ULTRASTRUCTURE OF PROJECTIONS TO THE OCULOMOTOR NUCLEUS AND INFERIOR OLIVE FROM VESTIBULAR AND CEREBELLAR NEURONS INVOLVED IN COMPENSATORY EYE MOVEMENTS

FOR THOSE WHO BELIEVE

" EL 'AANIGOO 'AHOOT' E "

(The truth is out there)

Dit proefschrift is tot stand gekomen binnen de afdeling Anatomie van de Erasmus Universiteit Rotterdam.

Aan de drukkosten werd bijgedragen door GABA B.V. te Almere

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ULTRASTRUCTURE OF PROJECTIONS TO THE OCULOMOTOR NUCLEUS AND INFERIOR OLIVE FROM VESTIBULAR AND CEREBELLAR NEURONS INVOLVED IN COMPENSATORY EYE MOVEMENTS

ULTRASTRUCTUUR VAN PROJECTIES NAAR DE OCULOMOTOR NUCLEUS EN DE OLIVA INFERIOR VANUIT VESTIBULAIRE EN CEREBELLAIRE NEURONEN DIE BETROKKEN ZIJN BIJ COMPENSATOIRE OOGBEWEGINGEN

PROEFSCHRIFT

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COPROMOTOR:	Dr. C.I. De Zeeuw

Deny everything !

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How does the brain work?
Altough notable progress has been made, the question
remains one of the profoundest confronting
modern science.

(D.H. Hubel in: The Brain, A Scientific American Book,)

Chapter I. General Introduction

a. Explanation of thesis subject
b. Organization and afferents of the OMN
c. Organization and afferents of the inferior olive
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Ia. Explanation of thesis subject

Early in the evolution of vertebrates eye movements were strictly primitive reflexes that were predominantly controlled by vestibular and visual sensory stimuli. Later during phylogeny, along with the development of the fovea of the retina, vertebrates acquired the ability to make voluntary eye movements (Büttner and Büttner-Ennever, 1988). In short, five types of eye movements can be distinguished. The first two are slow eye movements that compensate for movements of the head and the visual surround; these are named the vestibulo-ocular reflex (VOR) and the optokinetic reflex (OKR), respectively. Extended rotatory or visual stimulation results in a so-called vestibular or optokinetic nystagmus with a slow (compensatory) and a fast (reset) phase. The eye movements that operate predominantly under voluntary control are saccades, smooth pursuit, and convergence. Saccadic eye movements are fast conjugate eye movements which reset the eye position; smooth pursuit movements are used to follow a small moving visual target; and convergence movements are slow disconjugate eye movements enabling frontal-eyed animals to foveate near objects and establish stereoscopic vision.

The different types of eye movements are to a large extent mediated by separate anatomical pathways. The present study explores the neuronal circuitries underlying the VOR and OKR, which are particularly advantageous for investigating the operations of the brain at a systems level. The value of these circuitries as a model for sensorimotor transformation can be appreciated in terms of a problem articulated by Marr (1982). When analyzing the function of an information processing machine, one must achieve three levels of understanding. At the top level, one must determine the problem the machine solves, building a "computational theory" of the machine. At the intermediate level, one must specify how the machine encodes its inputs and outputs, and the algorithm the machine uses to transform one to the other. On the lowest level, which is the level of the present neuroanatomical study, one must determine how the algorithm is actually implemented in the hardware. Unlike investigations of many other brain functions, one of the major advantages of studying compensatory eye movements lies in our ability to answer the highest order question. The computational task of compensatory eye movements is to stabilize the eyes with respect to the surround so that movements of the head and body (VOR) or the environment (OKR) do not result in motion of the visual world across the retina (Figure 1). The brain's strategy is to measure the angular motion of the head around each of three linearly independent axes, to combine this information with a measurement of image velocity from the retina, and finally to excite the appropriate combination of extraocular muscles, counterrotating the eyes so as to minimize the retinal image velocity.

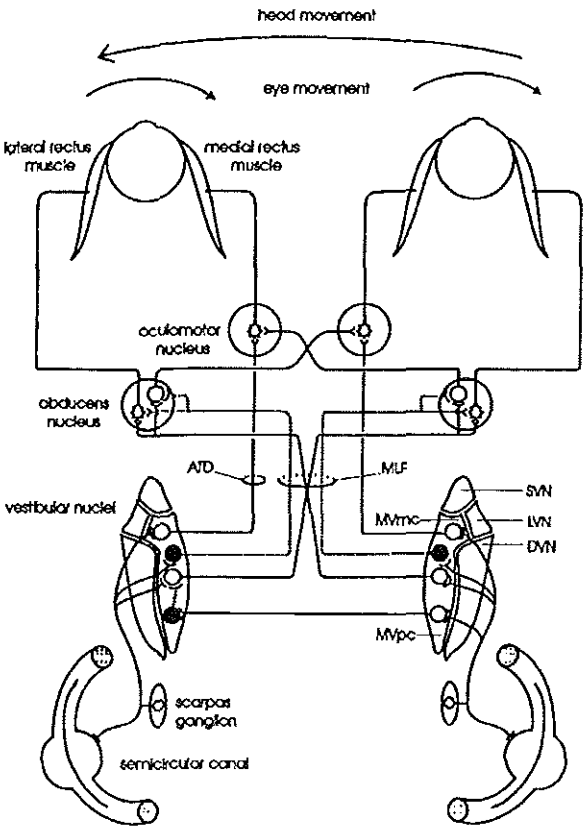

Figure 1. Horizontal VOR pathway. The major pathway that mediates the VOR is the three neuron arc of Lorente de Nó. This open loop is formed by the vestibular ganglion cells that receive input from the vestibular apparatus, the second order vestibular neurons which are innervated by the primary afferents from these ganglion neurons, and the oculomotor neurons that innervate the oculomotor muscles. For abbreviations, see list of abbreviations.

The inputs and outputs of the sensorimotor transformation during the VOR and OKR are relatively simple. A major input to the system comes from the vestibular labyrinth, a sense organ whose function, to a first approximation, is determined by the operations of elementary physics. The major input for the OKR comes from the visual system. The processing of the visual information is somewhat more complicated than that of vestibular information, since the registration of rotation of the visual world is a circuit property of the accessory optic system using information from movement detectors in the retina (AOS; Figure 2). Nevertheless, the visual processing is organized about a related, simplifying principle: the preferred axes of visual rotation correspond to the best response axes of the three semicircular canals in the vestibular labyrinth (Simpson et al., 1988). The output, i.e. motor, component of the eye movement

reflexes is also attractive in its simplicity. It is composed of a ball-in-socket joint, and the net torque upon the ball is determined by the action of only three muscle pairs, each of which rotate the eye about an axis that also corresponds to the best response axis of one of the three semicircular canals.

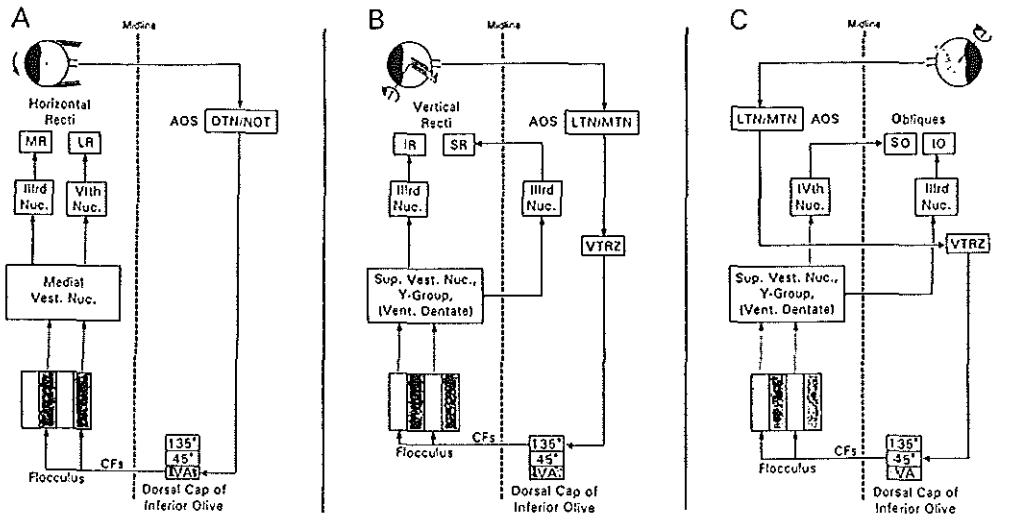

Figure 2. *The accessory optic system. One of the major pathways that mediates the OKR is the accessory optic system (AOS). The AOS is embedded in a closed loop that is formed by the retinal ganglion cells, several mesencephalic nuclei (AOSn) like the nucleus of the optic tract (NOT), the medial tegmental nucleus (MTN) and the visual tegmental relay zone (VTRZ) that transmit optokinetic signals to the inferior olive, the vestibulocerebellum that receives its climbing fibers (CF) from the inferior olive, and the complex of cerebellar and vestibular nuclei, which in turn innervate the oculomotor neurons. The AOS is organized according to the three axes of the semicircular canals; each panel describes the pathway for the control of the eye movement component of one particular axis. Other pathways that may be involved in OKR control are the optokinetic pathways from the AOSn to the nucleus prepositus hypoglossi, and/or from pontine nuclei to the vestibular nuclei or cerebellar cortex; these latter pathways are not indicated in this illustration. For other abbreviations, see list of abbreviations.*

The common reference frames used for the VOR and OKR probably result from a related sequential hardware implementation during development. The circuit underlying the VOR is phylogenetically ancient; newer systems may have been superimposed upon it. As such, the representations they employ may be grounded in the older paradigm. Thus, the coordinates of the AOS were arranged so as to mimic the coordinates of the semicircular canals.

Studying the circuitries underlying the VOR and OKR sheds light on the overall function of the cerebellum. The cerebellum may have originated as an adjunct to the vestibular system. In phylogenetically ancient vertebrates such as cyclostomes, the bulk of the protocerebellar tissue is limited to small centers dorsal to and intimately connected with the acousticolateralis nuclei, the forerunners of the vestibular nuclei of terrestrial vertebrates (Romer and Parsons, 1977). These centers evolved into the vestibulocerebellum, which includes the flocculus and nodulus and forms the ancient archicerebellum. The vestibulocerebellum possesses a remarkably orderly microstructure, which is conserved throughout the phylogenetically new cerebellar divisions (Eccles et al., 1967). If the function of the cerebellum is intimately related

to its special structure, then an understanding of one area could lead to an understanding of the generalized computational task of the entire organ.The vestibulocerebellum is the best area in which to begin because, as the phylogenetically oldest, it appeared in the simplest animals and thus might be expected to embody this hypothetical generalized cerebellar function in its simplest, unalloyed form. Furthermore, since it interfaces with compensatory eye movements, which are among the most analytically tractable instances of sensorimotor coordination, the investigations of the circuitry of the vestibulocerebellum benefits from all of the advantages of studying eye movement reflexes described above.

The vestibulocerebellum probably plays several roles in the control of compensatory eye movements (Ito, 1984; De Zeeuw et al., 1995b; Simpson et al., 1996). Its functions vary from regulation of adaptation of the VOR gain, and suppression of the VOR during smooth pursuit, to phase control, velocity storage, and recalibration of compensatory reflexes and saccadic eye movements. The nodulus and flocculus of the vestibulocerebellum receive various mossy fiber and climbing fiber inputs (Wylie et al., 1994; De Zeeuw et al., 1995b; Simpson et al., 1996). The climbing fibers are exclusively derived from the inferior olive (IOL); the mossy fibers originate from various brainstem nuclei. Whereas the flocculus receives a vestibular mossy fiber input from secondary vestibular neurons in the vestibular nuclei, the nodulus receives its vestibular mossy fiber input directly from primary afferents of the semicircular canals (for review, see Voogd et al., 1996). The flocculus also receives a prominent visual mossy fiber input; these signals are derived from basal and reticular pontine nuclei, which relay information from the frontal eye fields, the AOS and tectum. The majority of the climbing fiber inputs to the nodulus of the vestibulocerebellum carry vestibular or optokinetic signals, while those to the flocculus carry predominantly optokinetic signals.The vestibulocerebellum controls compensatory eye movements mainly via its Purkinje cell projections to the vestibular nuclei. The Purkinje cell output from the flocculus and nodulus reach different sets of neurons in the complex of vestibular nuclei. While floccular Purkinje cells inhibit the second order vestibular neurons and the neurons of the optokinetic system that directly innervate the oculomotor nuclei (OMN) (Highstein, 1972; Ito et al., 1977; Kawaguchi, 1985; Langer et al., 1985; De Zeeuw et al., 1994ab), the nodular Purkinje cells probably target for a large part the "vestibular only" neurons in the vestibular nuclei that do probably not substantially project directly to oculomotor neurons (Fuchs and Kimm, 1975; Haines, 1977; Epema et al., 1988; Scudder and Fuchs, 1992; Wylie et al., 1994).

Despite all the obtained knowledge on the connections and functions of the vestibulocerebellum, major links in the pathways underlying compensatory eye movements are unknown and/or have not been investigated with combined neuroanatomical immunocytochemical techniques. These missing links include the identification of neurotransmitters in various connections between the target nuclei of the vestibulocerebellum and the OMN and between the target nuclei of the vestibulocerebellum and the subnuclei of the IOL that are involved in compensatory eye movements. The rhombencephalon contains several areas in the complex of vestibular and cerebellar nuclei that project to both the OMN and IOL. It is the goal of this thesis to show at the morphological level 1) whether these connections are inhibitory or excitatory; 2) what the nature of their neurotransmiter is; and 3) whether individual neurons in the hindbrain can innervate both the OMN and IOL. Therefore, in the present thesis, these projections were investigated with the use of different combinations of tracing, lesions and immunocytochemistry in the rabbit. Special emphasis was put on the inhibitory components of the projections. Most experiments were carried out in the dutch pigmented rabbit, because a wealth of information concerning the electrophysiology and behavior of the system is present for this animal, which is mainly due to their inability to make smooth pursuit eye movements and their tendency not to make spontaneous saccades.

Ib. Organization and afferents of the OMN

Organization. The location of individual pools of motoneurons in the OMN of the rabbit has been described by Akagi (1978), Murphy et al. (1986), and Shaw and Alley (1981). The basic anatomical organization of the rabbit OMN follows that described for other mammals. Neurons in the OMN innervate the ipsilateral medial rectus (MR), inferior rectus (IR), inferior oblique (IO) and the contralateral superior rectus (SR) muscles. The mammalian OMN also includes motoneurons which innervate the contralateral levator palpebrae (LP) muscle. Although this muscle raises the upper eyelid instead of rotating the globe, the LP has much in common with extraocular muscles and motoneurons. Embryologically, the LP muscle arises from the SR muscle (Gilbert, 1947; Isomura, 1981; Leser, 1925). Physiologically, LP motoneurons discharge like SR motoneurons, increasing their level of activity with upward gaze (Bjork and Kugelberg, 1953). During a blink, however, LP motoneurons exhibit a cessation of activity while SR motoneurons show a burst of activity (Bjork and Kugelberg, 1953; Evinger et al., 1984). From rostral to caudal, the motoneuron populations in all the mammalian species follow an IR, MR, IO, SR (and LP) sequence (Figure 3).

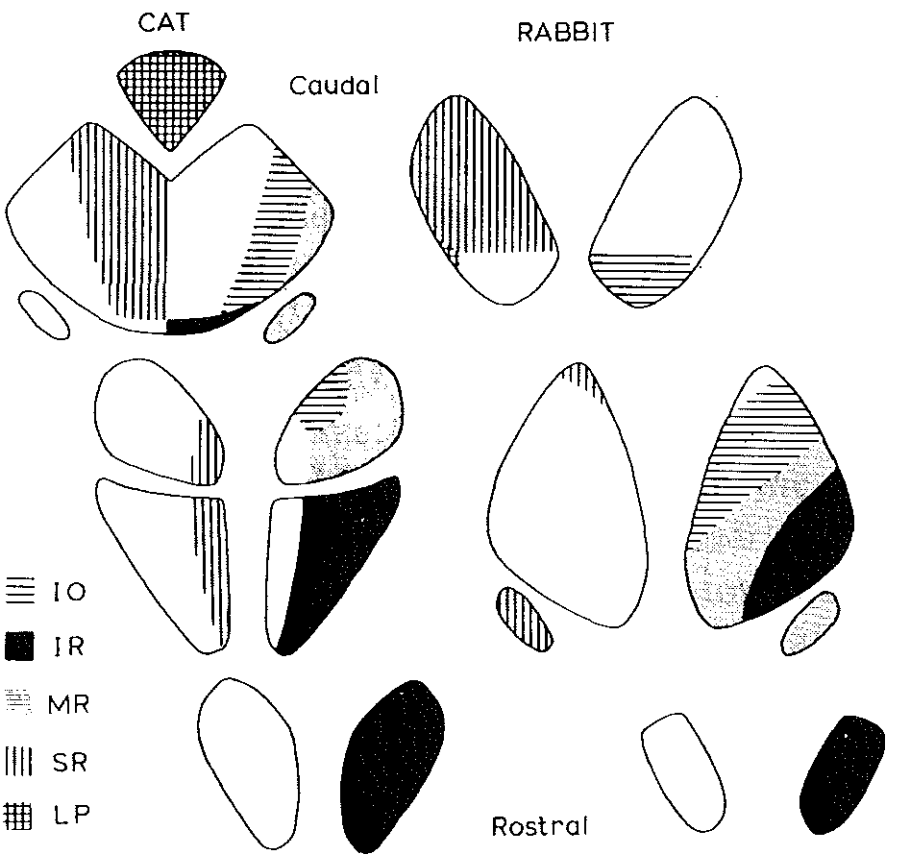

Figure 3. Coronal sections illustrating the location of motoneurons in the oculomotor nucleus of a frontal eyed animal (cat) and lateral eyed animal (rabbit). From Evinger (1988).

This ordered pattern of motoneuron organization occurs probably by allowing the most recently differentiated motoneurons to innervate the most recently differentiated muscles. For example, motoneurons in the caudal OMN will innervate SR muscles, and motoneurons in the rostral OMN will innervate IR and MR muscles. Thus, sharing a common ontogenetic sequence of motoneuron and muscle development would result in all mammals possessing similarly organized oculomotor nuclei.

One apparent difference in the organization of the OMN between primates and non-primate mammals is the existence of three anatomically distinct subpopulations of MR motoneurons in the primate (Büttner-Ennever and Akert, 1981; Porter et al., 1983). The ventral portion of the rostral two-thirds of the OMN (cell group A) contains the largest number of MR motoneurons. Smaller-diameter MR motoneurons located dorsally (cell group C) also extend throughout the rostral two-thirds of the nucleus. These latter motoneurons innervate the small orbital fibers of the MR muscle and are hypothesized to be significant for vergence movements (Büttner-Ennever and Akert, 1981). A third population of MR motoneurons (cell group B) occurs dorsolaterally in the caudal two-thirds of the OMN. It is probably erroneous, however, to assume that only the primate OMN contains multiple populations of MR motoneurons. For example, reanalysis of the cat OMN shows the existence of a small group of dorsolateral MR motoneurons (possibly homologous to cell group C of the primate) in addition to the principal population of ventrolateral MR motoneurons (Furuya and Markham, 1981; Miyazaki, 1985ab). Whether rabbits have a cell group C homologous to that in the primate is doubtfull since rabbits make relatively little vergence movements (Zuidam and Collewijn, 1979).

Apart from motoneurons, the OMN also contains oculomotor interneurons (OcIn). OcIn are neurons within and around the OMN that have intracranial projections. As with the cat, rabbits probably have several subpopulations of internuclear neurons with different intracranial projections. In rabbits, guinea pigs and rats OcIn are known to terminate in the abducens nucleus, the spinal cord, the cerebellum (Evinger et al., 1987; Weiss and Disterhoft, 1985), and the facial nucleus (Hinrichsen and Watson, 1983; Isokawa-Akesson and Komisaruk, 1987). Those within the cat OMN receive inputs from axon collaterals of oculomotor motoneurons (Evinger et al., 1981; Spencer et al., 1982), but lack a direct vestibular input (Highstein, 1977). OcIn in the reticular formation surrounding the OMN might not possess eye movement signals at all. Indeed, the diversity of OcIn projections and the distribution of OcIn outside the OMN suggest that only a small subset of OcIn may have a direct role in controlling eye movements.

Cytology and ultrastructure. The morphology of extraocular motoneurons has been examined both at the light and the electron microscopic level. At the light level, Golgi preparations (Abdel-Maguid and Bowsher, 1979, 1984; Ramon y Cajal, 1909) and intracellular injection of procion dyes (Grantyn and Grantyn, 1978) or HRP (Baker and McCrea, 1979; Destombes et al., 1983; Evinger et al., 1979, 1981, 1982, 1987; Evinger and Erichsen, 1986; Graf and Baker, 1985; Graf and mcGurk, 1985; Grant et al., 1979; Highstein et al., 1982; Letelier et al., 1987; McCrea et al., 1986; Russell-Mergenthal et al., 1986) have provided a description of the soma-dendritic organization of extraocular motoneurons in mammals, birds and teleost fish. Oculomotor motoneurons are approximately 30-50 mm in diameter and have 5-20 primary dendrites (Maciewiecz and Bowsher, 1979 and 1984; Evinger et al., 1987; Baker and McCrea, 1979). The dendrites of an individual oculomotor motoneuron in rabbits, cats, and guinea pigs extend over approximately one third of the OMN and into at least one adjacent population of motoneurons in mammals (Evinger et al., 1981, 1982, 1987). As mentioned above, in cat, their axons give off intracranial axon collaterals that terminate on OcIn (Evinger et al., 1981, 1982, and 1987; Spencer et al., 1982).

The ultrastructure of the mammalian OMN and/or abducens nucleus (AN) has been described for the cat (Tredici et al., 1976; Demêmes and Raymond 1980), rhesus monkey (Waxman and Pappas 1979), and rat (Soghomonian et al., 1989). Cat abducens motoneurons range from 15 to 60 mm in diameter and contain circular nuclei with smooth or slightly irregular nuclear membranes and extensive cisternae of granular endoplasmic reticulum. In comparison, most internuclear abducens interneurons (AbIn), which are known to innervate the contralateral MR subdivision, have fluted or deeply invaginated nuclear envelopes and poorly developed granular endoplasmic reticulum. Whether this morphological characteristic of AbIn also holds true for OcIn is not known. Synaptically, cat abducens motoneurons and AbIn are virtually identical. The average synaptic densities are 4.6 and 4.2 synapses/100 mm^2 for AbIn and abducens motoneurons, respectively (Destombes and Ripert, 1977; Destombes and Rouviere, 1981; Destombes et al., 1979, 1983; Maciewicz and Spencer, 1977; Spencer and Sterling, 1977). The types of terminals found in the OMN are the same as those observed in the AN (Destombes et al., 1979; Spencer and Sterling 1977) and TN (Bak and Choi 1974; Bak et al., 1976), and they are in line with the major characteristics of the terminations in the ventral horn of the spinal cord (Bodian 1966; Conradi 1969; Holstege and Kuypers 1987). In all areas the terminals are mostly contacting dendrites and soma's, but axo-axonal synapses also occur. Gap junctions and the concomitant presence of electrotonic coupling has only been observed in the oculomotor complex of nonmammalian vertebrates (Kidokoro 1969; Kriebel et al., 1969; Korn and Bennett 1972; Waxman and Pappas 1971; Sterling 1977; Saballus et al., 1984); no electrical synapses have been found in ultrastructural studies of cat, monkey, and rat (Tredici et al., 1976; Waxman and Pappas 1979; Soghomonian et al., 1989). In sum, the electron microscopic data have offered important insights into the cellular organization of extraocular motoneurons and internuclear neurons, but have afforded no information on the ultrastructure of the rabbit OMN.

Afferents. The afferents to the OMN are derived from many different areas. Its major inputs are derived from the AN, medial vestibular nucleus (MVN), superior vestibular nucleus (SVN), dorsal group y (dorsal y), ventral dentate nucleus (VDN), rostral interstitial nucleus of the MLF (rIMLF), interstitial nucleus of Cajal (INC), and PrH.

Abducens nucleus. By linking the AN innervating the LR of one eye to the MR motoneurons innervating the other eye, AbIn are the clearest anatomical substrate for the control of conjugate horizontal gaze (Furuya and Markham, 1981; Highstein et al., 1982; McCrea et al., 1986). The AbIn axon exits the AN medially, crosses the midline, and ascends in the contralateral MLF (Figure 1). In both cats and monkeys, about one-third to one-quarter of the AbIn axons give off a fine collateral to the caudal cell group of the paramedian tracts. In the monkey, some AbIn axons also arborize in the more rostral cell group of the paramedian tract. All AbIn axons continue rostrally to terminate in the OMN. In the cat, individual AbIn arborize extensively throughout the entire subdivision containing large MR motoneurons. In contrast, individual monkey AbIn have small terminal arborizations focussed on small clusters of MR motoneurons. This difference suggests that the monkey has a more precise control of conjugate gaze. In neither species, however, do AbIn arborize in the small cell C group of MR motoneurons that are postulated to participate primarily in vergence (disconjugate) eye movements. In Chapter IIc, we describe the projections from the AbIn to the OMN in the rabbit.

Medial and superior vestibular nucleus. The vestibular complex is located in the floor of the fourth ventricle and is composed of a few smaller subnuclei, like group x, y, and z, and four main subdivisions: the SVN, MVN, lateral vestibular nucleus (LVN), and descending vestibular nucleus (DVN). A few neurons in the LVN and DVN may project to the OMN, but the vast majority of the afferents of the OMN from the vestibular complex are derived from the MVN and SVN (Highstein and McCrea, 1988; Epema, 1990). The oculomotor projecting neurons in the MVN and SVN are involved in different types of eye movements; several classes of neurons within these vestibular nuclei can be identified (Chubb et al., 1984; Fuchs and Kimm, 1975; Henn et al., 1974; Lisberger and Miles, 1980; Miles, 1974; Shinoda and Yoshida, 1974; Tomlinson and Robinson, 1984; Reisine et al., 1981; Waespe and Henn, 1987). They include (1) vestibular-only responses, consisting of signals similar to those recorded from primary afferents, (2) vestibular pause cells, similar to those in 1 except that they pause for all saccades, (3) gaze velocity cells that modulate their rates in proportion to eye velocity in space, (4) position cells that change their rates with changes in eye position but do not burst or pause during saccades, (5) position-vestibular-pause cells (PVP) that carry signals proportional to eye position in the head, head velocity, and pause for all saccades, and (6) position-burst cells that carry an eye position signal but burst for saccades in one direction and pause for saccades in the opposite direction. Thus, the MVN and SVN are not merely involved in compensatory eye movements, but also in saccadic eye movements and smooth pursuit.

Nonetheless, the inputs from the neurons in the MVN and SVN to the OMN can best be described in perspective of their relation to the horizontal and vertical VOR (for HVOR, see Figure 1) (eg. Lorente de Nó, 1933; Szentágothai, 1943; Highstein and Ito 1971; Highstein 1973a; Ito et al., 1976a; Highstein and Reisine, 1979; Uchino et al., 1981 and 1982; Graf et al., 1983; Graf and Ezure, 1986; Iwamoto et al., 1990ab). Both the SVN and MVN are supposed to contain excitatory and inhibitory second-order vestibular neurons that relay information from the semicircular canals to the oculomotor complex. The excitatory second-order relay cells of the MVN can be activated by either one of all three semicircular canals. The neurons that are responsive to the anterior and posterior canals are located mainly in the parvocellular MVN (MVpc), (for rabbit see: Highstein and Ito 1971; Highstein 1973a; Ito et al., 1976a; Graf et al., 1983; for cat see Precht and Baker, 1972; Highstein and Reisine, 1979; Uchino et al., 1981 and 1982; Graf et al., 1983; Graf and Ezure, 1986; Iwamoto et al., 1990ab; monkey: McCrea et al., 1987b). Their fibers ascend predominantly in the contralateral MLF and terminate in the IR, IO, and SR subdivisions of the contralateral OMN as well as in the contralateral TN that innervates the SO, (for rabbit, see Thunnissen, 1990; for cat and monkey, see McMasters et al., 1966; Tarlov, 1970; Gacek 1971; Carleton and Carpenter, 1983; for opossum, see Henkel and Martin 1977). It has been demonstrated in cat that axons of excitatory secondary cells of the anterior canal circuit branch to innervate both ipsilateral SR and contralateral IO motoneurons, while axons of excitatory cells of the posterior canal circuit branch to innervate ipsilateral SO and contralateral IR motoneurons (Uchino et al. (1982; Graf et al., 1983, Graf and Ezure, 1986). The excitatory neurons that respond to signals from the horizontal canal and project to the OMN are probably located in all parts of the MVN: i.e. the MVpc, the caudal MVN (MVc), and the magnocellular MVN (MVmc; for definition, see Epema 1990) (for rabbit, see Highstein, 1973a; for cat, see Furuya and Markham 1981; Highstein and Reisine, 1979; Reisine and Highstein, 1979; Reisine et al., 1981; Uchino et al., 1982; for monkey, see McCrea et al., 1987a). The MVmc corresponds to pars alpha of the LVN in rabbit (Olszeweski 1949) and to the ventral LVN in cat (Brodal and Pompeiano, 1957). The neurons in the MVpc and MVc project to the contralateral AN whereas the fibers of the neurons in the MVmc enter the ascending tract of Deiters (ATD) and terminate either ipsilaterally or bilaterally in the MR subdivision (for cat and monkey, see McMasters et al.,

1966; Tarlov, 1970; Carpenter and Cowie, 1985a see also Büttner and Büttner-Ennever, 1988). Thus, in contrast to the neurons of the anterior canal or posterior canal circuit, the LR and MR motoneurons in the horizontal canal circuit are not driven by common branches, but only by distinct premotor secondaries. The neurotransmitter involved in the excitatory projections of the VOR is probably either aspartate or glutamate (Demêmes and Raymond 1982; Kevetter and Hoffman 1991).

The inhibitory second-order relay cells in the MVN can only be activated by the horizontal canal and are located between the excitatory horizontal canal neurons (Highstein 1973b; Uchino and Suzuki, 1983; McCrea et al., 1987a; Thunnissen 1990). These neurons probably do not project directly to the OMN, but mediate their inhibitory control by an inhibitory projection to the ipsilateral AN, which in turn exerts an excitatory effect on the contralateral MR motoneurons (Baker and Highstein 1975 and 1978; Highstein and Baker 1978). The neurotransmitter involved in the inhibitory projection to the AN is probably glycine (Spencer et al., 1989; Spencer et al., 1992).

The excitatory second-order neurons in the SVN are located in the dorsal and dorsolateral part of this nucleus, and receive afferents mainly from the anterior canal (Highstein and Ito 1971; Yamamoto et al., 1978; Highstein and Resine 1979; Uchino et al., 1982; Hirai and Uchino 1984). Their fibers enter the brachium conjunctivum (bc) and terminate in the contralateral IO and ipsilateral SR subdivisions (McMasters et al., 1966; Tarlov 1970; Lang et al., 1979; Carpenter and Cowie 1985a; Thunnissen 1990).

The inhibitory second-order neurons in the SVN are located more centrally, and are mainly activated by the anterior and posterior canal (for rabbit, see Highstein and Ito 1971; Ito et al., 1976b; Graf et al., 1983; for cat, see Uchino and Suzuki 1983; Mitasacos et al., 1983; Graf et al., 1983; Graf and Ezure 1986). Their axons ascend in the ipsilateral MLF and terminate in the IR, IO and SR subdivisions of the ipsilateral OMN and on motoneurons of the ipsilateral TN (Thunnissen 1990; McMasters et al., 1966; Tarlov 1970; Carpenter and Cowie 1985a).

Despite the fact that the inhibitory and excitatory nature of the different vestibular neurons involved in the VOR were shown in numerous physiological studies as described above, there is no direct anatomical demonstration of the postsynaptic distribution and the neurotransmitter of these afferents in the different sudivisions of the OMN. In Chapter IIc, double labeling techniques were used to determine directly and simultaneously the termination pattern and the neurotransmitter of the afferents from the SVN and MVN in the OMN of the rabbit.

Dorsal group y and ventral dentate nucleus. Dorsal y is one of the numerous small subgroups in the complex of vestibular nuclei, while the VDN is a cluster of predominantly small neurons that form the ventral part of the lateral cerebellar nucleus (eg. Highstein and McCrea, 1988). Dorsal y is located within the floccular peduncle and can be delineated by its composition of loosely packed, large, scattered, multipolar neurons that project to the OMN (Fuse 1912; Brodal and Pompeiano, 1957; Highstein, 1973a; Graybiel and Hartwieg, 1974; Hwang and Poon, 1975; Gacek, 1978; Pompeiano et al., 1978; Stanton, 1980; Tan et al., 1995). In the rabbit and the cat, dorsal y is much smaller than in the primate (Highstein and McCrea, 1988). Ventrally, dorsal y is bordered by the restiform body, and dorsally this nucleus merges with cell bridges that extend to the VDN. A projection from the VDN to the OMN was initially denied in the rabbit (Yamamoto et al., 1978), but later demonstrated in the monkey (Carpenter and Cowie, 1985b). Neither dorsal y nor the VDN receive primary afferents from the semicircular canals or the otoliths (Hwang and Poon, 1975; Gacek, 1978). The fibers emanating from dorsal y and probably also the VDN project to the same motoneuron pools as

the excitatory second-order neurons in the SVN, i.e. the contralateral IO and ipsilateral SR motoneurons (Sato and Kawasaki, 1987 and 1990).

According to Chubb and Fuchs (1982) dorsal y plays a prominent role in the control of vertical gaze. Recent studies in the rabbit support this hypothesis (De Zeeuw et al., 1994b; De Zeeuw et al., 1996a). Stimulation in zone C2 of the rabbit flocculus, which projects to both dorsal y and the interposed posterior nucleus, has been shown to evoke concomitant head and eye movements. In addition, recordings in dorsal y have demonstrated that its neurons respond optimally to vertical stimulation (Partsalis et al., 1995ab).

Rostral interstitial nucleus of the MLF and the paramedian pontine reticular formation (PPRF). The rIMLF and PPRF are involved in the control of vertical and horizontal, saccadic eye movements, respectively. Whereas short-lead burst neurons in the PPRF carry excitatory signals from the saccadic network to lateral rectus motoneurons and internuclear interneurons in the abducens nucleus, the rIMLF controls saccadic eye movements through the OMN (Büttner-Ennever, 1977; Büttner-Ennever and Büttner, 1978; Graybiel, 1977; Wang and Spencer, 1992 and 1996). In both cats and monkeys, the rIMLF projects to the TN and all subdivisions of the OMN except the MR subdivision (Büttner-Ennever, 1977, 1979; Graybiel, 1977; Graybiel and Hartwieg, 1974; Steiger and Büttner-Ennever, 1979). Electrophysio-logical studies in the cat demonstrate that most rIMLF neurons terminate on ipsilateral IR motoneurons, although a single rIMLF neuron may terminate in both the ipsilateral and contralateral IR subdivions of the OMN (Nakao and Shiraishi, 1983). These studies show that rIMLF neurons monosynaptically excite IR motoneurons and evoke a mixed IPSP-EPSP response in SR motoneurons (Nakao and Shiraishi, 1985). The importance of the rIMLF for vertical saccades is supported by recordings of rIMLF neurons in monkeys that exhibit a high frequency burst of activity 5 to 15 ms before vertically directed saccadic eye movements, while they are quiescent during all other eye movements (Büttner et al., 1977a,b; King and Fuchs, 1979). Consistent with the results from these experiments, bilateral lesions of the rIMLF in both humans and nonhuman primates produce long-lasting paralysis of downward gaze (Büttner-Ennever et al., 1982; Kompf et al., 1979; Trojanowski and Lafontaine, 1981) without eliminating the VOR (Büttner-Ennever et al., 1982).

Interstitial nucleus of Cajal. The INC contacts several nuclei involved in eye and head movements (for review see Fukushima, 1987). Motoneurons in the OMN receive direct bilateral projections from the INC (Büttner-Ennever, 1977; Fukushima et al., 1978; Graybiel, 1977; Graybiel and Hartwieg, 1974; King et al., 1980; Rutherford and Gwyn, 1982; Schwindt et al., 1974; Zuk et al., 1982). A small bilateral projection to the medial portion of the AN occurs in monkeys but not in cats (Langer et al., 1986). In addition to contacting motoneurons, neurons in the INC also terminate in the vestibular nuclei (for review see Fukushima, 1987; King et al., 1980), the perihypoglossal nucleus (McCrea and Baker, 1985), the periabducens region (Schwindt et al., 1974; Stanton and Greene, 1981), the nucleus of the posterior commissure (Carpenter et al., 1970), and weakly in the IOL (Zuk et al., 1982). To our knowledge the projections from the INC to the rabbit OMN have not been described.

While the involvement of the INC in vertical and torsional eye movements has long been accepted, the exact nature of its role is unclear. Early studies showed that unilateral INC lesions cause a chronic extorsion of the eye contralateral to the lesion and intorsion of the ipsilateral eye. In addition, cats with INC lesions hold their heads tilted down toward the side contralateral to the lesion. From these kinds of data, Szentágothai (1943) postulated that the INC works as a switching point between reflexive eye movements arising from the vestibular nuclei and voluntary movements originating in the frontal eye fields. Lesion studies (Anderson,

1981ab) suggest that the INC acts as an integrator for the VOR, because bilateral lesions of the INC cause an increased phase lead in the VOR. Further investigation (King and Leigh, 1982), however, showed that the INC is not only an integrator. First, INC lesioned animals make few vertical saccades. Second, INC lesions impair downward VOR more than upward VOR. Third, although reducing long-latency VOR responses, INC lesions do not affect the first beat of vestibular nystagmus evoked by either an upward or a downward step of head velocity (King and Leigh, 1982). This last result implies that direct INC control of extraocular motoneurons is less significant than indirect control through INC projections to other premotor afferents and reemphasizes the importance of indirect pathways in the oculomotor system.

Nucleus prepositus hypoglossi. The anatomy and physiology of the PrH suggests that it is an important nucleus in oculomotor functioning. The PrH projects to the OMN and is reciprocally connected with the cerebellum and vestibular nuclei (for review, see McCrea and Baker, 1985). In both cats and monkeys, the PrH projects bilaterally to all parts of all of the extraocular motor nuclei; the strongest projections are to the contralateral AN and the ipsilateral MR subdivision of the OMN (Baker and Berthoz, 1975; Baker et al., 1977; Carpenter and Batton, 1980; Gacek, 1977, 1979; Graybiel, 1977; Graybiel and Hartwieg, 1974; Hikosaka and Igusa, 1980; McCrea et al., 1979; McCrea and Baker, 1985; Steiger and Büttner-Ennever, 1979; Yingcharoen and Rinvik, 1982). This aspect of PrH connections supports the idea that the nucleus participates primarily in horizontal gaze. Nevertheless, PrH also projects to mesencephalic nuclei involved in vertical eye movements (see McCrea and Baker, 1985, for review). The fact that an individual PrH neuron may project to several mesencephalic nuclei, like the Edinger-Westphal nucleus and the periaqueductal gray dorsal to the OMN, demonstrates that the PrH can, like other premotor afferents, exert simultaneously a direct and an indirect influence on motoneurons (Hikosaka and Igusa, 1980; McCrea and Baker, 1985; McCrea et al., 1979).

The anatomical findings are not easily reconciled with the known physiology of the PrH. Most PrH neurons increase their firing rate as the eye moves ipsilaterally (Lopez-Barneo et al., 1982), which suggests that if their synaptic effect on motoneurons is excitatory (Baker et al., 1977; McCrea et al., 1979), the appropriate connectivity would be to the *ipsilateral* AN and *contralateral* MR subdivision. Since the opposite pattern of connectivity is present, one suspects either that many PrH neurons make inhibitory synapses on motoneurons, whose synaptic action is disguised in electrophysiological experiments by EPSPs evoked by antidromic activation of the caudal collaterals of internuclear neurons which terminate near the PrH (McCrea et al., 1986), or that the signals carried by PrH neurons are out of phase with other, more powerful inputs to motoneurons. This latter possibility is supported by the fact that removal of the PrH causes an increased phase lead in the horizontal VOR (Baker et al., 1981; Cannon and Robinson, 1987; Cheron et al., 1986). As described above for the INC, this finding also supports the hypothesis that the PrH serves as an integrator.

Alignment of semicircular canals and oculomotor muscle pairs. For the principal VOR pathways described above to produce perfect compensatory eye movements, the pulling directions of the extraocular muscles would have to precisely match the plane of rotation, which produces maximal semicircular canal excitation. In fact, the planes for canal excitation and for muscle pulling direction rarely align exactly. For example, in cats, the plane of rotation which excites the posterior canal forms a 52° angle with respect to the midsagittal plane but contraction of the SO muscle moves the eye in a plane forming a 68° angle with respect to the midsagittal plane (Ezure and Graf, 1984a; Graf and Simpson, 1981; Simpson and Graf, 1981, 1985). Both anatomical (Ezure and Graf, 1984a; Graf et al., 1983) and physiological data

(Uchino and Hirai, 1984; Uchino et al., 1980a,b, 1981, 1982) demonstrate that realignment of the plane of eye rotation with the plane of posterior canal activation occurs by small modifications in the principal vestibulo-ocular connections (for vestibulo-ocular projections in rabbit, see Thunnissen, 1990). As well as disynaptically exciting the contralateral SO and IR motoneurons, posterior canal driven vestibular neurons disynaptically excite ipsilateral IR motoneurons. The posterior canal driven secondary vestibular neurons disynaptically inhibit 26% of the contralateral IO and 87% of the contralateral SR motoneurons. The anterior canal driven secondary vestibular neurons of the cat inhibit 96% of the contralateral IR motoneurons and 21% of the ipsilateral SR motoneurons. These additions to the principal vestibulo-ocular projections probably compensate for imperfect matches between the planes of rotation evoking maximal canal excitation and muscle pulling direction (Ezure and Graf, 1984b; Graf and Ezure, 1986, for review). In contrast to the vertical and oblique muscles, the pulling directions of the cat LR and MR muscles closely match the plane of activation for the horizontal canals. It is to be expected, therefore, that secondary vestibular neurons activated by the horizontal canal do not appear to contact vertical or oblique extraocular muscles (McCrea et al., 1980, 1981). Thus, in addition to the principal connections of semicircular canals to extraocular motoneurons, weaker secondary connections exist which serve to bring the plane of eye rotation into register with the activation plane of the semicircular canals.

Since rabbits and cats exhibit quantitatively different mismatches between the planes of semicircular canal and the pulling directions of extraocular muscles (Graf and Simpson, 1981; Simpson and Graf, 1981, 1985), it is to be expected that these species have slightly different patterns of secondary vestibular neuron termination. For example, intracellular staining reveals two types of posterior canal activated secondary vestibular neuron in the cat. The first type terminates on contralateral SO and IR motoneurons. The second type contacts not only contralateral SO and IR motoneurons but also ipsilateral IR motoneurons (Graf et al., 1983; Graf and Ezure, 1986). In contrast, there is only one type of rabbit posterior canal activated secondary vestibular neuron. Like the first type of cat neuron, the rabbit secondary vestibular neuron arborizes in the population of contralateral SO and IR motoneurons. Rabbit posterior canal neurons, however, do not project to both the contralateral and ipsilateral IR motoneurons. Another difference between rabbit and cat posterior canal activated vestibular neurons is that only in the rabbit do secondary vestibular neurons contact contralateral AN motoneurons as well as the usual vertical motoneurons (Graf et al., 1983). Thus, frontal-eyed cats and lateral-eyed rabbits share the same principal vestibulo-ocular projections, posterior canal to contralateral SO and IR motoneurons, but each species has secondary connections to other extraocular motoneurons consistent with differences in the orientation of the extra-ocular muscles and semicircular canals.

