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Pontomedullary reticular formation neurones : A study of microanatomy, transmitter sensitivity and connections from the substantia nigra pars reticulata.

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by

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Abstract

This investigation examines certain aspects of the medial pontomedullary reticular formation (PMRF) microanatomy and neurotransmission and also the connections between the substantia nigra pars reticulata (SNr) and the PMRF in the rat.

The anatomical distribution of the population of PMRF neurones was determined by combining physiological identification with electrical stimulation and retrograde HRP tract-tracing. A dual stimulating/deposition electrode was used to combine antidromic stimulation of PMRF cells with optimal retrograde labelling. 139 PMRF neurones were identified by means of their stereotaxic location and physiological criteria, namely; spontaneous discharge, polymodal sensory responses and large receptive fields. 91/139(65%) cells in the ipsilateral PMRF were activated antidromically or orthodromically from stimulation sites within the ventromedial funiculus of the cervical spinal cord at the level C2-C4. 74/91(81%) cells were activated antidromically and 17/91 (19%)cells were activated orthodromically. Deposition of horseradish peroxidase (HRP) at the site of optimal antidromic activation labelled approximately 80% of cells predominantly in the ipsilateral and approximately 40% of cells in the contralateral RGC. However, since 35% of neurones were not activated antidromically or orthodromically and approximately 20% were not retrogradely labelled, suggests that many PMRF neurones project contralaterally or may not possess any caudal projections.

The detailed morphology of PMRF neurones was studied by the intracellular ionophoresis of HRP in 6 cells and 2 axons. The microanatomy of 4 HRP filled cells was completely reconstructed by a 3-dimensional reconstruction technique. The microanatomy conforms broadly to the description of intracellular HRP filled giant cells in the cat. Two basic pattens of dendritic branching are revealed. Three neurones with a low dendritic index (DI = 4) indicating sparse branching and one neurone with a high DI = 14, showing profuse branching. Some individual dendrites display complex patterns of branching. The detailed morphology was used to examine the passive electrical properties of these cells. Assuming values for the specific membrane resistance (Rm) and specific axial resistance (Ri) the electrotonic lengths of the cell were approximately = 1.0 and their input resistance (Rin) between 5 and 11 MΩ.

The cholinergic neurotransmission of PMRF cells was examined by the ionophoretic deposition of nicotinic and muscarinic agonists of acetylcholine. Previous studies showed predominantly excitatory effects of acetylcholine on PMRF neurones. However 38/68(56%) cells showed mainly excitation to nicotinic agonists, and 35/68(51%) cells showed mainly inhibition to muscarinic agonists. In 53/68(78%) PMRF neurones, both the nicotinic and the muscarinic agonists were active on the same neurone. It is therefore likely that the majority of PMRF neurones have both acetylcholine receptor types present on the same cell. However, the possibility of a presynaptic location for one of the receptors cannot be excluded. Since many cholinoceptive cells showed excitation and inhibition on the same cell the

question arises as to whether acetylcholine is the only neurotransmitter exerting inhibition on PMRF neurones or whether GABA is also active. Consequently the effects of GABA were examined. 17/18 (94%) cholinoceptive cells were also inhibited by GABA suggesting that these cells are subject to dual inhibition by acethylcholine and GABA.

A further 76 neurones were challenged by ionophoretic application of GABA, and some by combinations of GABA, bicuculline and baclofen. 75/76 (99%) PMRF neurones were profoundly inhibited by GABA. Similarly the GABA agonist, baclofen caused inhibition of a high percentage of neurones (41/41, 100%), but these responses were characterized by slow onset and long duration. In 24/25 (96%) of cases bicuculline reversed the inhibitory effect of GABA. Bicuculline applied on its own caused excitation in 24/25 (96%). These data indicate that in addition to the inhibitory effects of acetylcholine via muscarinic and nicotinic receptors, PMRF cells may also be subject to tonic GABA inhibitory control via a GABAa and/or GABAb receptor mechanism.

The presence of GABAergic afferents contacting PMRF neurones was confirmed by the immunocytochemical technique for the demonstration of GABA. Three single giant cells were selected randomly from the PMRF in 3 different rats and subjected to highly specific GABA-immunocytochemistry. Terminals containing electron dense immunogold particles with a ratio of 5:1 with respect to surroundings, contact the somata and proximal dendrites in all three cases, confirming the presence of GABA-like immunoreactive (GLIR) terminals within the PMRF. As no GLIR positive cells are observed within the PMRF it is likely that these are projection fibers. One likely source of these GLIR fibers is the GABA-rich SNr, since retrograde WGA-HRP injected into the cat PMRF have demonstrated the presence of HRP filled cells in the substantia nigra pars reticulata.

Electrical stimulation of the SNr evoked short latency (< 2 ms) predominantly short duration inhibition (< 10 ms) in 32/58(55%) PMRF cells. However short duration (<10ms) inhibitory responses were also elicited from stimulation sites in cerebral peduncle in 9/20(45%) cells and in 18/25(72%) cells from substantia nigra pars compacta stimulation. These data question the specificity of SNr projections. The anatomical basis for these effects were therefore examined by applying a highly specific anterograde tract technique.

8 rats were injected with Phaseolus vulgaris leucoagglutinin (PHA-L) in SNr sites from which electrical stimulation evoked PMRF inhibitory responses. In all cases of successful intra SNr depositions of PHA-L with discrete localization, no PHA-L stained fibers or terminals were observed in the PMRF, despite good uptake by many other SNr fibers known to be GABAergic. Consequently these data are unable to confirm a pathway between the SNr and PMRF in the rat and it is concluded that the SNr is not the source of the GLIR terminals observed within the PMRF.

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Glossary of Abbreviations

3n	oculomotor nerve
bic	brachium inferior colliculus
cp	cerebral peduncle
CPu	caudate putamen
EP	entopeduncular nucleus.
GP	globus pallidus
IC	inferior colliculus
ic	internal capsule
IO	olivary nucleus
IPC	interpeduncular nucleus, central
LPB	lateral parabrachial nucleus
LRt	nucleus reticularis lateralis
LV	lateral ventricle
MCa	nucleus reticularis magnocellularis pars alpha
MCb	nucleus reticularis magnocellularis pars beta
MGD	medial geniculate nucleus
ml	medial leminiscus
mlf	medial longitudinal fasciculus
MPB	medial parabrachial nucleus
NGc	nucleus reticularis gigantocellularis
NGcV	nucleus reticularis gigantocellularis pars ventralis
NRTP	nucleus reticularis tegmenti pontis
PAG/CG	periaqueductal gray
PCR	nucleus reticularis parvocellularis
PCRt	parvocellular reticular nucleus
PGi	nucleus reticularis paragigantocellularis
PGiD	nucleus reticularis paragigantocellularis dorsalis
PGiL	nucleus reticularis paragigantocellularis lateralis
PL	paraleminiscal nuclei
PM	paramedian reticular nucleus
PMRF	medial pontomedullary reticular formation
PMRt	nucleus reticularis paramedianus
PNc	nucleus reticularis pontis caudalis
PNo	nucleus reticularis pontis oralis
PNv	nucleus reticularis pontis ventralis
PPT	pedunculopontine tegmental nucleus (PPTg)
RN	red nucleus
RD	ucleus reticularis dorsalis
RF	reticular formation
RMg	nucleus raphe magnus
RMg	raphe magnus nucleus
RNC	red nucleus magnocellular
ROb	nucleus raphe obscurus
RPa	nucleus raphe pallidus
RRF	retrorubral field
RVa	nucleus reticularis ventralis pars alpha
RVb	nucleus reticularis ventralis pars beta
Sc	superior colliculus
Sge	Supragenículate nucleus of the pons

SNc	Substantia nigra pars compacta
SNL	substantia nigra pars lateralis
SNr	Substantia nigra pars reticularis
STh	subthalamic nucleus.
tfp	transverse fibres pons
Th	thalamic region
VP	ventral pallidum
VTg	ventral tegmental nucleus

The above are abbreviations mainly according to Paxinos and Watson (1986)

Abbreviations according to Newman (1985) for coronal diagrams through medullary reticular formation (p19 and 20)

7	facial motor nucleus
Amb	nucleus ambiguus
IO	inferior olive
LRt	lateral reticular nucleus
ml	medial leminiscus
mlf	medial longitudinal fasciculus
py	pyramidal tract
pyx	pyramidal decussation
RAmb	nucleus retroambiguus
RD	nucleus Reticularis Dorsalis
Re7	retrofacial nucleus
RGC	nucleus Reticularis Gigantocellularis
RMca	nucleus Reticularis magnocellularis pars alpha
RMcb	nucleus Reticularis Magnocellularis pars beta
RPc	nucleus Reticularis Parvocellularis
RPgcl	nucleus Reticularis Paragigantocellularis lateralis
RVa	nucelus Reticularis Ventralis pars alpha
RVb	nucleus Reticularis Ventralis pars beta
SO	superior olive
Sp5	spinal tract of the trigeminal nerve
Sp5c	nucleus of the spinal tract of the trigeminal nerve pars caudalis
Sp5I	nucleus of the spinal tract of the trigeminal nerve pars interpolar
Sp5O	nucleus of the spinal tract of the trigeminal nerve pars oralis.
Su7	supra facial nucleus.

Note: The abbreviation (RGC) for nucleus reticularis gigantocellularis was mainly used in this study.

Miscellaneous

5-HT	5-hydroxy tryptamine
ACh	acetylcholine
AchE	acetylcholinesterase
BAC	baclofen

BIC	(-)-bicuculline methiodide
CARB	carbamylcholine chloride
ChAT	Choline acetyl transferase
CNS	central nervous system
DA	dopamine
DAB	di-aminobenzidine tetrahydrochloride
DMPP	dimethyl-4-phenyl-piperazinium iodide
EPSP	excitatory post synaptic potential
IPSP	inhibitory post synaptic potential
GABA	gamma-amino-n-butyric acid
GABA-T	GABA-transferase
GAD	gamma-amino decaroxylase
GLIR	GABA-like immunoreactivity
HRP	horseradish peroxidase
MUS	muscarine chloride
NIC	nicotine sulphate
PHA-L	phaseolus vulgaris-leucoagglutinin
TBS	tris-buffered saline

CONTENTS

Page

Section 1.3.0 Results and discussion

Introduction

Background to this thesis

The neurones of the medial pontomedullary reticular formation (PMRF) have been one of the main research interests of the Neurophysiology Division in the Department of Physiology at the University of Cape Town during the past 8 years. The studies relating to the properties of these neurones have been made with a number of different approaches in mind. It therefore follows that various aspects of PMRF physiology have been studied intensively.

General research interest in this particular field has progressed, as appears to be the trend in Neuroscience, in cycles fluctuating between intense interest and relative indifference from the late 1950's to the early 1970's. The present status of the general organizational principles of the reticular formation, established at the beginning of this decade, is unclear. Research on brain stem neurones during the past decade has not kept pace with the intensive research in other brain areas. Since "The reticular formation revisited" by Hobson and Brazier in 1980, outlining the basic principles governing the function of the reticular formation, no major definitive studies have emerged. A possible reason for this setback may stem from the fact that the enormous resurgence of interest in "connectionism" as well as artificial neuronal networks is more likely to be studied in central nervous system structures where the network architectures are built from layers of these processing elements. Areas composed of well defined structural elements therefore lend themselves to this type of investigation. The cytoarchitecture of the reticular formation is vastly different and is therefore not readily accessible to this type of investigation.

The importance of these PMRF cells is thought to lie in their ability to perform the task of sensory and motor processing. There is much anatomical and electrophysiological data demonstrating reciprocal connections with various levels of the spinal cord that support this belief. In addition there is abundant evidence implicating cells of the medial reticular formation in a host of other functions. Some of these will be discussed further in the review section.

Neurones lying within the medial pontomedullary reticular formation (PMRF) have been shown by others, as well as workers in this laboratory, to be responsive to a wide range of sensory stimuli. In particular PMRF neurones have been shown to be responsive to thermal stimulation (Farham and Douglas, 1986).

Since these neurones belong to the reticular activating system and are therefore thought to perform a co-ordinating role, it is still not understood how the population of cells communicate to fulfill this function. This picture is further

complicated by the fact that these cells are phylogenetically primitive and rather homogeneous in terms of overlapping functional zones, with ill-defined anatomical zones. Given these ingredients it is clear that these cells present a challenging experimental problem. A single cell cannot play a role in the processing of information without interacting with other neurones of the intrinsic circuits. Hence there is an urgent need to establish the intraregional connections, their neurotransmitter characteristics and spatial arrangements of synapses, if the computational mechanisms of PMRF cells are to be understood.

Arising out of this is the requirement for a synthesis of computational theory and clear neurobiological data. Much of the latter category is still outstanding, eg. precise and detailed dendritic patterns, synaptic properties, transmitter properties and relationships and the intrinsic membrane properties of the individual cell.

In working toward such a synthesis there are still many fundamental questions to be answered. Although this study does not examine all of these aspects it should be seen in the broader context of the areas still requiring detailed study. This study is a journey down that road.

In the last 6 years, 4 Honours theses and one PhD thesis concerning various aspects of PMRF physiology have been completed. The four Honours theses were concerned with:-

(1) Thermal responsiveness of PMRF neurones (Farham, 1984);
(2) Cholinoceptive transmitter properties (Markram, 1985);
(3) Rostral projections of PMRF cells (Sefton, 1985);
(4) Thermal responsiveness of PMRF neurones comparing stimulation of glabrous and hairy skin (Francis, 1987).

The Doctoral thesis by Douglas, (1984) examined the temporal structure of action potential discharge.

The current research program on the reticular formation takes the form of another doctoral study, which extends the observations on the thermal properties of these neurones, and a masters thesis which examines the interactions between PMRF neurones utilizing cross-correlation techniques.

The work covered in the present study was therefore performed against a background of general research on the functional interactions of these cells. My interest in the reticular formation grew from my involvement with a number of collaborative studies aimed at the problem of teasing out functional neuro-circuitry, employing techniques such as electrophysiology and tract-tracing.

Scope of thesis and problems

This study covers various aspects of microanatomical structure, transmitter sensitivity and connectivity with the substantia nigra pars reticulata (SNr). I have adopted a multidisciplinary approach in an attempt to build a broad profile of some characteristic features of these neurones. I have endeavoured where possible to apply an integrated approach to the problem by a combination of various techniques. It was the overall aim of this study to systematically examine a number of properties of single identified neurones to gain further insights into their computational complexity.

To date, the bulk of histological data concerning reticular formation neurones is based on general staining procedures such as Golgi, Nissl and global horseradish peroxidase (HRP) staining. In order to understand the functional properties as well as connectivity of these cells with other target neurones, it is essential to have a clear picture of single cell morphology. Increasingly, researchers have become aware of the important role the dendritic morphology plays in the overall function of the cell. Therefore there is an urgent need to understand the detailed anatomy of the cell. An appraisal of the detailed electrical properties of a cell relies to a great extent on an intimate knowledge of its dendritic architecture. The implications of such detailed studies are far-reaching in the field of computational neuroscience.

The best way of achieving this goal is via the intracellular approach, which involves the deposition of dyes such as HRP into the neurone, thereby illuminating its entire structure. At the time the present series of experiments were performed, the microanatomy of PMRF cells had not been investigated by means of the intracellular injection of horseradish peroxidase, and most anatomical descriptions reported in the literature were limited to those based on Golgi and Nissl staining techniques. Recently PMRF cells in the cat have successfully been injected with intracellular HRP. These studies do not however combine their intracellular data with electrophysiology and furthermore these cells are reconstructed by the camera lucida technique, and therefore only in a single plane. A relatively new approach for reconstituting the structure of an intracellularly stained cell from serial histological sections is by means of a computerized 3-dimensional reconstruction technique, thereby enabling a more detailed structural examination than hitherto possible. I have employed the 3-dimensional reconstruction method here. A new system called "Traka", (recently developed by Prof. R. J. Douglas and Mr. D. Botha), was specially developed for the microanatomical reconstruction of intracellularly stained cells. Since the accurate dimensions of the dendritic tree are measured, the cable properties of these cells may be calculated by employing measured as well as

assumed membrane electrical parameters. The intent of this section of the thesis is to gain some insight of their electrotonic behaviour.

Many electrophysiological studies are beset with the problem of exact identification and location of the recorded cell. The problem is compounded where electrostimulation techniques are also involved. This technique is essentially "blind" and does not permit a description of the underlying connection. Since PMRF cells belong to the reticulospinal system, stimulation of their caudal projections is a means of electrically identifying the cell population. A novel technique, combining horseradish peroxidase, (HRP) tract-tracing and electrical stimulation was devised in order to map both the route of their axons and the distribution of retrogradely filled PMRF cells. This section of the thesis therefore fulfills an important requirement of this study, namely, positive confirmation of the stereotaxic location and procedures for the exteroceptive identification of these cells. Furthermore this method permits accurate tracing of the projection pathway from the PMRF to the cervical spinal cord.

The impetus for this approach in part stemmed from the unreliable marker techniques which employ extracellular dyes for marking recording locations. As the majority of cells in this study were identified by their gross stereotaxic location as well as other criteria, sound anatomical confirmation correlated with these identifying criteria is essential. At best, histological confirmation of the location of fine glass electrode tracts can only provide the approximate location of the recorded cell. With the incorporation of marker dyes in the recording electrode there is necessarily a trade-off between quality of recording characteristics and end-result of marker dye deposition. Since this study has a strong anatomical emphasis the omission of extracellular marker dyes may appear to be out of context with the central theme of this study. Some of my first endeavours were aimed at finding a reliable method for identification of recording sites. However, sacrifice of recording quality for the sake of unreliable and only intermittent demonstration of the sites of extracellular marker dyes was not considered a viable proposition. For this reason the problem was approached globally as outlined above. I found that Pontamine sky blue and or Evans blue both proved to be unreliable for marking of extracellular recording locations, therefore this direction was not pursued.

The transmitter properties of individual PMRF cells are largely unknown. To date few definitive *in vivo* studies on their transmitter properties are available. I have examined the sensitivity of PMRF neurones *in vivo* by means of the ionophoretic deposition of two classical neurotransmitters namely, acetylcholine and GABA as well as their respective agonists. The reason for selecting only these two transmitters in the face of a large transmitter diversity, arises partly from some earlier findings and a collaborative study performed in this laboratory. Our preliminary data suggested that acetylcholine, which is known

to operate via either nicotinic or muscarinic receptors, may play a unique role in the PMRF insofar as it employs both receptor mechanisms. It is therefore important to test the *in vivo* responsivity of these cells to muscarinic and nicotinic agonists in order to ascertain their broad anatomical distribution.

The data from our collaborative study show that the effects of acetylcholine are mainly excitatory. Taken together with the majority of evidence which indicates that inhibitory effects within the PMRF are in the minority, these data question the role of the major inhibitory neurotransmitter, γ-amino butyric acid (GABA), within the PMRF. It is thus also important to establish the role of GABA in the overall performance of these cells.

The technique of ionophoresis has a limited degree of precision. However it is the only *in vivo* method available for ascertaining, albeit indirectly, information pertaining to the transmitter and receptor properties of neurones. At best it is a qualitative technique with a number of factors affecting the ejection of substances through the fine orifices of the deposition electrode. Although it is possible to calculate the amount of drug ionophoresed, this was not attempted in the present study. The pharmacodynamics of ejected substances were therefore not explored quantitatively. As the answers to some basic questions concerning the transmitter sensitivity of these neurones were sought, the study was limited purely to a qualitative description of the responsivity of these cells to various ionophoresed transmitter substances.

The investigation of the GABAceptive properties was taken a step further, since the ionophoretic data supported the proposal that PMRF neurones may be under the control of an inhibitory GABAergic input. In order to demonstrate conclusively whether GABAergic terminals do contact PMRF neurones, a highly specific immunocytochemical technique was employed and the reaction product visualised by means of electron microscopy.

My interpretation of the GABA ionophoretic and immunocytochemical data led me to address the role of basal ganglia involvement in the control of PMRF activity. It is a well known principle that the basal ganglia exert an inhibitory influence on their target neurones. The functions of the extrapyramidal system in the rat have received a large amount of experimental attention in recent years, yet it is far from clear how supraspinal systems are integrated with the reticulospinal system in the execution of various motor programs. There is much evidence available to support the notion that the basal ganglia must exert an effect on the spinal cord that is not mediated via rostrally projecting nigrofugal projections. There is little doubt too that basal ganglia influences are mediated by way of the reticulospinal system, and there is general consensus that these effects are mediated via a nigral relay. However, another body of opinion supports the existence of a direct pathway between the substantia nigra and the pontomedullary reticular formation. Although the electrophysiological evidence is convincing, the neuroanatomical literature in support of such a connection is

contentious. In this section of the study two approaches were adopted for examining these putative connections. Firstly, electrical stimulation of the substantia nigra pars reticulata (SNr) combined with precise anatomical reconstruction of the electrode stimulation sites, was aimed at establishing the incidence of inhibitory effects on PMRF neurones correlated with the exact site of stimulation. The significance of this part of the study lies in the interpretation of electrical stimulation studies performed by previous workers. These data are unconvincing due to insufficient evidence of the anatomical locations of their stimulation sites. This is especially important in view of the unwanted stimulation of the large contingent of fibers en passant. The second approach aimed at demonstrating the anatomical substrate underlying SNr and PMRF interaction. This involved the use of a highly specific anterograde tract-tracing method employing the ionophoretic deposition of phaseolus vulgaris-leucoagglutinin (PHA-L) within the SNr, thereby testing for the presence of a fiber tract arising from SNr neurones.

History of this study

I commenced work in the Neurophysiology Division of the Physiology Department in November 1982. At that time I occupied the post of Technical Officer. My main duties were to provide postgraduate research support. In addition to these duties I collaborated on a number of research projects between 1983 and 1986, involving the Hyrax visual system, hippocampal brain slice technique, basal ganglia physiology (utilizing the rotating rat model) and brain stem *in vivo* studies involving cholinergic transmitter properties and tract-tracing.

I registered for and commenced part-time doctoral studies in 1985. Since that time I have held the full-time posts of Technical Officer, Senior Scientific Officer and, from October 1988 till present, the post of Chief Scientific Officer in the Department of Physiology. In addition to regular laboratory and administrative duties, I assist with senior practical classes and tutorials. For the last 6 years I have given a series of Neuroscience lectures to students majoring in Physiology as well as at second year level.

I executed all aspects of the work covered in this study and acknowledge with gratitude data capture programs written by Prof. R. J. Douglas and Dr. A. George.

The bulk of the work was performed at UCT, but some aspects of this thesis were carried out during a 3 month period of Study Leave at the MRC Anatomical Neuropharmacological Unit (ANU), Department of Pharmacology, University of Oxford, U.K. The purpose of the visit was to learn from experts in particular fields of research, new and specialized techniques that would be of benefit in our

postgraduate research program at UCT. Some of these techniques were directly applicable to my own research project and as such proved to be invaluable.

In order to demonstrate the presence of GABAergic fibers within the PMRF, the application of a highly specific antibody to GABA, as well as instruction on the finer points of this technique, was required. Since this antibody is not routinely used in our electrophysiological laboratory nor easily obtainable, a small amount was obtained for a once-off collaborative immunocytochemical study in the Department of Pharmacology at the University of Oxford. The antibody was kindly donated by Prof. P. Somogyi.

The technique, involving the injection of PHA-L, also relies on immunocytochemical principles and is a specialized technique not readily available in this country. As this technique is also routinely employed in visual tract-tracing experiments at the MRC ANU, it too was an opportunity for applying this technique to my particular experimental requirement, with a specialist in this field on hand to advise on problems.

While at the ANU, I endeavoured to improve the yield of intracellularly injected HRP neurones. At that stage of the study, only two neurones and two axons had been injected with HRP at our laboratory at UCT. It was suspected that a possible reason for the low yield was lack of robustness of the stereotaxic equipment and lack of experience on the part of the author. At the ANU, a number of experienced researchers routinely perform intracellular recordings from brain slices as well as *in vivo* cortical neurones. Drawing on the collective experience at Oxford, and application of many useful suggestions, the success rate was still low; in fact, only a further three cells were successfully injected with HRP.

Since the Traka program for reconstructing the 3-D morphology of intracellularly labelled cells was available, together with a microscope, fitted with a motorized stage and z-transducer, for routine reconstructions of cortical neurones at the MRC ANU, the opportunity was used to reconstruct all the HRP injected cells using these facilities. These two essential components for reconstructing the HRP filled PMRF cells in 3-dimensions are unavailable in our laboratory at UCT.

During the course of the last 5 years the progress of the study was presented in the form of short papers and or poster displays at the annual meetings of the Physiological Society of South Africa and the Neurosciences Group.

All the experiments were carried out on anaesthetized rats which were sacrificed at the end of an experimental session. Where the nature of the experiment required that the rat recover after initial surgery and treatment, the necessary precautions were taken to ensure that the animals suffered minimal discomfort. The animals were injected with a "cocktail" anaesthetic (Equithesin) from which recovery was fairly rapid. During the recovery period the animals were inspected regularly to ensure that there was no unnecessary

suffering. All the animal experimental methods and techniques met with the criteria acceptable to the University of Cape Town Faculty of Medicine Animal Research Review Committee and the United Kingdom Home Office.

Section 1.1.0

Literature Review

The brain stem reticular formation

The brain stem is composed of a number of important nuclei that perform various communicative and integrative functions. Within the core of the brain stem lies the reticular formation and, as the name implies, is comprised of a cellular network consisting of neurones with prolific dendritic processes and long axons with rich collateralization. Due to these anatomical features, the aggregates of reticular neurones form nuclei with unclear lines of demarcation. The reticular formation (RF) has been shown to acquire input signals from diverse and widely spread areas of the central nervous system. Information of all sensory modalities, spinal cord, cranial nerve nuclei, as well as from cerebral cortex, diencephalon and septal areas are received. Due to the overlapping arrangement of the neurones within the RF, interactive links are permitted between projections. Furthermore, information is then dispersed to most other portions of the brain.

Over the last few decades the reticular formation has been shown to serve a general, activating function and, in particular, is associated with the maintenance of consciousness.

As indicated by numerous studies, the brain stem reticular formation influences a variety of spinal cord activities as well as a number of behaviours, viz:

(a) somatomotor activity (Magoun and Rhines, 1946; Peterson et al., 1975; Siegel and Tomaszewski, 1983; Vertes, 1979)

(b) eye movement in waking (Kaneko et al., 1981) and eye movement during sleep (Pivik et al., 1977)

(c) vestibular and visuomotor reflexes (Peterson and Fukushima, 1982)

(d) muscle atonia (Sakai et al., 1982; Sakai et al., 1979)

(e) modulation of nociceptive input (Basbaum et al., 1981; Watkins et al., 1980)

(f) modulation of autonomic activity (Dampney et al., 1982; Caverson et al., 1983).

The volume of physiological and anatomical information about the reticular formation that has accrued since the classical studies of Magoun and Rhines, (1946) and Moruzzi and Magoun, (1949) is enormous. As the above list of heterogenous functions attest, it is not unexpected that the reticular formation would be divisible into functional areas. However most of the boundaries amongst reticular nuclei are indistinct and the majority of reticular nuclei are not cytoarchitecturally homogenous. Thus functional maps of the reticular formation frequently do not coincide with borders derived from anatomical studies.

With the advent of recent sophisticated neuronal staining and immunocytochemical techniques, the brain stem reticular formation has revealed more of its subtle features enabling identification of additional morphological features, and therefore giving rise to further new subdivision. Indeed in recent years additional morphological details have been revealed by combined anatomical techniques such as Golgi and Horseradish Peroxidase (HRP) tract-tracing, or Golgi and intracellular HRP. These features and further subdivisions of classical brain stem nuclei will be discussed briefly in the following account.

As is well recognized, nuclear boundaries are not clearly delineated by any single or combined method. At best, the definition of boundaries is open to interpretation and

thus remains subject to argument. The rat brain atlas of Paxinos and Watson, (1982) is popularly used by anatomists and electrophysiologists. Certainly there are many other popular atlases e.g. Pellegrino et al., (1979) and Albe-Fessard et al., (1966) . The various subdivisions of the reticular nuclei that appear in the following description have apparently not gained general recognition and therefore many of the various subdivisions currently in use do not appear in the atlases of Paxinos and Watson, (1986) and Pellegrino et al., (1979).

The current edition of Paxinos and Watson, (1986) similarly does not recognize some of the subdivisions of reticular nuclei suggested by Newman, (1985a; 1985b) and Andrezik and Beitz, (1985). Thus extreme caution has to be exercised when ascribing functions to a particular nucleus. The following is thus an attempt at characterizing briefly the main features of some of the pontomedullary reticular formation nuclei surrounding the nucleus gigantocellularis which is the nucleus of particular interest in this study.

(a) Anatomical boundaries of the pontomedullary reticular formation nuclei

The brain stem nuclei surrounding the gigantocellular reticular nucleus, the area which is the subject of this dissertation, are discussed in this section in the context of their cytoarchitecture and anatomical boundaries. The morphological features of the neurones comprising these nuclei apply specifically to the rat unless otherwise indicated. Listed below are some of the brain stem nuclei and their subdivisions.

The nomenclature and abbreviations are similar to the rat brain atlas of Paxinos and Watson (1982). This atlas is based on the delineation of nuclei in coronal and parasagittal sections by means of combined Acetylcholinesterase (AchE) and Nissl staining techniques. However in the light of more recent anatomical studies (Newman, 1985; Andrezik and Beitz, 1985), employing combinations of Nissl, Golgi and Horseradish peroxidase techniques, further differentiation is possible. Thus the abbreviations and subdivisions for some nuclei are different and do not appear in Paxinos and Watson. In the list below are some of the abbreviations commonly seen. It is beyond the scope of this dissertation to deal exhaustively with all the anatomical differences of each individual brain stem nucleus. Perforce this is a brief description of some of the nuclei in the immediate vicinity of RGC which are relevant to this study. Listed below are some of the major nuclei of the medulla and pons.

Medullary reticular formation

Nucleus reticularis dorsalis	(RD)
Nucleus reticularis ventralis pars alpha	(RVα)
Nucleus reticularis ventralis pars beta	(RVb)
Nucleus reticularis Parvocellularis	(RPc/PCRt)
Nucleus reticularis Paramedianus	(RPm/PMRt)
Nucleus reticularis paragigantocellularis	(RPgc/PGi)
Nucleus reticularis Paragigantocellularis lateralis and dorsalis	(RPgcl/PGiL) (RPgcd/PGiD)
Nucleus reticularis gigantocellularis	(RGc/RGC/Gi)
Nucleus reticularis gigantocellularis pars ventralis	(NGcV/RGcv)
Nucleus reticularis magnocellularis pars beta & alpha	(RMca/RMcb) (MCα/MCb)
Nucleus raphe magnus	(RMg)
Nucleus raphe obscurus	(ROb)
Nucleus raphe pallidus	(RPa)

Pontine reticular nuclei

Nucleus reticularis pontis caudalis	(PNc)
Nucleus reticularis pontis ventralis	(PNv)
Nucleus reticularis pontis oralis	(PNo)
Ventral tegmental nucleus	(VTg)
Pedunculopontine tegmental nucleus	(PPTg)
Supragenicuiate nucleus of the pons	(SGe)
Paralemniscal nuclei	(PL)

Fig 1.1.1. Illustrates subdivisions according to the above nomenclature. The diagrams are adapted from Newman (1985) and show the caudal to rostral extent of the medulla in the rat. The following section discusses some of the identifying features of these nuclei.

Nucleus reticularis ventralis [pars α and beta] (RVα and RVb)

RV appears to be the rostral continuation of the lamina VII of cervical gray matter. It is distinguishable from RD by its larger number of medium sized neurones and greater amount of neuropil. At the level of the caudal pole of the IO it is divisible into ventral (pars α) and dorsal (pars beta) portions. Towards its rostral pole RVb merges with the caudal end of NGC. The approximate extent of this nucleus is as follows:- rostral extent: to caudal half of the inferior olivary nucleus (IO); caudal

extent: spinomedullary transition commencing at the pyramidal decussation; medial boundary: medial longitudinal fasciculus; lateral boundary: lateral reticular nucleus.

The cellular morphology distinguishes the two zones. RVb contains polygonal multipolar neurones of all sizes. Larger in the rostral zone, 45 to 65m with polygonal somata bearing 4 to 6 long dendrites. These cells have radial symmetry in their dendritic aborizations. RVα on the other hand contains predominantly small and medium fusiform neurons widely separated from one another by large quadrangular patches of neuropil. There is no particular orientation of the dendrites. Axons of the RVb neurones course dorsomedially and enter the MLF, projecting to the spinal cord with a weak ipsilateral predominance, while axons of the RVα neurones similarly project to the MLF. These neurones project caudally with a strong ipsilateral predominance.

Nucleus reticularis dorsalis (RD)

This nucleus lies in the caudal medulla and along its length lies ventral and medial to the spinal trigeminal nuclear complex and dorsolateral to nucleus reticularis ventralis pars beta. At the caudal level it commences at approximately the spinomedullary transition. The neuropil is homogeneously distributed thoughout most of this nucleus, but cellular density increases more rostrally, so much so that at the level of the area postrema this nucleus consists almost entirely of small densely packed cells. Golgi stained sections indicate that dendritic arborizations of a majority of these neurones exhibit a ventrolateral to dorsomedial orientation. The dorsomedially directed dendrites frequently enter the corticospinal tract at a level where the latter fibers gain entry to the dorsal funiculus. The following areas demarcate the approximate boundaries of the RD nucleus:- the rostral extent: level of the area postrema where it is replaced by RPc; caudal extent: spinomedullary transition; medial boundary: RVb; lateral boundary: nucleus of the spinal tract of the trigeminal nerve, pars caudalis.

Retrogradely filled RD cells from HRP deposition sites in the cervical spinal cord demonstrate a strong ipsilateral staining. The axons arising from RD neurones project dorsomedially to the dorsal funiculus.

Nucleus reticularis gigantocellularis (Gi/RGC)

RGC is situated mostly in the rostral medulla oblongata. Andrezik and Beitz, (1985) have subdivided NGC into three parts, viz: gigantocellular reticular nucleus (Gi); Gi ventral part (GiV); and Gi pars alpha (Giα) on the grounds of different cytology. Considering the nucleus without regard for its proposed subdivisions, the caudal border is approximately at the middle portion of the inferior olivary nucleus (IO) (Newman, 1985). Andrezik and Beitz, (1985) however, place the commencement of RGC at the rostral tip of IO and its rostral pole at the level of the caudal pole of the superior olivary nucleus. According to Newman the RGC extends rostrally to the level of the rostral pole of the facial nucleus. The latter landmark, according to the

Atlas of Paxinos and Watson, (1982) is, however, still well represented at the level at which nucleus pontis caudalis commences. Thus the rostral termination of RGC as indicated by Andrezik and Beitz is more in agreement with that of Paxinos and Watson, (1982).

At its caudal end RGC merges with RVb ventrally, and is bounded dorsomedially by the nucleus of Roller. Andrezik and Beitz have indicated at mid-medullary level, the nucleus of Roller (Ro) and the intercalated nucleus of the medulla oblongata (In), as being amongst those nuclei comprising the dorsomedial roof of the medulla. As reference points, the position of these two nuclei do not coincide with the anatomical positions indicated by Paxinos and Watson, (1982). According to this atlas these two dorsally located nuclei are in the most caudal part of the medulla and not at the mid-medullary level as indicated by Andrezik and Beitz.

Along most of its medial extent the RGC is bordered by the medial longitudinal fasciculus (MLF). At its caudal end the medial surface abuts on the paramedian reticular nucleus (PM), and at its rostral end the medial suface abuts on the fibers of the predorsal bundle. It borders on the parvocellular reticular nucleus (RPc) laterally. The ventral boundary comprises Nucleus reticularis ventralis pars α and beta.

Although the nucleus derived its name from the prominent giant cells, (some of them measuring 70 x 30μ) within its boundaries, these large cells do not make up the majority of cells. By far the most common (approximately 70%) are the medium and small sized cells(20 - 40μ). The smaller cells are more densely packed in the lateral parts of the nucleus. The majority of giant cells lie medially. The cells are typically polygonal and multipolar and, broadly speaking, divisible into 2 classes based on the structure of their dendritic trees; those with few primary dendrites (±4) and those with many more primary dendrites (±11). The dendrites ramify predominantly in the transverse plane and some extend rostrally and cuadally for considerable distances. A noticeable feature of these cells is the absence of spines and/or excrescences in the majority of cells(Newman and Kiddy, 1981).

RGC has been further subdivided along its ventral aspect by Andrezik and Beitz, (1985) on the basis of differential Nissl staining and connections with the spinal cord, into a ventral zone termed (RGiV). A distinguishing feature of this area, and yet a further justification for this subdivision is that RGC neurones are packed more densely in this region and preferentially project to the lamina 9 area of the spinal cord (Martin and Waltzer, 1984).

Another subdivision of the RGC, termed RGiα by Andrezik and Beitz occupies the ventral medulla but lies immediately rostral to the RGiV. This area stretches from the rostral IO to the level of SO. The equivalent area in the cat described by Basbaum et al., (1978) is the nucleus reticularis magnocellularis. Although Watkins et al., (1980) have also defined the corresponding area in the rat as the nucleus reticularis magnocellularis (RMC), the general opinion seems to be that RGia is very different from nucleus magnocellularis. On the basis of the descending projections from the rostral medullary reticular formation in the cat , studied by means of ionophoretic HRP depositions, Abols and Basbaum, (1981) provide further support for a subdivision of the rostral medullary area into a dorsal and ventral zone, RGC and RMC, respectively. It has therefore been suggested that the nomeclature of these two areas should for the present time remain unaltered. The latter study also

demonstrates that the distinction between RMC and NRM on the basis of their afferent connections is not clearly defined. By means of the neurone labelling pattern derived from deposition sites in the spinal cord of slow release HRP gels, Watkins et al., (1980) have shown that this subdivision of RGC, i.e. RMC may share some common connectional features with nucleus raphe magnus (NRM) and have therefore suggested yet another name for this functional unit, "nucleus raphe alatus". However this suggestion apparently has not gained wide acceptance on the grounds that RMC does not contain serotonergic neurones, whereas NRM does (Basbaum et al., 1980; Glazer et al., 1980). All of the aforementioned, however, does serve to illustrate that traditional subdivisions and classification of groups of neurones, on the grounds of cytoarchitectural features alone, may exclude groups of neurones that have a common functional connection.

Nucleus reticularis parvocellularis (RPc)

This nucleus lies medial to the spinal trigeminal complex, but comes to occupy a relatively larger mediolateral position as it extends rostrally. RPc replaces RD rostrally. Caudally the nucleus is composed of densely packed small cells and more rostrally from about the level of the obex, 2 cell types with differing mediolateral distribution are evident, viz., densely packed medium and small cells lying medially and loosely packed small cells lying in the lateral portion. The larger cells of the medial RPc are distinguishable from those of the RGC by a relatively large area of neuropil.

In the region of the pontomedullary junction distinct features become blurred. Larger neurones are present and the neuropil has a dispersed appearance. This portion of the nucleus is very different in character to the more caudal portion of small compact cells, and the maximum size of somata are reached at the level of the facial motor nucleus. The approximate extent of this nucleus is as follows:- medial border: RVb caudally, and RGC more rostrally; lateral border: nucleus of the spinal tract of the trigeminal nerve, stretching from pars interpolaris (Sp5I) to rostral pole of this nucleus (Sp5O). The somata are usually oval to triangular and bear short, thin dendrites. These are orientated in one of two patterns, either dorsomedially or ventrolaterally. Some cells with the former dendritic orientation project to RGC. However the majority of RPc dendrites terminate in the same nucleus. Similar to RV neurones, the axons of RPc neurones project in a dorsomedial direction and via the medial longitudinal fasiculus terminate primarily in the upper cervical spinal cord. However, in contrast to RV there is no lateral predominance of projections to the spinal cord.

Nucleus reticularis magnocellularis (RMc)

According to Adrezik et al., (1981) the boundaries of nucleus reticularis magnocellularis are not well defined in any species. Consequently they and other workers omit to give an account of this nucleus in any detail (Andrezik and Beitz,

1985; Valverde, 1961; Brodal, 1981b). In a subsequent study by Valverde, (1962) it was noted however that the dorsal aspect of the rostromedullary reticular formation is composed of larger cells than the ventral area which is composed of densely packed medium sized cells. No distinction was made between these two cell groups. However in the cat the distinction is apparently clearer and the equivalent area is divided into giganto and magnocellular tegmental fields.

Newman, (1985) on the other hand provides a reasonably precise description of the nucleus. The following account is based largely on his histological data. RMc lies ventral to RGC and extends from mid pontine levels caudally to approximately the middle region of the medulla. It commences caudally at a level approximately the same as that at which RGC starts, i.e. the rostral pole of the lateral reticular nucleus. RMc is divided into a dorsal and ventral zone, beta and alpha respectively, along the greater part of its length. At its caudal extremity RMcbeta is sandwiched between the inferior olivary complex ventrally and NGc dorsally. RMcα commences at a level slightly rostral to the retrofacial nucleus. The maximal size of these two nuclei is reached at the level of the rostral medulla.

The neurones comprising RMcα are densely packed, with smaller neurones in the caudal regions changing to larger soma diameters more rostrally. The majority (79%) of magnocellular somata ranged between 20 - 40μ are flattened or elliptical, with an average of 5 dendrites orientated mainly in the horizontal plane (Newman and Kiddy, 1981). In RMcbeta the somata are mostly small, spindle-shaped and orientated horizontally. The dorsal border of RMc at lower pontine level is formed by nucleus reticularis pontis caudalis (PnC). Rostral extent: RMcα extends further than RMcbeta, the former having dispersed at approximately midway along the trigeminal motor nucleus.

RMcα and RMcbeta are distinguishable on the grounds of their soma shape and size. The latter being comprised mainly of elongated triangular or polygonal somata, with dendritic arbors compressed in the dorsoventral plane. As a result dendritic arbors are either horizontally or dorsomedial to ventrolaterally orientated. The dendrites of the dorsomedially orientated neurones frequently enter RGC while the ventromedially slanting neurones often enter RMcα. The two divisions of this nucleus are also distinguishable on the basis of their staining characterisitics with HRP. RMcbeta appears as a dark band of cells, whereas RMcα is a paler wedge shaped area lying ventral to RMcbeta. Axons from both these areas course to the MLF or the lateral edge of the brain stem with mainly ipsilateral projections to the spinal cord.

Nucleus reticularis paragigantocellularis lateralis (RPgcl) and dorsalis (RPgcd)

At its most developed region, approximately the rostral pole of the inferior olive, this nucleus fills the ventrolateral region of the medulla with a lattice-like appearance. It commences at the rostral pole of the LRN and extends rostrally to approximately the level of the genu of the facial nerve, where it lies medial to and is largely replaced by the facial nucleus. The medial border is the inferior olivary complex and the lateral border the spinal trigeminal nucleus.

Towards the rostral border the cellular density increases and cells are medium large. However the majority of cells are small and four types of neurone are recognizable on the basis of their dendritic pattern. HRP labelled neurones from spinal cord injection sites are observed throughout the nucleus, the most numerous being present in the rostral part. On the basis of this staining it is evident that RPgcl project bilaterally with a slight predominance of fibers ipsilaterally. Most fibers project caudally via the lateral funiculus, but some project via the MLF.

Another dorsally located division of this nucleus, bounded by RGC ventrally and medially by MLF, has been named by Newman,(1985) nucleus reticularis paragigantocellularis dorsalis (RPgcd). It extends rostrally from about the rostral pole of the hypoglossal nucleus to about the rostral pole of the facial nucleus, and is coextensive with nucleus prepositus hypoglossi (PrH). By comparison with RGC this nucleus has a much lower cell density than RGC as it is traversed by coarse fiber bundles. The somata show many variations in shape with dendrites frequently entering RGC and PrH. Axonal projections are directed caudally via the MLF with a strong contralateral preference. This is in contrast to most other reticulospinal neurones originating in the medulla. Different populations of neurones were retrogradely filled from different sites in the spinal cord, thus making a case for differential projections to cervical and thoracic spinal cord.

Nucleus reticularis paramedianus (RPm)

RPm is situated medially to RVb and immediately lateral to the midline raphe nuclei. This nucleus commences at about the level of the obex and extends rostrally to the rostral pole of the hypoglossal nucleus. The predominantly small neurones comprising this nucleus are intricately surrounded by fibers in the MLF and characterized by cruciform dendrites coursing in a straight dorsomedial and mediolateral plane.

Other medullary nuclei

Nuclei of the raphe system, viz. nucleus raphe obscurus, pallidus and magnus will not be dealt with in this dissertation. For a detailed description see Newman, (1985a) and Andrezik and Beitz, (1985). Briefly however, these nuclei whose names were derived from early cytoarchitectural studies, have generally become synonymous with the classification based on the serotonin fluorescent histochemical studies by Dahlström and Fuxe, (1965).

Pontine Nuclei

The medial pontine tegmentum is comprised mainly of two nuclei, the nucleus reticularis pontis caudalis (PNc) and oralis (PNo) and both these nuclei are contributors to the reticulospinal system.

Nucleus reticularis pontis caudalis (PnC)

This nucleus lies immediately rostral to the RGC. The boundary between the two nuclei is indistinct due to the fact that it also contains giant neurones which are comparable in size to those of RGC. It extends rostrally from about the level of the caudal pole of the trapezoid body to the rostral division of the middle third of the motor nucleus of the trigeminal nerve (Mo5).

The cells comprising this nucleus consist mainly of small to medium sized neurones and, spread out amongst them, a few giant cells located mainly in the medial portion of the nucleus. Distinguishing the giant cells within PnC from those in RGC is difficult. However there are subtle difference in the Nissl staining characteristics and position of the nucleus of the respective cell types which enables a cytoarchitectural distinction to be made. In general Nissl substance within PNc is evenly distributed throughout the cytoplasm showing a tendency toward smaller units of Nissl substance. A further distinction is the eccentric placement of the nucleus as compared to neurones in RGC.

Nucleus reticularis pontis oralis (PnO)

This nucleus occupies the most rostral part of the pontine tegmentum. It extends rostrally from the rostral pole of the PnC which is approximately from the middle third of the motor nucleus of the trigeminal nerve (Mo5), to the level of the trochlear nucleus. The raphe nuclei form its medial border, while lateraly lie the paralemniscal nuclei. The PnO abuts ventrally on the reticulotegmental nucleus of the pons (RtTg) and the pontine nuclei (Pn).

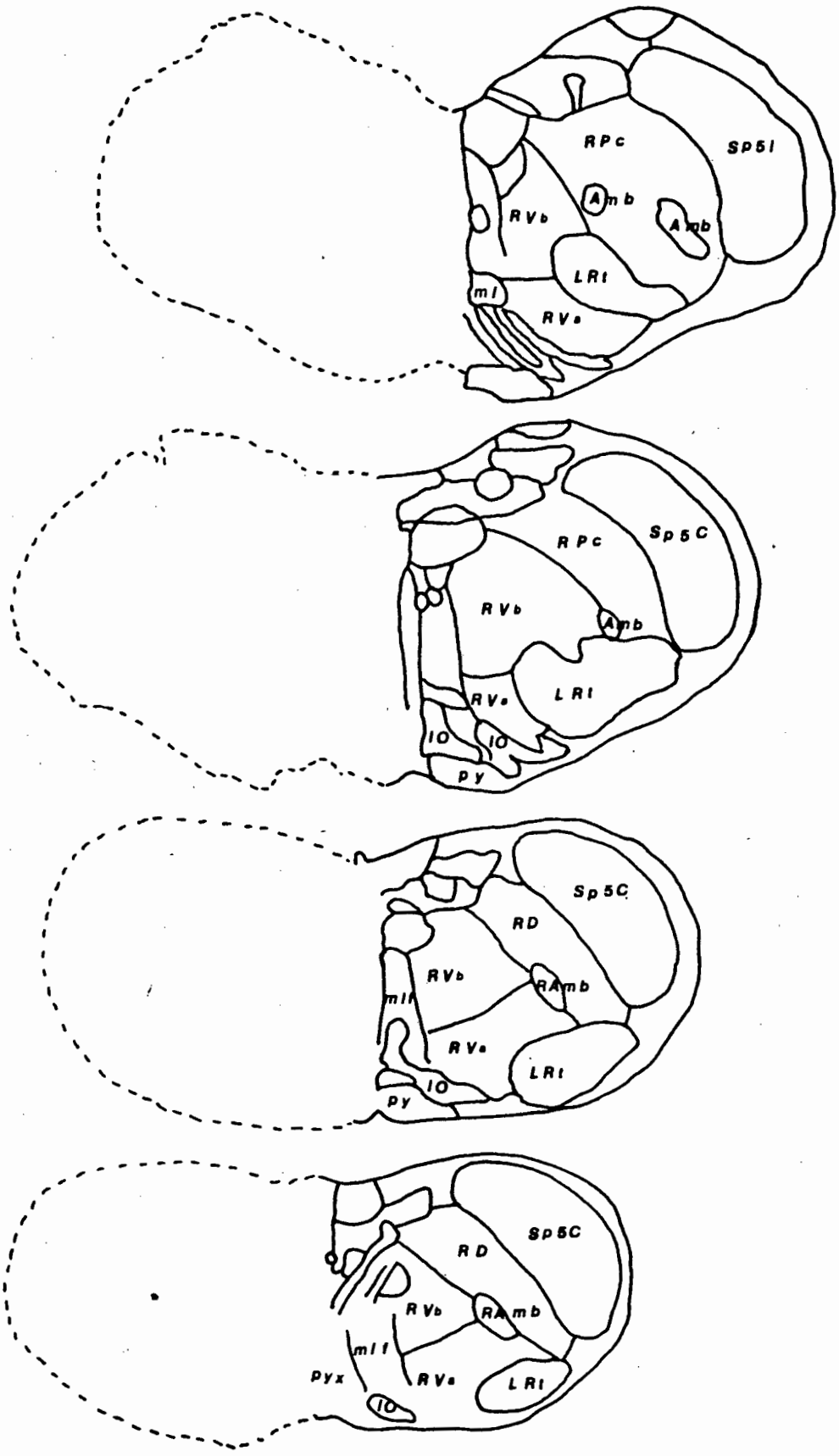
The bulk of cells contained in PnO are small, with only a few medium sized cells. However there are also some giant cells with distinguishing features such as peripheral Nissl substance distributed amongst the small cells which also helps to identify this nucleus (Valverde, 1961; Taber, 1961).

General observations

If the above description of nucleus reticularis gigantocellularis is singled out, it serves to demonstrate, firstly, the conflicting opinions held by various authors with regard to the exact anatomical location and boundaries of this nucleus and, secondly, highlights the general case of the ill-defined nature of nuclear boundaries within the reticular formation.

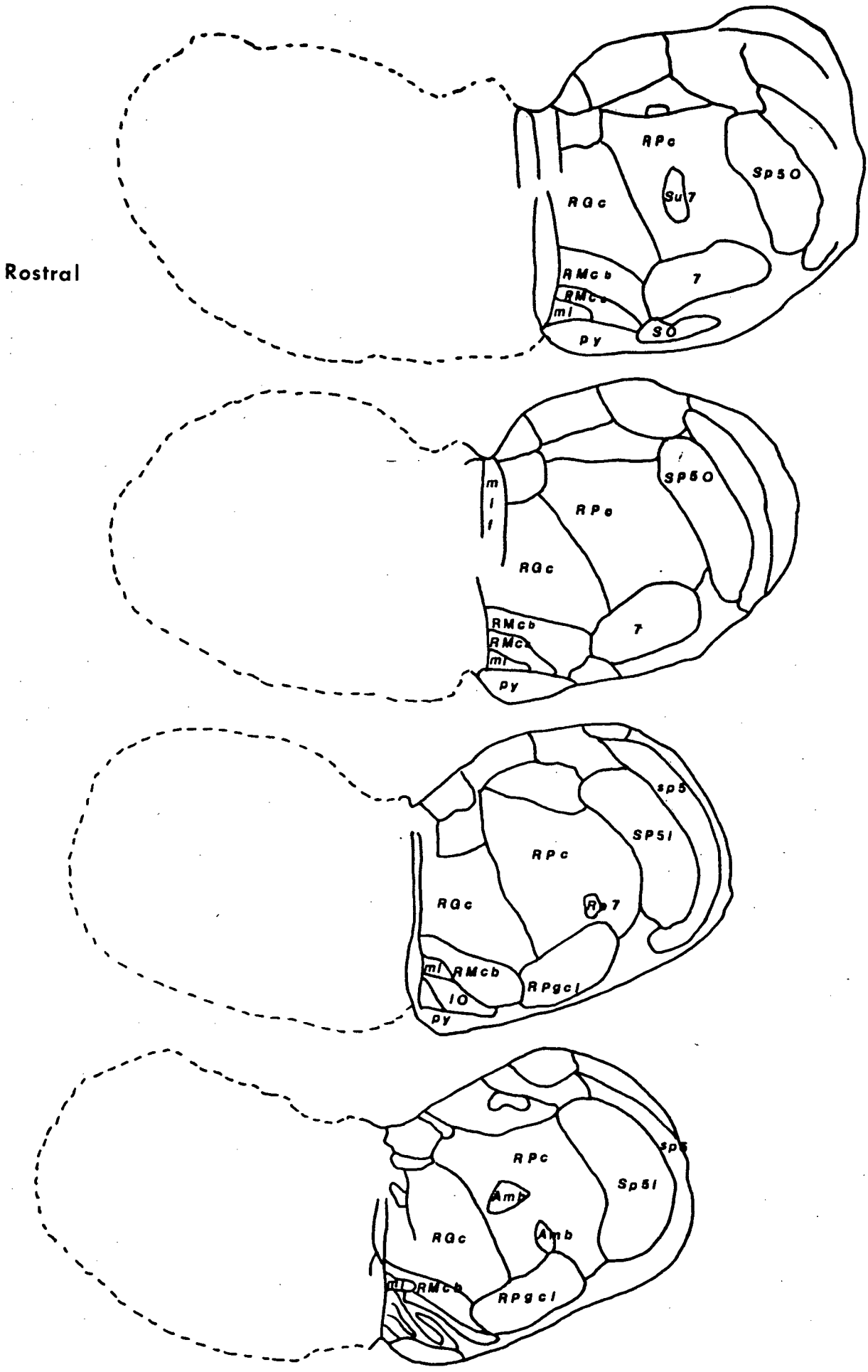
As the reticular formation is both cytoarchitecturally and chemically heterogenous and as more data emerge showing regional differences in connectivity, the criteria for delineating boundaries between nuclei become more complex. If all of these facts are taken into consideration and general consensus can be reached then hopefully a more meaningful system of nomenclature will evolve.

