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# PROJECTIONS FROM THE LATERAL MAMMILLARY NUCLEUS TO THE ANTERODORSAL THALAMIC NUCLEUS IN THE RAT[i]

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## INDEXING WORDS

anterodorsal thalamic nucleus; lateral mammillary nucleus; mammillothalamic tract; fluorescent tracers; albino rat

## SYNOPSIS

There has been an abundance of research on the connections of the mammillary bodies but the projections from the lateral mammillary nucleus to the anterodorsal thalamic nucleus has remained a gray area due to a dearth of material which directly addresses the details of this pathway. This study seeks to further define the nature of this particular nerve connection within the mammillothalamic tract. The technique employed is fluorescent nerve tract tracing using two fluorescent tracers implanted separately into each anterodorsal thalamic nucleus then followed retrogradely to the soma of the neurons in the lateral mammillary nucleus.

Fluorescent photomicrography allowed us to document the single and double labeled cells of the lateral mammillary nucleus. The single labeled cells can be categorized into ipsilaterally projecting neurons and contralaterally projecting neurons. About half of all labeled cells were bilaterally projecting double-labeled, a third was ipsilaterally projecting single-labeled and the remainder were contralaterally projecting single labeled-cells. There were no labeled cells traced to the medial mammillary nucleus.

The mammillary bodies play an important role in the limbic circuitry and a part of the so-called "Papez Circuit". The pathway by which the mammilary body projects to the other structures of the limbic system and the way it connects the limbic system to other parts of the brain like the tegmentum is not fully

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understood. This clarification of the connection between the lateral mammilary nucleus and the anterodorsal thalamic nucleus is but one of the contemplated pathways.

## INTRODUCTION

The afferent and efferent connections of the mammilary bodies has been extensively explored for a long time in various animals demonstrating its wide connectivity in the brain [1-8,15-18,19,24-26]. In particular the fibers from the neurons of the lateral mammillary nucleus to the bilateral anterodorsal thalamic nuclei has consistently been demonstrated [5,6,18,19,24,25]. In spite of these many investigations, the exact nature of this projection from the lateral mammillary nucleus to the bilateral anterodorsal thalamic nuclei has remained a gray area because of findings that do not address explicitly this pathway or findings that do not completely reconcile with each other.

This investigation was carried out to demonstrate the nature of the afferent projections from the lateral mammillary nucleus to the anterodorsal thalamic nuclei bilaterally. We did this by using two different retrograde fluorescent tracers in the albino rat.

## MATERIALS AND METHODS

*Dye Implantation*

Six Wistar rats (Japan Clea) were used. These were adults ranging in weight from 350 gms. to 450 gms. They were anesthetized with 10% chloral hydrate given intraperitoneally at a dose of 0.3 gms./kg BW. The head was mounted on a Narishige (SR6) stereotaxic apparatus. An incision was made in the midline of the scalp to expose the area of the bregma and the sagittal sutures. Two burr holes were then drilled through the parietal bones using a hand held dental drill. The site of the holes were plotted according to coordinates from Paxinos' Rat Brain Atlas[10] in order to approach both anterodorsal thalamic nuclei.

Fine glass capillaries (tip diameter about 50 μm) were then inserted into each anterodorsal thalamic nuclei target. The fluorescent tracer dye, 10% Fluoro-Ruby (FR) [14] or Fluoro-Red (FL-Re) was then implanted into the right anterodorsal thalamic nucleus. On the contralateral side the fluorescent tracer dye, 4% Fluoro-Gold (FG) [12,13] or Fluoro-Green (FL-Gr) was then implanted into the left anterodorsal thalamic nucleus. The skin incision was then sutured and the wound swabbed with antiseptic.

The fluorescent dyes, FL-Re and FL-Gr[iii] were injected using a 1 μl Hamilton syringe connected via plastic tubing to the fine glass capillaries. About 0.02μl was very slowly injected after the glass capillaries were stereotaxically positioned. After about 10-15 minutes equilibration time to localize the injection site the glass pipette was then slowly withdrawn over another 3-5 minutes. The fluorescent dyes

[iii] Details of these two new dyes have been submitted for possible publication.

FR and FG were iontophoretically deposited using a regulated DC source (voltage and pulses per minute can be set) followed by 10-15 minutes equilibration time and the pipette was withdrawn over 3-5 minutes. The optimal survival time for FG was found to be from 2-3 days while for FR it was 6 days. Subsequently FR was injected 6 days prior to sacrifice while FG was injected 2-3 days prior to sacrifice for the same animals. The survival time for both FL-Re and FL-Gr was from 2-3 days.

*Perfusion & Sectioning*

After the appropriate survival time the rats were deeply anesthetized again with intraperitoneal 10% chloral hydrate and mounted onto a perfusion board. The left ventricle was then cannulated and the right atrium opened after starting with a vascular rinse of 0.9% NaCl followed by at least 1 liter of 10% formol-saline over 45-60 minutes. The brain was then extracted intact and placed in a cryostat. The brain was serially sectioned coronally at 30 μm thickness and every third section was mounted onto glass slides.

