Excitatory and Inhibitory Vestibular Pathways to the Extraocular Motor Nuclei in Goldfish

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Graf, Werner, Robert Spencer, Harriet Baker, and Robert Baker. Excitatory and inhibitory vestibular pathways to the extraocular motor nuclei in goldfish. J. Neurophysiol. 77: 2765 ± 2779, 1997. Electrophysiological, ultrastructural, and immunohistochemical techniques were utilized to describe the excitatory and inhibitory vestibular innervation of extraocular motor nuclei in the goldfish. In antidromically activated oculomotor motoneurons, electrical stimulation of the intact contralateral vestibular nerve produced short-latency, variable amplitude electrotonic excitatory postsynaptic potentials (EPSPs) at 0.5–0.7 ms followed by chemical EPSPs at 1.0–1.3 ms. Stimulation of the ipsilateral vestibular nerve produced small amplitude membrane hyperpolarizations at a latency of 1.3–1.7 ms in which equilibrium potentials were slightly more negative than resting potentials. The inhibitory postsynaptic potentials (IPSPs) reversed with large amplitudes after the injection of chloride ions suggesting a proximal soma-dendritic location of terminals exhibiting high efficacy inhibitory synaptic conductances. In antidromically identified abducens motoneurons and putative interneurons, electrical stimulation of the contralateral vestibular nerve produced large-amplitude, short-latency electrotonic EPSPs at 0.5 ms followed by chemical depolarizations at 1.2–1.3 ms. Stimulation of the ipsilateral vestibular nerve evoked IPSPs at 1.4 ms that were reversed after injection of current and/or chloride ions. γ-Aminobutyric acid (GABA) antibodies labeled inhibitory neurons in vestibular subdivisions with axons projecting into the ipsilateral medial longitudinal fasciculus (MLF). Putative GABAergic terminals surrounded oculomotor, but not abducens, motoneurons retrogradely labeled with horseradish peroxidase. The spatial distribution of GABAergic neurons and terminals appears highly similar in the vestibuloocular system of goldfish and mammals. Electron microscopy of motoneurons in the oculomotor and abducens nucleus showed axosomatic and axodendritic synaptic endings containing spherical synaptic vesicles establishing chemical, presumed excitatory, synaptic contacts with asymmetric pre- and/or postsynaptic membrane specializations. The majority of contacts with spherical vesicles displayed gap junctions in which the chemical and electrotonic synapses were either en face to dissimilar or adjacent to one another on the same soma/dendritic profiles. Another separate set of axosomatic synaptic endings, presumed to be inhibitory, contained pleomorphic synaptic vesicles with symmetric pre- and/or postsynaptic membrane specializations that never included gap junctions. Excitatory and inhibitory synaptic contacts appeared equal in number but were more sparsely distributed along the soma/dendritic profiles of oculomotor as compared with abducens motoneurons. Collectively these data provide evidence for both disynaptic vestibular inhibition and excitation in all subdivisions of the extraocular motor nuclei suggesting the basic vestibulooculomotor blueprint to be conserved among vertebrates. We propose that unique vestibular neurons, transmitters, pathways, and synaptic arborizations are homologous structural traits that have been essentially preserved throughout vertebrate phylogeny by a shared developmental plan.

INTRODUCTION

Early electrophysiological work in the vestibulooculomotor system of mammals persuasively argued that the most strikingly feature of synaptic organization might be the presence of reciprocal excitatory and inhibitory pathways (Baker et al. 1973; Highstein 1973; Ito et al. 1973a,b; Magherini et al. 1974; Precht and Baker 1972). Primary vestibular afferents in each of the three ipsilateral semicircular canal related circuits were hypothesized to contact inhibitory and excitatory second-order neurons in the ipsilateral vestibular nuclei that would inhibit ipsilateral, and excite contralateral, motoneurons. For instance, ipsilateral posterior canal afferents projecting to the superior and medial vestibular complex would inhibit ipsilateral superior rectus and inferior oblique motoneurons and excite contralateral trochlear and inferior rectus motoneurons (Graf et al. 1983). Subsequent work showed that vestibular signals originating from each of the six canals formed nearly exclusive contralateral/excitatory and ipsilateral/inhibitory three neuron arcs terminating in the appropriate subgroups of extraocular motoneurons (reviewed in Evinger 1988; Highstein and McCrea 1988). To date, the only exception to this scheme has appeared to be the presence of ipsilateral excitation and lack of inhibition to the medial rectus subdivision from the horizontal canal (Baker and Highstein 1978).

Structure/function work using intracellular horseradish peroxidase (HRP) as a marker corroborated the electrophysiology and further suggested that second-order vestibular neuron projections may differ little between closely related vertebrates, because only minor differences in species-specific arborization patterns were found for individual vertical and horizontal vestibular neurons, for example cats and rabbits (Graf and Ezure 1986) compared with squirrel monkey (McCrea et al. 1987). Based on single-cell techniques, reciprocal vestibular innervation also was postulated in the distantly related species of flatfish (Graf and Baker 1983) and recently, this plan has been suggested in other lower vertebrates by the use of pathway tracers (e.g., lamprey, Pombal et al. 1994; elasmobranchs, Puzdrowski and Leonard 1994). In a particularly relevant study, a direct parallel was shown between the amphibian (Straka and Dieringer 1993) and...
mammalian (Spencer and Baker 1990) horizontal canal system in respect to the putative ipsilateral, inhibitory glycinergic and contralateral, excitatory glutamatergic input to both abducens internuclear and motoneurons. Nevertheless, a broad analysis including the anatomy, physiology, and transmitter histochemistry of canal-specific second-order projections to each subdivision of extraocular motoneurons has not been carried out for any taxa evolutionarily distinct from mammals.