Fig 1.1.1



Caudal

Fig 1.1.1


Rostral
RPc
Sp5O
RGc
Su7
7
RMcb
RMcr
ml
py
SO
mlf
Sp5O
RPc
RGc
7
RMcb
RMcr
ml
py
sp5
SP5I
RPc
RGc
R 7
ml
RMcb
RPgcl
IO
py
sp5
RPc
Sp5I
Amb
RGc
Amb
ml
RMcb
RPgcl

(b) Ascending and Descending projections of the RGC

The neurones of the medial reticular formation are known to project widely throughout the nervous system. There are substantial projections to the cerebellum, diencephalon and motor nuclei of the cranial nerves. These neurones are located laterally and medially to the hypoglossal nerve, as well as rostral pons (Martin and Walzer, 1984). Autoradiographic studies in cats have demonstrated crossed reticulo-reticular connections in the medulla, pons and mesencephalon. Neurones of the reticular formation influence many nuclei in the brain stem via axon collaterals (Scheibel and Scheibel, 1958), but precise localization of these target structures have only recently been investigated. Sotgui and Margnelli, (1976) have identified PMRF neurones projecting directly into the dorsal column nuclei (i.e. cranial sensory and motor nuclei) by electrophysiological means. A large area of the medial and dorsal medullary areas contribute axons to the medial forebrain bundle (Vertes, 1980, 1981, 1982), and amongst these areas RGC also contributes substantially to a bundle of fibers in the midbrain which divides into a dorsal and a ventral tract. Vertes et al., (1984) using autoradiographic techniques describe the dorsal branch as projecting to the dorsal thalamic area, and the ventral one to the hypothalamus, zona incerta and Fields of Forel. More recently by means of small amounts of Phaseolus Vulgaris leucoagglutinin (PHA-L) injected into the cat RGC, Matsuyama et al., (1988) have identified terminations from RGC. Deposition sites were within the area encompassed by the RGC, i.e. all of the reticular formation dorsal to the rostral part of the inferior olive and labelling cells diffusely distributed over a relatively large area. PHA-L filled fibers arising in RGC terminated in a wide area of the pons within PNo, PNc and RGC as well as in the cranial motor nuclei in the brain stem, thus indicating extensive intrareticular connections. Other terminations were found in locus coeruleus and raphe nucleus. Diencephalic projections were seen projecting to non-specific and specific thalamic nuclei and hypothalamus. The projections to the mesencephalic area are mainly to the periaqueductal gray and the red nuclei. With the PHA-L tracing method, ascending projections were both ipsi and contralateral as compared to previous methods where projections were found to be predominantly ipsilateral. These findings confirm and extend the earlier autoradiographic studies of Martin and Walzer, (1984). It is of special interest to note that of mesencephalic structures receiving projection fibers from RGC, the substantia nigra pars reticulata is not mentioned. It is therefore apparent that this mesencephalic area does not receive a direct projection from RGC.

According to Pardey-Borrero and Gonzales-Vegas, (1980) their electrophysiological data indicates that reciprocal connections may exist between the SNr and the RGC. This implies that neurones of the RGC send their axons directly to the SNr. The projection patterns from the PHA-L deposition sites of Matsuyama et al's (1988) study do not provide an anatomical explanation for the findings of the Pardey-Borrero and Gonzales-Vegas study as no RGC fibers could be demonstrated projecting to SNr.

The rostral projections of RGC have still to be examined in greater detail and, as the above references attest, have only recently been examined systematically with more

powerful tract-tracing techniques. This stands in contrast to the caudal projections which have been extensively studied since the early description of reticulospinal projections by Torvik and Brodal (1957). The area generally recognised as giving rise to the reticulospinal tract occupies a medial position within the brain stem reticular formation. This corresponds to the area in Brodal's (1957) study which includes nucleus reticularis ventralis (RV), nucleus reticularis gigantocellularis (RGC), nucleus reticularis pontis caudalis (PNc) and nucleus reticularis pontis oralis (PNo). This region is classically regarded as the origin of the reticulospinal tracts which are involved in body movements (Torvik and Brodal, 1957). More than 50% of neurones of the pontine and medullary reticular formation send their axons into the spinal cord. The boundary zones between all these areas as decribed in the previous section are to a geater or lesser degree indistinct.

According to Brodal, (1957) the boundary between RGC and PNc is defined as the most caudal point at which fibers of the abducens nerve run through the RF. On the other hand, using more modern anatomical techniques combined with the classical methods, Andrezik and Beitz, (1985) put the rostral commencement of the PNc, and hence the division between RGC and PNc, at the caudal pole of the trapezoid body. These definitions overlap broadly, and further serve to illustrate the difficulty in defining boundaries within the reticular formation and hence the precise origins of fiber tracts.

Nevertheless it is generally accepted that the pontomedullary region of the reticular formation is the source of an extensive system of reticulospinal projections. These projections have been demonstrated both anatomically (Torvik and Brodal, 1957; Valverde, 1962; Petras, 1967) and by means of physiological studies (Magni and Willis, 1963; Peterson et al., 1974).

Early reports (Torvik and Brodal, 1957) claimed, on the basis of lesion studies, that there was no somatotopic projection of the reticulospinal projection. Although somatotopic organization of brain stem projections are not as marked as in, say, the striatonigral projection, the emerging picture of reticulospinal projection patterns to the spinal cord and other areas such as the cerebellum is rapidly changing. Lesion and silver impregnation studies in the cat conducted by Nyberg-Hansen, (1965) showed that reticulospinal fibers of pontine origin descended mainly in the ipsilateral ventral funiculus of the cords, whereas the medullary fibers descend bilaterally in the lateral funiculus to all levels of the cord. The electrophysiological studies of Peterson et al., (1975) have shown that the spinal projections of RGC and RPc display some degree of somatotopy. Fibers arising from RGC descend in the lateral funiculus which is in keeping with the results from the Nyberg-Hansen, (1965) study. But some fibers also course in the ventral funiculus (Basbaum et al., 1978).

Investigation of the effects on spinal motor neurones produced by reticular formation stimulation, Jankowska et al., (1968) and Wilson and Yoshida,(1969) have shown that the medial reticular formation is divided into a rostral excitatory and a caudal inhibitory zone. Peterson et al., (1974) have shown that reticulospinal neurones, identified by antidromic activation of cervical and lumbar spinal cord, located in the caudal part of RGC, had slower conduction velocities than axons of neurones in more rostral regions. This could mean that the two zones have different

physiological properties, and may go some way in explaining the properties of reticular inhibition of motoneurones.

The giant neurones of RGC contribute axons to the ventral and lateral funiculi. The projection is characterized by being bilateral but with an ipsilateral predominance (Martin et al., 1985; Zemlan and Pfaff, 1979). These authors have demonstrated that RGC axons project the length of the cord to the medial zone of laminae 8, 9 and 10 most commonly. However axons also innervate laminae 3 to 7 , intermediolateral area and the sacral parasympathetic nucleus. The fibers innervating the lumbar area of the cord appear to arise preferentially in the RV (Holstege and Kuypers 1982). Furthermore the fiber projections to laminae 9 and 10 are serotonergic and some of the neurones in RGC, RV and RPg have been shown to be serotonergic as well (Björklund and Skagerberg 1982). However not all the projection fibers from RGC are serotonergic as Johannessen et al (1981) have shown that fibers projecting through the dorsal part of the lateral funiculus are non-serotonergic.

In addition to the somatotopy of reticulospinal projections from the various pontomedullary nuclei, the axon termination sites are not randomly dispersed in the spinal gray matter. In cat (Nyberg-Hansen, 1965) pontine reticulospinal contacts were found more ventrally in laminae 8 and 7, while medullary fibers end more dorsally and chiefly in lamina 7. In a more recent study of projection sites and terminations of RGC there is further evidence that within a particular nucleus there may be somatotopic organization of its terminals at the target site (Martin et al., 1985).

The approach used by Kuypers and Maisky, (1975) to study the projections of cat brain stem cells was by use of HRP hemi-infiltrated into upper cervical or thoracic spinal segments of the spinal cord. They showed that all brain stem nuclei that were labelled retrogradely with HRP from cord deposition sites gave rise to the descending pathways which compared favourably with the results obtained from retrograde degeneration studies.

Projection of coritcoreticular, corticobulbar corticospinal fibers

The neurones of the RGC are well placed to receive fibers from many parts of the nervous sytem, as indeed they do. Fibers enter RGC from all areas of the brain stem. As these neurones are widely implicated in motor control, it is not suprising therefore that they receive a generous input from areas that regulate somatic motor activity such as the motor cortex (Valverde, 1962; Peterson et al., 1974).

The inclusion of this section has special relevance to the interpretation of data from the study on electrical stimulation of the substantia nigra pars reticulata (Section 1.3.6). This stems from the possible involvement of corticofugal fibers, coursing in the cerebral peduncle, which may be affected from stimulation sites within the SNr. The ventral portion of each peduncle, at about the level of the mesencephalon, is a broad compact crescentic structure lying immediately ventral to the SNr, and so is at risk of being stimulated by placement of an electrode at the ventral surface of the SNr. In any discussion concerning cortical input to the reticular formation the use of the terms "corticobulbar" and "pyramidal" interchangeably might cause confusion. It is

thus best to define these terms. Fibers within the pyramidal tract course through the medullary pyramids with the major component being made up of corticospinal fibers originating in the cortex and projecting to the spinal cord. The corticospinal tract of the rat originates in layer V of the sensorimotor cortex, decussates in the medullary pyramids and then travels via the contralateral ventral region of the dorsal funiculus for the whole length of the spinal cord (Brown, 1971; Ullan and Artieda, 1981; Leong, 1983).

Other fibers that constitute the pyramidal tract but exit at the medullary pyramids and pass to the cranial motor nerves and other bulbar nuclei are often referred to as corticobulbar fibers (Brodal, 1981b). The other term frequently used in the literature with regard to cortical input to the reticular formation is "corticoreticular connections". These fibers arise in the sensorimotor cortex, descend via the internal capsule and the cerebral peduncle and distribute bilaterally to two distinct target areas in the pontomedullary reticular formation which are known sources of reticulospinal fibers (Brodal, 1981b). Antidromic stimulation studies conducted by Peterson et al., (1974) and Pilyavsky, (1975) demonstrated that reticulospinal neurones can be monosynaptically excited from the cerebral cortex.

Therefore there are two major routes whereby neurones in the cerebral cortex can affect the activity of cells within the reticular formation: the pyramidal tract and the cerebral peduncle. It was previously assumed that cells giving rise to activation of the pontine neurones i.e. corticopontine cells were the same as those giving rise to the corticospinal fibers. This does not appear to be the case since in man the number of fibers in the cerebral peduncle is much greater than that of the corticospinal fibers in the pyramids. (Brodal, 1981a).

Other early experimental evidence for the trajectory of corticobulbar fiber (Mettler, 1939; Verhaart, 1948) showed that "direct corticobulbar fibers" originating in the inferior frontal gyrus pass through the anterior crus of the internal capsule and thence to the innermost part of the basis pedunculi to the medial and dorsomedial pontine nuclei. The existence of this tract is firmly established in monkeys. However this terminology dates back some 40 years or more and in all probability refers to the corticoreticular tract (see Brodal, 1981a).

The lower brain stem also has extensive connection with the cortex via the pyramidal tract. It was recognized at the beginning of the 18th Century that the pyramidal tract was a decussating fiber bundle en route to the spinal cord carrying fibers from the cerebral cortex Kuypers, (1958) using the Marchi method for the demonstration of sites of termination of degenerating fibers, showed extensive distribution of fibers to the pons and lower brain stem in cats. The degenerating fibers resulted from lesions placed in the motor cortex. The pyramidal tract contained degenerating fibers which were distributed throughout the pontine and medullary tegmentum. Although there were regional differences in density of distribution as well as laterality, there was good representation of bilateral projections. However there was a predominance of contralateral projections especially in the caudal regions. The lateral reticular nucleus, especially the magnocellular part, as well as the spinal trigeminal nucleus received a large number of degenerating fibers. Other projection sites were less well described possibly because brain stem nuclei were not as fully described at the time these early studies were performed.

The corticopontine projection was also studied in the rat (Wise and Jones, 1977) and in the monkey (Jones and Wise, 1977) and found to originate throughout the cerebrum mainly from layer V pyramids in the primary somatosensory cortex. The pontine nuclei receive the majority of their afferents from the cerebral cortex but the fibers enter the pons via a different route. The fibers descend in the internal capsule and the cerebral peduncle and terminate in the ipsilateral pons (Nyby and Jansen, 1951). Fibers from the occipital and temporal lobes were found to end mainly in the lateral pontine nuclei, each area showing considerable overlap with other projection areas in the lateral pons. This may be due in part to collateral projections of corticopontine fibers. For a more comprehensive review of other cortical projection fibers see Brodal, (1981a).

Peterson et al., (1974) and Magni and Willis, (1964) have shown that many reticular neurones receive short latency excitation from the cerebral cortex. However the wide dispersion of EPSP latencies observed when either the cortex or the cerebral peduncle was stimulated probably signifies that a number of reticular neurones receive only indirect cortical excitation. The studies of Pilyavsky, (1975) show that many brain stem RF neurones are directly activated from cortical sites. These findings may be accounted for in terms of the stimulation sites that were employed, thereby preferentially affecting neurones giving rise to fibers which may course either in the pyramidal tract or in the corticoreticular tract. In cat, cortical neurones projecting to the medial reticular formation have their origin mainly in area 6 (Berrevoets and Kuypers, 1975). In the pyramidal tract many neurones project to the reticular formation area as collateral branches from relatively slowly conducting pyramidal tract neurones (Jinnai, 1984). Part of the cortical input may be multisynaptic thereby permitting other systems to modulate cortical excitation of reticular neurones.

Many fiber systems project to the PMRF besides cortical fibers. A major interpretive difficulty is caused by the fibers en passant. McCarley et al., (1987), for example, were unable to differentiate between activation of mesencephalic reticular formation sites of stimulation and fibers of passage. Edwards and de Olmos, (1976) found a dense ipsilateral projection from nucleus cuneiformis to the giant cell tegmental field. PMRF neurones are antidromically excited by stimulation of the MLF (Remmel et al., 1978; McCarley et al., 1987; Shammah-Lagnado et al., 1987). HRP studies show a very dense projection from MRF to the PMRF with a strong ipsilateral predominance estimated as 3:1. It is estimated that about 15% of all PMRF afferents arise from neurones in the MRF.

Tecto-reticular connections

In addition to the generally accepted somatomotor function of the reticular formation, reticulospinal neurones have been shown to play a role in a major class of movements related to orientating of the head and body (Straschill and Schick, 1977). Co-ordinated head and eye movements can be elicited by stimulation of the deep and intermediate layers of the superior colliculus by applying the stimulus to the layers that show bursts of activity prior to rapid orientating movements (Roucoux et al., 1980). Anatomical studies in the cat have revealed a crossed tecto-reticular pathway

originating from neurones in the dorsal tectum and coursing through deeper layers en route to the contralateral side (Kawamura et al., 1974). The superior colliculus sends an extensive crossed fiber projection to the PMRF which activates a large number of reticulospinal neurones. Stimuli applied to either the ipsilateral or contralateral superior colliculus, may evoke monosynaptic EPSPs in reticular neurones lying medially (Udo and Mano, 1970).

There may be several pathways whereby the colliculus influences head and body movements (Altman and Carpenter, 1961; Kawamura et al., 1974; Edwards and Henkel, 1978). There is good evidence that afferent fibers constituting this tecto-reticulospinal pathway are the most significant route for activation of the neck motoneurones. Anderson et al., (1971) demonstrated that the effects of tectoreticular tract stimulation were unaffected after transection of tectospinal and interstitiospinal tracts at the level of the obex. Ipsi- and contralateral projections have been shown to originate from various dorso-ventral levels of the mesencephalic tectum. Peterson et al., (1974) observed maximal firing of reticular neurones that corresponded to a zone of dense terminations of tectal fibers identified by Kawamura et al., (1974).

In contrast to the crossed tectal pathway, Kawamura et al., (1974) described terminations in nucleus reticularis pontis oralis from ipsilateral tectum, but no terminations on neurones in pontis caudalis or gigantocellularis. Although Peterson et al., (1974) observed monosynaptic EPSPs in these regions following ipsilateral tectal stimulation, these effects could have been due to stimulation of a pathway running from the sub-tectal tegmentum to the ipsilateral ponto-medullary reticular formation.

In addition to tectal neurones projecting to the medial reticular formation, another group of nuclei involved in the control of somatic musculature are the vestibular nuclei. Stereotyped movement of the eyes, head and body, due to electrical or natural stimulation of the semi-circular canals, may reach spinal motor neurones via interstitiospinal neurones. A connection exists between vestibular nucleus neurones, (which carry signals from vertical semi-circular canals) and the interstitial nucleus of Cajal (Fukushima et al., 1980) or indirectly via a vestibulo-reticulospinal pathway (Ladpli and Brodal, 1968; Peterson and Abzug, 1975). Both the latter authors have provided evidence for anatomical connections and physiological responses as between the vestibular nuclei and the medial pontomedullary reticular formation.

Overall, several lines of evidence demonstrate the involvement of medial reticulospinal neurones in both orientating and vestibular reflexes. Furthermore, on the basis of experimental evidence indicating tectal and vestibular responses in the same reticulospinal population, it has been suggested that PMRF neurones may function as a "common premotor element" in both tecto-reticulospinal and vestibulo-reticulospinal pathways (Peterson and Fukushima, 1982).

Cerebellar connections

The lateral reticular formation (LRN) is one of the few areas in the brain stem which displays some somatotopic organization of its efferent output. There is a somatotopic projection from the LRN to the cerebellum that is in register with the segmental

pattern of termination of the spinal afferents to the LRN. The inclusion of this RF nucleus in the present review, besides illustrating the complex role of the RF in motor output, serves to illustrate as well, how application of precise tract-tracing studies has prompted revision of previously held ideas concerning the organization of RF nuclei.

The lateral reticular nucleus, nucleus reticularis tegmenti pontis (NRTP) as well as the paramedian nucleus is part of the system described as the "precerebellar nuclei". This group of nuclei send most of their efferent projections to the cerebellum. Another reticular nucleus, the nucleus reticularis tegmenti pontis (NRTP) has been found to be related to cerebro-ponto-cerebellar pathways and, more recently, to eye movements. This nucleus has been subdivided into two regions based on the respective density of projections to the cerebellum derived from retrograde HRP studies (Torigoe et al., 1986a).

The two cytoarchitectonically distinct portions consist of a central subdivision (NRTPc) and a pericentral area (NRTPp). The NRTPc is located dorsal to the medial lemniscus and pyramidal tracts over the caudal 2/3 of the pons. It extends caudodorsally to the region just rostral and ventral to the abducens nerve. The NRTPp lies adjacent to the lateral margins of the NRTPc rostrally and ventral to the caudal portions of the NRTPc.

By means of HRP mapping Corvaja et al., (1977) have shown that the projection from the lateral reticular nucleus is largely bilateral with an ipsilateral predominance. The superficial layers called the parvicellular region are supplied by afferents from the lumbar levels, whereas the more medial area, the magnocellular portion, receives afferents mainly from the cervical cord. The topographic pattern of projection is to the anterior and paramedian lobes of the cerebellum which are areas representing both forelimb and hindlimb, but with considerable overlapping. However, there is a complex inter-relationship between the termination pattern of spinal afferents in the LRN and its projection to the cerebellum that explain stimuli from the fore or hindlimbs. The cerebral cortical afferents to the NRTP examined by small ionophoretic injections of HRP into its two subdivisions showed that pyramidal cells, exclusively of layer V, were HRP filled in 3 major cortical areas: (1) ipsilateral prefrontal cortex; (2) ipsilateral motor and somatosensory cortices; and (3) the bilateral cingular cortex. Torigoe et al., (1986b) have amply demonstrated that cortical afferents to NRTP are topographically organized in the rat.

Precise somatotopic organization of the cortico-NRTP-cerebellar projection has also been observed in the monkey (Brodal, 1980) and the cat (Hartmann-von Monakow et al., 1981). It has been suggested that NRTP is the relay for connecting phylogenetically similar areas of the cerebral cortex and the cerebellum. Its anatomical connections with a large number of afferents especially to the medial regions of the NRTP arise from visual or eye-movement-related nuclei. There are many examples supporting the view that NRTP neurones are involved in a number of aspects of oculomotor function (Torigoe et al., 1986a).

On the basis of the above experimental evidence it is clear that precise tract-tracing and electrophysiological techniques have provided further insight into the arrangement of reticular fibers and the distribution of their terminations in target areas. The somatotopic arrangememt of a number of reticular nuclei stands in

contrast to the originally held view that the reticulospinal pathway did not display any somatotopic organization.

In turn RGC also receives fibers from the cerebellum. In the monkey RGC receives fibers particularly from the contralateral cerebellar fastigial nucleus (Asanuma et al., 1983). Besides this projection the medial RF also receives fibers from the dentate and deep cerebellar nuclei (Bantli and Bloedel, 1975). Stimulation of the fastigial nucleus gives rise to monosynaptic excitation of neurones in the contralateral medial reticular formation (Ito et al., 1970). The cerebellum is thought to play an important role in controlling the activity of reticulopspinal neurones during locomotion and active tilt.

Collateralization of PMRF neurones

Reticular formation neurones have been shown to receive input from a number of sources; it is thus highly likely that these same neurones also innervate more than one target. In order to achieve this it would mean that axons arising from these neurones have special features such as long collateral branches. The term 'collateral' originally applied to those branches of the axon that ramified close to the soma, whereas the main axon projected out of the immediate vicinity. It has become clear with the advent of intracellular dye techniques that many axons in various parts of the brain bifurcate and then project to different areas of the brain. Both the aforementioned branching patterns of axons have been termed collaterals. Some authors have chosen to make a distinction between axons ramifying close to the soma and those that project out of the immediate vicinity, by the terms "local axon collaterals" and"projection axons" respectively (White, 1989).

For the purposes of this discussion the term "collateral" will apply to all branches of the axon whether they occur close to the soma or at a distance. It was recognized by the early anatomists that neurones within the reticular formation possessed long axonal projections and, whether projecting rostrally or caudally, gave rise to collateral branches. The long ascending fibers have been shown to arise mainly in the medial 2/3's of the reticular formation particularly from caudal RPc, caudal RGC and rostral RV. These neurones send projections to the mesencephalic RF, thalamus and cerebellum. While the long descending fibers arise chiefly from neurones in RGC, rostral RV and medial Lateral Reticular nucleus. These contribute fibers bilaterally mainly to the lateral funiculus. Collaterals also affect rostral structures and/or structures in brain stem. Collaterals of RGC neurones project to the dorsal column nuclei (Sotgui and Marginelli, 1976).

A similar pattern of collateralization is present in the corticospinal system (Shinoda and Yamaguchi, 1978). Electrophysiological evidence in cats indicates that corticospinal axons provide collaterals to different segmental levels of the spinal cord and that provision of these branches to the various segments can be correlated with a reduction in the conduction velocity of the stem axon. This is correlated with the diameter of the axon, insofar as the main axonal branches have large diameters and travel much longer distances than their collaterals. More recent techniques (Huisman et al., 1981) employing multiple fluorescent retrograde tracers have shown that a

number of pontomedullary neurones project the length of the spinal cord giving off collaterals to more than one level. This is in agreement with earlier lesion and Golgi studies respectively (Scheibel and Scheibel, 1958).

The pattern of reticulospinal collateralization to the spinal cord was also investigated by means of the double labelling technique (Martin and Walzer, 1984; Martin et al., 1985). These studies have provided further anatomical evidence for the collateralization of the reticulospinal system and confirm some of the earlier observations concerning the projection pattern of these cells. As the neurones of the RGC have been shown to project to the spinal cord, the diencephalon and cerebellum, Walzer and Martin, (1984) applied a double labelling technique to known target sites in order to test whether the single cells project to more than one target. Their data collectively showed that only some of the neurones that project to the spinal cord project to either the cerebellum or the diencephalon. It does not appear that single reticulospinal neurones target spinal cord, cerebellum and the diencephalon simultaneously (Bentivoglio and Molinari,1984; Walzer and Martin, 1984). Some PMRF neurones may directly affect their own activity via collaterals projecting back to their own soma or dendrites or via collaterals of RSTi. By comparison, this pattern of branching is particularly evident in the primary visual cortex of a number of species (White, 1984) besides also having long projection axons. The frequency with which this occurs in the brain stem RF is unknown.

What is the functional significance of this connectional heterogeneity? The answer to this question is still not clear but it is commonly suspected that the long collaterals may serve to integrate function and/or activate widely separated areas. Indeed some neurones provide collaterals to widely divergent areas. But many reticular neurones have much more restricted fields. Local axon collaters, i.e. those ramifying within the vicinity of the soma, may be involved with certain aspects of neuronal processing, eg. in cortex local axon collaters from pyramidal cells may be involved in the intracortical shaping of receptive fields. The general morphology of the efferent connections of the medial pontomedullary reticular formation neurones thus support the idea that influences are closely correlated in both a rostral and caudal direction. The frequency with which this occurs in the brain stem RF is unknown.

(c) Transmitter properties of pontomedullary reticular formation neurones

The elucidation of pontomedullary neurotransmitters is linked closely to the history of spinal cord neurotransmitters insofar as the spinal monoamine transmitter system was shown to have been derived from the bulbospinal projection system. These initial conclusions were derived from spinal cord transection studies where it was demonstrated that the amines, serotonin and noradrenalin, collected proximally to the transection with distal depletion (Carlsson et al., 1963; Magnusson and Rosengren, 1963).

With the advent of histofluorescent, immunofluorescent and immunocytochemical techniques for identifying the transmitter properties of cells and terminals in the central nervous system as a whole, and the reticular formation in particular, a vast amount of information pertaining to transmitters has accrued during the last two

decades. However, a major problem of interpreting chemical neuroanatomical data has arisen because studies that report the locations of transmitter specified cells within the reticular formation, in the majority of cases, do not conform to recognised cytoarchitectonic subunits originally classified by means of Golgi, Nissl and silver staining methods.

The monoamine transmitters

The foundations to the investigation of the monoamine system were laid by Carlsson et al., (1963) and Magnussen and Rosengren, (1963) and expanded by the subsequent histofluorescent studies of Dahlström and Fuxe, (1965). Their studies showed cell bodies localised primarily in the serotonergic medullary cell groups B1, B2 and B3. The designation "B" refers to the 5-HT containing neurone population only and because these neurones do not correspond exactly to classical neuroanatomical areas they have been arbitrarily numbered, e.g. the B3 cell group corresponds to cells lying within nucleus raphe magnus (NRM) and nucleus reticularis magnocellularis (RMc). Overall, this scheme therefore includes nucleus raphe magnus, obscurus and pallidus respectively, all of which are situated in the medullary reticular formation. The serotonergic cells which project primarily to the spinal cord are thus found in the caudal pons and the medulla.

The lateral tegmental system (LTS) is composed of a widely dispersed system of cells which can be divided into a medullary and pontine part respectively. In Dahlström and Fuxe's terminology the former consists of groups A1 and A3 and the latter part of groups A5 and A7. The noradrenergic (NA) cell groups, designated A1 and A2 respectively have axons that descend in the ventral and lateral funiculi and innervate the spinal cord at all levels. The neurones of RGC are known to contribute axons to the ventral and lateral funiculi with a strong ipsilateral predominance (Martin et al., 1985), suggesting that at least some of the neurones within RGC are adrenergic.

Another major source of spinal noradrenergic (NA) afferents arises in the pontine NA cell groups (locus coeruleus , nucleus subcoeruleus and the lateral pontine A5 cell group (Satoh et al., 1977; Lowey et al., 1979). Ross et al., (1981) have demonstrated that noradrenergic fibers arise mainly in the pons and Westlund et al., (1981) provide evidence for the medullary origin of adrenergic fibers. Neurones projecting to the spinal cord which are situated in the ventrolateral medulla, include adrenalin containing neurones belonging to the C1 group. These neurones are involved in vasomotor activity and, together with the serotonin neurones in the lateral B1 and B3 groups, form part of the bulbospinal group of modulating vasomotor activity at the spinal level (Howe, 1985). These are intimately involved in the control of blood pressure. Part also of this system are the cells of the serotonin projections from the medullary raphe, medial B1 and B2 cell groups.

Serotonergic neurones are also found in the medullary area of the brain stem (Björklund and Skagerberg, 1982). Neurones within the RGC, RV as well as in PGi have been shown to contain serotonin (Bowker et al., 1981a; Bowker et al., 1981b). As the RGC is implicated in balance and postural control these cells may be involved in serotonergic control of some motor behaviours.

Acetylcholine

Acetylcholine has long been known to be a central transmitter (Fonnum, 1975). Although there has been a continual reinvestigation of cholinergic neurones in the CNS there is still no direct method of in situ localization of acetylcholine. This has necessitated the identification of cholinergic neurones by use of techniques for the detection of other markers such as acetylcholinesterase (AChE), its degrading enzyme and choline acetyltransferase (ChAT), its synthesisizing enzyme. A major disadvantage in the use of the AChE as a marker for cholinergic neurones is that the enzyme is not limited to cholinergic neurones (Koelle, 1963 and Butcher and Woolf, 1984). If the intention is to demonstrate the presence of cholinergic neurones then this technique has an obvious shortcomining. However, insofar as discerning various CNS nuclei, AChE has considerable advantages over conventional Nissl staining techniques for mapping neuronal structures (Paxinos and Watson, 1982).

The most reliable marker for acetylcholine currently favoured is one of its biosynthetic enzymes, namely, choline acetlytransferase (ChAT), which is detectable by immunocytochemical methods.

This enzyme is associated with cholinergic neurones as opposed to AChE which is found in both cholinergic and cholinoceptive neurones (Hoover et al., 1978). However, the ChAT method also has certain disadvantages in that there is no discrimination of the structures that contain the enzyme i.e. whether somata, fibers or terminals. However the major advantage of this method lies in the detection of actual cholinergic neurones.

Within the pontomedullary reticular formation, some cell groups tested with various transmitter substances and/or their pharmacological agonists, show responsiveness to the ionophoretic application of these agonists. There have been few systematic studies aimed at directly characterizing the neurotransmitters affecting medial pontomedullary reticular formation neurones. More recently, however, Green and Carpenter, (1985) have attempted to identifiy the neurotransmitters affecting a small population of neurones in the para-abuducens reticular formation (PARF). The responsiveness of a neurone to application of a neurotransmitter does not necessarily imply its use as an intrinsic neurotransmitter. To illustrate this point Bradley et al., (1964) showed that a large majority of pontomedullary reticular formation neurones are cholinoceptive, i.e. responsive to acetylcholine, but it is not known what fraction of cells in the PMRF are actually cholinergic.

The presence of serotonin and norepinephrine terminals has also been demonstrated throughout the pontine medial reticular formation by histofluorescence methods (Hösli et al., 1970; Boakes et al., 1971). It is thus not suprising to find neurones within the medial reticular formation that are serotonoceptive and cholinoceptive.

Furthermore when analogues of acetylcholine were tested, namely nicotinic and or muscarinic agonists, the cells were responsive to both of these classes of agonist suggesting that both muscarinic and nicotinic receptors were present in the brain stem. However the locations of these receptors were not specified by Bradley et al., (1964); Bradley et al., (1966) and Bradley and Dray, (1972).

The transmitter actions on pontine neurones in the cat have been studied more recently by Green and Carpenter, (1985). Their study was limited to neurones in the

caudal third of the pons, immediately ventral to the abducens nerve (V1), termed the para-abducens reticular formation (PARF), which are thought to behave as generators of desynchronized sleep. Although a proportion of their cells could be driven antidromically from the spinal cord and are therefore broadly classified as reticulospinal cells, the population of cells in their study is not the same as the one in this study. However all the PARF cells studied were (a) activated by glutamate, (b) acted upon by both acetylcholine and serotonin (showing either fast inhibition , slow and long lasting excitation, or a biphasic combination of excitation-inhibition) (c) fast inhibitory responses to GABA, glycine and norepinephrine.

Where is the source of this cholinergic innervation? Since the locomotor studies of Shik and Orlovsky, (1976) have shown that the pedunculopontine tegmental nucleus (PPTg) projects to the reticulospinal cells in the medial brain stem region, a possible source of cholinergic fibers resides here. Butcher and Woolf, (1984) have shown that there is intense AChE staining in neurones of the PPTg. PPTg neurones have also been shown to be ChAT positive (Sofroniew et al., 1985). In the pons and the medulla there are many ChAT-positive neurones in various reticular nuclei, several ventral pontine nuclei and the neurones of origin of the cranial nerves (Sofroniew et al., 1985). According to their maps of ChAT-positive perikarya the medial pontomedullary reticular formation is sparsely populated or devoid of this type of neurone. The available evidence is thus not overwhelmingly in support of the presence of acetylcholine as the major transmitter of PMRF neurones.

Gamma-amino butyric acid (GABA)

GABA has been shown to be present in some brain stem reticular formation nuclei, and although Mugnaini and Oertel, (1985) have demonstrated GAD positivity within the reticular formation, their neurochemical maps indicate that the distribution of cells containing GAD is generally sparse. In support of their data, Placheta and Karobath, (1979) have shown that the lowest density of tritiated GABA binding sites occur in the pons and medulla as well as in the spinal cord. Although these authors have provided clear evidence for a regional distribution of GABA receptor type as well as a heterogeneity of GABA receptors, the regional distribution of benzodiazepine (BZ) receptors do not always correlate positively with the intensity of tritiated GABA binding sites. However the BZ-binding sites in the pons and medulla are also low. Taken together, the overall evidence suggests that GABA activity in the brain stem is generally low, and therefore by simple inspection noticeably different from areas of high GABA activity such as the substantia nigra and cerebellar cortex.

Mugnaini and Oertel, (1985) have constructed a comprehensive map of rat brain areas containing GAD positive cell bodies and axon terminals. Glutamic acid decarboxylase (GAD) is the biosynthetic enzyme for GABA, which is produced by means of decarboxylation of glutamic acid by GAD. This was used as a marker for evidence of GABAergic activity. The distribution GAD was graded according to a density score. Thus precise estimation of the proportion and relation of pre and postsynaptic GAD-immunoreactive components in a particular brain region was not

done in this study. GAD immunohistochemical staining does not always conform to known anatomical borders, as outlined by the age-old Nissl technique, in that in certain areas "constellations" of neurones are seen which do not coincide with generally accepted nuclear borders. It was suggested by Mugnaini and Oertel, (1985) that new subdivisions of certain brain areas may be proposed; however they cautiously point out that more precise maps of GABAergic neurones need to be established by combining a number of methods, such as neurotransmitter, neuropeptide and receptor labelling and tract-tracing procedures. Such an operation, although arduous, is essential for re-evalution of the present classification system. It has to be borne in mind however, that the concentration of GAD in cell bodies is low in comparison to the levels in synaptic endings. This factor must therefore be taken into consideration in interpretation of the data.

Their enormous study has revealed that some brain areas contain proportionally high levels of GABAergic neurones and/or axon terminal density, e.g. cerebellar cortex, olfactory bulb and thalamus. According to their scheme, RGC contains between 5 and 15% GAD-positive cells of all the neurones in this nucleus. The respective density of GAD-positive axon terminals is similarly low, being categorized as "few and very few". Nucleus reticularis medullae pars ventralis (RV) is similarly low in GAD activity. Whereas nucleus reticularis paragigantocellularis (PGi) has a large amount of GABAergic cells and a "medium" amount of GABAergic terminals. As a comparison, GABAergic neurones in the substantia nigra pars reticulata are of the order of 90% and the density of GABAergic terminals is very high.

Only a few discrete regions of the pontomedullary reticular formation are rich in GABAergic neurones, the nucleus reticularis paragigantocellularis (PG), being the most strongly GAD-immunoreactive. There are other high density GAD-immunopositive areas of the RF but these are not of significance to this study and thus will be omitted from the discussion. Of particular relevance to this study is the general paucity of strongly GAD-immunoreactive nuclei within this area. Areas within the reticular formation cited specifically as being "poor" in GAD-immunoreactive neurones are:-

N.reticularis medullaris pars dorsalis and ventralis; N.reticularis gigantocellularis (RGC); N.reticularis paramedianus; N.reticularis parvocellularis (PCRt); N.reticularis lateralis (NRL); N.reticularis pontis caudalis (RPC); N.reticularis tegmenti pontis.

Areas also significantly poor in axon terminals are RGC, NRL and RPC. Thus on the basis of GAD-immunoreactivity Mugnaini and Oertel, (1985) have shown that certain brain stem nuclei in the rat are remarkably poor in both GABAergic neurones and terminals.

The traditional view considered GABA a transmitter of inhibitory interneurones, and that GABAergic neurones were mainly functional as local circuit neurones. GABAergic projection neurones have been known for some time, such as the striato-nigral neurones (Kim et al., 1971; Bunney and Aghajanian, 1976) and cerebellar Purkinje cells (Ito and Yoshida, 1964; Saito et al., 1974). These were thought to be the exceptions rather than the rule. However, more recently further types of GABAergic projection neurones have been identified, e.g. in the hippocampus, neurones in the dentate gyrus (Seress and Ribak, 1983) and neurones within the basal

ganglia amygdala complex (Fonnum et al., 1978; Dray and Oakley, 1978; Waddington and Cross, 1978; Ribak et al., 1981). More recently GABAergic projections neurones to the brain stem in the rat have been identified in the cerebello-olivary projection in the rat (Nelson et al., 1984) and the rabbit (Mugnaini et al.,1982).

There are many other GAD-immunoreactive areas outlined in the Mugnaini and Oertel, (1985) study, as well as areas that potentially contain GABAergic projection neurones. Due to the availability of a number of new labelling techniques this list is growing, attesting to the fact that GABAergic neurones are no longer thought of as exclusively local circuit neurones.

GABA as well as GAD are found in high concentration in axon terminals (Salganikoff and De Robertis, 1965). Their map of GAD-immunoreactive neurones demonstrates a tremendous variation in GAD-positive axon terminal density. Thus there are clearly demarcated areas of high and low density GAD-immunoreactive terminals. The reticular formation, specifically the RGC, nucleus reticularis lateralis and nucleus reticularis pontis caudalis, is listed in their study as brain stem areas poor in GABAergic axon terminals. Other areas listed as being poor in GAD-immunoreactive terminals include (a) many of the thalamic nuclei, and (b) certain strata of superior colliculus, to mention but two areas of relevance to this study.

Another means for demonstrating the presence of GABAergic neurones is via the histochemical detection of 4-aminobuyterate 2-0xoglutarate transaminase (GABA-T), an enzyme that specifically catabolizes the neurotransmitter GABA (Nagai et al., 1985). A procedure for its histochemical detection and thus localization of GABA containing neurones has been described previously by Nagai et al., (1983). The method involves systemic administration of the irreversible GABA-T inhibitor Gabaculine, and the detection some time afterwards of the newly synthesized GABA-T by histochemical means. These two studies provide further confirmation of the GAD-immunohistochemical procedure employed by Mugnaini and Oertel, (1985). Non-GABA neuronal groups shown in the latter study were negative for GABA-T staining; however, in addition, further correlations with known GABA neurones are provided in rat pons and medulla. Within the reticular formation, GABA-T positive cells were distributed throughout the dorsal tegmental area. Many cells were present in the nucleus reticularis pontis oralis, some in the pontis caudalis area, and a "rich" distribution in NGC (medium to large cells). In the ventral part of the medulla, the area corresponding to nucleus reticularis magnocellularis, larger GABA-T cells were present. The medulla reputedly contains a relatively high concentration of GABA. Although the distribution of GABA-T confirms that belief, it does not concur with the distribution of GAD in the brain stem, which as outlined above is shown to be sparse (Mugnaini and Oertel, 1985). It has to be borne in mind that GABA-T has a different sub-cellular localization from GAD. It is assocaited with glial cells and the postsynaptic process.

The GAD immunoreactive terminals show specific patterns, characterizing well defined regions, on the basis of their terminal size, mode of distribution and the specific relationship betweeen terminal fibers and target profiles. These patterns include, amongst others: (a) bands of terminals, e.g. retina; (b) axonal perisomatic arrays, e.g. cortical pyramidal cells and hippocampal pyramidal cells; (c) axonal

peridendritic arrays which are the most common type of GABAergic synapse in the CNS; and (d) dendritic peridendritic arrays, e.g. dendrodendritic contact on granule cells.

The data regarding the distribution of GABA in the brain stem is fragmentary; however a number of reports provide evidence for the existence of GAD immunoreactive elements in various areas of the reticular formation. An exception to the general picture is nucleus reticularis paragigantocellularis which showed strong GAD positivity.

In agreement with Mugnaini and Oertel's findings, Lovik, (1987) has shown that certain pools of functionally distinct neurones within the nucleus paragigantocellularis, concerned with antinociception and vasomotor activity, are regulated by a tonic inhibitory GABAergic influence. However the source of this GABAergic input is not specified and certainly there is no mention of GABAergic interneurones of the Golgi type II variety. Thus the strong GAD positivity observed by Mugnaini and Oertel is probably due to a substantial GABAergic input to the neurones of nucleus reticularis paragigantocellularis.

Border and Mihailoff, (1985) have shown that somata, axons and axon terminals in the basilar pontine nuclei (BPN) and nucleus reticularis tegmenti pontis (NRTP) are GAD-positive. The source of the GAD-positive terminals are unknown but appear to be afferent projections to BPN and NRTP. The GAD labelled neuronal somata in the BPN and NRTP comprise a population of GABAergic neurones. These neurones might give rise to some local axon collaterals and are therefore a possible secondary source of GAD-positive axons in the PMRF.

A few studies have positively identified other brain stem nuclei containing GABAergic neurones by means of highly specific immunocytochemical methods. Amongst these nuclei are the neurones of the basilar pontine gray (Thier and Koehler, 1987). It has also been reported that GABA plays a role in the regulation of blood pressure, depression of heart rate and depression of respiration (De Freudis, 1982).

Although the location of GABA's modulation of autonomic function is not precisely known, there is good evidence to suggest that the nucleus of the solitary tract is one of the locations in the medulla of GABAergic neurones. Maley and Newton, (1985) using the GABA-immunocytochemical technique, have shown that the rostral ventrolateral medulla and nucleus of the solitary tract, two brain stem regions involved in respiratory and cardiovascular control, utilize GABA as a neurotransmitter.

A recent study by Drower and Hammond, (1988) using microinjections of the GABAa receptor agonist, THIP, into the RMg and RGC pars α, indicate that neurones in these two nuclei, which are involved in the regulation of nociceptive threshold are subject to an inhibitory GABAergic input mediated by a GABAa receptor, which does not appear to be tonically active. It is highly unlikely that GABA may play a role in interneuronal interactions, as the presence of small Golgi type II interneurones which usually contain GABA have not been shown to be present in the rat brain stem (Scheibel and Scheibel, 1958; Leontovich and Zakova, 1963). However, Their and Koehler, (1987) have described small neurones with GABA-like immunoreactivity in the pontine gray of the monkey which conform to the general description of type II neurones in Golgi material. The function of these particular cells has not been

established but their presence in this area of the brain stem challenges the traditional view that these brain stem neurones function purely as a relay between cortex and cerebellum, and that possibly some processing of information occurs at the level of this relay station.

GABA has also been shown to be present in midbrain raphe nuclei (Nishikawa and Scatton, 1985) where it is has been shown to exert inhibitory control over central serotonergic neurones arising in the midbrain raphe nuclei.

It thus appears that there are very few systematic and conclusive reports on the presence of GABAergic neurones within the medial brain stem reticular formation. The majority of reports indicate that there are regional GABAergic inputs to this area but there is little evidence in support of large numbers of brain stem neurones having GABAergic properties. Although widely recognised as the major and ubiquitous inhibitory neurotransmitter in the CNS, the exact role of GABA inhibitory neurotransmitter in the brain stem reticular formation is uncertain.

Glycine

The other CNS amino acid inhibitory neurotransmitter, glycine, is usually associated with spinal cord mechanisms, being confined to regions of the cord where interactions between afferent and efferent fiber systems are maximal. It has also been shown to be present at the level of the pons and medulla (Curtis and Johnston, 1974). Its action is similar to that of GABA, in that glycine also activates a Cl- ionophore, but is distinguishable from GABA on the grounds of it being non-responsive to bicuculline. Similar to GABA its role in the pons and medulla has not been defined to date. Green and Carpenter, (1985) have however demonstrated that neurones in the reticular formation, in the ventral periabducens area are inhibited by glycine, suggesting that glycine receptors are preset on pontine neurones.

Classical neurotransmitters coexisting with peptides

One of the most important developments of recent years in neurobiology has been the recognition of the widespread distribution of neuroactive peptides in the nervous system. The nomenclature of these substances often reflects the organ in which it was first found, or the physiological action that was first studied (For review of details of early studies: Reichelt and Edminson, 1977). Serotonin is known to co-exist with several peptides namely substance P (SP), thyrotropin releasing hormone (TRH) and enkephalin. These peptides have been shown to be present in the medulla or medulla and pons (Lovik and Hunt, 1983; Johansson et al., 1981; Hunt and Lovik, 1982).

A substantial number of SP containing somata have also been found in the RGC and in the mesencephalic reticular formation, especially in the nucleus cuneiformis (Lungdahl et al., 1978). A few cholycystokinin-like (CCK) immunoreactive neurones in the nucleus reticularis medullae oblongata pars medialis and ventralis have been demonstrated by Vanderhaeghen et al., (1980).

Within RGC as well as in the raphe neuronal system, in addition to the classical transmitter serotonin, there is also evidence suggesting its co-existence with several peptides (Hökfelt et al., 1982). The 5-HT neurones in the lower brain stem, many of which project to the spinal cord, represent a group of neurones which are heterogeneous not only with regard to their anatomical locations but also on the basis of their content and type of peptide. Although it has been demonstrated that some peptides are usually associated with serotonin, not all of the peptides shown to be present in the reticular formation, are associated with serotonin. The functional significance of the presence of all these peptides is not yet clear.

The above evidence thus supports the idea that at least serotonin and possibly noradrenalin, in additon to a number of peptides, are present in the population of RGC. However it has also been shown that many of the neurones of RGC projecting through the dorsal part of the lateral funiculus are not serotonergic (Johannessen et al., 1981).

Whether separate serotonin and noradrenalin containing cell groups are present within the RGC population or whether these classical neurotransmitters co-exist here is unknown. The lateral reticular areas of the caudal medulla are known to be catecholamine containing neurones and although they have been shown to play a role in tonic descending inhibition their exact contribution is not known.

What of the role of these neuropeptides? Not much is known of their role generally; however evidence suggests that in the visual cortex the peptides may be largely co-localized with GAD-immunoreactive cells (Hendry et al., 1984).

The current view (Hökfelt, 1987) on the role of peptides in the CNS is based on observations of peripheral nerve interactions; two types of interactions between co-existing neuropeptides and classical neurotransmitters have been proposed:
(1) the two types of compounds act synergistically and complementarily;
(2) the neuropeptides counteract the action of the classical neurotransmitters by inhibition of their release.

The above data clearly indicate that a number of classical neurotransmitters as well as a substantial neuropeptide component are present in the PMRF. However, the exact localization, distribution and relative weighting of the classical neurotransmitters in PMRF nuclei is largely unknown.

On the basis of these aforementioned studies it is clear that the distribution of specific brain stem neurotransmitters do not occupy discreet areas, but rather a broad segmental division of the neurotransmitter distribution exists within the reticular formation. A source of much confusion therefore arises from the fact that, in many instances, studies involving neurochemical analysis do not conform precisely to defined anatomical boundaries. This makes comparisons with studies from other fields somewhat difficult. It is evident that as more data is accumulated concerning brain stem reticular nuclei and their functional distinctions, the need arises for re-evaluating the present system of categorization of nuclei taking into account anatomical position, boundaries, morphology, projection targets and type of neurotransmitter substance.

Previous studies have demonstrated that neurones, especially in nucleus raphe magnus and the ventral medulla, are subject to tonic inhibitory noradrenergic input (Hammond et al., 1980; Sagen and Proudfit, 1985; Wessendorf and Anderson, 1983) as well as a serotonergic input (Wessendorf and Andersen, 1983; Willcockson et al., 1983) and an excitatory cholinergic input (Brodie and Proudfit, 1984). The more recent study of Drower and Hammond, (1988) is one of the few studies systematically demonstrating that neurones of the NRM and RGCγ involved in the regulation of nociceptive input are subject to an inhibitory GABAergic input mediated via a GABAa receptor.

In the lateral reticular formation in the caudal medulla it is well established that catecholamine containing neurones occur in this area and that on the basis of lesion experiments tonic inhibitory influences descend from this area , but their contribution to tonic descending inhibition is not well understood.

Concluding remarks

The task of identifying the many components of fundamental PMRF circuitry is extremely complex due to the many unknown factors related to the detailed connectivity of the various nuclei, the spatial distribution and types of receptor present, the complexity of the synaptic organization (largely an unknown factor), the type and distribution of intrinsic neurotransmitters, to mention a few areas that still require purposeful investigation. At best, only simplistic models of the inter-relationships of these cells can be built at present.

The Substantia nigra

One of the focal points of interest in this study concerns the effects of basal ganglia on pontomedullary reticular formation neurones. It is of particular importance to have a clear understanding of the anatomy and physiology of these nuclei. As the literature on basal ganglia is vast, only aspects of direct relevance to this study are discussed. This covers essentially the structure of substantia nigra and its efferent connections.

The great advantage of experimental procedures incorporating direct correlations between morphology, connectivity and histochemistry is now a widely recognised principle. This is attributable directly to the large number of methodological advances in the last decade. Certainly such experiments, although technically demanding, have shed enormous light on the intrinsic organisation of various parts of the basal ganglia circuitry and neurochemistry (Bolam, 1984; Somogyi et al., 1979).

The substantia nigra (SN) is located in the ventral mesencephalon and includes the dopamine (DA) rich pars compacta (SNc), the diffuse and cell poor pars reticulata (SNr) and a small group of cells called the pars lateralis (SNL). Although the SNL is an identified area of the substantia nigra in mammals, including the rat, most authors include this region as an integral part of the SN. The SNL is the smallest subdivision of the substantia nigra.

The SNc is composed of a cell rich sheet of cells which caps the cell sparse but neuropil rich SNr. The substantia nigra (SN) together with the entopeduncular nucleus (EP) are the two major output nuclei of the basal ganglia in the rat (Ricardo, 1980).

Falk, (1962) and Carlsson et al., (1962) discovered that by exposing freeze dried brain tissue to formaldehyde vapour, a fluorescent condensation with monoamines could be formed. This could readily be visualized by fluorescent microscopy. By means of this type of study monoamine cells in their greatest concentration were found in the SN, midbrain tegmentum and the locus coeruleus in the pons.

A nomenclature to describe cell groups containing monoamines was proposed by Dalhstrom and Fuxe, (1964). It has thus been accepted generally that cell groups containing the catecholamine class of neurones (dopamine and norepinephrine) or (epinephrine) is classified "A" and "C" respectively, whereas the indoleamine (serotonin) is given the "B" classification. Thus the dopamine (DA) containing cells of the SN are classified as A9 cells while those of the VTA and retrorubral field are called the A10 and A8 cell groups respectively.

Most morphological studies of neurochemically identified DA neurones have been carried out in the A9 region using either catecholamine histofluorescence (Lindvall and Björklund, 1978) or intracellular injection of fluoresecent dyes such as Lucifer Yellow (Grace and Bunney, 1983). The SNc also contains a number of non-DA interneurones and projection neurones. Several subtypes of Golgi-stained neurones have also been identified on the basis of size and shape in the SNc (Danner and Pfister, 1982; Juraska et al., 1977). There is general consensus that the larger

neurones of the SNc as well as those of the rostral SNr and SNL are dopaminergic cells lying most ventrally in the SNc and having dendrites that enter the dorsal layers of the SNr (Fallon et al., 1978b). The more dorsal layers of the SNc extend into the medial lemniscus, the caudal extension of the SN (retrorubral field) and the ventral tegmental area, as well as projections to limbic and cortical structures (Fallon and Moore, 1978a; Fallon et al., 1978b). In the rat the SNr has a population of cells which are broadly divided into 2 zones on the basis of size:-

(1) a ventromedial zone consisting of widely spaced neurones

(2) relatively clustered, medium to large neurones lying more dorsolaterally (Hanaway et al., 1970).

Throughout the length of the SNr the dendrites, mainly of the larger cells, course in the antero-posterior and dorso-ventral direction and are contained only within the structure (Juraska et al., 1977).

As this thesis is concerned only with the caudal outflow pathways of the SNr, it is not within the scope of this study to descibe in full all the basal ganglia connections. Therefore, merely the highlights will be mentioned, but pertinent issues will be dealt with more fully.

Efferent connections of the DA cells

The mesencephalic dopaminergic neurones have local connections as well as major ascending and descending fibers. Of these the most important is the large ascending mesotelencephalic system, composed of the meso or nigrostriatal, mesocortical and mesolimbic pathways (Lindvall and Björklund, 1983; Moore and Bloom, 1978). All these pathways originate from the A8, A9 and A10 cell groups which form the continuum of the SN - VTA. These projections are not directly relevant to this study and will not be discussed.

The nigrostriatal pathway

Neurones lying in the ventral SNc and the cell group comprising the ventrolateral paranigral area i.e. VTA, SNL and rostral SNr, are mainly dopaminergic, and to a lesser degree non-dopaminergic. Thus the classic dopaminergic pathway also contains nondopaminergic neurones (Moore and Bloom, 1978; Hattori et al., 1979; Hökfelt and Ungerstedt, 1969; Lindvall and Björklund, 1974; Lindvall and Björklund, 1983; Swanson, 1982). The nigrostriatal tract is composed of both an ipsilateral and contralateral component (Fass and Butcher, 1981; Pritzel et al., 1983; Loughlin and Fallon, 1982; Douglas et al., 1987). Evidence from tract-tracing experiments shows that the contralateral component arises almost entirely from unilaterally projecting neurones.

The DA terminals appear to synapse with GABAergic and cholinergic neurones in striatum. The striatum has been the most intensively examined dopaminergic target area, due in all likelihood to the clinical significance of striatal dopaminergic

neurones in the pathogenesis of dyskinetic syndromes and Parkinson's disease. The fundamental issue of whether DA is excitatory or inhibitory is still controversial.

There are many reports citing an inhibitory action of DA applied to striatal cells (Moore and Bloom, 1978; Bernardi et al., 1978; Herrling and Hull, 1980). However, excitatory effects have also been observed (Kitai et al., 1976; Norcross and Spehlmann, 1978). Similarly striatal responses to SN stimulation using intracellular recording techniques have also been shown to be excitatory (Wilson et al., 1982). It is thought that a possible explanation for some of these apparently contradictory responses can be explained in terms of regional effects of DA, i.e. the effect of DA on the soma is hyperpolarization, but on distant sites, e.g. on the dendrites, the effect is depolarization. This differential effect of a neurotransmitter, dependent upon a specific site of application, has a possible parallel in the hippocampus with respect to GABA. Thus the majority of evidence indicates that dopamine acts on the striatum in an inhibitory manner. However ascribing a purely inhibitory role to DA is probably an over-simplification of the situation.

