Afferent Projections from Motoneurons Innervating Extraocular Muscles to the Cerebellum Demonstrated by the Retrograde Double-Labeling Technique

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The objective of this study was to investigate the characteristics and distributions of neuronal origin of cerebellar afferents from motor cranial nerve nuclei innervating extraocular muscles by the method of retrograde transport of two fluorescence tracers in rats. Under deep anesthesia and aseptic conditions, 5 l of 3% solution of Fluoro-Gold (FG) in phosphate buffer solution (PBS) was injected into the bellies of the six extraocular muscles to study the labeling of motoneurons innervating corresponding extraocular muscles. The cerebellum was exposed by craniotomy, and 0.3 l of 10% solution of Dextran Tetramethyl Rhodamine Biotin (Micro Ruby: or MR) in PBS was injected into many regions of the anterior vermis (lobule I, II) and the posterior vermis (lobule VI, VII, IX, X),the flocculus, the paraflocculus and the deep cerebellar nuclei. Multiple injections were made to cover the entire cerebellum in order to obtain a near maximum labeling of cerebellar afferent neurons. In other cases, only small single or a few injections were made in specific areas of the cerebellum to study specific distributions and topographic organization. In one group of rats, injections were made both in the extraocular muscles with FG and in the cerebellum with MR to study the double labeling of neurons, which project their axons to both the extraocular muscle and the cerebellum. Another group of rats were injected in both sites with only PBS and served as the control for autofluorescence background. After 3 days postoperative survival time, all animals were deeply reanesthetized and perfused with heparinized normal saline solution, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, and 30% sucrose solution in PBS. The brainstem and the cerebellum were removed immediately, and stored in sucrose solution in PBS at 4 C. Serial transverse sections of the brainstem and sagittal sections of the cerebellum were obtained by a freezing microtome at 40 m thickness, collected on uncoated glass slides, and immediately dried. All sections were examined under an epifluorescence or confocal microscope equipped with filter systems for FG and MR. The presence of both single and double retrograde labeled neurons in the Oculomotor (CN 3), Trochlear (CN 4) and Abducens (CN 6) nuclei was recorded, photographed, stored as computer images files and printed out as hard copies. The labeling neurons in the vicinity of the CN 3, 4, 6 from all sections were plotted onto diagrams and counted. Neurons labeled only with MR retrogradely transported from injection sites in the cerebellum were found bilaterally and scattered throughout in the Oculomotor, Trochlear and Abducens nuclei. These neurons labeled only with MR were small and medium-sized interneurons and represented only a small proportion of the entire population. Neurons labeled only with FG retrogradely transported from injection sites in the extraocular muscles were the most numerous, and distributed almost throughout the entire population of small, medium-sized and large motoneurons, which innervate the extraocular muscles. A smaller proportion of small and medium-sized FG labeled neurons within these nuclei were also double labeled with MR, indicating that they project their axon collaterals to both extraocular muscles and the cerebellum. In conclusion, the present findings provide clear anatomical evidence that a small population of motoneurons in the Oculomotor, Trochlear and Abducens nuclei of the rat project their axon collaterals directly to the cerebellum and the extraocular muscle, in addition to the cerebellar afferents from other interneurons within these nuclei. The findings also indicate that cerebellar neuronal circuits play more direct roles in monitoring and controlling eye movements than previously known.

Keywords: *Cerebellum, Extraocular muscle, Motoneurons, Double labeling technique*

J Med Assoc Thai 2005; 88 (12): 1905-15
Full text. e-Journal: *http://www.medassocthai.org/journal*

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Cerebellar afferents from the extraocular motor nuclei have been demonstrated by using retrograde transport of horseradish peroxidase (HRP) or HRP-wheat germ agglutinin (HRP-WGA). A direct projection from the oculomotor, trochlear, and abducens nucleus to the flocculus was first described by Kotchabhakdi & Walberg in 1977[1], they also described that the motor nuclei of all cranial nerves were large or medium-sized motoneuron-like cells. Subsequently Blanks et al in 1983[2] demonstrated a substantial number of afferents arise bilaterally from several of cranial motor nuclei including the abducens to the flocculus. Some authors found labeled neurons in the abducens nucleus and in other motor nuclei of cranial nerves after horseradish peroxidase (HRP) or HRP- wheat germ agglutinin (HRP-WGA) injection into the cerebellum[3,4].

Rodella, et al 1995[5] found neurons in the abducens nucleus present bilaterally and localized principally in the dorsomedial area of the cranial half of each nucleus but did not display the typical ultrastructural features of motoneurons, and after that in 1996 they could identify the afferent projections from the rat abducens nucleus to the flocculus be cholinergic neurons by using double labeling of retrograde transport of HRP and choline acetyltransferase (ChAT) immunohistochemistry. Their finding showed the double-labeled neurons bilaterally in the rostral half of abducens nucleus and these abducens motor neurons also used acetylcholine as a neurotransmitter (Rodella, et al 1996)[6].

The other group of researchers investigated on cerebellar afferents from neurons in the extraocular motor nuclei in sheep by using a fluorescent retrograde double-labeling. A fluorescent tracer was injected into the extraocular muscles (EOMs) and the other into bilateral points of the vermal folia II-V, paramedian lobule, or into the vermal folia VI, VIIA, VIIB, or into the fastigial nuclei of cerebellum. They found almost bilaterally labeling neurons in the oculomotor, trochlear, and abducens nuclei and only a few cells were into the cerebellum. But never found double-labeled neurons[7].

