Joseph Gauthier and his son, Bill, for instructing me in the use of that system. Custom software used in volume measurements was designed by Jill Gemmill of UAB's Neurobiology Research Center.

Through the years I was fortunate to be assisted by many fine laboratory technicians: Jeanette Milligan, Linda Hayley, Tammy Carson, and, most recently, Jeff Ervin, one of the most patient and cooperative individuals I've ever encountered.

Finally, thanks go to four of my fellow graduate students for their unending and invaluable moral support: Joanne Sims, Sara Stratton, Vicki Michela, and Gregg Steele.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3-AP. 3-acetylpyridine

β. . . . . beta nucleus of inferior olivary nucleus

DAO . . . . . dorsal accessory olive

dc. . . . . dorsal cap of inferior olivary nucleus

dt. . . . . dystonic

DCN . . . . . deep cerebellar nuclei

GABA. . . . . gamma aminobutyric acid

GAD . . . . . glutamate decarboxylase

GU. . . . . glucose utilization

HPLC-EC. . . . . high performance liquid chromatography with electrochemical detection

INT a. . . . . anterior interpositus nucleus

INT p . . . . . posterior interpositus nucleus

IO. . . . . inferior olivary complex

LAT . . . . . lateral nucleus

MAO . . . . . medial accessory olive

PO. . . . . principal olive

VEST. . . . . vestibular nuclei

INTRODUCTION

Compared to other regions of the central nervous system, cerebellar development begins relatively late. Immunohistochemistry using an antibody for gamma-amino-butyric acid (GABA), the principal neurotransmitter of cerebellar neurons, reveals no reactivity in cerebellum of rats until the 2nd week of a 3-week gestational period (Aoki, Semba, & Kashiwamata, 1989). Although mature deep cerebellar nuclear (DCN) cells are in place at birth (Altman & Bayer, 1978, 1985) development continues postnatally, in stages, until at least the 3rd week of life. At this time adult firing patterns can be recorded in cerebellar Purkinje cells, the primary target cell for input and modulation in the cerebellar cortex and the only output neuron (Crepel, 1972; Shimono, Nosaka, & Sasaki, 1976).

The cerebellum plays a major role in the regulation and coordination of motor activity. Cerebellar damage during early development impairs normal locomotion and, more specifically, disturbs the normal equilibrium behavior required for locomotion (Auvray, Caston, Rebar, Stelz, 1989; Gramsbergen, 1982). Neurotoxic lesions that destroy climbing fibers, a major source of input to the cerebellum, produce a motor syndrome which is marked by ataxia and axial twisting (Sukin, Skedros, Beales, Stratton, Lorden, & Oltmans, 1987).

A variety of murine mutant strains with neurological motor syndromes show histological evidence of cerebellar defects (Sidman, Green, & Appel, 1965). Studies of mouse mutants with motor deficits have been useful as natural experiments to

investigate the organization and development of systems with obvious abnormalities of specific cell types. However, less attention has been paid to the functional significance of these abnormalities.

The *dystonic* (*dt*) rat is an autosomal recessive mutation of the Sprague-Dawley strain which displays a neurologically based motor syndrome (Lorden, Mceon, Baker, Cox, & Walkley, 1984). Initially, behavior of the *dt* rat pup does not differ from that of its normal littermates. However, at postnatal day 9-10, the phenotype is expressed as a progressively developing, generalized movement disorder with ataxia, axial twisting, and involuntary, uncoordinated movement. Although light microscopic analysis of the *dt* rat indicates normal morphological development, biochemical, electrophysiological, and pharmacological studies specifically designed to examine motor systems in these animals indicate functional abnormalities in the cerebellum (Beales, Lorden, Walz, & Oltmans, 1990; Brown & Lorden, 1989; Lorden & Ervin, 1990; Lorden et al., 1984, Lorden, Oltmans, McKeon, & Lutes, 1985; Lutes et al., 1987; Oltmans, Beales, Lorden, & Gordon, 1984; Oltmans, Beales & Lorden, 1986; Stratton, Lorden, Mays, & Oltmans, 1988). These methods have not, however, allowed us to identify specifically the source of such an abnormality.

The set of experiments presented in this study has examined the anatomy of the GABAergic Purkinje cell system in the cerebellum of two different groups of animals: the *dt* rat, where abnormal Purkinje cell function is indicated, and rats with 3-acetylpyridine (3-AP) lesions, which destroy a major source of input to Purkinje cells. These experiments, taken together with biochemical and electophysiological data, have allowed us to advance our understanding and description of the functional abnormalities underlying the motor syndromes that have been observed in these two groups of animals.

Cerebellum

The cerebellum regulates and plans motor activity as well as participates in the regulation of muscle tone. Anatomically, it has been well-described and is known to be organized into sagittal zones (Eccles, Ito, Szentagothai, 1967; Ito, 1984). In addition, the cerebellar cortex is divided into three clearly defined layers containing five major cell types: Purkinje, basket, stellate, Golgi, and granule (Figure 1). The Purkinje cell is the focus of cerebellar activity, receiving input from all other cell types, acting as the principal target cell for extracerebellar afferents and serving as the sole efferent from the cerebellar cortex. In the rat there are three pairs of DCN, located in the white matter under the cortex, that receive topographically organized projections from cerebellar Purkinje cells (Korneliussen, 1968). From medial to lateral these are the fastigial, interpositus (often divided into anterior and posterior), and dentate nuclei. These nuclei are also described and named in terms of their location, i.e., medial, interpositus, and lateral.

Mossy fibers and climbing fibers provide the two major sources of excitatory input to the cerebellar cortex (Colin, Manil, & Desclin, 1980). Climbing fibers arise from cells in the inferior olivary nucleus (IO) and enter the contralateral cerebellar cortex via the inferior cerebellar peduncle after sending collaterals to the DCN (Campbell & Armstrong, 1983a). Within the cortex a single climbing fiber axon winds around the dendrites of a single Purkinje cell, making multiple synapses and providing a major source of excitatory input. Studies of both olivocerebellar and corticonuclear topography reveal ordered zones of projection (Oscarsson, 1979) (Figure 2). Climbing fiber projections from the IO terminate in the cerebellar cortex with a longitudinal pattern of organization, i.e., the caudal portion of the medial accessory olive (MAO) projects to the medial portion of the cerebellum, the vermis;

Figure 1. *Cells of the cerebellar cortex.* There are five major cell types in the cerebellar cortex. The Purkinje cell is the only output neuron, sending projections, primarily, to the DCN. The two primary sources of excitatory input to the Purkinje cell are climbing fibers and mossy fibers. *Black rectangles* represent GABAergic synapses.

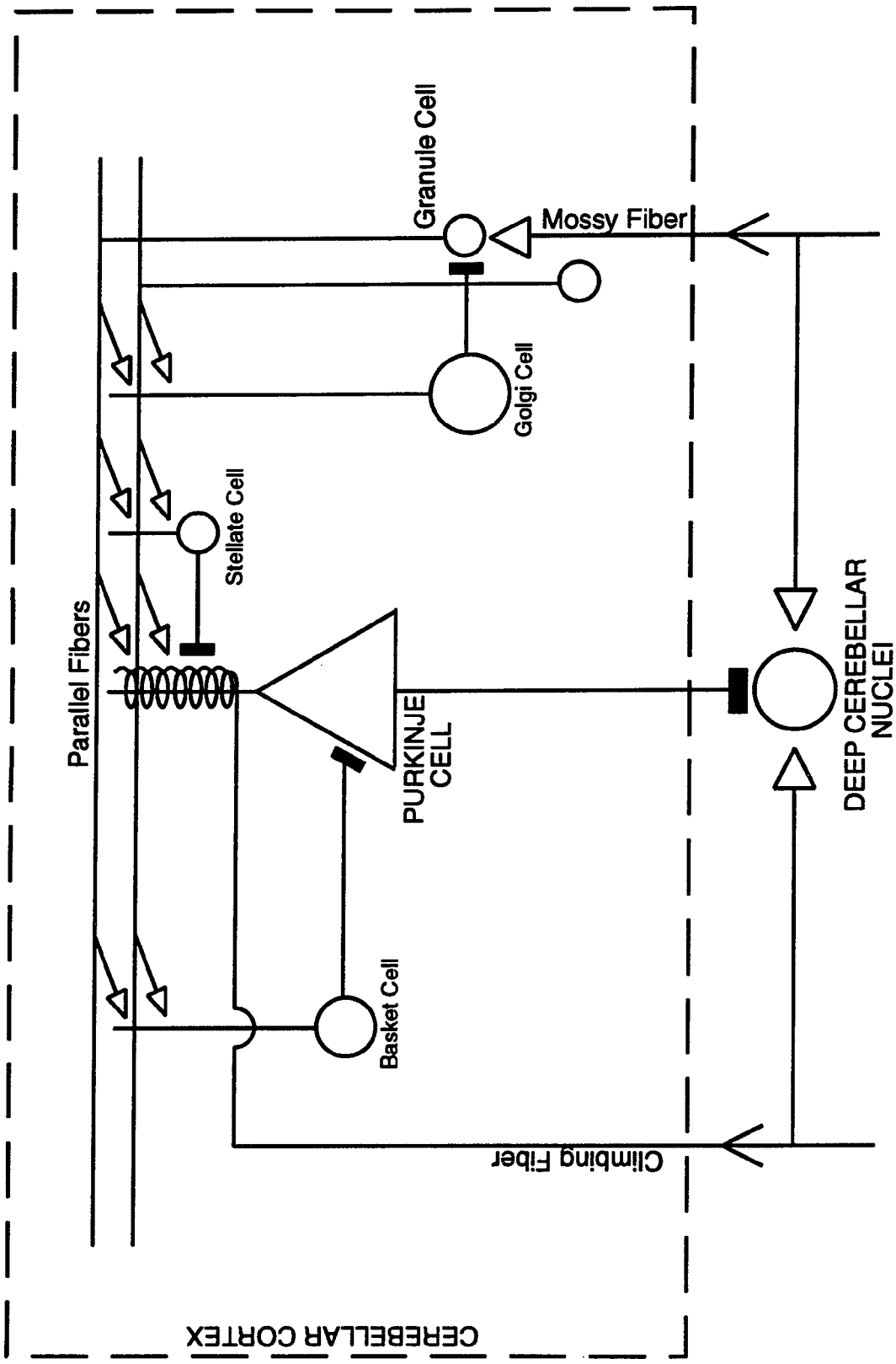
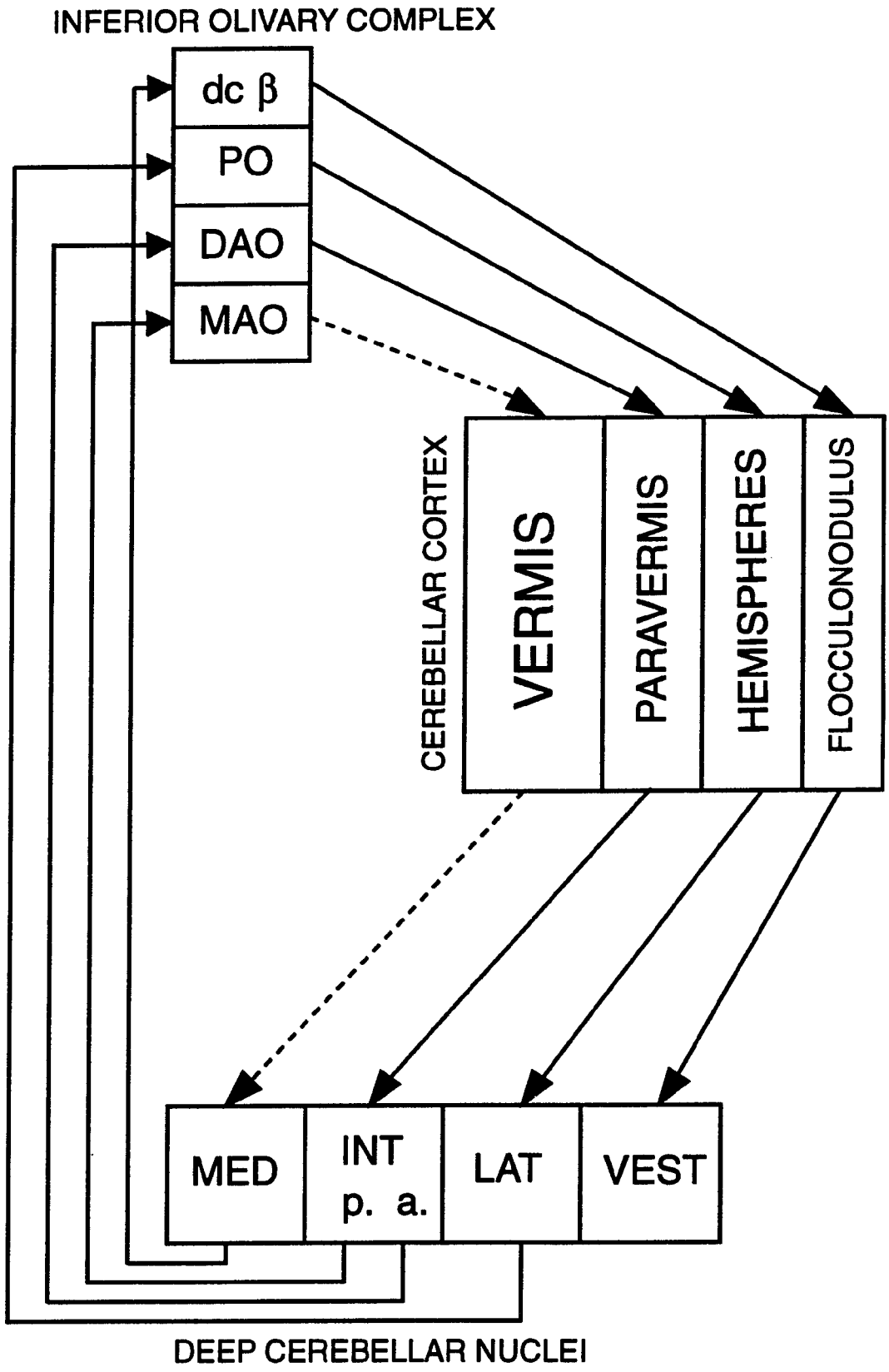

Granule Cell
Mossy Fiber
Golgi Cell
Stellate Cell
Parallel Fibers
PURKINJE CELL
DEEP CEREBELLAR NUCLEI
Basket Cell
Climbing Fiber
CEREBELLAR CORTEX

Figure 2. *Olivo-cortico-nuclear connections.* Climbing fibers project from inferior olivary nuclei to the cerebellar cortex with a mediolateral pattern of organization. Purkinje cell axons also project from the cortex to the DCN in a mediolateral pattern. In addition, there is also a rostrocaudal order of projection, not shown here, in both cases. *IO divisions: dc*, dorsal cap; β β nucleus, *PO*, principal olive, *DAO*, dorsal accessory olive, *MAO*, medial accessory olive. *DCN divisions: MED*, medial nucleus, *INT p. a.*, posterior and anterior interpositus nucleus, *LAT*, lateral nucleus. *VEST*, vestibular nuclei. *dotted line*, path of harmaline sensitivity.


INFERIOR OLIVARY COMPLEX
dc β
PO
DAO
MAO
CEREBELLAR CORTEX
VERMIS
PARAVERMIS
HEMISPHERES
FLOCCULONODULUS
MED
INT
p. a.
LAT
VEST
DEEP CEREBELLAR NUCLEI

lobules I-VI of the paravermis receives projections from the lateral dorsal accessory olive (DAO), and lobules VI-VIII in the posterior lobe of the paravermis receive projections from the medial DAO (Campbell & Armstrong, 1983b; Furber & Watson, 1983). Finally, the caudal principal olive (PO) and the MAO at the same level send projections to lobules I-VIII in the lateral zone, and the rostral portion of all 3 olivary nuclei sends projections to the most lateral portion of the cerebellum, the hemispheres. The organization established in olivocerebellar projections is maintained in projections from the cerebellar cortex to the DCN. Corticonuclear projections show mediolateral as well as rostrocaudal localization both between and within nuclei (Armstrong & Schild, 1978a, 1978b; DeCamilli, Miller, Levitt, Walter & Greengard, 1984; Goodman, Hallett, & Welch, 1963). Bernard (1987) described distinct modules made up of a specific inferior olivary region projecting in an orderly fashion to a distinct cortical region, which, in turn, projects to a precise region of one of the cerebellar and/or vestibular nuclei.

