Form Approved Through 09/30/2007 OMB No. 0925-0001

Department of Health and Human Services
ublic Health Services

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PI: **MUSTARI, MICHAEL J** Council: 10/2005

2 R01 EY013308-04 A1 IPF:2384501

Dual:

IRG: ZRG1 IFCN-A(02) M Received: 03/14/2005

1. TITLE OF PROJECT *(Do not exceed 81 characters, including spaces and ...)*

Neural Control of Visual Vestibular Behavior

2. RESPONSE TO SPECIFIC REQUEST FOR APPLICATIONS OR PROGRAM ANNOUNCEMENT OR SOLICITATION ☒ NO ☐ YES *(If "Yes," state number and title)*

Number: Title:

3. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR		New Investigator ☒ No ☐ Yes	
3a. NAME (Last, first, middle) MUSTARI,Michael J.		3b. DEGREE(S) Ph.D.	3h. eRA Commons User Name
3c. POSITION TITLE Associate Professor, Neurology		3d. MAILING ADDRESS *(Street, city, state, zip code)* YERKES Natl Primate Res.Cen 954 Gatewood Road, EMORY UNIV Atlanta, Georgia 30329	
3e. DEPARTMENT, SERVICE, LABORATORY, OR EQUIVALENT Neurology			
3f. MAJOR SUBDIVISION School of Medicine			
3g. TELEPHONE AND FAX *(Area code, number and extension)* TEL: 404: 727-9194 FAX: 404:727-9294		E-MAIL ADDRESS: mjmustar@rmy.emory.edu	
4. HUMAN SUBJECTS RESEARCH ☒ No ☐ Yes	4b. Human Subjects Assurance No. FWA0005792	5. VERTEBRATE ANIMALS ☐ No ☒ Yes	
	4c. Clinical Trial ☒ No ☐ Yes 4d. NIH-defined Phase III Clinical Trial ☒ No ☐ Yes	5a. If "Yes," IACUC approval Date	5b. Animal welfare assurance no. A-3180-01
4a. Research Exempt ☐ No ☐ Yes	If "Yes," Exemption No.		

6. DATES OF PROPOSED PERIOD OF SUPPORT *(month, day, year—MM/DD/YY)*		7. COSTS REQUESTED FOR INITIAL BUDGET PERIOD		8. COSTS REQUESTED FOR PROPOSED PERIOD OF SUPPORT	
From	Through	7a. Direct Costs ($)	7b. Total Costs ($)	8a. Direct Costs ($)	8b. Total Costs ($)
12/01/2005	11/30/2010	$250,000	$381,734	$1,250,000	$2,071,734

9. APPLICANT ORGANIZATION	10. TYPE OF ORGANIZATION
Name Emory University Address Office of Sponsored Programs 1784 N. Decatur Road, Suite 510 Atlanta, GA 30322	Public: → ☐ Federal ☐ State ☐ Local Private: → ☒ Private Nonprofit For-profit: → ☐ General ☐ Small Business ☐ Woman-owned ☐ Socially and Economically Disadvantaged
	11. ENTITY IDENTIFICATION NUMBER DUNS NO. 066469933 Cong. District 04

12. ADMINISTRATIVE OFFICIAL TO BE NOTIFIED IF AWARD IS MADE	13. OFFICIAL SIGNING FOR APPLICANT ORGANIZATION
Name Marilyn Surbey Title Associate VP for Finance and Research Address Emory University, Office of Sponsored Programs 1784 N. Decatur Road, Suite 510 Atlanta, GA 30322 Tel: (404) 727-2503 FAX: (404) 727-2509 E-Mail: osp@emory.edu	Name Holly Sommers Title Assistant Director for Research Address Emory University, Office of Sponsored Prog. 1784 N. Decatur Road, Suite 510 Atlanta, GA 30322 Tel: (404) 727-2503 FAX: (404) 727-2509 E-Mail: osp@emory.edu

14. PRINCIPAL INVESTIGATOR/PROGRAM DIRECTOR ASSURANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties. I agree to accept responsibility for the scientific conduct of the project and to provide the required progress reports if a grant is awarded as a result of this application.	SIGNATURE OF PI/PD NAMED IN 3a. *(In ink. "Per" signature not acceptable.)*	DATE 03/02/2005
15. APPLICANT ORGANIZATION CERTIFICATION AND ACCEPTANCE: I certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.	SIGNATURE OF OFFICIAL NAMED IN 13. *(In ink. "Per" signature not acceptable.)*	DATE 3/10/05

DESCRIPTION: See instructions. State the application's broad, long-term objectives and specific aims, making reference to the health relatedness of the project (i.e., relevance to the **mission of the agency**). Describe concisely the research design and methods for achieving these goals. Describe the rationale and techniques you will use to pursue these goals.

In addition, in two or three sentences, describe in plain, lay language the relevance of this research to **public** health. If the application is funded, this description, as is, will become public information. Therefore, do not include proprietary/confidential information. **DO NOT EXCEED THE SPACE PROVIDED.**

Primate gaze (line of sight in space) movements require coordinated interactions between visual, vestibular and oculomotor systems. The long-term goal of this study is to define the role of different components of the cortico-ponto-cerebellar system in gaze behavior. Our preliminary studies, employing multiple retrograde and anterograde tracers, indicate considerable specificity in anatomical connections between different regions of cortex and the basilar-pontine nuclei including the dorsolateral pontine nucleus (DLPN) and nucleus reticularis tegmenti pontis (NRTP). These pontine nuclei are thought to play a critical role in processing gaze-related signals from the frontal eye field (FEF) and medial superior temporal (MST) cortex and delivering these signals to different regions of the cerebellum (e.g., ventral paraflocculus and vermis). Our preliminary results support the suggestion that DLPN and rNRTP play differential roles in smooth pursuit and gaze movements. The FEF and MST cortical areas appear to have biased inputs to the NRTP and DLPN, respectively. These findings along with functional differences in response properties FEF, MST, NRTP and DLPN neurons support the suggestion that there could be separate gaze-related channels of information in the cortical-pontine system. Therefore, our studies are designed to compare and contrast gaze-related information carried in FEF-NRTP and MST-DLPN pathways in awake, behaving macaques. To accomplish this goal we will use quantitative methods (e.g., multiple linear -regression modeling) to define gaze-related signals (visual-, eye- and head-motion) carried in FEF, MST, DLPN and rNRTP neurons. We will include comparative analysis of gaze signals in different regions of FEF-NRTP and MST-DLPN pathways during gaze movements. Because only some cortical neurons project to the brainstem, we will use electrical stimulation of NRTP and DLPN to antidromically activate FEF and MST neurons. We will then be able to characterize gaze-related signals in these identified neurons. Our preliminary results indicate that neurons in the FEF-NRTP pathway provide information especially related to the initial phase of gaze movements. In contrast, neurons in the MST-DLPN pathway appear to provide signals related to maintaining gaze movements. Successful completion of our studies will provide new information that could help in the diagnosis and potential treatment of gaze disorders in patients.

PERFORMANCE SITE(S) (organization, city, state)

YERKES National Primate Research Center, EMORY University, Atlanta, Georgia 30329

KEY PERSONNEL. See instructions. *Use continuation pages as needed* to provide the required information in the format shown below. Start with Principal Investigator. List all other key personnel in alphabetical order, last name first.

Name	eRA Commons User Name	Organization	Role on Project
MUSTARI, Michael J.		YERKES/Emory Univ.	Principal Investigator
		YERKES\Emory Univ.	CO-Investigator
		YERKES\Emory	Res.Assocociate.

OTHER SIGNIFICANT CONTRIBUTORS

Name	Organization	Role on Project

Human Embryonic Stem Cells ☒ **No** ☐ **Yes**

If the proposed project involves human embryonic stem cells, list below the registration number of the specific cell line(s) from the following list: http://stemcells.nih.gov/registry/index.asp. *Use continuation pages as needed.*

If a specific line cannot be referenced at this time, include a statement that one from the Registry will be used.

Cell Line

Disclosure Permission Statement. Applicable to SBIR/STTR Only. See instructions. ☐ Yes ☐ No

The name of the principal investigator/program director must be provided at the top of each printed page and each continuation page.

RESEARCH GRANT
TABLE OF CONTENTS

(Items A-D: not to exceed 25 pages*)

* *SBIR/STTR Phase I: Items A-D limited to 15 pages.*

Appendix *(Five collated sets. No page numbering necessary for Appendix.)*

Check if Appendix is Included

Appendices NOT PERMITTED for Phase I SBIR/STTR unless specifically solicited ☒

Number of publications and manuscripts accepted for publication *(not to exceed 10)* 5

Other items (list):

BUDGET JUSTIFICATION PAGE
MODULAR RESEARCH GRANT APPLICATION

	Initial Period	2nd	3rd	4th	5th	Sum Total (For Entire Project Period)
DC less Consortium F&A	250,000 *(Item 7a, Face Page)*	250,000	250,000	250,000	250,000	1,250,000 *(Item 8a, Face Page)*
Consortium F&A	0	0	0	0	0	0
Total Direct Costs	250,000	250,000	250,000	250,000	250,000	$ 1,250,000

Personnel

Principal Investigator (Michael J. Mustari, Ph.D.): Salary support (effort) is requested for Dr. Mustari who will be responsible for all phases of this project. This includes conducting single unit recording, antidromic activation studies and anatomical analysis. The P.I. will work closely with all members of our research team to ensure that we are successful in obtaining our research objectives and publishing results.

Co-investigator (: Salary support (effort) is requested for who will assist with modeling studies in our behavioral and single unit research. is a Bioengineer with a strong background in computational and control-systems modeling, applied to vestibular-ocular research.

Research Associate: (: Salary support (effort) is requested for a dedicated Research Associate who has contributed significantly to the goals of our project. will devote full-time effort on this project. He will play a major role in all single unit recording studies

Animal Laboratory Technologist effort) This project involves extensive, demanding work associated with neurophysiological research in trained rhesus monkeys. The major responsibilities of the research technician will include, animal training, animal care management and general laboratory management such as manufacturing electrodes, ordering supplies, etc. The laboratory technician is essential to the successful completion of the proposed studies.

Histologist To be Appointed (B.S.): 50% effort Year 1, 100% effort year 2-5. Our neuroanatomical research under Specific Aim 2 involves extensive amounts of histological preparation. In year one of our project our anatomical workload will be less than in other years. Therefore, we have asked for 50% support in the first year and 100% in the remaining years.

Consortium

N/A

Fee (SBIR/STTR Only)

N/A

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2.
Follow this format for each person. **DO NOT EXCEED FOUR PAGES.**

NAME **MUSTARI, Michael J.**	POSITION TITLE
eRA COMMONS USER NAME MJMUSTAR@RMY.EMORY.E	**Associate Professor of Neurology**

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Southern Illinois University, Carbondale	B.A.	1970	Physiology
Southern Illinois University, Carbondale	M.A.	1971	Physiology
University of Washington, Seattle	Ph.D.	1976	Neuroanatomy

A. Positions and Honors

1976-78 Post-doctoral Fellow, Dept of Psychology, Dalhousie University, Halifax, Nova Scotia.
1979-82 Research Fellow, Dept of Physiology, Australian National University, Canberra, Australia.
1982-84 Research Fellow, Department of Physiology and Biophysics and Regional Primate Research Center, University of Washington, Seattle, WA.
1984-90 Principal Investigator, Department of Physiology and Biophysics and Regional Primate Research Center, University of Washington, Seattle, WA.
1990- 98 Associate Professor, Department of Anatomy & Neuroscience, University of Texas Medical Branch, Galveston, TX.
1998-present Associate Professor, Department of Neurology, Adjunct Assistant Professor, Department of Ophthalmology, Emory University, Atlanta, GA.
1998-present Core Staff, Visual Sciences Division, Yerkes National Primate Research Center, Emory University, Atlanta, GA.
1999-present Chief, Visual Sciences Division, Yerkes National Primate Research Center, Emory University, Atlanta, GA.
2001-2002 President, Atlanta Chapter of Society for Neuroscience

Other Experience and Professional Memberships

1975 - Present Member, Society for Neuroscience (SFN)
1982 - Present Member, Association for Research in Vision and Ophthalmology (ARVO)
1982 - Present Member, American Association for the Advancement of Science (AAAS)

B. Selected peer-reviewed publications (in chronological order)

1. Price N. Ibbotson M., Ono S. and Mustari MJ. Rapid processing of retinal slip during saccades in macaque area MT. J Neurophysiol in-press, 2005.
2. Stewart M, Mustari MJ and Perachio AA. Visual-vestibular interaction during vestibular compensation: Role of the NOT in hVOR recovery after hemilabyrinthectomy (HL). J Neurophysiol doi:10.1152, /jn00739, 2005).
3. Ono S, Das VE and Mustari MJ. Modeling smooth pursuit related neuronal responses in the DLPN and NRTP of Rhesus Macaque. J Neurophysiol. 93: 108-116, 2005.
4. Akao T., Mustari MJ, Fukushima J., Kurkin S., and Fukushima K. J. Discharge characteristics of MST pursuit neurons during vergence eye movements. Neurophysiol 110.1152/jn.01028, 2004.

RESOURCES

FACILITIES: Specify the facilities to be used for the conduct of the proposed research. Indicate the performance sites and describe capacities, pertinent capabilities, relative proximity, and extent of availability to the project. Under "Other," identify support services such as machine shop, electronics shop, and specify the extent to which they will be available to the project. Use continuation pages if necessary.

Laboratory:

Dr. Mustari's laboratory comprises at Yerkes National Primate Research Center (YNPRC). The laboratory comprises . Currently, we have two fully equipped neurophysiology setups and 1 animal training setup and separate laboratory space (500 square feet) for neuroanatomical research.

Clinical:

No Human Studies

Animal:

Animals are housed in special animal quarters Members of the YNPRC Animal Resource Division perform all activities associated with animal maintenance. All primate surgical procedures are performed by the P.I. in a dedicated sterile surgical suite maintained by YNPRC. The surgery is fully implemented with essential support personnel and equipment.

Computer:

We use current Pentium computers equipped with appropriate hardware (e.g., Cambridge Electronics Design, Power1401 CED) and software for experimental control, data acquisition/analysis (MAtlab) and visual stimulation (computer driven mirror galvanometers/optic bench or Cristie DLP, Mirage 2000).

Office:

Dr. Mustari and each have separate office space (approx 100 square feet each) on the same floor as the laboratory in YRPRC.

Other:

Machine and Electronics shop support is available on a fee for service basis at Yerkes and at other shops at Emory. Part-time secretarial support is provided for faculty by YNPRC, Division of Visual Sciences.

MAJOR EQUIPMENT: List the most important equipment items already available for this project, noting the location and pertinent capabilities of each.

We have two fully equipped visual-oculomotor recording laboratories, allowing simultaneous single unit recording sessions in awake, behaving monkeys. A third laboratory is setup for training animals in gaze-pursuit tasks. Each experimental setup is equipped for eye movement detection with magnetic search coil systems (3-foot CNC thee-field coil system, CNC-Engineering, Seattle, WA.). We have multiple phase-detectors (CNC) for each experimental setup, so that we can record simultaneously from each eye and head in our experiments. Visual Stimuli are rear projected on a high quality screen (Stewart Film Screen) using dual optic benches each equipped with x,y mirror galvanometers (General Scanning). Visual stimuli can also be presented on a dedicated computer controlled digital light projector (Cristie DLP Mirage 2000). The DLP has true vertical synchronization so that we can generate disparity based stimuli for vergence testing or visual motion in depth. The second recording setup includes a 60 ft•lb torque motor and slip-rings (Neurokinetics) to allow horizontal vestibular stimulation about the vertical axis (continuous or periodic). Standard anatomical facilities are available to support any of our proposed studies. This includes all necessary sectioning, staining and microscopy equipment.

3 pages redacted--response to reviewers' critiques

A. SPECIFIC AIMS:

Primate gaze (line of sight in space) movements require interactions between visual, vestibular and oculomotor systems. A considerable body of evidence indicates that different cortical areas, including supplementary eye fields (SEF), frontal eye fields (FEF), medial superior temporal (MST) and middle temporal (MT), contribute to producing smooth pursuit and gaze behavior. Gaze movements require sensory-motor transformations of signals in cortex, related brainstem and cerebellar centers. The long-term goal of this project is to define the role of different components of the cortico-ponto-cerebellar system in gaze movements. Our central hypothesis is that gaze behavior is supported by different cortical areas providing complimentary information to specific regions of the dorsolateral pontine nucleus (DLPN) and rostral nucleus reticularis tegmenti pontis (rNRTP), which in-turn provide essential inputs to gaze-related regions of the cerebellum (e.g., flocculus, ventral paraflocculus and vermis). These cerebellar regions can affect coordination of gaze by projections to the vestibular nucleus, deep cerebellar nuclei and thalamus. Our proposed studies address a central component of this circuitry, specifically, what are the signals carried in different FEF-rNRTP and MST-DLPN neurons during gaze behavior. We will primarily use single unit recording, antidromic activation and anatomical studies to define the functional organization cortical-pontine circuits in gaze.

