METABOLIC MAPPING OF THE CAT BRAINSTEM USING 2-DEOXY-D-GLUCOSE DURING MESENCEPHALIC LOCOMOTOR REGION INDUCED FICTIVE LOCOMOTION

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Jack J. Kettler

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Note to the reader: Figure legends are located on the left hand page facing the appropriate figure.

ABSTRACT

This study was designed to look at the areas of the brainstem activated by stimulation of the mesencephalic locomotor region (MLR). A modification of the 2-deoxy-D-glucose (2-DG) method (Sokoloff et al. 1977) was employed. Cats were placed in a stereotaxic head frame, decerebrated at the precollicular-postmammillary level and then electrically stimulated with a monopolar electrode placed in the MLR. Once locomotion was induced the animals were paralyzed with gallamine triethiodide to inhibit afferent input and injected with tritiated 2-DG intravenously. Fictive locomotion was maintained for 45 minutes and neurograms from the cut nerves to tibialis anterior and lateral gastrocnemius were recorded bilaterally as a monitor of locomotion. Control animals were injected with 2-DG but were not stimulated subsequent to the injection. The brainstem of each animal was rapidly frozen and processed for autoradiography using tritium sensitive X-ray film. Both the stimulus site and the contralateral cuneiform nucleus (which corresponds to the MLR) were found to have increased metabolic activity. Increased activity was also observed in the midline reticular formation of the pons and medulla, the ventral tegmental area of Tsai, the substantia nigra and the periaqueductal gray. Statistical analysis revealed the above mentioned structures were significantly more active in the experimental animals. These results are consistent with the

suggestion that stimulation of the MLR excites reticulospinal cells in the pons and medulla which relay information to the spinal cord for the initiation of locomotion (Steeves and Jordan 1984, Shefchyk et al. 1984). The spinal nucleus of the trigeminal nerve demonstrated increased activity in one experimental animal. This result suggests that the ponto-medullary locomotor strip (PLS) may correspond to the spinal nucleus of the trigeminal nerve and is activated by stimulation of the MLR.

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INTRODUCTION

The purpose of this thesis is to examine the regions of the brainstem involved in the production of locomotion during stimulation of the mesencephalic locomotor region. It has been known since the early 1900's that the spinal cord is able to produce coordinated locomotion without the aid of higher centers. Graham Brown demonstrated that decerebrated, lightly anesthetized cats with both hindlimbs deafferented could walk when their spinal cord was transected as long as they were supported (Brown, 1911). This finding led him to believe there is a stepping generator intrinsic to the spinal cord.

In 1940 it was found that stimulation of the subthalamic region in lightly anesthetized cats suspended by a hammock turned on the spinal stepping generator resulting in locomotion (Waller, 1940). Acute cats decerebrated at the rostral border of the mammillary bodies walk spontaneously and during stimulation of the subthalamic region (Orlovsky, 1969). If the subthalamic region is destroyed bilaterally in the decerebrate preparation the animal will not walk spontaneously but can be induced to walk by stimulation of a region of the midbrain called the mesencephalic locomotor region (Sirota and Shik, 1973). It appears then that the subthalamic region is involved in the initiation of locomotion in premammillary cats but does not have to be intact for locomotion to occur.

Electrical stimulation (30 Hz, 0.5 ms) of the mesencephalic locomotor region (MLR) located beneath the inferior colliculi in the precollicular postmammillary decerebrate cat preparation will result in coordinated locomotion (Shik et al., 1966). The stereotaxic coordinates of the MLR are P2, L4, depth 4-6 mm, and this site has been anatomically identified as the caudal end of the cuneiform nucleus. Stimulation of the MLR in intact cats will result in locomotion if the subthalamic region or centrum medianum-nucleus parafascicularis complex of the thalamus is destroyed bilaterally (Sirota and Shik, 1973). On the other hand bilateral destruction of the MLR will not prevent walking in otherwise intact animals (Shik et al., 1968). Thus the MLR can be used to initiate locomotion in postmammillary cats or cats with specific lesions but it is not necessary for locomotion.

Deafferentation of both hindlimbs has little effect on MLR induced locomotion (Grillner and Zangner, 1975). Postmammillary decerebrate cats can be induced to walk by MLR stimulation even after bilateral destruction of the red nucleus (Shik, 1968). Removal of the superior colliculi also has little effect on MLR induced locomotion (Jell et al., 1985). The lack of an effect indicates that afferent input as well as rubrospinal and tectospinal fibres are not involved in MLR locomotion in decerebrate cats.

It was thought that if the level of the transection in the postmammillary preparation was moved 2-3 mm caudally MLR

stimulation would no longer produce locomotion (Shik et al., 1967). A more recent study demonstrated MLR induced locomotion in cats with intercollicular decerebrations (Jell et al., 1985). Thus centers located in the region of the postmammillary transection do not appear to be involved in the initiation of locomotion by MLR stimulation.

An important question arises as to whether the MLR consists mainly of cell bodies or fibres of passage. Electrical stimulation will excite both fibres and cell bodies. Garcia-Rill recorded extracellularly in the MLR of premammillary decerebrate cats (Garcia-Rill et al., 1983a). These cats walked spontaneously on a treadmill, and nearly half the cells demonstrated rhythmic firing patterns during locomotion. This group has also induced locomotion by stimulation of the MLR chemically using the GABA antagonist picrotoxin, which is considered to preferentially effect cell bodies by removing inhibition (Garcia-Rill et al., 1983b). Thus it appears that cell bodies rather than fibres in the MLR are involved in the initiation of locomotion.

Electrophysiologists have tried to determine what areas of the brainstem receive projections from the MLR. Monosynaptic projections from the MLR to the spinal cord were sought but not found (Orlovsky, 1969b). Stimulation of the MLR did result in monosynaptic excitation of cells in the medial reticular formation of the pons and medulla (Orlovsky, 1970a, 1970b). This work demonstrated that the MLR was possibly relaying information through other

structures.

Further work was done to determine if the vestibulospinal and rubrospinal systems were also involved. It was learned that vestibulospinal neurons are maximally active during the stance phase of the step cycle or when extensor muscles are active, but the activity of the vestibulospinal neurons appears earlier than the muscle activity (Orlovsky, 1972a). Vestibulospinal neurons project from Dieter's nucleus, one of the vestibular nuclei, and MLR induced locomotion is still possible after destruction of this nucleus (Jell et al., 1985). Thus vestibulospinal systems may be involved in MLR induced locomotion but they are not required. Rubrospinal neurons are active during the swing phase of the step cycle, but bilateral destruction of the red nucleus does not prevent MLR induced locomotion (Orlovsky, 1972b). Stimulation of these pathways during locomotion enhanced the EMGs during the phase of the step cycle in which that particular pathway demonstrated increased activity.

Two important factors demonstrate that the reticulospinal, rubrospinal and vestibulospinal systems are only present as modulators but are not necessary for initiation or maintaining locomotion. Firstly if one hindlimb is stopped during the step cycle the rhythmic descending influences of these systems cease (Orlovsky, 1972a, 1972b). When these descending systems are stimulated during locomotion there is no change in the speed of

locomotion or length of the swing and stance phases of the step cycle (Orlovsky, 1972c).

Neuroanatomical tracing methods have been employed to determine the projections to and from the MLR. Anterograde transport of tritium labelled leucine injected into the region of the MLR revealed projections to the dorsolateral reticular formation of the pons and medulla, Probst's tract, the gigantocellular reticular nucleus, the substantia nigra and the nucleus centrum medianum (Garcia-Rill and Skinner, 1983). The injection site was 2.7 mm lateral to the midline which is more medial than the area usually stimulated to produce locomotion. Retrograde transport labelled structures rostral to the level of transection of postmammillary cats as well as the substantia nigra and central gray. In a more recent study injection of tritiated proline was restricted to a site 4 mm lateral, within the "classical" MLR. Projections were mainly to the ipsilateral gigantocellular and magnocellular reticular formation of the pons and medulla, the dorsal tegmental reticular nucleus and the nucleus raphe magnus (Steeves and Jordan, 1984). These studies localize first-order projections of the MLR but do not tell us which areas are functionally related to locomotion. It should be noted that the medial injection mainly labelled lateral brainstem sites while the lateral injection mainly labelled medial sites.