Climbing fibers originate from one site and synapse directly onto Purkinje cells on a one-to-one basis. Mossy fibers, however, the other source of excitatory input to the cerebellum, have multiple sites of origin in pontine and reticular formation nuclei as well as in the spinal cord (Schulman, 1983) and provide input through the granule cells to multiple Purkinje cells. The cerebellar surface is folded into cortical ridges or folia, and Purkinje cells lay perpendicular to the folium. Axons of granule cells bifurcate in the molecular layer into parallel fibers which run longitudinally along the folium synapsing on dendrites of numerous Purkinje cells.

The two major extrinsic inputs to Purkinje cells are excitatory; however, communication within the cerebellar cortex is all inhibitory (Schulman, 1983). Golgi cells make inhibitory connections onto granule cells while basket and stellate cells

furnish inhibitory input to Purkinje cell bodies and dendrites, respectively. Purkinje cells, in turn, inhibit target cells in the DCN (Ito, Yoshida, & Obata, 1964, 1970; McDevitt, Ebner, & Bloedel, 1987a, 1987b). The principal neurotransmitter used by these cells is the inhibitory amino acid GABA (Rea, McBride, & Rohde, 1981). The synthesizing enzyme for GABA, glutamic acid decarboxylase (GAD), has been localized in Purkinje, basket, stellate, and Golgi cells as well as in cells of the DCN (Chan-Palay et al., 1981; Fonnum & Walberg, 1973; McLaughlin et al., 1974; Oertel, Mugnaini, Schmechel, Tappaz, & Kopin, 1981; Saito et al., 1974).

GABA is widespread in the cerebellum and appears early in development. GABA is present in the central nervous system prenatally, even before the development of appropriate receptors (Aoki et al., 1989; Lauder, Han, Henderson, Verdoorn, & Towle, 1986). GABA receptors are present in the cerebellum within 24 hours of birth, and GAD activity shows a similar early appearance (Rothe, Middleton-Price, & Bigl, 1988). In fact, GAD synthesis is evident even before synapses are formed and is not localized to synaptic terminals until postnatal week 2-3 (McLaughlin, Wood, Saito, Roberts, & Wu, 1975). It has been hypothesized that two forms of GAD exist, an unstable newborn form with a high affinity for its cofactor, pyridoxal phosphate, and a more stable adult form with a low affinity for the cofactor (Denner & Wu, 1985; Tapia & Meza-Ruiz, 1975). It is, therefore, possible that GAD plays some early, non-synaptic, role in development.

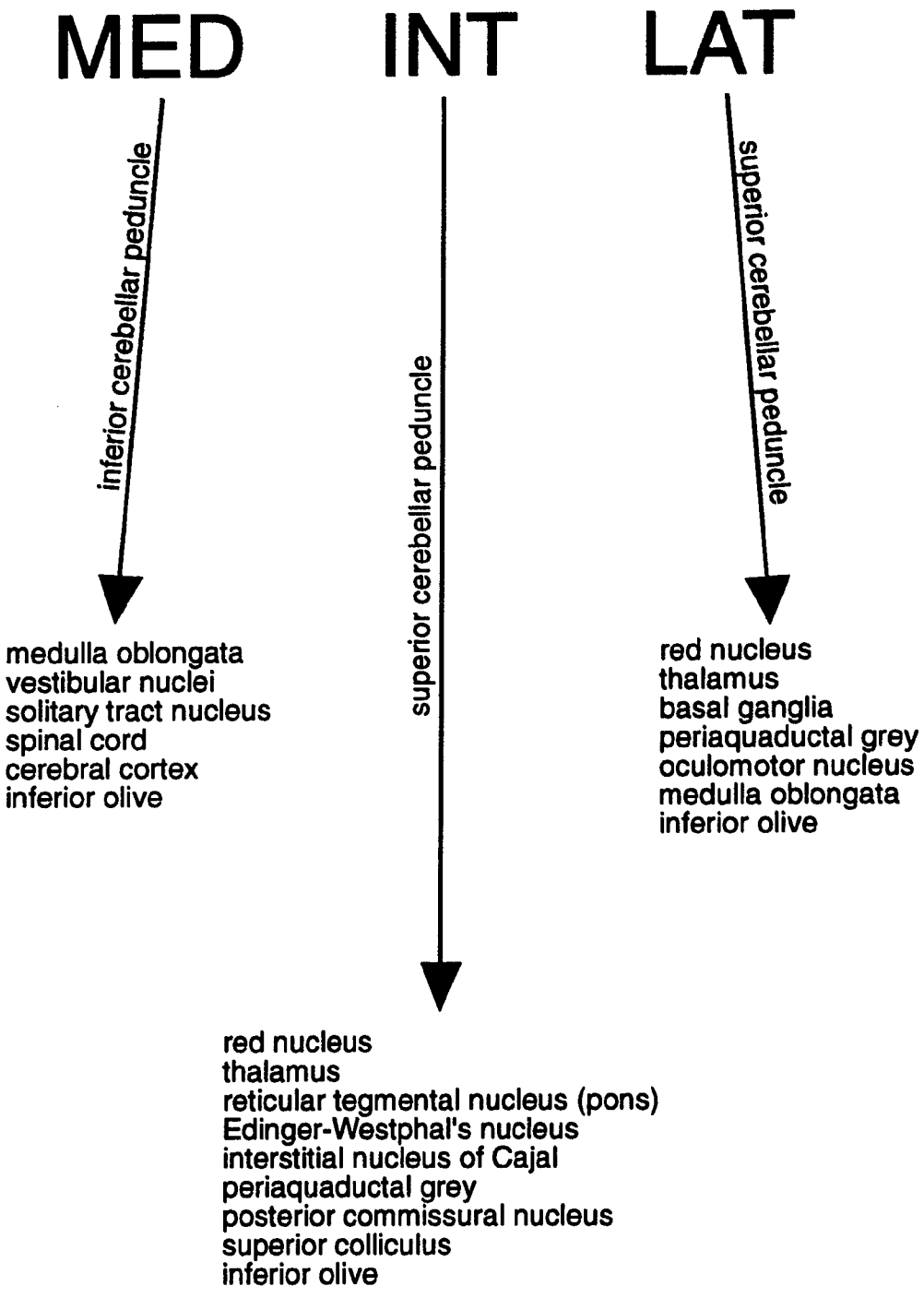
Cerebellar development is not complete at birth and proceeds in successive stages over a period of 3 weeks (Altman, 1969; 1972). Purkinje cells complete their migration to the cerebellar cortex at embryonic day 18 (Morris, Beech, Barber, & Raisman, 1985), but growth of the dendritic tree does not begin until almost two weeks later when progressive growth, lobule to lobule, occurs between postnatal days

4-9 (Goodlett, Hamre, & West, 1990). Although spontaneous Purkinje cell activity can be measured almost immediately after birth, synapses are not established until postnatal day 3 (Shimono et al., 1976; Woodward, Hoffer, & Lapham, 1969; Woodward, Hoffer, Siggins, & Bloom, 1971) when climbing fiber responses can be measured (Crepel, 1971; Puro & Woodward, 1977; Shimono et al., 1976). During the first 2 weeks of life more than 50% of the population of Purkinje cells receive input from two or more climbing fibers (Crepel, Mariani, & Delhaye-Bouchaud, 1976). The one-to-one relationship between climbing fiber and Purkinje cell is not established until after the 2nd week of life (Mariani & Changeux, 1981). In addition, some axons display dual morphology; typical mossy fiber and climbing fiber morphology is not apparent until postnatal day 5 (Mason & Gregory, 1984).

Purkinje cell maturation is primarily a postnatal event. Cells of the DCN, however, begin differentiation in the embryo and have reached a stage of advanced maturity at the time of birth (Altman & Bayer, 1978, 1985). *In vitro* studies indicate that climbing fiber and mossy fiber collaterals form functional synapses in the DCN within 24 hours of birth, at least 2 days before Purkinje cell/DCN synapses become functional (Gardette, Debono, Dupont, & Crepel, 1985).

At maturity, axons from the DCN leave via the superior and inferior cerebellar peduncles in both ascending and descending projections to establish connections with multiple sites of motor control (Faull, 1978; Faull & Carman, 1978) (Figure 3). Axons from all three nuclei project to the upper brain stem and down to the medulla; those from the medial nucleus project to vestibular nuclei and the pontomedullary reticular formation (Chan-Palay, 1977; Faull, 1978). DCN axon collaterals from all three nuclei send heavy projections to the diencephalon (primarily the thalamus), midbrain, medulla and cervical spinal cord (Bentivoglio & Kuypers,

Figure 3. *DCN projections.* Cerebellar efferents project from the deep cerebellar nuclei to sites throughout the nervous system. Axons from the medial nucleus (*MED*) exit via the inferiorcerebellar peduncle. Axons from the interpositus (*INT*) and lateral (*LAT*) nuclei exit via the superior cerebellar peduncle.


MED
INT
LAT
inferior cerebellar peduncle
superior cerebellar peduncle
superior cerebellar peduncle
medulla oblongata
vestibular nuclei
solitary tract nucleus
spinal cord
cerebral cortex
inferior olive
red nucleus
thalamus
basal ganglia
periaquaductal grey
oculomotor nucleus
medulla oblongata
inferior olive
red nucleus
thalamus
reticular tegmental nucleus (pons)
Edinger-Westphal's nucleus
interstitial nucleus of Cajal
periaquaductal grey
posterior commissural nucleus
superior colliculus
inferior olive

1982; Faull & Carman, 1978); other projections extend to lumbar spinal levels (Achenback & Goodman, 1968). The DCN and cerebellar cortex are linked by both cortico-nuclear fibers and nucleo-cortical fibers. Although topographical reciprocity exists between these paths, there are also a large number of non-reciprocal connections (Hess, 1982). Therefore, DCN neurons may provide a path of communication from one cerebellar cortical area to another via collaterals of nucleofugal axons that project to the cortex as mossy fibers (Hess, 1982). As previously mentioned, collaterals from the IO project to the DCN; there is also a substantial GABAergic projection of DCN fibers projecting back to the IO (Figure 2) (Achenbach & Goodman, 1968; Castro, 1978; Angaut and Cicirata, 1982; Angaut and Sotelo, 1987; Buisseret-Delmas, Batini, Comopoint, Daniel, & Menetray, 1989; Haroian, 1982; Nelson & Mugnaini, 1989).

Damage to the DCN, with such a broad array of connections, could conceivably produce major impairment in coordinated movement. Imperato, Nicoletti, Diana, Scapagnini, and DiChiara (1984) have demonstrated that either damage to cell bodies of the medial nucleus or direct GABA agonist receptor stimulation within this nucleus produces temporary deficits in limb tone and posture. Although damage to many areas of the nervous system before or during development often produces a more hopeful prognosis than later insult, this is not the case in the cerebellum. Even though DCN cells form new projections following early cerebellar hemispherectomy (Castro, 1978; Gramsbergen & IJkema-Paasen, 1982, 1987), these new connections produce aberrant patterns of electrical activity and are more disruptive to normal locomotion than lesions occurring late in development (Gramsbergen, Schuling, & Vos, 1984). Cerebellar hemispherectomy at less than 10 days postnatal produces long-lasting impairment in locomotion and equilibrium

behavior; removal of a hemisphere after the development of mature Purkinje cell/DCN synapses has much less effect than that observed in younger animals with a similar lesion (Auvray et al., 1989; Gramsbergen, 1982; Gramsbergen & IJkema-Paasen, 1985).

Dystonic rat (*dt*)

The motor syndrome of the *dt* rat begins to appear at approximately 9-10 days postnatal after a period of apparently normal development (Lorden et al., 1984). As in the human genetic disorder, *dystonia musculorum deformans*, no central or peripheral nervous system pathology has been revealed. In the absence of obvious pathology, pharmacobehavioral, biochemical, and electrophysiological studies have been employed in an effort to pinpoint the principal site of the defect. Studies in all three categories, comparing dystonic animals with their normal littermates, consistently point to the cerebellum, as opposed to other motor areas, as a potential site of dysfunction.

Pharmacobehavioral studies have revealed an insensitivity to the tremorogenic effects of the drug harmaline in the *dt* rat. In normal animals harmaline acts on the cells of the IO to produce a generalized tremor (8-12 Hz) within 5 min of systemic administration (Busby & Lamarre, 1980; DeMontigny & Lamarre, 1973; Mariani & Delhaye-Bouchaud, 1978). This tremor is believed to be produced by the rhythmic activation of an olivo-cerebellar-bulbar pathway for which the IO serves as a pacemaker. There is normally a rhythmic increase in frequency in IO cells following administration of harmaline. This effect is most obvious in the caudal medial accessory olive (cMAO) and caudal dorsal accessory olive (Bernard, Buisserret-Delmas, Compoint, & Laplante, 1984; DeMontigny & Lamarre, 1973). Recordings at subnucleus b of the MAO before and during harmaline administration have shown

that this response to harmaline occurs in *dt* rats (Figure 4) (Stratton & Lorden, in press). However, even with normal activation of IO cells, harmaline fails to produce a tremor in *dt* rats. This is specific to harmaline and not a general inability to tremor. The *dt* rat tremors in response to drugs such as oxotremorine, which act on cholinergic systems (Lorden, Oltmans, McKeon, & Lutes, 1985) and quipazine, a serotonin receptor agonist (Michela, Stratton, & Lorden, 1990). Although harmaline is not presumed to have any direct action on Purkinje cells, in normal rats, activation of the olive by harmaline produces rhythmic complex spikes in Purkinje cells. In *dt* animals, this drug does not produce the expected rhythmic activation of Purkinje cells. In addition, spontaneous Purkinje cell activity measured in 20-25-day-old *dt* rats is low relative to the firing rate of cells from unaffected littermates (Figure 5). These differences in firing rate appear to represent a lower frequency of both mossy fiber-induced simple and climbing fiber-induced complex spikes (Stratton et al., 1988). Climbing fibers from cMAO sites, which are most clearly affected by harmaline, project to Purkinje cells in the cerebellar vermis (Figure 2) (Brown, 1980; Campbell & Armstong, 1983a, 1983b). In normal animals, after harmaline administration, there is an increased frequency and rhythmicity of climbing fiber-induced complex spikes coupled with either complete or intermittent suppression of mossy fiber induced simple spikes. Although Purkinje cells in the cerebellum of the *dt* rat respond to harmaline with intermittent simple spike suppression, high frequency rhythmic complex spikes, coupled with complete suppression of simple spikes, are seen significantly less often in Purkinje cells of the *dt* rat (Stratton et al., 1988). Given a normal IO response to harmaline and an abnormal Purkinje cell response, a cerebellar cortical defect, rather than an olivary defect, is indicated.

