Reciprocal Pattern of Excitation and Inhibition

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Vestibuloocular reflex of the adult flatfish. III. A species-specific reciprocal pattern of excitation and inhibition. J Neurophysiol 86: 1376–1388, 2001. In juvenile flatfish the vestibuloocular reflex (VOR) circuitry that underlies compensatory eye movements adapts to a 90° relative displacement of vestibular and oculomotor reference frames during metamorphosis. VOR pathways are rearranged to allow horizontal canal-activated second-order vestibular neurons in adult flatfish to control extracocular motoneurons innervating vertical eye muscles. This study describes the anatomy and physiology of identified flatfish-specific excitatory and inhibitory vestibular pathways. In antidromically identified oculomotor and trochlear motoneurons, excitatory postsynaptic potentials (EPSPs) were elicited after electrical stimulation of the horizontal canal nerve expected to provide excitatory input. Electrotonic depolarizations (0.8–0.9 ms) preceded a small amplitude (<0.5 mV) chemical EPSPs at 1.2–1.6 ms with much larger EPSPs (>1 mV) recorded around 2.5 ms. Stimulation of the opposite horizontal canal nerve produced inhibitory postsynaptic potentials (IPSPs) at a disynaptic latency of 1.6–1.8 ms that were depolarizing at membrane resting potentials around −60 mV. Injection of chloride ions increased IPSP amplitude, and current-clamp analysis showed the IPSP equilibrium potential to be near the membrane resting potential. Repeated electrical stimulation of either the excitatory or inhibitory horizontal canal vestibular nerve greatly increased the amplitude of the respective synaptic responses. These observations suggest that the large terminal arborizations of each VOR neuron imposes an electrotonic load requiring multiple action potentials to maximize synaptic efficacy. GABA antibodies labeled axons in the medial longitudinal fasciculus (MLF) some of which were hypothesized to originate from horizontal canal-activated inhibitory vestibular neurons. GABAergic terminal arborizations were distributed largely on the somata and proximal dendrites of oculomotor and trochlear motoneurons. These findings suggest that the species-specific horizontal canal inhibitory pathway exhibits similar electrophysiological and synaptic transmitter profiles as the anterior and posterior canal inhibitory projections to oculomotor and trochlear motoneurons. Electron microscopy showed axosomatic and axodendritic synaptic endings containing spheroidal synaptic vesicles to establish chemical excitatory synaptic contacts characterized by asymmetrical pre/postsynaptic membrane specializations as well as gap junctional contacts consistent with electrotonic coupling. Another type of axosomatic synaptic ending contained pleomorphic synaptic vesicles forming chemical, presumed inhibitory, synaptic contacts on motoneurons that never included gap junc-

INTRODUCTION

Flatfish are a natural paradigm to study a developmental adaptation to a changing living situation. Larvae begin their lives in an upright body posture with the eyes placed on the left and right sides of the head (Fig. 1A). At this stage, vestibuloocular reflex (VOR) eye movements elicited during swimming involve detection of horizontal head rotation about the vertical axis by the horizontal semicircular canals. Horizontal conjugate eye movements are produced by the lateral and medial rectus eye muscles in the earth-horizontal plane (Graf and Baker 1985a) (Fig. 1A). The neuronal circuitry mediating this reflex involves the classical thalamic–neuron arborizations along with the specialized abducentns intercerebellar pathway (Fig. 1A).

During metamorphosis, flatfish rotate 90° about the longitudinal axis to become better adapted to an adult lifestyle on the bottom. The eyes ipsilateral to the side that now faces the sea bottom migrates across the dorsal aspect of the head to the upper side of the flatfish (Fig. 1B). The eyes, including the extraocular muscle apparatus retain their original orientation with respect to the environment; however, since the labyrinths remain in their original position in the head (Fig. 1B), the horizontal canals detect swimming motions that now occur in the vertical plane. The appropriate compensatory eye movements, by contrast, are parallel backward and forward rotations of the eyes produced by contraction of vertical eye muscles. This behavior requires a principal VOR circuitry connecting the horizontal canals to vertical extracocular muscles as shown in Fig. 1B (Graf and Baker 1983, 1985b). The previous neuroanatomical experiments described the structural basis of the...
novel horizontal canal-to-vertical eye muscle connections, and the current work addresses the electrophysiology of the excitatory and inhibitory pathways.

In all vertebrates, VOR pathways exhibit a reciprocal excitatory and inhibitory innervation of extraocular motoneurons in which a stereotyped, constant relationship is maintained between each of the three semicircular canals (anterior, posterior, and horizontal) and one extraocular muscle pair (reviewed in Evinger 1988; Graf 1988; Graf and Ezure 1986; Graf et al. 1985b). Excitatory and inhibitory 2nd-order vestibular neurons were labeled from the upside (right) horizontal canal system of winter flounders (Graf and Baker 1986; Graf et al. 1991). Together, these anatomical reconstructions unveiled a bilaterally symmetric innervation scheme of flatfish horizontal canal VOR pathways that would be well suited for the requirements of compensatory eye movements in postmetamorphic animals within the conceptual framework of reciprocal excitatory/inhibitory innervation of the VOR.

