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LOCALIZATION OF MOTONEURONS INNERVATING THE EXTRAOCULAR MUSCLES OF THE SHEEP BY RETROGRADE FLUORESCENT TRACERS

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INTRODUCTION

The pattern of extraocular muscle motor innervation has been extensively studied in the last decade with retrograde labeling techniques from lower vertebrates such as fishes (see 13) up to several species of mammals (see 6). This aspect of the oculomotor system, however, has been completely neglected in the Ruminants.

To fill this gap and extend the comparative analysis of the oculomotor system we have investigated the organization of the motoneurons innervating the extraocular muscles (EOMs) in the sheep. For all the eight EOMs, that is the rectus dorsalis (RD), rectus medialis (RM), rectus ventralis (RV), obliquus ventralis (OV), levator palpebrae superioris (LP), obliquus dorsalis (OD), rectus lateralis (RL) and retractor bulbi (RB) muscles, the location of the innervating motoneurons has been investigated by using the retrograde transport technique of fluorescent dyes (2, 8). The fluorescent substances show some advantages if compared with other retrograde labeling techniques. In fact, they make it possible the simultaneous injection of different EOMs with different tracers so that, in the same section, the corresponding motoneurons for each injected muscle can be observed. This system allows also to verify any possible overlapping among groups of motoneurons innervating different muscles.

METHODS

For the injections of fluorescent tracers into the EOMs, seven sheep (weighing 10-15 kg) were used. In the animals, anesthetized with both Ketamine (4 mg/kg, iv) and diazepam (2 mg/kg, im), the LP muscle was injected through multiple penetrations into the upper portion of the superior eyelid, whereas the other EOMs to be injected were exposed via conjunctival incision. After the incision, the soft tissue of the orbit was extracted and the eyeball was partially collapsed, by the aspiration of the aqueous humor with a syringe, care being taken not to damage the nerves, the blood vessels or the EOMs. Each muscle selected for the injection was carefully isolated and a plastic film as well as cotton wool were interposed between it and the eyeball.

Four fluorescent tracers were used: 2% Fast Blue (FB), 10% Evans Blue (EB), 2% Diamidino Yellow dihydrochloride (DY), and 2% Propidium iodide (PI). The muscles were slowly injected with 20-30 μl of tracer suspension at different sites along their longitudinal axis using Hamilton microsyringes. In order to obtain the location of all the labeled neurons for each muscle in the right oculomotor nucleus, the RV, OV, and RM muscles of the right side were injected, whereas the RD and LP muscles were contralaterally injected; it is generally accepted, in fact, that RD and LP muscles are mostly innervated by the contralateral nucleus. Also, the OD muscle was injected contralaterally in order to label the right trochlear nucleus. In the same animal more than one muscle was injected, as shown in the scheme reported in Table 1.

Table 1. — *Experimental protocols.*

Sheep no.	Muscle injected with FB	with EB	with DY	with PI
1	RM	LP*	RV	RL
2	RV	OV	RD*	OD*
3	OV	RD*	RM	RB
4	RD*	RL	OD*	RV
5	OD*	RB	RL	RM
6	LP*	OD*	OV	RV
7	RB	RL	LP*	RD*

* Contralaterally injected.

After a survival time of 3-4 days, each animal was perfused, under deep anesthesia, through the common carotid arteries with heparinized saline solution followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brain stem was then removed, postfixed in the same fixative for 3-4 hours and left overnight in 10% buffered sucrose. Serial cross sections of the brain stem (60 μm thickness) passing through the oculomotor, trochlear, and abducens nuclei were cut with a freezing microtome, immediately mounted from distilled water, air dried and coverslipped with Eukitt. The sections were examined with a Leitz Ploemopack fluorescence microscope at 360 nm excitation wavelength (filter system A), which elicits the blue FB fluorescent labeling of the cytoplasm and the yellow DY labeling of the nucleus, and at 550 nm excitation wavelength (filter system N2), which elicits the red fluorescence characteristic of EB and the orange-red fluorescence of PI.

One out of every nine sections was not coverslipped, but after observation under the fluorescent microscope was counterstained with 0.2% neutral red. These sections were utilized to draw the profiles of the nuclei on which the labelings collected from 9 adjacent sections were mapped and summarized. Counts of FB, EB, and PI labelings were effected taking the perikaryon as the unit, whereas labeled nuclei were counted in the case of DY tracer.