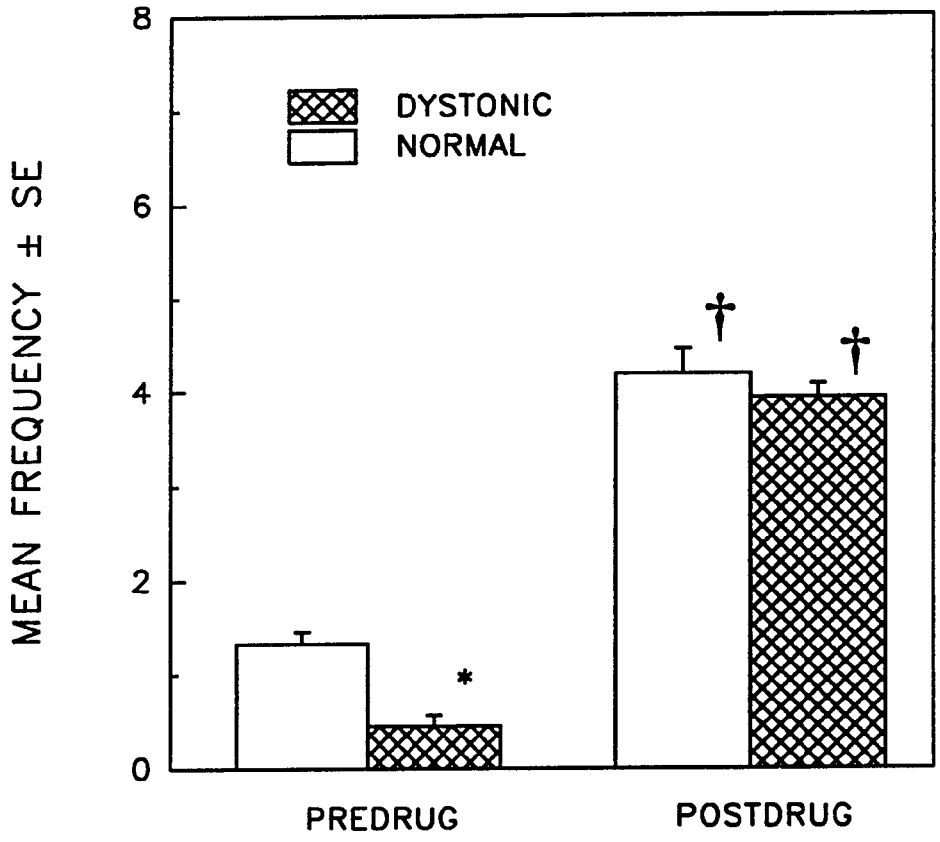
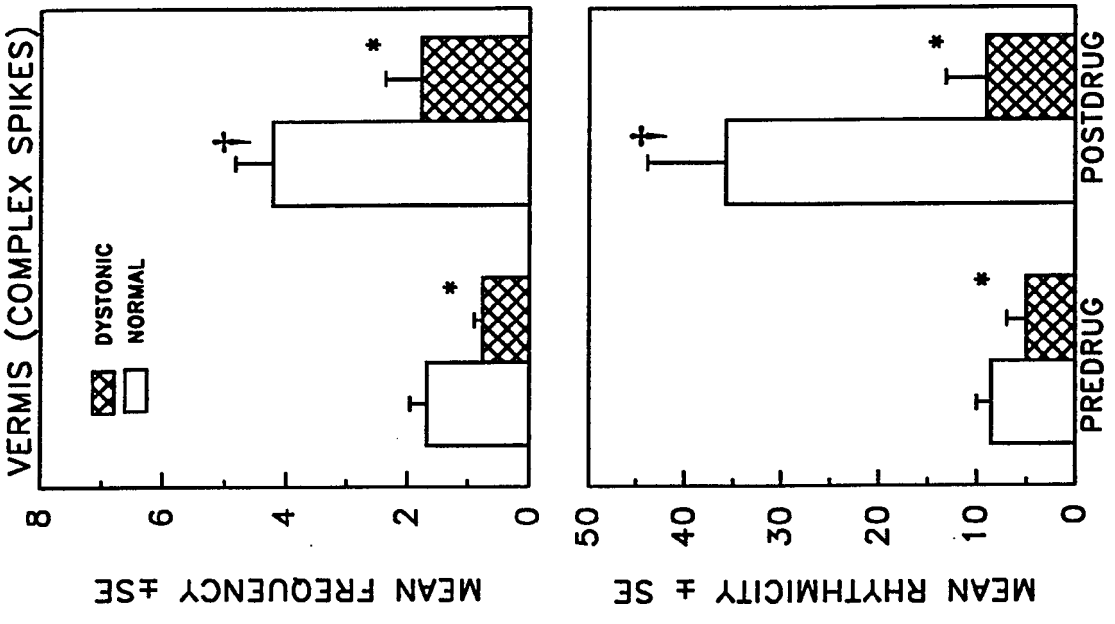


Figure 4. *Firing rates of cells in the inferior olivary nucleus of normal and dystonic animals.* The spontaneous firing rate is significantly lower in *dt* rats ($p<.05$). Harmaline administration produces an increase in frequency of single unit activity in the caudal medial accessory olive of both normal and mutant animals ($p<.05$). Postdrug firing rates of olivary neurons in *dt* rats are not significantly different from normal. (Redrawn, with permission, from Stratton & Lorden, in press)



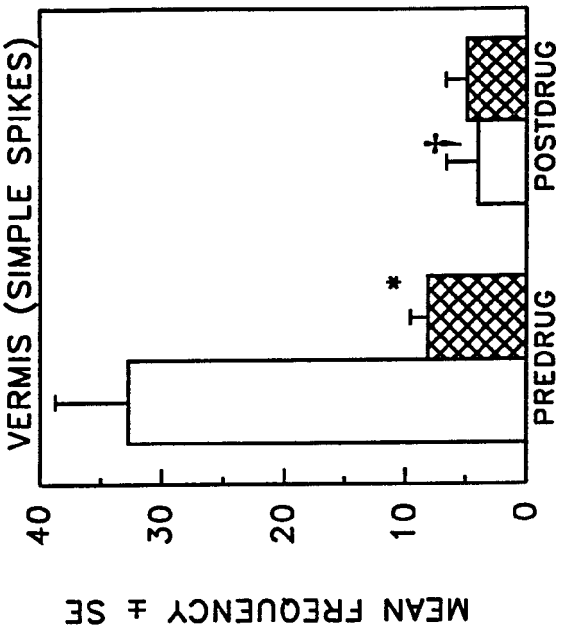


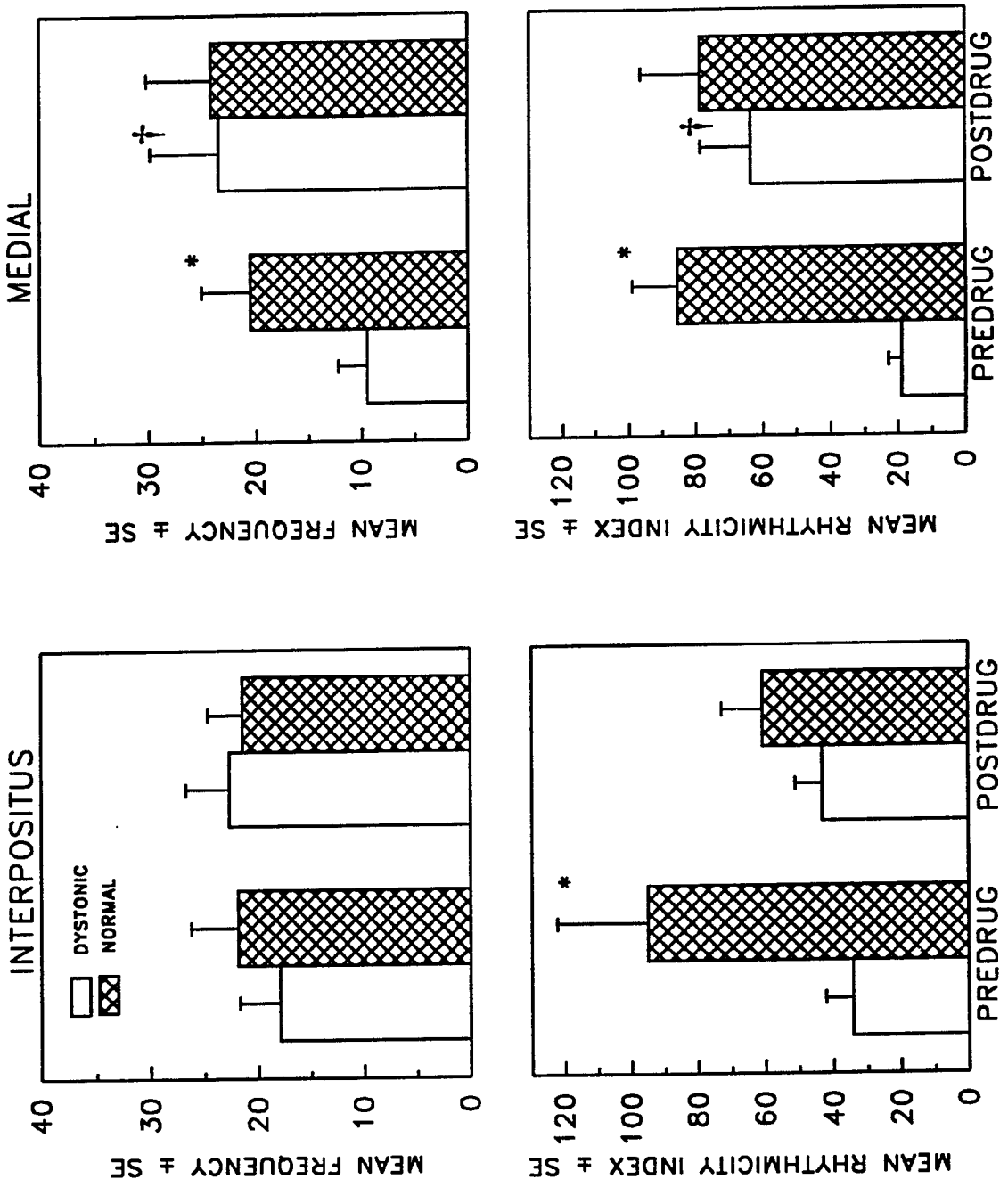
Figure 5. *Firing rates of Purkinje cells in the vermis of normal and dystonic animals.* Spontaneous firing rates are significantly reduced in the *dt* rat ($p<.05$). In normal animals harmaline administration significantly increases the frequency and rhythmicity of complex spike activity with suppression of simple spike activity ($p<.05$ for all 3 measurements). In *dt* rats only slight changes are detected even though afferent olivary neurons are appropriately activated by the drug. (Redrawn, with permission, from Stratton et al., 1988)

Recent studies (Lorden & Ervin, 1990) have examined electrical activity in the next group of cells along the harmaline-sensitive olivo-cerebellar-bulbar pathway. In line with the topographical corticonuclear organization, Purkinje cells of the vermis, which are most affected by harmaline's action, project to the medial nucleus (Figure 2) (Armstrong & Schild, 1978a, 1978b; Bernard 1987; DeCamilli et al., 1984). Normally, animals show significantly increased frequency and rhythmicity of firing in the medial nucleus of the DCN in response to harmaline administration. However, both of these measures are already significantly increased in untreated *dt* rats, and, following treatment with harmaline, no significant effect is observed in these animals (Figure 6). These findings suggest disinhibition of DCN cells. This could be due to an insensitivity of these cells to GABA or a decreased release of GABA by Purkinje cells. The output of cells in the medial nucleus of the DCN is evidently not modulated by changes in climbing fiber input to the cerebellar cortex. Direct climbing fiber input to the DCN apparently has no significant effect either. This input is excitatory, and DCN cells show no significant change in activity in either direction following harmaline administration.

Further evidence for abnormal activity in the DCN has been revealed in studies of ^{3}H-muscimol binding in the cerebellum of 25-day-old mutant and normal rats. Muscimol labels the agonist binding site on the GABA receptor. Dystonic rats were found to have decreased muscimol binding in both the lateral (dentate) and interposed nuclei (Beales et al., 1990; Lutes et al., 1987). This may account in part for the higher spontaneous firing rate of DCN cells.

Biochemical abnormalities that have been found in the *dt* cerebellum include increased GAD activity in the DCN, which appears to be paradoxical in relation to other findings (Beales et al., 1990; Oltmans et al., 1984, 1986). These increases are

Figure 6. *Firing rates of cells in the medial and interpositus DCN of normal and dystonic animals.* Cells in the medial nucleus of *dt* rats exhibit increased frequency and rhythmicity of spontaneous firing when compared to normal animals ($p<.05$). However, harmaline produced significant increases in these measurements in the cells of normal animals ($p<.05$),but failed to have an effect on *dt* rats. In the interpositus nucleus of *dt* rats,cells showed greater rhythmicity of spontaneous firing compared to controls ($p<.05$). However, these cells were not strongly affected by harmaline administration. (interpositus: n=8 normal, 13 dystonic; medial: n=9 normal, 7 dystonic) (Redrawn, with permission, from Lorden & Ervin, 1990)


MEDIAL
INTERPOSITUS
DYSTONIC
NORMAL
MEAN FREQUENCY ± SE
MEAN RHYTHMICITY INDEX ± SE
PREDRUG
POSTDRUG
0
10
20
30
40
20
40
60
80
100
120
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first detectable in the nucleus interpositus at postnatal day 16. Like the motor syndrome, enzyme increases are progressive. By postnatal day 20, when Purkinje cell firing in these animals is decreased, GAD activity in all three cerebellar nuclei is significantly higher in *dt* rats when compared with normal littermates (Oltmans et al., 1986). In addition, the magnitude of elevation continues to increase postnatally until at least day 25 (Beales et al., 1990). These changes in GAD activity appear to be specific to the DCN and have not been detected in other regions examined, including cerebellar cortex and basal ganglia. GAD activity changes may be primary changes that reflect the activity of the Purkinje cells themselves. However, GABAergic cells with recurrent axon collaterals have been shown to exist in the DCN (Matsushita & Iwahori, 1971). Changes in GAD activity may be secondary changes, resulting from a decrease in inhibitory Purkinje cell input to these GABAergic DCN cells. Increased DCN GAD activity, coupled with evidence of a decrease in GABA receptors at these sites, suggest a down-regulation of receptor number in response to increased GABA synthesis and release.

Recent 2-deoxy-D-glucose autoradiography studies have provided further evidence for abnormal functioning in the DCN of dystonic rats. Neural activity in various brain regions of *dt* rats, phenotypically normal littermates, and normal non-littermates was assessed through glucose utilization (GU) measurements (Brown & Lorden, 1989). These studies revealed, in both the *dt* rat and its phenotypically normal littermates, significantly increased GU in the DCN when compared to nonlittermate controls. Out of a total of 61 sites measured, the DCN region was one of a small number of sites showing abnormal activity. In the DCN, GU in *dt* rats was increased by 30-40% over littermate controls. In addition, the increase in GU was consistent across *dt* animals, whether they were moving or quiet.

3-Acetylpyridine

Studies in the *dt* rat suggest that the olivocerebellar system may be implicated in the motor syndrome. Therefore, we have asked whether a lesion in this pathway will produce similar effects. Administration of the neurotoxin 3-AP is an experimental method used widely to obtain rapid destruction of climbing fibers that project from the IO to the cerebellum (Desclin, 1974; Desclin & Colin, 1980; Llinas, Walton, Hillman, & Sotelo, 1975). Although this neurotoxin produces minor evidence of degeneration in other areas of the central nervous system, the climbing fibers are the only cerebellar afferents that are affected (Balaban, 1985). The 3-AP lesion in normal animals produces behavioral and biochemical effects that are, in many cases, similar to those observed in the *dt* rat. For example, a distinct motor syndrome that includes pivoting, ataxia, and axial twisting begins to appear within hours of intraperitoneal (i.p.) injection of this drug (Batini & Billard, 1985; Sukin et al., 1987). Although the dystonic motor syndrome is progressive, motor symptoms in 3-AP animals are in time attenuated. Symptomology in animals with this lesion is similar, whether the toxin is administered to pups or to adult rats; however, young animals do not seem to be as severely affected, nor are their symptoms as long lasting (Oltmans, Lorden, & Beales, 1985; Sukin et al., 1987). In line with these observations, Anderson & Flumerfelt (1984) observed that the immature IO neuron is less sensitive to the toxic effects of 3-AP. Doses that produce total cell death in adults yield some sparing of cells in young rats.