The largest body of nonmammalian oculomotor related literature has been accumulated in teleostean fish, primarily cypriniformes (includes goldfish, catfish, and zebrafish), possibly because this order exhibits robust oculomotor performance with eye movements comparable with those observed in mammals (Easter 1972; Pastor et al. 1992; Schaier and Bennett 1986). In the goldfish, neurons within the vestibular complex, notably the anterior, descending, and tangential octaval nuclei (McCormick and Braford 1994), have been found to be distributed consistent with putative separate inhibitory and excitatory projections to oculomotoneurons (Allum et al. 1981; Torres et al. 1992). Although initial intracellular records in goldfish oculomotoneurons did not report inhibition (Korn and Bennett 1971), structural and neurophysiological observations in the goldfish horizontal canal system indicated reciprocal inhibition and excitation (Baker et al. 1986, 1987a, 1994). Eye velocity and position signals have been recorded from hindbrain neurons presumed homologous to those in the mammalian vestibular and prepositus nuclei, suggesting similar central processing of vestibular and visual signals (Pastor et al. 1994). Collectively, these data provide sufficient rationale to suggest that it would be useful to directly compare the electrophysiology and anatomy between teleosts and mammals.

The presence of electrotonic coupling reported in teleost motor pathways, including extraocular motoneurons, provides a noteworthy difference between mammals and fish (Korn and Bennett 1972). Gap junctions were postulated to be largely localized on soma and proximal dendrites to facilitate initiation of dendritic spikes thereby synchronizing motoneuron discharge [e.g., during fast phases of nystagmus (Korn and Bennett 1975)]. Because the caudal subgroup of abducens motoneurons was observed to feature more gap junctions than those in the rostral subdivision (Sterling 1977), it was predicted to contain more phasic motoneuronal responses (Gestrin and Sterling 1977). However, the physiological profiles of motoneurons identified in the two subdivisions were demonstrated to be the same (Pastor et al. 1991) as were the observed distribution of gap junctions (present study). Data herein also will show that electrotonic coupling is present in all of the semicircular canal pathways that produce compensatory, i.e., not rapid, eye movements. Hence one goal was to achieve a consensus with prior work by employing a broad approach to analyze all of the vestibuloocular circuitry.

In this study we evaluate the evidence for reciprocal excitatory and inhibitory synaptic connections to extraocular motoneurons on the basis of electrophysiological, structural, and immunohistochemical methods. In comparison with mammals, postsynaptic inhibition appeared to be of smaller amplitude and efficacy because of less negative equilibrium potentials rather than changes in inhibitory synaptic conductance. Electrotoneic coupling was highly variable between individual oculomotor subgroups, but always more robust in abducens motoneurons and internuclear neurons. Overall, our data strongly suggest that the pattern of vestibuloocular innervation from the three semicircular canal is surprisingly well conserved between distantly related taxa. Preliminary results have been published previously in abstract form (Baker et al. 1986, 1987b).

METHODS

Surgical procedures

All preparative surgery was done under MS222 (1:2,000 wt/vol) anesthesia and immobilization with gallamine triethiodide (Flaxedil; 2 mg/kg). Twenty-four goldfish were mounted in a customized fish holder system, and aerated water was continuously circulated to the gills by an electric pump through a tube fitted to the mouth. Incisions were infiltrated with local anesthetics (2% lidocaine with epinephrine). Access to the labyrinths was obtained by bilateral removal of the bone overlying the hindbrain and cerebellum. Individual branches of the anterior, horizontal, and posterior semicircular nerves were exposed from the side of the brain stem. The bone overlying the midbrain also was removed so the oculomotor and trochlear nuclei could be reached either through the intertemporal cleft or by direct visualization after removal of the tectal commissure and valvula. For gross antidromic identification of all extraocular motoneurons, bipolar silver ball stimulation electrodes were implanted in both orbits. Orthodromic vestibular activation was attained by bipolar silver wire electrodes placed at the most peripheral location surrounding the three ampullary nerves. In most experiments, one or more of the individual extraocular muscles and labyrintheine nerve branches was dissected free from overlying tissue, and a set of smaller bipolar electrodes was utilized to obtain more selective stimulation.

Electrophysiological recordings and data analysis

Glass microelectrodes (0.5–1-μm tip size), containing either 3 M K Acetate, 2 M LiCl, or 3 M KCl were used for intracellular recordings from extraocular motoneurons. Oculo motor, trochlear, and abducens motoneurons were identified by antidromic action potentials following electrical stimulation in the orbit. Synaptic potentials, either excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs, respectively), were recorded after vestibular nerve stimulation. Latency for antidromic invasion ranged from 0.3 to 0.5 ms and for orthodromic disynaptic activation varied between 1.0 and 1.7 ms. During recording, the animals were kept under light pentobarbital sodium (15–20 mg/kg) anesthesia.

The electrophysiological data were recorded either on magnetic tape (Neurorecorder: Neurodata), or magnetic disks (Nicolet 4066, Data 6000) and later read out on a laser printer for analysis and documentation. Some electrophysiological records were photographed directly from a Tektronix oscilloscope (Figs. 1–3) and others assembled by computer-assisted graphics (Fig. 4).