The present studies therefore were aimed at corroborating this reciprocal excitatory/inhibitory projection onto oculomotor motoneurons in adult flatfish with electrophysiology and anatomy. Evaluating the pattern of excitatory and inhibitory postsynaptic potential (EPSP and IPSP, respectively) allowed the operational mode of this species-specific pathway to be tested as well as a basis for structure/function comparison to be established with the reciprocal excitatory/inhibitory organization of other vertebrate eye movement circuits (Graf and Ezure 1986; Graf et al. 1997). The physiology of the excitatory/
inhibitory innervation pattern of VOR pathways in flatfish was extended with immunohistochemistry and electron microscopy for comparison to similar integrated approaches undertaken in oculomotor and postural control in goldfish (Aksay et al. 2000; Baker et al. 1998; Faber et al. 1989; Korn et al. 1992; Kumar and Faber 1999; Straka and Baker 1998; Suwa et al. 1999).

Preliminary results of this study have been previously summarized in abstract form (Graf and Baker 1984; Graf et al. 1985; Spencer et al. 1986).

**METHODS**

All experiments were performed at the Marine Biological Laboratory, Woods Hole, Massachusetts, in 26 adult (25–30 cm body length), right-sided winter flounders, *Pseudopleuronectes americanus*. Neither left-sided examples of this species (Policanski 1982) nor the indigenous left-sided summer flounder, *Paralichthys dentatus* were examined in this study. Experimental flounders were wild-caught by the trawler of the institution in the Atlantic Ocean off Cape Cod and kept in large indoor-outdoor tanks with cooled running seawater of 18°C.

**Surgical procedures**

Animals were mounted in a “vertical” position in a fish-holder system (Graf and Baker 1985b), and cooled (18°C), aerated seawater was continuously circulated over the gills by an electric pump through a tube in the mouth. Animals were kept immersed as deeply as possible in water to submerge the gills. All surgery and physiological experiments were done under intracranial methanesulfonate (1:20,000 w/v) anesthesia. In addition, incisions were infiltrated with local anesthetics (2% lidocaine with epinephrine). During recording, animals were immobilized with gallamine triethiodide (Flaxedil). Access to the lacrimal ducts was obtained by removal of the skull bone overlying the fourth ventricle. Individual labyrinthine nerve branches to the bilaterai anterior, horizontal, and posterior canal ampullae, as well as the utricular, saccular, and lagena endorgans were dissected and exposed. The bone overlying the midbrain was removed, so the medial longitudinal fasciculus (MLF) could be reached through the intertecal cleft. Bipolar electrodes were positioned on the ampullary nerves for orthodromic activation of vestibular pathways to extracellular motoneurons. The stimulation electrodes were held in place by Narishige microdrives. The horizontal canal nerves were selectively isolated and stimulated in 19 experiments, the vertical canals in 5 cases, and the entire vestibular nerve in the 2 other experiments. In all experiments, two silver ball electrodes were placed into the orbits of both eyes to activate antidromically extracellular motoneurons.

**Electrophysiology**

Vestibular synaptic input to oculomotor motoneurons was recorded with glass micropipettes containing 1 M KC1 or 1 M KAcetate (approximately 20–30 MΩ resistance, 0.5–1.0 μm tip size). The recording electrodes were positioned and advanced by a three-axis micromanipulator (Narishige, Canberra type). Motoneurons were penetrated in the oculomotor and the trochlear nucleus. These nuclei were reached through the intertecal cleft that was widened by cutting the commissural fibers for direct visualization of the third ventricle. Earlier neuroanatomical studies had indicated the location of the various motoneuron pools (Graf and Baker 1985a), which were identified by their antidromic potentials following electrical activation (70 μA) via the stimulation electrodes located in the orbits. Subsequently, synaptic potentials were elicited by electrical stimulation from the labyrinthine electrodes (20 μA). Each penetrated motoneuron could be identified by its location within the oculomotor nuclei with respect to the eye from which it was antidromically activated. The electrophysiological data were recorded either on magnetic tape (Necorder: Data 6000), or on magnetic disks (Nicolet 4096), and later printed out on a laser printer for analysis and quantification.

**Neurotransmitters**

Protocols for GABA immunohistochemistry were described in detail in Graf et al. (1997) and are briefly summarized here. Animals were perfused with fixative solution containing 4.0% paraformaldehyde in 0.1 M phosphate buffer with 0.002% calcium chloride (pH 7.2). Vibratome sections were obtained through the abducens, trochlear, and oculomotor nuclei (25–50 μm) and subsequently processed for immunohistochemical localization of GABA using an antibody generated in rabbit against GABA conjugated to bovine serum albumin (Immunonuclear). Following primary and secondary antibody incubation, the tissue was incubated in an avidin/biotin-HRP complex (Vector). Addition of the chromogen 3,3’-diaminobenzidine and hydrogen peroxide produced a brown diffuse reaction product. The material was mounted for light microscopic examination using bright-field or Normarski differential interference contrast optics.

**Electron microscopy**

Protocols were identical to those described in Graf et al. (1997) and are summarized here briefly. Animals were anesthetized and perfused with 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 M phosphate buffer with 0.002 calcium chloride (pH 7.4). Serial coronal sections (50–75 μm) through the oculomotor nuclei were cut on a vibratome. Sections containing the oculomotor nuclei were trimmed, postfixed in 1% osmium tetroxide, stained en bloc with 0.5% uranyl acetate, dehydrated, and embedded in resin between vinyl plastic microslide and cover slips.