Animals with 3-AP lesions show immediate changes in Purkinje cell activity as a result of the loss of climbing fiber-induced complex spike activity. Destruction of climbing fibers produces a 100% increase in simple spike firing rate of cerebellar Purkinje cells (Colin et al., 1980; Montarolo, Palestini, & Strata, 1982; Savio &

Tempia, 1985). These increases in firing rate are accompanied by increased GAD activity in the DCN, which begins to appear in adult rats as early as 6 hr following the lesion, and is significantly elevated at 48 hr post-lesion. GAD activity peaks 12 days later, and remains elevated for as long as 43 days following treatment (Sukin et al., 1987). Seven days after 3-AP administration, animals show a 58% elevation in GAD activity levels (Litwak, Mercugliano, Chesselet, & Oltmans, 1990). When GAD mRNA in Purkinje cell bodies was measured in these same animals, levels were found to be significantly increased to 42% above control. This suggests an induction of GAD mRNA transcription, brought about by increased Purkinje cell firing rates, leading to increased GAD availability in Purkinje cell terminals. However, although GAD activity remains elevated for more than a month following lesion, simple spike activity returns to normal levels by 25 days post-lesion (Benedetti et al., 1984). In young 16-day-old rats, significant increases in GAD activity, over and above developmental increases, are apparent in the DCN 24 hr post-lesion and continue to increase for at least 4 weeks, at which time progressive enzyme increases in the cerebellar vermis also become significant (Oltmans et al., 1985). Thus, increases in GAD activity, once established, may become uncoupled from Purkinje cell firing rate. It is possible that after Purkinje cell activity declines, GAD is stored in terminals in an inactive apoenzyme form, or that the measure of GAD activity at different times relative to the lesion represents different molecular forms of GAD that have been shown to exist (Martin, 1987; Martin et al., 1990b).

Other observed similarities between 3-AP animals and *dt*s are decreased levels of cerebellar cGMP, insensitivity to the tremorogenic effects of harmaline (Guidotti, Biggio, & Costa, 1975; Simantov, Snyder, & Oster-Granite, 1976), and increased GU in the DCN (Bardin et al., 1983).

Rationale for proposed study

Studies of the *dt* rat point to the presence of functional abnormalities in the cerebellum of this mutant. More specifically, there is a strong suggestion of abnormal functioning of cerebellar Purkinje cells.

In pharmacological studies, *dt* rats are insensitive to the tremorogenic effects of the drug harmaline. Administration of this drug normally initiates a tremor, produced by activation of an olivo-cerebello-bulbar pathway. Interruption of any portion of that pathway disrupts the tremor. Under conditions of harmaline stimulation, increased activity in IO cells produces an increase in the frequency and rhythmicity of complex spikes in Purkinje cells. This rhythmic activity is carried downstream to the spinal cord. Production of tremor is the result of coactivation of alpha- and gamma-motoneurons (Lamarre & Weiss, 1973). Recordings in *dt* rats at olivary sites before and during harmaline administration indicate a normal increased frequency response to the drug at this site. However, recordings in the cerebellar cortex of these mutants reveal both a lower rate of spontaneous Purkinje cell firing and an abnormal firing response to harmaline. These Purkinje cell observations are not in agreement with increased GAD activity and increased GU, which have been observed in the DCN of dystonic animals. Recent studies of the spontaneous and harmaline-stimulated firing rate of cells in the DCN, the target site of Purkinje cells, show an increase in spontaneous firing in *dt* rats at this site in comparison with normal controls, suggesting disinhibition of DCN cells. Harmaline, however, which produces increased frequency and rhythmicity of firing in DCN cells of normal animals, failed to generate significant increases above baseline in dystonic animals. The drug had no effect on firing of DCN cells in the *dt* rat (Table 1).

Table 1

Summary of Abnormalities in the Dystonic Rat

insensitivity to tremorogenic effects of harmaline
↓ firing rate of inferior olive cells (harmaline stimulated firing = normal)
↓ firing rate of Purkinje cells in cerebellar vermis (↓ response to harmaline)
↑ firing rate of cells in DCN (no further ↑ with harmaline)
↑ GAD activity in DCN
↑ GU utilization in DCN
↓ muscimol (GABA receptor) binding in DCN

Similarities have been observed between *dt* rats and normal rats whose climbing fiber system has been destroyed by administration of the neurotoxin 3-AP. These rats are predictably insensitive to the tremorogenic effects of harmaline, which acts on climbing fibers. In addition, they initially display a behavioral syndrome similar to that observed in *dt* rats. In the DCN of these animals, GAD activity and GU are increased (Bardin et al., 1983; Oltmans et al., 1985). The same observations have been made in the *dt* rat. However, in animals with 3-AP lesions, these increases are understandable in light of increases in simple spike activity. In the mutant, with increased DCN GAD activity and GU, Purkinje cell activity is also altered, but in the opposite direction. These discrepancies need to be resolved.

Electrophysiology is limited in its ability to serve that purpose due to a sampling bias that may occur if data taken from a limited number of single cells do not adequately represent the overall functional characteristics of a larger system of like cells. Biochemical methods, on the other hand, provide information about an entire anatomical area of choice, but fail to furnish adequate resolution regarding functional operation within that area. Methods designed to examine particular aspects of anatomy within a given system offer an opportunity to examine more clearly functional components of that system.

The current set of experiments used quantitative anatomical techniques to investigate the morphology of the cerebellum in these two animal models of movement disorder. Measurements were made in both Nissl-stained sections and sections stained immunocytochemically with antibodies for GAD. Anatomical examination of the brains of *dt* rats has not previously revealed abnormalities. However, past studies have been limited to a search for obvious signs of lesion and degeneration at the light microscopic level. Purkinje cell soma size in 20-day-old *dt* rats has previously been shown to be significantly reduced (Lorden et al., 1985), and this may be an indication of degeneration of these cells. Therefore, the present studies examined the anatomy of Purkinje cell input to the DCN in more detail, using anatomical techniques and immunohistochemistry. Results lend additional support to a working hypothesis of Purkinje cell dysfunction in the genetically *dt* rat.

In these studies, the following questions regarding the source of increased electrical activity in the DCN of *dt* rats were asked:

1. Is there decreased Purkinje cell input to the DCN as a result of a decrease in the number of Purkinje cells in the *dt* cerebellum?

2. Is Purkinje cell terminal arborization, as reflected in volume measurements in individual DCN of the *dt* rat, altered in relation to decreased Purkinje cell firing and/or increased DCN enzyme activity?

3. Is cell size in the DCN altered as a consequence of altered Purkinje cell input, enzyme activity, or intrinsic neuron activity?

4. Is there an alteration in the size or density of putative Purkinje cell terminals at DCN sites?

5. Are changes in electrical activity or in the activity of neurotransmitter synthesizing enzymes in the DCN reflected in changes in neurotransmitter levels?

This set of experiments, designed to investigate the morphology of cerebellar Purkinje cells and their target cells in two animal models of movement disorder, served a twofold purpose. It uncovered information regarding the relationship between altered electrical activity and synthesizing enzyme activity changes in GABAergic Purkinje cells. More specifically it has enabled us to better understand the role of the Purkinje cell in the production of the *dt* syndrome and has provided additional evidence to support a hypothesis of defective Purkinje cell function in this mutant, as opposed to a morphological defect similar to those seen in several mouse mutants.

PROCEDURES AND METHODS

Experimental animals

Male and female, normal and *dt* Sprague-Dawley rat pups were obtained from a breeding colony maintained at the University of Alabama at Birmingham. At 20 or 25 days of age, *dt* pups were deeply anesthetized and perfused as described below. At these ages highly significant ($p<0.01$) increases in DCN GAD activity have been observed in these mutants (Oltmans et al., 1984).

Administration of 3-AP to young animals also produces significant increases in DCN GAD activity beginning 24 hr post-lesion and continuing for up to 4 weeks (Oltmans et al., 1985). To study the effects of 3-AP on GAD-containing cells in the DCN, phenotypically normal littermates from the same colony were injected with the neurotoxin (65 mg/kg, i.p.; concentration = 8.4 mg/ml isotonic saline). To confirm behaviorally the effectiveness of the injection, animals were observed for abnormal locomotor function at ≤4 hr following injection. Symptoms at this time are often mild; however, animals were observed again, and rated at 24 and 48 hr post-injection, and, for 14-day survival rats, at 1 and 2 weeks post-injection. Rating was subjective on a scale from 1-5, based on axial twisting, pivoting, falls to the side, and dragging of thorax and/or hind limbs, with 1 being most severe, and 5 showing no obvious impairment. The degree of impairment is a good indication of the completeness of the lesion. Therefore, to reduce variability, more animals than necessary were injected, and the most severely affected were chosen for inclusion in the experiments.

Animals in the 2-day and 2-week post-lesion survival groups were initially rated at 24 hr post-lesion, as at least a 3, and, more commonly, as a 1-2. However, animals with 2-week post-lesion survival times always showed attenuation of symptoms, regardless of the initial severity.

EXPERIMENT #1 - Purkinje cell counts

Is altered electrical activity in DCN cells of the *dt* rat the result of changes in the number of Purkinje cells that are afferent to this site?

Purkinje cells are a primary source of input to the DCN. Results of experiments with the *dt* rat indicate abnormal Purkinje cell input to the DCN. DCN cells could be disinhibited because of a functional defect in the Purkinje cells or because of a loss of Purkinje cells. Other neurological mutants have been found to have decreased numbers of Purkinje cells as either a primary or secondary defect (Caddy & Biscoe, 1979; Heckroth, Goldowitz, & Eisenman, 1989; Mullen, Eicher, & Sidman, 1976). Therefore, Purkinje cell number and density were estimated in Nissl stained sections of 20-day-old *dt* rats and age-matched, phenotypically normal littermates.

Methods

Animals were deeply anesthetized with i.p. injection of sodium pentobarbital and perfused with 10% formalin after a physiological saline flush. Whole brains were removed and kept in perfusate overnight. Beginning the next day, brains were thoroughly rinsed with running water and a series of dehydration/infiltration steps for paraffin (Paraplast) embedding was begun. Tissue was first immersed in increasing concentrations of ethanol (70% for 2 hr, 80% for 1 hr, 95% for 9-10 hr). For the next 16 hr, brains were moved through two changes of butanol. The preceding steps were all done at room temperature, and tissue was next immersed

in 50% butanol/50% paraffin and placed in a 60-62°C oven for 8-9 hr followed by 1 hr in paraffin alone, at the same oven temperature. Finally, tissue was immersed in fresh paraffin and placed in a vacuum oven, at the same temperature and 15-17 lbs of pressure, for 5-6 hr. After removal from the oven, the cerebellum was dissected out from the rest of the brain and hemisected. Each cerebellar hemisphere was then placed in a separate mold, which was subsequently filled with molten paraffin.

Each hemicerebellum was sliced sagittally, no less than 24 hr following embedding, on a rotary microtome set at 10 μm. Every section was saved and mounted on albumin-coated slides. Following overnight drying at room temperature and 1 hr of drying in a low temperature (37° C) oven, slides were soaked in xylene for removal of paraffin, and subsequently rinsed in decreasing concentrations of ethanol before Nissl staining in cresyl violet.

The cerebellar cortex is a convoluted sheet of tissue made up of three layers, with the Purkinje cell layer existing as a single cell layer located at the junction of the molecular layer and the granule cell layer. In Nissl-stained sections, the densely packed granule cell layer appears much darker than the molecular layer, and at low magnifications the junction between these two layers, and, hence, the Purkinje cell layer itself, is easily identified. At a final magnification of 58x (objective = 1.6x), the total length of the Purkinje cell layer was measured in sections at intervals of approximately 200 μm throughout half of each cerebellum. Measurements were begun at a point estimated to be the midline of the whole cerebellum. A section known to be near the midline was selected as being representative of the true midline when the length of the Purkinje cell layer in that section was longer than that of adjacent sections.

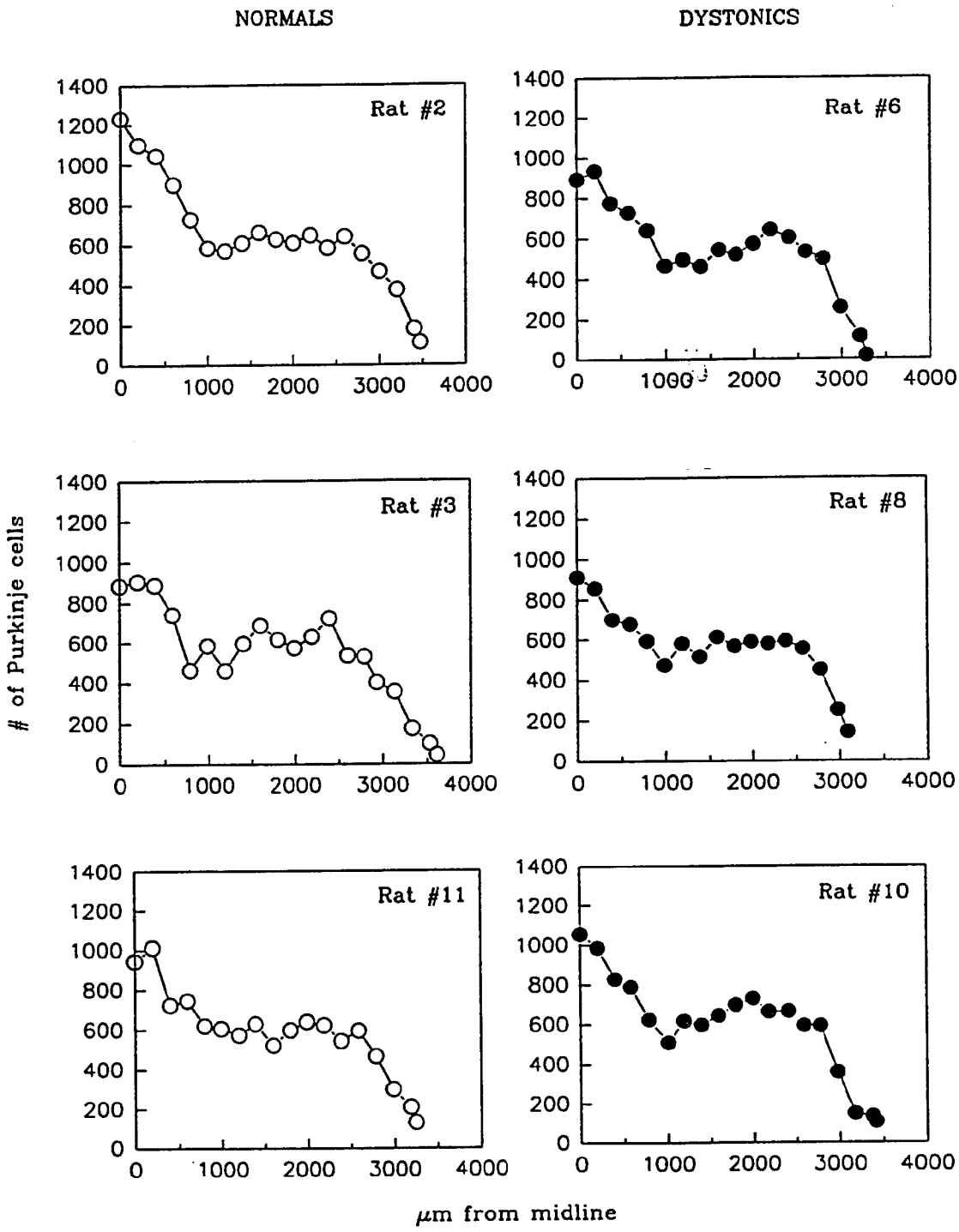
At high magnifications, the Purkinje cell itself is easily identifiable due to its location, and, in the same sections at a final magnification of 1760x (objective = 50x), every Purkinje cell with a visible nucleus was counted. Counts such as this are always considered to be an overestimate (Abercrombie, 1946). Therefore, in order to establish a correction factor for estimating Purkinje cell numbers as described below, the minor diameter of all counted Purkinje cell nuclei was measured in a section lying in a plane approximately half way through the hemicerebellum. Measurements and counts were made using an R & M Biometrics Bioquant System IV with Houston Instruments HiPad digitizing tablet.