Substantia nigra efferent projections: nondopaminergic pathways

In addition to the substantial dopaminergic projection from the SN, the major non-DA output pathways of the basal ganglia, besides that of the entopeduncular nucleus (EP), arise in the SNr. A considerable proportion of the SNr neurones have branching axons (Steindler and Deniau, 1980). Many of these branches terminate in several areas which are known to be involved in the integration of sensorimotor activities.

The nigral cells, projecting to the tectum and the thalamus, are located in separate longitudinal zones, or "subnuclei" of the SNr and receive afferents from different regions of the striatum (Faull and Mehler, 1978; Graybiel and Ragsdale, 1979; Mehler 1981).

The ordered topographical striatonigral projection contrasts with the widely divergent and complex efferent projections of SNr neurones. However, certain zones within the SNr have been recognized as having efferents that project to defined areas. The major recipients of these efferent projections are the ventromedial thalamus (VM), superior colliculus (SC), mesencephalic reticular formation (MRF), and brain stem pedunculopontine nucleus (PPN). A reciprocal link between the SNr and more caudal brain stem areas such as the NGC has been reported by Pardey-Borrero and Gonzalez-Vegas, (1980) using electrical stimulation techniques. However there is little anatomical evidence to substantiate this claim.

Nigrothalamic projections

The rostral and central area of the SNr gives rise to the nigrothalamic projection, which innervates the ventromedial nucleus of the thalamus. Axons course dorso-rostrally distributing collaterals, at first locally to the surrounding SNr and SNc and then to the midbrain reticular formation (Beckstead et al., 1979; Faull and Mehler, 1978; Bentivoglio et al., 1979). By means of combined anterograde and

immunofluorescent tracer techniques a non-DA pathway arising in the VTA and projecting to the locus coeruleus has also been outlined (Swanson, 1982). Combined anterograde and retrograde techniques have revealed other thalamic target nuclei, namely the mediodorsal and anterodorsal nuclei receiving bilateral projections from the SNr-VTA area (Simon et al., 1979).

The transmitter involved in the nigrothalamic projection is inhibitory and has been shown to be GABA (Di Chiara et al., 1979; Kilpatrick et al., 1980).

Nigrotectal projections

Faull and Mehler, (1978) have shown by means of autoradiographic and HRP tract-tracing techniques that the nigrotectal tract arises from the most ventral part of the SNr, distinct from the source of nigral neurones projecting to the thalamus. Their studies have revealed 3 dorsal-to-ventral zones in the SN as projecting to the striatum, thalamus and the superior colliculus respectively. More recently with intracellular HRP techniques it has been shown that the rostral ventromedial and caudal ventrolateral areas of the SNR project to the superior colliculus bilaterally (Grofova et al., 1982; Gerfen et al., 1982). Similar to the nigrothalamic projections, lesion and GAD studies of nigrotectal projections have shown that inhibitory effects are mediated by GABA (Vincent et al., 1978; Chevalier et al., 1981a).

Since it was shown by Wurtz and Albano, (1980) that the superior colliculus (SC) exerts an influence on head motoricity, it has led to the belief that the SNr and striatal neurones might influence head movement via the superior colliculus. This belief was based on a number of observations concerning the connectivity of these areas.

It is well established that the SNr projects to the intermediate and deep layers of the superior colliculus (SC) (Gerfen et al., 1982; Rhoades et al., 1982; Gaybiel, 1978). This projection exerts a potent inhibitory influence on the collicular neurones (Chevalier et al., 1981a; Deniau et al., 1978b) through the release of GABA (Chevalier et al., 1981a). Hikosaka and Wurtz, (1983) have provided strong evidence that the reciprocal firing of SNr and superior colliculus cells reflect a functional relationship between these two areas. SN cells may tonically inhibit superior colliculus neurones, as it was shown that eye movement related firing of SC neurones can only occur when the SN cells shut down.

In the lateral portion of the SC lie the tectospinal neurones which represent one of the efferent pathways whereby the SC can influence lower motor centres. By means of selective lesioning of the SC (Kilpatrick et al., 1982) it is clear that the lateral portion of the SC, in contrast to the medial portion, is involved in the mediation of either striatal and/or nigral induced head turning.

Grantyn and Grantyn, (1982) have shown that in the cat the tectospinal neurones give off a network of axon collaterals to the ponto-bulbar reticular core. This is thought to be an important collicular efferent system to promote head and eye movements. Thus it is conceivable that via a nigrotectospinal/tectoreticular link, the basal ganglia influences both cephalic and ocular motricity. It has also been shown that tonically active inhibitory nigrotectal neurones receive a potent inhibitory influence from the striatum (Deniau et al., 1978b). The discharge of nigrotectal

neurones provoke a phasic arrest when the striatum is stimulated. Hikosaka and Wurtz, (1980) have speculated that the SN tectal neuronal circuit exerts a gating effect on the excitability of tectospinal neurones via a disinhibiting mechanism. They showed that in monkeys which were performing orienting tasks, the SC neurones increased their firing rate before targeting movements, while the SNr cells exhibited a phasic arrest of their tonic spontaneous discharge. SNr cells in the cat similarly exhibit a phasic arrest of their tonic spontaneous discharge immediately preceding eye/head orientating movements (Joseph and Boussaoud, 1982). Thus the scheme whereby the orientating movement is achieved is thought to be via the tectum, which upon being activated by the striatum and the SNr, switches on an appropriate sensorimotor link, which in turn targets a significant stimulus for an orientating movement.

More recently Chevalier et al., (1984) have demonstrated that the tectospinal neurones are one of the cellular populations in the tectum on which SNr exerts its influence. It is generally accepted that the SNr projects to the intermediate and deep layers of the SC (Gerfen et al., 1982; Graybiel, 1978). However, the lateral portion of the SC, in contrast to the medial one is involved in the mediation of basal ganglia induced head turning (Di Chiarra et al., 1982; Kilpatrick et al., (1982). In the lateral position lie the tectospinal neurones which are thought to be the SC efferent pathway by which lower motor centres are influenced. Chevalier et al., (1984) have demonstrated that the SNr influences the responsiveness of antidromically identified reticulospinal to their peripheral sensory inputs. These authors thus believe that the basal ganglia, and in particular the SNr, are involved in the integrative sensorimotor processes which underly head orientating movements. The tectal effects on the lower motor centres are more complex than would at first be apparent. Grantyn and Grantyn (1982) have shown in the cat that the tectospinal neurones give off a network of collaterals to the pontobulbar reticular core. It is therefore likely that the basal ganglia effects on occular and head movements occur via a complex nigrotectospinal/tectoreticular system of fibers. The current view is that the basal ganglia act on the tectal neurones to engage an appropriate sensorimotor link whereby a particular stimulus is used as a target for an orientating movement.

Nigrotegmental projections

Nigrotegmental projections innervate a number of areas within the mesencephalic tegmentum. This was recognised by early workers such as Cajal, (1911) and in the subsequent years became known by the collective phrase "courant de la callote". With the advancement of technology their existence has become firmly established. A sizeable part of the nigrotegmental projection system arises from the SNc (Hopkins and Niessen, 1976). However nigrotegmental fibers arising in the SNr have been shown to project to the nucleus tegmenti pedunculopontis (PPN) (Graybiel and Ragsdale, 1979).

The projection of the SNr to the pedunculopontine area within the tegmentum is of considerable importance in as much as this area is known to express extrapyramidal influence on the reticulospinal system, which in turn innervates spinal motor

Substantia nigra pars reticulata target sites

Anatomy and connections of mesencephalic target areas receiving substantia nigra pars reticulata efferent projections: mesencephalic locomotor region and pedunculopontine nucleus and angular complex.

The mesencephalic locomotor region (MLR) is an important mesencephalic relay of the substantia nigra pars reticulata (SNr). The MLR in turn forms an important link with the medullary reticular formation, the latter being the main effector component of MLR stimulation under certain experimental conditions. The reticular formation areas receiving MLR efferent fibers include different subdivisions of the medullary RF. The reticulospinal recipient areas include the anteroventral NGC, posteroventral nucleus reticularis ventralis or nucleus magnocellularis. According to Garcia-Rill and Skinner, (1987) the reticulospinal fibers originating in the medulla, project along the ventrolateral funiculus to the spinal cord. On the basis of conduction velocities measured by them, the population of reticulospinal neurones include both large and small neurones, with a preponderance of smaller neurones constituting the MLR-reticulospinal system. A more complete description of the reticulospinal system is given in section 1.1.1).

With the advent of modern tract-tracing and electrophysiological techniques, the pathways between the substantia nigra and other mesencephalic projection sites have been delineated only recently. As the emphasis in this section of the study is on the efferent output of the substantia nigra, the following literature review of these anatomical connections will be dealt with in greater detail than the other basal ganglia afferent and efferent connections.

The efferent output of the SNr is generally recognised as projecting to:
(1) the superior colliculus (SC) as the nigrotectal pathway
(2) the pedunculopontine nucleus (PPN) - a group of cells lying amongst fibers of the superior cerebellar peduncle
(3) the periaqueductal grey (PAG)
(4) the reticular formation adjacent to PAG, a poorly defined area, christened the "Angular complex" (AC).

Furthermore, there is now good evidence that descending and ascending efferents of the substantia nigra are GABAergic (Garcia-Rill et al., 1985; Childs and Gale., 1983; Kilpatrick et al., 1980; MacLeod et al., 1980). Garcia-Rill et al., (1985) convincingly demonstrated that locomotion could be induced or blocked by localized infusion of GABA agonists and antagonists in the cat precollicular-postmammillary transected preparation.

An important physiological area encompassing at least some aspect of all of the aforementioned sites, has become known as the midbrain or mesencephalic locomotor region (MLR). The abovementioned projection sites of the SNr all lie within the MLR. In broad terms, the (MLR) is described in the literature, as an area which is "physiologically identified" within the posterior mesencephalon of a cat, with a transection at precollicular-postmammillary level, from which site locomotion can be induced by electrical stimulation. However, two further conditions have to be met,

i.e. that the cat's weight has to be supported, and that the treadmill has to be moving. (Shik et al., 1966; Shik and Orlovsky, 1976; Mori et al., 1983). This scheme implies that the spinal locomotion oscillators, under the influence of peripheral inputs, are also modulated by the MLR (Anderssen and Grillner, 1981).

For an objective view of the extent of the area that is called the MLR, the anatomical boundaries of this mesencephalic projection site have to be considered. The area adjacent to the periaqueductal gray (PAG) has been variously designated nucleus cuneiformis (Taber, 1961; Valverde, 1962) or alternatively the deep mesencephalic nucleus pars medialis (Veazy and Severin, 1982).

The original locomotor studies performed by Shik et al., (1966) involving the MLR, placed its anatomical position in the area of the cuneiform nucleus (CN) at the level of the posterior mesencephalon, ventral to the inferior colliculus. However the entire cuneiform nucleus does not constitute the MLR, as subsequently it has been demonstrated that the MLR is situated further towards the ventrolateral boundary of the CN, bordering on the dorsal brachium conjunctivum (Grillner, 1975; Jordan et al., 1979 and Garcia-Rill, 1986).

The area adjacent to the PAG has been variously designated, nucleus cuneiformis (Taber, 1961 and Valverde, 1962) or the deep mesencephalic nucleus pars medialis (Veazey and Severin, 1982). The mesencephalic tegmental cells within this area are relatively undifferentiated and occupy an area from the level of the pretectal area rostrally to the IC caudally and is bounded medially by the PAG, laterally by the medial geniculate nucleus, dorsally by the superior colliculus (SC) and ventrally by the medial lemniscus.

This is not however the only area within the mesencephalic tegmentum from which locomotion can be elicited. Other locomotion inducing sites have been identified, viz:

(a) Along the medial aspect of the trigeminal nucleus at the edge of the central gray nucleus (Garcia-Rill et al., 1983b)

(b) Anteriorly along the ventral brachium conjunctivum (Skinner and Garcia-Rill, 1984(a) and Skinner and Garcia-Rill, 1984(b).

Thus in the rat, at least these anteroventral sites are part of the MLR and appear to overlap the MLR areas originally described in the cat.

The Pedunculopontine Nucleus

A region adjacent to the superior cerebellar peduncle is known as the nucleus tegmenti pedunculopontinus or pedunculopontine nucleus (PPN). The PPN forms the posterior part of the MLR both in the rat (Saper and Lowey, 1982) and in the cat (Garcia-Rill et al., 1981). As it appears to be a part of the MLR, it seems likely that the MLR and the PPN function in a complementary manner. By means of Golgi and HRP staining techniques, the anatomical boundaries of the PPN have recently been defined by Newman, (1985). The nucleus is divided into two areas on the basis of neuronal morphology; the PPN pars compacta and PPN pars dissipatus. The former area lies along the ventrolateral edge of the brachium conjunctivum, and the latter lies

more ventrally and anteriorly. It is not clear whether independant stimulation of these two areas produces different motor effects.

The nucleus can be further categorised into two neuronal populations on the basis of their transmitter and staining properties. Double labelling with HRP and ChAT have revealed that 2 populations of neurones within the PPN correspond to areas demarcated by Nissl staining. The neurones that stain positively with ChAT correspond to the group of neurones within the PPN area called pars compacta, (the zone demarcated by Nissl staining), while the area known as pars dissipatus do not show any positive staining with ChAT. However, these cells are demarcated on the basis of being retrogradely labelled with HRP from deposition sites in the superior colliculus (Beninato and Spencer, 1986).

On the basis of Nissl stained sections Sugimoto and Hattori, (1984) have described three cell types which comprise the PPN. Whether each of these neuronal populations are preferentially involved with specific connections of one or more of the various nuclei to which the PPN is known to be linked and what transmitters they utilize, has yet to be established.

The SNr is known to project to the PPN. Hedreen, (1971) identified a non-dopaminergic pathway, between the SNR and PPN, on the basis of these neurones being resistent to 6-hydroxydopamine (6-OHDA) lesioning. Later, autoradiographic studies by Beckstead et al., (1979) showed that the PPN is the recipient of a substantial projection from the SNr. More recently anatomical connections between the PPN/MLR and the output nuclei of the basal ganglia have been established by use of retrograde tracers injected into identified MLR sites with subsequent recovery of labelled neurones located in the EN and SN. In the cat precollicular-postmammillary transection model the only known afferent projection to the MLR lying posterior to the transection is the substantia nigra, and furthermore is the most likely source of tonic GABAergic input to the MLR. (Garcia-Rill et al., 1983a; Garcia-Rill et al., 1983b; Spann and Grofova, 1984; Garcia-Rill et al., 1985). The PPN is also unique in that efferent projections from this nucleus contact a number of basal ganglia targets such as the pallidal complex, subthalamic nucleus as well as the substantia nigra (Gonya-Magee and Anderson, 1983; Scarnati et al., 1986). This area is thus linked by multiple ties to the basal ganglia, and constitutes one of the so-called loop nuclei related to basal ganglia circuitry.

The PPN is also reciprocally linked to the substantia nigra (Saper and Lowey, 1982; Moon-Edley and Graybiel, 1983). The nature of the connection between the PPN and the SNc is excitatory and the neurotransmitter involved appears to be the excitatory amino acid, glutamate or a closely related amino acid (Scarnati et al., 1986). On the other hand certain areas of the PPN projecting to the SNr are sensitive to the ionophoretic application of acetylcholine, and may thus be cholinergic.

Acetylcholinesterase (AChE) has been localized in the PPN extending from the caudal pole of the substantia nigra in a caudal direction to the level of the parabrachial nuclei. This AChE positive area within the pedunculopontine nucleus has also been shown to be in register with neurones which show ChAT immunopositivity. (Beninato and Spencer, 1986; Armstrong et al., 1982).

Beninato and Spencer, (1986) have also demonstrated that the PPN is a source of cholinergic input to the superior colliculus. As there are a substantial number of

efferent fibers to the medial pontomedullary reticular formation arising from the PPN (Aghajanian and Bunney, 1974) it is likely that these caudally projecting neurones may also be cholinergic.

The PPN, by virtue of its position in the mesencephalon forms a logical starting point for descending fibers from the basal ganglia leading to the spinal cord. The PPN in fact lies near to the so-called MLR (Grillner and Shik, 1973). Large injections of labelled amino acids into the area reveal descending pathways to the reticulospinal circuitry of the medulla (Graybiel, 1977).

The "Angular Complex"

Another neighbouring site within the mesencephalon receiving projections from the SNr is the periaqueductal gray matter (PAG) (Hopkins and Niessen, 1976). Leigh et al, (1983) have named the adjacent mesencephalic reticular formation together with the PAG recipient area the angular complex (AC). In the rat the whole angular complex receives a projection from the SNr (Beckstead et al., 1979). This area is a recipient of afferent fibers from a wide source namely, the hypothalamus, a number of thalamic nuclei, fields of Forel and the zona incerta.

What of the caudally directed projections of these SNr mesencephalic projection sites? Certainly Miller and Sinnamon, (1980) have shown that neurones of the pontomedullary reticular formation in the rat, receive projections from the AC. However up to about 1983 the caudal projections of the PPN were unknown, but Leigh et al., (1983) had preliminary HRP data that showed neurones from this area projecting to giant neurones in the PMRF.

Thus in the rat at least the link between the spinal motor mechanisms and the angular complex appears to be the reticulo-spinal neurones. There is thus overwhelming evidence in support of connections between the SNr and the AC, and between the AC and the medial pontomedullary reticular formation. There appears, however, to be a species related difference in that, in the cat, opossum and monkey (Tohyama et al., 1979; Martin et al., 1979; Castiglioni et al., 1978), respectively, the AC also projects directly to the spinal cord. In the rat this direct spinal projection has not been demonstrated. Thus the AC forms an important relay between the basal ganglia and the spinal motor mechanisms. In the rat the AC has one further relay station, in the form of the reticulospinal neurones. The other species mentioned above have, in addition, a direct connection between the AC and the spinal cord. The significance of these differences is not clear.

Thus although there are three areas within the mesencephalon individually recognised as receiving projections from the SNr, these areas share common boundaries, and may overlap to some degree. Thus caution has to be exercised when claiming a specific function for each of the areas. There is no doubt that the SNr efferents relay in the mesencephalon. These pathways are thus well recognised for mediating basal ganglia influence at the level of the brain stem reticular formation.

The MLR (per se) does not send fibers directly to the spinal cord but controls activity of the reticulospinal neurones. Tritiated amino acids injected into the MLR in the cat reveal bilateral distribution of MLR efferents to the medioventral medulla

(Garcia-Rill et al., 1983b). This may probably be an explanation of the finding whereby stimulation of one MLR is able to produce bilateral movement. However, the placement of the stimulating electrode critically influences reticulospinal cells. Cells stimulated by this procedure are either predominantly on the ipsilateral or contralateral side. This is borne out by both autoradiographic studies and anterograde tracing techniques (Steeves and Jordan, 1984 and Garcia-Rill and Skinner, 1987), which showed that when injections were placed in the lateral MLR the projections were mainly ipsilateral, while more medially located injections resulted in contralateral predominance.

The MLR projects to two major sites within the pontomedullary reticular formation: (a) the NGC, i.e. the medioventral area referred to above, and (b) the trigeminal nucleus, especially the medial edge along Probst's tract. Fibers from the latter site course the length of the medulla along the medial edge of the spinal tract of the trigeminal nerve nucleus and send axons the length of the spinal cord. This area forms part of another inter-related system, the pontobulbar locomotor strip (Shik and Yagodnitsyn, 1976), which when stimulated electrically also causes locomotor behaviour, even after the MLR has been lesioned.

The MLR is thus an important site of termination for basal ganglia outflow, the latter being a modulator of spinal locomotion oscillators. The weight of anatomical evidence suggests that the MLR is under direct SNr GABAergic influence. The overall function of the MLR is also probably regulated via subthalamic feedback pathways. Since this area also receives information from the motor cortex , together with the basal ganglia input to this area it is thought to act as a relay for information related to motor activity concerned with postural functions and locomotion.

Gamma-amino butyric acid (GABA): mechanisms and receptors

As one of the main interests in thus study concerns the role of GABA, the focus of this short review will be on current views of GABA receptors and GABAergic mechanims.

One of the major neuroactive inhibitory substances in the CNS is the ubiquitous neurotransmitter, GABA. Obata and Takeda (1969) provided the first convincing proof that GABA was an inhibitory neurotransmitter in mammalian CNS. When GABA is applied to the cell membrane, just about every neurone shows a response to GABA with greatest sensitivity at postsynaptic membranes, especially in the region where GABAergic innervation is known to take place (Enna 1983). GABA localization is probably best documented and studied in the cortex, where GABA has only been localized within GABA terminals that form symmetrical synapses, or in somata and dendrites of neurones whose axons form symmetrical synapses. Glutamate and aspartate, considered to be excitatory transmitters are associated with neurones whose axons form asymmetrical synapses.

It is widely accepted that GABA is implicated in numerous functional circuitry, e.g. cerebellum, visual cortex, hippocampus, thalamus to mention but a few. It is estimated that the percentage of neurones throughout the nervous system that utilize GABA may be as high as 40%. As the literature concerning GABA abounds with examples of areas containing GABA, the reticular formation is noticeably absent from the list of the many GABA containing structures. This may stem from the fact that the brain stem is not an easy system to use as a model and is therefore not a popular choice for studying GABA receptor physiology for a number of possible reasons viz; experimental access to this area is technically difficult; the brain stem has not yet yielded to the brain slice technique and in addition, since the neural organization is not well ordered it has not been mapped as extensively as, say, cortex or cerebellum.

Of great pharmacological, and hence clinical significance, is the abundant evidence that GABA synapses are likely to be the site of action of many depressant and excitatory drugs e.g. barbiturates and benzodiazepines (Olsen, 1987). This has probably been the central drive for research on this inhibitory neurotransmitter. There is also growing recognition that neurological and psychiatrical disorders in humans are related to altered GABAergic function.

Synthesis

GABA is synthesized from L-Glutamic acid under the influence of glutamic acid decarboxylase (GAD). It is thought that the biosynthetic enzyme GAD is expressed only in neurones that are GABAergic (Bachelard, 1981; Wu et al., 1982). As antibodies raised against the purified enzyme and then tagged in certain ways, have been used to localize GAD, this has been the main marking system used for demarcation of GABAergic neurones in the nervous system until recently. There is

good evidence that GAD is selectively localized in neurones (Saito et al., 1974; McLaughlin et al., 1975). However caution has to be exercised in the interpretation of the results achieved with GAD immunocytochemistry as it is not the neuroactive particle itself which is localized but rather the enzyme. The ratio of the concentrations of GAD:GABA may differ in different cellular compartments, thus the distribution of GABA may be very different from that of GAD. It also has to be borne in mind that the enzyme GAD is sensitive to fixation (Oertel et al., 1982) and its distribution may consequently be altered or destroyed. Furthermore, if the intention is to use this method in combination with other procedures, there is a further limitation that one should be aware of, that being the loss of demonstrable GAD immunocytochemistry in tissues postfixed with Osmium tetroxide (Hodgsen et al., 1985).

However, only recently, since the advent of successfully raising antibodies to the small GABA molecule, can its localization be demonstrated more reliably by direct immunohistochemical methods. This has only become possible due to a number of workers who have developed an antiserum to GABA (Sequela et al., 1983; Hodgsen et al., 1985 and Somogyi et al., 1985b). These highly specific antisera thus allow the demonstration of immunoactive GABA in neurones containing other neuroactive substances. This is an important consideration, especially in GABAergic neurones which contain and probably secrete neuroactive peptides. The beauty of this technique thus lies in its high selectivity.

Mechanism of GABA action

It has been establsihed that GABA operates via a minimum of two distinct receptor sub-types. Both differ structurally and functionally and are sensitive to different agonists and antagonists (Borman, 1988). Thus, like the expression of acetylcholine via its nicotinic and muscarinic receptors, the action of GABA is not limited to a single synaptic mechanism. The most well characterized have been called GABAa and GABAb, on the basis of their differential responses to bicuculline and baclofen. The GABAa receptors are those sensitive to bicuculline and activated by muscimol, (this being the major receptor type) whereas the GABAb receptors are those activated by baclofen and insensitive to bicuculline.

With ionophoretic techniques, Krnjevic and Schwartz, (1966) showed that GABA mimicked the activity of inhibitory neurones of the cerebral cortex via increased chloride conductance, thus hyperpolarizing the membrane potential. Using the radioactive tracer chlorine-36 it has been shown in crayfish muscle fiber (Ticku and Olsen, 1977) and in rat brain-slice (Olsen et al., 1984) that GABA regulates an ion channel that is specific for chloride. GABA is bound to the membrane receptors with high and low affinity. The receptors in turn control the ionophore (conductance channel) for Cl^-. This is the ion that moves during the GABAergic IPSP. When GABA occupies its receptor there is a rapid and brief increase in membrane conductance which involves a conformational change in the ion channel protein. The chloride equilibrium potential is close to the resting membrane potential, thus the opening of the chloride channels stabilizes the membrane potential close to its resting potential. The ultimate effect of this action, by preventing the depolarizing response of excitatory neurotransmitters, is to inhibit tonic firing of neurones. GABA is cleared from the synaptic cleft by uptake into the presynaptic terminal as well as by glia cells.

Certain drugs which show no chemical similarity to GABA, such as picrotoxin, are able to block the coupling of GABA to its ionophore. Both picrotoxin and bicuculline (GABAa antagonist) act at a specific site on the protein receptor complex. These sites are distinct from the GABA receptor sites, but by means of binding studies have been shown to be allosterically coupled to the receptor (Olsen 1982). The CNS depressant drugs such as the benzodiazepines act at different sites to produce depression of GABAergic synpases. Benzodiazepines and barbiturates enhance the function of GABA via a mechanism of increased GABA-activated chloride channel opening (Barker et al., 1984). The receptor sites for GABA, benzodiazepines and picrotoxin as assayed by radio-ligand binding, show that these three receptor sites share mutual chloride-dependent allosteric interactions with one another. They are also allosterically modulated by the barbiturates.

These findings have given rise to a hypothetical model of a GABA receptor-ion channel complex. The macromolecular complex model proposed by Olsen, (1981) and Olsen, (1982), as above, is thought to consist of four receptor sites: the GABA receptor; the benzodiazepine receptor; the barbiturate receptor; and the chloride channel or picrotoxin receptor. Lending substance to this theory, a relatively simple oligmeric protein has been isolated with binding and allosteric interactions preserved

(Siegel at al., 1983). Further progress in the elucidation of its structure has been achieved in the production of monoclonal antibodies against the benzodiazepine/GABA receptor protein (Schoch et al., 1985). Monoclonal antibodies are currently the best methods of detecting sites of GABA binding or the localization of the molecule itself and ultimately holds the key to unlocking the maze of receptor distribution.

Distribution of GABA receptors: The mismatch problem

The study of cellular receptors has required the development of increasingly sophisticated methods for examining their biochemical properties and distribution. Receptor mapping by means of the autoradiographic technique has been the most popular method for mapping neuronal receptors for some years (Kuhar, 1987). However the main disadvantage of this technique is that the direct association between specific binding sites and specific cells is often impossible to determine. It was still assumed until only recently that receptors were localized at the synapses. Only with the development of highly specific techniques have the ideas of the receptor mechanism begun to change. These techniques are: immunocytochemical labelling, site selective fluorescent probes that bind specific receptor sites on macromolecules, and a very recently developed technique named in situ hybridization, which concerns the production of nucleic acid probes that hybridize with mRNAs for a particular receptor.

GABA has been shown to act both presynaptically and postsynaptically, but GABAergic mechanisms and underlying principles are not completely understood. It is generally accepted that whether activation of the receptor takes place via the GABAa or GABAb mechanism the responses of the postsynaptic cell, with respect to excitatory signals are substantially modified. In keeping with the wide distribution of GABA in the CNS it is likely that all neurones express at least GABAa receptors. However, the cellular and subcellular distribution of the receptor is not well known. Only recently has the functional organization of especially GABAa mediated interactions come under intensive investigation (Connors et al., 1988).

To date one of the most intensively investigated and probably best understood circuits involving the inhibitory neurotransmitter GABA, is the visual cortex, and associated pathways involving GABA circuitry. Somogyi, (1989) has recently reported on the extensive investigation of GABAa-receptor mediated responses by means of high resolution immunocytochemical identification of the receptor complex in the visual pathways.

The distribution of cellular and sub-cellular GABAa/benzodiazepine/receptor/chloride channel complexes has been studied with a highly specific monoclonal antibody, named "bd-24" (Somogyi, 1989). These studies reveal that the receptor immunoreactivity was always associated with membranes whether at a subcellular or extracellular level. A particularly noticeable feature of the immunoreactive distribution of the receptor was that the subcellular distribution did not correlate positively with the extracellular receptor immunoreactivity. The immunopositive receptor complex is present, as expected, at synaptic specializations. However the presence of GABA-receptor immunoreactivity at non-synaptic junctional sites on the soma as well as on the dendrites is an unexpected feature of their distribution, e.g. cells of the dorsal LGN. The GABAa receptor proteins are present at the synaptic junctions as well as at the non-junctional sites in every area of the brain examined to date (Soltesz et al., 1989; Somogyi et al., 1989).

Results from receptor immunocytochemical studies applied to the dorsal LGN cells, an area known to have high levels of GABAergic activity as well as a population of identifiable GABA-negative cells, showed contrary to expectation, that the latter cell type was strongly immunoreactive on their somata and proximal dendrites. However they did not demonstrate any intracellular immunoreactivity.

In the light of recent evidence it is highly unlikely that nonjunctional immunopositivity is due either to cross-reactivity of the antibody with other membrane constituents and/or due to diffusion artefact, inherent in immunocytochemical methods. There are many technical reasons and/or biochemical explanations (e.g. low-affinity, non-functional, prebound receptors, etc.) which could explain possible mismatches between neurotransmitter and receptor locations. Kuhar, (1985) argued that fundamental reasons such as the aforementioned may account to a large degree for an exaggerated mismatch between receptors and neurotransmitters. Although these factors may affect the outcome and thus should be taken into account, recent evidence discounts such factors as being the likely explanation for mismatches. If the distribution of markers for receptors and terminals is compared, it is often noted that these occupy adjacent and non-interconnected areas (Herkenham 1987). For example the bulk of evidence to date for the CNS peptides and their receptors indicate that the receptors are not anatomically related but are distributed throughout the brain in an independent manner. The plasma membrane at nonjunctional areas is often positively immunoreactive for GABA in spite of the absence of any junction reaction. Similarly GABA is heterogeneously distributed throughout the CNS with varying concentration.

The anatomical localization of GABA has been achieved with antibodies either to GAD or GABA itself. A typical example of mismatch between GABA receptors and terminals occurs in the cerebellum. Specifically, the GABA receptor complex is much in evidence on the somata of cerebellar granule cells, where they never receive GABAergic synapses (Somogyi et al., 1989). Cerebellar GABA terminals are far more dense in the molecular layers, which in turn are receptor poor relative to the receptor rich granule layer. Also in the hypothalamus, SN and Globus pallidus, GABA levels are reported to be very high while receptors are moderate to sparse. Thus by comparing the relative levels of GABA vs receptor in numerous brain areas it has become apparent that a quantitative relationship between these two entities does not hold. Furthermore, the situation is made more complex as the opposite also holds i.e. in some nuclei (anteromedial and posterior nuclei) the concentration of GABA terminals is the lowest, but the receptors are the most dense. Another example is the septal area where GABA terminals are rare but receptor density is extremely high (Onteniente et al., 1986).

The highest concentration of GABAb receptors in the CNS resides in the nucleus interpeduncularis (Bowery et al., 1984). This nucleus also possesses the highest density of GAD positive cells and GAD-positive axon terminals. The physiological role of GABAb receptors is not well characterized. However there are certain areas that possess high density GAD-immunoreactivity in axon terminals and somata, but are devoid of GABAb receptors such as the striatum and pallidum (Bowery et al., 1984). Thus the relationship between GAD-immunoreactive intensity and the type of

receptor present on the neurone in a particular area does not necessarily hold, certainly with respect to GABAb type receptors.

What is the explanation for the widespread, extrasynaptic distribution of the receptor complex? Somogyi, (1989) suggests that the GABA gated Cl-channel may function as a regulator of the dynamic range of neurones, and that the two populations of receptors may be used under different levels of neuronal activity thus providing a mechanism for adaption.

At low levels of neuronal activity GABA acting at synaptic junctions provides sufficient inhibitory control of the neurone. The GABA released under these conditions can be removed by glia cells or reuptake - leaving the other reactive sites available without a possible desensitization of the receptors. At increased excitatory input the channels operating at the synaptic junctions may not be adequate (or some receptors may have become desensitized) hence the synaptic receptors would not be able to keep the activity of the cell in the optimal range of sensitivities. Under these conditions the channels at the extrasynaptic sites would be opened by GABA diffusing from the release sites. Non-junctional receptors are therefore associated with increased excitatory input.

If the hypothesis holds, then one of the consequences is that the topography of the GABAergic effects would be regulated primarily by the precise placement of the GABA releasing terminals. A second consideration is the need for feedback to the GABA releasing neurone to inform it of the intensity of the excitatory input to that part of the postsynaptic membrane. Converging feedforward and feedback connections with different gains could provide the GABAergic cell with the ability to release GABA in proportion to the efficacy of the excitatory drive reaching their target cells.

The bulk of evidence suggests that CNS peptides and their receptors are also not anatomically related but appear to be distributed in the brain in an indepedent maner. The physiological significance of this type of arrangement between a neuroactive substance and its receptor has still to be formulated.

Co-localization

It is generally accepted that GABAergic neurones interact with neurones using amino acid neurotransmitters and biogenic amines. These features differ according to the specific brain area. The incidence of co-localization of neuropeptides /neuroactive substances in the same neurone is reviewed by Herkenham, (1987) and Millhorn and Höckfelt, (1988). Mugnaini and Oertel, (1985) also provide a comprehensive list of areas displaying neuropeptide and GAD-immunopositive coexistence e.g., in cortex, GAD-immunoreactivity is associated with peptides (Hendry et al., 1984). Examples of association of GABAergic neurones and other neurones containing different transmitters in same region are the dopaminergic and GABAergic neurones within the substantia nigra (Oertel et al., 1982). Double immunocytochemical staining techniques have also demonstrated catecholaminergic and GABAergic anatomical relationships in the substantia nigra , locus coeruleus , median eminence (Berod et al., 1984). Taken a step further, some experiments have employed double labelling at an ultrastructural level. e.g. GABAergic monosynaptic input and neuropeptide -Y containing neurones in the nucleus accumbens (Massari et al 1984). The role of these neuropeptides as well as their mode of action is still not understood. With respect to GABA neurotransmission the role of these particular combinations may be one of modulation and or synergism. GABA may have transient effects whereas the neuropeptides may potentiate longer lasting effects, possibly by altering the binding characteristics of the postsynaptic receptors. The realization in the last few years that coexisting chemical messengers may be co-released has revolutionized the classical role of the transmitters. This has led to the revision of the traditional view of "one cell one transmitter".

Synaptic Relations

A lot has been written on GABA inhibitory synapses since the terms symmetrical and asymmetrical were first applied by Collonier, (1968) in describing the most frequent morphological type of synapses observed in aldehyde -fixed cat visual cortex. This section briefly mentions some of these and is not intended to be a comprehensive description.

The basic elements differentiating the class of synapse consists of the clustering of vesicles type at the presynaptic membrane, synaptic cleft size and distribution of electron dense material on the post synaptic membrane. GABAergic neurotransmission involves all types of synaptic arrays commonly found in vertebrate CNS, but GABAergic synaptic structures show comparatively less heterogeneity. GAD -immunoreactive terminals most commonly contain pleomorphic synaptic vesicles, and form symmetric (Gray II type) synaptic junctions. It is generally accepted that boutons containing pleomorphic vesicles and having Gray II type synaptic junctions are associated with inhibitory function. However, Gray I type synapses have been observed in axon terminals of basket and stellate cells contacting the dendritic spines of Purkinje cells in the cerebellar cortex (Oertel et al., 1981), as

well as in the substantia nigra (Ribak et al., 1976), and in the dentate gyrus (Kosaka et al., 1984).

It has also been demonstrated by Ribak et al., (1981) that in the substantia nigra, 85% of GAD positive axon terminals make symmetrical synaptic contacts while the remainder made asymmetrical synapses. It is thus apparent that although in many brain areas the majority of GAD positive axon terminals form symmetrical (Gray type II) synapses with their target neurones, GAD positive terminals forming asymmetrical synapses are not as uncommon as previously thought. Both glutamate and aspartate, which are excitatory neurotransmitters, are associated with neurones whose axons form asymmetrical synapses. As GABAergic symmetrical synapses have generally been associated with inhibitory effects, it was this relationship which gave rise to the general assumption that symmetrical synapses are always inhibitory and asymmetrical synapses are always excitatory. Thus in the light of the recent information, assigning functional aspects to synaptic structures on the basis of their ultrastructure alone, should be exercised with some circumspection. At present the significance of this type of synapse is unknown. The latter type of synapse has also been identified in the substantia nigra.

A complex relationship exists between the relationship of GAD-positive soma density and axon terminal density, e.g. low GABAergic soma density and high axon terminal density (lateral vestibular nucleus) vs low GABAergic soma density and low GAD-positive axon terminal density (lateral reticular nucleus). These relationships are characteristic for certain areas. From a host of electron microscopic data it is suggested that GAD-immunoreactive neurons are involved in:-

(a) presynaptic inhibition
(b) postsynaptic inhibition
(c) recurrent/lateral inhibition
(d) feed-forward inhibition
(e) feed-back inhibition
(f) disinhibition.

On the grounds of morphological investigations, postsynaptic inhibition appears to predominate in most areas of the brain. This is mediated via axosomatic and axodendritic GABAergic terminals. GABAergic contact on the target cell may exercise a powerful modulatory effect on the spiking activity of that cell depending on where the contact occurs. In order of effectiveness, on generation of action potential: initial segment, soma, basal dendrite spines, and dendritic branching sites. For example the axon hillock of hippocampal pyramidal cells (Somogyi et al., 1983) are covered by axon terminals of which the majority are GAD-immunoreactive. Furthermore a relatively large percentage of these axon terminals are derived from a single axo-axonic cell, and these cells may contact the initial segment of many hundreds of pyramidal cells (Somogyi et al., 1989). Thus GABAergic hippocampal axo-axonic cells may synchronize inhibition of the output of pyramidal cells. GABAergic synapses on peripheral dendrites or on their spine apparatuses may also be involved in subtle modulation of local currents without the involvement of spiking activity.

It could be speculated that if intensity of terminal GAD staining were related to the extent of GABA synthesis and/or release then the regions of the rat brain where terminals are most densely stained may represent regions of intense inhibitory modulation. But what the various degrees of GAD-immunoreactivity within soma indicate is still poorly understood. There are a number of factors possibly affecting GAD-immunoreactivity. These are as follows:-

(a) ratio between synthesis of GAD and transport rate

(b) the relationship between synthesis of GAD to total cell volume

(c) electrical properties of pre and post-synaptic neurones (i.e.firing characteristics)

(d) distribution and type of GABA receptors

(e) unknown parameter

All of these factors have to be taken into consideration before speculating on the regional variation in intensity of GAD-immunoreactivity.

Section 1.2.0

General Methods

As many different techniques are used in this study this section describes only the methods commonly employed. In each experiment requiring specialized techniques the pertinent methods are described in detail.

General stereotaxic procedure

Choice of anaesthesia

The main criteria for choosing a suitable anaesthetic is that the anaesthetic agent will not materially affect the neuronal system being investigated, or that it does so in a predictable and consistent manner. Barbiturate anaesthetics are known to cause depression of discharge rate in some neural systems eg. cortex. In preliminary experiments it was noted that barbiturate administration reduced the incidence of spontaneous activity of PMRF neurones. Except in animals where tract-tracing substances were injected and necessitated recovery of the animal from the surgical procedure, all the rats were injected with urethane (Ethyl Carbamate, 1.2g/kg body weight, i.p.). Although the use of urethane has generally fallen into disfavour because of its carcinogenic properties, urethane was administered only in non-recovery experiments. Urethane is favoured in our laboratory since there is little apparent variation in depth of anaesthesia judging by stability of blood pressure and other vital signs. It also has the advantage that maintenance doses are not usually required during the experiment. However with prolonged periods (>8 hours) of urethane anaesthesia there are accompanying changes in acid-base balance with deleterious effects. In cases where prolonged anaesthesia was required the rats were artificially ventilated. Expiratory $C0_2$ levels, continuously sampled on a Beckman $C0_2$-analyzer, were kept within 4.0% by small adjustments of tidal volume. Urethane has been shown to be without effect on the spontaneous activity in isolated hypothalamic islands but to be selectively suppressive in other brain areas (Mercer et al., 1978). However, observations in single unit PMRF activity over prolonged periods (in some cases, longer than 1 hour) in the course of the present study and others in our laboratory do not indicate supressive effects of urethane on spontaneous activity of PMRF neurones.

At the risk of under anaesthetizing the animals and causing them unnecessary stress during surgery, animals were observed for responses to stimulation viz; paw pinch, ear pinch and presence of blink reflex as general indicators of inadequate anaesthesia. If the rat responded to these stimuli either more anaesthetic was administered, or a longer time was allowed for induction of surgical levels of anaesthesia. During surgical procedures and experimentation close scrutiny of the animal ensured that adequate levels of anaesthesia were maintained. Signs such as blinking, back arching, piloerection and changes in respiratory rate during experimentation were regarded as the main indicators of inadequate anaesthesia, and the appropriate action was taken. In experiments necessitating administration of paralytic agents in combination with anaesthetics the standard practise was applied, of first determining the adequate anaesthetic dose before administration of the paralytic agent. In cases

of terminal experiments, adequate levels of anaesthesia were maintained right up to the time the animal was perfused.
In recovery experiments the anaesthetic of choice was Equithesin. This anaesthetic "cocktail" was made up as follows:-

81ml Sodium Pentobarbitone (60mg/ml)
21g Chloral hydrate
10.6g Magnesium sulphate
198ml Propylene glycol
50ml Absolute alcohol

Solution made up to 500ml with dH_2O and mixed on a mechanical stirrer. Solution stored in dark.
Anaesthetic dose; 0.3 to 0.4ml/100gBWt, administered i.p.

Choice of animals and stereotaxic atlas

Extracellular action potentials were recorded in nucleus reticularis gigantocellularis (RGC) of the brain stem reticular formation of urethane (1.2g/kg body weight, i.p.) anaesthetised female rats. All the rats used in these experiments were in the weight range 290-350 grams, the majority being closer to 320grams. Female rats were preferred as the transverse sinus, lying appoximately ventral to the transverse suture of the skull was found to be positioned slightly more anteriorly in female compared to male rats, thereby permitting easier access to the anterior border of the RGC in the rostral pontomedullary reticular formation. Once the sinus was damaged by electrode puncture, bleeding was incessant with the usual deterioration of the recording conditions.

In a further attempt to avoid damaging the transverse sinus the head was tilted forward by positioning the incisor bar of the stereotaxic apparatus, (Narishige), 5mm below the interaural line. The interaural line was used as the antero-posterior co-ordinate zero in preference to Bregma due to the variable position of the latter with age and sex. Therefore coronal histological sections were cut in a different plane to those of the atlas of Pellegrino et al., (1979), in which the head is tilted upward with respect to the interaural line. An atlas was therefore constructed for coronal PMRF sections cut in the plane as described above (Douglas and Farham, 1983). A close approximation to the latter is the Atlas of Paxinos and Watson, (1982) where the incisor bar is positioned at a level 3,3mm below the horizontal zero, thereby achieving the "flat-skull" position. The coronal sections of this atlas are very similar to that of the Douglas and Farham atlas.

Surgical techniques

A number of different surgical procedures were used in this study. These are described in further detail in the relevant sections. However a few general points will be mentioned in this section. The rats were anaesthetized via intra-peritoneal administration of anaesthetic agents. Once surgically anaesthetized they were placed on a heating pad. A thermistor probe inserted into the rectum provided the input signal for the heating pad controller. Rectal temperature was maintained at ±37.5°C. The trachea was exposed and an endotracheal cannula inserted to facilitate clear air passages. The incision was sutured to prevent fluid loss. This also provided the link between the rat and a small animal respirator when required. In protracted experiments blood pressure was monitored via an indwelling catheter placed in the femoral artery. Blood pressure in excess of 75mmHg diastolic and heart rate, (recorded via ECG leads) between 300-400 beats/minute were regarded as evidence for an acceptable physiological status. An intravenous catheter was also inserted for administration of paralytic agents and/or 10% dextrose solution for prolonged experiments. Cranial surgery was performed once the animal was mounted in the stereotaxic apparatus. In cases where excessive bleeding occurred, or the blood pressure fell below 40mmHg diastolic, the experiment was terminated and the animal sacrificed by an overdose of barbiturate anaesthetic.

Microelectrodes

(a) Glass

Microelectrodes for recording single unit extracellular activity were constructed from borosilicate glass fiber filled capillary tubes (GC100F-10, GC120F-10; Clarke Electromedical Instruments). Fine tipped microelectrodes were pulled on an electrode puller (C.F.Palmer Vertical puller; customized horizontal puller; Kopf 750 Vertical puller). The very fine tipped long shanked (approx 10mm) electrodes were broken back under microscopic guidance to yield tip diameters between 0.5 and 2.0μ. These were filled via the butt-end, by means of a fine polyethylene cannula, with 2.0 to 3.0M NaCl.

The constructed electrode was fitted into the electrode carrier on the stereotaxic apparatus, (Narishige), and coupled to the recording apparatus by means of a chlorided silver wire. The electrode was positioned under stereotaxic guidance in the medial pontomedullary reticular formation at a depth of 6.5 to 8.0mm, within 1.5 to 2mm of the midline; antero-posterior co-ordinates (AP) extended rostrally to the level of the genu of the facial nerve and caudally to the level of the retrofacial nucleus, which is approximately between AP -1.5 to -3.0, i.e. posterior to interaural line.

(b)Insulated Tungsten stimulating electrodes

These electrodes were manufactured from tungsten wire (0.001 ins, Clarke Electromedical Instruments). The wires were cleaned in distilled water and dried by sequential dipping in absolute alcohol and acetone. The wires were inserted into rubber cork. This was attached to a suitable holder and wires were dipped into Epoxylite resin (Clarke Electromedical Instruments) taking care to prevent globule formation on the electrode shaft. These were then baked in an oven for curing of the resin as follows:

30 minutes at 100°C to drive off solvents
30 minutes at 175-190°C to harden resin

This procedure provided satisfactory insulation. The tips of the electrode were exposed by scraping off insulation under microscopic guidance.

Recording protocol

The electrode was advanced fairly rapidly through the cerebellum. En route the recording characteristics of the electrode were established in the various nuclear layers of the cerebellum. Once the 4th ventricle had been traversed, (signalled by a reduced activity of the recording electrode), the electrode was then advanced through the brain stem extremely slowly until extracellular action potentials were recorded from gigantocellularis neurones. These were identified as large well isolated units, 0.5 - 3.5mV, with a duration greater than 0.5ms. Units displaying rapid rise times and short duration extracellular action potentials were regarded as fibers and ignored. As logic pulses generated by a window discriminator were used to log unit activity, only extracellular signals with a signal/noise (S/N) ratio of >3 were recorded due to the discriminator's inability to discriminate reliable lower S/N ratios.

As the majority of cells (76%) within the PMRF have been shown to respond to more than one sensory modality (Siegel, 1979), the cells in the present study were categorized according to their responsiveness to several stimuli. Typically these cells displayed large receptive fields (approximately 20% of the body surface). Receptive field testing carried out on each cell was as follows:-

(a) Non-noxious pressure applied to the tendoachilles (TA) region of the hindleg: applied bilaterally. In an attempt to standardize the stimulus, a large pair of forceps was always used and, by subjective assessment, the same pressure was applied during each test for a period of 2 to 3 seconds.

(b) Non-noxious pressure applied to the mid-region of the tail (Tail): pressure applied with same forceps and exerting an equivalent pressure, according to a subjective rating, for 2 to 3 seconds.

(c) Light stroking of the fur (Fur) in the midline and lateral sides of the body in a postero-anterior direction, and or a gentle air stream directed over the fur by blowing over rat in a caudo-rostral direction. Each of the above body areas was tested for every cell encountered by the electrode within the area delimited by the stereotactic co-ordinates mentioned above. The response to each stimulus was graded according to an acoustic subjective rating of the change in discharge rate of the cell, viz:-

+ or - Weakly responsive
(just noticeable change in firing rate)
++ or -- Moderately responsive
+++ or --- Strongly responsive
++++ or ---- Extremely responsive

Only PMRF cells which were responsive to one or more of the above stimuli were included in this study unless otherwise indicated. The cell's performance as assessed by the acoustic rating was used in conjunction with simple inspection of the basal firing rate as displayed on a digital panel meter (DPM). The output from the pulse ratemeter was led to the DPM for continuous digital display of the basal firing rate. The pulse rate meter could be set to count the number of events in 1, 2, 5 and 10 second bins. In addition to listening to the audio-amplifier output, the change in basal firing rate could also be observed to confirm the acoustic impression. The digital readout therefore greatly assisted in classification of cellular responses at the lower end of the scale.

Histological procedures

General comments

Since a number of histological methods and techniques are used and described in detail in relevant sections of this thesis and as the choice of method has a strong bearing on the outcome of the experiment, a number of general points are pertinent.

In the first instance, any selected method of tissue preparation for a subsequent histological investigation is of neccessity a compromise between good structural preservation and the desire to demonstrate *in situ* certain properties of the cell. As a number of experiments in this study relate directly to certain aspects of fixation involving the sensitivity and application of immunocytochemical techniques, the type of fixation and the subsequent processing of the materials is of great importance.

Fixation is essential to immobilize antigens thereby avoiding their artefactual diffusion and preserving the detailed morphology of structures. However it has to be borne in mind that fixation itself constitutes a major artefact since living tissue is surrounded by fluid in its natural state. The main goal therefore is to arrest enzymatic activity rapidly, sufficient to prevent structural decomposition, due to osmotic damage, autolysis and mechanical manipulations. Furthermore the intent is to arrest the diffusion of peptides and proteins in and out of the cell and to bind irreversibly any tract-tracing material that may have been injected prior to fixation. There are also many serious deleterious effects at the various stages of tissue preparation, viz: cutting, dehydration, clearing and embedding, all of which may introduce artefacts.

The immobilization of peptides and various proteins are best achieved by the cross-linking fixatives para-formaldehyde and glutaraldehyde with the added advantage of good cellular preservation. However cross-linkages due to fixation lead to a deterioration of antigenic properties due to changes of conformational features. Large protein antigenicity depends on primary structure as well as conformation. Since glutaraldehyde forms extensive protein cross-linkages, masking of antigenic sites may occur due to steric hinderance. Considering all these facts it is clearly unrealistic to hope for an all-embracive procedure. Therefore the choice of technique is a trade off between structural preservation on the one hand and immobilization and preservation of antigenicity on the other. Interpretation of experimental results can only be meaningful if the aforementioned limitations are borne in mind. Guided by the particular goal of the investigation, extreme care was therefore exercised in the choice and use of tried and practised techniques for tissue preparations.

In the immunocytochemical methods employed in this study to identify phaseolus vulgaris leucoagglutinin (PHA-L) labelled projections and

GABAergic terminals, the balance between the lowest concentration of fixative material compatible with the most reasonable tissue preservation and maximal retention of antibody binding capacity, were determined in each case by laborious empirical testing in the respective laboratories. The appropriate combination of specifications was employed in the present study.

Hanker Yates reaction for the demonstration of intracellular horseradish peroxidase injected by ionophoresis

It is essential that cellular and ultrastructural preservation of brains containing cells injected with HRP intracellularly be of the highest quality. An improvement on the routine fixation process is the replacement of Technical Grade Glutaraldehyde by an EM-Grade Glutaraldehyde in the Karnovsky's Fixative. In the retrograde HRP experiments, where HRP was deposited extracellularly, the same technique was applied but standard grade Glutaraldehyde was used instead for routine fixation.

The Karnovsky's Fixative is made up as follows:-
80ml Glutaraldeyde (20%, EM-Grade)
10g Paraformaldehyde (dissolved in 400ml double distilled water heated to 60°C. Solution is cleared by addition of 1 pellet NaOH at a time until solution has cleared).
500ml 0.2M Phosphate buffered saline (PBS)
pH of final solution, 7.4 - 7.6.

The delivery of the fixative was either by pressure injection from a very large syringe or entirely via hydrostatic feed. The former method was mainly used for the intracellular HRP experiments and the latter for routine anterograde HRP tract-tracing.

Perfusion method
Expose the heart by making a transverse incision just below the thorax. Make a transverse incision in the diaphragm and then cut through the ribs laterally. Reflect the thorax towards the head and clamp with large artery forceps. Inject Heparin (approx 100 Units) into the left ventricle. Clamp the descending aorta and insert a 18 Gauge needle into the left ventricle which is attached via a flexible piece of tubing to a 100ml syringe containing normal saline. As the heart distends with the injected saline, a slit is made in the right atrium, to allow blood and perfusate to escape. The animal is then rapidly pressure perfused transcardially with 200ml normal saline, followed immediately with 200ml Karnovsky's Fixative. Fasciculation should occur within seconds of the fixative entering the vasculature. This is a good indication of the outcome of the perfusion. In my experience poor fixation of neural tissue is usually correlated with weak fasciculation, thus possible remedial action may be taken at this crucial stage to improve one's chances of a successful perfusion. A further 300ml of Karnovsky's fixative over a period of 20 to 30 minutes is admininstered by slow hydrostatic feed.

Histological procedure

Two methods of preparing sections were used in this study.

(1) Frozen sections

Some of the brains that were injected with HRP were cut using the freezing stage sledge microtome. These brains, prior to cutting in this manner, were cryoprotected by immersing in 30% sucrose phoshate buffer overnight, or until they had sunk to the bottom of the container. This step is necessary for reducing ice-crystal artefact.

(2) Freshly fixed sections

A number of brains were cut using the Oxford vibratome. Immediately after the brains were blocked out, sections were cut.

Method

Glue the blocks of tissue to the Teflon mounting block with a drop of cyanoacrylate glue (Cyanon, Locktite or Blitzstick are suitable). If the block of tisssue is particularly large it may tend to distort while being cut. This may be remedied by stabilizing the block with a 4-6% agar in saline solution. As the agar cools down, pour around the base of the block, building up the layer of agar around the tissue in a pyramidal form. The intention is to have as broad a base as posssible. Trim away the excess agar once it has solidified. Set the vibratome to cut sections 75μ thick. Using the speed control, set for rapid advance, trim away the excess tissue, reducing the speed of advance as the target area is approached. At speed setting 2 the blade cuts through the block in approximately 20 seconds. This was found to be optimal. Note: higher speed increases frictional drag, causing distortion of the tissue. Float the section off the blade into the surrounding buffer, transfer to a petri dish containing 0.1M PSB and wash off any adherent agar. Maintaining strict sequence, place the section in a well in the staining rack and ensure that the sections are covered with buffer. Repeat the procedure until the entire block has been cut.