γ-Aminobutyric acid (GABA) immunohistochemistry

The extraocular muscles of four goldfish were injected with 2 μl of 10% HRP. After a survival time of 1 day, animals were reanesthetized and perfused with fixative solution containing 4.0% paraformaldehyde in 0.1 M phosphate buffer with 0.002% calcium chloride (pH 7.4). Vibratome sections (25–50 μm) were prepared through the abducens, trochlear, and oculomotor nuclei. The sections were first reacted for HRP (Pastor et al. 1991; Spencer and Baker 1990) to identify extraocular motoneurons, then washed through several changes of 0.1 M sodium phosphate buffer for up
FIG. 1. Postsynaptic potentials in right-side oculomotor neurons (rOc) following vestibular nerve stimulation. A and B: inferior rectus motoneuron recorded with a KCl electrode. A: antidromic identification. B: excitatory postsynaptic potential (EPSP) elicited from the contralateral (left, lVes), and inhibitory postsynaptic potential (IPSP) from the ipsilateral (right, rVes), vestibular nerves. Unitary potentials indicated by arrows. Electrotonic coupling occurred at 0.9 ms (short arrow) and chemical conductances at ~1.3 ms (long arrow). C–E: inferior oblique motoneuron recorded with a K Citrate electrode. IPSPs were evoked by ipsilateral (rVes) stimulation during hyperpolarizing (C and D) and depolarizing (E) current injection. IPSP equilibrium potential was found at ~4.0 nA (D). Small arrows in D indicate the axonal (M) spike and initial segment/soma dendritic (IS-SD) invasion inflections during antidromic invasion that was blocked in C during hyperpolarizing current injection. The long vertical line indicates IPSP onset. F–J: superior rectus motoneuron recorded with a K Acetate electrode. F: IPSP elicited by ipsilateral (rVes) stimulation at twice threshold intensity (2×T) followed by the antidromic response indicating intrasomatic recording. G: EPSP following contralateral (lVes) stimulation at 4 times threshold (electrotonic component is indicated by the small arrow). H and I: responses to stimulation of the ipsilateral vestibular nerve (rVes) at 4 times threshold and 10 times threshold, respectively, indicating no change in IPSP amplitude. J: determination of IPSP latency (small vertical double arrow) by comparison of intracellular (In) and extracellular (Ex) records. Amplitude calibrations for A–E are shown in A and for F–J in F. Time scales are indicated in the 1st record for each set of traces.

to 4 h. Subsequently, the sections were processed for the immunohistochemical localization of GABA using an antibody generated in rabbit against GABA conjugated to bovine serum albumin (Immuno Nuclear). Sections were immersed for 1 h in 10% normal goat serum (NGS) in phosphate-buffered saline (PBS; 0.01 M) containing 0.1% Triton X-100, and then incubated in primary antibody (1:2,000 to 5,000) containing 1% NGS and 0.1% Triton X-100 in PBS for 18–24 h at 4°C with constant agitation. As a control, some sections were incubated in the same solution without either the addition of primary antibody or with antibody preabsorbed with GABA. After the incubation, sections were washed for 30 min in three changes of PBS, and immersed in biotinylated anti-rabbit IgG (1:200; Vector) in PBS with 1% NGS for 90 min. After the secondary antibody incubation, the tissue was washed in PBS and immersed in a avidin:biotin-HRP complex (1:100; Vector) for 90 min. Sections then were washed through two changes of PBS over 20 min followed by two changes of 0.1 M phosphate buffer (pH 7.4) for 20 min. This chromogen produced a brown diffuse reaction product. The material was rinsed through several changes of 0.1 M phosphate buffer, and then placed on pretreated microscope slides, dehydrated, and mounted with Permount for light microscopic examination using brightfield and Nomarski differential interference contrast optics.

Electron microscopy

The extraocular muscles of 10 goldfish were injected with 2 μl of 10% HRP. After a survival time of 1–3 days, the animals were deeply reanesthetized and perfused with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer with 0.002 calcium chloride (pH 7.4). After fixation, the brains were removed and immersed in 0.1 M phosphate buffer with 8% dextrose overnight. Serial 50- to 75-μm coronal sections through the oculomotor nuclei were cut on a vibratome, and reacted with tetramethylbenzidine or diaminobenzidine to visualize the extraocular motoneuron pools (Graf and McGurk 1985). Sections containing the oculomotor nuclei were trimmed, postfixied for 2 h in 1% osmium tetroxide.
in 0.1 M phosphate buffer with 7% dextrose and 1.5% potassium-ferricyanide, stained en bloc for 2 h with 0.5% uranyl acetate in maleate buffer, dehydrated in methanol and propylene oxide, and embedded in TAAB 812 resin between vinyl plastic microscope slides and coverslips.

Selected loci containing oculomotor neurons were analyzed by serial ultrathin sectioning and electron microscopy. Serial 5-µm sections through the plastic-embedded vibratome sections were cut, examined by light microscopy, and remounted on blank BEEM capsules from which ultrathin sections were cut and collected on single-slot Formvar-coated grids. Sections were then stained with uranyl acetate and lead citrate, and examined and photographed with a Zeiss EM-10CA electron microscope at $1,500$, $8,000$, and at sites of synaptic contact zones, at $25,000$ (Spencer and Baker 1990).

RESULTS

Localization of oculomotor nuclei

At the outset of each experiment, the trochlear nucleus was located first by the characteristic antidromic field potential profile initiated from the contralateral orbit (Baker et al. 1973). Subsequently, superior rectus motoneurons were identified in the caudal oculomotor complex after stimulation of the contralateral orbit. Inferior rectus (rostrodorsal) and inferior oblique (caudoventral) motoneurons were recognized by antidromic activation from the ipsilateral orbit along with the rostral and caudal subgroups of abducens motoneurons in the posterior brain stem. Stimulation of the ipsi- and contralateral vestibular nerves also distinguished the MLF borders.

Antidromic oculomotor field potentials following electrical stimulation of the orbit were found to extend from 0.75–1.0 mm lateral from the visually observed midline of the oculomotor complex. Most of the respective motoneuron somata are located directly adjacent to the midline (Fig. 5, D and F) except for the dorsal and lateral extensions to either side (Graf and McGurk 1985; Pastor et al. 1991). Intracellular records from somata are illustrated in Figs. 1 and 2, even though the most frequent intracellular recording site was from dendrites (as in Fig. 3). Motoneuron somata were identified by antidromic activation characterized by initial segment / soma dendritic invasion (IS-SD) followed by a large after depolarization typical of an intrasomatic penetration (Fig. 1, A, D, and F).