Counted cells from the left hemisphere of each rat were plotted as a function of distance from midline. Using a method described by Wetts and Herrup (1982), the total area under the plotted curve was determined to be proportional to the total number of Purkinje cells in that hemisphere (Figure 7). The Abercrombie correction method was used to calculate a corrected estimate (Abercrombie, 1946). The total number of cells in each hemisphere was computed, as well as Purkinje cell density. This was done by dividing the corrected number of cells in each section by the Purkinje cell length of that section. A mean density for each rat was then determined using all counted sections in that rat.

Results

As described in Methods, the number of cells in each counted section was plotted against the distance from the midline of that section and the area under the resultant curve was then computed and corrected. From this area it was possible to calculate an estimate of the total number of Purkinje cells in a counted hemisphere. Student's t-test was used to compare the estimated number in 20-day-old *dt* rats versus normal littermates of the same age. No significant difference in the total

Figure 7. *Purkinje cell counts in the left hemisphere of 20-day-old normal and dystonic animals.* The number of cells in a given section were plotted against the distance from midline of that section. The total area under the plotted curve of each animal was then determined and was used to compute the total number of Purkinje cells in that hemisphere. This number was corrected using the Abercrombie (1946) correction method.


NORMALS
DYSTONICS
Rat #2
Rat #6
Rat #3
Rat #8
Rat #11
Rat #10
# of Purkinje cells
μm from midline
0
200
400
600
800
1000
1200
1400
1000
2000
3000
4000

number of Purkinje cells was found between these two groups of animals (Table 2). While great care was taken to begin counting at the midline and to use tissue spaced throughout the extent of a hemisphere, two possible sources of variance were acknowledged: the midline may not have been estimated exactly, and slicing may not have proceeded to the most lateral extent in every case. Therefore, slices which contained the beginning and end of the DCN were found in each animal. These sections were then spotted on the computed curve, and the total number of Purkinje cells between these two spots on the curve was also computed. Using the same statistical tests, once again, no significant difference was found between *dt* rats and their age-matched littermates (Table 2).

The density of Purkinje cells in the Purkinje cell layer (mean cells/mm) was also computed, as described in Methods. Once again, no significant difference was found between dystonic and normal animals (Table 2). These results indicate that observed increases in the activity of DCN cells in the *dt* rat are not due to a reduction in the number of Purkinje cells available for inhibitory input.

Table 2

Purkinje Cell Counts and Density (mean ± S.E.M.) in Left Hemisphere of 20 day old Normal and Dystonic Animals

	Total	Total in DCN sxns.	Mean cells/ mm P.C. layer
Normal	116,352 ±4,442.80	70,989 ±3,157.70	11.67±.47
Dystonic	107,383 ±4,840.30	69,692 ±3,827.56	11.69±.58

n=3/group

No significant differences

EXPERIMENT #2 - DCN volume

Is there a reduction in Purkinje cell terminal arbors in the DCN of the *dt* rat?

Purkinje cell degeneration (*pcd*) mutant mice lose essentially all Purkinje cells between postnatal days 17 and 45. Consequently, cells of the DCN lose their major source of afferent input. One result of this deafferentation is a 22% reduction in DCN volume (Triarhou, Norton, & Ghetti, 1987). Experiment #1 shows that Purkinje cells are not missing in the *dt* rat; however, there may be a change in terminal arborization. An indirect way to assess this possibility is to look for a change in DCN volume. Therefore, in this experiment, volume measurements of individual DCN were made in both 20-day-old mutants and their age-matched littermates.

Methods

Animals were deeply anesthetized with i.p. injection of sodium pentobarbital and perfused intracardially with 0.1 M phosphate buffer followed quickly by a 30-min perfusion with Stefanini fixative (Stefanini, DeMartino, & Zamboni, 1967), a picric acid-formaldehyde-phosphate buffer fixative that has the advantage of rapid tissue penetration. The whole brain was removed from the skull, stored for at least 1 week in picric acid fix, and then sliced into 30 mm thick coronal sections on a vibratome. Every section was saved. Sliced sections were mounted and stained for Nissl substance with thionine. Measurements were made using custom software installed in a VAX-II\750 computer with Ultrix operating system. Accessories included a

Marzhauser motorized stage for X-Y encoding, Numonics digitizing tablet, Evans & Sutherland PS-330 colorgraphics work station, and a Nikon microscope with 4X objective.

Individual nuclei were drawn, and area measurements were made throughout the extent of the DCN. In any one rat, measurements were made in 19-35 sections (mean number of sections ± S.E.M.: normals: 28.3 ± 2.29; dystonics: 29.2 ± 1.28). We attempted to measure every other section. If a section was damaged and could not be used, the next consecutive section was measured instead. Only rarely was it necessary to skip more than two sections. For volume computation the sections were listed serially, with each missing section indicated. The total volume was computed, using custom software, as the sum of a series of section volumes as follows:

1. If the section area exists, compute section volume as area x thickness.
2. Estimate missing areas using a weighted average as follows:

$$\textit{estimated area} = \sum_{j=1}^{\textit{no. missing}} \left[\textit{old area} - j \left(\frac{\textit{old area} - \textit{new area}}{\textit{missing} + 1} \right) \right]$$

where "old area" and "new area" are the two known measurements and "j" is the number of missing sections between the two.

3. Compute estimated volume for each missing section as follows:

$$\textit{estimate volume} = \textit{estimated area} \left(\frac{\textit{old thickness} + \textit{new thickness}}{2} \right)$$

(i.e., multiply by the average thickness of the two known thicknesses.)

4. For total volume, sum all section volumes.

Results

Volume differences were seen between individual nuclei in that interpositus nucleus > lateral nucleus > medial nucleus. This conforms with the appearance and extent of nuclei, as seen in mounted sections. These differences appeared in both the normal group and the mutant group, and provided a verification of the method used to measure volumes. Multivariate analysis of variance (MANOVA) to compare DCN volume of mutant rats and age-matched controls revealed only marginally significant differences [$F_{(3,7)} = 3.21704, p < .09$]. As a follow-up, specific DCN nuclei of mutant rats and age-matched controls were then compared using the student's t-test, and no significant difference was found in any nucleus (Table 3). The greatest difference was found in the lateral nucleus ($p < .12$), and power analysis revealed that a sample size of 15 per group would be necessary for this difference to be significant at a .05 level. Therefore, decreased DCN input from Purkinje cells does not, at this stage of the syndrome, produce a significant change in the overall volume of any specific DCN.

Table 3

Deep Cerebellar Nuclei Volume (mm^3 ± S.E.M.) of 20-day-old Normal and Dystonic Animals

	NUCLEUS		
	Lateral	Interpositus	Medial
Normal n=6	.3972±.0199	.4609±.0461	.3530±.0348
Dystonic n=5	.3451±.0216	.4257±.0379	.3111±.0178

No significant difference between *dts* and normals.

EXPERIMENT #3 - DCN cell size

Are individual cells of the DCN of either *dt* rats or rats with 3-AP lesions altered in size as a result of changes in input, electrical activity and/or enzyme activity?

Volume measurements may be an indirect indication of a lack of change in terminal arborization of Purkinje cells. However, terminal arborization might still be altered in conjunction with accompanying changes in DCN cell size. Multiple changes such as these would be masked in measures of volume. Reduced cell size has been found in the DCN of the staggerer, a mutant mouse with Purkinje cells of reduced size and number (Roffler-Tarlov & Herrup, 1981) and in *pcd* mice, which lose all Purkinje cells by 45 days of age (Triarhou et al., 1987). Thus, deafferentation may produce changes in target cell size. Although Purkinje cell number is not changed in the *dt* rat, functional deafferentation is indicated by a reduction in the firing rate of Purkinje and olivary cells, both of which are afferent to the DCN. In animals with 3-AP lesions, olivary cell destruction produces the loss of one source of input to the DCN, while inhibitory input from Purkinje cells is increased. It is not known what effect this change in input might have on DCN cells. Therefore, as an additional check for deafferentation of DCN cells in the *dt* rat, and to investigate the morphological effect of changes in input on target cells, we looked for changes in cell size in all three DCN, as well as in the LVN, of both mutant rats and animals with 3-AP lesions. To examine the possibility of progressive changes in either group, in

this study we examined both 20- and 25-day-old mutants and normal animals at both 2 days and 2 weeks post-lesion.

Methods

Two different perfusion protocols were employed in this experiment. Twenty-day-old and 32-day-old animals from all groups were deeply anesthetized with ether inhalation and perfused as in Experiment #2. The whole brain was removed from the skull and immersed in perfusate overnight at 20° C. Twenty-five-day-old animals were deeply anesthetized prior to perfusion with 0.9% NaCl, followed by perfusion with zinc salicylate mixed in the following proportions:

100 ml commercial formalin

500 ml 1% zinc salicylate

50 ml 9% NaCl

d-H_20 to 1000 ml

This perfusate was filtered, and the pH adjusted to either 4.0 or 6-6.5 prior to use. Following a 30-min perfusion, the brain was allowed to sit *in situ* for 30 min to 1 hr before being removed from the cranium and sliced that same day, or left in 0.9% NaCl overnight for slicing on the next day. In either case, immediately before slicing, the brain was hemisected sagittally, and the cerebellum and brain stem removed. One half of the cerebellum and brain stem were sliced sagittally into 30-μm thick sections on a Vibratome with 0.1M phosphate buffer (pH 7.4) in the reservoir. Every fourth section was left in 10% formalin for at least 1 week, then mounted in serial order and stained with thionine. From each animal, 11 sections that offered a representative and well-spaced sampling from all four nuclei of interest, were selected from throughout the cerebellum. Sections were selected at the same medio-lateral level in all animals examined. In certain of the selected sections, more than

one nucleus was visible; therefore, measurements for lateral nuclei were made in five sections, interpositus nuclei in six sections, medial nuclei in four sections, and LVN in three sections. Measurements were made with the R & M Biometrics Bioquant System IV with Houston Instruments HiPad digitizing tablet. Cells in the DCN included small, medium and large-sized multipolar neurons (Figure 8). A 100x objective, producing a final videoscreen magnification of 3575x, was used to measure the cross-sectional area in all cells with a minor diameter of ≥ 6 μm as well as a visible nucleolus. This cut-off was intended to exclude glia from measurements. The mean number of cells ($\pm$ S.E.M.) measured in specific nuclei is shown in Table 4.

Results

In this experiment, dystonic and normal animals as well as animals with 3-AP lesions were compared. In each condition, at least two ages were examined. Measurements were taken from *dt* rats at both 20 days of age and 25 days of age. Lesions were induced with 3-AP at 18 days of age, and these animals were sacrificed either 2 days or 2 weeks later, resulting in a 20-day-old group and a 32-day-old group. Normal animals were sacrificed at 20, 25, and 32 days to give an age-matched control for each of the experimental groups.

Separate MANOVA used to compare all animals and all nuclei within any one age group revealed a significant difference in DCN cell size in 20-day-old animals. Student's t-test was used to compare individual nuclei from any one age of any one experimental group to an age-matched control group. A significant difference in cell size was found to exist in the medial nucleus of 20-day-old *dt* rats, i.e., cells in this nucleus were significantly smaller in mutant rats than in normal controls ($n=4$) at an alpha level of $p<.05$ (Table 5). However, for this type of analysis, that alpha level

Figure 8. *Nissl stained sections in DCN of 20-day-old dystonic and normal animals.* A similar distribution of small, medium, and large-sized cells was observed in animals from all groups. Cell size measurements were made of all cells with a minor diameter ≥6 μm and a visible nucleolis. The arrow in *A* indicates a typical medium-large sized cell. This same cell, at a higher magnification, is indicated by the arrow in *B*. *A. Scale bar = 100 μm B. Scale bar = 50 μm*

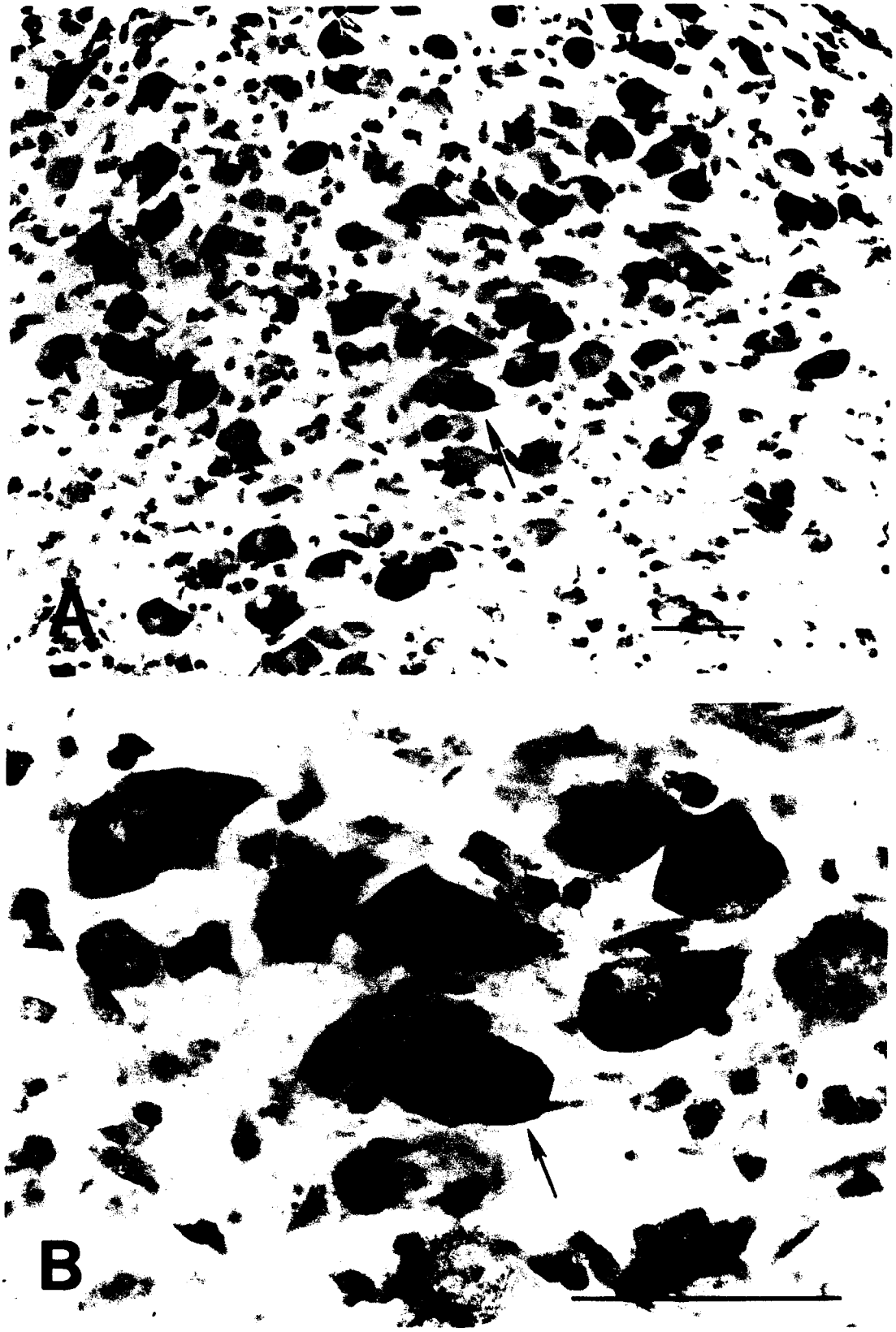

A
B

Table 4

Mean Number of Cells (± S.E.M) Measured/Rat for Cell Size Measurements in Deep Cerebellar and Lateral Vestibular Nuclei

	NUCLEUS			
	Lateral	Interpositus	Medial	LVN
Normals				
20 d.o	152.25 ± 17.40	177.75 ± 1.16	91.50 ± 11.93	58.17 ± 7.25
25 d.o	240.50 ± 32.84	270.75 ± 19.03	108.75 ± 10.14	60.25 ± 9.55
32 d.o.	143.00 ± 4.16	183.67 ± 8.09	88.67 ± 12.91	45.33 ± 4.70
Dystonics				
20 d.o.	153.75 ± 19.19	171.00 ± 20.58	109.00 ± 2.31	51.67 ± 8.16
25 d.o.	228.00 ± 18.93	302.25 ± 32.55	177.25 ± 10.80	53.50 ± 14.96
3-AP				
18 + 2	153.00 ± 30.04	164.25 ± 36.50	106.50 ± 20.60	35.60 ± 6.56
18 + 14	114.75 ± 6.06	168.50 ± 36.75	83.75 ± 16.94	25.25 ± 4.25

is only marginally significant. Similar differences between mutants and normals were not found in other divisions of the DCN and were not present at 25 days of age. Because 20-day-old animals and 25-day-old animals were perfused with different fixatives, it was not possible to make comparisons of cell size between the two ages. However, further analysis of dystonic animals versus normals was carried out by computing z scores for each animal and combining the two age groups for MANOVA. Using this method, no significant differences were found between normal and *dt* rats.