Both electrophysiological and neuroanatomical evidence show us that other areas are connected to the MLR. It was

found that decerebrate cats with a medullary pyradotomy and bilateral destruction of the MLR could be made to walk by stimulation of corticofugal fibres at the level of the pons (Shik et al., 1968). Other regions involved in initiation of locomotion would explain the ability to elicit locomotion in the absence of the MLR as well as the absence of the pyramids. Mori discovered a region extending from the MLR ventrally that then turns caudally and passes through the lateral pons which was called the pontine locomotor strip (PLS) (Mori et al., 1977). Stimulation of the PLS results in coordinated treadmill locomotion in postmammillary cats and subthreshold MLR stimulation combined with subthreshold PLS stimulation also elicits locomotion. The PLS appears to lie 3-4 mm lateral to the midline and about 2-3 mm below the surface of the IV ventricle. Recent work using injections of picrotoxin into the PLS have resulted, in locomotion implying it is a group of cell bodies rather than fibres (Noga et al., 1984). Bilateral lesions of the locomotor strip less than 1.5 mm in diameter do not inhibit locomotion induced by stimulation of more rostral regions of the PLS, also implying that cell bodies are involved (Budakova and Shik, 1978). Thus the PLS can be stimulated to produce locomotion and is made up of a chain of cells.

The previously mentioned neuroanatomical findings indicate that the medial and lateral MLR may be separate structures. Reversible cooling of the midline reticular formation can block locomotion induced by stimulation of the

lateral MLR, 3.5-4 mm lateral (Shefchyk et al., 1984). On the other hand, the same study showed that medial MLR induced locomotion could be blocked by cooling the PLS. Both MLR sites produce locomotion, but it seems there are at least two areas that the information is passing through.

The purpose of this study is to determine the areas of the brainstem that demonstrate increased metabolic activity during stimulation of the MLR. We attempted to find not only first-order projections from the MLR but also areas further downstream that are functionally active during MLR induced locomotion. A modification of the 2-deoxy-D-glucose (2-DG) method developed by Sokoloff to measure metabolic activity in the central nervous system was employed (Sokoloff et al., 1977). The 2-DG method is based on the properties of this compound, which is radioactively labelled. 2-deoxy-D-glucose is transported bidirectionally across the blood brain barrier by the carrier that transports glucose, and when it enters the tissue it is phosphorylated by hexokinase. Once phosphorylated the 2-deoxyglucose-6-phosphate becomes trapped in the cells because it can not be converted to fructose-6-phosphate by glucose isomerase. Thus the 2-deoxyglucose-6-phosphate is held in the tissue. Nevertheless, it will eventually be metabolized so the length of the experiment must be kept short. Since nervous tissue depends mainly on glucose as a source of energy the accumulation of 2-DG over a short period of time can be used as a measure of metabolic activity.

METHODS

I. Animal Preparation

The experiment was performed on 18 cats of either sex weighing between 2.0 and 2.5 kilograms. Eight of the 18 cats were given the tritiated 2-deoxy-D-glucose (2-DG). The rest of the animals used either did not survive the surgery or the locomotion was unreliable.

The cat was placed in a wooden box and anesthesia induced using a mixture of 70% nitrous oxide (N_2O), 30% oxygen (O_2) and 1.0-3.0% halothane delivered from a Drager vaporizer. Once the cat was anesthetized it was removed from the box and maintained under anesthesia through a face mask placed over its head. The body temperature of the cat was maintained within normal limits during surgery with a heating pad.

An incision was made into the ventral surface of the neck and a loose tie was placed on one common carotid artery with which one could occlude the artery if excessive bleeding occured during the decerebration. The other common carotid artery was cannulated using silastic tubing filled with heparin in lactated Ringer's solution (1:4) This cannula was then attached to a Statham pressure transducer connected to a Grass polygraph to monitor the blood pressure. The external jugular vein on one side was cannulated using polyethylene tubing filled with lactated Ringer's and was used to infuse fluids and the tritiated 2-

DG. Next the trachea was cannulated for continued anesthesia and the face mask was removed. The neck was then closed using wound clips and white hospital adhesive tape was placed around the neck to support the tracheal cannula.

Leg nerves in both hindlimbs were dissected for use as monitors of locomotion. Nerves to lateral gastrocnemius-soleus (LG), medial gastrocnemius (MG) and tibialis anterior (TA) were dissected free using fine spring scissors and glass rods to avoid damaging the nerves. The posterior biceps muscle was removed to facilitate placement of plexiglass nerve boats against the hindlimbs. Once the nerves were isolated they were placed on moistened latex to protect them from damage. During the nerve dissection warm saline was used to keep the nerves warm and moist.

An incision was made in the back and a silver wire electrode wrapped in moist gauze was tied to the muscle under the skin. The skin was closed with wound clips and the wire was connected to ground during stimulation and recording.

Another incision was made in the surface of the back at the thoracic level and a clamp was placed on the spinous processes of the verterbral column. This clamp was used to support the cat once it was placed in the Transvertex frame in the shielded room.

II. Craniotomy and Decerebration

The cat was carried from the surgery table into the

shielded room and placed into a modified Transvertex frame. Ear bars were inserted via the external auditory meatus to hold the head in the stereotaxic headholder. The back clamp was secured to the frame, and hip pins placed against the iliac crest of the pelvis were used to support the cat.

An incision was made into the dorsal surface of the scalp, and the skin was pulled back to expose the top of the skull. The bone covering the temporal and parietal lobes was removed. Bone wax was employed to control bleeding. The dura was reflected, and a blunt spatulla was used to perform a precollicular postmammillary decerebration extending from the the rostral edge of the superior colliculi to the caudal edge of the mammillary bodies. Once the decerebration was started the anesthetic was removed. Gauze pads, Surgicel (absorbable hemostat), and Avitene (microfibrillar collagen hemostat) were all used to control bleeding. Once the bleeding was stopped the cranium was filled with a cooled 4% agar in saline solution to keep the surface of the brain moist and prevent bleeding. If excessive bleeding occurred and the blood pressure fell below 50mmHg Dextran 75 and/or lactated Ringer's were infused until the pressure increased.

The animal was allowed to recover for a minimum of 90 minutes after the decerebration before stimulation was started. During this time plexiglass nerve boats were attached to each hindlimb to act as a reservoir for mineral oil. Each dissected nerve trunk was placed in a nerve boat, the contact of the nerve boat with the muscles of the

hindlimb was sealed using Elastomer, and then the boat was filled with warm mineral oil. A thermostatically controled heat lamp maintained the temperature of the oil in the nerve boats at 30-37 ^{o}C. The nerves were then mounted on silver wire hook electrodes connected to preamplifiers. These were in turn connected to Grass EEG amplifiers, and the activity of the nerves was monitored using an oscilloscope. The activity of the nerves was recorded on FM tape using an eight channel Vetter tape recorder. Four of the six nerves were monitored in each experiment.

III. Fictive Locomotion

Once the animal had stabilized it was paralyzed using 2-3 ml of gallamine triethiodide (Flaxedil 100, 8mg/ml) in saline and artificially ventilated using a respirator. Fictive locomotion was then evoked by stimulation of the mesencephalic locomotor region (MLR) (Shik et al., 1966). The locomotion is fictive because the animal is paralyzed and we are monitoring the locomotion via the dissected leg nerves (Jordan et al., 1979). An insulated monopolar stimulating electrode (exposed tip 0.25 mm, diameter 0.1 mm) was used to stimulate the MLR. The electrode was placed 4 mm lateral to the midline of the superior colliculus and 1 mm caudal to the junction of the inferior and superior colliculus. Square wave pulses (duration .5 ms, 30 Hz) were passed through a constant current, unit and the electrode was gradually lowered towards the MLR. The region which

displayed the lowest threshold for eliciting fictive locomotion (less than 100 microamps) was used as the stimulation site.

IV. 2-DG and Tissue Preparation

Once reliable fictive locomotion had been obtained, tritiated 2-DG suspended in sterile saline was infused via the venous canulla. Of the 8 animals used the first 4 received 200uCi/100gm body weight and the second 4 received 300uCi/100gm body weight. The FM tape deck was turned on prior to the administration of the 2-DG and recorded the nervous activity in the hindlimb nerves for the duration of the experiment. Each experimental animal was stimulated for 45 minutes. Control animals were stimulated prior to but not during or after receiving the 2-DG. The control animals were left in the frame for the 45 minute experimental period.

Forty-five minutes after the infusion of the 2-DG the brainstem was removed in four sections consisting of the superior colliculi, the inferior colliculi, the pons and the medulla. The pieces of brainstem were removed individually and placed in cold saline. Two pieces of the lumbar spinal cord, one in the region of L6 and one at L7 were also removed after a rapid laminectomy. Each piece of tissue was placed on a chuck and completely coated with Tissue Teck II embedding medium, then it was slowly lowered into a metal cup filled with Freon 22 cooled to $-70^{o}C$ using liquid nitrogen. If the tissue was lowered into the Freon 22 too

rapidly it would crack. Once all the tissue was frozen and covered with Tissue Teck II it was placed in a -70 ^{o}C freezer until sectioning.