Moreover, detailed knowledge of the organisation of the motoneurons innervation the extraocular muscles has been sought by many researchers. Labandeira et al, 1983 used retrograde transport of HRP to locate the motoneurons controlling the extraocular muscles of the rat[8]. Murphy et al, 1986 investigated in the rabbit and they examined neurons belonging to the medial rectus muscle found throughout the length of the ipsilateral oculomotor nucleus, the motoneurons innervating the lateral rectus muscle found in abducens nucleus, and indicated that the majority of the neurons of the trochlear nucleus innervate the contralateral superior oblique muscle[9].

However, previous studies on the cerebellar afferent system indicated that the cerebellum might play a more active monitoring role in the integrated command impulses which are sent down to the motoneuron pool, thus controlling the activities in the axons of the motoneurons[1]. Since the significance of cerebellar afferent from the extraocular motoneurons has not been elucidated experimentally or systematically analysis of the total afferent projecting to cerebellum. In addition, there are a few studies on the anatomical and physiological of the eye movement that is related to the cerebellum, and with regard to the type of neurotransmitters types used in this projection, no data are available in the literature. To verify the possibility that the cerebellum might receive information directly from the motor neurons innervating the extraocular muscles, the present study will undertake a fluorescent double-labeling study on the rat.

Material and Method

Experiments were performed on a total of 32 adult male Wistar rats, weighing 250-350 g. They were deeply anaesthetised with sodium pentobarbital (nembutal) 60 mg/kg. Craniotomies were made with a 1 mm diameter dental burr. The dura over the brain was carefully removed with a pair of fine forceps under an operating microscope.

Group I: 10 animals were injected with 0.3 ml (10% MR in 0.1M PBS) in the anterior vermis (lobule I, II), posterior vermis (lobule VI, VII, IX, X), paraflocculus, flocculus, and deep cerebellar nuclei.

Group II: 10 animals were injected with 5 ml (3% FG in 0.9% normal saline) in the belly of the superior oblique muscle, the medial rectus muscle and the lateral rectus muscle.

Group III: 10 animals were injected with 0.3 ml (10% MR in 0.1 M PBS) into the cerebellum and 5 ml (3% FG in 0.9% normal saline) into the bellies of the superior oblique muscle, the medial rectus muscle and the lateral rectus muscle.

Group IV: The injections were made with 0.3 l normal saline into the anterior vermis (lobule I, II), posterior vermis (lobule VI, VII, IX, X), paraflocculus, flocculus, and deep cerebellar nuclei.

Group V: The injections were made with 5 l normal saline into the belly of the extraocular muscle (the superior oblique, the medial rectus and the lateral rectus).

Following injection of the tracer, the exposure was closed with silk sutures. After an adequate survival time for transport of the fluorescent tracers, the animals were anaesthetized again, heparinized and perfused over a period of 30 min, followed consecutively by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brainstem and the cerebellum were removed from the skull and post fixed for 3 hrs. in fixative, and subsequently rinsed and stored in 30% sucrose solution at 4 C until the tissue had sunk (24-48 hrs). Transverse serial section with freezing microtome into 40 mm thick and examined under the epifluorescence microscope equipped with a Leitz Ploemopack filter system providing excitation wavelength 360-370 nm, emission 395-490 nm, appropriate filter for FG and wavelength 515-560 nm, and an emission 610 nm appropriate filter for MR.

The presence of both single or double-retrograde label neurons in the Oculomotor, Trochlear, and Abducens nuclei were photographed, store and printed out as computer image files. The distribution of single and double-retrograde labeled neurons were mapped on standard diagrams of the rat brainstem motor nuclei.

Results

A total of 32 animals in different groups had successful injections of both tracers, Dexran Tetramethyl Rhodamine Biotin (Micro-Ruby or MR) in the cerebellum; Fluoro-Gold (FG) in the extraocular muscles.

A. The injection sites

Cerebellum

In all cases they encompassed the vermal and paravermal regions bilaterally. The injection site showing a central, intensely labeled core surrounded by a more weakly stained area (Fig. 1).

These injections were generally massive and mostly involved the cerebellar cortex subjacent white matter, and often also, the fastigial and interposed nuclei. The injection sites in all animals differed between cases, but each was confined to the margins of the cerebellum. For example, the injection sites of MR extended to the flocculus. According to Larsell (1970)[10], the cerebellar flocculus of the rat consists of a single folium situated ventromedial to the ventral paraflocculus (PFL_v). In order to inject this structure, microsyringe filled with MR were inserted through the PFL_v. The injection sites of MR extended to the large vermis, a microsyringe filled with MR was inserted through large sites of the cerebellum.

Fig. 1 Drawing of sagittal section of the cerebellum, showing the injection sites (black areas) in different lobules of the cerebellar cotrtex and cerebellar nuclei

Extraocular muscles

The fluorescent tracer injections in the extraocular muscles, are in the superior oblique, the medial rectus and the lateral rectus muscle.