Table 5

Cell Size (Area-μm^2 ± S.E.M.) in Deep Cerebellar Nuclei of Normal Rats, *dt* Rats, and Rats With 3-AP Lesions

	NUCLEUS			
	Lateral	Interpositus	Medial	LVN
20-day-old animals*				
Normal	283.80±18.55 n=4	250.51± 4.45 n=4	294.92±11.48† n=4	430.80±12.79 n=6
Dystonic	272.44± 6.92 n=4	251.75± 5.87 n=4	267.90± 4.61 n=4	458.05±29.99 n=6
3-AP 18 +2	286.76 ± 3.89 n=4	289.18±12.35‡ n=4	280.97±11.31 n=4	420.57±18.77 n=5
25-day-old animals**				
Normal	326.03±12.48 n=4	301.40± 9.61 n=4	326.10±10.19 n=4	526.11±20.81 n=4
Dystonic	314.59±16.05 n=4	295.60±13.21 n=4	299.52±16.68 n=4	554.29±58.76 n=4
32-day-old animals*				
Normal	287.73± 6.11 n=3	261.90± 7.57 n=3	300.60± 4.51 n=3	403.83±30.58 n=3
3-AP 18 +14	293.63± 4.13 n=4	259.69± 8.07 n=4	293.64±10.76 n=4	417.71± 26.23 n=4

*Steffanini fix (picric acid/2% paraformaldehyde)
**zinc salicylate fix (10% formalin)
†20 d.o. norm > 20 d.o. *dt*, $p < .05$
‡3-AP 18 + 2 > 20 d.o. norm, $p < .025$

When comparisons were made between 20-day-old control animals and animals sacrificed 2 days after 3-AP administration, cells of the interpositus nucleus were found to be significantly larger in animals with lesions ($p < .025$) (Table 5). However, this difference did not exist at 14 days post-lesion, and may have been a transient response to the increases in Purkinje cell activity, which occur immediately after the lesion, but which soon begin to decline back to normal levels.

EXPERIMENT #4 - GAD+ puncta in DCN

Is increased DCN cell activity in the *dt* due to a change in GABAergic innervation, as represented by the number or size of GAD-positive (GAD+) puncta?

Electron microscopic examination of puncta positively stained for GAD has shown them to be axon terminals synapsing on large neuronal cell bodies and primary dendrites. Similar puncta staining in the neuropil has been determined to be boutons that make contact with medium and small sized dendrites (Oertel et al., 1981).

Immunocytochemistry used to examine the collective DCN of animals almost totally devoid of Purkinje cells has revealed the presence of some GAD+ puncta (Wassef, Simons, Tappaz, & Sotelo, 1986). However, cerebellar Purkinje cells are the primary source of GAD activity in the DCN. Therefore, it is possible to examine Purkinje cell innervation of the DCN through the use of GAD immunocytochemistry. Changes in input have been shown to alter the size and density of GAD+ puncta at several sites. When the IO is deprived of GABAergic input by cerebellar hemispherectomy, there is a near total loss if GAD+ puncta in most divisions of the IO nuclei (Nelson & Mugnaini, 1989). In rats enuculated at birth, deprivation of sensory input results in a significant reduction in GAD+ puncta density in the visual cortex (Ribak & Robertson, 1986). Deafferentation of red nucleus cells by lesions to the interpositus nucleus results in collateral sprouting from local GABAergic

neurons, producing an increase in GAD+ puncta density in the red nucleus (Katsumaru, Murakami, Wu, & Tsukahara, 1986).

Because *dt* animals and animals with 3-AP lesions display different Purkinje cell electrophysiology, different patterns of terminal staining might be expected. Decreased Purkinje cell activity, such as that found in the mutant animal, might produce decreases in puncta size or density; whereas, increased Purkinje cell activity, as seen in animals with 3-AP lesions, might produce increased puncta size or density. Additionally, 3-AP lesions deprive the DCN of innervation by climbing fiber collaterals from the IO, raising the possibility of collateral sprouting by local GABAergic neurons in the DCN. Thus, in this experiment we looked at both the size and density of GAD+ puncta in the DCN of both *dt* and 3-AP animals, in order to investigate the consequences of changes in input.

Methods

Two different GAD antibodies with two different perfusion protocols and labeling procedures were used to identify terminals of GABAergic cells in the DCN of four experimental and three control groups of animals.

Twenty-day-old and 32-day-old animals from all groups were sedated with ether inhalation and perfused intracardially with a picric acid perfusate as described in Experiment #2. The brain was removed from the skull and immersed in perfusate overnight at 20°C. On the following day, the brain was hemisected sagittally and the cerebellum and brain stem removed. One half of the cerebellum and brain stem was sliced sagittally into 30-μm thick sections on a Vibratome with 0.1M phosphate buffer (pH 7.4) in the reservoir. Sections were saved for immunocytochemistry, with every fourth section saved for Nissl staining (Experiment #3). Within 24 hr of slicing, sections were incubated in 0.1 M phosphate buffer-1% normal goat serum (NGS) for

one hr. At this and all other stages, tissue was agitated continuously. The tissue was then incubated in the primary GAD antiserum (#434-Fab, kindly furnished by C. Brandon, Chicago Medical School) diluted in 0.1 M phosphate buffer-1% NGS (1:375) for 48 hrs at 4° C. Replacing this step, in the final stage, with 1% NGS, rather than the primary antibody as a control for specificity of staining, resulted in light background staining only. The preparation of this antibody has been described and its specificity has been established (Brandon, 1986). The smaller molecular size of Fab fragments results in increased tissue penetration and, hence, greater depth of immunostaining along with decreased background staining (Brandon, 1985). Although detergents such as Triton-X also allow increased penetration, there is a greater possibility of lowered tissue antigeneity with their use.

The tissue was processed in Coors crucibles, and, in initial runs, any one crucible contained tissue from animals of two different groups in order to control for variability in staining across animals.

On the 3rd day, tissue was washed for 5 min, 5x, in 0.1 M phosphate buffer and incubated for 1 hr in goat anti-rabbit IgG-Fab (#0019; ICN Immuno-biologicals), diluted with 0.1 M phosphate buffer/1% NGS (1:40) at room temperature. Once again tissue, was rinsed five times in buffer, and then incubated for 1 hr in rabbit PAP-Fab (#4397 or #10217; Jackson Immunoresearch), diluted with 0.1 M PB/1% NGS (1:100) at room temperature. Two more 10-min washes followed this incubation before the tissue was reacted with fresh diaminobenzidine (DAB) (50 mg DAB/100 ml 0.1m PB) and .03% H_2O_2, augmented with cobalt/nickel intensification to produce a darker DAB reaction product (Adams, 1981).

GAD in 25-day-old animals was visualized using GAD antiserum #1440-4, kindly provided through the Laboratory of Clinical Science, NIMH.[1] Labeling with this antiserum was optimized using the zinc-aldehyde fixation procedure of Mugnaini and Dahl (1983). With this procedure, animals were deeply anesthetized with either ether or an i.p. injection of sodium pentobarbital, and perfused as described in Experiment #4. The brain was either left overnight in 0.9% NaCl or sliced the same day. Slicing was done at 20-25 μm on a vibratome with ice cold .09% NaCl in the reservoir. Tissue was incubated overnight at room temperature in primary antibody at a concentration of 1:10,000. Substitution of pre-immune serum for primary antibody in this step produced no specific staining in the final stage.

Labeling with this antibody was done using the Avidin Biotin Complex (ABC) procedure. Components for this procedure were obtained from Vector Laboratories (Irvine, CA), and the steps are as follows (all steps under constant rotary table agitation; all rinses in 0.01 M PB):

1 - IgG at 1:200 - 45 min

2 - Rinse 3 x 5 min

3 - ABC - 45 min

4 - Rinse 3 x 5 min

5 - Recycled IgG - 30 min

6 - Rinse 3 x 5 min

7 - Recycled ABC - 30 min

8 - Rinse 3 x 5 min

---

[1]This antiserum was developed under the supervision of Dr. Irwin J. Kopin with Drs. Wolfgang Oertel, Donald E. Schmechel and Marcel Tappaz. Assistance in its effective use in immunocytochemistry was provided by the laboratory of Enrico Mugnaini (University of Connecticut, Storrs).

Tissue was finally reacted with fresh DAB and .03% H_2O_2.

Using either antibody, tissue was rinsed several times in phosphate buffer, sorted into d-H_20, and mounted on gelatin-coated slides. Slides were finally air-dried and coverslipped using DPX. Staining produces a dark brown reaction product that clearly labels areas known to contain GAD (Figure 9).

GAD+ puncta are easily distinguished in material labeled with either of the GAD antibodies. They can be seen surrounding somata and throughout the neuropil (Figures 10 and 11). The puncta seen throughout the neuropil was both counted and measured in this material. A square with the area 466.394 μm^2 was drawn and saved into an image analysis system that used BioScan software. The system was installed in a Compaq deskpro 386s personal computer and used a Leitz microscope, a MTI video camera, a PIP Video Digitizer Board frame grabber, and a Microsoft inport mouse. Each section of tissue from which measurements would be taken was examined, and two noncontiguous areas from any one section were chosen for actual measurements at a final screen magnification of 2400x (microscope objective = 100x). Areas of the neuropil were chosen that were most typical of the section being examined and contained a population of relatively distinct GAD+ puncta. This criteria was established to avoid measurement of clustered or overlapping puncta. Chosen areas were free of blood vessels and cell body profiles. The chosen area was then moved, with the microscope stage, to a position under the square drawn on the monitor. Puncta that fell within that square were both measured and counted. Area measurements were made as follows.

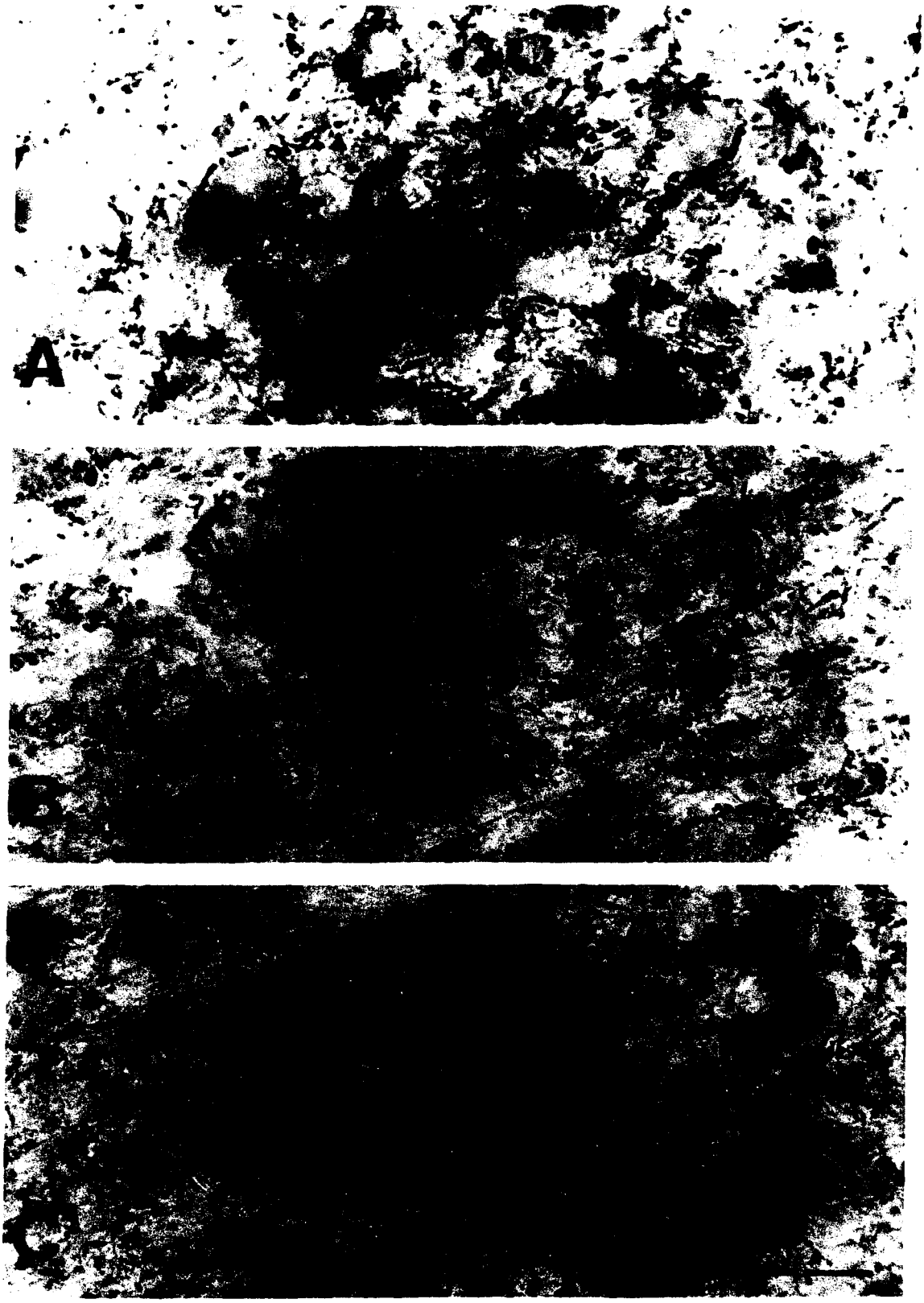
An optical density threshold was chosen such that the maximum number of puncta were highlighted. With highlighted puncta projected onto the screen, a Bioscan option that outlines all highlighted areas was selected. An additional option

Figure 9. *GAD immunocytochemical staining of the medial nucleus of 25-day-old animals.* GAD containing areas are clearly stained with a dark brown reaction product. *Arrows* indicate Purkinje cells. *A.* 25-day-old normal rat *B.* 25-day-old dystonic rat. *Scale bar = 100 μm*

Figure 10. *Immunocytochemical staining of GAD+ puncta in the medial nucleus of 25-day-old animals.* Labeled GAD+ puncta in *A)* 25-day-old normal rat and *B)* 25-day-old *dt* rat. *Arrowheads* indicate puncta which are found encircling somata and throughout the neuropil. The density of GAD+ puncta located in the neuropil of both the medial and interpositus division of the DCN in *dt* rats of this age is significantly less than that of age-matched controls. *Scale bar = 50 μm.*


A
B

Figure 11. *Immunocytochemial staining of GAD+ puncta in the medial nucleus of 20-day-old animals.* Labeled GAD+ puncta in medial nucleus of *A)* 20-day-old normal rat, *B)* 20-day-old *dt* rat, and *C)* 20-day-old normal rat 2 days after administration of 3-AP. *Arrowheads* indicate puncta which are found encircling somata and throughout the neuropil. At this time the size of GAD+ puncta located in the neuropil of both the medial and interpositus divisions of the DCN is significantly larger in animals with 3-AP lesions when compared to both normal and *dt* rats of the same age. *Scale bar = 50 μm.*


A

allowed the experimenter to point to individual puncta. The cross-sectional area of the selected puncta was computed and reported by Bioscan. Overlapping and clustered puncta were omitted from measurements of puncta size. However, as many puncta as possible were measured, providing 4-10 measurements per square. In the medial nucleus, the nucleus of primary interest, four different sections evenly spaced through the sagittal extent of the nucleus were chosen for measurements. Two separate squares of 466.394 μm^2 were measured in each section, for a total of 932.79 μm^2 per section. This represented from 0.2-1.1% of the total area of the DCN in the section being measured. However, any one DCN cross-section is made up of somata, blood vessels, and areas of neuropil. Therefore, while the percentage of neuropil measured was not computed, it represented a proportion of neuropil of a magnitude much greater than 0.2-1.1%. Four to 11 puncta were measured in each square (mean puncta/square for all animals, all nuclei, all squares = 7.89 $\pm$ 1.72, n=449). Therefore, in the medial nucleus, measurements were taken of 32-88 puncta in each animal. In the interpositus nucleus, three evenly spaced sections were chosen and one representative section each in the lateral DCN and lateral vestibular nuclei. (The latter two nuclei were sampled only for preliminary information.) All puncta within the square were counted for computation of density measurements.

