INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

**Commissural Projections of the Nuclei of the Lateral Lemniscus and
Neuronal Degeneration Following Midline Transections in the Adult Rat**

by

Brian Anthony van Adel
B.Sc. (Hons) Carleton University, 1995

A thesis submitted to the faculty of Graduate Studies and
Research in partial fulfillment of the requirements of the
degree of

Master of Science
Specialization in Neuroscience

Department of Biology

Ottawa-Carleton Institutes of Biology and Neuroscience
Carleton University
Ottawa, Ontario
May, 1998

© copyright
1998, Brian Anthony van Adel

National Library
of Canada

Acquisitions and
Bibliographic Services

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale
du Canada

Acquisitions et
services bibliographiques

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

L'auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-32433-8

The undersigned hereby recommend to
the Faculty of Graduate Studies and Research
acceptance of the thesis.

**“Commissural Projections of the Nuclei of the Lateral Lemniscus and
Neuronal Degeneration Following Midline Transections in the Adult Rat”**

submitted by
Brian Anthony van Adel, B.Sc. (Hons)

in partial fulfillment of the requirements for
the degree of Master of Science
Specialization in Neuroscience

Chair, Department of Biology
Thesis Supervisor
Thesis Co-Supervisor

Carleton University
May 1998

ABSTRACT

The normal neuroanatomical organization of the rat's nuclei of the lateral lemniscus (nLL) was investigated (Experiment 1) as a prerequisite for a time course analysis of retrograde changes in commissural projecting lemniscal neurons following midline surgical transection of their axons (Experiment 2). In Experiment 1, conventional neuroanatomical tract tracing techniques were employed to determine the efferent and afferent projections of the nLL. The results suggest that in the rat the nuclei of the lateral lemniscus (nLL) can be divided into three distinct cell groups (DNLL, INLL, and VNLL) based on the differential patterns of innervation each receives from auditory brainstem nuclei. Of the three lemniscal nuclei, only the DNLL receives bilateral inputs from lower auditory nuclei. Furthermore, it is the only nucleus within the LL that projects bilaterally to the ICC and to the opposite LL through the commissure of Probst (CP).

In Experiment 2, the anatomical consequence of surgical transection of the commissure of Probst was investigated. Responses to injury were investigated for survival times ranging from 1 day to 12 weeks after complete CP transection. Transection of the CP completely eliminated the transport of Fluorogold (FG) from the central nucleus of the inferior colliculus (ICC) to the opposite DNLL, and blocked the anterograde transport of biotinylated dextran (BD) to both contralateral DNLL and ICC. Axotomized DNLL neurons prelabeled with FG appeared normal 2 weeks post-transection, but by 4 weeks a significant loss of FG-prelabeled contralaterally projecting DNLL neurons was observed. Some fluorescent labeling was observed in animals that survived for 4 weeks or longer, but this was attributed to microglia which sequestered FG via phagocytosis. Cell counts of FG-prelabeled neurons in the DNLL indicated that no contralaterally projecting neurons survived CP transection. Immunoreactive astrocytes were labeled with anti-GFAP, revealing a pattern of anisomorphic fibrous gliosis at the wound site, along the CP and bilaterally in DNLL. These results suggest that transection of the CP is a valuable *in vivo* model of neuronal degeneration and time-dependent responses to traumatic brain injury.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Jack B. Kelly for his excellence in guiding me through all aspects of my research and academic studies. In addition, I would also like thank Dr. M. Tenniswood for allowing me to carry out a portion of my studies in his laboratory and for the input into this research, and Dr. J.J. Cheetham for many thought provoking conversations. Cheng Huang for all the help with the electron microscopy work involved in this thesis. Adam Baker and Cheng Huang for all the help preparing many of the photographic figures presented in this thesis.

I also wish to thank the members of our laboratory, and support staff in Life Sciences Research Building (LSRB) who have made my studies enjoyable and rewarding; and Dr. Makoto Ito from Kanazawa, Japan, who refined my neuroanatomical techniques and more importantly, for his friendship; Christine McGregor, Patrice Jacobson and Teresa Fortin for their excellent assistance in histology for making the lab seem so friendly and cheerful.

In addition, I would like to thank the following people: Heather McNeely, my siblings, my mom and grandparents, Sean Kidd, Rose Labelle, Colm Morrissey, Chris Davidson, Yves Bureau, Liang Li, Jennifer Arnold, Heather Lennox, Jim Carleton, the folks at Vittoria's on Bank Street, and every single person who has been through the LSRB since my first day - I have gained insight (scientific or otherwise) from each of you.

Many thanks to all the Columbi of the world. I now realize that exploring new territory inevitably evokes the Columbus response: shaking heads and muttering as you disappear over the horizon and a hero's welcome when (if) you return.

Thanks are also extended to Graduate Studies at Carleton University for financial support of my M.Sc. studies.

In memory of Raymond J. van Adel. Thanks for teaching me how to fly Grandpa.

TABLE OF CONTENTS

	Page
Title Page	i
Acceptance sheet	ii
Abstract	iii
Acknowledgments	iv
Table of Contents	v
List of abbreviations	vi
List of Figures	viii
List of Tables	xiii
List of Appendices	xiv
Chapter 1 Introduction	1-13
Chapter 2 Neuroanatomical investigation of the nuclei of the lateral lemniscus: a search for subdivisions and commissural projections using anterograde and retrograde tract tracing techniques in the adult rat, <i>norvegicus</i> (Experiment 1)	14-108
Chapter 3 Neuronal degeneration in the dorsal nucleus of the lateral lemniscus after surgical transection of the commissure of Probst in the adult rat, <i>Rattus norvegicus</i> (Experiment 2)	109-185
General Conclusions	186
Appendix 1 Surgical procedure for transection of the commissure of Probst	187-193
Appendix 2 Publication List	194-196
References	197-215

LIST OF ABBREVIATIONS

AMPA	a-amino-3-hydroxy-5-methyl-4-isoxazolepropionic
cAMP	adenosine 3',5',-cyclic monophosphate
Aq	aqueduct (cerebral)
ATP	Adenosine triphosphate
AVCN	anteroventral cochlear nucleus
BBB	blood-brain barrier\
BC	brachium conjunctivum
BD	biotinylated dextran
BIC	brachium of the inferior colliculus
c	contralateral
cic	commissure of the inferior colliculus
CN	cochlear nucleus
CP	commissure of Probst
DNLL	dorsal nucleus of the lateral lemniscus
DCIC	dorsal cortex of the inferior colliculus
DCN	dorsal cochlear nucleus
ECIC	external cortex of the inferior colliculus
EE (EE/F)	binaural response type - cell excited by stimulation of either ear alone.
EEA	excitatory amino acids
EEAC1	excitatory amino acid transporter
EI (EO/I)	binaural response type - cell excited by contralateral stimulation and inhibited by ipsilateral stimulation.
EO (EO/O)	binaural response type - cell excited by contralateral stimulation and ipsilateral stimulation has no effect.
FB	Fast Blue
FG	Fluoro-Gold
FR	Fluoro-Ruby
GABA	γ -gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GFAP	glial fibrillary acid protein
GS	glutamine synthetase
GLAST	glutamate astrocyte specific transporter
GLT-1	glutamate transporter

H ₂ O ₂	hydrogen peroxide
HRP	horseradish peroxidase
i	ipsilateral
IC	inferior colliculus
ICC	central nucleus of the inferior colliculus
IID	interaural intensity difference
INLL	intermediate nucleus of the lateral lemniscus
ITD	interaural time difference
KYNA	kynurenic acid
LL	lateral lemniscus
LSO	lateral superior olivary nucleus
MGB	medial geniculate body
MLF	medial longitudinal fasciculus
MSO	medial superior olivary nucleus
nLL	nuclei of the lateral lemniscus
NMDA	<i>N</i> -methyl-D-aspartic acid
NO	nitric oxide
nNOS	neuronal NOS
NOS	NO synthase
O ₂ ⁻	superoxide radical
ONOO	peroxynitrate
PARS	poly (ADP-ribose) synthase
PCD	programmed cell death
PL	paralemniscal zone
PS	phosphatidylserine
PVCN	posteroventral cochlear nucleus
Sag	sagulum
SC	superior colliculus
SOC	superior olivary nucleus
SPN	superior paraolivary nucleus
tz	trapezoid body
VNLL	ventral nucleus of the lateral lemniscus
VNTB	ventral nucleus of the trapezoid body

LIST OF FIGURES

Figure		Page
2.1	A schematic illustration of the anterograde and retrograde tract tracing procedures used for each experimental group from Experiment 1.	46
2.2	Cresyl violet stained coronal section through the rat's ICC, nLL and surrounding structures.	48
2.3	Anterograde labelling in a normal animal after a unilateral injection of BD into the left ICC.	50
2.4	Anterograde labeling in a normal animal after a unilateral injection of BD into the left DNLL.	52
2.5	The extent of efferent fibers contained within the commissure of Probst after an injection of BD into the right DNLL of a normal animal.	54
2.6	The pattern of retrograde labelling in a normal animal after a unilateral injection of FG into the left ICC.	56
2.7	Low power photomicrographs of the rostral-to-caudal extent of retrograde labeling, bilaterally in the nLL after a unilateral injection of FG into the left ICC of a normal animal.	58
2.8	Low power photomicrographs of the rostral-to-caudal extent of retrograde labeling in the ipsilateral nLL after a unilateral injection of FG into the left ICC.	60
2.9	Percentage of ipsilaterally and contralaterally labeled DNLL neurons after restricted injections of FG into the ICC of normal animals.	61
2.10	Low and high power photomicrographs of bilateral FG retrograde labeling in the nLL after injection into the ICC of a normal animal used for cell counting data.	63

2.11	Low and high power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FR into the left ICC.	65
2.12	The pattern of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left DNLL of a normal animal.	67
2.13	Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left DNLL of a normal animal.	69
2.14	Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left INLL of a normal animal.	71
2.15	Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left VNLL of a normal animal.	73
2.16	Contrasting patterns of retrograde labeling in the ipsilateral and contralateral SOC of normal animals after a left unilateral injection of FG into one of the following structures: ICC, DNLL, INLL, or VNLL.	75
2.17	Schematic diagram summarizing the contrasting patterns of retrograde labeling seen in auditory brainstem of normal animals after unilateral injection of FG into one of the following structures: ICC, DNLL, INLL, or VNLL.	77
2.18	The pattern of retrograde labeling in the auditory brainstem after bilateral injection of FG into the left ICC and FB into the right ICC in a normal animal	79
2.19	The pattern of retrograde labeling in the lateral lemniscus after bilateral injection of FG into the left ICC and FB into the right ICC in a normal animal	81
2.20	The pattern of retrograde labeling in the lateral lemniscus after restricted bilateral injections of FG into the left ICC and FB into the right ICC in an normal animal.	83

2.21	Fluorescent double labeling in the right DNLL after an injection of FG into the left ICC and an injection of FR in to the left DNLL.	85
2.22	Schematic diagram summarizing the results of animals with bilateral ICC injections of different fluorescent retrograde tracers.	87
2.23	Schematic diagram summarizing the results of animals with unilateral ICC and DNLL injections of using different fluorescent retrograde tracers.	89
2.24	A diagram illustrating the major and minor commissural projections of the nuclei of the lateral lemniscus through the commissure of Probst.	91
3.1	A schematic illustration of the anterograde and retrograde tract tracing procedures used in Experiment 2.	135
3.2	A schematic diagram of a sagittal section demonstrating the procedure of transecting the commissure of Probst	136
3.3	Schematic drawing showing the rostrocaudal extent of the damage to the CP produced by microsurgical midline knifecuts.	138
3.4	Photomicrographs of frontal sections through the midbrain tegmentum illustrating Nissl-staining of the CP and the DNLL in a surgical control animal (A and B) and a CP transection animal (C and D).	140
3.5	Anterograde postlabeling with BD in a surgical control animal and an animal that survived 1 week after CP transection.	142
3.6	The pattern of retrograde labeling in the brainstem auditory nuclei after a large FG injection into the right ICC of a sham operated animal (Columbus-19).	144
3.7	The pattern of retrograde labeling in the brainstem auditory nuclei after a large FG injection into the right ICC of an animal (Columbus-10) with a complete transection of the CP.	146

3.8	FG-postlabeling in DNLL and SOC after an injection of FG into the left ICC in a surgical control animal (A-D) and an animal which survived 8 weeks following complete transection of the commissure of Probst (E-H).	148
3.9	HRP and FR-postlabeling in the DNLL of different animals which survived 8 weeks following complete transection of the commissure of Probst.	150
3.10	Percentage of ipsilaterally and contralaterally projecting DNLL neurons in normal animals and surgical control animals.	152
3.11	Summary of FG-postlabeling in the DNLL from surgical control animals, CP transected animals, and normal unoperated animals	154
3.12	Schematic diagram summarizing the patterns of anterograde and retrograde postlabeling in the auditory brainstem of surgical control animals and CP transected animals.	156
3.13	Fluorescence photomicrographs showing the pattern of FG-prelabeling in the ipsilateral and contralateral DNLL at various times after CP transection.	158
3.14	Time course of neuronal death in the DNLL after surgical transection of the commissure of Probst.	160
3.15	FG-prelabeling in VNLL, SOC and CN after an injection of FG into the left ICC in animals which survived one week (A-D), and 8 weeks (A'-D') following complete transection of the commissure of Probst.	162
3.16	High power fluorescence photomicrographs further demonstrating the extent of retrograde degeneration observed in FG pre-labeled contralaterally projecting DNLL neurons.	164
3.17	Graphs showing the time course of neuronal degeneration in the DNLL after surgical transection of the commissure of Probst.	166

3.18	Fluorescence photomicrographs demonstrate the selective ability of activated microglia to phagocytose only transected contralaterally projecting DNLL neurons.	168
3.19	Schematic summary of fluorescent double-labeling experiments demonstrating the selective ability of activated microglia to phagocytose only transected contralaterally projecting DNLL neurons.	169
3.20	Ultrastructure of DNLL neuronal nuclei 4 weeks after surgical transection of the commissure of Probst	171
3.21	Photomicrographs of GFAP immunoreactivity in the DNLL four weeks after complete surgical transection of the commissure of Probst.	173
3.22	Schematic representation of neuronal degeneration of transected contralaterally projecting DNLL neurons and the activation of reactive astrocytes and phagocytic microglia in the DNLL following surgical transection of the commissure of Probst.	175
Appendix	Surgical procedure for midline transection of the commissure of Probst in adult albino rats.	193
1		
A1.1		

LIST OF TABLES

Table		Page
2.1	Summary of experimental groups and surgical treatments used for normal animals from Experiment 1	43
2.2	Cell counts from six representative cases with unilateral FG injections restricted to the central nucleus of the inferior colliculus (ICC).	44
3.1	Summary of experimental groups and surgical treatments used for animals from Experiment 2	130
3.2	Counts of retrogradely postlabeled DNLL neurons in surgical control animals with midline transection of the cerebellum.	131
3.3	Counts of retrogradely postlabeled DNLL neurons in CP transected animals.	132
3.4	Counts of FG-prelabeled DNLL neurons and FG-labeled phagocytic microglia in CP transected animals.	133

LIST OF APPENDICES

	Page
Appendix 1 Surgical procedure for transection of the commissure of Probst	187-193
Appendix 2 Publication List	193-196

Chapter 1

Introduction

Nuclei of the Lateral Lemniscus

The nuclei of the lateral lemniscus (nLL) are embedded within the fibers of the lateral lemniscus as they carry both excitatory and inhibitory inputs from lower central auditory structures to the central nucleus of the inferior colliculus (ICC). Anatomical pathways originating or ending in the nLL were first reported more than a century ago (Ramon y Cajal, 1909; Held, 1893; Lewandowsky, 1904; Probst, 1902; van Gehuchten, 1906; von Monakow, 1890) and have continued to be explored with the development of modern neuroanatomical tract-tracing methods in different mammalian species such as the cat (Adams, 1979; Brunso-Bechtold et al., 1981; Glendenning et al., 1981; Goldberg and Moore, 1967; Henkel and Shneiderman, 1989; Hutson et al., 1991; Kudo, 1981; Oliver and Shneiderman, 1989; Shneiderman et al., 1988; Stotler, 1953), the ferret (Moore, 1988), the opossum (Willard and Martin, 1983, 1984), various species of bats (Casseday et al., 1988; Covey and Casseday, 1991; Huffman and Covey, 1995; Vater et al., 1995; Yang et al., 1996; Zook and Casseday, 1979, 1982) and in several species of rodents: the rat (Bajo et al., 1993; Beyerl, 1978; Carey and Webster, 1971; Coleman and Clerici, 1987; González-Hernández et al., 1996; Kelly et al., 1998; Merchán et al., 1994; Merchán and Berbel, 1996; Tanaka et al., 1985; Zhang et al., 1998), the mouse (González-Hernández et al., 1986), the guinea pig (Schofield and Cant, 1997; Saint Marie and Baker, 1990), the mole (Kudo et al., 1990), the gerbil (Nordeen et al., 1983) and the chinchilla (Saint Marie, 1996). However, it has been shown only recently how the mammalian nLL are involved in the processing of acoustically guided information (Aitkin et al., 1970; Batra and Fitzpatrick, 1997; Brugge et al., 1970; Buckthoight, 1993; Covey, 1993; Covey and Casseday, 1991; Markovitz and Pollak, 1994; Yang and Pollak, 1994a, 1994b). Despite the numerous anatomical and physiological studies of the nLL, only three ablation-behavioural studies with rats have demonstrated the importance of these structures for

accurate auditory spatial discrimination (sound localization) in the horizontal plane (Ito et al., 1996; Kelly et al., 1996; Zrull and Coleman, 1997).

Cytoarchitecture and Anatomical Organization of the nLL

Although the nuclei of the lateral lemniscus form a major part of the ascending auditory system in all mammals, reports about the cytoarchitecture and anatomical organization of the nLL are controversial. In most mammalian species, the nLL have been divided into at least two subdivisions (dorsal and ventral nuclei of the lateral lemniscus, or DNLL and VNLL respectively) based on cytoarchitectonic criteria, neurochemical content, anatomical (efferent) projections to the inferior colliculus (IC) as well as ascending (afferent) inputs from lower auditory brainstem nuclei. In Nissl-stained sections, DNLL neurons are easily distinguished from bordering nuclei within the LL by their large size and darkly Nissl-stained cytoplasm. The borders between the DNLL and more ventral nLL are distinguished from one another by a narrow band of tightly grouped neurons referred to as the horizontal cell group of the lateral lemniscus, first described by Ruiz-Gómez (1988), further detailed by Bajo et al. (1993) and given its present name by Caicedo and Herbert (1993).

However, a recent controversy has arisen over the division of the group of LL neurons ventral to this horizontal group, commonly referred to as the VNLL. Based on cytoarchitectonic criteria from various mammals, the VNLL has been defined as having one (Hutson et al., 1991; Kudo, 1981, Merchán and Berbel, 1996; Nordeen et al., 1983; Saint Marie and Baker, 1990; Willard and Martin, 1983), two (Brunso-Bechtold et al., 1981; Glendenning et al., 1981; Ito et al., 1996; Kelly, et al., 1998; Kudo et al., 1990), or three (Adams, 1979; Schofield and Cant, 1997; Zook and Casseday, 1982) subdivisions. For example, many investigators using experimental rats have divided the LL into the DNLL and VNLL (Friauf, 1992; Merchán and Berbel, 1996; Sommer et al., 1993; Tanaka et al., 1985; Wynne et al., 1995), whereas some investigators (Caicedo and Herbert, 1993;

González-Hernández et al., 1996; Ito et al., 1996; Kelly, et al., 1998; More and More, 1987; Saint Marie, 1996; van Adel et al., 1998; Wynne et al., 1995; Zhang et al., 1998) have distinguished the dorsal part of the VNLL as a separate nucleus, the intermediate nucleus of the lateral lemniscus (INLL). Furthermore, this controversy is not restricted to different investigators, because different studies by the same laboratory have at different times reported the lateral lemniscus as having two subdivisions, DNLL and VNLL (Friauf, 1992) or three subdivisions, DNLL, INLL, and VNLL (Lohmann and Friauf, 1996; Piechotta and Friauf, 1998). Thus, it appears that the controversy surrounding the anatomical organization of the lateral lemniscus has resulted from individual investigators conveniently subdividing those neurons ventral to the DNLL into one nucleus (VNLL) or two distinct nuclei (VNLL and INLL) based on specific results from a particular study.

Neurochemical Characteristics of the nLL

Ultimately, one should be able to address the question of the function(s) of the nLL by knowing the specific neurotransmitters, receptors, and neuropeptides used by its cells. Several recent studies of the neurotransmitter systems of the nLL have demonstrated neurochemical evidence for the existence of three distinct nuclei based on the distribution and content of γ -gamma-aminobutyric acid (GABA) and/or glycine along the dorsoventral extent of the lateral lemniscus. Riquelme et al. (1998), using both immunohistochemistry and in situ hybridization histochemistry, showed that the rat's DNLL is the only homologous nucleus within the LL. All DNLL neurons contained strong immunoreactivity for GABA and not glycine, and additionally a strong mRNA signal for GAD-65 (glutamic acid decarboxylase, a GABA synthesizing enzyme found in neurons) was detected. In contrast, lemniscal neurons ventral to DNLL displayed a heterogeneous mixture of excitatory and inhibitory neurotransmitters. The dorsal portion of the VNLL (which I will refer to as the INLL) contained a large percentage of neurons immunonegative for both GABA and glycine, whereas the majority of neurons in the ventral portion of the VNLL

contain both GABA and glycine immunoreactivities and a strong mRNA signal for GAD-65. A similar result was demonstrated in the adult rat by Oliver and Bishop (1998) using a GABA and glycine double-labeling immunofluorescence procedure. These two studies have provided neurochemical evidence that the rat's VNLL is not a homologous structure, however, Riquelme *et al.* (1998) have incorporated both areas in their definition of VNLL.

In contrast to Riquelme *et al.* (1998) Oliver and Bishop (1998) have argued that there is a fundamental distinction between INLL and VNLL in the rat and consider the rat's LL to consist of a DNLL, an INLL, and a VNLL. Using *in situ* hybridization histochemistry, Wynne *et al.* (1995) examined the distribution of GABA_Aα1 receptors and GAD in the auditory brainstem of the adult rat. Although they only identified DNLL and VNLL as lemniscal nuclei, they showed that almost all DNLL neurons contained GABA_Aα1 mRNA, while in VNLL very few neurons were labeled for this inhibitory receptor. The distribution of GAD mRNA was denser in DNLL than the VNLL. As with GABA immunolabeling studies of VNLL, Wynne *et al.* (1995) showed that only neurons in the ventral division of the VNLL were labeled for GAD mRNA and no neurons were labeled in the dorsal division of the VNLL. Additional support for a distinction comes from, Fubara *et al.* (1996) who used receptor autoradiographic ligand binding to compare the distribution of GABA_A, GABA_B, and glycine receptors in the auditory brainstem of the big brown bat (*Eptesicus fuscus*), which unlike the rat has large and highly developed lemniscal nuclei. Fubara *et al.* (1996) demonstrated that the DNLL contains a mixture of GABA_A, and glycine receptors, whereas the INLL and VNLL contain mostly glycine receptors. Very sparse GABA_B receptor binding was observed in each lemniscal nucleus. These results are in agreement with previous neurochemistry studies that have demonstrated that the vast majority of DNLL neurons are GABAergic only and not glycinergic (Adams and Mugaini, 1984; Aoki *et al.*, 1988; González-Hernández *et al.*, 1996; Moore and Moore, 1987; Roberts and Ribak, 1987; Shneiderman *et al.*, 1993; Thompson *et al.*, 1985; Vater *et al.*, 1992; Winer *et al.*, 1995; Zhang *et al.*, 1998), whereas

structures ventral to the DNLL contain a mixture of inhibitory and excitatory neurotransmitters (Saint Marie, 1993, 1996; Roberts and Ribak, 1987; Saint Marie and Baker, 1990).

In addition to the distribution of neurotransmitters and receptors, two separate studies by Friauf, (1994) and Lohmann and Friauf (1996) analyzed the spatiotemporal distribution of calcium-binding proteins, (parvalbumin, calretinin, and calbindin) in developing and adult rats using immunohistochemistry. These studies showed that parvalbumin was an excellent marker to distinguish DNLL from INLL and INLL from VNLL. Calbindin only labeled DNLL in the adult rat and calretinin was absent in the LL of adult rats. At postnatal days 8 and 12, however, calretinin was seen in INLL and VNLL but not DNLL.

In summary, despite all the controversy over the actual number of LL subdivisions, the results from many neurochemistry studies have clearly demonstrated a neurochemical difference of the major subdivisions of the rat's LL. In addition to cytoarchitectonic criteria from Nissl-preparations, groups of neurons positioned dorsoventrally along the LL can be differentiated from one another based on the localization and expression levels of specific neurotransmitters, receptors, and neuropeptides. Simply identifying specific neurotransmitter systems is essential for understanding the physiological function(s) of each lemniscal nucleus. In addition, tracing the efferent projections of the nLL will further increase our understanding of how lemniscal nuclei and their different neurotransmitter systems shape acoustical responses in their postsynaptic targets.

Efferent Projections of the nLL.

In addition to the neurochemical studies described above, several studies have compared the efferent projections of the DNLL, INLL and VNLL using modern neuroanatomical techniques. In the rat, the DNLL sends projections to the ipsilateral ICC as well as projections to the contralateral DNLL and ICC through the commissure of Probst

(Bajo et al., 1993; González-Hernández et al., 1996; Ito et al., 1996; Kelly et al., 1998; Labelle and Kelly, 1996; Merchán et al., 1994; Tanaka et al., 1985; van Adel et al., 1996, 1997; van Adel and Kelly, 1998). Previous studies in rats have demonstrated that the ipsilateral and contralateral projections from the DNLL to the IC arise from separate populations of neurons in DNLL and only a small percentage of neurons give rise to projections to both sides of the brain (Ito et al., 1996; Merchán et al., 1994; Tanaka et al., 1985; van Adel et al., 1997). Cell counting data indicates that contralaterally projecting DNLL neurons in the rat account for approximately 70-75% of the cells projecting to IC via the CP (González-Hernández et al., 1996; Ito et al., 1996; Tanaka et al., 1985) and a slightly lower percentage of 60% was reported for the cat (Hutson et al., 1991). Transection of the rat's commissure of Probst and the behavioural and electrophysiological responses to such axotomy were first described by Ito et al. (1996) and van Adel et al. (1997). Using a stereotaxic knifecut, fluorescent retrograde tracers, and Nissl-staining they observed that after CP transection marked cell loss is apparent in the DNLL on both sides of the brain but many neurons remain intact due to their uncrossed connections to the ipsilateral ICC (Ito et al. 1997; van Adel et al. 1997). In addition to showing both the behavioural and physiological importance of this pathway for binaural processing and sound localization, anatomical data from these studies showed that the commissure of Probst is an obligatory pathway for contralaterally projecting DNLL neurons.

Anterograde tract tracing studies have been employed to characterize the pathway of fibers decussating through the commissure of Probst and to study the morphology of anterogradely labeled axonal endings and their arrangement on postsynaptic targets (Bajo et al., 1993; Labelle and Kelly, 1997; Oliver and Shneiderman, 1989; Shneiderman and Oliver, 1989). In a compelling light microscopic analysis of DNLL efferents in the rat, Bajo et al. (1993) identified three distinct fiber bundles leaving the DNLL medially, ventrally and dorsally. In particular, fibers running medially along the commissure of Probst turned slightly rostrally as they crossed the midline and then turned back caudally to

enter the DNLL where many gave rise to axon collaterals before turning dorsally to terminate in the ICC. Ultrastructural analysis in cats (Shneiderman and Oliver, 1989; Oliver and Shneiderman, 1989) and in rats (Labelle and Kelly, 1997) have shown that most (>90%) of the crossed projections to the opposite DNLL and ICC contain pleomorphic synaptic vesicles and form symmetric synaptic contacts suggestive of inhibitory transmission. In contrast, DNLL projections to the ipsilateral ICC were found to contain two types of axonal endings, those with pleomorphic synaptic vesicles forming symmetrical contacts (60-80%) and those with round synaptic vesicles forming asymmetrical synapses (20-40%), the latter being associated with excitatory transmission.

Anterograde and retrograde tract tracing studies have shown that in the rat both INLL and VNLL project heavily to the ipsilateral ICC, which contrasts sharply with the bilateral connections of the DNLL (Beyerl, 1978; Ito et al., 1996; Kelly et al., 1998; Merchán and Berbel, 1996; Tanaka et al., 1985). Ipsilateral projections from INLL and VNLL to the ICC have been described for other mammalian species including the cat and the bat (Adams, 1979; Brunso-Bechtold et al., 1981; Covey and Casseday, 1991; Goldberg and Moore, 1967; Glendenning et al., 1981; Hutson et al., 1991; Kudo, 1981; Nordeen et al., 1983; Roth et al., 1978; Saint Marie and Baker, 1990; Shneiderman et al., 1988; Whitley and Henkel, 1984; Willard and Martin, 1983; Zook and Casseday, 1979, 1982). In the cat, the INLL apparently has a contralateral as well as an ipsilateral projection to ICC whereas the VNLL does not (Brunso-Bechtold et al., 1981; Glendenning et al., 1981).

Several anatomical studies have confirmed these efferent projections as well as the specific transmitter systems of the nLL using either a single technique in which tritiated neurotransmitters ($[^3\text{H}]\text{GABA}$: Hutson, 1988; $[^3\text{H}]\text{glycine}$: Saint Marie and Baker, 1990; $[^3\text{H}]\text{aspartate}$: Saint Marie, 1996) are injected into the ICC and detected autoradiographically, or by combining retrograde tract tracing with immunohistochemical staining (González-Hernández et al., 1996; Zhang et al., 1998). First, Hutson (1988)

showed that DNLL neurons were bilaterally labeled and VNLL neurons were ipsilaterally labeled after [^3H]GABA injections into ICC. Second, Saint Marie and Baker (1990) used [^3H]glycine to selectively label glycinergic cells in the auditory brainstem and demonstrated that 68% of retrogradely labeled neurons were located in the ipsilateral VNLL, whereas no [^3H]glycine retrogradely labeled neurons were seen in the ipsilateral or contralateral DNLL. Additionally, Saint Marie (1996) used [^3H]aspartate to identify potential glutamatergic connections of the chinchilla ICC and found very few retrogradely labeled neurons in the nLL with the exception of a small cluster of neurons near the boundary of INLL and VNLL which would presumably excite their postsynaptic targets. Finally, studies by González-Hernández et al. (1996) and Zhang et al. (1998) showed GABA immunoreactivity in the same neurons retrogradely labeled with tracer bilaterally in the DNLL, and in the ipsilateral VNLL. According to González-Hernández et al. (1996) the VNLL is the largest source of GABAergic input to the ICC. All of these studies confirm earlier immunohistochemistry studies which showed that the vast majority of DNLL neurons are GABAergic (Adams and Mugaini, 1984; Moore and Moore, 1987; Roberts and Ribak, 1987; Shneiderman et al., 1993), and that neurons ventral to DNLL displayed a heterogeneous mixture of excitatory and inhibitory neurotransmitters (Oliver and Bishop, 1998; Riquelme et al., 1998; Saint Marie, 1993, 1996; Saint Marie and Baker, 1990; Wynne et al., 1995). Furthermore, the [^3H]GABA transport study by Hutson (1988) supports ultrastructural studies by Labelle and Kelly (1997), Oliver and Shneiderman (1989), and Shneiderman and Oliver (1989), which showed that the majority of axonal endings originating from the contralateral DNLL, and to a lesser extent, from the ipsilateral DNLL, are morphologically linked to inhibitory synapses. These observations, and the fact that few nLL neurons have excitatory characteristics suggest that the nLL must play a significant role in inhibitory processes in the ICC (which will be discussed later). However, insight into the functional significance of these inhibitory projections to the ICC can be gained by first examining the sources and organization of inputs to each subdivision of the nuclei of the lateral lemniscus.