B. Retrograde labeling with cerebellum and the extraocular muscles injection

All successful cerebellum and the extraocular muscles injections produced similar labeling patterns and differed only in terms of the number and size of labeled neurons and the degree to which each neuron was labeled.

Single-labeling of the cerebellar injections

Retrogradely labeled cells were found in all 20 cases. The presence of such widespread labeling served as a general indicator of the effectiveness of uptake and retrograde transport of the tracer from terminals and axons in a large area of the cerebellum. Neurons labeled only with MR retrogradely transported from the injection sites in the cerebellum were found bilaterally in rostral half regions and scattered throughout in the oculomotor, trochlear and abducen nucleus, as well as other previously known brainstem nuclei. Those neurons labeled only with MR appeared to be different

in size. Some were large but most were small and medium sized with a diameter between 20-60 mm. These neurons could potentially represent a motoneurons and represented only a small sub-population of the oculomotor, trochlear and abducen neurons (Fig. 2).

Single-labeling of the extraocular muscles injection

Retrogradely labeled neurons were found in all 5 cases. Neurons labeled only with FG retrogradely transported from the injection sites in the extraocular muscles (superior oblique, medial rectus and lateral rectus) were numerous and appeared to be medium-sized and large motoneurons, and were distributed bilaterally among almost the entire population of corresponding motoneurons which innervate the extraocular muscles (Fig 3).

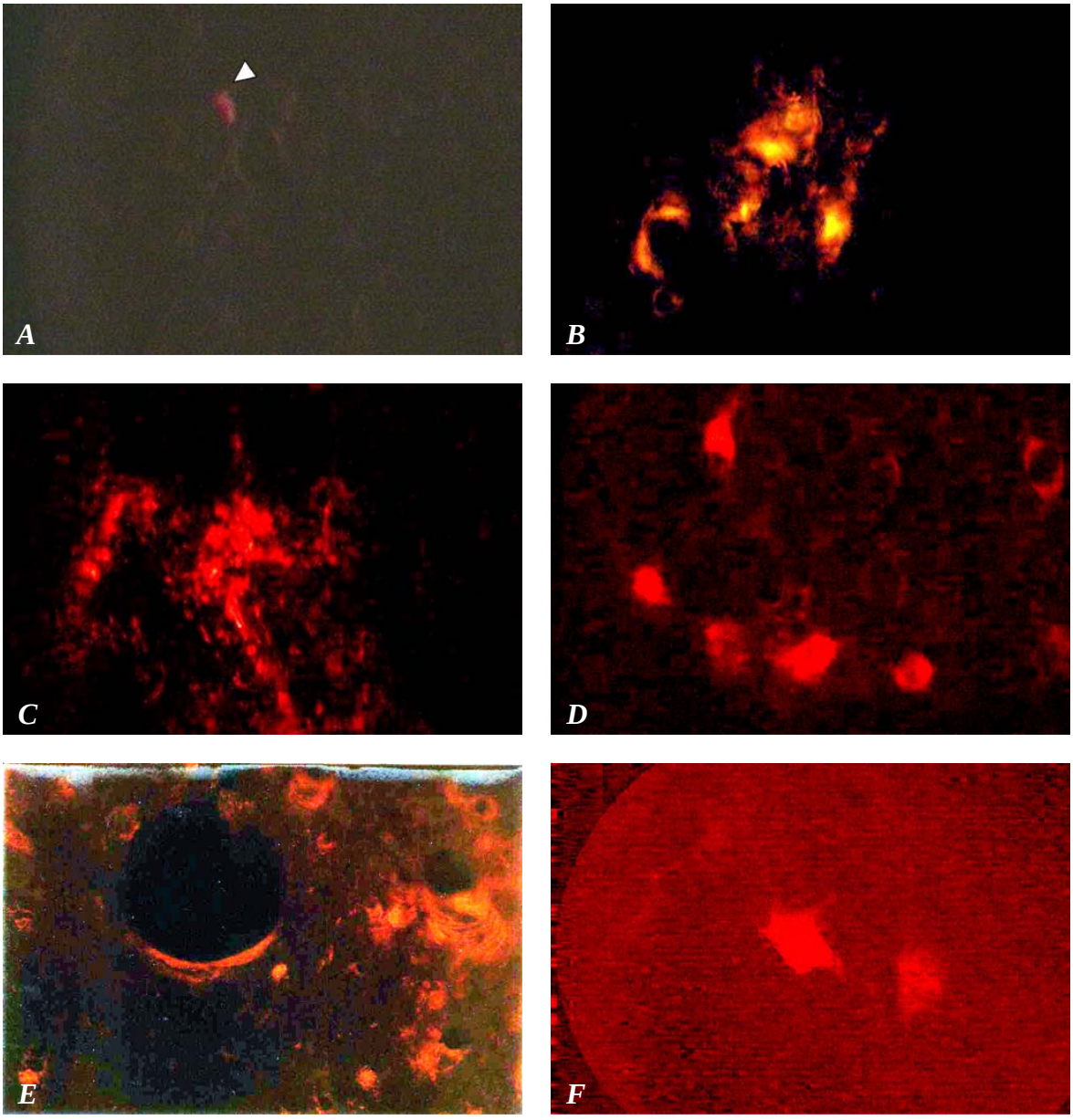

Fig. 2 A fluorescence photograph of labeled neurons in the Oculomotor nucleus CN III (A, B), the trochlear nucleus CN IV (C, D), and the abducens nucleus CN VI (E, F) after injection of MR into the cerebellum

C. Double-Labeling studies

All 5 cases had successful double labeling injection. A much smaller proportion of FG- labeled neurons within the oculomotor, trochlear and abducen nucleus were also double-labeled with MR, indicating that they projected their axon collaterals to both the extraocular muscles and the cerebellum (Fig. 4, 5 and 6).