Selected loci containing oculomotor neurons were analyzed by serial ultrathin sectioning and electron microscopy. Serial sections (5 μm) through the plastic-embedded vibratome sections were 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, examined, and photographed with a Zeiss EM-10CA electron microscope. The same procedure was followed in three animals who had received, 1 day earlier, an intracellular injections of HRP into second-order vestibular neurons of the horizontal canal system. The brains were also treated for HRP histochemistry before selected sections were prepared for for electron microscopy (Graf and Baker 1985a,b).

**RESULTS**

A total of 102 extraocular motoneurons was recorded in the trochlear, oculomotor, and abducens nuclei. The latency for antidromic invasion averaged 0.7 ms. Disynaptic depolarizing synaptic potentials were observed in all motoneurons following whole vestibular nerve branch stimulation with latencies between 1.2 and 1.8 ms. As expected, selective activation of individual canal nerves also evoked disynaptic depolarizations that were shown to be EPSPs in the appropriate populations of motoneurons (Figs. 2–4). However, stimulation of the presumed antagonistic canal nerve supposed to provide inhibitory input, typically produced membrane depolarizations as well (Figs. 2, C and D, and 4C). Hyperpolarizing IPSPs were recorded in only nine motoneurons (i.e., 9% of all cases; Fig. 3, B and D), and these IPSPs were about equally distributed between the vertical oculomotor subgroups (2 each in IR, IO, and SR motoneurons, and 3 in SO motoneurons). The existence of inhibition was supported by finding GABAergic contacts on
FIG. 2. Synaptic potentials in 2 left-side trochlear motoneurons (ITro) following horizontal canal nerve stimulations. A: antidromic identification of somatic recording showing IS-SD break and M-spike (top and bottom arrows, respectively). Top and bottom traces are AC- and DC-coupled records, respectively. B: AC recordings of an excitatory postsynaptic potential (EPSP) following contralateral (right-side) horizontal canal nerve (rHCe) stimulation with double shocks at 2 times threshold (2 × T). Averaged (n = 8) and single sweeps are shown in the top and bottom traces, respectively. Electrotonic coupling appears at 0.8 ms and the onset of chemical depolarization at 1.6 ms. Action potentials are shown in the lower DC-coupled record labeled ITRO Mn. An intra-axonal DC record from a VOR neuron following rHCe stimulation is shown in the 3rd trace (VOR axon in medial longitudinal fasciculus (MLF)). The vertical arrows point out synchronization between the 1st vestibular action potential and the electrotonic potential (E). Calibrations for A are shown in B. C: comparison of synaptic potential latencies elicited from stimulation of the excitatory (rHCe; top 3 traces) and the inhibitory sides (lHCi; bottom 3 traces). AC recordings are averaged (n = 8) with the 2nd and 3rd traces showing single AC and DC sweeps, respectively. EPSPs following rHCe stimulation (1.5 × T) exhibited electrotonic coupling (E) at 0.8 ms, and a small chemical depolarization (C) at 1.6 ms before onset of the larger EPSP at 2.4 ms. Inhibitory postsynaptic potentials (IPSPs) elicited from lHCi appeared as membrane depolarizations (C) at 1.8 ms. Vertical arrows illustrate the latencies of the respective synaptic potentials. D: AC recording (n = 8; 1st trace) and single AC and DC sweeps (2nd and 3rd traces) showing EPSPs (electrotonic, E, and chemical, C, at 1.5 × T) and presumed IPSPs (chemical, C) following rHCe and lHCi nerve stimulation, respectively. IPSPs appeared as membrane depolarizations at a latency of 1.8 ms following lHCi activation at graded stimulus strengths (1.5–10 × T). Vertical arrows illustrate the latencies of the respective synaptic potentials.
extraocular motoneurons (Fig. 5) and by ultrastructural anatomical features associated with inhibitory synapses (Figs. 7 and 8).

**Neurophysiology**

Synaptic potentials recorded in extraocular motoneurons could be compared with those recorded in goldfish (Graf et al. 1997) as well as interpreted with respect to the vestibulooculomotor pathways shown earlier in flatfish (Graf and Baker 1985b) (see also Figs. 1B and 9). To do so, it was necessary to recognize the recorded motoneuron pools (Graf and Baker 1985a). Hence, in each experiment the trochlear nuclei (SO motoneurons) were located first by the antidromic field potential profile initiated from the contralateral orbits (Fig. 4A) (Baker et al. 1973; Graf et al. 1997). SR motoneurons were identified in the caudal oculomotor complex following stimulation of the contralateral orbits. IR and IO motoneurons were recognized by antidromic activation from the ipsilateral orbits, while abducens (LR) motoneurons were recorded in the posterior brain stem. Motoneuron somata were located adjacent to the midline (Fig. 5A), and antidromic intracellular records were characterized by initial segment–soma dendritic (IS-SD) invasion followed by a large afterdepolarization typical of an intrasomatic penetration (Figs. 2A and 3A and C).