Specific Aim 1: COMPARE AND CONTRAST GAZE-RELATED SIGNALS IN FEF-NRTP AND MST-DLPN PATHWAYS.

Project 1: By characterizing neuronal properties in MST, FEF, NRTP and DLPN in each animal during the same behavioral paradigms, we have a chance to directly compare information content in these pathways.

In contrast, neurons in the MST-DLPN pathways may play a larger role in maintenance of tracking (e.g., gaze-velocity).

Project 2: We will identify which cortical gaze-related signals are relayed to pontine neurons.

In contrast, neurons in MST and DLPN feature visual and eye motion signals.

Specific Aim 2: DEFINE THE ANATOMICAL ORGANIZATION OF CORTICO-PONTO-CEREBELLAR PROJECTIONS.

B. BACKGROUND AND SIGNIFICANCE:

Visual-Vestibular Interactions (Behavior):

The main goal of our proposed studies is to investigate the role of specific cortical and basilar-pontine centers in gaze-pursuit behavior. Generation of gaze movements depends on coordinated function of visual, vestibular and oculomotor systems. We have chosen to focus our neurophysiological studies on FEF, MST, NRTP and DLPN because of strong anatomical and physiological evidence indicating that these areas play complimentary roles in gaze behavior. Figure 1 provides a simplified anatomical diagram showing pathways under consideration in our studies. Our studies and this review are specifically directed at the role of cortical-pontine signals in gaze-pursuit. Rapid gaze shifts may be supported by other regions of the cortical-ponto-cerebellar system. **We realize that cortical areas MST and FEF have projections outside the NRTP and DLPN including projections to the vestibular nuclei. However, our single unit recording and anatomical studies are focused on cortical-ponto-cerebellar pathways.**

Stable gaze during locomotion is necessary to maintain the visual image of the world from slipping on the retina (Walls 1962; Carpenter 1991). Previous studies in humans have shown that slip of greater than 2-4°/s causes degradation in visual performance (Westheimer and McKee 1975; Burr and Ross 1982). The main gaze stabilizing reflex is the vestibulo-ocular reflex (VOR). The VOR generates compensatory eye movements during head movements, such that subjects are able to maintain their line of sight, or gaze, on a stationary object. Linear and rotational accelerations of the head are sensed by otolith-related and semicircular canals-related neurons respectively (see Goldberg et al. 1990; Leigh and Zee 1999 for review). Vestibular afferents carry a head velocity signal to central vestibular neurons, which in turn drive motor neurons to produce the vestibular ocular reflex (VOR). This simple three-neuron VOR arc can be adjusted by visual inputs traveling in relatively direct and indirect pathways (see du Lac et al. 1995, for review). VOR gain (i.e., ratio of eye velocity to head velocity) is typically ~0.80 when measured in darkness. However, in the presence of a visual target, the "visually-enhanced" VOR (VVOR) gain is ~1.0. Visual enhancement of the VOR could be due at least in part to a linear summation of the VOR and visually mediated tracking including smooth pursuit or optokinetic responses generated in response to retinal image slip. Such visual following responses could contribute the additional eye velocity (~20%) of the VVOR to maintain clear vision (Robinson 1977; Raphan et al 1977; Lau et al. 1981; Lisberger et al. 1981; Baloh et al., 1981 1986). In addition, visual error signals are essential for adjusting VOR gain in response to physiological (Lisberger et al. 1983; Lisberger et al. 1994b; Raymond and Lisberger 1998) or pathological challenges to vestibular–ocular function (e.g., Stewart, Mustari et al. 2005; Appendix Manuscript). Most evidence points to the accessory optic system and NOT as sources of error signals for visual modification of the VOR (see Raymond and Lisberger 1998 for review; Yakushin et al. 2000; Stewart, Mustari et al 2005).

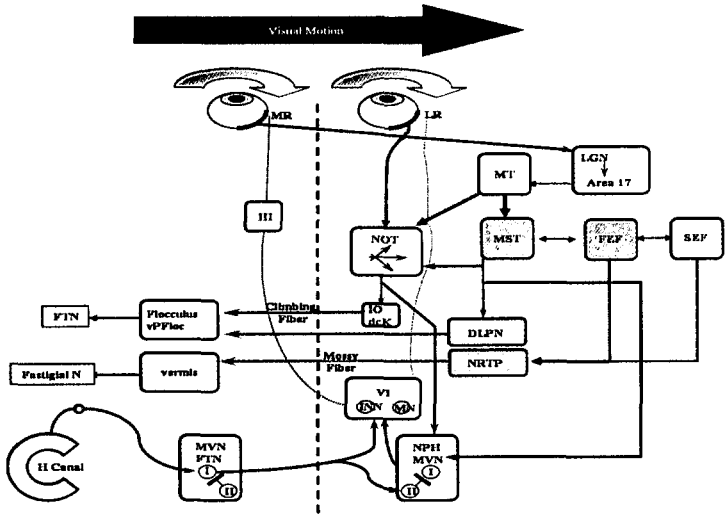

Figure 1: Simplified diagram indicating the potential flow of **cortico-ponto-cerebellar** signals essential for gaze behavior. Our studies will compare and contrast the signals carried in the FEF-rNRTP and MST-DLPN pathways. NOT signals drive complex-spike activity via connections with the inferior olive. DLPN and NRTP signals drive simple spike activity through mossy fiber connections with granule cells. Gaze-related signals carrying retinal error information (position, velocity and acceleration) are processed mostly in the NOT and DLPN. Gaze-related signals carrying mostly head or eye motion (position, velocity and acceleration) are carried in rNRTP and DLPN pathways. Specificity in ponto-cerebellar connections (flocculus, vPFloc and vermis) will be examined in new anatomical studies using multiple retrograde and anterograde tracers. **Specific subregions of FEF, MST (MSTd, MSTl, MSTf) will be examined.**

Exploring the visual environment requires reorienting gaze towards objects of interest. This is a deliberate process requiring participation of different cortical, brainstem and cerebellar areas in selecting targets and generating appropriate pursuit and gaze movements.

Cancellation of the VOR during Gaze Behavior:

Cancellation of the VOR is required to execute appropriate gaze movements in certain behavioral contexts. For example, when an object of interest moves in the same direction as the head, the VOR acting alone would move the eyes off target (Cullen and McCrea 2000; see Leigh and Zee 2000 for review). The primary method of cancellation could be an algebraic summation of the VOR with smooth-pursuit eye movements (Robinson 1982, Lisberger et al. 1981, Huebner, et al. 1992; Meng et al., 2005). However, linear superposition of VOR and smooth pursuit may not be the only cancellation mechanism because step changes in head velocity during VOR cancellation show that cancellation occurs at latencies shorter than can be attributed to smooth pursuit in both human and non-human primates (Lisberger 1990, Cullen et al. 1991, Johnston and Sharpe 1994). Quantitative testing of a linear model using parameter estimation techniques showed that there was a short-latency modulation of VOR gain in addition to summation of VOR and SP eye movements (Huebner et al. 1992). Potentially, gaze-related neurons in the cortico-pontine pathways could play a role in short-latency cancellation of the VOR. One theory that has been developed to explain short-latency cancellation of the VOR is that there is a reduction in head movement sensitivity within the PVP neurons in the vestibular nuclei (Cullen and McCrea 1993a, b; see Cullen and Roy 2004, for review). The trigger to this reduction in head movement sensitivity is unknown but could involve cortico-pontine inputs to the cerebellum that affect neurons in the vestibular nucleus. For example, recent work by Belton and McCrea (2000) demonstrated that the flocculus is essential for visual cancellation of the VOR. They found that after unilateral muscimol inactivation of the flocculus that squirrel monkeys were unable to use visual targets to cancel the VOR. *Our recent studies have identified the DLPN as, at least, one source of signals essential for VOR cancellation. This is because cancellation was defective after inactivation of the DLPN (see Appendix manuscript, Ono et al., 2003; see Fig. 14).* ***Our single unit studies will define information related to visual, eye and head movements in neurons in specific subregions of FEF-rNRTP and MST-DLPN pathways. Defining these signals is essential for us to understand the neural substrate supporting gaze behavior.***

Gaze-related signals in MT, MST and FEF Cortex:

Early studies in humans and monkeys demonstrate that extrastriate cortex is essential for smooth pursuit and potentially for gaze movement. Unilateral cortical lesions produce ipsilesional deficits (see Wurtz et al. 1990 for review; Baloh, et al. 1980; Lynch and McLaren 1983; Bogousslavsky and Regli 1986; Leigh and Tusa 1985; Zee, et al. 1987; Tusa et al. 1989). Lesions placed in the cortical visual motion processing areas MT and MST produce different deficits in smooth pursuit eye movements (Dursteler et al. 1987; Dursteler and Wurtz 1988) and defective visual motion perception (Newsome et al. 1985; see Britten et al. 1992 for review). Lesions of MT produce a "retinotopic deficit" such that visual motion is underestimated and pursuit is deficient when a target moves in the portion of the visual field represented at the lesion site (Newsome et al. 1985). In contrast, lesions of MST produce a "directional deficit" where smooth pursuit has reduced gain during ipsiversive target motion (Dursteller et al. 1987). These deficits comprise reduced gain of ipsilesional smooth pursuit. MT neurons receive direct inputs from special complex cells in layer-4B of striate cortex that are highly sensitive to component visual motion (Movshon and Newsome 1994). **It is important for our studies to compare and contrast functional properties of neurons in different subregions of MST. For example, we know that MSTd and MSTl contain different functional classes of visual-oculomotor or visual neurons. For example, neurons in MSTd carry extraretinal signals related to smooth pursuit or vergence (e.g, Newsome et al., 1988; Akao, Mustari Fukushima et al., 2004). MSTl neurons often have visual receptive fields with a "center-surround" like organization, ideally suited for processing local motion information. One hypothesis is that MSTd and MSTl play complimentary roles in processing motion due to our own movements (MSTd) or movement of objects in the environment (MSTl; see Eifuku and Wurtz 1998 for review). We hypothesize that different subregions of MST target different regions of the pontine nuclei (e.g., DLPN, NRTP).** Psychophysical and single unit studies have demonstrated that visual motion perception is well matched to the

properties of neurons in MT and MST (Celebrini and Newsome 1994; see Shadlen et al. 1996 for review). For example, in a two-alternative forced-choice paradigm, monkeys were required to indicate the direction of coherent motion in a random dot display. Not only did MT and MST neuronal response match psychophysical performance but motion perception could be biased toward the directional preference of neurons by delivering low current electrical stimulation through the recording electrode (Celebrini and Newsome 1994). The response properties of MT and MST neurons can also be influenced by attention to a particular stimulus or stimulus feature (Treue and Maunsell 1999; Seidemann and Newsome 1999; Recanzone and Wurtz 2000). Attention effects are strong when stimuli move within the receptive field of MT neurons in their preferred direction (Treue and Maunsell 1999).

For the cortex to play a role in gaze-pursuit, information regarding current head movement must be taken into account. Vestibular information is known to reach cortical levels over different pathways. For example, Ebata and colleagues (2004) recently reported that electrical stimulation of the vestibular nerve resulted in short latency activation in FEF and in cortex along the nearby principle sulcus. This short latency vestibular activation indicates rather direct inputs to the FEF, possibly by way of the vestibular nucleus and thalamus. What was not demonstrated in these studies was whether the inputs were from otolith and canals. Early reports of interactions between rotational-VOR and visual motion were described in area-7, probably including MST (e.g., Sasaki et al. 1984; Kawano et al. 1984). Evidence for MST neurons with gaze-sensitive properties comes from work of Duffy and colleagues (e.g. 1998). They found some MST neurons had strong interactions between otolith mediated linear-VOR and visual flow-fields. They hypothesized that MST neurons selectively activated by appropriate combinations of head translation and optic-flow could play role in discrimination of heading directions. For example, some populations of MST neurons might be especially sensitive to optic-flow expansion field that would be selectively activated during translation and perhaps especially during active translation (Duffy et al. 2003). In summary, neurons in the MT and MST cortex are sensitive to direction, speed, acceleration, disparity and other aspects of visual, eye and head motion essential for generating gaze-pursuit (Anderson et al. 1990; Maunsell and Newsome 1987; Wurtz and Duffy 1990; Kawano et al. 1992; Lisberger and Movshon 1999). **Area MST has connections with the FEF, where visual, eye and head motion related neurons have been described.** ***We suggest that the FEF uses information derived at least in part from MSTd to produce gaze-pursuit (see Preliminary Data).***

Neurons in the frontal eye field cortex have been shown to carry signals related to smooth pursuit (Keating 1991; Keating et al. 1993; Gotlieb et al. 1993) and gaze movements (Fukushima et al. 2004 for review). Fukushima and colleagues (2002) have demonstrated that FEF contains neurons related not only to smooth pursuit and gaze but to vergence as well. Therefore, the FEF may play a role in all aspects of gaze-pursuit. At least some of the gaze and vergence related activity of the FEF may be derived from MST cortex. This is because we recently obtained evidence that neurons in area MSTd play a role in vergence tracking (Fukushima et al. 2003; Akao, Mustari, Fukushima et al., 2004; Preliminary Data, Fig. 8). We found not only frontal pursuit and visual disparity sensitive neurons in MSTd but also neurons that responded specifically during vergence-position or vergence-velocity (see Preliminary Data, Fig. 8). Lesion studies have provided further support for a FEF role in smooth pursuit (Keating et al. 1991; 1993; see Keating et al. 1996 for review; Shi et al. 1998). Micro-electrical stimulation (ES) has proven to be a powerful tool in developing insights into potential roles of FEF in gaze behavior. When ES is delivered to the FEF cortex specific aspects of smooth pursuit eye movements are affected. For example, Tanaka and Lisberger (2001; 2002a) demonstrated that ES of the FEF had the largest effect during maintained smooth pursuit. Enhancements of both the direction and gain of pursuit were observed. Gain-control is essential when tracking specific targets among an array of possible targets, as is often required in natural viewing conditions. Electrical stimulation in the smooth pursuit part of FEF during a double-target task (where two identical targets move in opposite directions) produces a bias in the direction of the smooth eye movement performed. This bias was in the same direction as the eye movement elicited during fixation (Tanaka and Lisberger 2002b). Similarly, when the saccadic region of the FEF was stimulated in a double-target task, the target "chosen" for smooth pursuit tracking was the one closest to the endpoint of the electrically elicited saccade (Gardner and Lisberger 2002). Whether FEF is able to support target selection alone or whether other areas such as the SEF play a role in this process remains uncertain. In complimentary studies, ES of the SEF significantly

alters anticipatory or predictive smooth pursuit (Missal and Heinen 2001). The SEF provides strong input to FEF and also to the NRTP. The largest effect of SEF stimulation occurred when the stimulus was delivered immediately before pursuit initiation in a paradigm where the monkey used a predictive pursuit strategy. **Neurons in SEF could modulate gaze through connections with FEF. Alternatively, rNRTP may integrate signals from SEF and FEF at the level of individual neurons or simply relay separate channels of information to the cerebellum. Our modeling, antidromic activation and anatomical studies will provide new data to determine whether pontine nuclei function primarily as integrative or relay centers.**

Learning can be demonstrated in the smooth pursuit system under appropriate conditions. For example, Chou and Lisberger (2004) used a double-velocity step in a step-ramp smooth pursuit paradigm to determine whether neurons in the FEF changed their properties during pursuit learning. In this paradigm, smooth pursuit initial velocity gradually changes according to the magnitude of the second velocity step. Although FEF stimulation elicited much stronger eye movements after learning then before learning, neuronal properties of FEF neurons did not appear to change following learning. This led these investigators to argue that learning occurred downstream from FEF (Chou and Lisberger 2004). We suggest that signals derived from FEF and perhaps MST could play a role in learning in the double-velocity step paradigm. Other pathways including ones carrying visual motion information (e.g., MT-NOT) could also play a role in learning. The site of pursuit learning appears to be located, at least partially, in the vermis. This is because learning in a double-velocity step paradigm was found to be defective after lesions of the vermis (Takagi et al. 2000). As reviewed above, the rNRTP projects strongly to the vermis.