V. Processing of Tissue and Autoradiography

The frozen tissue, subbed slides and microtome knife were placed in an American Optical Cryo-Cut Microtome set at -22 ^{o}C and left to acclimatize for 45 minutes. Once the tissue was the same temperature as the cryostat, 10um sections were cut. The first 6 sections were discarded and the next 2 were picked up on a cold subbed slide using a fine brush. The slide was then transfered to a 70 ^{o}C hot plate to rapidly dry the section and prevent diffusion of the 2-DG. We obtained 12 slides for each mm of brain tissue sectioned.

The slides were then taped with two-sided tape to a piece of cardboard which was then placed in a Kodak X-ray exposure holder with the sections facing up. LKB Ultrafilm, a tritium sensitive film, was placed with the emulsion against the sections, in total darkness. The X-ray exposure holders were closed , taped shut, placed in green garbage bags and sealed. The holders were then put in a wooden press for 4 to 6 weeks to ensure even exposure of the film. The press ensured good contact between the sections and the emulsion on the films. Each cassette also contained a slide with tissue that did not contain 2-DG as a control against pressure or chemical artifacts.

The films were removed and developed in total darkness using Kodak D-19 developer at 20 ^{o}C for 5 minutes. The film was then placed in Kodak rapid fix for 5 minutes and washed in running water for 20 minutes. The films were hung up on clips and left to dry.

To identify the level of the sections representative slides were stained using the Kluver and Barrera method for frozen sections (Kluver and Barrera, 1953). This method involves staining the tissue overnight using Luxol Fast Blue followed by Cresyl Violet and differentiation. The result is the myelin is stained blue and the cell bodies are stained violet.

VI. Density Analysis and Analysis of Data

Each film had autoradiographs of control and experimental animals from the same level of the brainstem or spinal cord and was visually inspected to identify areas of increased uptake of 2-DG. By having experimental and control animals on the same films we were able to compare them to each other.

A microdensitometer used for the optical density readings was constructed as described by Bryant and Kutyna (1983). A solid state detector was attached to the camera port of a Wild M8 zoom stereomicroscope. The output of the photodetector was displayed digitally on a voltage meter adapted for this purpose. The densitometer was initially calibrated in terms of optical densities using a strip of

film with known densities. A nonlinear regression model was selected using a Hewlett Packard 9836 statistics package to determine the calibration constants needed to convert raw data to optical densities. The strip of film with the known densities was also used to standardize the densitometer after each film was read. The viewing field of the densitometer was 0.3 mm.

All structures that appeared to show uptake and that were visually identifiable on the film were analyzed. Some structures were not identifiable on certain films and thus no reading was obtained for particular structures on particular animals. The structure in the brainstem was identified then a total of 5 readings from at least 2 different sections were taken using the densitometer. These readings were recorded and then analyzed as follows. The density of the background of each film was recorded as well as the white matter for the control and experimental animal on each film. The pyramidal tract was used as representative white matter. The optical density(O.D.) ratio of a structure was determined using the following formula.

$$\text{O.D.ratio} = \frac{\text{O.D.structure} - \text{O.D.background}}{\text{O.D.white matter} - \text{O.D.background}}$$

The optical density ratios were used as a measure of increased uptake of 2-DG. A 2 sample t-test was done to determine if any difference existed between the average optical density ratio of a structure in control as compared to experimental animals.

ABBREVIATIONS

BC	brachium conjunctivum
BP	brachium pontis
caud	caudal to the junction of the superior and inferior colliculi
CB	cerebelum
CNF	cuneiform nucleus
cont	contralateral
ENG	electroneurogram
FTC	central tegmental field
FTG	gigantocellular tegmental field
FTP	paralemniscal tegmental field
GABA	gama-aminobutyric acid
gm	gram
Hz	hertz
IC	inferior colliculus
IP	interpeduncular nucleus
ipsi	ipsilateral
keV	kilo electron volts
L	left
LC	locus coeruleus
LG	lateral gastrocnemius
LLD	dorsal nucleus of the lateral lemniscus
LLV	lateral nucleus of the lateral lemniscus
ml	milliliter
MLR	mesencephalic locomotor region

mm	millimeter
O.D.	optical density
OLIVE	superior and inferior olive
P	pyramidal tract
PAG	periaqueductal gray
PLS	pontine locomotor strip
R	right (when prefix for TA or LG)
R,RN	red nucleus
RF	reticular formation
RM	raphe
SC	superior colliculus
SN	substantia nigra
SNC	substantia nigra, compact division
SNR	substantia nigra, reticular division
SOL	lateral nucleus of the superior olive
TA	tibialis anterior
TB	trapezoid body
V4	fourth ventricle
VTA	ventral tegmental area of Tsai
um	micrometer, micron
uA	microamp
2-DG	2-deoxy-D-glucose
3	oculomotor nucleus

RESULTS

I. Stimulus Site

Locomotion was induced in 8 precollicular postmammillary decerebrate cats by electrical stimulation of the MLR. Of these cats 3 were used as controls and the MLR was not stimulated after infusion of the 2-DG. Five were used as experimentals and were stimulated after infusion of the 2-DG.

The area classically defined as the MLR and most effective for producing locomotion in decerebrate cats is located at P2, L4, H0 (Shik et al., 1967). We located the MLR by placing an electrode 4 mm lateral to the midline, 1 mm caudal to the junction of the superior and inferior colliculi and started stimulating at a depth of 4 mm below the surface using square wave pulses of 0.5 msec duration and a frequency of 30 Hz. The stimulus strength required to initiate and then maintain fictive locomotion ranged from 60-200 uA as shown in Table I. If the first placement of the electrode failed to induce locomotion the electrode was moved to a site 0.5 mm medial. In all the cats used we were able to induce and maintain locomotion from sites 3.5 to 4 mm lateral to the midline and at a depth of 4 to 6 mm below the surface of the colliculi. These sites would appear to lie in the area which has been identified as the MLR.

The length of time the animal walked fictively during the 45 minute experimental period from the infusion of the

2-DG until the animal was sacrificed varied from 64% to 95% of the total time (Table I). The variability was probably due to the condition of each animal or the viability of the stimulus site. In one experiment recordings from L6 and L7 ventral root filaments were used as monitors of the fictive locomotion. In the rest of the experiments ENGs were recorded bilaterally from the TA nerves and LG nerves. As shown in Table I, activity was not obtained from all 4 nerves during each experiment. Damage to the nerve during the dissection may be the cause of this, but rhythmic activity in even one nerve tells us the MLR is activating some descending systems. Figure 1 is an example of rectified ENGs from right LG, right TA and left TA during stimulation of the MLR after infusion of 2-DG in experiment #15 and clearly shows rhythmic activity similar to locomotion. These results show us that the animals were made to walk fictively for a reasonable length of time during each experiment.

II. Autoradiographs

a) Stimulus Site

The autoradiographs of the experimental and control animals were compared visually. It is immediately obvious that at the level of the stimulus site increased uptake of 2-DG occurred dramatically in the ipsilateral MLR and also in the contralateral MLR (Figure 2). This was expected since we are electrically stimulating the ipsilateral MLR, and anatomical studies have shown connections between the two

MLRs (Steeves and Jordan, 1984). The O.D. ratio of the ipsilateral MLR in control animals (Table II) ranged from 2.17 to 3.50 while experimental animals (Table III) ranged from 4.37 to 9.66. Table II and Table III list the O.D. ratios for the structures examined in each animal. Structures like the ipsilateral MLR in cat #2 have more than one O.D. ratio, and the reason for this is cat #2 was used as a control for more than one experimental animal and a new O.D. ratio was also obtained from each film analyzed. Since there was no significant difference between the ipsilateral and contralateral MLR in control animals, they were grouped together for statistical analysis. A two sample t-test was done to compare the mean of the O.D. ratios of the ipsilateral MLR (stimulus site) to the MLR in control animals and indicated a significant difference with a $p \leq 0.01$. The contralateral MLR in the experimental group had O.D. ratios ranging from 3.5 to 9.99 (Table II). The O.D. ratio for this region in experiment #3 was 9.99. The mean of the O.D. ratios of the contralateral MLR was also found to be significantly different from controls ($p \leq 0.01$). The O.D. ratios clearly tell us both the ipsilateral and contralateral MLR of the experimental animals had increased uptake of 2-DG due to stimulation of the MLR.

b) Structures Rostral to Stimulus Site

Structures which we examined rostral to the MLR included the ventral tegmental area (VTA), substantia nigra

(SN), periaqueductal gray (PAG) and the red nucleus (RN). We examined these structures because visually these areas appeared darker in the experimental group (Figure 3).