Afferent Projections to the nLL

Despite the abundant studies of the efferent projections of the nLL, only a few studies have examined the afferent inputs to the nLL from lower auditory brainstem structures (Glendenning *et al.* 1981; Huffman and Covey, 1995; Kudo, 1981; Labelle and Kelly, 1996; Schofield and Cant, 1997; Shneiderman *et al.*, 1988; Yang *et al.*, 1996). Glendenning *et al.* (1981) used cytoarchitectonic criteria to distinguish three subdivisions (DNLL, VNLL and INLL) within the cat's LL, and using both anterograde and retrograde tract tracing methods demonstrated differential projections to each subdivision based on the patterns of labeling. Following local injections of HRP into DNLL of the cat, retrogradely labeled neurons were seen in the contralateral DNLL, the ipsilateral INLL, and VNLL, both ipsilateral (LSO and MSO) and contralateral (LSO) SOC, and the contralateral cochlear nucleus (CN). Injections of HRP into the INLL or VNLL retrogradely labeled only certain subdivisions of the ipsilateral SOC (primarily the medial nucleus of the trapezoid body, MNTB) and the contralateral CN. The ascending projections to the INLL and VNLL contrast sharply with the ascending projections to the DNLL, which in many ways parallel the projections that ascend to the cat's ICC (Glendenning *et al.* 1981; Shneiderman *et al.*, 1988). Thus, it appears on the basis of differential ascending inputs to the cat's LL, that three distinct subdivisions exist, namely the DNLL, INLL and VNLL, respectively. Huffman and Covey (1995) showed very similar inputs to the nLL of the big brown bat (*Eptesicus fuscus*) with the exception of a bilateral projection from MSO to the DNLL which is not common to most mammalian species (see Willard and Martin, 1985). Yang *et al.* (1996) identified an anterior and posterior subdivision of the mustache bat's DNLL and showed that each subdivision receives differential inputs from neuronal subpopulations of lower auditory brainstem nuclei, the same auditory structures that project to the cat's DNLL. With regard to the rat, only the afferent projections to the DNLL have been investigated (Bajo *et al.*, 1993; Labelle and Kelly, 1996) and appear identical to the afferent inputs to the cat's DNLL. The rat's DNLL receives most of its afferent inputs from the

contralateral CN, from both ipsi- and contralateral LSO, and from the ipsilateral MSO, SPN, VNLL and INLL, as well as a heavy projection from the contralateral DNLL, and like the cat parallels the afferent projections to the ICC. Indeed, several studies have determined that the bulk of ascending afferents innervate the DNLL via axon collaterals before terminating in the ICC (Bajo et al 1993; Henkel, 1997; Iwahori, 1986; Merchán et al., 1994; Zhao and Wu, 1998). However, so far there have been no comprehensive tract tracing experiments to demonstrate differential afferent projections to the INLL and VNLL in the rat nor to determine the extent of lower auditory brainstem nuclei participating in collateral innervation of the DNLL and ICC. Further studies of the afferent inputs to the mammalian nLL may provide insight into the physiological contribution of each lemniscal subdivision in processing acoustical information.

Neurophysiological Properties of the nLL

Neurophysiological studies of the nLL have demonstrated a contrast between the binaural sensitivity of DNLL neuronal response patterns, and the monaural sensitivity of responses recorded from INLL and VNLL neurons. This is consistent with what would be expected based on the anatomical data of the afferent inputs to the nLL.

The majority of DNLL neurons (over 70%) are excited by sounds from the contralateral ear and inhibited by sounds from the ipsilateral ear (EO/I cells), while a few are excited by stimulation of either ear (EE cells, 10%) (Aitkin et al., 1970; Buckthought, 1993; Covey and Casseday, 1991). A small percentage of DNLL neurons (10-20%) are monaural and are excited by stimulation of the contralateral ear only (EO/O cells). In contrast, the majority of INLL (over 60%) and VNLL (greater than 90%) neurons are functionally monaural (EO/O cells), responding only to contralateral sounds (Aitkin et al., 1970; Covey and Casseday, 1991). Recent evidence suggests that at least a few neurons in the INLL and the medial part of the VNLL are specialized for binaural processing (Batra and Fitzpatrick, 1997; Buckthought, 1993). Based on response properties, each cell group

within the LL is clearly differentiated from the other based on the ratio of binaurally sensitive neurons to monaurally sensitive neurons. Indeed, electrophysiological properties of the nLL are consistent with neuroanatomical investigations of the afferent inputs to the nuclei of the lateral lemniscus in mammals. In particular, the DNLL possesses mainly binaural response properties and receives heavy projections from binaural nuclei in the SOC (e.g., LSO and MSO), whereas the INLL and VNLL display primarily monaural response properties and receive direct projections from the contralateral cochlear nucleus and very little projections from binaural nuclei in the SOC.

Purpose of this Study

The following series of experiments investigate the neuroanatomical organization of the nuclei of the lateral lemniscus in the normal adult Wistar rat (*Rattus norvegicus*). Particular emphasis was placed on the commissural and non-commissural projections, as a prerequisite to the subsequent experiment which was conducted to determine the anatomical consequences of transecting commissural projections of the lateral lemniscus.

In Experiment 1, conventional neuroanatomical tract tracing techniques were employed to determine the afferent and efferent projections of the normal rat's nLL. The experimental strategy was based on the hypothesis that the auditory lower brainstem nuclei could be recognized by comparing the patterns of efferent and afferent labeling after injections of anterograde and retrograde tracers into stereotaxically-defined regions of the nLL and ICC. In other words, to identify differences within the lemniscal nuclei of the rat on the basis of afferent and efferent innervation patterns at various dorsoventral levels of the lateral lemniscus. Of particular interest was the crossed efferent projections of the DNLL to the opposite DNLL and ICC, via the commissure of Probst. Cell counting data was used to determine the percentage of DNLL neurons projecting ipsilaterally and contralaterally to the ICC in normal animals.

In Experiment 2, the same anterograde and retrograde tract tracing procedures were used to investigate the anatomical consequences of surgically transecting the commissure of Probst in adult rats. The goal was to provide a detailed qualitative and quantitative description of axotomy-induced cell death in the DNLL. Based on our recent finding that CP transection results in DNLL neuronal loss, Experiment 2 tested the suitability of the CP transection as an experimental paradigm to identify mechanisms which lead to neurodegeneration following traumatic brain injury.

Chapter 2

Neuroanatomical investigation of the nuclei of the lateral
lemniscus: A search for subdivisions and commissural
projections using anterograde and retrograde tract tracing
techniques in the adult rat, *Rattus norvegicus*

Experiment 1

EXPERIMENT 1

In contrast to the large amount of anatomical data accumulated on ascending projections to the SOC and the ICC, only a few studies have investigated the ascending projections to each lemniscal nuclei in cat and bat (Glendenning et al., 1981; Huffman and Covey, 1995; Kudo, 1981). Similarly, only a few studies have investigated the afferent projections to the DNLL in the adult rat (Bajo et al., 1993; Labelle and Kelly, 1996). Of the projections that have been shown to terminate in the lemniscal nuclei, it remains to be determined if innervation of the nLL from midbrain and hindbrain auditory structures arises from collaterals of fibers proceeding to the inferior colliculus. Information concerning this innervation, will further elucidate the anatomical organization of one of the least known pathways in the central auditory system. As well, such extensive normative neuroanatomical data can be used for comparison to changes resulting from injury to these pathways.

Purpose of Experiment 1

The following series of experiments investigates the neuroanatomical organization and commissural and non-commissural projections of the nuclei of the lateral lemniscus in the normal adult Wistar rat (*Rattus norvegicus*).

MATERIALS AND METHODS

Subjects

Seventy naive, male Wistar rats were obtained from Charles River Canada (St. Constant, Quebec) at four weeks of age (150 g). Animals were acclimatized to the animal facility for approximately 2 weeks prior to surgical procedures. The animals were housed individually in shoe box cages in the Carleton University Life Science Building vivarium. They were maintained in good health under the care of a veterinarian for the duration of the experiment. All rats were maintained on a 12 hour light-dark cycle (lights on at 7:00 a.m.) and both food and water were available *ad libitum*. Experimental animals were divided into four groups depending on the location(s) and type of anatomical tracer(s) injected. Table 2.1 and Figure 2.1 provide a summary of the number and surgical treatment of the four groups of animals used in Experiment 1.

Rationale for Tracer Selection

Several classes of anterograde and retrograde axonal tracers have been developed and used to determine the connectivity of specific brain regions. In both this study (Experiment 1) and the following study (Experiment 2) several different anatomical tracers were selected based on their individual physico-chemical properties, uptake and transport mechanisms, and non-cytotoxic storage within tracer-filled nerve cells. Brief descriptions of the anterograde and retrograde tracers selected for use in this study (as well as Experiment 2) are provided below.

Anterograde tract tracing

The anterograde tracer Biotinylated Dextran (BD, Molecular Probes, USA,) was chosen because of several distinct properties which support its use for axonal tracing (Rajakumar et al., 1993). BD can be injected using pressure or iontophoretic techniques, and provides quick anterograde (a small percentage is transported retrogradely) labeling

within 1 day and remains unchanged two weeks after the injection. BD is easily visualized using various avidin-conjugated markers and can be combined with other morphological methods for light and electron microscopic study. Furthermore, unlike other anterograde tracers, BD shows excellent anterograde labeling in both young and aged animals and more importantly, Rajakumar et al., (1993) demonstrated that when BD is delivered slowly and with minimal tissue damage, the uptake and subsequent axonal transport of BD by undamaged fibers-of-passage through the injection site is negligible. Thus, BD-labeled fibers result from only those neuronal cell bodies in the vicinity of the injection site that have sequestered BD (via endocytosis) and transported the tracer anterogradely to label specific efferent pathways in the CNS.

Retrograde tract tracing

Retrograde transport studies were carried out using three different fluorescent compounds: Fast-Blue (FB, Sigma Biochemicals, Canada), Fluoro-Gold (FG, 2-hydroxy-4,4'-diamidinostilbene, 204 M_w , Fluorochrome, Inc., USA), and Fluoro-Ruby (FR, tetramethyl rhodamine-dextran-amine, 10,000 M_w , Molecular Probes, USA), and one non-fluorescent compound, horseradish peroxidase (HRP, 40,000 M_w , Sigma Biochemicals, Canada). The use of fluorescent axonal tracers is becoming more and more popular for a number of reasons including high sensitivity, simplicity, and compatibility with other techniques. FG is a fluorescent stilbene derivative (2-hydroxy-4,4'-diamidino-*trans*-stilbene), which consists of two benzene rings connected to a vinyl linkage. The alternating single and double bond configuration of the FG molecule results in a resonant structure where π -orbital clouds contain the outer most electrons. Exposure to ultraviolet (UV) light excites these outer electrons to higher energy levels, and when returned to a lower energy level, light is emitted which can be visualized by means of fluorescence microscopy.

FG is commonly used to demonstrate long-term retrograde labeling without degradation or exocytosis. It is resistant to fading (because of the stability of the trans configuration of the molecule), it is easily released by iontophoresis, and more importantly it is not taken up by undamaged fibers-of-passage (Divac and Jesper, 1990; Schmued, 1990). In addition, our laboratory has previously used FG as a retrograde tracer to study tonotopic projections to the ICC (Kelly et al., 1998) and to characterize lesions in the auditory brainstem (Ito et al., 1996; Kelly et al., 1996; van Adel et al. 1997; van Adel and Kelly, 1998). Two other fluorescent tracers, FB and FR, were used in combination with FG to identify collateral projecting auditory neurons and to differentiate between two populations of neurons in the DNLL. Retrograde tracing studies with HRP, unlike fluorescent neuronal tracers require extensive histochemical processing, but have the advantage of easy combination with other morphological methods for light microscopic study. However, HRP is lysosomally degraded within 72 hours after uptake (Kristensson and Olson, 1976), and because of the long postlesion survival times required in Experiment 2 (see Table 2.1), this tracer was not suitable for cases that required cell counting of long-term (> 2 weeks) retrogradely labeled auditory neurons. Instead, FG was selected because of its superiority over all other commercially available retrograde tracers. Thus, in order to standardize cell counting procedures as described below (under histology), only cases with unilateral injections of FG into the ICC were used in Experiment 1 and Experiment 2.

Surgical Procedures

Tracer injections

The animals (200-250 g) were deeply anesthetized with Somnotol (sodium pentobarbital, 60 mg/kg ip) and prepared for tracer injection(s). Animals were placed in a stereotaxic headholder, and a midline incision was made in the scalp, the skin retracted laterally and a craniotomy was performed. The location of ICC, DNLL, INLL, and VNLL injection sites were determined stereotaxically using Paxinos and Watson's (1987)

stereotaxic atlas of the rat brain. For injections made into the central nucleus of the inferior colliculus (ICC), the injection pipette was positioned 2.0 mm lateral to lambda, 0.4-0.5 mm rostral to lambda, and then lowered vertically (90°) into the right ICC a total distance of 3.5 mm from the surface of the brain. For placement of injection pipettes in the DNLL, INLL, or VNLL the pipette was tilted 30° against the sagittal plane and lowered into the brain from a point 6.7-7.5 mm lateral and 0.4-0.5 mm rostral to lambda. Upon completion of tracer(s) injection(s) incisions were cleaned and sutured, and animals return to the vivarium.

Anterograde tract-tracing procedures, Group 1

In six animals BD was injected in the right or left DNLL; and in three animals BD was injected in the left ICC. BD was iontophoretically injected (alternating 6.0-8.0-mm DC positive current, 7 seconds on/7 seconds off) into the ICC or DNLL through a stereotaxically positioned glass micropipette (inner tip diameter 30-35 mm) for a total of 15-20 minutes. The pipette was left in place for 5 minutes without current to prevent leakage of the tracer along the injection tract. Confined injections were made to ensure a small distribution of the tracer throughout the rostral-caudal extent of the nucleus which would result in specific labeling of mainly efferent pathways.

Retrograde tract-tracing procedures, Groups 2, 3, & 4

Fifteen animals received unilateral injections of 2% FG into the central nucleus of the inferior colliculus (ICC). Five animals received a unilateral injection of 10% FR in the ICC, and in three others 20% HRP was injected unilaterally in the ICC. In ten animals, different fluorochromes (FG and FB, or FR) were injected into different locations of the ICC either unilaterally or bilaterally in the same animal. In ten animals multiple tracers (i.e., FG, and 3% FB, or FR) were injected into the ICC and DNLL on the same side of the brain, ipsilateral ICC, and ipsilateral DNLL. Six animals received unilateral injections of FG in the left or right DNLL; three additional animals received unilateral DNLL

injections of FR. In three other animals, FG was injected into the INLL, and in three others FG was injected into the VNLL. Under stereotaxic guidance, FG, FB, FR, and HRP were iontophoretically delivered through a glass pipette. FG and HRP injections were made by passing a continuous positive 5.0-7.5 μ A DC current through the pipette for a period of 10-15 minutes, whereas FR and FB injections required passing an alternating (7 seconds on/7 seconds off) 6.0-8.0- μ A DC positive current through the pipette for 30 minutes. For each tracer injection, the pipette was left in place for 5 minutes without current to prevent leakage of the tracer along the injection tract. Cases used to establish the ratio of ipsilateral/contralateral projecting DNLL neurons received large unilateral injections into the ICC to heavily label both ipsilateral and contralateral DNLL neurons. Injections into the nLL were considerably smaller to prevent leakage of tracer into neighboring structures.

Histology

Perfusion

Following 5-7 days after BD injections, 2-3 days after HRP injections and 1-12 weeks after injection(s) of fluorescent tracers (FG, FB, and FR), animals were re-anesthetized with an overdose of Somnotol (120 mg/kg). They were perfused transcardially with phosphate buffered saline (100 mM PBS, pH 7.4) followed by a fixative consisting of 4% paraformaldehyde and 0.5% gluteraldehyde, in 100 mM phosphate buffer (PB; pH 7.4) for BD animals or 4% paraformaldehyde HRP, FB, FG, and FR animals. The brains were removed, postfixed for 4 hr at 4°C in the same fixative and cryoprotected overnight in 25 % buffered sucrose and then cut serially at 40 μ m in the frontal plane on a freezing microtome. One series was mounted on gelatinized slides, air dried overnight, and stained with cresyl violet and coverslipped with Permount.

BD histochemistry

BD labeled neurons, fibers and terminal fields were visualized using a variation of the methods described by Wu and Kelly (1995). Briefly, free floating sections were rinsed 3 times (15 min./rinse) in PBS (100 mM, pH 7.4), then incubated in 0.5% H₂O₂ for 20 minutes followed by a 0.5% Triton-X 100 (in 100 mM PBS, pH 7.4) solution. The sections were incubated overnight with avidin conjugated horseradish peroxidase solution from a standard Vectastain ABC immunolabeling kit (Vector Laboratories, PK-4000, USA). Sections were then rinsed 3 times (15 min./rinse) in 100 mM PB (pH 7.4) before peroxidase reaction with diaminobenzidine (DAB, Sigma Chemicals, USA.) as a chromagen followed by nickel-cobalt intensification (Adams 1981). Sections were mounted on gelatinized slides, air dried overnight, alternating slides were counterstained with neutral red or cresyl violet, dehydrated in a graded series of alcohols and coverslipped with Permount.

HRP histochemistry

To demonstrate the distribution of HRP, sections were treated with tetramethylbenzidine (TMB) as a chromagen and processed with a 5% aqueous solution of ammonium molybdate for 15 minutes to stabilize the reaction product as described by Kelly *et al* (1998). Sections were then mounted onto gelatinized slides, air dried, cleared in alcohols and coverslipped with Permount for observation and photography of retrograde labeling. Some sections were stained with cresyl violet rather than with neutral red to facilitate black and white photography.

FB, FG, and FR processing

Fluorescent labeled sections required no further histochemical processing after sectioning and thus could be mounted directly onto gelatinized slides, air dried overnight, then cleared in a graded series of alcohols and coverslipped with one of two types of

fluorescent mounting media: DPX (British Drug House, BDH, London, England) or Vectashield (Vector Laboratories, USA). The results with the DPX were superior in that very faintly labeled neurons were clearly identifiable for all three fluorochromes. Furthermore, DPX reduced the pericellular fluorescent "bloom" prominent in many auditory brainstem nuclei containing clusters of tightly packed FB, FG, or FR labeled neurons and provided excellent differentiation of dendrites and soma. For example, FG labeled neurons appeared a brilliant white on a dark bluish-black background with the DPX mountant, whereas in the Vectashield-mounted sections FG-labeled neurons appeared blue on a faint blue background. Therefore, in cases with single FG injections into ICC used for cell counting data (as described below under cell counting), and cases with FG and FB or FG and FR used in combination, DPX was the mounting medium of choice.

Data analysis

Paxinos and Watson's (1986) stereotaxic atlas of the rat was used to identify brainstem auditory nuclei. Slides were examined for retrograde transport of FG, FR, FB, and HRP or anterograde transport of BD using a Zeiss Axioplan light and epifluorescent microscope equipped with a drawing tube attachment and a photomicrographic camera. Fluorescent labeled sections were observed with filter systems U and G and photographed for further analysis. FG and FB were viewed with a filter having an excitation frequency of 340-380 nm and a barrier filter frequency of 430 nm, whereas FR was visualized using a filter system with an excitation frequency of 530-560 nm and a barrier filter of 580 nm. Photomicrographs were taken using several different colour films (Kodak Elite 400, Ektachrome or Fuji 400 tungsten sensitive slide film) and two different black and white films (Kodak TMAX 100 or Ilford Delta 400) using varying magnifications (25-400 X) and exposure times (0.01-60.0 seconds).

Cell counting

As described below the criteria for counting ipsilaterally and contralaterally projecting neurons in the DNLL were carefully considered to provide a count of the two populations. In addition, only those cases with unilateral injections of FG into the central nucleus of the inferior colliculus were considered for counting data, and not animals with FG injections located in the external or dorsal cortices of the IC (ECIC and DCIC respectively). Although the populations of FG-labeled neurons in other central auditory structures were not counted in this study, they served as a reference as to the similarity of FG retrograde labeling between the cases and to rule out differences between the cases due to factors other than the locus of the injection site. Specifically, the pattern of FG-labeling in the contralateral ICC, and the ipsilateral and contralateral SOC and CN served as internal controls to establish counting criteria for this experiment (and Experiment 2).

The total number of DNLL neurons ipsilateral and contralateral to the ICC FG-injection site were counted from every fourth section (i.e., counts were made 160 μm apart). Since the majority of DNLL neurons are between 15–40 μm in diameter in the rat (Bajo et al., 1993) it is improbable that neurons were counted twice with this methodology. FG labeled neurons were recognized by the appearance of fluorescence in the perinuclear cytoplasm and proximal dendrites of FG positive cells. It was often not possible to discern nuclei in fluorescently-labeled neurons, unless the neuron was sectioned through the nucleus, which would appear as a blank area located centrally in the FG-labeled neuron. Only the profiles of DNLL neurons that included a filled soma greater than 15 μm in diameter (or where the nucleus was visible) were counted; those profiles containing fragments of a soma or proximal dendrites were not counted. Given these stringent criteria, the cell counts present may be a relatively conservative estimate.

Statistical Analysis

Statistical analysis of the results was performed using either Statview 512 (Brain Power Inc., Calabasas, CA) software for MacIntosh computers or SPSS for windows for IBM computers. Cell counts of the number of ipsilaterally and contralaterally FG-labeled DNLL neurons were entered into a computer spreadsheet. This data was analyzed using Student t-tests, with a p value <0.05 considered significant. This would indicate a significant lateralized difference in the number of FG-labeled DNLL neurons following unilateral injections into the ICC in normal animals.

RESULTS

The results of Experiment 1 have been divided into three parts. In the first part the cytoarchitectonic features of the nuclei of the lateral lemniscus are briefly described. In the second part, evidence from anterograde tract tracing experiments is presented. In the final section data from retrograde tract tracing experiments are presented to confirm the prior anterograde experiments, to add additional details about commissural and non-commissural projections of the nLL, and to differentiate between closely juxtaposed subdivisions of the lateral lemniscus. It should be pointed out that no attempt was made to describe the tonotopic (frequency) organization of the efferent and afferent projections of the lateral lemniscus based on anterograde and retrograde tract tracing data, as the issue of tonotopicity was not a goal of this experiment. Several previous studies have addressed this issue in the rat's ICC and nLL by microelectrode mapping techniques (Buckthout, 1993; Clopton and Winfield, 1973; Flammino and Clopton, 1975; Kelly et al., 1991; Preuß, 1991), using physiologically-defined retrograde tracer injections (Kelly et al., 1998), by stereotaxically-defined tracer injections (Merchán et al., 1993, Merchán and Berbel, 1996; Saldaña and Merchán, 1992), and using both c-fos and 2-deoxyglucose mapping (Coleman et al., 1982; Friauf, 1982; Huang and Fex, 1986).

Cytoarchitecture of the nLL

Since neuroanatomical data obtained from both anterograde and retrograde tract tracing experiments rest heavily on precise stereotaxic placements and the ability to accurately identify the subdivisions of the nLL in Nissl-stained materials, it is important to describe the cytoarchitectonic features of the nuclei of interest in this study. The cytoarchitectural boundaries and subdivisions of the rat's CP, LL, and ICC were first described by pioneering neuroanatomists (Held, 1891, 1893; Ramón y Cajal, 1899, 1904; Probst, 1902; Woollard and Harpman, 1940; Van Noort, 1969), and more recent descriptions in different mammalian species (Kudo, 1981; Morest and Oliver, 1984;

Tanaka et al., 1985; Iwahori, 1986; Shneiderman et al., 1991; Hutson et al., 1991; Saldaña and Merchán, 1992; Bajo et al., 1993; Merchán et al., 1994; Merchán and Berbel, 1996; Yang et al., 1996; Paxinos and Watson, 1997; Schofield and Cant, 1997) have been adopted for these experiments.

The location of IC, nLL and surrounding structures are shown in Figure 2.2A. The DNLL is a clearly defined, circular collection of nerve cells situated dorsally among the fibers of the lateral lemniscus and located ventrally from the IC. The area just ventral to the IC and dorsal of the DNLL is almost completely void of Nissl-stained materials and provides a useful reference to establish the ventral border of the IC and the dorsal border of the DNLL. This region of brainstem parenchyma dorsal to the DNLL belongs to the subcollicular tegmentum. The sagulum nucleus (Sag) bounds the DNLL laterally and the commissure of Probst (CP) enters and exits the DNLL medially and bifurcates the midline just ventral to the medial longitudinal fasciculus. The intermediate and ventral nuclei of the lateral lemniscus (INLL and VNLL) are located ventral to the DNLL and an area of acoustically responsive neurons, the paralemniscal zone (PL), is situated medial to the main lemniscal pathway, abutting chiefly the INLL and DNLL.

In Nissl-stained sections, DNLL neurons are easily distinguished from bordering nuclei within the LL by their large size and darkly Nissl-stained materials, whereas the nucleus sagulum, INLL, and VNLL contain relatively small and pale Nissl-stained neurons. In frontal sections like the example shown in Figure 2.1, many neurons in the DNLL appear elongated and horizontally oriented in a perpendicular fashion to the fibers of the LL. The border between the DNLL and more ventral nLL is distinguished by a narrow band of tightly grouped neurons referred to as the horizontal cell group of the lateral lemniscus, first described by Gómez (1988) further detailed by Bajo et al. (1993) and given their present name by Caicedo and Herbert (1993). The fibers of the commissure of Probst are easily identifiable by an absence of Nissl-stained materials but are seen as dense fiber bundles using a histological fiber stain.

-----Insert Figure 2.2 About Here-----

Anterograde Tract Tracing with Biotinylated Dextran, (BD), Group 1.

Both small and large iontophoretic injections of BD into the ICC or DNLL resulted in discrete, dense deposits of BD around the pipette tip, suggesting that the passive diffusion of this tracer within the neuropil was confined to a relatively small area (200-500 μm in diameter). BD was used as an anterograde tracer, however, a small percentage of BD was found to be transported retrogradely to neurons projecting to the injection site. Viewed with a light microscope, BD labeled neurons, fibers and terminals were identified as a black amorphous reaction product evenly distributed throughout axons and soma of labeled nerve cells.

ICC injections

Three animals received unilateral BD injections into either left or the right ICC. Figure 2.3 illustrates a representative case with a BD injection into the left ICC. From the ICC injection site, anterograde transport of BD was observed in many efferent axons leaving the ICC injection site via the following routes: medially along the commissure of the inferior colliculus (cic); laterally to the ipsilateral brachium of the inferior colliculus (BIC); and descending ventrolaterally through the ICC and into the ipsilateral LL. BD injections into the ICC resulted in intense retrograde labeling of neurons in both ICC and to a lesser extent in the ipsilateral and contralateral DNLL. Ascending fibers projecting medially through the cic could be followed as they crossed the midline to give rise to intense terminal labeling in the contralateral ICC. The ascending fibers projecting laterally to the ipsilateral BIC continued rostrally to form dense terminal fields in the ipsilateral medial geniculate body (MGB). BD-labeled fibers projecting ventrally from the injection site were observed to course through the nuclei of the lateral lemniscus to form small

clusters of terminal fields restricted to the Sagulum and DNLL - very few were seen in the INLL or VNLL. Only a few of these descending fibers turned medially to project through the commissure of Probst, and are likely the result of retrogradely transported BD to neurons of the contralateral DNLL. The numerous BD labeled fibers in the LL were seen traveling further ventrally, then entering the trapezoid body (TB) and into the ipsilateral SOC where terminal fields were seen in the superior paraolivary nucleus (SPN) and ventral nucleus of the trapezoid body (VNTB). At the level of the SOC some of these ventrally descending fibers from the ICC continued along the trapezoid body to cross the midline to form a small cluster of terminal varicosities in the contralateral dorsal cochlear nucleus (DCN).

---Insert Figure 2.3 About Here----

Injections of BD into the ICC demonstrate that the majority of efferent projections of the ICC are to the contralateral ICC, through the cic, and to the ipsilateral MGB, via the BIC. The efferent projections of the ICC that form the descending pathway are minor in comparison to those of the ascending pathway and more importantly very little of these descending efferents decussates across the midline at any level along the lateral lemniscus.

DNLL injections

Figure 2.4 illustrates the anterograde transport of BD in a normal animal following a single unilateral injection into the left DNLL. As mentioned above, BD injections resulted in intense anterograde labeling of efferent fibers and terminal varicosities as well as retrograde labeling of neurons. BD injections into the DNLL resulted in intense retrograde labeling of DNLL neurons at the injection site as well as the contralateral DNLL and to a lesser extent in ipsilateral and contralateral ICC. At the DNLL injection site, anterograde transport of BD was observed in many efferent axons leaving the DNLL injection site via three distinct directions (medial, dorsal, and ventral) and could be followed to their targeted

nuclei, where densely labeled terminal fields were observed. Fibers projecting medially through the commissure of Probst could be followed as they crossed the midline to terminate in the contralateral DNLL or continued dorsally to form large terminal fields in the ICC contralateral from the injection site. BD labeled fibers running dorsally from the DNLL injection site to the ipsilateral ICC formed much smaller terminal fields compared to those observed in the contralateral ICC. In addition, only one or two labeled fibers continued dorsally, then turning medially to cross the midline through the cic and terminate in the contralateral ICC. Descending fibers were observed to course ventrally along the nuclei of the lateral lemniscus to form terminal fields in the MSO and SPN of the ipsilateral SOC (data not shown).

---Insert Figure 2.4 About Here----

Figure 2.5 shows a series of photomicrographs from an animal with a restricted injection of BD into the right DNLL. The anatomical location and rostral-to-caudal extent of the commissure of Probst can easily be seen in these series of frontal sections. From caudal sections, heavily labeled fibers can be seen leaving the DNLL medially and running rostrally through the brachium conjunctivum (BC) and decussating across the midline ventral to the cerebral aqueduct (Aq, or 4th ventricle), central gray and the medial longitudinal fasciculus (MLF). Upon crossing the midline, labeled fibers turned back slightly caudally and ran laterally through the BC again to enter the contralateral DNLL. At more rostral levels labeled fibers traveled medially from the injection site through the CP and terminated in the contralateral DNLL and ICC. At the most rostral levels many fibers were found to project across the midline and terminate in the deep layers of the superior colliculus (SC).

---Insert Figure 2.5 About Here----

Injections of BD into the DNLL demonstrate that the majority of efferent projections of the DNLL are to the contralateral DNLL and ICC through the commissure of Probst. In the contralateral DNLL, BD-labeled varicosities were seen in close proximity to BD retrogradely labeled neurons, indicating a reciprocal circuitry between each DNLL. The efferent projections of DNLL to the ipsilateral ICC form a minor projection in comparison to fibers passing through the CP to the contralateral DNLL and ICC. Finally, BD was effective for mapping the rostral-to-caudal extent and path of DNLL efferent fibers projecting medially across the midline via the commissure of Probst, which was critical for establishment of surgical procedure for Experiment 2.