As soon as possible after cutting the sections they should be reacted for HRP. My subjective impression is that sections left too long in buffer have a relatively weaker reaction to HRP, and hence are much fainter.

HRP reaction according to the method of Hanker et al., (1977)

0.3gm pyro-catechol
0.15gm para-phenylenediamine
In 300ml Cacodylate buffer (pH 5.1)

For nickle intensification
1.2 gm Nickle Ammonium
1.8 gm Cobalt Chloride
In 300 ml distilled water

Osmication

As osmium tetroxide is highly volatile and extremely hazardous, adequate safety precautions have to be observed at all times. Stock is kept as a 4% solution of osmium tetroxide in a sealed dessicator jar in the fridge. For protection of hands, disposable rubber gloves should be worn at all times and to prevent mucous membrane damage fumes should not be inhaled. The reagent should therefore only be handled in a fume cupboard, and rubber gloves worn at all times.
For the reaction a 1% solution is made up in 0.1M phosphosaline buffer (PSB) (4ml of 4% OsO_4, 4 ml ddH_20 and 8ml 0.2M PSB).
Serially cut sections were then transferred to this osmium solution and incubated in the fume cupboard for 40 mins, with intermittent agitation. However shorter incubation times (25 minutes) were found by trial and error to be suited to brain stem sections. Longer incubations tended to stain sections too darkly. After the incubation, the sections were rinsed three times for 10 minutes periods in 0.1M PSB.

Dehydration sequence

Transfer sections to 50% alcohol for 10 minutes.

Incubate sections in 70% alcohol containing 1% Uranyl Acetate for 45 minutes in the total darkness. Immediately prior to use, dissolve uranyl acetate in 70% alcohol with high speed stirring in the dark. Then the following sequence is followed:-

90% alcohol - 10 minutes

95% alcohol - 10 minutes

Absolute alcohol - 2 x 15 minutes
(Absolute alcohol dried with anhydrous Copper Sulphate)

Transfer sections rapidly to glass test-tubes containing Propylene Oxide - 2 x 15 minutes.

Extreme care should be exercised at this stage and subsequently, as sections are extremely brittle and easily damaged.

Transfer sections rapidly from the propylene oxide to the containers with the mounting medium (Durcupan; ACM Fluka). Leave overnight (minimum of 12 hours) in an oven at 35 deg C.

As the embedding medium is carcinogenic in the uncured form it is advisable to dispose of it and its container only after it has been fully polymerized by being baked in an oven at high temperature. For this purpose one can fashion containers economically from tin foil. The appropriate shape is in the form of a narrow boat of approximately the following dimensions: 8 x 10 x 100 mm. Serial sequence is strictly maintained in the "boats" containing Durcupan.

Finally, transfer sections to absolutely clean glass slides which have been siliconized by wiping with a silicone impregnated cloth.

Mounting technique (Method employed at MRC ANU Oxford)

The main pitfall of this technique is that an excessive amount of Durcupan mounting medium may be inadvertantly transferred onto the slide during the mounting of the sections. The effects are twofold, and both result in an unsatisfactory or even unusable end product. Firstly, a thick layer of mounting medium tends to run when the slide is fitted with a cover slip and as a result the sections become re-arranged, with possible disruption of the sequence. This is particularly irritating when the microanatomy of a neurone has to be reconstructed microscopically. Secondly, a thick layer of mounting medium negatively affects the resolution of the section. Thus an excessive amount of mounting medium is to be avoided.

Transfer sections with a thin paint-brush, orientate each section correctly and place on the siliconized slide. A number of sections are usually arranged on a single slide, thus the quantity of mounting medium transferred with the section and the brush is usually sufficient. The sections are transferred from a warmed boat (37°C), thus reducing the viscosity of the mounting medium, and hence facilitating manipulation of the sections. Slides may also be laid on the hot-tray; this assists orientation of sections on the slide. A note of caution: Durcupan will start to cure if the temperature is too high (e.g., hotspots on the warming tray).

Once the sections are positioned, distribute the Durcupan with the brush evenly over the slide amongst the sections, avoiding bubble formation. A clean siliconized cover slip is positioned at one end of the slide, and very gently lowered onto the sections. The Durcupan should flow freely and without bubbles. Any bubbles that are formed should be cleared immediately by gentle pressure on the cover slip with a dissecting needle.

If a bubble does form under the cover slip, the latter can be lifted at one edge by inserting a blade under it. A small amount of Durcupan may now be run in from that side. This procedure executed incorrectly is potentially very harmful to the fragile sections.

Once the cover slip is in position, put it on a warming plate and, with thin strips of Whatman number 1 filter paper, draw off the excess mounting medium. This effectively removes most of the excess Durcupan. The end point is reached when the Durcupan just starts to withdraw from the edges of the cover slip.
Put slides into an oven at approx 65°C and permit to polymerize overnight.

HRP procedure for tract-tracing experiments

The same basic procedure for demonstration of HRP was also employed where HRP was used for anterograde tract-tracing. Except for the previously mentioned differences in fixation, the cutting and histochemical procedures were identical. However, after the HRP reaction procedure, the sections were mounted on gelatinized glass slides and left to air-dry overnight. The sections were then lightly counterstained by means of the routine Cresyl Fast Violet staining procedure, dehydrated and then mounted with DPX mounting medium. None of the tract-tracing HRP sections were exposed to the osmication and special dehydration sequence used on the intracellular HRP sections.

Ionophoresis and multibarrel glass electrode construction techniques

Ionophoresis, or iontophoresis, are terms used to describe the electrophoretic migration of ions by current flow. This technique is applicable to the administration of substances to either the intracellular or extracellular milieu of cells and can be conveniently used in combination with electrical recording of cellular responses from muscle, glandular and nervous tissue.

The particular advantage of the ionophoresis technique lies in the relative ease with which the diffusion and ejection of substances from micropipettes with small (typically 1-2μ) orifices can be controlled. The substances can be injected intracellularly or upon localized membrane areas extracellularly, with minimal tissue damage, and is the only method available for testing neuronal responses to transmitter substances, their agonists and antagonists *in vivo*. The major advantage of this technique is that extracellular activity can be monitored constantly while ionophoresing the test substance.

There is still a large amount of uncertainty as to the correct biophysical description of the passage of ions through small orifices. The general consensus is that Hirtoffs Law best describes the passage of ions through the pipette. For review see Globus, (1973) and Purves, (1981).

The electrode best suited to this type of study was the customized multibarrel glass, filament-filled electrode. The 7-barrel glass capillary (7GC100F-10, Clarke Electromedical) configuration was favoured as opposed to a 5-barrel configuration, the reason being that the symmetrical arrangement of the barrels permitted easier insertion and holding in the chuck of the C.F. Palmer electrode puller.

Construction of the electrode

The construction of the multibarrel electrode suitable for simultaneous ionophoresis and recording is critical. The success of the experiment ultimately depended on good recording and ejection characteristics of the electrode; thus discussion of the construction technique would not be out of place. All the multibarell electrodes were pulled on the C.F.Palmer vertical puller. It is absolutely critical that the chucks be aligned with respect to each other before the electrode is clamped into place, failing which, a torsional stress would be imparted to the glass, causing the glass to shatter, or in the worst case, causing the barrels to be twisted.

The solenoid microswitches were positioned so that the pull was initiated after an initial short free-fall. In addition altering either the solenoid pull or the coil heater setting, the shape of the electrode could be altered to suit ones particular requirements. The approximate settings used in this study were: solenoid 63, and heater 92. Under these conditions the relatively high heat setting yielded an electrode with a relatively long shank (16mm), with a gradual taper, ending in

an extremely fine, high impedance tip. This type of tip was found to be unsuitable for extracellular recording and ionophoresis, so the tip was routinely broken back manually under microscopic control. The tip size found to be ideally suited to the experimental procedures employed in all the ionophoretic studies had an overall tip diameter of 8-12μ. This gave an orifice size of approximately 1-2μ for each individual barrel. The breaking back of the tip is a trial and error procedure. It was undesirable to attempt breaking back the tip to the desired size repeatedly as the repetitive impacts probably caused very fine cracks along the individual barrels. Invariably electrodes manufactured by this means gave noisy low signal to noise recording and poor ionophoretic characteristics.

As a precaution against confusing the contents of the ionophoretic barrels, individual barrels containing test substances were colour-coded and matched to a specific ionophoresis pump of the Neurophore. The central barrel was filled with 2-3M NaCl and served as the recording electrode, and one of the outer barrels served as a balance channel for the ionophoresis and was similarly filled with 2-3M NaCl. The remaining barrels were filled with the test substances according to the chosen colour code.

Multibarrel filling technique

The filling procedure was also a critical step for successful ionophoretic deposition. Filling was achieved by means of a finely pulled polyethylene cannula attached to a 1cc syringe which could be inserted as far as the shoulder of the electrode. As the tip of the filling cannula was difficult to see in the solution of the barrel, it was marked with permanent ink. Great care was exercised to avoid air-bubble formation and spillage. This was achieved by expelling fluid as the cannula was withdrawn and only filling the barrel about halfway. This precaution was necessary to minimise the chances of spillage of contents into neighbouring barrels. The butt-end of the electrode was then blotted with tissue pledgets and swabbed with iso-propyl alcohol. Ambient air drying was found to be appropriate. The filled electrode was then secured in the headstage holder and the connections made between the recording system, ionophoretic current source (Neurophore) and solutions in the barrels by means of silver-silver chloride wires. These were inserted into the respective colour coded barrels under microscopic guidance.

If test solutions were inadvertently spilled across the top of the barrels, fluid was invariably drawn to the tips of the vacant barrels by capillarity, aided by the fiber. If such spillages occurred the electrode was abandoned. Furthermore, electrolytes remaining on the butt-end of the electrode were the probable cause of short-circuiting between the barrels, and over-ranging of the Neurophore. The wires in the respective barrels were then glued in place with clear silicone

glue at their point of entry, ensuring that there was no contact between them. As leakage from the tip orifices could conceivably also occur before the braking currents were applied, placing glue on the butt end of the electrode effected a good seal.

Testing procedure

The electrode was lowered onto the surface of the cerebellum and inserted to a depth of about 1mm. It was left in this position for a few minutes to allow the silicone glue to cure. The performance of the ionophoretic and recording barrels was then assessed. As the retaining currents had been running for a long time, the ejection currents for each barrel were left on for a few minutes. If the performance was satisfactory the electrode was advanced to the target area. Generally, ejection currents were cationic and varied between 20 and 100nA, whereas retaining currents were of opposite polarity and usually of the order of 5nA-15nA (negative).

Details of the respective solutions used in the ionophoretic barrels is given in the relevant sections of thesis. Concentrations of drugs reported in the literature usually ranged between 5mM and 3M, those used in this study were in the lower end of this range. Commercially obtainable transmitters are usually available as relatively acid salts and some researchers attempt to acidify the solution further with dilute HCl with the aim of increasing their dissociation. As this contributes to possible pH effects on neuronal activity, all drug solutions used in this study were made up as a 10% solution in double distilled H_2O.

Section 1.3.0

Results and Discussion

Caudal projections of the medial pontomedullary reticular formation neurones mapped by combined electrophysiological and retrograde horseradish peroxidase techniques

Introduction

The electrical activity of neurones of the medial pontomedullary reticular formation is correlated with both sensory (Carpenter et al., 1962; Carpenter et al., 1966) and motor mechanisms Pompeiano (1973).

There have been many studies concerned with the organization of the pontomedullary reticular formation, some purely descriptive of the anatomical organization, e.g. noteably the early studies of Taber, Brodal, etc. in the 1960's and some of the more recent studies by Kuypers and Maisky, (1975) and Edwards et al., (1987) and then those which can be broadly grouped as specifically aiming to ascribe functional organization of the cells within this area, noteably Basbaum et al, (1978). Although their functional connectivity has not been completely identified, it is widely accepted that these neurones have a major projection to spinal segmental levels (Peterson et al., 1975; Burton and Loewy, 1977). Evidence for spinal projections of the PMRF neurones was obtained originally by electrophysiological studies investigating their sensory properties referred to above and axonal degeneration studies (Torvik and Brodal, 1957). Early anatomical methods such as the Marchi and Golgi techniques have contributed significantly to the basic understanding of the anatomy and organization of the brain stem reticular formation and many of the earlier findings are still widely recognised today.

The reticular connections may be broadly divided into two groups on the basis of data obtained by the older degeneration techniques and the study of retrograde changes due to lesions. Abols and Basbaum, (1981) using the autoradiographic technique have demonstrated that the rostral medulla in the cat can be divided into three subzones on the basis of their differential projections descending to the spinal cord. The midline nucleus raphe magnus NRM projects to the spinal cord via the dorsolateral funiculus, terminating extensively in the Rexed lamina of the spinal dorsal horn; the adjacent nucleus reticularis magnocellularis (RMC) projecting via both the dorsal and the ventrolateral funiculi and terminating ipsilaterally in both the dorsal and ventral horn; the nucleus reticularis gigantocellularis (RGC) projecting both via the ipsilateral ventrolateral funiculus and the contralateral ventral funiculi, terminating in a restricted zone within the ventral horn.

More recently autoradiographic tract-tracing techniques applied to brain stem (Martin et al., 1985) have shown that the dorsomedial parts of both the nucleus pontis caudalis and oralis project ipsilaterally to the spinal cord, terminating in lamina 8 and 7. This projection like the axons of RGC extend the length of the spinal cord and are thought to be involved with somatic motor function (Peterson, 1980).

Although the origin and course of the spinal projections of reticular nuclei in the PMRF have been investigated by histological procedures such as autoradiography (Martin et al 1985) and HRP (Zemlan and Pfaff, 1979), there have been very few studies, however, which demonstrate a correlation between the physiological and

anatomical methods. This is particularly a problem where tracing compounds are applied "blindly", using only stereotaxic co-ordinates as a criterion for location of the injection site. More recently Zemlan et al (1984) have used the evoked peripheral motor activity obtained from a stimulating electrode as a criterion for the correct functional placement of the deposition site.

The aim of this investigation was therefore twofold: firstly, to identify the distribution of PMRF neurones displaying classical sensory responsiveness, by combined retrograde HRP tract-tracing and electrical stimulation of their caudal projections; and secondly, to trace the route of their HRP stained projections.

Methods

21 Rats were used in this study. Rats were anaesthetised with either urethane (1.2g/kg) or chloral hydrate (450mg/Kg) and then fitted, firstly, with a short polyethylene endotracheal tube to facilitate clear airways and thereby also enabling ventilation of the rat by attaching it to a small animal ventilator via the endotracheal tube and, secondly, with an intravenous femoral catheter for chronic administration of muscle relaxant and or 0.9% saline as required. The rats were then mounted in a stereotaxic frame (Narishige) and muscle relaxed by infusion of Pancuronium Bromide at a rate of 0.03mg/ml/hr by means of an infusion pump (Harvard Apparatus). The paralysed rats were maintained on positive pressure ventilation using a small animal respirator. Expiratory CO_2 was kept within 4.0% by adjusting the stroke volume (3-8 cc). A laminectomy was performed at C4-5, and a burrhole was constructed in the occipital bone overlying the rostral cerebellum.

A combined stimulating and horse-radish peroxidase (HRP) deposition cannula was constructed from a 30G stainless-steel needle. The cannula was insulated over it's complete outer surface with vinyl except for the bevelled tip. HRP was delivered via a length of polyethylene tubing attached to a 10ml Hamilton syringe. The cannula was also connected to one pole of a stimulus isolation unit.

Glass microelectrodes, filled with 3M NaCl and typically with tip impedances of 5-10 MΩ, were used to record extracellular action potentials from neurones of the medial pontomedullary reticular formation. The neurones were identified as described below. The cannula was positioned in the ventromedial funiculus of the spinal cord under microscopic and stereotaxic control. Stimuli of 0.15 ms duration and amplitude of 20-600 mA, provided by a stimulus isolation unit were used to activate the MPRF neurones. 2-4ml of 4% HRP (Sigma type VI) were deposited at the stimulation site which evoked optimal antidromic activity. The animal was then maintained under anaesthetic and muscle relaxant for the duration of the experiment.

At the end of the experiment the final co-ordinates of the stimulating and recording electrodes were carefully noted. The rat was then removed from the stereotaxic apparatus and perfused transcardially with Karnovsky's fixative. The brain stem with the spinal cord attached was then blocked out on the stereotaxic apparatus and immersed in 20% sucrose phosphate buffer until the block had sunk. 50-100μ frozen sections of spinal cord and brain stem were then cut in the coronal plane and immediately reacted for HRP according to the procedure of Hanker et al,(1977). Once the sections were mounted on gelatinized slides they were left to dry overnight.

The sections were then counterstained with Cresyl Fast Violet, mounted with DPX mounting medium for a permanent record and examined under a light microscope. The labelled axons and somata of the PMRF neurones were identified by the retrograde uptake of HRP. Some sections were photographed via the camera attachment, or drawn by means of the camera lucida.

Protocol and calculations

PMRF neurones were identified by their stereotaxic position and their sensory responses to non-noxious tail pinch, tendo-achilles pressure, and gentle fur stroke and/or a gentle air stream directed postero-anteriorly over the animal. Identification of these cells was also confirmed electrophysiologically on the basis of their large biphasic extracellular action potentials, typically, of 1.5 - 2.5msec duration and 2.0-5.0mV amplitude. Extracellular signals with rapid rise times (±1.5 ms) were regarded as signals arising from fibers and ignored. Futhermore, PMRF cells were also identified on the basis of antidromic activation by a focal stimulating electrode located in the spinal cord at the cervical level C4-5. Once the sensory response of the cells had been recorded, and the stimulating electrode optimally placed for antidromic activation of a number of cells, HRP was then deposited via the stimulating electrode in small incremental pulses (±0.5ml) over 10mins. The electrode was then left in this position for the duration of the experiment. Survival times ranged from 4-20 hours.

The electrodes were then repositioned at their final co-ordinates. The difference between the horizontal planes of each electrode tip as well as the difference in antero-posterior positions of each electrode were accurately measured. These values were used to calculate the linear distance between the electrode tips by means of the Pythogorean theorem. This value was then used in each case as a close approximation of the distance the antidromic pulse and HRP had to travel and was used to compute the conduction velocity and HRP transport rate.

Results

The responses of PMRF cells to electrical stimulation of the spinal cord are summarized below in the Table 1.3.1(i)

Table 1.3.1(i)

Non responsive	Anti or ortho	Anti	Ortho	Anti+Ortho
49/139	91/139	74/91	17/91	16/91
35%	65%	81%	19%	18%
Mean Latency(ms)		1.00	1.99	
±Sem .		±0.048	±0.143	

Where:
Anti or Ortho = PMRF cell activated either by antidromic or orthodromic stimulation or both
Anti = antidromic activation
Ortho = orthodromic activation
Anti + Ortho = PMRF cell activated both antidromically or othodromically

The above table shows the typical responses of PMRF neurones to stimulation of the cervical spinal cord in the ventromedial funiculus. Typical PMRF extracellular action potentials to antidromic or othodromic electrical stimulation traces are shown in Fig 2.1.3a and 2.1.3b. The latencies of the responses are also reported in Table 1.3.1(i)

The reponses of 139 PMRF neurones rats were tested to stimulation of the ventral spinal cord. 91/139(65%) PMRF cells could be activated either antidromically or orthodromically. Of those 74/91 (81%) were activated antidromically, while 17/91(19%) were activated othrodromically. Antidromically activated cells had a mean latency of 1.00 ±0.048ms, while othrodromically activated cells had a mean latency of 1.99 ±0.143 ms. A small number 16/91(18%) of these cells, were activated by both antidromic and orthodromic stimulation. In 9 rats where the distance between the stimulating electrode and the average position of the recording electrode was reliably measured, the conduction velocity of these fibers was computed from the latency measurements of antidromically activated units.

The mean conduction velocity of reticulospinal fibers in these 9 cases was 9.15m/s ±0.619 . This value is considerably lower than those reported for typical reticulospinal fibers. The majority (90%) of reticulospinal fibers are reported to have

conduction velocities in the range 90 - 130m/sec (Magni and Willis 1963). Medial reticulospinal tract fibers (MRST) are reported to have conduction velocities ranging from 14 to 150 m/sec, with a median value of 101 m/sec (Peterson et al., 1975). RGC fibers also contribute to the ventrolateral funiculus mainly on the ipsilateral side and like MRST fibers these fibers also have a wide range of conduction velocities viz., 11 to 150 m/sec, with a median value of 69 m/sec (Peterson et al., 1975). It is well known that fibers of many varying diameters comprise the reticulospinal tract and these are positively correlated with the size of the source cell. This probably accounts for the large range in reported conduction velocities. However in the present study the calculation of the conduction velocity from the latency of the antidromically activated cell and the distance between the recording electrode and stimulating electrode is fairly crude. The large margin or error inherent in this technique may account for this discrepancy.

21 animals were injected with HRP at approximately C4-C5. In 13 of these animals the HRP was deposited in the ventral cord at a site from which neurones of the MRF could be antidromically activated from the stimulator tip of the HRP injection cannula. In the remaining 8 animals, the HRP was deposited in the ventromedial funiculus on the basis of stereotaxic co-ordinates only, and no antidromic identification was attempted.

Survival times ranged from 4 to 20 hours. At shorter survival times the HRP extended up the cord, and sometimes into the caudal medulla, but did not necessarily label somata. The time of arrival of HRP at the caudal end of the medulla was measured in one rat where the time of HRP injection and exact time of sacrifice was known. The distance from the injection site to the furthest level showing HRP stained axons was measured as accurately as possible. According to these measurements the transport rate of HRP is of the order of 1.6mm/hr. This is only an approximate value and was used to ascertain the minimum time required for HRP to reach the pontomedullary level. This value does not include the time required for maximum accumulation of HRP and consequently optimal staining of these neurones. This value is lower than reported values of 2.9 to 5mm/hr for retrograde HRP transport (Warr et al., 1981) and can probably be accounted for in terms of different criteria employed.

However on the basis of these measurements the minimum time for HRP arrival in the caudal medulla was estimated to be of the order of 5 hours. Hoewever, only those animals with survival times in excess of 8 hours exhibited optimal labelling of the PMRF neurones. Therefore in general, the anticipated relationship between survival time and the extent of the HRP displacement towards the brain stem was observed.

The siting of the electrode tip, although obscured by a region of non-specific HRP staining, could be determined with a reasonable degree of certainty by examination of adjacent sections which contained a cluster of heavily labelled axons coursing in the same ventral position corresponding to the more caudal deposition site. Figure 2.1.2A and Fig 2.1.4 show a camera lucida reconstruction and photograph respectively, of typical coronal cord sections slightly rostral to the HRP deposition sites confirming the region of effective uptake in the ventromedial funiculus. In 8 cases retrogradely filled cells showing strong HRP uptake the deposition sites were

located in the ventromedial funiculus (VMF), while in the remaining rats the deposition site was in the region of the ventral white and gray commissures of the cord.

In the 8 animals where the tip of the combined stimulation/HRP electrode was placed in the VMF, the HRP tracer was largely restricted to the thick axons of the ventromedial funiculus (Fig 2.1.4). The labelled axons are largely ipsilateral, but a small fraction of contralateral fibers were also labelled. The majority of labelled axons maintained this location in the cord as they coursed rostrally to the craniocervical junction. Two features of these projections were noted: (a) many axons lay in the coronal plane of the spinal cord sections, extending from the VMF into the ventromedial gray matter, and also more dorsally into the intermediate zone, and (b) a small fraction of labelled axons crossed from the ipsilateral VMF to the ventral region of the contralateral cord. It was not clear what the destination of this latter group of fibers was. No labelled somata were observed in the surrounding spinal gray. Although it may have been possible that those that were labelled were masked by the non-specific staining at the HRP deposition site.

At the craniocervical junction the axons course dorsomedially to gain access to the dorsal region of the medial longitudinal fasiculus (MLF). The fibers were particularly dense along the lateral border of the MLF (Fig 2.1.5). At this point the number of ipsilateral versus contralateral labelled fibers was even greater than that observed near the injection site at cord level.

As the axons course rostrally from the craniocervical junction their positions are maintained in the dorsal half of the MLF. Above AP -6.0 some of the axons begin to branch out of the MLF. The branches take three routes (Fig. 2.1.2c):- (1) swinging out into the ipsilateral surrounding region of giant somata; (2) gaining entry to the contralateral giant field by curling over the dorsal extremity of the MLF and then descending ventrolaterally into the giant cell region; and (3) a few fibers cross the midline within the MLF. This is a feature of the most rostral 'dispersal' of the axons.

The dominant population of labelled somata were those of the giant cells located between the genu of the facial nerve rostrally and the caudal extent of the retrofacial nucleus posteriorly. The rostral limit of labelling coincided with a sharp reduction in the fraction of giant somata in the parasagittal fields. Very few small somata were labelled in this region. The most caudal limit of labelled somata was variable. This may be correlated with either the rostrocaudal position of the injection site, and/or the location of labelled fibres in the MLF. Mainly giant cell somata in the parasagittal fields were labelled. In addition to the labelled giant somata, some labelling was detected in the vestibular nucleus complex. This labelling was bilateral, even in those instances in which the MLF contained almost exclusively ipsilateral labelled axons.

In 4 animals where HRP was deposited via stereotaxic co-ordinates only, the bulk of the deposition occurred in relation to the ventral white and gray commissures of the cord. The labelling from this site had two major features: (a) bilateral labelling of axons in the VMF which then coursed rostrally by the same route, and with the same features as that described above for the MLF Group, and (b) labelling of axons and somata whose range was restricted to a few 100μ sections above the deposition site.

The labelled somata of the latter group (b) were disposed mainly in Rexed layers 8 and 9 bilaterally. Labelled axons coursed in the plane of the section throughout the spinal gray, and in the anteroposterior axis in a narrow band surrounding the spinal gray from the ventrolateral aspect of the ventral horn, posteriorly to the lateral aspect of the dorsal horn. These longitudinal axons also extended only a few sections (100m thick) beyond the HRP deposition site.

Discussion

The reticulospinal neurones in the medullary area which give rise to spinal cord projections lie in the ventral and dorsal medullary nuclei as well as in the RGC, RGC pars α, ventral reticular nucleus and in the paragigantocellular nucleus (Basbaum et al., 1978; Basbaum and Fields, 1979; Huisman et al., 1981). Neurones within RGC contribute axons bilaterally to the ventral and lateral funiculi with a predominance of ipsilateral fibers (Zemlan and Pfaff, 1979; Martin et al., 1985).

In the present study HRP labelled axons were confined predominantly to the ventromedial funiculus ipsilateral to the injection site. The axons coursed rostrally into the dorsal part of the MLF and then swung sharply in a ventral and lateral direction. HRP labelling was confined predominantly to the giant cells of RGC and RPC, but not all of the giant cells were labelled with HRP. Approximately 80% of the giant neurones on the ipsilateral side were labelled while about 40% on the contralateral side were also labelled.

The projections of the RST neurones have been determined using histological tract-tracing techniques in the rat (Leichnetz et al., 1978; Zemlan et al., 1979; Zemlan et al., 1984) and in the cat (Basbaum and Fields, 1979; Holstege and Kuypers, 1982). In the study by Zemlan et al., (1979) comparison of their results with the present study is difficult due to the different approach used. These authors placed unilateral spinal transection which included either the lateral or ventral columns, or both, at the cervical level C2-C3 and thoracic level (T10). The most medial portions of the ventral columns were spared by their transections, and so were not labelled with HRP; furthermore HRP was applied directly to the cut axons. This must have resulted in huge numbers of non-specifically labelled axons. Their diagrams show labelling restricted to axonal fields only, without any labelling of the central gray. These data are difficult to reconcile with the large areas of non-specific HRP labelling. Application of HRP to ventral and lateral columns at C2-3 lead to labelled somata throughout the ventral portion of ventral reticular nucleus (NRV). Labelling of this area is not observed in the present study. At a more rostral level Zemlan et al., (1984) observed dorsal and ventromedial labelled somata on the ipsilateral side. In the present study ventro-medial cells are not as prominant.

At the level of the facial nucleus (V11), labelled somata are also present in the contralateral medial RF. More rostrally the cells dissipate bilaterally. In this study labelled somata are encountered predominantly in the dorsal and medial areas, with extension across the medial pontomedullary reticular formation in a ventrolateral direction. Anterior to the facial nucleus labelled somata begin to fade out.

Application of HRP to the lateral column alone leads to similar labelling of the NRV and ventral RGC as that seen in the more extensive application i.e ventral and lateral columns in the aforementioned study. However, both ipsi and contralateral dorsomedial somata were not labelled. These authors therefore deduced that the dorsomedial RGC somata project in the ventral columns, which is consistent with the findings in the present study.

Application of HRP to the ventral columns at T10 did not yield labelled somata in the dorsomedial RF. The authors therefore concluded that the dorsomedial RF projects only to the rostral cord. The HRP tract-tracing studies of Satoh, (1979) show that neurones in the dorsal division of the RGC were almost equally labelled after injection of HRP at all spinal cord level. However Satoh's study demonstrates that cells in the ventral part of RGC show distinctly less labelling after cervical cord injection compared to thoracic cord injection. These data suggest that the giant multipolar cells found in the more dorsal division of RGC and those in the more ventral zone (RGC pars fα) which are the small to medium sized cells, form two distinct divisions within the RGC. The cells in these divisions may project differentially to two different terminal fields and innervate different levels of the spinal cord. However, Satoh's study does not trace the route of the fiber pathway and the cervical HRP injections into the cord do not appear to differentiate between the medial and lateral reticulospinal tracts.

More recent autoradiographic studies by Martin et al., (1985) have shown that neurones of the RGC contribute axons bilaterally to the ventral and lateral funiculi, with a strong ipsilateral predominance. RGC axons extend the length of the cord distributing axons to lamina 7, 8 and 10. In particular the ventral region of RGC has been seen to innervate motorneurones in the lumbar cord (Holstege and Kuypers, 1982). This study is therefore in broad agreement with Satoh, (1979) in that the ventral zone of RGC seems to project further caudally than the rostral zone.

The anterograde degeneration techniques used by Nyberg-Hansen, (1965) and Petras, (1967) demonstrate the existence of 2 reticulospinal projection systems, one originating in the pons and the other in the medulla. Electrophysiological mapping of these projectons by Ito et al., (1970) and Peterson et al., (1975) confirm and extend these findings. These studies have demonstrated more specifically the existence of three topographically distinct regions within the reticulospinal system that project via both the ventromedial funiculus (VMF), ipsilateral (VLFi) and contralateral ventrolateral funiculus (VLFc). The neurones projecting via the VMF are located primarily in the rostrodorsal zone, and those projecting via the VLFi occur mainly in the caudoventral zone. According to this scheme rostrodorsally located neurones project mainly to the upper cervical regions via the VMF. This is in broad agreement with the findings of Zemlan et al., (1979).

In Zemlan et al's (1979) study the ventral column lesion at the level of T10 spared the most medial region of the column, and also its extension dorsally along the sulcus (Zelman et al., 1979, Fig 2e). The material from the present study demonstrates that an appreciable fraction of the RST fibers are located in that region. By analogy with the gracile and cuneate components of the dorsal columns, it is likely that the most caudal projections of the RST could be located in the most medial region of the anterior axonal fields, which were spared in the T10 HRP applications in the Zemlan

et al., study. In support of Zemlan et al., (1979) is Zemlan et al's (1984) observation that descending RST fibres observed by autoradiographic tracing became sparse at thoracic levels. However, Zemlan et al. do not describe the path of the HRP labelled axons in cord or brain stem.

In the more recent study by Zemlan et al., (1984) autoradiographic techniques were applied to define the projection sites of NMC and RGC. Their injections into RGC and NMC were guided by evoked response to PMRF stimulation. However, no details of this technique are provided in the article, nor do they appear in the previous work which they reference. Cervical application of HRP revealed retrogradely stained HRP cells in the ventral part of nucleus reticularis ventralis, lateral reticular formation, RGC and in the magnocellular area. The number of cells was greatest at rostral levels on the ipsilateral side, but RGC cells were also labelled bilaterally. Comparing the labelling from more caudal deposition sites Zemlan et al., (1984) concluded that the dorsolateral RGC projects only to rostral levels of the cord and ventral RGC projects to both rostral and caudal levels. In their study HRP was applied non-specifically to one hemisection of the spinal cord. Therefore, although the present data is in broad agreement with their findings, direct comparison is complicated by the fact that HRP, applied to the whole hemisection of the spinal cord, labels cells projecting to the ventromedial and ventrolateral divisions of the spinal cord.

Conclusions

Since a large component of ipsilateral cells in the nucleus reticularis gigantocellularis were labelled retrogradely from ipsilateral deposition of HRP within the ventromedial funiculus and approximately 60% fewer were labelled on the contralateral side, it is clear that there are bilateral caudal projections from the medial pontomedullary reticular formation. Cells lying in this area project to the ventromedial funiculus at the level of the cervical cord with an ipsilateral predominance.

From an electrophysiological point of view, only PMRF neurones, located on the basis of their stereotaxic co-ordinates and typified by large (≥1.5mV) extracellular action potentials, spontaneous activity and large polysensory receptive fields, were recorded in this study. Many cells defined by these criteria could also be activated antidromically and/or orthodromically from stimulation sites in the ventromedial funiculus. The placement of HRP was guided in some cases by the optimal site of antidromic activation of PMRF cells. Retrograde labelling confirms that these cells occupy a region within the medial pontomedullary reticular formation lying predominantly within the RGC.

Since some neurones are not labelled and some neurones could not be activated by antidromic stimulation sites in the cervical cord, there must be a number of neurones that project only unilaterally, or do not have caudal projections that reach the cervical level of the spinal cord or, in the extreme case, do not have any caudal projections. The technique of combining electrophysiological recordings with HRP tract-tracing in the same preparation is a novel approach and serves to define the population of cells which are the subject of this study.

Cytoarchitecture of single pontomedullary reticular formation neurones stained by means of the intracellular horseradish peroxidase technique

Introduction

The PMRF is composed of many large and small neurones interwoven in a complex network with ill-defined boundaries. To understand the operation of these cells as a group, first requires precise knowledge of their cytoarchitecture and detail of their connectivity with other cellular elements. The computation properties of neurones depends to a large extent on the structure of the dendritic tree. It is here that the neurone receives a vast amount of its input. Therefore to study the dendritic arbor in any detail requires that its structure be accurately known.

The previous section detailed the caudal projection pathway of these PMRF neurones and as such afforded a view of their general morphology due to their retrograde uptake of HRP, but this type of technique does not permit a detailed description of their microanatomy. The main reason being the mass of neurites stained by the HRP as well as the many other background counterstained elements, both of which obscure the detailed cytoarchitecture of the single cell.

The cytoarchitectonics of neurones of the pontomedullary reticular formation have been extensively investigated by a number of researchers using a variety of anatomical techniques based largely on the Nissl and Golgi staining techniques (Taber, 1961).

Few intracellular studies concerning the microanatomy of PMRF neurones have been attempted. Recently however Edwards et al., (1987) have reported on the detailed microanatomy of pontomedullary reticular formation neurones achieved by the intracellular HRP method in the cat. In the rat the microanatomical description of PMRF cells based on the HRP intracellular technique has not been reported to date. Furthermore at the time of doing this study no literature reports were available on intracellular HRP stained PMRF cells in the rat. Consequently the intracellular HRP technique for the investigation of the microanatomy of PMRF neurones was favoured as it has several distinct advantages over the Golgi methods, viz:

(a) The physiological response can be obtained before the cell is filled with HRP;

(b) Ionophoretically applied HRP is confined to a particular cell, compared to the random labelling pattern of neurones achieved with Golgi staining;

(c) Dendritic fields in Golgi stained sections may be partially obscured by other stained processes and/or the Golgi stain may fail to impregnate entire dendritic fields;

(d) The Golgi stain may impregnate some cell types selectively;

(e) The HRP filled cell can be serially sectioned and its anatomy reconstructed by following its processes for considerable distances, whereas with Golgi stained neurones it is difficult or impossible to differentiate between impregnated processes and to trace them reliably from section to section;

(f) HRP stains myelinated axons, whereas myelinated axons do not impregnate with Golgi stain thereby imposing a serious limitation on its use (Feldman, 1984).

Considering these facts together, intracellular HRP offers a more reliable and complete assessment of the morphology of the neurone than obtainable with the Golgi method. Intracellular HRP also has its limitations though, in that it is a technically

demanding procedure, with a low recovery rate and it is generally known that some neurones are more difficult to fill than others (Peters, 1984).

If a neurone is successfully injected with an intracellular dye and successfully recovered in the histological procedure one is then faced with the problem of reliably displaying its spatial characteristics. With the development of sophisticated software there is an option between reconstructing the cell by a graphical reconstruction such as the camera lucida, or using a computer method and mathematical algorithim. With the latter method the image of the cell is represented by a series of points corresponding to their 3-dimensional coordinates in space. The image in each section is traced and fed into a computer. From such a series of 3-D co-ordinates a model of the cells structure can be reconstituted. The computer may then generate different views of the cell's morphology.

Most reports in the literature on cellular reconstructions still generally employ the camera lucida technique for reconstruction of the cellular detail. Although extremely useful, this technique is limited in that only a two-dimensional image can be displayed. Only recently have reports appeared describing cellular morphology using the computer-aided microscope for three dimensional reconstruction. A relatively new 3-dimensional reconstruction technique, achieved by means of a microscope with a motorized microscope stage, in combination with a new digitizing software package called Traka affords a more complete description of a neurone and novel way of examining the cell.

The reconstructed HRP filled cell not only provides a comprehensive description of the geometric distribution of the dendritic surface area but since the entire structure is digitized the accurate dimensions of all the reconstructed elements, not possible by conventional means, are provided. One is therefore able to examine some of the passive electrical properties of these HRP filled cells. An appraisal of the electrical properties of a neurone is impossible if the detailed structure is not known. By combining the accurate geometry with electrical measurements such as input resistance, the electrotonic behaviour of the cell can be predicted.

This study was therefore undertaken with the specific aim of investigating the detailed microanatomy of intracellular HRP filled PMRF cells reconstructed in 3-dimensions by means of a digitizing package. This permits the accurate geometry of the cell to be combined with its passive cable properties.

Methods and material

Female Long-Evans (UCT) and Wistar (MRC.ANU Oxford) rats weighing between 280 and 320 grams were anaesthetized with either Urethane (1.2 g/Kg BWt i.p.) or Equithesin (0.3ml/100gBWt ip.- MRC ANU) The anaesthetized rats were mounted in a stereotaxic apparatus and a burr-hole constructed in the bone overlying the target area. Once the dura was exposed and the cavity thoroughly cleaned of bone fragments and dried, a plastic cylinder constructed from a 2.0ml syringe was cemented to the skull with Fastcure (methylmethacrylate - Kerr Sybron). Once the cement had hardened the dura was incised with a sharp 24 gauge hypodermic needle. Excess CSF was removed with a tissue pledget and the pia incised to provide a small

"key-hole" overlying the target area. Attempts to remove the entire pia or to expose a large area of the underlying cerebellar cortex invariably caused excessive brain movement. However it is essential to puncture the pia before inserting the electrode otherwise "dimpling" of the surface results and the incidence of blocked or damaged electrodes is high. A warm solution of 3-4% Agar in 0.9% saline was injected into the container to a height of approximately 1cm, avoiding air bubble formation. Ideally this should solidify within a few minutes. The aim of this procedure was to minimize brain movement within the calvaria during recording. Once the agar had set, one of two procedures was then adopted. Either the electrode was positioned at a specific co-ordinate and then slowly lowered directly to the region of the brain containing the target area, or the electrode tip was inserted into the superficial layers of the cerebellar cortex and a thin film of low melting point wax then poured onto the surface of the set agar while advancing the electrode as the wax solidified. This method provided far greater stability of the brain than with the agar surface alone and markedly reduced respiratory induced brain movement. The electrode was then advanced by means of a hydraulic drive in steps of 2-5μ into the vicinity of the target cell.

Intracellular PMRF recordings

Intracellular recordings were obtained from giant cells using a glass micropipette electrode filled with HRP solution (4-6% HRP Sigma type V1, in Tris/KCl buffer pH 7.9). The recording electrodes were thus also used as HRP ionophoretic electrodes. The tips were bevelled on a bevelling apparatus to give a tip resistance of 20-80 MΩ. Bevelling was performed either in an Alumina slurry (in 0.9% saline or 2MKCl) or on a saline wetted lapping paper. While continuously measuring the tip resistance the electrode was lowered onto the surface of the slurry or alumina disc at an angle of 45°. The direction of the approach follows the direction of the rotating disc. Both the slurry and the disc were contained in the rotating glass trough of the bevelling apparatus. The electrode tip was then bevelled to the desired tip resistance under controlled conditions. As a general rule low resistance electrodes filled with HRP solution provide better recording and current passing characteristics than very fine tipped high resistance electrodes.

Protocol for intracellular-HRP ionophoresis

The electrode was advanced slowly through the brain stem into close proximity with a PMRF cell until an optimal extracellular action potential could be recorded. The cells were identified in terms of their stereotaxic location and their polymodal sensory receptive fields. If the receptive fields conformed to the criteria as established in the previous studies, one of two procedures was then adopted viz:-

(1) The cell was impaled and HRP immediately ionophoresed. The quality of the penetration was assessed by monitoring a DC trace of the membrane potential, displayed continuously on an oscilloscope. Loss or deterioration of the penetration

was indicated by a sudden or gradual decrement in membrane potential. Assessment of this parameter determined whether the HRP ejection was continued or not and therefore further determined whether the electrode was advanced or withdrawn and moved to a new site. The step change in the DC trace upon withdrawal of the electrode tip from the cell after HRP ionophoresis was also monitored. A good penetration typically showed ±40 mV deflection of the baseline when the electrode was withdrawn. This criterion was used as indication of an acceptable penetration. Only the minority of penetrations were characterized by deflections ≥40mV. Rarely were intracellular penetrations accompanied by shifts in membrane potential greater than 40mV encountered in this study.

(2) A similar procedure to the above was adopted, except that the current passing characteristics of the electrode were first established once the receptive field of the neurone was established. This was an attempt, firstly, to improve the success rate of the intracellular HRP injection and, secondly, to assess the performance of the electrode intracellularly in order to calculate some of the cable properties of the PMRF cells. These steps were necessary for the construction of current-voltage curves and the measurement of the input resistance of the cell. Usually a satisfactory electrode could pass approximately 1nA pulsed current without the tip becoming polarized. An active bridge circuit was used to allow simultaneous passage of current and voltage measurement via the same microelectrode.

HRP was ejected from the tip of the electrode by passing a series of bridge-balanced, hyperpolarizing current pulses through the recording electrode. The amount of current injected and the corresponding change in membrane potential were displayed on an oscilloscope. At the MRC ANU these parameters were digitized via a CED 1401 (Cambridge Electronic Design) interface (Sampling rate 10KHz) and then stored on an AT computer for subsequent analysis.

To ensure positive identification of the HRP filled cells, all penetrations were spaced at least 0.5mm apart.

Method for reconstructing detailed anatomy of HRP-filled cells

(a) Some important considerations

The first problem encountered in a study of this type is that of measuring and encoding the morphology of the neurone. Any compartmental model that accurately represents the details of the neurone's morphology requires that precise morphometric data must be obtained. It is thus necessary that a single neurone be stained in such a way so as to render it easily identifiable in order that the dimensions of the soma, the lengths and diameters of all the dendrites, as well as the axon can be measured accurately. To date the best method for achieving the detailed visualization of cellular morphology is by means of injecting HRP intracellularly, and then reconstructing the cell from serial histological sections. For the purposes of this study, and future computer simulations, it is essential that each of the dendritic branches is uniquely defined. (See system of nomenclature adopted in this study: Appendix Vol. ii).

In making these quantitative measurements a number of inherent errors are involved. These are as follows:-
(a) limitation of the staining technique; e.g. incomplete filling of cell;
(b) shrinkage of tissue during the fixation process;
(c) inherent limits imposed by the measuring system that "discretizes" a continuous structure;
(d) representation of irregularly shaped neuronal segments by geometric entities such as cylinders and spheres.

With regard to (a) and (b) above, possible sources of error were minimized by exercising extreme caution. After the final HRP injection it was ensured that adequate time was permitted for complete uptake and distribution of the tracer. In osmicated sections shrinkage is negligible and therefore not a significant source of error. However for non-osmicated tissue sections, cut from frozen blocks containing the HRP filled cells, i.e. NGC1 and NGC2, an error due to non-uniform shrinkage was present. Therefore to correct for the shrinkage error a correction factor was applied to the reconstructed cell. The shrinkage was calculated at 11% on the nominal thickness of the 100μ slice, (i.e. in the z-plane). Non-uniform shrinkage of the section was assumed, as adherence of the tissue to the slide probably minimized shrinkage in the the x and y planes.

For the purpose of this study it was assumed that both the cellular morphology outlined by means of the intracellular HRP as well as the measurement of the somata and dendrites recorded by Traka were reliable. For a much broader discussion of these points see Rall, (1959) and Clements and Redman, (1989).

(b) Reconstruction technique

Reconstruction of the morphology of the two giant cells injected with HRP at UCT was initially done by means of the camera lucida attachment to the microscope. This method is the only one available in our laboratory at UCT. The main disadvantages of this method are the constraints of having to depict the morphology in 2-dimensions, only being able to reconstruct the morphology in either the coronal or para-sagittal plane and the huge time investment of having to reconstruct the cell by means of an entirely manual operation. Essentially this entails high-power tracing of all the HRP filled fragments of the cell, contained in the serial sections, onto individual sheets of tracing-paper and then reconstructing the eell by transposing all its pieces drawn on the individual sheets onto a mastercopy.
The second method for reconstructing the cellular anatomy was by means of a suite of 3-D reconstruction programs, named Traka. This software package was designed and developed by R.J. Douglas and D. Botha at the MRC ANU, OXFORD. Traka, has been extensively used for the anatomical reconstruction of visual cortical neurones at the MRC ANU (Douglas and Martin; personal communication). Traka is essentially an integrated database system that enables rapid and precise measurements to be made of neuronal profiles. Traka is executed via a PC that is linked to a motorised microscope stage (measuring in the X/Y plane) and a transducer (measuring displacement in the Z plane). A neurone is digitized via a graphics cursor which is

superimposed on the HRP filled neurone. A pointing device controls the motorised stage and the cursor. The data records consist of x,y and z co-ordinates , and a code that denotes a number of parameters (morphological features, diameters, laminar boundaries etc). The data records are linked in a binary-tree data structure. To ensure that the neuronal structure is consistent, recursive techniques are employed to log and manipulate the neuronal data. Once all the neuronal data is digitised in serial sections, the abutting ends are joined individually under interactive control. For display or plotting, a general 3-D module is used to rotate the entire neurone.

HRP filled neurones NGC1 and NGC2 were initially reconstructed by means of the camera lucida and perforce, depicted in a single parasagittal plane. The original diagrams of these first camera lucida reconstructions are not included for discussion as these cells were reconstructed again at the MRC ANU in Oxford, by means of the 3-D Reconstruction program, Traka (Figs 2.2.3 and 2.2.8). It is worth mentioning the tremendous time and labour saving advantage provided by Traka as opposed to the camera lucida reconstruction technique. Reconstruction of NGC1 and NGC2 by means of the latter technique took approximately 3 months to complete, whereas using the 3-D Traka reconstruction technique, the same task was completed in about 14 days.

The other major advantages of the Traka reconstruction system is that accurate anatomical measurements of the soma and dendritic arbor can be made. The digitized dendritic measurements made by means of Traka provides the basis for the cable analysis. Furthermore the digitized neurone, (stored on disc) can also be rapidly accessed, displayed and rotated about certain axes to gain an instantaneous view of its 3-dimensional orientation. The numerous advantages of such a system over the conventional camera lucida representations therefore need not be stressed.

Two of three cells (NGC3 and NGC4) which were injected intracellularly with HRP at the MRC ANU were also fully reconstructed using the Traka program.

Measurements on HRP filled cells

The dendritic ratio (DR) broadly classifies the pattern of dendritic branching as either round, oval or flattened, by expressing the mediolateral spread with respect to the dorsoventral spread of the dendritic arbor. DR thus provides information about the overall shape of the dendritic arbor. Ratios closer to 1 are equivalent to round dendritic trees, while ratios of 2 or greater are indicative of oval to elongated dendritic arbors or dendritic arbors flattened in the mediolateral direction.

The dendritic index (DI) is equal to the total number of terminal branches at the ends of the dendrites divided by the number of primary dendrites (i.e. trunks). The DI is a convenient way of expressing the complexity of the dendritic branch pattern.

All the soma perimeters were digitized by running the cursor along the cell body contour. Where soma/dendritic junctions were encountered the perimeter was estimated to lie at the initial point of deviation from the smooth cell body contour. The x and y co-ordinates at two opposing points on the long axis, and on the short axis were measured by means of Traka. From these measurements the axial lengths

and widths were computed by the Pythagorean rule for each of the cells. These values are reported in the Table 1.2.1.

Cable analysis

In order to predict the effect of synaptic inputs on the soma of the cell which are located on the peripheral dendrites it is necessary to measure the electrotonic length (X). This is usually achieved by deriving the specific membrane resistance (Rm) from the input resistance and the detailed geometry of the cell. The electrotonic properties of the cell can then be computed from the geometry and Rm.

The input resistance of a cell (Rin) is a measurement commonly used in electrophysiological studies. Rin is a significant factor determining the current strength required for eliciting the discharge of the cell, (i.e. the synaptic properties of the cell), and is therefore a useful parameter for predicting neuronal behaviour in the face of an electrical input. Furthermore this measurement reflects the electrotonic structure of the cell. The whole cell input resistance (Rin) of a cell is defined as the dc-resistance offered by the cell to the flow of current between an intracellular electrode and a reference electrode placed outside the soma. The standard method for measuring Rin is by generating a current-voltage plot. Small hyperpolarizing or depolarizing current pulses of low amplitude are passed across the cell membrane and the voltage developed by the transmembrane currents either in the depolarized or hyperpolarizing direction is measured. Rin is obtained from the linear region of the slope of the graph. For an accurate assessment of this parameter the electrode should pass current linearly in the range required to polarize the cell by a few tens of millivolts.

The membrane resistance (Rm) can be derived from the measurment of Rin if the detailed morphology is known. Rm in turn is needed to calculate the length constant (lambda) of the neurone. The length constant defines the spread of electrotonic potential. The electrotonic length normalized against the actual length is known as the electrotonic length, X.

Lambda, the length constant, is defined as the distance at which the change in transmembrane voltage has fallen to 0.37 (1/e, where e = 2.718, the base of natural logarithms) of the maximum value. It is thus a quantitative measure of the extent of current spread in the steady state. How far the current will spread is determined by the relative values of membrane and intracelullar axial resistance. If the value of lambda is known for the cell then the distance along the dendrite at which the electronic potential will have decayed to 0.37 of its value at the origin is also known. Therefore it follows that the electrotonic distance falls to 37% of its original amplitude at lambda = 1. It is usually considered that an electrotonic distance within 1 lambda is the optimal range for effective interaction of a synapse with the impulse generation site in the soma.

The primary objective was to measure the input resistance of the cell directly and then calculate the membrane resistance. However, a number of complicating factors were experienced during these intracellular studies. The main ones were electrode instability, bridge balance and poor current passing ability. Reliable current voltage

plots were not obtained for the present series of HRP filled cells. Furthermore great difficulty was experienced in achieving stability of the intracellular penetration for sufficiently long to ensure successful HRP deposition, that no time was lost in filling the cell if the penetration looked promising and the current passing capability looked reasonable. In most cases it was a trade-off between making the electrical measurements at the risk of losing the penetration before HRP could be injected, or immediately injecting the HRP if the penetration looked promising and foregoing the electrical measurements. Consequently, in these 4 cells, no direct measurement of the input resistance of the cell was made.

Since no reliable values of Rin were obtained, and none were available in the literature, reasonable values of Rm and Ri were assumed so as to examine the electrotonic implications of the HRP derived morphology. Theoretical values of specific membrane resistances (Rm) in the range 1.0, 2.0, 5.0 and 10.0 $k\Omega c^2$ were employed. These theoretical values were selected because values for Rm commonly cited in the literature for many cell types range from 500 to 4000 Ωcm^2. Since the value of the specific intracellular axial resistance (Ri) is also required for calculating the length constant, and hence the electrotonic length, this value was also assumed. In agreement with the literature it was assumed to be 100 Ωcm (Shepherd and Koch, 1990).

The whole cell input resistance Rin was calculated from the 3-D geometry and assumed Ra and Rm values using the method of Koch and Poggio, (1985) for the limited case of zero frequency. Tables 2.2.2, 3, 4 and 5 report the electrotonic lengths and input resistances calculated for each value of the Rm.

Results

(a) Detailed morphology of the reconstructed HRP-filled cells

A total of 6 cells and two axons were successfully injected with HRP. Of these, 4 PMRF cells were completely reconstructed by means of the Traka technique. All HRP filled cells were found in the nucleus reticularis gigantocellularis as depicted by the camera lucida inserts, Fig 2.2.2, 7, 12, 17. The intracellular HRP filled cells were all of the multipolar variety, as shown in the photographs shown in the above figures as well as in the Traka reconstruction diagrams. Overall, the dimensions of the somata extend over the range of reported sizes typical of PMRF cells (Edwards et al., 1987). The size of the somata, reported in Table 1.2.1, show a large variation, and range between typical giant size, 34x70μ and small 18x35μ.

Table .1.2.1.
Summary of measurements of dendritic parameters

Cell	SS μ	DN	PSLμ Sem	DI	DR
NGC1	36.0 19.2	5	66.41 ±42.12	4	1:4
NGC2	35.3 24.9	7	203.91 ±89.77	3	1:4
NGC3	70.0 29.5	7	51.18 ±15.7	14.1	1:1.5
NGC4	35.4 13.8	7	50.79 ±20.38	4.6	1:1.5

Where:
SS = soma size (long axis; width)
DN = dendritic number
PSL = proximal segment length (μ)
DI = dendritic index
DR = dendritic ratio

The somata of the 4 cells bear between 5 and 7 primary dendrites. In order to establish whether a particular pattern of orientation of the dendritic arbor is common to the above examples, the dendritic ratios were calculated. The dendritic ratios (DR) are reported in Table 1.2.1. NGC1 and NGC2 both show dendritic ratios of 1:4 respectively which indicates flattening of the arbor in the mediolateral plane, whereas NGC3 and NGC4 show dendritic ratios of approximately 1:1.5 and therefore less pronounced flattening of the dendritic arbor in the mediolateral plane. It is noteworthy that the two neurones with high dendritic ratios were cut from tissue prone to shrinkage, whereas the other two cells with much smaller dendritic ratios were cut from osmicated tissue in which it is generally considered that shrinkage is minimal. Although a correction factor for shrinkage (11% in the z-plane) was applied to the former two cells, both show pronounced mediolateral compression of their dendritic arbors. Thus on the basis of their overall shape, the RGC neurones in this study may be broadly classified into two groups, those with mediolaterally flattened dendritic arbors and those tending to rounded dendritic arbors. Although

the number of examples from the two treatment regimens is small, dramatic alterations of their microanatomy due to shrinkage cannot be discounted.