Topographic arrangement

The general topographic findings were covers made in the single labeled injection by the results of the cerebellar injections. Detailed description of the FG injection sites and the findings in each positive case are summarized in Table 1.

In MCB 40, after bilateral injections of FG in the large vermis found a lot of labeled neurons in the

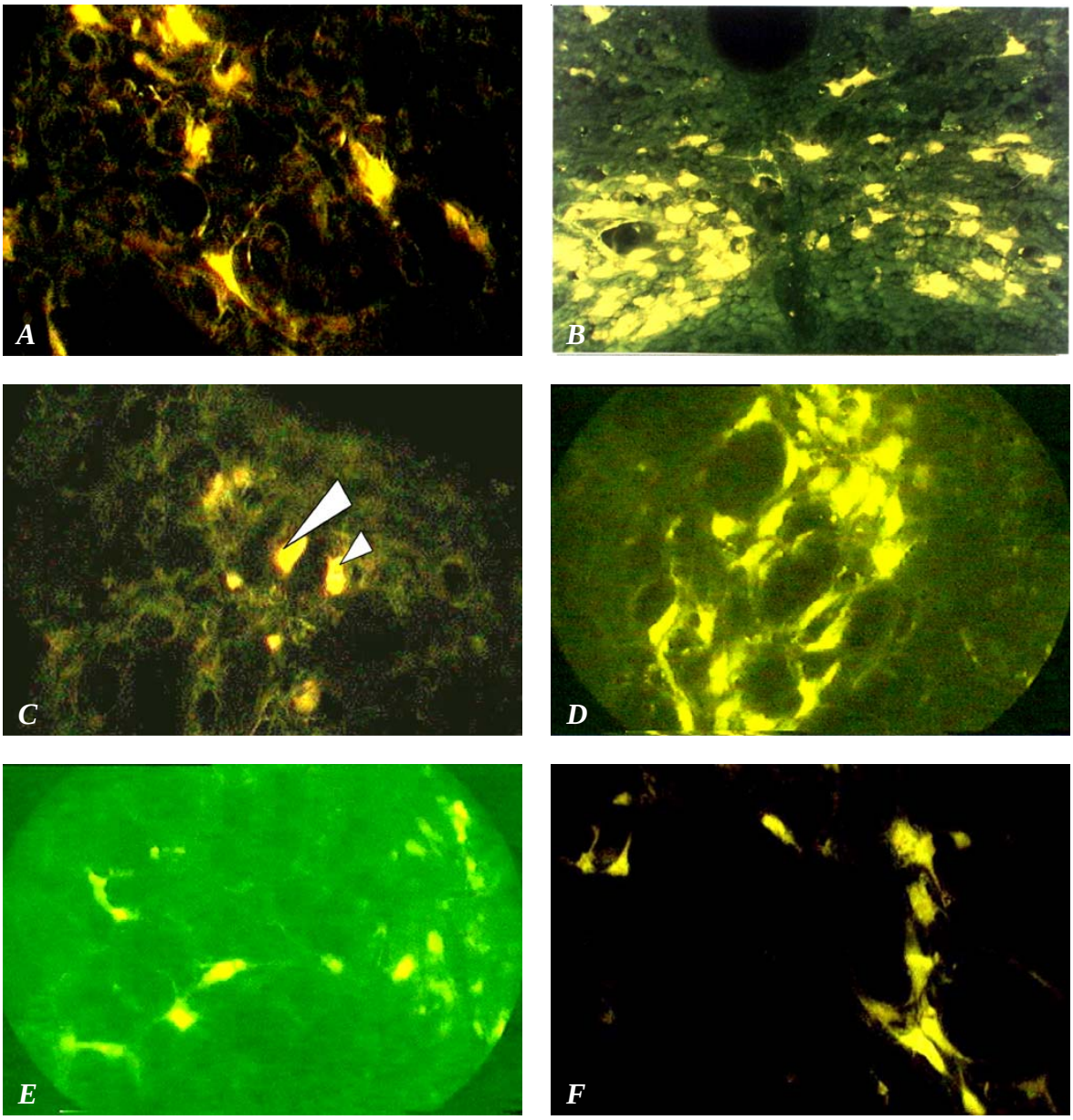

Fig. 3 A fluorescence photomicrograph of labeled neurons in the oculomotor nucleus (CN III) after injection of FG into the medial rectus muscle (A, B),in the trochlear nucleus (CN IV) after injection of FG into the superior oblique muscle (C, D), and the abducens nucleus (CN VI) after injection of FG into the lateral rectus muscle (E, F)