*Fluorescent Microscopy*

Fluorescent microscopy was then done using an Olympus AX-80 equipped with three filter systems. The G filter system with main excitation wave length 546 nm was used to view FR and FL-Re. The UV filter system with main excitation wave length 365 nm was used to view FG. The B filter system with main excitation wave length of 515 nm was used to view FL-Gr.

The accuracy and extent of the implantation or injection sites were first examined with the proper filter pack before the dyes were traced to the lateral mammillary nucleus. Photomicrographs were taken at the injection sites while labeling of the neurons in the lateral mammillary nucleus were documented with both single and double exposure fluorescent microscopic techniques. The number of single and double labeled neurons were then counted per photomicrograph, summed for each rat and percentages estimated.

## RESULTS

*Injection Sites*

An experiment was deemed successful only if: 1.) each tracer was found in each anterodorsal thalamic nucleus without significant involvement of adjoining areas and 2.) the labeling of the anterodorsal nucleus is adequate. Fig. 1 illustrates this point.

*Labeling in Lateral Mammillary Nucleus*

Both single and double-labeled cells were seen in both the right and left lateral mammillary nuclei. Fig. 2 depicts the labeled cells from the right lateral mammillary nucleus labeled with FL-Re and FL-Gr from the ipsilateral and contralateral anterodorsal thalamic nucleus respectively. The single labeled cells

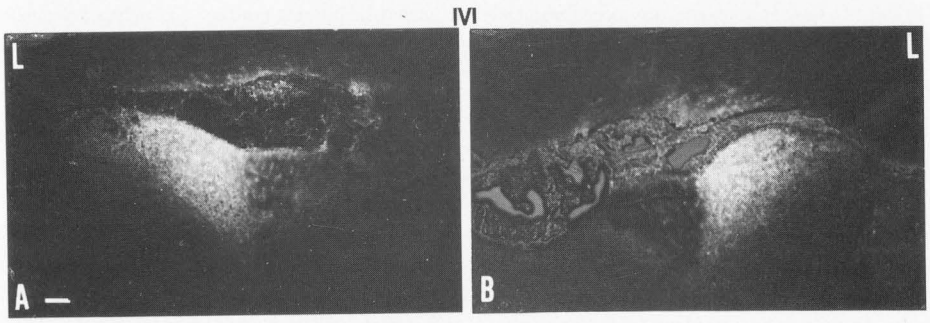


**Fig. 1. INJECTION SITES. A. FR** tracer in the right anterodorsal thalamic nucleus. **B. FG** tracer in the left anterodorsal thalamic nucleus. Bar scale ≅ 0.1 mm.

were of two types: the first set were the cells labeled from the ipsilateral anterodorsal thalamic nucleus and the second set were the cells labeled from the contralateral anterodorsal thalamic nucleus. In our best case injected with FL-Re and FL-Gr into the right and left anterodorsal thalamic nuclei respectively, the bilaterally projecting double-labeled cells accounted for about half of all labeled cells, the ipsilaterally projecting single-labeled cells constituted about a third of all labeled cells while the remainder were contralaterally projecting single-labeled cells. There were no labeled cells in the whole medial mammillary nucleus.

## DISCUSSION

Due to the small area of the anterodorsal thalamic nucleus the two most crucial variables to regulate were the volume of the dye and the placement of the tip of the fine glass capillary pipettes. FG and FR had the advantage of being iontophoretically injected which in our experimental setup translated to better controlled injection site size. Although both had its advantages, the main drawback of using FR was the longer survival time required compared to FG necessitating two surgeries 3-4 days apart. We found that the tendency for diffusion from the injection site for both FG and FR was acceptable. If we now turn our attention to the target lateral mammillary nucleus we found that FR labeling was at times inconsistent, i.e. even with what seems to be adequate labeling of the anterodorsal thalamic nucleus, most especially if the survival time was less than 6 days. FG is an excellent retrograde tracer, enabling us to control the area and size of the injection site. We found that its tendency to fade under fluorescent light accelerates if survival