In summary, single unit recording, electrical stimulation and lesion studies demonstrate that different cortical areas including SEF, FEF, MST and MT play complimentary roles in smooth pursuit, vergence and gaze movements. ***Our studies are designed to specifically characterize gaze-related signals (eye, head and retinal error motion) in rNRTP, DLPN and in FEF and MST (MSTd, MSTl, MSTf) neurons that project to these pontine centers.***

Gaze-related signals in NRTP and DLPN:

Virtually all potential gaze-related regions of the cortex including cortical areas including the SEF, FEF, MST and MT have strong differential projections to NRTP, DLPN and NOT (Gotlieb et al. 1993; May and Andersen 1986; Glickstein et al. 1980 1994; Distler et al. 2001). Cortically derived visual-oculomotor signals are processed in different brainstem regions, including the NOT (see Fuchs and Mustari 1993 for review), DLPN (Suzuki and Keller 1984; Mustari et al. 1988; May et al. 1988; Suzuki et al. 1990) and NRTP (Crandall and Keller 1985; Yamada et al. 1996; Suzuki 1996; et al. 1999). Recently, we have shown that the NOT and DLPN receive projections from different populations of MT and MST neurons (see Fig. 2; Distler et al. 2002). We suggest that the DLPN and rNRTP play complimentary roles in smooth pursuit and gaze control (Suzuki et al. 1992; Their et al. 1988; Mustari et al. 1988; Ono et al. 2004). The DLPN sends mossy fiber projections to the contralateral ventral paraflocculus and dorsal paraflocculus (Glickstein et al. 1994; Nagao et al. 1997) and some input to vermal lobule VI and VII (Brodal 1979; Brodal 1982; Langer et al. 1985). The NRTP receives inputs from FEF and supplementary eye fields (SEF) (Kunzle and Akert 1977; Brodal 1980a; Huerta et al. 1986; Shook et al. 1990; Giolli et al. 2001) and sends strong projections to the oculomotor vermis (lobules VI and VII; Brodal 1980b; Brodal 1982; Glickstein et al. 1994). Therefore, the cerebellum receives information from numerous cortical areas that could play a role in gaze. This information could be used by the cerebellum to construct internal models useful for controlling and modifying specific motor output (see Wolpert et al. 1998 for review).

Previous lesion and electrical stimulation studies established that the DLPN and rostral region of the nucleus reticularis tegmenti pontis (rNRTP) both play a role in smooth pursuit eye movements (Suzuki and Keller 1984; Mustari et al. 1988; Thier et al. 1988; Suzuki et al. 1990; Yamada et al. 1996; Suzuki et al. 1999). Further evidence for postulating an expanded role of DLPN and rNRTP in eye movements comes from single unit studies (Mustari et al. 1988; Thier et al. 1988; Suzuki and Keller 1984; Suzuki et al. 1990; Suzuki et al. 2002; Ono et al. 2003). These single unit recording studies show that different DLPN and rNRTP neurons could be classified as preferentially sensitive to smooth pursuit eye-velocity, position (Mustari et al. 1988; Thier et al. 1988) or acceleration (Suzuki et al. 2002; Ono et al. 2004). The DLPN and NRTP may play complimentary roles in

pursuit eye movement control, with the rNRTP having an expanded role involving gaze control (Yamada et al. 1996; Ono et al. 2003; Preliminary Data). Some NRTP neurons respond during changes associated with the near or far response and electrical stimulation among these neurons elicits vergence eye movements (Gamlin and Clarke 1995). These vergence-related neurons may receive signals from MST (Fukushima et al. 2003; Akao, Mustari, Fukushima et al., 2004; Preliminary Data) and FEF where vergence related neurons have been described (Fukushima et al. 2002). **A missing component in some of the early studies of NRTP and DLPN was that contributions from visual, eye and head motion were rarely examined in the same studies.** ***Our studies specifically target this deficiency. We have recently discovered that neurons in both rNRTP and DLPN actually discharge in relation to gaze movements and not just during smooth pursuit.***

Cortical-pontine signals are then relayed to the cerebellar flocculus, ventral paraflocculus and vermis (see Gerrits et al. 1995 for review) to eventually effect smooth eye movements (Lisberger and Fuchs 1978; Goldreich et al 1996; Shidara et al. 1993). Different regions of the basilar pons such as NRTP and DLPN and subregions within these areas have highly specific functional properties. For example, the NRTP has clearly separate saccadic and smooth pursuit regions (rNRTP). Anatomical studies using anterograde tracers report patchy cortical projections to the basilar pontine nuclei (e.g., Glickstein et al. 1980; Brodal et al. 1980; Distler, Mustari, Hoffmann 2002). Therefore, there appears to be clear anatomical and functional segregation in the cortical-pontine system with the rNRTP and DLPN being specialized for slow eye movements (smooth pursuit and gaze) and the caudal NRTP for rapid eye movements (saccades and gaze). Our single unit recording and anatomical studies are specifically designed to test this hypothesis.

Lesion or reversible inactivation of the DLPN results in deficits in smooth pursuit (May et al. 1988) cancellation of the (Ono et al. 2003), optokinetic nystagmus (May et al. 1988) and ocular following, (Kawano et al. 1992) for ipsilesional motion. The NRTP has been shown to play a role in smooth pursuit eye movements in addition to its well-known role in saccadic eye movements (Crandall and Keller 1985; Hepp and Henn 1983). In a series of studies by Suzuki and colleagues, the rNRTP has been shown to play a role in vertical smooth pursuit by reversible inactivation (Suzuki et al. 1999), electrical stimulation (Yamada et al. 1996) and single unit recording studies (Suzuki et al. 1996). The NRTP appears to receive cortical inputs from regions of the frontal eye fields, possibly including those related to smooth pursuit (Gottlieb et al. 1994; Distler et al. 2001). The DLPN may be specialized as a link for visual motion and smooth pursuit processing areas of the cortex with the cerebellum. Our preliminary studies using antidromic activation indicate that different regions of FEF, MST (MSTd, MSTl) and MT target different regions of NRTP and DLPN.

Cerebellum and Gaze:

The flocculus, ventral paraflocculus and vermis of the cerebellum have been shown to play major and complimentary roles in visual-vestibular behavior, control of smooth eye movements and vergence. Early lesion studies show that removal of the cerebellum including the flocculus and ventral paraflocculus produce profound deficits in smooth pursuit (Zee et al. 1981; Waespe et al. 1983) and adaptive plasticity of the VOR (Robinson 1976; Lisberger et al. 1984). Early studies by Lisberger and Fuchs 1978; Miles and Lisberger 1980) discovered that a class of Purkinje cell in the flocculus discharged in relationship to gaze movements during visual-vestibular behavior. These so-called horizontal gaze-velocity Purkinje (HGVP) cells discharge during smooth pursuit with the head stationary and during cancellation of the VOR. HGVP cells receive inputs from the visual system over climbing- and mossy-fiber pathways and vestibular and eye movement related signals over mossy fiber pathways (Lisberger and Fuchs 1978; Stone and Lisberger 1990). Therefore, HGVP cells receive appropriate signals necessary to play a role in normal visual-vestibular function, including visually guided motor learning in the VOR. Belton and McCrea provided single unit data documenting two types of Purkinje cells in the squirrel monkey flocculus that could play a role in visual-vestibular interaction and VOR cancellation. Eye-velocity Purkinje cells (EVPC) evinced strong visual sensitivity, showing reduced sensitivity during VOR in the dark. The other cell type resembled macaque HGVP cells. The contribution of these different P-cell types to visual-vestibular function is probably similar in squirrel monkeys and macaques. *Our studies under Specific Aim 1 will determine the visual, eye and head sensitivity (position, velocity, acceleration) of FEF-rNRTP and MST-DLPN*

neurons during gaze movements. Signals carried in these pathways are likely to make a significant contribution to the response properties of cerebellar Purkinje cells (e.g. HGVP).

Recent lesion studies have shown that the oculomotor vermis plays a significant role in smooth pursuit initiation and short-term adaptive plasticity in a double velocity-step smooth pursuit paradigm (Takagi et al. 2000). Smooth pursuit was particularly impaired during the first 100ms during step-ramp tracking. When monkeys were trained to adapt to a velocity perturbation during step-ramp tracking there was no early response. This deficit coupled with our findings that rNRTP neurons have significant eye acceleration sensitivity suggests that the FEF-rNRTP-oculomotor vermis might mediate the early response. A role for the vermis in smooth pursuit is supported by single unit studies showing that simple spike discharge of vermal Purkinje cells is related to smooth pursuit. This pursuit role may be supported by projections of the vermis to the caudal fastigial nucleus (cFN). Single unit recording, muscimol inactivation and lesion studies have provided strong evidence that the cFN plays a role in smooth pursuit initiation and maintenance (Fuchs et al. 1994). For example, lesions of the cFN lead to deficits in smooth pursuit consistent with a role in accelerating contralateral and decelerating ipsilateral smooth pursuit. Fuchs and colleagues (e.g. 1994) have provided evidence that timing of cFN discharge either early or late with respect to smooth pursuit initiation may provide the control signals required to produce differential effects on ipsi-and contralateral pursuit.

Gaze Pursuit: In a natural setting gaze movements are actively produced using different combinations of head and eye movements. For volitional gaze movements to be performed successfully, the cortex must be kept apprised of current head position and movement. Vestibular information can reach cortex through several pathways including projections from posterior tier of thalamic nuclei (ventro-posterior complex), medial pulvinar and potentially through the oculomotor thalamus (Schwartz and Fredrickson 1971; Büttner and Buettner 1978; Grüsser et al. 1990; Akbarian et al. 1992; see Guldin et al. 1992 for review). Such vestibular information could support gaze behavior and vestibular perception essential for spatial location (see Fukushima et al. 1997; Bremmer et al. 2002 for review). Neurons with vestibular sensitivity (e.g., VORd) have been reported in parietal cortex including areas MST and VIP (Sasaki et al. 1984; Page and Duffy 2003) and in the frontal cortex (e.g., Fukushima et al. 2000; Ebata et al. 2004). There is clear evidence that neurons in the flocculus (Belton and McCrea 1999) and vestibular nucleus (McCrea et al. 1999) have different properties during active and passive head movements (see Cullen and Roy 2004 for review). Some of these differences could be associated with signals carried in cortical-pontine pathways, where visual, eye and head signals are available.

In summary, our preliminary anatomical data indicates that NOT, DLPN and NRTP receive projections from mostly separate populations of MT, MST and FEF neurons. These results support the hypothesis that there are separate channels of gaze-related information traveling in FEF-rNRTP and MST-DLPN pathways (see Fig. 2; Distler, Mustari Hoffmann 2001). Our proposed studies will significantly extend these results to include examination of afferent and efferent connectivity of different regions of the pontine nuclei with different regions of cortex (SEF, FEF, VIP, MST, MT) and cerebellum (vermis, floccular complex). Control experiments will examine projections from specific regions of MT to DLPN and NOT. In our antidromic activation studies we will specifically identify, for the first time, the information carried in gaze-related neurons in FEF and MST (MSTd, MSTl, MSTf) neurons projecting to the NRTP and DLPN. Since our first submission of this competing renewal we have added additional antidromic data that further supports our hypothesis that FEF neurons contribute acceleration related information to the rNRTP (see figures 12-14).

C1. PROGRESS REPORT:

We have made considerable progress during the first 2 years of this new project directed at defining the role of pretectal nucleus of the optic tract (NOT) and basilar pontine (NRTP and DLPN) neurons in visual-vestibular behavior. We have completed studies that demonstrate that the DLPN and NOT play different roles in supporting normal and plastic visual-vestibular behavior. We propose to extend these studies by examining the role of cortical-ponto-cerebellar circuits in gaze behavior. We have recently published studies related to the role of MSTd cortex in generating vergence eye movements. We will compare and contrast the signal content in FEF-rNRTP and MST-DLPN neurons during gaze behavior. Since the previous submission of this proposal we have completed three additional studies, two published and another that is in-press (Stewart et al 2005).

1. **Role of the DLPN in short-term adaptation of the horizontal VOR** (Ono, Das & Mustari, J Neurophysiol. 89: 2879-2885 2003). McCrea and colleagues have shown that the flocculus is essential for cancellation of the VOR. The source of signals essential for this function is not fully understood. We demonstrated that the DLPN, which provides strong inputs to the floccular-complex, was essential for cancellation of the VOR. However, the DLPN was not necessary for short-term modification of the VOR in a visual-vestibular mismatch paradigm. We argue that those signals are provided by the NOT.
2. **Gaze-related response properties of DLPN and NRTP neurons.** (Ono, Das and Mustari, J Neurophysiol. 91: 2484-2500 2004). We used multiple-linear regression modeling to compare gaze-related responses in NRTP and DLPN during sinusoidal testing. We found differences in the sensitivity of neurons in these areas to position, velocity and acceleration of either eye or retinal error. NRTP neurons were best modeled using eye motion parameters. In contrast DLPN neurons typically had mixed eye and retinal image motion sensitivity
3. **Discharge characteristics of MST pursuit neurons during vergence eye movements.** Akao T., Mustari MJ, Fukushima J., Kurkin S., and Fukushima K. J Neurophysiol 110.1152/jn.01028, 2004. We demonstrated for the first time that monkey MSTd contains neurons with appropriate response dynamics to participate in vergence eye movements. We found neurons with sensitivity to vergence-position and vergence-velocity. Some carried an extraretinal signal related to vergence, supporting the suggestion that MSTd neurons may carry a signal related to reconstructed target motion in three-dimensional space.
4. **Modeling smooth pursuit related neuronal responses in the DLPN and NRTP of Rhesus Macaque.** (Ono, Das and Mustari, J Neurophysiol. 93: 108-116, 2005). We found that neurons in the rNRTP were best modeled using eye motion parameters in contrast to neurons in the DLPN which had significant visual and eye motion sensitivity. Furthermore, our data supports the suggestion that the rNRTP plays a larger role in smooth pursuit initiation then DLPN perhaps due to enhanced eye acceleration sensitivity.
5. **Visual-vestibular interaction during vestibular compensation: Role of the NOT in hVOR recovery after hemilabyrinthectomy (HL).** (Stewart, Mustari and Perachio, J Neurophysiol doi:10.1152, /jn00739, 2005). Following HL humans and monkeys undergo a process of compensation over the course of months such that spontaneous nystagmus is reduced and VOR gain recovers substantially. Fetter and Zee (1988) showed that visual signals were essential for vestibular compensation following HL. They did this by removing the occipital lobes of monkeys. However, their studies were not specific enough to define the actual source of visual signals. We demonstrated that the pretectal nucleus of the optic tract (NOT) plays an essential role in VOR plasticity following hemi-labyrinthectomy (HL). We accomplished this by comparing post HL recovery with and without the NOTs. Lesion of the NOT severely impaired the process of vestibular compensation.

C2. PRELIMINARY STUDIES and PROGESS:

Cortical Inputs to NRTP, DLPN and NOT:

Our recently published studies show that there are gaze-related neurons in rNRTP and DLPN (Ono et al. 2004). We illustrate some of our recent findings below to indicate how our new studies will progress. Our preliminary data demonstrate that there are also different gaze-related signals in MSTd and FEF and these areas may target different regions of DLPN and rNRTP (see below). We recently published a comparison of anatomical projections from MT and MST to NOT and DLPN respectively (see Appendix

Manuscript 1; Distler et al. 2002). Using multiple retrograde tracer injections, we performed double-labeling studies to test the hypothesis that the NOT and DLPN receive inputs from different populations of neurons in area MT and MST. We injected dextrane-rhodamine in the NOT and granular-blue in the basilar pons including the DLPN on the same side in each of three animals. Representative plots from two of our cases are shown in figure 2. The NOT and DLPN receive extensive projections from the middle temporal visual area (MT) and MST respectively but we found very few (<8%) double-labeled neurons. These data provide support for the suggestion that the cortical projections to the brainstem (NOT, DLPN and NRTP) provide separate channels of information to support gaze behavior. We also conducted studies of connectivity patterns of NRTP compared to DLPN and NOT. We found strong labeling in the FEF after NRTP injections. However, those results must be viewed as preliminary because our injections did not target only the smooth pursuit part of NRTP (i.e., rNRTP; see Experimental Design). We will refine our studies by using multiple retrograde/anterograde tracer injections in functionally defined regions of NRTP, DLPN, MST and FEF (Experimental Design).