General organization of inhibitory and excitatory vestibular pathways

Postsynaptic potentials following vestibular nerve stimulation were recorded in inferior rectus, inferior oblique, and superior rectus motoneurons in Fig. 1, A and B, C–E, and F–J, respectively. EPSP and IPSP characteristics varied between motoneuronal populations; however, the ipsi- and contralateral spatial organization of inhibition and excitation was invariant between experiments. At normal resting membrane potential, stimulation of the contralateral vestibular nerve produced a short-latency depolarization consisting of a presumed electrotonic and a subsequent chemical component initiated at $0.9$ and $1.3$ ms, respectively (Fig. 1B, IVes). Stimulation of the ipsilateral vestibular nerve produced a small-amplitude 1-mV hyperpolarization, typically at a latency of 1.3 ms (Fig. 1B, rVes). In general, after 5–10 min of intrasomatic recording with KCl electrodes, small-amplitude, all-or-none depolarizations and hyperpolarization (arrows in Fig. 1B) were recorded (see also Fig. 2, E and...
The leakage of chloride ions from the microelectrode appeared to affect the unitary IPSPs in a nonuniform fashion (Korn et al. 1992).

Because the major goal of these electrophysiological experiments was to establish the presence of inhibitory synaptic potentials in extraocular motoneurons, intrasomatic records were obtained with electrodes filled with either K Citrate (Fig. 1, C–E) or K Acetate (Fig. 1, F–J). Large hyperpolarizing IPSPs were recorded in the presence of extrinsic membrane depolarization (+4.0 nA, Fig. 1E). The application of hyperpolarizing current (−4.0 nA, Fig. 1D) demonstrated the inhibitory synaptic effect to be associated with a membrane conductance exhibiting an equilibrium potential slightly more negative than the resting potential. In fact, the IPSP equilibrium potential was near the point where the antidromic invasion of the IS-SD spike was blocked (arrows in Fig. 1D). Further injection of hyperpolarizing current (−6.0 nA, Fig. 1C) reversed the inhibitory synaptic conductance to a depolarization, and the antidromic invasion of the IS-SD component was completely blocked leaving only the M spike. Spontaneous and/or evoked unitary synaptic potentials like those illustrated in Figs. 1B and 2, E and F, were absent from recordings obtained with acetate or citrate filled electrodes.

**Vestibular input to identified superior rectus and trochlear motoneurons**

The presence of contralateral excitation and ipsilateral inhibition was also documented in a superior rectus motoneuron illustrated in Fig. 1, F–J. This series of intracellular records was obtained with a K Acetate electrode that did not appear to affect the equilibrium potential for the IPSP (Fig. 1, H and I). Stimulation of the ipsilateral vestibular nerve (rVes) at two times threshold (2 × T) produced a hyperpolarizing IPSP preceding, but not blocking, antidromic activation of the superior rectus motoneuron (Fig. 1F). Stimulation of the contralateral vestibular nerve (lVes) at 4 times threshold evoked a short-latency electrotonic depolarization (arrow in Fig. 1G) followed by a more slowly
rising EPSP that initiated action potentials. Stimulation of the ipsilateral vestibular nerve at 4 times threshold and 10 times threshold (H and I) did not result in larger amplitude potentials indicating that the IPSP was already near maximum at 2 times threshold. The 1.3-ms latency for this IPSP was determined by comparing the adjacent intra- (In) and extracellular (Ex) responses (Fig. 1J).

Trochlear motoneurons were the easiest to identify because peripheral current spread to the IIIrd nerve was minimal (Fig. 2, A–F). These motoneurons always exhibited large-amplitude inhibitory (reversed IPSPs) as well as excitatory potentials following ipsilateral and contralateral vestibular stimulation, respectively (Baker et al. 1973; Llinas and Baker 1972; Precht and Baker 1972). A rather distinct electrotonic component was always associated with excitatory potentials (Fig. 2, A and B, see also Fig. 1B). Trochlear motoneurons were frequently recorded after chloride loading as had been previously done in mammalian trochlear neurons (Llinas and Baker 1972). Seemingly much smaller amounts of chloride (<1 min injection) reversed the equilibrium potential for the IPSP to values close to that presumed for the EPSP; however, little change was noted in the electrotonic and/or chemically evoked EPSP (compare Fig. 2, A and B).

After intrasomatic chloride loading of trochlear motoneurons, electrical stimulation of the ipsilateral vestibular nerve always produced large-amplitude IPSPs that even could sustain action potentials in the soma (Fig. 2, D and E; also see Fig. 3, E–H). These action potentials were superimposed on large-amplitude (30–40 mV) depolarizations including evoked unitary IPSPs ranging from 5 to 15 mV in amplitude (Fig. 2E). As had been previously demonstrated in mammalian trochlear motoneurons (Llinas and Baker 1972), inhibitory synaptic transmitter (GABA) appeared to be continuously released from presynaptic terminals (Fig. 2F).

Intradendritic recording from oculomotor and trochlear motoneurons

The majority of intracellular records from extraocular motoneurons were obtained from presumed dendritic, and not somatic, penetrations (Fig. 3). This assessment was based on electrophysiological criteria as well as comparison between medial and lateral MLF penetration sites. Dendritic, as opposed to somatic, records occurred in a ratio of at least 5:1. The EPSP and IPSP profile, including the response to current clamp conditions, differed from somatic sites (Fig. 3, G and H), except for the rapid and sustained response to chloride injection (H).