Since synaptic potentials recorded in all vertical oculomotor motoneurons exhibited similar profiles, the electrophysiology is thus illustrated for four key somatic recording sites (2 left-side trochlear motoneurons, Fig. 2, and 1 left-side and 1 right-side SR motoneuron, Fig. 3) and one intradendritic recording from a right-side trochlear motoneuron (Fig. 4). The cases selected were stable penetrations with resting potentials between $-50$ and $-60$ mV. Membrane depolarization was elicited from stimulation of the right horizontal canal labyrinthine nerve branch (rHCe) predicted to provide excitatory input to trochlear motoneurons (Figs. 1B and 9). Stimulation of the prospective left horizontal canal inhibitory input (lHCi; Fig. 1B, blue pathway) did not produce significant membrane hyperpolarization at this level of resting potential. In fact, in 93/102 cases, the stimulus that should have produced a hyperpolarizing IPSP induced membrane depolarization (Fig. 2, C and D). In only nine cells could hyperpolarizing IPSPs be demonstrated without the application of extrinsic current following stimulation of the appropriate canal nerves (Fig. 3, B and D). Depolarizing current injection between $+5$ and $+15$ nA, in the absence of action potentials, showed that stimulation
of the vestibular nerve branch expected to provide inhibitory input, produced IPSPs associated with a membrane conductance. This observation suggested that the IPSP equilibrium potential might be close to this motoneuron’s resting potential. In these cases, the synaptic potentials could also be re-reversed to depolarization by injection of hyperpolarizing current ranging from $-4$ to $-11 \text{ nA}$. Injection of chloride ions also significantly displaced the IPSP equilibrium potential in some motoneurons to a level equal to that of the contralateral EPSP (not illustrated). However, in contrast to goldfish motoneurons, use of depolarizing current and chloride injection to change the membrane resting potential and/or IPSP equilibrium potential did not reveal either robust hyperpolarizing IPSPs or modified EPSP amplitudes (Graf et al. 1997).

Chemically elicited EPSPs were always preceded by a short-latency electrotonic potential at a latency of 0.8–0.9 ms (Figs. 2, B–D, 3B, and 4C). The ubiquitous presence of electrotonic coupling indicates distributed gap junction connections throughout the extraocular motoneuron pools that was corroborated by electron microscopy (Figs. 6–8). Electrotonic coupling could be demonstrated by collision block of the action potential elicited from direct activation with that from anti-

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**FIG. 4.** Dendritic recording from a right-side trochlear motoneuron neuron (rTro) with a KCl electrode. A: antidromic response with $-2.0 \text{ nA}$ membrane hyperpolarization. Arrow indicates the somadendritic all-or-none action potential. Inset illustrates the antidromic field potential elicited from contralateral orbit stimulation. B: EPSPs recorded after ipsilateral (right-side) horizontal canal nerve (rHCe) activation at $2 \times T$. Top traces are 2 single sweep AC-coupled records, and the bottom trace, a DC-coupled record of the EPSP producing action potentials. C: comparison of synaptic potentials elicited from the excitatory (rHCe) and inhibitory sides (lHCi). The top and the middle traces are averages of the AC recording ($n = 8$). The bottom trace is a single AC sweep. Arrows indicate onset of electrotonic coupling (E) at 0.9 ms and chemical depolarization (C) at 1.5 ms for stimulation of the excitatory (rHCe) horizontal canal. The IPSP following stimulation of the lHCi at $2 \times T$ (C) arrived at 1.8 ms, but was reversed due to chloride leakage and shift of the equilibrium potential. Note the different latencies for the evoked excitatory electrical potential (E) at 0.5 ms, excitatory chemical potential (C) at 1.5 ms, and inhibitory chemical potential (C) at 1.8 ms. Amplitude calibrations and time scale for B are shown in A.
dromic invasion (Fig. 3C). Following electrical activation of the horizontal canal nerve, small-amplitude chemical depolarizations were recorded with latencies of 1.2–1.6 ms (Figs. 2, B–D, and 3B). Larger excitatory depolarizations, presumed to be EPSPs, occurred from 2.2 to 2.6 ms (Fig. 2, B–D). Stimulation of a vestibular nerve branch expected to produce IPSPs, in general, also produced small membrane depolarizations beginning at a latency of 1.6–1.8 ms that gradually increased in amplitude resulting in larger depolarizations at 2.6 ms (Figs. 2, C and D, and 3B).

Intra-axonally recorded action potentials in the MLF (Fig. 2B) from horizontal canal vestibular neurons were recorded at a latency of 0.6–0.8 ms after stimulation (Fig. 2B). The first vestibular action potential was coincident with the electrotonic synaptic potential at 0.8 ms (Fig. 2, B and E), whereas the second vestibular neuron action potential overlapped with both an electrotonic and chemical depolarization at 1.6 ms (Fig. 2, B and C). In these experiments, multiple HC stimuli were required to produce a large enough EPSP to initiate oculomotor motoneuronal action potentials (Fig. 2B, ITro Mn).