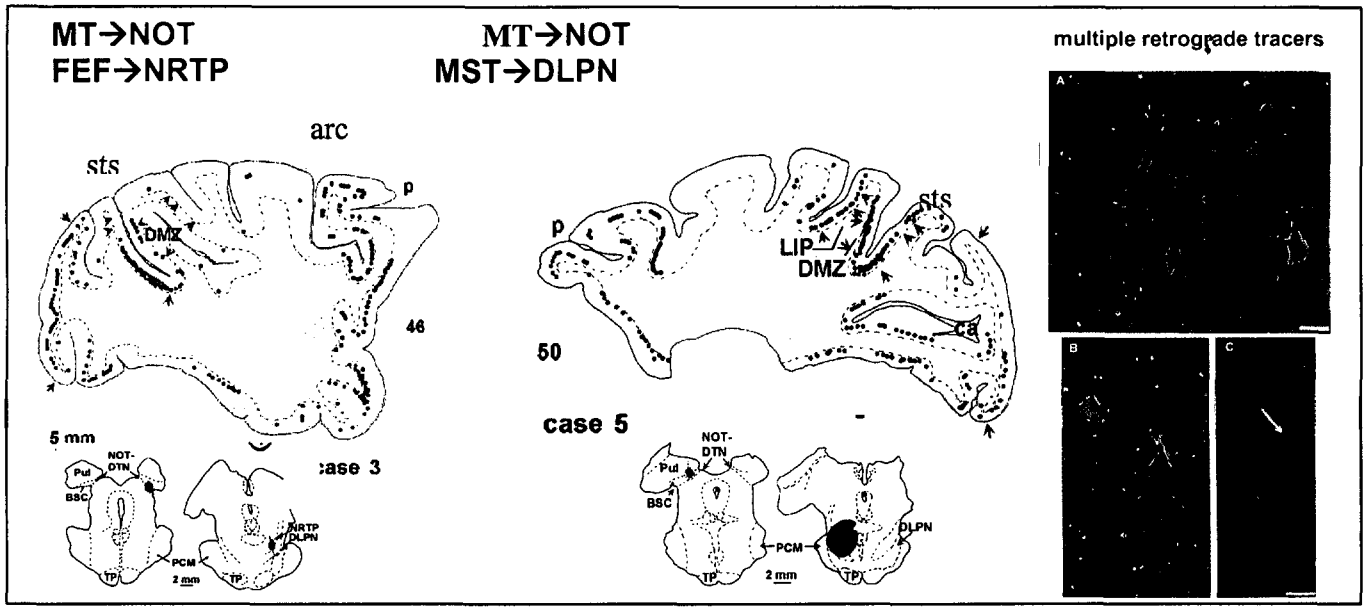

Figure 2: Cortical-Pontine and Cortical-NOT Pathways: Results from multiple, retrograde tracer study designed to demonstrate sources of cortical input to the NOT and DLPN. Dextrane-rhodamine (red) was injected in the NOT and granular-blue in the vicinity of the NRTP with either little involvement of the DLPN (left column) or significant involvement of DLPN and NRTP (middle panel). Injection sites are indicated in the brainstem drawings (lower panels). Neurons labeled from the NOT (red dots), DLPN (blue dots) and double-labeled neurons (green dots) are indicated. Injection of NRTP mostly labeled neurons in the FEF (left panel). We found mostly separate neurons projected to NOT, DLPN and NRTP with only rare doubled labeled neurons (arrow, right panel). Preliminary data indicates that the NRTP has a bias of input from the FEF (near the arcuate sulcus). MT provides the strongest input to NOT and MST the strongest input to DLPN. (Appendix manuscript-1; Distler, Mustari and Hoffmann, 2002).

***Single Unit Recording in rNRTP and DLPN**: Gaze-Related Activity:*

We have successfully recorded from rNRTP and DLPN units during visual, smooth pursuit and vestibular (whole-body rotation) gaze paradigms. We have used both sinusoidal and step-ramp conditions to examine gaze-related activity of rNRTP and DLPN neurons. Figure 3 shows a Nissl stained section from one of our pontine animals. Electrode tracks to the NRTP and DLPN are visible including one with marking lesions (red arrows) placed dorsal and ventral to the location of gaze related neurons in rNRTP. We can also use MRI to localize recording sites (fig 16).

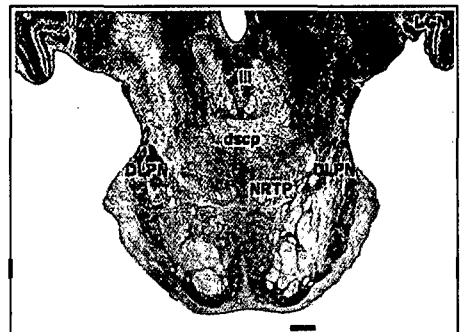

Figure 3: Photomicrograph of Nissl stained, 50μm section cut in the coronal stereotaxic plane. Abbreviations; LGN, lateral geniculate nucleus; III, oculomotor nucleus; dscp, decussation of superior cerebellar peduncle. Scale bar=2mm.

DLPN and NRTP Neuronal Response During Smooth Pursuit and Gaze

We use extensive visual, oculomotor and vestibular testing in our single unit studies to determine the gaze-related information carried in these neurons (see Experimental Design). Figure 4 shows examples of DLPN and rNRTP neurons tested during five standard sinusoidal conditions. 1) Smooth pursuit of a small target over a dark background (top panels), 2) VOR in complete darkness (VORd), 3) VOR with a visual target (VORL), 4) VOR while tracking a small target moving 180 out-of-phase with the head (VOR x2) and 5) cancellation of the VOR while tracking a target moving exactly with the head (VOR x0). We also include smooth pursuit trials where the target spot is briefly extinguished (see below). We have found that VORd testing must be performed in complete darkness, otherwise, some neurons may appear to be modulated during head or eye motion when, in fact, they are simply visually driven (see Experimental Design for further discussion). Figure 4 documents that DLPN and rNRTP neurons have significant differences in gaze-related response sensitivity. First, we find that both DLPN and rNRTP neurons carry information related to gaze and not just smooth pursuit. Second, rNRTP neurons often discharge in-phase with eye acceleration (Fig. 4, right column; Fig. 5).

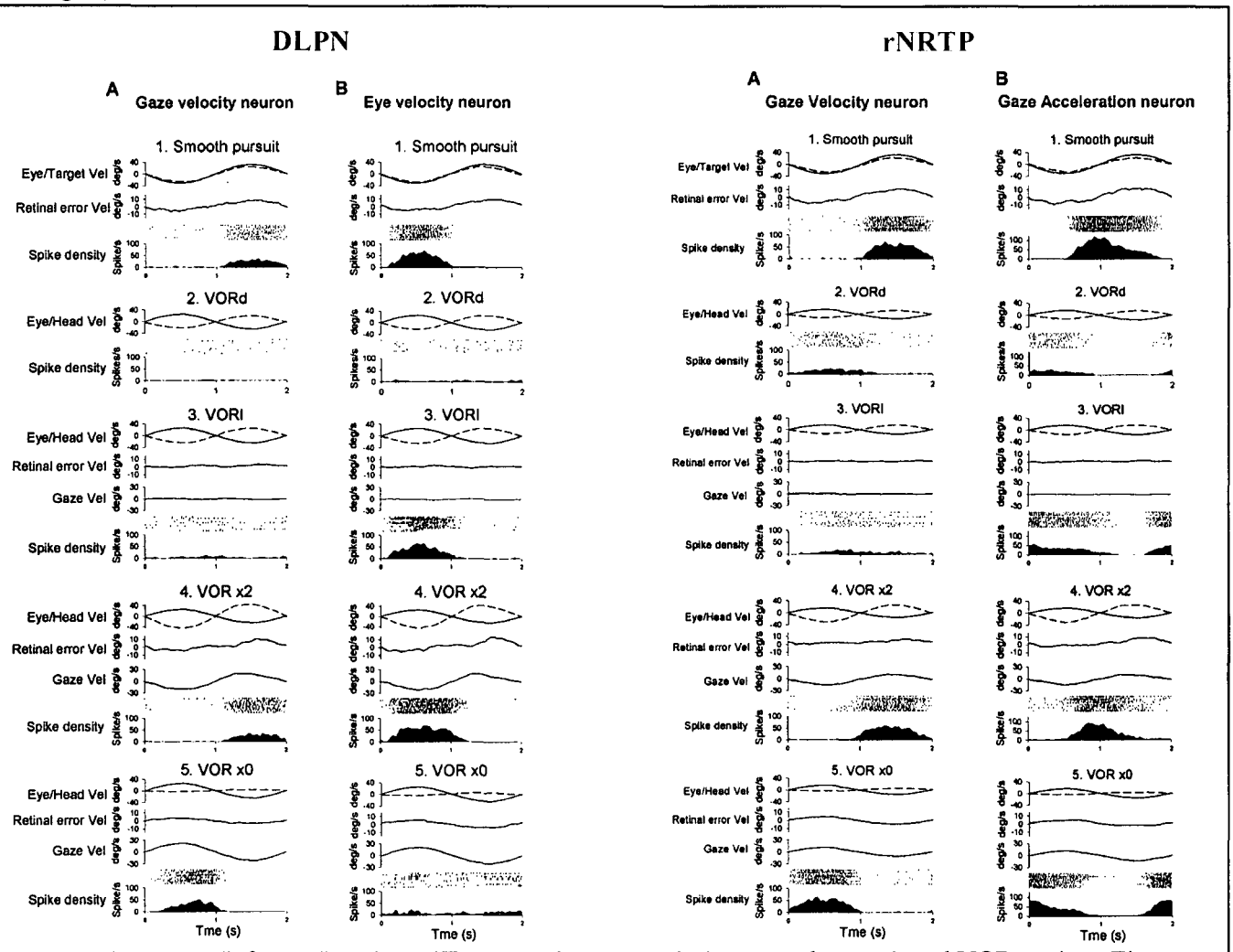

Figure 4: DLPN (left panel) and rNRTP neuronal response during smooth pursuit and VOR testing. Five standard conditions are illustrated as described in the text. Data from at least ten trials in each condition are shown. Saccades were removed prior to averaging. Neuronal response saturated for the DLPN eye-velocity neuron during condition 4.

Although we found gaze-velocity sensitive neurons in both DLPN and rNRTP, there were significant qualitative and quantitative differences in neuronal sensitivities. We have not observed significant eye-acceleration sensitivity in DLPN or MST (see below) neurons but we find this property in a significant fraction of our rNRTP and FEF neurons (Fig. 6, see below). Figure 5 illustrates the eye-acceleration related response of a typical rNRTP neuron tested in two different ways. First, the peak firing-rate of this neuron is in-phase with eye acceleration as frequency of tracking is changed (0.25, 0.5, 0.75 Hz; ±10°). Second, to further verify that peak-firing rate was in phase with eye acceleration and not just position, we had the monkey track a target moving over the same frequency and amplitude (0.5Hz; ±10°) but with different offsets (-10°, 0°, +10°). In each condition, peak firing remained in-phase with eye acceleration (not position). Figure 6 provides a summary of the types of neuronal response we have encountered, so far, in our DLPN and rNRTP gaze studies. To provide a more quantitative basis for comparing gaze-related responses of DLPN, rNRTP, FEF and MST neurons, we have applied multiple linear regression modeling of visual, eye and head motion sensitivity (see below, Experimental Design, Specific Aim 1). We also use non-sinusoidal testing to examine the response of DLPN, rNRTP, MST and FEF neurons (see below and Experimental Design). In summary, our preliminary comparative analysis demonstrates the rNRTP and DLPN neurons could provide different gaze-related signals to different regions of the cerebellum. We hypothesize that these differences in neuronal sensitivity are at least in part due to different balances of cortical inputs suggested by our anatomical studies (see Figure 2).

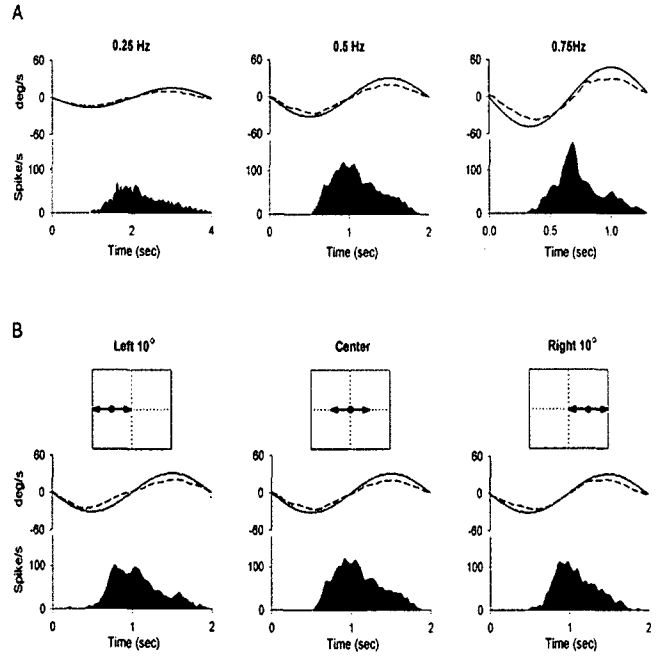

Figure 5: Eye-acceleration-related response of rNRTP neuron. Peak firing is in-phase with acceleration regardless of frequency (A, top panel) or orbital position (bottom panel). Position of the tracking target is indicated above the averaged data in B. Traces are eye (dashed line) and target velocities. Unit firing illustrated as spike-density functions.

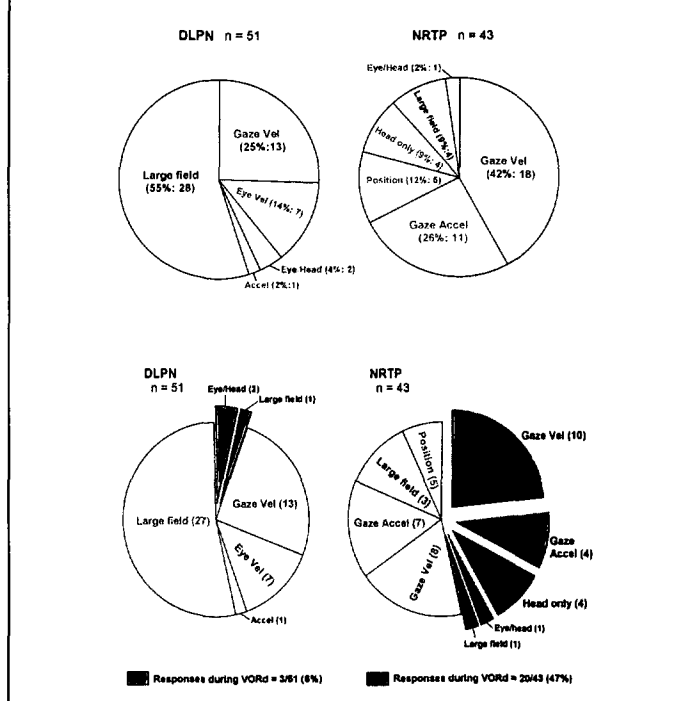

Figure 6: Relative proportions of neuronal response types in DLPN and rNRTP. Gaze-velocity neurons are found in both DLPN and rNRTP. Large-field visual neurons are common in DLPN but not in rNRTP. Neurons with response during VORd conditions are rare in DLPN and common in rNRTP.

We have applied the same testing conditions in our recording experiments in MST and FEF as used in DLPN and rNRTP studies. We have developed effective techniques for recording in DLPN, NRTP, MST and FEF of the same monkey. This is essential for conducting our antidromic activation studies (see Experimental Design).

We have found significant differences between the neuronal responses of MST neurons and those in rNRTP. For example, we have not encountered neurons that were modulated during VORd conditions in large parts of MSTd and MSTl, while this property is common in rNRTP and FEF. We suspect that this lack of VORd response may be due to under-sampling specific regions of MST. We are now working to sample the full extent of MST (MSTd, MSTl). In fact, our recording chambers are large enough to allow sampling the full extent of MT, MST, area 7a and VIP. Figure 7 shows three different MSTd neurons during 5 standard testing conditions. An important result is that we found MSTd smooth pursuit neurons that discharged through a target blink period (indicating the existence of an extraretinal signal) were not modulated in the VORd condition. This finding strongly suggests that the so-called extra-retinal signal in at least some MSTd neurons is not simply an efference copy of an eye movement command but could be either a partially formed smooth pursuit command or reconstructed target motion in space signal.