Experiments with Single Retrograde Tracer Injection, Group 2.

In this section, retrograde tract tracing experiments were used to map the ascending inputs from the nLL to ICC, and from lower auditory brainstem structures (SOC and CN), and to differentiate between closely juxtaposed subdivisions of the lateral lemniscus. In addition, retrograde labeling was examined in the contralateral lateral lemniscus to identify the various LL subdivisions that project to the contralateral LL or ICC. The pattern and the extent of retrograde labeling in the SOC proved to be the most useful lower auditory brainstem data to analyze and identify differential inputs to LL subdivisions.

ICC injections

The pattern of FG labeling after unilateral injection into the ICC of a normal rat is presented in Figure 2.6. After a large injection of FG into the left ICC, numerous positively labeled nerve cells were found in the contralateral ICC and in the ipsilateral and contralateral dorsal nucleus of the lateral lemniscus (DNLL). The number of FG labeled neurons was always higher in the contralateral DNLL than in the ipsilateral DNLL (this point is quantitatively described below). In the ipsilateral SOC heavy labeling was seen in each of the three main subdivisions: the lateral superior olive (LSO), medial superior olive

(MSO), and superior paraolivary nucleus (SPN). In the contralateral SOC, many neurons of the VNTB were labeled and the LSO was heavily labeled. Comparison of the extent of FG labeling in the ipsilateral and contralateral subdivisions of the SOC, confirmed that only the LSO projects bilaterally to the ICC. All three levels of the CN (AVCN, PVCN, and DCN) contralateral to the ICC injection site contained numerous FG labeled neurons whereas only a few labeled cells were seen in the ipsilateral CN.

----Insert Figure 2.6 About Here----

At low magnification, the pattern of FG labeling in the nLL ipsilateral and contralateral to the ICC injection site was found to be quite different throughout the rostrocaudal sequences of frontal sections as presented in Figures 2.7 and 2.8. As described above, FG labeled neurons were found bilaterally only in the DNLL, whereas in the INLL and VNLL, FG labeled neurons were found exclusively in these nuclei ipsilateral to the ICC injection (see Figure 2.7). Because no labeling was seen in the subcollicular tegmentum or in the horizontal cell group, retrograde labeling of the nLL after unilateral FG injections into ICC was effective for determining the rostral-to-caudal extent of retrograde labeling as well as a means of differentiating the DNLL from the INLL and the INLL from the VNLL. At caudal levels the DNLL was heavily labeled bilaterally and the INLL was labeled ipsilaterally to the ICC injection site. At this level the ventral division of the nLL (or VNLL) contains very few retrogradely labeled neurons, whereas at more rostral levels this structure is the most heavily labeled structure within the ipsilateral LL (see Figure 2.8). At rostral levels the ipsilateral DNLL was found to contain no labeling, yet the contralateral DNLL remained heavily labeled several sections beyond the ipsilateral DNLL.

From these results it is clear that of the three nLL subdivisions only the DNLL projects bilaterally to the ICC. The labeling seen in the contralateral DNLL is much greater than that observed in the DNLL ipsilateral to the injection site. The results also show that,

below the level of the DNLL, many labeled neurons are observed in the INLL and VNLL ipsilateral to the ICC injection site. Finally, based on retrograde transport studies of animals with unilateral FG injections into the ICC it was possible to identify three distinct subdivisions (i.e., DNLL, INLL and VNLL) within the lateral lemniscus.

----Insert Figure 2.7 and Figure 2.8 About Here---

Animals with FG injection positioned centrally within the ICC were used to establish the ratio of ipsilateral/contralateral projecting DNLL neurons. Cell counts data from 6 representative cases are summarized in Table 2.2. A t-test for related samples was performed to determine if there were differences in the number of ipsilaterally and contralaterally labeled neurons in the DNLL. A significant difference was found between the number of ipsilaterally labeled DNLL neurons and contralaterally labeled neurons ($t_{(6)} = 11.67$, $p < 0.0001$). Figure 2.9 shows that approximately 70% of DNLL neurons project contralaterally to the ICC, and only 30% of DNLL neurons project ipsilaterally to the ICC. Figure 2.10 shows, low and high power photomicrographs of the DNLL from one of the six representative cases used for cell counting. From this case it is clear that the contralateral DNLL contains more FG labeled neurons which form concentric ring-like structures, whereas smaller clusters of FG labeled neurons were found in the ipsilateral DNLL. Although the actual number of ipsilaterally and contralaterally labeled DNLL neurons varies from case to case, the percentage of neurons projecting to the ICC from the ipsilateral and contralateral DNLL does not vary, suggesting that the stereotaxic deposition of the tracer was similar for each case. The variation in neuronal counts between cases can be attributed to the size and amount of tracer injected into the ICC and not the locus of the injection. Furthermore, only cases with identical patterns of FG-labeling in lower auditory brainstem structures were considered for counting in this experiment.

----Insert Table 2.2, Figure 2.9 and 2.10 About Here----

Similar results were obtained using retrograde tracers other than FG, however, all seemed inferior to the quantity and quality of neuronal labeling observed when using FG. For example, Figure 2.11 illustrates the pattern of labeling after injection of FR into the ICC. At the ICC injection site many FR labeled fibers can be seen leaving the injection site medially and traveling through the cic and into the contralateral ICC where FR-labeled neurons, fibers and terminals were found, indicating that FR is transported both anterogradely and retrogradely. However, very few FR-labeled neurons were seen in the ipsilateral DNLL, INLL, VNLL, and SOC, or the contralateral DNLL, SOC and CN indicating the poor transport and labeling qualities of this tracer.

----Insert Figure 2.11 About Here----

DNLL injections

As Figure 2.12 shows, the pattern of FG retrograde labeling in animals with unilateral injections into the DNLL parallels the distribution of labeling in lower brainstem auditory structures after unilateral ICC injections. This animal received a left DNLL injection of FG, which labeled neurons in the ipsilateral ICC, and very few in the contralateral ICC. The most intense labeling was seen in the contralateral DNLL, where an FG labeled sphere of neurons is seen and is the only structure labeled in the contralateral nLL. Similar to ICC injections, FG injections into DNLL resulted in labeling in each of the three main subdivisions of the ipsilateral SOC: the lateral superior olive (LSO), medial superior olive (MSO), and superior paraolivary nucleus (SPN). In the contralateral SOC, many neurons of the VNTB were labeled and the LSO was heavily labeled. The pattern of labeling in the CN (AVCN, PVCN, and DCN) contralateral to the ICC injection site was similar to cases with unilateral ICC injection, however, fewer neurons were labeled.

----Insert Figure 2.12 About Here----

At lower magnifications, it is possible to present a complete view of FG labeling in the ipsilateral and contralateral LL, SOC and CN. Figure 2.13 shows low power photomicrographs of the pattern of retrograde labeling in the auditory brainstem after an FG injection into left DNLL. Just ventral from the DNLL injection site many ipsilaterally labeled neurons are found throughout the INLL and VNLL, whereas the DNLL is the only LL subdivision labeled in the contralateral LL. The pattern of labeling in contralateral DNLL forms a spherical structure with many labeled neurons throughout the rostral-to caudal extent of the nucleus. Cases with smaller and more restricted (no leakage into INLL) injections into the DNLL, resulted in labeled neurons forming concentric ring-like structures in the opposite DNLL, similar in appearance to the concentric pattern of FG labeling seen in DNLL after FG injections into the ICC. In addition, both the ipsilateral and contralateral SOC and CN are similar to the patterns of FG retrograde labeling seen in these structures after unilateral ICC injections.

----Insert Figure 2.13 About Here----

INLL injections

Several animals were intended INLL injections, but only a few cases had injections that did not spill over into the DNLL or VNLL. As Figure 2.14 shows, cases with injections restricted to INLL produced a unique pattern of labeling that was different for cases with DNLL injections (as presented above) or VNLL injections (see below). In cases with small amounts of FG leaking into DNLL proper, a few labeled cells were found in the contralateral DNLL, whereas in cases with restricted injections into INLL an absence of labeling in the contralateral DNLL was observed. In addition, FG labeled neurons were

observed in the ipsilateral ICC, whereas the contralateral ICC was totally devoid of any FG labeled neurons. A few cells were labeled in the contralateral lateral lemniscus forming a narrow band running parallel to LL fibers and positioned just medial to VNLL and contained within the medial edge of the paralemniscal zone. A few labeled cells were seen in the ventral division of ipsilateral VNLL. Indeed, the pattern of labeling in the SOC confirmed that these cases were unique and that the patterns of labeling were novel. FG labeled cells were only seen in the major subdivisions of the SOC ipsilateral to the injection site; the fact that no labeled cells were seen in the contralateral SOC clearly separates these cases from DNLL or ICC unilateral FG injection cases. Many labeled cells were seen in the ipsilateral LSO, MSO, SPN and the MNTB. Furthermore, the labeling in the ipsilateral MNTB is unique since this structure is not labeled with DNLL or ICC retrograde tracer injections. A few labeled cells were seen in the ipsilateral CN, whereas extensive labeling was seen in the contralateral anteroventral and posteroventral cochlear nucleus (AVCN, and PVCN) and to a lesser extent in the dorsal cochlear nucleus (DCN).

----Insert Figure 2.14 About Here----

VNLL injections

Stereotaxic placements in the VNLL proved more difficult than any other nucleus within the LL. Like the injections into DNLL and INLL, the approach can only be from a large angle to prevent extensive damage to fibers-of-passage through the LL en route to structures dorsal of the injection site, mainly the ICC. Thus a craniotomy was made on the side of the skull to allow for a penetrating angle of 30°. Despite the technical difficulties of these injections, the pattern of retrograde labeling was clearly different for injections into INLL or DNLL as described above. Figure 2.15 illustrates that after injections into the VNLL, very few neurons were labeled in the ipsilateral DNLL and ICC and a complete absence of labeling was seen in the contralateral DNLL and ICC. At the level of the SOC,

no FG-labeled neurons were seen in any subdivisions of the SOC contralateral to the VNLL injection site. However, some labeled neurons were found in the ipsilateral SOC, but were confined to the MNTB. The contralateral AVCN and PVCN were heavily labeled. Injections of FG into the VNLL resulted in more labeling of the contralateral CN than seen in any cases with FG injections to either the ICC, DNLL, or INLL. Thus, the VNLL unlike the INLL or DNLL, receives mainly ascending afferents directly from the contralateral cochlear nucleus as well as a minor projection from the ipsilateral MNTB. The most striking difference in these cases was absence of labeling in either the ipsilateral MSO and SPN, and the ipsilateral and contralateral LSO.

----Insert Figure 2.15 About Here----

Summary of Retrograde Labeling with Single Tracer Injections.

Based on the retrograde tract tracing data presented above it is possible to subdivide the lateral lemniscus into three distinct nuclei. Figure 2.16 demonstrates that the patterns of retrograde labeling in the ipsilateral and contralateral SOC provide the most convincing evidence that the DNLL, INLL and VNLL are differentially innervated by hindbrain auditory nuclei. The photomicrographs of the left and right SOC presented in Figure 2.16 were taken from each of the single tracer injection cases (ICC, DNLL, INLL and VNLL) presented above. The patterns of retrograde labeling in the ipsilateral and contralateral SOC are identical for both ICC and DNLL injection cases. However, the pattern of labeling in the SOC following INLL or VNLL FG injections is drastically different, as noted by a complete absence of retrograde labeling in the contralateral SOC. In addition, the differential patterns of labeling seen in the SOC, clearly indicate that neuronal labeling from fibers of passage, or tissue damage from penetrating pipettes into lemniscal fibers did not contaminate the results seen with FG injections into the different subdivisions of the lateral lemniscus.

----Insert Figure 2.16 About Here----

A summary of the data obtained from retrograde tract tracing from animals with single unilateral FG injections into the ICC and nLL is illustrated in Figure 2.17. This is the first evidence from the rat that the three nuclei of the lateral lemniscus are differentially innervated from hindbrain auditory nuclei. The data based on evidence from retrograde tract tracing experiments with single tracer injection suggest that the inputs to the VNLL, INLL and DNLL differ from one another.

----Insert Figure 2.17 About Here----

Retrograde Tract Tracing with Multiple Tracer Injections, Groups 3 & 4.

The fluorescent double-labeling technique

As demonstrated above, unilateral injections of anterograde tracer into the DNLL revealed many labeled axons crossing the midline via the commissure of Probst, to the opposite DNLL where many of these fibers emitted an axonal collateral before turning dorsally and terminating in the ICC contralateral to the injection site. Furthermore, single unilateral retrograde tracer injections into either ICC or DNLL resulted in a large number of fluorescently labeled neurons in the contralateral DNLL. In fact, injections of retrograde tracers into either ICC or DNLL resulted in virtually identical patterns of labeling in midbrain and hindbrain auditory brainstem structures.

Several questions arise in regards to the afferent and efferent connectivity of the DNLL. In particular, (1) are the ascending inputs to the DNLL simply axon collaterals that continue to the ICC; and (2) does DNLL contain two anatomical distinct populations of neurons based on differential efferent projections? In order to address these questions, the technique of fluorescent double-labeling was employed. This technique makes use of the

fact that different fluorochromes can be transported retrogradely through divergent axon collaterals to label the same parent neuronal cell body, where they may be visualized by means of fluorescence microscopy. This technique is also useful for differentiating between two neuronal populations in the same nucleus. If one fluorochrome such as FG is combined with a second fluorochrome such as FB or FR, two general approaches can be employed depending on the physico-chemical properties of the second fluorochrome. One approach is to choose a second fluorochrome that is excited by the same wide-band UV light as FG, which allows both tracers to be visualized simultaneously and captured on a single photomicrograph. The alternative approach is to combine FG with a fluorescent tracer that is excited by longer wavelengths such as FR, which allows the detection of both FG and FR in the same neuron by using different excitation filters for separate photomicrographs.

In this study three different fluorescent retrograde tracers were used in the following combinations: bilateral injections into the ICC, that is, a single injection of FG into right ICC, and one FR or FB injection into the left ICC; or unilateral injections into ICC and DNLL on the same side of the brain, such as FG injection into the left ICC and FR, or FB into the left DNLL. Cases with FG and FB could be viewed simultaneously under the same UV filter because they are both illuminated with incident light of 360 nm wavelength, whereas FG and FR were visualized independently because FR requires a different filter system since it has an excitation frequency of 550 nm.

Bilateral ICC injections (ICC-ICC)

Figure 2.18 shows the pattern of labeling in the lower brainstem auditory system after an injection of FG into the left ICC and FB into the right ICC. In these cases, single labeled neurons appeared white when labeled with FG and a deep blue colour with FB; and when colocalized, double labeled neurons fluoresced an "icy-blue" colour. Both FG and FB labeled neurons were found bilaterally in the DNLL as would be expected from a single

unilateral injection of either tracer into the ICC. A closer examination of either the right or the left DNLL showed an overlapping of the distribution of FG and FB single labeled neurons with only a few neurons being double labeled. In all cases examined only a few FG and FB double labeled neurons were seen in either DNLL. In both the right and left SOC only the LSO contained both FG and FB fluorescently labeled neurons, whereas the MSO and SPN contained fluorescently labeled neurons of the tracer injected into the ICC on the same side of the brain. As with the overlapping pattern of FG and FB in the DNLL, no double labeled (FG-FB) neurons were found in either the right or left LSO. Each CN contained fluorescently labeled neurons of the tracer that was injected into the contralateral ICC.

----Insert Figure 2.18 About Here----

In contrast, both the INLL and VNLL contained only one type of fluorescently labeled neuron, which was always the fluorescent tracer injected into the ICC on the same side of the brain as shown in Figure 2.19. Looking closely at the patterns of labeling in the INLL and VNLL provided further evidence that the INLL and VNLL are different populations of neurons. First, the pattern of labeling in the INLL on both sides of the brain appeared at more caudal levels than the VNLL which is consistent with single unilateral injections of retrograde tracer into the ICC. Secondly, fluorescently labeled neurons in the INLL formed clusters of tightly packed cells in the shape of a sphere which at caudal levels were the only group of neurons labeled ventrally from DNLL. Finally, the labeling that did appear in the VNLL occurred at more rostral levels and resulted in a "stair-cased" pattern of fluorescently labeled neurons.

----Insert Figure 2.19 About Here----

Another representative bilaterally injected animal (FG and FB) is shown in Figure 2.20. In this case the injections of each fluorescent retrograde tracer into the left (FG) and

right (FB) ICC are smaller than in the previous case and are both positioned along the same dorsoventral and rostrocaudal axis of the ICC. Since fewer cells were retrogradely labeled in this case, it is possible to see the concentric organization of the DNLL first proposed by Merchán et al. (1993) and the helical organization of the VNLL, latter proposed by Merchán and Berbel (1996). On both sides of the brain, ring-like labeling was found in the DNLL and a helical-shaped pattern of labeling was found in the VNLL. Like all of these cases (bilateral ICC injections) only the DNLL contained labeled neurons from either fluorescent tracer, whereas the INLL and VNLL contained fluorescently labeled neurons of the tracer injected ipsilaterally into the ICC.

----Insert Figure 2.20 About Here----

Unilateral ICC and DNLL injections (ICC-DNLL), Group 4

The goal of this procedure was to make restricted injections of FG into the left ICC and FB or FR into the DNLL on the same side of the brain and look for double labeled neurons in the contralateral DNLL and lower auditory brainstem nuclei, which would suggest collateral projections to both targets. In these cases many intensely labeled neurons were seen in the contralateral DNLL using either combination of tracers, however, the results were more convincing using both FG and FR, since these tracers are excited at different wavelengths. This allowed a view of the pattern of FR labeling in the contralateral DNLL, followed by a view of FG labeling in the same tissue section. In Figure 2.21, although more contralaterally projecting DNLL neurons are labeled using FG, all of the FR contralaterally labeled DNLL neurons are colocalized with the same FG labeled neurons. Similar results were obtained when FB was substituted for FR, however, since both FG and FB are simultaneously excited under the same UV filter, it was more difficult to detect double labeled neurons. As mentioned previously, FG labeled neurons fluoresced white, FB labeled neurons appeared deep blue in colour, and if both tracers were colocalized

neurons fluoresced an "icy-blue" colour. In these cases many single and double labeled neurons appeared in the INLL and VNLL ipsilateral to the injection sites, and no labeled neurons were seen in the contralateral INLL or VNLL. In addition, many double labeled cells were seen bilaterally in the LSO, unilaterally in the MSO and SPN, and contralaterally in CN.

----Insert Figure 2.21 About Here----

Summary of Multiple Retrograde Tract Tracing Experiments

In the present study, fluorescent retrograde double-labeling with FG and FR or FB was employed to determine the extent of axon collaterals from ascending auditory structures innervating the DNLL and ICC. In one group of animals, when two different fluorescent tracers were injected into each ICC, only a few double-labeled neurons were seen bilaterally in DNLL and LSO. Both LSO and DNLL contained numerous neurons single-labeled with both tracers, which indicates that only a small percentage of LSO and DNLL neurons give rise to projections to both sides of the brain (see summary Figure 2.22). The remaining auditory nuclei appeared identical to cases with single unilateral injection of a retrograde tracer. The bilateral projections of both the LSO and the DNLL probably reflect the importance of these nuclei for processing binaural information and the ability to accurately localize sounds in space.

----Insert Figure 2.22 About Here----

In contrast, when FG was iontophoretically injected into the ICC and FR or FB was iontophoretically injected into the DNLL on the same side of the brain numerous neurons double labeled with both tracers (i.e., FG and FR, or FG and FB) were observed in the contralateral DNLL, LSO and CN, and in the ipsilateral INLL, VNLL, LSO, MSO, and SPN (see summary Figure 2.23). This might explain the similarity of the patterns of

retrograde labeling seen in cases with either unilateral ICC or DNLL injections, because injection of retrograde tracer into either ICC or DNLL results in virtually identical patterns of labeling in auditory brainstem structures.

----Insert Figure 2.23 About Here----

Summary of Commissural Projections of the Lateral Lemniscus.

The primary goal of this study was to determine the anatomical organization of the nuclei of the lateral lemniscus as well as lemniscal nuclei with commissural projections through the commissure of Probst. The data obtained from this experiment were to serve as the basis for Experiment 2, a time course analysis of retrograde changes in commissural projecting neurons following surgical transection of the commissure of Probst. Commissural and non-commissural projections of the nLL are summarized in Figure 2.24. Data from both anterograde and retrograde tract tracing studies demonstrated that the fibers of the commissure of Probst originate almost exclusively from the DNLL. Only a few neurons from the PL and INLL may send an axon through the commissure of Probst to project to the contralateral PL and the INLL. Cell counting data revealed that over 70% of DNLL neurons project contralaterally to the opposite ICC and very few neurons were found to project to both ipsilateral and contralateral ICC. In the DNLL a separate population of neurons, approximately 30%, projects ipsilaterally to the ICC. In contrast, over 90% of neurons in the INLL and >99% of in the VNLL project ipsilaterally to the ICC on the same side of the brain. It is safe to assume that transection of the commissure of Probst would result in injury to contralaterally projecting neurons of the DNLL and not neurons of the INLL and VNLL, with the exception of the few neurons in these nuclei that project through the commissure of Probst.

----Insert Figure 2.24 About Here----

Table 2.1 Number and surgical treatment for experimental animals in Experiment 1.

Experimental Group	Number of Animals	Anatomical Tracer	Injection Site
Group 1: Anterograde tracing - unilateral injections, one tracer	3	BD	ICC
	6	BD	DNLL
Group 2: Retrograde tracing - unilateral injections, one tracer	15	FG	ICC
	5	FR	ICC
	3	HRP	ICC
	6	FG	DNLL
	3	FR	DNLL
	3	FG	INLL
	3	FG	VNLL
Group 3: Retrograde tracing - bilateral injections - two tracers	5	FG FB	L-ICC R-ICC
	5	FG FR	L-ICC R-ICC
Group 4: Retrograde tracing - unilateral injections, two tracers	5	FG FR	L-ICC L-DNLL
	3	FG FB	L-ICC L-DNLL

Abbreviations: BD, biotinylated dextran; DNLL, dorsal nucleus of the lateral lemniscus; FB, Fast-blue; FG, Fluoro-Gold; FR, Fluoro-Ruby; ICC, central nucleus of the inferior colliculus; L, left; R, right.

Table 2.2. Cell counts from six representative cases with unilateral FG injections restricted to the central nucleus of the inferior colliculus (ICC). Note that every fourth section was counted for the entire rostrocaudal extent of FG labeling in the ipsilateral and contralateral DNLL. Consistently each case showed that approximately 72% of DNLL neurons project contralaterally to the ICC and only 28% project ipsilaterally to the ICC.

Subject ID	FG labeled Neurons		% IPSI Projecting	% CONTRA Projecting
	IPSI	CONTRA		
Columbus-109	218	494	31	69
Columbus-111	143	413	26	74
Columbus-168	192	577	25	75
Columbus-268	109	308	26	74
Columbus-101	222	505	31	69
Columbus-185	229	531	30	70
MEAN $\pm$ S.E.M.				
	185 $\pm$ 20.0	471 $\pm$ 39.4	28.0 $\pm$ 1.1	72.0 $\pm$ 1.1

Figure 2.1: A schematic illustration of the anterograde and retrograde tract tracing procedures used for each experimental group from Experiment 1.

Group 1: anterograde tract tracing studies with BD injected unilaterally into the ICC or DNLL. **Group 2:** retrograde tract tracing studies with FG, FR, or HRP injected unilaterally into either the ICC, DNLL, INLL, or VNLL. **Group 3:** both FG and FR, or FG and FB combinations injected into the same side of the brain in one or two locations. **Group 4:** two different retrograde tracers each injected into one side of the ICC.

Symbols for tracer injection sites: BD, gray filled circle; FG, yellow filled circle; FR, red filled circle; FB, blue filled circle; HRP, black filled circle.

Anterograde and Retrograde Tract Tracing

Anterograde Axonal Tracing

● BD Injection sites

Retrograde Axonal Tracing

⊖ FG Injection sites

● FR Injection sites

● FB Injection sites

● HRP Injection sites

Figure 2.2: A low power photomicrograph of the rat's ICC, nLL, and surrounding structures and higher power photomicrographs of the DNLL and CP. A: Nissl- stained frontal section of the auditory midbrain showing the anatomical location of the ICC, DNLL, INLL and VNLL. The dorsomedial orientation of the section is shown in the lower left corner. **B:** DNLL neurons darkly stained with cresyl violet. **C and D:** The fibers of the commissure of Probst are not stained with cresyl violet (C), but intensely stained using a protargol fiber stain. Scale bar in A = 200 μ m, and in B, C, D 100 μ m.

Figure 2.3: Anterograde labeling in a normal animal after a unilateral injection of BD into the left ICC. A: BD labeling in the ipsilateral MGB. **B:** an absence of labeling in the contralateral MGB. **C:** BD injection site into the left ICC. **D:** normal labeling in the contralateral ICC **E:** BD labeled fibers originating from the left ICC can be followed medially along the CIC. **F:** only a few BD labeled fibers can be seen crossing the midline via the commissure of Probst. **G:** BD labeled fibers were seen traveling ventrally along the ipsilateral LL, some terminals were seen in the ipsilateral DNLL. **H:** a complete absence of BD labeled fibers, terminals, and neurons are seen in this frontal section of the contralateral DNLL. Scale bars, 200 μm .

Figure 2.4: Anterograde labeling in a normal animal after a unilateral injection of BD into the left DNLL. A: BD injection site into the left DNLL. At the injection site, anterograde transport of BD was observed in many efferent axons leaving the DNLL injection site via three distinct directions, medially, dorsally, and ventrally respectively. B: labeled fibers originating from the left DNLL (injection site) were followed medially through the commissure of Probst to the opposite DNLL and ICC. C: BD labeled fibers and terminals in the contralateral (right) DNLL. Many fibers continued dorsally to terminate in the ICC. Scale bars, 200 μm .

Figure 2.5: The extent of efferent fibers contained within the commissure of Probst after an injection of BD into the right DNLL. Rostral-to-caudal series of frontal sections cut 160 μm apart, with top, rostral and bottom caudal. Scale bar, 200 μm .

Figure 2.6: The pattern of retrograde labeling in a normal animal after a unilateral injection of FG into the left ICC. A: FG injection site in the left ICC. B: FG labeling in the contralateral ICC. C and D: FG labeling in the DNLL ipsilateral (C, left) and contralateral (D, right) to the injection site. Note the greater number of labeled neurons in the contralateral DNLL. E and F: FG labeling in SOC ipsilateral (E) and contralateral (F) to the ICC injection site. Note that in the contralateral SOC only the LSO contains FG labeled neurons. G: absence of FG labeling in the ipsilateral CN. H: Extensive labeling with FG in the contralateral CN. Scale bar, 200 μ m.

Figure 2.7: Low power photomicrographs of the rostral-to-caudal extent of retrograde labeling, bilaterally in the nLL after a unilateral injection of FG into the left ICC. A-D: FG labeling in ipsilateral nLL. E-H: FG labeling in the contralateral nLL is restricted to the DNLL.

Figure 2.8: Low power photomicrographs of the rostral-to-caudal extent of retrograde labeling in the ipsilateral nLL after a unilateral injection of FG into the left ICC. A-F: FG labeling in ipsilateral nLL. A and B: At very caudal levels the DNLL appears as a small cluster of cells and the INLL appears as a cluster of many small FG labeled cells. Ventral in this section the caudal aspect of the VNLL emerges, however, a large portion of the LL is not labeled between the VNLL and INLL. C and D: In these frontal sections the DNLL appears as a concentric ring with an absence of labeling where the fibers of the CP enter and exit the DNLL medially. At these levels the INLL and VNLL appear as one group of cells. E and F: At more rostral levels a large population of labeled cells is seen in the ipsilateral VNLL. Note: Each section is separated by 80 μ m. Scale bar = 1000 μ m.

Figure 2.9: Percentage of ipsilaterally and contralaterally FG labeled DNLL neurons after restricted injections of FG into the ICC of normal animals.

Figure 2.10: Low and high power photomicrographs of bilateral FG retrograde labeling in the nLL after injection into the ICC of Columbus-285 used for cell counting data. A: ipsilateral labeling in the DNLL, INLL and VNLL. **B:** contralateral labeling in the nLL is restricted to the DNLL. **C:** higher power photomicrograph of the same section in A shows that ipsilaterally projecting DNLL neurons are labeled centrally in the DNLL. **D:** high power photomicrograph of the DNLL shown in plate B demonstrates the concentric organization of contralaterally projecting DNLL neurons. Notice that the contralateral DNLL has far more FG labeled cells than the ipsilateral DNLL (see table 2.2 for counting data).

Figure 2.11: Low and high power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FR into the left ICC.

A: FR injection site located centrally in the left ICC. **B:** FR labeling in fibres of the commissure of the inferior colliculus (cic). Many labeled axons, terminals, and neurons were seen in the contralateral ICC. **C:** FR neuronal and axonal labeling in the ipsilateral DNLL. **D:** FR labeled neurons in the contralateral DNLL. **E and F:** High power photomicrographs illustrating ICC neurons (E) and contralateral projecting DNLL neurons (F) labeled with FR. The retrograde transport of FR produced extensive dendritic filling with a Golgi-like appearance in FR labeled neurons. Scale bars = 100 μ m.

Figure 2.12: The pattern of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left DNLL of a normal animal. A: FG labeled neurons in the ipsilateral ICC. **B:** no FG labeling in the contralateral ICC. **C:** FG injection site in the left DNLL. **D:** FG labeling in the contralateral DNLL. **E:** FG labeling in the ipsilateral SOC (LSO, MSO, and SPN are all labeled). **F:** in the contralateral SOC only neurons in the LSO are labeled. **G:** absence of FG labeling in the ipsilateral CN. **H:** Many FG labeled neurons are seen in the contralateral CN. Scale bar, 200 μm .

Figure 2.13: Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left DNLL of a normal animal. A: FG injection site in the left DNLL and FG labeled neurons in the INLL and VNLL. B: FG labeling in the contralateral lateral lemniscus is restricted to the DNLL which forms a sphere of FG labeled neurons. C: in the ipsilateral SOC numerous FG labeled neurons are found in the LSO, MSO, and SPN. D: in the contralateral SOC only neurons in the LSO are labeled. E: no FG labeled neurons were seen in the ipsilateral CN. F: Many FG labeled neurons are seen in the contralateral CN.

Figure 2.14: Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left INLL of a normal animal. A: FG labeled neurons in the ipsilateral ICC. **B:** very few FG labeled neurons in the contralateral ICC. **C:** FG injection site in the left INLL. **D:** FG labeling in the contralateral lateral lemniscus is confined to a narrow band of cells in the paralemniscal zone (PL) and a few in the INLL. **E:** FG labeling in the ipsilateral SOC (LSO, MSO, and SPN are all labeled). **F:** very sparse FG labeling in the contralateral SOC. **G:** a few FG labeled neurons are seen in the ipsilateral AVCN. **H:** Extensive labeling of the contralateral AVCN. Scale bar, 200 μm .

Figure 2.15: Low power photomicrographs of retrograde labeling in the auditory brainstem after a unilateral injection of FG into the left VNLL of a normal animal. A: FG injection site in the left VNLL. B: no FG labeling in the contralateral nLL. C: in the ipsilateral SOC, FG labeled neurons were restricted to the MNTB. D: a complete absence of labeling in the contralateral SOC. E: a few FG labeled neurons were seen in the ipsilateral CN. F: Many FG labeled neurons are seen in the contralateral CN.