Ic. Organization and afferents of the inferior olive

Organization. The IOL is one of the precerebellar nuclei. The cerebellum receives information about peripheral events and central nervous processes through numerous precerebellar systems, which terminate in different layers of the cerebellar cortex as climbing and mossy fibers, and as multilayer afferents which include the monoaminergic afferents. The IOL gives rise to all the climbing fibers innervating the Purkinje cells, while the Purkinje cells themselves are the sole source of the ouput signals of the cerebellar cortex that reach the central cerebellar and vestibular nuclei. The IOL is located in the ventral part of the medulla oblongata and is composed of the principal olive and two accessory olives (Kooy, 1916; Brodal, 1940; Whitworth and Haines, 1986). The principal olive (PO) consists of a folded sheet of grey

matter. Rostrally a dorsal and a ventral lamella can be distinguished. Caudally the PO is located next to the ventrolateral outgrowth (VLO) and the dorsal cap of Kooy (1916) (dc), which are the olivary subnuclei involved in the control of the OKR. The medial accessory olive (MAO) includes several subnuclei. The Beta-nucleus and the dorsomedial cell column are located at its caudal and rostral medial side, respectively; these olivary subnuclei receive indirectly vestibular signals. The dorsal accessory olive (DAO) is located dorsally from the PO. The projection from these subnuclei to the cerebellum has been studied mainly in the cat (for reviews see Voogd, 1964; Brodal and Kawamura, 1980; Voogd and Bigaré, 1980), but also in primates and subprimates (for reviews see Brodal and Brodal, 1981 and 1982; Whitworth et al., 1983; Whitworth and Haines, 1986), and rats (for reviews see Flumerfelt and Hrycyshyn, 1985; Voogd, 1995; Ruigrok et al. 1995). Tan et al. (1995) studied the efferent projections from the dc, VLO and beta nucleus to the vestibulocerebellum in the rabbit. In general, each olivary subnucleus projects contralaterally to one or more longitudinal strips of Purkinje cells and gives off primary collaterals to that part of the central cerebellar nuclei (Groenewegen et al., 1979; Andersson et al., 1987), which receives its main input from the same zone (Voogd and Bigaré, 1980). Since the cerebellar nuclei in turn project to the olivary subnucleus from which they receive collaterals (Tolbert et al., 1976; Dietrichs and Walberg, 1985 and 1986; Dietrichs et al., 1985 and 1986), the direct connections between them are reciprocally and topographically organized.

Cytology and ultrastructure. The population of olivary neurons is not homogeneous. Apart from a few interneurons, which may be GABAergic (Nelson et al., 1988; Walberg and Otterson, 1989), it is composed of two main types of neurons. The first type (type I) is equiped with relatively long and diffuse, sparsely branched, spiny dendrites radiating away from the soma. The dendritic field of type I cells is large and it mainly occurs in the caudal parts of the accessory olives. The second type (type II) is a neuron with a spherical cell body with a diameter (15 to 30 um) somewhat larger than that of the first type (Ruigrok et al., 1990), and with an arbor of complex, spine-bearing dendrites which are highly branched and tend to turn back toward the soma, at times creating spirals. This type of neuron occupies a relatively small three dimensional area. It represents the predominant cell type in the PO, the rostral part of the MAO and the DAO. What types of neurons are most prominent in the VLO, dc and Beta-nucleus is unknown. Both cell types are probably projecting neurons, i.e. give rise to the climbing fibers, since the axons of both types of cells leave the neuropil of the adult IOL without giving off collaterals (Foster and Peterson, 1986; Ruigrok et al., 1990).
The ultrastructure of the IOL has been described in many studies of various animals including the opossum (Bowman and King, 1973; King, 1980), rat (Gwyn et al., 1977; De Zeeuw et al., 1990c), rabbit (Mizuno et al., 1974), cat (Walberg, 1963; Walberg, 1964; Walberg, 1966; Sotelo et al., 1974; De Zeeuw, 1990), and monkey (Rutherford and Gwyn, 1980). In general, most neural elements in the olivary neuropil are enveloped in thin processes of fibrous astrocytes. The neuronal somata are oval or round in shape, and their cytoplasm contains subsurface cisterns of the endoplasmic reticulum, spherical inclusions and deposits, and the complement of organelles characteristic of most neurons in the central nervous system. Somata occasionally are apposed to dendrites without any membrane specialization.The segments of olivary dendrites bear simple, pedunculated club-shaped, and/or racemose spiny appendages (Sotelo et al., 1974; Gwyn et al., 1977; Ruigrok et al., 1990; De Zeeuw et al., 1990ab), and they sometimes contain varicose dilatations packed with mitochondria or dendritic lamellar bodies (De Zeeuw et al., 1995a). These dendritic lamellar bodies can be associated with gap junctions, which are predominantly located in complex synaptic arrangements called glomeruli.

The glomeruli form the most characteristic feature of the olivary neuropil (Nemecek and Wolff, 1969; Bowman and King, 1973; Sotelo et al, 1974; King et al., 1975; King, 1976; Gwyn et al, 1977; Rutherford and Gwyn, 1977; De Zeeuw et al., 1990ab), and to less extent the dendritic thickets (Sotelo et al., 1974; Molinari, 1987). The dendritic thickets are formed by several (up to four) serial dendrites in direct apposition with each other, but without any dendrodendritic membrane specialization. The glomeruli contain a core of several dendritic appendages surrounded by terminals and glia. The spines in the centre of these complex synaptic structures are frequently linked by the dendrodendritic gap junctions, and they all receive both excitatory and inhibitory terminals (De Zeeuw et al., 1990abc). It was suggested by De Zeeuw et al. (1990a) that this combined inhibitory and excitatory input innervating the dendritic and probably also axonal spines may regulate the electrotonic coupling between olivary cells as well as their firing frequency in a timing sensitive manner, and provide, in this way, an instrument for the timing of a motor response. Benardo and Foster (1986) showed in slices of the guinea pig inferior olive that intracellular injected Lucifer yellow can pass from one cell to the other through the gap juctions. They were able to label aggregates containing up to six coupled olivary cells. Harmaline and picrotoxin induced synchronous firing suggests that in the intact IOL the coupled cellular aggregates can extend to a much larger size (Llinás and Volkind, 1973; Sjölund et al., 1980; Lang et al., 1996); in fact, it has recently been shown that an ensemble of coupled olivary neurons can even extend beyond the midline (De Zeeuw et al., 1996b). Until now, gap junctions have been found in each olivary subnucleus except for the dc (Mizuno et al., 1974). It was one of the specific aims of the present thesis to demonstrate the presence of gap junctions in the dc (see part IIIa).
Terminals on neurons of the IOL differ as to their morphology, localization, their membrane specializations, their neurotransmitter and their origin. More than half of the olivary terminals contain rounded vesicles (Bowman and King, 1973; Mizuno et al., 1974; Sotelo et al., 1974; Gwyn et al., 1977; Rutherford and Gwyn, 1980; De Zeeuw et al., 1989ab). These terminals usually are associated with asymmetric synapses (for definitions of morphology of synapses, see Gray, 1959 and Gray and Guillery, 1966). The second largest group of terminals contains pleomorphic vesicles and is provided with symmetric synapses, they are roughly of the same size as the round vesicle containing terminals. A small minority of the terminals contains either dense core vesicles or only flattened vesicles (Bowman and King, 1973; Gwyn et al., 1977; De Zeeuw et al., 1989). The large majority of the terminals contact the distal dendrites and/or their spines, whereas the somata receive relatively few terminals. The somatic terminals consist mainly of terminals with pleiomorphic vesicles. Special types of synapses, including synapses associated with subsynaptic densities as described by Taxi (Taxi, 1961) and so-called crest synapses (Milhaud and Pappas, 1966ab; Akert et al, 1967) are present in the IOL of several animals (Mizuno et al, 1974; Gwyn et al, 1977; Rutherford and Gwyn, 1980; Cintas et al, 1980; Sotelo et al, 1986). Chemical dendrodendritic synapses in the IOL have only been observed in the opossum (King, 1980), while axo-axonal contacts have not been described, apart from those of recurrent collaterals in the hypertrophic IOL (De Zeeuw et al., 1990d).

Afferents. The afferent systems of the IOL have been reviewed for cat (Brodal and Kawamura, 1980), rat (Brown et al., 1977; Flumerfelt and Hrycyshyn, 1985; Ruigrok et al., 1995), and opossum (Martin et al., 1980). Apart from the cerebello-olivary projection mentioned above, several afferent systems can be distinguished. The first one consists of the projections from the cerebral cortex most of which are interrupted in a set of nuclei at the mesodiencephalic border. They include the nucleus of Darkschewitsch and the parvocellular red nucleus, and nearby regions like the nucleus of Bechterew, the nucleus interstitialis of Cajal, the tegmental field of Forel, the zona incerta, the subparafascicularis nucleus, and the

suprarubral reticular formation (Ogawa, 1939; Walberg, 1956; Brown et al., 1977; Leonard et al., 1978; Cintas et al., 1980; Saint-Cyr and Courville, 1980 and 1982; Kawamura and Onodera, 1984; Ruigrok et al., 1988). These nuclei receive afferents from the motor- and premotorcortex and to a limited degree from the superior parietal lobule (Rinvik and Walberg, 1963; Niimi et al., 1963; Mabuchi and Kusama, 1966; Kuypers and Lawrence, 1967; Hartmann-von Monakow et al., 1979; Nakamura et al., 1983; Saint-Cyr, 1987), but also from the cerebellar nuclei (Voogd, 1964; Kievit, 1979; Courville, 1966; Kawamura et al., 1982; Sugimoto et al., 1982; De Zeeuw et al.,1989; De Zeeuw and Ruigrok, 1994). They project predominantly to the ipsilateral, rostral MAO and PO, and to a lesser extent to the ipsilateral, caudal MAO (Walberg, 1974; Walberg, 1982; Swenson and Castro, 1983; Onodera, 1984; Holstege and Tan, 1988). The anterior and posterior sigmoid gyrus of the cerebral cortex itself projects directly with an ipsilateral preponderance to the caudal MAO and the border region of the more rostrally located ventral lamella of the PO and the DAO (Sousa-Pinto and Brodal, 1969; Bishop et al., 1976; Saint-cyr and Courville, 1980; Saint-Cyr, 1983; Swenson and Castro, 1983). The second group of afferent pathways includes several sensory relay nuclei, which project predominantly contralaterally, like the dorsal horn of the spinal cord, the dorsal column nuclei and the spinal trigeminal nucleus (Brodal et al., 1950; Mizuno, 1966; Boesten and Voogd, 1975, Groenewegen and Voogd, 1977; Berkley and Hand, 1978; Armstrong et al., 1982; Swenson and Castro, 1983; Gerrits et al., 1985a; Huerta et al., 1985). The vestibular nuclei (with exception of the lateral vestibular nucleus), the medial and descending vestibular nuclei (Saint-cyr and Courville, 1979; Gerrits et al., 1985b), the parasolitarius nucleus (Ebbeson, 1968; Loewy and Burton, 1978; Nelson and Mugnaini, 1987), group Y (Gerrits et al., 1985b; McCrea and Baker, 1985), and the superior colliculus (Hoddevik et al., 1976; Weber et al., 1978) give rise to projections to certain subdivisions of the MAO including group Beta and the dorsomedial cell column. Visual centres involved in the OKR, like the nucleus of the optic tract (NOT), the medial and dorsal nuclei of the accessory optic tract (MTN, DTN), visual tegmental relay zone (VTRZ), and the PrH project to the dc and VLO (Mizuno et al., 1973; Walberg et al., 1981; Itoh et al., 1983; Gerrits et al., 1985b; McCrea and Baker, 1985).

Physiology of visual and vestibular afferents. The projections to the dc, VLO and Beta-nucleus have also been demonstrated at the physiological level; visual and vestibular stimulation are known to evoke olivary activity. Light flashes evoke responses in the dc (Barmack and Hess, 1980; Gellman et al., 1983) and large climbing fiber potentials on the vermis (Buchtel et al., 1972) and flocculus (Maekawa and Simpson, 1973). Floccular climbing fiber activity mediated through the dc can be modulated in relation to speed and direction of movement of large patterns over the visual field (Simpson and Alley, 1974; Waespe and Henn, 1981; Blanks and Precht, 1983; Watanabe, 1984; Stone and Lisberger, 1986). The caudal dc is related to the horizintal OKR, whereas the VLO and rostral dc are involved in the vertical OKR. In the flocculus four zones have been identified, which also respond differentially to optokinetic stimuli moving in vertical or horizontal planes (Simpson et al., 1981; Leonard et al., 1988; Simpson et al., 1989; Graf et al., 1988; De Zeeuw et al., 1994a). The preferred axis of each of these zones are closely aligned with those of the semicircular canals of the vestibular apparatus. Most of the neurons in the dc are not sensitive to vestibular stimulation (Barmack et al., 1989; De Zeeuw et al., 1995b). Caloric, electrical and natural stimulation of the vestibular labyrinth itself evokes action potentials in and near the Beta-nucleus (Ferin et al., 1971; Precht et al., 1976ab; 1977; Ghelarducci et al., 1975; Robinson, 1987). The cells of the beta-nucleus are sensitive to low frequency yaw oscillations in a specific direction, but do not signal very well the magnitude of movement, speed or acceleration (Robinson et al., 1988). The neurons in the Beta-nucleus that are sensitive to vestibular stimulation about the longitudinal axis, are

inhibited when the rabbit is rolled ipsilaterally (Barmack et al., 1989). The vestibular receptive cells in the IOL are not responsive to somatosensory or visual stimuli (Gibson and Gellman, 1987; Barmack et al., 1989) indicating that these afferent systems do not converge onto the same olivary neurons.

The behavioral effects of the actvities in the neurons of the dc, VLO, and Beta-nucleus can only be understood, if one understands the effects of their climbing fibers. Signal processing in their climbing fibers from neurons in the dc, VLO, and Beta-nucleus follows the rules that apply to all climbing fibers: the climbing fibers have an extremely powerful excitatory action on the Purkinje cell resulting in a burst discharge, its complex spike, and they have an extremely low firing frequency (Eccles et al., 1966; Thach, 1967). The limited frequency range of olivary discharge stands in contrast to the range of several hundred spikes per second for the Purkinje cell simple spikes whose excitatory drive is conveyed by the mossy fiber - parallel fiber input (Eccles et al., 1967). However, despite decades of investigation, the issue of the consequences of the climbing fiber contributions to cerebellar transactions is still unresolved. For many researchers, the climbing fibers signal errors in motor performance, either in the conventional manner of frequency modulation or as a single announcement of an "unexpected event". Marr (1969) and Albus (1971) hypothesized that the cerebellum learns to perform motor skills through climbing fiber induction of long-term changes in the strength of parallel fiber synapses on Purkinje cells. Ito (1970a) put this idea into a specific context by proposing that the flocculus adjusts the performance of compensatory eye movements in response to climbing fiber signals of "visual blur" functioning as a teaching input. Thus, the later discoveries by Simpson and Alley (1974) and Barmack et al. (1989) that the signals carried by climbing fibers from the dc/VLO and Beta-nucleus encode respectively, retinal slip and vestiblar instability, is perfectly in line with the learning hypothesis. However, other investigators studying different areas of the cerebellum found that climbing fiber activity leads to a short-lasting enhancement of the responsiveness of Purkinje cells to mossy fiber inputs (Ebner and Bloedel, 1981a,b, and 1984; Ebner et al., 1983), or that the climbing fibers serve internal timing functions through their capacity for synchronous and rhythmic firing (Llinás et al., 1974; Llinás, 1985; Llinás and Welsh, 1993; Welsh et al., 1995). This timing hypothesis is supported by the presence of electrotonic coupling and gap junctions in the IOL (Sotelo et al., 1994; De Zeeuw et al., 1989 and 1995a).

Taken together, one can conclude that consensus exists about the type of message that is carried by the climbing fibers, including those of the dc, VL and Beta-nucleus, but not what the consequences of these messages are in terms of electrophysiological activity in the cerebellar cortex or in terms of behavior. One of the clues to understanding the function of the vestibulocerebellum may lie in unraveling its connections with the IOL. As noted above, the inhibitory inputs from the cerebellar nuclei to the PO, MAO and DAO have been elucidated (De Zeeuw et al., 1989ab; Fredette and Mugnaini, 1991). However, the inhibitory inputs to the olivary subnuclei involved in compensatory eye movements are unknown. Chapter IIIa deals with the source of the inhibitory inputs to the olivary subnuclei involved in the horizontal OKR (caudal dc) and in the VOR (Beta-nucleus); chapter IIIb deals with the source of the inhibitory input to the olivary subnuclei involved in the vertical OKR (rostral dc and VLO).

Id. Physiology of compensatory eye movements

As an introduction to the current morphological experiments, a short review of what is known about compensatory eye movements is in order. The principal compensatory eye movements are the VOR and the OKR. Both reflexes result in rotation of both eyes to stabilize the visual world on the retina. The stimulus for the VOR is angular head acceleration, which is sensed by the six semicircular canals located in the petrous temporal bones of the head. The stimuli for the OKR are the patterns of image motion across the retina ("visual flows") that are produced by head rotation. In both reflexes the first stage of processing involves decomposing the total head rotation into motion about each of three axes. Both reflexes measure rotation about similar axes - the axes defined by the normals to the planes of the horizontal, anterior vertical, and posterior vertical semicircular canals (Simpson et al., 1988a). The VOR is the simpler of the two reflexes, since the sensing of acceleration and its decomposition are achieved by the geometrical and mechanical properties of the semicircular canals. In contrast, the construction of the rotational signals from visual information is performed predominantly by neuronal circuitry. In the rabbit this circuitry is contained within the AOS (Oyster, 1968; Oyster et al., 1972 and 1980; Simpson, 1984; Soodak and Simpson, 1988; Simpson et al.,1988ab. The elaboration of the rotational signals involves a number of steps, including: 1) the property of directional selectively is created from the simple luminance sensitively of the retinal photoreceptors; 2) the small (approximately 5° in the rabbit) receptive fields of the ganglion cells are summed to create large receptive fields appropriate for detecting movements of the entire visual world; 3) the unidirectional response of the ganglion cells is converted to a bidirectional response (i.e., capable of being both excited and suppressed); 4) "bipartite" receptive fields are constructed in the channels that synergize with the outputs of the vertical canals, i.e., response types are created in which different directions of visual motion are preferred in different parts of the receptive fields. Further complications of the OKR include the fact that it is an open-loop system with a strong nonlinearity, which greatly increases the amount of information required to describe the input signal (Oyster, 1968; Collewijn, 1981). The greater complexity of the input stages of the OKR is evident from the fact that it cannot be tested, in contrast to the VOR, in unconscious patients (Leigh et al., 1984). In addition, it is reflected in the latencies of the OKR and the VOR. In the rabbit, about 75 msec elapses between the onset of motion of the visual surround and the onset of the eye movement response (Collewijn, 1981). The latency of the VOR, measured in the monkey, is only 14 msec (Lisberger, 1984). Since the two reflexes share many of the same vestibular nucleus neurons, and the same motoneurons and oculomotor plant, the difference in latencies stems entirely from the different complexities of the input stages.

The comparative simplicity of the VOR input stage makes it more analytically tractable. From each semicircular canal signals are transmitted via afferent vestibular nerve fibers to the vestibular nuclei. The appropriate stimulus for the canals is angular head acceleration. In order to obtain the eye position related signal found in oculomotor neurons, a twofold integration (acceleration - velocity - position) has to take place. The first integration is determined mechanically by the cupula-endolymph system (torsion-pendulum model; Steinhausen, 1933). Accordingly, a 'head velocity' signal can be recorded from afferent vestibular nerve fibers at frequencies above 0.1 Hz (Fernandez and Goldberg, 1971). The dynamics of the cupula-endolymph system do not allow vestibulo-ocular compensatory eye movements in the low-frequency range (below 0.1 Hz). This can best be demonstrated during constant velocity rotation in the dark. The initially high slow-phase eye velocity slowly returns to zero with a time constant of 12 - 20 sec, depending on the species and stimulus conditions (Robinson, 1976;Malcolm and Melvill-Jones, 1970). Thus, the time constant of the oculomotor response

to a vestibular stimulus is considerably longer than the time constant of 4-6 sec found in primary vestibular afferents (Fernandez and Goldberg, 1971; Büttner and Waespe, 1981). This - in relation to the primary vestibular nerve signal - extended performance of the VOR in the low-frequency range is the result of the so-called *'velocity storage' mechanism* (Raphan et al., 1977). Its operation is reflected in the activities of vestibular nuclei neurons of alert animals. The anatomical substrate for the 'velocity storage' mechanism is not clear, but it is under the control of the nodulus (Waespe et al., 1985).

Apart from an integration, which transforms acceleration into a velocity signal, a second integration (velocity to a position signal) has to take place to obtain an accurate eye position related signal for the motoneurons. This step is not only necessary for the VOR (Fuchs and Kim, 1975), but for all eye movements including the OKR (Waespe and Henn, 1987), saccades (Fuchs et al., 1985), and smooth pursuit eye movements (Lisberger and Fuchs, 1978; Miles et al., 1980). Thus, for all eye movements eye velocity is the oculomotor parameter that has been found to be encoded in the premotor neurons. Original experiments performed by Robinson (1975) in monkey suggest that all these types of eye movements are controlled by a common *'neural integrator'*. However, lesion and recording studies indicate at least two distinct sites of integration for horizontal and vertical eye movements; the PrH and INC, respectively (Cannon and Robinson, 1987; Fukushima, 1987). Moreover, lesions of the flocculus of the cerebellum also affects the neural integration process (Zee et al., 1981). This leads to a gaze-evoked nystagmus, i.e. a centripetal drift of the eyes. The postsaccadic drift after cerebellar lesions has a time constant of more than 1.3 sec, which is considerably longer than the 200 msec found after lesions of the PrH (Cannon and Robinson, 1987). Thus, cerebellar lesions only make the neural integrator 'leaky' and residual integration remains in the brainstem.

Finally, it should be noted that both the VOR and OKR are not fully compensatory by themselves; for example, even in the range of naturally occurring head movements (0.1 - 3 Hz) the gain (eye velocity/stimulus velocity) of the VOR is not 1. In humans, the gain of the VOR is around 0.8 - 0.9, but it can vary considerably among species and is particularly dependent on the state of alertness (Collins, 1962; for rabbits see Baarsma and Collewijn, 1974; De Zeeuw et al., 1995b). Thus during VOR in the light, visual signals have to be utilized in addition to achieve a fully compensatory VOR. This visual enhancement is probably mediated through circuits in the flocculus of the cerebellum (Ito et al., 1974 and 1982; De Zeeuw et al., in press). The limitations of the VOR are also evident from studies which showed that the direct route of the three-neuron arc accounts for only the most rapid component of the reflex (Lisberger, 1984; Lisberger and Pavelko, 1986). While fast, the VOR is not necessarily accurate. In the monkey, adjustments to the VOR gain reflecting the adaptive state of the animal appear approximately 5 msec after the earliest response, and may involve additional synapses. Compensation for subtle spatial problems requires even more time-consuming processing. For instance, the ideal gain for the VOR varies as a function of the position of the axis of rotation in space, the distance of the visual target, and the separation of the eye and labyrinth. In the monkey, the correction of the VOR gain for these factors is not complete until 45-100 msec after the onset of head acceleration.

Chapter II. Ultrastructure of the Inhibitory Inputs to the Oculomotor Nucleus

a. Gabaergic and glycinergic inputs to the oculomotor nucleus with special emphasis on the medial rectus subdivision
b. Colocalization of gaba and glycine in the oculomotor nucleus
c. Synaptic inputs to the oculomotor nucleus from the medial vestibular nucleus and superior vestibular nucleus

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IIa. Gabaergic and glycinergic inputs to the oculomotor nucleus with special emphasis on the medial rectus subdivision

Abstract

Contradictory results have been reported about the inhibitory input to the medial rectus subdivision of the oculomotor nucleus of the cat. In the present ultra-structural study, we quantified the GABAergic and glycinergic terminals in the various subdivisions of the rabbit oculomotor nucleus with the use of post-embedding immunocytochemistry combined with retrograde tracing of horseradish peroxidase. The density of the GABAergic input to the medial rectus subdivision was as substantial as that to the other subdivisions and the postsynaptic distribution of the GABAergic and glycinergic innervation did not differ among the different oculomotor subdivisions.

Introduction

GABA is the main inhibitory transmitter in the vestibular pathways to the oculomotor complex of several species (Lanoir et al. 1982; De La Cruz et al. 1992; Spencer and Baker 1992; Spencer et al. 1992; Wentzel et al. 1995) . The larger part of this GABAergic input is mainly derived from the superior vestibular nucleus (SVN), which seems to subserve vestibular control over all subdivisions of the oculomotor nucleus (OMN) except the medial rectus (MR) motoneurons (Baker and Highstein 1978; Wentzel et al. 1995). Inhibitory vestibular control over MR motoneurons is maintained through a projection from inhibitory medial vestibular nucleus (MVN) neurons to internuclear cells located in the ipsilateral abducens nucleus (AN) which, in turn, excite the contralateral MR motoneurons (Baker et al. 1975; Baker and Highstein 1978; Highstein and Baker 1978) . The inhibitory neurotransmitter involved in this projection to the abducens nucleus is probably glycine (Spencer et al. 1989; Spencer and Baker 1992). Thus, while the inhibitory input to oculomotor nucleus and trochlear nucleus is predominantly GABAergic (Spencer et al. 1989; De La Cruz et al. 1992; Wentzel et al. 1993 en 1995) , the abducens nucleus (AN) contains more glycinergic than GABAergic boutons (Spencer et al. 1989) .

Contradictory results have been reported with respect to the distribution of the GABAergic innervation among the different oculomotor subdivisions of the cat. Spencer et al. (Spencer and Baker 1992) reported a lack of GABA in the MR subdivision, whereas De La Cruz et al.

(De La Cruz et al. 1992) showed that the density and the postsynaptic distribution of the GABAergic terminals in the MR region in this species was not significantly different from other OMN subdivisions. In the present study we investigated and compared the GABAergic and glycinergic input to the different subdivisions of the rabbit OMN at the ultrastructural level using postembedding immunocytochemistry. Special emphasis was put on the MR subdivision, the motoneurons of which were retrogradely labeled with horseradish peroxidase (HRP) from the MR muscle.(De Zeeuw 1989)

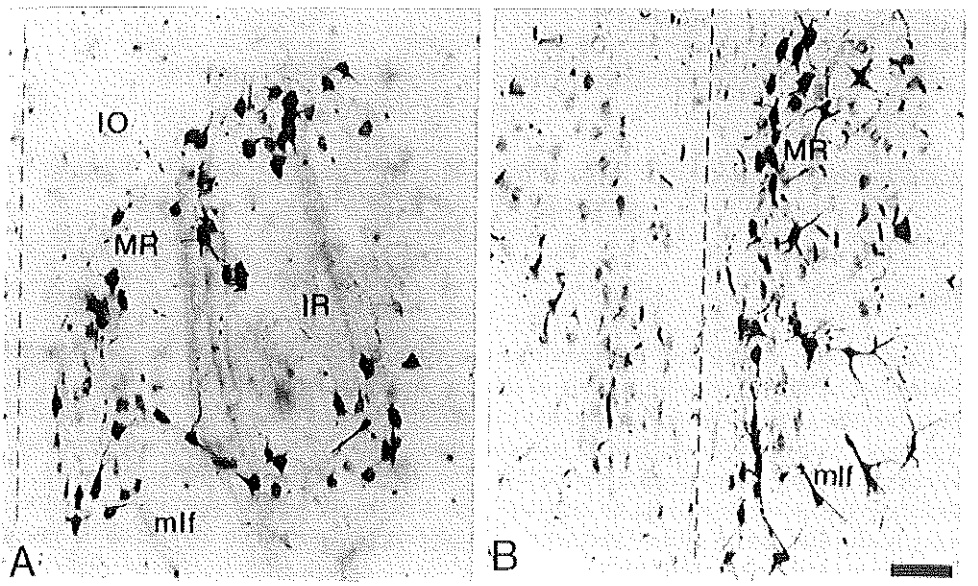

Figure 1. A: caudal part of the OMN showing the dorsolateral to ventromedial and the ventrolateral to ventromedial bands of MR motoneurons, labeled with HRP from the contralateral MR muscle. B: Photomicrograph of the more rostral part of the OMN showing the dorsolateral to ventromedial band of HRP-labeled MR motoneurons. Bar equals 0.1 mm.

Material and methods

HRP was injected in the MR muscle of four adult pigmented Dutch belted rabbits in order to demarcate the MR motoneurons that are typically distributed between the IR and the IO and SR subdivisions (Akagi 1978; Murphy et al. 1986) . Subsequently, the tissue was processed for electron microscopy and treated for GABA and glycine immunocytochemistry.

General anaesthesia was maintained with a halothane-oxygen mixture (Fluotec Mark II) administered through an endotracheal tube. The MR muscle was injected with 1-5 µl of HRP (30% in saline Miles) with the use of a Hamilton syringe. We reduced the spread of HRP by isolating the muscle or by reducing the volume injected (for details see (Murphy et al. 1986)). After a survival time of 2 days, the animals were premedicated with Hypnorm (0.25 ml/kg ip.), deeply anaesthetized with sodium pentobarbital (Nembutal; 60 mg/kg ip.), and perfused with 100 ml saline followed by 2 liters of 5% glutaraldehyde in PB. The dissected brains were kept in the same fixative for one hour. Tissue containing the OMN was cut on a vibratome in 70 µm sections. These sections were collected in PBS, incubated with TMB and H_2O_2, stabilized

with DAB in a cobalt solution(Lemann et al. 1985; De Zeeuw et al. 1988) , rinsed in a glucose solution in PB, postfixed for one hour in 1% osmium tetroxide in the same solution, and rinsed in PB and distilled water. Subsequently, these sections were chemically dehydrated with acidified dimethoxy-propane (Muller and Jacks 1975) , and flat embedded in araldite on slides between polyethyleen foils. Ultrathin sections were cut from pyramids of the OMN on a Reichert ultratoom, and mounted on formvar coated nickel grids. The grids were rinsed in a solution of Tris buffer containing 0.9% NaCl and 0.1% Triton-X100 at pH 7.6 (TBST), and incubated overnight in a droplet of GABA (1:2000 in TBST) or glycine (1:250 in TBST) antiserum. The GABA and glycine antisera were generously supplied by Dr. R.M. Buijs and Dr. R.J. Wenthold, respectively (for specificity tests see (Seguela et al. 1984; Wenthold et al. 1987)). The next day the grids were rinsed twice with TBST and stored for 30 minutes in the rinsing solution. After rinsing with TBST (pH 8.2) the grids were incubated for one hour in a droplet of goat anti rabbit IgG labeled with 15 nm gold particles (Janssen Pharm.) diluted 1: 25 in TBST. Subsequently, the grids were rinsed with TBST (pH 7.6) and distilled water, counterstained with uranyl acetate and lead citrate, and examined in a Philips electron microscope.

Pyramids of the OMN ipsilateral to the injected eye muscle were made. The tissue was cut in such a way that they contained besides the MR subdivisions also either the IR or IO subdivision, or both the IO and SR subdivisions. For each OMN, ultrathin sections were made from at least 2 pyramids of different rostrocaudal levels. At least 2 non-serial ultrathin sections from each pyramid were analyzed for GABA-immunoreactivity, while another nonserial section of these pyramids was analyzed for glycine-immunoreactivity. The boundaries of the respective subdivisions were determined from photographs of semithin (2μm) sections. To avoid mistakes in the identification of the borders of the subdivisions we used a safety margin of approximately 100 μm from each border in which no terminals were counted for analysis. Within the boundaries we randomly sampled the presence of GABA and glycine immuno-positive terminals as well as the nonlabeled terminals. A terminal was considered GABA positive or glycine positive, when the number of gold particles overlying it was at least eight times higher than the number of particles overlying an equal surface area of surrounding nonlabeled structures (for details about counting procedure see (De Zeeuw et al. 1989; Holstege and Bongers 1991)).

Results

Injection of HRP in the MR muscle resulted in strong labeling of motoneuronal cellbodies throughout the rostrocaudal extent of the ipsilateral OMN. The HRP labeled cells in the caudal part of the OMN were arranged in an oblique band that extended from the ventromedial to the dorsolateral borders of the OMN (Fig. 1A). In addition, many labeled cells were found among the fibres of the medial longitudinal fasciculus (MLF). More rostrally, the labeled motoneurons approximate the midline of the OMN (Fig. 1B). The semithin sections used to delineate the boundaries between the oculomotor subdivisions showed the same pattern.

GABA immunopositive terminals ranged from 1 to 4 μm in diameter and contained many mitochondria. Their vesicles were predominantly flattened and their synaptic contacts were usually multiple and symmetric (F-type (Holstege and Calkoen 1990), Fig.2). In all oculomotor subdivisions approximately 50% of the encountered axon terminals were GABA positive (Table 1). No major differences were observed between the subdivisions.In all subdivisions, the GABAergic terminals were similarly distributed within the neuropil: on average 9.5% of the terminals contacted somata, and 20.2% and 70.3% contacted proximal and distal dendrites, respectively (Tables 2 and 3; Fig. 2). Sometimes, the GABAergic and nonGABAergic

terminals were apposed to spines that arose from cellbodies or dendrites (Fig.3).These spines sometimes showed accumulations of clear and coated vesicles in the spine head and the interneuronal cleft was unusually straight.

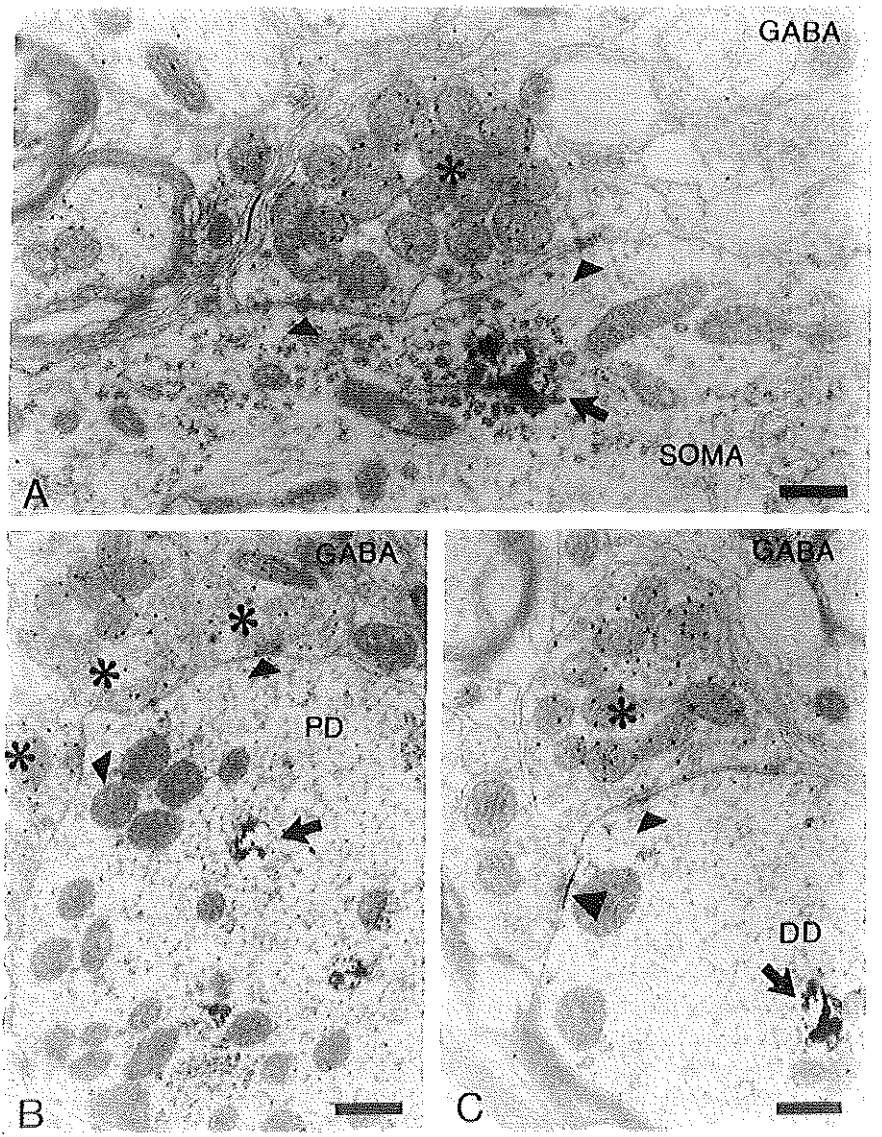

Figure 2. Electron micrographs of GABA positive axon terminals adjacent to HRP labeled structures in the MR region of the OMN. Gold particles (diameter equals 15nm) indicate the presence of GABA. Synaptic contacts and HRP/DAB-Cobalt reaction product are indicated by arrowheads and arrows, respectively. The axon terminals contain small flattened vesicles and establish symmetric synaptic contacts. Panel A illustrates a contact with a soma; B demonstrates a contact with a large proximal dendrite (PD) and C a synaptic contact with a small dendrite(DD). Bar equals 0.5μm.

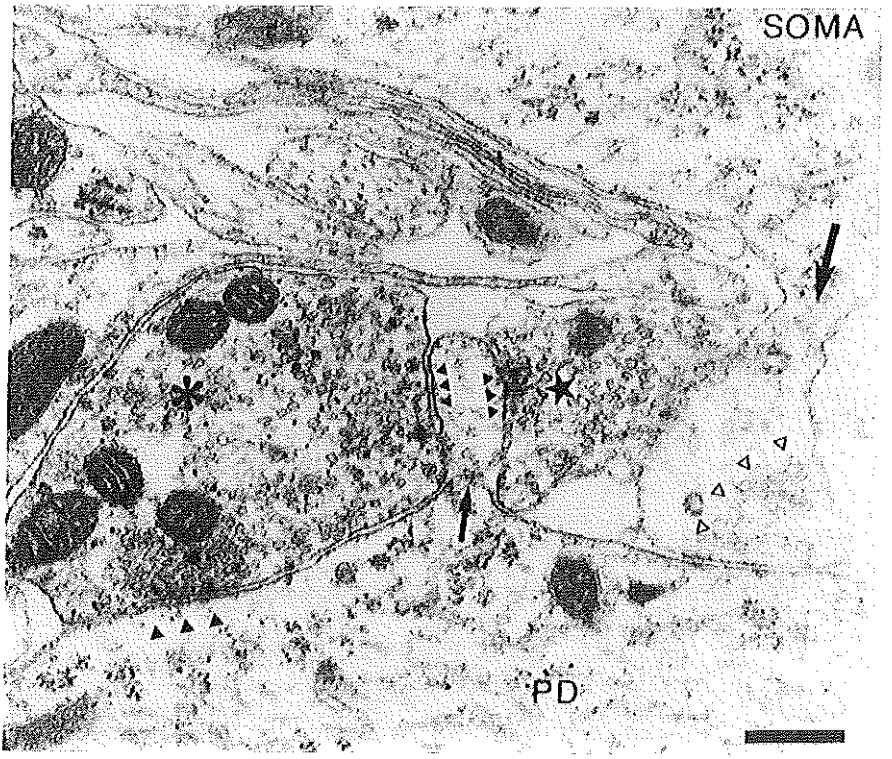

Figure 3. Electron micrograph of a GABA positive terminal (asterisk) and an unlabeled terminal (star) that make synaptic contacts (arrowheads) with an unlabeled proximal dendrite (PD) and dendritic spine (small arrow) respectively. The large arrow indicates the entrance of a somatic spine that is apposed to the same PD. The open arrowheads point at the vesicles accumulated in the spinehead. Bar equals 0.5 μm.

In all subdivisions only few terminals were glycinergic (3% to 5%; Table 1). The labeling of the glycinergic profiles differed somewhat from that of the GABAergic profiles.The glycinergic axons usually contained a larger number of gold particles than the GABAergic axons, while the glycinergic terminals usually carried fewer gold particles. The morphological characteristics of the glycinergic and GABAergic profiles were similar. The glycine immuno-reactive terminals in the OMN were small to medium sized F-types with many mitochondria and symmetric synaptic contacts (Fig. 4). In all subdivisions the distribution of the glycinergic terminals in the neuropil was similar to that of the GABAergic terminals (Tables 2 and 3). Most of the glycinergic terminals were apposed to distal dendrites (74.5%), while 9.6% and 16.9% were apposed to somata and proximal dendrites, respectively.

To find out whether different neurons within the MR subdivision may receive different inputs we compared the synaptic input of motoneurons retrogradely labeled with HRP from the MR muscle with that of nonlabeled neurons in the MR subdivision. The percentage of GABA positive (43.3%) and glycine positive (7.7%) boutons contacting motoneurons (n = 50) that were retrogradely labeled with HRP was similar to that of GABA positive (39.7%) and

glycine positive (6.4%) boutons contacting nonlabeled somata (n = 50). These data suggest that the synaptic input of motoneurons is not substantially different from that of the non-motoneurons of the oculomotor complex.

Table 1.

Percentages of immunopositive and immunonegative, GABAergic (A) and glycinergic (B) terminals (n = 4878) in different oculomotor subdivisions.

A	GABA+ (%)	GABA- (%)	*B*	gly+ (%)	gly- (%)
MR	48	52	MR	4	96
IR	52	48	IR	5	95
IO	45	55	IO	3	97
SR	49	51	SR	4	96

Table 2.

Postsynaptic distribution of randomly sampled GABA and glycine immunoreactive terminals.

			Soma (%)	Prox.dendr. (%)	Dist.dendri. (%)
MR	GABA+	n = 348	9.8	18.8	71.4
	GABA-	n = 416	9.6	26.0	64.4
	glycine+	n = 15	6.7	13.3	80.0
	glycine-	n = 364	9.9	20.0	70.1
IR	GABA+	n = 416	8.7	20.7	70.6
	GABA-	n = 384	7.3	15.6	77.1
	glycine+	n = 23	4.3	17.4	78.3
	glycine-	n = 443	9.0	22.1	68.9
IO	GABA+	n = 360	9.4	17.3	73.3
	GABA-	n = 440	9.3	24.1	66.6
	glycine+	n = 13	15.4	15.4	69.2
	glycine-	n = 361	8.3	22.7	69.0
SR	GABA+	n = 392	10.2	24.0	65.8
	GABA-	n = 408	11.7	21.6	66.7
	glycine+	n = 17	11.8	17.6	70.6
	glycine-	n = 396	9.6	22.0	68.4

It was determined for each oculomotor subdivision what percentage of the terminals contacted somata, and proximal or distal dendrites.

Table 3.
Presynaptic distribution of randomly sampled somata and proximal dendrites in the different oculomotor subdivisions

A		GABA+(%)	GABA-(%)	B	gly+(%)	gly-(%)
MR	soma	43.3	56.7		7.7	92.3
	prox.den	48.5	51.5		13.2	86.8
IR	soma	38.8	61.2		5.4	94.6
	prox.den	62.4	37.6		11.2	88.8
IO	soma	41.2	58.8		4.7	95.3
	prox.den	70.6	29.4		14.7	85.3
SR	soma	44.5	55.5		10.2	89.8
	prox.den	66.6	33.4		10.3	89.7

In the sections processed for GABA (A) and glycine (B) immunocytochemistry of each subdivision we analyzed the terminals on 50 somata and 100 proximal dendrites. Note that the GABAergic and glycinergic groups of terminals each total 100%.

Figure 4. Electron micrograph of a glycine positive axon terminal contacting a HRP labeled proximal dendrite. Gold particles indicate the presence of glycine. Arrowheads indicate synaptic contacts; arrows indicate HRP/DAB-Cobalt reaction product. Bar equals 0.5 μm.

Discussion

In the present study we demonstrated that MR motoneurons of the rabbit oculomotor nucleus receive a prominent GABAergic input. The density and postsynaptic distribution of the GABAergic innervation in the medial rectus motoneuron group was similar to that of the other subdivisions of the OMN, and the morphological characteristics of the GABAergic synaptic endings in the different subdivisions were the same. These observations are in agreement with the findings of De La Cruz et al. (De La Cruz et al. 1992) in the oculomotor nucleus of the cat.

The major source of the GABAergic innervation of the MR region remains to be determined. The SVN gives rise to a small part of the GABAergic input to the MR subdivision (Wentzel et al. 1995) , but it has not been established whether these terminals innervate MR motoneurons, MR interneurons or dendrites of motoneurons of adjacent subdivisions that extend into the MR subdivision. The direct input from the SVN to MR motoneurons is probably not very substantial since IPSP19s have not been found in MR motoneurons following vestibular stimulation (Baker and Highstein 1978; Highstein and Reisine 1979). Possible sources for the GABAergic input to the MR motoneurons include the pontine and mesencephalic reticular formation, prepositus hypoglossi nucleus and oculomotor internuclear neurons (for review see (Barmack et al. 1989; De La Cruz et al. 1992) .