MST Neuronal Response During Gaze Testing

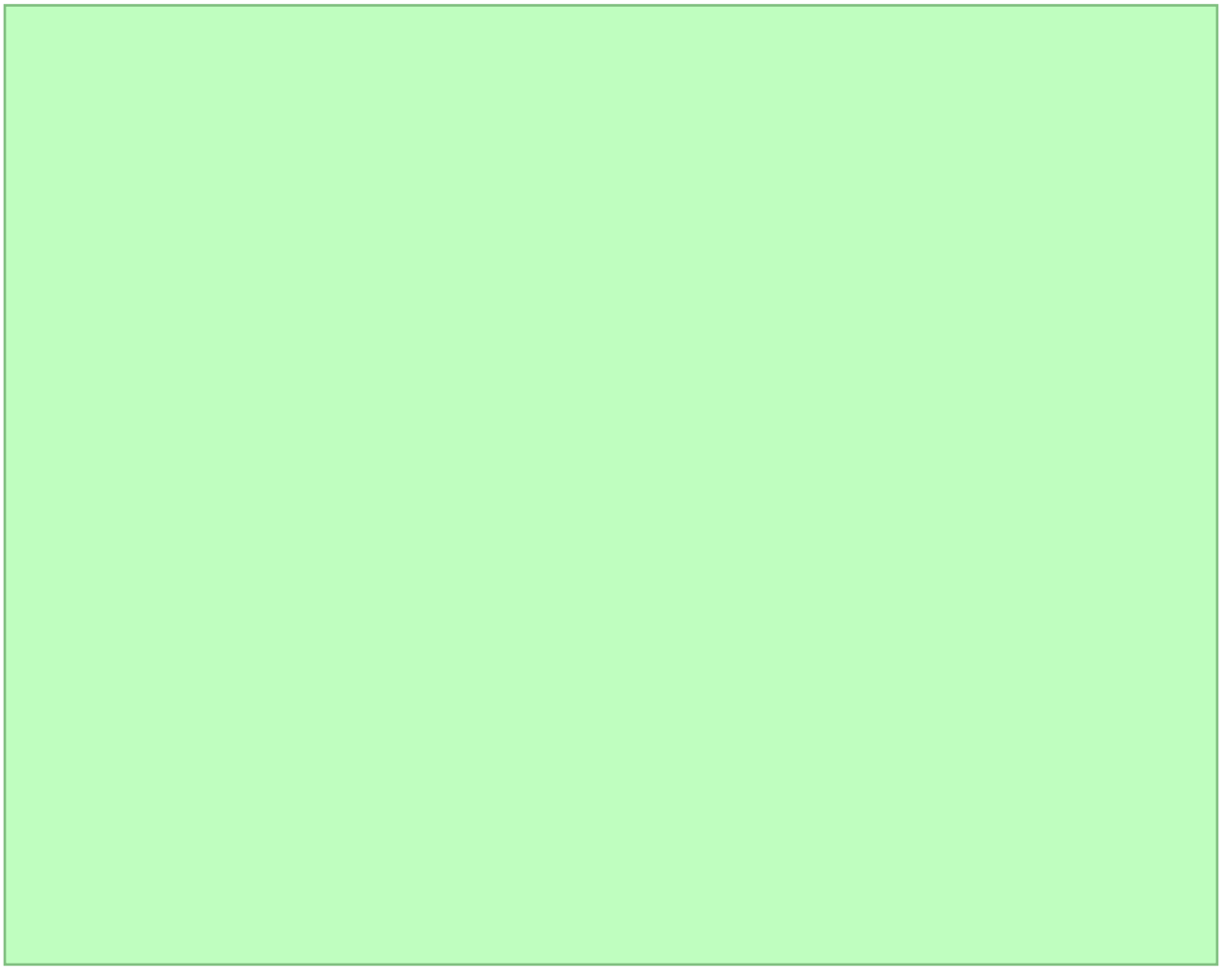

Figure 7: Three different MST units tested during sinusoidal smooth pursuit, VORd, VORL, VOR x2 and cancellation of the VOR. Testing was over the same amplitude and frequency for each illustrated neuron (± 10°; 0.5 Hz). The neuron in the left panel only responded during smooth pursuit. The neuron in the middle was modulated during gaze. The neuron in the right panel was modulated in relation to eye- and retinal-error velocity. The choppy response of this neuron may be related to a combination of eye- and retinal-error velocity. Ordinate, target, head and eye velocity; abscissa, time (s). Traces; target or head velocity (green), eye (blue), spike-density function (red). Firing-rate (spikes/s) as indicated on lower left. Other conventions as in figure 4.

Vergence plays an essential role in gaze behavior and vergence signals in FEF and MSTd are likely to project to different regions of rNRTP and DLPN. The vergence state modifies VOR gain and active changes in gaze in a natural setting must often include a vergence component. We have recently examined the potential contribution of MSTd neurons to disparity-vergence eye movements (Akao, Mustari, Fukushima et al., 2004; see Experimental Design). Figure 8 shows examples of vergence related neurons in MSTd cortex. Monkeys performed vergence tracking of a virtual-target moving sinusoidally in depth (see Experimental Design). We created this motion using frame-sequential display of targets presented through ferro-electric shutters synchronized to alternate video frames. This produces a virtual target in depth ideally suited to driving vergence and neurons in MSTd. We are using a Cristie Digital Light projector (Mirage 2000) to provide motion stimuli for vergence testing (see below). In Figure 8, we illustrate 3 different MSTd neurons with vergence related responses. The left panel illustrates examples of neurons that were modulated with vergence-velocity. These particular neurons did not have any smooth pursuit or visual sensitivity, other MSTd neurons had these such sensitivity (see Akao, Mustari, Fukushima et al 2004 for details). The neuron in the right panel of Figure 8 was modulated in relation to vergence position. **This neuron also maintained its vergence-related response when the target was extinguished, leaving the monkey in complete darkness. This indicates that some neurons in MSTd carry extraretinal signals perhaps related to a partially formed vergence command or target motion in three-dimensional space.** ***Our study provides the first demonstration of vergence-related neurons in MSTd.***

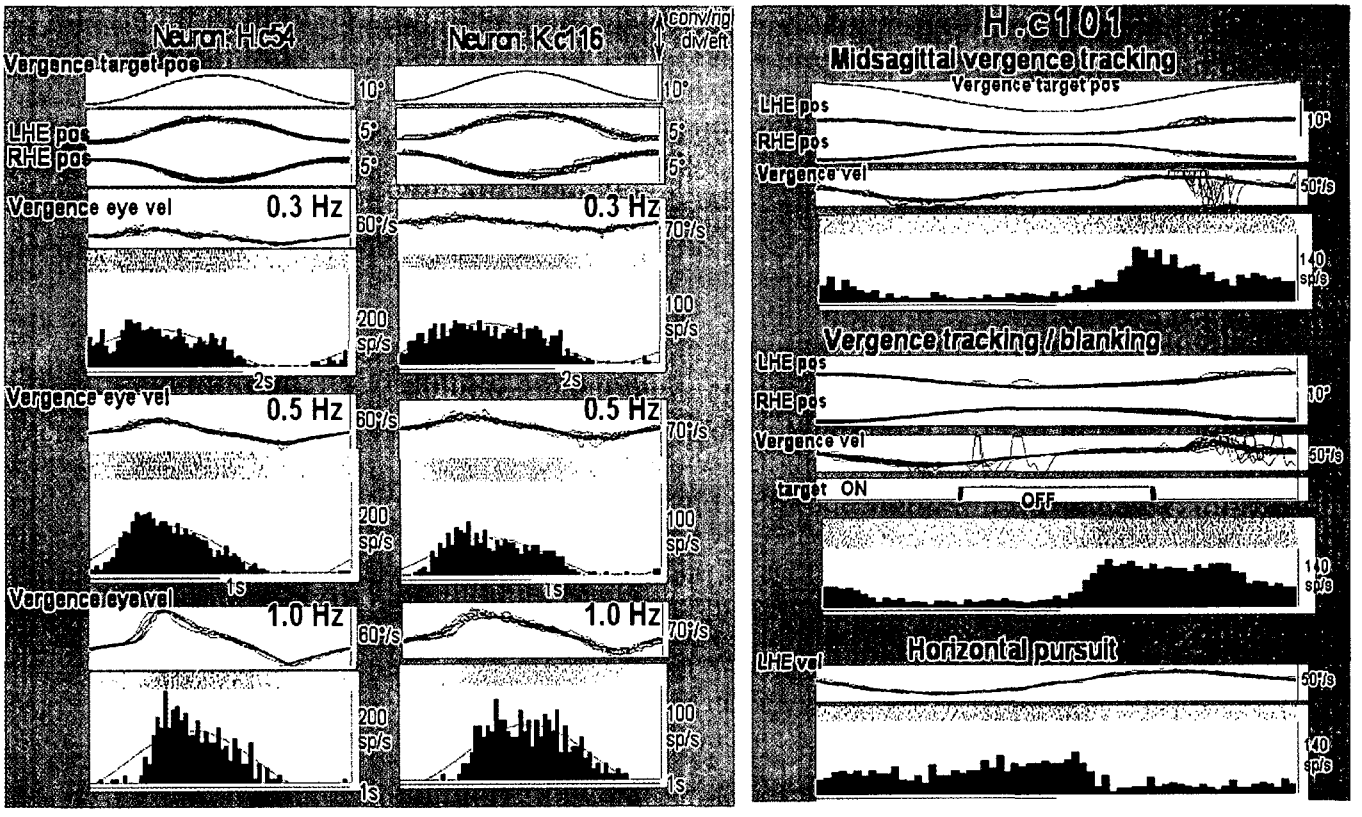

Figure 8: Vergence-related neurons in MSTd cortex. Left panel illustrates 2 different MSTd neurons during vergence tracking at 3 different frequencies (0.3, 0.5 and 1.0 Hz). Peak neuronal response is in-phase with **vergence velocity**. The neuron illustrated in the right panel responds during convergence in-phase with **vergence position**. This neuron maintains firing during a target blink indicating the presence of an **extraretinal signal**.

Figure 9 shows an example of a FEF neuron tested during sinusoidal smooth pursuit and different VOR conditions. We also have begun modeling FEF neurons during step-ramp smooth pursuit (see Experimental Design). This neuron was not well-modulated during VORd conditions but other FEF neurons were. Step ramp conditions have some advantages over sinusoidal testing including clear separation of acceleration and position components. The FEF neuron illustrated in figure 9 has a gaze-related response with strong modulation for leftward gaze movements (smooth pursuit and cancellation of the VOR). We do not yet know which types of FEF neurons project to either rNRTP or DLPN. Since the first submission of our competing renewal we have made advances in identifying FEF projection neurons (see below).

The FEF neuron shown in figure 12 (left panel) was antidromically activated following low current (50μA) stimulation in the rNRTP (see below for details). We will examine MST (MSTd, MSTl, MSTf) and FEF neurons antidromically activated from DLPN and/or rNRTP (see Experimental Design). In the Experimental Design section, we show examples of FEF neurons with gaze-acceleration sensitivity similar to that which we have observed in rNRTP but not DLPN neurons. Below we also show the results of modeling FEF neurons that were shown by antidromic activation to project to the rNRTP.

D. EXPERIMENTAL DESIGN AND METHODS

We propose experiments designed to determine the relative roles of neurons in different compartments of cortical-pontine circuits in gaze movements. Our studies will focus on FEF, MST (MSTd, MSTl, MSTf), NRTP and DLPN. We describe our Experimental Design in the order of our Specific Aims. Techniques that are specific to each part of the research will be described first, followed by a description of General Methods that are applicable to all the neurophysiological studies. Although our experimental approach is ambitious, we feel that we have already made enough significant progress in the first two years of this new project to feel confident that we will be successful in achieving our goals.

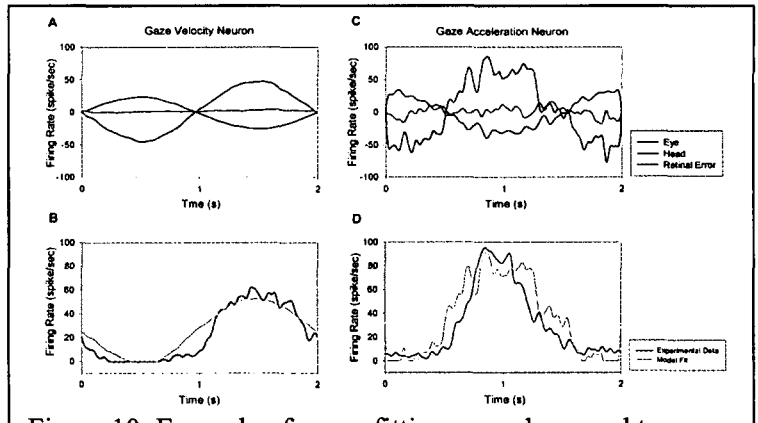

Figure 10: Example of curve-fitting procedure used to identify model parameters and estimate C.D.s for rNRTP neurons. Similar methods will be applied for modeling rNRTP, DLPN, MST and FEF neurons during active and passive gaze movements.

Specific Aim 1: CHARACTERIZATION OF GAZE RELATED NEURONS IN FEF-NRTP AND MST-DLPN PATHWAYS.

RATIONALE: Neurons in different cortical and pontine areas are sensitive to different combinations of visual, eye and head motion during smooth pursuit, vergence and gaze movements. Our hypothesis is that there is specificity in the functional role played by neurons in these different centers. By characterizing neuronal properties in MST, FEF, NRTP and DLPN in each animal during the same behavioral paradigms, we will be able to directly compare information content in these pathways.

Earlier studies on basilar-pontine neurons did not include combined visual, smooth pursuit, vergence and vestibular testing.

Our preliminary single unit modeling studies demonstrate that we are successfully pursuing this objective.

A major innovation in our studies is that we are able to record from multiple areas (FEF, MST, NRTP and DLPN) in the same animals. Our brainstem chamber (e.g., Figure 3) allows us to access all regions of the NRTP (rostral-caudal), DLPN (rostral-caudal) and pretectal NOT. Our two cortical chambers are implanted so that a posterior chamber allows us to record from the full extent of MT/MST as well as neighboring areas as needed (e.g., VIP). Our rostral chamber permits access to FEF. We have experienced no technical problems with our multi-chamber preparation.

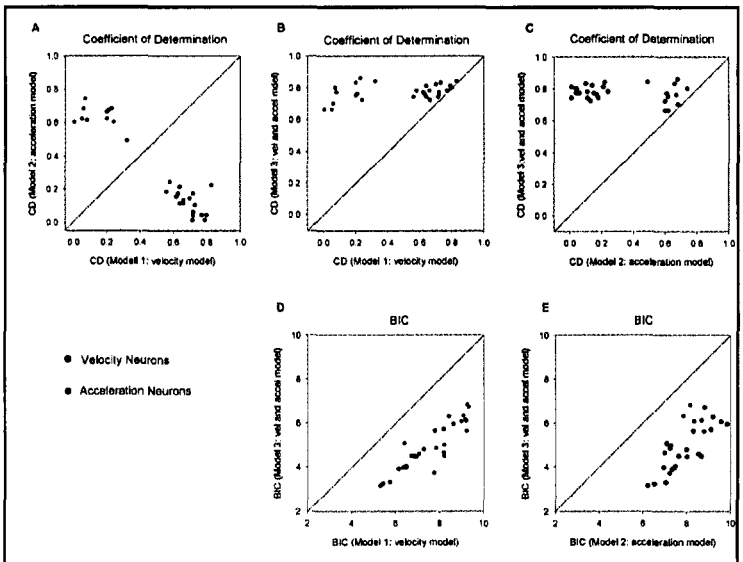

Figure 11: Comparison of models for rNRTP neurons indicates two populations of neurons (gaze-velocity and gaze-acceleration). Using a Bayesian information criteria index (BIC), we observed a decrease in BIC as CDs increased in the more complex models. This provides support for including the additional terms in our models.

EXPERIMENTAL POTOCOL:

We have already been successful in comparing the response properties of neurons in rNRTP and DLPN during both sinusoidal smooth pursuit and hVOR testing. We will employ single unit recording and antidromic activation to investigate the information carried in FEF-NRTP and MST-DLPN pathways. All neurons will be characterized using comparable methods whether they are antidromically activated or not. We have found that by carefully matching functional regions in the pontine nuclei and cortex (MST and FEF) that we are activating a significant fraction of our neurons.

Gaze Testing During Whole-body Rotation and Pursuit

Behavioral paradigms

During experiments using head-restrained monkeys, the head is stabilized in the horizontal stereotaxic plane. Neurons in the DLPN and rNRTP are first classified as either large-field or parafoveal depending on the relative size of their visual fields and their response during smooth pursuit. Neurons that respond strongly for motion of a large-field (75° x 75°) stimulus while the monkey fixates a centrally located stationary spot (~0.2° diameter) are classified as large-field sensitive neurons. Neurons that respond during high frequency oscillation of a small laser spot against a dark background and also during smooth pursuit of a small diameter (0.2°) target spot moving at low frequency (0.1-0.75 Hz; ±10°) are classified as smooth pursuit or parafoveal neurons (May et al. 1988; Mustari et al. 1988). Visual receptive field mapping is accomplished with appropriate stimulation during fixation (see General Methods). We subject smooth pursuit neurons to further testing to determine whether neuronal response is related to eye position or eye acceleration. For this testing, we require the monkey to fixate at static locations (-10, 0, +10) and plot a rate-position curve for each neuron. If a neuron shows no static rate-position sensitivity, modulation during sinusoidal smooth pursuit could be related to eye velocity or eye acceleration. For all neurons modulated during horizontal smooth pursuit, we employ four vestibular testing conditions (typically 0.5 Hz; ±10°) including (1) sinusoidal whole-body rotation in darkness (VORd), (2) viewing an earth-stationary target during sinusoidal chair rotation (VORl), (3) viewing a target that moves exactly in-phase with the head to allow the monkey to cancel his VOR (VORx0) and during (4) viewing a target that moves equal and opposite to the head to

produce VOR enhancement (VORx2). We also will test neurons during non-sinusoidal conditions such as step-ramp tracking (see below).

