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"Latent" inhibitory connections become functional during activity-dependent plasticity

(Mauthner cell/inhibition/long-term potentiation/escape reflex reaction)

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ABSTRACT **Simultaneous pre- and postsynaptic recordings from identified glycinergic inhibitory interneurons and the Mauthner cell showed that 25% of the afferents produced no or extremely small postsynaptic responses. Morphological determination of the number of contacts made by these cells on the Mauthner cell revealed a connectivity similar to that of functional neurons which always produce clear inhibitory postsynaptic potentials, suggesting that most of the endings made by weak interneurons are silent. Intraaxonal injection of 4-aminopyridine or Ca^{2+} greatly enhanced transmission at functional connections but did not modify those which were ineffective. However, after eighth nerve tetanic stimuli, transmission at the weak connections was unmasked or enhanced for prolonged periods and was twice as likely to be potentiated, with a 6-fold greater mean enhancement than the potent ones. This result provides additional support for long-term potentiation of inhibitory synapses. Furthermore, weakly functional junctions represent a "reserve" pool which can be critical for the expression of plasticity within a network, and, consequently, for setting the threshold of reflex activities such as the escape reaction mediated by the Mauthner cell.**

Inhibitory inputs to the goldfish Mauthner cell (M-cell) (1) encompass a wide range of synaptic strengths (2), as is true for afferents to principal neurons in other systems (3). In the M-cell network, which apparently controls the threshold and direction of the escape reaction of the fish (4, 5), some inhibitory cells represent one extreme of that range, having been described as silent because with simultaneous pre- and postsynaptic recordings, their activation did not produce detectable postsynaptic responses (6). It has been suggested that weak or inactive connections might constitute a functional reserve (7). Therefore the present study focused on the properties of this apparent lack of transmission, and we asked whether the efficacy of transmission involving these neurons was susceptible to activity-dependent potentiation, as has been shown for effective synapses during inhibitory long-term potentiation (LTP) (8).

MATERIAL AND METHODS

Electrophysiological Procedures. Experiments were performed on goldfish (*Carassius auratus*) anesthetized with MS222 (70 mg/liter) and immobilized with *d*-tubocurarine. Electrodes filled with 3 M KCl had resistances of 20–50 and 2–4 MΩ for recording from the presynaptic axon and the M-cell soma, respectively. The latter was localized electrophysiologically and interneurons were recognized by the presence of a spinal cord evoked passive hyperpolarizing potential (PHP), which is due to a field effect and indicates that they are inhibitory to the M-cell (9). Fig. 1*A* shows that they belong to two classes which can be distinguished electrophysiologically, (*i*) crossed commissural and (*ii*) collateral interneurons (1). In some cases, the presynaptic pipette contained either 30 mM 4-aminopyridine in 1.5 M KCl or 0.2—1 mM $CaCl_2$ in 3 M KCl solution. Chloride ion injections were used to displace the IPSP reversal potential and unitary IPSPs were depolarizing. Most recordings were obtained by using an Axoprobe-1A amplifier (Axon Instruments, Burlingame, CA). Amplitudes of unitary IPSPs were expressed in mV ± SD and were normalized—i.e., given in percent of the amplitude of the full-sized antidromic IPSP (termed V_{coll}) evoked by activation of the entire pool of collateral interneurons (Fig. 1*B*) (10). This normalization procedure allows for comparisons of synaptic