The ventral tegmental area is a midbrain region located near the midline ventral to the red nuclei. The O.D. ratios of experimental animals ranged from 3.62 to 5.63 while the controls ranged from 2.51 to 3.7. The means of these values are different with $p \leq 0.075$, and it should be noted the mean of the experimentals was 4.38±1.09 and the mean for the control group was 3.07±0.60. The substantia nigra, which is also at the midbrain level, was darker in experimentals than in controls. The mean of the O.D. ratios of the SN in control animals was 2.74±0.26, and the mean for experimental animals was 3.70±0.89, and these are different with $p \leq 0.075$. The PAG was found to be significantly different with $p \leq 0.05$ and the mean of the O.D. ratio of the controls was 2.78±0.51 and the mean of the experimentals was 4.38±1.28. Thus during stimulation of the MLR the VTA, SN and PAG demonstrate increased uptake of 2-DG. The red nucleus was also examined in the midbrain but no significant difference was found between experimentals and controls. Figures 5 and 6 clearly show these results and indicate which structures were significantly different in experimentals as compared to controls.

c) Structures Caudal to Stimulus Site

The structures we examined caudal to the stimulus site

included the reticular formation (RET), the superior and inferior olive (OLIVE), the spinal nucleus of V, the raphe nucleus (RM) as well as lamina I and II and lamina IX of the lumbar spinal cord. The O.D. ratios were determined for the structures after they were visually identified on the films.

The reticular formation of the pons and medulla demonstrated increased activity during MLR stimulation. This was observed when the films were viewed (Figure 4). The mean of the O.D. ratios for the controls was 2.28±0.39, and the mean for the experimentals was 3.18±0.66. These values are significantly different with $p \leq 0.05$, and this significance is probably due in part to the small standard deviations. The superior and inferior olive were considered one structure and appeared to be darker in the experimental group, but the mean of the O.D. ratios for controls was 3.94±1.56, and experimentals were 4.45±1.84 which is not significant. The mean was higher for the experimentals, but the variability from animal to animal increased the standard deviations and reduced the significance.

The next structure we examined was the spinal nucleus of V, which was visibly darker in one experiment, #12 (Figure 4). The stimulus site was 3.5 mm lateral in this case, and we may have been stimulating a portion of the medial division of the MLR. The mean of the O.D. ratios of this structure was 3.38±0.36 for all the controls and 4.24±1.50 for all the experimentals, which is not significantly different. The midline raphe nuclei were

visible but also found not to be significantly different. The mean of the O.D. ratios for the controls was 2.10±0.29 and 2.56±0.82 for the experimentals. The last structures we looked at were lamina I and II and lamina IX of lumbar spinal cord segments. The mean O.D. ratios were greater for experimentals than for controls (table II and III) but were not significantly different. In all cases mentioned the mean O.D. ratio for experimentals was larger than for the controls.

TABLE I

EXP.	STIMULUS SITE	STRENGTH	MONITORS	% TIME WALKED
#3	3.5 lat/1caud/depth5	60-125uA	L7,L6 filaments	>95%
#5	4 lat/1caud/depth4.5	100-200uA	RTA ENG	75%
#12	3.5 lat/1caud/depth5-6	200uA	RTA ENG	64%
#15	4 lat/1caud/depth5	150-175uA	RTA,RLG,LTA ENG	67%
#17	4 lat/1caud/depth4.2	150uA	RTA,LTA ENG	89%

caud caudal to junction of inferior and superior colliculi

ENG electroneurogram

lat lateral to midline

LTA left tibialis anterior

RLG right lateral gastrocnemius

RTA right tibialis anterior

TABLE II

OPTICAL DENSITY RATIOS: CONTROL ANIMALS

STRUCTURE	CAT#2	CAT#13	CAT#16	MEAN
MLR IPSI	2.52,2.25, 2.21	3.5	2.17	2.46±.52
MLR CONT	2.66,1.88			
RAPHE	2.26,2.52, 1.82	2.02	1.88	2.10±.29
VTA	3.00	3.70	2.51	3.07±.06
SN	2.81	2.96	2.46	2.74±.26
OLIVE	2.02,3.32, 6.28,2.30, 3.13	5.06,5.62	3.76	3.94±1.56
RED N.	1.49	3.87	2.53	2.63±1.19
LAMINA I,II	2.38	3.67	2.39	2.81±.74
LAMINA IX	2.15	3.24	2.14	2.51±.63
PAG	2.99,3.20	2.89	2.03	2.78±.51
SPINAL V	3.90,3.29	3.06,3.29		3.38±.36
RET	2.52,1.83	2.67	2.09	2.28±.39

TABLE III

OPTICAL DENSITY RATIOS: EXPERIMENTAL ANIMALS

STRUCTURE	#3	#5	#12	#15	#17	MEAN
MLR IPSI	4.37		7.62	9.66	5.78	6.86±2.29
MLR CONT	9.99	3.50			4.33	5.94±3.53
RAPHE	2.49	2.14	4.00	2.05	2.15	2.56±.82
VTA			5.63	3.89	3.62	4.38±1.09
SN			3.82	4.52	2.76	3.70±.89
OLIVE	3.01 6.91	2.24		3.31 4.82 6.84	4.04	4.45±1.84
RED N.			2.93	3.32	3.34	3.20±.23
LAMINA I,II			3.95	4.78	2.48	3.74±1.16
LAMINA IX			3.71	5.51	2.16	3.79±1.68
PAG		4.50	4.79	5.65	2.60	4.38±1.28
SPINAL V			6.11 4.80	3.09 2.96		4.24±1.50
RET	2.61		2.91	4.12	3.06	3.18±.66

Figure 1: Example of fictive locomotion monitored with peripheral nerve recordings from an experimental animal, #15. The tibialis anterior (TA) is rhythmically active bilaterally and the right lateral gastrocnemius (LG) is also rhythmically active.

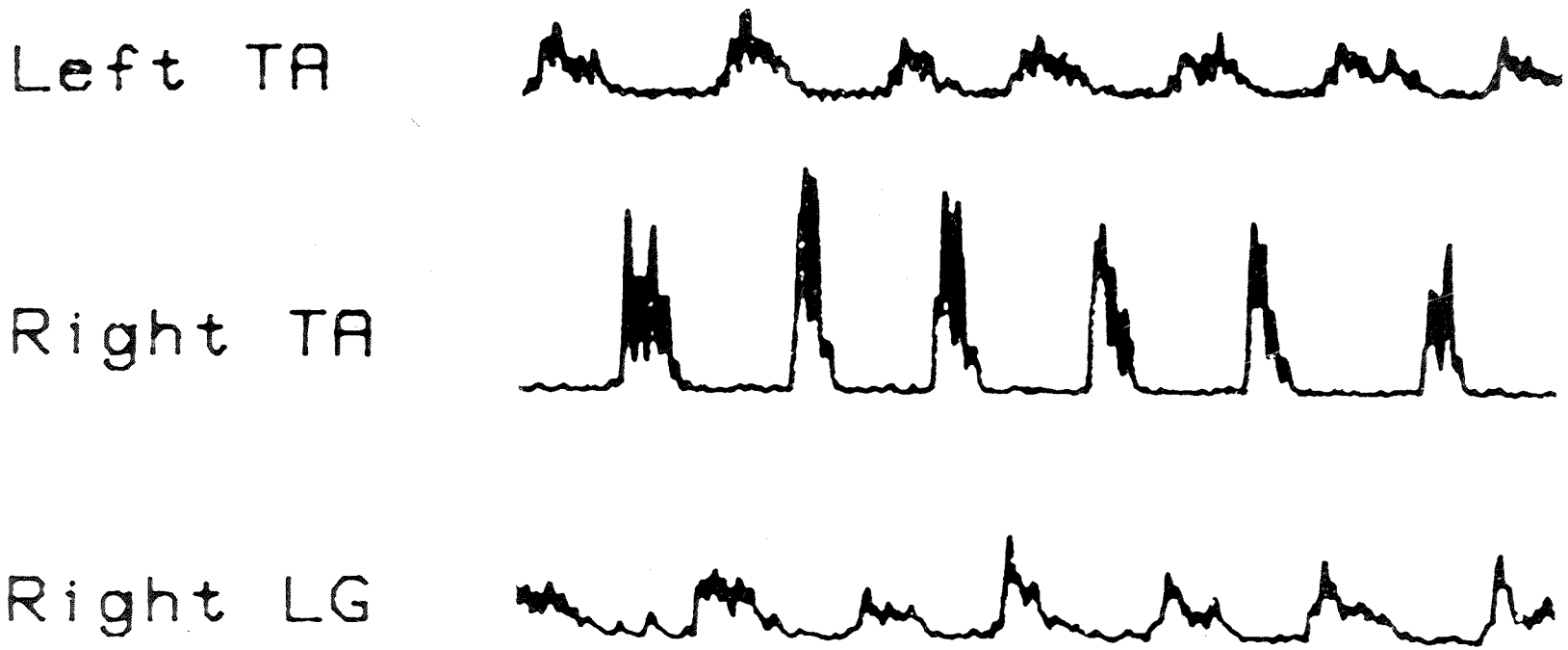
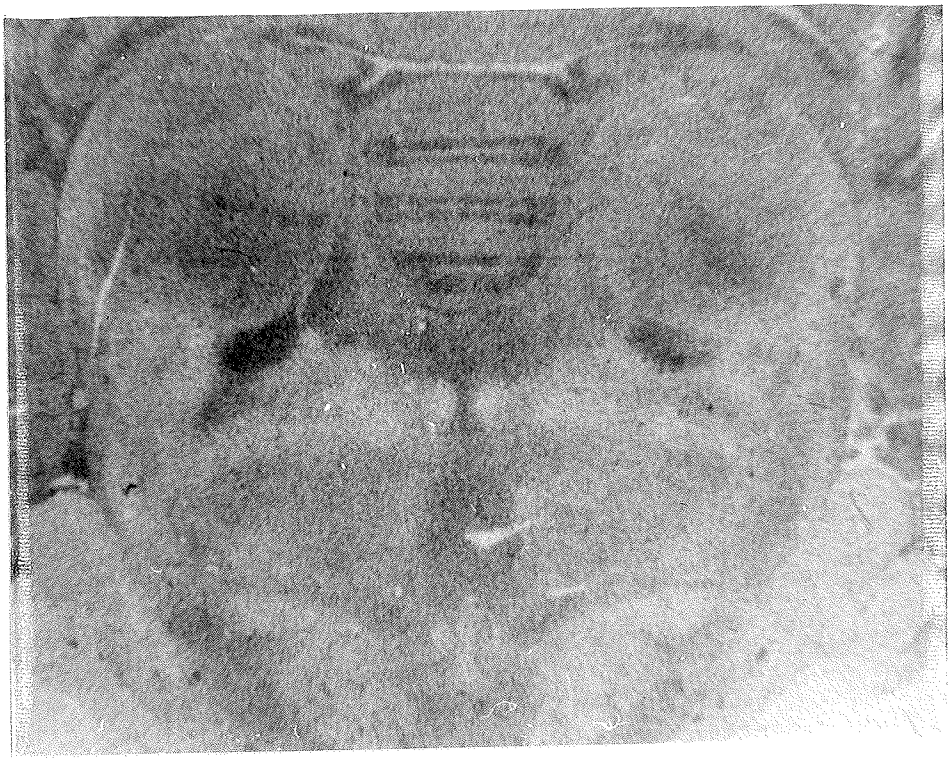