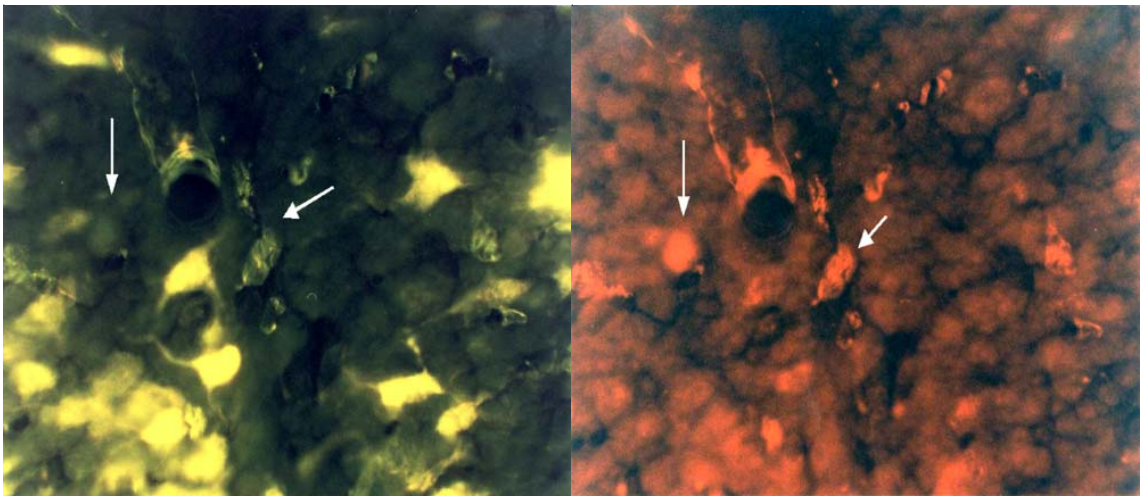

Fig. 4 A photograph of the double retrogradely labeled cell within the oculomotor motoneurons

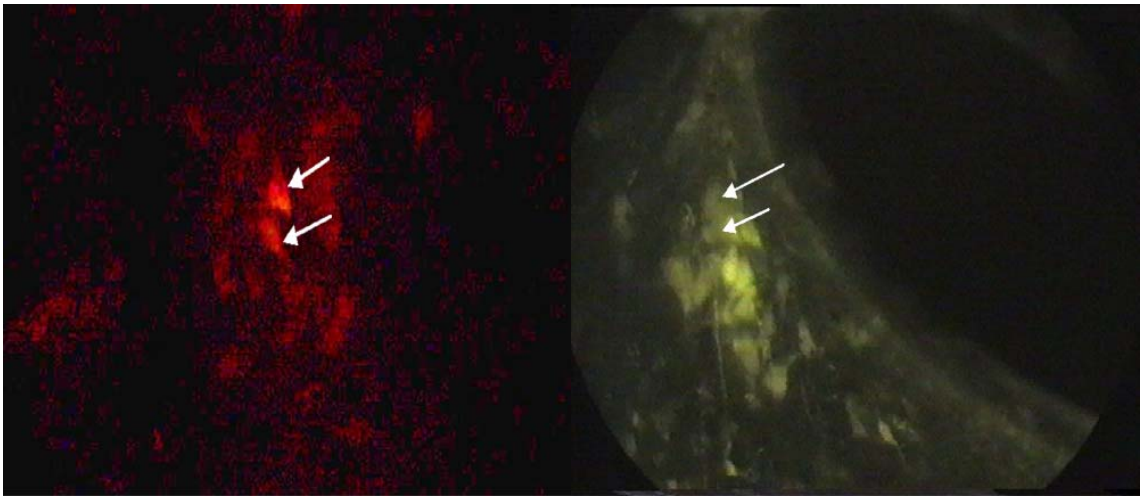

Fig. 5 A photograph of the double retrogradely labeled cell within the trochlear motoneurons

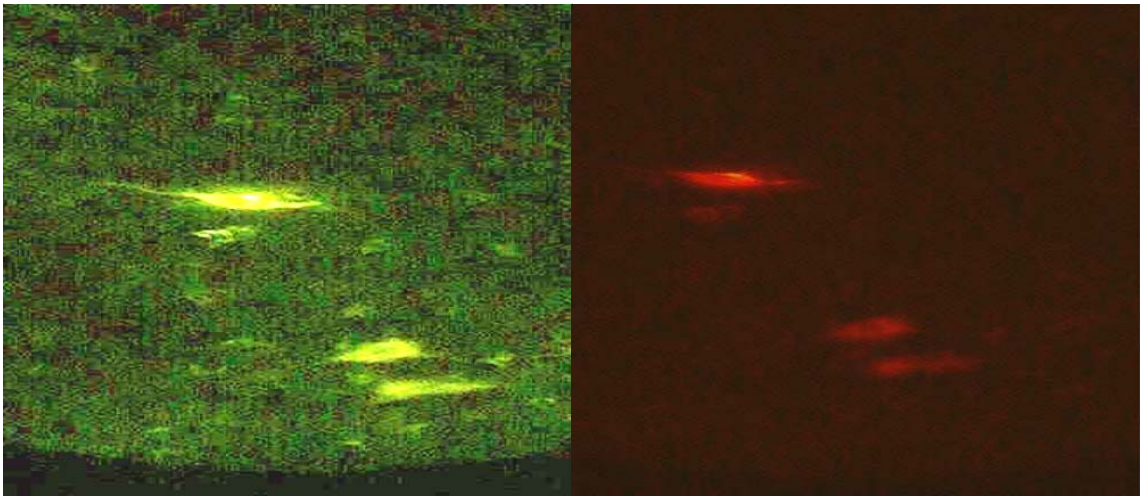

Fig. 6 A photograph of the double retrogradely labeled cell within the abducens motoneurons