On the basis of their dendritic ratios it is apparent that the dendritic arbors tend to be oval or rounded. Simple inspection of the figures showing the dendritic branching pattern in the coronal (Figs 2.2.3, 2.2.8, 2.2.13 and 2.2.18) parasagittal (Figs 2.2.4, 2.2.9, 2.2.14 and 2.2.19) horizontal planes (Figs 2.2.5, 2.2.10, 2.2.15 and 2.2.20) for each of the cells further supports the conclusions arrived at by calculation of the dendritic ratios.

The proximal dendrites of the cells are asymmetrically arranged. In two examples the dendritic long axes are orientated perpendicular to the longitudinal axis of the brain stem. Characteristically the dendrites are smooth and the absence of dendritic spines is marked in all of the HRP filled cells. No spines are detected anywhere along the lengths of the dendrites. The proximal segments display a large variation in length as well as thickness (reported in Appendix 1.). Cell NGC2 has long proximal dendrites which radiate, on average, a distance of 203μ ±89.77 before branching, whereas the other cells have much shorter proximal dendritic lengths of ranging between 50 and 66μ, with their initial branches closer to the cell body. The branches continue to radiate outwards.

The dendritic branch patterns of the reconstructed cells show considerable variation. This can be ascertained from inspection of the reconstructed morphology, e.g. Fig 2.2.3, 8, 13 and 18. However the logical tree representation which details the dendritic arbor gives a rapid impression of the complexity and branching pattern. Figs. 2.2.6, 11, 16 and 21 respectively, depict the dendritic branch pattern of each of the filled cells in the form of a logical tree, clearly demonstrating the branching pattern of individual dendrites, number of dendritic branches and branch lengths in each of the cells.

Table 1.2.1 reports the values of the dendritic index (DI) of the 4 reconstructed cells. A high DI is indicative of an increased number of branches at the ends of dendrites, or alternatively the presence of fewer primary dendrites relative to terminal branches. For the estimation of DI, the dendritic terminals, irrespective of whether they are natural endings (ne) or artificial endings (ae), were all considered to be the terminations of a particular dendrite. The rationale for this was that the distal terminations were sometimes impossible to detect at slice interfaces. Where this was the case the ending was designated as an artifical end (ae). Therefore natural endings (ne) were considered together with artificial ends.

Both the relatively low DI (3.0) of NGC 2 and the high DI (14.1) of NGC3 demonstrate the differences in the relative densities of branch points within the dendritic arbors of these PMRF cells. The low number of branch points in cells, NGC1, NGC2 and NGC4 correlate with the low DI's of these cells. Although the number and variety of cells injected in this study is low, it is tempting to speculate that a correlation may exist between the soma size and the numbers of dendrites/dendritic branches. It is noteworthy that the cell with the largest soma dimensions is also the cell with the highest dendritic index (14.1). The dimensions of the other three somata are all relatively small and all have low dendritic indices (approximately 4.0).

The extent of the dendritic fields (reported in Table 1.2.2) was estimated by measuring the axial distances of the extreme points of the dendritic arbors.

Table 1.2.2.
Dimensions of dentric arbors

Cell	AP μ	ML μ	DV μ
NGC1	500	282	1165
NGC2	1090	236	1039
NGC3	1547	840	1290
NGC4	439	309	488

Where: AP, ML and DV are anteroposterior, mediolateral and dorsoventral estimates of the spread of the dendritic arbor.

The dendritic arbor of cell (NGC3) has the largest extent, where the dendrites extend approximately 1.5mm in the anteroposterior plane and approximately 1.2mm in the dorsoventral plane. The smallest cell of this series (NGC 4) by comparison has an arbor that extends only about 0.5 mm in both the anteroposterior and dorsoventral plane. These two cell types may represent typical features of cells belonging to separate classes within RGC based on their morphological features.

The axons of these cells arise either from the soma or the proximal dendrites. In all cases the axon trajectories were not followed to their ends, and neither was the entire axon trajectory drawn by means of camera lucida, or digitized via Traka. As the sections were cut in the parasagittal plane the axons could be followed for long distances in both the rostral and caudal directions. These were seen to ascend and/or descend in the longitudinal axis of the brain stem, where they joined the MLF axonal trajectories.

The axon trajectories appear to be similar in all 4 HRP injected NGC cells, although the axon for NGC1 was not digitized, and therefore not included on the reconstruction diagram. The axons course toward the dorsal surface of the brain stem where they may enter the medial longitudinal fasiculus and bifurcate. This was confirmed in NGC1 and NGC2, but not in the remaining two cells. The branches then course in the rostral and caudal directions respectively. The axons of NGC 1 and NGC 2, were initially reconstructed by means of the camera lucida. Although these diagrams are not included in this study, inspection of their axonal trajectories clearly demonstrate the presence of recurrent collaterals. In each case the collateral branches turned back from the main trajectory toward the dendritic tree of the parent soma. However as no contacts with their own dendrites or parent soma could be demonstrated at the light microscope level, this aspect of their microanatomical detail

cannot be resolved until further EM studies have been performed on these collateral branches. In the case of NGC 3 and NGC4 no axon collaterals were present.

(b) Cable measurements on the reconstructed HRP-filled cells

In each of the 4 cells the detailed geometry was employed to calculate the electrotonic lengths of the dendrites according to the methods outlined above. Figs 2.2.23, 24, 25 and 26, show the frequency histograms of the electrotonic lengths (X) calculated for each value of the specific membrane resistance (Rm) viz; 1, 2, 5, 10 $k\Omega cm^2$.
The mean X is reported opposite each set of histograms in Tables 2.2.2, 3, 4 and 5, respectively.

Theoretical calculations have shown that the electrotonic potential falls to 37% of its original amplitude at lambda = 1. At lambda = 2 the electrotonic potential would have decayed by 15%. It is generally considered that the synaptic potential of a synapse placed within an electrotonic distance of 1 lambda from the soma will effectively interact with the impulse generation site. At 2 lambda the synaptic potential is at the limit of effectiveness. Therefore it is widely accepted that cells with electrotonic lengths of 1 would permit effective interaction of synpases placed on their extremities with the soma. On this basis, the most appropriate value of the electrotonic length (X) is 1. For each of the cells the calculated mean electrotonic lengths ranged from <1 to >>1 for each of the assumed values of specific membrane resistance (Rm) i.e.,1, 2, 5 and 10.$k\Omega cm^2$. Since the calculated value of Rin also depends on the geometry and the value of Rm from which X = ±1 was calculated, the corresponding values of calculated input resistances are also reported in Table 1.2.3.

As X and Rin values calculated for each specific Rm are reported for each individual cell in the abovementioned tables, the values of Rm and Rin corresponding to X approximately equal to 1 are summarized in the table below.

Table 1.2.3
Summarized values of data appearing in Tables 1.2.1 and 2.2.2, 3, 4 and 5

Cell	soma size μ	$Rm\Omega cm^2$	Mean X	Rin MΩ
NGC1	36x19.2	2000	1.12	11.5
NGC2	35.3x24.9	2000	1.26	5
NGC3	70x29	5000	1.12	5.3
NGC4	35.4x13.8	1000	1.1	5.6

Where:
Rm = specific membrane resistance Ωcm^2
X = electrotonic length
Rin = input resistance MΩ

These data show that for each cell where the geometry of the cell was employed to calculate the electrotonic length and for reasonable values of specific membrane resistance (Rm) all the cells, irrespective of size, have electrotonic lengths approximately equal to 1. The calculated input resistances of these cells range from 5 to 11.5.

Discussion

The appearance of the HRP filled PMRF cells reconstructed by means of Traka conform to the general description of neurones in the NGC as seen in the cat by means of intracellular HRP (Edwards et al., 1987) and in the rat by means of the Golgi technique (Newman, 1985; Scheibel and Scheibel, 1958). NGC1 and NGC2 were originally fully reconstructed by means of the camera lucida in a single plane. From these a few basic measurements could be made, viz: approximate dendritic lengths; the branching pattern; the approximate soma dimensions and the dendritic ratio. However by employing Traka the measurements of the dendritic lengths and diameters are accurately computed by digitizing the cells in 3-D thus enabling computation of some of the cable properties of that cell, given certain electrical measurements, from the exact dimensions of the cell.

As there is no systematic intracellular HRP description of brain stem cells in the rat, most of the anatomical comparisons will perforce be confined to the intracellular HRP descriptions available for cat brain stem neurones (Edwards et al., 1987). Their material was derived from brains similarly fixed in Karnovsky's fixative. Some of cells were reconstructed from frozen sections and some from vibratrome cut sections. Direct comparison with the present study is difficult as these authors do not differentiate between cells that were obtained by the two different regimens.

The dendritic ratios for these 4 cells fall into two groups; 2 cells showed marked flattening of the dendritic arbor in the mediolateral plane, and 2 cells showed dendritic arbors tending to round. In the recent study by Edwards et al., (1987) the predominant cell type shows compression of its mediolateral dendritic arbor. However some cells in this study were characterized by either rounded or slightly oval dendritic arbors. These were orientated parallel to the coronal plane.

Part of their reticular formation cell sample was taken from RGC. In the cat the giant cell soma sizes have been variously reported as being anything between 40 and 70μ in diameter (Edwards et al., 1987). McCarley et al., (1987) suggested that at least 80% of the labelled neurones had somata >40μ. In the rat retrogradely filled HRP neurones in RGC are reported to be in the range of 60 to 80μ (Newman, 1985). According to their studies there may be an anatomical arrangement of the cells within the nucleus according to the size of RGC cells since neurones in the lateral part of RGC rarely exceed 40μ. The giant somata are more numerous at the level of the facial nerve.

As the somata in the present study were not rounded but tended to be polygonal and elongated, both measurements of the long and short axis of the cell are cited for purposes of conveying a clearer picture of the actual morphology. The cells reconstructed by means of Traka all appeared to have oval-shaped somata, which may

be slightly misleading. The reason for this is that the digitized soma perimeter has been optimized, thus presenting a smooth profile. For purposes of direct comparison, it may be better to consider differences in size in terms of the somatic surface area. According to Edwards et al., (1987) there is little variation in cellular morphology and detail within the giant cell tegmental field. Their sample was however drawn from NMC, NPG and to a great extent from NGC. The sample size in the present study is too small to enable similar conclusions to be made. However, it is apparent that the cells do not differ in any major respects from those reported in the literature.

The dendritic branching pattern has been shown by Newman, (1985) to be correlated with soma size. In his combined Golgi and HRP studies, cells with small somata had 2 to 4 primary dendrites, whereas cells with large/giant somata had up to 10 dendrites. In the present study, although the number of HRP cells is small there may be a similar correlation between dendritic number and soma size, as NGC 1, 2 and 4 have low DI's and relatively small somata, all of comparable size (Table 1.2.1), whereas NGC3 had a much larger soma and dendritic index of 14.1, indicating profuse branching. On the other hand Edwards et al., (1987) could not demonstrate any correlation between dendritic index, dendritic ratio and soma size in the cat brain stem. Furthermore, no consistently different feature of the cellular morphology could be detected in HRP filled cells located in NMC, NPGD, NPM as well as NGC, although the majority of samples were in NGC.

Although Edwards et al., (1987) could find no correlation between the cell body size and the dendritic index, they did find a relationship between the dendritic branching pattern and the numbers of dendrites. The densely branched cells were larger and these were largely confined to RGC. The small to medium sized cells were found in all reticular nuclei in the cat. However the sparsely branched cells were represented by all sizes of cells. Only one of the cells (NGC3) in the present study could be described as large and densely branched. Two of the cells may be classified as sparsely branched and medium sized whereas the remaining one is small and sparsely branched.

In Newman's study PMRF cells were classified on the basis of the dendritic structure into two broad classes (a) sparsely branched cells, with long, radiating multipolar, dendrites with very few if any dendritic spines, dendritic tree orientated parallel to coronal plane, or (b) highly branched, relatively numerous dendrites (5-8) with dendritic lengths of up to 900 microns in the dorsoventral plane. The cells in this study may belong to either of these categories.

In the present study the dendrites of all the cells stained by means of the intracellular HRP technique as well as those cells retrogradely filled from deposition sites in the spinal cord, were remarkable in that they all lacked specialized structures such as dendritic spines. In contrast to Edwards et al., (1987) study, spines were present on cells stained by means of the intracellular filled HRP technique. Distribution of spines appeared to be correlated with the branching pattern of those neurones. They showed that on highly branched neurones (high mean DI of 14.4), the presence of spines was more common than in cells with lower DIs. However spines were absent on somata. The representative in the present study of this category, NGC3, had smooth dendrites and no spines. On any given neurone characteristically bearing dendritic spines, the size, shape and distribution of spines

may vary considerably. However there is a fairly consistent relationship between the number of dendritic spines and the distance from the soma. In the general case there are no or very few spines on the proximal portions of the dendrites with the majority occurring on the middle portions, and then declining towards the distal ends of the dendrites. The functional significance of this distribution has not yet been established. However, it has to be borne in mind that in spine bearing neurones the principal synaptic surface occurs on the spines. In sparsely branched cells Edwards et al., (1987) found that the cells in this category in the cat were not as richly endowed with spines as the large highly branched cells.

The absence of dendritic spines on HRP filled neurones in this study does not appear to be related to the pattern of distribution on the dendrites. Neither the periphery nor the mid-portion of any dendritic branch showed evidence of spines. The lack of dendritic spines on the PMRF cells may be related to the age of animals, as all the rats used in this study were mature. Scheibel, (1973) noted an age related disappearance of dendritic spines. Disappearance with increasing age of spines is correlated with loss of afferent inputs to various CNS structures, e.g. in the cortex spine exuberance in immature animals is well established and maturity is correlated with a decrement of dendritic spine numbers. Furthermore dendritic spines are thought of as labile structures in the sense that some of them may disappear after the animal is sensory deprived and also after deafferentation. In contrast to these cells of the brain stem, the Purkinje cells of the cerebellum are well known for their dendritic spines (llinas, 1975; Collonier, 1981). In the Edwards et al., study the cats were all mature, as were the rats in the present study. This might mean that there is a species difference with regard to the presence of spines on RGC neurones.

It is therefore likely that the pattern of synaptic contacts on the giant cells will be completely different to those of spine bearing neurones. Although investigation of the distribution of synapses was not part of this study, this type of information has important consequences if the cable properties of these neurones are to be studied in greater detail. However the functional significance of this is not well understood.

The lengths of the primary dendritic branches (proximal segment) as measured by Traka are reported in Table 1.2.1. These dendritic segments show an enormous variation in length. In the case of NGC2, these are directed away from the cell body for a long distance before branching. On the other hand all the cells had at least one dendrite showing extensive branching close to the soma. There is a large overlap of proximal dendrite lengths with neurones in other areas, e.g. the primary branch lengths of the basal dendrites of rat sensory cortex pyramidal cells (Lindsay, 1977). Therefore the arrangement of the PSL is not markedly different from other areas.

The emphasis in this section of the study was on the morphology of the dendritic tree, hence the trajectories of the entire axon were not recorded by Traka reconstruction for a great distance. However typical axonal trajectories were observed in more detail in an earlier phase of this study, (Section 1.3.1) where their projections to the spinal cord were examined by means of combined retrograde HRP uptake and antidromic activation. Tracing the course of these axons in the aforementioned as well as the present set of cells, revealed that the axons leave the immediate vicinity of the soma or proximal dendrites and then travel in a dorsal

direction before bifurcating. The branches then course in a rostral and caudal direction respectively within the medial longitudinal fasciculus (mlf).

Although the full axonal trajectories are not depicted on all the reconstructions of the HRP filled cells, all the cells showed axons ascending in a dorsal direction. All of them eventually turned to descend or ascend in the long axis of the brain stem. In the case of NGC1 and NGC2 the axons coursing in a dorsal direction bifurcate and send each branch in a rostral and caudal direction. NGC1, (axon not drawn in the reconstruction) and NGC2 both have axons that ascend and enter the medial longitudinal fasciculus. This information was ascertained from previous camera lucida reconstructions of these two cells which have not been included in this study. Both these cells also showed axon collaterals. In the case of NGC2, a collateral branched off outside the field of the dendritic arbor and then looped back toward the dendritic arbor in the vicinity of the soma. The other collateral brached off some distance from the cell. This is broadly in keeping with the finding of Edwards et al. Newman (1985) however, noted the paucity of recurrent collaterals in rat brain stem neurones. However Scheibel and Scheibel, (1958) demonstrated an abundance of collaterals from reticular neurones in neonatal Golgi preparations.

The overall pattern of dendritic arbor arrangement was originally described in Golgi stained preparations by Scheibel and Scheibel, (1958) and subsequently Valverde, (1961). These early studies noted that dendritic arbors were orientated in the transverse plane. Ramon-Moliner and Nauta, (1966) developed the general theme that the reticular formation neurones possess characteristic somadendritic morphology which distinguishes them from the surrounding motor and sensory nuclei. The general view is that reticular neurones express regional differences in soma and dendritic morphology. The characteristic compression of the dendritic arbors in the rostrocaudal plane in rat Golgi material gave birth to the "poker-chip" analogy of Scheibel, (1984). This description was based on the claim that the large neurones in the pontomedullary areas possess rostrocaudally flattened dendritic "envelopes". This subsequently gave rise to the concept that such a "modular" organization of the brain stem units may be the anatomical substrate for segregation of inputs in an area known for its multiple convergence of heterogeneous afferents. Although this concept has provided a means for a more manageable description of its cellular arrangements, it still remains to be shown what defines the limits of these so-called "modules".

As these concepts were largely based on Golgi stained material, where the individual cell characteristics are largely obscured, this concept may have to be revised in terms of new information regarding cell microanatomy derived from intracellular studies. A strong case cannot be built on the basis of the small number of HRP filled neurones as in the present study. Nevertheless one of the cells conforming to the classical size of a giant cell (NGC3) showed a dendritic arbor tending to be round and had no indication of flattening in the antero-posterior plane. Similarly the smallest HRP injected cell also shows a dendritic arbor which tends to be round. However the other two neurones show marked compression in the mediolateral plane. It is noteworthy that these two neurones which differ markedly in their dendritic arrangement were obtained from histological material that was handled in a different manner to the histological material from which NGC3 and

NGC4 were obtained, even though a correction factor for shrinkage was applied to NGC1 and NGC2. The influence of artefactual effects on the overall structure of this area cannot be totally discounted. In these two cells the dendritic arbors were compressed in the mediolateral plane and showed fairly extensive anteroposterior and dorsoventral extension of their respective dendritic arbors. Although there may be slight distortion in the z-plane, compression in the rostrocaudal plane is not evident. The 3-dimensional orientation of these HRP-filled cells are contrary to Scheibel's generally accepted "poker-chip" analogy. It could be argued that the dendritic arbors are distorted in the z-plane, but it is unlikely that shrinkage can totally account for these findings. However many more intracellular HRP cells will have to be examined in 3-D before a more definitive statement can be made about the general orientation of the dendritic arbors of these cells and consequently the functional implications.

Discussion of cable analysis

The 4 HRP stained cells show a large variation in size and spread of the dendritic tree. In cell NCG3 the dendritic arbor extends for distances up to 1547μ in the AP plane and 1290μ in the dorsoventral plane, it also has a high dendritic index. Similarly NGC1 has dendrites extending 1165μ in the dorsoventral plane. In comparison to other brain areas these dendrites spread for considerable distances. Early Golgi studies also noted that the dendrites of RGC neurones are particularly long and enter adjacent areas of the brain stem. These early studies intuitively suggested that afferent input to the extensive dendritic arbors permitted interaction from many different areas. Since it is generally believed that the large spread of the dendritic arbor enables the cell to receive input from a large area, the question thus arises as to what effect peripheral synapses will have on the soma in a cell with the dimension of that of the above. Since the dendrites are exceptionally long, synaptic effects may have decayed to near zero before they reach the soma. In fact, Valverde (1961) suggested in early early Golgi studies that the considerable lengths of dendrites may be an inherent mechanism of reticular formation neurones for specifically reducing the effectiveness of distally situated synapses.

By employing the detailed geometry of the cell to calculate the electrotonic length, it is revealed that for reasonable Rm values the calculated electrotonic values fall within the range of 1. These cells, which in some cases are exceptionally large, therefore possess dendrites perfectly capable of transmitting synaptic effects from distal sites. These findings have profound functional implications and are compatible with previously held views that the extensive branching pattern diplayed by reticular formation cells permits integration of information from a large area.

With recent technological advances both in the field of microelectrodes and intracellular dye injection techniques combined with the use of large-memory microcomputers, it has become possible to obtain a realistic representation of the architecture of the dendritic tree. Combining these techniques with the classical cable theory pioneered by Rall, (1959) has led to new concepts of individual neurone function. It is fast being realized that the neurone's operation depends to a large

degree on its membrane properties as well as its 3-dimensional geometry. Virtually all neurones in the CNS possess extensive dendritic trees. Since the dendrites contain the majority of the non-axonal cell membrane it accordingly receives most of the synaptic input. Each dendrite with its unique geometry behaves as a specific information processing channel. The arrangement of specific inputs and the detailed geometry of the dendritic tree combine with the electrotonic properties ensuring that individual dendrites function semi-independently. This has given rise to the concept that each dendrite possesses its own "electrical personality" (Gogan and Tyc-Dumont, 1989). Of the many electrical parameters that can be computed for every dendrite, the measurements of input resistance and electrotonic length give some insight into the electrical image of the dendritic arbor. As a detailed description of the computational nature of dendritic integration is beyond the scope of this thesis, the reader is referred to Shepherd and Koch, (1990) for a review of this subject.

The logical tree representations show that the cells have dendritic branching patterns that range from simple (low branch order) to complex (high branch order). This raises the question as to the functional implications of this particular type of branching pattern. Previous investigators have suggested that the extension of dendrites over wide areas provide the PMRF cell with numerous sources of heterogenous input. Conceiveably particular dendrites may receive selective input from specific areas.

There are a number of other mechanisms for regulation of the integration of signals such as synapses that operate via conductance decreases as well as a variety of voltage-gated channels that may modulate membrane conductance at sites on the dendritic arbor. An in-depth discussion of these aspects of synaptic organization is also not within the scope of this study as it is largely unknown what the spatial arrangement of excitatory and inhibitory synapses are on these cells.

In the present study, (Section 1.3.5) it was shown that GABA immunopositive terminals contact these cells on the proximal dendrites and somata. These data provide some evidence for the possible siting of inhibitory synapses. It is thus likely that some inhibitory synapses occur on the cell at these locations but does not exclude the possibility that inhibitory synapses also occur on the distal dendrites. From the conclusions of Koch et al., (1982) their dendritic tree model suggests that the most effective site for inhibitory synapses is between the excitatory synapse and the soma, or even at the same site as the excitatory input. Inhibition sited at the soma is the most effective means for reducing EPSPs. The few observations regarding the GLIR terminals on these cells might be in keeping with the scheme suggested by Koch et al., (1982). Inhibitory contacts may also be spatially segregated on individual dendrites thereby allowing selective switching off, of some dendrites. However these suggestions are purely speculative and will require substantiation by in-depth mapping of the arrangement of synaptic inputs to these cells. The spatial arrangement of excitatory inputs to these cells is largely unknown. However, the early studies by Bowsher and Westman, (1970, 1971) showed that in general the large polydendritic neurones in the caudal and rostral brain stem receive about 80% or more of the synaptic input. The smaller oligodendrites have considerably less synaptic coverage. Furthermore their studies showed that relatively few synaptic contacts occur on the dendrites and that the majority of synaptic contacts were on the soma and

proximal dendrites. It is unknown if this is the case for the majority of large cells within the PMRF.

Recent investigations of dendritic spines have revealed that spines have very complex effects within the parent dendrite as well as on neighbouring spines. Spines are very high resistance pathways and thus impose some very complex electrotonic properties on the dendrites. However as is evident from these intracellular HRP stained cells no spines are present on any of these cells. The functional consequences of such an arrangement are unknown, but it does suggest that the information processing occurs at a different level of complexity compared to cortical cells where spines are abundant, and many spines have both excitatory and inhibitory contacts.

In order to understand information processing and gain a deeper insight into the computational complexity of these neurones, future research will have to investigate the significance of the different morphological features of the dendrites and the arrangement of excitatory and inhibitory synaptic locations on the cell. The HRP filled neurones in the present study partly lend themselves to such a study, since a second phase of this study could be aimed at mapping the spatial arrangements of the synapses by doing serial electron micrograph reconstructions of the dendritic branches. With such data it will be possible to construct models of the computational abilities of these cells.

Conclusion

The detailed 3-D reconstruction of the 4 HRP filled cells reveals typical multipolar cells ranging in size from small to very large with extensive dendritic arbors. In the case of the large cell the dendrites extend for up to 1.5mm. The dendritic branch pattern varies from simple branching with low dendritic index (DI = 3) to a more complex highly branched pattern (DI = 14.1). The overall shape of these cells conform broadly to neurones described by the Golgi technique in the rat and intracellular HRP in the cat.

However as two of these cells were obtained from histological material prone to shrinkage and the other two from tissue where shrinkage is minimal, the profile of the dendritic arbor may have been influenced in the former two. Although a correction factor for shrinkage was applied to these two cells, shrinkage effects have to be taken into consideration since they may give rise to erroneous conclusions regarding the compression of the dendritic arbor. Nevertheless in these 4 cells the anteroposterior compression of their dendritic arbors as described in Golgi preparations is not striking.

In order to establish if Scheibel's "poker-chip" analogy of the dendritic arrangements of these cells is in fact a general organizational pattern of PMRF cells, future 3-D reconstructions of intracellularly injected cells will have to be examined in osmicated tissue which is free of shrinkage.

Cable measurements employing the detailed geometry of the cell show that these cells have electrotonic lengths approximately = 1.0. This indicates that synaptic influences from distal locations on the long dendrites are capable of interacting with the soma.

Muscarinic, nicotinic and GABA sensitivity of cholinoceptive medial pontomedullary reticular formation neurones.

Introduction

The neurones of the medial brain stem reticular formation have been implicated in a number of different behavioural roles (Siegel, 1979). Besides their clear involvement in motor output (Peterson et al., 1978; Peterson et al., 1979) many reports in the literature (Segundo et al., 1967 ; Siegel, 1979; Farham and Douglas, 1985; Markram et al., 1986b) provide unambiguous support for their sensitivity to polymodal sensory input. The results from the previous section in the present study are in agreement with these findings. Collectively these data suggest that PMRF neurones may subserve the function of primitive sensorimotor integration.

Neurones of the medial reticular formation display some other very interesting properties. Systematic observations of single unit activity have shown that most RF neurones display decreased responsiveness to repeated stimuli (Peterson et al., 1976). The "habituation" response, as it has been called by many investigators of this process, is not generally regarded as resulting from an inhibitory mechanism. IPSP's have not been observed during habituation (Segundo et al., 1967) and furthermore there is no decrease in the spontaneous activity during habituation (Peterson et al., 1976). It has therefore been proposed that, for habituation to occur, the pathways between sensory receptors and RF neurones must somehow "alter" their activity.

A number of reports cited by both Siegel, (1979) and Chase, (1980) also attest to the existence of a strong correlation between the level of medial RF activity and arousal state of the animal. Single unit studies in the unrestrained cat have shown a strong correlation between the level of activity of RF neurones and the arousal state of the animal (Kasamatsu, 1970). Numerous studies with as many different experimental approaches have clearly established a strong relationship between the level of activity of the neurones medial reticular formation and the arousal state of the experimental animal. Arguably then, the basis of integration of all these behavioural relationships may be due to the organization of cell types into discrete anatomical zones within the reticular formation. However this does not appear to be the case, as there is complete anatomical overlap of all the functional subdivisions (Siegel, 1979). In this respect all cells of the medial reticular formation appear to be a homogenous population.

In order to perform the functions associated with sensorimotor integration, sleep pattern alteration, habituation and state selection, it would be necessary for the PMRF to influence the spinal cord and or the higher centers in such a manner that the most appropriate motor and sensory program is selected for a particular set of environmental circumstances. A possible mechanism whereby the specific output properties could be achieved is by fractionation of the neuronal pool into different groups with similar discharge profiles. The PMRF might signal a change of state simply by altering the mean discharge rate of particular groups of cells. An alternative mechanism might operate via an internal coding of the discharge. Douglas, (1984) examined the discharge properties of PMRF neurones in order to demonstrate whether coding existed. His study revealed that no significant coding within the discharge of these neurones took place. The findings in this study also

have implications in the analysis of PMRF responses. If these neurones displayed discharge coding then the interspike intervals would have to be logged. Since no significant coding characterises their discharge profile, the mean discharge rate is an appropriate signal for examination of their properties. It is difficult to explain how subsets of the PMRF neuronal population gain control and so engage a particular behavioural state.

For such an integrative process to occur, one can infer from general principles that the neurones must rely on a scheme of subtle neurotransmitter interactions. Exactly how the individual neurone participates and which transmitters are responsible for the production of these states is not known. However it seems likely that PMRF cells are influenced by neighbouring cells within the PMRF as well as those located in nuclei outside of this area. Both classes of neurones may contribute to the evolution of a particular state by means of the specific neurotransmitter released at their terminals. There have been as many investigations as there have been differing experimental approaches concerning the properties of cells in the reticular formation. Suprisingly a systematic investigation of the transmitter types involved in the function of neurones within the medial pontomedullary reticular formation is still outstanding to date.

Early neurotransmitter studies concerning reticular formation function suggested that acetylcholine, amongst others, is primarily involved in the mechanism and generation of desynchronised cortical EEG (Moruzzi and Magoun, 1949). Since this was the first substance to be recognised as a chemical neurotransmitter in the central nervous system it is hardly suprising that the candidate neurotransmitter underlying reticular formation activity was thought to be acetylcholine. These authors described an ascending system within the reticular formation which acted to desynchronize cortical EEG activity via a relay in the thalamus. The extensive cholinergic pathway from the pontomesencephalic reticular formation which relays in the thalamus is thought to play an essential role in regulating cortical activity (Mesulam et al., 1983). Indeed acetylcholine has been shown amongst other neurotransmitters to be involved in the medial pontine reticular formation mechanism affecting desynchronized sleep. Hobsen and McCarley's (1977) model proposes that neurones within the medial pontine reticular formation be cholinergic, and that triggering of the desynchronized sleep is induced by cholinergic activity within the pontine reticular formation.

A few studies have shown that the majority of PMRF neurones lying caudal to the pontomesencephalic acetylcholine-rich area are cholinoceptive (Bradley et al., 1965; Bradley and Wolstencroft, 1967; Markram, 1985). However, this area is not generally recognised as being richly endowed with cholinergic neurones. The various markers for the detection of acetylcholine, especially acetyl cholinesterase (AChE), are now known to be widely distributed within the central nervous system, and not necessarily associated with cholinergic neurones. The emerging picture today regarding the distribution of acetylcholine, questions the interpretation of some of the earlier cholinergic data (Greenfield, 1984). Many studies have employed histochemical techniques for demonstrating its presence via its biosynthetic enzyme (choline acetyltransferase, ChAT) and its inactivating enzyme (acetyl cholinesterase, AChE). These techniques have severe limitations however, in that they do not enable one to discriminate between specific cholinergic sites and non-specific metabolic

sites. The demonstration of acetylcholine localization has therefore only been achieved by indirect means to date. There is no available technique that directly demonstrates the presence of acetylcholine as yet. However, with the advent of specific monoclonal antibodies against ChAT, developed by Eckenstein and Thoenen, (1983) it has become possible to construct maps of acetylcholine distribution by means of this immunological probe (Cuello and Sofroniew, 1984).

In the brain stem ChAT-positive neurones have been confirmed in the nuclei of origin of motor and autonomic nerves. In addition to this a diffuse reticular system in the upper pons, lying medial to the lateral lemniscus, extends to the periaqueductal regions. This area spreads rostrally to within close proximity of the substantia nigra. Cholinergic neurones also spread from the lateral wings of the dorsal raphe to the parabrachial nuclei (pedunculopontine area). In the medullary area, cholinergic neurones spread "loosely" from the level of the facial nerve nucleus through the reticular formation towards nucleus of the solitary tract and from the ambiguus nucleus in a caudal direction (Cuello and Sofroniew, 1984).

Therefore, although maps of cholinergic neurones demonstrate the existence of ACh within the PMRF area, the exact anatomical location of these cholinergic neurones remains uncertain. There is evidence to suggest that both noradrenergic and serotonergic activity in the locus coeruleus and raphe nucleus respectively are also involved in the medial pontine reticular formation mechanism affecting desynchronized sleep. Acetylcholine, however, is of special interest as it is widely known that its action is expressed via two different receptor types, namely muscarinic and nicotinic receptors. Systematic and detailed neurotransmitter studies employing ionophoretic techniques applied to medial brain stem neurones are few. Although a number of different transmitters are active at reticular formation sites there are not many detailed studies on the actions and inter-relationships amongst these transmitters.

The majority of PMRF neurones are excited by acetylcholine (ACh) but a number are also inhibited (Bradley and Wolstencroft, 1967). Preliminary studies conducted by Markram (1985) in this laboratory, showed that 86% of PMRF neurones are cholinoceptive and that the responses of these neurones are influenced by muscarinic and nicotinic agonists of acetylcholine, which is in broad agreement with reports by Bradley and Dray, (1970, 1972). The distribution of nicotinic and muscarinic receptors within the PMRF is largely unknown. However, our preliminary studies suggested that nicotinic analogs were excitatory and muscarinic analogs were inhibitory and that single neurones may be responsive to both the nicotinic and muscarinic analogs of acetylcholine. These data do not agree with the earlier held view that most CNS cholinoceptive neurones contain both types, with one or the other predominating (Koelle, 1975). However the distribution of receptors at peripheral sites may differ from their central distribution, e.g. autonomic ganglion cells contain predominantly nicotinic receptors and some muscarinic receptors, whereas motor end plate receptors are exclusively nicotinic.

The possibility therefore exists that acetylcholine, which possesses the ability to function both as an excitatory and an inhibitory neurotransmitter, may provide the necessary permutations underlying the activity of medial RF function. However, as cited above, other studies as well as those performed in this laboratory have shown

acetylcholine to be predominantly excitatory. The available evidence does not support the presence of strong inhibitory effects within the PMRF. IPSP are evident within the reticular formation in the minority of cases (Segundo et al., 1967; McCarley et al., 1987; Ito and McCarley, 1987). It is arguable then, that acetylcholine which possesses only minimal inhibitory properties may not provide the inhibitory effects within the PMRF exclusively. It is also intriguing that the PMRF is generally recognized as having no typical Golgi type II inhibitory interneurones (Scheibel and Scheibel, 1958). Although GABA is a ubiquitous inhibitory amino acid it may therefore not be widely distributed in the PMRF. Extensive GABAergic mapping of the CNS in the rat does not support the presence of widespread GABA activity in the medial pontomedullary reticular formation (Mugnaini and Oertel, 1985).

The aim of this study is to demonstrate responsiveness of PMRF cells *in vivo*, firstly, to various acetylcholine analogs in order to confirm and extend our preliminary findings with regard to the cholinoceptive properties of PMRF neurones and, secondly, to establish whether both GABA and acetylcholine affect the discharge rate of PMRF cells.

Methods

28 female Long-Evans rats (280- 320 g) were used in this study. The rats were anaesthetized with urethane (1.2g/kg, i.p.). Body temperature was maintained at 37.0 ±0.5°C.) using a feed-back controlled heating pad via an intrarectal thermistor probe. Some rats were fitted with an endotracheal tube in order to maintain unobstructed airways. The head was mounted in a Narishige stereotaxic apparatus. A burr-hole (±3.0mm diameter) was made through the occipital bone exposing the dura. The latter was incised and reflected to the sides of the burr-hole, but the underlying cerebellum left intact. In order to minimize brain stem and cerebellar movement, a thin layer of ±3% agar in normal saline was poured into the burr-hole around the electrode. Extracellular recordings were derived from the central barrel of a 7-barrel electrode constructed on a C.F. Palmer vertical puller. For a detailed description see general methods section 1.2.0. The recording barrel contained 2-3 M NaCl and the ionophoretic barrels contained combinations of acetylcholine chloride (Sigma, 10 %; 0.6M, pH 4.1); Dimethyl-4-phenyl-piperazinium iodide (DMPP) (Sigma, 10%; 0.3M, pH 7.8); Muscarine chloride (Sigma, 10%; pH 5.4); carbamylcholine chloride (carbachol) (Sigma, 10%; 0.6M, pH 6.8); nicotine sulphate (Sigma, 10%; 0.24M, pH 6.3) and GABA (Sigma, 10%; 1M, pH 6.7).

The Neurophore provided the constant currents necessary for ionophoresis. Another barrel of the electrode (also containing 2-3M NaCl) was used to satisfy the current balance circuitry of the ionophoretic apparatus (Neurophore). The ejection currents for all the test substances ranged between 20-100 nA, and retention currents were usually between 5 and 10nA. Conventional extracellular recording procedures were used. The block diagram (Fig 2.3.1) shows the basic layout of the electronic circuitry. The electrode signal was continuously monitored on a dual beam oscilloscope, and only units with good signal to noise ratios and good signal isolation from other units were accepted for investigation. The output of the window discriminator was led to a peristimulus histogram (PSTH) generator (Neurolog). The window discriminator output was also monitored closely on the oscilloscope to ensure validity of translation of action potentials to logic pulses. The PMRF discharge patterns were recorded on a X/Y plotter as rate histograms (10sec bins).

PMRF units were identified by their stereotaxic position, their large (0.5 to 5 mV) spontaneous extracellular action potentials, and their polysensory responses. All signals with rapid rise times were regarded as fibers and ignored. PMRF units responded to one or more of light tactile stimulation (fur-stroke, gentle air-stream directed over the back of the rat, mechanical pressure of tendo-achilles area, and or/tail). Only neurones that were spontaneously active and exhibited typical polysensory responses were challenged with the test substances.

Results.

A total of 68 cells were investigated. In the first part of this study, combinations of acetylcholine, nicotinic and muscarinic analogs of acetylcholine were ionophoresed onto single PMRF cells. Two typical responses to the application of acetylcholine, DL muscarine and DMPP are shown in Figs 2.3.2 and 2.3.3. In both cases the responses to acetylcholine are rapid initial excitation followed by slow inhibition of activity to below basal levels in one case, and a similar decline in activity in the other, with a return to slightly elevated basal discharge rate. Both these cholinoceptive cells from two different animals were responsive to DMPP and muscarine. Fig 2.3.2 demonstrates the sudden and profound inhibitory response to muscarine. The inhibition lasted approximately 40 secs after termination of muscarine application. The response to DMPP is excitatory, with gradual onset, rising to a peak. After termination of the application a sustained suprabasal discharge rate is present for the duration of the recording.

Inhibitory effects were elicited from the second cholinoceptive cell in response to ionophoresis of both the agonists (Fig 2.3.3). In response to DMPP, after a short delay, C2B102 shows rapid and profound inhibition lasting ±40 seconds followed by a slow return and overshoot of basal firing rate. This effect of DMPP is opposite to the excitation elicited from the previous cell, C8C112. However the effect of muscarine was comparable, in that it causes a slow onset and profound inhibition of discharge rate. The inhibitory effect continued after termination of the application with a similar slow return and overshoot of basal discharge rate. In some cases the responses to acetylcholine and its agonists were not as dramatic as those shown. These effects may be related to the ionophoretic characteristics of the electrode as well as the position of the electrode tip with respect to the cell. However, where any doubt existed as to the ionophoretic performance of the electrode the recording was abandoned and the electrode replaced.

39/48 (81%) of PMRF cells responded to both DMPP and muscarine (Table 2.3.1). 8/48 (17%) cells were unresponsive to both DMPP and muscarine, and in one case only to DMPP and not to muscarine. In 28/48 (58%) of cases DMPP elicited mainly excitatory responses. In 4/48 (8%), initial excitation was followed by inhibition during the application of DMPP. These typically biphasic responses were therefore in the minority in this study. This finding is different to the preliminary studies conducted by Markram, (1985) where many responses to DMPP were of a biphasic nature. This may be explained on the basis of the different ionophoretic conditions employed in his study. The main differences were the high retention currents together with high ionophoretic currents.

Muscarinic effects on PMRF cells are mainly inhibitory. In this study 24/48 (50%) of cells tested exhibited inhibitory responses. However in 5/48 (10%) cases, clearcut muscarinic excitation was observed as well. 10/48 (21%) cells displayed biphasic responsiveness to muscarine. These were usually characterized by initial excitation followed by inhibition. 9/48 (19%) of cells were unresponsive to muscarine and 8/48 (17%) cells were unresponsive to both DMPP and muscarine.

Figs 2.3.4, 5 and 6 respectively show responses of three individual cells to muscarinic and nicotinic application. In these examples two of the cells, C1A22 and C6B1312, show excitatory responses to muscarine, while the responses to DMPP are excitatory. Figs 2.3.4 and 2.3.6 therefore demonstrate two individual cases where the nicotinic and muscarinic agonists have the same effect on the cell. The responses of the cell shown in Fig 2.3.3 are similar however, in this case both agonists are inhibitory.

These data therefore clearly demonstrate the presence of both nicotinic and muscarinic effects on a high percentage 81% (39/48) of PMRF cells. It is therefore evident that nicotinic and muscarinic receptors may be present on the same cells, although a presynaptic location for one of the receptors cannot be ruled out altogether. The respective location of these receptor sites on PMRF cells is unknown. However, it has been shown that nicotinic receptors are situated mainly on postsynaptic membranes, therefore muscarinic acetylcholine receptors may be located presynaptically.

Another combination of nicotinic and muscarinic analogs was tested to confirm the above pattern of responses. In this series of tests DMPP was replaced with nicotine sulphate as the nicotinic analog and muscarine with carbamylcholine chloride (carbachol) as the muscarinic analog. 14/20 (70%) PMRF cells responded to both nicotine sulphate and carbachol. Thus the overall reponsiveness of PMRF cells to this combination of agonists is comparable. However 6/20 (30%) cells tested with nicotine sulphate were either unresponsive or only marginally responsive. This might be due to the weaker agonist properties of nicotine sulphate. 10/20 (50%) responses to nicotine are excitatory, and 1/20 (5%) showed biphasic excitation followed by inhibition. In 3/20 (15%) cells nicotine elicited only inhibition. Carbachol on the other hand caused mainly inhibition in 11/20 (55%) of PMRF neurones. 6/20 (30%) of cells showed biphasic response characterized by initial excitation followed by inhibition. In one case carbachol elicited only excitation. 2/20 (10%) cells were unresponsive to both nicotine and carbachol, and a total of 6 cells only to nicotine.

Rate histograms for this series of trials are not shown in the Figures (Vol II), since the pattern of responses elicited by these two agonists are essentially similar to the previous two agonists. Therefore except for minor differences the overall pattern of responses to the ionophoretic application of these two agonists are in good agreement with the previous combination of agonists.

Table 2.3.1

Percentages of PMRF cells responding to nicotinic and muscarinic analogs of acetylcholine

DMPP	MUSCARINE	NICOTINE	CARBACHOL
39/48(83%)		14/20(70%)	
(+)	(-)	(+)	(-)
28/48	24/48	10/20	11/20
(58%)	(50%)	(50%)	(55%)
ss			
(-)	(+)	(-)	(+)
8/48	5/48	3/20	1/20
(17%)	(10%)	(15 %)	(5%)
(+;-)	(+;-)	(+;-)	(+;-)
4/48	10/48	1/20	6/20
(8%)	(21%)	(5%)	(30%)
(0)	**(0)**	**(0)**	**(0)**
8/48	9/48	6/20	2/20
(17%)	(19%)	(30%)	(10%)

Where;
(+), (-), (+;-) and **(0)** denote excitation, inhibition, biphasic excitation and inhibition and no response, respectively. 83% and 70% respectively, refer to the total number of cells in each group showing responsiveness to both nicotinic and muscarinic agonists.

Taken together, 53/68 (78%) PMRF cells tested are responsive to both nicotinic and muscarinic agonists. As the above table lists only the overall effects of both sets of agonists it is not evident from this what the characteristics of individual cell responses are to the pair of nicotinic and muscarinic agonists. Inspection of individual cell responses to the pair of agonists indicate that in 36/53 (68%) of the cases nicotinic and muscarinic agonists elicit opposite effects from the same cell. However in 17/53 (32%) of PMRF cells the responses evoked by the pair of agonists are either both excitatory, both inhibitory or both biphasically excitatory and inhibitory. Roughly equal proportions of PMRF cells were excited or inhibited by both the agonists. Figs 2.7.3a, b, and c, show the response of 3 individual cells in two rats to ionophoretic application of acetylcholine and GABA. These examples show the inhibitory and excitatory effects of acetylcholine, whereas GABA consistently caused rapid and

profound inhibition with rapid return to basal firing rate. In 17/18 (94%) cells tested with this combination of transmitters one cholinoceptive cell was unresponsive to GABA, whereas in the remainder of cases, GABA causes rapid and complete inhibition of discharge rate for the duration of the ejection phase, with rapid return to basal discharge rate after terminating GABA ejection. Therefore a high proportion of PMRF cells are cholinoceptive as well as GABAceptive. In agreement with previous studies acetylcholine effects on PMRF cells were predominantly excitatory (10/18, 56%) whereas 4/18 (22%) cells which were inhibited. However, 4/18 (22%) PMRF cells showed biphasic excitation and inhibition in response to acetylcholine.

Discussion

These data show that the majority of cells within the PMRF are cholinoceptive as they are responsive to nicotinic and muscarinic agonists of acetylcholine and or acetycholine itself. In the majority of cases individual cells show responsiveness to both types of agonist, suggesting that nicotinic and muscarinic receptors of acetylcholine are fairly uniformly distributed throughout the PMRF. In many cases the two classes of agonists provoked opposite effects on the discharge rate of the cell. These results are thus in broad agreement with the preliminary study conducted by Markram, (1985). Ionophoresis of acetylcholine in close proximity to the cell evoked either excitation or inhibition. In Markram's study a high percentage (86 %) of PMRF cells were excited by acetylcholine, whereas only 14 % were inhibited.

In the present study PMRF responses to acetylcholine are qualitatively similar to the cholinoceptive responses derived from neurones in a neighbouring area (Green and Carpenter, 1985). These authors demonstrated that neurones in the caudal pons immediately ventral to the abducens nerve, (para-abducens area) were either excited or inhibited by acetylcholine in addition to being responsive to a host of other transmitter substances. Some of these were identified as reticulospinal cells, but a number were unidentified. However these authors do not make a distinction between nicotinic or muscarinic cholinergic responses. In an earlier study on cat reticulospinal neurones Green and Carpenter (1981), demonstrated a high percentage of cholinoceptive cells where the response patterns were excitation alone, inhibition alone and biphasic inhibition-excitation. They concluded that these responses were due to mixed nicotinic-muscarinic behaviour, but did not actually test the responses of these cells to acetycholine analogs. Furthermore their recordings were derived from predominantly silent cells which were driven by constant application of glutamate. All the cells in the present study were spontaneously active, so are probably different to the reticulospinal population in their study.

In some of these studies the responses to acetylcholine were biphasic. The biphasic excitatory-inhibitory response to acetycholine may be explained on the basis of a number of factors. These probably include receptor type prevalence and or spatial segregation on the soma and dendritic tree, their relative densities as well as accessibility to the ionophoresed acetylcholine. In the visual cortex a different pattern of responses to acetycholine is evident since 92% of cells also showed modification of responses to the application of acetylcholine. However, the responses were either excitatory (61%) or inhibitory (31%). No biphasic

cholinoceptive responses were shown to be present as is the case with brain stem neurones. Furthermore the responsivity of these cholinoceptive cortical cells can be discerned according to discrete anatomical areas within visual cortex (Sillito and Kemp, 1983). Similarly these authors did not determine whether the cholinergic responses were due to anatomical segregation of the cholinergic receptor types.

In general the density of muscarinic receptor sites within the CNS is much higher than that of nicotinic receptors as defined by α-bungarotoxin (Salvaterra and Foders, 1979). In the present study both nicotinic and muscarinic responses were elicited from a single PMRF cell in the majority of cases, suggesting that nicotinic and muscarinic receptor sites are present in roughly similar proportions. It is likely that the receptors mediating excitation and inhibition are located on the post-synaptic membrane of the same cell, although a presynaptic origin for these responses cannot be ruled out.

It could be argued that the inhibitory action of acetylcholine is mediated by cells in the immediate vicinity of the recording electrode as a result of diffusion of acetylcholine to the surrounding area. During a number of recording sessions more than one unit could often be detected by the recording electrode, although it was usually possible to get good isolation of the cell under investigation. Although it was not possible to get a simultaneous recording from these "background" units, observation of the discharge rate of these cells does not support indirectly mediated inhibition of PMRF cells. The discharge rate of the background unit was unaffected by the ionophoretic deposition of acetylcholine. Conceivably the "background" cholinoceptive cell would increase its discharge rate with a simultaneous decrease in firing rate of the recorded cell if they were responsible for mediation of the observed inhibition. This was never observed. It therefore seems unlikely that the inhibitory responses were mediated by the diffusion of acetylcholine to neighbouring cells.

The finding that PMRF cholinoceptive properties are mediated by both muscarinic and nicotinic receptors therefore has profound implications for the PMRF's function as a sensorimotor integrator. Although there has been awareness recently of a mixed population of nicotinic and muscarinic receptors on central neurones, this characteristic of central cholinergic distribution has not been investigated in any detail and it is not known how commonly this may occur. Ligand-binding studies have indicated that in many brain areas muscarinic and nicotinic receptors are not distributed in parallel. According to these studies in major brain areas the muscarinic binding sites are far greater in number than the nicotinic binding sites. It appears that the general pattern of cholinergic receptor distribution is organized according to discrete anatomical locations. In an early experiment Stone, (1972) showed that excitation evoked by acetylcholine in deep cortical layers is mimicked by muscarinic agonists, whereas a strong inhibitory effect mediated by muscarinic agonists is found in more superficial cortical layers

More recently however, the inhibitory effects of acetylcholine on cortical pyramidal cells have been shown to be mediated via excitation of intrinsic inhibitory interneurones (McCormick and Prince, 1986b). These recent data suggest that acetylcholine is exclusively an excitatory transmitter in cortex, and that inhibitory effects can be accounted for in terms of cholinergic excitation of inhibitory interneurones. In the pontomedullary reticular formation there is no anatomical

evidence for inhibitory interneurones. The organization of cholinergic receptors in thalamus is somewhat different. In various thalamic nuclei acetylcholine has inhibitory or excitatory effects depending on the anatomical location of the cell (Dingledine and Kelly, 1977; McCormick and Prince, 1987). Therefore in the case of cortex, only one acetylcholine receptor type appears to be present, whereas in thalamus the receptor types are not mixed, but distinguishable on the basis of their anatomical location. The mixed population of nicotinic and muscarinic receptor types and their distribution on PMRF neurones may therefore be peculiar to this part of the nervous system. The functional significance of this particular distribution pattern has yet to be examined in far greater detail, but as suggested above may be a unique mechanism underlying the activity of large networks of PMRF cells.

The incidence of neuronal excitation within the PMRF is high. Certainly in cats, intracellular PMRF recordings indicate a high degree of mainly excitatory monosynaptic input from the bulbar reticular formation (Ito and McCarley, 1987). It could be argued that if the interaction between PMRF neurones were only excitatory, then in the first instance the output of the overall neuronal population would be scalar. This would mean that the information contained in the population discharge profile would be limited to the mean level of the discharge rate of the population. At some critical point the whole population would be recruited, attaining the maximum fully active state. However there is no systematic evidence in the literature to suggest that this may be the case. If both excitatory and inhibitory modes of interaction between PMRF neurones are present then a more complex mechanism of activity within the population would be permitted. The findings in the present study where the two receptor types, presumably on the same neurone, have predominantly opposite effects would therefore subserve such a function. Although it is still uncertain which neurotransmitters are intrinsic to PMRF cells themselves, if the communication between PMRF cells depended only on acetylcholine then a fairly complex system of interactions would be permitted on the basis of the combinations of receptors with opposing and like actions. This stands in sharp contrast to a system that is primarily excitatory.

Conceivably then, acetylcholine could singly subserve the dual role of both excitatory or inhibitory transmitter, depending on the combination and spatial distribution of receptor types that are activated. Such a system, besides subserving the function of excitation or inhibition, would also enable fine tuning of the responsiveness of PMRF neurones. However this scheme may be far more complex in that the present data show that not only does nicotine and muscarine have exclusively inhibitory and/or excitatory effects respectively, but in addition, nicotine and muscarine have combined effects on single cells which are either both excitatory, or both inhibitory. This might reflect yet another permutation of receptor organization. Conceivably the PMRF may be partitioned into subpopulations of neurones, segregated according to the predominant type of cholinergic receptor present on the membrane. Thus small groups of cells might be either excitatory or inhibitory, whereas the major cell type present would be of the mixed variety. However this does not discount the possibility that some of these effects may be mediated by receptors located on the presynaptic membrane.

As a small percentage of cells showed responses opposite to the predominating effect elicited from either the muscarinic or nicotinic agonist, implies that these "atypical" agonist responses are mediated by nicotinic and muscarinic receptor sub-types. These may be distributed to a greater or lesser degree amongst the predominant acetylcholine receptor type, or may occur only on specific PMRF neurones. Indeed, by means of pharmacological agents such as pirenzepine which binds to muscarinic receptors in different affinities, it has been shown that there are two muscarinic receptor sub-types, M_1 and M_2 present in nervous tissue. In addition at least 4 different muscarinic receptor genes have been cloned (Taylor and Brown, 1989). Similarly multiple subtypes of nicotinic receptors have been identified (Bolter et al., 1986). Probably the most thorough biochemical investigation of the acetylcholine receptor has been performed on the nicotinic acetylcholine receptor of the elasmobrach, where it is available in large quantities. In this species the nicotinic receptor is made up of 4 subunits. The number of subunits may vary according to the species. The electric organs of the Torpedo are a rich source of nicotinic receptors and are composed of 5 distinct subunits (Brisson and Unwin, 1985). The binding of acetylcholine to the receptor, (one subunit is recognition site for agonist) changes the properties of the ion channel so that small cations may move more readily through the membrane. However, the number of subunits making up the nicotinic receptor in vertebrates may be different. Furthermore there are different pharmacological properties of the receptor between species (Colquhoun et al., 1987). In the last few years a number of new subunits on the neuronal nicotinic acetylcholine receptor of the vertebrate have been discovered. To date, the functional properties and the physiological implications of this diversity of stuctural type is unknown. In terms of function, it is also unknown how many neuronal nicotinic receptors can be distinguished. However, recent patch-clamp data on neuronal and muscle nicotinic receptors has revealed that subtle differences between receptors exist viz., differences in voltage dependence of gating, single channel conductance, burst duration and open channel duration. On the basis of these differences detected in the properties of nicotinic receptor it appears that some cells may have more than one type of nicotinic receptor present (Steinbach and Ifune, 1989). The biggest unresolved question concerns what structure of the neuronal nicotinic receptor is correlated with a particular functional property.