A large-amplitude, stable resting membrane potential of −65 to −72 mV was encountered immediately after successful penetration of a dendrite. Frequently, orbital stimulation at a low-intensity straddling antidromic threshold produced small-amplitude all-or-none action potentials exhibiting a fast rise time and two separate exponential decays (Fig. 3, A and B). Stimulation at a slightly higher intensity (2 times threshold) produced a large-amplitude all-or-none action potential with a uniform, fast rising phase exhibiting either two (Fig. 3B) or three (Fig. 3C) component, exponential-like falling phases. Generally, slight membrane hyperpolarization (Fig. 3D, −5.0 nA) could block the large-amplitude invasion leaving only a small-amplitude, all-or-none (5–20 mV) depolarization. We interpret this type of recording as a sequential invasion of the antidromic action potential of the initial segment (Fig. 3D) and the soma-dendritic compartment (Fig. 3, B and C). This action-potential profile was largely only seen during antidromic activation when the action potential progresses from the axon toward the dendrites. By contrast, contralateral vestibular nerve stimulation (rVES) produced electrotonic (arrows in Fig. 3, E and F) and chemical EPSPs eliciting action potentials with fast rising phases and a single exponential falling phase. These observations suggest that in this case the regenerative current was being initiated distal in the dendritic tree and propagated toward the soma.

In representative intradendritic records, irrespective of microelectrode type, electrical stimulation of the ipsilateral (inhibitory) vestibular nerve (rVES) produced a short-latency (1.3 ms), small-amplitude membrane depolarization (Fig. 3, G and H). After recording with a chloride microelectrode, for only 1 min, the reversed IPSPs also initiated action potentials (Fig. 3G). An active chloride injection (3 min −5.0 nA) altered the IPSP equilibrium potential such that rVES stimulation at 4 times threshold produced a high-frequency discharge of the proximal soma-dendritic compartment in the presence of a −2.0-nA holding current (Fig. 3H). During chloride loading, rVES stimulation at 4 times threshold elicited EPSPs with either single or multiple dendritic action potentials (Fig. 3F).

In summary, disynaptic inhibition and excitation including electrotonic coupling could be documented in all vertical recti (exclusive of the medial rectus (cf., however, Suwa and Baker 1996)) and oblique motoneurons. Although the equilibrium potential for the IPSP, around −65 mV, was never as negative as that observed in mammalian oculomotor motoneurons, −80 mV (Llinas and Baker 1972), the effect of chloride loading in either the soma or dendrite was comparable. In all oculomotoneurons, IPSPs were quickly reversed and the membrane depolarization could initiate action potentials spreading through the soma-dendritic compartment.

Intracellular records from abducens motoneurons and internuclear neurons

Abducens motoneurons were antidromically identified in the rostral and caudal subgroups of the abducens nucleus in the posterior brain stem (Pastor et al. 1991). Intracellular records were also obtained from nonantidromically activated putative abducens internuclear neurons located adjacent to, and somewhat dorsolateral to the caudal subgroup of abducens motoneurons. In both types of neurons, electrical stimulation of the contralateral vestibular nerve (rVES) evoked robust, short-latency electrotonic EPSPs with a latency of 0.5 ms followed by chemical depolarizations at 1.1–1.3 ms (Fig. 4A). Stimulation of the ipsilateral vestibular nerve revealed IPSPs with a latency of 1.4 ms that initially were always hyperpolarizing in direction. Soon after penetration with chloride containing electrodes, IPSPs were quickly reversed. As illustrated in Fig. 4B, injection of hyperpolarizing current from either K Acetate or K Citrate electrodes shifted the membrane resting potential toward the IPSP equilibrium potential, and the hyperpolarization could be entirely reversed from an intrasomatic electrode location. Like in the
mammalian abducens nucleus (Spencer et al. 1989), the efficacy of inhibition could be demonstrated by the blockade of the antidromic field potential following electrical stimulation of the ipsilateral horizontal canal nerve (not illustrated).

After contralateral vestibular nerve stimulation (IVes) EPSPs with electrotonic and chemical components were also recorded from abducens internuclear neurons in both the rostral and caudal subgroups (Baker et al. 1994). Large amplitude hyperpolarizing IPSPs were recorded after ipsilateral vestibular nerve (rVes) activation (Fig. 4C). In summary, these electrophysiological data document a pattern of reciprocal canal-related disynaptic excitation and inhibition in all extraocular subdivisions including the abducens internuclear neurons. As noted in the Discussion, the synaptic profiles in each subgroup were also found to be remarkably similar to their mammalian homologues.

**GABA immunohistochemistry of vestibulooculomotor pathways**

In five goldfish, GABA antibodies were used in conjunction with retrograde labeling of oculomotor and abducens motoneurons to localize putative inhibitory neurons in the vestibular nuclei, identify their axonal trajectories in the MLF, and establish which subdivisions of the extraocular motor nuclei contained terminal arborizations. Figure 5A shows a low magnification coronal section containing the entire hindbrain and cerebellum. GABA immunostaining is notably robust in the Purkinje cell and external granular cell layer of the cerebellum (CC and EG) along with the octavolateral column including the vestibular and mechanosensory medial nuclei (VN and M) (McCormick and Bradford 1994). The brain stem consisted of largely unlabeled fiber tracts and few GABAergic cells in the medulla, but terminal arborizations were present in most mid- and hindbrain nuclei.

A higher magnification photomicrograph through the caudal end of the anterior octaval nucleus, located just dorsal to the descending trigeminal (V) and secondary gustatory (trg) tract, always showed vestibular neurons with intense reaction product (inset in Fig. 5B). In general, GABAergic neurons appeared to be more dense in the anterior octaval complex and relatively sparser in the caudal descending octaval subdivisions. Axons of inhibitory vestibular neurons could be followed through the vestibular commissure into the MLF (Fig. 5C). The rostral MLF axonal trajectory tended to show a preferential location to the ventral MLF, especially closer to the oculomotor complex (Fig. 5C). Distinctly fewer GABAergic axons were found in the descending MLF (not illustrated).