We also test visual motion sensitivity during fixation (see below). We have found that even minimal amounts of diffuse light can produce visually driven modulation during VOR testing. We have observed this effect in MT, MST, FEF, rNRTP, DLPN and NOT.

Our recording booths are designed to allow a completely dark environment for VORd or blink testing during pursuit. We first made this point in our initial study of NOT neurons (Mustari and Fuchs, 1990). We demonstrated that smooth pursuit related neurons in the NOT were not modulated during VORd. We also found that their modulation during VOR-cancellation was visually contingent because blinking the target spot off during cancellation produced a large drop in firing rate. More recently, we used multiple-linear regression modeling to show that smooth pursuit related neurons in the NOT were always modeled best using retinal image motion parameters rather then eye motion (Das et al. 2000). We obtained similar results in our modeling studies of NOT neurons during ocular following (Inoue et al. 2000).

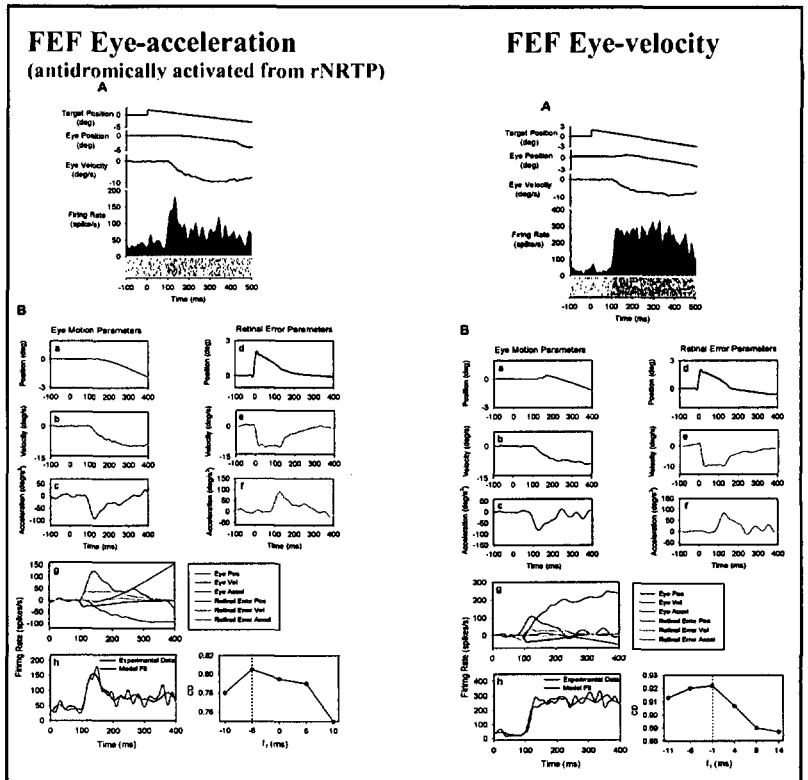

Figure 12: Examples of FEF neurons antidromically activated (left panel) or non-activated (right panel) following stimulation of the rNRTP. Top panels show response during step-ramp tracking. Below are the response components (position velocity and acceleration) that contribute to the models. This and other activated neurons tend to have strong eye-acceleration sensitivity.

Data collection and analysis

Eye movements are detected and calibrated using standard electromagnetic methods (Fuchs and Robinson 1966) using precision hardware (CNC Electronics, Seattle, WA). Motion of the laser spot is controlled by a two-axis mirror galvanometer (General Scanning, Watertown, MA). Visual motion stimuli are also presented with a Digital Light Projector (Christie Mirage 2000).

Vestibular stimulation is provided by a servo-controlled 60ft-lb DC torque motor (Neurokinetics, Pittsburgh, PA) that oscillates the chair sinusoidally or in a ramp trajectory about the earth-vertical axis. All stimulus generation is computer-controlled using custom Labview software and National Instruments hardware (Austin, TX). Eye, head and target position feedback signals are processed with anti-aliasing filters at 200 Hz using 6-pole Bessel filters prior to digitization at 1 KHz with 16-bit precision. Velocity arrays are generated by digital differentiation of the position arrays using a central difference algorithm in Matlab (Mathworks, Natick, MA). Unit activity is recorded using custom-made, glass-coated tungsten or epoxy-coated tungsten electrodes (Frederick-Haer, Brunswick, ME). The impedance of our electrodes is typically in the 1-3 MOhm range. Single unit action potentials are detected with either a window discriminator (Bak Electronics, Mount Airy, MD) or template matching algorithm (Alpha-Omega, Nazareth, Israel) and represented by a TTL level that is sampled at high precision as an event mark in our data acquisition system (CED Power1401, Cambridge, England). During analysis, neuronal response is represented as a spike density function, generated by convolving spike times with a 5ms Gaussian (Richmond et al. 1987).

Localization of NRTP, DLPN, MST and FEF

We use both functional and anatomical criteria for localization of units in the rNRTP, DLPN, MST and FEF (see General Methods). Brainstem recording chambers are stereotaxically implanted and aimed such that a track located in the center of the chamber intersects a point near the oculomotor nucleus (e.g., Fig. 3). We first map the location of oculomotor neurons before running tracks to deeper sites either in the NRTP or DLPN. Because we use a 20° angle for our tracks, we can reach both the NRTP and DLPN on each side of the brain using a single chamber (see Figure 3). During recording, we map the saccade related region of the NRTP and the more rostral smooth pursuit related region (rNRTP). We now can use MRI to confirm our recording location in FEF and MST while animals are still involved in experiments (see Figure 16, General Methods).

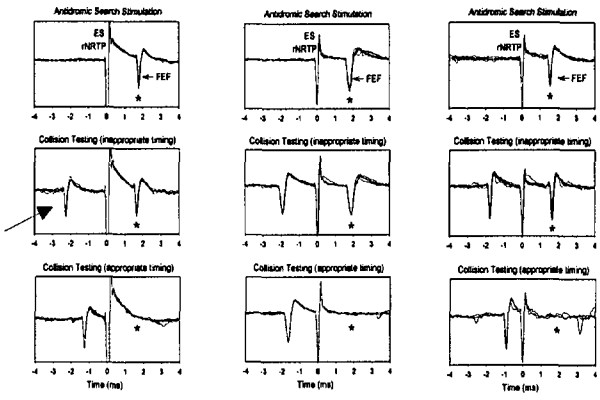

Figure 13: Three examples of FEF neurons antidromically activated from the rNRTP. The top panels show the **search condition** with 5 consecutive stimulus trials aligned on stimulation artifact (ES rNRTP). Antidromically activated spikes are indicated in the top panel and during collision testing middle panel with inappropriate timing to collide the **naturally occurring spike** (e.g., arrow) and the activated spikes (*). In the bottom panels appropriate timing for collision was used verifying antidromic activation. Currents used were low ranging from 100 to 30 μA. Red asterisk indicates the location of the antidromic spike or its expected location based on timing in the lower panels.

Model fitting and optimization

From previous studies (e.g., Preliminary Results) and our quantitative characterization, it is clear that there are several different unit types (e.g., visual, eye and head movement) with complex characteristics in the DLPN and NRTP. We also have initiated comparable studies in FEF and MST (see below). To provide a more objective method for unit classification and to consider possible combinations of signals, we have used a model, parameter-estimation procedure to investigate potential information encoding within the individual response profiles of smooth pursuit related units in the DLPN and rNRTP. We have previously used a similar method to study information coding in parafoveal smooth pursuit related cells in the NOT (Das et al 2000) and large-field NOT neurons during ocular following (Inoue et al. 2000). Eye, head and retinal error velocity data are filtered using an 80-point finite impulse response (FIR) digital filter with a bandpass of 0-50 Hz. Saccades are marked with a cursor on eye velocity traces and removed. After de-saccading, the missing eye data is replaced with a linear-fit connecting the pre- and post-saccadic regions of data using Matlab (Mathworks, Natick, MA).

We apply our modeling procedure after pooling data obtained during smooth pursuit, VORx0 and VORx2. These conditions (VORx0 and VORx2) are most important for classifying neurons as gaze related. Previously, we excluded VORd and VORl conditions from our modeling studies, because gaze velocity is close to zero in those two conditions. Averaged data from at least ten trials when the eye is on target are used to identify coefficients in the models 1-3 (see below).

Where, FR(t) is the estimated value of the unit spike density function at time "t", E(t) the eye motion at time "t", H(t) the head motion at time "t" , R(t) the retinal error motion at time "t" and A - G are constants that specify the coefficients in the models. Therefore, model 1 relates unit response to eye, head or retinal error velocity parameters. Model 2 relates unit response to eye, head or retinal error acceleration parameters, while model 3 relates unit response to eye, head or retinal error velocity and acceleration parameters, i.e., a combination of model 1 and 2.

$$\textit{Model 1}\quad FR(t) = A + B\dot{E}(t) + C\dot{H}(t) + D\dot{R}(t)$$

$$\textit{Model 2}\quad FR(t) = A + B\ddot{E}(t) + C\ddot{H}(t) + D\ddot{R}(t)$$

$$\textit{Model 3}\quad FR(t) = A + B\dot{E}(t) + C\dot{H}(t) + D\dot{R}(t) + E\ddot{E}(t) + F\ddot{H}(t) + G\ddot{R}(t)$$

$$\mathbf{BIC = \log\,(1/N\ \textstyle\sum[\,M(i) - data(i)]^2) + P/2\log\,(\log N/N)}$$

The goodness of fit is determined by calculating coefficients of determination (CD). Since simply increasing the number of terms in the model could lead to improvement in CD, we also calculate a Bayesian information criteria (BIC) index between the experimentally observed unit data and the model estimated fit. The BIC measure serves as a cost index that penalizes adding new terms in the model (Angelaki and Dickman 2003; Cullen et al. 1996). BIC are calculated as described below;

where *data (i)* represents the firing rate modulation obtained experimentally during visual-vestibular behavior, *M (i)* the corresponding values estimated from the model fit, *N* the number of trials of sinusoidal smooth pursuit tracking or VOR task, and *P* the number of the model parameters fit. For an increase in complexity of the model to be valid (e.g. model 3 compared to model 1), there must a relative increase in the CD and a relative decrease in the BIC index. We also calculate coefficients of partial determination (partial r^2 values) as another indicator of the relative importance of each term (eye, head and retinal error velocity and acceleration) to the firing rate of the neuron.

Eye and Retinal Error Motion Sensitivity of FEF

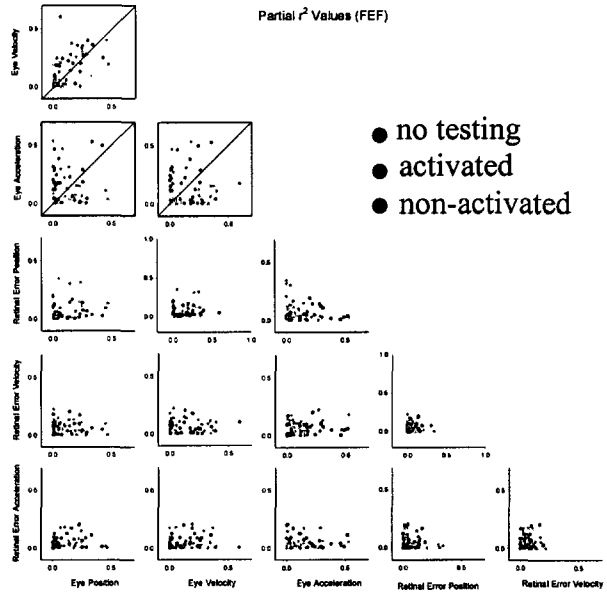

Figure 14: Response sensitivity of FEF neurons to different components of motion during step-ramp smooth pursuit as indicated by calculating partial-r^2 values. FEF neurons antidromically activated from the rNRTP are indicated by red dots. These neurons have strong sensitivity to eye acceleration.

Step-ramp pursuit: Model fitting and optimization

We will also use a model estimation procedure to define smooth pursuit related signals in DLPN, rNRTP, FEF and MST during step-ramp tracking. Briefly, we reconstruct the individual neuronal response profiles of smooth pursuit-related neurons by using combinations of position, velocity and acceleration. Similar procedures have been used with success in other parts of the oculomotor system including the cerebellum, oculomotor nuclei, the nucleus of the optic tract and MST cortex (Shidara et al. 1993; Sylvestre and Cullen 1999; Das et al. 2001; Inoue et al. 2000). Position, velocity and acceleration data are filtered and de-saccaded as described above. Averaged data, taken from at least ten trials in which the animal performed high quality smooth pursuit, are then used to identify coefficients in the following models.

$$\textit{Model 1}\quad FR(t-\tau) = A + BE(t) + C\dot{E}(t) + D\ddot{E}(t)$$

$$\textit{Model 2}\quad FR(t-\tau) = A + BR(t) + C\dot{R}(t) + D\ddot{R}(t)$$

$$\textit{Model 3}\quad FR(t) = A + BE(t-\tau 1) + C\dot{E}(t-\tau 1) + D\ddot{E}(t-\tau 1) + ER(t+\tau 2) + F\dot{R}(t+\tau 2) + G\ddot{R}(t+\tau 2)$$

In the equations described above, E(t) denotes the eye position at time "t", R(t) denotes the retinal error position at time "t" and FR(t) is the estimated value of the unit spike density function at time "t". Coefficients in the models are defined by terms A-G. Therefore, this model attempts to relate unit response to eye-motion and retinal-error motion parameters. The latency value of the unit response with respect to pursuit (eye) onset and retinal error onset is represented by the "*τ1*" and "*τ2*" terms, respectively. Therefore, "*τ1*" represents a lag and "*τ2*" represents a lead. To model the response associated with retinal error, we align unit response onset with retinal error onset at a fixed latency. A set of coefficients (A-G) and coefficients of determination (CD) are calculated for a series of pursuit onset latencies in steps of 5 ms. The latency of pursuit onset and coefficients for any particular eye latency that yielded a maximum CD are used as the final coefficients for a particular model. Retinal error parameters are calculated as the difference between target and eye motion parameters. Since rNRTP and DLPN units are generally unresponsive to large velocities, the impulse in target velocity due to differentiation of the step in target position is removed in software prior to presenting the data to the modeling algorithm. Further, target acceleration was assumed as $0°/s^2$, since differentiation of a step in target velocity results in zero steady state target acceleration. We calculate partial r^2 values for eye motion and retinal-error motion parameters, and for each component (eye and retinal error position, velocity and acceleration) to estimate the relative contribution of eye- and retinal-error (position, velocity and acceleration) to the firing rate of the neuron. Comparisons of partial r^2 values between eye and retinal error motion parameters are performed using a paired *t*-test. Statistical tests are executed with a significance value of at least 0.05.