Left TA
Right TA
Right LG

Figure 2: Autoradiographs of sections from a control (bottom) and experimental animal (top) at the level of the inferior colliculus (P 2.1). Increased activity is evident bilaterally in the nucleus cuneiformis (MLR) of the experimental animal. The inferior colliculus in both animals show increased uptake as compared to other regions.



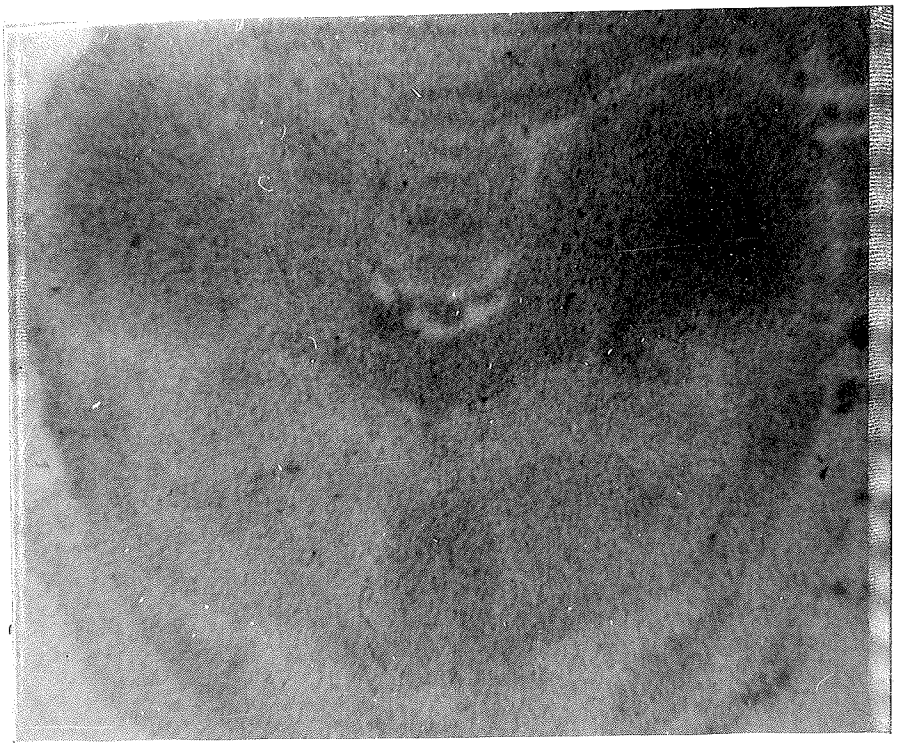
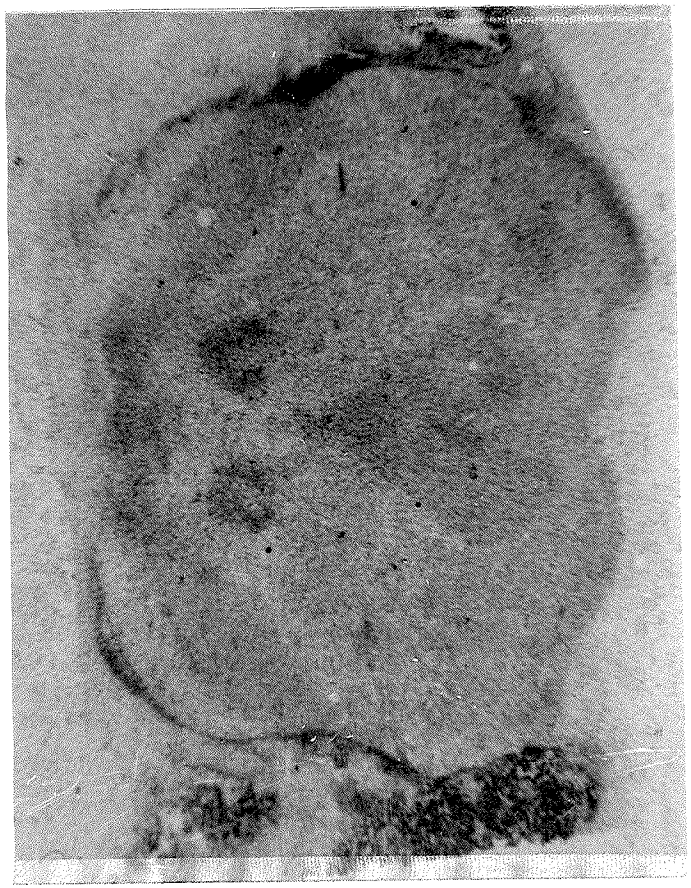


Figure 3: Autoradiographs of sections from a control (bottom) and experimental animal (top) at the level of the superior colliculus (A 3.3). The substantia nigra, ventral tegmental area and periaqueductal gray demonstrate increased uptake of 2-DG in the experimental animal. The red nucleus shows increased uptake on both sections.