RESULTS

1. *Oculomotor nucleus.* — The somatic oculomotor nucleus (ON) of the sheep is located in the central gray of the mesencephalon, ventrally to the cerebral aqueduct, at the level of the rostral colliculi. It consists of a column of cells located near the midline, and extending for about 4.8 mm from the rostral third of the trochlear nucleus to 1 mm rostrally to the cranial extremity of the red nucleus. Each cell

column is always ventrolaterally indented by the medial longitudinal fasciculus (MLF) and oculomotor neurons intermingle with the fibers of the MLF.

The nucleus is irregularly shaped along its rostrocaudal extent (Fig. 1). In cross sections taken at the rostral tip (Fig. 1 A) of the ON the motoneurons are distributed over an area which appears ovoid. Along the rostral third of the nucleus (Fig. 1 B, C) the area swells ventrolaterally, whereas through the middle third (Fig. 1 D-F) and the rostral part of the caudal third (Fig. 1 G) it enlarges dorsolaterally; finally, it shrinks abruptly towards the caudal end (Fig. 1 H, I), where

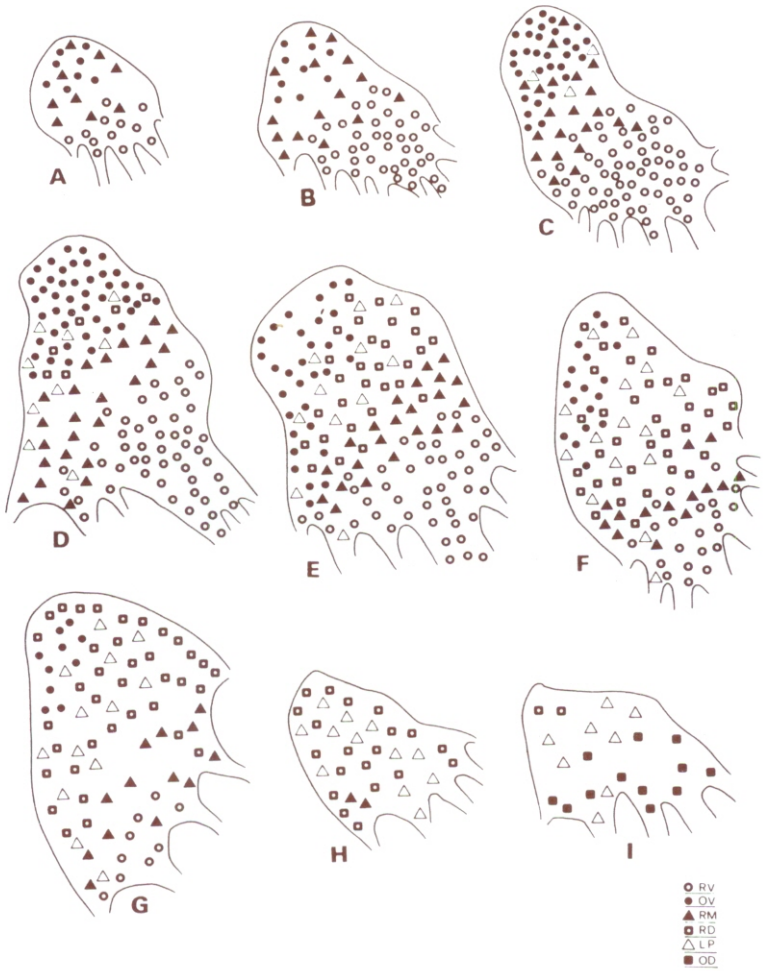

Fig. 1 – *Location of retrogradely labeled extraocular muscle motoneurons in the right oculomotor nucleus of the sheep.*

A-I drawings represent sections of the nucleus arranged from rostral to caudal with 540 μm intervals. The medial border of the nucleus is located on the left side of each drawing. Cumulative plots of labeled cells from experiments are displayed in each drawing. One symbol corresponds to approx. nine labeled cells.
○, rectus ventralis; ●, obliquus ventralis;
▲, rectus medialis; □, rectus dorsalis;
△, levator palpebrae superioris; ■, obliquus dorsalis.

the caudalmost neurons of the ON are intermingled with the rostralmost ones of the trochlear nucleus (Fig. 1 I). In cross sections at any level of the ON, the motoneurons are scattered throughout the nuclear area and no clear boundaries can be recognized between the groups of cells.