An extensive immunocytochemical, electrophysiological-pharmacological and autoradiographic study in the cat (Spencer et al. 1989) has revealed that glycine is utilized in inhibitory connections from the prepositus hypoglossi nucleus, parts of the reticular formation and second-order vestibular neurons to the abducens nucleus. Thus, while GABA may control mainly the inhibition of vertical eye movements, glycine is probably the major inhibitory transmitter of horizontal eye movements. In agreement with studies in the cat and monkey (Spencer et al. 1989; Spencer and Baker 1992) , the OMN of the rabbit contains only few glycine-immunoreactive boutons. In contrast to these studies however, the glycine-immunoreactive boutons in the OMN of the rabbit are distributed through all subdivisions including the MR region. The source and functional implications of this innervation remains unclear. Considering the low occurrence of glycine in the OMN, and the fact that most, if not all, of the glycine immunoreactive terminals also contain GABA (Wentzel et al. 1993 and 1996), we cannot exclude the possibility that the function of glycine is merely metabolic.

IIb. Colocalization of gaba and glycine in the oculomotor nucleus

Abstract

In the present study we examined the possible colocalization of the inhibitory neurotransmitters glycine and GABA in the oculomotor nucleus of the rabbit. Serial sections were processed alternately for glycine and GABA postembedding immunocytochemistry. Ultrastructural analysis revealed that all terminals that showed glycine positive immunoreactivity were also GABA positive; up to 5% of the GABA positive terminals were also glycine positive.

Introduction

GABA and glycine are the inhibitory neurotransmitters in the oculomotor nucleus (OMN). The presence of GABA within terminal profiles in the OMN and trochlear nucleus (TN) has been established with uptake of tritiated GABA (Lanoir et al. 1982) and immunocytochemistry in several species (Wentzel et al. 1989; De La Cruz et al. 1992).The GABAergic input to the inferior oblique, inferior rectus and superior rectus subdivisions of the OMN is dense (Wentzel et al. 1989; De La Cruz et al. 1992; Spencer and Baker 1992; Spencer et al. 1992) . Reports on the density of the GABAergic input to the medial rectus subdivision are controversial. Spencer and colleagues reported for cat and monkey that this input is sparse (Spencer and Baker 1992; Spencer et al. 1992) , while De La Cruz et al. in the cat (De La Cruz et al. 1992) and Wentzel et al. in the rabbit (Wentzel et al. 1989) did not observe a difference between the medial rectus and other subdivisions. One source of the GABAergic input to motoneurons within the inferior oblique, inferior rectus and superior rectus subdivisions lies in the ipsilateral superior vestibular nucleus (SV) (Wentzel et al. 1989) . These anatomical findings confirmed the earlier physiological studies demonstrating that GABA antagonists like bicuculline and picrotoxin or lesions of the ipsilateral medial longitudinal fasciculus, block the inhibitory postsynaptic potentials in the OMN after stimulation of the vestibular nerve (Ito et al.1970; Obata and Highstein 1970; Precht et al. 1973; Ito et al.1976b). Glycinergic terminals are distributed throughout all subdivisions of the OMN in cat and rabbit, but compared to GABA this input is weak (Wentzel et al. 1990; Spencer and Baker 1992; Wentzel et al. 1993 and 1996) .The source of the glycinergic input to the OMN is unknown. Recent studies have shown that GABA and glycine coexist in terminals and somata in different parts of the nervous system (Ottersen et al. 1988; Yingcharoen et al. 1989; Walberg et al. 1990; Wentzel et al. 1990; Helfert et al. 1992; Proudlock et al. 1993). Whether glycine is active as a neurotransmitter where it is colocalized with GABA is not yet clear. In the present study, we tested the possibility of colocalization of GABA and glycine in the OMN.

Material and Methods

Tissue was collected from 4 adult pigmented Dutch-belted rabbits (1,8-2,3 kg). The animals were deeply anaesthetized with sodium pentobarbital (Nembutal; 60 mg/kg iv). Heparin and $NaNO_3$ were injected into the ear-vein to facilitate perfusion. The animals were perfused intracardially with 500 ml of 0.8% NaCl in phosphate buffer at pH 7.4 (PB) followed by 2 l of 5% glutaraldehyde in PB. The brains were removed and immersed in the same fixative for 1 h. The tissue containing the OMN was cut on a Vibratome in 70 μm sections. The

sections were collected in PB, rinsed in 8% glucose in PB, postfixed for 1 h with 1% osmium tetroxide in 8% glucose in PB, and rinsed in PB and distilled water. Subsequently, they were chemically dehydrated with acidified dimethoxy-propane (Muller and Jacks 1975) , and flat embedded in Araldite between polyethyleen foil. Serial ultrathin sections were cut from pyramids of the OMN on a Reichert ultratome, and mounted on Formvar coated nickel grids. The grids were rinsed in a solution of Tris buffer containing 0.9% NaCl and 0.1% Triton-X100 at pH 7.6 (TBST), and incubated overnight in a droplet of GABA (1:3000 in TBST) or glycine (1: 250 in TBST) antiserum. The GABA and glycine antisera were generously supplied by Dr. R.M. Buijs and Dr. R.J. Wenthold, respectively (for specificity tests see refs (Seguela et al. 1984) and (Wenthold et al. 1987)). The grids were rinsed and then stored for 30 min in TBST (pH 7.6). After rinsing with TBST (pH 8.2) the grids were incubated for 1 h in a droplet of goat anti-rabbit IgG labeled with 15 nm gold particles (Janssen Pharm.) diluted 1:25 in TBST (pH 8.2). Finally the grids were rinsed in TBST (pH 7.6) and distilled water, counterstained with uranyl acetate and lead citrate, and examined in a Philips electron microscope.

From each animal, ultrathin sections were made from at least 3 pyramids at different rostrocaudal levels to include all subdivisions of the OMN. Of every set of two adjacent sections one was processed for GABA while the other was processed for glycine immunocytochemistry. Structures were considered GABA- or glycine-positive, (GABA(+) /gly(+)), when the number of overlying gold particles was at least 8 times higher than in an equal surface area of surrounding non-labeled structures (for details about counting procedure see (De Zeeuw et al. 1989; Holstege and Bongers 1991)).

Results

Examination of the ultrathin sections of the OMN showed glycine and GABA immunoreactivity in myelinated axons, preterminal axons and terminal profiles. While GABA was infrequently observed over dendrites and small somata, glycinergic neurons were not observed. The gly(+) terminals were equally distributed over the different subdivisions of the OMN. Glycine immunoreactivity was mostly present in small to medium sized (1.5- 2.5 mm) terminals filled with flattened vesicles and an abundancy of mitochondria (Figs. 1 and 2). The gly(+) terminals were mostly apposed to distal and proximal dendrites, only a few to somata. All observed synapses were symmetric. Remarkably, myelinated axons were labeled with gold particles much heavier than terminals. GABA(+) terminals were also distributed equally over the different subdivisions of the OMN. They were more variable in size than the gly(+) terminals with a diameter up to 3.5 μm. Most of the GABA(+) terminals contained flattened or pleiomorphic vesicles and showed symmetric synapses with dendrites and somata. In contrast to the gly(+) myelinated axons, the GABA(+) ones were not labeled heavily with gold particles. Comparison of adjacent sections treated for GABA and glycine immunocytochemistry showed that GABA positive profiles outnumbered those containing glycine by a factor 20. Within the OMN of the four rabbits a total of 234 glycinergic terminals were observed of which 183 were recovered in the adjacent section. Terminal profiles with glycine immunoreactivity in one section always showed GABA immunoreactivity in the adjacent section (Figs. 1 and 2). Thus, GABA and glycine coexist in all glycine positive terminals, but only a small proportion of the GABA(+) terminals contain glycine.

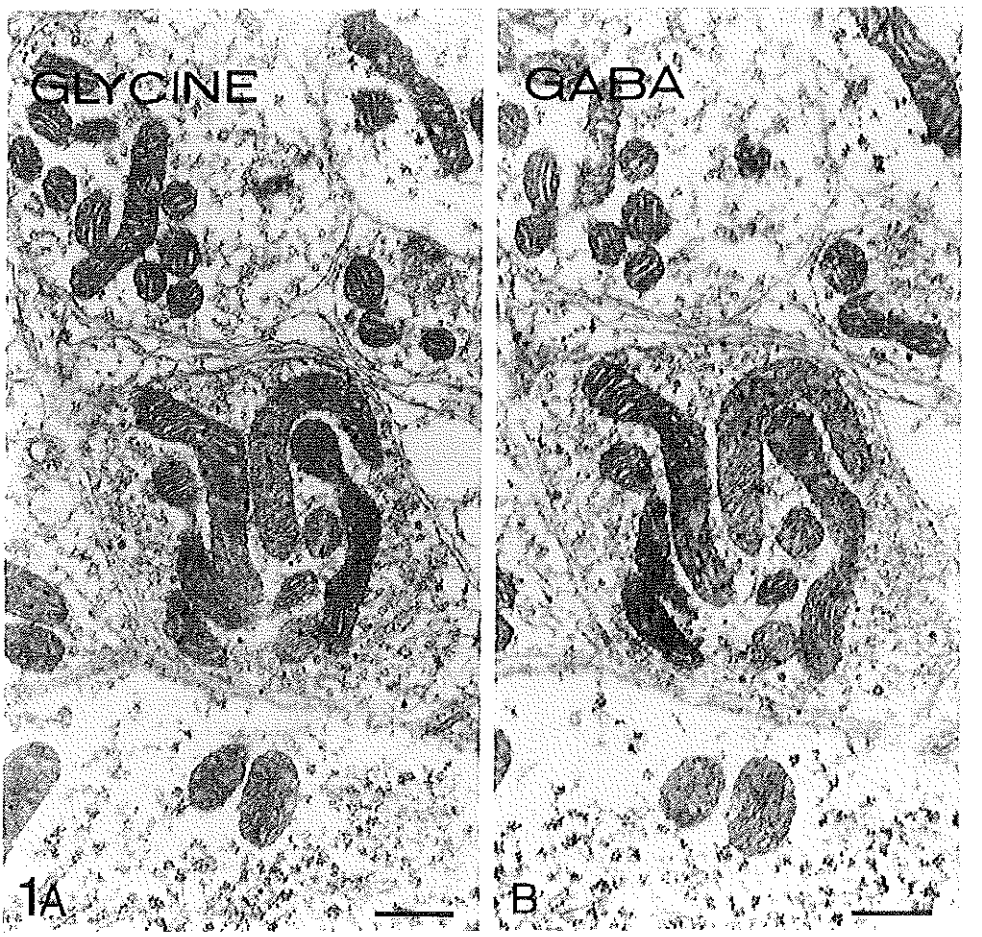

Figure 1. Electron micrographs of two adjacent sections through the OMN treated for glycine (A) and GABA (B) immunocytochemistry, respectively. A: gly(+) terminal apposed to a proximal dendrite in the IO subdivision of the OMN. B: the same terminal as in A after GABA immunocytochemistry. The surrounding terminals are neither gly(+) nor GABA(+). Bar = 0.4 μm.

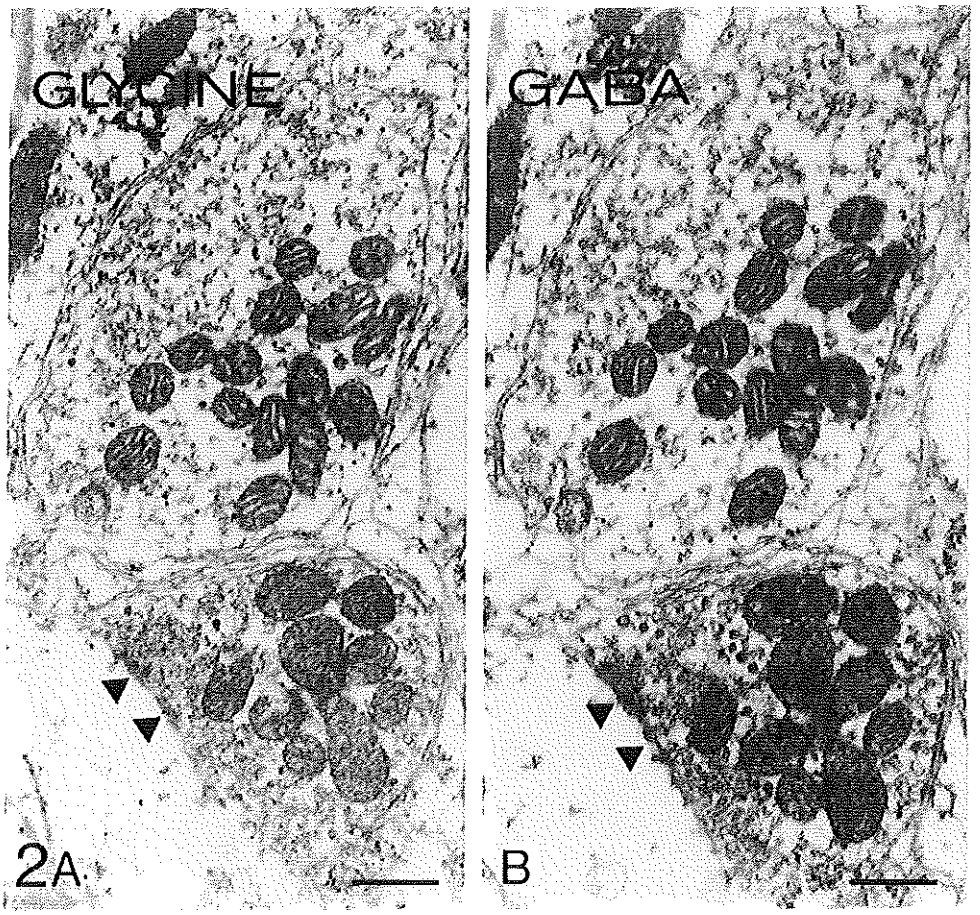

Figure 2. Electron micrographs of two adjacent sections through the OMN showing a gly(+) (A) and two GABA(+) (B) terminals in the MR subdivision. The gly(+) terminal contains flattened vesicles (A) and also shows GABA immunoreactivity (B). The non-glycinergic GABA(+) terminal (B) establishes symmetrical synapses (arrowheads) with a distal dendrite. Bar = 0,5 µm.

Discussion

A question emerging from the present study refers to the source of the continued GABA/ glycinergic input to the OMN. Analysis of the present material provided no evidence for the existence of glycinergic (inter)neurons in the OMN, indicating that the origin of the GABA/ glycinergic projection is most likely in brainstem nuclei. Data available from literature on the most important sources of OMN afferents make clear that not a single source can meet the criterion of having an inhibitory connection as well as colocalization of GABA and glycine. Of the four OMN related vestibular nuclei, the superior (SV), medial (MV) and descending (DV) nuclei and group Y, the SV can be excluded because glycinergic neurons have not been found in the SV (Walberg et al. 1990) and all terminals in the OMN from the SV have been found to be non-glycine immunoreactive (Wentzel et al. 1989). The MV and DV both contain neurons with colocalization of GABA and glycine (Walberg et al. 1990) and group Y contains GABA-ergic neurons (Mugnaini and Oertel 1985) ,but these nuclei can also be excluded since there is no evidence for an inhibitory component in their ascending connections (Ito et al. 1976b; Highstein and McCrea 1988). Similar arguments can be found against a localization of the GABA/glycine source in other OMN projecting nuclei.The prepositus hypoglossal nucleus (Prh) has a mixed population of GABA- and glycinergic neurons but no colocalization (Spencer et al. 1989; Yingcharoen et al. 1989; De Zeeuw et al. 1993), while the connections of the interstitial nucleus of Cajal (INC) with the OMN are excitatory (Schwindt et al. 1974; Nakao and Shiraishi 1985). The rostral interstitial nucleus of the medial longitudinal fasciculus (riMLF) remains a possible candidate since it shows an inhibitory (GABA) projection to the OMN (Wang and Spencer 1992 and 1996) but unfortunately the occurrence of glycine has not yet been studied in this nucleus.

IIc. Synaptic inputs to the oculomotor nucleus from the medial vestibular nucleus and superior vestibular nucleus

Abstract

Studies of the pathways involved in the vestibulo-ocular reflex have suggested that the projection from the superior vestibular nucleus to the ipsilateral oculomotor nucleus is inhibitory, whereas the medial vestibular nucleus, the abducens nucleus, and the contralateral superior vestibular nucleus most likely exert excitatory effects on oculomotor neurons. In order to determine directly the termination pattern and the neurotransmitter of these afferents, we studied their input to the oculomotor nucleus in the rabbit at the light microscopical level with the use of anterograde tracing of *Phaseolus vulgaris*-leucoagglutinin combined with retrograde tracing of horseradish peroxidase from the extraocular muscles, and at the ultrastructural level with the use of anterograde tracing of wheatgerm agglutinated horseradish peroxidase combined with GABA and glycine postembedding immunocytochemistry. The general ultrastructural characteristics of the neuropil and the types of boutons observed in the rabbit oculomotor nuclei are in general agreement with the descriptions for the oculomotor complex of other mammals.

The superior vestibular nucleus projected bilaterally to the superior rectus and inferior oblique subdivisions, and ipsilaterally to the inferior rectus and medial rectus subdivision; the medial vestibular nucleus projected bilaterally to the medial rectus, inferior oblique, inferior rectus and superior rectus subdivisions with a strong contralateral predominance. The abducens nucleus projected contralaterally to the medial rectus subdivision. More than 90% of all the anterogradely labeled terminals from the ipsilateral superior vestibular nucleus were GABAergic. These terminals were characterized by flattened vesicles and symmetric synapses, and they contacted somata, as well as proximal and distal dendrites of motoneurons. All terminals derived from the medial vestibular nucleus, the abducens nucleus, and the contralateral superior vestibular nucleus were non-GABAergic. These non-GABAergic terminals showed spherical vesicles and asymmetric synapses, and they contacted predominantly distal dendrites. None of the anterogradely labeled terminals from the studied vestibular nuclei or abducens nucleus were glycinergic.

The present study provides the first direct anatomical evidence that most, if not all, of the synaptic input from the superior vestibular nucleus to the ipsilateral oculomotor nucleus is GABAergic, and that the medial rectus subdivision is included in the termination area. Furthermore it confirms that the projections from the medial vestibular nucleus, the abducens nucleus, and the contralateral superior vestibular nucleus are exclusively non-GABAergic.

Introduction

Vestibular neurons that are involved in the vestibulo-ocular reflex (VOR) are mainly located in the medial vestibular nucleus (MV) and the superior vestibular nucleus (SV). Both nuclei contain the excitatory and inhibitory second-order relay neurons that link the afferents from the semicircular canals with (subsets of) motoneurons in the oculomotor complex; i.e. the oculomotor (OMN), trochlear (TN) and abducens (AN) nuclei (Büttner and Büttner-Ennever 1988; see also: Büttner-Ennever 1992;).

A comparison of the literature on the projection of vestibular neurons to motoneuron pools which innervate the individual eye muscles shows that the experimental data obtained on the MV are in general agreement. Excitatory second-order relay neurons of the MV (for the subdivision of the MV see (Epema et al. 1988)) can be activated by all semicircular canals.

Neurons responsive to anterior and posterior canal stimulation are located preferentially in the parvo-cellular MV (MVpc) (Highstein and Ito 1971; Highstein 1973a; Ito et al. 1976a; Uchino et al. 1981; Uchino et al. 1982; McCrea et al. 1987a). Neurons that respond to signals from the horizontal canal have a more wide-spread distribution and are located in all parts of the MV: i.e. the MVpc, the caudal MV (MVc), and the magnocellular MV (MVmc) (Highstein 1973a; Uchino et al. 1982; McCrea et al. 1987b) .

The inhibitory second-order relay cells in the MV can only be activated by the horizontal semicircular canal and are located between the excitatory horizontal canal neurons (Highstein 1973b; Uchino and Suzuki 1983; McCrea et al.1987b). There is strong physiological and anatomical evidence that these neurons mediate their inhibitory control by a glycinergic projection to the ipsilateral AN (Spencer et al. 1989), which in turn exerts an excitatory effect on contralateral MR motoneurons (Baker and Highstein 1975; Highstein and Baker·1978) .

Thus, the ascending connections of the MV to the TN and OMN may be considered to carry exclusively excitatory signals, with either aspartate or glutamate as neurotransmitter (Demêmes and Raymond 1982; Kevetter and Hoffman 1991). Different anatomical studies agree on a predominantly crossed, but bilateral projection through the medial longitudinal fascicle (mlf) to the superior oblique (SO), inferior rectus (IR), medial rectus (MR), inferior oblique (IO) and superior rectus (SR) motoneuron pools (McMasters et al. 1966; Tarlov 1970; Carleton and Carpenter 1983; Thunnissen 1990). The SR subdivision seems to receive the weakest contribution of all (McMasters et al. 1966; Thunnissen 1990). The same authors also agree on a, most likely, excitatory (Highstein and Reisine 1979) projection to the contralateral AN from all MV subnuclei.

In contrast to the general agreement on the MV, the literature on the SV reveals a clear discrepancy between anatomical and physiological data. The excitatory second-order neurons in the SV receive afferents mainly from the anterior canal and project to the contralateral OMN by way of the brachium conjunctivum Highstein and Ito 1971; Yamamoto et al. 1978; Uchino et al. 1982; Hirai and Uchino 1984). Neurons with similar connections with the contralateral OMN are located in the dorsal group Y (Sato and Kawasaki 1987). Although Tarlov (Tarlov 1970) reported SV termination throughout the OMN, later studies showed that the terminals were restricted to the IO and SR subdivisions (Carpenter and Cowie 1985a; Thunnissen 1990).

The inhibitory second-order neurons in the SV have a more medial location in the central part of this nucleus. They can be activated by both the anterior and posterior canals and their axons ascend in the ipsilateral mlf (Highstein and Ito 1971; Ito et al. 1976b; Uchino and Suzuki 1983). Whereas termination of this pathway on the SO motoneurons of the TN (Tarlov 1970; Carpenter and Cowie 1985a; Thunnissen 1990) is not disputed, the distribution of terminals in the OMN is not agreed upon. The IO (McMasters et al. 1966) and IR (McMasters et al. 1966; Carpenter and Cowie 1985a) subdivisions undoubtedly receive input from the SV but Tarlov(Tarlov 1970) and more recently Thunnissen(Thunnissen 1990) demonstrated a more extensive distribution including the SR and MR subdivisions. In particular the SV projection to MR motoneurons is peculiar since horizontal canal relay neurons have not been reported from the SV and a direct inhibitory input violates the general opinion that MR inhibition is relayed exclusively through the AN.

Electrophysiological studies in combination with lesion and selective blocking techniques have suggested GABA as the neurotransmitter in the inhibitory pathways relaying the vertical components of the vetibulo-ocular reflex (Ito et al. 1970; Obata and Highstein 1970; Precht et al. 1973). This suggestion was confirmed by immunocytochemical and autoradiographic studies that showed the presence of GABA in axon terminals in the OMN (Lanoir et al. 1982; Soghomonian et al. 1989; De La Cruz et al. 1992; Spencer and Baker 1992). Recently we studied the distribution of GABA and glycine in the OMN and our expectation to find a

difference between MR and other subdivisions was not confirmed, e.g. the abundance of GABAergic terminals in the MR subdivision was similar to that in other motoneuron subdivisions (Wentzel et al. 1993).

Direct anatomical evidence of the presynaptic distribution of GABA in identified axon terminals is not available. Therefore, the present study combines anterograde tracing from the SV, MV and AN with GABA and glycine immuno-cytochemistry and quantification of terminals in the OMN at the ultrastructural level, to determine whether or not (i) the ipsilateral SV projection to the OMN is completely GABAergic, (ii) the MV, AN and crossed SV projections to the OMN are non GABAergic, (iii) the GABAergic terminals in the MR subdivision have an origin in the SV. The projections were studied simultaneously with lightmicroscopy.

Material and Methods

The terminal distribution of projections from the SV, MV and AN to the OMN were studied at the light microscopical level with anterograde transport of *Phaseolus vulgaris* - leuco-agglutinin (PHA-L). To provide an anatomical basis for the localization of terminals in individual motoneuron groups, the animals were simultaneously injected with horseradish peroxidase (HRP) in the medial rectus eye muscle, which resulted in retrogradely labeling of MR motoneurons. For the ultrastructural analysis the OMN afferents were labeled with anterograde transport of wheatgerm-agglutinated horseradish peroxidase (WGA-HRP) which was combined with postembedding GABA or glycine immunocytochemistry.

Light microscopy

Surgery and perfusion. The animals were premedicated with Hypnorm (0.25 ml/kg, i.p.) and general anaesthesia was maintained with a halothane-oxygen mixture (Fluotec Mark II) administered through an endotracheal tube. After placement in a Wells rabbit head holder, the occipital bone was freed of neck muscles and the foramen magnum was enlarged to expose the brainstem. Injections were made under stereotactic guidance.

Before the transcardial perfusion, the animals were premedicated with Hypnorm (0.25 ml/kg, i.p.), intravenously injected with Heparin (0.5ml) and 1% $NaNO_2$, and deeply anaesthetized with Nembutal (200 mg/kg, i.p.).

Anterograde tracing of Phaseolus vulgaris-leucoagglutinin combined with retrograde tracing of horseradish peroxidase. Six adult Dutch belted rabbits (BMI Helmond, The Netherlands) were injected with PHA-L and HRP. Injections with PHA-L in the SV were combined with retrograde transport of HRP from the ipsilateral MR muscle; the PHA-L injections in the MV and AN were combined with retrograde transport of HRP from the contralateral MR muscle. Injections were made in the SV, MV, or AN with a glass micropipette (tip diameter 8-15 µm) filled with a solution of 2.5% PHA-L (Vector) in 0.05 M Tris-buffered saline (pH 7.4). The tracer was delivered by means of a positive current (4-8 µA), pulsed 7 sec on - 7 sec off, for a total of 30 min. Seven days later the animals were reanaesthetized and the MR muscle was injected with 1-5 µl of 20% HRP (Miles) in distilled water with a Hamilton syringe. The spread of HRP was reduced by isolating the muscle from the surrounding tissue and delivering the tracer in small deposits (Murphy et al. 1986) . After a survival time of two days, the animals were perfused with 0.5 l 0.8% NaCl in phosphate buffer (PBS) followed by 2 l PBS containing 0.5% depolymerized paraformaldehyde, 2.5% glutaraldehyde and 4% sucrose. The dissected brains were kept in the same fixative for 2 h, transferred to a 10% sucrose solution in PBS until they sank, and embedded in gelatine. The

embedded brains containing the OMN and injection sites were cryoprotected in a 30% sucrose solution in PBS, and cut in serial coronal sections (40 µm) on a freezing stage microtome.

To reveal the HRP, the sections were incubated with tetramethylbenzidine (TMB) and H_2O_2 in acetate buffer, rinsed in the same buffer, and stabilized with diaminobenzidine (DAB; Sigma) in a cobalt solution (Rye et al. 1984) . To visualize the PHA-L, the sections were subsequently rinsed in Tris-buffered saline (TBS, pH 7.6), incubated in goat-anti-PHA-L (Vector) in TBS containing Triton X-100 (TBST pH 7.6), rinsed in TBST, incubated in rabbit-anti-goat IgG (Sigma) in TBST, rinsed in TBST, and incubated in goat PAP (Nordic) in TBST (Gerfen and Sawchenko 1984) . The sections were rinsed in Tris-HCl and incubated with DAB and H_2O_2 in Tris-HCl. After a final rinsing in Tris-HCl, the sections were mounted, counterstained with cresyl violet and coverslipped. Injection sites are illustrated in figures 1 and 2.

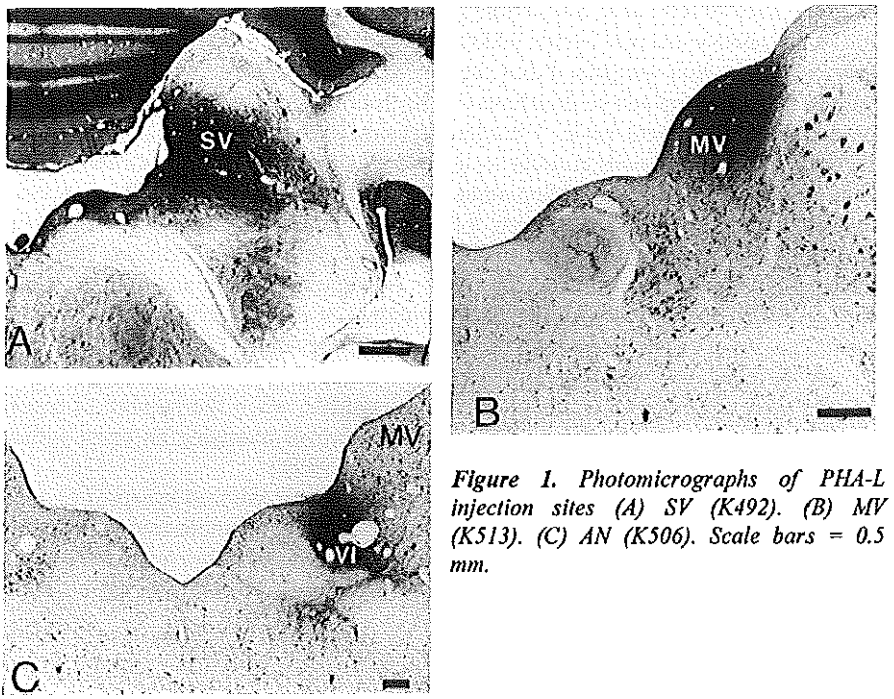

Figure 1. Photomicrographs of PHA-L injection sites (A) SV (K492). (B) MV (K513). (C) AN (K506). Scale bars = 0.5 mm.

Electron Microscopy

Anterograde tracing of wheatgerm-agglutinated horseradish peroxidase. The experiments were carried out in 10 adult pigmented Dutch belted rabbits. Unilateral WGA-HRP injections were made in the SV (five cases), the MV (three cases) and the AN (two cases). Injections of 0.2 µl 7% WGA-HRP in saline were made with a glass micropipette (tip diameter 25-30 µm) connected to a hydraulic system. After a survival time of three days the animals were perfused with 0.1 l saline followed by 2 l PBS with 5% glutaraldehyde. Tissue containing the injection sites was processed for light microscopy as described for HRP. Injection sites are illustrated in figure 1. Tissue containing the OMN was cut on a vibratome in 70 µm sections. These sections were collected in PBS, incubated with TMB and H_2O_2, stabilized with DAB in a cobalt

solution (Lemann et al. 1985; De Zeeuw et al. 1988), rinsed in 30% glucose PBS, postfixed for 1 h in 1% osmium tetroxide in the same glucose PB solution, and rinsed in PBS and distilled water. The sections were chemically dehydrated with acidified dimethoxy-propane (Muller and Jacks 1975), and flat embedded in araldite on coverslips between polyethyleen foils. Ultrathin sections were cut from pyramids of the OMN on a Reichert ultratome, and mounted on formvar coated nickel grids.

GABA and glycine immunocytochemistry. The grids were rinsed (TBST), and incubated overnight in a droplet of GABA (1:3000 in TBST) or glycine (1:250 in TBST) antiserum. The GABA and glycine antisera were generously supplied by Dr. R.M. Buijs and Dr. R.J. Wenthold, respectively (for specificity tests see (Seguela et al. 1984; Wenthold et al. 1987)). The grids were rinsed twice in TBST and stored for 30 minutes in the rinsing solution. After rinsing with TBST (pH 8.2) the grids were incubated for 1 h in a droplet of goat-anti-rabbit IgG labeled with 15 nm gold particles (Janssen Pharm.) diluted 1:25 in TBST (pH 8.2). Finally, the grids were rinsed twice with TBST (pH 7.6) followed by distilled water, counterstained with uranyl acetate and lead citrate, and examined in a Philips electron microscope.

Data collection and analysis. From the SV and MV experiments separate pyramids were made of the ipsilateral and contralateral OMN; from the AN experiments pyramids of only the contralateral OMN were made. Ultrathin sections were made from at least 3 pyramids of different rostrocaudal levels in order to include all subdivisions of the OMN. At least 2 non-serial ultrathin sections from each pyramid were processed for GABA immunocytochemistry, while another section was processed for glycine immunocytochemistry. Following random sampling of WGA-HRP labeled terminals the proportion of the terminals that were double labeled for WGA-HRP and GABA or glycine was determined. In addition, the percentage of both double and single WGA-HRP labeled terminals making synaptic contacts with either somata, proximal dendrites, or distal dendrites was determined (Table 1).

A terminal was considered: i) WGA-HRP labeled, when crystalline electron dense deposits were observed; ii) GABA- or glycine-positive, when the number of overlying gold particles was at least eight times higher than the number overlying an equal surface area of surrounding non-labeled structures (De Zeeuw et al. 1989) ;
iii) axosomatic, when there was direct apposition (with or without synapse) to a neuron; iv) axodendritic when apposed to proximal or distal dendrites, characterized by a diameter larger or smaller than 5 μm, respectively. The data for each type of information were averaged over all ultrathin sections and the standard error of the means (SEM) was calculated. Statistical analysis was performed with Student's t-test.

Results

Light Microscopy

Injection of HRP in the MR muscle of all six rabbits resulted in strong and similar labeling of cell bodies throughout the rostrocaudal plane of the ipsilateral OMN (Fig. 3). Most caudally in the OMN, many labeled cells were found interspersed between the fiber bundles of the mlf. As seen in transverse sections, the majority of HRP labeled neurons were arranged in an oblique band extending from the ventromedial to the dorsolateral border of the OMN. In rostral direction this band progressively approached the midline. This characteristic distribution of MR motoneurons agrees well with the findings of Akagi (Akagi 1978) and Murphy et al. (Murphy et al. 1986) and provides a clear separation between the ventrally located IR and the dorsally located IO and SR subdivisions (Akagi 1978; Murphy et al. 1986) (Figs 3, 4).

A

		Total number of WGA-HRP-labeled terminals	WGA-HRP+ GABA double labeled	WGA-HRP single labeled
SV	ipsi	1556	92.5±1.5	7.5±1.5
	contra	1297	2.1±0.5	97.9±0.5
MV	ipsi	1019	7.9±3.5	92.1±3.5
	contra	820	---	100
AN	contra	500	---	100

Table 1. Distribution of single wheat-germagglutinated horseradish peroxidase and double wheatgerm-agglutinated horse-peroxidase GABA-labeled terminals in the oculomotor nucleus (A). In (B) the proportional distribution of labeled terminals contacting distal (dd) or proximal dendrites (pd), or cell bodies (cb) is indicated (± values are SEM).

B

		WGA-HRP + GABA labeled			WGA-HRP labeled		
		dd	pd	cb	dd	pd	cb
SV	ipsi	60.9+1.7	35.7+3.7		3.4+1.1	61.4+0.6	35.5+0.4 3.1±0.2
	contra	---	---	---	85.0+1.9	13.0+1.9	2.0+0.2
MV	ipsi	60.3+1.5	34.4+1.8		5.3+0.4	71.6+1.6	22.3+1.7 6.1+0.2
	contra	---	---	---	81.4+0.5	13.8+0.6	4.8+1.0
AN	contra	---	---	---	79.6+0.6	15.7+0.4	4.7+0.3

Projections from the superior vestibular nucleus. The results in the two cases (K489, K492) with PHA-L injections including the central and rostral parts of the SV were very similar. The injection site of K492 is illustrated in Fig. 1A and 2A. Labeled axons were seen to ascend ipsilaterally in the lateral aspect of the medial longitudinal fascicle (mlf) entering the OMN at caudal levels. The termination in the TN was almost completely ipsilateral. The termination in the ipsilateral OMN was mainly in the IR, IO and SR subdivisions, but a small number of terminals was found in the MR subdivision (Figs 3, 4). PHA-L labeled boutons were often seen in the vicinity of of HRP labeled neurons (Figs 3, 4). Furthermore labeled fibers from the SV were observed in the brachium conjunctivum. Their termination was nearly completely restricted to the IO and SR subdivisions of the contralateral OMN, although a few terminal ramifications were located in the MR subdivision. Some terminal ramifications were present in the midline between the rostral poles of the OMN (Fig. 3).

Projections from the medial vestibular nucleus. The injection sites of the experiments with PHA-L in the MV (K512, K513) were located primarily in the MVc and MVpc, with the boundaries touching MVmc. The injection site of K513 is illustrated in figures 1B and 2A. In both cases labeled fibers from the MV were observed in the compact medial part of the contralateral mlf. A few labeled fibers were present in the lateral part of the ipsilateral mlf. The termination area was extensive on the contralateral side and included the TN and all subdivisions of the OMN. The SR region received the smallest number of terminals (Fig. 3). Ipsilaterally, the termination was concentrated in the MR subdivision with some spread into the adjacent IO and IR regions and an occasional terminal ramification in the SR.

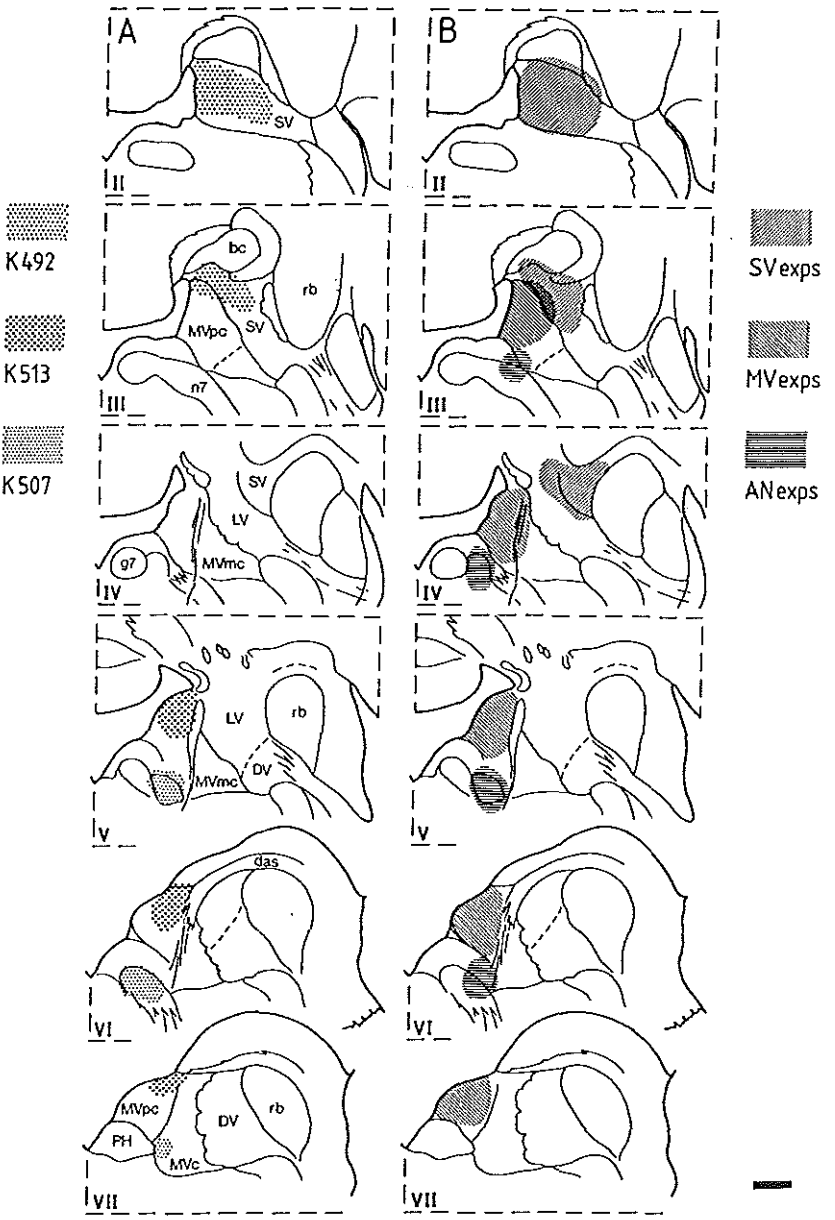

Figure 2. (A) Diagrammatic representation of transverse sections through the vestibular nuclei (VN) showing the extent of the injection sites of PHA-L in the SV (K492), MV (K513) and AN (K507) used for light microscopic analysis of the OMN projections. (B) Diagrammatic representation of transverse sections through the vestibular nuclei showing the total area covered by the injections of WGA-HRP in the SV (five animals), MV (five animals) and AN (two animals) used for electron microscopic analysis. bc, brachium conjunctivum; das, dorsal acoustic striae; DV, descending vestibular nucleus; g7, facial genu; LV, lateral vestibular nucleus; Scale bar = 0.5 mm. The diagrams of the vestibular nuclei were adopted from Epema 1990.

Projections from the abducens nucleus. The PHA-L injections aimed at the AN (K506 and K507) were situated in the centre of this nucleus with some spread of tracer into the MV. The injection site of K507 is illustrated in figures 1C and 2A. The labeled AN fibers ascended exclusively through the contralateral mlf. Figure 5 shows a large number of PHA-L labeled terminals in the MR region, a moderate number in the IR and few in either the SR or IO region.

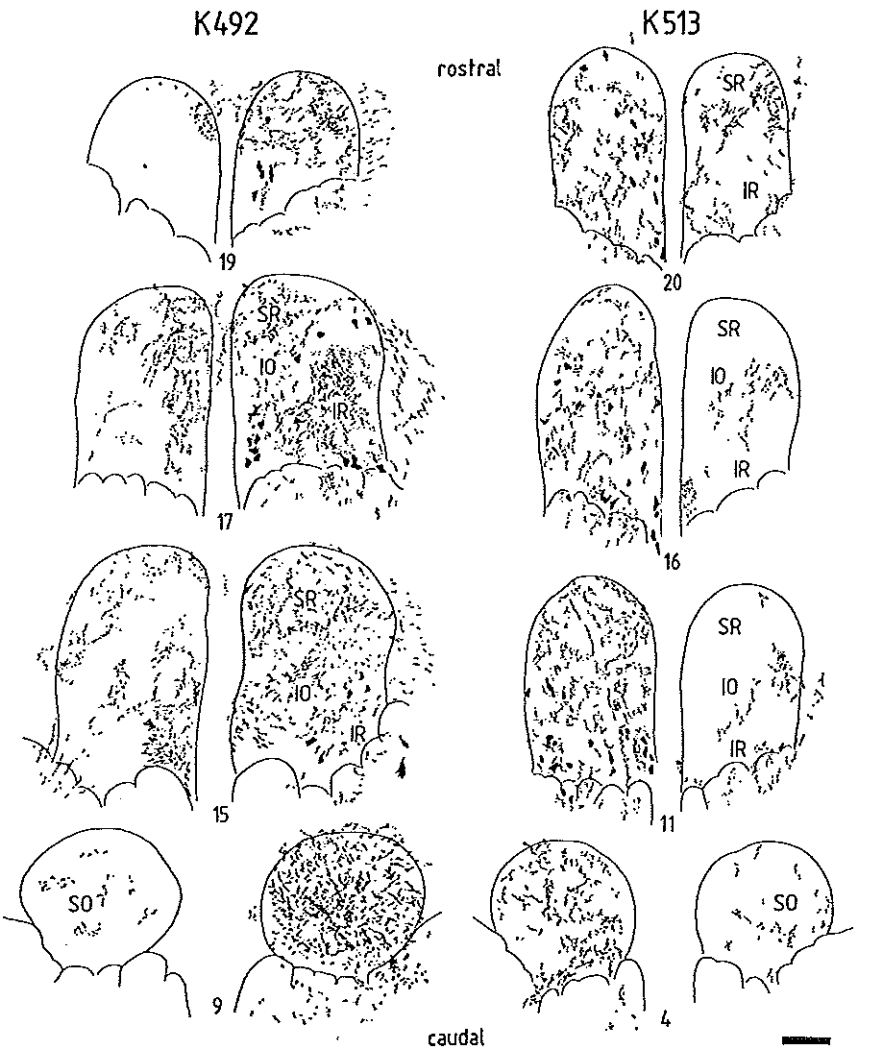

Figure 3. Distribution of terminal labeling in comparable transverse sections through the OMN and TN following injection of PHA-L in the right SV (K492) and right MV (K513), combined with retrogradely labeled MR motoneurons following simultaneous injection of the ipsilateral (K492) and contralateral (K513) MR muscle with HRP. Scale bar = 0.2 mm. Individual section numbers represent sections spaced at 160 μm.