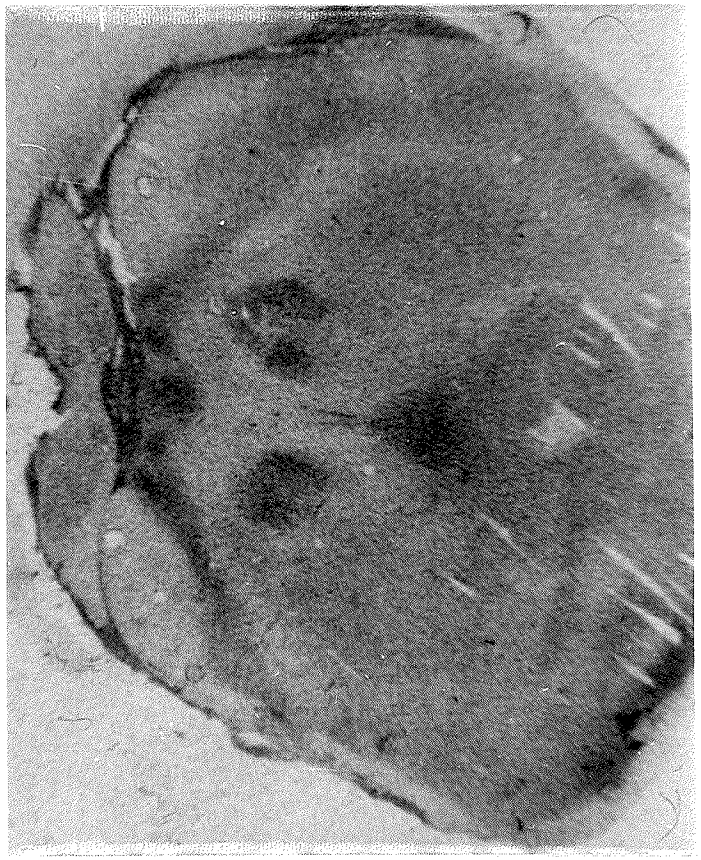
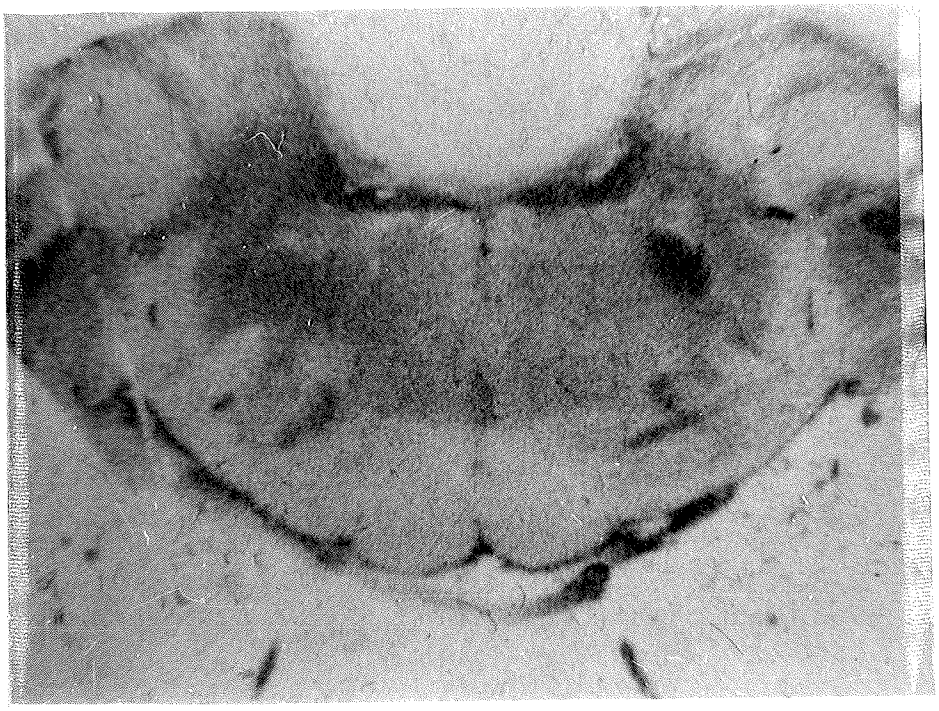


Figure 4: Autoradiographs of sections from a control (bottom) and experimental animal (top) at the level of the pons (P 5.5). Areas corresponding to the magnocellular and gigantocellular fields of the reticular formation show increased uptake of 2-DG in the experimental animal. The pontine locomotor strip (PLS) located in the area of the spinal nucleus of V showed uptake in this experimental animal.



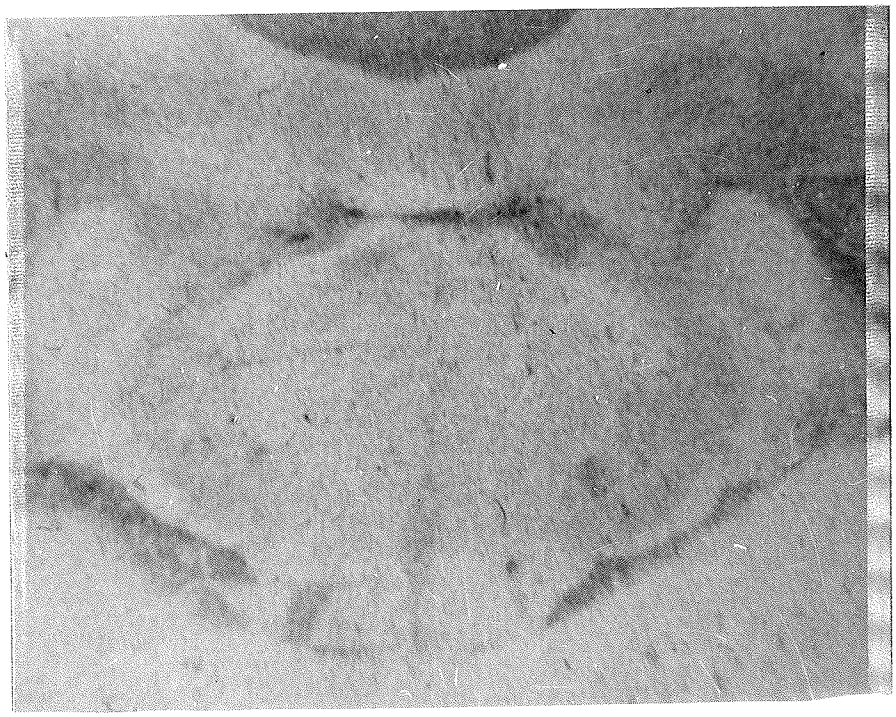


Figure 5: Optical density (O.D.) ratios of experimental animals (n=5) compared to control animals (n=3). The standard deviation and significance of the ratios are illustrated.

●● $p \leq 0.05$

● $p \leq 0.10$

This graph clearly shows the significant uptake of 2-DG in both MLRs.

CONT contralateral, IPSI ipsilateral, MLR mesencephalic locomotor region, PAG periaqueductal gray, RF reticular formation, VTA ventral tegmental area of Tsai