The ON is composed of neurons varying in size (from 10 to 35 μm) and shape. The majority of them are large to medium sized (20-35 μm) and multipolar in shape, and are intermingled with triangular or fusiform cells. The latter neurons are mostly numerous among the fibers of the MLF.

2. *Location of RV, OV, RM, RD, and LP muscle motoneurons. — RV muscle motoneurons (Fig. 1 A-G).* These motoneurons are present throughout the length of the ipsilateral ON with the exception of the caudalmost levels. The rostral two thirds of the ON contain about 94% of the labeled cells. RV motoneurons occupy the ventrolateral aspect of the nucleus and several cells lie among the fibers of the MLF. Throughout all their rostrocaudal distribution RV motoneurons show some intermingling with RM motoneurons (Fig. 2); in the caudalmost sections of the middle third they intermingle also with LP motoneurons.

OV muscle motoneurons (Fig. 1 A-G). The motoneurons belonging to the OV muscle extend rostrocaudally in the ipsilateral ON as far as those of RV muscle. The labeled cells have the highest concentration in the middle third (60%). In

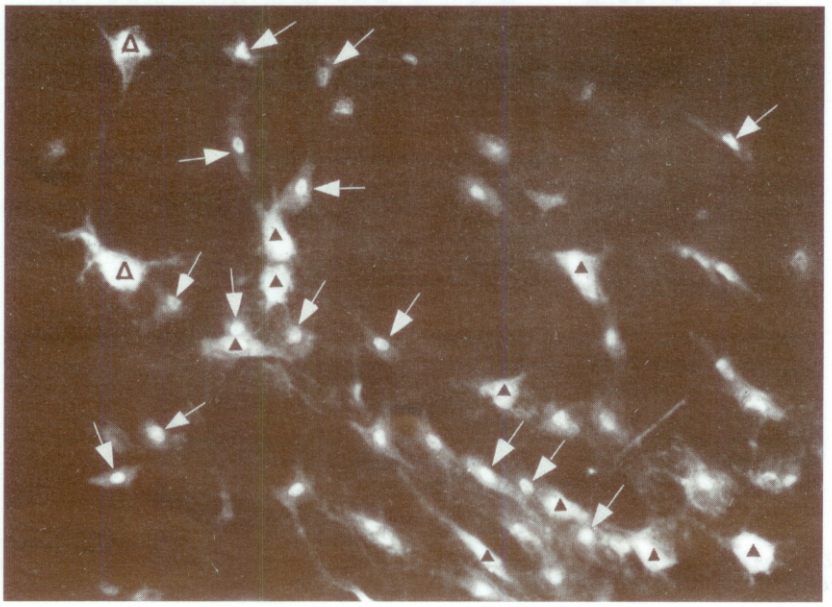

Fig. 2 – *Oculomotor nucleus of the sheep no. 1.*

Intermingling of rectus ventralis, rectus medialis and levator palpebrae superioris muscle motoneurons can be seen. White arrows = DY-labeled rectus ventralis muscle motoneurons. ▲, FB-labeled rectus medialis muscle motoneurons. △, EB-labeled levator palpebrae superioris muscle motoneurons.
Filter system combination A + N2. 250×.

the rostral third the OV motoneurons are located in a dorsal position and are intermingled with RM motoneurons. In the middle third the large dorsal cell cluster extends ventromedially to form a narrow cellular band which occupies almost completely the medial side of the nucleus. Throughout the middle third and the rostral tip of the caudal third the OV motoneurons present some intermingling with the RD and LP cells. Some labeled cells (approx. 1%) were found in the contralateral ON.

RM muscle motoneurons (Fig. 1 A-H). The motoneurons innervating the RM muscle are present ipsilaterally in all the transverse sections through the ON. The maximum number of labeled cells was counted in the middle third (50%). In the rostral third the RM labeled cells are mingled with either the dorsally located OV motoneurons or the ventrally placed RV motoneurons. In the middle third, the RM motoneurons tend to be clustered in an intermediate region of the nucleus, which extends obliquely from a dorsolateral point to a ventromedial one. Towards the caudalmost sections of the middle third and also in the caudal third, because of the massive presence of RD and LP motoneurons, the RM motoneuron pool is shifted more ventrally. Some labeled cells lie among the fibers of the MLF, often intermingled with the RV motoneurons.