Figure 4. *Photomicrograph of terminal axons in the ipsilateral OMN, labeled with PHA-L following injection in the SV (K492) and retrogradely labeled motoneurons following injection of HRP in the ipsilateral MR muscle. Same section as Fig. 2, K492 section 17.*

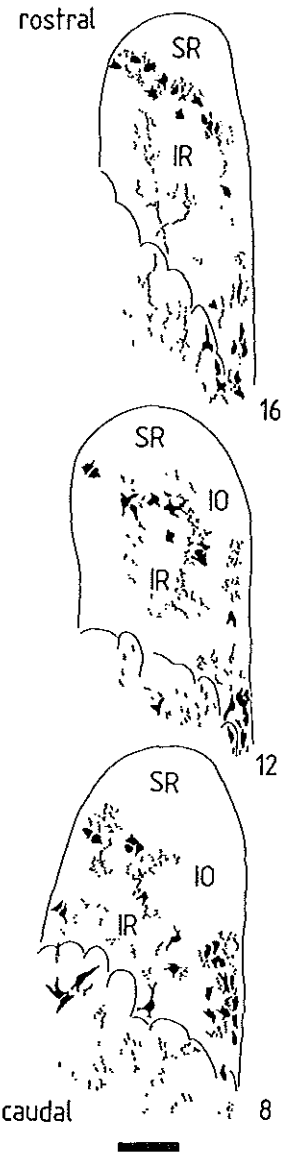

Figure 5. Distribution of terminal labeling in transverse sections of the OMN (levels comparable to those illustrated in Fig. 3) following injection of PHA-L in the AN (K507), combined with retrogradely labeled MR motoneurons following simultaneous injection of the contralateral MR muscle with HRP. Abbreviations as in Fig. 3. Scale bar = 0.2 mm. Individual section numbers represent sections spaced at 160 μm.

Electron Microscopy

Examination of the WGA-HRP injections in the SV (K258, K259, K266, K267, K313), showed that the central and dorsolateral parts of the SV were always included in the injection site. There was either some spread of tracer into the most rostral part of the MVpc (K258) or into the LV and group y (K259, K266). The total area covered by the SV injections is illustrated in figure 2B. The fiber trajectory and termination in these experiments closely matched the results obtained with the PHA-L experiments described above. In addition, some scattered retrogradely WGA-HRP labeled neurons were observed bilaterally in the SR subdivision.

The injection sites of the MV experiments (K229, K279, K383) always included a considerable portion of the MVpc and extended to a variable though small degree into the adjacent MVc (K229, K279) or MVmc (K229). In the cases K229 and K279 some DAB-reaction product was also present in the ventromedial part of the SV. The total area covered by the MV injections is illustrated in Fig. 2B. The fiber course and termination agreed well with the results of the PHA-L experiments. In all cases a few retrogradely labeled neurons were present bilaterally in the OMN.

Only one of the AN injections was completely restricted to this nucleus (K373), whereas the other (K384) included part of the MVpc and MVc (Fig. 2B). In both cases labeled fibers ascended in the contralateral mlf and terminated in the contralateral MR subdivision. In case K384 termination was also observed in the ipsi- and contralateral SR and IO subdivisions as well as in the ipsilateral IR subdivision. In both cases some small retrogradely WGA-HRP labeled neurons were present bilaterally in the OMN.

Ipsilateral projection from the superior vestibular nucleus. The morphological characteristics of WGA-HRP labeled terminals in the ipsilateral OMN were uniform. The terminals contained, apart from a few large dense core vesicles, predominantly clear flattened vesicles (F-type terminals) (Holstege and Calkoen 1990) . Often, several mitochondria were included, and sometimes the terminals contained neurofilaments in their preterminal segments. The diameter of the terminals ranged from 1.0 to 4.0 μm. While the distally located labeled terminals usually formed only a single synaptic junction (Fig. 6A), the proximal ones frequently had more than one synapse with an individual cell body or primary dendrite (Figs 6B, C). Both, the distally and proximally located labeled terminals were regularly of the "en passant" type, and both sometimes contacted spines. These spines were usually small, without constrictions and without a spine apparatus. The cell bodies contacted by labeled terminals were never immunoreactive for GABA nor retrogradely labeled with WGA-HRP. All of them were large (>25 μm) and contained a well developed granular ER apparatus and a smooth nucleus.

Of all the WGA-HRP labeled terminals in the ipsilateral OMN 92.5% were double labeled for WGA-HRP and GABA (Table 1). The remaining 7.5% did not meet the GABA-positive criterium and were considered single WGA-HRP labeled (Table 1), although the number of goldparticles was usually above background level. About 55% of the WGA-HRP/GABA labeled terminals established synaptic contacts, most of which were symmetrical. The majority of these (60.9%) contacted distal dendrites, while 35.7% and 3.4% were apposed to proximal dendrites and somata, respectively (Table 1). None of the terminals or axons were double labeled for WGA-HRP and glycine.

Contralateral projection from the superior vestibular nucleus. Only 2% of the WGA-HRP labeled terminals from the SV were double labeled for GABA (Table 1). The single WGA-HRP labeled terminals mostly contained spherical vesicles and showed asymmetric synapses

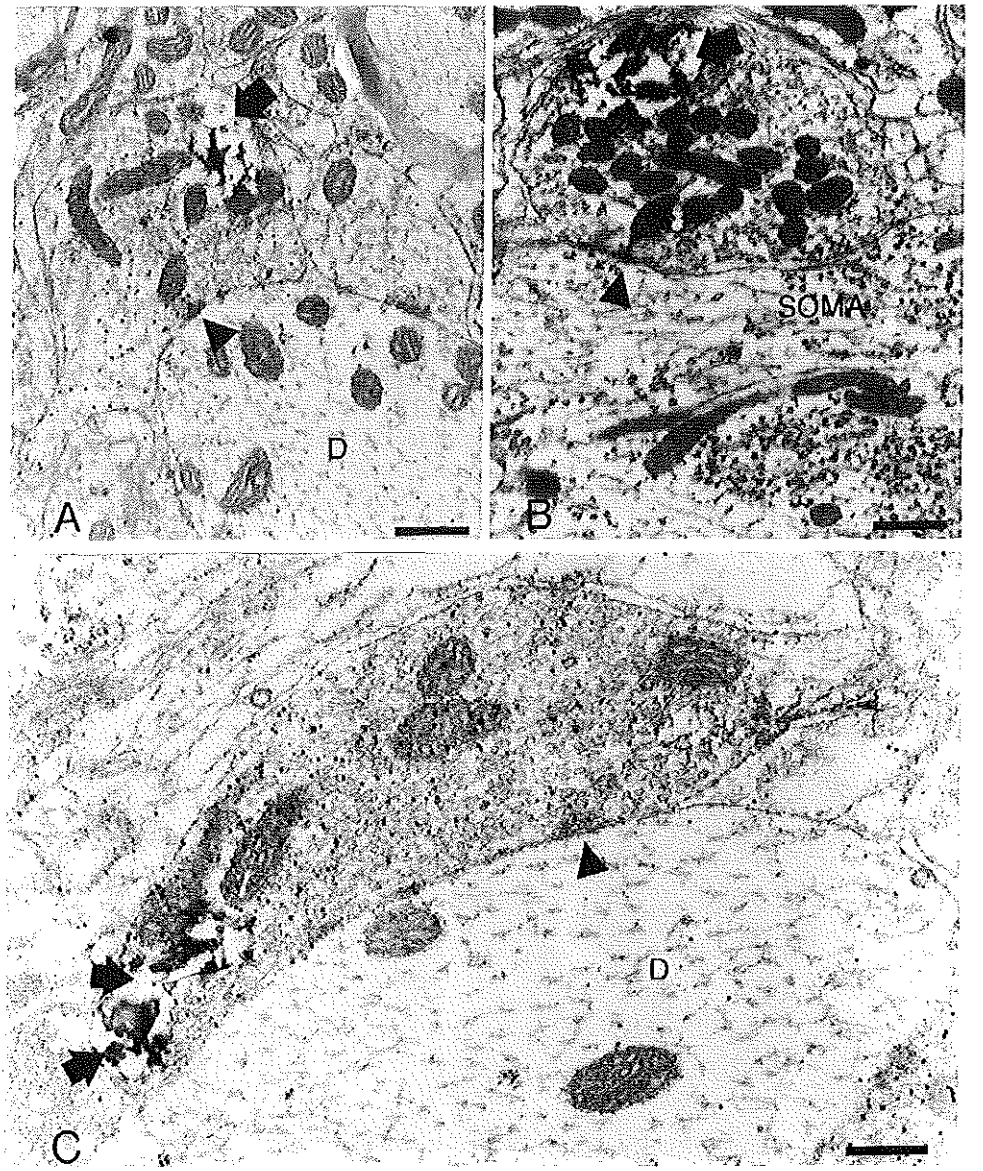

Figure 6. *Electron micrographs of axon terminals labeled from the SV with WGA-HRP. Gold particles (size = 15 nm) indicate the presence of GABA. In all panels, synaptic contacts are indicated by arrowheads and WGA-HRP/DAB-Cobalt reaction product is indicated by arrows. The axon terminals contain small flattened vesicles and establish symmetrical synaptic contacts. A demonstrates a contact with a small dendrite; B illustrates a contact with a soma in the IO region of the OMN; C illustrates a contact with a large dendrite (D) in the IR region of the OMN. Scale bars = 0.5 μm.*

(S-type terminals (Holstege and Calkoen 1990)). Their diameters ranged from 1 to 2.5 μm, and they usually contained one or two mitochondria. The terminals with synaptic contacts (51%) were mostly apposed to distal dendrites (85%) and less frequently to proximal dendrites (13%) or somata (2%) (Table 1). The WGA-HRP/GABA labeled terminals contralateral to the SV showed the same morphological characteristics as those on the ipsilateral side. None of the terminals or axons showed double labeling with WGA-HRP and glycine.

Ipsilateral projection from the medial vestibular nucleus. The labeled terminals ipsilateral to the injected MV belonged to either the S-type or the F-type. Morphologically they were indistinguishable from similar terminals labeled bilaterally in the OMN following SV injections.

The S-type terminals were always single labeled with WGA-HRP and constituted 92.1% of the labeled population. The majority of this group established synaptic contacts with distal dendrites (71.6%), while the remaining contacts were with proximal dendrites (22.3%) and somata (6.1%) (Table 1).

The F-types formed 7.9% of the labeled terminals and were double WGA-HRP/GABA labeled (Table 1). Synaptic contacts were with distal dendrites (60.3%), proximal dendrites (34.4%) and somata (5.3%) (Table 1). The WGA-HRP/GABA terminals were only present in the cases K229 and K279, in which a small part of the SV was included in the injection site; they were not found in case K383 in which the injection site was restricted to the MV nor in case K384 where the MV was partly included in the AN injection site. None of the WGA-HRP labeled structures was double labeled with glycine.

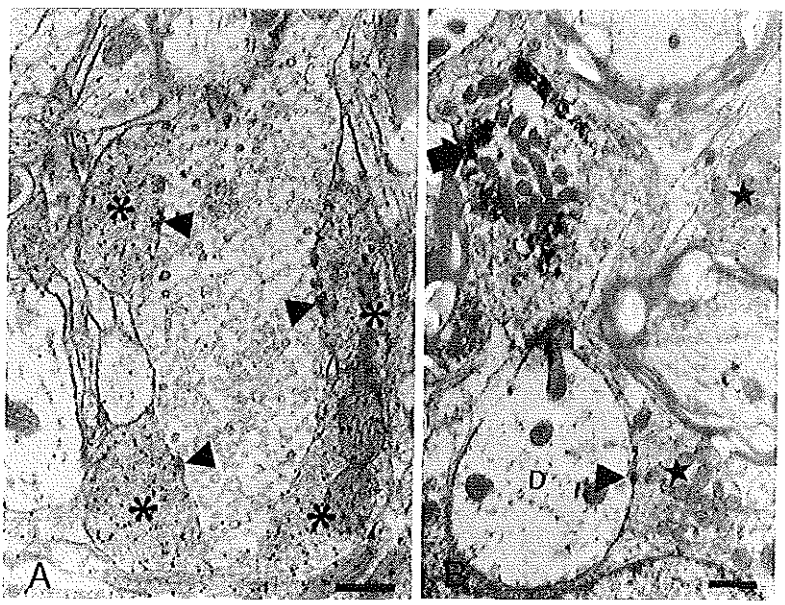

Figure 7. (A) Electron micrograph of a large non-GABAergic terminal in the OMN of the rabbit, that is surrounded by four smaller terminals (asterisks), all labeled for GABA, of which three establish symmetric synaptic contacts (arrowheads). Size of gold particles = 15 nm. Scale bar = 0.5 μm. (B) Electron micrograph of a single WGA-HRP-labeled terminal in the MR region of the OMN following injection of WGA-HRP in the MV. The WGA-HRP-labeled terminal shows an asymmetrical synaptic contact with a small dendrite (small arrow), while one of the two GABAergic terminals (asterisks) on the bottom shows a symmetric synapse (arrowhead) with the same dendrite. Large arrow points to the WGA-HRP/DAB-colbalt reaction product. Size of gold particles = 10 nm. Scale bars = 0.5 μm.

Contralateral projection from the medial vestibular nucleus. Labeled terminals contralateral to the injected MV showed the typical S-type morphology described above, and contained WGA-HRP exclusively (Fig. 7B). They reacted neither in GABA nor in glycine immunochemistry (Table 1). The majority of postsynaptic elements were distal dendrites (81.4%) and only 18.6% of the terminals were apposed to proximal dendrites or cell bodies. Some terminals, characterized as T-types (Holstege and Calkoen 1990), contained subsynaptic densities. One single C-type axosomatic terminal was observed, characterized by subsurface cisterns and a ribosomal apparatus along the entire length of the synapse (Holstege and Calkoen 1990) .

Contralateral projection from the abducens nucleus. All the WGA-HRP labeled terminals from the AN were single WGA-HRP labeled and belonged to the S-type category. The postsynaptic distribution of these terminals was similar to that of the S-type terminals labeled from the MV (Table 1). None of the terminals reacted with either GABA or glycine immunochemistry.

Unlabeled GABAergic and glycinergic terminals. Several types of GABAergic terminals were observed that were never labeled with WGA-HRP, the majority belonging to the F-type category. An approximately similar number of F-type terminals reacted positive to glycine. Other GABAergic terminals were characterized by an abundance of large granular vesicles (G-type (Holstege and Calkoen 1990); type IV (Bak and Choi 1974; Demêmes and Raymond 1982)) or by subsynaptic densities (Taxi 1961) (T-type (Holstege and Calkoen 1990); (Lanoir et al. 1982) ; type III (Bak and Choi 1974), type VI (Demêmes and Raymond 1982)). Several GABAergic axo-axonal terminals were observed next to an axon hillock, while others made symmetric synaptic contacts with large boutons (Fig. 7A). These presynaptic terminals were morphologically similar to the axo-axonal synapses that have been described for the OMN of the cat and the monkey (Tredici et al. 1976; Waxman and Pappas 1979), and the TN of the cat (Bak and Choi 1974), and they have been referred to as P-type (Holstege and Calkoen 1990).

Discussion

The major findings of the present study are that the projection from the SV to the ipsilateral OMN is completely GABAergic and includes a relatively minor projection to the MR subdivision, whereas the crossed projections from the SV and AN, as well as the bilateral MV projection to the OMN are non-GABAergic. The terminals of these GABAergic and non-GABAergic projections have their own morphological characteristics and postsynaptic distribution which will be discussed below.

Ipsilateral projections from the superior vestibular nucleus. The majority of all WGA-HRP labeled terminals from the ipsilateral SV contained GABA. The morphology and postsynaptic distribution of the single WGA-HRP and double WGA-HRP/GABA labeled terminals did not differ significantly. Moreover, the fact that most of these single WGA-HRP labeled terminals contained a subthreshold but higher than background amount of gold particles justifies the conclusion that the entire projection from the SV to the ipsilateral OMN is GABAergic. This is in agreement with several physiological studies which demonstrated the inhibitory effect of GABA in the ipsilateral SV projection (Ito et al. 1970; Obata and Highstein 1970; Ito et al. 1976b).

The terminals from the uncrossed SV projection were rather large, contained flattened vesicles and established symmetric synapses. This F-type terminal (Holstege and Calkoen 1990; Wentzel et al. 1993), corresponds to the types I and II described for the TN (Bak and Choi 1974) and OMN (Demêmes and Raymond 1982) in the cat. These terminals also frequently

established multiple synaptic contacts. The morphology of the WGA-HRP/GABAergic terminals matched that of terminals in the cat, labeled either by injection of radioactive substances in the vestibular nuclei (Demêmes and Raymond 1982), intra-axonal injection of HRP in inhibitory second-order vestibular neurons (Spencer and Baker 1983), GAD- and GABA-immunocytochemistry (De La Cruz et al. 1992; Spencer and Baker 1992), or 3H-GABA uptake (Lanoir et al. 1982; Soghomonian et al. 1989).

The somata contacted by WGA-HRP/GABA labeled terminals showed the characteristics of motoneurons. Similar to the description of Tredici (Tredici et al. 1976) they were usually large, contained a well developed granular ER apparatus and a smooth nucleus. In addition they were not GABAergic and never retrogradely labeled with WGA/HRP from the injections in the vestibular nuclei.

Two observations support the direct projection from the SV to the MR subdivision, which is small compared to input from the MV or AN. The light microscopic experiments showed terminal ramifications and boutons labeled with PHA-L in the MR subdivision, sometimes in close apposition to retrogradely labeled motoneurons. Secondly, our electron microscopic analysis of the MR subdivision showed boutons that were double labeled with WGA/HRP and GABA. These findings support data obtained in the rabbit (Thunnissen 1990) but disagree with earlier studies in the monkey (McMasters et al. 1966) and the cat (Carpenter and Cowie 1985a).

Medial vestibular, abducens nucleus and contralateral superior vestibular nuclei projections. The different positions within the mlf of crossed and uncrossed ascending fibers has been reported before (Carleton and Carpenter 1983; Thunnissen 1990). The ipsilateral fibers with a preferential termination in the MR subdivision were usually described as the ascending tract of Deiters, which has its origin in the MVmc (McMasters et al. 1966; Reisine and Highstein 1979; Carleton and Carpenter 1983; Thunnissen 1990).

Virtually all terminals of the crossed projections from the SV, MV, and AN contained spherical vesicles, made asymmetric synaptic contacts, and were neither GABAergic nor glycinergic. This S-type terminal (Holstege and Calkoen 1990), most likely corresponds to the type III terminals described for the TN (Bak and Choi 1974) and OMN (Demêmes and Raymond 1982) in cat. Their morphology and the absence of inhibitory transmitters stresses their excitatory nature (Uchizono 1965). Involvement of the SV in some of the MV injection sites may explain the presence of the small proportion of WGA-HRP/GABA labeled F-types in the ipsilateral OMN. The similarity of the ipsi- and contralateral MV projection strongly suggests a total excitatory character.

Our methods do not allow a precise explanation for the small amount of terminal axonal ramifications observed between the rostral poles of the OMN. They may either represent (re)crossing fibers or just terminations; judged by their appearance we favor the latter. Uchino et al. (Uchino et al. 1994) reported in the cat, that all fibers from the SV representing the anterior canal pathway which projected through the brachium conjunctivum to terminate in the contralateral OMN, gave off collaterals that recrossed the midline to terminate in the ipsilateral OMN. Similar recrossing behaviour was observed for the posterior canal related projection in the cat, but not in the rabbit (Graf et al. 1983).

The majority of WGA-HRP labeled S-type terminals contralateral to the injection sites made asymmetric synaptic contacts with distal dendrites. This is in line with results obtained with anterograde tracing with tritiated aminoacids (Demêmes and Raymond 1982). S-type terminals labeled from the ipsilateral MV were more often apposed to proximal structures. This difference in ipsi- and contralateral terminal preference was also reported in a physiological study (Baker and Highstein 1978).

GABAergic input in the oculomotor nucleus. In the present study, 39.1% of the postsynaptic elements of the GABAergic terminals from the SV were proximal dendrites or somata. In a previous study it was shown that 23% of the GABAergic boutons in the OMN (without regard to their specific origin) contacted proximal dendrites or somata (Wentzel et al. 1993). Thus, although the majority of SV boutons contacts distal dendrites there is a significant input to proximal structures. From these data it can be concluded that the GABAergic input to the distal dendrites is partly derived from another source than the SV. This agrees well with recent findings that demonstrate that the rostral interstitial nucleus of the mlf provides a GABAergic input to the distal dendrites of the oculomotor neurons Spencer and Baker 1992). In the cat, the differential input to the proximal and distal structures is even more pronounced since only 45% of the terminals from the ipsilateral vestibular nuclei contact distal dendrites (Demêmes and Raymond 1982; Spencer and Baker 1983).

Two findings of the present study support the conclusion that the ipsilateral SV is not the only source of GABA in the OMN. Firstly, the already mentioned G-, T- and P-type GABAergic terminals that were never WGA-HRP labeled. Secondly,
the finding that SV terminals are never glycinergic combined with the fact that glycine was always found to be colocalized with GABA (Wentzel et al. 1993). This indicates that at least some of the non-WGA-HRP labeled GABA positive F-type terminals originate outside of the SV.

Conclusions

GABA and glycine immunocytochemistry together with morphological data showed that the projection from the SV to the ipsilateral OMN is completely GABAergic whereas the projection from the entire MV and the crossed projections from the SV, and the AN are non-GABAergic. Furthermore there exists a large pool of GABAergic axon terminals throughout the OMN that does not belong to SV, MV or AN neurons. Several glycinergic terminals were observed but they also did not belong to vestibular nuclear neurons and they were always double labeled with GABA (Wentzel et al. 1993).

These data agree with the physiological studies in rabbit and cat which showed that inhibitory postsynaptic potentials in the motoneurons of the OMN were exclusively induced via the ipsilateral SV, whereas the excitatory postsynaptic potentials were evoked by stimulation of the ipsilateral and contralateral MV, the contralateral brachium conjunctivum or AN (Highstein and Ito 1971; Baker and Highstein 1975; Baker and Highstein 1978; Highstein and Baker 1978; Yamamoto et al. 1978; Uchino et al. 1982).

Signals are sent from one neuron to another by diverse chemical transmitters. These chemical systems, overlaid on the neuronal circuits of the brain, add another dimension to brain funcion.

(L. I. Iversen in: The Brain, A Scientific American Book,)

Chapter III. Ultrastructure of the Inhibitory Inputs to the Subnuclei of the Inferior Olive Involved in Compensatory Eye Movements

a. Gabaergic inputs from the nucleus prepositus hypoglossi and medial vestibular nucleus to the caudal dorsal cap and beta nucleus
b. Gabaergic inputs from dorsal group y and the ventral dentate nucleus to the rostral dorsal cap and ventrolateral outgrowth

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IIIa. Gabaergic inputs from the nucleus prepositus hypoglossi and medial vestibular nucleus to the caudal dorsal cap and beta nucleus

Abstract

The dorsal cap of the inferior olive is involved in the control of eye movements and is excited by inputs from the midbrain. In the present study an attempt was made to find out in the rat and rabbit where the inhibitory input to this nucleus is derived from.

The projection from the nucleus prepositus hypoglossi to the dorsal cap was studied in the light microscope by anterograde tracing of Phaseolus vulgaris-leucoagglutinin and lesion induced depletion of glutamic acid decarboxylase immunoreactivity, and in the electron microscope by anterograde tracing of wheat germ agglutinin-coupled horseradish peroxidase combined with GABA-immunocytochemistry. We show that the nucleus prepositus hypoglossi projects bilaterally to the dorsal cap, contralaterally to the ventrolateral outgrowth and ipsilaterally to the medial accessory olive. After lesioning the nucleus prepositus hypoglossi the caudal dorsal cap was depleted of most of its glutamic acid decarboxylase immunoreactive terminals while the rostral dorsal cap and the ventrolateral outgrowth were depleted for a minor part. Ultrastructural analysis indicates that the majority, but not all, of the terminals from the nucleus prepositus hypoglossi in the dorsal cap are GABA-positive. These GABA-positive and GABA-negative terminals form predominantly symmetric and asymmetric synapses; most of them synapse on dendrites outside and inside glomeruli, frequently in association with dendrodendritic gap junctions, while a small minority are axosomatic. None of the terminals from the nucleus prepositus hypoglossi was found to form a crest synapse, although synapses of this kind were predominantly formed by GABAergic terminals.

This study shows that the dorsal cap receives a major inhibitory input from the nucleus prepositus hypoglossi, the terminals of which are located at strategic positions on the olivary neurons.

Introduction

The inferior olive (IOL) provides the climbing fibers to the Purkinje cells of the cerebellar cortex and gives off axon collaterals to the neurons of the cerebellar nuclei (Szentágothai and Rajkovits, 1959; Eccles,1966; Murphy et al.,1973; Desclin,1974; Van der Want et al.,1989). The IOL consists of three main subnuclei, namely the principal olive (PO), the dorsal accessory olive (DAO) and the medial accessory olive (MAO), (Kooy,1916; Brodal,1940; Whitworth

and Haines,1986; Nelson and Mugnaini,1988; Ruigrok and Voogd,1990). The MAO is continuous with the dorsal cap (dc) of Kooy and ventrolateral outgrowth (VLO), (Azizi and Woodward,1987; Buisseret-Delmas,1988a,b; Bourrat and Sotelo,1991) which are known to innervate Purkinje cells in the flocculo-nodular lobe (Alley et al.,1975; Groenewegen and Voogd,1977; Gerrits and Voogd,1982; Gerrits,1985; Ruigrok et al.,1992).

The dc is involved in the visual control of eye movements. In rabbit, the neurons of this subnucleus respond to slow movements of large patterns over the visual field (Simpson and Alley,1974; Simpson et al.,1981; Leonard et al.,1988; Simpson et al., 1989; Graf et al., 1988; for rat see Blanks and Precht,1983; for monkey see Waespe and Henn,1981). Three different areas in the dc can be identified depending on response preferences for rotation of optokinetic stimuli about particular axes of physical space (Simpson et al.,1981; Leonard et al., 1988): the caudal part of the dc responds optimally to field movements around the vertical axis while two more rostrally located areas, each dominated by a different eye, respond optimally to images moving around an axis in the horizontal plane. This organization agrees with the differential inputs to the dc. The caudal half of the nucleus receives an ipsilateral projection from the dorsal terminal nucleus of the accessory optic system (AOS) and the pretectal nucleus of the optic tract, whereas the rostral half of the dc receives an ipsilateral projection predominantly from neurons situated in a region extending from the medial terminal nucleus of the AOS to the tegmental region ventromedial to the red nucleus (Mizuno et al.,1973; Takeda and Maekawa,1976; Maekawa and Takeda,1977 and 1979; Walberg et al.,1981; Holstege and Collewijn,1982; Simpson, 1984; Giolli et al.,1985; Simpson et al.,1988). The projections of these areas to the dc are non-GABAergic (Horn and Hoffmann,1987; Nunes-Cardozo and Van der Want,1990) and, according to the morphology of their terminals, presumably excitatory (Mizuno et al.,1974). In addition, both the caudal and rostral half of the dc are innervated by small neurons situated in the nucleus prepositus hypoglossi (PrH), (Gerrits et al.,1985; McCrea and Baker, 1985; Barmack et al.,1991).

Like other olivary subdivisions, the dc is provided with a substantial GABAergic innervation (Mugnaini and Oertel,1985; Sotelo et al.,1986; Nelson and Mugnaini, 1988; Nelson et al., 1989), which is probably inhibitory (Krnjevic and Schwartz,1966; Roberts,1974). Most of the GABAergic input to the major olivary subnuclei is derived from cerebellar or vestibular nuclei (Nelson et al., 1984; Nelson et a l., 1986; Angaut and Sotelo,1987 and 1989; De Zeeuw et al., 1988a; Buisseret-Delmas et al.,1989; Fredette and Mugnaini,1991). The GABAergic terminals in these subnuclei are frequently located within glomeruli and some of them appear strategically apposed to dendritic profiles coupled by gap junctions (Sotelo et al.,1986; De Zeeuw et al.,1988b; De Zeeuw et al., 1989a; Angaut and Sotelo,1987 and 1989; Fredette and Mugnaini,1991). A double labeling electron microscopic technique showed that these GABAergic terminals near the gap junctions are derived from the cerebellar nuclei (De Zeeuw et al.,1988b; De Zeeuw et al.,1989a; Angaut and Sotelo,1989). Since GABA-antagonists as well as lesions of the cerebellar nuclei influence the synchrony between olivary neurons, these GABAergic terminals may modulate the electrotonic coupling within the IOL (Sasaki and Llinás, 1985; Lang et al., 1989; Sasaki et al., 1989; Llinás and Sasaki, 1989; Lang et al., 1990). GABAergic terminals unrelated to gap junctions may be responsible for other forms of inhibitory modulation in the IOL (Andersson and Hesslow,1986 and 1987; Andersson et al.,1988; Barmack et al.,1989). Concerning the dc, however, it is not known whether gap junctions are present in its neuropil (Mizuno et al., 1974) and whether the GABAergic terminals are located in the vicinity of gap junctions. Moreover, the origin is of the GABAergic input to the dc is still unknown.

In the present study, an attempt was made to find out whether the PrH is a major source of the GABAergic input to the dc, and whether its terminals are associated with gap

junctions. These issues were extensively studied and quantified in the rat with various neuroanatomical approaches, such as anterograde labeling with Phaseolus vulgaris-leuco-agglutinin (PHA-L), lesion-induced depletion of glutamic acid decarboxylase(GAD)-immunoreactivity, and a combination of anterograde labeling with wheat germ agglutinin coupled horseradish peroxidase (WGA-HRP) and GABA-immunocytochemistry. The salient points were confirmed in the rabbit.

Material and Methods

Light Microscopy

Anterograde Tracing with PHA-L. Two adult male Wistar rats and two adult male Dutch belted rabbits were used for the PHA-L experiments. The animals were anesthetized with sodium pentobarbital (Nembutal, 120 mg/kg, i.p.). After mounting in a stereotaxic apparatus, the occipital bone was freed of neck muscles and the foramen magnum was enlarged. A stereotaxic injection was made in the PrH with a glass micropipette (tip diameter 8-15 μm) filled with a 2.5% PHA-L (Vector) solution in 0.05 M Tris-buffered saline (pH 7.4). The tracer was injected by means of a positive current (4-8 μA) which pulsed 7 sec on- 7 sec off, for a total of 30 minutes. Afterward the pipette was withdrawn, the wound was sutured and the animal was allowed to recover. After a survival time of 7 (rats) or 10 (rabbits) days the animals were anesthetized with Nembutal (200 mg/kg), and perfused according to the following protocol (Ruigrok and Voogd,1990). Blood was rinsed out with 200 ml 0.05 M phosphate buffer (pH 7.4) containing 0.8% NaCl, 0.8% sucrose, and 0.4% d-glucose; this was followed by 1 liter of a fixative consisting of 0.5% depolymerized paraformaldehyde, 2.5% glutaraldehyde and 4% sucrose in the same buffer. The dissected brains were kept in the same fixative for two hours, transferred to a 10% sucrose solution until they sank, and embedded in 10% gelatin dissolved in the same sucrose solution. The gelatin was hardened in 4% paraformaldehyde for 3 hours. Finally the embedded brains were transferred to a 30% sucrose solution in phosphate buffer (pH 7.4, 4C) in which they were stored until they sank. Serial coronal sections (40 μm) of the brainstem were cut on a freezing stage microtome and processed to reveal the PHA-L (Gerfen and Sawchenko, 1984). The sections were collected in Tris-buffered saline (TBS: 0.9% NaCl in 0.05 M Tris-HCl, pH 7.4), rinsed in TBS (3 x 15 min), and incubated overnight in the primary antiserum (Goat anti-PHA-L, Vector), diluted 1:2000 in TBS containing Triton X-100 (TBS+)(0.9% NaCl, 0.2% - 0.4% Triton X-100 in 0.05 M Tris-HCl, pH 8.6). Subsequently, the sections were rinsed in TBS+ (3 x 15 min), incubated for 2 hours in rabbit antigoat IgG (Sigma, 1:200 in TBS+), again rinsed in TBS+ (3 x 15 min), and incubated for 2 hours in goat PAP (Nordic, 1/400 in TBS+). Finally, the sections were rinsed in Tris-HCl (0.05 M, pH 7.6) and incubated with 0.05% DAB (3,3'-diaminobenzidine-tetrahydrochloride, Sigma) and 0.01% H_2O_2 in Tris-HCl for 30-45 minutes. Following thorough rinsing in Tris-HCl, sections were mounted, counterstained with either cresyl violet or neutral red, and coverslipped.

Lesion-induced depletion of GAD immunoreactivity. To find out whether the PrH is the source of the GABAergic terminals in the dc, unilateral lesions were made in adult Sprague-Dawley albino rats, two of which were selected for this study. The rats were anesthetized with a mixture of ketamine (30 mg/kg), acepromazine (0.3 mg/kg) and xylazine (5 mg/kg). After exposing the cisterna magna, the cerebellum was lifted and the PrH was damaged mechanically by a knife. The animals were allowed to survive for 10 days, which is the optimal survival time for inducing depletion of GAD immunoreactivity in parts of the IOL after a lesion of the cerebellum (Fredette and Mugnaini,1991). Subsequently, the rats were anesthetized with

Nembutal (200 mg/kg), and perfused transcardially with 200 ml saline followed by 1 liter of 0.5% zinc salicylate, 4% formaldehyde and 0.9% NaCl (pH 4.5, room temperature). The brainstems were removed 1 h after perfusion, cryoprotected in saline containing 30% sucrose, and cut coronally at 25 µm on a freezing microtome. To stain the remaining GABAergic terminals the sections containing the IOL were processed for immunocytochemistry with a GAD antiserum raised in sheep (Oertel et al.,1981; Nelson et al.,1989; Fredette and Mugnaini,1991). The sections were blocked in rabbit serum, and incubated in GAD antiserum (1:2000), rabbit anti-sheep (1:50), and goat PAP (1:100). Finally, the sections were incubated in DAB and H_2O_2. In addition, some of the brainstem sections were processed for GAD immunocytochemistry to show the presence of GABAergic neurons in the PrH. This procedure is slightly different from the one that shows immunoreactive terminals (Mugnaini and Oertel,1985). Briefly, animals were perfused with a zinc-formol fixative at pH 6.8, and the brainstem sections were immunostained by a double cycle PAP procedure, without any detergent permeabilization.

Electron Microscopy

Tissue processing. A total of 4 adult male Wistar rats and 4 adult male Dutch belted rabbits were used for electron microscopy. Two specimens of each species were used for combined WGA-HRP anterograde tracing from the PrH and GABA-immunocytochemistry, while the remaining specimens were perfused with an aldehyde fixative as specified below, and the slices of the IOL were postfixed with ferrocyanide-reduced osmium tetroxide (Karnovski,1971) and used for standard electron microscopy. All animals were anesthetized with Nembutal (120 mg/kg, i.p.). The rats and rabbits used for the combination experiment were operated upon like the animals that received the PHA-L injections. WGA-HRP (0.1µl) was injected stereotaxically in the PrH by means of a hydraulic pressure system. After a survival time of 2 (rats) or 3 (rabbits) days the animals were anesthetized with Nembutal (200 mg/kg), and perfused transcardially with 100 ml 0.9% saline in 0.18 M cacodylate buffer at pH 7.3, followed by 1 (rats) or 2 (rabbits) liter(s) of 5% glutaraldehyde in the same buffer. The brainstems were kept in the same fixative for two hours and coronally sectioned on a Vibratome at 70 µm. The sections were processed with a procedure combining WGA-HRP histochemistry and postembedding GABA-immunostaining. Detailed protocols are published elsewhere (De Zeeuw et al.,1988a). Briefly, Vibratome sections were incubated with tetramethylbenzidine and stabilized with DAB-cobalt (Rye et al.,1984; Lemann et al.,1985), osmicated (in 8% glucose solution), block stained in uranyl acetate (UA), directly dehydrated in dimethoxypropane and embedded in Araldite. Guided by semithin sections, we prepared pyramids of the dc, and, for comparison, of the ß-nucleus. From these tissue blocks ultrathin sections were cut, mounted on Formvar coated nickel grids and processed for GABA-immunocytochemistry with a rabbit antiserum to a GABA-glutaraldehyde conjugate (for details about the specificity of the antibody, see Seguela et al.,1984; and Buijs et al.,1987). GABA-antibody binding sites were revealed with a goat anti-rabbit (GAR) antiserum (Janssen) labeled with 15 nm gold particles, diluted 1:40. After rinsing, the sections were counterstained with UA and lead citrate, and examined in an electron microscope operated at 80 kV.

Collection and analysis of the data. The fine structural examination was subdivided into a general and a quantitative analysis. In the general analysis the main features of the neuropil of the dc were studied, and special emphasis was placed on terminals apposed to neuronal elements linked by gap junctions (Peters et al.,1970; Sotelo et al.,1974) and on terminals constituting the so-called crest synapses (Milhaud and Pappas, 1966a, b; Akert et al, 1967).

The quantitative analysis was performed as follows: from both the caudal and rostral dc of each of the two rats with injections of WGA-HRP in the PrH, two embedded tissue blocks with substantial anterograde labeling were selected. In the rat, the trimmed pyramid of each tissue block, and hence the ultrathin sections, contained both the ipsi- and contralateral dc (the latter being marked by a clear cut in the Araldite). Of each of the two rabbits with injections of WGA-HRP in the PrH only two tissue blocks were analyzed. The pyramids of these blocks included the rostral contralateral dc, which was the area with the most heavy anterograde labeling. In both the rat and the rabbit a random sample of terminals was collected from two non-serial ultrathin sections obtained from each tissue block. In each sample, we determined what percentage of the WGA-HRP labeled terminals was GABA-negative (single labeled) and GABA-positive (double labeled). In addition, it was calculated what percentages of both single and double labeled terminals were situated within glomeruli, in the neuropil outside the glomeruli, and apposed to somata (see Table 1). In this analysis a terminal was considered: 1) WGA-HRP labeled, when crystalline electron dense deposits and/or increased darkening of the vesicles were observed; 2) GABA positive, when the number of gold particles overlying it was at least eight times higher than the number of particles overlying an equal surface area of surrounding non-GABAergic structures (for details about counting procedure see De Zeeuw et al.,1989a); 3) glomerular, when, alone or together with other terminals, it joined a core of at least three small dendritic elements, the synaptic complex having a glial sheath (de Zeeuw et al.,1989b; 4) extraglomerular, when a neuropil terminal did not fulfill these criteria; and 5) axosomatic, when it was directly apposed to a neuronal cell body. The percentages of the features mentioned above were averaged for all ultrathin sections and, if necessary for the statistical analysis, the standard errors of the means (SEM) were calculated.

In addition, some tissue blocks of the ß-nucleus of one of the rats were analyzed. Since they were studied for comparison, they were processed for GABA-immunocytochemistry in parallel with sections of the dc. Data from the caudal and rostral, ipsi- and contralateral dc, and those from the ß-nucleus were statistically compared by Student's t test (Glantz,1981).

Results

Light Microscopy

Injection and projection sites of PHA-L and WGA-HRP. After injection of PHA-L in the PrH of the rat and the rabbit, labeled neurons were present in the PrH and the most medial part of the medial vestibular nucleus (MVe) (Fig. 1A-B), but not in other surrounding areas (Fig. 2A). The contralateral PrH contained only anterogradely labeled fibers, that had crossed the midline through and beneath the hypoglossal nucleus. Labeled fibers projecting to the ipsilateral IOL coursed ventrally and ran directly to the olivary subnuclei. The fibers projecting to the contralateral IOL in part crossed the midline ventral to the hypoglossal nucleus and proceeded along a trajectory similar to that of the ipsilateral fibers, and in part crossed the midline near the IOL itself. Analysis of the projection sites indicated that the PrH provides the caudal dc with a substantial bilateral input (Figs. 1C and 2C). In the rostral dc of the rat, the anterograde labeling was less substantial than in the caudal dc and was located predominantly on the contralateral side. In the rabbit, the projection from the PrH to the contralateral rostral dc appeared relatively dense (Figs. 2B and 3). Both in rat and rabbit, the contralateral VLO

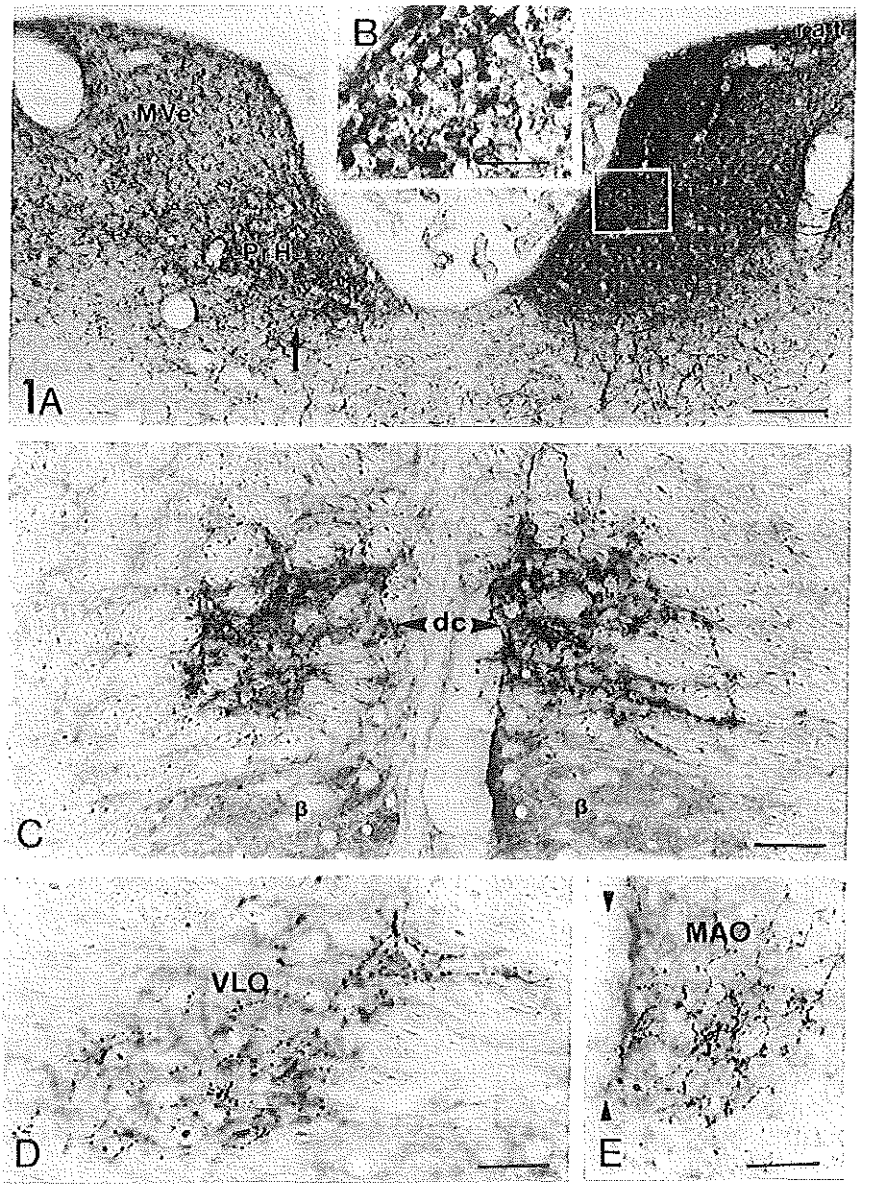

Figure 1A-E. Injection site and projection sites in a rat after a unilateral injection of PHA-L in the PrH. A: Injection site on the right; note that the contralateral PrH only contains anterogradely labeled fibers and varicosities (arrow). B: Magnification of area boxed in A. Note the presence of neurons which took up PHA-L. C: Bilateral anterograde labeling in the caudal dc. Note that the labeling in the dc is slightly denser on the ipsilateral side (right) than on the contralateral side (left), and that there is no labeling in the β-nucleus. D: Anterograde labeling in the contralateral VLO. E: Labeling in the ipsilateral MAO near the midline which is marked by arrow heads. The micrographs D and E are taken from the same section, i.e. they are from the same level of the rostrocaudal axis. Scale bar in A is 154 μm, in B is 51 μm, in C is 61 μm, in D is 20 μm, and in E is 22 μm.