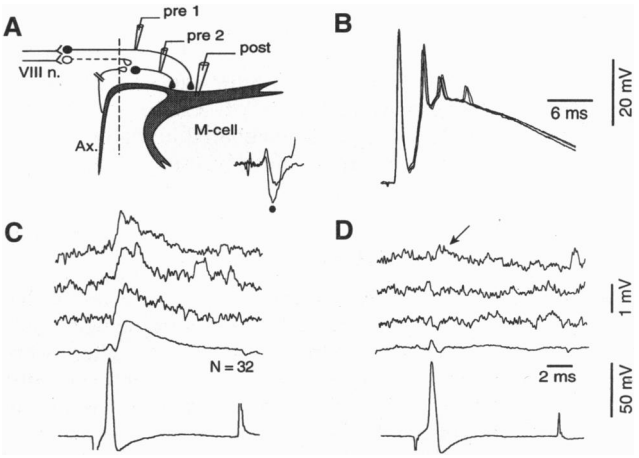

FIG. 1. Electrophysiological evidence for ineffective synaptic transmission. (*A*) Experimental set-up and diagram of presynaptic inhibitory network. Paired recordings were obtained from the M-cell and the commissural (pre 1) and collateral (pre 2) interneurons identified by a PHP (*Inset*; see *Material and Methods*). Cell bodies of commissural cells are located in the vestibular nucleus and are activated monosynaptically by the posterior eighth nerve (VIII n.), while collateral neurons are excited disynaptically via axon collaterals of the M-cell's axon (Ax.) and polysynaptically via the eighth nerve (dashed horizontal line; vertical one indicates midline). (*B–D*) Intracellular recordings from one M-cell and two PHP neurons during the same experiment. (*B*) Superimposed traces of the antidromic Mauthner action potential and the subsequent collateral inhibitory postsynaptic potential (IPSP), showing stability of both. When the IPSP triggered spikes, as here, its amplitude was taken as the peak of the depolarizing envelope underlying them. (*C* and *D*) Sample recordings (upper three traces) and averages of 32 responses (fourth trace from top) produced by direct spikes (lower traces) in a commissural (*C*) and collateral (*D*) interneuron. Unitary IPSPs are visible in all sweeps in *C*; presynaptic impulses failed to evoke IPSPs in *D*; the deflection above the noise level (arrow) could be a spike-evoked response.

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Abbreviations: M-cell, Mauthner cell; IPSP, inhibitory postsynaptic potential; LTP, long-term potentiation; PHP, passive hyperpolarizing potential; V_{coll}, amplitude of the full-sized recurrent collateral IPSP; 4-AP, 4-aminopyridine.

strength between experiments which have different degrees of Cl^- loading of the M-cell. A single test presynaptic spike was evoked twice per second through the intracellular microelectrode. Tetanic stimulation consisted of short trains of 10–20 pulses at 150–300 Hz applied to the posterior branch of the contralateral eighth nerve, at 2-s intervals for 1–2 min. The stimulus strength was adjusted so that every pulse activated the presynaptic neuron.

Morphological Methods. Biocytin was injected iontophoretically in the presynaptic neurons as described elsewhere (11). After 15 min, the fish were perfused intracardially with cold freshly prepared 4% paraformaldehyde and 0.1% glutaraldehyde in 0.12 M sodium phosphate buffer, pH 7.4, for 20 min. After three washes in 0.12 M sodium phosphate-buffered 0.9% saline, pH 7.4 (PBS), slices (60 μm) were left to react for 30 min with 0.03% H_2O_2 to reduce endogenous peroxidase activity. After washing, they were incubated overnight in 1% avidin–biotin complex (ABC elite, Vector Laboratories) in the presence of 0.12% gelatin and 0.25% Triton X-100. After three rinses in PBS and Tris, they were incubated in diaminobenzidine (DAB) for 30 min and then stained by using a DAB–H_2O_2 kit (Sigma Fast).

RESULTS

Definition of Weak Connections. In a first series of 200 paired recordings, the majority of presynaptic neurons produced mean responses 0.15–5 mV in amplitude. These were unambiguously visible on single sweeps after each presynaptic action potential (Fig. 1*C*). However, responses could not reliably be distinguished from background noise for a special group of 45 cells (22.5%) (Fig. 1*D*). For 19 of them, signal averaging gave no evidence of a response. The mean control responses found in the remaining 26 cases (m = 0.64% ± 0.26% of V_{coll}) never exceeded the normalized size of even one inhibitory quantum previously determined in this system (1, 6, 12). As noted above, the normalization uses the compound collateral IPSP (Fig. 1*B*), that is V_{coll}, as an indicator for the Cl^- driving force. We found no significant difference in this parameter between neurons giving obvious responses (20.0 ± 6.3 mV) and those which were weak or silent (17.6 ± 4.7 mV; P = 0.08, t test) meaning that ineffectiveness was not due to an insufficient driving force for the generation of IPSPs.