Figure 2.16: Contrasting patterns of retrograde labeling in the ipsilateral and contralateral SOC of normal animals after a left unilateral injection of FG into one of the following structures: ICC, DNLL, INLL, or VNLL. A and B: the pattern of FG labeling in the ipsilateral SOC (A, iSOC) and contralateral SOC (B, cSOC) after an unilateral injection of FG into the left ICC. **C and D:** FG labeling in iSOC (C) and cSOC (D) after unilateral FG injection into DNLL. **E and F:** FG labeling in iSOC (E) and cSOC (F) after unilateral FG injection into the INLL. **G and H:** FG labeling in iSOC (G) and cSOC (H) after unilateral FG injection into VNLL. **Notice** that the pattern of FG labeling in the iSOC and cSOC are very similar for cases with either ICC or DNLL injections, whereas case with FG injections into the INLL or VNLL results in no labeling in the cSOC and labeling of the MNTB in the iSOC. Magnification 250X.

Figure 2.17: Schematic diagram showing the contrasting patterns of retrograde labeling seen in auditory brainstem of normal animals after unilateral injection of FG into one of the following structures: ICC, DNLL, INLL, or VNLL. A: the pattern of FG labeling in animals with unilateral retrograde tracer injections into the ICC. B: FG retrograde labeling in the auditory brainstem is very similar in cases with unilateral injections into the DNLL. C: FG injections restricted to the INLL result in contrasting patterns of labeling compared to ICC or DNLL injections. D: FG injections into the VNLL labeled mainly monaural structures in the auditory brainstem. Both the ipsilateral MNTB and contralateral CN were labeled in these cases. Notice in each case that the contralateral CN is always labeled, whereas the pattern of retrograde labeling in the ipsilateral and contralateral SOC and contralateral nLL vary drastically depending on the placement of the injection site in the nLL. Symbols: FG injection site, black hatched filled circle; FG labeled nuclei, gray shaded areas.

Origins of Afferent Projections to the nLL and ICC

- FG Injection Site
- FG Labeled Nuclei

Figure 2.18: The pattern of retrograde labeling in the auditory brainstem after bilateral injections of FG into the left ICC and FB into the right ICC in an normal animal. In this animal FG was injected into the left ICC and FB was injected into the right ICC. Each tracer produced the same pattern of labeling in the auditory brainstem in cases with single unilateral retrograde tracer injections into the ICC. **A:** the left DNLL contains ipsilaterally FG labeled neurons surrounded by contralaterally FB labeled neurons. **B:** in the right DNLL the reverse pattern shown in A is seen, ipsilaterally FB labeled neurons surrounded by contralaterally FG labeled neurons. **C and D:** rostral frontal sections of the SOC show that the MSO contains only neurons of the tracer injected into the ipsilateral ICC. **E and F:** in caudal sections of the SOC only the LSO contains neurons labeled with either tracer. Both the MSO and SPN possess labeled neurons of the fluorescent tracer injected into the ipsilateral ICC. **G and H:** each CN contains only fluorescent labeled neurons from the tracer injected into the contralateral ICC, the left CN is labeled with FB and right CN is retrogradely labeled with FG. Scale bar, 200 μm .

Figure 2.19: The pattern of retrograde labeling in the lateral lemniscus after bilateral injections of FG into the left ICC and FB into the right ICC in normal animals. A-D: low power photomicrographs of the DNLL and INLL from caudal sections. Notice that the DNLL contains FG and FB labeled neurons and that the size of these neurons are much larger than FB labeled neurons in the ipsilateral INLL. **E and F:** Low power photomicrographs of the nLL. **G and H:** High power photomicrographs of the DNLL (G) and the VNLL (H) demonstrates the contrasting organization of lemniscal efferent projections. The DNLL is concentrically organized and projects bilaterally to the ICC; the VNLL appears as a staircase and projects ipsilaterally to the ICC. Magnifications: A-D = 100x; E and F = 50x; and G and H = 300x.

Figure 2.20: The pattern of retrograde labeling in the lateral lemniscus after restricted bilateral injections of FG into the left ICC and FB into the right ICC in a normal animal. A and B: the DNLL contains FG labeled neurons and FB labeled neurons, and all labeled neurons ventral to the DNLL contain fluorescent retrograde tracer from the ipsilateral ICC injection site. C: Higher power photomicrograph of A shows that the ipsilateral (left) VNLL contains only FG labeled neurons. D: High power photomicrograph of B shows that the ipsilateral (right) VNLL contains only FB labeled neurons. Notice that the DNLL is concentrically organized and contains a mixture of FG and FB labeled neurons, whereas the VNLL is helically organized and contains fluorescently labeled neurons of the tracer injected into the ipsilateral ICC. Magnification A and B, 100x; C and D, 300 x.

Figure 2.21: Fluorescent double labeling in the right DNLL after an injection of FG into the left ICC and an injection of FR in to the left DNLL. A and B: fluorescent frontal sections showing the distribution of contralaterally FR labeled DNLL neurons following an injection of the tracer into the left DNLL. **A' and B':** the same frontal section showing the distribution of FG labeled neurons. Arrows point to neurons that are double labeled with both FR and FG. Notice that almost all of the FR labeled neurons are colocalized with FG labeled neurons. Magnification 500x.

Figure 2.22: Schematic diagram summarizing the results of animals with bilateral ICC injections of different fluorescent retrograde tracers. Distributions of labeling in the auditory midbrain and the lower brainstem in cases with FG (gray shaded circles) injected into the left ICC and FB (black filled circles) injected into the right ICC. Notice that the DNLL and the LSO are the only two nuclei in the auditory brainstem with both FG and FB labeled neurons and that no neurons in these structures are double labeled with both tracers. The INLL, VNLL, MSO, and SPN are labeled with the tracer injected into the ipsilateral ICC and the CN is labeled with the fluorescent retrograde tracer injected into the opposite ICC.

Bilateral ICC Injections

Figure 2.23: Schematic diagrams summarizing the results of animals with unilateral ICC and DNLL injections of using different fluorescent retrograde tracers. A: Injections of FG into the ICC and FB or FR into the DNLL on the same side of the brain result in many double labeled neurons throughout the auditory brainstem. **B:** a schematic diagram shows the extent of collateral projections to the DNLL. The double labeling technique used in this study shows that the majority of afferent inputs innervate the DNLL via axon collaterals from the same fibers ascending to targets in the ICC.

A

Fluorescent Double-Labeling Paradigm

B

Collateral Projections to DNLL and ICC

Figure 2.24: A diagram illustrating the major and minor commissural projections of the nuclei of the lateral lemniscus through the commissure of Probst. The diagram is a summary of the data obtained from both anterograde and retrograde tract tracing studies which demonstrated that the fibers of the commissure of Probst originate almost exclusively from the DNLL (closed circles). Only a few neurons from the PL and INLL may send an axon through the commissure of Probst to project to the contralateral PL and the INLL (hatched circles). Each subdivision of the lateral lemniscus contains neurons that ascending dorsally through the lateral lemniscus to innervate the ipsilateral ICC (shaded circles).

Commissural Projecting Neurons of the Lateral Lemniscus

- Non-commissural Projecting Lemniscal Neurons
- Commissural Projecting Lemniscal Neurons
(major source of CP fibers)
- Commissural Projecting Lemniscal Neurons
(minor source of CP fibers)

DISCUSSION

The purpose of this study was to determine the normal afferent and efferent anatomical projections of the rat's nuclei of the lateral lemniscus (nLL). In addition, commissural and non-commissural projections of the nLL were investigated as a prerequisite for a time course analysis of retrograde changes in commissural projecting lemniscal neurons following midline surgical transection of their axons (Experiment 2). The cytoarchitecture, efferent, afferent, collateral, and commissural projections of the nuclei of the lateral lemniscus, and the tracers used to map these pathways will be discussed below.

Cytoarchitecture of the nLL

In Nissl-stained sections, DNLL neurons are easily distinguished from bordering nuclei within the LL by their large size and darkly Nissl-stained materials, whereas the nucleus sagulum, INLL, and VNLL contain relatively small and pale Nissl-stained neurons and are difficult to distinguish from one another. The horizontal cell group of the lateral lemniscus served as a convenient landmark to separate DNLL neurons from more ventrally positioned neurons of the lateral lemniscus. Nissl-staining was useful for differentiating DNLL neurons from other neurons embedded within the fibers of the lateral lemniscus but not useful for differentiating INLL from VNLL. Therefore, cytoarchitectural analysis from Nissl-preparations does not warrant subdividing the VNLL into two distinct subdivisions (i.e., INLL and VNLL). Based on Nissl staining alone, it is not surprising that no agreement exists concerning the number of lemniscal subdivisions ventral to DNLL, as indicated in the literature (see: Friauf, 1992; Glendenning et al., 1981; González-Hernández et al., 1996; Ito et al., 1996; Kelly, et al., 1998; Kudo et al., 1990; Merchán and Berbel, 1996; Saint Marie and Baker, 1990; Schofield and Cant, 1997; Tanaka et al., 1985; van Adel et al., 1998; Zhang et al., 1998; Zook and Casseday, 1982)

As numerous reports have shown, the lemniscal nuclei form part of the ascending auditory system in all mammals, but their relative size and degree of differentiation show considerable variation between species, specifically the INLL and VNLL. Several investigators using rats consider the VNLL to be a single nucleus (Friauf, 1992; Merchán and Berbel, 1996; Sommer et al., 1993; Tanaka et al., 1985; Wynne et al., 1995), whereas other investigators regard the dorsal part of the VNLL as a separate nucleus, the INLL. (Caicedo and Herbert, 1993; González-Hernández et al., 1996; Ito et al., 1996; Kelly, et al., 1998; Moore and Moore, 1987; Saint Marie, 1996; van Adel et al., 1998; Wynne et al., 1995; Zhang et al., 1998). In various species of bats, the INLL is hypertrophied and has a substantially different morphology than in the cat or rat. For example, in the echolocating big brown bat (*Eptesicus fuscus*) the DNLL, INLL and VNLL are especially large and well differentiated, and there is almost a complete segregation of specific cell types (Huffman and Covey, 1995). Based on comparative anatomical data from many mammalian species, the DNLL is clearly differentiated. In contrast the INLL and VNLL vary considerably in the degree of differentiation, and although present in all mammals they are clearly segregated structures in species such as echolocating bats and in most rodent species like the rat. In this study further differentiation of nLL was only possible by analyzing patterns of retrograde labeling of the efferent and afferent projections of the nLL; thus the terms INLL and VNLL have been retained for reasons that will become apparent as discussed below.

Efferent Projections of the Nuclei of the Lateral Lemniscus

In the present study both retrograde and anterograde tract tracing data support previous anatomical data from the rat and cat, showing that the commissure of Probst (CP) is the exclusive pathway for all crossed projections from DNLL to the opposite DNLL and IC (Bajo et al., 1993; Glendenning and Masterton 1983; Goldberg and Moore, 1967; Hutson et al., 1991; Ito et al., 1996; Kudo 1981; Merchán et al., 1994; Shneiderman et al.,

1988; Tanaka et al., 1985; van Adel et al., 1997). As in the case of other mammalian species, the rat's DNLL projects bilaterally to the central nucleus of the inferior colliculus with a larger proportion of neurons projecting contralaterally through the commissure of Probst (Adams, 1979; Bajo et al., 1993; Beyerl, 1978; Brunso-Bechtold et al., 1981; Coleman and Clerici, 1987; Covey and Casseday, 1991; González-Hernández et al., 1996; Hutson et al., 1991; Ito et al., 1996; Kudo, 1981; Shneiderman et al., 1988; Tanaka et al., 1985; Zook and Casseday, 1979, 1982). In the present study, cell counting was carried out for six animals; each received a unilateral injection of FG into the ICC, and in each case approximately 70 % of FG-labeled neurons were found in the DNLL contralateral to the ICC injection site. Similar cell counting data from previous studies in the rat have shown that contralaterally projecting DNLL neurons account for approximately 70-75% of the cells projecting to IC via the CP (González-Hernández et al., 1996; Ito et al., 1996; Tanaka et al., 1985). Previous estimates were based on only two normal animals in the studies by Ito et al. (1996) and Tanaka et al. (1985), and three by González-Hernández et al. (1996). Hutson et al. (1991) applied HRP flakes directly to freshly cut ends of CP fibers (cut and fill technique) and showed that a slightly lower percentage (60%) of DNLL neurons project contralaterally in the cat, but cautioned that this could be an underestimate because it could not be determined that every neuron with an axon in the CP was successfully labeled. This technique was useful in showing that the fibers in the CP originate exclusively from the DNLL, because HRP-labeled neurons were seen bilaterally only in DNLL, and not in the ICC, INLL, or VNLL. Furthermore, unilateral injection of the anterograde tracer BD into the DNLL labeled more fibers and terminals in the contralateral ICC than in the ipsilateral ICC, which is consistent with the results reported by Bajo et al. (1993). Anterograde axonal tracing with BD proved especially useful for mapping the rostrocaudal extent of the commissure of Probst which would ultimately make transection of this pathway a much easier task and provided for a more precise surgical procedure for use in Experiment 2. Additionally, only a couple of BD labeled fibers were seen in the commissure of the IC

(cic) in animals with DNLL injections, indicating that most contralateral projections from the DNLL to the ICC decussate across the midline through the commissure of Probst. Similarly, after unilateral injections of BD or FR into the ICC many labeled fibers were found in the cic and very few in the commissure of Probst. However, since both BD and FR are transported in the anterograde as well as retrograde directions, the few labeled fibers seen in the CP could have resulted from retrograde transport of these tracers to neurons in the contralateral DNLL, and not anterogradely labeled axons of descending projections from the ICC to the contralateral DNLL.

Double labeling after injections into left and right ICC in the cat indicates that the ipsilaterally and contralaterally projecting neurons form essentially separate populations with less than 10% of the cells giving rise to projections to both sides of the brain (Hutson et al., 1991). A similar pattern seems likely in the rat based on cases from this study with FG and FB injected into the left and right ICC; virtually no double labeled neurons were seen in the DNLL, indicating that the ipsilaterally and contralaterally projecting neurons are clearly separate populations in the rat. Tanaka et al. (1985) reported a very similar finding in the rat after injecting diaminidophenylindol (DAPI) and propidiumiodine (PI) fluorescent dyes into each ICC. In their double labeling study a very small percentage of DNLL neurons were found to be labeled with both DAPI and PI. Similarly, Willard and Martin (1985) found very few double labeled neurons in the DNLL of the North American Opossum following injection of the fluorescent retrograde tracers, True Blue, into one ICC and Nuclear Yellow into the opposite ICC.

In the present study animals with either unilateral ICC or bilateral ICC retrograde tracer injections demonstrated that the both INLL and VNLL project ipsilaterally to the ICC. Previous studies in rats have shown similar results, both INLL and VNLL form considerable projections to the ipsilateral ICC with little or no projection to the contralateral ICC (Beyerl, 1978; Ito et al., 1996; Kelly et al., 1998; Merchán and Berbel, 1996). The laterality of this pathway contrasts sharply with the bilateral connections of the DNLL to the

ICC. With regard to the laterality of the ipsilateral projection to ICC there is no obvious distinction between INLL and VNLL and the retrogradely labeled neurons are similar in appearance. Indeed, Merchán and Berbel (1996) have argued on the basis of cytoarchitecture and connectivity that there is no fundamental distinction between INLL and VNLL in the rat and have incorporated both areas in their definition of VNLL. However, data from the present study after unilateral and bilateral retrograde tracer deposits were made into the ICC clearly showed that at the most caudal levels of the lateral lemniscus the INLL is a distinct cluster of neurons positioned just ventral to the DNLL. Furthermore, results from retrograde transport studies of the nLL show that each lemniscal nuclei is differentially innervated by lower auditory brainstem structures (as discussed below). Therefore, I have retained the terms INLL and VNLL to differentiate lemniscal nuclei positioned dorsoventral along the lemniscal pathway.

Several other studies have described ipsilateral projections from INLL and VNLL to ICC in the cat and bat both of which are slightly different than the rat. (Adams, 1979; Brunso-Bechtold et al., 1981; Covey and Casseday, 1991; Goldberg and Moore, 1969; Glendenning et al., 1981; Nordeen et al., 1983; Roth et al., 1978; Saint Marie and Baker, 1990; Whitley and Henkel, 1984; Willard and Martin, 1983; Zook and Casseday, 1982). For example, in the cat the INLL apparently has a contralateral as well as an ipsilateral projection to ICC whereas VNLL does not (Brunso-Bechtold et al., 1981; Glendenning et al., 1981); However, as indicated in the present study there is no evidence for this connection in the rat (Ito et al., 1996; Kelly et al., 1998; Merchán and Berbel, 1996).

In summary, the efferent projections of the nLL ascend to the ICC via two distinct fiber paths. Ipsilaterally projecting neurons in the VNLL (100%), INLL (>90%), and DNLL (30%) have efferent axons traveling along the lateral lemniscus that terminate in the IC on the same side of the brain, and contralaterally projecting neurons in the DNLL (70%) have axons running across the midline in the commissure of Probst to terminate in the IC on the opposite side of the brain.

Afferent Projections to the Nuclei of the Lateral Lemniscus

Since very little is known about the origins of ascending projections to each lemniscal subdivision in the rat, small iontophoretic injections of the retrograde tracer, FG were made into DNLL, INLL, and VNLL of adult rats. Considerable effort was made to insure that restricted injections were made into each lemniscal nucleus. From this study we can conclude that the major sources of afferents to the rat's DNLL are from the contralateral DNLL through the commissure of Probst, the contralateral LSO and CN via the trapezoid body and lateral lemniscus, and from the ipsilateral INLL, VNLL, LSO, MSO and SPN via the lateral lemniscus on the same side of the brain. Major sources of afferents to the INLL are from the ipsilateral LSO, MSO, SPN and MNTB and from the contralateral CN. The VNLL receives the least amount of diverse inputs, with major projections from the ipsilateral MNTB and from the contralateral CN. The most noticeable difference between cases with different injection sites along the LL was the pattern of labeling in the ipsilateral and contralateral SOC. Indeed, the SOC proved most useful for determining the differential projections from binaural and monaural structures in the auditory brainstem to the nuclei of the lateral lemniscus. Patterns of labeling in the contralateral LL were useful for differentiating DNLL from INLL and VNLL, but not INLL from VNLL, because only cases with retrograde tracer deposited in the DNLL produced labeling in the contralateral LL.

The differential patterns of innervation of the rat's nLL are consistent with similar anatomical studies carried out in the cat (Glendenning et al., 1981; Goldberg and Moore, 1967; Shneiderman et al., 1988; Oliver and Shneiderman, 1989), in echolocating bats (Covey and Casseday, 1985; Huffman and Covey, 1995; Vater et al., 1995; Yang et al., 1996), and in the rat (Labelle and Kelly, 1996). Glendenning et al., (1981) used cytoarchitectonic criteria to distinguish three subdivisions (DNLL, VNLL and INLL) within the cat's LL. In their study both anterograde and retrograde tract tracing methods

demonstrated differential projections to the DNLL, INLL and VNLL. Local injections of HRP into DNLL of the cat resulted in intense labeling of the contralateral DNLL, LSO and CN, and the ipsilateral INLL, VNLL, LSO, MSO, and SPN whereas injections of HRP into the INLL or VNLL retrogradely labeled the ipsilateral MNTB and the contralateral CN.

The afferent patterns of innervation of the rat's nLL are consistent with previous studies that examined the electrophysiological properties of the rat's nLL (Buckthout, 1993). The DNLL possesses mainly binaural response properties and receives a projection from binaurally sensitive auditory nuclei (i.e., opposite DNLL, both LSO, and the ipsilateral MSO), whereas the INLL and VNLL display primarily monaural response properties and receive projections from monaural auditory nuclei (MNTB and CN). The findings that each lemniscal nucleus receives a unique ratio of binaural to monaural afferents from various sources suggests that acoustical information processing is different in each nucleus, but currently it is not at all that clear how these differential inputs (monaural and binaural) contribute to the observed response properties associated with the rat's nLL. Further anatomical and physiological studies are needed to determine how connections, the morphology of presynaptic terminals, and intrinsic membrane properties shape acoustical response properties in the nLL. Such extensive anatomical and physiological studies have been carried out on the rat's DNLL (anatomical: Bajo et al., 1993; González-Hernández et al., 1996; Kelly et al., 1998; Labelle and Kelly, 1996; Merchán et al., 1994; Tanaka et al., 1985; Zhang et al., 1998; ultrastructural studies: Labelle and Kelly, 1997; intrinsic membrane properties: Fu et al., 1997; Wu and Kelly, 1995a, 1995b, 1996), whereas such studies of the rat's INLL and VNLL have only begun (anatomical: Merchán and Berbel, 1996; Zhao and Wu, 1998) (see functional implications below).

Afferent Collateral Projections to DNLL and ICC

In the present study, retrograde tract tracing with single unilateral injections into the ICC or DNLL demonstrate that afferent projections to the rat's DNLL are virtually identical to the afferent projections that ascend to the ICC. Consistently, other studies have also shown that the ascending projections to the DNLL parallel the projections that ascend to the ICC in the majority of mammalian species (Glendenning *et al.* 1981; Huffman and Covey, 1995; Labelle and Kelly, 1996; Shneiderman *et al.*, 1988). Furthermore, several recent studies have suggested that the similarity between the inputs to both DNLL and ICC arise from ascending fibers in the LL that give off axon collaterals in the DNLL before terminating in the ICC (Bajo *et al.* 1993; Henkel, 1997; Iwahori, 1986; Merchán *et al.*, 1994; Zhao and Wu, 1998). Firstly, Iwahori (1996) observed that a large percentage of ascending lemniscal axons impregnated by the Golgi method give off one or more thin collaterals. Secondly, Henkel (1997) made small deposits of anterograde tracer into the MSO of cats and showed that most, if not all ascending labeled fibers innervate the ipsilateral DNLL via right-angle axon collaterals before terminating in ICC. Bajo *et al.* (1993) and Merchán *et al.* (1994) have demonstrated that contralaterally projecting DNLL neurons traveling through the CP and entering the opposite DNLL give rise to axon collaterals before turning dorsally to terminate in the ICC. Additionally, Zhao and Wu, (1998) showed that some *in vitro* intracellularly Neurobiotin-labeled VNLL neurons give rise to short axon collaterals in the DNLL before ending in the ICC of young rats.

In order to determine the which auditory brainstem nuclei participate in collateral innervation of the DNLL and ICC, the technique of fluorescent double labeling. Injection of one fluorescent retrograde tracer was made into the ICC (e.g., FG) and injection of a different fluorescent retrograde tracer injected was made into the DNLL (e.g., FR or FB) on the same side of the brain. Fluorescence microscopic analysis revealed many neurons labeled with both tracers in the contralateral DNLL, LSO and CN, and in the ipsilateral INLL, VNLL, LSO, MSO, and SPN. Of particular interest for this study was the fact that,

in the DNLL opposite to the tracer injection almost every neuron was double-labeled with both tracers, which suggests that the majority (if not all) of contralaterally projecting DNLL neurons innervate the opposite DNLL and ICC via axon collaterals. Convincingly, results using multiple neuroanatomical techniques indicate that the majority of afferent inputs innervate the DNLL via axon collaterals from the same fibers ascending to targets in the ICC. These findings support the idea that most efferent projections of neurons in the central auditory system possess extensive networks of local axon collaterals (Oertel and Wu, 1989; Oertel et al., 1990; Oliver et al., 1991; Sprangler et al., 1985; Yang et al., 1996).

Results from the present double labeling studies were crucial for the design of Experiment 2 in which all of these commissural projecting axons were transected using a stereotaxic procedure developed by Ito et al. (1996). Considering that one population of neurons in the DNLL has an exclusively contralateral projection through the commissure of Probst, and that the same axons innervate both DNLL and ICC allows one to easily prelabel this population of neurons by injecting retrograde tracer into either the DNLL or ICC. However, ICC injections of retrograde tracers would prelabel both ipsilaterally and contralaterally projecting DNLL neurons, which would permit a comparison of prelabeled injured DNLL neurons (axotomized) versus prelabeled uninjured DNLL neurons.

Anatomical Tract Tracing and Technical Considerations

Tracer transport properties

In the present study both retrograde and anterograde tract tracing techniques were used to determine specific anatomical projections to and from the nLL and ICC. BD and FR were found to be transported in the anterograde and retrograde directions, which is in agreement with previous studies that have extensively characterized these tracers (Rajakamar et al., 1993; Schmued et al., 1990). Schmued et al. (1990) characterized FR for use *in vivo* and proposed that it is the lysine groups conjugated to the molecule that may be instrumental in facilitating anterograde axonal transport because similar lysine and

biotin-lysine conjugates undergo anterograde axonal transport. Iontophoretic injections of BD were similar to FR injections, in the sense that labeled cells in the area of the injection site generally displayed well-filled processes. In animals with BD or FR injections, many labeled axons were seen leaving the injection site and could be followed in the same tissue section where anterogradely labeled terminals and retrogradely labeled neurons were found in auditory nuclei. The retrograde transport of both BD and FR appeared qualitatively superior to FG or FB: each produced extensive dendritic filling with a Golgi-like appearance in labeled neurons. However, in terms of the number of cells that could be labeled, both BD and FR were quantitatively inferior to FG or FB. In contrast to BD and FR which are transported anterogradely and retrogradely, both FG and FB were transported exclusively retrogradely over long distances to label hindbrain and midbrain auditory structures in animals surviving extended periods of time. Unlike BD or FR, both FG and FB were found only in neurons, and not in axons. Both FG and FB appeared qualitatively similar in retrogradely labeled neurons, although the dendritic fill was considerably greater in FG labeled neurons than FB labeled neurons, which may have been because of the greater fluorescent "bloom" given off by FB labeled neurons.

Leaky tracers

Of great concern to the present study was leakage of tracer out of labeled fibers, terminals and neurons and subsequent uptake by neighboring neurons or glial cells. Diffusion of tracer may occur *in vivo* prior to sacrifice and perfusion or at various stages of histological processing. Diffusion of the tracer can result in a complete or partial loss of the dye or a decreased intensity with selective loss of weakly labeled neurons, creating the potential for false negative results. Also, exocytosis or transneuronal uptake can produce false positive results. None of the tracers used in the present study were found to leak from labeled structures at any survival time. Both BD and HRP required shorter survival times because both of these tracers are subject to proteolytic degradation within a few days

after application. Divac and Mogensen (1990) have reported that FG and different fluorescent rhodamine microspheres are capable of labeling neuronal perikarya in rats that have lived for one year after the surgical injection of these tracers. Schmued et al. (1990) made a similar report for the long-term transport properties within the CNS using various fluorescent dextrans including Fluoro-Ruby.

The possibility of transneuronal transport of retrograde tracers was thoroughly addressed in this study. Auditory structures with multiple subpopulations of neurons such as in the SOC and LL, were very useful for demonstrating the lack of transneuronal transport with the retrograde tracers used in this study. Injection of retrograde tracer into either the ICC or DNLL labeled neurons in the contralateral DNLL and LSO, which were the only nuclei labeled within the contralateral LL and SOC. Had transneuronal transport occurred labeled neurons would have been seen in neighboring nuclei; however, even with survival times as long as 12 weeks, labeling was restricted to the contralateral DNLL and LSO. Furthermore, in structures with multiple labeled nuclei, such as the ipsilateral LL and SOC, transneuronal transport was ruled out because labeling in these structures was restricted to the cytoarchitectonic borders of each labeled nucleus. For example, unilateral injections of retrograde tracers into the ICC resulted in concentric rings in the DNLL, a staircase or helical pattern in the VNLL, and a distinct S-shape in the LSO. Furthermore, in the ipsilateral LL there was an absence of labeling in the horizontal cell group, the narrow band of cells between the DNLL and the INLL. Finally, long-term survival times used in the present study suggest that retrograde labeling of auditory neurons is possible using one or a combination of the fluorescent neuronal tracers tested in this study over long periods of time.

Fibers of passage

Another potentially confounding factor can be the uptake of anterograde and retrograde tracers by intact fibers of passage projecting through the injection site. This was

not of great concern for injections made into the ICC because most ascending auditory brainstem projections terminate in the inferior colliculus (Kelly et al., 1998). However, most ascending fibers from the ipsilateral INLL, VNLL, LSO, MSO, SPN, and the contralateral DNLL, LSO and CN travel through the nLL to terminate in the ICC, it is possible that these fibers could be labeled accidentally with injections of anterograde or retrograde tracers made into the nLL.

In the present study, injections of anterograde tracer into the DNLL always labeled more fibers and terminals in the contralateral DNLL than the ipsilateral DNLL, which is consistent with anterograde transport studies (Bajo et al., 1993; Labelle and Kelly, 1997) and retrograde transport studies that have demonstrated that over two thirds of contralaterally projecting DNLL neurons are labeled after ICC injections (González-Hernández et al., 1996; Hutson et al., 1991; Ito et al., 1996; Tanaka et al., 1995). If BD was transported anterogradely by fibers of passage more fiber and terminal labeling would have been observed in the ipsilateral ICC and not the contralateral ICC.

Uptake of retrograde tracers by fibers of passage through the lateral lemniscus could potentially produce erroneous patterns of labeling in the cochlear nucleus and superior olivary complex. However, several observations indicated that the patterns of retrograde neuronal labeling reported in this study were not due to uptake by fibers of passage projecting through the injection site. For example, if an injection into the INLL or VNLL had produced retrograde labeling from uptake of tracers by fibers of passage, numerous labeled neurons would have been observed in the contralateral superior olivary complex, specifically the contralateral LSO. In this study, no FG retrogradely labeled neurons were found in the contralateral SOC of animals with INLL or VNLL injections of FG. A second potential indication of transport by fibers of passage would be labeling in various subdivisions of the ipsilateral superior olivary complex. DNLL injections labeled the ipsilateral LSO, MSO and SPN, and not the MNTB, whereas injections into either INLL or VNLL always labeled the MNTB. Furthermore, injections into the INLL labeled

the ipsilateral LSO, MSO and SPN, whereas none of these nuclei were labeled in cases with VNLL injections. Therefore, the differential patterns of retrograde labeling in the ipsilateral and contralateral SOC that resulted from unilateral injections of retrograde tracer into the DNLL, INLL, and VNLL strongly suggest that the uptake of tracer and transport by fibers of passage was negligible.