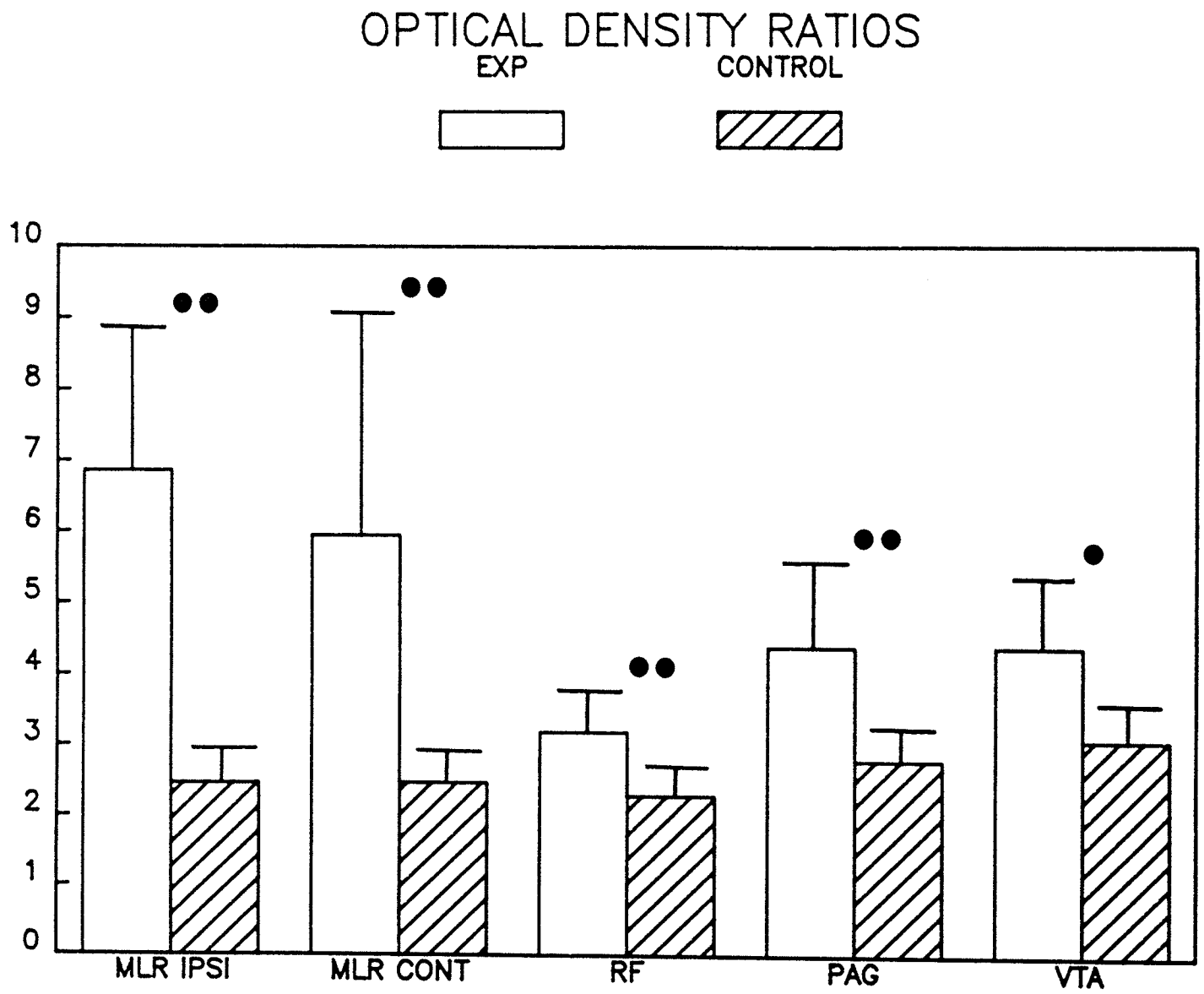

OPTICAL DENSITY RATIOS
EXP
CONTROL
10
9
8
7
6
5
4
3
2
1
0
MLR IPSI
MLR CONT
RF
PAG
VTA

Figure 6: Optical density (O.D.) ratios of experimental animals (n=5) compared to control animals (n=3). The standard deviation and significance of the ratios are illustrated.

●● $p \leq 0.05$

● $p \leq 0.10$

OLIVE superior and inferior olive, PLS pontine locomotor strip, RM raphe, RN red nucleus, SN substantia nigra

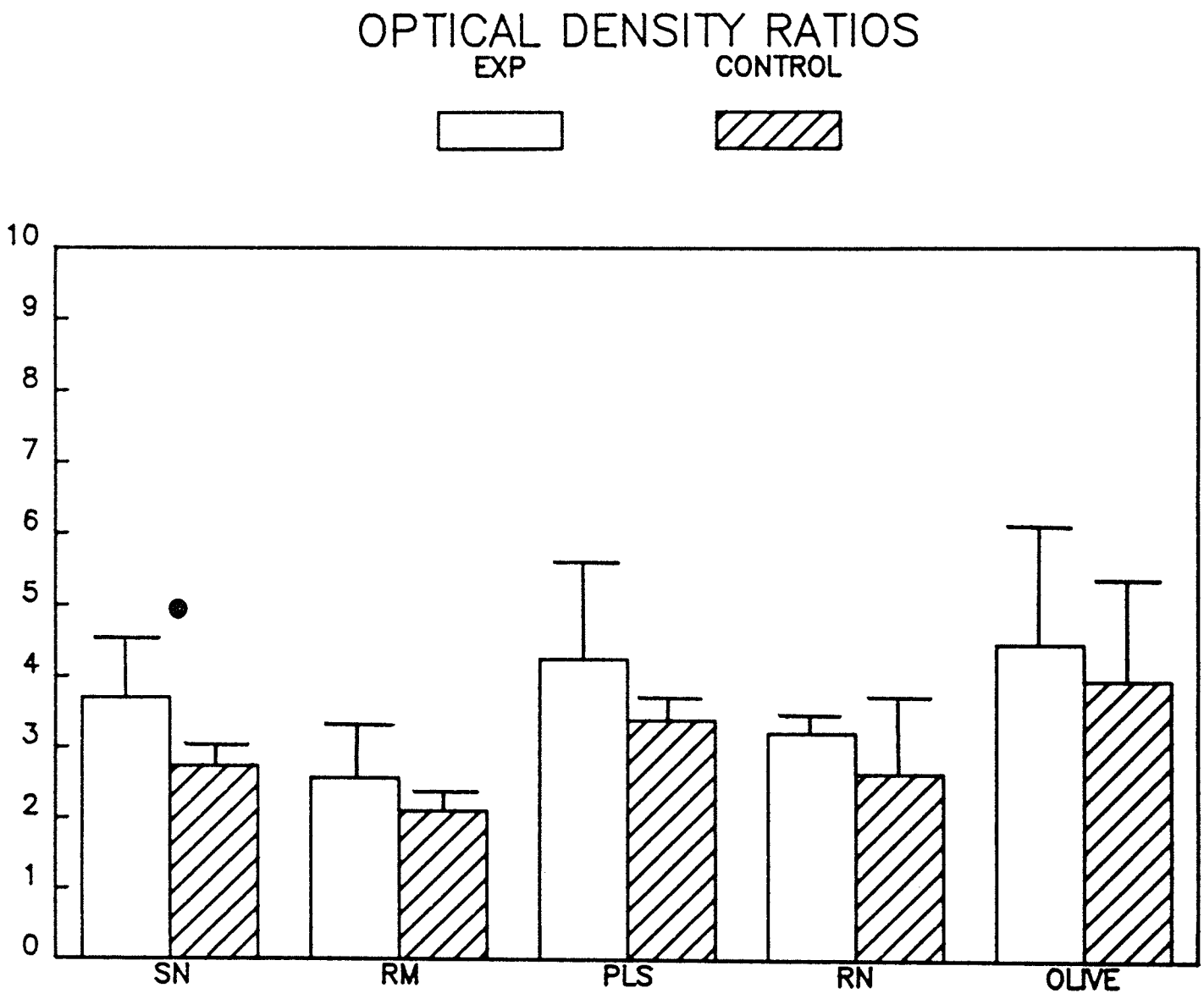
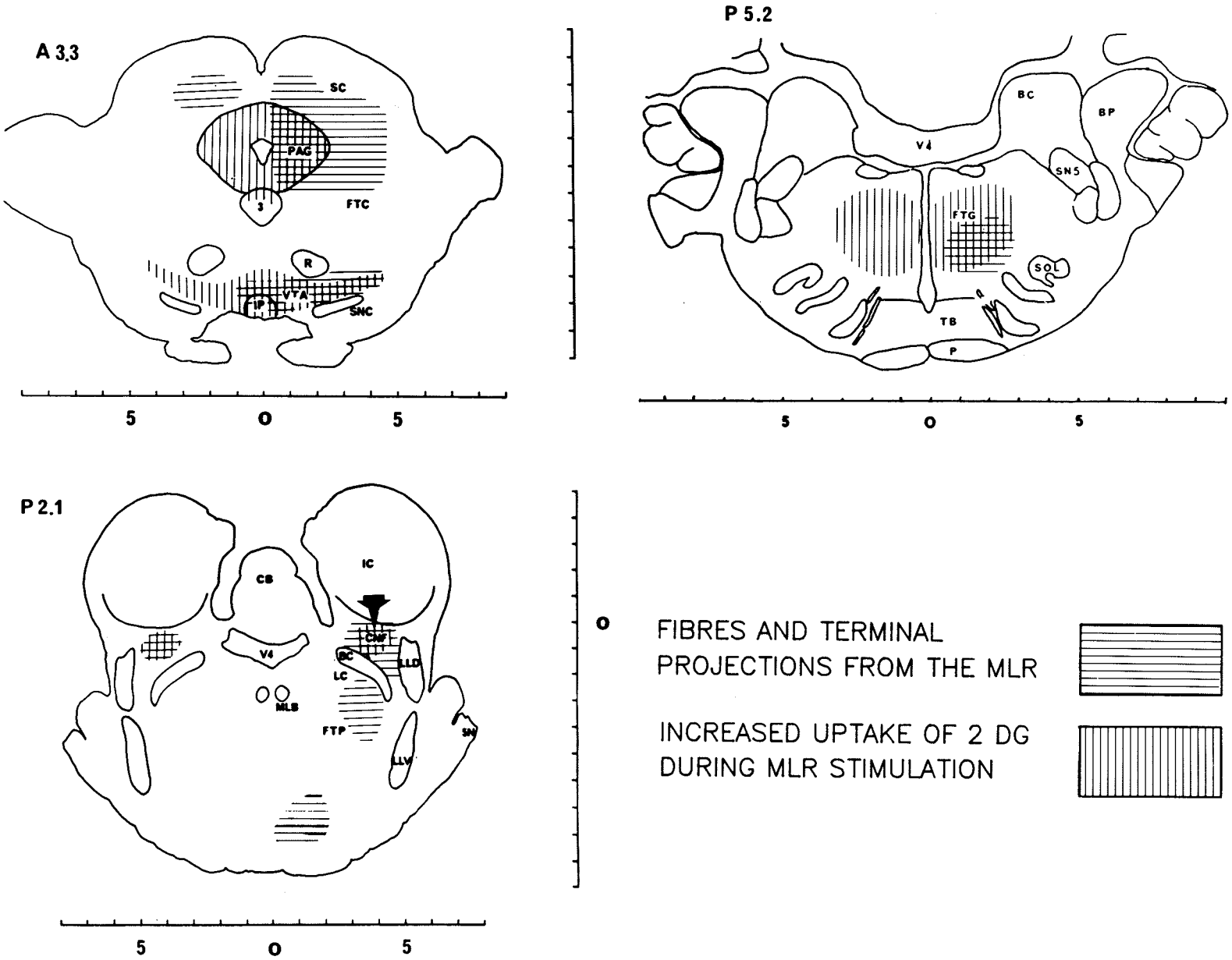

OPTICAL DENSITY RATIOS
EXP
CONTROL
10
9
8
7
6
5
4
3
2
1
0
SN
RM
PLS
RN
OLIVE

Figure 7: Illustration of fibres and terminal projections from the MLR (Steeves and Jordan, 1984) and areas demonstrating uptake of 2-DG during MLR stimulation. The arrow on the section (P 2.1) indicates the stimulus site. Cross hatching shows areas that encompass both, indicating that they may be relay sites to the spinal cord for initiation of locomotion.