RD muscle motoneurons (Fig. 1 D-I). The labeled cells are present in the middle and caudal thirds of the contralateral ON, extending caudally as far as the rostral tip of the trochlear nucleus. They are intermingled with the OV motoneurons and occupy a broad area of the nucleus, dorsally to the RM motoneuron pool. Throughout their rostrocaudal distribution RD motoneurons are also intermingled with LP motoneurons. The middle third of the ON contains about 53% of the RD labeled motoneurons. Up to 1.5% of the RD labeled cells have been found ipsilaterally to the site of injection.

LP muscle motoneurons (Fig. 1 C-I). The motoneurons innervating the LP muscle have their highest concentration in the middle third of the contralateral ON (54%) and extend caudally as far as the rostral tip of the trochlear nucleus. Throughout their rostrocaudal extent, LP motoneurons are intermingled with RD and OV cells. Several LP motoneurons are present among the fibers of the MLF. Up to 2% of LP motoneurons have been found ipsilaterally to the site of injection.

3. *Trochlear nucleus.* — The trochlear nucleus (TN) consists of a column of cells which extends from the middle third of the caudal colliculi to the caudal pole of the ON. The rostral tip of the TN overlaps for about 450 μm the caudal pole of the ON. Throughout its rostrocaudal extension the TN is located dorsally to MLF and some of its cells can be found also among the MLF fibers. The rostral portion of the nucleus is placed ventrolaterally to the ON, then the TN expands dorsally to reach its greatest extension and an ovoid shape in its middle third. Both small and large motoneurons are present in this nucleus (from 10 to 35 μm).

The injections of fluorescent tracers into the OD muscle result in a consistent labeling of cells throughout the rostrocaudal extension of the contralateral TN.

Only occasionally labeled motoneurons are present in the ipsilateral TN. Some labeled cells have been found among the MLF fibers.

4. *Abducens and accessory abducens nuclei.* — The abducens nucleus (ABDN) lies within the caudal third of the pons and its rostrocaudal extension is of about 1.8 mm. In the rostral portion it is located below the genu and descending branch of the facial nerve; in its caudal tract, the ABDN shifts in a dorsolateral direction so as to lie laterally to the genu of the facial nerve and reach the floor of the fourth ventricle. The nucleus is ovoid in shape and is composed of small, and medium-sized cells (range of diameter: 15-30 μm), the majority of which are 20 μm in diameter. The cells can be multipolar, triangular or fusiform; these latter are numerous near the genu of the facial nerve.

The accessory abducens nucleus (ACCN) is a small column of cells approximately at the same rostro-caudal level of the ABDN, but ventrolateral to it. It is composed of small, and medium-sized cells (range of diameter: 15-30 μm). Most of the cells are larger than ABDN cells, and multipolar shaped with long dendrites.

The injection of fluorescent tracers into the RL muscle resulted in a consistent labeling of cells throughout the rostrocaudal extension of the ipsilateral ABDN, whereas no labeled motoneurons were found in the ACCN.

After injection of fluorescent tracers into the RB muscle almost all the cells of the ipsilateral ACCN were labeled. Also in the ipsilateral ABDN labeled cells were found scattered throughout its rostrocaudal extension, intermingled with RL motoneurons. The labeled cells of the ACCN represented about the 38% of RB motoneurons. No labeled cells were detected in the ON.

DISCUSSION

The general location of extraocular motoneurons in the sheep is similar to that of other mammals. Concerning the ON, its motoneurons form a coalesced mass, without distinct subnuclei or subdivisions, and innervate the ipsilateral RV, OV, and RM muscles and the contralateral RD and LP muscles. Nevertheless, few OV labeled cells can be found in the contralateral nucleus, whereas a small proportion of RD and LP motoneurons are located in the ipsilateral one, analogously to what observed in the rat (10), guinea pig (7), rabbit (12), and cat (11).

In the ON of the sheep the EOM motoneurons have a location similar to that of the cat (11) and rabbit (12). In fact, RV motoneurons lie ventrally in the rostral two-thirds of the nucleus, whereas OV motoneurons lie mainly dorsally. The RM motoneurons tend to occupy the medial portion of the ON in its rostral two-thirds, and approximately the same position, though more caudally, is occupied by RD motoneurons. However, although the motoneurons innervating a given EOM tend to be clustered together, no neat boundaries can be traced defining the territories of the various muscles. In fact, the cell groups intermingle with each other. An overlapping of motoneuron clusters had been observed also in