In all 102 motoneurons, the presence of excitatory and putative inhibitory synaptic potentials correlated well with the proposed innervation scheme of horizontal canal second-order input to oculomotor neurons in flatfish. Activation of the left horizontal canal nerve produced EPSPs in superior rectus and inferior oblique motoneurons on both sides, whereas IPSPs were elicited from the right horizontal canal (cf. Figs. 1B; 3, B and D; and 9). Similarly, the bilateral trochlear (superior oblique) and inferior rectus motoneurons received excitatory input from the right horizontal canal nerve and inhibitory input from the left horizontal canal nerve (Figs. 1B, 2, B–D; 4, B and C; and 9). Synaptic responses from the anterior and posterior canals (not illustrated) were qualitatively similar to those observed in mammals and goldfish (Graf et al. 1997).

In all animals, averaging of synaptic potentials following single shock stimuli was sufficient to determine latency and polarity (Figs. 2, B–D; 3B; and 4C); however, multiple shock stimulation was required to elicit synaptic potentials large enough to activate spikes in the penetrated motoneurons (Figs. 2B and 4B). This phenomenon, in part, may have been due to either anesthetic (MS222 and Flaxedil) and/or surgical effects on central vestibular excitability. Alternatively, the small synaptic potentials, especially IPSPs, after single electrical stimuli could be due to a larger than normal electrotonic load provided by the dense, bilateral terminal arborization (Figs. 1B and 9; see also DISCUSSION) (Graf and Baker 1985b).

![Figure 6](http://jn.physiology.org/)

**FIG. 6.** Electron microscopic reconstruction of flatfish 2nd-order vestibular axon terminal identified after IHC activation and stained by intracellular injection of HRP. A: low-power electron micrograph of an inferior oblique motoneuron (IO MN) on the left side showing a labeled axosomatic synaptic contact in the bottom right corner below the label of soma. B–D: serial reconstruction of the labeled terminal shown in A. B and C: chemical (arrows) and electrical (arrowheads) axosomatic synaptic contacts (B). D and E: axospinodendritic contacts established only chemical contact zones (arrows). Synaptic vesicle morphology and number are obscured by the dense HRP reaction product inside the axon terminal. Calibrations are 5 μm in A, and 0.5 μm in B–E. d, dendrite; sp, spine.
Corroborative evidence for vestibular inhibition of oculomotor neurons was provided by the immunohistochemical localization of GABA, which is the inhibitory transmitter in the vertical VOR of higher vertebrate species (Precht et al. 1973; Spencer et al. 1989, 1992). GABA immunohistochemistry was used in goldfish to demonstrate the axonal trajectories of inhibitory vestibular neurons in the MLF, as well as terminations on extraocular motoneurons in the trochlear and oculomotor nucleus (Graf et al. 1997). In flatfish, axons of HC inhibitory neurons were physiologically identified to course in the MLF (Graf and Baker 1985b), and the GABA reaction product was also found in the transversely cut axonal profiles in the MLF (Fig. 5, C–F).

Unlike in goldfish, these HC inhibitory axons did not travel together in bundles, but were dispersed throughout the coronal sections of this pathway (compare Fig. 5D with Fig. 5C of Graf et al. 1997). In the flatfish oculomotor nucleus, GABA-positive boutons were observed on the motoneurons in all subdivisions (Fig. 5, A and B). Terminal arborizations were distributed largely on the somata and proximal dendritic trees (Fig. 6, B and E).

Electron microscopy

To provide evidence for the presence of electrotonic and chemical excitation and chemical inhibition, the pattern and mode of terminal arborizations were studied in the oculomotor nuclei of four flatfish after intracellular HRP labeling of physiologically identified HC activated neurons (Graf and Baker 1985b) (Figs. 6 and 7). Axons of horizontal canal VOR neurons were injected in the MLF for 3–5 min to minimize masking the cytoplasmic details of the contacts onto oculomotor neurons. The somata of neurons in individual subnuclei were identified first in thick plastic sections (e.g., Fig. 7A) that subsequently were thin sectioned for electron microscopy (Spencer and Baker 1983). Cytoplasmic organelles typical of other vertebrates, e.g., Golgi apparatus and mitochondria, were observed in the motoneuron somata (Figs. 6A and 7B). In addition, cisternal arrays of granular endoplasmic reticulum typical of mammalian oculomotor motoneurons were well-developed (Spencer and Baker 1983).

A moderate to high density of axosomatic synaptic endings was found in all oculomotor subdivisions and on trochlear motoneurons (e.g., arrow in Fig. 6B). Electron microscopic reconstruction of synaptic contacts on oculomotor neurons showed axosomatic and axodendritic synaptic endings that contained spheroidal synaptic vesicles and established chemical (presumed excitatory) synaptic contacts characterized by asymmetrical pre/postsynaptic membrane specializations (Fig. 8, B and D). Many HRP-labeled HC axosomatic synaptic endings also exhibited gap junction contacts consistent with electrotonic coupling (Figs. 7F and 8, C and D). Chemical synaptic contacts exhibited an intersynaptic space of approximately 20 nm, while gap junction contacts displayed a