The present data, which shows that in some cases both muscarinic and nicotinic agonists respectively, elicit excitatory effects from one cell and inhibitory effects from another cell, may be explained in terms of the presence of a particular receptor subtypes. These particular receptor subtypes may also be spatially segregated on particular areas of the membrane. However, their distribution and how they are regulated is largely unknown. The existence of so many different nicotinic subunits is enigmatic. Many more studies combining electrophysiology, neuroanatomy and immunocytochemistry will be required to demonstrate the functional significance of this structural diversity.

With the advent of new probes for the investigation of neuronal acetycholine receptors, recent evidence sheds new light on the regulation of receptor number and function. There is good experimental evidence to suggest that the neuronal receptors are regulated by both presynaptic input and retrograde signals arising from the

synaptic targets. These findings may have important synaptic regulatory properties (Darwin et al., 1989).

As a general concept it is widely recognized that some CNS areas utilize various transmitter systems which possess potent and versatile transmitters through which the processing and excitability of neurones are controlled. Typical examples of such systems are the neocortical pyramidal neurones and hippocampal neurones where both acetylcholine and nor-adrenalin (NA) have potent and long duration effects. The same combination of neurotransmitters, i.e. Ach and NA have been shown to play a key role in the shift to waking and attentiveness in thalamocortical processing (McCormick, 1989). The thalamocortical system which promotes a state of cortical excitability may therefore be similar in function to the reticular activating system which since the early experiments of Moruzzi and Magoun, (1949) and by many others since, is known to control excitability in the forebrain. The transmitter mechanism whereby this occurs may nevertheless be quite different.

Extensive immunocytochemical tests will have to be undertaken in order to determine the relative spatial distribution of the cholinergic receptor types on PMRF neurones. Such an arrangement of receptor types could explain the absence of inhibitory interneurones, as acetylcholine alone would mediate the interaction between PMRF neurones. This scheme therefore also implies that the neurotransmitter utilized by PMRF neurones is in fact acetylcholine. As mentioned before there is no conclusive evidence that the transmitter involved in PMRF interactions is acetylcholine, only that these neurones are cholinoceptive. Indeed recent investigations by Sofroniew et al., (1985) have revealed that ChAT positive somata are extremely sparse in the PMRF. These data do not argue in favour of a large population of intrinsically cholinergic PMRF cells.

It is therefore likely that cholinoceptive PMRF cells receive a cholinergic input either from those PMRF cells that are cholinergic, or from a source outside the immediate area. It is likely that cholinergic afferents may be derived from the pedunculopontine nucleus (PPN) since large areas of this nucleus stain positively for acetylcholinesterase and it has been established that the PPN has a large efferent fiber system making contact with reticulospinal neurones (Sofroniew et al., 1985). Furthermore it has also been shown by means of ChAT immunocytochemical methods combined with retrograde HRP uptake from the superior colliculus, that many of the cells in PPN are ChAT positive (Beninato and Spencer, 1986). However as only the pars compacta division of cells within PPN are ChAT positive, there is no clear evidence that cholinergic PPN neurones project to reticulospinal neurones. However in an early report by Aghajanian and Bunney, (1974) an afferent fiber tract from PPN to the medial pontomedullary reticular formation is described. However the nature of the transmitter in the pathway described by them is uncertain. Cholinergic projections from the pedunculopontine nucleus (PPN) have also been shown to affect a wide region of the forebrain (Mesulam, 1988). In common with neurones from many other regions affecting the forebrain, PPN neurones discharge at a slow regular rate, which increases concomitantly with the animal's state of arousal. Thus from a behavioural point of view the PPN acetylcholine system may too be implicated in the brain stem arousal system (McCormick and Prince, 1986a). These

fibers are therefore strong candidates as a possible source of cholinergic inputs to the PMRF.

The general picture with regard to the cholinergic innervation and cholinoceptive properties of some neuronal populations in the brain is perplexing, as in some cases the arrangement between neuronal receptivity and innervation appear to be ambiguous. Some areas of the brain have been shown to receive no cholinergic input despite the fact that the neurones are cholinoceptive. Crawford et al., (1966) demonstrated that cholinoceptive cerebellar Purkinje cells stimulated via their afferent input, are not modified by cholinergic agents. On the other hand nigrostriatal cells have been shown to be cholinoceptive in addition to possessing a large amount of AChE, but do not have an identifiable afferent input which is cholinergic (Greenfield, 1984). It has therefore still to be confirmed whether neurones in the PMRF fall into this category.

Some electrophysiological studies point to a predominantly excitatory effect within the PMRF (Ito and MacCarley, 1987). Furthermore Markram's ionophoretic study showed that the predominant effect of acetylcholine ionophoresis was excitatory. On the basis of the scheme outlined above, it is quite feasible to have one transmitter such as acetycholine subserving both excitatory and inhibitory effects. However on the basis of GABA being the major and ubiquitous inhibitory transmitter in the CNS, it is likely that GABA may also be active within the PMRF.

This indeed appears to be the case as approximately 95% of the cholinoceptive neurones are also inhibited by GABA. There is thus an additional strong inhibitory effect on PMRF neurones. As all the cholinoceptive neurones show a high incidence of GABAceptivity it is likely that PMRF neurones also possess GABA receptors. Insofar as the percentage of inhibition caused by acetylcholine is low and that caused by GABA is high, these data indicate that the cholinergic system is unlikely to be the only inhibitory mechanism operative within the PMRF.

Since the immunocytochemical detection of acetylcholine is not possible by direct means and the factors governing the localization of acetycholinestrase (AChE) are uncertain, the most reliable means presently available for identifying acetylcholine in these neurones rests upon the detection and demonstration of choline acetyltransferase (ChAT). This, together with highly specific monoclonal antibodies to the nicotinic and muscarinic receptors of ACh will have to be employed for elucidation of the cellular localization of the transmitter and its receptors.

Conclusion

This study confirms our preliminary findings of the presence of both nicotinic and muscarinic effects on individual PMRF neurones. However the present data, indicating non-uniform distribution of excitatory and inhibitory effects of both agonists suggests that the cholinoceptive receptor mechanisms are more complex than previously anticipated.

As a high percentage of PMRF cells show responsiveness to both agonists it is likely that the majority of cholinoceptive cells possess both nicotinic and muscarinic receptor types on the same cell. This does not exclude the possibility of a presynaptic location of one of the receptors. Since the majority of cells showed nicotinic and muscarinic effects which are opposed, these properties might serve to mediate fine control of the discharge of the individual cell. However a small percentage of cells demonstrate exclusive excitatory or inhibitory responses to both analogs. This suggests the existence of a small population of cells possessing sub-types of the acetylcholine receptor.

In addition to the nicotinic and muscarinic effects a high proportion of cholinoceptive cells also demonstrate GABAceptivity, suggesting the presence of a dual inhibitory control mechanism within the PMRF

Although this study demonstrates responsiveness to ionophoretic application of both nicotinic and muscarinic acetylcholine analogs as well as to GABA, it is not possible to specify the exact location of these receptors. A classical pharmacological approach such as ionophoresis makes it difficult to characterize fully the relevant receptor. However, these *in vivo* data set the stage for further studies, which would employ techniques such as radioligand binding and new molecular probes enabling discrimination of the type of receptors co-existing on PMRF cells, as well as their spatial distribution.

GABAceptive properties of PMRF neurone

Introduction

The previous data indicate that a high percentage of cholinoceptive PMRF cells are also GABAceptive. Extensive immunocytochemical mapping of the distribution of glutamic acid decarboxylase (GAD) in the rat brain has revealed a low incidence of GAD positive somata within the PMRF and especially within nucleus reticularis gigantocellularis (Mugnaini and Oertel, 1985). According to their study the respective density of GAD positive terminals are also few. A notable exception is nucleus paragigantocellularis which according to the comparative scale employed by these authors has a large amount of GAD positive cells and a "medium" amount of GAD positive fibers. Therefore according to the GAD distribution maps of Mugnaini and Oertel the PMRF is relatively poor in GAD positive somata and terminals. This suggests that the neurones in the nucleus reticularis gigantocellularis are themselves not GABAergic and that they do not receive an abundant GABAergic input. Earlier quantitative biochemical studies by Tappaz et al, (1976) showed low GAD activity in nuclei of the pons and the reticular formation in general. These findings are in broad agreement with those of Mugnaini and Oertel, (1985).

Although the PMRF is composed of a relatively large number of small cell types, no cells belonging to the group of neurones classified as Golgi type II inhibitory interneurones, have been demonstrated in the PMRF (Brodal, 1969; Cajal, 1952; Scheibel and Scheibel, 1958). Furthermore McCarley et al., (1987) and Ito and McCarley, (1987) have demonstrated by means of intrareticular microstimulation studies in the cat, that predominantly excitatory effects are elicited from PMRF neurones. Therefore biochemical, anatomical and electrophysiological evidence attest to the low overall incidence of inhibitory effects within the PMRF. On the basis of the paucity of GAD positive fibers and cells in the PMRF, it could be argued that the responsiveness of PMRF neurones to GABA observed in the previous experiment may be due to nonspecific GABA effects, as virtually all neurones in the CNS respond to GABA.

As there are no data currently available which systematically demonstrate the nature of the GABA sensitivity of PMRF neurones, the aim of this study is to investigate the GABA sensitivity of PMRF neurones and their responsiveness to ionophoretic application of bicuculline, a GABAa competitive inhibitor, and baclofen, a GABAb agonist.

Methods

20 rats were used in this study. All rats received i.p. injections of urethane (1.2g/kg body weight). Once the rat was surgically anaesthetised a tracheotomy tube was inserted to facilitate open airways. A burrhole approximately 3mm in diameter was constructed over the target area and both the dura mater and pia mater were incised and reflected to the edges of the burrhole. Extracellular recordings were derived

from multibarrel glass electrodes. The central barrel, filled with 3M NaCl was used as the recording electrode while the surrounding barrels were used for ionophoresis. For a full description of the construction of the multibarrel electrodes, see General Methods (Section 1.2.7). The ionophoretic barrels each contained, according to a colour code system, one of the following solutions:
GABA (Sigma, 10%; 1.0M, pH 6.7) ejection currents, 15-50 nA; -(-)Bicuculline methiodide (Sigma, 10%; 0.2M, pH 5.6) ejection currents, 25-100 nA; Baclofen (Ciba-Geigy, 10%; 0.5M, pH 4.9) ejection currents, 50-100 nA; Acetylcholine chloride (Sigma, 10%; 0.55M, pH 4.1) ejection currents, 50-100nA. In all the above cases the ejection currents were cationic. The retention currents were all between 5 and 15 nA and of opposite polarity to ejection current.

In the case of baclofen and bicuculline respectively great difficulty was experienced in dissolving these drugs. A number of buffers with different pH ranges were used in an attempt to dissolve baclofen and bicuculline with little success. A relatively high concentration of the drug was therefore used, as indicated above. The solution was mixed on a mechanical stirrer for a number of hours, left to stand overnight and then thoroughly mixed once again. It was then centrifuged for a few minutes and the supernatant decanted. This supernatant was used in the ionophoretic barrels. Therefore the final concentration in the ionophoretic barrels of the multibarrel electrode was much less than 0.2M and O.5M for bicuclline and baclofen respectively.

The balance barrel contained the same solution as the recording barrel. The ionophoretic and balance currents were provided by the Neurophore ionophoretic pumps. PMRF neurones were located by their stereotaxic position and their polymodal sensory responses. All units showing short duration extracellular action potentials with rapid rise-times were considered to be fibers and rejected. The electrode was slowly advanced until a PMRF unit was encountered that responded to at least two of the following stimuli:
(1) Non-noxious tendoachilles pressure; (2) fur stroke and or gentle air currents (blowing) over the back and sides of the rat; (3) non-noxious tail pinch.
Standard electrophysiological recording techniques as used previously were employed and rate histograms of the cellular responses to ionophoretically applied test substances were plotted on an XY recorder (see circuit diagram Fig 2.3.1). The location of the electrode penetrations were confirmed by subsequent histological inspection in rats perfused transcardially with 10% buffered formalin.

Protocol

Once a PMRF neurone was encountered that responded to the exteroceptive stimuli as outlined above, its spontaneous basal firing rate was allowed to stabilize at basal discharge rate for a five minute period. Thereafter the ionophoretic test substances were all first applied individually with a 10 minute recovery period between applications. The sequence of application of test substance was randomized. A minimum of 10 minutes was allowed for recovery between applications of test substances.

Results

76 PMRF neurones were tested by extracellular ionophoretic deposition of GABA. 75/76 (98%) of the neurones showed rapid and profound reduction in discharge. The reduction of the basal discharge occurred within seconds of applying the ionophoretic current to the GABA barrel, followed by complete inhibition in the majority of cases. Basal discharge resumed in a similarly short period upon termination of the delivery. Figs 2.4.1a, b and c, show the typical responses of 3 individual PMRF neurones to the application of GABA, bicuculline and baclofen. In almost all the cells tested with this combination 27/30 (90%) responses were typically rapid GABA inhibition, slow and prolonged baclofen inhibition and large excitatory response to bicuculline. These typical responses to the GABA antagonist and agonist respectively, therefore suggest that a large percentage of these neurones probably contain both GABAa and GABAb receptor types.

In 24/25 (96%) PMRF cells the GABA-induced inhibition was antagonised by bicuculline (Fig 2.4.4). In these tests bicuculline was ionophoresed as soon as a clear cut GABA inhibition was established. A noticeable feature of this inhibition, clearly seen in Fig 2.4.4, was that the firing rate of the test neurone in all these cases rose higher than the control rate. This is also observed in the majority of cases. The inhibition was re-asserted immediately the bicuculline was withdrawn. When the GABA ejection was terminated the discharge rate again rose to levels greater than the control rate, and then gradually decayed to control levels over a number of minutes. These data indicate that in addition to antagonising the inhibitory GABA effect, bicuculline enhanced the discharge rate of most PMRF neurones tested. When bicuculline was applied on its own, (Fig 2.4.2) the response was a marked increase in discharge rate that decayed slowly after cessation of the ejection of bicuculline. This may be an explanation for the rebound and protracted decays seen after withdrawal of GABA in the combined test.

41 neurones were tested by the application of baclofen. 41/41 (100%) PMRF neurones showed slow, and in some cases, complete inhibition. The inhibition was typically prolonged and in some cases lasted for minutes, with a slow resumption of basal firing rate. Fig 2.4.3 shows typical cellular responses of individual cells to the ionophoretic application of baclofen. As can be seen, the PMRF inhibitory responses to the GABA agonist are markedly different compared to GABA elicited inhibition. A possible explanation for this might be the preferential stimulation by GABA of GABAa receptors. The time course of GABAb activation appears to be much longer. GABA was applied for short periods, 10 to 15 seconds, whereas baclofen was applied for 50 to 60 seconds to achieve full effect.

Table 1.3.4(i) below, shows the mean percentage increase above mean basal discharge rate of 25 cells tested with ionophoretically applied bicuculline. With the exception of 1 cell which showed a reduction in discharge rate, 22/25 (88%) of PMRF neurones tested showed an enormous range (20% to >100%) of increased excitatory response with respect to control levels when challenged with bicuculline. The increased discharge rate of PMRF cells to bicuculline, in 16/25 (64%) of cases was characterized by a large percentage increase ranging from 50% to >100% with respect

to basal discharge, while 6/25 (24%) showed percentage increases ranging from 20%-50%.

Table 1.3.4(i)

Difference in mean number of events during bicuculline application expressed as a percentage of control

Cell n=25	Control	Treated	Difference	%Control
17/3C10	13	95	82	630%
17/3C3	125	202	77	62%
14/3C6	98	141	43	44%
17/3C1	64	111	47	73%
16/3C3	57	156	99	174%
9/3C3	21	109	88	419%
9/3C2	76	115	39	51%
5/3C7	81	118	37	46%
29/10C1	93	121	28	30%
28/10C6	54	71	17	31%
28/10C5	46	69	23	50%
28/10C4	90	138	48	53%
28/10C1	8	74	66	825%
21/10C1	64	71	7	11%
21/10C2	23	49	26	113%
29/9C6	60	48	-12	*
29/9C5	10	22	12	120%
29/9C4	19	59	40	210%
29/9C3	54	150	96	178%
29/9C2	15	69	54	360%
29/9C1	62	86	24	39%
27/8C3	61	86	25	41%
27/8C1	24	75	51	212%
26/9C4	66	75	9	14%
24/9C1	70	112	42	60%

Where:
Control = mean number of events in control period
Treated = mean number of events during bicuculline application.
Difference = difference between control and treated
%Control = % difference with respect to control
* = decreased discharge rate

In 16/18 (89%) of cells tested the enhanced discharge rate of PMRF neurones due to the application of bicuculline could also be reversed by the ionophoretic application of GABA. Figures 2.4.5a and b, show typical patterns of PMRF responsivity to the application of both bicuculline and GABA. Once a definite excitatory response was elicited by bicuculline, i.e. ±50% suprabasal increase in firing rate, GABA was applied. GABA effectively blocked the excitatory action bicuculline in the majority of cases tested. The low ejection currents needed to achieve these effects (Figs 2.4.5a and e) attest to the exquisite sensitivity of some PMRF neurones to the agonist and antagonist. In the majority of cases tested there was a very marked excitatory rebound in the presence of bicuculline when GABA application was terminated. This excitation persisted and commonly only reverted to basal levels some minutes after bicuculline was withdrawn.

Discussion

These data clearly demonstrate that the majority of PMRF neurones are sensitive to GABA. It is likely that two GABAergic inhibitory mechanisms may be active *in vivo* within the PMRF, firstly, since GABA inhibition is reversed by bicuculline which is known to selectively block GABAa receptors and, secondly, since the PMRF neurones are inhibited by baclofen, a GABAb agonist, known to act specifically at bicuculline insensitive GABAb receptor sites.

The bicuculline-induced excitation may reflect disinhibition of PMRF neurones that are normally subject to tonic GABAergic inhibition. Although the excitatory properties of bicuculline have been well known for the past two decades, this property has not detracted from its value as a tool for assessing whether or not a particular inhibitory pathway could function by releasing GABA as a transmitter. The reliability of bicuculline as a specific antagonist of GABA has been a controversial issue since the early seventies. Although it has been clearly established that bicuculline has more than one effect on the response of cortical neurones to GABA, (Straughan et al., 1971), these authors concluded that the antagonistic effects of bicuculline on the responses to GABA were probably due to its postsynaptic action, but that direct non-specific effects could not be discounted. Bicuculline has also been shown to have a direct excitatory action on neurones in both the invertebrate preparation (Walker et al., 1971) as well as in the vertebrate nervous system (Curtis et al., 1971). Bicuculline often enhanced the firing rate of cat cerebellar, Purkinje, hippocampal pyramidal and frequently cortical cells. A more critical examination of their study revealed that in most cases the excitatory effects of bicuculline typically evolved over a period of 1 to 2 minutes with bicuculline ejection currents ranging from 80-150nA. Although a direct comparison of the concentration of bicuculline delivered at the tips of their electrodes and those in the present study is not possible, in this study the majority of PMRF excitatory responses to bicuculline ionophoresis evolved rapidly with a sharp rise in discharge rate. Peak discharge rate, reflecting

50to >100% increase above basal levels in 64 % of cases tested, usually evolved within 30 to 60 seconds. Excitatory effects of bicuculline on cortical or hippocampal neurones did not show increases in discharge rate as large as those observed in this study. Furthermore the excitatory bicuculline effects were observed at relatively lower ejection currents with the onset of raised discharge rate occurring within the first few seconds of starting the bicuculline ionophoresis. Therefore although these excitatory effects of bicuculline are a widely acknowledged property of bicuculline, its use as a highly specific GABA antagonist as shown by radioactive ligand binding studies (Olsen and Snowman, 1983; Möhler and Okada, 1978) cannot be denied. The high incidence of bicuculline induced excitation observed in this study may therefore not be due exclusively to antagonism of an ongoing tonic GABAergic input, but may parallel non-specific excitatory effect on these neurones.

It was demonstrated in the previous section on the basis of responses to nicotinic and muscarinic analogues, that a large percentage of neurones in the PMRF are cholinoceptive. Futhermore GABAceptivity was demonstrated on all of the neurones displaying cholinoceptive properties. It is also possible that the excitatory effects caused by bicuculline are due to its interaction with cholinergic neurotransmission (Svenneby and Roberts, 1974). It is therefore also likely that bicuculline could potentiate the excitatory effects of acetylcholine. This effect has been observed in the medial septal nucleus (Miller and McLennan, 1974). The cholinoceptive cells inhibited by GABA in Section 1.3.1c demonstrate that acetylcholine does not have exclusively excitatory effects on PMRF neurones and that acetylcholine was capable of suppressing the discharge of 8/18 (44%) PMRF neurones. It can therefore be argued that if bicuculline does potentiate and/or increase the availability of acetylcholine then it could be expected that bicuculline would have suppressed the discharge of approximately the same percentage of PMRF neurones that were tested, i.e. 44%. This was not observed. Table 1.3.4(i) indicates that except for one case, bicuculline causes predominantly excitatory effects on PMRF neurones.

On the basis of these responses it is therefore highly unlikely that the effects of bicuculline are mediated via potentiation of the acetylcholine system, and activation of the inhibitory cholinergic receptors. The entire dynamics of the bicuculline response were not studied exhaustively, as the main purpose was to demonstrate antagonism of the GABA inhibition. The ionophoretic current of the bicuculline barrel was merely left on for as long as it was required to demonstrate a clear antagonism of the GABA inhibition. However in a few cases responses to bicuculline are biphasic. Once the peak excitatory response of the PMRF neurone had been established, further application of bicuculline caused a decline in the neurone's excitability. The effects of prolonged bicuculline application on PMRF responses were not examined any further in this study. These biphasic responses during ionophoretic application of bicuculline are puzzling, especially since rebound excitation is present after termination of the bicuculline ionophoresis. It is not known how bicuculline is metabolised by the cell and/or surrounding neuropil, but judging from the prolonged, (of the order of minutes), raised basal firing rate after cessation of the bicuculline ionophoresis in most cases, it is apparent that bicuculline either diffuses away and/or is metabolised very slowly. Under usual testing conditions the basal firing rate resumed only after many minutes.

As a general principle it is accepted that each neurotransmitter must have at least one specific receptor but that many neurotransmitters are associated with multiple receptor types (McGeer et al., 1987). However one striking fact emerges from the present study. Neurones of the PMRF may not only have multiple receptor types for a particular neurotransmitter but in addition have receptor types associated with at least two neurotransmitter types. Indeed Green and Carpenter, (1985) have shown that neurones in the rostral and dorsal reticular formation demonstrate receptivity to many classes of transmitter. Their ionophoretic study of inhibitory neurotransmitters, viz., GABA, glycine and noradrenalin on neurones in the para-abducens reticular formation demonstrate that all of the aforementioned transmitters elicited fast inhibitory responses from all the neurones tested. PMRF inhibitory responses to GABA are similar. It was also shown by these authors that cells in this area responded to acetylcholine.

A number of differences in experimental technique between the present study and their study exists. Although the sample of cells in their study are from a neighbouring area, most of the cells in their study were activated by glutamate, whereas all the PMRF cells tested are spontaneously active. A number of combinations of transmitter substances were tested in their study but GABA and acetylcholine responses were not apparently tested together on the same neurone, nor were any of the nicotinic and muscarinic analogues of acetylcholine tested. However judging from the large numbers of neurones that were responsive to GABA and acetylcholine, it is likely that individual neurones in the para-abducens reticular formation (PARF) area too are receptive to both the transmitters. Certainly, in the previous study, (Section 1.3.1) it was shown that all 18 cells tested responded to the GABA and acetylcholine combination. It therefore seems likely from these collective data that cells in the medial zones of pontomedullary reticular formation may have multireceptor properties insofar as all classes of neurotransmitters tested elicited either excitatory or inhibitory responses. For technical reasons both in the present and aforementioned study there is a limit to the number of substances that could be ionophoresed on any single neurone during a recording session.

Although receptivity to neurotransmitter substances does not necessarily imply intrinsic use, the current view on the distribution of transmitter substances holds, that many transmitters may be co-localized within the same neurone in many areas of the brain. The views on the relationships between transmitters and their receptors have also undergone complete revision in the last 10 years. There has been a growing awareness that CNS peptides and their receptors are not anatomically related but appear to be distributed in the brain in an independent manner. More recently it has been shown that GABA, too, is heterogeneously distributed throughout the CNS with varying concentration. In the cerebellum in particular, there is a clear mismatch between the GABA receptors and GABA terminals (Somogyi et al., 1989). Studies comparing the relative levels of GABA versus receptor in numerous brain areas have shown that a quantitative relationship between these two entities does not hold (Herkenham, 1987). In the septal area for example GABA receptors are particularly dense but GABA terminals are a rare occurrence (Onteniente et al., 1986). As no such quantitative study on the relationship between receptors and terminals has been performed on the PMRF cells, the present data is difficult to interpret.

Whether the observed GABA effects are mediated via a synaptic or non-junctional GABA receptor site is open to speculation. The view that the observed bicuculline effect could be mediated via these non-junctional receptors for GABA cannot therefore be rejected out of hand. Furthermore, in view of the very large excitatory effects observed on many of the PMRF cells, seemingly in the face of a sparse GAD presence in the area, (Mugnaini and Oertel, 1985) it appears unlikely that these large excitatory effects would have occurred in the absence of an intrinsic GABAergic input.

If the presence of GABA terminals can be shown to contact PMRF cells, then it is likely that the large excitatory response of these cells to bicuculline application could in part be due to the removal of a tonic inhibitory effect.

Conclusion

The majority of PMRF neurones are profoundly inhibited by GABA and its agonist baclofen. In the majority of cases tested the inhibitory effects of GABA were antagonised by bicuculline. Since it is widely accepted that bicuculline is active at GABAa receptor sites, and that baclofen is active at GABAb receptor sites, it is likely that PMRF cells are inhibited via both GABAa and GABAb inhibitory mechanisms. Furthermore, as demonstrated in the previous section, acetycholine also exerts inhibitory effects on the PMRF cells via both its nicotinic and muscarinic receptors. The contribution to the inhibitory effects seen at the level of the PMRF neurone will therefore ultimately depend on the distribution of inhibitory nicotinic and muscarinic receptors as well as GABAa and GABAb receptors.

The large excitatory responses of PMRF cells to the application of bicuculline may be due in part to antagonism of tonic inhibitory input to PMRF neurones.

GABA-like immunoreactivity of terminals contacting giant cells within the pontomedullary reticular formation

Introduction

Since the majority of PMRF neurones in this study demonstrate rapid and profound inhibition of discharge rate in the face of ionophoretically applied GABA it is probable that PMRF cells are influenced by a GABAergic input. However, since GABA receptors are distributed widely throughout the nervous system it does not necessarily mean that the GABAceptivity reflects innervation. The large excitatory effect on PMRF cells in response to bicuculline application on its own, may reflect tonic control of PMRF discharge by GABAergic input, taking into account the well known excitant effect of bicuculline on the discharge rate of some neurones (Curtis et al., 1971).

Although it is well known that GABA receptors may not be in register with GABAergic terminals, the nature of the responses elicited from bicuculline ionophoresis, suggest that these effects may be mediated via GABAergic terminals contacting PMRF neurones. This assumption may hold provided GABAergic terminals can be demonstrated. To make the case that the excitatory effects are mediated via inhibition of the GABAa receptor would require the demonstration of sub-synaptic GABAa receptors. As this is beyond the scope of the present study the aim is to demonstrate the presence of GABAergic contacts on PMRF cells by means of a highly specific GABA-immunocytochemical technique.

Methods

In order to test for the presence of GABAergic terminals within the PMRF the immunocytochemical method for the detection of GABA followed the procedure of Somogyi and Hodgsen, (1985). The immunogold technique introduced about a decade ago employs small gold particles as electron dense markers for the ultrastructural localization of cellular antigens. The small gold particles offer a very specific high resolution label. However, as with other immunocytochemical procedures, one is confronted with the problem of obtaining good ultrastructural preservation together with the retention of high levels of protein integrity and hence immunoreactive sites. Optimal ultrastructural preservation is usually obtained with osmium tetroxide (OsO_4) employed in the fixation procedure. This fixative is known to drastically inhibit immunocytochemical labelling of proteins (Bendayan et al., 1980; Roth et al., 1981). On the other hand non-osmication seriously compromises structural preservation. However, recently developed techniques involving the usual tissue osmication steps have included a step whereby osmication is followed by treatment with strong oxidizing agents. These are capable of unmasking the protein antigenic sites on glutaraldehyde-fixed, post-osmicated tissue. This improved technique (Bendayan and Zollingen, 1983) has the advantage that the immunogold technique can be used in routinely fixed tissues, resulting in good ultrastructural preservation and therefore superior resolution of labelling.

Animal preparation.

Rats weighing approx 250g were anaesthetised with Sagatal (35mg/Kg Bwt) and perfused transcardially with Karnovsky's fixative. Coronal sections (75μ thick) were cut on an Oxford vibratome from a block of fixed tissue containing the brainstem area. The coronal sections were flat-embedded in Durcupan and mounted on glass slides. The giant cells within the PMRF were microscopically identified in these sections. Identical procedures in each of 3 rats were adopted and single histologically identified NGC cells in the rostral PMRF each of a single rat were selected at random and excised from the flat-embedded Durcupan sections.

Each piece of excised tissue was orientated and embedded in a resin block for ultramicrotomy. Serial ultrathin (silver) sections were cut through each of the selected neurones on an ultramicrotome. The silver ultrathin sections were mounted on Formvar coated single slot gold or nickle grids. Copper grids are unsuitable for immunostaining procedures due to copper's reaction with periodate. All the cut sections were stored in labelled containers specially designed for housing the Formvar grids. The immunostaining procedure was carried out in droplets (approximately 40 μl) of reagent deposited on strips of clean laboratory parafilm in a glass petri dish. A coding system was employed in order to identify the sections. Thus the droplets were arranged in a convenient order and a strict sequence was adhered to during transferral of the grids from droplet to droplet during the staining procedure. The reactions took place at room temperature and all the grids were floated on the droplets face down. As an additional precaution all reagents and washing solutions were filtered through 0.22μ Millpore filters. The following immunocytochemical procedure was employed.

Post-embedding immunogold GABA-immunocytochemistry

This technique relies entirely on the ability of the antibody to react specifically with their corresponding antigen. As such, the specificity (i.e. absence of cross reactions of the 1st antibody) of the antiserum has undergone rigorous testing (Somogyi and Hodgsen 1985a; Somogyi et al., 1985b). The latter workers have been responsible for the development of GABA antiserum (code number 9 antibody) . This antibody which has been extensively used in studies concerning GABA-immunoreactivity of cells in the visual cortex (Somogyi and Hodgson, 1985; Kisvarday et al., 1986) was used in the present study. The tests for specificity of the GABA-9 antibody were as follows;

(1) absorption of antibodies with corresponding antigens;

(2) use of pre-immune sera, i.e. incubation of some cells which had been pre-incubated with GABA-antiserum pre-incubated with GABA coupled to polyacrylamide beads.

(3) omission of the first step of the incubation procedure, i.e. alternate sections were stained in solutions where the antibody was omitted.

In the Department of Pharmacology, University of Oxford the above GABA-9 antibody has been routinely employed in immunocytochemical studies involving the visual system. Its highly specific nature has been well established.

Staining protocol

(a) 10 ml of 1% Periodic acid in double distilled water (ddH_2O) and 10ml of 1% Sodium periodate in ddH_2O respectively, were prepared shortly before use. These solutions have to be prepared fresh on each occasion.
(b) Single drops of the 1% periodic solution were deposited onto the parafilm strips in the petri-dish by means of a syringe fitted with the aforementioned Millipore filter. The periodic acid effectively removes the resin from the sections. The grids were floated onto the droplets of periodic acid for a period of 10 minutes. Timing of the respective incubation periods is critical; thus a 20 second interval was allowed to elapse between the transferral of each grid from solution to solution.
(c) After the 10 minute incubation period the first grid was transferred through 3 washes of ddH_2O.
(d) After the third rinse the grids were placed on a droplet of ddH_2O and left for 5 minutes.
(e) At 20 second intervals the grids were transferred to droplets of sodium periodate for a period of 10 mins.
This reagent oxidises osmium complexes within the exposed tissue. Excess water transferred between the arms of the forceps must not dilute the sodium periodate solution . This was remedied by absorbing the water with strips of filterpaper before positioning on the next droplet.
(f) At the end of 10 minutes the grids were rinsed in ddH_20. After 5 minutes on the ddH_2O droplet, the grids were again transferred to a humidified chamber containing droplets of Tris Phosphosaline buffer (TPBS) pH 7.4.
(g) Grids were rinsed in 4 changes of TPBS, twice for 3 minutes and twice for 7 minutes. All the subsequent steps were performed in humidified chambers.
(h) Grids were transferred to drops of 5% Normal Goat serum (NGS) in TPBS for a 10 minute period. This diminished non-specific binding.
(i) Then for 90 minutes the grids were incubated on droplets of primary antibody. The primary antibody, GABA-9, kindly donated by Prof.P.Somogyi, was made up in filtered 1% NGS, in TPBS at a dilution of 1;2000 which was filtered before it was added.
(j) Grids were then removed from the primary antibody and washed twice for 3 minutes on droplets of a solution of 0.5mg/ml polyethylene glycol (PEG) in Tris HCL buffer pH 7.4. This was followed by a further two rinses of 7 minutes each. These steps were carried out to avoid the mixing of high concentrations of salt solution in the TPBS with the protein coated colloidal gold in the next step.
(k) The final incubation was performed in droplets of Goat Anti-Rabbit immunoglobulin coated colloidal gold (GAR) at a dilution of 1:10 in PEG/Tris HCL solution for 90 minutes.

(l) At the end of this period the grids were washed in 5 changes of filtered ddH_20, dried using filter paper to remove excess water and then finally dried with a hair-drier, set to create a gentle stream of warm air. The grids were then stored in a labelled grid-box.
(m) Before examination under EM the sections were stained with uranyl acetate and alkaline lead citrate to increase the contrast of the sections.
(n) Tissues fixed in $Os0_4$ or glutaraldehyde stain so intensely that the staining times are relatively short, approximately 5 minutes and the lead citrate is usually diluted, (up to 1:1000) in 0.01 N Sodium Hydroxide. This generally reduces or prevents overstaining (Reynolds, 1968).
(o) For optimal results a test run was performed . Grids with immunostained sections were floated on drops of staining solution for the appropriate time .
(p) Following staining the grids were washed sequentially in 0.02N Sodium Hydroxide followed by several rinses with ddH_20 and once again dried in a stream of warm air.
(q) The sections were examined under the electron microscope for GABA-like immunoreactivity.

Sites containing the electron dense gold particles were photographed at various magnifications (up to 22000X) for a permanent record of GABA-like immunopositive terminals.

Quantification of gold particles

Since background labelling is a general problem in any immunocytochemical technique, an attempt was made at quantifying the relative densities of immunogold particles contained within the terminals. Electron dense gold particles (20nm diameter) were counted in the surrounding tissue and in the terminals by 3 independent viewers. These counts were averaged, the relative densities of gold particles computed and then expressed as a ratio.

Results

Electron microscope examination of the sections containing the three randomly selected PMRF cells, revealed the presence of many electron dense gold particles and therefore GABA-like immunoreactivity (GLIR), within terminals which contact the 3 randomly selected PMRF cells. Fig. 2.5.1a and Fig. 2.5.1b are electron micrographs of the giant cells cut at different levels. In these three examples GLIR terminals are seen contacting the proximal dendrite and the soma respectively in one of the cells, and the proximal dendrite in the other. The positions of the GABA-like immunopositive terminals are indicated by arrows in these figures. In all the micrographs showing GABA-like immunoreactive terminals the density of electron dense gold particles contained within the terminals were compared to the density in the surrounding tissue. In all cases where electron dense gold particles were detected in terminals abutting on PMRF cells, the ratio of gold particles in the terminal to that

of the surrounding area were of the order of 5:1. This high incidence of immunogold label within a defined structure was taken as clear evidence for GABA-like immunoreactivity within the PMRF associated with the giant cells. Although all electron micrograph details of contacts with all three cells are not shown the overall picture was similar in all of the cases. As no PMRF somata containing immunogold particles in the immediate vicinity of the GLIR terminals displayed particle densities per unit area of neuropil similar to that within the GLIR-terminals, it is unlikely that the cells themselves contain significant amounts of GABA-like immunoreactivity, suggesting that these giant cells are not GABAergic. The above three cells subjected to immunocytochemical staining were selected randomly from coronal sections of the RGC under low power examination.

These cells were similar in morphology to the intracellularly filled HRP cells described in this study (e.g. Fig. 2.2.2,.7, 12, 17) The cells are large stellate, multipolar (polydendritic) neurones lying within the medial and rostral PMRF. These neurones also conform to those described in the cat by Bowsher and Westman, (1970) and Edwards et al.,(1987) as well as Golgi and Nissl stained PMRF cells described by Newman, (1985) in the rat.

The presynaptic terminals were identified by the presence of synaptic membrane specialisations and vesicles, where they contacted either the proximal dendrites or the soma of the giant cells. As an added precaution against false positive immunoreactions the terminals were judged to be immunopositive only if the electron-dense gold particles were consistently observed within the same terminal and in consecutive sections which were reacted similarly, but with a different batch of immunocytochemical reagents. The GLIR terminals containing the electron-dense gold particles (small dense spheres) are clearly demonstrated at higher magnification in electron micrographs (Fig. 2.5.2 to Fig. 2.5.4).

Fig. 2.5.2A and Fig. 2.5.2B show a section through the periphery of the gigantocellular soma from one rat. At this level of peripheral ultrathin sectioning, the nucleus of the cell was not revealed. Fig. 2.5.2A shows a presynaptic GLIR-terminal (marked by an arrow) seen making contact on the proximal dendrite with a postsynaptic protruberance, known as a gemmule. Although "spine-like" in appearance, a spine apparatus was not observed. The arrow position (Fig 2.5.2b) indicates slight membrane thickening and therefore a possible site of a synaptic contact.

Fig. 2.5.3 shows another example of a GLIR terminal (marked by an arrow in Fig 2.5.1a, D) making axosomatic contact. This ultrathin serial section was cut at a level which passes through the nucleus of the cell. In this case there is no protruberance similar to the gemmule as in the previous example and the terminal makes direct contact with the soma. Fig. 2.5.3A and Fig. 2.5.3B show successive examples through the same axosomatic contact at higher magnification. In the successive sections of the same terminal immunogold particles are clearly visible. As in the above case Fig. 2.5.4A and Fig. 2.5.4B show successive sections through the same GLIR terminal. Similarly the immunogold particles are present within the terminals at a ratio of 5:1, as in the previous examples. In these examples possible synaptic contacts are also indicated by arrows. The examples shown are for two cells

of the three examined in this study. However, the same overall picture was observed in all three of the experimental rats.

In all the electron micrographs shown the possible sites of synaptic formations are not all that distinct. However on the gemmule (Fig. 2.5.2B) and in the other example Fig. 2.5.3) the so-called synapses have the appearance of symmetrical synapses. There is no indication of dense postsynaptic material, so these synapses probably conform to the description of Gray type 1 or symmetrical synapse. The synaptic vesicles tend to be pleomorphic and many vesicles are irregularly shaped. Another feature common at symmetrical synapses is the presence of a clustering of vesicles in the immediate vicinity of the synapse. These are known as "synaptic complexes", and often encountered in terminals with symmetrical synapses (Peters, 1987).

Although the number of examples are low and not all that distinct, it is likely that the structures observed in this study are inhibitory synapses. It is well known that ultrastructural diagnosis of the class of neurotransmitter, based upon the characteristics of the synapse is highly speculative especially when the functional attributes of the synapse are under question. However it is tempting to speculate given the overall picture, that the observed synapses are inhibitory. A more elaborate study will have to be designed to test the frequency and occurence of these types of synapses on giant cells within the PMRF. The frequency and spatial distribution of these synapses on the giant cell is also unknown but certainly as has been demonstrated in these two examples, GLIR-terminals contact these cells on the soma and on the proximal dendrites. In the cortex where the distribution of synapses is probably best understood symmetrical synapses are most common on the initial portions of the dendrite whereas the population of synaptic contacts on the more distal portions of the dendrite are of the asymmetrical form. In addition the frequency of both synaptic types is much lower along sparsely spinous or smooth dendrites than on spiny dendrites (Peters, 1987). This may also be the case with large aspinous RGC cells encountered in this study .

These ultrastructural details together with the detailed microanatomical structure and interconnections of PMRF cells are prerequisites for constructing a model of PMRF activity. Many more cells will have to be examined in greater detail to determine the spatial distribution of these GLIR-terminals and synaptic contacts.

Conclusion

The presence of GLIR-positive terminals within the PMRF is confirmed by the demonstration of electron dense immunogold particles contained within terminals contacting the soma and/or dendrites of RGC cells. The giant cells themselves do not appear to be GABAergic. Since GLIR-positive terminals are observed contacting three randomly selected neurones from the nucleus reticularis gigantocellularis in three different rats, together with the fact that the majority of PMRF cells tested show GABAceptive properties, it seems likely that many RGC cells receive GABAergic terminals. Even though GABAergic terminals have been demonstrated making contact with PMRF cells there is still no certainty that the observed bicuculline effects are expressed via subsynaptic GABA receptors. However the presence of GABA-like immunoreactive terminals strongly suggest that PMRF cells may be under the influence of tonic GABAergic inhibition. Since the giant cells themselves are not GABAergic and collective evidence does not support the presence of Golgi type II interneurones within the PMRF, these GABAergic terminals probably belong to GABAergic projection neurones lying outside the PMRF.

Responses of PMRF neurones to electrical stimulation of the substantia nigra pars reticulata, pars compacta and cerebral peduncle.

Introduction

The data in the previous section provide strong support for a tonic inhibitory input to PMRF neurones. It is thus of importance to establish where these inhibitory inputs originate. As mentioned before there are no known inhibitory inputs to PMRF neurones in the form of local circuit, Golgi type II interneurones arising in the immediate vicinity of the PMRF. It is therefore likely that these GABAergic fibers arise in an area outside the medial pontomedullary reticular formation. There is a growing body of evidence suggesting that GABAergic neurones also represent a population of projection neurones that send their axons considerable distances to target sites. This, in particular, is the case for the substantia nigra pars reticulata (SNr). It is the richest GABA containing nucleus in the central nervous system across a broad spectrum of vertebrates, ie. rat, cat and rabbit. Furthermore it has been shown to send predominantly GABAergic fibers to a number of distant target sites, viz. thalamus, superior colliculus, pedunculopontine nucleus and angular complex amongst others. In recent years a few electrophysiological investigations have aimed at demonstrating a direct connection between the PMRF and the SNr (Pardey-Borrero and Gonzales-Vegas, 1980; Gonzales-Vegas, 1981; Perciavalle, 1987). However, to date, the exact status of this pathway is uncertain as it has not gained widespread acceptance, judging by the lack of reference to this pathway in recent literature regarding the rat nervous system and especially its omission from recent reviews on basal ganglia anatomy and physiology (Paxinos, 1985). More recently Perciavalle, (1987) demonstrated that a sustained inhibition of PMRF neurones could be achieved as a result of SNr stimulation. The latter report, as do the others, refers only to stereotaxic co-ordinates of stimulation but provides no histological evidence of the actual site of stimulation. Precise location of the stimulating electrode is essential for interpretation of the responses as electrical stimulation applied within the substantia nigra not only activates nigral neurones but fibers *en passant*, as well as those fibers in the immediate vicinity of the substantia nigra such as corticofugal fibers coursing in the cerebral peduncle. There is a large contingent of corticoreticular fibers coursing to the reticular formation in the adjacent cerebral peduncle that may be affected by the spread of the stimulating current used to excite SNr neurones. This problem is highlighted by the substantial amount of controversial data concerning the effects of electrical stimulation of the nigro-tectal pathway due to the possible effects of stimulation on the surrounding fibers (Chevalier et al., 1981b).

Although PMRF responses to electrical stimulation of the SNr have been tested in previous studies, demonstration of the exact histological sites of stimulation have not been provided by many of these authors. Since the interpretation of effects of electrical stimulation in this area is confounded by the numerous fibers in the vicinity of stimulation, and the unwanted stimulation of fibers lying in the vicinity is an ever present problem, it is essential to know the exact point of electrical stimulation

correlated with particular PMRF responses, as a basis for claiming a connection between the SNr and brain stem.

Therefore, the aim of this study is to characterize PMRF responses to electrical stimulation of known anatomical sites within the SNr as well as in the cerebral peduncle and SNc and furthermore to establish the incidence of inhibitory effects on PMRF neurones elicited by stimulation of these areas.

Methods

All rats were anaesthetized with Urethane (1.2g/Kg) and mounted in a stereotaxic frame. A burr-hole was constructed over the target areas, according to the method described in the general methods section. A second slot-like burr-hole was constucted over the underlying target area at the following co-ordinates; AP 2.5 - 3.8mm, Lat 0.5 to 3.5mm. This enabled the placement of a stimulating electrode within the substantia nigra pars reticulata according to the atlas of Paxinos and Watson, (1982). The position of the stimulating electrode varied between AP 2.8 and 3.2mm, Lat 1.5 and 3.0mm and usually between 7.0 and 8.0mm ventral to the surface of the brain. Two types of stimulating electrodes were used in this study. The first type was self-manufactured Epoxylite resin (Clarke-Electromedical) coated tungsten wire (0.01 inch diam. Clarke-Electromedical) insulated along the length of the shaft except for the tip (size approx 0.2mm) (see General Methods, Section 1.2.3 for details of construction). The second type of electrode used in the majority of studies was the concentric bipolar stimulating electrode (Clarke Electromedical Instruments: tip size 0.2 : 0.5mm). The latter was the preferred type in that its configuration offered the advantage of a much reduced and more discrete stimulus artefact. The signal was amplified and filtered in the standard way (see Schematic representation Fig 2.6.1). However the output from the Neurolog averager was displayed on an oscilloscope and logged on a BBC microcomputer, using a program designed and developed by Dr. A.George, (see Acknowledgements), for use in spike data capture. All data logged on the BBC was stored on floppy disks. These BBC PSTH data were then transferred from the BBC disks to a DOS disk using a dual disk drive and the "getfile" command in DOS Plus version 5. All the BBC PSTH data files were then transferred to hard disk on a Nimbus PC and converted to a form that could be imported into Excel, using a program (BBC PSTH) written by Prof. R.J. Douglas. PSTH graphs were generated using the "Freelance" graphics system. Hard copies were made on a Hewlett Packard 7470A pen Plotter or Laser Printer.

Protocol

Extracellular recordings from PMRF giant cells were recorded by means of glass electrodes filled with 2 to 3 M NaCl, and tip impedances of between 5 and 10 MW. Once an extracellular action potential of a PMRF giant cell was located the electrode position was adjusted to yield an optimal extracellular signal. The standard procedures for identifying the cell's receptive field were followed. Standard

electrophysiological recording procedures were used (Fig 2.6.1). Logic pulses from a window discriminator were led to the Neurolog averager and the binary output to the BBC. A control period of a few minutes duration was allowed for the basal firing rate to resume; the rats were stimulated via the implanted tungsten or bipolar electrode from a stimulus isolation unit driven by the Neurolog. The characteristics of the stimulus were as follows (square wave pulses, duration 250μs, and amplitude ranging between 200 and 600 μAmp). Peristimulus time histograms (PSTH) were constructed from a number of sweeps (200 - 500) consisting of a 50ms control period followed by the stimulus pulse of 250 μs duration and a further 50ms response period. All the PSTH's were logged on a BBC microcomputer and viewed before being stored on disk for later analysis.

Histological procedure

At the end of the recording session the bipolar, or insulated tungsten electrode was withdrawn. The rat was rapidly perfused by the transcardial route with heparinized saline to exsanguinate it, followed immediately by an initial rapid perfusion with 10% buffered formaldehyde,(150 - 200 ml), and then by a much slower perfusion (300ml). The fixed brains were blocked coronally in the same plane as the electrode and cut at 100μ thickness through both the mesencephalic stimulation sites and the PMRF recording sites, mounted on gelatinized glass slides and stained with Creysl Fast Violet. The stimulating electrode tracts were reconstructed by means of the camera lucida technique and the exact locations of the tips recorded. Fig. 2.6.2 shows the individual mesencephalic stimulation sites in 23 rats.

In some cases two different stimulation sites in the mediolateral plane were tested, as indicated by two dark dots, within the same coronal section (e.g. 10-3 and 9-3). The same cell could rarely be tested from two or more stimulation sites, as the movement imparted to the surrounding tissue as a result of relocating the stimulating electrode invariably caused tissue movement with the consequent destruction of the recording. The electrode was thus usually positioned at one location during the test period and the only manipulation attempted was the slight adjustment of the depth of the stimulating electrode.

Results

Fig 2.6.2 shows the camera lucida reconstructions of coronal sections of 22 rats indicating the exact location of the tip of the bipolar stimulating electrode (dark dot). In the majority of cases the electrode tip was situated in the substantia nigra pars reticulata or in its dorsal border, i.e. just ventral to the substantia nigra pars compacta, or on its ventral border ie. immediately dorsal to the cerebral peduncle. In two cases, 14-4 and 15-4, the electrode tip was positioned in the SNc, and in 4 cases, 16-6, 23-4, 13-4 and 22-4, the tip was initially positioned in the cerebral peduncle. Many accounts in the literature claim that mainly excitatory fibers course in the cerebral peduncle. Accordingly, when the stimulating electrode was placed in or very near to the cerebral peduncle mainly excitatory responses from the PMRF were observed. Where the intention was to avoid cerebral peduncle stimulation, the sudden appearance of large excitatory responses was used as an approximate indicator of the electrode tips position, and consequently the stimulating electrode was withdrawn to a more dorsal position. In cases 17-3 and 9-4, the electrode tip was initially positioned in the cerebral peduncle and then subsequently in the SNr. Final depth adjustments to the stimulating electrode in most cases were in steps of approximately 0.5 mm. However, unless specified, once the electrode was positioned according to the stereotactic co-ordinate, it remained in position for the duration of the experiment.

The responses of 128 PMRF neurones to electrical stimulation of the SNr, SNc and cp were tested at various anteroposterior, mediolateral and dorsoventral positions of the stimulating electrode within the SNr at the following stereotaxic co-ordinates; A.P 2.8 to 3.5mm; laterally between 1.5mm (medial zone) and 3.0mm (lateral zone) and in the dorsal, middle and ventral positions within the SNr. The responses of PMRF cells to stimulation of the SNc and the cp are also within these stereotaxic co-ordinates.

As the responses of PMRF cells are extremely variable in terms of timing and duration of the inhibitory episodes, the responses are grouped according to the duration of the inhibitory period, irrespective of the presence of excitatory peaks (primary, secondary or rebound). This was done in order to gain an impression of the incidence of inhibition arising from stimulation of the SNr and surrounding area.

The responses of 103 neurones to stimulation of histologically confirmed locations are reported in Table 1.3.6(i). The response profiles of the remaining 25 neurones have not been included in this discussion as the anatomical location of the stimulus sites is unconfirmed.

Table 1.3.6(i)

Summary of PMRF cells showing variable duration inhibitory episodes or excitatory responses grouped according to the electrical stimulation site within SNr, SNc and cp

SNr n=58	SNc n=25	cp n=20
Inhib >10ms 7/58(12%)	Inhib >10ms 6/25(24%)	Inhib >10ms 3/20(15%)
<10ms 7/58(12%)	<10ms 12/25(48%)	<10ms 6/20(30%)
<5ms 25/58(43%)	<5ms 6/25(24%)	<5ms 3/20(15%)
(n)Inhib 39/58(67%)	(n)Inhib 24/25(96%)	(n)Inhib 12/20(60%)
(n)SDI 32/58(55%)	(n)SDI 18/25(72%)	(n)SDI 9/20(45%)
Excit 18/58(31%)	Excit 1/25(4%)	Excit 6/20(30%)
(0) 1	(0) -	(0) 2(depress)

Where:
>10ms, <10ms and <5ms respectively = duration (ms) of inhibitory episodes shown by PMRF neurones
(n) Inhib = number of cells showing variable periods of inhibition
(n)SDI = number of cells showing short duration inhibition
Excit = cells showing excitatory responses
(0) = unresponsive, or showing marked depression

The responses of 103 PMRF cells reported in Table 1.3.6(i) to stimulation within SNr, SNc and cp for which the exact site of stimulation is known, show a high incidence of short latency, predominantly short duration inhibition elicited from all of these mesencephalic sites. The exact site of stimulation determined from camera lucida reconstructions is reported in Fig 2.6.2. The responses of 58/103 (56%) cells were recorded from stimulation of various sites within the SNr, 25/103(24%) from the SNc and 20/103(19%) from the cp. As the nature of the responses appear to differ widely according to the stimulation site, the PMRF responses elicited from SNr, SNc and cp were grouped according to the anatomical stimulation site and examined in detail, before attempting a global description of the responses. Consequently these data have been divided into three groups viz., A, B and C below:-

(A) Stimulation site: Various areas within the SNr.

Responses elicited from individual PMRF neurones in two rats (27/4 and 23/1) to stimulation of the SNr in the medial mid-zone serve to illustrate the different response profiles of cells to stimulation in this area. The responses of only 4 PMRF cells are shown in Fig 2.6.3a and b. These examples demonstrate short latency (1 to 2 ms), inhibitory responses with large variations in duration of the inhibitory period, ranging from approximately 40ms to <10ms. However the response profiles are also characterised by short duration excitatory peaks (Fig 2.6.3b). The response of cell (27/4C6) is characterised by two short duration excitatory peaks preceding the 13 ms inhibitory episode, with a short (<5ms) inhibitory period interposed between the excitatory peaks. Responses from cells to stimulation of an approximately equivalent site within the SNr in another rat (Fig 2.6.2, 29-7b) demonstrate a similar pattern of inhibitory responses. Of 4 PMRF cells tested in this rat, short latency, short duration inhibition (<5ms), similar to cell 27/4C7, as well as short latency, short duration excitatory peaks are evident. In the remaining cell the firing rate is unaffected. Therefore the 8 examples show variable duration inhibitory effects to stimulation in this area. The majority of inhibitory responses are of short duration only. However, short duration excitatory responses could also be elicited from this area of the SNr.