Functional Implications

Understanding both the physiological response patterns, as well as the afferent, efferent, and commissural projections of the nuclei of the lateral lemniscus will ultimately help to determine the contribution of these nuclei in processing acoustic information. Differential ascending inputs to lemniscal nuclei may explain the differences in the ratios of binaural/monaural properties seen in electrophysiological mapping studies of these nuclei. For example, the major sources of ascending afferents to the DNLL are from binaural structures within the SOC, and from the opposite DNLL via the commissure of Probst. In the rat, a strong contralateral projection from the opposite DNLL, from the ipsilateral projection from MSO and from bilateral projection of the LSO contributes to the majority of the binaural responses (over 70% EI) found in the DNLL (Buckthoight, 1993). The small percentage (10% EE, and 10-20% EO) of DNLL neurons that respond to monaural stimulation most likely arise from the direct monaural projections from the contralateral CN and indirect projections from the ipsilateral SPN, VNLL and INLL (Aitkin et al., 1970; Buckthoight, 1993; Covey and Casseday, 1991). In contrast, both the INLL and VNLL display primarily monaural response properties and receive direct projections from the contralateral cochlear nucleus and very little projections from binaural nuclei in the SOC (Glendenning *et al.* 1981; Huffman and Covey, 1995). The majority of INLL (over 60%) and VNLL (greater than 90%) neurons are functionally monaural (EO/O cells), responding only to contralateral sounds (Aitkin et al., 1970; Covey and Casseday, 1991). Recent evidence suggest that at least a few neurons in the INLL and the medial part of the VNLL

are specialized for binaural processing (Batra and Fitzpatrick, 1997; Buckthout, 1993). This small group of binaurally sensitive neurons may be the same group of contralaterally labeled neurons in the INLL and PL seen in cases with FG injections restricted to INLL. Likewise, FG injections into the INLL did label binaural nuclei in the ipsilateral SOC, specifically, the LSO and MSO. Indeed, electrophysiological properties of the nLL are consistent with neuroanatomical investigations of the afferent inputs to the nuclei of the lateral lemniscus in mammals. In particular, the DNLL possesses mainly binaural response properties and receives heavy projections from binaural nuclei in the SOC (e.g., LSO and MSO) and from the opposite DNLL, whereas the INLL and VNLL display primarily monaural response properties and receive direct projections from the contralateral cochlear nucleus and very little projections from binaural nuclei in the SOC or contralateral nLL.

A second approach to understanding the function(s) of the lateral lemniscus has been to examine the neurotransmitters, receptors and neuropeptides used by its cells. Both neurochemical and ultrastructural studies have identified that the vast majority of neurons in the DNLL exhibit GAD, GABA immunoreactivity, and a strong mRNA signal for GABA_Aα1 receptor. Furthermore, DNLL neurons are retrogradely labeled with [³H]GABA, contain pleomorphic synaptic vesicles and make symmetrical synaptic contacts. As such these neurons would be expected to contribute an inhibitory influence on neurons with which they make synaptic contact (i.e., the contralateral DNLL and ICC) (Adams and Mugnaini, 1984; Glendenning and Baker, 1988; González-Hernández et al., 1996; Hutson, 1988; Moore and Moore, 1987; Oliver and Shneiderman, 1989; Shneiderman and Oliver, 1989; Roberts and Ribak, 1987; Thompson et al., 1985; Vater et al., 1992; Winer et al., 1995; Wynne et al., 1995; Zhang et al., 1998). On the other hand, neurons ventral to the DNLL contain a mixture of inhibitory and excitatory neurotransmitters (Oliver and Bishop, 1998; Riquelme et al., 1998; Roberts and Ribak, 1987; Saint Marie, 1993, 1996; Saint Marie and Baker, 1990). Two recent studies in the rat demonstrate that the dorsal portion of the VNLL (or as some authors prefer, the INLL)

contains a large percentage of neurons that are immunonegative for both GABA and glycine, whereas the majority of neurons in the ventral portion of the VNLL contain both GABA and glycine immunoreactivities and a strong mRNA signal for GAD-65 (Oliver and Bishop, 1998; Riquelme et al., 1998). This mixture of inhibitory and excitatory transmitters found in lemniscal nuclei ventral to DNLL is not limited to the rat. Saint Marie and Baker (1990) used [^3H]glycine to retrogradely label glycinergic inputs to the ICC of cats and, approximately 68% of retrogradely labeled neurons were located in the ipsilateral VNLL. In a later study, Saint Marie (1996) used [^3H]aspartate to identify potential glutamatergic connections of the chinchilla ICC and found very few retrogradely labeled neurons in the nLL with the exception of a few labeled neurons in the INLL. Since neurochemical data suggest that few lemniscal neurons have excitatory characteristics, it may be assumed that the nLL must play a significant role in inhibitory processes in the ICC.

The functional significance of lemniscal inhibitory inputs to the ICC, specifically the commissural projections via the commissure of Probst, has been studied by various manipulations (pharmacological blockade, neurochemical lesion, and mechanical lesions) that activate or shutdown these pathways, dramatically altering the acoustic responses properties of collicular neurons. For example, electrophysiological studies with rats have shown that *in vivo* pharmacological blockage of the DNLL using kynurenic acid (KYNA), a specific glutamate antagonist, alters both sound localization cues, interaural intensity differences (IIDs) and interaural timing differences (ITDs) in the contralateral ICC (Li and Kelly, 1992; Kelly and Li, 1997, Kidd and Kelly, 1996). The extent of binaural inhibition produced by varying the IID or ITD of sound stimuli delivered simultaneously to the two ears is greatly attenuated by injection of KYNA into the DNLL contralateral to the recording site. It is believed that injection of KYNA into the DNLL drastically reduces the ratio between excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) of neurons in the opposite ICC, and shifts the control of the membrane potential in favour of excitatory potentials for reaching spike threshold when the ear ipsilateral to the

recording electrode is stimulated. In addition, no change is seen in the IID or ITD curves obtained from neurons in the ICC ipsilateral to the KYNA injection site in DNLL (Li and Kelly, 1992; Kelly and Li, 1997, Kidd and Kelly, 1996). A similar effect has been reported by Faingold *et al.* (1993) after blockade of activity in DNLL by local injection of lidocaine. Inversely, an injection of the excitatory amino acid agonist kainate into the DNLL enhances ipsilateral suppression of binaural responses recorded in the contralateral ICC (Faingold *et al.* 1993). Furthermore, neurochemical destruction of DNLL using kainic acid affects binaural evoked responses recorded from the rat's primary auditory cortex (Glenn and Kelly, 1992). Interaural timing difference functions are shifted in the hemisphere contralateral to the lesion and the response suppression produced by stimulating with the ipsilateral ear leading the contralateral ear is drastically reduced.

The disruptive effect on either IIDs or ITDs following pharmacological blockage, neurochemical destruction of DNLL, suggests that the contralateral projection from DNLL via the commissure of Probst may be an important and necessary substrate for accurate sound localization by refining physiological responses to binaural cues. Therefore, complete destruction of the DNLL or transection of its crossed efferent projections in the CP would presumably cause substantial deficits in sound localization and a degradation in the accuracy of auditory spatial discrimination. Indeed, two recent studies successfully demonstrated that unilateral and bilateral kainic acid lesions of the DNLL, or a midline transection of the CP produces severe deficits in midline sound localization and an elevation in auditory spatial acuity in the horizontal plane (Kelly *et al.*, 1996; Ito *et al.*, 1996). In general, behavioural studies support the view that disruption of the auditory brainstem at levels necessary for binaural processing produces deficits in sound localization and auditory spatial acuity, which is most likely due to changes of IID and ITD sensitivities in ICC neurons (SOC lesions: Kavanagh and Kelly, 1992; van Adel and Kelly, 1998; DNLL lesions: Kelly *et al.*, 1996; CP lesions: Ito *et al.*, 1996; IC lesions: Kelly and Kavanagh, 1994).

Conclusions

The nuclei of the lateral lemniscus can be divided into three distinct subdivisions within the LL, abbreviated : DNLL, INLL, and VNLL, based on the anatomical organization as revealed using conventional anterograde and retrograde transport studies. The DNLL has a bilateral projection to the ICC and is organized in a roughly concentric fashion containing two populations of neurons. The majority (70%) of DNLL neurons have crossed projections through the commissure of Probst to the opposite DNLL and ICC, and a smaller population (30%) of DNLL neurons has uncrossed projections to the ipsilateral ICC. The DNLL receives projections from the opposite DNLL through the commissure of Probst and receives mainly binaural inputs from the lower brainstem. The INLL and VNLL have ipsilateral projections to ICC and receive mainly monaural inputs from lower auditory brainstem structures. The observed pattern of retrograde labeling in the SOC is most useful for differentiating the afferent inputs to the three subdivisions of the lateral lemniscus.

Chapter 3

Neuronal degeneration in the dorsal nucleus of the lateral
lemniscus after surgical transection of the commissure of
Probst in the adult rat, *Rattus norvegicus*

Experiment 2

EXPERIMENT 2

In Experiment 1 anterograde and retrograde tract tracing procedures were used to study the anatomical organization and commissural projections of the nuclei of the lateral lemniscus (nLL). The results showed that the commissure of Probst is a fiber bundle which carries efferent axons from one DNLL to the opposite DNLL and ICC. Cell counts demonstrated that 70% of the neurons in the DNLL project contralaterally, whereas only 30% of DNLL neurons project ipsilaterally. In addition, using a fluorescent double-labeling paradigm, it was demonstrated that ipsilaterally and contralaterally projecting DNLL neurons form essentially separate populations of neurons and that very few neurons in the DNLL project bilaterally to the ICC. Injections of retrograde tracer along the dorsoventral extent of the lateral lemniscus clearly demonstrated that the commissure of Probst is the exclusive pathway for contralaterally projecting DNLL neurons. The anatomical organization of the DNLL and its efferent projections have been extensively studied and seem to be very similar in different mammalian species (Adams, 1979; Bajo et al., 1993; Beyerl, 1978; Brunso-Bechtold et al., 1981; Coleman and Clerici, 1987; Covey and Casseday, 1991; González-Hernández et al., 1996; Hutson et al., 1991; Ito et al., 1996; Kudo, 1981; Shneiderman et al., 1988; Tanaka et al., 1985; Zook and Casseday, 1979, 1982).

Over a century ago, Held (1893) transected the lateral lemniscus at the level of the DNLL and noticed extensive degeneration along commissurally projecting fibers that traveled through the dorsal tegmentum to cross the midline just below the medial longitudinal fasciculus and continued laterally and entered the contralateral DNLL and then turned dorsally to terminate in the contralateral ICC. A few years later, Probst (1902) demonstrated that a bundle of commissural fibers underwent extensive degeneration after damage to the lateral lemniscus at the level of the DNLL. In Marchi-stained materials, Probst (1902) documented that these commissural projecting axons originated from the DNLL and ventral IC, and crossed the midline to terminate in the contralateral DNLL and

ICC. Two years later this commissure was named the "commissure of Probst" by Lewandowsky (1904). Over sixty years later, Goldberg and Moore (1967) repeated these experiments and concluded that the commissure of Probst originated from the DNLL and terminated in both the contralateral DNLL and ICC. These early studies relied on the augmentation of tissue present and the removal of tissue of interest, followed by careful microscopic work dependent on artfully conceived tissue fixation and histochemical staining. Their findings were based on an anatomical technique whereby histochemical visualization of degenerating fibers was used to determine the origins and terminations of the commissure of Probst. More recently, these earlier reports have been confirmed by studies that employed both anterograde and retrograde techniques for tracing fiber projections (Bajo et al. 1993; Hutson et al. 1991; Ito et al. 1996; Kudo et al. 1981; Shneiderman et al., 1988).

Transection of the commissure of Probst was first reported by Hutson et al. (1991) who made large midline transections of the commissure of Probst in adult cats and applied HRP flakes directly to freshly cut ends of CP fibers and showed that the fibers in the CP originate exclusively from the DNLL. In frontal sections HRP-labeled neurons were seen bilaterally only in DNLL and sagulum, and not in any other auditory nuclei. The studies by Ito et al. (1996) and van Adel et al. (1997) are the only other studies to describe surgical transections of the commissure of Probst. Transection of the rat's commissure of Probst and the behavioural and electrophysiological responses to such axotomy were recently described by Ito et al. (1996) and van Adel et al. (1997). In addition to showing both the behavioural and physiological importance of this pathway for binaural processing and sound localization, anatomical data from these two studies showed that midline transection of the CP selectively eliminated all crossed projections of DNLL while ipsilateral projections seemingly remained intact, due to their uncrossed connections to the ipsilateral ICC. In a separate study double-labeling (fluorescent retrograde tract tracing combined with immunohistochemistry or histochemistry) demonstrated that transection of the

commissure of Probst resulted in a dramatic increase in the expression of neuronal nitric oxide synthase (nNOS) in contralaterally projecting FG-prelabeled DNLL neurons but not in ipsilaterally projecting FG-prelabeled DNLL neurons (van Adel et al., 1996). Taken together, our initial descriptive reports demonstrated behavioural and electrophysiological consequences of CP transections, and extensive neuronal loss in the DNLL, which was possibly mediated by excessive free-radical production (nitric oxide, NO). Our recent discovery that CP transection results in DNLL neuronal loss (Ito et al., 1996; van Adel et al., 1997) suggested that this phenomenon might be useful as a model of selective, experimentally controlled neuronal death possibly relevant to neurodegenerative disorders. However, our previous studies provided no details of the anatomical features of this process. As such the present study investigated the neuronal and non-neuronal responses to injury, cellular selectivity, and the time course of this phenomenon.

Purpose of this Study

The aim of the present study was to provide a detailed qualitative and quantitative description of axotomy-induced cell death in the DNLL using a combination of neuroanatomical and histochemical techniques. Both neuronal and non-neuronal responses to injury were investigated for survival times ranging from 1 day to 12 weeks after complete surgical transection of the commissure of Probst.

MATERIALS AND METHODS

Subjects

Animals were obtained and housed as per Experiment 1. Experimental animals were divided into five groups depending on their surgical treatment, survival time and histological processing. Table 3.1 and Figure 3.1 provide a summary of the number of animals, surgical treatments, anatomical tract tracing procedures, and histological processing required for each experimental group.

-----Insert Table 3.1, and Figure 3.1 About Here-----

Surgical Procedures

Transection of the commissure of Probst

Experimental animals (Groups 2-5) were anesthetized with Somnotol (sodium pentobarbital 65 mg/kg, ip), shaved and placed in a stereotaxic head holder. A midline incision was made in the scalp, the trapezius musculatures were retracted from the skull and a craniotomy was performed above the midline of the cerebellum. The dura was cut and a microknife (Fine Science Tools, Inc., Canada) was lowered stereotaxically to a depth of 6.0 mm with the animal's head flat and the microknife tilted backward 45° relative to the frontal plane (see Figure 3.2). The blade was drawn 3 mm in the rostral direction and then slowly lifted dorsally and removed. For control purposes, thirteen animals (Group 1) received a midline cut through the cerebellum with no damage to the auditory brainstem. A detailed description of the surgical procedure for transecting the commissure of Probst is provided in Appendix 1.

-----Insert Figure 3.2 About Here-----

Anatomical Tract Tracing Procedures

For Experiment 2, the same anterograde and retrograde tract tracers, surgical procedures, injection parameters, and histological processing were used as per Experiment 1. In the present experiment, anterograde and retrograde tract tracing procedures were used to study the anatomical consequences of transecting the commissure of Probst in adult rats. Normative data obtained with these tracers (see Experiment 1) was used to compare to the patterns of labeling seen in animals with transection of the commissure of Probst and surgical control animals with a midline cut through the cerebellum. The use and experimental goal of each tracer are briefly described below and are summarized in Table 3.1 and Figure 3.1.

Biotinylated Dextran postlabeling after CP transection

Animals were surgically prepared for tracer injections as described in Experiment 1. Prior to BD injections into the DNLL, animals received midline surgical transection of the commissure of Probst and survived a specific postoperative time period (1, 2, 4, 6, and 12 weeks, minimum of n=2 per survival time). For control purposes, three animals received a midline cut of the cerebellum approximately eight weeks prior to BD injections into the DNLL. BD was iontophoretically injected (as per Experiment 1) into the right DNLL of experimental and surgical control animals, four days prior to the end of their postoperative survival time. The rationale was to make restricted injections to ensure a small distribution of the tracer throughout the rostrocaudal extent of the DNLL which would result in specific labeling of remaining intact efferent pathways. This was performed to demonstrate the loss of terminal fields in both the contralateral DNLL and IC and determine if the CP was capable of regeneration.

Retrograde tracer injections: postlabeling after CP transection

Iontophoretic injections of different retrograde tracers (FG, FR, and HRP) were made into the ICC (Group 2) in 15 animals that survived for 8 weeks after CP transection and six surgical control animals that received a midline cut of the cerebellum. In both CP transected animals and surgical control animals retrograde tracer was injected into the ICC seven weeks postoperatively, and allowed one week for retrograde transport. Neuronal counts of ipsilaterally and contralaterally retrogradely postlabeled DNLL neurons were taken from CP transected and surgical control animals which were then compared to normal animals from Experiment 1.

Retrograde tracer injections: prelabeling before CP transection

Animals from group 3 received unilateral injections of retrograde tracer into the left ICC seven days prior to CP transection surgery. Animals were prepared for tracer injection as per Experiment 1. In this series of experiments, the goal was to obtain a time course of cell death. To accomplish this both ipsilaterally and contralaterally projecting DNLL neurons were prelabeled before transecting their axons, then the surviving population of prelabeled neurons in the ipsilateral and contralateral DNLL could be counted one to eight weeks later. Because of the long postoperative survival intervals selected in this study, HRP, which begins to be lysosomally degraded within 72 hours after uptake, was not suitable as a retrograde for prelabeling experiments. Instead, FG was chosen for the present time course study because of its longevity, intracellular stability, and more importantly, for its non-toxic properties (see Experiment 1) (Divac and Mogensen, 1990; Schmued et al., 1990). In order to standardize cell counting protocols, only those animals (experimental, surgical control, and normals) with unilateral FG injections prior to CP cutting were considered for quantitative analysis of the time course of axotomy-induced neuronal death in the DNLL.

Animals from group 4 were used to demonstrate the ability of activated microglia to selectively phagocytose degenerating DNLL neurons. This experimental paradigm is illustrated in Figure 3.1A. In these experiments, bilateral injections of fluorescent retrograde tracers (using either FG and FB, or FG and FR combinations) were made into the ICC one week prior to CP transection. Experimental animals in this group were allowed a three week survival period. The anatomical position and injection parameters were matched for each ICC to try and deliver equal amounts of fluorescent tracer to each ICC.

Histology

Perfusion and tissue sectioning

After their survival period (refer to Table 2.1) animals were re-anesthetized with an overdose of Somnotol (sodium pentobarbital, 120 mg/kg ip) and perfused transcardially with 100 mM phosphate buffered saline (PBS; pH 7.2) followed by a fixative consisting of 4% paraformaldehyde, in 100 mM PB. Animals that received BD injections were perfused with a slightly modified fixative consisting of 4% paraformaldehyde and 0.5% glutaraldehyde, in 100 mM PB. The brains were removed, postfixed for 4 hr at 4°C in the same fixative, and then stored overnight in 20% buffered sucrose. The brains were then cut serially (4 parallel series) at 40 μ m in the frontal plane on a freezing microtome and immediately processed for Nissl staining, BD and HRP histochemistry as per Experiment 1. The remaining series were collected free-floating in a cryoprotectant solution containing polyethyleneglycol and stored in a freezer at -20°C. A few sections from two animals were saved for ultrastructural studies as described below (under Ultrastructural Analysis of Degenerating DNLL Neurons). The brains from animals in Group 5 were removed, postfixed for four hours and stored overnight in 10 mM PBS. The following day, brains were trimmed into blocks containing the auditory brainstem and were paraffin embedded and later cut at a thickness of 5 μ m in the frontal plane on a rotary microtome. Paraffin

sections were mounted on coded gelatinized slides and stored in a protective slide box until processed for GFAP immunohistochemistry.

Ultrastructural analysis of degenerating DNLL neurons

For ultrastructural analysis of neuronal degeneration in the DNLL, tissue sections (40 μm) were saved from two rats that survived four weeks after CP transection. The tissue was postfixed overnight with fresh 2% paraformaldehyde and 1.0% glutaraldehyde, in 100 mM PBS. The following day tissue was washed several times in 100 mM PBS and the DNLL dissected into small blocks and treated with 2% osmium tetroxide in 100 mM PBS for two hours at room temperature. Thin sections (0.05 μm) were cut with an ultramicrotome, mounted on thin grids, stained with uranyl acetate and lead citrate, and photographed with a Philips CM12 transmission electron microscope (TEM) in the Carleton University Electron Microscopy Laboratory.

GFAP immunohistochemistry

Thin paraffin sections were deparaffined, and washed with 100 mM PBS before being incubated overnight at 4°C in monoclonal GFAP (1:600, Sigma Chemicals, U.S.A.). For controls, the primary antibody was omitted from the incubation solution. Peroxidase activity was demonstrated using DAB (in 50 mM Tris-buffer, pH 7.4, with 0.003% H_2O_2) as a chromagen followed by nickel-cobalt intensification (Adams 1981). All sections were counterstained with Pyronin Y or Neutral Red in order to differentiate immunostained astroglia from Nissl-stained neurons within DNLL, IC, and fiber tracts of the CP, and LL. Finally, sections were dehydrated in a graded series of ethanol, cleared in xylene and coverslipped.

Cell counting

The morphological criteria for counting ipsilaterally and contralaterally projecting prelabeled and postlabeled DNLL neurons were the same as described for Experiment 1. Neuronal counts of retrogradely prelabeled and postlabeled DNLL neurons were determined for each postoperative survival time. As in Experiment 1, retrogradely labeled DNLL neurons ipsilateral and contralateral to the ICC tracer-injection site were counted from every fourth section (i.e., counts were made 160 μm apart). Retrogradely prelabeled and postlabeled neurons were recognized by the appearance of fluorescence in the perinuclear cytoplasm and in proximal dendrites. Only the profiles of DNLL neurons that included a filled soma greater than 15 μm in diameter (or the nucleus was visible) were counted; those profiles containing fragments of a soma or proximal dendrites were not counted. Microglial cells, are capable of removing fluorescent debris (FG, FR, or FB), which accumulate in phagosomes and thus become fluorescently labeled themselves. Fluorescently labeled microglia were easily distinguished from fluorescently labeled DNLL neurons. Since degeneration of fluorescently-prelabeled axotomized DNLL neurons results in the phagocytosis-dependent labeling of activated microglia, cell counts of fluorescently labeled microglia were possible based on their ramified morphology identified under epifluorescence microscopy.

Statistical Analysis

Statistical analysis was performed as per Experiment 1. Cell counts of the number of ipsilaterally and contralaterally FG-labeled DNLL neurons from control and CP transected animals were entered into a computer spreadsheet. This data was analyzed using either one-way, or repeated measures analysis of variance (ANOVA), with a p value <0.05 considered significant. A post-hoc Tukey test was also conducted on the time course data to further explore observed differences between FG-prelabeled DNLL neurons and controls.

RESULTS

The results of Experiment 2 have been divided into four parts. In the first part the extent of damage caused by microsurgical knifecuts in CP transected animals is briefly described. In the second part, neuroanatomical data are presented from postlabeling experiments using both anterograde and retrograde tract tracing procedures in control animals and CP transected animals. The third part is a complete time course analysis of neuronal death of fluorescently prelabeled DNLL neurons. In the fourth part non-neuronal responses to CP transections are described based on histological examination of reactive astrocytes labeled with anti-GFAP serum, and the ability of phagocytic microglia to selectively sequester fluorescent retrograde neuronal tracers from retrogradely prelabeled axotomized DNLL neurons.

Surgical Transection of the Commissure of Probst

Before presenting neuroanatomical tract tracing results it is necessary to provide a detailed anatomical description of the lesion produced by the CP surgical technique. The extent of damage to the commissure of Probst produced in all experimental animals is illustrated in Figure 3.3. In all cases the stab wound extended rostrally to the level of the red nucleus and the dorsal tegmental decussation. A typical wound produced by CP transection was restricted to about 300-400 μm of the midline and damage lateral to the knifecut was minimal. In all cases relatively large lesions were made to ensure that all the fibers of the commissure of Probst were transected, yet these lesions were restrictive in that they did not extend dorsally to include the commissure of the inferior colliculus. Figure 3.4 shows Nissl-stained frontal sections through the auditory midbrain of the DNLL and the CP as it decussates through the midbrain tegmentum at the level of the medial longitudinal fasciculus (MLF). In Nissl-stained frontal sections from control animals with midline transection of the cerebellum there was no damage to the CP or any other auditory brainstem nuclei. In contrast, a typical wound produced by a knife cut through the CP was

easily distinguishable as a glial scar in Nissl-stained sections of experimental rats. In addition, there was an substantial cell loss (approximately 70%) in Nissl-stained sections of the DNLL from animals that survived four-eight weeks after CP transection.

-----Insert Figure 3.3, and 3.4 About Here-----

Postlabeling with Neuronal Tracers After CP Transection, Groups 1 & 2.

Anterograde tracers injections into DNLL

As shown in Figure 3.5, complete transection of the commissure of Probst affected only commissural fibers exiting the DNLL medially via the CP. BD-postlabeled commissural fibers could only be followed along the path of the CP to the wound site and did not decussate across the midline as seen in control animals. CP transected animals showed normal patterns of BD labeling in the ipsilateral ICC and SOC. In CP transected animals from each survival time (1-12 wk) there was a complete absence of BD labeled neurons, fibers, and terminals in the contralateral DNLL and ICC. In contrast, surgical controls demonstrated a pattern of anterograde labeling similar to that observed in normal animals. These data demonstrate that, the commissure of Probst is a fiber path for contralaterally projecting DNLL neurons to the opposite DNLL and ICC.

-----Insert Figure 3.5 About Here-----

Retrograde tracer injections into ICC

Retrograde tract-tracing procedures were useful in characterizing the extent and selectivity of CP lesions. Figures 3.6, and 3.7 illustrate the pattern of retrograde postlabeling in the auditory brainstem of a surgical control animal (Figure 3.6) and in a CP transected animal (Figure 3.7). In each case a large FG injection was made into the right ICC. In the control animal there was intense retrograde FG labeling in the contralateral ICC, DNLL, LSO, and CN, and in the ipsilateral DNLL, INLL, VNLL, LSO, MSO, and

SPN. The pattern of FG labeling was virtually identical to normal animals with unilateral retrograde tracer injections into the ICC as presented in Experiment 1.

----Insert Figure 3.6 About Here----

As shown in Figure 3.7, animals with complete transection of the CP had no FG-postlabeled neurons in the contralateral DNLL, although a large number of labeled cells were found in the ipsilateral DNLL. The distribution of FG labeling in the contralateral ICC was similar to that found in surgical control cases, which indicates that CP lesions did not extend dorsally to include the commissure of the inferior colliculus (cic). The pattern of FG labeling in the contralateral LSO and CN, and the ipsilateral INLL, VNLL, LSO, MSO, SPN, and CN resembled patterns of retrograde FG transport seen in surgical control animals and normal animals from Experiment 1.

----Insert Figure 3.7 About Here----

Figure 3.8 shows retrograde postlabeling in control and CP transected animals with retrograde tracer injection into the left ICC. (Figures 3.6 and 3.7 presented above had FG injections into the right ICC). Control animals showed intense retrograde labeling of DNLL neurons both ipsilateral and contralateral to the injection site, whereas CP transected animals had no FG-labeled neurons in the contralateral DNLL. Similar results were obtained using retrograde tracers other than FG. For example, Figure 3.9 illustrates the pattern of postlabeling in the ipsilateral and contralateral DNLL in CP transected animals following injection of either HRP or FR into the right ICC. As with FG-postlabeling cases, no FR-postlabeled neurons or HRP-postlabeled neurons were seen in the contralateral DNLL, whereas many retrogradely labeled neurons were found in the DNLL ipsilateral to the ICC injection site.

----Insert Figure 3.7, 3.8, and 3.9 About Here----

Retrograde postlabeling neuronal counts

Cell counts from six surgical control animals are summarized in Table 3.2 and Figure 3.10. Since the procedure for neuronal counting in the present study was identical to the procedure used in Experiment 1, the number of retrogradely postlabeled neurons in the ipsilateral and contralateral DNLL of surgical controls and CP transected animals was compared to normal animals from Experiment 1. As shown in Table 3.2, the number of neurons found in the ipsilateral and contralateral DNLL in surgical controls was similar to that found in normal animals. Although the actual number of ipsilaterally and contralaterally labeled DNLL neurons varied from case to case, the percentage of neurons projecting to the ICC from the ipsilateral (30%) and contralateral DNLL (70%) did not vary from case to case. The analysis of variance shows that the number of retrogradely postlabeled neurons in the ipsilateral and contralateral DNLL was not significantly different in surgical control and normal animals ($F_{(1,10)} = 0.89$, $p > 0.05$). Figure 3.10 shows that no significant differences were found for the percent of ipsilaterally and contralaterally labeled DNLL neurons between surgical controls and normal animals. These data suggest that the stereotaxic deposition of tracer was similar for each case and that the variation in neuronal counts between cases is most likely attributed to the size and duration of the ICC injection and not the locus of the injection or surgical transection of the cerebellum.

----Insert Table 3.2, and Figure 3.10 About Here----

In animals with complete transection of the commissure of Probst, there was a remarkable cell loss in the DNLL as shown in Table 3.3 and Figure 3.11. By eight weeks, virtually no contralaterally projecting DNLL neurons survived CP transection as

demonstrated by a complete absence of retrograde transport following unilateral injection of tracer into the ICC. In contrast, the number and distribution of ipsilaterally, retrogradely postlabeled neurons remained virtually unchanged. Figure 3.11A shows that there was no significant difference ($F_{(2,20)} = 0.45$, $p > 0.05$) between the number of ipsilaterally labeled neurons in normal animals from Experiment 1 and surgical control animals and CP transected animals from the present study. Indeed, as Figure 3.11B shows, only CP transected animals showed no retrograde postlabeling in the contralateral DNLL. Figure 3.12 summarizes the results of postlabeling with anterograde and retrograde tracers in the auditory brainstem following control and CP transection surgeries.

----Insert Table 3.3, Figure 3.11, and 3.12 About Here----

Prelabeling with Retrograde Tracers Before CP Transection, Groups 3 & 4.

Neuronal responses to injury and time course analysis

A qualitative and quantitative time course analysis of neuronal degeneration was accomplished by first retrogradely prelabeling auditory neurons with FG. Examination of FG-prelabeled, axotomized DNLL neurons provided additional evidence that only contralaterally projecting DNLL neurons are affected by CP transection. Figure 3.13 demonstrates the pattern and distribution of FG-prelabeled neurons seen bilaterally in the DNLL after FG injection into the left IC of animals with varying postsurgical survival times (1, 2, 3, 4, and 8 week). Table 3.4 provides cell counts of ipsilaterally and contralaterally prelabeled DNLL neurons for each survival group.

One and two weeks following CP transection, the pattern and distribution of FG-prelabeled neurons seen bilaterally in the DNLL appeared similar to control animals. At both survival times (1 and 2 weeks) slightly more than 30% of FG-prelabeled neurons were found in the ipsilateral DNLL and slightly less than 70% in the contralateral DNLL. Experimental animals surviving three weeks following CP transection the number of FG-

prelabeled ipsilaterally projecting DNLL neurons (44%) was slightly less than the number of FG-prelabeled contralaterally projecting DNLL neurons (56%). By four weeks after CP transection, there was fewer FG-prelabeled neurons in the contralateral DNLL (25%) compared to the number of FG-prelabeled neurons in the ipsilateral DNLL (75%). In animals that survived eight weeks after CP transection, virtually no contralaterally FG neurons were found (12%). A one-way analysis of variance was performed to compare the number of contralaterally FG-prelabeled neurons for controls and CP transected animals at different survival times. A significant difference in the number of FG-prelabeled contralaterally projecting DNLL neurons was found for CP transected animals compared to controls ($F_{(5,35)} = 12.70$, $p < 0.0001$). A post-hoc Tukey indicated that there were significant reduction in the number of FG-prelabeled contralaterally projecting DNLL neurons after survival times of 3, 4, and 5 weeks compared to controls (see Table 3.4 and Figure 3.14). There was a slight decrease in the number of ipsilaterally FG-prelabeled DNLL neurons in CP transected animals surviving four to eight weeks, however the extent of cell loss was minimal in comparison to neuronal death that had occurred in the contralateral DNLL for these survival times. A one-way analysis of variance indicated that there was no significant differences in the number of FG-prelabeled ipsilaterally projecting DNLL neurons for each survival time compared to normals ($F_{(5,35)} = 1.53$, $p > 0.05$). The reduced ipsilateral cell numbers found at these survival times may have arisen, not due to cell death, but because there were only three animals in this group each had a slightly small injection than controls, which would have prelabeled fewer neurons both ipsilaterally and contralaterally. Furthermore, postlabeling data demonstrated that ipsilaterally projecting neurons survive CP transection with no significant cell loss comparing normal, control, and CP transected animals ($F_{(2,20)} = 0.45$, $p > 0.05$). Figure 3.14 shows the time course of DNLL neuronal death based on the counts of FG-prelabeling data. As survival time increases, the number of contralaterally projecting neurons drastically decreases, beginning

at two weeks after CP transection. By eight weeks after CP transection, virtually all contralaterally prelabeled DNLL neurons had died.