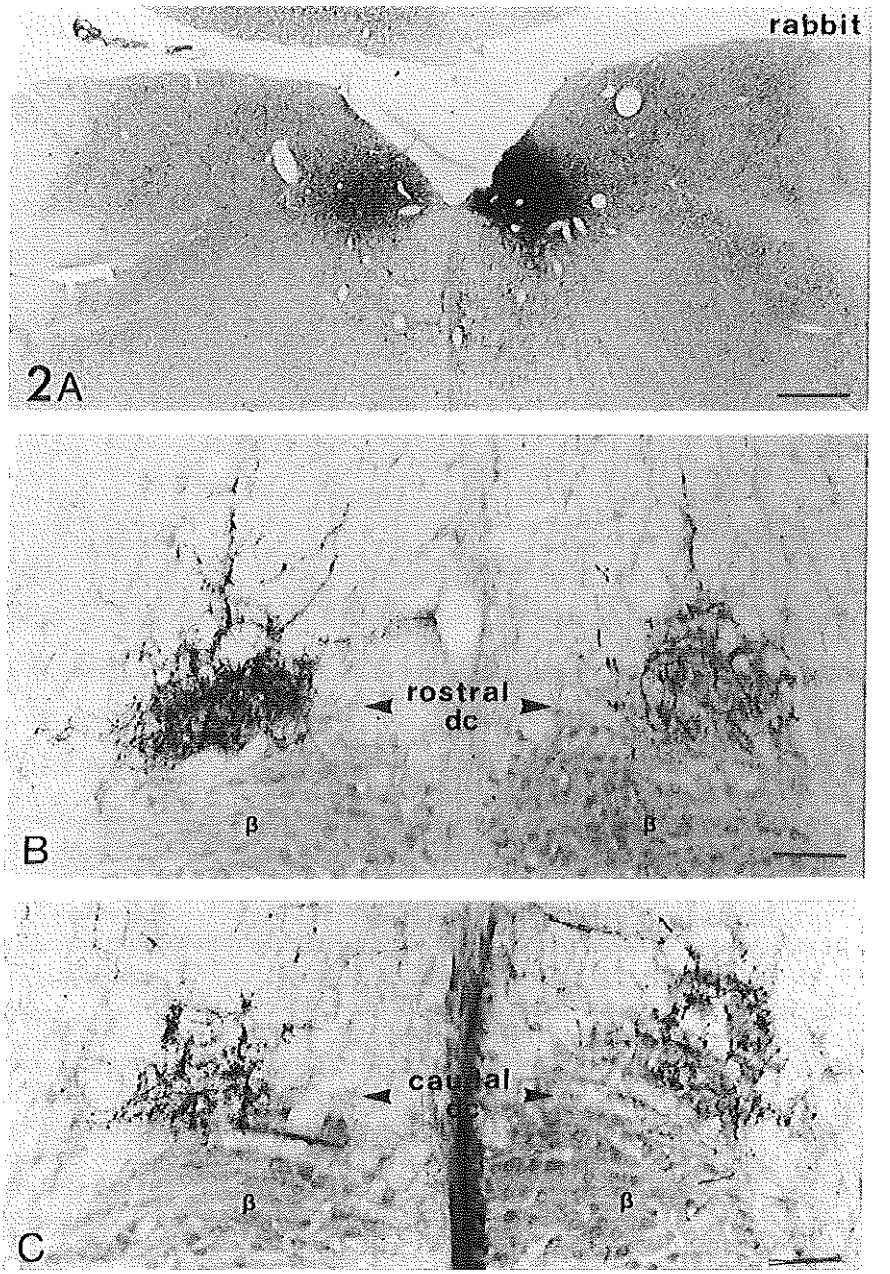

Figure 2A-C. Injection site and projection sites in a rabbit after a unilateral injection of PHA-L in the PrH. A: The injection site, which is restricted to the PrH is visible on the right. The contralateral PrH contains, like in the rat (Fig. 1), only anterograde labeling. B: Bilateral anterograde labeling in the rostral dc. C: Bilateral anterograde labeling in the caudal dc. Note that the difference in density of the projection between the ipsilateral (right) and contralateral (left) side is greater in the rostral dc than in the caudal dc. Scale bar in A is 580 μm, in B is 110 μm, and in C is 90 μm.

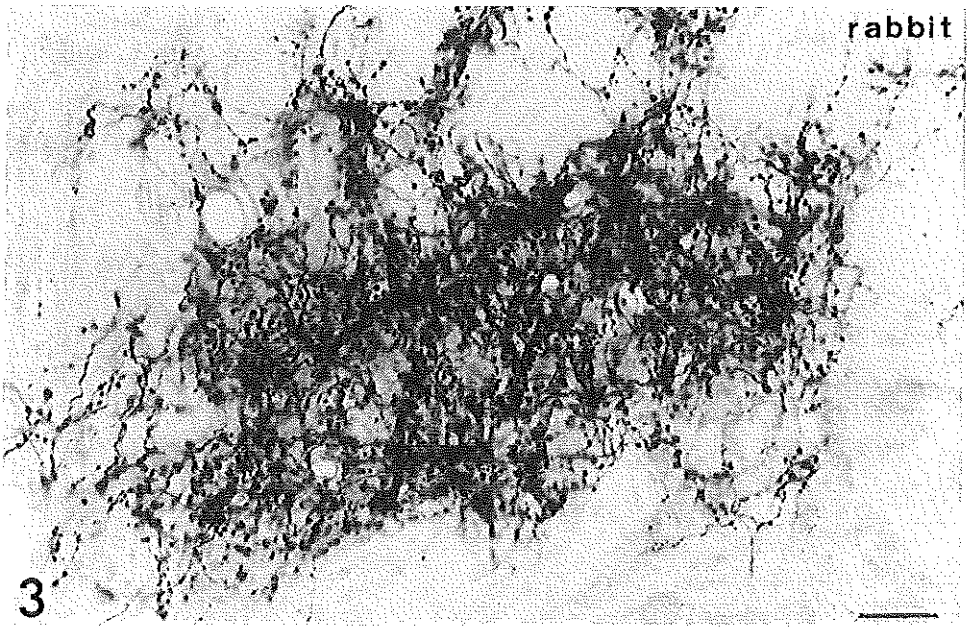

Figure 3. Higher magnification of the contralateral dc illustrated in Figure 2B; note the high density of labeled boutons present in this section. Scale bar is 23 μm.

and medial part of the ipsilateral MAO at the rostrocaudal level of the VLO received a sparse innervation (for rat see Figs. 1D and 1E).

The WGA-HRP injections in the rats and the rabbits, which were used for the electron microscopic double labeling experiments, were centered in the PrH, and the effective injection sites were estimated not to cross the midline (Figs.4A-B). The distribution of the anterogradely labeled terminals was in agreement with the results of the PHA-L experiments described above. In one of the rats parts of the MVe and spinal vestibular nucleus (SpVe) were included in the injection site (Fig. 4A). In this case, the ipsilateral ß-nucleus was anterogradely labeled as densely as the dc, thereby providing a good area for control (see below).

Lesion-induced depletion of GAD immunoreactivity. The unilateral lesions made in the hindbrain of the rats were centered in the caudal part of the PrH (Fig. 5). The lesions also damaged the directly surrounding areas, including part of the region where the fibers from the contralateral PrH cross to the ipsilateral side. Compared to the normal control (Fig. 6A), the ipsilateral caudal dc of the lesioned rat was depleted of most of its GABAergic innervation (Fig. 6B) while the contralateral caudal and rostral dc and the contralateral VLO were depleted of a smaller proportion of the GABAergic terminals. In the MAO no evident differences were observed.

The tissue processed to label the GABAergic cell bodies showed that the PrH contains an abundant number of immunoreactive neurons distributed rather equally at all rostrocaudal levels (Figs. 7A-B). The vast majority of the GAD-positive cell bodies were small but some large GAD-positive cell bodies were observed. Among the GAD-positive cells many cells of various sizes were present that did not show any immunoreactivity.

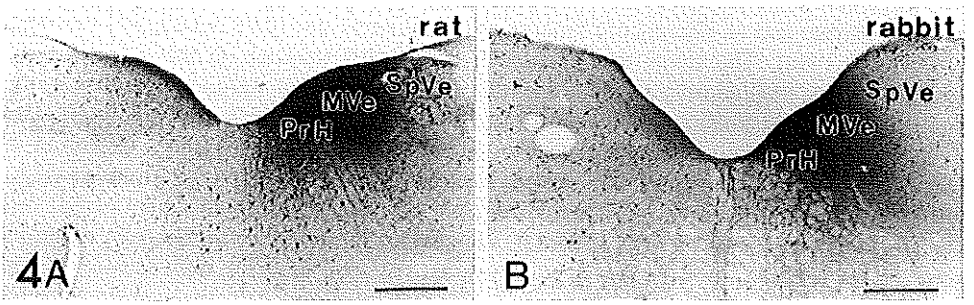

Figure 4A-B. Injection sites of WGA-HRP in the PrH in a rat (A) and rabbit (B). Note that the injection site in the rat extends to the MVe and SpVe. Scale bar in A is 230 μm, and in B is 510 μm. PrH, MVe and SpVe are indicated.

Electron Microscopy

General features. In both the rat and the rabbit the dc contained several types of axon terminals. Most of the terminals contained either rounded or pleomorphic vesicles and showed asymmetric or symmetric synapses respectively (for characteristics see Gray, 1959; Gray and Guillery,1966). However, some of the terminals contained mainly dense core vesicles. The vast majority of the terminals in the dc neuropil contacted intermediate and distal dendritic elements. The intermediate dendrites were often grouped together in thickets (see Sotelo et al., 1974) while the smaller dendrites and their spines were frequently incorporated in glomeruli. These glomeruli were usually rather small, often containing no more than 2 terminals and 3 spines (Figs. 8A-B). Gap junctions were regularly seen (Figs. 8A, B and C). These junctions occurred mostly within glomeruli, and less frequently between primary dendrites or between a primary dendrite and a soma (Fig. 8C). The somata were usually contacted by a few terminals, most of which had pleomorphic vesicles and formed symmetric synapses. Apart from the classical synapses described above, crest synapses were observed (Figs. 9A, B and C). These special synapses, which consistently exhibited asymmetric junctions, were formed with terminals containing pleomorphic vesicles as well as with terminals containing round vesicles (Fig. 9A). In only one case did one of the participating terminals contain mainly large granular vesicles. The narrow dendritic elements involved in the crest synapses always had a narrow width of approximately 0.26 μm (± 0.03 μm). With regard to the general characteristics of the neuropil, no major differences were seen between the caudal and rostral dc.

In the rats and rabbits in which WGA-HRP anterograde tracing from the PrH was combined with GABA-immunocytochemistry, ultrathin sections of the dc did not show any labeled cell bodies or dendrites but included numerous single GABA labeled (Figs. 8A-B and 9B-C), double GABA/WGA-HRP labeled (Figs. 10 and 11), and single WGA-HRP labeled (Fig. 12) terminals, as well as myelinated axons and preterminal axon segments.

At least 3 different kinds of GABAergic boutons were observed. The first type comprised most of the single GABA labeled and all the double GABA/WGA-HRP labeled terminals. Terminals of this kind ranged in size from 1 to 3.5 μm, often contained mitochondria and a few large dense core vesicles (approximately 90 nm in diameter) in addition to clear pleomorphic vesicles, and established standard types of synapses, mostly the symmetric category (Figs. 8A-B; Figs. 10A-C; Figs.11A-B). While the smaller double GABA/WGA-HRP labeled terminals usually formed only one synaptic junction, the larger ones frequently had

more than one synapse. Sometimes the same terminal established a symmetric synapse with one dendritic element and an asymmetric synapse with a different one (data not shown) (see also Sotelo et al.,1986). The single and double GABA labeled terminals in the neuropil were located inside as well as outside the glomeruli. The terminals that were encapsulated in the glomeruli frequently contacted dendritic elements linked by gap junctions (Figs. 8A-B; Fig. 10A). The extraglomerular terminals were often apposed to dendritic profiles organized in thickets (Fig. 11A-B). Some double labeled axosomatic terminals were also observed (Figs. 10B-C). Another subclass of GABAergic terminals in the dc was involved in special cell junctions called crest synapses (Figs. 9B-C). With regard to size, these GABAergic terminals were indistinguishable from the first type. However, the crest synapses were located almost exclusively outside the glomeruli, and none of their associated terminals was ever found to be double labeled. In the rats, a total of 27 crest synapses were observed; 17 were formed with 2 GABAergic terminals, 7 were formed with 1 GABAergic and 1 non-GABAergic terminal, and 3 were formed with 2 non-GABAergic terminals. In the one case, in which 3 crest synapses were part of the same dendritic structure, all the involved terminals were GABAergic (Fig.9B). In the rabbit, a total of 31 crest synapses were found. Of these crest synapses 24 were made with only GABA-positive terminals whereas the remaining 7 were formed with 1 GABAergic and 1 non-GABAergic terminal. Overall, 76% of the 54 terminals involved in the crest synapses of the rat were GABAergic while the corresponding figures for the rabbit were 89% of 62 terminals. By comparison, in the ß-nucleus of the rat only 1 crest synapse was observed. This crest synapse was formed with 2 GABAergic terminals. The third type of GABAergic bouton was rarely encountered and was never found to be double labeled. It was characterized by numerous large dense core vesicles, interspersed between tubulovesicular elements and clear pleomorphic vesicles. These terminals, which ranged in diameter from 0.7 µm to 3.5 µm, usually did not exhibit a synapse and were rarely located within a glomerulus. In one case this type of GABAergic terminal was located within a glomerulus and established an asymmetric synaptic contact with a spine in the vicinity of a gap junction (Fig. 10A).

The morphology of the single WGA-HRP labeled terminals (Fig. 12) differed from that of double GABA/WGA-HRP labeled terminals. The single WGA-HRP labeled terminals usually contained round synaptic vesicles, mitochondria and an occasional large dense core vesicle. The size of these terminals was rather variable, ranging from 1 µm to 4 µm in diameter. Most of these terminals formed asymmetric synapses with spines and medium sized dendrites, some of which were encapsulated within glomeruli and linked by gap junctions. Single WGA-HRP labeled terminals were observed together with GABAergic terminals in the same glomerulus (Fig. 12). In addition, they also occurred in dendritic thickets and contacted cell bodies.

Quantitative analysis. A total of 498 WGA-HRP labeled terminals were photographed from the caudal (n=324) and rostral (n=174) dc of the rats. The exact data about the distribution of these terminals are given in Table 1 and schematically represented in Figure 13. In the ipsilateral caudal dc (n=195) 37% of the WGA-HRP labeled terminals were labeled with only WGA-HRP while 63% were double labeled with WGA-HRP and GABA-immunoreactivity. In the contralateral caudal dc (n=129) 13% were GABA-negative and 87% were GABA-positive. In the rostral dc only 8% of the WGA-HRP labeled terminals were GABA-positive on the ipsilateral side (n=86), whereas on the contralateral side 79% were double labeled (n=88). The differences between the ipsilateral and contralateral side were significant for both the caudal ($p<0.005$) and rostral ($p<0.005$) dc (for SEM19s see Table 1). In addition, the difference between the ipsilateral caudal and rostral dc was significant ($p<0.005$). In all dc areas studied only a minority of the single and double WGA-HRP labeled terminals in the neuropil was

TABLE 1. Populations of single WGA-HRP (S) and double WGA-HRP/GABA (D) labeled terminals randomly collected from ultrathin sections of the caudal and rostral, ipsilateral (IPSI) and contralateral (CONTRA) dorsal cap of rats following injection of WGA-HRP in the PrH combined with GABA immunocytochemistry. For further details see "Collection and analysis of the data" in the Material and Methods.

	CAUDAL DORSAL CAP				ROSTRAL DORSAL CAP			
	IPSI		CONTRA		IPSI		CONTRA	
	S	D	S	D	S	D	S	D
Mean (%)	37	63	13	87	92	8	21	79
SEM	2.7	2.7	2.9	2.9	2.9	2.9	5.7	5.7
n	76	119	21	108	78	8	19	69
Glomerular (%)	11	16	14	15	13	13	16	15
Extraglomerular(%)	85	78	86	79	84	87	84	78
Axosomatic(%)	4	6	0	6	3	0	0	7

located inside a glomerulus (for details see Table 1). Hence the vast majority of these terminals was extraglomerular. Only a small minority of the single and double WGA-HRP labeled terminals was apposed to a perikaryon. The quantitative data of the dc obtained from the rat, in which the WGA-HRP injection was restricted to the PrH, were not significantly different from those obtained from the rat in which the injection site included also part of the MVe and SpVe. In the ß-nucleus, which was obtained from the rat with the large injection, 93% of the WGA-HRP labeled terminals (n=78) were GABA-positive. This percentage was significantly higher than that of double labeled terminals on the ipsilateral side of both the caudal ($p<0.005$) and rostral ($p<0.005$) dc. Of the double WGA-HRP labeled terminals in the ß-nucleus 34% were classified as glomerular, 61% extraglomerular and 5% axosomatic. In the contralateral dc of the rabbit, 92 WGA-HRP labeled terminals were analyzed, 87% of which were GABA-positive. Of these double labeled terminals 17% were glomerular, 80% extraglomerular and 3% axosomatic.

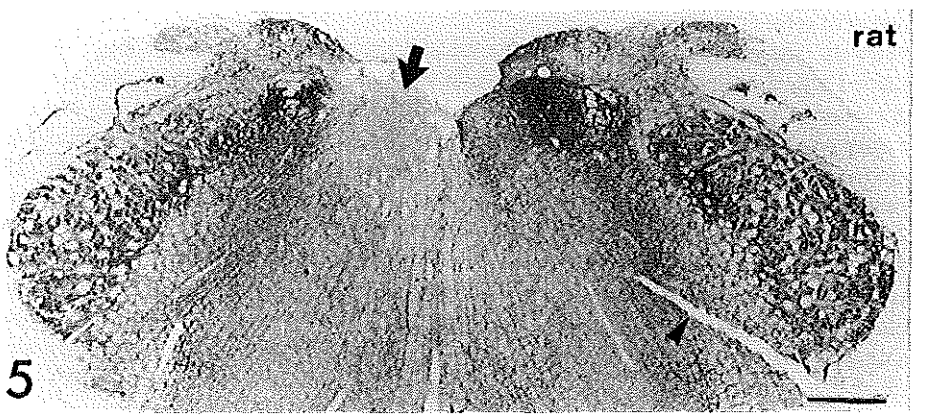

Figure 5. Site of lesion in the caudal PrH of the left side (arrow)in a rat. Note that the lesion extends rather ventrally, i.e. the pathway of the fibers which course from the contralateral PrH to the ipsilateral side in the hindbrain is affected. This lesion, therefore, should be expected to interrupt a large proportion of both the ipsilateral and contralateral projections to the left dc and only partially the projections to the right dc. The arrow head indicates the cut that was used to mark the right side of the brain. Scale bar is 520 µm.

Discussion

The present study indicates that the principal ultrastructural features of the rat and rabbit dc of Kooy qualitatively are the same as those of the other olivary subdivisions: the neuropil contains extraglomerular and glomerular synaptic fields; the neurons are coupled by gap junctions; and the nucleus contains numerous GABA-positive and GABA-negative axon terminals. Part of both types of terminals in the dc are derived from neurons of the PrH. They are situated in the glomeruli next to dendrites linked by gap junctions, but they also form synapses with extra-glomerular dendrites and neuronal cell bodies. In addition, we found that the dc contains relatively many crest synapses, and that these specialized junctions are predominantly formed with GABAergic terminals, whose origin remains to be established.

Electron Microscopy

General features. Most of the terminals in the dc of the rat and the rabbit contained either rounded or pleomorphic vesicles and formed, respectively, asymmetric or symmetric synapses with intermediate and distal dendritic elements inside or outside the glomeruli. Some of the terminals contacted neuronal cell bodies. Our results agree with the study of the dc in rabbit by Mizuno and colleagues (1974). In addition, the present study shows that gap junctions are present in the dc of both the rat and the rabbit, thereby indicating that neurons in the dc are electrotonically coupled. This is in line with a study by Takeda and Maekawa (1989) who showed "olivary reflexes" (Armstrong et al.,1973) within the flocculo-nodular lobe, a phenomenon which is now generally accepted as being due to electrotonic coupling in the IOL (Llinás et al.,1974; Llinás and Sasaki,1989). These results indicate that the dc is organized like other olivary subnuclei, as described in rat (Gwyn et al.,1977; Sotelo et al.,1986), cat (Walberg,1963, 1966; Nemecek and Wolff,1969; Sotelo et al.,1974), opossum (Bowman and King,1973; King,1976 and 1980), and squirrel monkey (Rutherford and Gwyn,1977 and 1980). Quantitatively, however, two features of the neuropil of the dc differ from those of the other olivary subnuclei: i) the relatively small size of the glomeruli; and ii) the prominent presence of crest synapses.

Glomeruli. The glomeruli in the dc of the rat and the rabbit were usually small; they often contained only 3 spines and 2 axon terminals in single sections, which means they are smaller than glomeruli in the MAO and PO of the cat and the rat (De Zeeuw et al.,1990 a,b, and c). For example, the glomeruli of the feline MAO contain an average of 6 spiny profiles in single sections. The percentage of terminals within the glomeruli is obviously related to the size of these synaptic nests. Quantitative analysis of the ultrathin sections of the dc of the rats and rabbits showed that only about 15% of the terminals anterogradely labeled from the PrH were located within glomeruli. This held true for both the single WGA-HRP and the double GABA/WGA-HRP labeled terminals. In ß-nucleus of the rat, however, 34% of the double labeled terminals from the vestibular nuclei were located within glomeruli. Previous quantitative analysis of the cat and the rat showed that in the MAO and PO approximately one third of the terminals derived from the mesodiencephalic junction (non GABAergic) and the central cerebellar nuclei (GABAergic) were located within glomeruli (De Zeeuw et al.,1989a,b; De Zeeuw et al.,1990a; for comparison see qualitative data of rat in PO by Angaut and Sotelo,1987). It appears, therefore, that fewer boutons are situated in the glomeruli in the dc than in other olivary subnuclei.

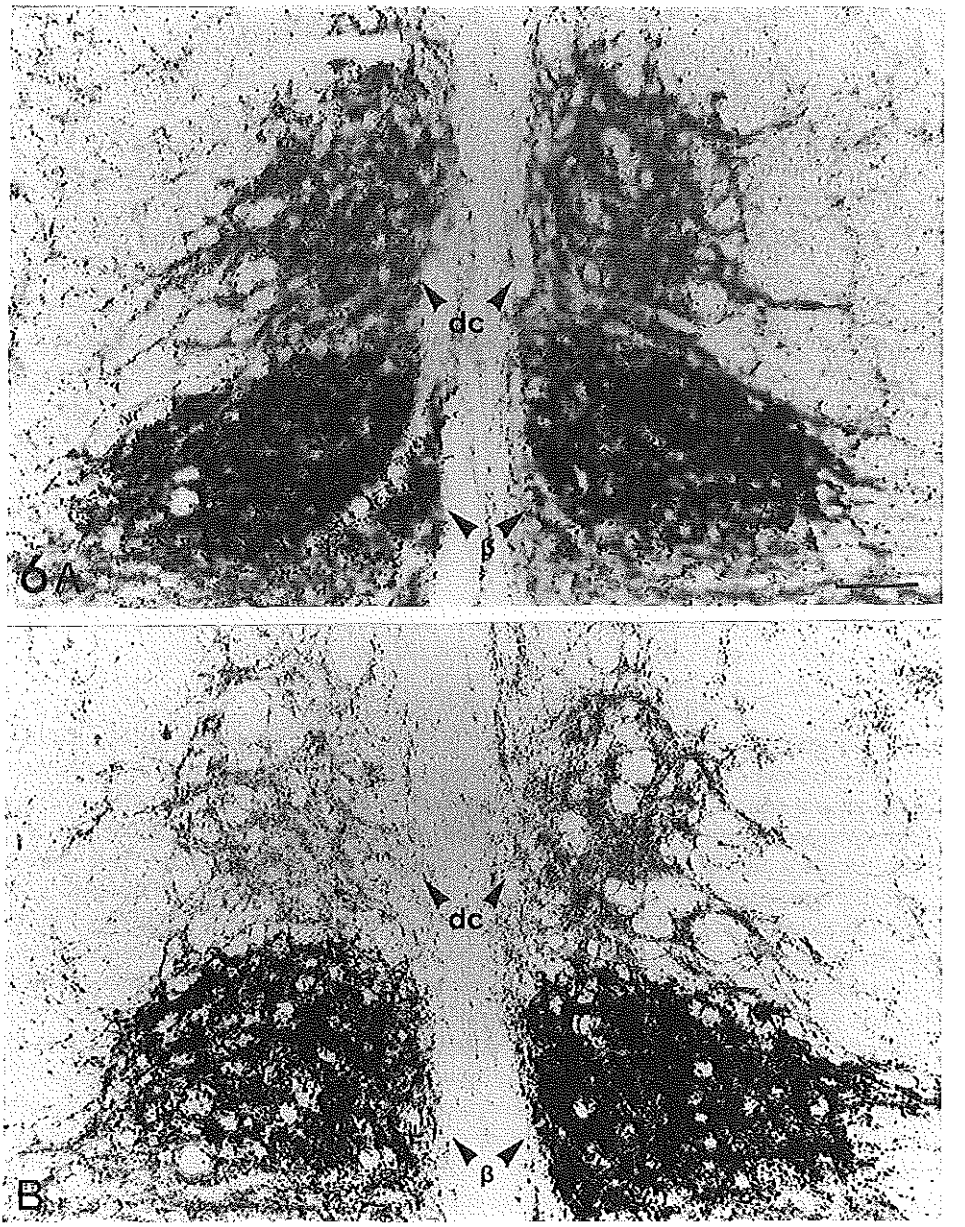

Figure 6. *Lesion (given in Fig. 5) induced depletion of GAD in the rat. A: GAD terminal labeling in the dc and β-nucleus of a normal rat used for control. B: Depletion of GAD in the dc. Note that the ipsilateral side (left) is depleted of most of its GAD positive input whereas the contralateral side still contains a substantial number of GAD immunoreactive boutons. Scale bar in A is 56 μm, and in B is 53 μm.*

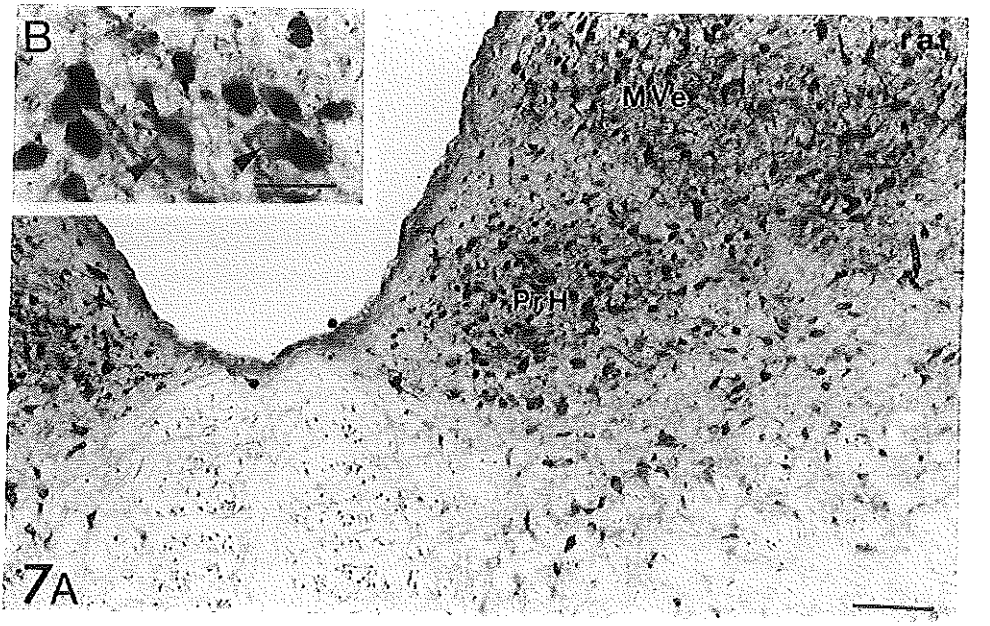

Figure 7A-B. GAD positive and negative neurons in the PrH of the rat. B is a detail of A. Arrow heads indicate GAD negative neurons. Scale bar in A is 139 μm, and in B is 36 μm.

Crest synapses. The presence of crest synapses in the dc was first described by Mizuno et al. (1974) for the rabbit. The present study confirms and extends their observations. In the areas from the rat and rabbit dc sampled for our quantitative analysis, we observed a total of 27 and 31 crest synapses, respectively, while only 1 such synapse was observed in the ß-nucleus. Since the total sampled area in the rabbit was about 4 times smaller than in the rat these numbers suggest that relatively many crest synapses are present in the rabbit. In the MAO and PO of the cat and the rat a maximum of 6 crest synapses was observed in spite of much larger sample areas (for details, see De Zeeuw et al.,1989a; De Zeeuw et al.,1990a). Thus the density of crest synapses in the dc of both the rabbit and the rat appears to be higher than in other olivary subnuclei. The vast majority of the dc terminals engaged in the crest synapses were GABAergic in spite of the fact that the synaptic junctions consistently belonged to the asymmetric category. The few crest synapses found in the previous study of the MAO and PO of the cat (De Zeeuw et al.,1989a) were likewise associated with GABAergic terminals. Sotelo et al. (1986) have also illustrated a crest synapse formed with two GAD positive terminals in an unspecified region of the rat IOL The GABAergic terminals associated with the crest synapses were rarely located within glomeruli and they were never found to originate from the PrH (present study) or the cerebellar nuclei (De Zeeuw et al.,1989a). These findings may be due to a sampling bias but they offer the possibility that the GABAergic terminals associated with the crest synapses are derived from a different origin. Other areas where crest synapses have been found include the habenula (Milhaud and Pappas,1966a,b), interpeduncular nucleus (Milhaud and Pappas,1966a,b; Lenn,1976; Murray et al.,1979), subfornical organ (Akert et al., 1967), suprachiasmatic nucleus (Guldner,1976), and locus ceruleus (Mizuno and Nakamura,

1972). The crest synapses in the interpeduncular nucleus are formed with one terminal derived from the ipsilateral habenula and the other from the contralateral habenula (Murray et al., 1979). In addition, crest synapses have been observed in the avian ciliary ganglion (Mugnaini, unpublished observations) where they are formed by excitatory, cholinergic endings derived from the Edinger-Westphal nucleus (Pilar and Johnson,1988).

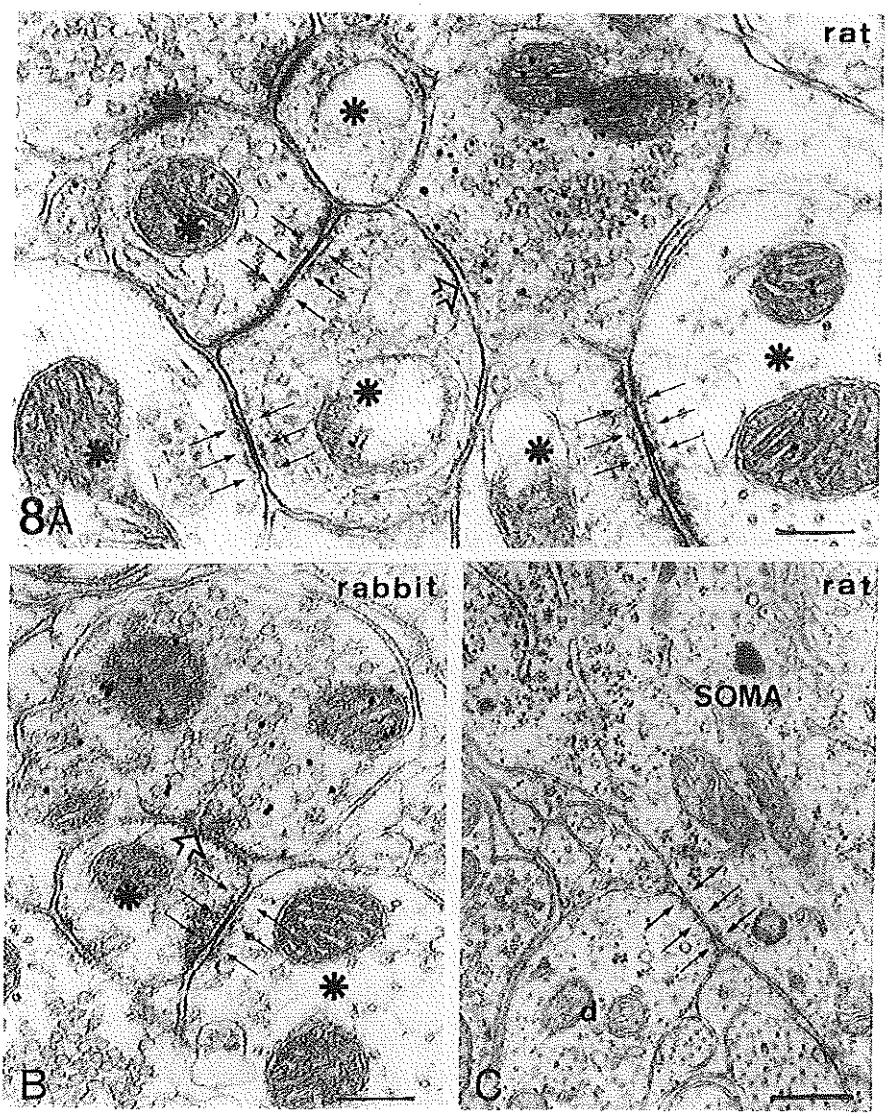

Figure 8A-C. Gap junctions in the dc of the rat (A and C) and the rabbit (B). In A and B a GABAergic terminal is apposed to one or more dendritic elements (asterisks) that are coupled by gap junctions (small arrows). Note that in A the GABAergic terminal is located in the vicinity of 3 gap junctions. In C the soma is coupled by a gap junction with a primary dendrite (d). Open arrows indicate symmetric synapses. Scale bar in A is 0.28 μm, in B is 0.31 μm, and in C is 0.61 μm.

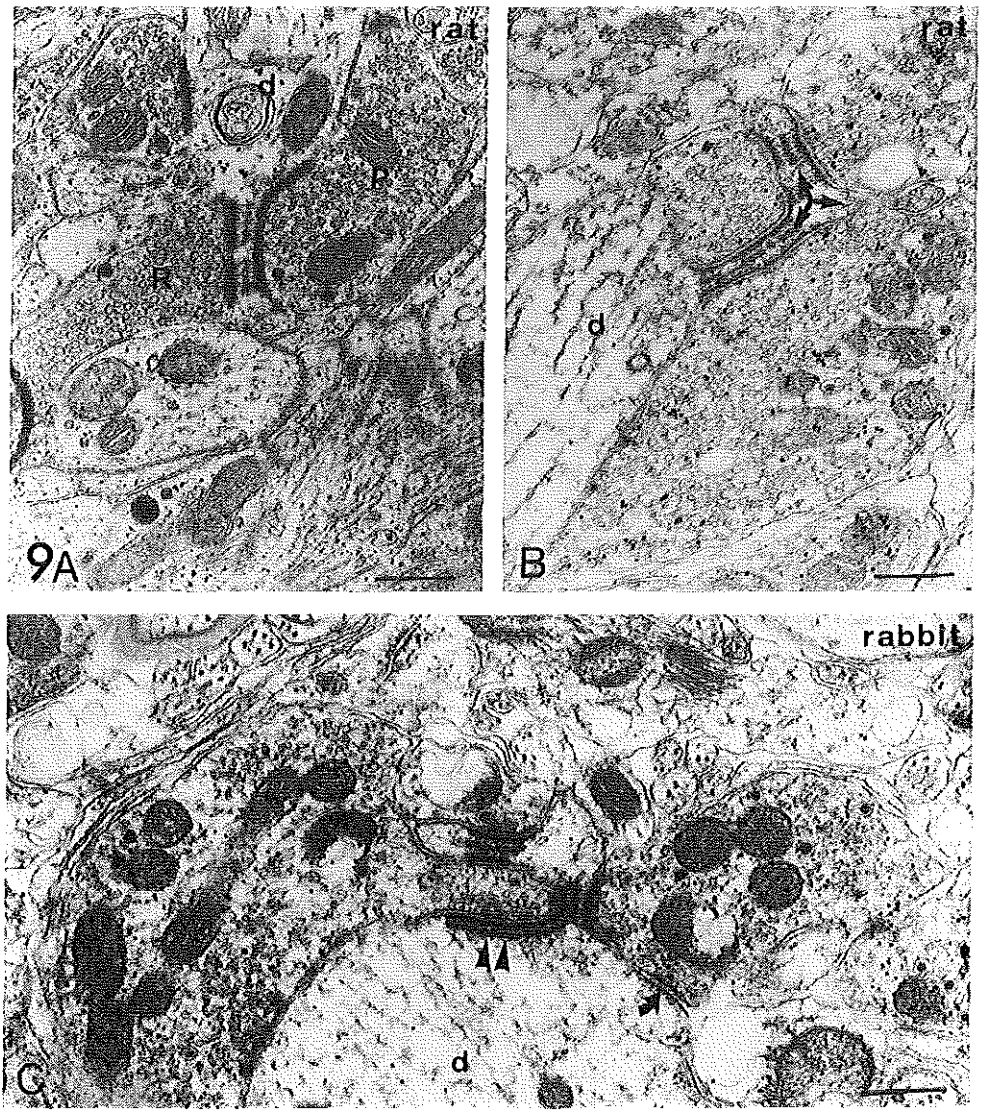

Figure 9A-C. Crest synapses in the dc of the rat (A and B) and the rabbit (C). In A the crest synapse is formed with a terminal containing round vesicles (R) and a terminal containing pleomorphic vesicles (P). The micrograph of Figure 9A is obtained from an ultrathin section that is not processed for GABA immunocytochemistry. The spots visible in this micrograph presumably represent glycogen particles preserved by the ferrocyanide-reduced osmium. The terminals forming the crest synapses in B and C are all GABAergic. The arrows in B indicate branching of the dendritic process involved in the crest synapse. Small d indicates the dendritic shaft from which the narrow evagination forming the crest synapse originates.The arrow in C indicates a punctum adherens. Note that the synaptic junction and the row of subsynaptic densities belonging to the crest synapse in C is continuous with the synaptic junction and subsynaptic densities in the primary dendrite (arrow heads). Scale bar in A is 0.46 μm, in B is 0.48 μm, and in C is 0.54 μm.

Projection from the PrH to the dc

Distribution. The experiments in which PHA-L and WGA-HRP were injected into the PrH of the rat and the rabbit showed that: i) this nucleus provides a bilateral input to the caudal dc; ii) the input to the rostral dc is predominantly contralateral; iii) the projection to the VLO is exclusively contralateral; and iv) the input to the MAO is ipsilateral. These results confirm and extend previous studies in cat that indicated the PrH projects mainly to the contralateral dc and ipsilateral MAO (Saint-Cyr and Courville,1979; McCrea and Baker,1985; Gerrits,1985). Since the dc and the VLO are now considered as being continuous with the MAO (Azizi and Woodward,1987; Buisseret-Delmas,1988a,b; Bourrat and Sotelo,1991) it appears that the PrH projects exclusively to areas of the MAO. Our finding that the PrH bilaterally innervates neurons in the caudal dc that are known to respond optimally to movements of the visual field around the vertical axis (Leonard et al.,1988), is in line with the fact that the projection from the PrH to the extraocular motornuclei is mainly to the abducens nucleus and the medial rectus subdivision of the oculomotor nucleus (McCrea and Baker,1985).

Neurotransmitters. Following a lesion of the PrH in the rat most of the GABAergic terminals in the caudal dc and a smaller fraction of these terminals in the rostral dc and VLO disappeared (see also Fig. 13). The lesions affected the ipsi- and contralateral fibers of the lesioned PrH as well as those fibers of the contralateral PrH that cross the midline and innervate the dc on the side of the lesion. However, the lesions did not damage the fibers of the contralateral PrH that do not cross the midline and innervate the dc contralateral to the side of the lesion. Therefore, it is understandable that the caudal dc contralateral to the lesioned PrH was only partly depleted of GABA. In the ultrathin sections from the rats in which WGA-HRP anterograde tracing from the PrH was combined with GABA-immunocytochemistry, it was found that the vast majority of the WGA-HRP labeled terminals on the contralateral side were GABAergic, both caudally and rostrally, whereas on the ipsilateral side the double labeled GABAergic terminals were predominantly present at the caudal level. In the contralateral dc of the rabbit it was also found that the majority of the WGA-HRP labeled terminals were GABA positive. These data indicate that the PrH is a major source of the GABAergic input to the caudal dc, and is also one of the sources of the GABAergic innervation of the rostral dc and VLO. This finding is in agreement with the observations of McCrea and Baker (1985) in cat that PrH cells projecting to the dc are small and that the PrH of the rat contains many small GABAergic neurons (Mugnaini and Oertel,1985; present study; for comparison with cat, see Yingcharoen et al.,1989). Moreover, it is in line with the finding that the PrH contains retrogradely labeled neurons following large injections of WGA-HRP covering the entire IOL and that these retrogradely labeled neurons can be GAD positive (Nelson and Mugnaini,1989).

Whether the MVe and SpVe also contribute to the GABAergic input to the dc can not be excluded from the present study. However, this projection would probably not affect our results since the data obtained from the dc of the rat, in which the WGA-HRP injection was restricted to the PrH, were not significantly different from those obtained from the rat in which the injection site included also part of the MVe and SpVe. Moreover, the existence of a GABAergic input from the MVe and SpVe to the dc seems unlikely because Gerrits (1985) showed in cat that these vestibular nuclei project to the ß-nucleus and the dorsomedial cell column but not to the dc.

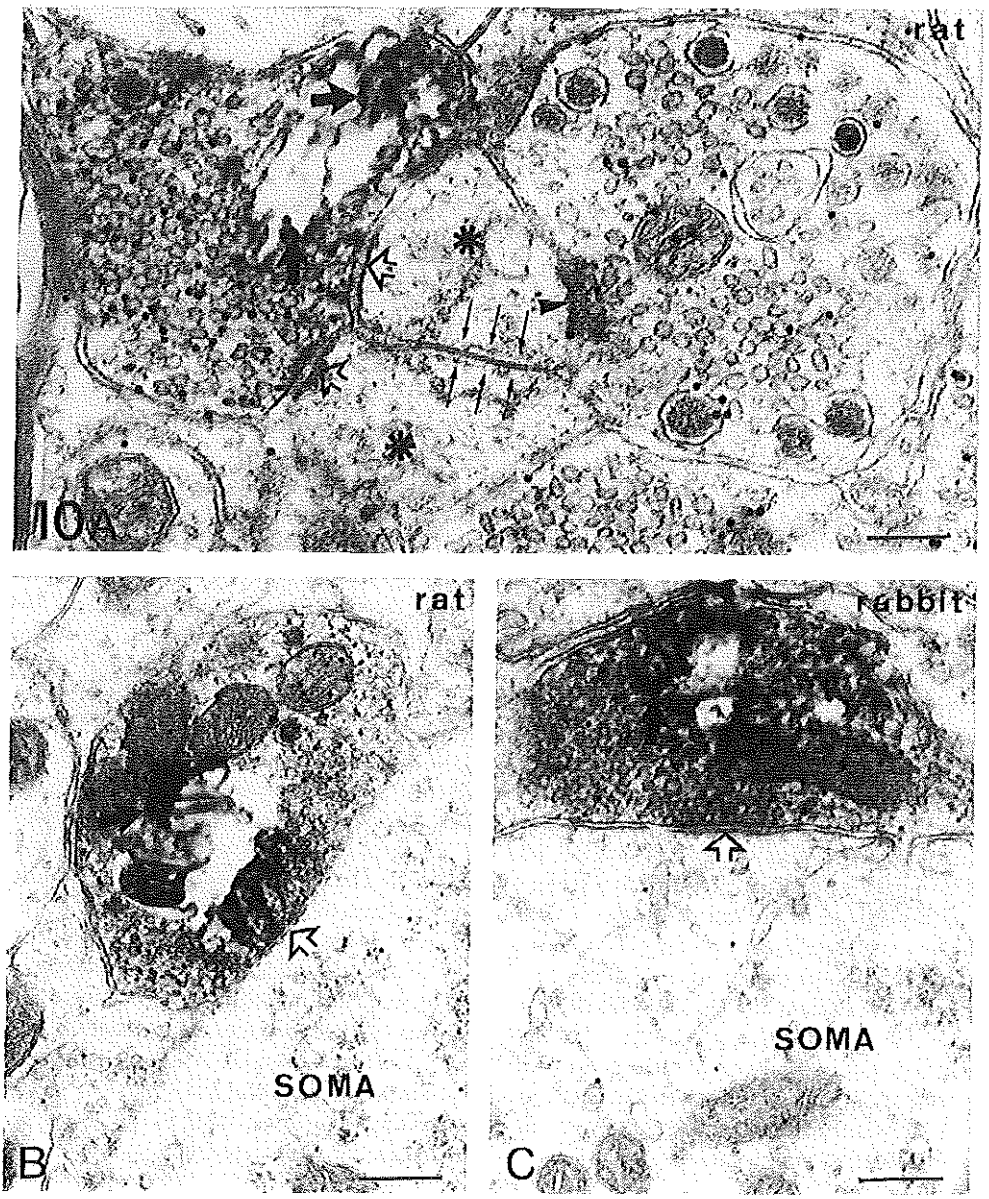

Figure 10A-C. Double labeled boutons (GABA and anterogradely transported WGA-HRP) from the contralateral PrH terminate on distal and proximal portions of neurons in the dorsal cap of the rat (A and B) and the rabbit (C). In A, a double labeled terminal (left) and a GABAergic large granular terminal (right) are apposed to dendritic spines coupled by a gap junction. In B and C, double labeled terminals are apposed to somata. The large arrows indicate WGA-HRP reaction products and open arrows indicate symmetric synapses. In A, the arrow head indicates an asymmetric synapse, the small arrows indicate a gap junction, and the asterisks mark dendritic spines. Scale bar in A is 25 μm, in B is 41 μm, and C is 37 μm.