Table 1. Summary of the findings from positive cases in which labeled cells were observed in motor nuclei of the oculomotor nucleus, the trochlear nucleus, and the abducens nucleus
(The number indicated in the table represents the total number of labeled cells in each nucleus counted from every section.Abbreviation : L=left, R=right)

Case	Injection site	nucleus of CN VI		nucleus of CN IV		nucleus of CN III	
		L	R	L	R	L	R
MCB 40	large vermis	228	122	99	75	215	230
MCB 43	flocculus & Ant.lobe I-II	24	12	16	20	8	10
MCB 45	deep cer.nuclei	12	8	-	-	-	-
MCB 50	flocculus	20	16	8	32	24	12

abducens nucleus in the left site found a total of 228 neurons and in the right site found 122 neurons, in the trochlear nucleus found 99 labeled neurons in the left site and 75 labeled neurons in the right site, and in the oculomotor nucleus found labeled neurons in the left site 215 neurons and in the right site found 230 neurons. The present study injected the FG in several areas of the cerebellum to reconfirm the efficiency of the tracers and the site of labeled neurons in the cranial nerve motor nuclei. The other cases, like MCB 43 injected in the anterior lobule I and II and the flocculus found the labeled neurons in the abducens nucleus in the left site 24 neurons and in the right site 12 neurons, labeled neurons in the trochlear nucleus found in the left site 16 neurons and in the right site 20 neurons, and in the oculomotor nucleus found in the left site 8 neurons and in the right site 10 neurons.

From injections, the labeled neurons were found throughout the nucleus of oculomotor, trochlear, and abducens. The number of retrograde labeled neurons overall seemed to be greater with injections that also encompassed the cerebellar nuclei, particularly for the caudal in most parts of the abducens nucleus.

The quantitative observation were extended in 2 cases (MCB 39, MCB 40) by making cell counts of the nuclei of labeled cells in 40 mm sample sections at different rostrocaudal levels throughout the oculomotor, the trochlear and the abducens nucleus. The plots of the average total number of labeled neurons in cerebellar injections and the extraocular muscles injection are shown in Table 2.

In the cerebellar injection the labeled neurons were more concentrated in the abducens nucleus. In the extraocular muscles injection the average total number of neurons in all cases after injection in the seperated muscle were found as in Table 3.

Table 2. The average total number of labeled neurons in the CN III, CN IV, and CN VI after cerebellar injections and the extraocular muscles injection both in the right site

Cranial nerve motor nuclei	Total number of labeled neurons	
	contralateral	ipsilateral
oculomotor nucleus	2	1
trochlear nucleus	3	2
abducens nucleus	3	3

Table 3. The average total number of labeled neurons in the CN III, CN IV, and CN VI after injection in the extraocular muscles

The muscle injections	Total of labeled neurons		
Medial rectus	CN III	left 83	right 144
Superior oblique	CN IV	left 218	right 263
Lateral rectus	CN VI	left 315	right 351

Discussion

In the present study, the afferent projections to the cerebellar from motoneurons innervating the extraocular muscles of the rats were studied using double retrograde axonal transport of Fluoro-Gold and Micro-Ruby. The projections to the cerebellum are considered to originate from cranial nerve motor nuclei [1, 2, 5-7]. For the oculomotor, the trochlear and the the abducens nucleus, Kotchabhakdi and Walberg[1] reported that large labeled cells were found in the nucleus of abducens nerve and also found in the trochlear and oculomotor nuclei following injection in the nodulus and the flocculus. In some mammalian species, cerebellar

afferents from the extraocular motor nuclei have been demonstrated by using the other fluorescent tracers; fast blue and diamidino yellow dihydrochloride, the labeling produced by cerebellar injections in the sheep was distributed bilaterally within all the extraocular motor nuclei[7].

This present study confirms the above studied and provides clear evidence that cerebellar afferents from the cranial nerve motor (CN III, IV and VI) in the rat originate from both small motoneurons and interneurons and also indicated that two tracers could be transported retrogradely and accumulate be visualized in the same cell.

Therefore, the present results confirm the existence of cerebellar projections from the oculomotor, the trochlear, and the abducens nuclei in rat and demonstrated the small population of motoneurons in the oculomotor, trochlear and abducens nucleus of the rat project their axon collaterals directly to the cerebellum and the extraocular muscles, in addition to the cerebellar afferent from other interneurons within these nuclei (Fig. 7).

As expected, many brainstem nuclei that are known to project to the injected sites of the cerebellum[11-17] were labeled in this experiment, giving evidence of the efficiency of the tracer in filtration within the cerebellum.

Along the length of the mesencephalon at different levels, firstly the present study has considered the cerebellar projecting neurons located within the somatic divisions of the nucleus. In the rats, oculomotor neurons labeled by injections into the cerebellum are scattered among motor neurons and are located mostly in the rostral half aspect of the nucleus, where it is indented by the medial longitudinal fasciculus fibers. In the sheep, this area contains the motor neurons innervating the inferior rectus muscle, which in the caudal pole of the nucleus are intermingled with the neurons supplying the media rectus muscle[18].