-----Insert Table 3.4, and Figure 3.13, and 3.14 About Here-----

Figure 3.15 shows the pattern of FG-prelabeling in the auditory brainstem of CP transected animals with short (1 wk) or extended (8 wk) postsurgical survival times. In contrast to the neurodegenerative changes observed in the DNLL as survival times increased, other auditory brainstem nuclei showed little or no difference in FG retrograde labeling at any of the survival times examined in this study. Specifically, the patterns of retrograde labeling in the ipsilateral INLL and VNLL, the ipsilateral and contralateral SOC, and the contralateral CN, did not change over time and appeared the same as that in surgical control animals or unoperated normal animals.

-----Insert Figure 3.15 About Here-----

Visual examination of fluorescently prelabeled frontal sections reveals considerable neuronal degeneration in the DNLL based on the loss of neuronal FG-prelabeled neurons and an increase in the number of fluorescently labeled microglial cells. Figure 3.16 shows a series of high power photomicrographs of contralaterally FG-prelabeled axotomized neurons at different survival times. The number of contralaterally FG-prelabeled DNLL neurons was unchanged one and two weeks after CP transection. However, the pattern of FG labeling observed between three and eight weeks after CP transection was characteristic of degenerating neurons as noted by a drastic reduction in the number of FG-prelabeled neuronal perikarya. Further examination of this series of high power photomicrographs shows phagocytosis-dependent FG-labeled microglia in close proximity to degenerating DNLL neurons. Cell counts of activated (ramified morphology) microglia were obtained

from the same tissue samples used to make neuronal counts for each postoperative survival time. Microglial cell counts from the ipsilateral and contralateral DNLL are included in Table 3.4 with the neuronal counts from the same group of animals, and summarized in Figure 3.17A. Figure 3.17B shows that the time course of activated phagocytic microglia in the DNLL was coincident with the time course of neuronal death. The fact that no FG-labeled microglia were found in the DNLL ipsilateral to the ICC injection site provides further evidence that ipsilaterally projecting DNLL neurons survive CP transection. The absence of activated microglia in the DNLL ipsilateral to the ICC injection site further supports the result of cell counting from postlabeling data presented earlier in this study. The ability of activated microglial cells to selectively phagocytose only contralaterally projecting axotomized DNLL neurons was further examined using the technique of fluorescent double-prelabeling.

-----Insert Figure 3.16, and 3.17 About Here-----

Selective microglial phagocytosis

The ability of microglial cells to selectively phagocytose only those neurons destined to die was investigated further. Both ipsilaterally and contralaterally projecting DNLL neurons were prelabeled with different fluorescent tracers before CP lesions were made. Figure 3.18 shows the pattern of double fluorescent prelabeling in CP transected animals (one week survival, Figure 3.18A and A'; three week survival, Figure 3.18B and B') with injection of FG into the left ICC and FR into the right ICC. In both cases, ipsilaterally projecting DNLL neurons were prelabeled with FR and contralaterally projecting DNLL neuron were prelabeled with FG. In the animal that survived for one week after CP transection, both FG and FR-prelabeled DNLL neurons appeared morphological normal (see Figure 3.18A and A'). However, in the animal that survived for 3 weeks after CP transection, the contralaterally FG-prelabeled axotomized DNLL neurons appeared abnormal, whereas the ipsilaterally FR-prelabeled non-axotomized

DNLL neurons appear similar to fluorescently retrogradely labeled neurons in control and normal animals. Furthermore, microglia in this case were labeled with FG only, no FR-labeled microglial cells were detected. Similar results were obtained using double-labeling procedures with a combination of FG and FB (data not shown). Figure 3.19 summarizes the results obtained from double-labeling techniques demonstrating the selective ability of activated microglial cells to sequester fluorescent retrograde tracer from dying prelabeled neurons. These data strongly indicate that microglial cells selectively sequester fluorescent tracer exclusively from the injured contralaterally projecting DNLL neurons, which are destined to die, and not from uninjured ipsilaterally projecting DNLL neurons even though both types of neurons are closely juxtaposed.

-----Insert Figure 3.18, and Figure 3.19 About Here-----

Ultrastructural Morphology of Axotomy-Induced Cell Death in the DNLL.

Transmission electron microscopy

As mentioned above, an extensive morphometric analysis of the ultrastructural changes in axotomized DNLL neurons was limited to two animals that survived for 4 weeks after CP transection. These cases were used for further study of the neuronal degenerative changes of axotomized DNLL neurons. Representative electron micrographs of DNLL neurons are provided in Figure 3.20. Four weeks after CP transection, numerous axotomized DNLL neurons were observed at different stages of neuronal degeneration. A few morphologically normal neurons can be seen and are presumably intact ipsilaterally projecting neurons. Many neurons contained an irregularly shaped nucleus with condensed chromatin and relatively intact organelles, which are morphological features of apoptotic cell death. However, with such a small sample size, the presence or absence of necrotic cells cannot be confirmed. Further work is need in order to determine

the time course of neuronal degeneration of contralaterally projecting DNLL neurons at the ultrastructural level.

-----Insert Figure 3.20 About Here-----

Astrocyte reactivity after CP Transection, Group 5.

In uninjured animals the commissure of Probst, DNLL, and IC did not show GFAP immunoreactive astrocytes. However, one day after transection of the commissure of Probst, robust reactive GFAP-positive astrocytes were observed throughout the midbrain tegmentum with the greatest concentration near the wound site. This same pattern of GFAP labeled astrocytes was observed for all survival times, indicating that reactive astrocytes display an early and a late response to CP transection. Figure 3.21 shows GFAP-staining in an animal that survived four weeks after CP transection. This case shows that transection of the commissure of Probst results in a dramatic increase in GFAP-positive astrocytes at the wound site, along the fibers of the CP, and bilaterally in both DNLL.

-----Insert Figure 3.21 About Here-----

Summary of Results

In the present study the use of anterograde and retrograde tract tracing procedures were effective in determining both the outcome of CP transection and the time course of neuronal degeneration in the DNLL. The pattern of pathology reflects the complete and selective retrograde degeneration of those DNLL neurons that had a contralateral projection through the commissure of Probst. Ipsilaterally projecting DNLL neurons were spared by transection of the commissure of Probst as revealed by the pattern of anterograde and retrograde postlabeling. FG-prelabeling was effective in following the progressive changes in contralaterally and ipsilaterally projecting DNLL neurons and for studying the activation of phagocytic microglia. Time course analysis of neuronal degeneration in the DNLL

revealed a process of delayed neuronal death, which began 2 weeks after the CP was transected and continued for the next 6 weeks and resulted in the loss of virtually every contralaterally projecting DNLL neuron. Fluorescent double-labeling techniques confirmed that contralaterally projecting DNLL neurons were phagocytosed by activated (ramified morphology) microglial cells while intact ipsilaterally projecting DNLL neurons were not. Astrocytes showed an early response to CP transection as evident by a large glia scar that had formed at the site of the wound as early as one day after CP transection and remained unchanged the 12 weeks. GFAP-immunoreactive astrocytes were detected along the length of the commissure of Probst and bilaterally in the DNLL for all survival times studied in Experiment 2. Figure 3.2

Table. 3.1: Summary of experimental groups and surgical treatments used for animals from Experiment 2.

Experimental Group	Surgical Treatment	Number of Animals ¹	Survival Time (wk)	Histological Processing	Experimental Goal
Group 1 Surgical Controls	Cerebellum Transection & FG Postlabeling (ICC Injection)	n=10	8	Anterograde & Retrograde Tracers	Control group to demonstrate the pattern of FG and BD postlabeling in surgical control animals with midline transection of the cerebellum.
	Cerebellum Transection & BD Postlabeling (DNLL Injection)	n=3	8	Nissl-Staining	
Group 2 CP Axotomy & Postlabeling	Midline CP Transection & FG, HRP, & FR Postlabeling (ICC Injection)	n=15	8	Anterograde & Retrograde Tracers	To determine the pattern of anterograde and retrograde post labeling in CP transected animals
	Midline CP Transection & BD Postlabeling (ICC Injection)	minimum of n=2 per survival time	1, 2, 4, 6, & 12	Nissl-Staining	
Group 3 CP Axotomy & Prelabeling	Midline CP Transection & FG Prelabeling (ICC Injection) ²	minimum of n=3 per survival time	1, 2, 3, 4, & 8	Retrograde Tracer Nissl-Staining	Neuronal responses to injury - time course analysis of FG prelabeled DNLL neurons
Group 4 CP Axotomy and Reactive Microglia	Midline CP Transection - FG & FB or FR bilateral ICC Injections ²	n=6	3	Retrograde Tracer Nissl-Staining	To demonstrate the selective ability of activated Microglia to phagocytose only injured (axotomized) DNLL Neurons
Group 5 CP Axotomy and Reactive Astrocytes	Midline CP Transection	minimum of n=3 per survival time	1 day, 1, 2, 3, 4, 8, & 12 weeks	GFAP, GS, & Nissl Staining on thin paraffin sections	To investigate the role of reactive astrocytes in neuronal injury

¹ All animals included in this study were adult male Wistar rats

² Tracer was injected into the ICC 1 week prior to CP transection

Table 3.2: Counts of retrogradely labeled DNLL neurons in surgical control animals with midline transection of the cerebellum. **A:** the number of ipsilaterally and contralaterally labeled DNLL neurons in surgical control animals. In this group of animals FG was injected into the left ICC seven weeks postoperatively and then allowed one week for retrograde transport. **B:** for comparative purposes normative data is shown from Experiment 1. **Note:** there was no significant difference in the number of ipsilaterally and contralaterally postlabeled DNLL neurons among surgical controls and normal animals. Cell counts are from every fourth section, values are means $\pm$ S.E.M. for $n = 6$ for each group. $P > 0.05$ for surgical controls vs. normals by ANOVA.

A Surgical Control Subjects: Experiment 2

Subject ID	FG labeled DNLL Neurons		% ipsi	% contra
	ipsi	contra		
Columbus-194	232	615	27	73
Columbus-196	239	647	27	73
Columbus-103	199	366	33	67
Columbus-106	114	210	35	65
Columbus-119	84	242	26	74
Columbus-130	90	196	30	70
MEAN $\pm$ SEM	160 $\pm$ 29.0	379 $\pm$ 83.0	30.3 $\pm$ 1.7	69.7 $\pm$ 1.7

B Normal Subjects: Experiment 1

	FG Labeled DNLL Neurons		% ipsi	% contra
	Ipsi	Contra		
MEAN $\pm$ SEM	185 $\pm$ 20.0	471 $\pm$ 39.0	28.0 $\pm$ 1.1	72.0 $\pm$ 1.1

($F_{(1,10)} = 0.89$, $p > 0.05$, n.s)

Table 3.3: Counts of retrogradely postlabeled DNLL neurons in CP transected animals. Note: Cell counts are from every fourth section, values are means $\pm$ S.E.M.

Subject	Survival Time	Tracer Injected	Ipsi DNLL Labeling	Contra DNLL Labeling
Columbus-257	8 weeks	FG	163	0
Columbus-260	8 weeks	HRP	122	7
Columbus-262	8 weeks	HRP	103	0
Columbus-264	8 weeks	HRP	140	0
Columbus-282	8 weeks	FG	275	0
Columbus-283	8 weeks	FG	264	0
Columbus-284	8 weeks	FG	191	0
Columbus-285	8 weeks	FG	217	0
Columbus-286	8 weeks	FG	274	1
Columbus-134	8 weeks	FG	141	0
Columbus-310	8-weeks	FG	181	2
MEAN $\pm$ S.E.M.			188.3 $\pm$ 18.7	0.9 $\pm$ 0.6

Table 3.4: Counts of FG-prelabeled DNLL neurons and FG-labeled phagocytic microglia in CP transected animals. Note: Cell counts of ipsilaterally and contralaterally FG-prelabeled DNLL neurons and FG-labeled microglia (via phagocytosis) are from every fourth section, values are means $\pm$ S.E.M.

Subject ID	Survival Time	FG-Prelabeled Neurons		Phagocytic Microglia	
		iDNLL	cDNLL	iDNLL	cDNLL
Columbus-207	1 week	191	412	0	0
Columbus -217	1 week	241	482	0	0
Columbus -281	1 week	95	223	0	0
Columbus -282	1 week	118	285	0	0
Columbus -283	1 week	159	363	0	0
Columbus -251	1 week	152	267	0	0
MEAN $\pm$ S.E.M.		159 $\pm$ 21	339 $\pm$ 40.0	-	-
Columbus -218	2 weeks	292	463	0	59
Columbus -204	2 weeks	248	399	0	46
Columbus -279	2 weeks	105	226	0	0
Columbus -280	2 weeks	212	406	0	0
Columbus -219	2 weeks	110	269	0	0
MEAN $\pm$ S.E.M.		193 $\pm$ 37	352 $\pm$ 45	-	21 $\pm$ 13
Columbus -206	3-weeks	187	152	0	105
Columbus -208	3 weeks	223	278	0	80
Columbus -271	3 weeks	145	141	0	74
Columbus -253	3 weeks	122	256	0	60
Columbus-256	3 weeks	87	115	0	73
MEAN $\pm$ S.E.M.		153 $\pm$ 24	188 $\pm$ 33	-	78.4 $\pm$ 7
Columbus -209	4 weeks	199	84	0	219
Columbus -210	4 weeks	131	45	0	239
Columbus -228	4 weeks	90	27	0	82
Columbus -229	4 weeks	87	45	0	165
Columbus -276	4 weeks	108	33	0	203
MEAN $\pm$ S.E.M.		123 $\pm$ 21	47 $\pm$ 10	0	182 $\pm$ 30
Columbus -219	8 weeks	75	7	0	231
Columbus -220	8 weeks	70	9	0	162
Columbus-221	8 weeks	143	19	0	373
MEAN $\pm$ S.E.M.		96 $\pm$ 24	12 $\pm$ 4	0	255 $\pm$ 62

Figure 3.1: A schematic illustration of the anterograde and retrograde tract tracing procedures used in Experiment 2. **A:** DNLL neurons were prelabeled with fluorescent retrograde tracers 1 week prior to cutting the commissure of Probst. Prelabeling DNLL neurons prior to transecting their neurons provided a time course analysis of the rate of neuronal degeneration (cell death) in the DNLL. The technique of double labeling was used to prelabel both ipsilaterally and contralaterally projecting neuronal populations in the DNLL. These cases were used to study the ability of activated microglia to selectively sequester fluorescent neuronal tracers from axotomized neurons. **B:** Postlabeling studies with both anterograde and retrograde neuronal tracers were carried out to determine auditory structures affected by surgical transection of the commissure of Probst. Note that the same procedures were carried out in surgical control animals that received midline transection through the cerebellum.

A

Prelabeling Before CP Transection

B

Postlabeling After CP Transection

Figure 3.2. A schematic diagram of a sagittal section demonstrating the procedure of transecting the commissure of Probst.

Surgical Transection of the Commissure of Probst

Figure 3.3: Schematic drawing showing the rostrocaudal extent of the damage to the CP produced by microsurgical midline knifecuts.

Restricted Transection

Extensive Transection

Figure 3.4: Photomicrographs of frontal sections through the midbrain tegmentum illustrating Nissl-staining of the CP and the DNLL in a surgical control animal (A and B) and a CP transection animal (C and D). Scale bars, 200 μm .

Figure 3.5: Anterograde postlabeling with BD in a surgical control animal and an animal that survived 1 week after CP transection. A: BD-labeled fibers of the commissure of Probst. **B:** BD injection site into the right DNLL. **C:** BD-labelling in the contralateral DNLL **D:** BD-labeled fibers originating from the right DNLL can only be followed medially along the CP to the site of injury. **E:** BD injection site into the right DNLL of a CP transected animal. **F:** Complete absence of labeling in the contralateral DNLL.

Figure 3.6: The pattern of retrograde labeling in the brainstem auditory nuclei after a large FG injection into the right ICC of a sham operated animal (Columbus-19). A: FG injection site. B: contralateral ICC. C and D: FG labeling in DNLL contralateral (left) and ipsilateral (right) to the injection site. E and F: FG labeling in SOC contralateral and ipsilateral to the ICC injection site. G: contralateral CN. H: ipsilateral CN. Scale bars, 200 μm .

Figure 3.7: The pattern of retrograde labeling in the brainstem auditory nuclei after a large FG injection into the right ICC of an animal (Columbus-10) with a complete transection of the CP. A: FG injection site in the right ICC. B: contralateral ICC. C and D: FG labeling in DNLL contralateral (left) and ipsilateral (right) to the injection site. E and F: FG labeling in SOC contralateral and ipsilateral to the ICC injection site. G: contralateral CN. H: ipsilateral CN. Scale bars, 200 μm .

Figure 3.8: FG-postlabeling in DNLL and SOC after an injection of FG into the left ICC in a surgical control animal (A-D) and an animal which survived 8 weeks following complete transection of the commissure of Probst (E-H). A and B: FG postlabeling in DNLL ipsilateral (left) and contralateral (right) to the injection site. C and D: ipsilateral and contralateral SOC. E and F: ipsilateral and contralateral DNLL. No labeled neurons were seen in the contralateral DNLL. G and H: ipsilateral and contralateral SOC. Scale bars, 200 μ m.

Figure 3.9: HRP and FR-postlabeling in the DNLL of different animals which survived 8 weeks following complete transection of the commissure of Probst. A and B: HRP postlabeling in DNLL ipsilateral (left) and contralateral (right) to the HRP injection site in the ICC. C and D: FR postlabeling in DNLL ipsilateral (left) and contralateral (right) to the FR injection site in the ICC. Magnification, 300x.

Figure 3.10: Percentage of ipsilaterally and contralaterally projecting DNLL neurons in normal animals and surgical control animals. The percentages of ipsilaterally and contralaterally projecting neurons were determined by counting the number of FG-labeled neurons in the ipsilateral and contralateral DNLL in normal animals (n=6), and surgical control animals (n=6) with midline transection of the cerebellum. The percentages given represent the means and standard errors. $P>0.05$ for surgical control vs. normals by ANOVA.

Figure 3.11: Summary of FG-postlabeling in the DNLL from surgical control animals, CP transected animals, and normal unoperated animals A: The number of FG labeled neurons in the DNLL ipsilateral to the injection site is the same in normal animals (n=6), control lesioned animals (n=6), and CP transected animals (n=11). **B:** Summary of DNLL retrograde labeling experiments in normal, control lesioned, and CP transected animals. Note that there is no significant differences in the number of ipsilaterally FG-postlabeled neurons between the groups. CP transection results in a complete absence of FG retrograde labeling in the contralateral DNLL of experimental animals. Data are given as mean $\pm$ S.E.M. Note: in a $P > 0.05$ for CP transected vs. surgical control and normals by ANOVA.

Figure 3.12: Schematic diagram summarizing the patterns of anterograde and retrograde postlabeling in the auditory brainstem of surgical control animals and CP transected animals. A and A': the pattern of BD postlabeling in surgical control animals (A) and CP transected animals (A'). **B and B':** FG postlabeling in the auditory brainstem surgical control animals (B) and CP transected animals (B').

Notice how transection of the CP completely eliminated the transport of Fluorogold (FG) from the central nucleus of the inferior colliculus (ICC) to the opposite DNLL and blocked the anterograde transport of biotinylated dextran (BD) to either contralateral DNLL or ICC and not in control animals with surgical midline transection of the cerebellum.

Summary of BD and FG Postlabeling in Experimental Animals

Figure 3.13: Fluorescence photomicrographs showing the pattern of FG-prelabeling in the ipsilateral and contralateral DNLL at various times after CP transection. 1wk and 2wk: FG-prelabeled neurons in the ipsilateral (left) and contralateral (right) DNLL seem morphologically normal. Note the well-labeled cell bodies and the extensive filling of long primary dendrites. **4wk:** at this survival time the number of FG-prelabeled neurons in the contralateral DNLL is markedly reduced. **8wk:** no FG labeled DNLL neurons are detected. Scale bar, 200 μm .

Figure 3.14: Time course of neuronal death in the DNLL after surgical transection of the commissure of Probst. A: delayed neuronal death in the contralateral DNLL after surgical transection of the commissure of Probst. Closed circles represent fluorescent cell counts from the contralateral DNLL (cDNLL), and open circles represent counts of ipsilateral DNLL (iDNLL). Values are expressed as means $\pm$ S.E.M.

DNLL DELAYED NEURONAL DEATH

Figure 3.15: FG-prelabeling in VNLL, SOC and CN after an injection of FG into the left ICC in animals which survived one week (A-D), and 8 weeks (A'-D') following complete transection of the commissure of Probst. No secondary neuronal degeneration or FG-positive microglia were detected in other auditory brainstem nuclei in animals with CP lesions.

Figure 3.16: High power fluorescence photomicrographs further demonstrating the extent of retrograde degeneration observed in FG pre-labeled contralaterally projecting DNLL neurons. A: One week after CP transection. B: three weeks after CP cutting, C: four weeks after CP cutting. D: By 8 weeks the number of FG labelled DNLL neurons is markedly reduced. Note, the densely fluorescent cells are presumably microglia, which have sequestered FG from degenerated neurons. Scale bar, 200 mm.

Figure 3.17: Graphs showing the time course of neuronal degeneration in the DNLL after surgical transection of the commissure of Probst. A: Time course of activated microglia in the DNLL at different survival times following CP transection. Closed squares, cDNLL, and open squares, iDNLL. **B:** Summary of axotomy-induced neuronal degeneration in the DNLL. As survival times increase the number of FG-prelabeled neurons (closed circles) decrease with coincidental increase in the number of FG-labeled activated microglia (closed squares). Values are expressed as means $\pm$ S.E.M.

Figure 3.18: Fluorescence photomicrographs demonstrate the selective ability of activated microglia to phagocytose only transected contralaterally projecting DNLL neurons. A: FG pre-labeling of transected contralaterally projecting DNLL neurons in an animal that survived 1 week after CP transection. **A': FR labeling** of uninjured ipsilaterally projecting DNLL neurons in the same tissue section following a large injection of this tracer into the right IC. **B:** by three weeks post-transection **microglia** appear to have selectively sequestered FG from contralaterally projecting (injured) neurons prelabeled with FG. **B':** no FR-labeled microglial in the same tissue section.

Figure 3.19: Schematic summary of fluorescent double-labeling experiments demonstrating the selective ability of activated microglia to phagocytose only transected contralaterally projecting DNLL neurons.

Figure 3.20: Ultrastructure of DNLL neuronal nuclei 4 weeks after surgical transection of the commissure of Probst. A: a normal DNLL neuron. B, C, and D: examples of DNLL neurons at various stages of degeneration. Many neuronal nuclei were seen with highly condensed chromatin and indentation of nuclei. Magnification: 82000x in A and D; 10200x in B and C.

Figure 3.21: Photomicrographs of GFAP immunoreactivity in the DNLL four weeks after complete surgical transection of the commissure of Probst. **A:** GFAP-reactivity can be seen at the wound site and throughout the surrounding parenchyma. **B and C:** higher magnification of cells in A reveals GFAP immunoreactive astrocytes in the DNLL (B) GFAP-immunoreactive astrocytes at the lesion site (C). Scale bar, 200 μm .

Figure 3.22: Schematic representation of neuronal degeneration of transected contralaterally projecting DNLL neurons and the activation of reactive astrocytes and phagocytic microglia in the DNLL following surgical transection of the commissure of Probst.

- 1) Axotomized contralaterally projecting DNLL neurons are vulnerable to high extracellular glutamate concentrations after neuronal injury.
- 2) Overstimulation of NMDA-receptors initiates a series of events that leads to delayed neuronal death via nitric oxide-mediated neurotoxicity (see Dawson et al., 1993).
- 3) Energy depletion leads to loss of membrane phospholipid asymmetry and translocation of phosphatidylserine (PS) to the outer leaflet of the plasma membrane (see Bennett et al., 1995).
- 4) Activated microglia recognize exposed PS on the outer leaflet of plasma membrane and selectively phagocytose only injured (axotomized) DNLL neurons.
- 5) Uninjured ipsilaterally projecting neurons survive CP transection based on retrograde tract tracing studies.
- 6) Reactive astrocytes increase expression of GFAP which may lower extracellular glutamate concentrations in the vicinity of uninjured DNLL neurons.
- 7) The interaction between astrocytes and microglia following traumatic brain injury is not fully understood and further studies are needed to elucidate molecular signaling between these two types of glial cells.
- 8) Finally, our results suggest that surgical transection of the commissure of Probst is an excellent *in vivo* model to investigate neuronal and non-neuronal response to traumatic brain injury.

Axotomy-Induced Delayed Neuronal Death in the DNLL

DISCUSSION

The principal findings of Experiment 2 were that the commissure of Probst is the exclusive pathway for contralaterally projecting DNLL neurons and that surgical transection of the commissure of Probst results in extensive neuronal death in the DNLL. These findings are further discussed below.

Transection of the commissure of Probst: Anatomical Considerations.

Postlabeling with neuronal tracers

Anatomical data from the present study indicate that in the rat, as in the cat (Glendenning et al., 1981; Hutson et al., 1991), all crossed DNLL projections are routed through the CP. In control animals with large unilateral injections of FG into the ICC, both contralateral and ipsilateral DNLL were heavily labeled. In addition, normal patterns of labeling were seen in other brainstem auditory nuclei such as the contralateral ICC, both contralateral and ipsilateral SOC, and the contralateral CN. In CP transected animals normal patterns of FG labeling occurred in all auditory brainstem nuclei with the exception of the contralateral DNLL where no FG-labeled neurons were seen. Cell counts showed that no contralaterally projecting DNLL neurons regenerated an axon to their original target in the contralateral ICC. Furthermore, cell counts showed that there was no loss of ipsilaterally projecting DNLL neurons in CP transected animals.

A similar absence of labeling in the contralateral DNLL was seen using anterograde tract tracing techniques following injections of BD into the right DNLL of CP transected animals. In control animals, anterograde transport of BD was observed in many efferent axons leaving the DNLL injection site via three distinct directions (medial, dorsal, and ventral) and could be followed to targeted nuclei, where densely labeled terminal fields were observed in the ipsilateral ICC and in both the contralateral DNLL and ICC; an identical pattern was observed in normal animals from Experiment 1. Anterograde

transport of BD to the contralateral DNLL and ICC was completely eliminated by midline transection of the CP for all survival times in this study.

Retrograde prelabeling experiments

The pattern of FG-prelabeling in control and CP transected animals was also effective for demonstrating that the CP is an obligatory pathway for neurons projecting from DNLL to the contralateral IC in the rat. The pattern of FG-prelabeling in control and CP transected animals was similar with the exception of the contralateral DNLL. Eight weeks after transection of the CP no contralaterally projecting DNLL neurons were seen, whereas all other structures usually labeled after ICC injections appeared normal. Projections to the ICC from the contralateral IC via the commissure of inferior colliculus, the ipsilateral DNLL, INLL, and VNLL, the SOC and CN were all intact in CP transected rats. This demonstrates that all other projections of the auditory brainstem remain intact in animals with extensive lesions of the commissure of Probst.

Together, both anterograde and retrograde tract tracing data from the present study confirm previous anatomical data that a specific population of DNLL neurons projects contralaterally to the DNLL and ICC via the CP, and that a much larger proportion of the DNLL neurons project contralaterally (Bajo et al. 1993; Hutson et al. 1991; Ito et al. 1996; van Adel et al., 1997). It seems reasonable to conclude that the vast majority of the crossed ascending projections from the DNLL to the contralateral DNLL and IC pass through the commissure of Probst and that in adult rats no reinnervation occurs in this system after transection.

Selectivity of Transection of the Commissure of Probst

Perhaps the most striking feature of this study is the selectivity in the time course of neuronal death seen in FG-prelabeled, contralaterally projecting DNLL neurons. Selective DNLL neuronal death is delayed for at least two weeks following CP transection and then

continues rapidly over the next few weeks. Cell counts of FG-prelabeled contralaterally projecting DNLL neurons showed a progressive reduction in numbers with increasing survival times, with the greatest rate of loss occurring between two and four weeks. Cell counts of prelabeled ipsilaterally projecting neurons showed little or no cell loss four and eight weeks after CP transection. Cell counts of postlabeled ipsilaterally projection DNLL neurons showed no significant difference between the number of ipsilaterally projecting, retrogradely labeled neurons in the DNLL of normal, control, and CP transected animals that survived for eight weeks.

In addition to the selective neuronal response to CP transection, the response of non-neuronal cells, both microglia and astrocytes, showed similar patterns of selectivity in their response to CP transection. Microglia and astrocytes alter their structural and physiological capacities to form part of the complex cellular and molecular mechanisms associated with neuronal injury (Aschner, 1998; Kreutzberg, 1996; Minghetti and Levi, 1998). Microglia which can phagocytose necrotic and apoptotic neuronal debris, may also contribute to death of viable neurons when they become activated after neuronal injury. Astrocytes, on the other hand, hypertrophy, increasing their expression of glial fibrillary acidic protein (GFAP), and glutamine synthetase (GS) which may have neuroprotective benefits.

In the present study it was shown that CP transection results in the activation of phagocytic microglia which were capable of selectively sequestering retrogradely prelabeled DNLL neurons. The death of FG-prelabeled DNLL neurons resulted in the phagocytosis-dependent fluorescent labeling of ramified microglial cells. Cell counts of fluorescently-labeled microglia indicated that these cells are capable of selectively sequestering retrograde tracer from degenerating DNLL neurons and that the temporal pattern of phagocytosis-dependent labeling of microglia is coincidentally similar to the time course of neuronal degeneration in the DNLL. Double-fluorescent labeling procedures further demonstrate the selective ability of microglia to participate in active phagocytosis of dying neurons that are

in close proximity to intact non-axotomized neurons. The ability of microglial cells to selectively sequester neuronal and axonal debris has been described in other axonal injury studies (Rinaman *et al.*, 1991; Kreutzberg, 1996). A number of fundamental questions regarding the role of microglial cells in neurodegenerative disorders might be answered by examining these cells while they interact with prelabeled, axotomized DNLL neurons.