Results

GAD immunocytochemistry produced a pattern of staining in all three DCN consisting of dark dots, or puncta, scattered or clustered throughout the neuropil and also arranged in continuous rows apparently surrounding somata (Figures 10 and 11). In other ultrastructural studies, dots such as these have been identified as axon terminals (Oertel et al., 1981; Wassef et al., 1986). Direct comparisons could only be made between groups using the same antibody. Therefore, 25-day-old *dt* rats

could only be compared to 25-day-old normals, whereas, all remaining groups used the same antibody and could be compared to one another where necessary.

Two-way analysis of variance (ANOVA) (condition x DCN) was used to compare puncta size or density for all DCN in any one age group. Student's t-test was used as a follow-up to compare either *dt* rats or rats with 3-AP lesions to age-matched controls in any one DCN. At 25 days of age, puncta density was significantly effected by the condition of the animal ($F_{(1,18)}$, $p<.028$) (Figure 10). This effect was found to exist in the medial nucleus (norm>*dt*; $n=4$; $p<.05$) and marginally in the nucleus interpositus (norm>*dt*; $n=4$; $p<.10$) (Table 6). In normal animals, the density of puncta did not change from 20-days-old to 32-days-old. However, antibody #1440, which was used on the 25-day-old animals, stained more effectively, but still revealed decreased puncta density in 25-day-old *dt* rats when compared to age-matched controls. This provides good evidence for the authenticity of the observed effect at this age. In addition, while the type of fixation and/or antibody affected puncta size, this effect was seen in both normal and *dt* rats; i.e., both groups showed a similar increase in measured puncta size in tissue fixed with zinc salicylate perfusate when compared with tissue fixed with picric acid.

ANOVA in 20-day-old animals also revealed a significant effect for condition ($F_{(2,24)}$, $p<.001$). Student's t-test showed no significant difference between *dt* rats and normal rats. However, when animals with 3-AP lesions were compared to normal animals, significant differences in puncta size were found in both the medial (3-AP > norm; $df=5$; $p<.025$) and interpositus (3-AP > norm; $df=5$; $p<.05$) nuclei of animals sacrificed 2 days after administration of the 3-AP neurotoxin. As with cell size differences, these differences were not present at 14 days post-injection (Table 7).

Table 6

Puncta Density (puncta/466.394 μm^2 ± S.E.M.) in Medial and Interpositus DCN of Normal Rats, *dt* Rats, and Rats With 3-AP Lesions

	NUCLEUS	
	Interpositus	Medial
20-day-old animals GAD antibody #434		
Normal (n=4)	22.04±1.43	20.22±2.11
Dystonic (n=4)	24.46±2.51	19.57±1.45
3-AP 18+2 (n=3)	20.78±1.66	19.96±1.38
25-day-old animals GAD antibody #1440		
Normal (n=4)	29.75±1.24	26.63±1.21
Dystonic (n=4)	26.42±1.72**	21.72±2.07*
32-day-old animals GAD antibody #434		
Normal (n=2)	19.50±.17	16.69± .94
3-AP 18+14 (n=4)	19.63±.96	16.25± .56

*dt 25 < norm 25, $p<.05$
**dt 25 < norm 25, $p<.10$

Table 7

Puncta Size (Area-μm^2 ± S.E.M.) in the Medial and Interpositus DCN of Normal Rats, *dt* Rats, and Rats With 3-AP Lesions

	NUCLEUS	
	Interpositus	Medial
20-day-old animals GAD antibody #434		
Normal (n=4)	.506±.040	.530±.056
Dystonic (n=4)	.519±.091	.532±.113
3-AP 18 + 2 (n=3)	.743±.024*	.706±.046**
25-day-old animals GAD antibody #1440		
Normal (n=4)	.907±.069	.911±.086
Dystonic (n=4)	.938±.052	1.127±.130
32-day-old animals GAD antibody #434		
Normal (n=2)	.559±.068	.663±.035
3-AP 18 + 14 (n=4)	.677±.034	.759±.149

*3-AP 18+2 > norm 20, $p<.005$
**3-AP 18+2 > norm 20, $p<.025$

EXPERIMENT #5 - Cerebellar GABA Levels

Do increases in DCN GAD activity, which have been observed in both the *dt* rat and animals with 3-AP lesions, represent increases in GABA synthesis and, hence, GABA levels?

Experiments 1-3 provide quantitative measurements supporting the assumption of no morphological defect in the genetically *dt* rat; i.e., there is no evidence of missing Purkinje cells with a resultant loss of input to the DCN. Experiment #4, however, revealed a decrease in the density of GAD+ puncta in 25-day-old mutants in line with decreased firing rate of afferent cells. In the same study the size of puncta, which represent terminals of GABAergic cells, is increased in animals with lesions where the firing rate of those cells is increased.

Although immunocytochemical results are different in the two groups, increased GAD activity in the DCN of both groups suggests the possibility of increased levels of GABA. In addition, receptor binding studies using ^{3}H-muscimol have revealed decreased binding in the DCN of *dt* rats compared to normal littermates (Beales et al., 1990). Increased GAD activity coupled with decreased receptor binding at this site suggests a down-regulation of receptor number in the *dt* rat in compensation for an increase in GABA synthesis and release. If this hypothesis is correct, increased GABA levels are expected in the DCN of *dt* rats.nl line with immunocytochemical observations of increased GAD+ puncta size and eletrophysiological measurements of increased Purkinje cell firing rate in animals

with 3-AP lesions, we would also expect to find increased GABA levels in the DCN of these animals. Therefore, to confirm observations from the immunocytochemical studies in Experiment #4 and to examine further the hypotheses regarding GABA synthesis and release in the *dt* rat, this experiment made direct measurements of GABA levels in the DCN and cerebellar vermis of both *dt* rats and rats with 3-AP lesions.

Methods

Rats were killed by focused microwave irradiation. Their brains were dissected and immediately immersed in liquid nitrogen. The cerebellum was sliced and the DCN dissected out for later dissection into samples of vermal cerebellar cortex and DCN, as previously described (Oltmans et al., 1985). Dystonic rats and littermate controls were 20-23 days of age at sacrifice. Another group of normal rats were injected with 65 mg/kg 3-AP at 23 days of age and killed 24 hr later. Controls for this group were injected with equal volumes of saline.

GABA was assayed by high performance liquid chromatography with electrochemical detection (HPLC-EC). The HPLC-EC system consisted of a Beckman 112 pump; a 10 cm, 3 μm, C-18 reverse phase ODS column (Rainin); and a Bioanalytical Systems (BAS) amperometric detector. Quantification using an external standard calibration table was carried out with a Hewlett-Packard recording integrator.

Samples were homogenized in 500 μl of ice cold methanol and reacted with o-phthaladehyde reagent (OPA) to increase sensitivity for trace amounts (Peinado, McManus, & Myers, 1986). The OPA regent was made by dissolving 27 mg OPA in 500 μl absolute ethanol and adding 5 ml .1 M sodium tetraborate (pH=10), followed by the addition of 20 μl mercaptoethanol. This solution can be used for up

to 1 week if the bottle is wrapped in foil and kept at room temperature. One volume of sample was reacted with 4 volumes of OPA and injected onto the column exactly 2 min later. Composition of the mobile phase was as follows:

780 ml .1 M dibasic sodium phosphate

100 ml tetrahydrofuran

100 ml acetonitrile

20 ml propanol-1 (apparent pH = 5.35)

The mobile phase was pumped at a flow rate of 1.0 ml/min at ambient temperature, and the amperometric detector was set at 100 nA sensitivity using a 0.75 V potential. Protein was assayed with the Lowry technique (Lowry, Rosebrough, Farr & Randall, 1951).

Results

One way ANOVA with Tukey post-hoc comparisons were run separately for the DCN and the vermis. At both sites, rats treated with 3-AP showed a significant increase in GABA levels 1 day after treatment when compared to untreated controls (Table 8). This is in agreement with electrophysiological studies where the simple spike rate of Purkinje cells has been shown to be increased by over 100% in rats with this type lesion. GABA levels in *dt* rats, however, did not differ from control at either site. This finding, coupled with the differences in immunocytochemical staining between *dt* and 3-AP rats, suggests that the interpretation of increased GAD activity is not the same for both groups. In the immediate post-lesion period, GAD activity and GABA levels appeared to rise in unison in the 3-AP group. The increases in Purkinje cell firing at this time strongly suggest an increased demand for transmitter and, therefore, a resultant increase in GAD activity to meet this demand.

Table 8

Cerebellar GABA levels (μg GABA/mg protein ± S.E.M.) in Normal Rats, *dt* Rats, and Rats With 3-AP Lesions

	DCN	Cortex
20-24 day old normal n=18	1.3587 ± .0983	1.1149 ± .0661
20-23 day old dystonics	1.2797 ± .0690 n=6	.9728 ± .0913 n=9
3-AP at 23 d.o.+ 1 day n=8	1.9522 ± .1069*	1.4352 ± .0898**

* 3-AP > normal, $p<.002$
** 3-AP > normal, $p<.017$

The enlargement of immunoreactive puncta appears to be a morphological correlate of increased GABA synthesis.

In the *dt* rat, however, where Purkinje cell activity is low, GABA levels were normal and the density of stained puncta was reduced. This pattern suggests that the increased GAD in these animals is not due to a form of GAD important in transmitter synthesis.

DISCUSSION

Since identifying the mutant rat *dystonic*, attempts to locate the anatomical and/or functional site of origin of the behavioral phenotype have employed pharmacological, electrophysiological, histological, and biochemical methods. These studies have implicated the cerebellum, and particularly the Purkinje cell, as a potential site of dysfunction. For example, GAD activity in the DCN is increased and there is a decrease in DCN GABA receptor sites. Increased GAD activity in association with a decrease in receptor sites in the *dt* rat has been interpreted as a down-regulation of receptor number in response to increased transmitter release. This type of down-regulation has been shown to occur in other systems, e.g., lesions of the dopaminergic nigrostriatal pathway produce increased turnover of GABA in striatal neurons, a consequent decrease in GABA receptor sites in the striatum itself, and at a specific striatal projection area (Pan, Penney, & Young, 1985).

The experiments reported here set out to examine the inconsistencies which exist between the electrophysiological and biochemical abnormalities that have been observed in this mutant. By performing a thorough investigation of Purkinje cell morphology at the light microscopic level we were able to add additional support to the hypothesis of altered Purkinje cell function in the *dt* rat. For example, the increased activity of DCN cells that has been observed is not due to a loss of Purkinje cells and, therefore, suggests a functional defect. The volume of the nuclei is not altered, which, taken in conjunction with a lack of change in target cell size,

suggests that there is no change in Purkinje cell terminal arborization. Negative results must be interpreted with caution. However, similar studies have found significant differences in these measures in mutant mice. Because these methods have been found to be reliable, negative results in this case indicate that the difference in firing patterns of DCN cells between normal and *dt* rats is not due to deafferentation of DCN cells. It is more likely due to a decrease in Purkinje cell firing rate or a reduced sensitivity of DCN cells to Purkinje cell input. The decrease in GAD+ puncta density we observed in the medial and interpositus nucleus of 25-day-old mutants is consistent with the decreases in Purkinje cell activity and increases in DCN cell activity that have been measured previously. There was no significant difference from normal in puncta density in 20-day-old mutants; therefore, the change in density may be progressive. Although Purkinje cells in *dt* rats have already been shown to have decreased rate and rhythmicity of firing, at this time, the relationship between terminal number and firing rate is not known. However, because the decreases in puncta number are present at 25 days, but not at 20 days of age, decreased terminal number appears to be a secondary consequence rather than a primary effect of the syndrome. It would be reasonable to propose that as firing rate decreases, the need for transmitter decreases, resulting in a reduction in staining for the synthesizing enzyme.

Receptor-binding studies using muscimol have shown a decrease in the number of GABA receptors in the DCN of the *dt* rat. $GABA_A$ receptor autoradiography has been performed in various mutant mice with a variety of cerebellar cell abnormalities (Rotter & Frostholm, 1988; Rotter, Gorenstein, & Frostholm, 1988). The $GABA_A$ receptor is a GABA/benzodiazepine receptor complex, labeled by both ^{3}H-flunitrazepam binding and ^{3}H-muscimol binding. In

mouse DCN, flunitrazepam binding predominates, and in *pcd* mouse, where all Purkinje cells are lost by day 45, there is a 54% increase in receptor density (Rotter & Frostholm, 1988). It has been hypothesized that this is a form of denervation supersensitivity leading to an up-regulation of benzodiazepine receptors. Although in the past, decreased muscimol binding in the *dt* rat has been interpreted as a down-regulation in response to increased GAD activity associated with the synthesis of GABA, the experiments presented here revealed no elevation in DCN GABA levels.

Some studies have suggested the existence of pre-synaptic GABAergic autoreceptors (Brennan & Cantrill, 1979; Brennan, Cantrill, Krogsgaard-Larsen, 1981; Mitchell & Martin, 1978; Van der Heyden, Venema, & Korf, 1980). The possibility of a negative feedback system associated with this class of receptor suggests alternative hypotheses to explain increased GAD activity coupled with a decrease in GABA receptors. Purkinje cell terminals in the DCN of *dt* rats may develop a reduction in the number of putative GABAergic autoreceptors in response to decreased cell activity, allowing for a compensatory increase in GABA release. At this time, however, it is difficult to establish strong support for this hypothesis. While the existence of a GABAergic autoreceptor has gained wide acceptance, agreement concerning the nature of the receptor has not developed as readily. GABA receptors of at least two types, A and B, are known to exist. Raiteri, Bonanno, & Fedele (1989) supported the existence of a GABA autoreceptor feedback system with the demonstration that exogenous GABA inhibits the electrically stimulated release of [^{3}H]GABA in slices of rat tempero-parietal cortex. Additionally, their work showed this effect to be blocked by $GABA_B$ antagonists and mimicked by $GABA_B$ agonists; whereas, $GABA_A$ agonists and antagonists were ineffective. Conversely, Arbilla, Kamal, & Langer (1979) found that the $GABA_A$ receptor agonist, muscimol, acted

to inhibit K^+ stimulated release of GABA in substantia nigra slices where GABA release was Ca^{++} dependent; the same effect could not be demonstrated in slices from the occipital cortex, where GABA release was Ca^{++} independent. Differences within the study by Arbilla and associates may be attributed to differences in site and calcium dependence; differences between the studies of Arbilla et al. (1979) and Raiteri et al. (1989) may result from differences in site or in the type of stimulation used to evoke neurotransmitter release. *In vivo* operation of a $GABA_A$ type autoreceptor has also been demonstrated. Using a push-pull cannula for infusion and collection, Van der Hayden et al (1980) found that muscimol reduced the release of GABA in the corpus striatum, whereas the $GABA_A$ antagonist, picrotoxin, produced increased GABA release at the same site. Thus, GABAergic autoreceptors may play a role in neurotransmitter release. Studies of stimulated GABA release are needed in the *dt* rat to assess this possibility.