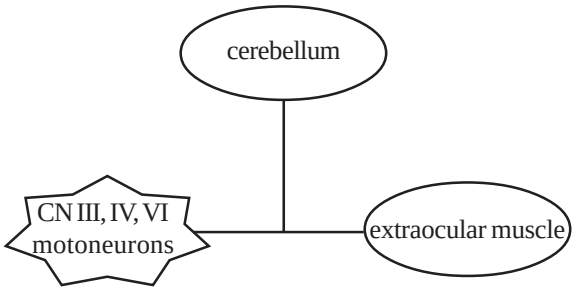

Fig. 7 Drawing of the diagram showing the cerebellar afferent projection from the cranial nerve III, IV and VI motoneurons

At every rostrocaudal level of the trochlear nucleus, the neurons projecting to the cerebellum occupy the region facing the medial longitudinal fasciculus fibers. On the other hand, the abducens nuclei are mainly distributed bilaterally within the dorsal and dorsomedial portions of the abducens nucleus that lies lateral to the genu of the facial nerve. This findings appears consistent with the observation of Blank et al, 1983[2]. These authors labeled abducens motoneurons were found after large injection sites within the flocculus.

In the oculomotor, trochlear, and abducens nuclei neurons projecting to the cerebellum are smaller than the neurons that innervate extraocular muscles. After seperately injecting the vermal folia, or the deep cerebellar nuclei, no consistent differences in number or location of labeled neurons were detected within the extraocular motor nuclei. This result leads to consider the nuclear afferents as collaterals of vermal mossy fibers; infact,for the climbing fibers, the inferior olive is the only source[19,20]; moreover the fastigeal afferents arise mostlly as collaterals of mossy fibers [16]. Moreover, oculomotor, trochlear and abducens neurons project to the nucleus prepositus hypoglossi[21,22].

Secondly, the present study considered with the extraocular injection;

Medial rectus muscle

Neurons belonging to the medial rectus muscle were found throughout the length of the bilateral oculomotor nucleus, but 90% of them were concentrated in the rostral two thirds. The most rostral cells appeared to be somewhat haphazardly distributed, some being located ventrolaterally. In the caudal half of the nucleus, the neurons tended to lie in the lateroventral region, though medially situated cells were also found. Some of the neurons lay among the fibers of the medial longitudinal fasciculus the greatest concentration showing in the central third of the nucleus.

Superior oblique muscle

The nucleus of the trochlear nerve occupied a hollow of the medial longitudinal fasciculus. It usually overlap about 40-80 mm of the caudal end of the oculomotor nucleus. The trochlear neurons lie lateroventrally with respect to those of the oculomotor nucleus. In all vertebrates studied, approximately 96-98% of the motoneurons in the trochlear nucleus innervate the contralateral superior oblique muscle.

The remaining motoneurons project to the ipsilateral superior oblique muscle[23-28]. The sole exception to this pattern, trochlear motoneurons in cats, where (1 to 12) neurons in the trochlear nucleus innervate the ipsilateral tensor typani muscle [29].

However, in the cat, labeled neurons were sporadically located more caudally along the medial longitudinal fascicle as tailing away from the trochlear nucleus[24]. This findings also indicates that labeled neurons were found bilaterally and did not have much difference in numbers of labeled neurons between both sides.

Lateral rectus muscle

The motoneurons innervating the lateral rectus muscle were spread over some 680-720 mm rostrocaudally. The nucleus of the abducens was located, in the rats, between the genu of the facial nerve and the medial longitudinal fasciculus. Its transverse section presented the general shape of a triangle pointing in the dorsal direction.

The results of this finding concerning the location of the innervating motoneurons may be seen to differ markedly from those of Glickman, 1980[30] with respect to both their rostrocaudal and transverse distribution. In the abducens nucleus, most of the motoneurons innervating the lateral rectus muscle appear in this experiment in the rostral two thirds as the same location as has been reported by Labandeira et al, 1983[8] in the rat and by Gacek;1972[31] in the cat and those which lie in the caudal third of the nucleus were found exclusively in lateral positions, ventrolateral to the genu of the facial nerve.

In the oculomotor and trochlear nuclei of the cat, it has been demonstrated that the internuclear neurons receive collaterals from motor neurons[23,32-35]. In the abducens nucleus of the cat, nearly half of the cells are internuclear neurons projecting to the medial rectus motor neurons, and almost the total number of abducens neurons is accounted for by the sum of motor neurons and internuclear neurons[36].

Undoubtedly, the diversity in the projections of the internuclear neurons suggests the existence of several populations of these neurons within the extraocular motor nuclei. Presently, the authors can state that the cells projecting to the cerebellum are internuclear neurons sending collaterals to the cerebellum. They are neurons with exclusively intracranial projections that establish connections between the extraocular motor nuclei and the cerebellar areas involved in eye movements.