Fig 2.6.4a and b, illustrate the responses of PMRF cells to stimulation of the mid SNr lying slightly towards the lateral zone. All the cells responded to stimulation of this area with short latency, short duration (<10ms) inhibition. However in one case (22/7C4) a large primary excitatory peak precedes the inhibitory episode.

Fig 2.6.5 and Fig 2.6.6 show the responses of 6 cells to stimulation of the more ventral aspect of the SNr. In one rat (Fig 2.6.5) the responses recorded are: no response from cell 11/3C1; primary excitation followed by slightly reduced activity and slight excitatory rebound from cell 11/3C4; long latency (approx 5ms) primary excitation and short duration (>10ms) inhibition from cell 11/3C4b. The latter response is from the same cell after altering the position of the stimulating electrode to a slightly more dorsal position. The amplitude of the excitatory peak evoked from the previous site was decreased and followed by a short duration (<10 ms) inhibitory period not present previously.

Fig 2.6.6 shows the responses of 2 PMRF cells in the second rat, to stimulation of the ventral area of the SNr, but at a location slightly more medial and posterior to the

previous position of the stimulating electrode. Both cells show short duration (<10ms) inhibitory periods.

As stimulation at different sites within the SNr may have a profound effect on the response profile of a PMRF cell, it is important to establish over what distance the effects of the stimulating electrode can alter the evoked PMRF response. Where possible, attempts were made to record from the same cell at various stimulation sites. The electrode was advanced in stages from a more dorsal position within the nucleus towards its ventral border with the cerebral peduncle.

Two such cases are illustrated. Figs 2.6.7a and b show the responses of cells 9/4C1 and 9/4C5 to successive stimulation trials of the SNr, at progressively deeper positions of the tip of the stimulating electrode. At the dorsal position of the electrode (border between SNc and SNr) there is short latency, short duration depression followed by large primary excitatory activity. The response profile is largely unaffected by stimulation of the more ventral position of the stimulating electrode. The large excitatory response is therefore elicited from both sites of stimulation. At the dorsal position of the electrode, cell 9/4C5 (Fig 2.6.7b) showed immediate depression of activity for about 10 ms after the stimulus. At a slightly deeper position, short latency multiple primary excitatory peaks of about 8msec in total duration precede a short duration inhibition (<5msec) and depressed episode lasting a further 10ms. This in turn is followed by rebound excitatory activity. At the most ventral position stimulated, short latency, short duration excitatory peaks are once again manifest. However, the number of peaks and duration (<5ms) of the excitatory episode are reduced, and the depressed activity gives way to complete inhibition lasting about 13ms. Similar rebound excitatory activity is present after the inhibitory episode.

These two cases therefore illustrate the dramatic effect on the overall response of the cell to stimulation of the dorsal and ventral border areas of SNr respectively. The response of cell 9/4C3 (Fig 2.6.7c) shows a similar overall pattern, as elicited from 9/4C5b, to equivalent stimulation sites in the middle zone of the SNr.

(B) Stimulation site: ventral border SNr and cerebral peduncle.

Cerebral peduncle stimulation at a more ventral position of the stimulating electrode evoked a short latency, short duration (<10ms) inhibitory response with no primary excitatory responses from cell 9/4C4, in contrast to the mixed excitatory/inhibitory responses observed in 9/4Cb and 9/4C5c. On the basis of these responses it is evident that stimulation of the more caudal SNr and the area bordering on the cerebral peduncle is capable of eliciting responses from PMRF cells that are not markedly different from stimulation sites within the bulk of the SNr.

Fig 2.6.8a and b and Fig 2.6.9 illustrate the response of 4 PMRF cells to stimulation of the caudal cerebral peduncle. All of the responses are characterised by short duration excitatory peaks occurring within 5ms of stimulation and inhibitory periods of <10ms in 3 cases. Cell 13/4C3b shows an inhibitory episode of 30ms duration. In another rat (Fig 2.6.9) stimulation of the caudal SNr/cp border similarly elicited short duration primary excitation followed by short duration (<10ms) inhibition. Another cell (23/4C8) showed short duration inhibition (<10ms) followed by rebound

excitatory activity and the other example showed only a small primary excitatory peak.

In a further two examples of responses to caudal SNr/cp border stimulation (Fig 2.6.10) both cells show large primary excitatory peaks occurring within the 10ms after stimulation. Thus stimulation of the caudal region of the SNr and cerebral peduncle elicits mixed effects from PMRF cells consisting of inhibition as well as excitation.

(C) Stimulation site: SNc.

The PMRF responses were tested in 2 rats to stimulation of the SNc. Fig 2.6.11a, b and c shows the response of 8 PMRF cells to stimulation of the medial SNc at approximately A.P 3.00 in both rats. In the examples shown, inhibitory responses were elicited from the PMRF cells with the exception of one cell. In 6/8 cases, clear short duration (<10ms) inhibitory periods were evident; however in one case (14/4C2) the duration of the inhibitory period was >50ms. The remaining cell (15/4C2) showed only a large primary excitatory peak occurring within 10ms after stimulation.

The remainder of cells within this group (PSTHs not shown) also demonstrate short duration inhibitory periods either preceded or followed by a large excitatory peak. Thus the majority of PMRF responses induced from SNc stimulation typically showed a high incidence of short duration inhibitory episodes. However, in another case, a long duration inhibitory episode (> 50ms) could also be elicited from stimulation of this site.

The incidence and duration of the various inhibitory episodes elicited from the respective stimulation sites are reported in Table 1.3.6(i). Overall, SNr stimulation evoked short latency, variable duration inhibitory effects from 39/58(67%) PMRF cells. This may be further subdivided on the basis of the duration of inhibition, where: 25/58 (43%) show short latency, <5ms inhibitory periods, 7/58 (12%) show short duration (<10ms) inhibitory responses and 7/58(12%) show slightly longer (10 to 50ms) inhibitory periods, of which one cell showed inhibition in excess of 50ms.

Stimulation of cerebral peduncle/SNr border area elicits short duration (<10ms) inhibitory responses in 12/20 (60%) cells and in 3/20(15 %) cells duration of lasting >10ms but <50ms.

SNc stimulation evoked variable duration inhibitory responses in 24/25(96%) cases and only excitation without any inhibitory phase in one case.

These data therefore attest to the widespread inhibitory effects on PMRF neurones achieved by SNr, SNc or cp stimulation.

Discussion

The data indicate that from all positions within the SNr, SNc as well as the cerebral peduncle, short duration (< 10 msec) inhibitory responses are elicited from a large percentage of PMRF neurones. Longer duration (>10ms) inhibitory responses are in the minority and in only 3/103 cells are the inhibitory periods ≥50ms.

In addition to the inhibitory responses, the response profiles of these cells show a large variation. In some cases the inhibitory responses are preceded by a large primary excitatory peak and/or followed by rebound excitation. In some cases the large primary excitatory peaks are absent and the response is characterized by short latency, short duration inhibition. With respect to the responses elicited from the SNr no particular response profile was preferentially elicited from a particular site within the SNr. There is a large variation of responses to broadly overlapping areas of stimulation within the SNr, and many of these are characterized by large excitatory responses either preceding or following a short inhibitory period. On the basis of these data, it is difficult to predict the type of evoked response that may be elicited from a PMRF cell stimulated from a particular area within the SNr. However, stimulation sites closer to the cerebral peduncle may be correlated with a higher incidence of PMRF excitatory responses, but the cp stimulation data also indicate that some fibers occupying the area in the region of the ventral SNr, bordering on the cerebral peduncle also exert clear inhibitory effects.

Judging from the mixed responses of the PMRF neurones to stimulation of the SNr, it is evident that a number of structures may be influenced by the stimulation electrode. Firstly, somata within the SNr itself and in the immediate vicinity, secondly the fibers *en passant* and thirdly collateral axons of the PMRF cells themselves. The stimulation data therefore have to take into account these alternative sources. The present data show that variable duration inhibition as well as excitation can be elicited from all these stimulaton sites, making it extremely difficult to predict the outcome of stimulation of PMRF cells from the various sites within the SNr, as well as from the immediate surrounding area. The varied responses elicited from substantia nigra and the cerebral peduncle stimulation therefore depends on the regional distribution of cellular and fiber components that are stimulated.

The cells investigated in the present study are comparable to those of Pardey-Borrero and Gonzalez-Vegas, (1980) as they also studied cells in the RGC; however as only 37 units were studied their sample was relatively small. Of these 22/37 (59%) showed periods of inhibition ranging in duration from 20 to 120 ms sometimes followed by excitation. The mean latency of the responses are 12.5 ms. Furthermore in their study 9/37 (24%) were excited and, in 2 of these, excitation was followed by inhibition. These inhibitory responses are somewhat different to those in the present study. Although it is not indicated what the relative percentage of long duration (120ms) inhibition is, only two cells in the present study showed inhibitory periods in excess of 50ms. Furthermore the majority of responses in the present study were characterized by short latency responses.

Perciavalle's (1987) study in the rat also examines the nigro-reticular connection. This study was conducted on reticulospinal neurones but most of the cells from which their responses were obtained comprised an area stretching from the rostral medulla

(at the level of the genu of the facial nerve) rostrally to the level of the inferior colliculus. Thus their sample of reticulospinal cells are composed of cells lying predominantly in the rostral reticular formation. The cell population in the present study lie mainly in the medullary region of the reticular formation. However, their data indicate that the majority (73%) of cells, from those of which inhibition is elicited, show an inhibitory period with a mean duration of approximately 55ms. Furthermore the latencies are of the order of 5ms. Perciavalle showed that the responses could be divided into three categories on the basis of their latencies and duration of inhibition. His overall findings indicate that mean short duration (10.5ms) inhibitory responses are displayed by only 20% of the neurones showing inhibition. It is thus clear from this study that the majority of inhibitory responses recorded in his study are quite different from those in the present study, as only 7/58(12%) of cells showed inhibitory periods >10ms. This difference in response can probably be explained on the basis of the different cell population and/or contingent of fibers being stimulated from within the SNr. Furthermore the connectivity of the rostral reticulospinal neurones with the SN and the surrounding mesencephalon may be different to those lying in the more caudal regions of the brain stem. No histological indication of the sites of stimulation are however provided in the Peciavalle study.

In the present study predominantly excitatory effects with no inhibitory episodes are observed in 31% of PMRF cells driven from SNr sites. These effects are probably due to spread of the stimulating current to corticoreticular fibers projecting in the cerebral peduncle. Indeed where ablations are placed in the sensorimotor cortex of the rat, excitatory effects on PMRF neurones are abolished (Perciavalle, 1987). However, the present data are in conflict with this finding as both excitatory and inhibitory effects on PMRF cells are elicited from cerebral peduncle stimulation. Predominantly short duration inhibitory periods are elicited from 9/20(45%) PMRF cells. However the responses are also characterized by the presence of large primary excitatory peaks. In some cases this preceded inhibition or followed short duration inhibition.

Where SN cells are destroyed by kainic acid lesions, only excitatory responses could be elicited from PMRF cells and no inhibition is evident (Perciavalle, 1987). These data indicate that fibers of passage are responsible for the excitatory effects observed, and that stimulation of SN cells underlie the inhibitory effects observed. Although these data indicate that PMRF inhibition can be derived from the SN stimulation it does not differentiate between the SNc or SNr population of cells, which may be the source of these inhibitory fibers, as the kainic acid lesions probably cause unselectively destructive of both SNc and SNr cells. Magni and Willis (1964) have shown that many reticular neurones receive short latency excitation from the cerebral cortex. However when the cerebral peduncle was stimulated they observed that there was a wide dispersion of latencies and suggested that many reticular formation neuones probably receive only indirect cortical excitation.

Barasi and Duggal, (1982) and Duggal and Barasi, (1983) reported that electrical stimulation, with low stimulus strengths, of the substantia nigra in the rat, evoked inhibitory responses from cells in both RGC and nucleus reticularis para-

gigantocellularis (RpGC) in roughly equal proportions. These authors showed that inhibitory effects elicited from mesencephalic reticular formation neurones were observed only when the stimulation electrode was placed in the "vicinity of the SN". In addition they reported long latency excitatory responses also derived from equivalent stimulation sites. Excitatory response could also be elicited from extranigral stimulation sites but these were of short latency. Their data are difficult to interpret in the context of the present findings as no indication of the exact site of stimulation is given. Duggal and Barasi, (1983) have also shown that cells in RGC and RpGC are not selectively influenced by stimulation of the substantia nigra or the immediate vicinity. Evoked responses of the medullary reticular formation cells in their study showed either excitation or inhibition, of which 9% of the evoked responses were inhibitory and 43% excitatory. In the present study the incidence of PMRF inhibition is much higher, being 67% and 96% for sites within SNr and SNc respectively. Very few neurones were selectively excited or selectively inhibited and most reponse profiles consisted of a sequence of excitatory and inhibitory events. Furthermore the latencies of responses in the present study are predominatly of short duration (of the order of 1 to 2 ms) whereas they report latencies of between 2 and 18ms. The longest duration of latency was observed in one case by stimulation within the SNr and not outside of this nucleus. Furthermore inhibitory responses were characterized by inhibitory periods ranging from 20 to 200 ms. By contrast, the present findings indicate that the majority of inhibitory responses have relatively short duration inhibitory episodes. If the PMRF inhibitory responses are considered together, irrespective of whether the SNr or SNc was stimulated, then 50/83 (60%) of cells showed periods of inhibition of less than 10ms, and only 13/78(7%) inhibitory periods of greater than 10ms. These findings thus differ substantially from those of Duggal and Barasi, (1983).

Although evoked activity was also recorded from RGC cells by Barasi and Duggal, these authors, firstly, do not clarify the exact position within the substantia nigra from which the stimulation took place and, secondly, do not describe the exact nature of the inhibitory responses especially of the RGC neurones. Although noting a large variation in the latency of responses, these authors do not comment on the duration of the inhibitory response.

As the response of these PMRF cells to mesencephalic stimulation can conceivably be affected by stimulation of SNr cells, fibers *en passant*, and collaterals from PMRF neurones it is a daunting task to tease out the respective contributory components. Pardey-Borrero and Gonzales-Vegas, (1980) also provided evidence for a reciprocal connection between the PMRF and the SN in that stimulation of RGC resulted in evoked activity in cells located in the SN and VTA and some of the units in this area showed antidromic activation, suggesting a monosynaptic connection between cells of PMRF and SN. This means that RGC neurones may also be affected via antidromic stimulation from sites in the SN and VTA.

However, Duggal and Barasi, (1983) could not demonstrate reticulo-nigral connections by stimulation of the ventral part of RpGC as only 1/75 SN neurones could be driven antidromically. This difference may be accounted for in terms of the different stimulation site and therefore activation of a different group of neighbouring cells. Double labelling fluorescent studies by Waltzer and Martin, (1984) suggest

that a few neurones in overlapping areas of the RGC provide collaterals to the cerebellum and diencephalon. In keeping with one of the suggested functions of the brain stem reticular formation of influencing widespread regions of the neural axis, it is conceivable that the observed responses in the SNr may be mediated by long collaterals from RGC neurones. Thus it is possible that the evoked responses observed are via collateral connections, but so far there has been no study confirming this.

Matsuyama et al., (1988) using the highly specific anterograde tracer, phaseolus vulgaris leucoagglutinin (PHA-L) injected into cat RGC, demonstrated ascending PHA-L fibers projecting to all the cranial motor nuclei of the brain stem, the locus coeruleus, and the raphe nucleus. At a diencephalic level PHA-L stained projections were located in both specific and non-specific thalamic nuclei. However at a mesencephalic level only moderate projections to the red nucleus were found. These authors do not mention any connections to the SN, however a dense projection of PHA-L stained fibers project to the PAG. Thus the electrophysiological evidence provided by Pardey-Borrero and Gonzalez-Vegas, (1980) for the activation of some cells in the SN and VTA are difficult to reconcile with these anatomical data achieved by means of specific anterograde PHA-L staining.

Shammah-Lagnado et al., (1987) have studied in detail the afferent connections of reticularis pontis oralis and caudalis in the rat. The afferent projections of these two nuclei were studied in detail after discrete injections of either free HRP or WGA-HRP deposited by microionophoresis. The location of labelled somata whether by HRP or WGA-HRP were found to be similar. Amongst the many different structures consistently taking up HRP, the HRP staining of SNc and SNr, (according to their subjective rating), ranged from moderate to low respectively, from deposition sites within pontis oralis. There was no evidence of labelling of SNr cells from more caudal deposition sites, such as the pontis caudalis. From this site only SNc were labelled and the intensity was rated as low. It therefore seems likely that the further caudal in the brain stem the injection site, the lower the incidence of staining of reticulata cells with HRP.

Petrovicky, (1988) injected HRP into the pontomesencephalic area, which included pontis oralis and pontis caudalis and the caudal parts of the nucleus cuneiformis, and similarly found that retrograde staining of the SNr was much less prominent than staining from more rostral depositions. Petrovicky also demonstrated the collateralized nature of the nigrostriatal projection, with collateral branches extending caudally.

These data therefore do not provide strong support for the existence of a direct connection between the PMRF and the SN. These data also do not support the existence of a projection coursing as far as the medullary reticular brain stem. Therefore electrical stimulation of the SN is unlikely to affect these caudal components which may have an influence on the overall response profile of these cells.

What of the wiring of connections between SNr and PMRF? On the basis of electrophysiological experiments showing RGC units antidromically activated from substantia nigra, (SN) Pardey-Borrero and Gonzales-Vegas, (1980) have postulated that there are direct monosynaptic connections between the SN and the RGC, and

that the pathway runs both ipsi and contralaterally. However these data are difficult to interpret as they do not provide evidence of the exact stimulation site.

In the microstimulation studies of McCarley et al., (1987) a high percentage (72%) of medial PMRF intracellular recordings showed EPSP responses to stimulation of the mesencephalic reticular formation. Although the exact histological placement of the stimulating electrode is not shown they claim to have positioned the electrode lateral to the medial longitudinal fasciculus in the gigantocellular tegmental field. The information obtained from these studies indicate that either a high percentage of fibers have their source in the mesencephalic tegmental field and/or that fibers *en passant* which are affected by the stimulating electrode are excitatory. Thus the inhibitory effects seen in the present study as well as those in the aforementioned ones are likely to arise in, or course through, a highly localized area in the mesencephalon in the vicinity of the substantia nigra.

The anatomical data concerning connections between the SN and the PMRF in either the rat or cat is contradictory. Schneider et al., (1985) have shown the presence of WGA-HRP containing somata in the SNr retrogradely labelled from injection sites in the medial gigantocellular tegmental field of the cat. Somata within the SNr were labelled with WGA-HRP on the ipsilateral side only, but no labelling of cells in the SNc and the VTA were observed. Labelled cells were also seen in the fastigial nucleus, oculomotor nucleus and deep layers of the superior colliculus (SC). Since WGA-HRP was injected into the gigantocellular tegmental field, it is conceivable that SNr cells may have been retrogradely labelled with WGA-HRP from a relay within the SC, as anatomical connection between SNr and SC have been clearly demonstrated Rinvik et al., (1976).

Although Hopkins and Niessen, (1976) have also demonstrated connections between the SN and lower brain stem of the cat, most of their HRP injection sites were however located in the parabrachial pontine region. Furthermore these authors have also provided evidence that injections of discrete amounts of HRP into various regions of the lower brain stem of the cat, monkey and rat have labelled cells within the SNr, thus supporting the presence of direct projections from the substantia nigra pars reticulata to the lower brain stem of all the aforementioned species. In the rat the HRP injection sites within the caudal brain stem region are not shown. It is therefore difficult to assess which nuclei within the brain stem were affected by the HRP deposition and its consequent spread to the immediate surroundings. In Hopkins and Niessen's (1976) study, one example in the rat showed SNr HRP labelling from an injection site within the pontine tegmentum; however the nuclei that were labelled within the tegmentum are not indicated. This, at least is indicative of a SNr projection to rostral levels of the lower brain stem. However, based on HRP injections at various levels within the brain stem, a broad classification of caudal projections from the SNr appears to exist, as SNr cells situated at rostral and caudal levels within the nucleus were labelled from more rostral and more caudal brain stem HRP deposition sites, respectively. Therefore in this study, in the cat, the monkey and the rat, there is clear evidence of labelling within the SN from deposition sites in the rostral brain stem. On the other hand, as mentioned above, two studies (Shammah-Lagnado, 1987; Petrovicky, 1988) employing ionophoretically applied

HRP as a tracer, have failed to show any nigral projection in the cat or the rat to the level of the medullary reticular formation.

Autoradiographic tracing with ^{3}H-leucine injected into the SNr of rats with destroyed dopaminergic cells show projections from non-dopaminergic cells to the region of nucleus tegmenti pedunculopontinus, lying close to the cerebellar peduncle (Wright and Arbuthnott, 1980). In this study there is no indication of any fiber projections lying caudal to the PPN. On the strength of these data, the effects seen on PMRF cells by stimulation of the SNr may occur via the nucleus tegmenti pedunculopontinus (PPN) relay, as there is good evidence in support of descending projections from PPN (Spann and Grofova, 1984). This may account for the long latency effects seen in some of the abovementioned studies.

The inhibitory effects on PMRF cells from stimulation sites within the SNr are clearcut, however the majority of evoked PMRF responses from SNr display only short latency, short duration inhibition (< 10msec), usually preceded or followed by excitatory peaks. The longest duration inhibition (>50ms) of two PMRF cells in this study was shown to be derived from stimulation of the SNc. Therefore extreme caution has to be exercised in interpreting the electrical stimulation data, since matters are complicated by the possible stimulation of fibers passing though this region and certainly those in the close vicinity.

The present study also indicates that the cerbral peduncle carries inhibitory fibers. In a study of the connections between the SN and the superior colliculus Chevalier et al., (1984) showed that in normal intact rats SN stimulation induced both excitatory and inhibitory responses in superior colliculus (SC) cells but after transection of the cerebral peduncle and optic tract fibers rostral to the SN, stimulation of the SN only induced inhibitory effects. These authors therefore demonstrated that the fibers comprising the nigrotegmental tract are predominantly inhibitory. In further experiments SN stimulation in rats in which nigral cells were destroyed by kainic acid produced only excitatory responses, thereby confirming that that nigrotectal projections are inhibitory. But stimulation of cerebral-peduncle and optic tract fibers induced both inhibitory and excitatory responses of SC cells. Suggesting therefore that the SC receives excitatory and inhibitory input from the cerebral peduncle and optic tracts and only inhibitory input from the SN.

Considering all these data in support of a direct connection between the substantia nigra pars reticulata and the medial ponto medullary region of the reticular formation in the rat it is apparent that these data are largely inconclusive. The earlier electrophsiological data claiming such a connection are also inconclusive due to the problem of unwanted stimulation of fibers in the immediate vicinity. The possible sites of origin of electrically acitvated fibers *en passant* are, cortical neurones, and various subthalamic areas such as zona incerta and fields of Forel, which have been shown by Shammagh-Lagnado et al (1987) to have a relatively dense ipsilateral projection to the PMRF. The majority of mesencephalic stimulation studies cannot differentiate between activation of fibers of passage and of mesencephalic reticular formation neuronal somata.

There is major uncertainty regarding the anatomical connections between the substantia nigra pars reticulata and the brain stem pontomedullary region. The electrophysiological data in general is in favour of a "nigro-reticular" connection

(Perciavalle (1987); Gonzalez-Vegas (1981) and Pardy-Borrero & Gonzales-Vegas 1980). However the anatomical data is conflicting since some reports support the presence of a connection between the SNr and PMRF and others do not.

Conclusion

Both inhibitory and excitatory effects on PMRF cells could be elicited from stimulation of SNr, SNc and cp. A high percentage of PMRF cells show short duration inhibitory responses to SN stimulation, and only a very few show sustained inhibition as reported in previous literature reports. These results differ somewhat from those in other studies. However, direct comparison of data is difficult owing to the lack of histological data on the stimulation sites used in many of these studies. Since similar inhibitory effects on PMRF cells could be elicited from all of the structures stimulated in the present study, the specificity of the SNr projections to the PMRF is doubtful.

Anterograde tract-tracing of neural connections between substantia nigra pars reticulata and the PMRF with highly specific anterograde tracer phaseolus vulgaris-leucoagglutinin (PHA-L)

Introduction

Central fiber connections can be traced with a high degree of accuracy using the technique based on the uptake, transport and immunocytochemical detection of injected kidney-bean lectin, Phaseolus vulgaris-leucoagglutinin (PHA-L). Lectins are plant proteins that are known to bind with various degrees of specificity to certain surface glycoproteins or glycolipids of neurones (Dumas et al., 1979; Gonatas et al., 1977). The uptake and transport of injected lectins is undoubtedly facilitated by this property. The lectin wheat-germ agglutinin (WGA) is transported in both an anterograde and retrograde manner (Sherk and Levay, 1979; Lechan et al., 1981; Gerfen et al., 1982). Subsequently Gerfen and Sawchenko, (1984) have largely been responsible for developing the technique based on PHA-L as a sensitive anterogradely transported marker.

As the general trend in neuroscience today emphasizes the necessity for combinations of techniques as the most desired analytical tool, the impression should not be gained that the use of PHA-L on its own is of limited use. Certainly, some cell groups that share biochemical specificity often give rise to fiber groups that become inextricably mixed. In such cases immunocytochemical techniques used in isolation, which allows cell bodies, fibers and terminals of antigen specific neuronal systems to be labelled exclusively may be of questionable value in tracing their axonal projections.

Recent techniques (Sawchenko and Swanson, 1981; van der Kooy and Steinbusch, 1980) involve the combination of various retrograde, anterograde and immunohistochemical markers permitting chemically specific pathways to be delineated more precisely. However in a situation where the route and or the destination of a fiber projection is largely unknown, the predominantly anterograde nature of PHA-L makes it a particularly useful tracer. It is this property that is applicable to the anatomical problem encountered in the present study.

The use of PHA-L offers several advantages over the other commonly used tracer techniques. The injection sites are discrete, compared with tracers such as HRP where the spread of the tracer is difficult to contain, and therefore makes the interpretation of tract-tracing data that much more difficult or inconclusive. With PHA-L there is very little uncontained spread to sites adjacent to the injection. At the site of injection there is complete labelling of neurones in the immediate vicinity (Wouterlood and Groenewegen, 1985). Furthermore the number of labelled neurones varies in relation to the dendritic topography of the neurones at the injection site. According to Gerfen et al., (1989) anterogradely transported PHA-L occurs only in those neurones incorporating the tracer at the time of injection. It has been reported that PHA-L stained somata, dendrites and their appendages, are comparable to

neurones that have been stained by means of Golgi impregnation. Axons with their varicosities and terminal boutons are well defined.

Another major significant feature of this technique, is ability of being able to inject PHA-L in sufficiently small quantities by ionophoretic means into circumscribed sites within the CNS. Furthermore, fibers en passant do not take up the tracer to any significant extent (Gerfen and Sawchenko, 1982; Gerfen and Sawchenko, 1984). Thus the properties of this tracer are well suited to the study of connections between the SNr and the PMRF. PHA-L has one disadvantage though in that it has a long optimal uptake time, of the order of 10 to 14 days.

The opinion regarding the existence of the pathway between the SNr and the PMRF has vacillated during the last few years between acceptance on the one hand (Gonzales-Vegas, 1981) and rejection on the other (Duggal and Barasi, 1983). Electrophysiological evidence in the rat however supports the existence of a direct pathway between the SNr and PMRF (Gonzales-Vegas, 1981; Pardy-Borrero and Gonzales-Vegas, 1980; Perciavalle, 1987). In the present study the electrical stimulation of the SNr and the surrounding area which was shown to elicit predominantly short latency, short duration inhibition, suggests that there are many inhibitory connections with PMRF neurones that either originate in and/or course through this area of the mesencephalon. Therefore, these data cannot discount the possibility that SNr fibers may at least be part of a group of inhibitory connections contributing fibers to the PMRF. However without specific disruption of known projection pathways, lesioning of parent somata and double labelling techniques the present stimulation data is difficult to interpret. Since the immunocytochemical data in the present study provide evidence for GABA-like immunoreactive terminals contacting PMRF neurones, it is likely that these inhibitory contacts may underly the inhibitory effects on PMRF neurones derived from electrical stimulation of the GABA-rich SNr.

The evidence for such a direct connection between the SNr and the PMRF was discussed in the previous section. Although some of the available electrophysiological evidence strongly favours a direct inhibitory connection between the SNr and the PMRF, to date there are only a few reports that describe this pathway. The anatomical evidence in support of a direct pathway between the SNr and PMRF is contradictory and there are no published data on the use of PHA-L in this context. However, both Leigh et al., (1985) employing anterograde and retrograde techniques in the rat and Rinvik et al., (1976) employing the technique of axonal transport of protein in the cat, provide some evidence for the anatomical connections between the SNr and the reticular formation. However the anatomical evidence for GABAergic terminal fields within the PMRF is unconvincing. Judging by a recent and comprehensive review on the rat nervous system by Heimer et al., (1985) it is apparent that the existence of this pathway has not gained general acceptance. No reference to the data of the above authors is cited. Furthermore a recent issue of Trends in Neuroscience, (1990) devoted entirely to descriptions of basic physiological principles and anatomical connections of the basal ganglia, similarly does not refer to this putative pathway. The exact status of the proposed connection between the SNr and the PMRF pathway in the rat is therefore uncertain at the present time.

This study therefore aims at tracing the anatomical pathway between the SNr and the PMRF by employing the high specific anterograde tract-tracer, phaseolus vulgaris leucoagglutinin.

Methods

Injection of PHA-L into the rat SNr

Rats were surgically anaesthetized with the anaesthetic "cocktail" Equithesin (0.3ml/100g BWt) and the head mounted in a stereotaxic apparatus. A small burr-hole (approx 1.5mm diameter) was constructed over the target area, and the underlying dura incised with a sharp 28 gauge needle. The target sites of PHA-L injection were aimed at the same locations within the SNr from which stimulating electrode placements elicited inhibitory responses from PMRF neurones. The arrangements of the electrode placements are similar to the SNr stimulation electrode position as used previously, except that the stimulating electrode was replaced with a glass microelectrode (20μ tip diameter) for the ionophoretic deposition i.e. PHA-L was deposited at the following stereotaxic co-ordinates; AP 2.7 - 3.2, Lat 2.0, D7.5 - 8.0, according to the atlas of Paxinos and Watson (1982).

Ionophoretic procedure

The injected PHA-L solution was made up as follows:
PHA-L (Vector Labs USA.), 2,5% solution made up in either 50mM Tris buffered saline, pH7.4, or in normal saline. PHA-L was deposited by means of ionophoresis via glass microelectrodes (Clarke Electromedical GC-100F) which were pulled on a vertical electrode puller (Kopf) to a fine tip. Under microscopic guidance the tip was broken back to between 20 and 25μ.

Ejection parameters:- PHA-L was injected by means of a pulsed current, delivered from a constant current source. The following parameters were employed; positive pulsed current; 2,5-5mA., 10sec ON/10sec OFF, for a period of 20 mins. If the monitored current tended to drop below 2.5mA during the ON phase, the amplitude was increased slightly. The electrode was withdrawn immediately after completion of the deposition. The burrhole was plugged with dental wax and the skin sutured. The rat was kept warm and recovery from anaesthesia monitored until it was fully recovered. It was then returned to the animal house. After a survival time of 11 to 14 days, rats were re-anaesthetised and rapidly perfused transcardially with heparinized normal saline (200ml) and then 300ml of fixative (see below). This was then followed by a very much slower perfusion of fixative (90 - 120 minutes; approx volume delivered was 700ml).

Fixative solutions

The fixative was made up, in all cases, shortly before the animal was perfused:-
4.0% paraformaldehyde
0.1 to 0.5% glutaraldehyde
0.2% picric acid
In 0.1M phosphate buffered saline pH 7.4

Brains were blocked out immediately after fixation and sectioned serially on an Oxford Vibratome at a thickness of 75μ. The sections were cut in a parasagittal plane with the exception of one brain which was cut parasagittally through the deposition site (mesencephalic area), and coronally through pontomedullary brain stem. Sections were kept in chilled Tris(hydroxymethyl methylamine) buffered saline (TBS) pH 8.6.

Immunocytochemical procedure

The following description is based on the method of Wouterlood and Groenewegen (1985). The essential features and stages of the immunocytochemical procedure for the PHA-L reaction are summarized diagramatically (Fig 2.7.2). The following is a detailed description of the PHA-L immunoreaction procedure;

All the subsequent steps in the sequence were performed under continuous agitation.

Sections transferred to TBS pH 8.6, to which had been added 0.05% Triton X-100 (TBS-T) and rinsed 3 x 5 minutes in this solution.

Thereafter sections were incubated in Goat anti-PHA-L (Vector labs. USA , dilution 1:1000 in TBS-T).
Incubation was performed at room temperature for 12 to 13 hrs.

Sections rinsed again 3 x 15 minutes in TBS-T.

This was followed by incubation (2 to 3 hours at room temperature) in rabbit anti-goat whole serum (Sigma) diluted 1:40 in TBS-T.

Sections rinsed again 3 x 15 minutes in TBS-T at room temperature.

After this step the sections were incubated (2-3 hours at room temperature) in Goat-peroxidase anti-peroxidase (Vector Laboratories) at a dilution of 1:400 in TBS-T.

Sections rinsed 3 x 15 minutes in TBS without Triton.

Final incubation in the chromogen solution 3.3' Di-aminobenzidine tetrahydrochloride (DAB)(BDH Chemicals, 0.05% in TBS pH 7.2-7.1 for 10 minutes).

At this stage of the procedure nickle intensification is optional, but recommended for enhancing the contrast of the sections.

After 7 minutes in the DAB solution, 0.5 ml of a 1% Nickle ammonium sulphate solution was added to each tray, per 1ml of DAB solution. At the end of this period 10 microlitres of a 0.01% hydrogen peroxide solution in double distilled water was added to each container.

Sections were incubated for 2 to 5 minutes or until colour had developed sufficiently. Microscopic assessment of the colour intensity was necessary for optimal results.

Final rinses 3 x 15 minutes in TBS pH 7.2-7.4.

The sections were transferred finally to 0.1M phosphate buffered saline and then mounted immediately on gelatinized slides, or stored overnight in the same buffer. Sections were then dried at room temperature for 24 hours, dehydrated, cleared, mounted in DPX mounting medium and then fitted with a cover slip for a permanent record.

Results and Discussion

In all the rats injected with PHA-L via ionophoresis, the deposition sites were discretely localised within the SNr with minimal contamination of adjacent structures. These sites were predominantly in the caudal part of the SNr with some spread to the rostral pole of the SNr. The electrode dimensions, ejection parameters and sizes of the resultant injection site are comparable to those of other studies (Groenewegen and van Dijk, 1984; Gerfen and Sawchenko, 1984). Similarly the 5 to 14 day post-injection survival times employed in these studies were comparable to those in the present study. With similar overall ejection parameters Groenewegen and van Dijk obtained injection sites with diameters of the order of 100 to 300μ. The PHA-L injection site from which uptake occurs is considered to be the area encompassed by darkly stained neurones. The general staining pattern presented by the PHA-L positive fibers in this study are also in agreement with that of PHA-L stained fibers in other areas (Gerfen and Sawchenko, 1984). Stained neurones revealed finest details of their projections, i.e. axon collaterals, en passant boutons, and terminal varicosities, suggesting complete filling of the SNr neurones and their projections. This in turn is comparable to the general features as revealed with intracellular HRP (Grofova et al., 1982).

In the present study there are also relatively large darkly stained areas devoid of cellular uptake. Similarly in Gerfen and Sawchenko's, (1984) study these large areas with a darkly stained background are evident. The evidence provided by their study shows that only darkly stained neurones give rise to axonally transported PHA-L, and

that the larger areas of dark staining probably represent nonspecific diffusion of the tracer. Fig. 2.7.3 and Fig. 2.7.4 show the deposition sites of PHA-L in serial sections cut in the coronal and parasagittal planes in two different rats. In these examples the stained cells are very well localised in the SNr and little contamination of adjacent areas i.e. SNc and cerebral peduncle is evident. Thus it is most likely that the fibers stained with PHA-L originate from soma contained within the SNr.

All the PHA-L injection sites examined are similar with some minor differences. The overall histological picture presented is that of PHA-L stained fibers projecting from intensely stained injection sites within the SNR. Numerous axons leave this area. Their fine structure is well defined, with greater detail of fibers and boutons visible in sections cut in the parasagittal plane. These projections can be traced for relatively long distances in a rostral and dorsal direction. A small number of fibers can be followed in the ventrocaudal direction to approximately the level of the rostral pons.

To provide a detailed description of the topography of all the efferent connections of the SNr as revealed by the PHA-L injections is beyond the scope of this study, as the aim is primarily to describe the projection fibers and their terminations in the PMRF. Overall the fiber projection pattern conforms to the descriptions of target areas of SNr neurones as described in other studies employing a variety of neuroanatomical tract tracing methods. It is widely recognized that a major non-dopaminergic pathway from the SNr innervates a number of structures. Briefly these are as follows;

(a) Nigrothalamic efferents run dorsally and rostrally especially to the VM nucleus of the thalamus (Deniau et al., 1982; Deniau et al., 1978a). These fibers have also been shown to be GABAergic (Di Chiara et al., 1979; Chevalier and Deniau, 1982; Chevalier et al., 1985).

(b) Nigrotectal fibers innervate the deep layers of the superior colliculus (Rinvik et al., 1976; Chevalier et al., 1981b). This has also been shown to be a GABAergic projection (Chevalier et al., 1981a and Kilpatrick et al., 1982). Nigrotectal neurones retrogradely filled with HRP from deposition sites within the SC, show a varied shape from fusiform to stellate, and these were found mainly in the rostroventral two-thirds of the SNc (Williams and Faull, 1985). The retrogradely filled HRP cells shown in their study have a very similar appearance to those of the PHA-L stained neurones in the present study, in that the shape varied from fusiform to stellate, with few short dendrites.

(c) Nigrotegmental projections mainly innervating parabrachial area of the pontomesencephalic tegmentum, especially the pedunculopontine nucleus (Saper and Lowey, 1982; Garcia Rill, 1986).

The above data provide evidence for a SN inhibitory influence on the PPN/MLR area which is mediated by GABA.

Thus the majority of evidence suggests that the SNr non-dopaminergic pathway is GABAergic. However the GABAergic nature of this pathway to certain areas such as the PPN/MLR has yet to be demonstrated conclusively.

In this study, PHA-L filled fibers are seen coursing to 3 main target areas (see Fig. 2.7.5 and Fig. 2.7.6) viz; rostrally to the thalamic area, dorsally to the tectum and then caudally into the mesencephalic tegmentum. This projection pattern conforms to the

SNr projections and target sites as outlined in the references above. A similar pattern of fiber projections, outlined by tritiated leucine and proline injections into the SNr of the rat, was observed by Beckstead et al., (1979). Isotope injections labelled sparse nigrostriatal fibers and numerous nigrothalamic fibers. Their study showed descending fibers exiting the SNr and bifurcating into a large nigrotectal and a smaller nigrotegmental component. The termination sites of the latter component occurred largely in the pedunculopontine nucleus of the pontomesencephalic tegmentum. No nigral efferent fibers could be traced in their study beyond the level of the locus coeruleus. In the cat however Rinvik et al., (1976) found HRP labelled fibers projecting caudally as far as the medulla. These fibers project from neurones in the medial half of the substantia nigra.

The typical boutons observed in PHA-L filled fibers are shown in Fig. 2.7.8A and Fig. 2.7.8B. These examples are taken from the fiber tracts coursing to the thalamus and the tectum. Some of the boutons (marked with arrows in Fig. 2.7.8B) are probably terminal boutons contacting tectal and thalamic cells respectively. As the sections have not been counterstained, this is inferential. On the basis of the above overall picture there is good PHA-L uptake from all the deposition sites limited largely to the SNr. These fibers therefore must have originated from parent soma within the SNr. All the PHA-L stained fibers and their projections conform to the pattern generally accepted as classical SNr projection sites. In addition the SNr also has the highest concentration of GABA (or GAD positive immunoreactivity) in the nervous system, and the majority of evidence indicates that SNr efferents are GABAergic. There is thus overwhelming evidence in favour of these stained elements also being GABAergic. It is therefore reasonable to expect that a similar pattern of GABAergic fibers project to the PMRF area of the brain stem.

However PHA-L stained fibers could not be detected at the level of the PMRF. A thorough histological examination of all the sections of injected brains revealed only a very few PHA-L filled fibers in the caudal brain stem. Furthermore their location is far lateral to the medial pontomedullary area. PHA-L stained fibers coursing in a ventrocaudal direction are at best sparse, and as shown in the camera lucida reconstruction (Fig. 2.7.5B) are only seen occurring singly. As there appears to be two types of PHA-L stained fibers present viz., relatively thick and dark staining, and very fine pale staining fibers that make up the SNr projection, due caution was exercised in the histological examination to ensure that the latter component was not overlooked.

Since the optimal uptake of PHA-L depends on a reasonably long uptake time, it could be argued that uptake time might have been insufficient for complete filling of these fibers, and that this may account for the lack of fibers seen in the caudal brain stem. If this was the case then no fibers as far caudal as the medullary level would have been visible. Fig. 2.7.5b depicts the lateral fibers seen coursing as far caudal as the ventral medulla. So the uptake time of 10 to 14 days employed in the present study appears to be adequate time for the PHA-L uptake to reach the level of the PMRF, and is probably not a significant factor affecting the interpretation of these findings. van Krosigk and Smith, (1988) utilizing the PHA-L method in the investigation of descending projections from the substantia nigra, have recently described a small fiber tract from the SNr to the lateral brain stem area. In their

study the PHA-L stained SNr fibers seen projecting in a caudal direction are located specifically in the lateral parvocellular reticular formation. These studies were performed using experimental parameters, such as injection sites and post-injection survival times (personal communication) that are comparable to the present study and as such constitutes a control for this study. No fibers or terminals are revealed in the area comprising the medial PMRF in their study and in this respect agrees with the findings in the present study.

A further possibility is that a specific zone or subdivision of cells within the SNr may give rise to fibers that course selectively to a specific target area of the brain. According to this scheme the projection to the PMRF would arise at only a specific site within the SNr. However the general arrangement of cells within the SNr projecting to the specific targets do not appear to be anatomically organised, at least in the rodent. The primate substantia nigra however shows output organization. This has been ascertained only recently by means of retrograde double labelling techniques (Parent A., 1990). In the rodent the neuronal population appear to be more homogenous. An important species difference therefore appears to exist.

Nigrothalamic and nigrotectal cells lie spread throughout the dorsoventral extent of the SNr (Grofova et al., 1982) . These neurones also possess axon collaterals that extend throughout the mesencephalic tegmentum as well as intra-SN. The intracellular HRP data of Grofova et al., (1982) supports the idea put forward by Faull and Mehler, (1978) of a layering of cells within the SNr. On the basis of their dendritic anatomy SNr cells are arranged in a dorsoventral manner. The cellular arrangements in the mediolateral plane are unremarkable, with the exception of the dorsolateral parts of the SNr. Although these so-called "subnuclei" may be involved in different and specific circuitries, the available evidence points to a mixed population of projection neurones. Judging from the electrical stimulation studies of SNr (Perciavalle, 1987; Gonzalez-Vegas, 1981) no distinction is made as to which population of SNr cells was being stimulated. However in a recent study by van Krosigk and Smith (unpublished data) retrograde labelling with very discrete WGA-HRP injections into the medullary reticular formation revealed labelled cells in the SNc and SNr, the majority of which are located in the lateral and caudal half of the SNr. These data may indicate a topographical organization of SNr cell groups, which give rise to fibers projecting to highly restricted zones within the brain stem. It appears therefore, that no general consensus exists with regard to this aspect of SNr cytoarchitecture.

Electrophysiological and pharmacological evidence in a recent study provides support for the presence of an inhibitory connection between the SNr and the PMRF (Perciavalle, 1987). Intracellular studies by Gonzales-Vegas, (1981) which demonstrated IPSPs recorded from cells within the nucleus reticularis gigantocellularis in cortical ablated rats, also suggest direct inhibitory contacts on these cells. Neither of these studies however demonstrate the exact histological site of the stimulating electrode. Since the present study demonstrates that the PHA-L deposition sites were all within the SNr, it is difficult to reconcile the present data with these findings. The electrical stimulation data in the present study demonstrate that 67 % of PMRF cells show inhibitory episodes to stimulation of the SNr. It is therefore reasonable to expect that if an inhibitory fiber projection from the SNr to the

medial pontomedullary reticular formation exists, then the density of the projections and terminals would be such that they are readily detectable, especially in tissue sections cut in the parasagittal plane. However as is evident from the present data this clearly is not the case.

It could be argued on the other hand that GABAergic SNr fibers which project to the PMRF selectively resist uptake of PHA-L. There is little reason to doubt that the majority of SNr fibers stained with PHA-L are predominantly GABAergic. Support for this belief lies in the observation that the SNr projections to thalamus , tectum and parabrachial areas are all considered to be GABAergic. As the pattern of PHA-L stained fiber tracts in this study agrees with widely cited projections to the classic target areas, these fibers are in all probability, GABAergic. These present findings therefore argue against some peculiar aspect of PHA-L uptake by GABAergic fibers.

An alternative explanation of the results may be that the pathway is so sparse that the few nigroreticular fibers that do stain with PHA-L remain undetected in the histological sections. This is unlikely to be the case because all the brains were cut serially, and in most cases in the parasagittal plane. In these sections fibers could easily be follwed, but only very few fibers were observed coursing caudally past the level of the pontomesencephalic area. All the brains examined which show PHA-L deposition sites within the SNr as well as clearly labelled cells and fibers show no fiber projections to the medial pontomedullary region. As the bulk of the injected PHA-L in some rats was located more toward the caudal zone of the SNr it may be possible that fibers projecting to the PMRF originate only in the extreme rostral zone of the SNr. The homogenous nature of the neurones within the SNr, as well as the undifferentiated output to the thalamus as well as the tectum argue against the likelihood of a group of neurones, spatially segregated, preferentially projecting to the PMRF. It is therefore unlikely that the nigroreticular pathway exists in the rat. These anatomical data therefore question the interpretation of the earlier findings of Gonzales - Vegas (1981) and Perciavalle (1978).

Conclusion

The absence of PHA-L stained fibers coursing from the SNr to the PMRF is striking, despite the presence of many stained SNr cells and fibers leaving the nucleus. Although PHA-L depositions overlapped the sites from which electrical stimulation evoked inhibitory PMRF responses, the existence of a fiber tract projecting to the PMRF containing PHA-L stained fibers is not revealed. Since the interpretation of these tract tracing data is not confounded by fibers *en passant* taking up the highly specific anterograde tracer, it is concluded that these data do not support the existence of a significant pathway between the SNr and the medial pontomedullary reticular formation. It is therefore unlikely that the SNr is the source of the GLIR terminals observed making contact with the giant cells.

Section 1.4.0

Summary and General Comments

Summary of the major findings of this study

(A) A high percentage (81%) of cells within the nucleus reticularis gigantocellularis (RGC), identified by their typical exteroceptive characteristics, could be driven antidromically from positions within the ventromedial cervical spinal cord. Some cells (19%) were activated orthodromically. In some cases HRP was deposited in the ventromedial funiculus at sites evoking antidromic activation of PMRF cells. The majority of retrogradely stained cells were within the ipsilateral RGC. The pathway of HRP filled axons can be traced in the ventromedial funiculus coursing rostrally to the craniocervical junction where the fibers enter the medial longitudinal fasciculus (MLF). These fibers course in the MLF with an ipsilateral predominance and enter the giant cell field mainly from the dorsal aspect of the MLF. As some cells were not stain with HRP or could not be antidromically activated it is concluded that these cells either do not have ipsilateral projections or alternately do not project to that level of the spinal cord.

(B) The intracellularly stained neurones conform broadly to the reported general characteristics of RGC cells within the RGC of the rat stained by the Golgi technique, and intracellularly stained RGC cells of the cat. One cell is typically large and the remaining three are small to medium sized. These cells are typically multipolar with radially projecting spineless dendrites with branching patterns of varying complexity. The dendritic indicies range from 3 to 14. The dendritic arbor tends to be either rounded or elongated. This may be related to the histological procedure employed or these cells may belong to a particular class within the RGC. The dendritic arbors extend up to 1.5mm in the AP plane, and approximately 1.2mm in the dorsoventral plane. The arbor is less extensive in the mediolateral plane. The detailed geometry and assumed values of Rm and Ri were used to calculate the electrotonic lengths of these cells. In the 4 examples the electrotonic lengths are all approximately equal to 1. The input resistances were also calculated from the precise dimensions of the dendritic tree and assumed Rm values. The cells all showed relatively low input resistances ranging between 5 and 11 MΩ. Some of these cells with very extensive dendrites therefore have the electrotonic capability of enabling distal synapses to interact with the soma.

(C) The majority of individual PMRF cells are responsive to both nicotinic and muscarinic agonists, suggesting that both receptor types are present on the same neurone. However, the possibility of a presynaptic location of one of the receptors cannot be excluded. Nicotinic agonists mainly cause excitation while muscarinic agonists cause inhibition. Although this is the general case, a small number of cells show exclusively excitatory or inhibitory responses to both agonists. This pattern of parallel nicotinic and muscarinic effects on the same PMRF neurone does not reflect the usual features of central cholinergic receptor distribution. Furthermore cholinoceptive cells are also GABAceptive, suggesting that PMRF cells have a dual inhibitory control mechanism. This is likely to have important consequences on the overall performance of these cells.

(D) The majority of PMRF neurones are inhibited by ionophoretic application of GABA. The inhibitory effects of GABA are reversed by bicuculline, suggesting that the responses are not purely non-specific metabolic effects on the cell membrane but may be mediated via a GABAa receptor mechanism. However, baclofen also exerts a profound inhibitory effect on all the cells tested. Baclofen is known to interact specifically with GABAb receptors. These data suggest that GABA acts via both GABAa and GABAb mechanisms on PMRF cells. As the majority of GABAceptive cells tested show responses to both bicuculline and baclofen, it is likely that the PMRF cells may have both receptor types present on the same cell. GABAceptive RGC neurones challenged with ionophoretic deposition of bicuculline show a large excitatory response, suggesting the existence of tonic GABAergic input to these cells. Since cholinergic cells are also GABAceptive, it is likely that PMRF cells contain both cholinergic receptor types as well as GABAa and GABAb receptor types.

(E) Highly specific GABA immunocytochemistry applied to ultrathin sections, cut from selected RGC cells in 3 different rats, demonstrate the presence of GLIR-terminals on the somata and proximal dendrites in all cases. The terminals contain electron dense immunogold particles with a density of approximately 5:1 with respect to the surrounding tissue. These data strongly suggest that GABAergic terminals contact giant cells. Since these giant cells were chosen at random in three different rats and as all show GLIR-terminals, it is probable that many PMRF neurones receive a GABAeric input. These GABAergic fibers may be responsible for tonic GABAergic inhibition of these cells

(F) Electrical stimulation of histologically confirmed sites within the substantia nigra pars reticulata evoked short latency, short duration inhibitory episodes in 55% of PMRF cells tested. However 45% of cells tested from stimulation sites in the cerebral peduncle also revealed short duration short latency inhibitory periods although a higher incidence of excitatory activity was also elicited. SNc stimulation similarly elicited inhibitory periods of variable duration in a high percentage of cases (72%). These data strongly support the presence of mesencephalic inhibitory fibers arising in or coursing through the substantia nigra. Therefore the existence of a specific inhibitory pathway between the SNr and the PMRF is unlikely. Since this area is known to have a high incidence of fibers *en passant*, many fibers are probably unselectively stimulated.

(G) The immunocytochemical detection of phaseolus vulgaris leucoagglutinin (PHA-L) stained fibers is a powerful technique for demonstrating anterograde connections. Deposition of PHA-L within the SNr shows many SNr cells and fibers taking up the stain. PHA-L stained fibers are observed projecting to all the thalamic area, tectum and mesencephalic tegmentum. No PHA-L stained fibers are observed coursing to the level of the medial pontomedullary reticular formation. These findings cast doubt on the existence of a pathway between the SNr and PMRF.

General comments

There are still many aspects of PMRF physiology and anatomy that have to be clarified. The rapid advances being made with new techniques in the fields of neuroanatomy, molecular biology and ion channel biophysics, to mention but a few, will no doubt contribute enormously to an understanding of the properties of individual cells. However, there is a pressing need to exploit all these contributions and to integrate these data at the level of discrete neural systems and local microcircuits. Worthwhile contributions to modern neuroscience will only be made in future by adopting a collaborative multidisciplinary approach.

Great advances in some areas of neuronal modelling have been made recently. These studies provide some insight to the computational mechanisms of the cell and their related networks. Models of PMRF cells are still outstanding, yet if the computational mechanisms of PMRF cells are to be understood, the route of investigation is clear. A synthesis is needed to bridge the gap between computational theory and neurobiological data. Only up until very recently the vast majority of neural network simulations neglected the effects of the dendritic pattern, synaptic properties and intrinsic membrane properties of the individual cell. As with all other neuronal models, an important goal is to establish specific operations of synaptic organization and then to incorporate these properties into more realistic networks. A single neurone cannot play a role in the processing of information without interacting with other neurones. In general, intrinsic circuits have complex levels of organization which are integrated with long distance connections. It is thus of paramount importance to establish intraregional connections. This is nowhere more evident than in the present study. I allude here to the presence of GLIR terminals on the giant cells of unknown origin.

With the advent of powerful tools such as the intracellular dye and 3-D reconstruction techniques and emergent new data, some of the older theories concerning the functional organization of the PMRF cells will, in all likelihood, have to be revised. The present 3-D data enable a deeper insight as to the individual cell's orientation. This is important as functional models have been constructed on the basis of organizational patterns of these cells. As stressed before, although few in number, the orientation of the cells in the present study does not resemble that of Scheibel's system where cells are stacked in "modules". However many more individual cells will have to be examined before the older theories can be challenged and the functional implication of specific orientation can be ascertained.

The data from the present study indicate that the giant cells with large spreading dendritic arbors are fully equipped electrotonically, for distally located synapses to have an effect on the soma. This has significant implications in PMRF processing, as many of these cells are large and often spread across distances of 2mm of the brain stem. These cells, which have the potential of receiving enormous and divergent input, have always been assumed to be capable of integration of input across the vast dendritic domains, thereby allowing integration of many different inputs without consideration for the cable properties of these cells.

New insight into the complexity of dendritic behaviour is emerging and this has an important bearing on the behaviour of single cells. In this respect an important theoretical parameter is the "weight" of the individual synapse, i.e., the size and the time course of potential change produced in the postsynaptic cell as well as the spatial arrangement of synaptic types. The general arrangement of synapses in the cortex show that Type II synapses tend to be nearer the axon hillock than Type I. This feature of their spatial organization also has profound effects on the dendrites, where an inhibitory synapse on a given dendrite can exercise a "partial veto" on more distal excitation. The exact arrangement of the various synapses is probably significant. Thus the emerging view is that a single neurone, rather than being a single integrating device, may be a more complex processing unit.