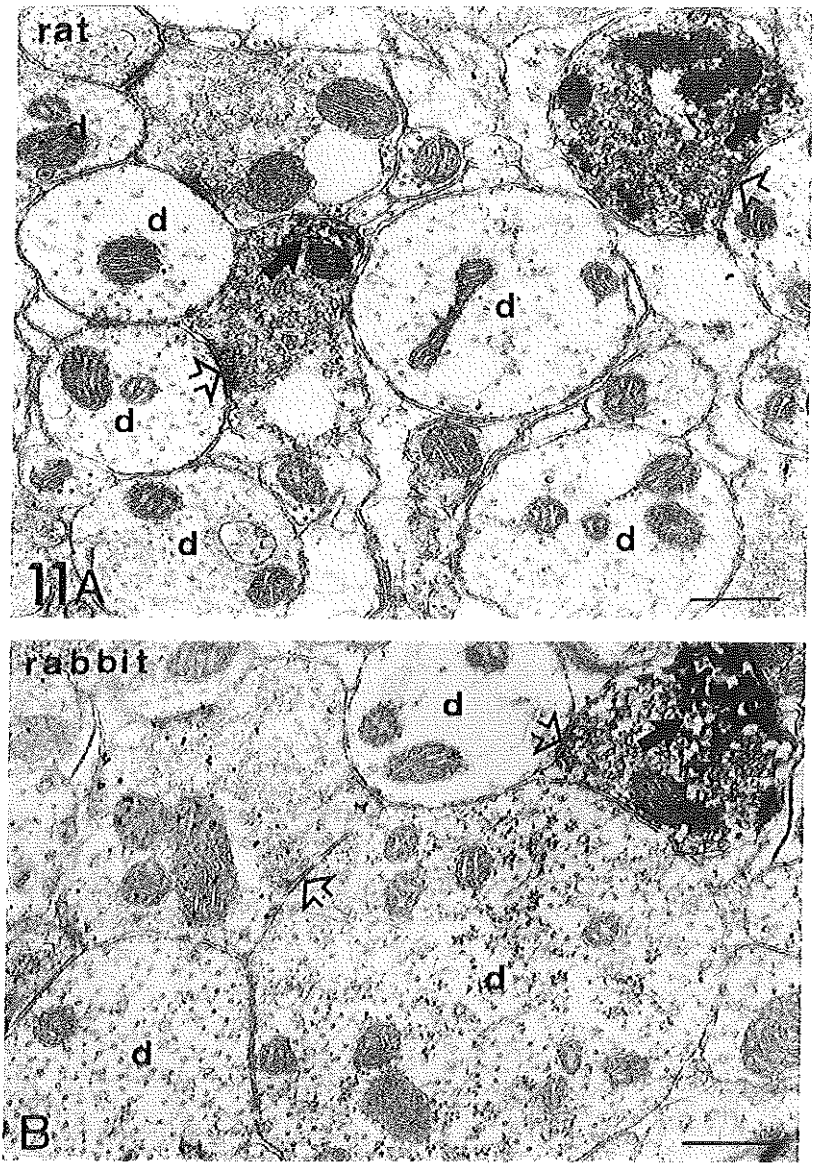

Figure 11A-B. Double labeled terminals (GABA and anterogradely transported WGA-HRP) from the contralateral PrH apposed to dendrites in the dc of the rat (A) and the rabbit (B). The terminals in A and B form symmetric synapses (open arrows). Note that the double labeled terminals are apposed to dendrites (d) that are organized in dendritic thickets (a group of dendrites located next to one another). Large arrows indicate WGA-HRP reaction products. Scale bar in A is 0.74 μm, and in B is 0.71 μm.

Significantly more WGA-HRP labeled terminals were GABAergic on the contralateral side than on the ipsilateral side. This difference was not only significant for the rostral dc but also for the caudal dc. In the rat ß-nucleus, in which the ipsilateral GABAergic projection from the MVe and adjoining SpVe was analyzed for control (see also Nelson et al.,1986; Barmack et al.,1989), significantly more WGA-HRP labeled terminals were GABA positive than in the ipsilateral dc. These data indicate that part of the ipsilateral projection from the PrH to the dc is non-GABAergic. This conclusion is supported by the observation that, unlike the GABAergic WGA-HRP labeled terminals, the non-GABAergic terminals from the PrH usually contained round synaptic vesicles and formed asymmetric synapses. Synapses of this kind are usually excitatory (Uchizono,1965). Moreover, the non-GABAergic WGA-HRP labeled terminals varied considerably in size, whereas the GABA positive terminals were usually of intermediate size. Barmack et al. (1991) have shown a cholinergic projection from the PrH to the ipsilateral dc. It is possible that at least a portion of the non-GABAergic terminals in the ipsilateral dc is actually made up of cholinergic terminals. On the other hand, it should be noted that cholinergic terminals in the rat cochlear nuclei can represent an heterogeneous population (Vetter et al.,1992), and that acetylcholine and GABA can coexist within the same neuron (Beaulieu and Somogyi,1991; Todd,1991).

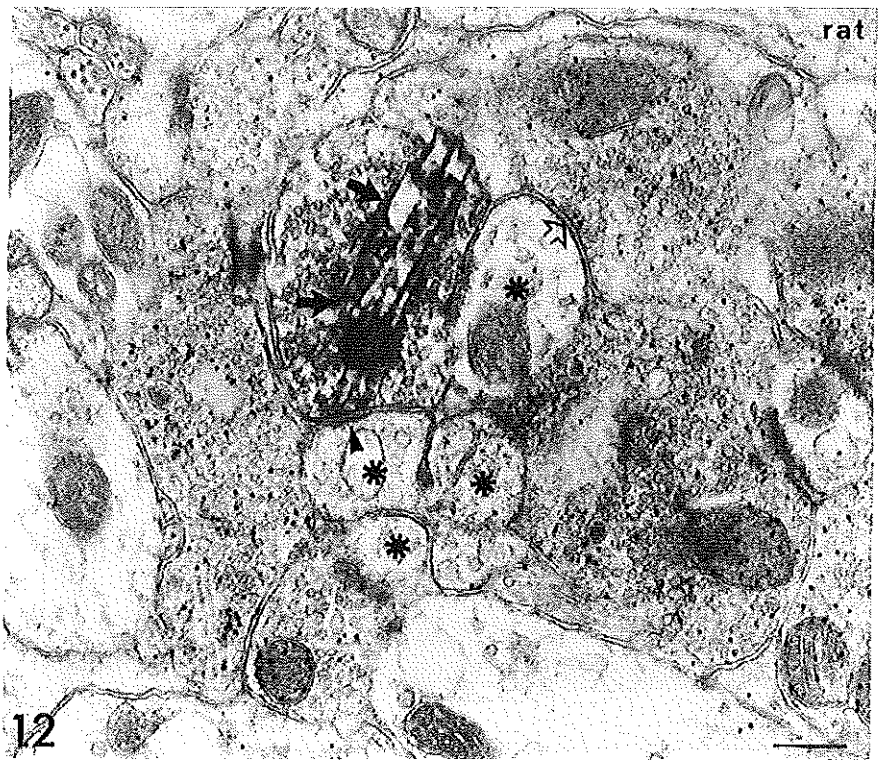

Figure 12. A single WGA-HRP labeled terminal (arrows) from the PrH and GABAergic terminals of unknown origin are located in the same glomerulus in the ipsilateral rostral dc of the rat. Dendritic spines are marked by asterisks. Note that the single WGA-HRP labeled terminal forms an asymmetric synapse (arrow head) with a dendritic spine. Open arrow indicates symmetric synapse. Scale bar is 0.39 µm.

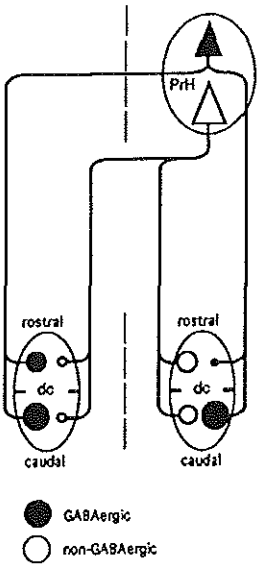

Figure 13. Schematic diagram showing the distribution of GABAergic and non-GABAergic projections from the PrH in the ipsilateral and contralateral, caudal and rostral dc in the rat. The surface areas of the different GABAergic and non-GABAergic terminal fields (black and white circles) are meant to represent differences in the relative proportions of the 2 terminal populations (for numerical data see Table 1) and not the sizes of individual boutons.

Functional implications. The PrH is involved in the control of eye movements and, to a lesser degree, in the control of related head movements (for review see McCrea,1988). However, nothing is known about the specific electrophysiological properties of the PrH neurons projecting to the dc. Therefore, several possible functional roles for the projection from the PrH to the dc should be considered.

Firstly, this innervation could send an efference copy signal to the dc that relays information about neural processing occurring in the caudal PrH (Baker,1977; Baker et al.,1981; McCrea and Baker,1985; McCrea,1988; Delgado-Garcia et al.,1989). The neural processing in the PrH, which includes integration in the mathematical sense, is thought to be necessary for maintaining eye velocity through "velocity storage" and for determining eye position in the vestibulo-ocular reflex (Collewijn,1972; Skavenski and Robinson,1973; Blanks et al.,1977; Robinson,1989). Another possible reason for sending an efference copy signal to the dc may be to suppress optokinetic reflexes during voluntary eye movements (Holst and Mittelstaedt,1950; McCrea and Baker,1985; McCrea,1988).

Secondly, the projection from the PrH to the dc can be compared with the cerebellar nuclear input to the IOL (De Zeeuw et al.,1988b; De Zeeuw et al.,1989a; Angaut and Sotelo,1989; Fredette and Mugnaini,1991). Each olivary subnucleus projects to one or more strips of Purkinje cells, and the Purkinje cells of each strip project to a particular cerebellar and/or vestibular nucleus, which in turn innervates the corresponding olivary subnucleus (Groenewegen and Voogd,1977; Groenewegen et al.,1979; for review see Voogd and Bigaré,1980). An analogous triangular pathway is represented by the dc, the flocculo-nodular lobe (including folium p) and the PrH, as demonstrated by studies in cat, rat and rabbit (Alley et al.,1975; Yamamoto,1979; Gerrits and Voogd,1982; Yingcharoen and Rinvik,1983; McCrea,1985; Ruigrok et al.,1992; and the present study). This analogy is supported by the findings that the GABAergic terminals derived from the PrH in the dc and the GABAergic terminals from the cerebellar nuclei in the MAO and the PO have very similar morphological characteristics, and that both types of terminals contact dendrites linked by gap junctions in the

glomeruli (for rat see Angaut and Sotelo,1987 and 1989; De Zeeuw et al.,1990a, for cat see De Zeeuw et al.,1989a and 1990a). Therefore, it seems possible that the GABAergic terminals from the PrH, like those from the cerebellum regulate the electrotonic coupling in the IOL (see also Sasaki and Llinás,1985; Lang et al.,1989; Sasaki et al.,1989; Llinás and Sasaki,1989; Lang et al.,1990). The hypothesis that the PrH functions partly as a cerebellar nucleus is not mutually exclusive with the first hypothesis mentioned above. In fact, the effectiveness of efference copy signals may very well depend on the level of synchronicity between the olivary neurons (see also Yarom,1992). Moreover, the cerebellar central nuclei may also send out efference copy signals to the IOL in order to prevent reflexes during voluntary movements (Gibson and Gellman,1987; Armstrong and Andersson,1987).

Thirdly, the projection could carry vestibular information. Although neurons in the dc generally do not seem to respond to vestibular stimulation (Barmack and Hess,1980; Barmack et al.,1989), Leonard and Simpson (1985) reported the existence of floccular Purkinje cells that showed climbing fiber modulation during natural vestibular stimulation in the dark. Since the dc does not receive a direct input from the vestibular nuclei (Gerrits,1985), while the PrH receives a strong input from these nuclei (McCrea,1988), it is attractive to speculate that the projection from the PrH to the dc relays vestibular information to the dc.

IIIb. Gabaergic inputs from dorsal group y and the ventral dentate nucleus to the rostral dorsal cap and ventrolateral outgrowth

Abstract

The dorsal cap and ventrolateral outgrowth of the inferior olive are involved in the control of eye movements. The caudal dorsal cap is predominantly involved in the horizontal optokinetic reflex; it receives most of its GABAergic input from the nucleus prepositus hypoglossi. In the present study, we determined the source of a major inhibitory input to the rostral dorsal cap and the ventrolateral outgrowth which are the olivary subnuclei mainly involved in the "vertical" optokinetic reflexes. We studied these subnuclei in the rabbit with the use of retrograde tracing of horseradish peroxidase and with the use of anterograde tracing of wheat germ agglutinin-coupled horseradish peroxidase combined with postembedding immunocytochemistry. The ventral dentate nucleus of the cerebellum and dorsal group y project contralaterally to the rostral dorsal cap and ventrolateral outgrowth; this projection is entirely GABAergic. The terminals of this input form predominantly symmetric synapses with extra- and intraglomerular dendrites; the remaining terminals are axosomatic. In addition, the dorsal cap and ventrolateral outgrowth contain significantly more crest synapses than any other olivary subnucleus. The terminals that form these crest synapses are derived from dorsal group y and/or the ventral dentate nucleus. None of the terminals in the dorsal cap or ventrolateral outgrowth was glycinergic.

Introduction

The dorsal cap of Kooy and the adjacent ventrolateral outgrowth (VLO) are subnuclei of the inferior olive (IOL; Kooy,1916; Brodal,1940) that are known to provide climbing fibers to the Purkinje cells in the flocculo-nodular lobe of the cerebellar cortex (Alley et al.,1975; Groenewegen and Voogd,1977; Gerrits and Voogd,1982; Gerrits et al.,1985; Ruigrok et al, 1992). Both subnuclei are involved in the visual control of eye movements. In the rabbit their neurons respond best to slow movements of large, textured patterns (Simpson and Alley,1974; Simpson et al.,1981; Leonard et al.,1988; Simpson et al.,1989; Graf et al.,1988; for rat see Blanks and Precht,1983). Three different regions within the dorsal cap and VLO can be identified on the basis of differences in eye dominance and the spatial orientation of the axis of monocular visual world rotation that results in the best response: the caudal dorsal cap is dominated by the contralateral eye and responds optimally to optokinetic stimuli rotating about the vertical axis; the caudal part of the rostral dorsal cap is dominated by the ipsilateral eye and responds preferentially to rotations about a horizontal axis at an azimuthal angle of 45° to the ipsilateral eye; and the rostral part of the rostral dorsal cap and the VLO are dominated by the contralateral eye and respond optimally to rotations about a horizontal axis at an azimuthal angle of 135° to the contralateral eye. A distinction between the caudal and rostral dorsal cap of the rabbit has also been demonstrated in relation to different extraocular muscles (Ito et al, 1977 and 1978). Electrical stimulation of the caudal dorsal cap inhibited the excitation of the contralateral medial rectus muscle produced by stimulation of the contralateral vestibular labyrinth. Stimulation of the rostral dorsal cap inhibited the excitation of the contralateral superior rectus muscle and of the inferior oblique muscle produced by stimulation of the contralateral vestibular labyrinth.

The visual sensory signal in the dorsal cap and VLO is encoded in an intrinsic reference frame that reflects the physical structure of the semicircular canals as well as the orientation of the extraocular muscle pairs (Simpson and Hess,1977; Simpson et al.,1981; Graf and Simpson, 1981; Graf et al.,1988; Van der Steen et al.,1991). The physiological organization of the dorsal cap and VLO follows from anatomical and physiological differences in their input sources. The caudal dorsal cap receives an ipsilateral projection from the dorsal terminal nucleus (DTN) of the accessory optic system (AOS) and the pretectal nucleus of the optic tract (NOT), whereas the rostral dorsal cap and adjacent VLO receive an ipsilateral projection predominantly from neurons situated in the visual tegmental relay zone (VTRZ), (Mizuno et al.,1973; Takeda and Maekawa,1976; Maekawa and Takeda,1977 and 1979; Walberg et al., 1981; Holstege and Collewijn,1982; Simpson,1984; Giolli et al.,1985; Simpson et al.,1988; Soodak and Simpson,1988). These projections are non-GABAergic and presumably excitatory (Mizuno et al.,1974; Horn and Hoffmann,1987; Nunes-Cardozo and Van der Want,1990). In addition, the dorsal cap and VLO are innervated by neurons situated in the nucleus prepositus hypoglossi (PrH), (cat:Gerrits et al.,1985; McCrea and Baker, 1985) that are partly cholinergic (rat, rabbit, and monkey: Barmack et al.,1993) and partly GABAergic (rat and rabbit: De Zeeuw et al.,1993). The laterality of the cholinergic projection from the PrH is unclear because contradicting results have been reported (Barmack et al.,1991; Barmack et al.,1993). The GABAergic projection from the PrH to the dorsal cap is bilateral with a contralateral predominance, while the projection to the VLO is contralateral. Lesion-induced depletion of glutamic acid decarboxylase immunoreactivity in rat showed that the caudal dorsal cap receives most of its GABAergic input from the PrH while the rostral dorsal cap and VLO receive less than half of their GABAergic input from this nucleus (De Zeeuw et al.,1993). The GABAergic terminals from the PrH are located in both the extra- and intraglomerular neuropil, but they do not form crest synapses (Milhaud and Pappas,1966; Akert et al.,1967; Mizuno et al.,1974) even though crest synapses in the IOL are formed mostly by GABAergic terminals (De Zeeuw et al.,1993).

In the present study, an attempt was made in the rabbit to find the source of the major GABAergic input to the rostral dorsal cap and VLO, and to examine whether this input gives rise to crest synapses. We explored the hindbrain by using retrograde transport of horseradish peroxidase (HRP) from the rostral dorsal cap and VLO, which were physiologically identified before injection by determining the response preferences of their neurons for rotation of optokinetic stimuli around the 45°/135° axis, as mentioned above. Subsequently, we studied the same projections 1) with the use of anterograde tracing of wheat germ agglutinin-coupled HRP (WGA-HRP) at the light microscopic level, and 2) with the use of anterograde tracing of WGA-HRP combined with GABA immunocytochemistry at the electron microscopic level. To find out whether glycine is also used as an inhibitory neurotransmitter we did postembedding glycine immunocytochemistry on the same tissue.

Material and Methods

Light Microscopy

Physiological identification and retrograde tracing of HRP from the IOL. These experiments were performed on four adult Dutch belted rabbits anesthetized with a mixture of Nembutal (15 mg/kg) and a-chloralose (60 mg/kg) injected into an ear vein. During the recording session the rabbits were immobilized with Flaxedil and artificially ventilated, and anesthesia was continuously infused intravenously at a rate of 15% of the initial dose per hour.

The dorsal neck muscles were retracted, the dura over the cisterna magna was removed, and the IOL was approached from the dorsal side. Unitary activity recorded extracellularly with the use of glass micropipettes filled with 2 M NaCl saturated with fast green dye was assignable to IOL neurons on the basis of the slow spontaneous firing rate (0.5-1.5 spikes/s) and the characteristic complex waveform (Crill,1970). With the microelectrode inclined at 30□ to the vertical and advanced in a parasagittal plane, the caudal pole of the dorsal cap was typically found 400 μm lateral and 800 μm rostral to the obex at a depth of 2.5-3.0 mm. In 3 animals the neurons of the rostral dorsal cap were identified by field potentials evoked by flash stimulation of the ipsilateral eye and/or by unitary olivary activity that modulated to rotations of a patterned visual field around a horizontal axis oriented at 45° ipsilateral azimuth / 135° contralateral azimuth (for details see Soodak and Simpson,1988; Leonard et al.,1988; Simpson et al.,1988). Visual stimulation was provided by large, textured patterns moving slowly at a speed of about 0.5°/s, which is the optimal speed for modulating the floccular visual climbing fibers (Simpson and Alley,1974; Barmack and Hess,1980). In one animal that was used as a control we identified the neurons of the caudal dorsal cap by contralateral flash stimulation and by modulation produced by visual field rotation about the vertical axis. HRP (30% in 0.05 M Tris buffer at pH 7.6 (TB) with 2% dimethylsulfoxide) was iontophoretically injected (+ 2 μAmp; 50% duty cycle for 15 min) in the identified areas. After a survival time of 2 days the animals were anesthetized with Nembutal (60 mg/kg, i.v.), and perfused transcardially with 500 ml of 0.9% saline, followed by 2 liters of 4% paraformaldehyde in 0.1M phosphate buffer (PB). The brain was embedded in gelatin, cut on a freezing microtome in 40 μm sections, and incubated with tetramethylbenzidine (TMB, Sigma) according to Mesulam (1978). Subsequently, the sections were rinsed, stained with neutral red, coverslipped, and analyzed.

Anterograde tracing of WGA-HRP. Two adult Dutch belted rabbits were anesthetized with Nembutal (20 mg/kg, i.v.) and mounted in a stereotaxic apparatus. The occipital bone was partly removed and WGA-HRP (0.1 μl) was injected stereotaxically into dorsal group y (dorsal y) and the ventral dentate nucleus of the cerebellum (VDN; also called parvicellular lateral cerebellar nucleus, Ruigrok et al.,1992) by means of a hydraulic pressure system. After a survival time of 2 days the rabbits were anesthetized (Nembutal, 60 mg/kg, i.v.), perfused, and processed for HRP histochemistry as described above, except that the sections with the injection sites were incubated with diaminobenzidine (DAB, Sigma).

Electron Microscopy

Tissue processing. Two adult Dutch belted rabbits were anesthetized with Nembutal and injected with WGA-HRP in dorsal y and the VDN as described above. After a survival time of 2 days the rabbits were anesthetized with Nembutal (60 mg/kg, i.v.), and perfused transcardially with 100 ml 0.9% saline in 0.18 M cacodylate buffer at pH 7.3, followed by 2 liters of 5% glutaraldehyde in the same buffer. The brainstems were coronally sectioned on a Vibratome at 70 μm. The sections were processed with a procedure combining WGA-HRP histochemistry and postembedding GABA-immunostaining. Detailed protocols are published elsewhere (De Zeeuw et al.,1988). Briefly, Vibratome sections were incubated with TMB and stabilized with DAB-cobalt, osmicated (in 8% glucose solution), block stained in uranyl acetate (UA), directly dehydrated in dimethoxypropane and embedded in Araldite. Guided by observations made in the semithin sections, we prepared pyramids of the olivary subnuclei of interest (see below). From these tissue blocks ultrathin sections were cut, mounted on Formvar coated nickel grids, and processed for GABA and glycine immunocytochemistry. The grids were rinsed in a solution of TB containing 0.9% NaCl and 0.1% Triton-X100 at pH 7.6

(TBST), and incubated overnight in a droplet of GABA (1:3000 in TBST) or glycine (1: 250 in TBST) antiserum. The GABA and glycine antisera were generously supplied by Dr. R.M. Buijs and Dr. R.J. Wenthold, respectively (for specificity tests see Seguela et al.,1984; Buijs et al.,1987; Wenthold et al.,1987). The next day the grids were rinsed twice with TBST and stored for 30 minutes in the rinsing solution. After rinsing with TBST (pH 8.2) the grids were incubated for one hour in a droplet of goat anti-rabbit IgG labeled with 15 nm gold particles (Janssen Pharm.) diluted 1: 25 in TBST. Subsequently, the grids were washed twice with TBST (pH 7.6) and distilled water, counterstained with UA and lead citrate, and examined in a Philips electron microscope.

Collection and analysis of the data. Two populations of terminals were analysed. The first population was a sample of WGA-HRP labeled terminals in the rostral dorsal cap and VLO, while the second population consisted of a random sample of all terminals (i.e. terminals with and without WGA-HRP reaction products) in the principal olive (PO), caudal dorsal cap, rostral dorsal cap, VLO, ß-nucleus (BETA), the caudal medial accessory olive (cMAO), and dorsal accessory olive (DAO).

The analysis of the first population was performed as follows: from both the rostral dorsal cap and VLO of each of the two rabbits, two embedded tissue blocks with substantial anterograde labeling were selected. A random sample of terminals was collected from at least three non-serial ultrathin sections obtained from each tissue block. In each sample, we determined what percentage of the WGA-HRP labeled terminals was GABA or glycine positive (double labeled). In addition, we calculated the percentages of both the single and double WGA-HRP labeled terminals that were situated within glomeruli, in the neuropil outside the glomeruli, or apposed to somata (for criteria see De Zeeuw et al.,1989b and 1993). In this analysis, a terminal was considered 1) glomerular, when it joined together with other terminals a core of at least three small dendritic elements, the synaptic complex having a glial sheath; and 2) GABA or glycine positive when the number of gold particles overlying it was at least 8 times higher than the number of particles overlying an equal surface area of surrounding dendritic and/or glial structures (for details about counting procedure see De Zeeuw et al.,1989a; Holstege and Bongers,1991). The percentages of the terminals having the characteristics mentioned above were averaged for all ultrathin sections (data presented in Table 1).

TABLE 1. Populations of double WGA-HRP/GABA (D) and single WGA-HRP (S) labeled terminals randomly collected from ultrathin sections of the contralateral rostral dorsal cap and VLO of rabbits following injection of WGA-HRP in the VDN and dorsal y combined with postembedding GABA immunocytochemistry. For further details see Material and Methods.

	rdc		*VLO*	
	D	S	D	S
mean(%)	93	7	95	5
n	189	15	273	14
Glomerular(%)	12	16	22	23
Extraglomerular(%)	85	78	75	81
Axosomatic(%)	3	6	3	0

In the analysis of the second population, we determined for each olivary subnucleus what percentage of the terminals formed crest synapses, and what percentage of the terminals involved in the crest synapses were GABAergic, glycinergic, and/or WGA-HRP labeled. A crest synapse is the synaptic arrangement of a narrow dendritic element with subsynaptic densities that is innervated by two facing terminals with asymmetric synapses. The samples of

all subnuclei were collected as in the first population, the average percentages were calculated in the same manner, and the standard errors of the means (SEM19s) were calculated. Statistical comparisons were made by Student's t test.

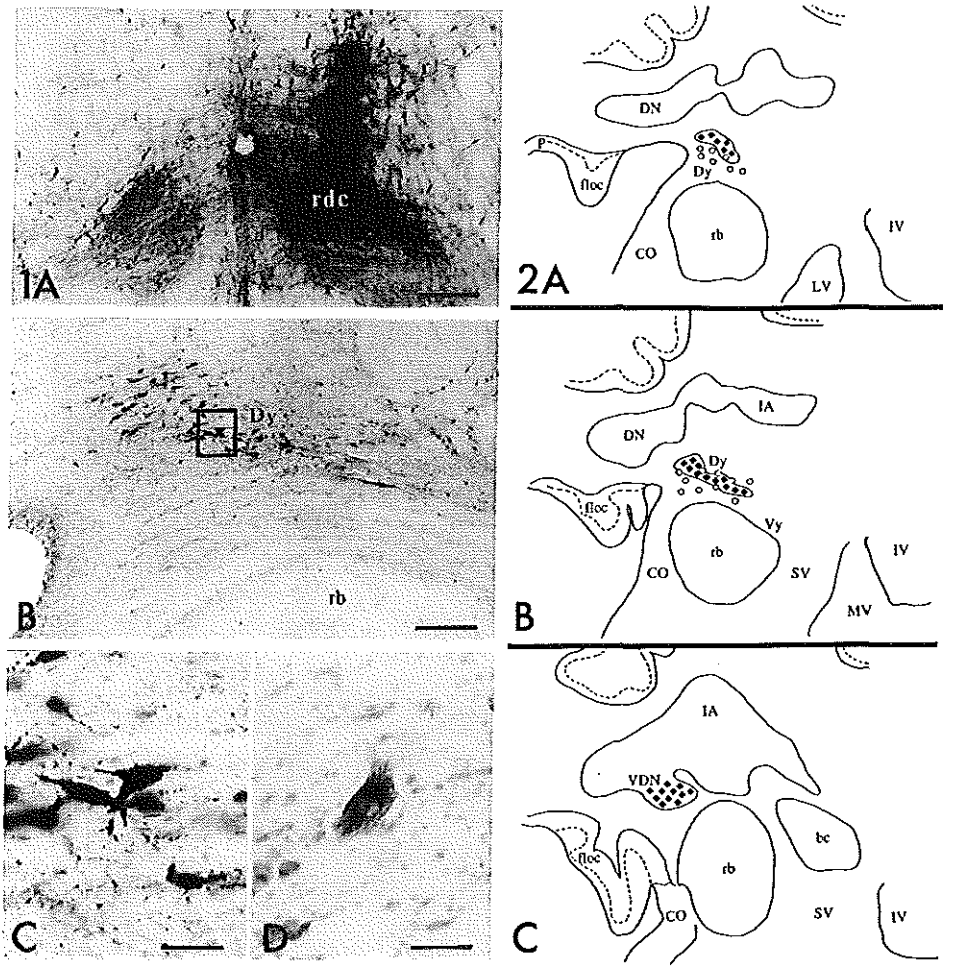

Figure 1A-D. Retrograde tracing of HRP from the rostral dorsal cap and VLO. A: Injection site in the right rostral dorsal cap. The injection was visualized using TMB as the chromogen. B: Retrogade labeling in the contralateral Dorsal y (Dy). C corresponds to the inset in B and shows a high magnification of the retrogradely labeled neurons in Dy. D: A large non-retrogradely labeled neuron in Dy; these large neurons characterizing Dy were never retrogradely labeled; note that the magnification is the same as in C. Scale bar in A is 210 μm; in B 130 μm; and in C and D, 26 μm.

Figure 2A-C. Drawings of the contralateral retrograde labeling in Dy and the VDN after the injection shown in Figure 1A. The black diamonds indicate the retrogradely labeled neurons while the open circles indicate the large neurons of Dy. At the most caudal level (A) the retrogradely labeled neurons are located dorsal to Dy, at the intermediate rostrocaudal level (B) the labeling mostly coincides with Dy, and rostrally (C) retrograde labeling is present only in the VDN. The drawing in B corresponds to Figure 1B. For abbreviations see list in the text. The black bar between A and B and between B and C indicates 4.5 mm.

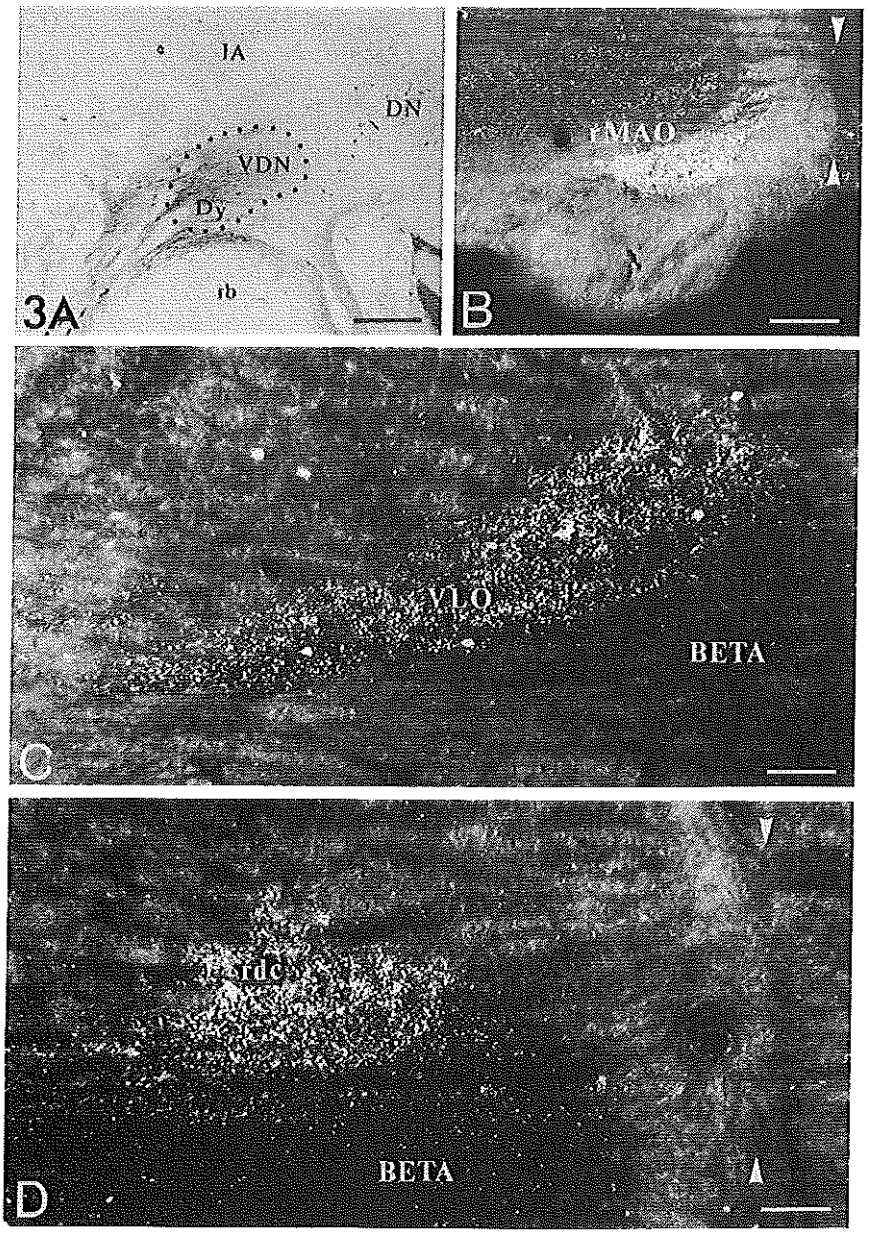

Figure 3A-D. *Anterograde tracing of WGA-HRP from Dy and VDN to the IOL. A: The core of the injection site in Dy and VDN is delineated by the dotted line; DAB was used as the chromogen. B-D: Dark field micrographs of anterograde labeling in the contralateral rMAO (B), VLO (C), and rostral dorsal cap (D); TMB was used as the chromogen. White arrowheads in B and D indicate the midline. Scale bar in A is 540 μm; in B, 180 μm; in C, 67 μm; and in D, 58 μm.*

Results

Light Microscopy

Retrograde tracing of HRP. The injection sites of the 3 animals injected in the rostral dorsal cap and VLO varied in size. In two cases, the injections covered both the rostral dorsal cap and VLO and extended to the contralateral dorsal cap, but not to the surrounding olivary nuclei (Fig. 1A). Examination of the brainstem and the cerebellum indicated that a high concentration of retrogradely labeled neurons was located in the contralateral and ipsilateral VDN and dorsal y (Fig. 1B). No retrograde labeling was present in the ventral group y, and the PrH of both sides contained only a few retrogradely labeled neurons. In addition, we observed many retrogradely labeled neurons in the ipsilateral and contralateral VTRZ, but hardly any in the NOT and DTN, thereby confirming that the injection was largely confined to the rostral dorsal cap and VLO (Simpson,1984; Giolli et al.,1985). The retrogradely labeled neurons in the VDN were continuous with those in dorsal y (Fig. 2A-C). In our material we distinguished dorsal y from the VDN by the presence, in dorsal y, of large neurons, which project to the contralateral oculomotor nucleus (Highstein and Reisine,1979). The labeled neurons in both the VDN and dorsal y were small to medium sized and somewhat elongated (Fig. 1C). The large neurons in dorsal y were not retrogradely labeled (Fig. 1D). In the third case, the injection site was nearly restricted to the rostral dorsal cap as it did not extend substantially into any other nucleus on the ipsilateral or contralateral side. Many retrogradely labeled neurons were observed in the contralateral VDN and dorsal y (VDN > dorsal y), but only a few labeled neurons were present in the ipsilateral VDN and dorsal y. The contralateral PrH and the ipsilateral VTRZ showed some scattered retrogradely labeled neurons. In the animal studied for control (data not shown; see also Barmack et al.,1993) the injection was centered in the caudal dorsal cap and did not extend into the rostral dorsal cap. In this case, no retrogradely labeled neurons were located in the VDN, dorsal y or VTRZ. Many labeled neurons were observed in a ventrolateral region of the caudal contralateral PrH while fewer were located in a dorsolateral region of the ipsilateral caudal PrH. On both sides the retrograde labeling reached the border with the medial vestibular nucleus. In addition, some retrogradely labeled neurons were present in the ipsilateral DTN and NOT.

Anterograde tracing of WGA-HRP. The injection site of one of the animals injected with WGA-HRP included the entire VDN and dorsal y as well as the adjacent part of the interposed posterior nucleus (IP; Fig. 3A). The injection site of the other animal included a minimal part of the VDN, the entire dorsal y, and a smaller part of the IP. In both animals the anterogradely labeled fibers passed through the brachium conjunctivum and descended to the IOL; the contralateral VLO, rostral dorsal cap, and the very rostral tip of the MAO (rMAO) contained dense anterograde labeling (Figs. 3B, C and D) while the rostral part of the contralateral caudal dorsal cap was less densely labeled. The ipsilateral rostral dorsal cap and VLO were not totally devoid of labeling, but the amount of WGA-HRP reaction product was small. Scattered retrogradely labeled neurons were observed in the contralateral VLO and rostral dorsal cap (VLO > rostral dorsal cap) as well as in the ipsilateral flocculus. No anterograde labeling was observed in areas directly surrounding the IOL.

The WGA-HRP injections in the rabbits used for the electron microscopic double labeling experiments were also centered in the VDN and dorsal y and they also extended into the IP. They were somewhat larger than those used for the light microscopic analysis and included part of the dentate nucleus (DN). The distribution of labeling in the dorsal cap and VLO was in general agreement with that found in the experiments described above.

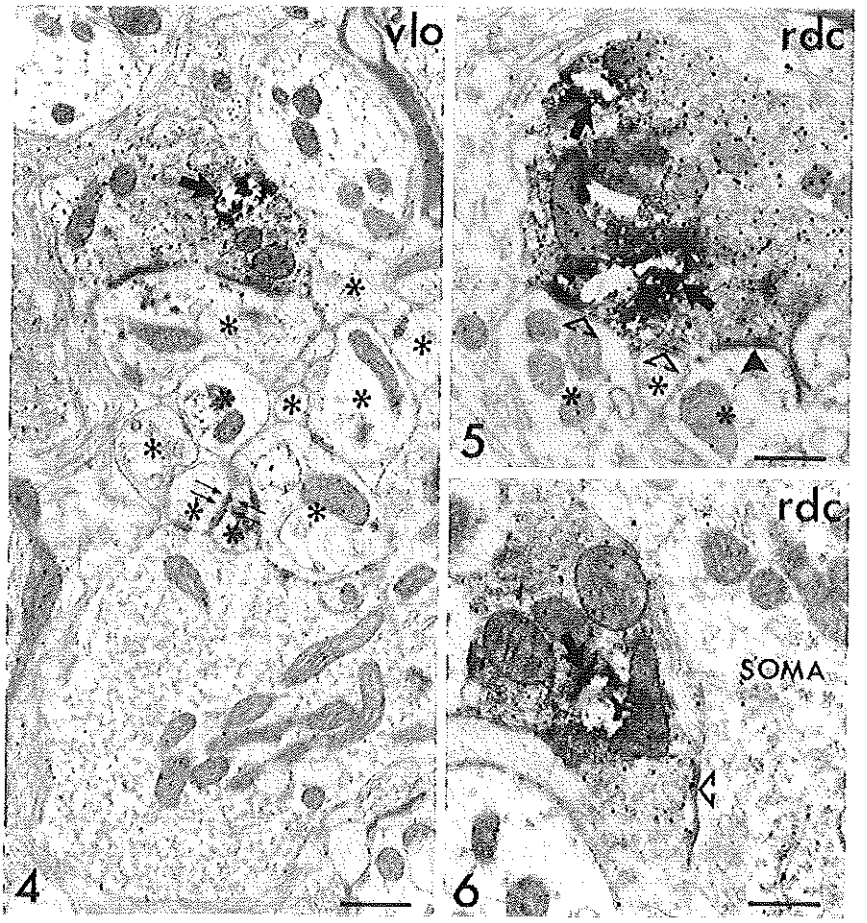

Figure 4. *Electron micrograph of a glomerulus in the VLO following anterograde transport of WGA-HRP from Dy and VDN combined with postembedding GABA-immunocytochemistry (small black dots are 15 nm immunogold particles). Note that the glomerulus is complex and has at least 10 different dendritic profiles (asterisks). The core of dendritic elements is contacted by a double labeled terminal at the top (large arrow indicates WGA-HRP reaction product) and a non-labeled terminal at the bottom. Small arrows indicate a gap junction. Scale bar is 0.7 µm.*

Figure 5. *Electron micrograph of a small glomerulus in the rostral dorsal cap following anterograde transport of WGA-HRP from Dy and VDN combined with postembedding GABA-immunocytochemistry. Note that the double labeled terminal participates in both symmetric (open arrowheads) and asymmetric (black arrowhead) synapses. Arrows indicate WGA-HRP reaction product and asterisks indicate dendritic elements. Scale bar is 0.43 µm.*

Figure 6. *Electron micrograph of a GABAergic terminal in the rostral dorsal cap that is anterogradely labeled from the area of Dy and VDN. This double labeled terminal makes a symmetric synaptic contact with a soma. Arrow indicates the WGA-HRP reaction product. Scale bar is 0.39 µm.*

Morphological characteristics. The ultrathin sections of the contralateral rostral dorsal cap and VLO included numerous single GABA labeled and double-labeled GABA/WGA-HRP myelinated axons, preterminal axon segments, and terminals (Figs. 4, 5, 6, and 7). In addition, some of the WGA-HRP labeled terminals were classified as non-GABAergic. The WGA-HRP reaction products observed in the ipsilateral rostral dorsal cap (see above) were predominantly located in myelinated non-GABAergic axons and occasionally in GABAergic terminals. The ultrathin sections of the other olivary subnuclei (PO, caudal dorsal cap, BETA, cMAO and DAO) also showed numerous GABAergic and GABA-negative terminals. Apart from some double-labeled GABA/WGA-HRP terminals in the caudal dorsal cap, these subnuclei did not contain WGA-HRP reaction product. None of the terminals in any of the olivary subnuclei was glycine positive, even though some glycinergic myelinated axons were observed.

The sample of the first population of terminals (see collection and analysis of the data) consisted of 491 WGA-HRP labeled terminals from the rostral dorsal cap (n=204) and VLO (n=287). Of these terminals 94% were double WGA-HRP/GABA labeled and 6% were single WGA-HRP labeled (for details see Table 1). Apart from a few large, dense core vesicles, the double-labeled WGA-HRP/GABA terminals contained predominantly clear pleomorphic vesicles. These terminals often included several mitochondria, and sometimes they contained neurofilaments where they emerged from their preterminal segments. The diameter of the terminals ranged from 1 to 3.5 μm. About 70% of these double labeled terminals were seen to establish synaptic contacts, most of which were symmetric. While the smaller double-labeled GABA/WGA-HRP terminals usually formed only one synaptic junction, the larger ones frequently had multiple synapses. Occasionally, such a terminal made both asymmetric and symmetric synaptic contacts (Fig. 5). The double labeled terminals in the neuropil were located inside as well as outside the glomeruli. The glomeruli of the VLO (Fig. 4) were usually more complex than those of the rostral dorsal cap (Fig. 5), and the percentage of glomerular terminals in the VLO (22%) was higher than in the rostral dorsal cap (12%; see Table 1). The terminals encapsulated in the glomeruli sometimes contacted dendritic elements linked by gap junctions whereas the extraglomerular terminals were occasionally apposed to dendritic profiles organized in thickets (a dendritc thicket consists of a group of dendrites that are located directly next to one another without spatial interference of glial elements, for details see Sotelo et al.,1974). Some of the double labeled terminals were apposed to cell bodies (rostral dorsal cap 3% and VLO 3%; Fig. 6). The morphological characteristics of the single WGA-HRP labeled terminals in the rostral dorsal cap and VLO did not differ from that of the double GABA/WGA-HRP labeled terminals. In addition, there were no significant differences in the postsynaptic distribution of both types of terminals (Table 1). Furthermore, the vast majority of the WGA-HRP labeled terminals that were classified as non-GABAergic contained 2 to 6 times more gold particles than most of the surrounding non-GABAergic structures that did not contain any WGA-HRP reaction products.