In two experiments, oculomotor (Fig. 5, D, F, and H) and abducens (Fig. 5, E and J) motoneurons were retrogradely labeled with HRP and followed by GABA localization. Dendrites of oculomotor neurons were found laterally in the MLF (Fig. 5D) and the axons leave via the ipsilateral oculomotor nerve. The coronal view at the mid level of the oculomotor complex in Fig. 5D is shown at higher power in Fig. 5F to indicate reaction product in the axons of the MLF and terminals in the area containing presumed inferior rectus, inferior oblique, and superior rectus motoneurons. GABAergic termination on a HRP retrogradely labeled motoneuron (Fig.
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Electron microscopy of oculomotor nuclei

To provide evidence for the presence of electrotonic excitation and chemical inhibition, the pattern and mode of terminal arborization was studied in both the oculomotor and abducens motor nuclei of six goldfish retrogradely labeled with HRP (Figs. 6A and 8A, insets, respectively). Oculomotor neurons were identified first in thick plastic sections (e.g., Fig. 6A, bottom inset) that were subsequently thin sectioned for electron microscopy (Spencer and Baker 1983). Cytoplasmic organelles typical of other vertebrates, e.g., Golgi apparatus and mitochondria, were observed (Fig. 6A). Cisternal arrays of granular endoplasmic reticulum (ger) were well developed, and in most instances the nucleus also exhibited the type of invaginations seen in mammalian oculomotor neurons (Spencer and Baker 1983). A moderately high density of axosomatic synaptic endings was found in all oculomotor subdivisions and trochlear motoneurons (e.g., arrows in top of Fig. 6A). The density of axosomatic contacts varied among motoneurons, but the number of axodendritic synaptic endings always appeared lower, despite the relatively large size and length of the dendrites (arrows in Fig. 6B). In general, the large-sized dendrites exhibited few axodendritic synapses (Fig. 6C). Most synaptic contacts were located on medium and small caliber dendrites in the dendritic neuropile lateral to the oculomotor nucleus. All such synapses contained spheroidal synaptic vesicles and possessed chemical contact zones characterized by asymmetric pre-/postsynaptic membrane specializations (Figs. 6C and 7, A and B). Gap junctions were rarely associated with axodendritic synaptic endings, particularly those on distal dendrites.

Two kinds of presumed excitatory synapses and one type of a presumed inhibitory synapse could be discerned on oculomotor motoneurons. Putative excitatory synaptic endings, in general, showed axosomatic and axodendritic synaptic endings (as in Fig. 6, B and C) and were either of pure chemical nature (Fig. 7, A and B) or exhibited chemical contacts and gap junctions consistent with electrotonic coupling (Fig. 7, E and F). Chemical synapses with spheroidal vesicles were characterized by asymmetric pre/postsynaptic membrane specializations (Fig. 7A, inset). Their ultrastructure was also characterized by the presence of pale matrix mitochondria (Fig. 7, A and B). These types of axosomatic endings that contained large numbers of synaptic vesicles were never observed to establish gap junction contacts.

Mixed chemical and electrotonic axosomatic synaptic endings contained few spheroidal vesicles. The chemical synaptic contacts were also characterized by asymmetric pre- and/or postsynaptic membrane specializations with an ~20-nm intersynaptic space (Fig. 7E, top left inset). Gap junction contacts displayed an ~2-nm separation between the pre- and postsynaptic membranes (Fig. 7E, bottom right inset). Both types of contacts were rarely observed in relationship to the same postsynaptic profile (arrows in Fig. 7F). In this case, as in other synaptic profiles, the two contacts were found to be spatially separated. The presumed inhibitory axosomatic synaptic endings (Fig. 7, C and D) were characterized by symmetric pre-/postsynaptic membrane specializations (Fig. 7D, inset). They contained pleomorphic vesicles and established only chemical synaptic contacts on motoneurons, i.e., these endings never exhibited gap junctions.

Abducens nucleus motoneurons (Fig. 8A) also showed typical cytoplasmic organelles similar to those observed in cats (Spencer and Baker 1983) including cisternal arrays of granular endoplasmic reticulum. In most instances, the nucleus also exhibited invaginations. As a rule, abducens motoneurons displayed a more densely packed arrangement of axosomatic synaptic endings than found in oculomotor motoneurons (compare Figs. 6B and 8B). In addition, synaptic endings were differentially distributed along soma, proximal dendrites, and distal dendrites in abducens motoneurons in respect to the content of spheroidal and pleomorphic vesicles (Fig. 8C). In general, a mixture of synaptic contacts containing pleomorphic and spheroidal vesicles was found at the soma and at the proximal dendrites (Fig. 8B), whereas predominantly spheroidal vesicle containing synapses were present at distal dendrites (Fig. 8C). Comparatively more synaptic contacts contained pleomorphic vesicles except those located on the dendritic areas.

Abducens motoneurons also possessed synaptic contacts of presumed inhibitory and excitatory nature containing pleomorphic vesicles and spheroidal vesicles, respectively.