Project 1b: COMPARE AND CONTRAST GAZE RELATED SIGNALS IN FEF AND MST NEURONS ANTIDROMICALLY ACTIVATED FROM THE RNRTP AND DLPN

RATIONALE: To identify the information carried in FEF and MST neurons that project to the rNRTP and DLPN, we need to antidromically activate these neurons. There is no better way to define the signal content carried in cortical-pontine neurons. Antidromically activated neurons will be characterized regarding of sensitivity to eye, retinal error and head motion as described above. **We will also test whether the same FEF or MST neurons project to both the rNRTP and DLPN. Our anatomical results indicate that this may not be the case.**

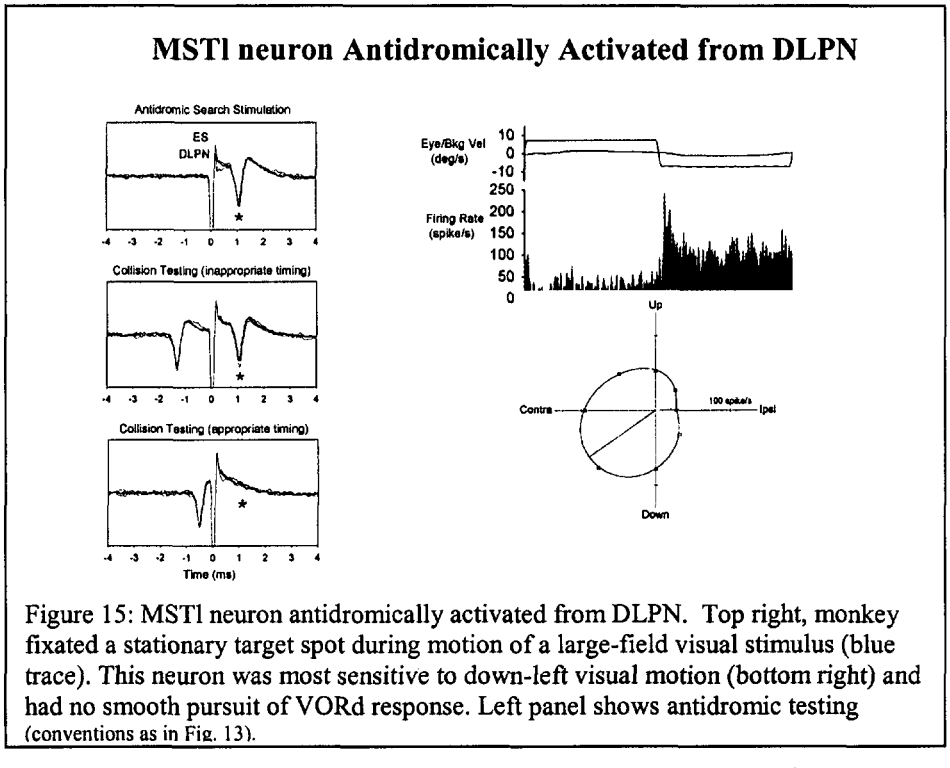

Figure 15: MSTl neuron antidromically activated from DLPN. Top right, monkey fixated a stationary target spot during motion of a large-field visual stimulus (blue trace). This neuron was most sensitive to down-left visual motion (bottom right) and had no smooth pursuit of VORd response. Left panel shows antidromic testing (conventions as in Fig. 13).

In new results since the last submission of our competing renewal, we have found that antidromically activated FEF neurons tend to have large amounts of eye acceleration sensitivity, similar to that observed in rNRTP neurons. We still do not have a large enough sample of antidromically activated FEF neurons to make strong conclusions about the net signal delivered to the rNRTP from FEF but we are confident that are studies are moving along well in this regard. Recently, we have been successful in antidromically activating MSTl neurons following DLPN stimulation (figure 15). So far we have activated MSTl visual direction selective neurons with out pursuit sensitivity. Figure 15 shows and example of an MSTl neuron

including antidromic testing (left panel), visual motion sensitivity during multiple trials of visual stimulation during fixation and a direction tuning curve calculated from 8 cardinal directions of visual motion.

EXPERIMENTAL POTOCOL:

We will antidromically activate FEF and MST neurons following electrical stimulation of the NRTP and/or DLPN. We have the antidromic activation paradigms working well. We are able to implant multiple chambers on our animals so that we can deliver antidromic stimulation in NRTP and DLPN through electrodes carried in one chamber while we record neurons in the FEF or MST in other chambers. We have considerable experience using electrical stimulation in visual pathways to activate identified visual cortical neurons (e.g., Mustari et al. 1982; Bullier et al. 1982; Henry et al. 1983) and to elicit eye movements with electrical stimulation delivered in the DLPN and NOT (e.g., Mustari and Fuchs 1990; Mustari et al., 2001). Figure 12 (left panel) shows an example of a FEF smooth pursuit–related neuron antidromically activated following stimulation delivered in the rNRTP. Figure 13 and Figure 14 show other activation examples and modeling statistics, respectively.

Our protocol for conducting antidromic activations is to first extensively map the smooth pursuit regions of the DLPN and pursuit and saccadic regions of the NRTP. For every isolated FEF and MST neuron, we will test for antidromic activation using a search protocol where stimuli (single biphasic pulses; 200μs duration; 20-200μA; <0.5 Hz) are delivered to check for time-locked activation of the cortical neurons (Fig. 13). The example neurons (Fig13 & 15) were activated at low current (50μA). Next, we use standard tests for antidromic activation, including collision block, following rate and latency reliability (Bishop, et al. 1962; Segraves and Goldberg 1987; Summer and Wurtz 2000). For collision testing, we use a naturally occurring spike in the cortical cell to trigger the basilar-pontine stimulus (Fig. 13, middle panel). By varying the delay between the triggering cortical spike and the electrical stimulus pulse, we can obtain collision, thus verifying a direct connection between FEF and rNRTP (Fig. 16, bottom panel). We find antidromic latencies of FEF neurons in the range of 2-5 ms. Our latency values are similar to those reported in other studies (e.g., Summer and Wurtz 2000). We have observed even shorter latencies for MSTl neurons (e.g., Fig. 15). We are placing recording and stimulating electrodes in the NRTP and DLPN to allow us to check a given cortical neuron for activation from both NRTP and DLPN.

POTENTIAL PROBLEMS, Specific Aim 1: We anticipate no major problems in completing our proposed studies. We anticipate no serious difficulties performing any of our antidromic activation and single unit recording studies in FEF or MST cortex (see Preliminary Data). This is because we are readily activating neurons in FEF and MST neurons following stimulation of rNRTP and DLPN respectively. Antidromic activation experiments are challenging but there is no better way to determine the actual gaze-related signals delivered to the DLPN and NRTP. We will be successful in activating only some FEF and MST neurons because only layer-V neurons project to the brainstem (e.g., see Mustari et al., 1994, Fig. 10; Distler et al., 2002). Using mobile stimulation electrodes allows us to check the specificity of our results by testing activation above and below the location of related gaze-related neurons in DLPN and NRTP. We have found that low current activation only occurs when the stimulating electrode is in the NRTP or DLPN not when above or below, even by a small distance (100-200μm). By using low currents and careful placement of stimulating electrodes we can reduce current spread and involve known structures. There is always some concern regarding potential damage to a structure when repeated penetrations are made on a daily basis. We have solved this problem by providing some behavioral training days between recording days or by moving between available sites. Using this approach we have been able to record from small delicate regions like the lateral terminal nucleus (Mustari and Fuchs, 1989) and NOT (e.g., Mustari and Fuchs, 1990; Mustari et al., 1997; Mustari et al., 2001) for over a year during which time unit isolation and function properties remained stable.

As reviewed in detail under Specific Aim 1, we plan to include combined visual, vestibular and oculomotor testing for all of our neurons. Multiple linear-regression modeling provides an effective approach for estimating the sensitivity of neurons to visual, head and eye motion. This method allows us to compare and contrast the potential roles of neurons in DLPN, rNRTP, MST and FEF related to gaze. We feel that it is important to focus our comparative studies in FEF-rNRTP and MST-DLPN pathways. We realize that other cortical areas (e.g., VIP) could also contribute to neuronal response properties of rNRTP and DLPN. Our anatomical studies indicate that the strongest inputs to rNRTP and DLPN are FEF and DLPN respectively (see Specific Aim 2). It is also possible that the hypothesized dichotomy of FEF-rNRTP and MST-DLPN pathways may not be supported once we accomplish our antidromic studies (see below).

approach. Experimental Approach - We will expand the testing regimen so that visual and motor signals provide a larger range of contributions than in a simple step-ramp task that we used in Ono et al (2004).

3) **Spot oscillation during fixation of a stationary target. This separates eye motion from parafoveal visual motion (see Akao, Mustari, Fukushima et al. 2004).**

Specific Aim 2: DEFINE THE ANATOMICAL ORGANIZATION OF CORTICO-PONTO-CEREBELLAR PROJECTIONS.

RATIONALE: For us to understand how gaze related signals are constructed, we must understand the underlying specific cortico-ponto-cerebellar pathways. Based on our preliminary anatomical data employing multiple retrograde tracers injections in NRTP, NOT and DLPN, we hypothesize there will be mostly separate populations of neurons in cortex (e.g., FEF, SEF, LIP, VIP, MST and MT) projecting to the rNRTP compared to caudal regions of NRTP and the DLPN. Similarly, we hypothesize that NRTP and DLPN project differentially to flocculus, ventral-paraflocculus and vermis to support gaze behavior. Much of the anatomical data available in earlier studies (see Background and Significance) was not guided by functional mapping. In fact, only our recent study employed multiple, retrograde tracers in NOT, DLPN and NRTP to address the question of whether there might be compartmentalization of cortical neurons projecting to these critical gaze-related sites in the brainstem (Distler et al. 2002). We did employ functional mapping of NOT and DLPN prior to placing injections (Distler et al. 2002). However, we did not specifically target different regions of the NRTP nor did we typically use combined anterograde and retrograde tracers in our studies. We can significantly improve our anatomical knowledge of gaze-related pathways by refining our approach. We will include injection of subregions of NRTP and DLPN using different tracers to determine specificity in connectivity. Using combined retrograde and anterograde traces e.g., rNRTP, we will map cortical projections and cerebellar targets. By limiting the size of our injections to well-mapped pontine areas we can significantly improve our understanding of gaze related

circuits. In our proposed studies, we will perform detailed analysis of other cortical areas likely to play a role in gaze and or perception of spatial location such as area VIP (see Bremmer et al. 2000 for review).

EXPERIMENTAL POTOCOL: Our procedure for conducting anatomical investigation of functional pathways is well established (e.g., Mustari et al. 1994; Distler et al. 2002). All of our injections are placed under physiological guidance after detailed mapping of a given structure. This is important because many studies in the literature placed injections without regard to functional segregation. This is an important issue especially for NRTP where separate saccade and pursuit areas have been identified. Our plan is to perform anatomical studies in each of our chronic neurophysiology cases. We also will use animals dedicated specifically for anatomy studies. The injection sites will be mapped as in physiological studies but without extensive neuronal testing. This is important to generate sufficient cases for study of anatomical connections. We will use tracers that are well established as being effective in the macaque. For retrograde tracing we have used 15% rhodamine-dextrane 1% cholera toxin-B, true-blue 2% granular-blue 2% WGA-HRP (anterograde & retrograde) and 4% diamidino-yellow. Other tracers have been shown to be effective including biotynilated-dextran amine. Our animals are implemented with brainstem chambers angled 20 degrees off the midline (e.g., Mustari et al. 1994; Distler et al. 2001) allowing us to reach the NRTP and DLPN bilaterally. Our procedure is to map each DLPN and NRTP completely before delivering injections at the site of smooth pursuit, saccadic, and visual motion neurons. Small injections (<200 nanoliters) are delivered slowly over a 5 minute time period. After 5 min the pipette is withdrawn after aspiration to minimize leakage of the tracer into the overlying tissue. We inject tracers with pressure using a pico-pump (WPI-PV830). We fabricate our own injection pipettes, which consist of a 33 gauge stainless steel needle fitted with a glass pipette pulled to a fine point (< 20μm). Using this system we can precisely control the volume and spread of injections. Because of our chamber placement we can deliver multiple tracers in each animal. We have delivered up to 5 different injections in the same animals distributed in the DLPN, NRPT, SC, Pulvinar, NOT on both sides of the brain. Our approach has been to use tracers with fast retrograde transport times (e.g., WGA-HRP) on one side of the brain and longer transport times on the other side (e.g., DY).

Control injections: As in our earlier studies (Mustari et al. 1994; Distler et al. 2001) we will include control injections in areas close to intended targets. Most important are brainstem areas that would also receive projections from layer-5 neurons (e.g., other basilar pontine regions, substantia nigra).

Histology: After appropriate survival times (see Distler et al. 2001) animals will be sedated with ketamine hydrochloride and sacrificed with an overdose of pentobarbital. They will be perfused through the heart with 0.9% NaCl containing 0.1% procain hydrochloride, followed by paraformaldehyde-lysine-periodate containing 4% paraformaldehyde. After postfixation overnight the tissue will be transferred to 0.1M PB containing 10% glycerol followed by 20% glycerol for cryoprotection. The midbrains will be cut in the coronal stereotaxic plane at 50μm to verify injection sites. The cortical hemispheres will be cut at 50μm in the frontal or the parasagittal plane. We have typically used 5 alternate series for reconstruction of cortical labeling. We will use at least 2 series for visualization of retrogradely labeled cells. The other series will be used for Nissl staining, myeloarchitecture, SMI 32- and Wisteria floribunda agglutinin histochemistry (Gallyas 1979, as modified by Hess and Merker 1983; Brückner et al. 1994; Hof and Morrison 1995). For visualization of fluorescent tracers the sections will be mounted from 0.45% NaCl immediately after cutting, dried on a hot plate, defatted in fresh xylene (2x1min), and coverslipped with DEPEX. Tetramethylbenzidine (TMB) will be used for visualization of WGA-HRP (after van der Want et al. 1997). For double labeling with choleratoxin, the TMB reaction product will be first stabilized with ammonium heptamolybdate followed by a second stabilization with diaminobenzidine. Then, CTB immunohistochemistry will be performed and CTB was visualized with streptavidin coupled to CY3 or CY2 (modified after Angelucci et al. 1996).

DATA ANALYSIS AND EXPECTED RESULTS: Retrogradely labeled neurons will be viewed with a fluorescence microscope (Zeiss Axioscope) and charted on enlarged drawings of the entire ipsilateral cortical

hemisphere at 1mm interval. Cortical areal borders will be determined based on the myeloarchitectonic characteristics as described in our earlier study (Distler, Mustari, Hoffmann 2002). The location of retrogradely labeled cells and areal borders will then be transferred onto two-dimensional maps derived from physically constructed three-dimensional wire models (van Essen and Maunsell 1980; Distler et al. 1993). To quantify the occurrence of double-labeled cells in relation to the overall number of neurons labeled following NRTP and DLPN injections, we will count labeled cells and express their density as cells per mm in layer-V of FEF, MST and MT. We will also chart neurons in other areas (e.g., LIP, VIP) because of their potential involvement in gaze behavior. Distribution of anterograde label in the floccular complex, vermis and other areas of the cerebellum will be charted to test our hypothesis that there is specificity not only in the cortical efferent projection to rNRTP and DLPN but also ion the projections of subregions of NRTP and DLPN to the cerebellum.

POTENTIAL PROBLEMS: In all anatomical studies spread of tracer from the desired injection site must be controlled and considered. We have given considerable discussion to this issue in Distler et al. 2001 (Appendix Manuscript). We have been able to specifically target NOT, DLPN and NRTP with discrete injections (see Preliminary Data, Fig. 2). We have found it most helpful to deliver our injections through fine tipped pipettes using a picoliter pump (WPI-PV830) so that injections can be delivered precisely and over the course of a few minutes. By placing multiple retrograde tracers in close proximity as describe above, we can verify that our injections are localized. We use these same injection pipettes for our muscimol injections and have been able to produce well-localized functional inactivation with these pipettes.

Experimental Schedule: Table 1 provides an estimate of how our work will proceed. Single unit recording and antidromic activation experiments will be conducted during the full course of the proposed studies. We conduct single unit recording sessions five days/week. Some animals are involved in behavioral training. At least 3 animals per year will be dedicated to single unit recording studies. Three animals/year will be dedicated to anatomical studies using multiple, retrograde and anterograde tracers to identify afferent and efferent connections of different regions of NRTP and DLPN. This will allow our research to proceed on the most efficient schedule possible.

Table 1: EXPERIMENT *YEAR*

	1	2	3	4	5
1) Compare and contrast signals in MST-DLPN and FEF-rNRTP pathways. (Difference in identified projections neurons and non-activated neurons).	X	X	X	X	X
2) Compare and contrast signals in antidromically activated FEF and MSTd and MSTl neurons with rNRTP and DLPN neurons (evidence for separate channels or integration of signals).	X	X	X	X	X
3) Multiple retrograde tracers (rNRTP; Caudal NRTP; DLPN). Evidence for or against double-labeled neurons projecting to both rNRTP and DLPN	X	X	X	X	
4) Bidirectional Tracers rostral NRTP; caudal NRTP; DLPN (evidence for separate channels of information directed at cortex and cerebellum e.g., vermis and ventral paraflocculus)	X	X	X	X	

GENERAL METHODS: Here we provide only a brief description of our general methods. Further details can be found in our published work (Mustari et al. 1988; Mustari and Fuchs 1989, Mustari and Fuchs 1990; Mustari et al. 1997; Inoue et al. 2000; Mustari et al. 2001; Das et al. 2001; Ono et al. 2003; Ono et al. 2004; see appendix manuscripts).

Surgical Procedures: All surgical procedures including implantation of dual eye coils, head stabilization post and recording chambers, follow protocols that are well-established in our laboratory. These protocols are approved by the IACUC of Emory University. All surgical procedures are performed in a dedicated primate surgery facility, under general inhalation anesthesia (isofluorane 1.5 - 2%) and when possible in a single

procedure. Standard anti-inflammatory and analgesic agents (e.g., Banamine and Buprenorphine) are provided in the first post-surgical week.