Increased GU has been observed in the DCN of the *dt* rat. GU is an indicator of neural activity in a particular region and can only identify a site of abnormality. The source of the increased GU in the DCN of the *dt* rat is not known. Although most studies indicate that the GU measured in 2-deoxyglucose studies is the result of metabolic activity in terminals (Kadekaro, Crane, & Sokoloff, 1985), it may indicate either increased excitation or increased inhibition. Increased GAD activity in the DCN of the *dt* rat suggests increased inhibition in this case. However, the reduction in GAD+ puncta density coupled with decreased Purkinje cell firing rates and increased DCN firing indicate that GU in the DCN of the *dt* rat probably does not represent Purkinje cell activity. Recordings from the IO also rule out climbing fiber collaterals as a source of increased GU. However, recurrent axon collaterals have been shown to exist in DCN (Matsushita & Iwahori, 1971). Thus,

either these DCN cell collaterals or mossy fiber collaterals would appear to be the only remaining candidates.

The DCN neuronal population consists of small interneurons and larger relay neurons receiving input from a variety of sources, including extracerebellar sites. However, all DCN neurons receive Purkinje cell innervation and a majority of terminals synapsing on all DCN neurons represent projections from cortical Purkinje cells (Chan-Palay, 1977). Other studies have shown decreases in DCN cell size and volume following loss of Purkinje cell input in the staggerer and in *pcd* mice (Roffler-Tarlov & Herrup, 1981; Triarhou et al., 1987). Although there is no evidence in the *dt* rat of a similar loss of Purkinje cells, the low firing rate of Purkinje cells in this mutant could be viewed as functional deafferentation. This spontaneous firing rate has, however, been observed in both the vermis and hemispheres. Thus, a widespread effect would have been expected if reduced activity were sufficient to alter DCN cell size. Although the medial nucleus in *dt* animals showed a marginal effect, this was not consistent across ages or nuclei, and cell-size measurements in the DCN of *dt* animals did not indicate the presence of any general degenerative processes.

Climbing fibers that project from the IO to the cerebellar cortex send collaterals to the DCN, and, therefore, 3-AP animals are also deprived of a source of DCN innervation. In animals with 3-AP lesions, a significant increase in the size of cells in the interpositus nucleus was found 2 days after the lesion. This change was not seen in other nuclei and had disappeared in the interpositus nucleus by 2 weeks post-lesion. Because the direction of both DCN cell size and puncta size changes over time follow the direction of changes in Purkinje cell firing, it is possible

that they are consequences of increased Purkinje cell firing in the early post-lesion period. As firing rates return to normal, these measures would also normalize.

Due to similarities between the *dt* rat and rats with 3-AP lesions, animals with this lesion have been useful as models for the formation of hypotheses regarding the progression of abnormalities in the mutant rat. Of particular interest is the manner in which observed changes progress following a 3-AP induced lesion. Although Purkinje cell activity in 3-AP animals is significantly increased immediately after the lesion, activity returns to normal by one month post-lesion. DCN cells are initially silenced due to increased inhibitory input from Purkinje cells (Batini & Billard, 1985; Batini et al., 1985); however, 1 month after the lesion, DCN firing rates have increased and returned to normal. This gradual increase then continues, overshooting normal levels, and Billard and Daniel (1988) have observed increased firing rates in the DCN of animals with 3-AP lesions up to 2 years after treatment with this neurotoxin. Normal biochemical activity is also disrupted by the lesion. Progressive increases in DCN GAD activity are first observed within 24 hr of 3-AP treatment, and activity remains at significantly elevated levels even after Purkinje cell firing rates have returned to normal. In line with increased Purkinje cell activity and increased DCN GAD activity, the current experiments have revealed significantly increased DCN GABA levels 24 hr post-lesion, along with increased GAD+ puncta size in two of the three DCN 2 days post-lesion. In our study puncta size had returned to normal by 2 weeks post-lesion. This may correlate with the normalization of Purkinje cell firing rates and DCN firing rates, which occurs by 1 month post-lesion. In light of the persistent DCN GAD activity that has been observed in these animals, it appears that the initial increase in Purkinje cell firing

triggers a change in GAD activity, which subsequently becomes uncoupled from Purkinje cell activity.

Multiple molecular forms of GAD have been identified in rat brain (Henry & Tappaz, 1989; Martin et al., 1990b; Spink, Porter, Wu, & Martin, 1987). In synaptosomes it is present predominately as an inactive, apoenzyme, form of 63kDA GAD (Martin, Martin, wu & Espina, 1990a). ApoGAD is rapidly activated to holoGAD by binding with its cofactor pyridosal phosphate (Porter & Martin, 1988). The 63kDA molecular form has been found to be heavily labeled by the #1440 (Oertel) GAD antibody used in our studies as well as several other antibodies. We do not have information regarding specificity of the #434 antibody that was used on 3-AP animals; however, staining for this antibody was similar to that found with #1440. Therefore, while initial increases in DCN GAD activity after 3-AP lesions may reflect increased neurotransmitter synthesis, the type of persistent changes that have been observed may be associated with some long-term non-synaptic change in the activity of the cells and their response to loss of IO input.

The sequence of events in 3-AP animals and the biochemical changes associated with extended post-lesion survival suggest hypotheses regarding the apparently paradoxical increases in GAD activity that have been observed in the DCN of the *dt* rat. Although Purkinje cell activity is depressed in the mutant animals by the time the phenotype becomes apparent, the increased GAD activity observed beginning on postnatal day 16 may have been triggered by earlier events. As in animals with 3-AP lesions, perhaps GAD activity becomes uncoupled from the electrophysiological activity of the cells. In biochemical assays performed on *dt* rats, measurement of GAD activity has been carried out using saturating levels of cofactor; therefore, total GAD is measured and it is not possible to tell in what

proportions inactive apoGAD and active holoGAD forms are represented. In addition, different molecular weight forms of GAD are not distinguished. However, the differences in GAD+ puncta which we have observed in the DCN of 25-day-old mutants was revealed with the use of an antibody which, as previously mentioned, labels the molecular weight of GAD, found primarily at terminal sites.

In mouse brain an unstable newborn form of GAD and a more stable adult form have been hypothesized (Tapia & Meza-Ruiz, 1975). The existence of a newborn versus an adult form may account for an age-dependent difference in sensitivity to Triton X-100, which has been observed in one set of studies. Successful activation of GAD in biochemical assays requires the presence of the detergent Triton X-100 and an age-dependent sensitivity to the concentration of Triton X used in these assays has been indicated; i.e., up until 17 days postnatal 0.1% and 0.5% Triton X produce similar results; after 17 days of age, 0.5% has a significantly greater effect on GAD activity than does 0.1% (Balcar & Johnston, 1987; Zetzsche,Balcar, & Wolff, 1985). This age-dependent difference suggests that the pool of GAD which has formed in older rats is associated with cellular structures more resistant to Triton X activation. Perhaps this pool is representative of GAD associated with structures in active GABAergic synapses, whereas, GAD in younger, early postnatal, animals is associated with non-synaptic structures more sensitive to Triton X liberation. GAD activity is present in the cortex prenatally before the appearance of GABAergic synapses. In rat cerebellar tissue, GAD has been found in growing axons during Purkinje and Golgi cell development and does not appear to become associated with synaptic nerve terminals until adulthood (McLaughlin et al., 1975). Therefore, the Triton X sensitive pool may represent a molecular form of GAD that plays some nonsynaptic role during development. Perhaps the increased GAD activity that has

been observed in the *dt* rat represents a lag in development. It is not out of the question to propose that this same pool of GAD comes into play following the kind of insult to the nervous system that occurs with administration of 3-AP. At this time the DCN must undergo some type of functional reorganization in response to loss of input from the IO. Electron microscopy studies of axon terminals in the DCN of 3-AP rats have shown long-lasting ultrastructural changes typical of hyperactivity in Purkinje cell terminals (Desclin & Colin, 1980; Rossi, Cantino, & Strata, 1987). These changes, like GAD activity, appear to outlast changes in Purkinje cell firing rates and could be associated with a shift in the predominant form of GAD present.

The identification of multiple forms of GAD suggests a new interpretation of differences in GAD activity in *dt* rats and rats with 3-AP lesions. Biochemical measures of total GAD may be misleading. During development, or in response to injury or genetically programmed abnormalities, different forms of GAD may be present in different amounts. The change in GAD+ puncta size in 3-AP rats at a time when Purkinje cell activity increases and GABA levels are elevated suggests the induction of a form of GAD used for transmitter synthesis in terminals. However, studies are needed to determine whether the same molecular forms of the enzyme are present as recovery progresses. Differences in GABA levels between the *dt* rat and 3-AP rats immediately post-lesion suggest that different forms of GAD are represented in the these two groups. However, additional studies are needed to test this hypothesis directly.

In conclusion, observations made in this set of experiments support the hypothesis that defects in the cerebellum of the *dt* rat are functional, rather than morphological, as in many mouse mutants. Furthermore, abnormalities in the *dt* rat resemble those seen following extended survival in animals with 3-AP lesions (Table

nine). Alterations in DCN GAD activity appear to be inconsistent with the reduction in Purkinje cell activity observed in *dt* rats. However, in animals with 3-AP lesions GAD activity remains elevated long after Purkinje cell activity has returned to normal. It is possible that the changes observed in the *dt* rat are persistent changes initiated by early elevation in Purkinje cell activity and that these changes are related, in both animals, to a shift in the form and function of GAD that predominates in Purkinje cells.

Table 9

Summary of Abnormalities in Dystonic Rats and Rats With 3-AP Lesions

	3-acetylpyridine		Dystonic
	Immediate	Late	
Purkinje cells			
firing rate	>>	<	<<
DCN cells			
firing rate	<<	>	>
DCN			
GABA levels	>	?	=
GAD activity	>	>	>
GAD+ puncta size	>	=	=

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APPENDIX
Rat Records

EXP	RAT #	LITTER (dam)	AGE (days)	CONDITION	WT.(g)
1	2L	1108	20	norm	26.7
	3L	1098	20	norm	26.6
	6L	1093	20	*dt*	26.1
	8L	1093	20	*dt*	28.0
	10L	1095	20	*dt*	27.0
	11L	1095	20	norm	39.0
2	1	1116	20	norm	29.0
	2	1117	20	*dt*	27.9
	3	1116	20	norm	26.4
	4	1117	20	*dt*	25.9
	5	1116	20	*dt*	23.4
	6	1116	20	norm	31.4
	7	1116	20	*dt*	23.3
	8	1116	20	norm	33.8
	9	1083	20	norm	51.6
	10	1098	20	norm	34.0
	11	1083	20	*dt*	37.5
3	21	1071	18	*dt*	26.7
	22	1071	18	norm	37.1
	24	1001	20	3AP 18+2	33.7
	40	1018	32	norm	87.3
	41	1065	20	3AP 18+2	39.5
	3C	1095	20	3AP 18+2	29.6
	1098/5	1098	20	norm	34.0
	1098/10	1098	20	*dt*	29.3
	20z	1095	25	norm	32.2
	22z	1095	25	norm	30.4
	23z	1095	25	norm	54.0
	24z	1090	25	norm	53.8
	25z	1090	25	norm	30.1
3,4	25	852	32	3AP 18+14	60.2
	26	852	32	norm	87.3
	27	1023	20	norm	44.9

	28	1023	20	*dt*		30.2
	29	1030	20	*dt*		26.3
	30	1062	19	3AP 17+2		28.3
	31	1027	32	3AP 18+14		81.6
	32	1020	20	3AP 18+2		41.5
	33	1020	20	*dt*		37.3
	35	992	20	norm		35.3
	36	1075	33	norm		104.5
	37	SD-4	20	norm		49.1
	38	1018	32	3AP 18+14		105.4
	39	1065	20	*dt*		34.8
	42	1079	32	3AP 18+14		56.5
	43	UF/SD-9	20	norm		43.5
	29z	1108	25	norm		24.0
	31z	1105	26	norm		49.9
	32z	1105	26	norm		51.2
4	34	992	20	3AP 18+2		29.6
	27z	1108	25	norm		49.5
	28z	1108	25	norm		52.0
	30z	1108	25	*dt*		24.9
	33z	1087	25	*dt*		47.2
	34z	1087	25	*dt*		39.0

	TUBE #					BRAIN WT.(mg) DCN	Cortex
5	1	673	24	3AP 23+1	37.9	14.8	60.4
	2	673	24	norm	36.4	24.6	48.7
	3	673	24	3AP 23+1	38.7	11.7	29.4*
	5	673	24	3AP 23+1	38.9	16.1	65.8
	6	673	24	norm	37.9	18.1	43.6
	8	673	24	norm	40.7	18.0	55.6
	9	673	24	3AP 23+1	39.5	21.9	74.7
	10	673	24	norm	35.4	13.5	47.4
	11	674	24	3AP 23+1	33.5	16.5	56.6
	12	674	24	norm	33.3	16.7	50.5
	13	674	24	3AP 23+1	35.3	13.7	40.8
	14	674	24	norm	35.3	13.6	53.4
	15	674	24	3AP 23+1	34.2	19.7	69.8
	17	674	24	3AP 23+1	34.0	16.7*	28.9
	18	674	24	norm	37.0	10.9	31.4
	19	674	24	3AP 23+1	36.4	16.0	45.5
	20	674	24	norm	33.0	11.6	79.9
	1	596	23	norm	n/a	18.7	59.4
	2	596	23	norm	n/a	17.4	51.2
	3	596	23	*dt*	n/a	15.1	56.3
	4	596	23	norm	n/a	11.6	41.5
	5	596	23	*dt*	n/a	21.0	53.7
	6	596	23	norm	n/a	16.8	58.1

7	642	21	*dt*	n/a	20.2	65.2
8	642	21	norm	n/a	11.7	61.0
9	661	21	norm	n/a	19.1	56.1
10	661	21	*dt*	n/a	13.7	43.2
11	661	21	norm	n/a	8.6	39.7
12	639	20	*dt*	n/a	13.3	47.7
13	639	20	norm	n/a	16.5	53.4
14	714	23	norm	n/a	11.7	42.8*
16	714	23	norm	n/a	19.4	58.0
17	714	23	*dt*	n/a	10.0*	17.5
19	714	23	*dt*	n/a	12.0*	44.3
21	714	23	*dt*	n/a	12.2*	22.4
22	714	23	norm	n/a	10.6*	45.1
23	714	23	*dt*	n/a	23.4	63.0

*section not included in data

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Jacqueline Lutes Goldstein

Major Subject Psychology - Behavioral Neuroscience Specialty

Title of Dissertation An Immunocytochemical and Morphological Examination of the Cerebellum of Mutant Dystonic (dt) Rats and Rats with 3-Acetylpyridine Lesions

Dissertation Committee:

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Director of Graduate Program _____

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