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เซลล์ประสาทที่เป็นต้นกำเนิดของเส้นประสาทเข้าสู่สมองส่วนซีรีเบลลั่มจากมอเตอร์นิวรอนที่ควบคุมกล้ามเนื้อนัยน์ตา

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การวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาลักษณะและการกระจายของเซลล์ประสาทที่เป็นต้นกำเนิดของเส้นประสาทจากกลุ่มมอเตอร์นิวรอนที่ส่งไปยังกล้ามเนื้อนัยน์ตาโดยอาศัยการลำเลียงแบบย้อนกลับในเส้นประสาทของสารเรืองแสงสองชนิดในหนู หลังจากทำให้หนูทดลองสลบ หนูกลุ่มหนึ่งฉีดสารละลาย 10% ของ Micro-Ruby (MR) ในฟอสเฟทบัฟเฟอร์ (PBS) ปริมาณ 0.3 ไมโครลิตรที่เวอร์มิสส่วนหน้า (lobule I, II), ส่วนหลัง (lobule VI, VII, IX, X), ฟลอคคูลัส, พาราฟลอคคูลัส และที่นิวเคลียสของซีรีเบลลั่ม การฉีดครอบคลุมพื้นที่หลายส่วนของซีรีเบลลั่มเพื่อให้ได้จำนวนเซลล์ประสาทที่ติดฉลากเป็นจำนวนมากที่สุด หนูในกลุ่มนี้บางตัวฉีดบริเวณเฉพาะบางส่วนของซีรีเบลลั่มเพื่อศึกษาการกระจายและลักษณะของเซลล์ประสาท หนูทดลองอีกกลุ่มหนึ่งทำการฉีดสารเรืองแสงสองชนิดคือ MR ที่ซีรีเบลลั่ม และฉีดสารละลาย 3% Fluoro-Gold (FG) ใน PBS ปริมาณ 5 ไมโครลิตรเข้าในแต่ละมัดกล้ามเนื้อนัยน์ตาซึ่งเลี้ยงโดยเส้นประสาทสมองคู่ที่ 3, 4, 6 แล้วติดตามการติดฉลากของสารเรืองแสงทั้งสองชนิดในซัยโตพลาสมของมอเตอร์นิวรอนในบริเวณกลุ่มเซลล์ประสาทออคูโลมอเตอร์ (CN 3) โทรเคลีย (CN 4) แอบดูเซนส์ (CN 6) หนูทดลองอีกกลุ่มหนึ่งฉีดสารละลาย PBS เพื่อใช้ในการตรวจสอบ background ของสารเรืองแสง ต่อมาอีก 3 วันหลังทำให้สัตว์ทดลองสลบอีกครั้งจึงทำการเก็บรักษาสมองโดยวิธีการ perfusion ด้วย 4% พาราฟอร์มาลดีไฮด์ แล้วนำเนื้อสมองส่วนซีรีเบลลั่มมาตัดเรียงตามแนวยาวและก้านสมองตัดเรียงตามขวางด้วยความหนา 40 ไมโครเมตร ก่อนส่องดูเซลล์ประสาทที่ติดฉลากด้วยกล้องจุลทัศน์ชนิดฟลูออเรสเซนด้วยแผ่นกรองแสงสำหรับ FG และ MR เก็บข้อมูลด้วยภาพถ่ายและเก็บไว้ในแฟ้มข้อมูลภาพคอมพิวเตอร์ เซลล์ประสาททั้งสามกลุ่มถูกนับจำนวนและวาดแบบพื้นที่สมองไว้ทุกชิ้นสไลด์ ผลการทดลองพบว่าเซลล์ประสาททั้งสามกลุ่ม CN 3, CN 4, CN 6 ทั้งสองข้างของก้านสมองติดสีแดงด้วย MR ที่ลำเลียงย้อนกลับมาจากซีรีเบลลั่มแต่อย่างเดียวเป็นอินเตอร์นิวรอนมีจำนวนไม่มากนักมีทั้งเซลล์ขนาดเล็กและกลางกระจายอยู่ระหว่างมอเตอร์นิวรอนขนาดกลางและใหญ่ที่ติดสีเหลืองทองของ FG ที่ลำเลียงย้อนกลับมาจากกล้ามเนื้อนัยน์ตา มีเซลล์ประสาทจำนวนหนึ่งที่มีทั้งขนาดเล็กและใหญ่อยู่ในตอนกลางของกลุ่มเซลล์ประสาทเหล่านี้ที่ติดทั้งสองสีในเซลล์ตัวเดียวกัน แสดงว่ามีมอเตอร์นิวรอนที่ส่งแขนงของเส้นประสาท (axon collaterals) ไปสู่ทั้งซีรีเบลลั่มและกล้ามเนื้อนัยน์ตา สรุปได้ว่า ต้นกำเนิดของเซลล์ประสาทจาก CN 3, CN 4, CN 6 ที่ส่งเส้นประสาทไปสู่ซีรีเบลลั่มมาจากทั้งกลุ่มมอเตอร์นิวรอนและอินเตอร์นิวรอน ที่มีบทบาทสำคัญในการควบคุมประสานงานการเคลื่อนไหวของนัยน์ตา ดังนั้น สมองส่วนซีรีเบลลั่มจึงสามารถควบคุมติดตามการเคลื่อนไหวของนัยน์ตาได้โดยตรงและรวดเร็วกว่าวงจรประสาทที่ทราบกันมาแต่ก่อนหน้านี้