A 3.3
SC
PAG
3
FTC
R
VTA
IP
SNC
5
0
5
P 5.2
BC
BP
V4
SN5
FTG
SOL
TB
P
5
0
5
P 2.1
CB
IC
V4
CNF
BC
LLD
LC
MLB
FTP
SN
LLV
5
0
5
0
FIBRES AND TERMINAL PROJECTIONS FROM THE MLR
INCREASED UPTAKE OF 2 DG DURING MLR STIMULATION

DISCUSSION

The aim of this thesis was to determine what regions of the brainstem are involved in MLR induced locomotion. Our results clearly show both the ipsilateral cuneiform nucleus (the stimulus site) and the contralateral cuneiform nucleus demonstrate increased uptake of 2-DG. The other regions that showed significant increases included the ventral tegmental area, substantia nigra, periaqueductal gray and reticular formation. The spinal nucleus of V demonstrated increased uptake in one experimental animal.

A modification of the 2-DG method developed by Sokoloff (1977) was used to measure cerebral metabolism in this study. Sokoloff's technique uses autoradiography to localize sites of isotope accumulation to discrete regions of the brain which are then analyzed by quantitative densitometry with reference to precalibrated standards. In this way the actual cerebral glucose utilization is determined for each structure examined. In our study we adopted a semi-quantitative analysis of the autoradiographs in which the optical density of a region of interest is divided by the optical density of a region believed to be constant to give an O.D. ratio. To obtain the O.D. ratio we divided the O.D. of the structure of interest by the O.D. of white matter from the same animal and same film. Each animal was being used as it's own control and the O.D. ratio is a reflection of activity in that animal and is thus not affected by time

of exposure, time of development or background activity on the films. A recent study stated that the relationship between isotope concentration and O.D. is not linear and depends on the exposure period (Kelly and McCulloch, 1983). This problem is avoided by zeroing the densitometer to the film background and by not exposing the film to the point of saturation (Mitchell and Crossman, 1984). The O.D. ratios are linearly related to local cerebral glucose utilization within a given animal but are not valid for comparison between animals in different physiological states. It has been demonstrated that anesthesia profoundly affects the relationship between O.D. ratios and local cerebral glucose utilization (Sharp et al, 1983). All animals used in our study were taken off the anesthetic at least two hours prior to stimulation to ensure they were all in the same physiological state.

The above mentioned studies including the work by Sokoloff all used carbon-14 labelled 2-DG. In our study we used tritium labelled 2-DG in an effort to improve the resolution. Both isotopes emit beta particles which expose silver grains on X-ray film but carbon-14 emits particles with an energy of 156 keV while tritium emits particles with an energy of 18.6 keV (Rodgers, 1979). This means the particles from a tritium source can travel 0.2-1.0 um through tissue while particles from a carbon-14 source may travel as far as 14 um. By using tritium we know the grains exposed on the film are less than 1.0 um from the source and

are less contaminated by radiation from adjacent sources. Another advantage of tritium is the density of the autoradiograph will not be affected by irregularities in the section thickness because only the 1.0 um of tissue in contact with the film can affect the film (Faraco-Cantin et al., 1980). The use of LKB Ultrafilm, a tritium sensitive film with very fine grains and no antiscratch layer over the emulsion, also increased the resolution. All these facts combined convinced us to use tritium labelled 2-DG for this study.

Studies have shown that electrical stimulation of the motor cortex which resulted in forelimb movements in restrained conscious rats caused increased uptake of 2-DG in numerous brain regions (Sharp, 1984). Our study also allowed demonstration of increased uptake in various brain regions and comparisons of these regions to similarly treated unstimulated controls. The animals in our study were paralyzed to decrease phasic afferent input and therefore isolate regions in the brainstem involved in MLR induced locomotion. Previous stimulation studies with 2-DG involving motor systems have been succesful, and our study specifically looks at a motor system which is isolated from afferent input.

Our results support the hypothesis that the MLR is a group of cell bodies rather than fibres of passage. Extracellular recording from cells in the MLR of spontaneously walking premammillary decerebrate cats showed

nearly half the cells had rhythmic firing patterns (Garcia-Rill et al., 1983a). This group has also used the GABA antagonist picrotoxin to chemically excite the MLR by removing inhibition (Garcia-Rill et al., 1983b). These studies are further supported by the present findings of increased uptake of 2-DG in the MLR indicating that cell bodies in the MLR are involved in the initiation and maintenance of locomotion. Our findings also demonstrated that the MLR is activated bilaterally when one MLR is electrically stimulated. Neuroanatomical tracing methods have demonstrated projections from the MLR to the contralateral MLR (see figure 7 and Steeves and Jordan, 1984). Thus our results show stimulation of the MLR excites cells in both the stimulus site and the contralateral MLR, in support of anatomical studies showing that they are interconnected. The two MLR's are able to work independently, however, because destruction of the MLR on one side does not inhibit the other MLR from producing locomotion (Shik et al., 1967).

The ventral tegmental area, periaqueductal gray and substantia nigra all demonstrated increased uptake of 2-DG during MLR induced locomotion, and their possible role in regard to locomotion must be considered. Projections from the VTA to the MLR have been demonstrated using flourescent dye injections (Garcia-Rill et al, 1983c). Bilateral microinjection of the GABA antagonist picrotoxin into the VTA has been shown to result in increased ambulatory

behaviour (Mogenson et al., 1979). This effect is believed to be due to disinhibition of dopaminergic neurons projecting to the nucleus accumbens. Injections of substance P into the VTA has resulted in increased locomotor activity (Treptow et al., 1983). Electrical stimulation of the PAG and SN can initiate locomotion in postmammillary decerebrate cats (Garcia-Rill et al., 1983d). Garcia-Rill also demonstrated retrograde transport of flourescent dye from the medial MLR to these structures. It seems reasonable to assume stimulation of the MLR activated these areas antidromically, and the fact they showed increased uptake of 2-DG does not prove these areas are necessary for locomotion. Jell et al. (1985) have shown that cats in which these structures have been removed by midcollicular decerebration can be induced to walk via MLR stimulation. This strongly suggests that the VTA, SN and PAG are not necessary in the production of MLR induced locomotion.

The fact that the reticular formation had increased uptake of 2-DG was not unexpected in view of other studies. Electrophysiological studies have shown monosynaptic excitation of cells in the medial reticular formation after stimulation of the MLR (Orlovsky, 1970a,1970b). Cells of the reticular formation are known to project through the ventral lateral funiculus of the spinal cord, and this area is required for MLR locomotion in cats (Steeves and Jordan, 1980). Thus the medial reticular formation is a good candidate as a relay site sending information to the spinal

cord to initiate locomotion. Blocking lateral MLR induced locomotion by reversible cooling of midline reticular formation structures also supports this (Shefchyk et al., 1984). Projections from the MLR to the reticular formation have been demonstrated (see figure 7) and all these factors together strongly suggest a role for the reticular formation in the initiation of locomotion.

The final structure which demonstrated increased uptake of 2-DG in one animal is the spinal nucleus of V. Injections of picrotoxin into this region have induced locomotion in decerebrate cats (Noga et al., 1984), and projections have been shown from the medial MLR to Probst's tract which lies in the region of the spinal nucleus of V (Garcia-Rill et al., 1983c). The fact that we only saw increased 2-DG uptake in one animal could be explained by our stimulus site being to far lateral to excite this structure in most experiments. This one animal does support the idea that there are two pathways projecting from the MLR through the brainstem, one pathway from the medial MLR and one from the lateral MLR.

Figure 7 summarizes the anatomical projections of the MLR as well as the areas of increased metabolic activity found in this study. Our results show the MLR is in fact a group of cells and is connected bilaterally. It also supports the idea the MLR is relaying information through the reticular formation and possibly via the spinal nucleus of V.

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