The response of astrocytes to CP lesions was determined by immunolabeling this form of glial cell with anti-serum to GFAP, the most commonly used marker for reactive astrocytes (Bignami *et al.* 1972). An increase in cytoplasmic glial fibers and formation of bundles of intermediate filaments (IF), are the main factors in the formation of glia scars at a site of injury. Astrocytes have been shown to proliferate following CNS injuries that disrupt the blood brain barrier (BBB), however, this phenomenon is mainly confined to the site of injury (Miyake *et al.* 1992). Following transection of the CP, extensive GFAP-positive astrocytes were seen around the wound site, and could be followed bilaterally along the CP into the DNLL and IC. A pattern of anisomorphic fibrous gliosis was observed around the CP wound site, which is normally seen in cases of severe tissue damage associated with disruption of the blood-brain barrier (BBB) (Bignami and Dahl, 1994). Finally, very few GFAP-positive astrocytes were found in lemniscal nuclei other than the DNLL of CP transected animals which further indicates that the commissure of Probst is the exclusive pathway for DNLL neurons.

Under normal physiological conditions, glutamate, the most common excitatory amino acid (EAA) in the brain, is maintained at relatively low concentrations by Na^+ - dependent transport into glia and neurons via specific EAA transporters (glutamate astrocyte specific transporter, GLAST, glutamate transporter, GLT-1, and excitatory amino acid transporter, EAAC1) (Hertz, 1979; Nichols and Attwell, 1990; Swanson *et al.* 1997). However, several neurodegenerative disorders (e.g., Huntington's disease, Alzheimer's disease, stroke, AIDS dementia, and epilepsy) including axonal transection result from elevated EAA levels, a condition that can be very excitotoxic to neurons and ultimately lead

to their death (Choi, 1988, 1992; Dawson et al., 1993; Meldrum and Garthwaite, 1990; Rothman and Olney, 1986). The exact mechanisms of neuronal cell death following EAA stimulation is as yet unresolved, although increased Ca^{2+} and Na^{+} fluxes, free radicals, catabolic enzymes, membrane failure, and cytoskeleton breakdown have all been implicated as being involved (Choi, 1992). Excessive glutamate is actively transported into astrocytes, via GLT-1 and GLAST transporters, and is then metabolized to glutamine by an upregulation of the astrocyte-specific enzyme glutamine synthetase (GS) (Hertz *et al.* 1978; Martinez-Hernandez *et al.* 1977; Torgner and Kwamme, 1990). Inhibition of the synthesis of glial glutamate transporters, GLAST and GLT-1, using antisense oligonucleotide administration results in elevated extracellular glutamate concentrations, and neurodegeneration characteristic of excitotoxicity (Rothstein et al., 1996). Several *in vitro* studies have demonstrated the neuroprotective effects of astrocytes under high glutamate concentrations when neurons are cultured with glia (Mattson and Rychlik, 1990; Sugiyama et al. 1989).

Previous *in vitro* and *in vivo* studies have shown the presence of two excitatory processes in DNLL which are mediated via NMDA and non-NMDA receptors (Wu and Kelly, 1996, Kelly and Kidd, 1997). In the presence of EAA agonists, L-glutamate, α -amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA), or *N*-methyl-D-aspartic acid (NMDA), DNLL neurons increases their excitability (Wu and Kelly, 1996, Kelly and Kidd, 1997). In addition, we recently reported that transection of the commissure of Probst induces a bilateral increase in neuronal nitric oxide synthase (nNOS). The upregulation of nNOS in the DNLL was colocalized with axotomized FG-prelabeled contralaterally projecting DNLL neurons and not intact ipsilaterally FG-prelabeled DNLL neurons (van Adel, et al., 1996). In addition, we showed that this upregulation occurred only a few days after CP transection, and peaked at two weeks, and was undetected in CP transected animals that survived four and eight weeks after CP transection. Several other studies have also reported an upregulation of nNOS after peripheral and central nervous

system axonal injuries (Herdegen et al. 1993; Kristensson *et al.* 1994; Wu, 1993; Yu, 1994).

Neuronal NOS is calmodulin dependent (a protein with four high affinity Ca^{2+} binding sites) and thus highly dependent on an influx of calcium (Ca^{2+}) which is caused by activation of NMDA and AMPA receptors (Garthwaite, 1991; Reif, 1993; Bruhwylter *et al.* 1993; Schuman and Madison, 1994; Vincent, 1994). Furthermore, axonal injury has been shown to lead to excessive Ca^{2+} entry and accumulation. Excessive Ca^{2+} accumulation will initiate free-radical production and energy depletion which will ultimately end in neuronal death. Activation of NMDA receptors is known to raise intracellular calcium (Ca^{2+}) concentrations and activate nNOS. Several *in vitro* studies have shown that overstimulation of NMDA receptors can result in neuronal death which can be prevented by several classes of nNOS inhibitors (e.g., L-N^G-nitroarginine and L-N^G-aminoarginine (Garthwaite, 1991; Reif, 1993; Dawson et al., 1993). Lesion-induced NOS in DNLL neurons is coincidental with the death of contralaterally projecting DNLL neurons. It is possible that NO-mediated neurotoxicity is a contributing factor to delayed neuronal degeneration seen in the DNLL. However it is not certain that the upregulation of NOS in these neurons is directly related to processes associated with their death. Further investigations are required to determine the role of NO-mediated neuronal degeneration in DNLL.

In the present study GFAP is upregulated in the DNLL of CP transected animals. It is likely that reactive astrocytes serve a protective role to prevent excessive glutamate activation. A similar protective role of reactive astrocytes has been reported in the facial motor nucleus following unilateral facial nerve axotomy and in the supraoptic nucleus in portacaval shunted rats, an experimental model that causes hepatic encephalopathy (Graeber and Kreutzberg, 1990; Suárez et al. 1996). Evidence to suggest that the extensive astrocyte response to CP transection serves a protective role in DNLL comes from retrograde tract tracing data using FG. Postlabeling with FG was only observed in

ipsilaterally projecting DNLL neurons in animals with CP lesions, suggesting that these neurons survive an extended period of high extracellular glutamate concentration. Furthermore, our previous study demonstrated that lesion-induced nNOS was only detected in contralaterally and not ipsilaterally projecting DNLL neurons.

The mechanisms of delayed neuronal death in the DNLL were not a focus of this study. However, initial pilot data obtained from ultrastructural analysis of neuronal degeneration does suggest that transection of the commissure of Probst could potentially serve as an *in vivo* model of apoptosis (programmed cell death). Cell death via apoptosis can be distinguished from necrosis, by differences in morphological, biochemical and molecular changes occurring in dying cells. Necrotic cell death can be induced at any age by severe injurious changes in normal physiological conditions such as, hypoxia, excessive inhibition, substrate deprivation, hyperthermia, and exposure to cytotoxins (Wyllie, 1981; Kerr and Harman, 1991). The initial stages of necrosis are reversible and are marked by disaggregated polysomes, focal chromatin margination, and a slight swelling of mitochondria. Unrepaired cells enter an irreversible stage in which the plasma membrane ruptures, recognizable organelles are lost, and nuclear dissolution occurs. On the other hand, apoptosis, or programmed cell death occurs mainly in embryonic stages of development, and functions to control cell numbers, and facilitate morphogenesis. It is considered a specific type of terminal cell differentiation (Miura *et al.* 1993). Apoptosis has been shown to proceed via biochemical cascades which are gene-directed (active process that requires specific gene expression and energy production). Because events leading to apoptotic cell death occur through the expression of specific genes, the process can be slowed by inhibitors of RNA and protein synthesis (Yuan *et al.* 1993; Miura *et al.* 1993). Morphologically and cytologically, programmed cell death is characterized by distinctive ultrastructural changes in the cell, such as, compaction and segregation of chromatin into narrow masses followed by condensation of the cytoplasm, nuclear fragmentation,

convolution of the cell membrane. Finally, membrane-bound apoptotic bodies are formed and phagocytosed by neighboring cells.

From the two tissue samples analyzed at the ultrastructural level in this study several neurons were detected at various stages of neuronal degeneration. These neurons were identified by their irregularly shaped nucleus with condensed chromatin and relatively intact organelles. Perhaps the most persuasive evidence to hint at apoptotic death in the DNLL is the selectivity, and the time-dependent responses of neuronal death seen in FG-prelabeled transected contralaterally projecting DNLL neurons. In contrast, necrosis usually causes large groups of cells in an anatomical region to die synchronously after injury a trend that was absent in this model of neuronal injury. Furthermore, the recognition and uptake of dying contralaterally FG-prelabeled DNLL neurons by phagocytic microglia, prevents the release of toxic intracellular contents which would be lethal to neighboring intact ipsilaterally projecting DNLL neurons. Although microglia actively participate in DNLL neuronal degeneration, evidence from this study would suggest that their participation seems to prevent further death of surrounding non-axotomized DNLL neurons. Taken together, the data from the present study hints that the delayed neuronal death occurring in the DNLL proceeds via apoptosis. However, it is premature at this stage to suggest that neuronal death in the DNLL is apoptotic until further work is completed.

In several *in vitro* and *in vivo* models of neurodegenerative disease, it has been proposed that weak NMDA-mediated excitotoxicity, possible linked to mitochondrial dysfunction, can initiate cytotoxic cascades, including overproduction of reactive oxygen species (ROS) (Beal, 1992). Injury-induced ROS production can intensify excitotoxicity thereby forming a cycle that will eventually end in cell death. In addition, excitotoxicity can induce necrosis or apoptosis depending on the intensity of the stimulus (Gwag et al., 1995). Recent studies by Lucius and Sievers (1996, 1997) showed that free radical production after neuronal injury may partly contribute to the failure of axonal regeneration in the adult mammalian CNS, via an apoptotic program. In addition, several other reports

indicate that ROS and oxidative stress induces apoptosis in different groups of neurons (Greenlund et al., 1995; Olanow, 1993; Ratan et al., 1994). It would be interesting to see if nNOS (and other ROS) are capable of inducing apoptosis in axotomized contralaterally projecting DNLL neurons. Further work is required to determine the molecular responses and mechanisms leading towards delayed neuronal death in the DNLL following CP transection. Figure 3.22 summarizes findings from this experiment and some of the events that are likely to lead to delayed neuronal death in the DNLL.

Within the brain proper very few commissural pathways provide a discrete bundle of axons that can be accurately and precisely transected. The crossed efferent projections of the DNLL, via the commissure of Probst, to the opposite DNLL and ICC is one major exception. The present results provide the first detailed anatomical description of this phenomenon, and indicate that transection of the commissure of Probst may be a promising new model with which to study cellular and molecular response to injury, as well as mechanisms that inhibit CNS regeneration, and processes that lead to neurodegeneration. This model could also be useful for testing experimental therapeutic approaches being developed to delay or prevent neuronal degeneration as well as therapies designed to promote neuronal growth and survival. The value of this model is enhanced by the fact that CP transections can be made with great precision, thus resulting in cell death in a highly selective, and predictable manner. Furthermore, the unique anatomical connections of the DNLL permit fluorescent prelabeling of two separate populations of neurons within a single nucleus of the DNLL. Quantification of cell number and survival is feasible and unequivocal identification of prelabeled axotomized neurons can be obtained, allowing a comparison between axotomized and non-axotomized neurons in terms of both biochemical and histological changes that occur in response to CP transections.

Conclusions

In summary, a time course analysis of neuronal degeneration has revealed that neuronal death in the DNLL is delayed and occurs bilaterally only in those neurons with crossed projections in the CP. Furthermore, ipsilaterally projecting neurons seem to be unaffected by CP transection, suggesting that neuronal death can occur in close proximity to uninjured neurons in the same CNS structure. Further work is required to determine the molecular responses and mechanisms leading towards delayed neuronal death in the DNLL of CP transected animals. It is possible that the DNLL cell death observed here is associated with apoptotic death and so involves a biochemical cascade relevant to programmed cell death and/or neurodegenerative diseases. If this is so, this suggests that surgical transection of the commissure of Probst may serve as a valuable experimental paradigm to identify neuronal and glia interactions as well as the cellular and molecular mechanisms which lead to neurodegeneration following traumatic brain injury.

General Conclusions

The primary purpose of this series of experiments has been to determine the projections of the CP and delineate the changes associated with its transection. The results have led to the following general conclusions:

- 1) The DNLL has a bilateral projection to the ICC and is organized in a roughly concentric fashion containing two separate populations of neurons.
- 2) Approximately 70% of DNLL neurons project contralaterally via the commissure of Probst.
- 3) The commissure of Probst is an obligatory pathway for neurons projecting from the DNLL to the contralateral DNLL and ICC.
- 4) The majority of contralaterally projecting DNLL neurons innervate the opposite DNLL and ICC via axon collaterals.
- 5) Transection of the commissure of Probst results in neuronal death in the DNLL.
- 6) Neuronal death in the DNLL is highly selective and occurs in a time-dependent fashion.

These data will be valuable in understanding the functional significance of the DNLL for processing acoustic information, particularly binaural cues involved in sound localization (Kelly, 1997). In addition, the pattern of projections from the DNLL to the ipsilateral and contralateral DNLL and ICC provides a promising model system for future studies of neuronal degeneration in the CNS.

Appendix 1

Surgical Procedure for Transection of the Commissure of Probst

Midline Surgical Transection of the Commissure of Probst

The results of Experiment 2 suggest that transection of the commissure of Probst is a valuable in vivo model to study processes related to neurodegeneration (neuronal and glia responses). Therefore a detailed description of this procedure is provided. Before employing this procedure, investigators should be aware the stereotaxic coordinates provided apply only to adult rats weighing between 200-400 grams. Additionally, although, several similar versions of microknives are commercial available, these coordinates provided may have to be modified depending on the size of surgical knife used. Materials required for CP transections are listed below, followed by illustrations and a detailed description of the stereotaxic surgical procedures (see Figure A1.1).

Materials

Somnotol (sodium pentobarbital, recommended)

Animal clippers

Microsurgical dissecting scope

Stereotaxic instrument with blunt ear bars

Squeeze bottle containing 70% (v/v) ethanol

Squeeze bottle containing 0.9% saline

Squeeze bottle containing 3% hydrogen peroxide (H_2O_2)

Beaker of Germex or 70% (v/v) ethanol

Scalpel handle with sterile No. 10 blade

Scalp retractors, e.g., two or three hemostats

2-inch x 2-inch gauze

Dental or hobby drill and several different sized drill bits

Fine jewelers forceps

Suture scissors

Silk suture thread and needle

Microsurgical knife (Fine Science Tools, Canada)

Stereotaxic Surgical Procedure

1. Sterilize all surgical instruments
2. Anesthetize the animal with a interperitoneal injection of sodium pentobarbital (65 mg/kg, i.p.). If needed, use supplemental injections (15 mg/kg) to maintain a state of areflexia.
3. Use clean hair clippers to shave the animal's head, beginning just above eye level and work to the base of the neck.
4. Position the animal in the stereotaxic head holder with the nose bar positioned 4 mm below horizontal zero (see Paxino and Watson, 1996). It is critical that the ear bars are blunt ended and care is taken not to damage the inner ear of the animal by excessive force.
5. Clean the scalp with (Hibitane), then disinfect the scalp with (Bridine), taking care to keep each solution from the animals eyes. These solutions provide an antiseptic treatment to the surgical site.
6. Using a sterile scalpel blade, make a straight skin incision beginning between the eyes to the nape of the neck. When making the incision use enough pressure to make one clean incision that cuts through the periosteum (the tissue attached to the surface of the skull) to the skull surface without cutting into the bone (Note: for older animals [> 4 months] the skull is thick and much harder than that of younger animals [between 6 - 16 weeks of age]).
7. Use a spatula to loosen the periosteum away from the midline. Retract the temporal and cerebellar musculature to expose the skull. To avoid cutting the muscle, simply separate it from the bone using the spatula to force the muscle from its insertion point(s) on the bone.
8. Clean the skull surface using hydrogen peroxide (the H_2O_2 will bubble profusely on contact with red blood cell catalase) and a sterile guaze pad. The hydrogen

peroxide step is the quickest and most effective way to completely clean the skull, exaggerate the visibility of the skull bone sutures (i.e., the sagittal, coronal, and lambdoid sutures), cauterize skull blood vessels and reduce skull bleeding, and provide an antiseptic treatment to the surgical site.

9. Since precise transections are required using stereotaxic methods, lambda serves as a convenient landmark from which to start. Since considerable variation for the intersection of the lambdoid and sagittal sutures is seen in different animals, lambda is identified by the midpoint of the curve of best fit along the lambdoid suture. Using a dissecting microscope and a scalpel blade mark lambda on the skull surface and then highlighted using a permanent marker. The skull surface to be removed is measured and identified with the marker to increase the accuracy of drilling.

10. Drilling the skull is one of the most crucial aspects of this surgical procedure. The craniotomy must be wide enough for the shaft of the micro-surgical knife to move freely, and the rostrocaudal and dorsoventral extent must be sufficient enough to allow passage of the knife to guarantee complete transection of the commissure of Probst.

11. Remove bone and tissue debris away from the craniotomy using sterile saline and fine forceps. Next, using fine forceps, carefully tear into the dura and remove as much as possible. Avoid puncturing the surface of the brain (and the venous sinus at the front of the craniotomy), which will cause extensive bleeding.

12. Mount the micro-surgical knife in a stereotaxic instrument holder secure to the table and butt up against the back of the animal stereotaxic holder.

13. Tilt the micoknife to 45° relative to the frontal plane and locate lambda with the tip of the microknife. Make sure not to damage the tip on the skull surface. Record the stereotaxic coordinates for this lambda position.

14. Raise the microknife 5.7 mm AP and then lower the microknife 5.7 mm ventrally to a reference point above the back of the skull. This point is the starting point of the descent of the microknife through the cerebellum and into the brainstem.

15 Advance the microknife slowly 11.2 mm ventrally through the cerebellum and into the brainstem to an arbitrary position referred to as the starting point. Beginning when the microknife first penetrates the brain parenchyma, it is crucial to advance the microknife slowly to avoid excessive brain movement.

16. The microknife is now positioned at the "starting point", posterior to the commissure of Probst. In order to make restricted lesions that completely transect the dorsoventral and rostrocaudal extent of the commissure of Probst, the microknife must be simultaneously advanced in the anterior direction 3 mm and in the ventral direction an additional 2 mm. Since the brain is soft and the microknife will cause brain movement, this motion must be repeated several times in order to completely transact the commissure of Probst.

17. After several passes have been completed return the microknife to the starting point and retract slowly out of the brainstem and cerebellum. It is important not to retract the microknife out of the brain from the anterior coordinates used for CP transection since this could rupture the venous sinus and cause extensive bleeding.

18. Fill the craniotomy is with Gelfoam or Bone Wax, and clean any excess fluids from the skull surface with sterile saline and a square guaze pad.

21. Close the skin wound is closed with sutures or wound clips. Administer gravol to the animal for postoperative recovery.

22. Monitor the animal until it has fully recovered before returning it to the vivarium.

Figure A1.1: Surgical procedure for midline transection of the commissure of Probst in adult albino rats.

- A) Make a straight skin incision from the eyes to the nape of the neck to expose the skull surface.
- B) Retract the trapezius musculatures from the skull and clean the skull surface using hydrogen peroxide to visualize the lambdoid (λ) and sagittal sutures (*)
- C) Identify lambda by the midpoint of the curve of best fit along the lambdoid sutures. The arrowhead marks the intersection of the lambdoid sutures and identifies the area of skull to be drilled with a permanent marker.
- D) Make a craniotomy is made posterior and perpendicular to the lambdoid sutures.
- E) Remove the skull bone to the edges of the rectangle and remove the dura carefully.
- F) Tilt the microknife backwards 45° relative to the frontal plane and position at lambda.
- G) Retract the microknife 5.7 mm AP, and then lower 5.7 mm to an arbitrary reference point above the back of the skull.
- H) From the reference point, slowly advance the microknife 11.2 mm AP through the cerebellum and into the brainstem to the starting point.
- I) Slowly advanced the microknife 3.0 mm in the rostral direction and at the same time 2 mm ventrally. Since the brain is soft this movement must be repeated several times to ensure complete transection of the commissure of Probst. With care and practice, transection of commissure of Probst can be achieved throughout the dorsoventral and rostrocaudal extent of this commissural pathway.
- J) Return the microknife to the start position and slowly retract out of the brainstem and cerebellum.
- K) Clean the wound and fill the cavity with saline-soaked Gelfoam (bone wax can also be used) and remove the excess fluid. Seal the skin wound with suture or wound clips (not shown). Scale bar: 0.5 cm

Appendix 2

Publication List

Articles in Refereed Journals:

- [1] van Adel, B.A., and J.B. Kelly. Kainic Acid Lesions of the superior olivary complex: effects on sound localization by the Albino Rat.. Behavioral Neuroscience, 112: 432-446.
- [2] Kelly, J.B., Liscum, A., van Adel, B.A., and M. Ito. Projections from the superior olive and lateral lemniscus to tonotopic regions of the rat's inferior colliculus. Hearing Research, 116: 43-54, 1998.
- [3] Ito, M., van Adel, B.A. and J.B. Kelly. Sound localization after transection of the commissure of Probst in the albino rat. Journal of Neurophysiology, 76: 3493-3502, 1996.
- [4] Kelly, J.B., Li, L. and B.A. van Adel. Sound localization after kainic acid lesions of the dorsal nucleus of the lateral lemniscus in the albino rat. Behavioral Neuroscience, 110: 1445-1455, 1996.

Abstracts in Conference Proceedings:

- [1] van Adel, B.A., and J.B. Kelly. Collateral Innervation of the dorsal nucleus of the lateral lemniscus and the inferior colliculus in the albino rat: A fluorescence double labeling study. Society for Neuroscience. Los Angeles, CA., November 7-12, 1998.
- [2] Kelly, J.B., van Adel, B.A., and S.H. Wu. Monaural and binaural innervation of the nuclei of the lateral lemniscus in the adult rat: A fluorescence retrograde transport study. Society for Neuroscience. Los Angeles, CA., November 7-12, 1998.
- [3] van Adel B.A and J.B Kelly. Accurate sound localization is dependent on binaurally sensitive auditory brainstem nuclei. Canadian Society for Brain, Behaviour and Cognitive Science. Ottawa, Canada, June 18-20, 1998
- [4] Kelly, J.B., and B. A. van Adel. Brainstem mechanisms for binuaral processing and sound localization. Canadian Society for Brain, Behaviour and Cognitive Science. Ottawa, Canada, June 18-20, 1998
- [5] van Adel B.A., Ito, M., and J.B Kelly. Glia reactivity in the dorsal nucleus of the lateral lemniscus following surgical transection of the commissure of Probst in the adult rat. Association for Research in Otolaryngology. Abstracts, Florida, USA, February 15-19, 1998.
- [6] van Adel B.A., Ito, M., and J.B. Kelly. Time course of neuronal degeneration and activation of astrocytes and microglia in the dorsal nucleus of the lateral lemniscus after transection of the commissure of Probst in adult rats. Society for Neuroscience. New Orleans, October 25, 1997.
- [7] van Adel B.A., Ito, M., and J.B. Kelly. Transection of the commissure of Probst in the adult rat: characterization of an in vivo model of delayed neuronal death. Neuronal Degeneration and Regeneration: From Basic Mechanisms to Prospects for Therapy. 20th International Summer School of Brain Research. Amsterdam, 25-29 August 1997.
- [8] van Adel, B.A., Labelle, R. E., McGregor, C., and J.B. Kelly. Neuroanatomical tools for the neuroscientist. Carleton University Biomedical and Engineering Research Afternoon. April 24, 1997.

[9] van Adel, B.A., Kidd, S A., and J.B. Kelly. Transection of the commissure of Probst affects interaural time difference sensitivity in the rat's inferior colliculus. Association for Research in Otolaryngology. Abstracts, 20, 351, 1997.

[10] van Adel, B.A., Ito, M., and J.B. Kelly. Transection of the commissure of Probst results in neuronal degeneration and expression of nitric oxide synthase (NOS) in Injured DNLL neurons of the albino rat. Association for Research in Otolaryngology. Abstracts, 19, 46, 1996.

[11] van Adel, B.A., and J.B. Kelly. Unilateral kainic acid lesions of the superior olivary complex (SOC): effects on midline sound localization in the albino rat. Association for Research in Otolaryngology. Abstracts, 19, 765, 1996.

[12] Ito, M., van Adel, B.A., and J.B. Kelly. Axotomy of the facial nerve induces nitric oxide synthase (NOS) in facial motor neurons of the albino rat. Association for Research in Otolaryngology. Abstracts, 19, 47, 1996.

[13] van Adel, B.A., Ito, M., and J.B. Kelly. Transection of the commissure of Probst induces a bilateral increase in nitric oxide synthase (NOS) in the dorsal nucleus of the lateral lemniscus as shown by NADPH-diaphorase histochemistry. Fourth International Brain Research Organization (IBRO) World Congress of Neuroscience Abstracts, 1995.

[14] Ito, M., van Adel, B.A., and J.B. Kelly. Effect of cutting the commissure of Probst on sound localization and auditory orientation response in the albino rat. Association for Research in Otolaryngology. Abstracts, 18, 249, 1995.

[15] Li, L., van Adel, B.A., and J.B. Kelly. Unilateral kainic acid lesions of the lateral lemniscus: effects on sound localization in the albino rat. Association for Research in Otolaryngology. Abstracts, 18: 250, 1995.

[16] Kelly, J.B., Liscum, A., van Adel, B.A., and M. Ito. Retrograde labeling in the rat's dorsal nucleus of the lateral lemniscus from frequency specific regions of the central nucleus of the inferior colliculus. Association for Research in Otolaryngology. Abstracts, 18, 157, 1995.

Research to be submitted for Publication:

Ito, M., van Adel, B.A., and J.B. Kelly. Axotomy of the facial nerve induces nitric oxide synthase in facial motor neurons of the albino rat. Paper in preparation (for Experimental Neurology), March, 1998.

van Adel, B.A., Ito, M., and J.B. Kelly. Afferent and efferent projections of the nuclei of the lateral lemniscus in the albino rat. Paper in preparation, January, 1998.

van Adel, B.A., Ito, M., and J.B. Kelly. Transection of the commissure of Probst in the adult rat: characterization of an *in vivo* model of delayed neuronal death. Paper in preparation, January, 1998.

van Adel, B.A., Ito, M., and J.B. Kelly. Expression of neuronal nitric oxide synthase (nNOS) in injured DNLL neurons of the albino rat. Paper in preparation, November 1997.

van Adel, B.A., Kidd, S A., and J.B. Kelly. Transection of the commissure of Probst affects interaural time difference sensitivity in the rat's inferior colliculus. Paper in preparation, November, 1997.

References

References

- Adams, J.C. (1979) Ascending projections to the inferior colliculus. *J. Comp. Neurol.* 183: 519-538.
- Adams J.C. (1981). Heavy metal intensification of DAB-based HRP reaction product. *J Histochem. Cytochem.* 3: 265-274.
- Adams, J.C. and Mugnaini, E. (1984). Dorsal nucleus of the lateral lemniscus: A nucleus of GABAergic projection neurons. *Brain Res. Bull.* 13: 585-590.
- Aitkin, L.M., Anderson, D.J. and Brugge, J.F. (1970). Tonotopic organization and discharge characteristics of single neuron in nuclei of the lateral lemniscus of the cat. *J. Neurophysiol.* 33: 421-440
- Aoki, E., Semba, R., Keino, R., Kato, K. and Kashiwamata, S. (1988). Glycine-like immunoreactivity in the rat auditory pathway. *Brain Res.* 442: 63-71.
- Aschner, M. (1998). Astrocytic functions and physiological reactions to injury: the potential to induce and/or exacerbate neuronal dysfunction - a forum position paper. *Neurotoxicol.* 19: 7-18.
- Bajo, V.M., Merchán, M.A., López, D.E. and Rouiller, E.M. (1993). Neuronal morphology and efferent projections of the dorsal nucleus of the lateral lemniscus in the rat. *J. Comp. Neurol.* 334: 241-262.
- Batra, R., and Fitzpatrick, D.C. (1997). Neurons sensitive to interaural temporal disparities in the medial part of the ventral nucleus of the lateral lemniscus. *J. Neurophysiol.* 78: 511-515.
- Beal, M.F. (1992). Does impairment of energy metabolism result in excitotoxic neuronal death in neurodegenerative illness? *Ann. Neurol.* 31: 119-130.
- Bennett, M.R., Gibson, D.F., Schwartz, S.M., and Tait, J.F. (1995). Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ. Res.* 77: 1136-1142.

- Beyerl, B.D. (1978). Afferent projections to the central nucleus of the inferior colliculus in the rat. *Brain Res.* 145: 209-223.
- Bignami, A., and Dahl, D. (1994). General pathology. In *Glia cells in the central nervous system and their reaction to injury*. Auston: R.G. Landes Company, pp. 77-92.
- Bignami, A., Eng, L.F., Dahl, D et al. (1972). Localization of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* 43: 429-435.
- Bredesen, D.E. 1994. Neuronal apoptosis: genetic and biochemical modulation. In apoptosis II: the molecular basis of apoptosis in disease. *Current Communications in Cell & Molecular Biology-Vol 8*. Cold Spring Harbor Laboratory Press. pp 397-421.
- Brugge, J.F., Anderson, D.J. and Aitkin, L.M. (1970) Responses of neurons in the dorsal nucleus of the lateral lemniscus of cat to binaural tonal stimulation. *J. Neurophysiol.* 33: 441-458.
- Brunso-Bechtold, J.K., Thompson, G.C. and Masterton, R.B. (1981). HRP study of the organization of auditory afferents ascending to the central nucleus of the inferior colliculus. *J. Comp. Neurol.* 197: 705-722.
- Buckthout, A.D. (1993). Electrophysiological investigation of the dorsal nucleus of the lateral lemniscus in the auditory system of the albino rat. Master's Thesis, Carleton University, Ottawa, Ontario, Canada.
- Caicedo, A., and Herbert, H. (1993). Topography of descending projections from the inferior colliculus to auditory brainstem nuclei in the rat. *J. Comp. Neurol.* 328: 377-392.
- Carney, L.H., and Webster, D.B. (1971). Ascending and descending projections of the inferior colliculus in the kangaroo rat (*Dipodomys merriami*). *Brain Behav. Evol.* 4: 401-412.

- Carney, L.H., and Yin, T.C.T. (1989) Responses of low-frequency cells in the inferior colliculus to interaural time differences of clicks: excitatory and inhibitory components. *J. Neurophysiol.* 62: 144-161.
- Choi, D W. (1988). Glutamate neurotoxicity and disease of the nervous system. *Neuron* 1: 623-634.
- Choi, D W. (1992). Excitotoxicity cell death. *J. Neurobiol.* 23: 1261-1276.
- Clopton, B.M., and Windfield, J.A. (1973). Tonotopic organization of the inferior colliculus of the rat. *Brain Res.* 56: 355-358.
- Coleman, J.R. and Clerici, W.J. (1987). Sources of projections to subdivisions of the inferior colliculus in the rat. *J. Comp. Neurol.* 262: 215-226.
- Coleman, J.R., Blatchley, B.J., and Williams, J.E. (1982). Development of the dorsal and ventral nuclei and effects of acoustic deprivation. *Dev. Brain Res.* 4: 119-123.
- Covey, E. (1993). Response properties of single units in the dorsal nucleus of the lateral lemniscus and paralemniscal zone of an echolocating bat. *J. Neurophysiol.* 69: 842-859.
- Covey, E., and Casseday, J.H. (1986). Connectional basis for frequency representation in the nuclei of the lateral lemniscus of the bat *Eptesicus fuscus*. *J. Neurosci.* 6: 2926-2940.
- Covey, E., and Casseday, J.H. (1991). The monaural nuclei of the lateral lemniscus in an echolocating bat: parallel pathways for analyzing temporal features of sound. *J. Neurosci.* 11: 3456-3470.
- Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhl, G.R., and Snyder, S.H. (1993). Mechanisms of nitric-oxide mediated neurotoxicity in primary brain cultures. *J. Neurosci.* 13: 2651-2661.
- Divac, I., and Mogensen, J. (1990). Long-term retrograde labeling of neurons. *Brain Res.* 524: 339-341.