Recent data by McCarley et al., (1987) provide further insight into the processing capabilities of these cells. Their data show that the PMRF is not organized into functional zones where some cells are specialized for input and some for output; instead individual members of PMRF behave as input and output neurones. Furthermore, as inhibitory interneurones are not present in the PMRF, this further strengthens the case put forward by McCarley et al., (1987) that individual cells perform the task of information processing. The present data regarding the neurotransmitter properties of PMRF cells do indicate that these cells are equipped with a rather unusual distribution of cholinergic receptor types. PMRF cells may have a dual cholinergic/GABAergic inhibitory mechanism although GABA containing inhibitory Golgi Type II interneurones are absent within the PMRF. These features probably have profound functional implications. By comparison, in terms of input/output organization, the cerebellar cells are clearly segregated for this purpose. The cerebral cortex is also well endowed with local circuit neurones which fulfill the role of intermediate processing. Clearly the neural arrangement in the medial reticular formation provides little support for local processing and is strikingly different from the two aforementioned areas, well known for processing information.

Although detailed information of single cells is now being assembled, there is still a tremendous amount of outstanding information. If the behaviour of single cells is still unclear, then their behaviour as a group is even more obscure. How networks of these neurones co-operate in transforming information still requires extensive investigation.

The brain stem has variously been implicated in the descending pathway for mediation of various motor acts. Previous data have suggested that the role of substantia nigra in postural, head, and eye movement control may be directed via a descending projection to the brain stem reticular formation, in view of persistent effects after ascending pallidothalamocortical pathways have been destroyed. Results from electrical stimulation of SN lend support to the existence of direct descending fibers to the medial brain stem area. However, the present tract-tracing data do not support the existence of such a direct pathway in the rat. The substantia nigra effects therefore probably only occur via mesencephalic reticular formation relay stations in the rat. The role of the brain stem in substantia nigra influence on eye movement, head turning and general behavioural orientation in the rat is still unclear. Direct contribution by the SNr is therefore unlikely to play an significant role in the control mechanisms of these motor behaviours.

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Appendix

Antidromic and orthodromic activation of PMRF cells

Code & Cell No.	Sweep (ms)	S.S A	Latency	Anti or Ortho.
TH1 C1.1	5	55	0.81	A
C1.2	5	55	2.27	O
C1.3	5	95	0.65	A
TH3 C3.1	5	185	0.71	A
C3.2	5	175	1.56	O
TH4 C1	5		1.8	O
C1	5		0.46	A
C2.1		110	0.89	O
C2.2		370	0.62	A
C2.3				O
TH5 C1.1	10	345	0.7	A
C1.2	10	100	1.58	O
C1.3	10	250		O
TH7 C3.1	10	107	3.95	O
C5.1	20	280	1.69	A
TH8 C2.1	10	150	1.34	A
C4.1	5	150	1.15	A
TH10 C1	10	130	0.76	A
C2.1	5	70	1.04	A
C2.2			1.06	
C4.1	10	180	1.64	O
C4.2			3.76	O
C9.1	5	290	0.60	A
C10.1	5	20	1.48	A
C11	10	40	2.27	O
TH11 C3.1	10		2.47	O
C5.1	5	120	0.75	A
C6.1	10	440	3.83	O
C10.1	5	90	1.02	A
C14	10	150	1.56	O
C16	5	80	1.46	A
C18	5	80	2.16	O
C19	5	100	0.87	A
C24	10	280	1.08	A
TH12 C2.1	10	65	1.68	A
C4.1	10	72	1.48	A
C5.1	5	180	1.04	A
C5.2				
C7.1	10	180	0.98	A
C8.1	5	120	1.17	A
C9.1	5	280	1.59	A

TH13	C3.1	10	115	0.77	A
TH15	C3	5	90	2.01	O
	C4	10	200	1.29	A
	C5	10	160	1.90	A
	C7.1	10	150	2.06	O
	C7.2	10	204	1.63	A
	C8.1	10	204	2.65	O
	C8.2	10	254	1.44	A
TH16	C1.1	10	340	3.15	0
	C2.1	10	180	0.75	A
	C3.1	10	200	1.03	A
	C4.1	10	130	0.72	A
	C5.1	10	110	1.47	O
	C5.2	5	140	0.73	A
	C7.1	10	200	1.62	O
	C7.2	10	380	0.70	A
TH17	C1	10	90	0.92	A
	C4.1	5	260	1.52	O
	C4.2	5	300	0.81	A
	C6	5	30	1.23	O
TH18	C1	5	72	1.52	O
	C4	5	92	1.66	A
	C5	5	610	0.75	A
	C7	5	30	0.76	A
	C8	10	180	1.74	O
	C9	10	60	0.72	A
TH19	C2	5	142	0.73	A
	C3	5	82	0.85	A
	C4	5	55	0.66	A
	C5	10	43	1.58	A
	C6	10	82	1.30	A
	C8.1	10	90	0.76	O
	C8.2	10	212	0.67	A
TH20	C2.1	10	160	1.61	O
	C2.2	10	204	0.72	A
	C3	10	130	0.66	A
	C4	5	250	0.68	A
	C5	5	450	0.67	A
TH21	C1	10	230	0.69	A
	C2	10	130	0.73	A
	C3	5	155	0.73	A
	C6.1	5	186	1.02	A
	C6.2	5	170	1.6	O
	C7	10	363	2.16	A
	C8	5	360	0.41	A
	C9	10	198	0.91	A

TH22	C1	10	185	1.49	A
	C2	10	520	1.64	O
	C3	10	250	1.32	A
	C4	10	82	0.53	A
	C5	10	320	2.79	O
TH23	C1	5	310	0.88	A
	C2	10	130	0.72	A
	C3.1	10	310	1.78	O
	C3.2	10	400	0.94	A
	C4.1	10	150	0.86	A
	C4.2	10	122	1.43	O
	C6	5	97	1.81	A
	C8	5	240	0.70	A
	C9	5	195	0.60	A
TH24	C1	5	90	0.66	A
	C2	5	190	0.65	A
TH25	C1	5	100	0.79	A
	C2.1	5	110	0.60	A
	C2.2	5	72	1.78	O
	C3.1	5	120	0.58	A
	C3.2	5	68	1.47	O
	C4	10	104	1.02	A

Linear Lengths (μ) of Primary Dendrite:

Cell	Code No.	Length μ	Mean Length ± SD & SEM
NGC1	1.1	9.23	Mean 66.41
	2.1	233.99	SD ±94.34
	3.1	21.16	SEM±42.12
	4.1	27.04	
	6.1	40.98	
NGC2	1.1	26.54	Mean 203.91
	4.1	65.01	SD ±237.9
	5.1	302.09	SEM±89.77
	6.1	53.43	
	7.1	156.61	
	8.1	122.14	
	3.1	701.55	
NGC3	2.1	120.46	Mean 51.18
	3.1	87.45	SD ±41.66
	4.1	50.91	SEM±15.7
	5.1	8.84	
	6.1	33.96	
	7.1	52.41	
	9.1	4.21	
NGC4	2.1	157.29	Mean 50.79
	4.1	15.68	SD ±54.00
	5.1	15.44	SEM±20.38
	6.1	35.77	
	7.1	22.61	
	8.1	18.06	
	9.1	90.70	

	A	B	C	D	E	F	G	H	I
1	branchID	order	length	diameter	totlen	X1	totX1	X2	totX2
2	1.1	0	0.002713	0.001164	0.002713	0.0503	0.0503	0.0356	0.0356
3	1.11	1	0.018846	0.000386	0.021559	0.6067	0.657	0.429	0.4645
4	1.111	2	0.037404	0.000217	0.058963	1.6059	2.2629	1.1355	1.6001
5	1.1111	3	0.001091	0.00005	0.060054	0.0976	2.3604	0.069	1.6691
6	1.1112	3	0.010302	0.000058	0.069265	0.8555	3.1184	0.605	2.205
7	1.112	2	0.023515	0.000088	0.045074	1.5854	2.2423	1.121	1.5856
8	1.12	1	0.001212	0.00079	0.003925	0.0273	0.0776	0.0193	0.0548
9	1.121	2	0.011317	0.000554	0.015242	0.3041	0.3817	0.215	0.2699
10	1.1211	3	0.017913	0.000163	0.033155	0.8874	1.269	0.6275	0.8973
11	1.1212	3	0.002353	0.00043	0.017595	0.0718	0.4534	0.0507	0.3206
12	1.12121	4	0.025942	0.000152	0.043537	1.3308	1.7842	0.941	1.2616
13	1.12122	4	0.024638	0.000311	0.042233	0.8836	1.337	0.6248	0.9454
14	1.121221	5	0.000451	0.000167	0.042684	0.0221	1.3591	0.0156	0.961
15	1.121222	5	0.012522	0.00014	0.054755	0.6693	2.0064	0.4733	1.4187
16	1.122	2	0.003858	0.000585	0.007783	0.1009	0.1784	0.0713	0.1262
17	1.1221	3	0.042883	0.000286	0.050666	1.6037	1.7822	1.134	1.2602
18	1.1222	3	0.034314	0.000237	0.042097	1.4097	1.5881	0.9968	1.123
19	3.1	0	0.070295	0.000265	0.070295	2.7311	2.7311	1.9312	1.9312
20	4.1	0	0.006573	0.000513	0.006573	0.1835	0.1835	0.1298	0.1298
21	4.11	1	0.054594	0.00021	0.061167	2.3827	2.5662	1.6848	1.8146
22	4.12	1	0.008917	0.000421	0.01549	0.2749	0.4584	0.1944	0.3241
23	4.121	2	0.004348	0.000203	0.019838	0.193	0.6514	0.1365	0.4606
24	4.122	2	0.013322	0.000309	0.028812	0.4793	0.9377	0.3389	0.6631
25	5.1	0	0.030312	0.000394	0.030312	0.9658	0.9658	0.6829	0.6829
26	5.11	1	0.006473	0.000067	0.036785	0.5001	1.466	0.3537	1.0366
27	5.12	1	0.017451	0.000218	0.047763	0.7475	1.7133	0.5286	1.2115
28	5.121	2	0.000706	0.000178	0.048469	0.0335	1.7468	0.0237	1.2352
29	5.122	2	0.023188	0.000126	0.070951	1.3065	3.0198	0.9238	2.1353
30	6.1	0	0.005531	0.0008	0.005531	0.1237	0.1237	0.0875	0.0875
31	6.11	1	0.033365	0.000383	0.038896	1.0783	1.2019	0.7624	0.8499
32	6.12	1	0.050359	0.000269	0.05589	1.9419	2.0656	1.3731	1.4606
33	7.1	0	0.015753	0.000179	0.015753	0.7447	0.7447	0.5266	0.5266
34	7.11	1	0.009053	0.000025	0.024806	1.1451	1.8898	0.8097	1.3363
35	7.12	1	0.010934	0.000135	0.026687	0.5952	1.3398	0.4208	0.9474
36	8.1	0	0.012577	0.000327	0.012577	0.4399	0.4399	0.311	0.311

	J	K	L	M	N
1	X5	totX5	X10	totX10	end
2	0.0225	0.0225	0.0159	0.0159	BP
3	0.2713	0.2938	0.1918	0.2078	BP
4	0.7182	1.012	0.5078	0.7156	BP
5	0.0436	1.0556	0.0309	0.7464	NE
6	0.3826	1.3946	0.2705	0.9861	NE
7	0.709	1.0028	0.5013	0.7091	NE
8	0.0122	0.0347	0.0086	0.0245	BP
9	0.136	0.1707	0.0962	0.1207	BP
10	0.3968	0.5675	0.2806	0.4013	NE
11	0.0321	0.2028	0.0227	0.1434	BP
12	0.5952	0.7979	0.4208	0.5642	BAE
13	0.3952	0.5979	0.2794	0.4228	BP
14	0.0099	0.6078	0.007	0.4298	NE
15	0.2993	0.8973	0.2117	0.6345	NE
16	0.0451	0.0798	0.0319	0.0564	BP
17	0.7172	0.797	0.5071	0.5636	NE
18	0.6304	0.7102	0.4458	0.5022	NE
19	1.2214	1.2214	0.8636	0.8636	NE
20	0.0821	0.0821	0.058	0.058	BP
21	1.0656	1.1476	0.7535	0.8115	BAE
22	0.1229	0.205	0.0869	0.145	BP
23	0.0863	0.2913	0.061	0.206	BAE
24	0.2144	0.4194	0.1516	0.2965	BAE
25	0.4319	0.4319	0.3054	0.3054	BP
26	0.2237	0.6556	0.1582	0.4636	NE
27	0.3343	0.7662	0.2364	0.5418	BP
28	0.015	0.7812	0.0106	0.5524	NE
29	0.5843	1.3505	0.4132	0.955	NE
30	0.0553	0.0553	0.0391	0.0391	BP
31	0.4822	0.5375	0.341	0.3801	TAE
32	0.8685	0.9238	0.6141	0.6532	NE
33	0.333	0.333	0.2355	0.2355	BP
34	0.5121	0.8451	0.3621	0.5976	NE
35	0.2662	0.5992	0.1882	0.4237	BAE
36	0.1967	0.1967	0.1391	0.1391	BAE

	A	B	C	D	E	F	G	H	I
1	branchID	order	length	diameter	totlen	X1	totX1	X2	totX2
2	2.1	0	0.012046	0.000285	0.012046	0.4513	0.4513	0.3191	0.3191
3	2.11	1	0.008154	0.000193	0.0202	0.3712	0.8225	0.2625	0.5816
4	2.111	2	0.023324	0.000212	0.043524	1.0131	1.8356	0.7164	1.298
5	2.1111	3	0.003224	0.000189	0.046748	0.1483	1.9839	0.1049	1.4029
6	2.1112	3	0.005358	0.000135	0.048882	0.2917	2.1273	0.2062	1.5042
7	2.112	2	0.007562	0.000088	0.027762	0.5098	1.3323	0.3605	0.9421
8	2.12	1	0.016786	0.000227	0.028832	0.7046	1.1559	0.4983	0.8174
9	2.121	2	0.005508	0.000151	0.03434	0.2835	1.4394	0.2005	1.0178
10	2.122	2	0.018316	0.00016	0.047148	0.9158	2.0717	0.6476	1.4649
11	2.1221	3	0.001877	0.000087	0.049025	0.1273	2.199	0.09	1.5549
12	2.1222	3	0.003562	0.000186	0.05071	0.1652	2.2369	0.1168	1.5817
13	2.12221	4	0.001051	0.000142	0.051761	0.0558	2.2927	0.0394	1.6212
14	2.12222	4	0.005118	0.000177	0.055828	0.2433	2.4802	0.172	1.7538
15	3.1	0	0.008745	0.000443	0.008745	0.2628	0.2628	0.1858	0.1858
16	3.11	1	0.03319	0.000138	0.041935	1.7869	2.0497	1.2635	1.4493
17	3.12	1	0.027374	0.000204	0.036119	1.2121	1.4749	0.8571	1.0429
18	3.121	2	0.027189	0.000196	0.063308	1.2283	2.7032	0.8685	1.9114
19	3.1211	3	0.005581	0.00016	0.068889	0.2791	2.9822	0.1973	2.1088
20	3.12111	4	0.00063	0.000083	0.069519	0.0437	3.026	0.0309	2.1397
21	3.12112	4	0.002438	0.000148	0.071327	0.1267	3.109	0.0896	2.1984
22	3.121121	5	0.018431	0.000145	0.089758	0.968	4.077	0.6845	2.8829
23	3.121122	5	0.003035	0.0002	0.074362	0.1357	3.2447	0.096	2.2944
24	3.121122	6	0.00046	0.0001	0.074822	0.0291	3.2738	0.0206	2.3149
25	3.121122	6	0.004369	0.000162	0.078731	0.2171	3.4618	0.1535	2.4479
26	3.1212	3	0.007181	0.000115	0.070489	0.4235	3.1267	0.2995	2.2109
27	3.12121	4	0.000492	0.000117	0.070981	0.0288	3.1555	0.0203	2.2313
28	3.12122	4	0.000705	0.00011	0.071194	0.0425	3.1692	0.0301	2.241
29	3.122	2	0.020106	0.000184	0.056225	0.9374	2.4124	0.6629	1.7058
30	3.1221	3	0.013206	0.000127	0.069431	0.7411	3.1535	0.5241	2.2299
31	3.1222	3	0.014087	0.000144	0.070312	0.7425	3.1548	0.525	2.2308
32	4.1	0	0.005091	0.000662	0.005091	0.1251	0.1251	0.0885	0.0885
33	4.11	1	0.045271	0.000193	0.050362	2.061	2.1861	1.4573	1.5458
34	4.12	1	0.003583	0.000402	0.008674	0.113	0.2382	0.0799	0.1684
35	4.121	2	0.005383	0.000308	0.014057	0.194	0.4322	0.1372	0.3056
36	4.1211	3	0.020925	0.000104	0.034982	1.2977	1.7299	0.9176	1.2232
37	4.1212	3	0.025006	0.000297	0.039063	0.9177	1.3498	0.6489	0.9545
38	4.12121	4	0.011396	0.000239	0.050459	0.4662	1.8161	0.3297	1.2841
39	4.121211	5	0.000641	0.0002	0.0511	0.0287	1.8447	0.0203	1.3044
40	4.121212	5	0.010467	0.000238	0.060926	0.4291	2.2452	0.3034	1.5876
41	4.121212	6	0.00064	0.00014	0.061566	0.0342	2.2794	0.0242	1.6118
42	4.121212	6	0.005244	0.000216	0.06617	0.2257	2.4708	0.1596	1.7471
43	4.121212	7	0.003057	0.000208	0.069227	0.1341	2.6049	0.0948	1.8419
44	4.121212	8	0.00555	0.0001	0.074777	0.351	2.9559	0.2482	2.0901
45	4.121212	8	0.009096	0.000203	0.078323	0.4038	3.0087	0.2855	2.1274
46	4.121212	7	0.032495	0.000181	0.098665	1.5276	3.9984	1.0802	2.8273
47	4.12122	4	0.024499	0.000192	0.063562	1.1182	2.4681	0.7907	1.7452
48	4.121221	5	0.008661	0.00012	0.072223	0.5	2.9681	0.3536	2.0988
49	4.121222	5	0.003836	0.000136	0.067398	0.208	2.6761	0.1471	1.8923
50	4.122	2	0.024978	0.000251	0.033652	0.9971	1.2353	0.7051	0.8735
51	4.1221	3	0.013531	0.000122	0.047183	0.7748	2.0101	0.5479	1.4213
52	4.1222	3	0.039133	0.000204	0.072785	1.7328	2.9681	1.2253	2.0988

	A	B	C	D	E	F	G	H	I
53	4.12221	4	0.000558	0.000285	0.073343	0.0209	2.989	0.0148	2.1136
54	4.12222	4	0.008997	0.000195	0.081782	0.4075	3.3756	0.2881	2.3869
55	4.122221	5	0.00531	0.000187	0.087092	0.2456	3.6212	0.1737	2.5606
56	4.122222	5	0.01142	0.00018	0.093202	0.5383	3.914	0.3807	2.7676
57	5.1	0	0.000884	0.001757	0.000884	0.0133	0.0133	0.0094	0.0094
58	5.11	1	0.028077	0.000288	0.028961	1.0464	1.0597	0.7399	0.7493
59	5.111	2	0.038642	0.000188	0.067603	1.7824	2.8421	1.2604	2.0097
60	5.1111	3	0.008876	0.00016	0.076479	0.4438	3.2859	0.3138	2.3235
61	5.11111	4	0.000307	0.00015	0.076786	0.0159	3.3018	0.0112	2.3347
62	5.111111	5	0.000372	0.000175	0.077158	0.0178	3.3196	0.0126	2.3473
63	5.111112	5	0.001108	0.000125	0.077894	0.0627	3.3645	0.0443	2.379
64	5.11112	4	0.002575	0.00019	0.079054	0.1181	3.4041	0.0835	2.407
65	5.1112	3	0.010421	0.000155	0.078024	0.5294	3.3715	0.3743	2.384
66	5.112	2	0.005991	0.000156	0.034952	0.3034	1.3631	0.2145	0.9638
67	5.1121	3	0.023901	0.000152	0.058853	1.2261	2.5892	0.867	1.8308
68	5.1122	3	0.017057	0.000132	0.052009	0.939	2.302	0.6639	1.6278
69	5.12	1	0.005672	0.000908	0.006556	0.119	0.1324	0.0842	0.0936
70	5.121	2	0.057344	0.000186	0.0639	2.6593	2.7917	1.8804	1.974
71	5.122	2	0.01407	0.000522	0.020626	0.3895	0.5219	0.2754	0.369
72	5.1221	3	0.038637	0.000165	0.059263	1.9024	2.4242	1.3452	1.7142
73	5.1222	3	0.005322	0.000569	0.025948	0.1411	0.663	0.0998	0.4688
74	5.12221	4	0.030933	0.00023	0.056881	1.29	1.953	0.9122	1.381
75	5.122211	5	0.006711	0.000116	0.063592	0.3941	2.3471	0.2787	1.6596
76	5.122211	6	0.002458	0.0001	0.06605	0.1555	2.5025	0.1099	1.7695
77	5.122211	6	0.000884	0.00009	0.064476	0.0589	2.406	0.0417	1.7013
78	5.122212	5	0.011555	0.00015	0.068436	0.5967	2.5497	0.4219	1.8029
79	5.122212	6	0.006374	0.000136	0.07481	0.3457	2.8953	0.2444	2.0473
80	5.122212	6	0.008409	0.00012	0.076845	0.4855	3.0352	0.3433	2.1462
81	5.12222	4	0.024971	0.000363	0.050919	0.8289	1.4919	0.5861	1.0549
82	5.122221	5	0.012974	0.000147	0.063893	0.6768	2.1687	0.4786	1.5335
83	5.122222	5	0.001067	0.00045	0.051986	0.0318	1.5237	0.0225	1.0774
84	5.122222	6	0.028244	0.000184	0.08023	1.3169	2.8406	0.9312	2.0086
85	5.122222	6	0.004816	0.000212	0.056802	0.2092	1.7329	0.1479	1.2253
86	5.122222	7	0.005936	0.000163	0.062738	0.2941	2.027	0.2079	1.4333
87	5.122222	8	0.010835	0.000105	0.073573	0.6688	2.6957	0.4729	1.9062
88	5.122222	8	0.033661	0.000149	0.096399	1.7441	3.771	1.2332	2.6665
89	5.122222	7	0.003165	0.000149	0.059967	0.164	1.8969	0.116	1.3413
90	6.1	0	0.003396	0.00069	0.003396	0.0818	0.0818	0.0578	0.0578
91	6.11	1	0.030706	0.000275	0.034102	1.1711	1.2528	0.8281	0.8859
92	6.111	2	0.012376	0.000153	0.046478	0.6328	1.8856	0.4475	1.3334
93	6.112	2	0.015093	0.000182	0.049195	0.7076	1.9604	0.5003	1.3862
94	6.1121	3	0.000782	0.000083	0.049977	0.0543	2.0147	0.0384	1.4246
95	6.1122	3	0.000356	0.00014	0.049551	0.019	1.9794	0.0135	1.3997
96	6.11221	4	0.001102	0.00018	0.050653	0.0519	2.0314	0.0367	1.4364
97	6.11222	4	0.00044	0.0003	0.049991	0.0161	1.9955	0.0114	1.411
98	6.112221	5	0.000898	0.000123	0.050889	0.0512	2.0467	0.0362	1.4473
99	6.112221	6	0.002678	0.000067	0.053567	0.2069	2.2536	0.1463	1.5936
100	6.112221	6	0.000757	0.00014	0.051646	0.0405	2.0872	0.0286	1.4759
101	6.112222	5	0.002198	0.000094	0.052189	0.1434	2.1389	0.1014	1.5124
102	6.12	1	0.027755	0.000241	0.031151	1.1307	1.2125	0.7996	0.8574
103	6.121	2	0.003269	0.000165	0.03442	0.161	1.3735	0.1138	0.9712
104	6.122	2	0.028873	0.000208	0.060024	1.2662	2.4787	0.8953	1.7527

	A	B	C	D	E	F	G	H	I
105	6.1221	3	0.004457	0.000167	0.064481	0.2181	2.6968	0.1542	1.9069
106	6.1222	3	0.007876	0.000211	0.0679	0.3429	2.8216	0.2425	1.9952
107	7.1	0	0.005241	0.000748	0.005241	0.1212	0.1212	0.0857	0.0857
108	7.11	1	0.009465	0.000438	0.014706	0.286	0.4072	0.2023	0.288
109	7.111	2	0.002377	0.00032	0.017083	0.084	0.4913	0.0594	0.3474
110	7.1111	3	0.02136	0.000244	0.038443	0.8648	1.3561	0.6115	0.9589
111	7.11111	4	0.011562	0.000235	0.050005	0.477	1.8331	0.3373	1.2962
112	7.111111	5	0.00429	0.000181	0.054295	0.2017	2.0348	0.1426	1.4388
113	7.111112	5	0.007793	0.00012	0.057798	0.4499	2.2831	0.3181	1.6144
114	7.11112	4	0.011523	0.000164	0.049966	0.5691	1.9252	0.4024	1.3613
115	7.1112	3	0.013244	0.000253	0.030327	0.5266	1.0179	0.3724	0.7197
116	7.11121	4	0.014064	0.000167	0.044391	0.6883	1.7062	0.4867	1.2065
117	7.11122	4	0.008087	0.000212	0.038414	0.3513	1.3692	0.2484	0.9681
118	7.111221	5	0.004078	0.000158	0.042492	0.2052	1.5743	0.1451	1.1132
119	7.111222	5	0.010885	0.000239	0.049299	0.4453	1.8145	0.3149	1.283
120	7.111222	6	0.00054	0.00014	0.049839	0.0289	1.8433	0.0204	1.3034
121	7.111222	6	0.003584	0.000143	0.052883	0.1896	2.004	0.134	1.4171
122	7.112	2	0.025314	0.0003	0.04002	0.9243	1.3316	0.6536	0.9416
123	7.1121	3	0.003242	0.000125	0.043262	0.1834	1.515	0.1297	1.0712
124	7.11211	4	0.02227	0.000182	0.065532	1.044	2.559	0.7382	1.8095
125	7.112111	5	0.000713	0.0001	0.066245	0.0451	2.6041	0.0319	1.8414
126	7.112112	5	0.000676	0.000167	0.066208	0.0331	2.5921	0.0234	1.8329
127	7.11212	4	0.002677	0.000075	0.045939	0.1955	1.7105	0.1382	1.2095
128	7.1122	3	0.004819	0.000276	0.044839	0.1835	1.515	0.1297	1.0713
129	7.11221	4	0.004357	0.000325	0.049196	0.1529	1.6679	0.1081	1.1794
130	7.112211	5	0.005916	0.000265	0.055112	0.2298	1.8977	0.1625	1.3419
131	7.112211	6	0.022716	0.000197	0.077828	1.0236	2.9213	0.7238	2.0657
132	7.112211	6	0.023635	0.000154	0.078747	1.2046	3.1023	0.8517	2.1936
133	7.112212	5	0.017333	0.000168	0.066529	0.8458	2.5136	0.598	1.7774
134	7.112212	6	0.001007	0.00016	0.067536	0.0503	2.564	0.0356	1.813
135	7.112212	6	0.005635	0.000218	0.072164	0.2414	2.755	0.1707	1.9481
136	7.112212	7	0.007413	0.000182	0.079577	0.3475	3.1025	0.2457	2.1938
137	7.112212	8	0.000807	0.0001	0.080384	0.051	3.1536	0.0361	2.2299
138	7.112212	8	0.002523	0.000133	0.0821	0.1384	3.2409	0.0978	2.2917
139	7.112212	7	0.001538	0.000198	0.073702	0.0691	2.8241	0.0489	1.997
140	7.112212	8	0.001059	0.000137	0.074761	0.0572	2.8814	0.0405	2.0374
141	7.112212	8	0.01017	0.000144	0.083872	0.536	3.3601	0.379	2.376
142	7.11222	4	0.009621	0.0002	0.05446	0.4303	1.9453	0.3042	1.3755
143	7.112221	5	0.017265	0.000141	0.071725	0.9196	2.8649	0.6502	2.0258
144	7.112222	5	0.030213	0.000182	0.084673	1.4164	3.3617	1.0016	2.3771
145	7.12	1	0.008416	0.000377	0.013657	0.2741	0.3953	0.1938	0.2795
146	7.121	2	0.007739	0.000251	0.021396	0.3089	0.7043	0.2185	0.498
147	7.1211	3	0.019519	0.000238	0.040915	0.8002	1.5045	0.5658	1.0638
148	7.12111	4	0.010006	0.000194	0.050921	0.4543	1.9588	0.3213	1.3851
149	7.12112	4	0.010084	0.000168	0.050999	0.492	1.9965	0.3479	1.4118
150	7.1212	3	0.017159	0.000141	0.038555	0.9139	1.6182	0.6462	1.1442
151	7.122	2	0.05971	0.0002	0.073367	2.6703	3.0656	1.8882	2.1677
152	9.1	0	0.000421	0.00065	0.000421	0.0104	0.0104	0.0074	0.0074
153	9.11	1	0.003701	0.000524	0.004122	0.1023	0.1127	0.0723	0.0797
154	9.111	2	0.014461	0.000376	0.018583	0.4717	0.5844	0.3335	0.4132
155	9.1111	3	0.004241	0.000323	0.022824	0.1492	0.7336	0.1055	0.5187
156	9.11111	4	0.030002	0.000164	0.052826	1.4817	2.2153	1.0477	1.5665

	A	B	C	D	E	F	G	H	I
157	9.11112	4	0.014214	0.000288	0.037038	0.5297	1.2633	0.3746	0.8933
158	9.111121	5	0.017528	0.000196	0.054566	0.7918	2.0552	0.5599	1.4532
159	9.111122	5	0.010504	0.000149	0.047542	0.5442	1.8076	0.3848	1.2781
160	9.1112	3	0.024542	0.000178	0.043125	1.1634	1.7478	0.8226	1.2359
161	9.112	2	0.003268	0.000504	0.00739	0.0921	0.2048	0.0651	0.1448
162	9.1121	3	0.014496	0.000411	0.021886	0.4522	0.657	0.3198	0.4646
163	9.11211	4	0.014398	0.000345	0.036284	0.4903	1.1472	0.3467	0.8112
164	9.112111	5	0.006825	0.000282	0.043109	0.257	1.4043	0.1818	0.993
165	9.112111	6	0.007228	0.000223	0.050337	0.3061	1.7104	0.2165	1.2094
166	9.112111	7	0.002461	0.000154	0.052798	0.1254	1.8358	0.0887	1.2981
167	9.112111	7	0.007533	0.000195	0.05787	0.3412	2.0516	0.2412	1.4507
168	9.112111	6	0.011192	0.000157	0.054301	0.5649	1.9692	0.3995	1.3924
169	9.112111	7	0.000547	0.000125	0.054848	0.0309	2.0002	0.0219	1.4143
170	9.112111	7	0.00537	0.000146	0.059671	0.2811	2.2503	0.1988	1.5912
171	9.112112	5	0.006224	0.00023	0.042508	0.2596	1.4068	0.1835	0.9948
172	9.112112	6	0.00183	0.000108	0.044338	0.1114	1.5182	0.0788	1.0735
173	9.112112	6	0.000742	0.000175	0.04325	0.0355	1.4423	0.0251	1.0198
174	9.112112	7	0.001901	0.000198	0.045151	0.0854	1.5277	0.0604	1.0803
175	9.112112	8	0.002859	0.000099	0.04801	0.1817	1.7095	0.1285	1.2088
176	9.112112	8	0.005758	0.00018	0.050909	0.2714	1.7992	0.1919	1.2722
177	9.112112	7	0.008422	0.000171	0.051672	0.4073	1.8496	0.288	1.3079
178	9.112112	8	0.000399	0.000075	0.052071	0.0291	1.8788	0.0206	1.3285
179	9.112112	9	0.000435	0.000075	0.052506	0.0318	1.9105	0.0225	1.3509
180	9.112112	9	0.000845	0.000075	0.052916	0.0617	1.9405	0.0436	1.3721
181	9.112112	8	0.00172	0.00011	0.053392	0.1037	1.9533	0.0733	1.3812
182	9.11212	4	0.016008	0.000117	0.037894	0.936	1.593	0.6618	1.1264
183	9.1122	3	0.021688	0.000125	0.029078	1.2269	1.4316	0.8675	1.0123
184	9.12	1	0.007536	0.000299	0.007957	0.2756	0.2861	0.1949	0.2023
185	9.121	2	0.014438	0.000118	0.022395	0.8406	1.1267	0.5944	0.7967
186	9.122	2	0.032893	0.000225	0.04085	1.3869	1.673	0.9807	1.183
187	9.1221	3	0.005814	0.000208	0.046664	0.255	1.9279	0.1803	1.3633
188	9.12211	4	0.003325	0.000224	0.049989	0.1405	2.0684	0.0994	1.4626
189	9.122111	5	0.000394	0.000195	0.050383	0.0178	2.0863	0.0126	1.4752
190	9.122112	5	0.037234	0.000176	0.087223	1.7751	3.8435	1.2552	2.7178
191	9.12212	4	0.012682	0.000115	0.059346	0.7479	2.6759	0.5289	1.8921
192	9.1222	3	0.021152	0.000142	0.062002	1.1226	2.7956	0.7938	1.9768

	J	K	L	M	N
1	X5	totX5	X10	totX10	end
2	0.2018	0.2018	0.1427	0.1427	BP
3	0.166	0.3678	0.1174	0.2601	BP
4	0.4531	0.8209	0.3204	0.5805	BP
5	0.0663	0.8872	0.0469	0.6274	NE
6	0.1304	0.9513	0.0922	0.6727	NE
7	0.228	0.5958	0.1612	0.4213	NE
8	0.3151	0.5169	0.2228	0.3655	BP
9	0.1268	0.6437	0.0896	0.4552	NE
10	0.4096	0.9265	0.2896	0.6551	BP
11	0.0569	0.9834	0.0402	0.6954	NE
12	0.0739	1.0004	0.0522	0.7074	BP
13	0.0249	1.0253	0.0176	0.725	NE
14	0.1088	1.1092	0.0769	0.7843	NE
15	0.1175	0.1175	0.0831	0.0831	BP
16	0.7991	0.9166	0.5651	0.6482	NE
17	0.5421	0.6596	0.3833	0.4664	BP
18	0.5493	1.2089	0.3884	0.8548	BP
19	0.1248	1.3337	0.0882	0.9431	BP
20	0.0196	1.3533	0.0138	0.9569	NE
21	0.0567	1.3904	0.0401	0.9831	BP
22	0.4329	1.8233	0.3061	1.2893	NE
23	0.0607	1.4511	0.0429	1.0261	BP
24	0.013	1.4641	0.0092	1.0353	NE
25	0.0971	1.5482	0.0687	1.0947	NE
26	0.1894	1.3983	0.1339	0.9888	BP
27	0.0129	1.4112	0.0091	0.9978	NE
28	0.019	1.4173	0.0134	1.0022	NE
29	0.4192	1.0788	0.2964	0.7629	BP
30	0.3314	1.4103	0.2344	0.9972	NE
31	0.332	1.4109	0.2348	0.9976	NE
32	0.056	0.056	0.0396	0.0396	BP
33	0.9217	0.9777	0.6517	0.6913	NE
34	0.0505	0.1065	0.0357	0.0753	BP
35	0.0868	0.1933	0.0613	0.1367	BP
36	0.5804	0.7736	0.4104	0.547	NE
37	0.4104	0.6037	0.2902	0.4269	BP
38	0.2085	0.8122	0.1474	0.5743	BP
39	0.0128	0.825	0.0091	0.5834	NE
40	0.1919	1.0041	0.1357	0.71	BP
41	0.0153	1.0194	0.0108	0.7208	NE
42	0.1009	1.105	0.0714	0.7813	BP
43	0.06	1.1649	0.0424	0.8237	BP
44	0.157	1.3219	0.111	0.9347	NE
45	0.1806	1.3455	0.1277	0.9514	NE
46	0.6832	1.7881	0.4831	1.2644	NE
47	0.5001	1.1038	0.3536	0.7805	BP
48	0.2236	1.3274	0.1581	0.9386	NE
49	0.093	1.1968	0.0658	0.8463	NE
50	0.4459	0.5524	0.3153	0.3906	BP
51	0.3465	0.8989	0.245	0.6356	NE
52	0.7749	1.3274	0.548	0.9386	BP

	J	K	L	M	N
53	0.0093	1.3367	0.0066	0.9452	NE
54	0.1822	1.5096	0.1289	1.0675	BP
55	0.1098	1.6195	0.0777	1.1451	NE
56	0.2408	1.7504	0.1702	1.2377	NE
57	0.006	0.006	0.0042	0.0042	BP
58	0.4679	0.4739	0.3309	0.3351	BP
59	0.7971	1.271	0.5637	0.8988	BP
60	0.1985	1.4695	0.1403	1.0391	BP
61	0.0071	1.4766	0.005	1.0441	BP
62	0.008	1.4846	0.0056	1.0497	NE
63	0.028	1.5046	0.0198	1.0639	NE
64	0.0528	1.5223	0.0374	1.0765	NE
65	0.2367	1.5078	0.1674	1.0662	NE
66	0.1357	0.6096	0.0959	0.431	BP
67	0.5483	1.1579	0.3877	0.8188	NE
68	0.4199	1.0295	0.2969	0.728	NE
69	0.0532	0.0592	0.0376	0.0419	BP
70	1.1893	1.2485	0.8409	0.8828	NE
71	0.1742	0.2334	0.1232	0.165	BP
72	0.8508	1.0841	0.6016	0.7666	NE
73	0.0631	0.2965	0.0446	0.2097	BP
74	0.5769	0.8734	0.4079	0.6176	BP
75	0.1762	1.0496	0.1246	0.7422	BP
76	0.0695	1.1192	0.0492	0.7914	NE
77	0.0264	1.076	0.0186	0.7608	NE
78	0.2669	1.1402	0.1887	0.8063	BP
79	0.1546	1.2948	0.1093	0.9156	NE
80	0.2171	1.3574	0.1535	0.9598	NE
81	0.3707	0.6672	0.2621	0.4718	BP
82	0.3027	0.9699	0.214	0.6858	NE
83	0.0142	0.6814	0.0101	0.4818	BP
84	0.5889	1.2704	0.4164	0.8983	NE
85	0.0936	0.775	0.0662	0.548	BP
86	0.1315	0.9065	0.093	0.641	BP
87	0.2991	1.2056	0.2115	0.8525	NE
88	0.78	1.6865	0.5515	1.1925	NE
89	0.0733	0.8483	0.0519	0.5998	NE
90	0.0366	0.0366	0.0259	0.0259	BP
91	0.5237	0.5603	0.3703	0.3962	BP
92	0.283	0.8433	0.2001	0.5963	NE
93	0.3164	0.8767	0.2238	0.6199	BP
94	0.0243	0.901	0.0172	0.6371	NE
95	0.0085	0.8852	0.006	0.626	BP
96	0.0232	0.9085	0.0164	0.6424	NE
97	0.0072	0.8924	0.0051	0.631	BP
98	0.0229	0.9153	0.0162	0.6472	BP
99	0.0925	1.0079	0.0654	0.7127	NE
100	0.0181	0.9334	0.0128	0.66	NE
101	0.0641	0.9565	0.0453	0.6764	NE
102	0.5057	0.5422	0.3576	0.3834	BP
103	0.072	0.6142	0.0509	0.4343	NE
104	0.5662	1.1085	0.4004	0.7838	BP

	J	K	L	M	N
105	0.0976	1.206	0.069	0.8528	NE
106	0.1534	1.2619	0.1084	0.8923	NE
107	0.0542	0.0542	0.0383	0.0383	BP
108	0.1279	0.1821	0.0905	0.1288	BP
109	0.0376	0.2197	0.0266	0.1554	BP
110	0.3868	0.6065	0.2735	0.4288	BP
111	0.2133	0.8198	0.1508	0.5797	BP
112	0.0902	0.91	0.0638	0.6435	TAE
113	0.2012	1.021	0.1423	0.722	NE
114	0.2545	0.861	0.18	0.6088	NE
115	0.2355	0.4552	0.1665	0.3219	BP
116	0.3078	0.763	0.2177	0.5395	NE
117	0.1571	0.6123	0.1111	0.433	BP
118	0.0918	0.7041	0.0649	0.4979	NE
119	0.1991	0.8115	0.1408	0.5738	BP
120	0.0129	0.8244	0.0091	0.5829	NE
121	0.0848	0.8962	0.0599	0.6337	NE
122	0.4134	0.5955	0.2923	0.4211	BP
123	0.082	0.6775	0.058	0.4791	BP
124	0.4669	1.1444	0.3302	0.8092	BP
125	0.0202	1.1646	0.0143	0.8235	NE
126	0.0148	1.1592	0.0105	0.8197	NE
127	0.0874	0.7649	0.0618	0.5409	NE
128	0.082	0.6775	0.058	0.4791	BP
129	0.0684	0.7459	0.0483	0.5274	BP
130	0.1028	0.8487	0.0727	0.6001	BP
131	0.4578	1.3065	0.3237	0.9238	NE
132	0.5387	1.3874	0.3809	0.981	NE
133	0.3782	1.1241	0.2675	0.7949	BP
134	0.0225	1.1467	0.0159	0.8108	NE
135	0.1079	1.2321	0.0763	0.8712	BP
136	0.1554	1.3875	0.1099	0.9811	BP
137	0.0228	1.4103	0.0161	0.9973	NE
138	0.0619	1.4494	0.0438	1.0249	NE
139	0.0309	1.263	0.0219	0.8931	BP
140	0.0256	1.2886	0.0181	0.9112	NE
141	0.2397	1.5027	0.1695	1.0626	NE
142	0.1924	0.87	0.1361	0.6152	BP
143	0.4112	1.2812	0.2908	0.9059	NE
144	0.6334	1.5034	0.4479	1.0631	NE
145	0.1226	0.1768	0.0867	0.125	BP
146	0.1382	0.315	0.0977	0.2227	BP
147	0.3579	0.6728	0.253	0.4758	BP
148	0.2032	0.876	0.1437	0.6194	NE
149	0.2201	0.8929	0.1556	0.6314	NE
150	0.4087	0.7237	0.289	0.5117	NE
151	1.1942	1.371	0.8444	0.9694	NE
152	0.0047	0.0047	0.0033	0.0033	BP
153	0.0457	0.0504	0.0323	0.0356	BP
154	0.2109	0.2613	0.1492	0.1848	BP
155	0.0667	0.3281	0.0472	0.232	BP
156	0.6626	0.9907	0.4686	0.7005	NE

	J	K	L	M	N
157	0.2369	0.565	0.1675	0.3995	BP
158	0.3541	0.9191	0.2504	0.6499	NE
159	0.2434	0.8084	0.1721	0.5716	NE
160	0.5203	0.7816	0.3679	0.5527	NE
161	0.0412	0.0916	0.0291	0.0648	BP
162	0.2022	0.2938	0.143	0.2078	BP
163	0.2192	0.5131	0.155	0.3628	BP
164	0.115	0.628	0.0813	0.4441	BP
165	0.1369	0.7649	0.0968	0.5409	BP
166	0.0561	0.821	0.0397	0.5805	NE
167	0.1526	0.9175	0.1079	0.6488	NE
168	0.2526	0.8807	0.1786	0.6227	BP
169	0.0138	0.8945	0.0098	0.6325	NE
170	0.1257	1.0064	0.0889	0.7116	NE
171	0.1161	0.6291	0.0821	0.4449	BP
172	0.0498	0.6789	0.0352	0.4801	NE
173	0.0159	0.645	0.0112	0.4561	BP
174	0.0382	0.6832	0.027	0.4831	BP
175	0.0813	0.7645	0.0575	0.5406	NE
176	0.1214	0.8046	0.0858	0.5689	NE
177	0.1822	0.8272	0.1288	0.5849	BP
178	0.013	0.8402	0.0092	0.5941	BP
179	0.0142	0.8544	0.01	0.6042	NE
180	0.0276	0.8678	0.0195	0.6136	NE
181	0.0464	0.8736	0.0328	0.6177	NE
182	0.4186	0.7124	0.296	0.5037	NE
183	0.5487	0.6402	0.388	0.4527	NE
184	0.1233	0.1279	0.0872	0.0905	BP
185	0.3759	0.5039	0.2658	0.3563	NE
186	0.6202	0.7482	0.4386	0.529	BP
187	0.114	0.8622	0.0806	0.6097	BP
188	0.0628	0.925	0.0444	0.6541	BP
189	0.008	0.933	0.0056	0.6597	NE
190	0.7938	1.7189	0.5613	1.2154	NE
191	0.3345	1.1967	0.2365	0.8462	NE
192	0.5021	1.2502	0.355	0.884	NE

	A	B	C	D	E	F	G	H	I
1	branchID	order	length	diameter	totlen	X1	totX1	X2	totX2
2	2.1	0	0.015729	0.000171	0.015729	0.7607	0.7607	0.5379	0.5379
3	4.1	0	0.001568	0.00047	0.001568	0.0457	0.0457	0.0323	0.0323
4	4.11	1	0.000348	0.00038	0.001916	0.0113	0.057	0.008	0.0403
5	4.111	2	0.024377	0.000226	0.026293	1.0255	1.0826	0.7252	0.7655
6	4.1111	3	0.001294	0.00013	0.027587	0.0718	1.1544	0.0508	0.8163
7	4.1112	3	0.001578	0.000067	0.027871	0.1219	1.2045	0.0862	0.8517
8	4.112	2	0.000986	0.000252	0.002902	0.0393	0.0963	0.0278	0.0681
9	4.1121	3	0.018801	0.000175	0.021703	0.8989	0.9952	0.6356	0.7037
10	4.11211	4	0.002368	0.00012	0.024071	0.1367	1.1319	0.0967	0.8004
11	4.11212	4	0.002796	0.00012	0.024499	0.1614	1.1566	0.1141	0.8178
12	4.1122	3	0.009354	0.000131	0.012256	0.5169	0.6132	0.3655	0.4336
13	4.12	1	0.010827	0.000229	0.012395	0.4525	0.4982	0.32	0.3523
14	4.121	2	0.006784	0.00012	0.019179	0.3917	0.8899	0.277	0.6293
15	4.122	2	0.006507	0.000124	0.018902	0.3696	0.8678	0.2613	0.6136
16	5.1	0	0.001544	0.00047	0.001544	0.045	0.045	0.0319	0.0319
17	5.11	1	0.001342	0.000215	0.002886	0.0579	0.1029	0.0409	0.0728
18	5.111	2	0.021518	0.000156	0.024404	1.0896	1.1925	0.7705	0.8432
19	5.1111	3	0.002654	0.0001	0.027058	0.1679	1.3604	0.1187	0.9619
20	5.1112	3	0.002607	0.000083	0.027011	0.181	1.3735	0.128	0.9712
21	5.112	2	0.001824	0.00023	0.00471	0.0761	0.179	0.0538	0.1266
22	5.1121	3	0.012197	0.000165	0.016907	0.6005	0.7795	0.4246	0.5512
23	5.11211	4	0.021517	0.000108	0.038424	1.3095	2.089	0.9259	1.4772
24	5.11212	4	0.021471	0.000101	0.038378	1.3512	2.1307	0.9554	1.5067
25	5.1122	3	0.016315	0.000115	0.021025	0.9622	1.1412	0.6804	0.807
26	5.12	1	0.011019	0.000205	0.012563	0.4867	0.5318	0.3442	0.376
27	5.121	2	0.025733	0.000149	0.038296	1.3333	1.8651	0.9428	1.3188
28	5.122	2	0.010084	0.000136	0.022647	0.5469	1.0787	0.3867	0.7627
29	6.1	0	0.003577	0.000299	0.003577	0.1308	0.1308	0.0925	0.0925
30	6.11	1	0.005134	0.00019	0.008711	0.2356	0.3664	0.1666	0.2591
31	6.111	2	0.010771	0.000113	0.019482	0.6408	1.0072	0.4531	0.7122
32	6.112	2	0.01033	0.000111	0.019041	0.6201	0.9865	0.4385	0.6976
33	6.12	1	0.001779	0.000156	0.005356	0.0901	0.2209	0.0637	0.1562
34	6.121	2	0.025793	0.000137	0.031149	1.3937	1.6146	0.9855	1.1417
35	6.122	2	0.01308	0.000134	0.018436	0.7146	0.9356	0.5053	0.6615
36	7.1	0	0.002261	0.000261	0.002261	0.0885	0.0885	0.0626	0.0626
37	7.11	1	0.001495	0.00025	0.003756	0.0598	0.1483	0.0423	0.1049
38	7.111	2	0.005471	0.000231	0.009227	0.2277	0.376	0.161	0.2659
39	7.1111	3	0.014035	0.000165	0.023262	0.691	1.067	0.4886	0.7545
40	7.1112	3	0.008493	0.000123	0.01772	0.4843	0.8603	0.3425	0.6083
41	7.112	2	0.004827	0.000161	0.008583	0.2406	0.3889	0.1701	0.275
42	7.1121	3	0.010152	0.000163	0.018735	0.5029	0.8918	0.3556	0.6306
43	7.1122	3	0.002878	0.000106	0.011461	0.1768	0.5657	0.125	0.4
44	7.12	1	0.012095	0.00014	0.014356	0.6465	0.735	0.4571	0.5197
45	7.121	2	0.003449	0.00011	0.017805	0.208	0.943	0.1471	0.6668
46	7.122	2	0.003363	0.000116	0.017719	0.1975	0.9325	0.1396	0.6594
47	8.1	0	0.001806	0.00031	0.001806	0.0649	0.0649	0.0459	0.0459
48	8.11	1	0.010116	0.000159	0.011922	0.5074	0.5723	0.3588	0.4046
49	8.12	1	0.002278	0.000174	0.004084	0.1092	0.1741	0.0772	0.1231
50	8.121	2	0.006264	0.000148	0.010348	0.3256	0.4997	0.2303	0.3534
51	8.1211	3	0.004277	0.00012	0.014625	0.2469	0.7467	0.1746	0.528
52	8.1212	3	0.008008	0.000114	0.018356	0.4744	0.9741	0.3354	0.6888

	A	B	C	D	E	F	G	H	I
53	8.122	2	0.012249	0.00013	0.016333	0.6795	0.8535	0.4804	0.6035
54	9.1	0	0.00907	0.000129	0.00907	0.5051	0.5051	0.3571	0.3571
55	9.11	1	0.002382	0.0001	0.011452	0.1507	0.6557	0.1065	0.4637
56	9.111	2	0.002237	0.0001	0.013689	0.1415	0.7972	0.1	0.5637
57	9.112	2	0.00151	0.0001	0.012962	0.0955	0.7512	0.0675	0.5312
58	9.12	1	0.020253	0.000114	0.029323	1.1997	1.7047	0.8483	1.2054
59	9.121	2	0.000052	0.00011	0.029375	0.0031	1.7079	0.0022	1.2077
60	9.122	2	0.000136	0.00011	0.029459	0.0082	1.7129	0.0058	1.2112

	J	K	L	M	N
53	0.3039	0.3817	0.2149	0.2699	TAE
54	0.2259	0.2259	0.1597	0.1597	BP
55	0.0674	0.2932	0.0476	0.2074	BP
56	0.0633	0.3565	0.0447	0.2521	NE
57	0.0427	0.336	0.0302	0.2376	NE
58	0.5365	0.7624	0.3794	0.5391	BP
59	0.0014	0.7638	0.001	0.5401	NE
60	0.0037	0.7661	0.0026	0.5417	NE

Duration of inhibitory periods of PMRF neurones to stimulation of cerebral peduncle (cp), substantia nigra pars reticulata (SNr) and compacta (SNc)

Cp	SNc	SNr	SNr
13.4C2 (<5ms)	14/4C3 (<5ms)	28/1C3 (<5ms)	27/4C2 (>10ms)
22/4C5 (<5)	14/4C10(<5)	28/1C6 (<5)	27/4C3 (>10)
23/4C5 (<5)	14/4C1 (<5)	28/1C7 (<5)	17/3C4 (>10)
	14/4C14(<5)	8/7C2 (<5)	17/3C6 (>10)
22/4C2 (<10ms)	14/4C6 (<5)	22/7C5 (<5)	22/7C1 (>10)
23/4C8 (<10)	15/4C7 (<5)	22/7C12(<5)	22/7C2 (>10)
13/4C6 (<10)		22/7C9 (<5)	22/7C6 (>10)
13/4C4 (<10)	14/4C5 (<10ms)	16/3C6 (<5)	22/7C10(<10)
22/C4 (<10)	14/4C7 (<10)	29/7C3 (<5)	10/3C4 (Excit)
22/4C6 (<10)	14/4C12(<10)	29/7C2 (<5)	9/4C3 (Excit)
	15/4C4 (<10)	17/3C5 (<5)	9/4C5 (Excit)
13/4C3 (>10ms)	14/4C13(<10)	8/7C1 (<5)	29/7C5 (Excit)
23/4C1 (>10)	14/4C4 (<10)	8/7C3 (<5)	10/3C2 (Excit)
23/4C7 (>10)	15/4C6 (<10)	8/7C5 (<5)	10/3C1 (Excit)
	15/4C1 (<10)	8/7C6 (<5)	16/3C7 (Excit)
13/4C8 (Excit)	15/4C8 (<10)	9/4C2 (<5)	17/3C3 (Excit)
9/3C1 (Excit)	15/4C11(<10)	9/4C4 (<5)	28/1C4 (Excit)
9/3C4 (Excit)	15/4C13(<10)	27/4C1 (<5)	22/7C8 (Excit)
9/3C5 (Excit)	15/4C14(<10)	27/4C4 (<5)	22/7C3 (Excit)
13/4C5 (Excit)		22/7C4 (<5)	17/3C7 (Excit)
13/4C7 (Excit)	15/4C12(>10ms)	22/7C7 (<5)	17/3C10(Excit)
	15/4C3 (>10)	22/7C11(<5)	29/7C4 (Excit)
22/4C3 (Depres)	15/4C9 (>10)	22/7C13(<5)	17/3C9 (Excit)
23/4C9 (Depres)	14/4C9 (>10)	29/7C1 (<5)	16/3C5 (Excit)
	14/4C2 (>50)	27/4C7 (<5)	29/7C6 (Excit)
Total = 20	15/4C5 (>50)		29/7C8 (Excit)
	15/4C2 (Excit)	28/1C2 (<10ms)	22/7C10(
		28/1C5.(<10)	10/3C3.(No)
	Total = 25	16/3C4 (<10)	
		9/4C5b(<10)	Total = 58
		27/4C6 (<10)	
		17/3C2 (<10)	