Eye Position Monitoring and Behavioral Training: We measure eye movements using an electromagnetic method (CNC Engineering, Seattle, WA) employing scleral search coils (Fuchs and Robinson 1966) implanted using the method of Judge et al. 1980. Calibration of the eye coil signal is achieved by rewarding the monkey with 0.1 ml of apple juice when it looks at a small diameter (0.25 °) target spot that is rear projected on a tangent screen through one optic bench system. Animals are trained to track target movements with sinusoidal, ramp, triangle, step and step-ramp target trajectories.

Vestibular Testing: We provide whole-body vestibular testing with a servo-controlled 60 ft•lb torque motor (Neurokinetics). The vestibular stimulator is equipped with a 15-inch CNC field coil (Robinson Configuration) and dual phase detectors. This setup is also equipped with a tangent screen for visual testing (see below). To measure the VOR, we will employ periodic or step rotations, about the earth-vertical axis. We have ensured that the axis of rotation lies midway between the ears so that there is minimal stimulation of the otolith organs. This minimizes the influence of otolith mediated target distance effects on our Visual-VOR measurements. Vestibular testing will includes all of the following tests: a) **Sinusoidal rotations** in the dark at 0.2 to ≤ 2 Hz with a peak velocity of 20 and 40°/s. b) Sinusoidal rotations at frequencies between 0.2 and 2Hz while viewing an earth stationary target with a peak velocity of 20 and 40°/s (VVOR). c) Sinusoidal rotations while viewing a head-fixed target at 0.2 to ≤ 2 Hz with a peak velocity of 20 and 40°/s (VOR Cancellation).

Visual stimulation during single-unit recording: For visual receptive-field testing, we require the monkey to fixate a small (0.25°) target spot while visual stimuli are presented using a second optic bench system. Both optic bench systems are computer controlled and equipped with separate X, Y mirror galvanometers (General Scanning) and digital light projectors. Stimuli are rear projected on a high quality screen (Stewart Film Screen) subtending a large (75° x 75) visual angle. Recently, we have implemented visual testing using a Digital Light Projector (Christie, Mirage 2000). Using the DLP, we can rapidly change visual stimulus configuration to plot a minimum response field, using optimal direction, speed, depth etc. Our computer based visual display allows us to present all of the visual stimuli we require in this project including virtual targets moving in depth to test vergence (see Fig. 8). All stimuli are presented interleaved and displayed on-line as peri-stimulus time histograms. Typically, we determine optimal direction and speed before mapping receptive fields. We then move to more complex visual testing as required in a given experiment.

Single Unit Recording and Data Analysis: We record extracellular single unit activity using tungsten microelectrodes advanced by a hydraulic micro-drive through a guide tube (Mustari et al. 1988; Mustari and Fuchs 1990; Mustari et al. 1997; Inoue et al. 2000; Mustari et al. 2001; Ono et al. 2004). As the electrode is advanced, units are tested while the monkey performs different tasks including smooth-pursuit, fixation and suppression of the VOR (see Experimental Design). All single unit data and necessary analog channels including target, eye and chair position signals are saved directly to disk for subsequent off-line data analysis. Signals are digitized to computers employing a CED Power-1401 (Cambridge, England) hardware. This is a highly capable system that allows us to precisely record all signal channels. We typically sample analog data representing eye and target position at 1 kHz. We also save the raw single unit channel at 50kHz to perform off-line spike sorting as needed. On-line, we use an Alpha-Omega hardware and template-matching algorithm to represent the time of occurrence of each well-isolated single unit. The occurrence of each well-isolated single unit spike is represented by a TTL pulse; acquired as a time event (CED Power1401). We can check our TTL time marks against the raw spike channel in each file. Our data analysis is performed in custom software written in Matlab (Mathworks).

Statistical Analysis: We use the most appropriate statistical tests to determine the significance of our results. All statistical tests will be conducted with a significance value of 0.05, t-tests will be used to compare two groups; for example to compare VOR gain while in complete darkness to VOR gain while viewing a stationary target. When there are three or more groups to be compared we will use ANOVA methods. Multiple comparisons will be made using Tukey's test. When multiple t-tests are used, we will use the Bonferroni correction to the significance value such that the overall significance value of the comparison is maintained at 0.05. If the data are not normally distributed, we will use the equivalent non-parametric methods of testing such

as the Mann-Whitney Rank Sum test and the ANOVA on Ranks. Regression analysis will be used wherever we attempt to determine a relationship between the variables.

Reversible and Permanent Lesions: *We have been able to create reversible lesion using muscimol injections in the NOT or DLPN units produced deficit in optokinetic nystagmus, the ocular following response towards the side of injection and cancellation of the VOR (Inoue et al. 2000; Mustari et al. 2001; Ono et al. 2003). For all lesion experiments, we first map the location of gaze-related neurons (e.g., rNRTP and DLPN). We then replace the recording electrode with a small tip (15μm) micropipette and advance to the depth of related units. Injection volumes (0.1 to 0.2μl) of muscimol (2%) delivered over 2 minutes, by pressure, with a pico-pump (W.P.I. PV 830) are highly effective and stable for hours. We use a similar procedure for creating a permanent lesion using ibotenic acid. Using this method we found that multiple injections of 15% ibotenic acid were required to remove the NOTs bilaterally. We will adjust the volume of ibotenic acid used for each structure under consideration.*

Anatomical Procedures: Anatomical procedures in this project will be directed at two fundamental problems. 1, determining the specificity in cortical-NRTP, and Cortical-DLPN projections (see above, Specific Aim 2. 2, identification of recording sites using microlesions at the end of an experimental series (e.g., Fig. 3).

MRI localization of recording sites: ***We have developed effective procedures for implanting our monkeys using MRI compatible titanium bolts and "Cilux" or titanium chambers (Crist Instruments). We now are able to localize recording sites while monkeys are still under study. Figure 16 shows and example of an MRI with the locations of the arcuate sulcus (arc) and superior temporal sulci (sts) indicated. The approximate locations of FEF and MST are also shown. This image was acquired using at 3T with a gradient echo (GE) image (192 x192 matrix). We do not plan to include functional-MRI studies in this project.*** Anatomical images will be acquired using T2-weighted RARE (fast spin echo) pulse sequence using a custom-designed volume coil. These images will be planned based on localizer (scout) images. The image parameters are: TR = 4s, TE (effective) = 65-100 ms, spectral width = 50 kHz, FOV = 8 cm x 8 cm, 22 coronal slices, slice thickness = 250 microns, data matrix = 256 x 256, 16 echo train length, 4-8 averages. We will adjust the coverage of the brain to produce the clear images of MST and FEF cortex.

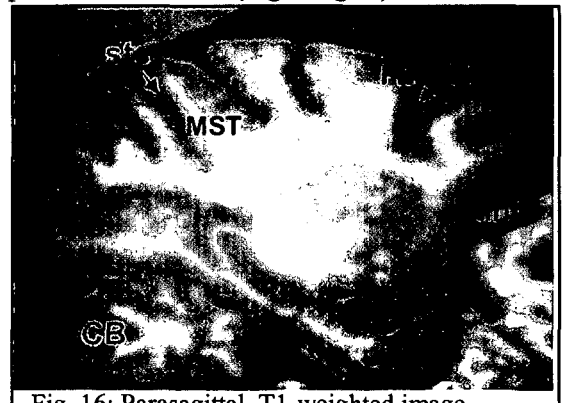

Fig. 16: Parasagittal, T1-weighted image indicating location of MST and FEF.

FUTURE DIRECTIONS AND EXPERIMENTS: Following the request of reviewers, we removed head-free studies from this proposal. **We know that in natural gaze tracking that the VOR must be suppressed in some conditions. It is** **For example, some neurons in FEF may play a role in predictive aspects of active gaze pursuit.** **We have taken the reviewer's comments to heart on this issue and will work to fully implement an efficient head-free testing capability to use in future studies. The P.I. has already visited the laboratories and consulted with several colleagues (Dr. Albert Fuchs, Seattle, Dr. Shawn Newlands, Galveston, Dr. Peter Thier, Tubingen and Dr. Ulrich Buettner, University of Munich) to develop and effective head-free preparation. We are very excited about the results we are obtaining in defining sensitivities of antidromically activated gaze-related neurons. We wish to continue this work and eventually extend our research to include studies in head-free preparations.**

E. Human Subjects

None.

F. Vertebrate Animals

1) Description of Animal Use

Three rhesus monkeys (Macaca mulatta) will be used for chronic recording in each year of this study. Animals are used as subjects in chronic single unit recording studies for approximately 10 months, preceded by 2-4 months associated with surgical preparation and behavioral training. In addition, we will perform anatomical studies on two monkeys per year to define the cortical-brainstem connectivity related to gaze.

2) Justification for Choice of Species and Number of Animals Used

Rhesus monkeys have been selected for use for several compelling reasons. First, smooth pursuit eye movements are made in this species whereas they are absent or primitive in lower mammals. We need to be able to assess the potential role of smooth pursuit in visual-vestibular gaze studies. Second, the large data base describing visual, vestibular and oculomotor system function already available in the research community, as well as in our own experience make it an ideal animal for study. Third, the similarity between the oculomotor, vestibular and visual systems of the rhesus monkey and humans make the macaque a particularly appropriate choice for attempting pioneering investigations of the visual-vestibular interactions. This allows our work to provide valuable insights important for diagnosis and treatment of clinically relevant problems in the visual-vestibular and vestibular-oculomotor functions.

3) Veterinary Care Information

The following explains Emory University's and Yerkes animal welfare compliance: The Yerkes National Primate Research Center (YNPRC) has the necessary facilities for the care and maintenance of non-human primates. The YNPRC operates to comply with the USDA Animal Welfare Act (Public law 89-544) as amended by PL91-579 (1970) PL94-279 (1976) and 45 CFR37618 (6-30-80); Health Research Extension Act of 1985 (Public Law 99-158); follows the Public Health Service Policy on Humane Care and Use of Laboratory Animals (revised September 1986); and the Guide for the Care and Use of Laboratory Animals DHEW (NIH) 1997. YNPRC is a registered Research Facility under the Animal Welfare Act. It has a current Letter of Assurance on file with the Office for Protection from Research Risks, in compliance with NIH Policy. YRPRC is accredited by AALAC. YNPRC is under the direction of a Doctor of Veterinary Medicine and staffed by veterinarians with training and experience in laboratory animal medicine, surgery, clinical care and diagnostic pathology. The animals are kept in cages in climate controlled quarters and are inspected daily. A clinical veterinarian oversees all inspections of the monkeys and provides any necessary medical care.

4) Procedures to Minimize Stress

All surgical procedures are done under full sterile conditions in a dedicated surgery room maintained by the YNPRC Animal Resource Center. Animals are anesthetized with isofluorane (1.5 - 2%) for longer procedures or Ketamine and Telazol for shorter procedures. All of these anesthetics allow rapid post-surgical recovery in a special padded cage, located in a recovery room under observation of veterinary staff and experimenter. Post-surgical anti-inflammatory and analgesic agents (e.g., Banamine and Buprenorphine) are administered for the first few days or week after surgery.

Our monkeys are adapted to handling and chairing before surgery. During behavioral training and single unit recording the monkeys head is stabilized and he sits in a primate chair facing a tangent screen. The animals work for a fortified applesauce (Formula 95 protein, Dr. Donsbach's; Dyne, Biolab Corp) or juice

reward, during training and recording. During the first two weeks of this training the animal's solid food access is controlled so that most feeding is done in the laboratory or after daily behavioral training. In all cases, where any reduction of food is considered, we take a one-week base-line weight and provide sufficient food to allow each animal to gain weight along an age and gender adjusted normogram for *Macaca mulatta.* Animal weight is not allowed to drop more then 10% of the age and gender adjusted baseline weight. Animals have free access to water at all times and the solid food ration is readjusted to near normal levels after the first two weeks of training. The monkeys used in these experiments gain weight at rates comparable to age matched, non-experimental animals.

5) Method of Euthanasia:

At the end of an experimental series, animals are prepared for euthanasia as follows. Sedation is accomplished with a single injection of Ketamine (25 mg/kg I.M.). Animals are then given an overdose of barbiturate (Nembutal 90 mg/kg I.V.; 3 times the surgical anesthetic dose). Following barbiturate overdose, animals are perfused transcardially with saline followed by paraformaldehyde fixation, to allow appropriate histological processing for anatomical studies and electrode track reconstruction (see Mustari et al. 1989; Mustari et al. 1994; Distler et al. 2002 for further details).

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H. Consortium/Contractual Arrangements: None

I. Consultants:

Appendix:
5 Manuscripts
3 figures

Principal Investigator/Program Director (last, First, Middle): MUSTARI, Michael J.

CHECKLIST

TYPE OF APPLICATION *(Check all that apply.)*

☐ NEW application. *(This application is being submitted to the PHS for the first time.)*

☒ REVISION of application number:

(This application replaces a prior unfunded version of a new, competing continuation, or supplemental application.)

☒ COMPETING CONTINUATION of grant number: EY 013308

(This application is to extend a funded grant beyond its current project period.)

INVENTIONS AND PATENTS *(Competing continuation appl. and Phase II only)*

☒ No ☐ Previously reported

☐ Yes. If "Yes," ☐ Not previously reported

☐ SUPPLEMENT to grant number:

(This application is for additional funds to supplement a currently funded grant.)

☐ CHANGE of principal investigator/program director.

Name of former principal investigator/program director:

☐ CHANGE of Grantee Institution. Name of former institution:

☐ FOREIGN application ☐ Domestic Grant with foreign involvement List Country(ies) Involved:

☐ SBIR Phase I ☐ SBIR Phase II: SBIR Phase I Grant No. ☐ SBIR Fast Track

☐ STTR Phase I ☐ STTR Phase II: STTR Phase I Grant No. ☐ STTR Fast Track

1. PROGRAM INCOME ***(See instructions.)***

All applications must indicate whether program income is anticipated during the period(s) for which grant support is request. If program income is anticipated, use the format below to reflect the amount and source(s).

Budget Period	Anticipated Amount	Source(s)
NA	$0.00	NA
NA	$0.00	NA
NA	$0.00	NA

2. ASSURANCES/CERTIFICATIONS ***(See instructions.)***

In signing the application Face Page, the authorized organizational representative agrees to comply with the following policies, assurances and/or certifications when applicable. Descriptions of individual assurances/certifications are provided in Part III. If unable to certify compliance, where applicable, provide an explanation and place it after this page.

•Human Subjects; •Research Using Human Embryonic Stem Cells• •Research on Transplantation of Human Fetal Tissue •Women and Minority Inclusion Policy •Inclusion of Children Policy• Vertebrate Animals• •Debarment and Suspension; •Drug- Free Workplace *(applicable to new [Type 1] or revised [Type 1] applications only)*; •Lobbying; •Non-Delinquency on Federal Debt; •Research Misconduct; •Civil Rights (Form HHS 441 or HHS 690); •Handicapped Individuals (Form HHS 641 or HHS 690); •Sex Discrimination (Form HHS 639-A or HHS 690); •Age Discrimination (Form HHS 680 or HHS 690); •Recombinant DNA Research, Including Human Gene Transfer Research; •Financial Conflict of Interest (except Phase I SBIR/STTR); •Smoke Free Workplace; •Prohibited Research; •Select Agents •STTR ONLY: Certification of Research Institution Participation.

3. FACILITIES AND ADMINSTRATIVE COSTS (F&A)/ INDIRECT COSTS. See specific instructions.

☒ DHHS Agreement dated: JULY 31,2003 ☐ No Facilities And Administrative Costs Requested.

☐ DHHS Agreement being negotiated with Regional Office.

☐ No DHHS Agreement, but rate established with Date

CALCULATION* *(The entire grant application, including the Checklist, will be reproduced and provided to peer reviewers as confidential information.)*

a. Initial budget period:	Amount of base $	191,000	x Rate applied	69.00	% = F&A costs	$	131,790
b. 02 year	Amount of base $	250,000	x Rate applied	69.00	% = F&A costs	$	172,500
c. 03 year	Amount of base $	250,000	x Rate applied	69.00	% = F&A costs	$	172,500
d. 04 year	Amount of base $	250,000	x Rate applied	69.00	% = F&A costs	$	172,500
e. 05 year	Amount of base $	250,000	x Rate applied	69.00	% = F&A costs	$	172,500
					TOTAL F&A Costs	$	821,790

*Check appropriate box(es):

☐ Salary and wages base ☒ Modified total direct cost base ☐ Other base *(Explain)*

☐ Off-site, other special rate, or more than one rate involved *(Explain)*

Explanation *(Attach separate sheet, if necessary.):*