FIG. 7. Electron microscopic reconstruction of flatfish 2nd-order vestibular axon terminal identified after rHC stimulation and stained by intracellular injection of HRP. A: light-microscopic image of an IO MN contacted by a labeled 2nd-order vestibular axon collateral (pta) in the right oculomotor nucleus. B: low-power electron micrograph of the IO MN and preterminal axon (pta). C–F: serial sections of axodendritic and axosomatic chemical synaptic junctions. C: the labeled synaptic ending containing pleiomorphic vesicles forms a chemical axodendritic contact (arrowhead). Spheroidal synaptic vesicles in the unlabeled terminal (top left) establish a chemical synapse on the IO MN soma. D: adjacent section showing the postsynaptic profile of the labeled ending (arrowhead). E: contact of the unlabeled (arrowhead) and labeled ending with the dendrite. F: formation of an axosomatic chemical contact with the dendrite (arrowhead) and an electrotonic contact (arrow) with the dendrite. Calibrations are 10 μm in A, 5 μm in B, and 0.5 μm in C–F. d, dendrite; pta, preterminal axon.
separation of approximately 2 nm between the pre- and postsynaptic membranes (Fig. 8D). Mixed chemical and electrotonic axosomatic synaptic endings contained fewer spheroidal vesicles than axosomatic endings establishing only chemical synaptic contacts (Fig. 8B). By contrast, axodendritic endings, including those on spines, established only chemical contact zones (Fig. 7E). When individual synaptic contacts were serially reconstructed, gap junctions were associated with other unlabeled axodendritic synaptic endings (Fig. 7F). Both types of contacts could be observed in relation to different (Fig. 7E) or the same (Fig. 8, C and D) postsynaptic profiles.

Other axodendritic and axosomatic synaptic endings contained pleiomorphic vesicles and established single chemical synaptic contacts (Figs. 7, C and D, and 8A). Synaptic contact zones exhibited symmetrical pre-/postsynaptic membrane specializations (Fig. 8A). These presumed inhibitory synaptic endings were never observed to establish gap junctions and were oriented either on somata or proximal dendrites (Fig. 7, B and D).

**Discussion**

**Summary**

In our previous morphological study, second-order VOR neurons were identified by electrical stimulation of the left-side (down-side) HC nerve, injected with HRP and their axonal trajectories and termination patterns reconstructed in the oculomotor complex (Graf and Baker 1983, 1985b). These observations suggested the presence of reciprocal HC species-specific excitatory and inhibitory vestibular projections to vertical extraocular motoneurons as illustrated in Fig. 9 (red and blue pathways, respectively). The electrophysiology (Figs. 2–4) and anatomy (Figs. 5–8) presented here strongly support this interpretation and, in particular, also confirm the presence of a second-order excitatory and inhibitory pathways originating from the right-side (up-side) HC nerve (Fig. 9, green and orange pathways, respectively). As a result, either right-side or left-side horizontal semicircular canal stimulation initiates a pattern of electrotonic/chemical synaptic depolarization and inhibition. This is consistent with the idea of equally weighted, bilaterally symmetric, reciprocal excitatory/inhibitory pathways contacting the four vertical oculomotor motoneuron pools. Since the ultrastructural and immunochemical synaptic profiles in flatfish were similar to some aspects of those described in the vertical VOR pathways of goldfish (Graf et al. 1997), we suggest that the development, hindbrain location and function of VOR neurons may be an unchanging trait in not only teleost fish, but also possibly throughout vertebrate phylogeny.

**Electrophysiology**

In antidromically identified oculomotor motoneurons innervating either the bilateral SO/IR or IO/SR muscle pairs, electrical stimulation of either the rHC or IHC vestibular nerve produced short-latency disynaptic electrotonic EPSPs (0.8–0.9 ms) followed by disynaptic chemical depolarizations with latencies of 1.2–1.8 ms, respectively. In each motoneuronal population, and for all experiments, the amplitude of the single shock EPSPs were small (<1 mV), whereas the depolarization was significantly enhanced in response to multiple stimuli. While the surgical and anesthetic conditions likely contributed to this reduced synaptic efficacy, an alternative, more physiological explanation might be related to the observation that unitary action potentials do not completely invade large terminal arborizations as seen, for example, in tectobulbospinal neurons (Grantyn and Grantyn 1982) and other neuronal models (e.g., Mackenzie and Murphy 1998; Toth and Cruenelli 1998). Intra-axonal records from HC excitatory and inhibitory vestibular neurons ascending in the MLF always responded with a single or double action potential of 0.6–0.8 ms latency after single shock stimulation of the appropriate HC nerve (Fig. 2B) (Graf and Baker 1985b). Multiple HC stimuli were re-
required, however, to produce additional action potentials that clearly were more effective in producing transmitter release as evidenced from the intracellular records in oculomotor neurons. The latter interpretation suggests that orthodromic invasion of the multiple branch points requires a short presynaptic interspike interval and/or a background of spontaneous activity likely present under natural conditions (Graf and Baker 1990).