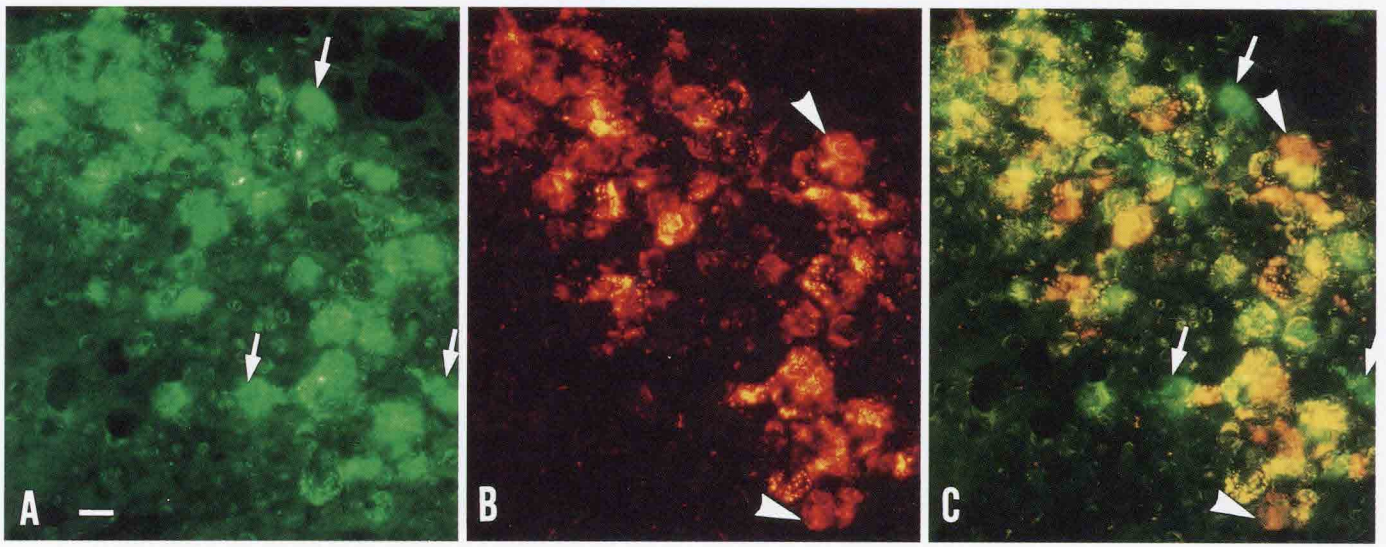


**Fig 2. RIGHT LATERAL MAMMILLARY NUCLEUS.** **A.** Cells labeled with Fl-Gr from contralateral anterodorsal thalamic nucleus using B filter system. **B.** Cells labeled with Fl-Re from ipsilateral anterodorsal thalamic nucleus using G filter. **C.** Double-exposed photomicrograph to demonstrate bilaterally projecting double-labeled cells (yellowish- green cells) with single labeled contralateral projecting cells (green cells; arrow) and single labeled ipsilaterally projecting cells (red cells; arrow head) using B and G filter for first and second exposures respectively. Bar scale ≅ 15 μm.

times were longer than 2-3 days and even then FG labeling of the target lateral mammillary nucleus could only be documented within the first 5 minutes or so.

Recently our laboratory tried to use two new fluorescent tracers, FL-Re and FL-Gr. These two tracers were delivered via pressure injection method but has very little tendency to diffuse so that the injection site is rather well circumscribed. In our experimental setup, the size of the injection sites for both FL-Re and FL-Gr were easier to control. Both dyes appear to be exclusively retrograde tracers. Traced to the target area the labeling of FL-Re under the G filter system appears as dark-red granules in the cytoplasm while for FL-Gr under the B filter system appears as green granules in the cytoplasm. Even after extended viewing under fluorescent illumination these two dyes do not seem to fade nor do they seem to diffuse from the labeled cells.

The "Papez circuit" [9] is still not completely elucidated and although fibers of the fornix [17,23], subiculum and presubiculum [18] have been documented to terminate in the mammillary nuclei, only the supramammillary subnuclei has efferents to the dentate gyrus [22] and none in the fornix [1]. There has been a suggestion thus that one pathway for the mammillary nuclei to project to the limbic structures is via the anterior thalamic nuclei [16] since the anterodorsal thalamic nucleus has been documented to receive afferent inputs from the ipsilateral retrosplenial, postsubicular and presubilar areas [15] and send efferents to the granular retrosplenial area [20,21].

Kooy et al., in 1978 was able to demonstrate both single and double labeled cells in the lateral mammillary nucleus of rats after retrograde transport of Evans Blue from one side and a mixture of DAPI-Primulin on the contralateral side of the anterior thalamus. However, these injection sites in the anterior thalamus were not confined to the anterodorsal thalamic nucleus alone and they inferred only that the double labeling came from the anterodorsal thalamic nucleus without any direct evidence to support this claim. In addition, there is no way to confirm whether there were contralateral or ipsilateral projecting single-labeled cells to the anterodorsal thalamic nucleus.

In guinea pig [16] and rat [11,18] there have been findings that the medial mammillary nuclei projects to all three nuclei of the anterior thalamus but this is not born out in this study because we were not able to find any labeled cells in any subnuclei of the medial mammillary nucleus as long as the dye was confined only to the anterodorsal thalamic nucleus.

Our findings show that about half of all neurons projecting to the anterodorsal thalamic nucleus does so bilaterally while about a third projects ipsilaterally only and the remaining one-sixth projects contralaterally only as far as the anterodorsal thalamic nuclei are concerned. Taking into consideration the assumptions and limitations of Fry & Cowan[3] in 1972 in the light of the present findings it would be possible to say that in the rat most of these single and double-labeled cells must at least also send fibers

down the mammillotegmental tract. Of course the only way to partly settle the matter is by triple labeling from both anterodorsal thalamic nucleus and each one of the terminations of the mammillotegmental tract to see if there are triple, double and single labeled cells in the lateral mammillary nucleus which we hold to very likely.

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