The sample of the second population of terminals (see collection and analysis of the data) consisted of a total of 4645 terminals collected from the PO (n=626), caudal dorsal cap (n=741), rostral dorsal cap (n=671), VLO (n=654), BETA (n=664), cMAO (n=639), and DAO (n=650). For this population we determined the percentage of terminals forming crest synapses. These synapses are characterised by two opposed, asymmetric, postsynaptic thickenings in a narrow dendritic stalk filled with subsynaptic densities (Fig. 7; see also Milhaud and Pappas,1966; Akert et al.,1967). The percentages of the terminals involved in the formation of crest synapses in these subnuclei were: PO, 0.31% (± 0.02%); caudal dorsal cap, 2.9% (±0.5%); rostral dorsal cap, 3.7% (± 0.4%); VLO, 2.7% (± 0.35%); Beta, 0.25%

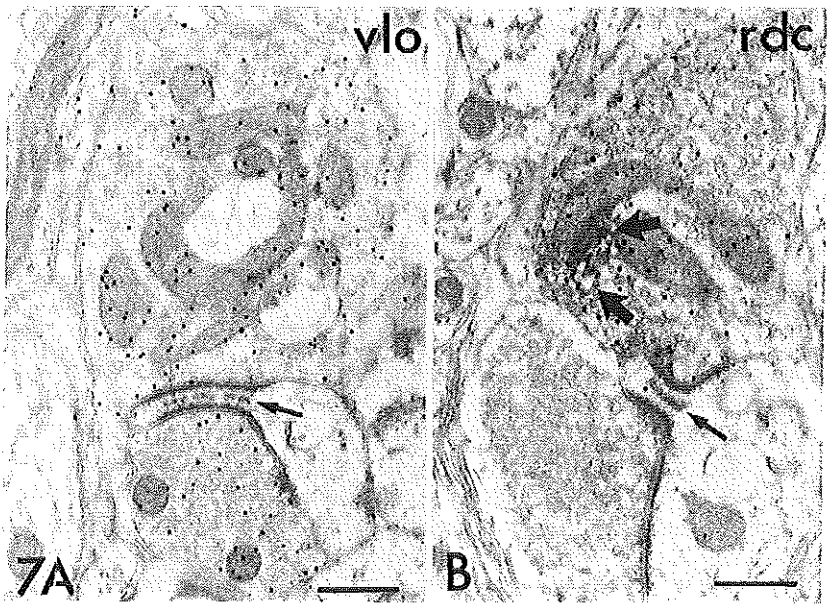

Figure 7A-B. Crest synapses (small arrows) in the VLO (A) and rostral dorsal cap (B). The crest synapse in A is formed with two GABAergic terminals. The crest synapse in B is formed with one GABAergic terminal from the area of Dy and VDN and with one non-labeled terminal. Arrows indicate WGA-HRP reaction product. Scale bar is 0.51 μm in A and 0.40 μm in B.

(±0.04%); cMAO, 0.1% (±0.01%); and DAO, 0% (Fig. 8). The percentages for the caudal dorsal cap, rostral dorsal cap and VLO were all significantly ($p<0.01$) higher than those for the other subnuclei. The few crest synapses observed in the PO, BETA, and cMAO were all formed by GABAergic terminals. In the caudal dorsal cap, rostral dorsal cap and VLO, respectively, 82%, 88% and 91% of the terminals that formed crest synapses were GABAergic (Fig. 8). Eight of the 42 GABAergic terminals involved in crest synapses in the rostral dorsal cap and VLO were double labeled (Fig. 7B), but these double labeled terminals were never involved in the same crest synapse. In the caudal dorsal cap we were unable to find any double labeled terminal contributing to a crest synapse. Throughout the IOL most of the terminals that formed crest synapses contained pleiomorphic vesicles and were located outside the glomeruli.

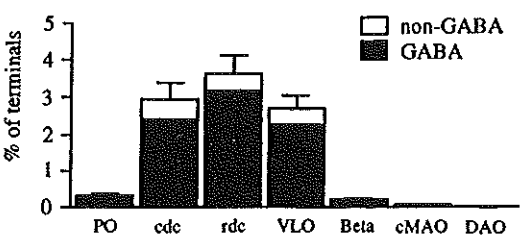

Figure 8. Percentages of terminals involved in crest synapses (total number of examined terminals = 4645). The percentages of terminals that form crest synapses in the caudal dorsal cap, rostral dorsal cap and VLO are all significantly higher ($p<0.01$) than in the other olivary subdivisions examined (PO, Beta, cMAO, and DAO). Bars indicate SEM's. Note that the great majority of the crest synapses in the caudal dorsal cap, rostral dorsal cap and VLO are formed with GABAergic terminals.

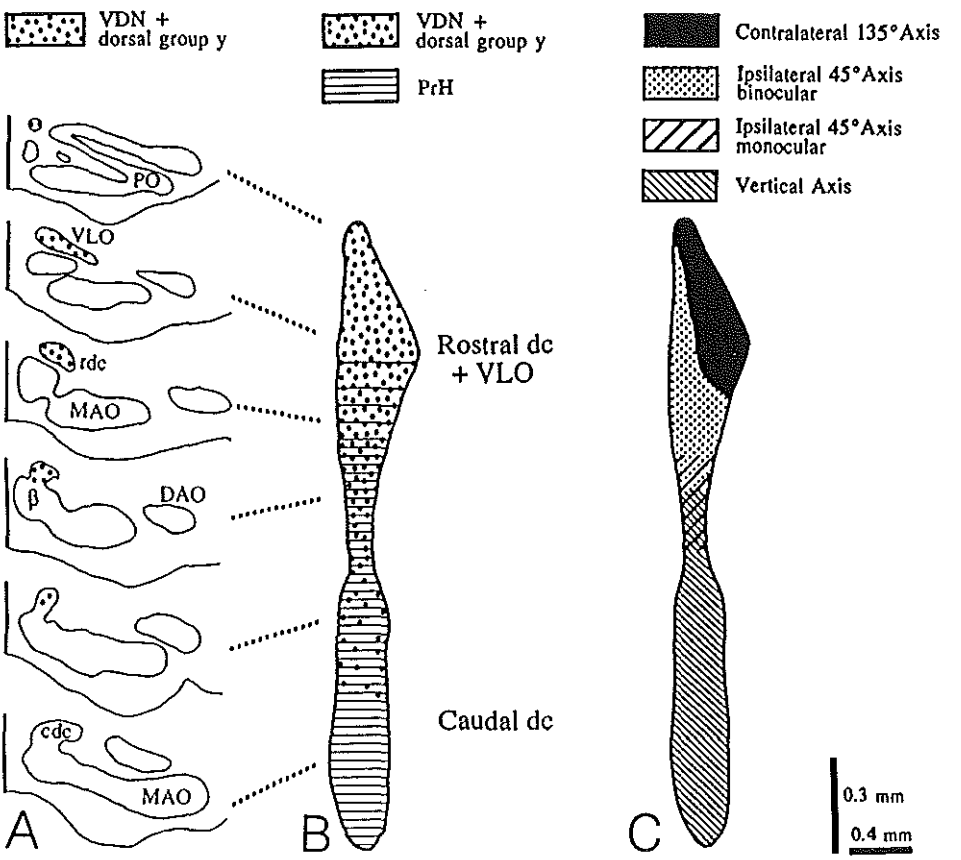

Figure 9A-C. Drawings of the GABAergic inputs to the dorsal cap and VLO (A and B), and the areas of the dorsal cap and VLO with different optimal axes for visual stimulation (C; modified from Leonard et al.,1988). A shows transverse sections through the dorsal cap and VLO with anterograde WGA-HRP labeling from Dy and VDN. B shows a dorsal view of a reconstruction of the dorsal cap and VLO with the input from Dy and VDN, and for comparison, with the input from the PrH (from De Zeeuw et al.,1993). Note that the PrH is the major source of the GABAergic input to the caudal dorsal cap while the Dy and VDN are the major sources of the GABAergic input to the rostral dorsal cap and the VLO. C depicts the different regions in the dorsal cap and VLO that respond preferentially to rotation of optokinetic stimuli about particular spatial axes. The caudal dorsal cap responds optimally to visual field rotations about the vertical axis, the rostral dorsal cap responds preferentially to rotations about a horizontal axis at an azimuthal angle of 45^0 to the ipsilateral eye while the rostral part of the rostral dorsal cap and the VLO respond optimally to rotations about a horizontal axis at an azimuthal angle of 135^0 to the contralateral eye.

Discussion

The major findings of this study are that the dorsal y and VDN of the rabbit innervate the rostral dorsal cap and VLO, and that this projection is GABAergic. In addition, it was found that the rostral dorsal cap and VLO contain significantly more crest synapses than the other olivary subnuclei, and that these synapses are derived from dorsal y and VDN.

Light Microscopy

In the present study we have shown that the VDN and dorsal y in the rabbit project to the VLO and rostral dorsal cap. The nucleo-olivary connections between VDN and the VLO have been shown before in cat (Tolbert et al.,1976; Dietrichs et al.,1985) and rat (Ruigrok and Voogd,1990; Ruigrok et al.,1992). In cat, the fastigial nucleus has been reported to project to the dorsal cap (Dietrichs and Walberg,1985), but in our material we observed no retrogradely labeled cells in the fastigial nucleus following injection of HRP in the dorsal cap. The projections from VDN and dorsal y to the rostral dorsal cap have not been reported before. Whether VDN or dorsal y individually project to both the rostral dorsal cap and VLO cannot be determined by the retrograde or by the anterograde experiments because the areas of origin and termination are both contiguous and small. The case in which the WGA-HRP injection site included the entire dorsal y but a minimal part of the VDN suggests that the dorsal y projects to both the rostral dorsal cap and the VLO. On the other hand, in the case in which we made a small injection of HRP that was almost restricted to the rostral dorsal cap, relatively few neurons were labeled in dorsal y compared to VDN.

The extent to which the VDN and dorsal y also project to the caudal dorsal cap was shown in the experiments in which WGA-HRP was anterogradely traced from the VDN and dorsal y to the IOL (see also Fig. 9). These experiments indicate that the rostral dorsal cap and VLO receive a substantial input from these nuclei while the caudal dorsal cap receives only a modest input in its most rostral part. These results are consistent with the observation that no neurons in the VDN and dorsal y were retrogradely labeled after injection of HRP in the caudal dorsal cap.

The experiment in which HRP was injected unilaterally in the rostral dorsal cap and retrogradely traced to the VDN and dorsal y, together with the experiments in which WGA-HRP was anterogradely traced from the VDN and dorsal y to the rostral dorsal cap and VLO, indicate that this projection is predominantly contralateral. The ultrastructural analysis of the ipsilateral dorsal cap showed that the WGA-HRP reaction products were located not only in GABAergic terminals but also in non-GABAergic myelinated axons. Therefore, the ipsilateral projection is even weaker than it appears in the light microscope because some of the labeling observed in the ipsilateral dorsal cap is likely from retrogradely labeled olivary axons that pass through the ipsilateral IOL and give off collaterals in the area of the VDN and dorsal y. Ipsilateral nucleo-olivary projections have been noted previously in various other species (rat: Brown et al.,1977; Chan-Palay,1977; Angaut and Circirata,1982; Swenson and Castro,1983; Ruigrok and Voogd,1990; cat: Beitz,1976; monkey: Chan-Palay,1977; Kalil,1979). In these studies, the ipsilateral labeling was usually also distributed as the mirror image of the contralaterally labeled areas.

The retrogradely labeled neurons in the VDN and dorsal y were small to medium sized and somewhat elongated. The electron microscopic analysis of their terminals (see below) showed that all these neurons were GABAergic and, therefore, inhibitory (Krnjevic and Schwartz,1966). These results agree with observations in rat and cat that the VDN and dorsal y contain small to medium sized GABAergic neurons (Mugnaini and Oertel,1985; Carpenter et

al.,1990). Moreover, the relation between cell size and the presence of GABA is in line with the present observation that the large neurons in dorsal y, which provide an excitatory input to the contralateral oculomotor nucleus (Graybiel and Hartwieg,1974; Highstein and Reisine,1979; Stanton,1980; Carpenter and Cowie,1985a; Highstein and McCrea,1988), were not retrogradely labeled.

Electron Microscopy

GABAergic projection. The quantitative analysis indicated that 94% of the WGA-HRP labeled terminals from the VDN and dorsal y in the VLO and rostral dorsal cap were also GABA labeled. Most of the WGA-HRP labeled terminals that were classified as non-GABAergic contained more gold particles than the surrounding non-GABAergic structures. In addition, the single WGA-HRP labeled terminals showed the same morphological characteristics and the same postsynaptic distribution (see Table 1) as the double GABA/WGA-HRP labeled terminals. These data strongly suggest that all terminals in the rostral dorsal cap and VLO derived from the VDN and dorsal y are GABAergic. Since the projection from VDN and dorsal y to the rostral dorsal cap and VLO is dense, they receive a major GABAergic input from these nuclei. The present finding that none of the examined terminals was glycinergic implies that glycine is not used as an inhibitory neurotransmitter in the dorsal cap and VLO. The positively labeled axons in the dorsal cap were probably glycinergic axons that pass through the IOL (see also Fort et al.,1990).

The PrH provides the major GABAergic input to the caudal dorsal cap and a substantial, but not dominating GABAergic input to the rostral dorsal cap and VLO (De Zeeuw et al.,1993). That finding is supported by the present observations that 1) following injection of HRP in the caudal dorsal cap the PrH contains many retrogradely labeled neurons while the VDN and dorsal y contain none, and 2) following injection of HRP in the rostral dorsal cap and VLO the PrH contains clearly fewer retrogradely labeled neurons than the VDN or dorsal y. Therefore, it appears that the VDN and dorsal y are the major sources for the GABAergic input to the olivary neurons involved in the "vertical" optokinetic reflexes (i.e. eye movements dominated by the vertical recti and oblique muscles) while the PrH is the major source for the GABAergic input to the olivary neurons involved in the horizontal optokinetic reflex (i.e. eye movements dominated by the horizontal recti muscles). These relations are summarized in Fig. 9. Our results agree with the finding that group y and the DN in monkey are involved in vertical eye movements (Chubb and Fuchs,1982). In addition, they agree with the observations in cat that the projection from the PrH to the extraocular motor nuclei is mainly to the abducens nucleus and to the medial rectus subdivision of the oculomotor nucleus (McCrea and Baker,1985; Delgado-Garcia et al.,1989) and that neurons in the PrH respond mainly to horizontal angular acceleration (Blanks et al.,1977). Moreover, the present results are in line with the observation that the Purkinje cells in the flocculus involved in the control of "vertical" compensatory eye movements project in part to the VDN and dorsal y (De Zeeuw et al.,1992) whereas the Purkinje cells in folium p that are innervated by the climbing fibers from the caudal dorsal cap, project to the PrH (Yamamoto,1978 and 1979; Tan et al., unpublished observations).

Postsynaptic distribution. The present electron microscopic observations showed that the GABAergic terminals in the rostral dorsal cap and VLO from VDN and dorsal y contain mostly pleomorphic vesicles and establish symmetric synapses primarily with glomerular and extraglomerular dendrites. The terminals in the IOL from other cerebellar and vestibular nuclei in cat and rat have the same morphology, neurotransmitter, and postsynaptic distribution as found in the present investigation of rabbit (Sotelo et al.,1986; Angaut and Sotelo,1989;

Fredette and Mugnaini,1991; De Zeeuw et al.,1989a and 1993). The terminals within the glomeruli may regulate the electrotonic coupling between olivary neurons (Llinás,1974; Llinás et al.,1974; Llinás and Sasaki,1989; Lang et al.,1989 and 1990). Terminals in the dorsal cap derived from the pretectum contain predominantly round vesicles, form asymmetric synapses, and do not contact the cell bodies (Mizuno et al.,1974).

Crest synapses. The rostral dorsal cap and VLO contain significantly more crest synapses than the other subnuclei. The vast majority of these crest synapses are formed by GABAergic terminals, and 8 of the 42 GABAergic terminals that formed crest synapses were double labeled. Since this double labeling technique labels at most about a third of the actual number of terminals (De Zeeuw et al.,1989a), these data strongly suggest that the VDN and dorsal y are the major sources of the terminals involved in the crest synapses in the rostral dorsal cap and VLO. Crest synapses are also present in the caudal dorsal cap, and in this subdivision they are also formed predominantly with GABAergic terminals (De Zeeuw et al.,1993; present study). Whether these terminals are also derived from the VDN and dorsal y could not be determined in the present study in which the embedded tissue blocks of the caudal dorsal cap contained relatively few WGA-HRP reaction products. However, since the crest synapses involve only a small minority of terminals, a contribution from the VDN and dorsal y cannot be excluded. The possibility that the PrH gives rise to the terminals forming crest synapses in the caudal dorsal cap seems unlikely. In a previous study of the dorsal cap of the rat and rabbit, in which the same tracing technique was used, we were unable to detect any terminal from the PrH that made a crest synapse (De Zeeuw et al.,1993). The non-GABAergic terminals involved in the crest synapses could be derived from the pretectum, but degenerated terminals making crest synapses have not been observed after pretectal lesions (Mizuno et al.,1974). The function of crest synapses is still unclear. It is also unknown whether the crest synapses observed in other areas of the mammalian nervous system, like the habenula (Milhaud and Pappas,1966), interpeduncular nucleus (Milhaud and Pappas,1966), subfornical organ (Akert et al.,1967), suprachiasmatic nucleus (Guldner,1976), and locus coeruleus (Mizuno and Nakamura,1972) are also formed predominantly with GABAergic terminals. The crest synapses in the interpeduncular nucleus are formed with a terminal from the ipsilateral habenula and a terminal from the contralateral habenula (Murray et al.,1979). The fact that we never found a crest synapse formed with two double labeled terminals, together with the finding that the VDN and dorsal y project bilaterally (although contralateral >> ipsilateral) to the dorsal cap and VLO, offers the possibility that the crest synapses in the dorsal cap and VLO receive a terminal from each side of the brain.

Chapter IV. Olivary Projecting Neurons in the Nucleus Prepositus Hypoglossi, Dorsal Group y and Ventral Dentate Nucleus do not Project to the Oculomotor Complex

Abstract

The nucleus prepositus hypoglossi, dorsal group y, ventral dentate nucleus, and medial vestibular nucleus all project to both the oculomotor complex and inferior olive. In the present study, we demonstrate with the use of retrograde double labeling techniques in rabbits and rats that the neurons in these preoculomotor nuclei that project to the inferior olive are intermingled with those that project to the oculomotor nucleus, but that virtually none project to both.

Introduction

The dorsal cap (dc), ventrolateral outgrowth (vlo) and Beta-nucleus are subnuclei of the inferior olive (IOL) that are involved in compensatory eye movements (Barmack et al., 1989, De Zeeuw et al., 1993). Both these olivary subnuclei and the oculomotor complex (OMC) receive synaptic inputs from the nucleus prepositus hypoglossi (PrH), dorsal group y, ventral dentate nucleus (VDN), and medial vestibular nucleus (MVN). Dorsal group y and the VDN project to the contralateral OMC (Carpenter and Cowie, 1985) and to the contralateral rostral dc (rdc) and ventrolateral outgrowth (vlo) of the IOL (De Zeeuw et al., 1994ab). The PrH projects bilaterally to both the OMC and the IOL; the projection to the OMC mainly involves the ipsilateral medial rectus subdivision (Highstein and McCrea, 1988; McCrea and Baker, 1985), while the projection from the PrH to the IOL predominantly involves the contralateral caudal dc (cdc), (De Zeeuw et al., 1993). The MVN projects predominantly to the contralateral OMC (Labanderia-Garcia et al., 1991; Wentzel et al., 1995a and 1996) and to the ipsilateral Beta-nucleus of the IOL (Barmack et al., 1989). It is unknown whether individual neurons in the preoculomotor nuclei mentioned above project to both the IOL and OMC. With the use of retrograde double labeling techniques in rabbits and rats we sought to answer this question.

Material and Methods

Three adult Dutch belted rabbits were injected with retrograde fluorescent tracers; two rabbits received an injection of Diamidino Yellow (DY) and Fast Blue (FB) in respectively the IOL and OMC, while in the third rabbit the injected tracers were reversed (see Table 1). The rabbits were anesthetized by a mixture of Ketamine (32 mg/kg), Acepromazine (0.32 mg/kg) and Xylazine (5 mg/kg). The OMC was identified by evoking eye movements with the use of low threshold (<15 μA) stimulation with tungsten electrodes, while the IOL was identified by recording the characteristic low firing rate (1-2 Hz) of its neurons (De Zeeuw et al., 1994ab) with glass micropipettes (2-3 M□). After the DY (3%) and FB (3%) injections (Bentivoglio et al., 1980; Keizer et al., 1983), the animals were allowed to recover and to survive for 7-11 days. Subsequently, the animals were anesthetized with Nembutal and perfused with 1.5 L

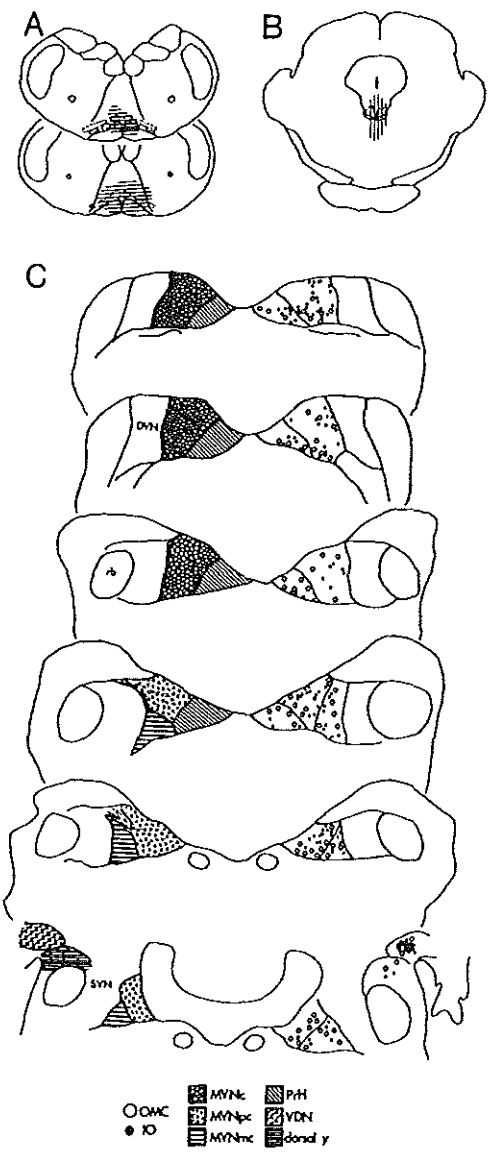

Figure 1. Injections of FB in the IOL (A) and DY in the OMC (B), and retrograde labeling in the PrH, VDN, dorsal y and MVN of rabbit K561 (C). The percentage of double retrogradely labeled neurons comprise less than 0.1% of the total number of retrogradely labeled neurons in this case; these neurons are not indicated.

0.9% saline followed by 2000 ml of 30% formalin in citrate buffer containing 8% sucrose. The brains were removed and transverse sections (40 μm thick) were cut on a microtome, mounted on slides, and analysed under an Olympus microscope with a Ploemopack fluorescence attachment. The position of the labeled neurons in the MVN, PrH, dorsal group y and VDN was plotted on a X-Y plotter connected to the microscope stage and the number of labeled cells was counted (Table 1). Subsequently, the slides were counterstained with Neutral Red, and the boundaries of the nuclei were plotted. The area of dorsal group y was identified by its large elliptic neurons that are known to project to the OMC (De Zeeuw et al., 1994ab).

Since the projection from the PrH to the IOL in rat has a complex distribution and since it employs both inhibitory and excitatory neurotransmitters (Barmack et al., 1993; De Zeeuw et al., 1993), the major outcomes were confirmed in rats. Two rats were anesthetized with Nembutal (60 mg/kg), and the OMC and IOL were identified as described above. Cholera toxin-b-subunit (CTB) was injected iontophoretically into the IOL (+4 μA, 30 min, 7 sec on-off cycle), while the OMC was injected with 100 nl of a gold-lectin conjugate (Ruigrok et al., 1995). After a survival time of 7-10 days, the animals were perfused with 300 ml of a 0.8% NaCl, 0.8% sucrose, 0.4% d-glucose solution in 0.05M phosphate buffer (pH 7.3) followed by 4% paraformaldehyde, 0.1% glutaraldehyde and 4% sucrose in the same buffer. The brains were removed and cut as described above, and the sections were incubated in anti-CTb (1:15000; List Laboratories) in Tris buffer containing 0.5 M NaCl and 0.5% Triton for 3 days. The sections were rinsed, incubated in biotinylated donkey anti goat (List Lab.; 1:2000), reacted with the avidine-biotine-complex (ABC Elite kit, Vector), reacted with diaminobenzidine, and finally silver intensified (Aurion), mounted, counterstained and coverslipped.

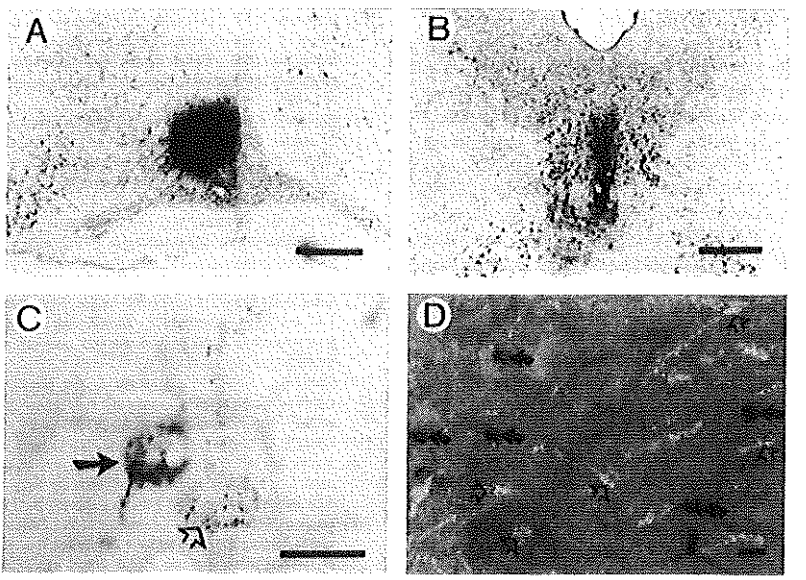

Figure 2. Micrographs of injection and projection sites of rat 517 following injection of CTB in the IOL and gold lectin in the OMC. The CTB injection (A) was largely confined to the left cdc and Beta-nucleus, while the gold lectin injection site (B) included both the left and right OMC. Figure C and D show single CTB (black closed arrows) and single gold lectin (open arrows) retrogradely labeled neurons in the PrH; double labeled neurons are absent. Scale bars in A and B are 500 mm and in C and D 20 mm.

Results

In case K561 the IOL injection (FB) included the left and right dc and Beta-nucleus as well as the left vlo (Fig. 1A), (for summary see Table 1). The OMC injection (DY) included the entire right OMC and the medial part of the left OMC (Fig. 1B). FB and DY retrogradely labeled neurons were observed in the left and right PrH, MVN, group y and VDN (Fig. 1C). Two double labeled neurons were observed; both were located in the MVN. In case K548 the IOL injection site (DY) included the left cdc, the left and right Beta-nucleus, but barely touched the left rdc and vlo. The OMC injection site (FB) was small and confined to the left side. DY retrogradely labeled neurons were observed in the left and right PrH and MVN; few DY labeled cells were seen in the right VDN and dorsal group y. FB retrogradely labeled neurons were observed predominantly in the right MVN, group y and VDN, and bilaterally in the PrH. All retrogradely labeled neurons were single labeled except one neuron in the MVN which was double labeled. In case K555 the IOL injection (FB) included the left dc and vlo, but spared the Beta-nucleus. The OMC injection (DY) was predominantly on the left side but spread across the midline. FB labeled neurons were observed bilaterally in the PrH and in the right group y and VDN; DY labeled neurons were observed in both the left and right MVN, PrH, group y and VDN. All analysed neurons were single labeled.

In the PrH, dorsal group y, VDN and MVN of the cases described above the olivary and oculomotor projecting neurons were found next to one another. However, in all these nuclei slight differences were observed. In the PrH, olivary projecting neurons tended to be located more caudally than neurons that project to the OMC, while in the MVN, IOL projecting neurons were often located more rostrally. In the VDN, the projection to the IOL was heavier than to the OMC, whereas in dorsal group y, the reverse was true (see Table 1).

Table 1. Frequency of single and double labeled neurons in the vestibular complex retrogradely labeled from the IOL and OMC. Because no MVN neurons were labeled with FB from the IOL in case K555, the MVN neurons labeled with DY from the OMC were not analysed in this animal.

		MVN	PrH	Group y	VDN
Rabbit	IOL (DY)	69	27	4	1
K548	OMC (FB)	45	16	7	7
	IOL+OMC	1	0	0	0
Rabbit	IOL (FB)	--	45	45	37
K555	OMC (DY)	--	60	18	4
	IOL+OMC	--	0	0	0
Rabbit	IOL (FB)	531	118	41	153
K561	OMC (DY)	562	239	522	75
	IOL+OMC	2	0	0	0
Total	IOL	600	190	90	191
	OMC	607	317	547	86
	IOL+OMC	3	0	0	0

In general, the retrogradely labeled neurons from the OMC were somewhat larger than the neurons that project to the IOL.

In the rats, the IOL injections (CTB) were largely confined to the left cdc and Beta-nucleus, while the gold lectin injection sites included both the left and right OMC (Figs. 2A and 2B). CTB retrogradely labeled neurons were located in the left and right PrH as well as in the left MVN (Figs. 2C and 2D); retrogradely gold labeled neurons were present in the PrH, MVN, and in the area of group y and the VDN. Double labeled neurons were scarce and only

observed in the MVN. Olivary projecting neurons in the PrH tended to be located more caudally than PrH neurons that project to the OMC.

Discussion

In this study we have shown that neurons in the PrH, dorsal group y, VDN and MVN can project to either the IOL or the OMC, but hardly ever to both. After injections of different retrograde tracers in the IOL and OMC no double labeled neurons were seen in the PrH, dorsal group y or VDN, while only a few MVN neurons were double labeled. The present data can be compared with electron microscopic and electrophysiological studies, which showed that the projections from the PrH, dorsal group y, VDN and MVN to the OMC and IOL are predominantly excitatory and inhibitory, respectively (De Zeeuw et al., 1993 and 1994ab; Graybiel and Hartwieg, 1974; Highstein and Reisine, 1979; Highstein and McCrea, 1988; McCrea et al., 1979; Stanton 1980; Wentzel et al., 1995). In addition, they agree with the observation that the GABAergic neurons in the PrH, dorsal y and VDN projecting to the IOL are small to medium sized neurons (De Zeeuw et al., 1993 and 1994ab), whereas neurons projecting to the OMC are generally larger (Graybiel and Hartwieg, 1974; Highstein and Reisine, 1979).

The demonstration that the projections to the OMC and IOL are isolated from one another emphasizes that the PrH, dorsal y, VDN and MVN may have dual functions in that they serve both as a preoculomotor nucleus and a preolivary nucleus (De Zeeuw et al., 1994ab). The function of these structures as preolivary nuclei resides within "closed" anatomical pathways: in general they receive input from particular zones in the flocculus and ventral nodulus, and project to the subnuclei of the contralateral IOL that send climbing fibres to the zone in question (De Zeeuw et al., 1994ab, Ruigrok and Voogd, 1980; Tan et al., 1995). The GABAergic terminals from the cerebellar and vestibular nuclei in the IOL are apposed to dendrites coupled by gap junctions (De Zeeuw et al., 1993 and 1994ab), and may regulate the electrotonic coupling in between the olivary neurons (Lang et al., 1989 and 1990, Llinás and Sasaki, 1989; Sasaki et al., 1989; Sotelo et al., 1974). The function of the dorsal y, PrH, VDN and MVN as preoculomotor nuclei resides within "open" anatomical pathways in which no short feedback projections are involved (Delgado-Garcia et al., 1989). Interestingly, individual Purkinje cell axons from the vestibulocerebellum are apposed to neurons with different shapes and sizes within the same cerebellar and vestibular nuclei suggesting that individual Purkinje cells can influence both the "open" and "closed" pathways (De Zeeuw et al., 1994ab).

Reflecting on itself, the human brain has uncovered some marvelous facts.
What appears to be needed for understanding how it works
is new techniques for examining it and new ways
of thinking about it.

(F.H.C. Crick in: The Brain, A Scientific American Book,)

Chapter V. Discussion

Both the oculomotor nucleus and the olivary subnuclei involved in compensatory eye movements receive afferents from various nuclei surrounding the fourth ventricle. In the present study the major afferents from this complex of cerebellar and vestibular nuclei to the oculomotor nuclei and dc, ventrolateral outgrowth, and Beta nucleus were investigated with the use of several double labeling techniques at the light microscopic and electron microscopic level in the rabbit. In addition, we described the general characteristics of the ultrastructure of these nuclei in the rabbit. Below we will discuss the major results and functional implications.

General ultrastructural characteristics of the oculomotor nucleus. The ultrastructural characteristics of the neuropil of the rabbit OMN generally agree with those described for the oculomotor complex of other mammals like the cat (Tredici et al., 1976; Demêmes and Raymond, 1980), rhesus monkey (Waxman and Pappas, 1979), and rat (Soghomonian et al., 1989). They show the same types of terminals as described for the AN (Destombes et al., 1979; Spencer and Sterling, 1977) and TN (Bak and Choi, 1974; Bak et al., 1976), and they are in line with the major characteristics of the ventral horn of the spinal cord (Bodian, 1966; Conradi, 1969; Holstege and Kuypers, 1987). The similarity in ultrastructural characteristics even extended to the observation of axo-axonal synapses. This type of terminal has been described for the OMN of the cat and the monkey (Tredici et al., 1976; Waxman and Pappas, 1979), the TN and AN of the cat (Bak and Choi, 1974; Destombes et al., 1979), and for the spinal ventral horn of different species (Bodian, 1966; Conradi, 1969; Holstege and Calkoen, 1990). Although many physiological (Kidokoro, 1969; Kriebel et al., 1969; Korn and Bennett, 1972) and electron microscopic (Waxman and Pappas, 1971; Sterling, 1977; Saballus et al., 1984) studies have demonstrated the presence of electrotonic coupling and concomitant gap junctions in the OMN of several inframammalian species, no electrical synapses were observed in the present study of the rabbit nor in previous ultrastructural studies of the cat, monkey, or rat (Tredici et al., 1976; Waxman and Pappas, 1979; Soghomonian et al., 1989).

Inhibitory inputs to the oculomotor nucleus. In the present study we demonstrated that all subdivisions of the rabbit OMN receive a prominent GABAergic input and a weak, but consistent, glycinergic input. Most of the GABAergic and glycinergic synaptic endings were characterized by pleiomorphic or flattened vesicles and symmetric synapses, and their postsynaptic distribution was the same for all oculomotor subdivisions. In addition, we were the first to show colocalization of GABA and glycine within the same terminals of the OMN; all glycinergic terminals were GABAergic.

GABAergic input. The observations on the GABAergic input to the rabbit OMN are in agreement with the findings by De La Cruz et al. (1992) in the cat, but they are at variance with earlier reports on the GABAergic innervation of MR motoneurons in the cat and monkey by Spencer (Spencer et al. 1989; Spencer and Baker 1992). These authors observed a relatively weak GABAergic input to the MR subdivision. One of the reasons for the different outcomes may result from the fact that both the study by De La Cruz and the present study used a combination of retrograde tracing from the oculomotor MR muscle with GABA-immunocytochemistry (Chapter IIa), whereas Spencer and colleages did not use a double labeling technique to identify the postsynaptic structure of the GABAergic terminals. Moreover, their initial quantifications of the GABAergic input to the cat OMN were based on a pre-embedding study using an antibody against GAD, the synthesizing enzyme of GABA. The preembedding method provides excellent results for qualitative descriptions, but the

quantification is hampered by a limited penetration of the antibody. In contrast, we employed postembedding immunogold labeling using an antibody against GABA (Buijs et al., 1987), which allows the identification of virtually all GABAergic terminals (De Zeeuw et al., 1988; De Zeeuw, 1990). On the other hand, differences between cat and rabbit should not be excluded. As outlined in the introduction of this thesis, frontal-eyed cats and lateral-eyed rabbits share the same principal vestibulo-ocular projections, but each species has secondary connections to other extraocular motoneurons consistent with differences in the orientation of the extra-ocular muscles and semicircular canals.

The question as to what the source is of the GABAergic input to the MR region, is one of the major issues raised by the findings presented in this thesis. The SVN gives rise to a small part of the GABAergic input to the MR subdivision (Wentzel et al. 1995; Chapter IIc) , but it has not been established whether these terminals directly innervate MR motoneurons, MR interneurons, or dendrites of motoneurons of adjacent subdivisions that extend into the MR subdivision. A direct GABAergic input from the SVN to MR motoneurons is questionable, because earlier tracing studies in the monkey and the cat failed to show this connection (McMasters et al. 1966; Carpenter and Cowie 1985), and IPSP19s have not been found in MR motoneurons following vestibular stimulation (Baker and Highstein 1978; Highstein and Reisine 1979) . Possible sources for the GABAergic input to the MR motoneurons include the pontine and mesencephalic reticular formation, PrH and oculomotor internuclear neurons (for review see: Barmack et al. 1989; De La Cruz et al. 1992; De Zeeuw et al., 1993; Chapters I and IIIa).

The bulk of the GABAergic input to the IR, IO, and SR oculomotor subdivisions was found to be derived from the ipsilateral SVN (Chapter IIc, Table 1). The terminals traced from the ipsilateral SVN were rather large, contained mainly flattened vesicles and established symmetric synapses. This F-type terminal (Holstege and Calkoen 1990; Wentzel et al. 1993) , corresponds to types I and II described for the TN (Bak and Choi 1974) and OMN (Demêmes and Raymond 1982) in the cat. Their morphology matched that of terminals in the cat, labeled either by injection of radioactive substances in the vestibular nuclei (Demêmes and Raymond 1982) , intra-axonal injection of HRP in inhibitory second-order vestibular neurons (Spencer and Baker 1983), GAD- and GABA-immunocytochemistry (De La Cruz et al. 1992; Spencer and Baker 1992), or ^{3}H-GABA uptake (Lanoir et al. 1982; Soghomonian et al. 1989) . The postsynaptic distribution of the GABAergic terminals in the OMN from the SVN in the rabbit differed somewhat from that in the cat. We observed that 39.1% of the postsynaptic elements of the GABAergic SVN terminals were proximal dendrites or somata (Chapter IIc). In the cat, the differential input to the proximal and distal structures is more pronounced; only 45% of the terminals from the ipsilateral vestibular nuclei were found to contact distal dendrites (Demêmes and Raymond 1982; Spencer and Baker 1983). The somata contacted by GABAergic terminals from the SVN in the rabbit OMN showed the characteristics of motoneurons. Similar to the description by Tredici in the cat (Tredici et al. 1976) they were usually large, contained a well developed granular ER apparatus and a smooth nucleus. In addition, they were not GABAergic and not retrogradely labeled with WGA/HRP from the injections in the vestibular nuclei.

The observation that the entire projection from the SVN to the ipsilateral OMN is GABAergic is in agreement with several physiological studies (Ito et al., 1970; Obata and Highstein, 1970; Precht et al., 1973; Ito et al. 1976b). These studies employed a combination of electrophysiological recordings with lesion and selective blocking techniques and showed at the physiological level that GABA is the inhibitory neurotransmitter in the VVOR pathways to the OMN. The GABAergic neurons in the SVN projecting to the OMN are probably the second-order neurons, which can be activated by both the anterior and posterior canal (for

rabbit see Highstein and Ito, 1971; Ito et al., 1976b; Graf et al., 1983; for cat see Uchino and Suzuki, 1983; Mitasacos et al., 1983; Graf et al., 1983; Graf and Ezure, 1986). They are located in the central part of the SVN.

Two findings of the present study support the notion that the ipsilateral SVN is not the only source of GABA in the OMN. First, F-type terminals were not the only GABergic terminals. G-, T- and P-type GABAergic terminals were also observed; none of these types of terminals were WGA-HRP labeled following anterograde tracing from the SVN (Chapter IIc). Second, the GABAergic terminals that were glycinergic, were never found to originate from the SVN either.

Glycinergic input. In agreement with studies in the cat and monkey (Spencer et al. 1989; Spencer and Baker 1992), the OMN of the rabbit contains relatively few glycine-immunoreactive boutons. In contrast to these studies however, the glycine-immunoreactive boutons in the OMN of the rabbit are evenly distributed through all subdivisions. The immunocytochemical, electrophysiological-pharmacological and autoradiographic experiments on the cat oculomotor complex suggests that glycine is utilized in inhibitory connections from the PrH, parts of the reticular formation and second-order vestibular neurons to the AN (Spencer et al. 1989). Thus, glycine may be the major inhibitory transmitter for the HVOR in cat. Whether glycinergic projections to the AN exist in the rabbit was not determined in the present study, but the inhibitory innervation of the MR motoneurons suggests that the role of the glycinergic input in the HVOR of the rabbit is not as prominent and specific as has been reported for the cat. The source and functional implications of the glycinergic innervation of the rabbit OMN remain unclear. Analysis of the present material provided no evidence for the existence of glycinergic (inter)neurons in the OMN, indicating that the origin of the GABA/glycinergic projection is most likely a brainstem nucleus. Data available from the literature on the most important sources of OMN afferents (see Chapter I) do not provide a single source that meets the criterion of both having an inhibitory connection with the OMN and showing colocalization of GABA and glycine. The SVN can be excluded, because glycinergic neurons have not been found in the SVN (Walberg et al. 1990) and all terminals in the OMN traced from the SVN were not immunoreactive for glycine (Wentzel et al. 1989; Chapter IIc) . The MVN and DV contain neurons with colocalization of GABA and glycine (Walberg et al. 1990) and group Y contains GABAergic neurons (Mugnaini and Oertel 1985) , but these nuclei can be excluded since there is no evidence for an inhibitory component in their ascending connections (Ito et al. 1976b; Highstein and McCrea 1988). Similar arguments can be found against other possible candidates for the source of the glycinergic input to the OMN. The PrH has a mixed population of GABA- and glycinergic neurons but no colocalization (Spencer et al. 1989; Yingcharoen et al. 1989; De Zeeuw et al. 1993) , while the connections of the interstitial nucleus of Cajal (INC) with the OMN have been reported to be excitatory (Schwindt et al. 1974; Nakao and Shiraishi 1985). The riMLF provides an inhibitory (GABA) projection to the OMN of the cat (Wang and Spencer, 1992 and 1996), but the occurrence of glycine has not yet been demonstrated in the riMLF. Moreover, the identification of this nucleus, which plays a prominent role in the control of vertical saccadic eye movements (Chapter I), remains to be established in rabbits.

Excitatory inputs to the oculomotor nucleus. These inputs include the afferents from the MVN, AN and contralateral SVN. Virtually all OMN terminals derived from these nuclei contained spherical vesicles, made asymmetric synaptic contacts with distal dendrites, and were neither GABAergic nor glycinergic. This S-type terminal (Holstege and Calkoen 1990) most likely corresponds to the type III terminals described for the TN (Bak and Choi 1974) and

OMN (Demêmes and Raymond 1982) in cat. Their morphology and the absence of inhibitory transmitters stresses their excitatory nature (Uchizono 1965).