In the appropriate motoneurons, stimulation of the vestibular nerve branch expected to be inhibitory produced small-amplitude depolarizations beginning at a latency of 1.6–1.8 ms that gradually increased in amplitude to peak at 3.0 ms. Direct evidence for disynaptic inhibitory input to extracellular motoneurons in the form of hyperpolarizing IPSPs was only seen in 9/102 motoneurons (i.e., 9%). Postsynaptic inhibition in teleost fish largely appears as a shunting membrane conductance at the soma and proximal dendrites to reduce the input resistance of the cell and thereby short-circuiting excitation arriving at, and from, the more distal dendrites (Faber and Korn 1987; Graf et al. 1997). The location of inhibitory (GABAergic) terminals overlying the somata and proximal dendrites supports a similar interpretation in flatfish (Figs. 5, 7, and 8A). Manipulation of membrane potential by current injection through the microelectrode demonstrated the short-latency polarization to be associated with a membrane conductance exhibiting an equilibrium potential slightly more negative than resting potential (Fig. 3, C and F). However, IPSP reversal by injected current appeared to be independent of either intrasomatic or intradendritic recording sites and, surprisingly, in neither case did the injection of chloride ions significantly displace the equilibrium potential.

Efficacy of the inhibitory synaptic conductance could not be assessed in flatfish as easily as in either goldfish extracellular motoneurons (Graf et al. 1997) or Mauthner cells (Faber and Korn 1975, 1987). The steep voltage dependence of chloride channels envisioned to increase the magnitude and duration of the inhibitory response in goldfish motoneurons (Graf et al. 1997) was not seen in flatfish motoneurons. Hyperpolarizing IPSPs also were not found in the VOR pathways of the puffer fish, a marine teleost (Korn and Bennett 1972, 1975). Perhaps sharp electrode intracellular recordings from hindbrain neurons in teleosts may not reflect sufficiently well the extent of inhibitory conductance because reconstructed cells exhibit an extensive cable-like appearance arguing against a uniform change in potential throughout all compartments (Graf and Baker 1985a; Highstein et al. 1992; Pastor et al. 1991; Straka and Baker 1998; Zottoli and Faber 1980). Nonetheless, in view of the voltage dependence of glycine-activated Cl− channels in which the kinetics of the synaptic response are enhanced in the face of excitation (Faber and Korn 1988), activation of the inhibitory HC canal nerve would be expected to reduce the firing frequency of oculomotor neurons. Indeed, in some isolated cases, when spontaneous activity was present in the recorded motoneuron, stimulation of the putative inhibitory vestibular nerve markedly reduced the firing rate.

The reciprocal semicircular canal inhibitory and excitatory pathways must play an important role in coordinated eye movements. If such reciprocity were not present, vestibular input would result in a counterproductive functional input to the oculomotor system (Graf and Baker 1990). Our current interpretation therefore is that the special nature of excitable membrane properties in fish permits an IPSP equilibrium potential close to membrane resting potential to be as effective as the much more negative equilibrium potential in mammalian oculomotor neurons (Llinás and Baker 1972).

**GABA immunohistochemistry and electron microscopy**

GABAergic terminals were found clustered around cell somata and proximal dendrites in a similar pattern as described for goldfish oculomotor and abducens nuclei (Graf et al. 1997). GABA antibodies localized inhibitory neurons in the vestibular nuclei (not illustrated) and, as in goldfish, axons headed toward and into the contralateral MLF. Terminal arborizations were observed to distribute primarily over the somata and proximal dendritic trees of trochlear (SO) and oculomotor motoneurons. Thus the presence, spatial distribution, and magnitude of GABAergic inhibition from presumed GABAergic neurons of the HC vestibular system in flatfish appears to be similar to the vertical VOR system in goldfish and mammals (Graf et al. 1997; Spencer and Baker 1992).

In the vestibuloocular motor system of mammals, the inhibitory transmitter related to vertical canal reflexes is GABA; that related to the horizontal canal reflex is glycine (Spencer et al. 1989). Thus an apparent paradox exists in the adapted vestibuloocular reflex circuitry of adult flatfish: our immunohistochemical data indicate the existence of a robust GABA termination in the oculomotor and trochlear nucleus, while the inhibitory input to these vertical eye muscle motoneurons is largely derived from HC second-order vestibular neurons. The solution to this apparent paradox will bear directly on the embryonic origin of the adapted VOR circuits, and several scenarios may be envisioned. If the novel VOR circuitry originates from extant classical and functional horizontal canal VOR neurons with terminals originally in the abducens nucleus where glycine exists as an inhibitory transmitter (Spencer et al. 1989), the neurotransmitter content must switch from glycine to GABA during metamorphosis when new contacts would be made with vertical extraocular motoneurons. Alternatively, these inhibitory neurons might arise from neurons formerly related to the vertical VOR, and secondarily became recruited by the horizontal canals. A more parsimonious hypothesis, however, would be that these second-order inhibitory neurons and their excitatory counterparts originate from entirely different embryonic progenitors. Preliminary results support the latter conjecture because newly born vestibular neurons appear at a time when the postmetamorphic connectivity formed (Graf et al. 1991). Establishing the embryonic origin of these novel VOR pathways will reveal the relevant hindbrain rhombomeres and genes responsible for flatfish-specific neuronal adaptation (Baker 1998).