the ON of other mammals, such as the baboon (1), macaque monkey (4), marmoset (5), cat (11), and rat (10). In the case of the sheep the degree and combination of the intermingling vary along the rostrocaudal extension of the ON in relation to the extension and location of the cellular pools within the nucleus. In the rostral third of the ON, in which the OV, RM, and RV motoneurons are represented, a considerable merging occurs in the dorsal area between OV and RM motoneurons. The RV motoneurons tend to be clustered in the ventrolateral side of the nucleus, but also intermingle with RM motoneurons. In the middle third of the ON all the muscles innervated by the oculomotor nerve are represented and the highest proportion of labeled cells was found for each of them. The OV and RV motoneurons tend to be clustered respectively in a dorsomedial and in a ventrolateral position. At this level, RD and LP motoneurons show an extensive intermingling between each other, with the OV motoneurons located dorsally and the RM motoneurons ventrally. In the caudal third of the ON, the motoneurons supplying the OV, RM, and RV muscles gradually disappear, whereas RD and LP cells, which are present in large number, occur as far as the trochlear nucleus and are extensively intermingled.

The superposition among the motoneuron pools of the ON represents the main characteristic of this nucleus in the sheep. Labandeira *et al.* (10), which observed an analogous situation in the rat, interpret this finding as the consequence of the scarce development of the extraocular musculature in the species they studied. This interpretation is not acceptable, however, in the case of the sheep, which has EOMs of strong consistency and thickness. More interesting appears to be the suggestion by Büttner-Ennever and Akert (4), which correlates the coexistence of RV motoneurons within the RM subgroup with their coactivation in many convergent movements. An analogous interpretation could be given also for the overlapping of RD with RM motoneurons. In the case of intermingling between OV and RD neurons, the explanation could be searched in the fact that when the eye is in some intermediate position the OV muscle collaborates with the RD muscle, the oblique muscles compensating with some abduction the adductor tendency of the recti (3).

According to the literature, the existence of EOM motoneurons among the fibers of the MLF has been observed, and present some differences in the various species. Precisely, the MLF contains RM motoneurons in primates and rabbit; RM, RV, and OV motoneurons in the cat; RD and LP motoneurons in the guinea pig; and RM, RD, RV, and LP motoneurons in the rat (see 6, 7, 10, 11). In our study, we have shown that in the sheep RM, RV and LP motoneurons occupy this location. Quite reasonable appears the hypothesis suggested by Miyazaki (11), who observed EOM cells with such an intimate relationship with the MLF in the cat. These neurons could have been trapped in the MLF during the development of the oculomotor nuclear complex.

Concerning the TN, we have shown that in the sheep, likewise in the rabbit (9), guinea pig (7), and rat (10), the rostral portion of this nucleus overlaps with the caudal pole of the ON, so that some RD and LP motoneurons are still observed

at the same levels where OD motoneurons start to be recognized. The OD muscle of the sheep, like that of all the other mammals studied (see 6), is innervated mainly by the contralateral TN, and only very few OD motoneurons are found in the ipsilateral TN.

Moreover, analogously to all mammals studied so far (see 6), the ABDN of the sheep innervates the ipsilateral RL muscle, whereas the RB muscle is innervated by both the ABDN and ACCN.

In conclusion, the fluorescent tracers, which have been considered more efficient than horseradish peroxidase to identify the motoneurons innervating EOMs (10), have enabled us to demonstrate in the sheep the intermingling between the motoneuronal pools innervating EOMs. Similar results were also obtained in the literature by using the same tracers in the rat (10).

SUMMARY

Retrograde transport of the fluorescent tracers Fast blue, Evans blue, Diamidino yellow dihydrochloride, and Propidium iodide was used to determine the location of the motoneurons innervating the extraocular muscles of the sheep. An extensive superposition among the motor pools of the oculomotor nucleus (ON) has been observed. In the rostral third of the ON, a considerable merging occurs between obliquus ventralis and rectus medialis motoneurons and also between rectus ventralis and rectus medialis motoneurons. In the middle third of the ON, rectus dorsalis and levator palpebrae superioris motoneurons are intermingled with each other, and also with obliquus ventralis motoneurons dorsally and with rectus medialis motoneurons ventrally. The rostral portion of the trochlear nucleus overlaps with the caudal pole of the ON. The motoneurons innervating the obliquus dorsalis muscle are mainly contralateral with few ipsilateral exceptions. The retractor bulbi muscle receive the innervation by both the abducens and accessory abducens nuclei.

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