The observation that the projection from the MVN to the ipsilateral and contralateral OMN is excitatory is supported by several physiological studies (eg. Highstein and Ito, 1971; Highstein, 1973a; Ito et al., 1976a). The non-GABAergic neurons in the MVN projecting to the OMN are probably mainly excitatory second-order neurons, which can be activated by all semicircular canals (see Table 1). The neurons that are responsive to the anterior and posterior canals are located mainly in the parvocellular MVpc, (for rabbit see: Highstein and Ito, 1971; Highstein, 1973a; Ito et al., 1976a; Graf et al., 1983; for cat see Precht and Baker, 1972; Highstein and Reisine, 1979; Uchino et al., 1981 and 1982; Graf et al., 1983; Graf and Ezure, 1986; Iwamoto et al., 1990ab; monkey: McCrea et al., 1987b). Their fibers ascend predominantly in the contralateral MLF and terminate in the IR, IO, and SR subdivisions of the contralateral OMN as well as in the contralateral TN (for connections in rabbit see Thunnissen, 1990; for cat and monkey see McMasters et al., 1966; Tarlov, 1970; Gacek, 1971; Carleton and Carpenter, 1983; for opossum see Henkel and Martin, 1977). The excitatory OMN projecting neurons that respond to signals from the horizontal canal are probably located in all parts of the MVN: i.e. the MVpc, the MVc, and the MVmc, (for rabbit see Highstein, 1973a; for cat see Furuya and Markham, 1981; Highstein and Reisine, 1979; Reisine and Highstein, 1979; Reisine et al., 1981; Uchino et al., 1982; for monkey see McCrea et al., 1987a). The neurons in the MVpc and MVc project to the contralateral AN, whereas the fibers of the neurons in the MVmc enter the ATD and terminate either ipsilaterally or bilaterally in the MR subdivision (for cat and monkey see McMasters et al., 1966; Tarlov, 1970; Carpenter and Cowie, 1985; see also Buttner and Buttner-Ennever, 1988). The neurotransmitter involved in these excitatory projections of the VOR is probably either aspartate or glutamate (Demêmes and Raymond, 1982; Kevetter and Hoffman, 1991).

The observation that the projection from the SVN to the contralateral OMN is excitatory is also supported by physiological studies (Highstein and Ito, 1971; Yamamoto et al., 1978; Highstein and Resine, 1979). The excitatory OMN projecting neurons in the SVN are located in the dorsal and dorsolateral part of this nucleus, and receive afferents mainly from the anterior canal (for rabbit see Highstein and Ito, 1971; Yamamoto et al., 1978; for cat see Highstein and Resine, 1979; Uchino et al., 1982; Hirai and Uchino, 1984). Their fibers enter the bc and terminate in the contralateral IO and ipsilateral SR subdivisions (for rabbit see Thunnissen, 1990; for cat and monkey see McMasters et al., 1966; Tarlov, 1970; Lang et al., 1979; Carpenter and Cowie, 1985).

Finally, the observation that the projection from the AN to the contralateral OMN is excitatory, is in agreement with physiological studies in cat by Baker and Highstein (1975), and Baker and McCrea (1979) who showed that the projection from AbIn is excitatory and restricted to MR motoneurons. In our light microscopic experiments, in which we combined retrograde tracing from the MR muscles with anterograde tracing from the AN, we observed that some of the anterogradely labeled fibers were located outside the MR subdivision (Chapter IIc). However, we cannot exclude the possibility that these fibers innervate dendrites of MR motoneurons extending in neighbouring subdivisions. The excitatory OMN projecting neurons in the AN of the rabbit and the cat intermingle among abducens motoneurons and do probably not receive afferents from the semicircular canals (Evinger, 1988).

Canal	Excites	Soma	Axon	Suppresses	Soma	Axon
AC	iSR	iSVN(MVN*)	ibc	iIR	iSVN	iMLF
	cIO	iSVN(MVN*)	ibc	cSO	iSVN	iMLF
PC	iSO	iMVN	cMLF	iIO	iSVN	iMLF
	cIR	iMVN	cMFF	cSR	iSVN	iMLF
HC	iMR	iMVN	iATD	iLR	iMVN	-
	cLR	iMVN	-	cMR	iSVN?	iMLF

Table I. The relationship between the secondaries of the 3-neuron arc in the MVN and SVN and their motoneuronal targets in the rabbit based upon previous physiological work by Highstein and others (Highstein and Ito, 1971a; Highstein et al., 1971; Highstein, 1971; Highstein, 1973ab) and the present morphological study. Highstein and colleages mapped the locations of the secondaries participating in the horizontal canal, anterior canal, and posterior canal VOR circuits in a series of studies using intra- and extra- cellular recording in conjunction with monopolar stimulation. In the present study, we investigated the same connections at the electron microscopic level using a combination of WGA-HRP anterograde tracing with postembedding immunocytochemistry (Chapter IIc). For each VOR circuit, the table lists the location of the soma and axon of the relevant excitatory and inhibitory vestibular nucleus cells. The data presented above applies only to cells of the three neuron arc, i.e., to those cells receiving monosynaptic input from the VIIIth nerve. In addition, it should be noted that the present table depicts the situation in the rabbit; in cat, the MVN is probably involved in the excitatory path from the AC to the SR and IO subdivisions (see asterisks).

General ultrastructural characteristics of the olivary subnuclei involved in compensatory eye movements. The present study indicates that the principal ultrastructural features of the dc, VLO, and Beta-nucleus are qualitatively similar to those of the other olivary subdivisions and that the fine structure of the IOL in the rabbit does not differ from that in other mammals. The neuropil of the dc, VLO, and Beta-nucleus contains extraglomerular and glomerular synaptic fields; the neurons are coupled by gap junctions; and the subnuclei contain numerous GABA-positive and GABA-negative axon terminals, but no glycinergic terminals.

Most of the terminals in the dc, VLO and Beta-nucleus contained either rounded or pleomorphic vesicles and formed, respectively, asymmetric or symmetric synapses with intermediate and distal dendritic elements inside or outside the glomeruli. Some of the terminals contacted neuronal cell bodies. In general, the terminals in the dc were smaller that those in the Beta-nucleus. These results agree with the study of the dc in rabbit by Mizuno and colleagues (1974). The present study was the first to show that gap junctions are present in the dc, VLO and Beta-nucleus of both the rabbit and the rat, thereby indicating that neurons in these olivary subnuclei are electrotonically coupled. This is in line with a study by Takeda and Maekawa (1989) who showed "olivary reflexes" (olive-relayed climbing fiber responses to stimulation of the cerebellar cortex; Armstrong et al., 1973) within the flocculo-nodular lobe, a phenomenon which is now generally accepted as being due to electrotonic coupling in the IOL (Llinás et al., 1974; Llinás and Sasaki, 1989). In addition, they agree with the ubiquitous presence of dendritic lamellar bodies in the IOL; these organelles can be associated with dendrodendritic gap junctions and are present in all olivary subdivisions (De Zeeuw et al., 1995a). Taken together, the present results indicate that the dc, VLO and Beta-nucleus are organized like other olivary subnuclei, as described in rat (Gwyn et al., 1977; Sotelo et al., 1986), cat (Walberg,1963, 1966; Nemecek and Wolff, 1969; Sotelo et al., 1974), opossum (Bowman and King, 1973; King, 1976 and 1980), and squirrel monkey (Rutherford and Gwyn, 1977 and 1980). Quantitatively, however, two major features of the neuropil of the dc differ

from those of the other olivary subnuclei: 1) the relatively small size of the glomeruli; and 2) the prominent presence of crest synapses.

Glomeruli. The glomeruli in the dc of the rabbit and the rat were usually small; they often contained only 3 spines and 2 axon terminals in single sections, which means they are smaller than glomeruli in the MAO and PO of the cat and the rat (De Zeeuw et al., 1990 a,b, and c). The percentage of terminals within the glomeruli is related to the size of these synaptic nests. Quantitative analysis of the ultrathin sections of the dc and VLO showed that only about 10-20% of the terminals anterogradely labeled from the PrH, VDN and dorsal y were located within glomeruli. In the Beta-nucleus, however, 34% of the double labeled terminals from the vestibular nuclei were located within glomeruli. Previous quantitative analysis of the cat and the rat showed that in the MAO and PO approximately one third of the terminals derived from the mesodiencephalic junction (non GABAergic) and the central cerebellar nuclei (GABAergic) were located within glomeruli (De Zeeuw et al., 1989a,b; De Zeeuw et al., 1990a; for comparison see qualitative data of rat in PO by Angaut and Sotelo, 1987). It appears, therefore, that fewer boutons are situated in the glomeruli in the dc and VLO than in other olivary subnuclei.

Crest synapses. The presence of crest synapses in the dc was first described by Mizuno et al. (1974) for the rabbit. The present study confirms and extends their observations. The dc and VLO contained significantly more crest synapses than the other subnuclei, including the Beta-nucleus. The vast majority of the dc terminals engaged in the crest synapses were GABAergic in spite of the fact that the synaptic junctions consistently belonged to the asymmetric category. The few crest synapses found in a previous study of the MAO and PO of the cat (De Zeeuw et al., 1989a) were likewise associated with GABAergic terminals. Sotelo et al. (1986) have also illustrated a crest synapse formed with two GAD positive terminals in an unspecified region of the rat IOL. About 20% of the GABAergic terminals that formed crest synapses in the rostral dc and VLO were double labeled with WGA-HRP from the VDN and dorsal y, whereas none could be traced from the PrH. Since the double labeling technique combining anterograde tracing of WGA-HRP with postembedding GABA-immunocytochemistry labels at most about a third of the actual number of terminals (De Zeeuw et al., 1989), these data strongly suggest that the VDN and dorsal y are the major sources of the terminals involved in the crest synapses in the rostral dc and VLO. Crest synapses are also present in the caudal dc, and in this subdivision they are also formed predominantly with GABAergic terminals (Chapter IIIa). Unfortunately, we have not been able to trace the origin of the crest synapses in the caudal dc. Some of the crest synapses in this olivary subdivision are formed by cholinergic terminals (Caffé et al., 1996). Because the PrH also contains cholinergic neurons, some of which show colocalization with GABA, that project to the IOL (Barmack et al., 1993; Caffé et al., 1996), we cannot exclude the possibility that some neurons in the PrH also give rise to terminals involved in crest synapses. The non-GABAergic terminals forming the crest synapses could be derived from the PrH or the pretectum. However, degenerated terminals making crest synapses have not been observed after pretectal lesions (Mizuno et al., 1974). The function of crest synapses has not been elucidated. It is also unknown whether the crest synapses observed in other areas of the mammalian nervous system, like the habenula and interpeduncular nucleus (Milhaud and Pappas, 1966ab; Lenn, 1976; Murray et al., 1979), subfornical organ (Akert et al., 1967), suprachiasmatic nucleus (Guldner, 1976), and locus coeruleus (Mizuno and Nakamura, 1972) are also formed predominantly with GABAergic terminals. Crest synapses have also been observed in the avian ciliary ganglion (Mugnaini, unpublished observations) where they are formed by excitatory, cholinergic endings derived from the Edinger-Westphal nucleus (Pilar and Johnson, 1988). The crest synapses in the interpeduncular nucleus are formed with a terminal from the ipsilateral habenula and a terminal

from the contralateral habenula (Murray et al., 1979). The fact that we never found a crest synapse formed with two double labeled terminals, together with the finding that the VDN and dorsal y project bilaterally (although contralateral >> ipsilateral) to the dc and VLO, offers the possibility that the crest synapses in the dc and VLO receive a terminal from each side of the brain.

Inhibitory inputs to the olivary subnuclei involved in horizontal compensatory eye movements. In Chapter IIIa we demonstrate that the GABAergic input to the caudal dc, which is mainly involved in the horizontal OKR, originates primarily in the PrH. The experiments in which PHA-L and WGA-HRP were injected into the PrH of the rabbit and the rat showed that: 1) this nucleus provides a bilateral input to the caudal dc; 2) the input to the rostral dc is predominantly contralateral; 3) the projection to the VLO is exclusively contralateral; and 4) the input to the MAO is ipsilateral. These results confirm and extend previous studies in cat that indicated the PrH projects mainly to the contralateral dc and ipsilateral MAO (Saint-Cyr and Courville, 1979; McCrea and Baker, 1985; Gerrits, 1985). Our finding that the PrH bilaterally innervates neurons in the caudal dc that are known to respond optimally to movements of the visual field around the vertical axis (Leonard et al., 1988), is in line with the facts that the projection from the PrH to the extraocular motornuclei is mainly to the AN and the MR subdivision of the OMN (McCrea and Baker, 1985; Delgado-Garcia et al., 1989) and that neurons in the PrH respond mainly to horizontal angular acceleration (Blanks et al., 1977).

Following a lesion of the PrH most of the GABAergic terminals in the caudal dc and a smaller fraction of these terminals in the rostral dc and VLO disappeared. The lesions affected the ipsi- and contralateral fibers of the lesioned PrH as well as those fibers of the contralateral PrH that cross the midline and innervate the dc on the side of the lesion. In addition, we found at the electron microscopic level that the majority of dc terminals anterogradely labeled from the PrH were GABAergic. The present findings are in agreement with the observations of McCrea and Baker (1985) in cat that PrH cells projecting to the dc are small and that the PrH contains many small GABAergic neurons (Mugnaini and Oertel, 1985; Chapter IIIa). Moreover, it is in line with the finding that the PrH contains retrogradely labeled neurons following large injections of WGA-HRP covering the entire IOL and that these retrogradely labeled neurons can be GAD positive (Nelson and Mugnaini, 1989).

However, the projection from the PrH to the IOL is not merely GABAergic. In the electron microscopic analysis of the dc following injection of WGA-HRP in the PrH significantly more WGA-HRP labeled terminals were GABAergic on the contralateral side than on the ipsilateral side. This difference was not only significant for the rostral dc but also for the caudal dc. In the Beta-nucleus, in which the ipsilateral GABAergic projection from the MVe and adjoining SpVe was analyzed for control (see also Nelson et al., 1986; Barmack et al., 1989), significantly more WGA-HRP labeled terminals were GABA positive than in the ipsilateral dc. These data indicate that part of the ipsilateral projection from the PrH to the dc is non-GABAergic. This conclusion is supported by the observation that, unlike the GABAergic WGA-HRP labeled terminals, the non-GABAergic terminals from the PrH usually contained round synaptic vesicles and formed asymmetric synapses. Synapses of this kind are usually excitatory (Uchizono, 1965). Moreover, the non-GABAergic WGA-HRP labeled terminals varied considerably in size, whereas the GABA positive terminals were usually of intermediate size. The non-GABAergic terminals may consist of cholinergic terminals, because Barmack et al. (1991; 1993) have shown a cholinergic projection from the PrH to the dc. It should be noted, however, that the cholinergic input to the dc does not only contribute to the non-GABAergic pool of terminals, since it was recently demonstrated that 29% of the

cholinergic terminals in the dc also contain GABA (Caffé et al., 1996). Colocalization of GABA and acetylcholine has also been demonstrated for other areas of the CNS (Beaulieu and Somogyi, 1991; Todd, 1991).

Inhibitory inputs to the olivary subnuclei involved in vertical compensatory eye movements. We demonstrated that the GABAergic input to the rostral dc and VLO, which are mainly involved in the vertical OKR, originates primarily in the VDN and dorsal y (Chapter IIIb), and that the GABAergic input to the Beta-nucleus, which is probably involved in the control of the vertical VOR, originates in the MVN and SpVe (Chapter IIIa).

The nucleo-olivary connections between VDN and the VLO have been shown before in cat (Tolbert et al., 1976; Dietrichs et al., 1985) and rat (Ruigrok and Voogd, 1990; Ruigrok et al., 1992). In cat, the fastigial nucleus has been reported to project to the dc (Dietrichs and Walberg, 1985), but in our material we observed no retrogradely labeled cells in the fastigial nucleus following injection of HRP in the dc. The projections from VDN and dorsal y to the rostral dc have not been reported before. Both the projections to the rostral dc and VLO are predominantly, but not exclusively, contralateral. Ipsilateral nucleo-olivary projections have been noted previously in various other species (rat: Brown et al., 1977; Chan-Palay, 1977; Angaut and Cicirata, 1982; Swenson and Castro, 1983; Ruigrok and Voogd, 1990; cat: Beitz, 1976; monkey: Chan-Palay, 1977; Kalil, 1979; De Zeeuw et al., 1996b). In these studies, the ipsilateral labeling was usually also distributed as the mirror image of the contralaterally labeled areas.

The retrogradely labeled neurons in the VDN and dorsal y were small to medium sized and somewhat elongated. The quantitative electron microscopic analysis of their terminals in the rostral dc and VLO showed that all these neurons were GABAergic. These results agree with observations in rat and cat that the VDN and dorsal y contain small to medium sized GABAergic neurons (Mugnaini and Oertel, 1985; Carpenter et al., 1990). Moreover, the relation between cell size and the presence of GABA is in line with the present observation that the large neurons in dorsal y, which provide an excitatory input to the contralateral OMN (Graybiel and Hartwieg, 1974; Highstein and Reisine, 1979; Stanton, 1980; Carpenter and Cowie, 1985; Highstein and McCrea, 1988), were not retrogradely labeled. In addition, the results agree with the findings that group y and the DN in monkey are involved in vertical eye movements (Chubb and Fuchs, 1982; Partsalis et al., 1995ab), and that the Purkinje cells in the flocculus involved in the control of vertical compensatory eye movements project to the VDN and dorsal y (De Zeeuw et al., 1994b).

In the present study, we demonstrated that the projection from the MVN and SpVe to the Beta-nucleus is ipsilateral and entirely GABAergic (Chapter IIIa). These results are in agreement with lesion induced GAD depletion studies by Fredette and Mugnaini (1991). Neurons in the Beta-nucleus respond to roll vestibular stimulation about the longitudinal axis (Barmack et al., 1989). They are excited and inhibited when the rabbit is rolled onto the contralateral and ipsilateral side, respectively. This modulation is probably partly due to the activity in the projection from the vestibular nuclei to the Beta-nucleus.

Functional implications of inhibitory inputs to inferior olive. The caudal dc, which is involved in the horizontal OKR, receives its major GABAergic input from the PrH; the rostral dc and VLO, which are mainly involved in the vertical OKR, receive their major GABAergic input from the VDN and dorsal y; and the Beta-nucleus, which is involved in the vertical VOR, receives its major GABAergic input from the MVN and SpVe (Chapter III). Interestingly, all the nuclei that project to the IOL, i.e. the PrH, VDN, dorsal y and MVN, are also all known to be involved in the control of eye movements (figure 1).

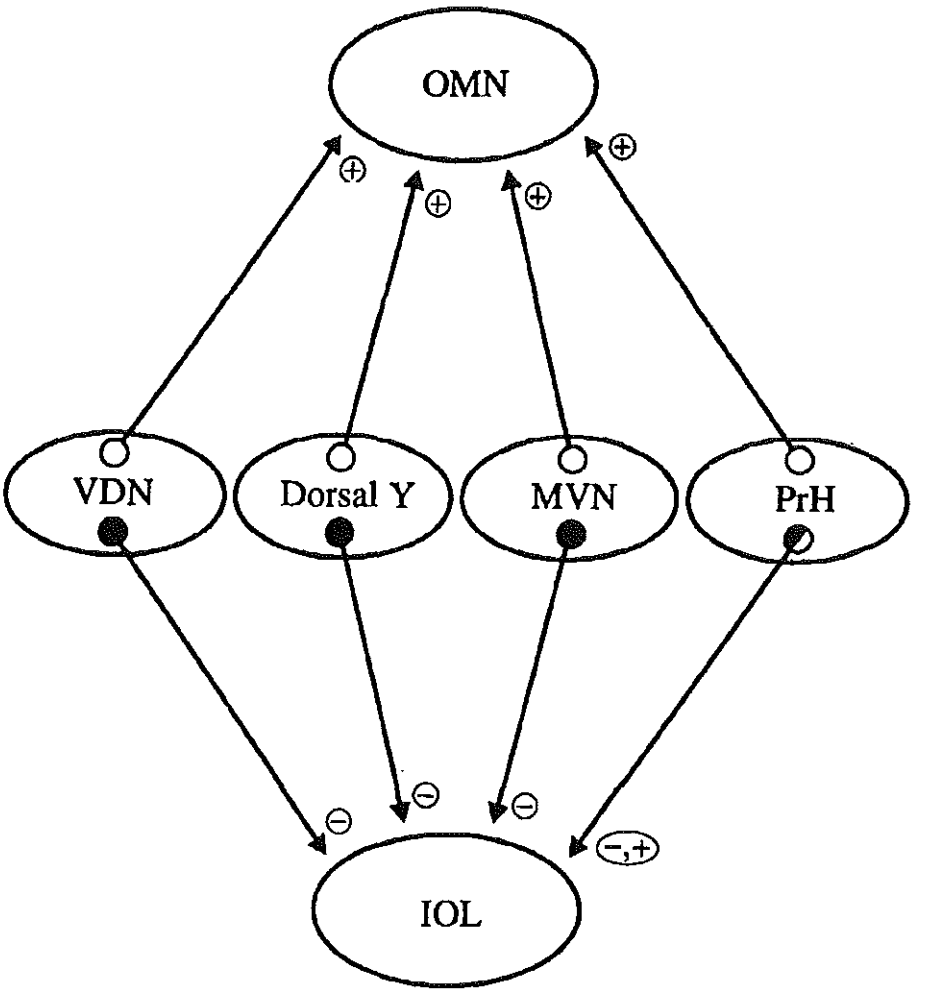

Figure 1. Illustration of the excitatory (o) and inhibitory (o) projections from the ventral dentate nucleus (VDN), dorsal group y (dorsal y), medial vestibular nucleus (MVN) to the oculomotor nucleus (OMN) and inferior olive (IOL). Note that the projections to the OMN are probably predominantly excitatory, whereas those to the IOL are mainly inhibitory.

Below we will discuss the possible functions of the inhibitory inputs from these nuclei to the IOL.

The heterogenous group of neurons forming the PrH is probably involved in maintaining eye velocity through "velocity storage" (an integration process which is reflected in the slow-phase velocity of postrotatory nystagmus or optokinetic after-nystagmus) and in determining eye position in the VOR (Chapter I; Skavenski and Robinson, 1973; Blanks et al., 1977; Robinson, 1989). At present, nothing is known about the specific electrophysiological properties of the neurons in the PrH that project to the dc. For example, it is not known whether signal processing in these neurons contributes to the integration necessary for the velocity storage. Therefore, several possible functional roles for this projection should be considered. First, the GABAergic input from the PrH could send an efference copy signal to the dc that relays information about neural processing in the PrH (Baker, 1977; Baker et al., 1981; McCrea and Baker, 1985; McCrea, 1988; Delgado-Garcia et al., 1989). Such an efference copy signal could be used to suppress OKR during voluntary eye movements (Holst and Mittelstaedt, 1950; McCrea and Baker, 1985; McCrea, 1988). Recent studies showed that, even though the projection is massive, lesions of the PrH in the *anaesthetized* rabbit do not influence the complex spike modulation of VA Purkinje cells in the flocculus and have only a modest impact on the spontaneous complex spike firing frequency in the dark (Arts et al., 1996). Thus, the effects of the input from the PrH onto the neurons in the dc may only become visible when the animal is alert and makes voluntary eye movements. The suppression of reflexes during voluntary movements is compatible with the learning theory on olivocerebellar function (see Chapter I). For example, if the signals encoding retinal slip would continue to be transmitted to the cerebellum during voluntary eye movements, the activity in the climbing fiber "teachers", would totally disrupt the completed heterosynaptic effects that were induced during the compensatory eye movements. Since the projection from the PrH to the IOL is bilateral, one of the voluntary eye movements that may be facilitated by suppression from the PrH is convergence. Second, the projection from the PrH could carry vestibular information. Although neurons in the dc generally do not seem to respond to vestibular stimulation (Barmack and Hess, 1980; Barmack et al., 1989), De Zeeuw et al. (1995b) demonstrated the existence of floccular Purkinje cells that showed climbing fiber modulation during natural vestibular stimulation in the dark in the high frequency range. Since the dc does not receive a direct input from the vestibular nuclei (Gerrits, 1985), while the PrH receives a strong input from these nuclei (McCrea, 1988), it is attractive to speculate that the projection from the PrH to the dc relays vestibular information to the dc.

In addition, the function of the GABAergic input from the PrH to the caudal dc, but also that of the GABAergic projections from dorsal y, VDN, MVN and SpVe to the rostral dc, VLO, and Beta-nucleus, can be compared to the general function of the input from the cerebellar nuclei to the IOL (De Zeeuw et al., 1988b; De Zeeuw et al., 1989a; Angaut and Sotelo, 1989; Fredette and Mugnaini, 1991). Each olivary subnucleus projects to one or more strips of Purkinje cells, and the Purkinje cells of each strip project to a particular cerebellar nucleus, which in turn innervates the corresponding olivary subnucleus (Groenewegen and Voogd, 1977; Groenewegen et al., 1979; for review see Voogd and Bigaré, 1980). Analogous triangular pathways are represented by the vestibular nuclei and PrH, the Purkinje cells in the vestibulocerebellum, and the dc, VLO and Beta-nucleus (Chapter III; Alley et al., 1975; Yamamoto, 1979; Gerrits and Voogd, 1982; Yingcharoen and Rinvik, 1983; McCrea, 1985; Ruigrok et al., 1992; De Zeeuw et al., 1994b). This analogy is supported by the finding that the morphology and postsynaptic distribution of the GABAergic terminals derived from the PrH, dorsal y, VDN and MVN in the dc, VLO and Beta-nucleus are similar to those of the GABAergic terminals from the cerebellar nuclei in the MAO and PO (Angaut and Sotelo,

1989; De Zeeuw et al., 1989a and 1990a); both the terminals from the cerebellar nuclei and the vestibular complex contact dendrites linked by gap junctions in the glomeruli. Therefore, it seems possible that the GABAergic terminals from the vestibulocerebellar nuclei, like those from the neocerebellum regulate the electrotonic coupling in the IOL (Sasaki and Llinás, 1985; Lang et al., 1989, 1990 and 1996; Sasaki et al., 1989; Llinás and Sasaki, 1989). Interestingly, the dendritic spines in the glomeruli of the dc, VLO and Beta-nucleus also receive, like those in the MAO and PO, a combined excitatory and inhibitory input (Chapter III; De Zeeuw et al., 1990abc). The source of the excitatory terminals in the caudal dc is probably the NOT, while the excitatory terminals in the rostral dc and VLO presumably originate from the pathway of the MTN and VTRZ (see Figure 2 in Chapter I); the possible source of excitatory terminals in the Beta-nucleus remains to be determined. The combined excitatory and inhibitory input to the olivary glomerular spines, which is unique in the CNS, has been proposed (De Zeeuw et al., 1990a) to serve as the interlocking gears of the olivary clockwork that may function as a timing device for motor behaviour (Llinás, 1989; Llinás et al., 1989; Llinás and Welsh, 1994; Lang, 1995; Welsh et al., 1995). This hypothesis is based on the fact that the integrative time constant of excitable spines that receive a combined excitatory and inhibitory input is extremely sensitive to the timing between these inputs (Segev and Rall, 1988). The importance of the combined excitatory and inhibitory input to the olivary spines is stressed by the finding that the excitatory projection to a particular olivary subdivision is mostly bilateral, when its inhibitory input is bilateral (De Zeeuw et al., 1996b). The timing hypothesis is not mutually exclusive with the efference copy hypothesis mentioned above. In fact, the effectiveness of efference copy signals may very well depend on the level of synchronicity between the olivary neurons (Yarom, 1992). Moreover, the cerebellar central nuclei may also send out efference copy signals to the IOL in order to prevent reflexes during voluntary movements (Gibson and Gellman, 1987; Armstrong and Andersson, 1987).

Projections from the rhombencephalon to the oculomotor nucleus and inferior olive are separated. In Chapter IV we demonstrated that neurons in the PrH, dorsal group y, VDN and MVN can project to either the IOL or the OMC, but hardly ever to both (Figure 1). This finding is also in line with the efference copy hypothesis presented above, because suppression of reflexes mediated via the IOL during voluntary eye movements would be useless if these reflexes were inhibited by neurons that also directly influence oculomotor activity; then the voluntary movements themselves would be equally affected. The observation that the projections to the OMC and IOL are isolated from one another emphasizes that the PrH, dorsal y, VDN and MVN may have dual functions in that they serve both as a preoculomotor nucleus and a preolivary nucleus (De Zeeuw et al., 1994b). The function of these structures as preolivary nuclei resides within the "closed" triangular anatomical pathways described above (Ruigrok and Voogd, 1990; De Zeeuw et al., 1994b; Tan et al., 1995b). The function of the dorsal y, PrH, VDN and MVN as preoculomotor nuclei resides within the "open" anatomical pathways in which no short feedback projections are involved (Delgado-Garcia et al, 1989). Importantly, individual Purkinje cell axons from the vestibulocerebellum are apposed to neurons with different shapes and sizes within the same cerebellar and vestibular nuclei suggesting that individual Purkinje cells can influence both the "open" and "closed" pathways (De Zeeuw et al., 1994b). Recently, it was demonstrated at the electron microscopic level that individual Purkinje cell axons indeed innervate directly both the excitatory and inhibitory neurons in the vestibular and cerebellar nuclei (De Zeeuw and Berrebi, 1995). Thus, this observation suggests that, if the efference copy hypothesis presented above is correct, the Purkinje cell output can control simultaneously a voluntary movement and the involuntary reflex that has to be suppressed during this voluntary movement.

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List of Abbreviations

IV	fourth ventricle
AbIn	abducens interneurons
AN	abducens nucleus
AOS	accessory optic system
bc	brachium conjunctivum
BETA	ß-nucleus
cdc	caudal dorsal cap
cMAO	caudal medial accessory olive
CO	cochlear nucleus
CTB	cholera toxin-b-subunit
DAB	diaminobenzidine
DAO	dorsal accessory olive
dc	dorsal cap
DN	dentate nucleus
DTN	dorsal terminal nucleus
dorsal y/Dy	dorsal group y
DY	diamidino yellow
GABA	gamma-aminobutyric acid
floc	flocculus
FB	fast blue
HRP	horseradish peroxidase
IA	interposed anterior nucleus
IO	inferior oblique
IOL	inferior olive
IP	interposed posterior nucleus
IR	inferior rectus
LVN	lateral vestibular nucleus
MLF	medial longitudinal fascicle
MR	medial rectus
MV(N)	medial vestibular nucleus
MVc	caudal MV
MVmc	magnocellular MV
MVpc	parvocellular MV
NOT	nucleus of the optic tract
OcIn	oculomotor interneurons
OMC	oculomotor complex (i.e. OMN + TN + AN)
OMN	oculomotor nucleus
p	folium p
PAP	peroxidase anti peroxidase antibody
PBS	phosphate buffered saline
PHA-L/PhaL	phaseolus vulgaris-leucoagglutinin
PO	principal olive
PrH	nucleus prepositus hypoglossi
rb	restiform body
rdc	rostral dorsal cap
rIMLF	rostral interstitial nucleus of the medial longitudinal fascicle
rMAO	rostral medial accessory olive
OKR	optokinetic reflex
SO	superior oblique
SR	superior rectus

SV(N)	superior vestibular nucleus
TBS	tris buffered saline
TBST	TBS containing 0.1% Triton X-100
TMB	tetramethylbenzidine
TN	trochlear nucleus
VDN	ventral dentate nucleus
ventral y	ventral group y
VLO/vlo	ventrolateral outgrowth
VOR	vestibulo-ocular reflex
VTRZ	visual tegmental relay zone
WGA-HRP	wheatgerm agglutinated horseradish peroxidase

Summary

The OMN and IOL receive afferents from various brainstem nuclei in the hindbrain that are involved in the regulation of compensatory eye movements (Chapter I). These nuclei include some of the vestibular and cerebellar nuclei as well as the PrH. For all these projections it is not known and/or it has not been shown at the morphological level 1) whether they are inhibitory or excitatory; 2) what the nature of their neurotransmiter is; and 3) whether individual neurons in the hindbrain can innervate both the OMN and IOL. In the present thesis, these projections were therefore investigated with the use of different combinations of tracing, lesions and immunocytochemistry in the rabbit. Special emphasis was put on the inhibitory components of the projections.

In Chapter II the projections to the OMN are described. The inhibitory inputs to the OMN are comprised of a dense GABAergic innervation and a moderate glycinergic input. The postsynaptic distribution of the GABAergic and glycinergic innervation did not differ among the different oculomotor subdivisions, and, despite earlier reports in the cat, the density of the GABAergic input to the medial rectus subdivision was as substantial as that to the other subdivisions. The GABAergic and glycinergic projections partly overlapped; ultrastructural serial section analysis revealed that all terminals with glycinergic immunoreactivity were also GABA positive, while up to 5% of the GABAergic terminals were glycine positive. Both types of terminals were characterized by flattened vesicles and symmetric synapses, and they contacted somata, as well as proximal and distal dendrites of motoneurons.

To determine the source of the GABAergic and glycinergic input to the OMN, we studied their terminals at the ultrastructural level following anterograde tracing of WGA-HRP from the SVN, MVN, and AN combined with GABA and glycine postembedding immunocytochemistry. The SVN projected bilaterally to the superior rectus and inferior oblique subdivisions, and ipsilaterally to the inferior rectus and medial rectus subdivision; the MVN projected bilaterally to the medial rectus, inferior oblique, inferior rectus and superior rectus subdivisions with a strong contralateral predominance. The AN projected contralaterally to the medial rectus subdivision. More than 90% of all the anterogradely labeled terminals from the ipsilateral SVN were GABAergic. All terminals derived from the MVN, the AN, and the contralateral SVN were non-GABAergic. These non-GABAergic terminals had a morphology characteristic of excitatory terminals: they showed spherical vesicles and asymmetric synapses, and they contacted predominantly distal dendrites. None of the anterogradely labeled terminals from the studied vestibular nuclei or AN was glycinergic.

In Chapter III the projections from the hindbrain to the olivary subnuclei involved in compensatory eye movements are described. These subnuclei include the dc, the VLO and Beta-nucleus. The dc and VLO are excited by neurons from the nucleus of the optic tract, medial tegmental nucleus and visual tegmental relay zone and they are involved in the optokinetic reflex; their climbing fibers carry signals that encode retinal slip and they innervate Purkinje cells in both the flocculus and nodulus. The Beta-nucleus is probably involved in the vestibulo-ocular reflex; their climbing fibers convey signals from the semicircular canals to the nodulus. In this thesis it was determined where the inhibitory inputs to these olivary subnuclei are derived from. We show that the PrH, dorsal group y, VDN and MVN are the sources of their GABAergic input. The PrH projects bilaterally to the dc, contralaterally to the VLO and ipsilaterally to the MAO. After lesioning the PrH the caudal dc was depleted of most of its glutamic acid decarboxylase immunoreactive terminals, while the rostral dc and the VLO were depleted for a minor part. Ultrastructural analysis indicated that the majority, but not all, of the terminals from the PrH in the dc are GABA-positive. These GABA-positive and GABA-negative terminals form predominantly symmetric and asymmetric synapses; most of them

synapse on dendrites outside and inside glomeruli, frequently in association with dendrodendritic gap junctions, while a small minority is axosomatic. None of the terminals from the PrH was found to form a crest synapse, although synapses of this kind were predominantly formed by GABAergic terminals. The VDN of the cerebellum and dorsal group y project contralaterally to the rostral dc and VLO; this projection is entirely GABAergic. The terminals of this input form predominantly symmetric synapses with extra- and intraglomerular dendrites, while most of the remaining terminals are axosomatic. In addition, the terminals derived from dorsal group y and the VDN are involved in the formation of crest synapses in the dc. The MVN projects ipsilaterally to the Beta-nucleus; this projection is also entirely GABAergic, and their terminals also form predominantly symmetric synapses with extra- and intraglomerular dendrites. None of the terminals in the dc, VLO or Beta-nucleus was glycinergic.

In Chapter IV we describe the topographical relation between the neurons in the hindbrain that project to the OMN and those that project to the IOL. The PrH, dorsal group y, VDN, and MVN all project to both the OMN and IOL. It was determined with the use of retrograde double labeling techniques whether individual neurons in these hindbrain nuclei can innervate both the OMN and IOL. We demonstrate that the neurons that project to the OMN are intermingled with those that project to the IOL, but that virtually none project to both.

In sum, in this thesis we provide the first direct anatomical evidence for the sources of the major inhibitory inputs to both the OMN and IOL. Possible consequences of interactions between both pathways are discussed in Chapter V.

Samenvatting

De oculomotor nucleus (OMN) en de inferior olive (IOL) ontvangen afferente vezels van diverse hersenstamkernen die betrokken zijn bij de regulatie van compenserende oogbewegingen (Hoofdstuk I). Deze groep van hersenstamkernen bestaat uit enkele vestibulaire en cerebellaire kernen en de nucleus prepositus hypoglossy (PrH). Van al deze projecties is onbekend, of is op morphologisch niveau nog niet aangetoond, 1) of zij inhibitoir of excitatoir zijn; 2) welke neurotransmitter zij gebruiken; en 3) of individuele neuronen in de achterhersenen zowel de OMN als IOL kunnen innerveren. In dit proefschrift zijn deze projecties bestudeerd in het konijn, met behulp van verschillende combinaties van tracing, lesie en immunocytochemische technieken. De nadruk lag op de inhibitoire componenten van de projecties.

In Hoofdstuk II zijn de projecties naar de OMN beschreven. De inhibitoire input naar de OMN bestaat uit een dichte GABAerge innervatie en een gematigde glycinerge input. Er zijn geen verschillen wat betreft de postsynaptische verdeling van de GABAerge en glycinerge innervatie tussen de diverse subdivisies van de OMN, en, ondanks eerder gepubliceerde onderzoeksgegevens in de kat, is de dichtheid van de GABAerge input van de mediale rectus even substantieel als die van de andere subkernen van de OMN. De GABAerge en glycinerge projecties overlappen elkaar gedeeltelijk: electronenmicroscopische analyse van ultradunne seriecoupes heeft aangetoond dat alle eindigingen met glycinerge immunoreactiviteit eveneens positief waren voor GABA, terwijl slechts 5% van de GABAerge eindigingen tevens glycine positief waren. Beide eindigings typen werden gekarakteriseerd door het bezit van afgeplatte blaasjes en symmetrische synapsen en zij maakten contact met somata en zowel proximale als distale dendrieten van motoneuronen.

Om de bron van de GABAerge en glycinerge input van de OMN op te kunnen sporen hebben we hun eindigingen op ultrastructureel niveau bestudeerd met behulp van anterograde tracing van WGA-HRP vanuit de superior vestibulair nucleus (SVN), de medial vestibulair nucleus (MVN) en de abducens nucleus (AN) in combinatie met GABA en glycine immunocytochemie. De SVN projecteert bilateraal naar de superior rectus en inferior oblique subdivisies en ipsilateraal naar de inferior en mediale rectus subdivisies; de MVN projecteert bilateraal naar de mediale rectus,inferior oblique, inferior rectus en superior rectus subdivisies, waarbij de contralateral projecties overheersen. De AN projecteert contralateraal naar de mediale rectus subdivisie. Meer dan 90% van alle anterograad gelabelde eindigingen van de ipsilaterale SVN waren GABAerg. Alle eindigingen afkomstig van de MVN, de AN en de contralaterale SVN waren niet-GABAerg. Deze niet-GABAerge eindigingen hebben de morfologische karakteristieken van excitatoire eindigingen: zij vertonen ronde blaasje en asym-metrische synapsen, en zij maken overheersend contact met distale dendrieten. Geen van de anterograad gelabelde eindigingen van de bestudeerde vestibulaire kernen of de AN waren glycinerg.

In Hoofdstuk III worden de projecties van de achterhersenen naar de subkernen van de olijf die bij compensatoire oogbewegingen zijn betrokken beschreven. Deze subkernen omvatten de dorsal cap (dc), de ventrolateral outgrowth (VLO) en de Beta nucleus. De dc en VLO worden geexciteerd door neuronen van de nucleus van de tractus opticus, de mediale tegmentum nucleus en de visual tegmental relay zone en zij zijn betrokken bij de optokinetische reflex: hun klimvezels dragen de signalen die coderen voor de retinale slip en zij innerveren Purkinje cellen in zowel de flocculus als de nodulus. De Beta-nuclei zijn waarschijnlijk betrokken bij de vestibulo-oculaire reflex:hun klimvezels transporteren signalen van de halfcirkelvormige kanalen naar de nodulus. In dit proefschrift is onderzocht waar de inhibitoire inputs van deze olijf subnuclei vandaan komen. Wij tonen aan dat de PrH, dorsale groep Y, de

ventral dentate nucleus (VDN) en MVN de oorsprong zijn van deze GABAerge input. De PrH projecteert bilateraal naar de dc, contralateraal naar de VLO en ipsilateraal naar de medial accessory olive (MAO). Na lesies in de PrH trad een sterke depletie op van GAD immunoreactieve eindigingen in de caudale dc, terwijl in de rostrale dc en de VLO slechts een klein deel van de GAD immunoreactieve eindigingen verdween. Analyse op ultrastructureel niveau toonde aan dat het grootste deel, maar niet alle, van de eindigingen afkomstig van de PrH in de dc GABA positief waren. Deze GABA-positieve en GABA-negatieve eindigingen vertonen respectievelijk symmetrische en asymmetrische synapsen: de meerderheid synapteert op dendrieten die gelegen zijn zowel binnen als buiten de glomeruli, vaak zijn dezen verbonden door dendrodendritische gap junctions, en slechts een klein deel maakt een axosomatisch contact. Geen van de eindigingen van de PrH vertoonde crest synapsen, ofschoon dit type synapsen hoofdzakelijk gevormd worden door GABAerge eindigingen. De cerebellaire VDN en de dorsale groep Y projecteren contralateraal naar de rostrale dc en de VLO; deze projectie is in zijn geheel GABAerg. De eindigingen van deze projectie vormen overheersend symmetrische synapsen met extra- en intraglomerulaire dendrieten, terwijl het merendeel van de overgebleven eindigingen axosomatisch synapteren. Bovendien zijn de eindigingen afkomstig van de dorsale groep y en de VDN betrokken bij de formatie van crest synapsen in de dc. De MVN projecteert ipsilateraal naar de Beta-nucleus; deze projectie is eveneens volledig GABAerg en de eindigingen vormen eveneens hoofdzakelijk symmetrische synapsen met synapsen met extra- en intraglomerulaire dendrieten. Geen van de eindigingen in de dc, VLO of Beta-nucleus was glycinerg.

In Hoofdstuk IV beschrijven we de topografische relatie tussen die neuronen in de hersenstam die projecteren naar de OMN en de neuronen die projecteren naar de IOL De PrH, dorsale groep Y, VDN, en MVN projecteren allen naar zowel de OMN als de IOL. Met behulp van retrograde dubbel labelling techniek werd bepaald of individuele neuronen in deze stamnuclei zowel de OMN als de IOL innerveren. Wij demonsteren dat de neuronen die naar de OMN projecteren vermengd zijn met de neuronen die naar de IOL projecteren, maar dat vrijwel geen van beide type neuronen naar beide kernen projecteert.

Samenvattend, in dit proefschrift geven wij het eerste directe anatomische bewijs voor de bronnen van de grote inhibitoire projecties naar de OMN en IOL. Mogelijke consequenties aangaande de interacties tussen beide projecties worden beschreven in Hoofdstuk V.

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Het boek kan in de kast.

Let's get on with our lives!

Curriculum Vitae

De schrijver van dit proefschrift werd geboren op 13 september in het jaar van de hond, 1958 te Amsterdam. Hij volgde vanaf 1970 de MAVO-4, daarna de HAVO en behaalde in 1978 het VWO diploma.
Na het behalen van het diploma werd hij opgeroepen voor militaire dienst Na 2 maal te zijn uitgeloot voor de studie diergeneeskunde en 1 maal voor biologie besloot hij als PL-1 programmeur te gaan werken bij de KLM. Een jaar later werd hij echter toch ingeloot voor de studie biologie. Van 1980 to 1987 studeerde hij medische biologie aan de Vrije Universiteit te Amsterdam, met als hoofdvak neuroanatomie/electronenmicroscopie en als bijvakken farmacologie en immunologie. Tussendoor behaalde hij de eerstegraads onderwijsbevoegdheid.
Drie maanden na het behalen van het doctoraal examen werd het promotieonderzoek aangevangen als AIO bij de afdeling Anatomie aan de Erasmus Universiteit te Rotterdam, o.l.v. Dr. J. Holstege, Dr. N. Gerrits, Dr. C.I. De Zeeuw en Prof.dr J.Voogd.
In 1994 verliet hij het Lab en behaalde hij het Horeca diploma, daarna heeft hij tot 1998 een eigen bedrijf gehad. Na zijn promotie zal hij werkzaam zijn in de informatica.

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