**Ultrastructure**

The electron microscopic visualization of flatfish oculomotor and trochlear nuclei revealed axosomatic and axodendritic synaptic endings containing spheroidal synaptic vesicles that established chemical, presumed excitatory, synaptic contacts characterized by asymmetrical pre/postsynaptic membrane specializations. In addition, many axosomatic synapses also showed gap junction contacts consistent with electrotonic coupling. The latter were similar in morphological profile to the “mixed” synapses described in the goldfish abducens nucleus.
(Graf et al. 1997; Sterling 1977). Other axosomatic synaptic endings contained pleomorphic synaptic vesicles establishing chemical synaptic contacts on motoneurons. These presumed inhibitory synapses were never observed to establish gap junctions. Comparison of flatfish oculomotor and trochlear nuclear complex to that in goldfish showed that the relative placement of chemical versus electrotonic contacts of excitatory second-order vestibular neurons was different (Spencer et al. 1986). In goldfish, chemical contacts were located at some distance from gap junctions (Graf et al. 1997), whereas in the flatfish, gap and chemical junctions were adjacent to each other (Fig. 8, C and D). In flatfish, the spatial relationship of chemical and electrotonic contacts was closer, and thus more like the arrangement in the goldfish abducens nucleus (Graf et al. 1997). The functional significance of this difference in chemical versus electrical junction placement is undetermined in both species as is the significance of electrotonic coupling per se in VOR behavior. A prior interpretation argued that electrotonic transmission played a more important role in goldfish VOR (Graf et al. 1997) than in synchronizing motoneuronal discharge during either fast phases or saccades (Korn and Bennett 1971). Nonetheless, electronic coupling apparently is not essential for normal VOR behavior in all vertebrates, because gap junctions are not found in mammals.

Reciprocal excitatory/inhibitory innervation

The pattern of excitation and inhibition in the vestibuloculomotor system of flatfish is in agreement with previously obtained morphological results and also supports the structural requirements to produce compensatory eye movements during swimming in the adult animals (Graf and Baker 1985b, 1986, 1990). In particular, HC innervation of the SR, SO, IR, and IO extraocular motoneurons is quite unique, but its blueprint fits well into the classical reciprocal excitatory/inhibitory innervation scheme of semicircular canal-related VOR function. In eye movement plants of upright symmetrical vertebrates, e.g., mammals and goldfish, excitation arrives from the contralateral, and inhibition from the ipsilateral labyrinth. The flatfish innervation pattern of the right-sided winter flounder can only be interpreted in a context-specific manner. In such case, the bilateral SR and IO motoneurons receive excitatory input from the downside (left) horizontal canal and reciprocal inhibitory input from the upside (right) horizontal canal. The motoneurons innervating the antagonists of these muscles, the bilateral SOs and IRs, would be innervated with opposite-directed signals, i.e., excitation from the (upsided) HC, and inhibition from the (downside) IHC. Unlike VOR organization in mammals and goldfish, wherein excitatory and inhibitory VOR neurons ascend on the contralateral and ipsilateral side, respectively, all ascending excitatory and inhibitory horizontal canal VOR neurons in flatfish ascend on the contralateral side (Figs. 1 and 9).

Spatial coordination of eye movements

VOR connections are quite remarkable, if not coincident, when classical co-contraction patterns of yoke eye muscles are compared between flatfish and other vertebrates (Szentágothai 1943, 1950). The SR muscle of one eye and the IO muscle of the other eye are defined as yoke muscles as in similar fashion are the SO and contralateral IR counterpart. Flatfish co-contract either the bilateral SR and IO for backward rotation (i.e., extorsion) or the bilateral SO and IR for forward rotation (i.e., intorsion) to produce compensatory eye movements during downward or upward head movements, respectively (Graf and Baker 1983, 1985b). In light of extraocular muscle kinematics (Graf and Baker 1985a), this arrangement is biologically meaningful and not different from either other upright fish or lateraled-eyed vertebrates (Graf and Brunken 1984; Graf and McGurk 1985; Simpson and Graf 1985). However, the similar VOR behavior in flatfish is brought about by a VOR innervation scheme that is different from all other vertebrates. In the latter case both anterior (vertical) canals provide excitatory signals to the bilateral SR and IO motoneurons during downward head movements. In flatfish, comparable excitation is transmitted from the downside horizontal canal to the bilateral SR and IO motoneurons. A similar scenario holds for posterior (vertical) canal reflexes in upright vertebrates as well as that elicited by the upside horizontal canal in flatfish. Inhibitory input from the same canals in the above-mentioned cases reaches the antagonists of the respective muscles. Thus to implement the evolutionarily adapted state, a previously established innervation scheme based on retained spatial geometry and orientation of the eye muscles is employed in the postmetamorphic flatfish VOR circuits. In upright vertebrates, the sensory information signaling upward and downward head movement arises from the four vertically oriented canals, whereas in flatfish, the two, now vertically oriented, horizontal canals fulfill this role (Fig. 1B).

Conclusion

The conceptual view of reciprocal excitatory-inhibitory organization of VOR circuits as necessary and essential for compensatory eye movement production has been upheld by electrophysiological, immunohistochemical, and ultrastructural evidence obtained from the species-specific expression found in flatfish. These observations also suggest that second-order vestibular neurons can use a novel developmental plasticity to achieve new structural and physiological requirements. Thus the unique VOR organization in flatfish can be used as a model to study the embryonic, endocrine, environmental, and genetic mechanisms underlying this pre- to postmetamorphic transformation.

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