- Faingold, C.L., Boersma-Anderson, C.A. and Randall, M.E. (1993). Stimulation or blockade of the dorsal nucleus of the lateral lemniscus alters binaural and tonic inhibition in contralateral inferior colliculus neurons. *Hearing Res.* 69: 98-106.
- Fitzpatrick, D.C., Kuwada, S., Batra, R. and Trahiotis, C. (1995). Neural responses to simple simulated echoes in the auditory brain stem of the unanesthetized rabbit. *J. Neurophysiol.* 74: 2469-2486.
- Flammino, F. and Clopton, B.M. (1975). Neural responses in the inferior colliculus of albino rat to binaural stimuli. *J. Acoust. Soc. Am.* 57: 692-695.
- Friauf, E. (1992). Tonotopic order in the adult and developing auditory system of the rat as shown by c-fos immunocytochemistry. *Eur. J. Neurosci.* 4: 789-812.
- Friauf, E. (1994). Distribution of calcium-binding protein calbindin-D_{28k} in the auditory system of adult and developing rats. *J. Comp. Neurol.* 349: 193-211.
- Fu, X.W., Wu, S.H., Bresden, B.L. and Kelly, J.B. (1997) Synaptic excitation in the dorsal nucleus of the lateral lemniscus: whole-cell patch clamp recordings from rat brain slice. *Neurosci.* 78: 815-827.
- Fubara, B.M., Casseday, J.H., Covey, E., and Schwartz-Bloom, R.D. (1996). Distribution of GABA_A, GABA_B, and glycine receptors in the central auditory system of the big brown bat, *Eptesiscus fuscus*. *J. Comp. Neurol.* 369: 83-92.
- Garthwaite, J. (1991). Glutamate, nitric oxide and cell-cell signaling in the nervous system. *Trends in Neuroscience.* 14: 60-67.
- Glenn, S.L. and Kelly, J.B. (1992) Kainic acid lesions of the dorsal nucleus of the lateral lemniscus: effects on binaural evoked responses in rat auditory cortex. *J. Neurosci.* 12: 3688-3699.
- Glendenning, K.K., and Baker, B.N. (1988). Neuroanatomical distribution of receptors for three potential inhibitory neurotransmitters in the brainstem auditory nuclei of the cat. *J. Comp. Neurol.* 275: 288-308.

- Glendenning, K.K., Brunso-Bechtold, J.K., Thompson, G.C., and Masterton, R.B. (1981). Ascending auditory afferents to the nuclei of the lateral lemniscus. *J. Comp. Neurol.* 232: 673-703.
- Glendenning, K.K., and Masterton, R.B. (1983). Acoustic chiasm: efferent projections of the lateral superior olive. *J. Neurosci.* 3: 1521-1537.
- Goldberg, J.M., and Moore, R.Y. (1967). Ascending projections of the lateral lemniscus in the cat and monkey. *J. Comp. Neurol.* 129: 143-156.
- González-Hernández, T., Mantolán-Sarmiento, B., González-González, B., and Pérez-González, H. (1996). Sources of GABAergic input to the inferior colliculus of the rat. *J. Comp. Neurol.*, 372: 309-326.
- González-Hernández, T.H., Meyer, G., Ferres-Torres, R., Castañeyra-Perdomo, and Pérez Delgado, M.M. (1986). Afferent connections of the inferior colliculus in the albino mouse. *J. Hirnforsch.* 28: 315-323.
- Graeber, M.B., and Kreutzberg, G.W. (1990). In advances in neural regeneration research. (Seil, F.J., Ed.), Wiley Liss, pp 215-224.
- Gwag, B.J., Lobner, D., Koh, J.T., Wie, M.B., and Choi, D.W. (1995). Blockade of glutamate receptors unmasks neuronal apoptosis after oxygen-glucose deprivation *in vitro*. *Neurosci.* 68: 615-619.
- Held, H. (1893). Die centrale Gehörleitung. *Arch. f. Anat. u. Physiol. Anat. Abtheil.* 17: 201-248.
- Henkel, C.K. (1997). Axonal morphology in fibrodendritic laminae of the dorsal nucleus of the lateral lemniscus: Afferent projections from the medial superior olivary complex. *J. Comp. Neurol.* 380: 136-144.
- Henkel, C.K., and Shneiderman, A. (1988). Nucleus sagulum: projection of the lateral tegmental area to the inferior colliculus in the rat. *J. Comp. Neurol.* 271: 577-588.
- Herdegen, T., Brecht, S., Mayer, B., Leah, J., Kummer, W., Bravo, R., and Zimmermann, M. 1993. Long-lasting expression of JUN and KROX transcription

- factors and nitric oxide synthase in intrinsic neurons of the rat brain following axotomy. *J. Neurosci.* 13: 4130-4145.
- Hertz, L., Schousboe, A., Boechler, N., Mujerki, S., and Fedoroff, S. (1978). Kinetic characteristics of the glutamate uptake into normal astrocytes in cultures. *Neurochem. Res.* 3: 1-14.
- Hertz, L. (1979). Functional interactions between neurons and astrocytes. Turnover and metabolism of putative amino acid transmitter. *Prog. Neurobiol.* 13: 277-323.
- Huang, C.M., Fex, J. (1986). Tonotopic organization in the inferior colliculus of the rat demonstrated with the 2-deoxyglucose method. *Exp. Brain. Res.* 61: 506-512.
- Huffman, R.F., and Covey, E. (1995). Origin of ascending projections to the nuclei of the lateral lemniscus in the big brown bat, *Eptesicus fuscus*. *J. Comp. Neurol.* 357: 532-545.
- Hutson, K.A. (1988). Connections of the auditory midbrain: efferent projections of the dorsal nucleus of the lateral lemniscus, the nucleus sagulum, and the origins of the GABAergic commissure of Probst. Doctoral dissertation. Tallahassee: Florida State University.
- Hutson, K.A., Glendenning, K.K. and Masterton, R.B. (1991). Acoustic chiasm IV: Eight midbrain decussations of the auditory system in the cat. *J. Comp. Neurol.* 312: 105-131.
- Irvine, D.R.F. (1992). Physiology of the auditory brainstem. In *The Mammalian Auditory Pathway: Neurophysiology* (Popper, A.N. and Fay, R.R., eds) New York: Springer Verlag, pp. 153-231.
- Ito, M., van Adel, B. and Kelly, J.B. (1996). Sound localization after transection of the commissure of Probst in the albino rat. *J. Neurophysiol.* 76: 3493-3502.
- Iwahori, N. (1986). A golgi study on the dorsal nucleus of the lateral lemniscus in the mouse. *Neurosci. Res.* 3: 196-212.

- Johnson, E.M. Jr., and Deckwerth, D.L. (1993). Molecular mechanisms of developmental neuronal death. *Annual Review of Neuroscience*. 16: 31-46.
- Kavanagh, G.L., Kelly, J.B. (1986). Midline and lateral field sound localization in the albino rat (*Rattus norvegicus*). *Behav. Neurosci.* 100: 200-205.
- Kavanagh, G.L., Kelly, J.B. (1992). Midline and lateral field sound localization in the ferret (*Mustela putorius*): contribution of the superior olivary complex. *J. Neurophysiol.* 67: 1643-1658.
- Kelly, J.B. (1974). Localization of paired sound sources in the rat: small time differences. *J. Acoust. Soc. Amer.* 55: 1277-1284.
- Kelly, J.B. (1997). Contribution of the dorsal nucleus of the lateral lemniscus to binaural processing in the auditory brainstem. In: J. Syka (Ed.) *Acoustical Signal Processing in the Central Auditory System*. Plenum Press, New York, pp. 329-352.
- Kelly, J.B., Glenn, S.L. and Beaver, C.J. (1991). Sound frequency and binaural response properties of single neurons in rat inferior colliculus. *Hearing Res.* 56: 273-280.
- Kelly, J.B., Kavanagh, G.L. (1994). Sound localization after unilateral lesions of inferior colliculus in the ferret (*Mustela putorius*). *J. Neurophysiol.* 71: 1078-1087.
- Kelly, J.B. and Kidd, S.A. (1997). NMDA and non-NMDA excitatory processes in the rat's DNLL shape binaural inhibitory responses in the contralateral inferior colliculus. *Assoc. Res. Otolaryngol. Abstr.* 20, 131.
- Kelly, J.B. and Phillips, D.P. (1991). Coding of interaural time differences of transients in auditory cortex of *Rattus norvegicus*: implications for the evolution of mammalian sound localization. *Hearing Res.* 55: 39-44.
- Kelly, J.B., and Li, L. (1997). Two sources of inhibition affecting binaural evoked responses in the rat's inferior colliculus: the dorsal nucleus of the lateral lemniscus and the superior olivary complex. *Hearing Res.* 104: 112-126.

- Kelly, J.B., Li, L. and van Adel, B. (1996) Sound localization after kainic acid lesions of the dorsal nucleus of the lateral lemniscus in the albino rat. *Behav. Neurosci.* 110: 1445-1455.
- Kelly, J.B., Liscum, A., van Adel, B. and Ito, M. (1998). Projections from the superior olive and lateral lemniscus to tonotopic regions of the rat's inferior colliculus. *Hearing Res.* 116: 43-54.
- Kerr, J.F.R., and Harman, B.V. (1991). Definition and Incidence of apoptosis: an historical perspective. In apoptosis: the molecular basis of cell death. *Current Communications in Cell & Molecular Biology*. Cold Spring Harbor Laboratory Press, pp 5-30.
- Kidd, S.A., Kelly, J.B. (1996). Contribution of the lateral lemniscus to binaural responses in the inferior colliculus of the rat: interaural time delays. *J. Neurosci.* 16: 7390-7397.
- Kristensson, K. Aldskogius, M., Peng, Z.-C., Olsson, T., Aldskogius, H., and Bentoivoglio, M. (1994). Co-induction of neuronal interferon-gamma and nitric oxide synthase in rat motor neurons after axotomy: a role in nerve repair or death? *Journal of Neurocytology.* 23: 453-459
- Kristensson, K., and Olsson, Y. (1976). Retrograde axonal transport of protein. *Brain Res.* 29, 363-365.
- Kreutzberg, G.W. (1996). Microglia: a sensor for pathological events in the CNS. *Trends Neurosci.* 19: 312-318.
- Kudo, M. (1981). Projections of the nuclei of the lateral lemniscus in the cat: an autoradiographic study. *Brain Res.* 221: 57-69.
- Kudo, M., Nakamura, Y., Tokuno, H., and Kitao, Y. (1990). Auditory brainstem in the mole (*Mogera*): nuclear configuration and the projections to the inferior colliculus. *J. Comp. Neurol.* 298: 400-412.

- Labelle, R.E., and Kelly, J.B. (1996). A retrograde transport study of the ascending projections to the dorsal nucleus of the lateral lemniscus in the albino rat. *Assoc. Res. Otolaryngol Abstract* 19: 481.
- Labelle R.E., Kelly, J.B. (1997). Efferent projections of the dorsal nucleus of the lateral lemniscus in the rat: a light and electron microscopic study. *Assoc. Res. Otolaryngol Abstract* 20: 754.
- Lewandowsky, M. (1904). Untersuchungen über die Leitungsbahnen des Truncus Cerebri und Ihren Zusammenhang mit denen der Medulla spinalis und des Cortex cerebri. *Jenaische Denkschriften.* 10: 63-148.
- Li, L. and Kelly, J.B. (1992a). Binaural responses in rat inferior colliculus following kainic acid lesions of the superior olive: interaural intensity difference functions. *Hearing Res.* 61: 73-85.
- Li, L. and Kelly, J.B. (1992b). Inhibitory influence of the dorsal nucleus of the lateral lemniscus on binaural responses in the rat's inferior colliculus. *J. Neurosci.* 12: 4530-4539.
- Lohmann, C., and Friauf, E. (1996). Distribution of the calcium-binding proteins parvalbumin and calretinin in the auditory system of adult and developing rats. *J. Comp. Neurol.* 367: 90-109.
- Lucius, R., Sievers, J. (1996). Postnatal retinal ganglion cells *in vitro*: protection against reactive oxygen species (ROS)-induced axonal degeneration by cocultured astrocytes. *Brain Res.* 743: 56-62.
- Lucius, R., Sievers, J. (1997). YVAD protect post-natal retinal ganglion cells against axotomy-induced but not free radical-induced axonal degeneration *in vitro*. *Mol. Brain Res.* 48: 181-184.
- Markovitz, N.S. and Pollak, G.D. (1994). Binaural processing in the dorsal nucleus of the lateral lemniscus. *Hear. Res.* 73: 121-140.

- Martínez-Hernández, A, Bell, K P, and Nrenberg, M D. (1977). Glutamine synthetase: glial localization in Brain. *Science*. 195: 1356-1358.
- Mattson, M.P., and Rychlik, B. (1990). Glia protect hippocampal neurons against excitatory amino acid-induced degeneration: involvement of fibroblast growth factor. *Int. J. Dev. Neurosci.* 8: 399-415.
- Meldrum, B.S., and Garwaite, J. (1990). Excitatory amino acid neurotoxicity and neurodegenerative disease. *Trends Pharmacol. Sci.* 11: 379-387.
- Merchán, M.A.E., Saldaña, E. and Plaza, I. (1994) Dorsal nucleus of the lateral lemniscus in the rat: concentric organization and tonotopic projection to the inferior colliculus. *J. Comp. Neurol.* 342: 259-278.
- Merchán, M.A., and Berbel, P. (1997). Anatomy of the ventral nucleus of the lateral lemniscus in rats: a nucleus with concentric laminar organization. *J. Comp. Neurol.* 372: 245-263.
- Miura, M., Zhu, H., Rotello, R., Hartwig, E.A., and J. Yuan. (1993). Induction of apoptosis in fibroblasts by IL-1 β -converting enzyme, a Mammalian Homolog of the *C. elegans* cell death gene *ced-3*. *Cell*. 75: 653-660.
- Miyake, T, Okada M, and Kitamura, T. (1992). Reactive proliferation of astrocytes studied by immunohistochemistry for proliferating cell nuclear antigen. *Brain Res.* 590: 300-302.
- Minghetti, L., and Levi, G. (1998). Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. *Prog. Neurobiol.* 54: 99-125.
- Moore, J.K. (1988). Auditory brainstem of the ferret: Sources of projections to the inferior colliculus. *J. Comp. Neurol.* 269: 342-354.
- Moore, J.K. and Moore, R.Y. (1987). Glutamic acid decarboxylase-like immunoreactivity in brainstem auditory nuclei of the rat. *J. Comp. Neurol.* 260: 157-174.

- Morest, D.K., and Oliver, D.L. (1984). The neuronal architecture of the inferior colliculus in the cat: defining the functional anatomy of the auditory midbrain. *J. Comp. Neurol.* 222: 209-236.
- Nichols, DG, and Attwell, D. (1990). The release and uptake of excitatory amino acids. *Trends Pharmacol. Sci.* 11:462-468.
- Noort, J. van (1969). The structure and connections of the inferior colliculus. Assen: van Gorcum, pp. 1-115.
- Nordeen, K.W., Killackey, H.P., and Kitzes, L.M. (1983). Ascending auditory projections to the inferior colliculus in the adult gerbil, *Meriones unguiculatus* . *J. Comp. Neurol.* 214: 155-172.
- Oertel, D., and Wu, S.H. (1989). Morphology and physiology of cells in the slice preparations of the dorsal cochlear nucleus of mice. *J. Comp. Neurol.* 283: 228-247.
- Oertel, D., Wu, S.H., Garb, M.W., and Dizack, C. (1990). Morphology and physiology of cells in the slice preparations of the posteroventral cochlear nucleus of mice. *J. Comp. Neurol.* 295: 136-154.
- Olanow, C.W. (1993). A radical hypothesis for neurodegeneration. *Trends Neurosci.* 16: 439-444.
- Oliver, D.L., and Bishop, D.C. (1998). Different proportions of GABA and glycine content distinguish the nuclei of the lateral lemniscus in the rat. A double-label immunofluorescence study. *Assoc. Res. Otolaryngol. Abstr.* 21: 371.
- Oliver, D.L. and Shneiderman, A. (1989). An EM study of the dorsal nucleus of the lateral lemniscus: inhibitory, commissural, synaptic connections between ascending auditory pathways. *J. Neurosci.* 9: 967-982.
- Oliver, D.L., Kuwada, S., Yin, T.C.T., Haberly, L.B., and Henkel, C.K. (1991). Dendritic and axonal morphology of HRP-injected neurons in the inferior colliculus of the cat. *J. Comp. Neurol.* 303: 75-100.

- Oppenheim, R.W., Schwartz, L.M., and C.J. Shatz. (1992). Neuronal death, a tradition of dying. *Journal of Neurobiology*. 23: 1111-1115.
- Paxinos, G. and Watson, C. (1987). The Rat Brain in Stereotaxic Coordinates. Second Edition. Academic Press, New York.
- Piechotta, K., Weth, F., and Friauf, E. (1998). In situ hybridization of glycine receptor subunits in the auditory brainstem of developing rats. *Assoc. Res. Otolaryngol. Abstr.* 21: 843.
- Pollak, G.D. (1988) Time is traded for intensity in the bat's auditory system. *Hear. Res.* 36: 107-124.
- Preuß, S. (1991). Elektrophysiologische und neuroanatomische charakterisierung der kme des lateralen lemniscus und der umgebenden auditorischen Areale bei der Ratte. Thesis, Universitat Tübingen.
- Probst, M. (1902). Experimentelle Untersuchungen über die Anatomie und Physiology der Leitungsbahnen des Gehirnstammes. *Arch. F. Anat. Physiol. Suppl.* 47: 147-254.
- Rajakumar, N., Elisevich, K., and Flumerfelt, B.A. (1993). Biotinylated dextran: a versatile anterograde and retrograde neuronal tracer. *Brain Res.* 607: 47-53.
- Ramon y Cajal, S. (1909). *Histologie du system nerveux de l'homme et des vertebres*. Vol. 1. Paris: A. Moloine.
- Ratan, R.R., Murphy, T.M., Baraban, J.M. (1994). Oxidative stress induces apoptosis in embryonic cortical neurons. *J. Neurochem.* 62: 376-379.
- Reif, D.W. (1993). Delayed production of nitric oxide contributes to NMDA-mediated neuronal damage. *Neuroreport*. 4: 566-568.
- Rinaman, L. Milligan, C.E., and Levitt, P. (1991). Persistence of fluoro-gold following degeneration of labeled motoneurons is due to phagocytosis by microglia and macrophages. *Neuroscience*. 44: 765-776.

- Riquelme, R., Merchán, M.A., and Ottersen, O.P. (1998). GABA and glycine in the ventral nucleus of the lateral lemniscus: an immunocytochemical and in situ hybridization study in the rat. *Assoc. Res. Otolaryngol. Abstr.* 21: 370.
- Roberts, R.C. and Ribak, C.E. (1987). GABAergic neurons and axon terminals in the brainstem auditory nuclei of the gerbil. *J. Comp. Neurol.* 258: 267-280.
- Ross, L.S., Pollak, G.D. and Zook, J.M. (1988). Origin of ascending projections to an isofrequency region of the mustache bat's inferior colliculus. *J. Comp. Neurol.* 270: 488-505.
- Roth, G.L., Aitkin, L.M., Andersen, R.A., Merzenich, M.M. (1978). Some features of the spatial organization of the central nucleus of the inferior colliculus of the cat. *J. Comp. Neurol.* 182: 661-680.
- Rothman, S M, and Olney, J W. (1986). Glutamate and the pathophysiology of hypoxic-ischemic brain damage. *Ann. Neurol.* 19: 105-111.
- Rothstein, J D, Dykes-Hoburg, M, Pardo, C A, Bristol, L A, Jin, L, Kuncl, R W, Kanai, Y, Hediger, M.A., Wang, Y, Schielke, J P, and Welty, D F. (1996). Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron.* 16: 675-686.
- Ruiz-Gómez, M.D. (1988). Estructura y conexiones de los núcleos del lemnisco lateral de la rata albina. M.D. Dissertation. Salamanca, Spain: Univeristy of Salamanca.
- Saint Marie, R.L. (1993). GABA and glycine immunoreactivities define principal subdivisions of the cat lateral lemniscal nuclei. *Soc. Neurosci. Abstr.* 19: 1204.
- Saint Marie, R.L. (1996). Glutamatergic connections of the auditory midbrain: selective uptake and axonal transport of D-[³H]Aspartate. *J. Comp. Neurol.* 373: 255-270.
- Saint Marie, R.L. and Baker, R. A. (1990). Neurotransmitter-specific uptake and retrograde transport of [³H]glycine from the inferior colliculus by ipsilateral projections of the superior olivary complex and nuclei of the lateral lemniscus. *Brain Res.* 524: 244-253.

- Saldaña, E., and Merchán, M.A. (1992). Intrinsic and commissural connections of the rat inferior colliculus. *J. Comp. Neurol.* 319: 417-437.
- Sally, S.L. and Kelly, J.B. (1992). Effects of superior olivary complex lesions on binaural interaction in rat inferior colliculus. *Brain Res.* 572: 5-18.
- Schmued, L., Kyriakdis, K., and Heimer, L. (1990). In vivo anterograde and retrograde axonal transport of the fluorescent rhodamine-dextran-amine, Fluoro-Ruby, within the CNS. *Brain Res.* 526: 127-134
- Schofield, B.R., and Cant, N.B. (1997). Ventral nucleus of the lateral lemniscus in guinea pigs: cytoarchitecture and inputs from the cochlear nucleus. *J. Comp. Neurol.* 379: 363-385.
- Shneiderman, A., Chase, M.B., Rockwood, J.M., Benson, C.G. and Potashner, S.J. (1993). Evidence for a GABAergic projection from the dorsal nucleus of the lateral lemniscus to the inferior colliculus. *J. Neurochem.* 60: 72-82.
- Shneiderman, A. and Oliver, D.L. (1989). EM autoradiographic study of the projections from the dorsal nucleus of the lateral lemniscus: a possible source of inhibitory inputs to the inferior colliculus. *J. Comp. Neurol.* 286: 28-47.
- Shneiderman, A., Oliver, D.L. and Henkel, C. (1988). Connections of the dorsal nucleus of the lateral lemniscus: An inhibitory parallel pathway in the ascending auditory system? *J. Comp. Neurol.* 276: 188-208.
- Silverman, M.S. and Clopton, B.M. (1977). Plasticity of binaural interaction. I. Effect of early auditory deprivation. *J. Neurophysiol.* 40: 1266-1274.
- Sommer, I., Lingenhöhl, K., and Friauf, E. (1993). Principal cells of the rat medial nucleus of the trapezoid body: an intracellular in vivo study of their physiology and morphology. *Exp. Brain Res.* 95: 223-239.
- Spangler, K.M., Warr, W.B., and Henkel, C.K. (1985). The projections of the medial nucleus of the trapezoid body in the cat. *J. Comp. Neurol.* 238: 249-262.

- Stotler, W.S. (1953). An experimental study of the cells and connections of the superior olivary complex of the cat. *J. Comp. Neurol.* 98: 401-432.
- Suárez, I., Bodega, G., Arilla, E., and Fernández, B. (1996). Long-term changes in glial fibrillary acid protein and glutamine synthetase immunoreactivities in the supraoptic nucleus of portacaval shunted rats. *Metabolic Brain Res.* 11: 369-379.
- Sugiyama, K., Brunori, A., and Mayer, M.L. (1989). Glial uptake of excitatory amino acids influences neuronal survival in cultures of mouse hippocampus. *Neurosci.* 32: 779-791.
- Swanson, R A, Liu, J, Miller, J W, Rothstein, J D, Farrell, K, Stein, B A, and Longuemare, M C. (1997). Neuronal regulation of glutamate transporter subtype expression in astrocytes. *J. Neurosci.* 17: 932-940.
- Tanaka, K., Otani, K., Tokunaga, A. and Sugita, S. (1985). The organization of neurons in the nucleus of the lateral lemniscus projecting to the superior and inferior colliculi in the rat. *Brain Res.* 341: 252-260.
- Thompson, G.C., Cortez, A.M. and Lam, D.M.K. (1985). Localization of GABA immunoreactivity in the auditory brainstem of guinea pigs. *Brain Res.* 339, 119-122.
- Torgner, I, and Kwamme, E. (1990). Synthesis of transmitter glutamate and the glial-neuron interrelationship. *Mol. Chem. Neuropathol.* 12: 11-17.
- Trapp, B.D., Peterson, J., Ransohoff, R.M., Rudick, R., Mork, S., and Bo, L. (1998). Axonal transection in the lesions of multiple sclerosis. *New England J. of Med.* 338: 278-85.
- van Adel B.A., Kelly, J.B. (1998). Kainic acid lesions of the superior olivary complex: effects on sound localization by the albino rat. *Behav. Neurosci.* 112: 432-446.
- van Adel, B.A. Kidd, S.A., Kelly, J.B. (1997). Transection of the commissure of Probst affects interaural time difference sensitivity in the rat's inferior colliculus. *Association for Research in Otolaryngology Abstracts.* 20: 351.

- van Adel, B.A., Ito, M., and J.B. Kelly. (1996). Transection of the commissure of Probst results in neuronal degeneration and expression of nitric oxide synthase (NOS) in Injured DNLL neurons of the albino rat. Association for Research in Otolaryngology Abstracts. 19: 46.
- van Gehuchten, A. (1906). Le system Nerveux de l'Homme, 4th ed. Louvain: Trois Rois.
- van Noort, J. (1969). The structure and connections of the inferior colliculus. An investigation of the lower auditory system. Assen: Van Gorcum.
- Vater, M., Casseday, J.H., and Covey, E. (1995). Convergence and divergence of ascending binaural and monaural pathways from the superior olive of the mustached bat. J. Comp. Neurol. 351: 632-646.
- Vater, M., Kössl, M. and Horn, A.K.E. (1992). GAD- and GABA-immunoreactivity in the ascending auditory pathway of horseshoe and mustached bats. J. Comp. Neurol. 325: 183-206.
- Vincent, S.R. 1994. Nitric Oxide: a radical neurotransmitter in the central nervous system. Progress in Neurobiology. 42: 129-160.
- von Monakow, C. (1890). Striae acusticae und untere Schliefe. Arch. Psychiatr. Nervenkrankh. 22: 1-29.
- Whitley, J.M., Henkel, C.K. (1984). Topographical organization of the inferior collicular projection and other connections of the ventral nucleus of the lateral lemniscus in the cat. J. Comp. Neurol. 229: 257-270.
- Willard, F.H., and Martin, G.F. (1983). The auditory brainstem nuclei and some of their projections to the inferior colliculus in the North American opossum. Neuroscience. 10: 1203-1232.
- Willard, F.H., and Martin, G.F. (1984). Collateral innervation of the inferior colliculus in the North American opossum: A study using fluorescent makers in a double labeling paradigm. Brain Res. 303: 171-182.

- Winer, J.A., Larue, D.T. and Pollak, G.D. (1995). GABA and glycine in the central auditory system of the mustache bat: structural substrates for inhibitory neuronal organization. *J. Comp. Neurol.* 355: 317-353.
- Woollard, H.H., and Harpman, J.A. (1940). The connexions of the inferior colliculus and of the dorsal nucleus of the lateral lemniscus. *J. Anat.* 74: 441-458.
- Wu, W. (1993). Expression of nitric-oxide synthase (NOS) in injured CNS neurons as shown by NADPH diaphorase histochemistry. *Experimental Neurology*. 120: 153-159.
- Wu, S.H. and Kelly, J.B. (1995). *In vitro* brain slice studies of the rat's dorsal nucleus of the lateral lemniscus. I. Membrane and synaptic response properties. *J. Neurophysiol.* 73: 780-793.
- Wu, S.H. and Kelly, J.B. (1995). *In vitro* brain slice studies of the rat's dorsal nucleus of the lateral lemniscus. II. Physiological properties of biocytin-labeled neurons. *J. Neurophysiol.* 73: 794-809.
- Wu, S.H. and Kelly, J.B. (1996). *In vitro* brain slice studies of the rat's dorsal nucleus of the lateral lemniscus. III. Synaptic pharmacology. *J. Neurophysiol.* 75: 1271-1282.
- Wyllie, H.A. (1981). Cell Death: a new classification separating apoptosis from necrosis. In: *Cell Death in Biology and Pathology*. Edited by I.D. Bowen and R.A. Lockshin. Chapman and Hall, New York, pp 9-29.
- Wynne, B., Harvey, A.R., Robertson, D., Sirinathsinghji, D.J.S. (1995). Neurotransmitter and neuromodulator systems of the rat inferior colliculus and auditory brainstem studied by in situ hybridization. *J. Chem. Neuroanat.* 9: 289-300.
- Yang, L., and Pollak, G.D. (1994a). The roles of GABAergic and glycinergic inhibition on binaural processing in the dorsal nucleus of the lateral lemniscus of the mustache bat. *J. Neurophysiol.* 71: 1999-2013.

- Yang, L., and Pollak, G.D. (1994b). Binaural inhibition in the dorsal nucleus of the lateral lemniscus of the mustache bat affects responses for multiple sounds. *Auditory Neurosci.* 1: 1-17.
- Yang, L., Liu, Q., and Pollak, G.D. (1996). Afferent connections to the dorsal nucleus of the lateral lemniscus of the mustache bat: evidence for two functional subdivisions. *J. Comp. Neurol.* 373: 575-592.
- Yin, T.C.T (1994). Physiological correlates of the precedence effect and summing localization in the inferior colliculus of the cat. *J. Neurosci.* 14: 5170-5186.
- Yu, W.A. (1994). Nitric oxide synthase in motor neurons after axotomy. *Journal of Histochemistry and Cytochemistry.* 9: 3908-3914.
- Yuan, J., Shaham, S., Ledoux, S., Ellis, H.M., and H.R. Hovitz. 1993. The *C. elegans* cell death gene *ced-3* encodes a protein similar to mammalian interleukin-1 β -converting enzyme. *Cell.* 75: 641-652.
- Zhang, D.X., Li, L., Kelly, J.B., and Wu, S.H. (1998). GABAergic projections from the lateral lemniscus to the inferior colliculus of the rat. *Hearing Res.* 117: 1-12.
- Zhao, M., and Wu, S.H. (1998) Intracellular labeling of cells in brain slice preparation of the rat's ventral nucleus of the lateral lemniscus. *Assoc. Res. Otolaryngol. Abstr.* 21: 368.
- Zook, J.M. and Casseday, J.H. (1979). Connections of the nuclei of the lateral lemniscus in the mustache bat, *Pteronotus parnellii*. *Soc. Neurosci Abstr.* 5: 34.
- Zook, J.M. and Casseday, J.H. (1982). Origin of ascending projections to inferior colliculus in the mustache bat, *Pteronotus parnellii*. *J. Comp. Neurol.* 207: 14-28.
- Zrull, M.C., and Coleman, J.R. (1997). Effects of tectal grafts on sound localization effects induced by inferior colliculus lesions in hooded rats. *Exp. Neurol.* 145: 16-23.

IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989

© 1993, Applied Image, Inc., All Rights Reserved