FIG. 5. Immunohistochemical localization of γ-aminobutyric acid (GABA). A: coronal section through the brain stem and cerebellum at the level of anterior octaval (AO) vestibular subgroup. B: high magnification of the AO and M subdivisions of the octavolateral complex (at arrow in A) with the inset showing cells containing immunoreaction product. C: coronal section showing presumed GABAergic axons in the medial longitudinal fasciculus (MLF) with the higher magnification inset indicated by the arrow. D–I: double-labeled sections showing horseradish peroxidase (HRP) retrograde identification of extraocular motoneurons with GABA staining. D: coronal section through the midbrain of the goldfish at the mid level of the oculomotor nucleus. E: coronal section through the rostral subgroup of abducens motoneurons. F: high magnification view of the bilateral oculomotor complex showing GABAergic reaction product in MLF axons and terminals surrounding HRP and unlabeled motoneuronal somata. G and H: GABA terminal patterns on 2 motoneurons indicated by arrows in F. I: high-magnification view of abducens motoneuronal somata as indicated by the arrow in E. Abd, abducens nucleus; AO, anterior octaval nucleus; CC, corpus cerebellum; EG, eminentia granularis; IV, 4th ventricle; LL, lateral lemniscus; MIV, trigeminal nucleus; M, medial nucleus; MLF, medial longitudinal fasciculus; nIII, oculomotor nerve; Oc, oculomotor nucleus; RF, reticular formation; trg, secondary gustatory tract; VN, vestibular nuclei; VIII, vestibular nerve; VII, facial nerve (abbreviations, after McCormick and Braford 1994).
FIG. 6. Light and electron microscopic visualization of oculomotor motoneurons illustrating the distribution of synaptic endings. A, top inset: darkfield photomicrograph of a 50-µm vibratome section through the mid level of the oculomotor nucleus containing motoneurons labeled by retrograde transport of HRP [tetramethylbenzidine (TMB) reaction]. Bottom inset: semithin (1 µm) section through 3 motoneurons [diaminobenzidine (DAB) reaction, toluidine blue counterstain]. The oculomotor neuron in the electron micrograph is indicated by the asterisk and the high density of axosomatic synaptic endings by arrows. ger, cisternal arrays of granular endoplasmic reticulum; G, Golgi apparatus; m, mitochondria; Nuc, nucleus. B: low-magnification electron micrograph of a primary dendrite (d) emanating from the soma (S) of a labeled oculomotor neuron with few synaptic contacts (arrows). C: low-magnification electron micrograph of the dendritic neuropile (d) lateral to the oculomotor nucleus with numerous axon contacts in the central core (arrows). Calibrations in A are 100 µm (top inset), 20 µm (bottom inset), and 5 µm (electron micrograph); B, 5 µm; and C, 1 µm.

(Fig. 9A and C). Excitatory synapses existed in the chemical only (Fig. 9, A–C), as well as the mixed chemical/electrotonic configuration (Fig. 9D). All chemical synaptic contact zones in the abducens nucleus were characterized by asymmetric pre-/postsynaptic membrane specializations (Fig. 9C). Gap junction contacts were separated by a 2-nm cleft between pre- and postsynaptic membranes (compare with Fig. 7E). In conclusion, the ultrastructure of terminal arborizations differed little between oculomotor and abducens motoneurons.

Discussion

Because all vertebrate species studied to date represent a widely divergent taxonomy exhibiting a wide repertoire of eye movements, we believe these new data support the hypothesis that the ancestral, hence primitive, vestibuloocular motor plan was based on the utilization of semicircular canal-specific disynaptic excitation and inhibition. The corroborative chemical, structural, and physiological evidence argues that the neurons, transmitters, pathways, and synaptic arborizations on extraocular motoneurons have been highly conserved and as a result are likely homologous traits that are embryologically linked throughout vertebrate phylogeny.

Electrophysiology

In antidromically identified oculomotor motoneurons innervating inferior/superior oblique and inferior/superior recti muscles, electrical stimulation of the contralateral vestibular nerve produced short-latency disynaptic electrotonic EPSPs (0.5–0.7 ms) followed by disynaptic chemical depolarizations with latencies of 1.0–1.3 ms (Figs. 1 and 2). Stimulation of the ipsilateral vestibular nerve produced small-amplitude hyperpolarizations beginning at a latency of 1.3–1.7 ms that gradually increased in amplitude to peak at 4.0–7.0 ms. Manipulation of membrane potential by current injection through the microelectrode demonstrated the short-latency ipsilateral hyperpolarization to be associated with a membrane conductance exhibiting an equilibrium po-
tential slightly more negative than resting potential. Reversal of this IPSP by injected current, as opposed to Cl injection, was highly dependent on intrasomatic (Fig. 1) as opposed to intradendritic (Fig. 3) recording sites.

Injection of chloride ions significantly displaced the equilibrium potential in the depolarizing direction to the extent that IPSP reversal was closer to the equilibrium potential for the contralateral EPSP. Following chloride loading, efficacy of the inhibitory synaptic conductance was demonstrated by the large difference in amplitude of the two evoked depolarizations. The ability to change the equilibrium potential with chloride ions is similar to that utilized to study recurrent and vestibular commissural inhibition in the goldfish Mauthner cell (Faber and Korn 1975). This model system has been immensely informative concerning inhibitory postsynaptic conductances in vertebrates, especially that related to glycinergic transmission (e.g., the abducens nucleus; Fig. 4) (Faber and Korn 1987, 1988). Of particular interest is the extrapolation of current- and voltage-clamp data showing that the chloride channels exhibit a steep voltage dependency such that the lower equilibrium potential in goldfish extraocular motoneurons as compared with mammals (e.g., \(-65 \text{ vs. } -90 \text{ mV}\) ) would not be expected to alter canal-related inhibition. Because both the magnitude and duration of inhibitory responses would in fact be noticeably increased in the presence of larger excitation, we conclude that the reciprocal semicircular canal inhibitory and excitatory pathways play an equally important role in coordinated eye movement.

In the horizontal canal system, intracellular records from antidromically identified abducens motoneurons revealed robust short-latency, disynaptic electrotonic EPSPs at 0.5-ms latency followed by disynaptic chemical depolarizations at 1.3 ms after electrical stimulation of the contralateral vestibular input to extraocular motor nuclei.
ular nerve. As expected, stimulation of the ipsilateral vestibular nerve revealed disynaptic IPSPs at 1.4 ms that were also reversed after injection of current and/or chloride ions. Because disynaptic inhibition and excitation was demonstrated for adjacent rostral and caudal subgroups of abducens internuclear neurons, the reciprocal horizontal canal organization appears to be similar in goldfish and mammals. The larger IPSP amplitude and more negative equilibrium potential for goldfish abducens versus oculomotor neurons suggests that either the density of inhibitory boutons and/or the amplitude of the channel conductances are larger for glycinergic versus GABAergic terminal arborizations, respectively. This difference in inhibitory efficacy between comparable motoneuron groups was not detected in mam-
mals (Spencer et al. 1992). By contrast, in all species, GABAergic inhibition has been found to be the most robust in trochlear motoneurons.

**Immunohistochemistry**

Double label experiments using GABA antibodies and HRP injections localized inhibitory neurons in the anterior octaval subdivision of the vestibular nucleus (Fig. 5) (McCormick and Brafard 1994). Axonal trajectories were identified toward, and into, the ipsilateral MLF along with observing terminal arborizations distributed largely on the somata and proximal dendritic trees of trochlear and oculomotor, but not abducens, motoneurons. Thus the presence, spatial distribution, and magnitude of GABAergic inhibition in oculomotor neurons produced by presumed GABAergic neurons of the vertical vestibular system also appears to be analogous between fish and mammals (Spencer et al. 1992).

Immunohistochemical observations in the abducens nucleus, especially the absence of GABA axons and terminals, was consistent with findings in mammals demonstrating glycine to be the inhibitory neurotransmitter of second-order vestibular neurons related to the horizontal vestibuloocular reflex (Spencer et al. 1989, 1992).

Recently both GABA and glutamic acid decarboxylase were localized in the extraocular motor nuclei of monkey and cat (Spencer et al. 1992). The densest GABA-related termination patterns were found in the trochlear nucleus, and the lightest, in the abducens nucleus. Comparison of single versus multiple postsynaptic profiles on respective somadendritic profiles suggested two separate inhibitory GABAergic synaptic inputs onto oculomotor and trochlear motoneurons. For example, in goldfish and mammals, vertical saccade-related premotor neurons in the rostral interstitial nucleus of the MLF and anterior octaval vestibular neurons are the most likely candidates. Hence it is well established
that GABA is the major inhibitory neurotransmitter utilized by premotor neurons involved in vertical eye movements. In contrast, glycine is the inhibitory neurotransmitter of most premotor neurons that are related to horizontal eye movements. On the basis of rationale related to the embryological origin of extraocular motoneurons and muscles, the differences in neurotransmitters utilized in the vertical and horizontal eye movement systems have been suggested to reflect the evolutionary history correlated to each extraocular subdivision (Baker 1991).

**Ultrastructure**

The electron microscopic visualization of goldfish oculomotor nucleus neurons revealed axosomatic and axodendritic synaptic endings containing spheroidal synaptic vesicles that established chemical, presumed excitatory, synaptic contacts characterized by asymmetric pre- and/or postsynaptic membrane specializations (Figs. 6 and 7). In addition, many axosomatic synapses also showed gap junction contacts consistent with electrotonic coupling. Other axosomatic synaptic endings contained pleiomorphic synaptic vesicles establishing chemical synaptic contacts on motoneurons. These presumed inhibitory endings were never observed to establish gap junctions.

Electron microscopy of HRP-labeled abducens motoneurons in the goldfish showed numerous axo-somatic/dendritic synaptic endings containing spheroidal synaptic vesicles establishing both chemical (asymmetric pre- and/or postsynaptic specialization) and extensive electrotonic (opposed membranes exhibiting gap junctions) synaptic contacts (Figs. 8 and 9). Other axosomatic synaptic endings containing pleiomorphic vesicles established only chemical synaptic specializations (Spencer and Baker 1983; Spencer et al. 1989).

Comparison of the flatfish oculomotor and trochlear nuclear complex with that in the goldfish showed a difference in the relative placement of chemical versus electrotonic contacts of excitatory second-order vestibular neurons (Spencer et al. 1986). In the goldfish, chemical contacts were located at some distance from gap junctions, whereas in the flatfish, gap and chemical junctions were adjacent to each other (compare Fig. 7, E and D, unpublished observations). By contrast to the goldfish abducens nuclei, the spatial relationship of chemical and electrotonic contacts was closer and like the arrangement in flatfish. The functional significance of this difference in chemical versus electrical junctional placement is as yet undetermined. Furthermore, the larger question concerns the significance of electrotonic coupling per se in the vestibuloculomotor system of fish. A prior interpretation argued that electrotonic transmission played an important role in synchronizing motoneuronal discharge during either fast phases or saccades (Korn and Bennett 1971) and that the site of coupling was more restricted to the soma rather than the dendrites (Korn and Bennett 1972). However, the ultrastructural data suggest that electrotonic coupling is restricted to excitatory boutons largely located on the proximal dendritic tree and they originate from second-order vestibular neurons that produce the slow, not fast, phase. Furthermore, electronic coupling is likely not necessary for appropriate vestibulococular reflex function because, mammals, for instance, do not have gap junctions anywhere in the oculomotor system. The electrophysiological role of gap junctions in oculomotor function remains to be determined.

The collective experimental evidence in goldfish and flatfish (unpublished observations) now provide electrophysiological, immunohistochemical, and ultrastructural evidence for second-order vestibular inhibitory pathways in two evolutionarily separate teleostean designs. These results point to a common organizational pattern of vestibuloculomotor connectivity across vertebrate species. First, excitatory synapses appear to be located predominantly on dendrites and inhibitory synapses on somata and proximal dendrites. Second, although detailed comparisons reveal quantitative differences in density of presumed inhibitory and excitatory contacts (including gap junctions between extraocular motoneurons), it is the overall similarity of the vertebrate vestibuloculomotor blueprint that is most striking. Third, on a phylogenetic scale, these observations on reciprocal inhibition and excitation are noteworthy, because each of the many species studied are quite divergent from lampreys which presumably exhibit the most primitive vestibulocular plan (Pombal et al. 1994). Fourth, we conclude that the conceptual view of reciprocal excitatory-inhibitory control of motoneurons may be consistent from inception throughout vertebrate phylogeny. If so, these relatively few and easily identifiable neurons in fish offer significant potential for pursuing long-standing questions concerning the visual and vestibular regulation of oculomotor function and the sites underlying adaptive plasticity.

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**REFERENCES**


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FABER, D. S. and KORN, H. Inputs from the posterior lateral line nerves upon the goldfish Mauthner cell. II. Evidence that the inhibitory components are mediated by interneurons of the recurrent collateral network. Brain Res. 96: 349–356, 1975.