Morphological Characteristics of Ineffective Neurons. The lack of significant transmission from this class of interneurons raised the question of their connectivity. This problem was addressed in eight pairs where collateral IPSPs were larger than average (21.2 ± 4.7 mV). As shown in Fig. 2, we found that the number and the distribution of the synaptic contacts on the postsynaptic cell of this series were similar to those of previously analyzed "potent" cells (ref. 1; see also *Discussion*). Most synaptic boutons had a characteristic end bulb shape (Fig. 2A_1), and they could form tightly packed clusters, which then made it difficult to count them (Fig. 2B_1). The number of boutons ranged from 7 to 20 and from 14 to 75 for the commissural (n = 5) and collateral (n = 3) cells, respectively. Sixty-five percent of them were grouped within the axon cap (Fig. 2A_2), where the majority synapsed on the M-cell soma. Outside the axon cap, 60% impinged on the soma and the remaining 40% were distributed on the lateral dendrite except in one case (Fig. 2B_2).

Presynaptic Manipulation of Synaptic Strength. Attempts were made to facilitate inhibitory transmission by injecting axons with substances that increase transmitter release, and they showed that functional contacts provide an appropriate material for studying *in vivo* the protein machinery involved in neuronal secretion. We first used 4-aminopyridine (4-AP), taking broadening of presynaptic impulses (14) as an indicator of successful injections. As expected, in 16 of the 21 functional cells which did develop a long-lasting "plateau potential" (Fig. 3A_1), this effect was associated with a large potentiation of the unitary responses. Immediately after impalement, the mean normalized unitary IPSP was 5.64% ± 4.4% of V_{coll}, and their amplitudes doubled during drug action (m = 200% ± 62.4%). In the case of weak connections (n = 7), when 4-AP effectively prolonged the duration of presynaptic impulses (n = 4),

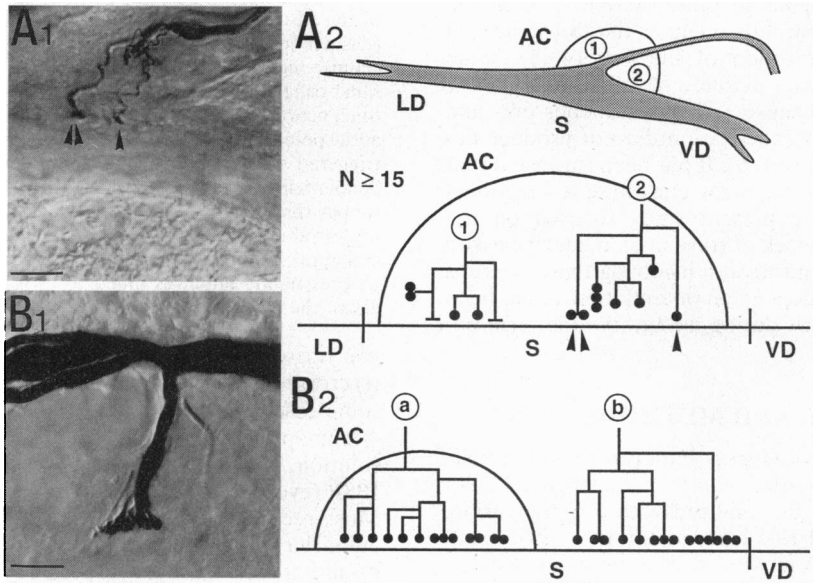

FIG. 2. Structural evidence for synaptic contacts between biocytin-filled weak inhibitory interneurons and the M-cell. (A_1) Light micrograph of a section used for assessing the number of synaptic boutons issued by a commissural interneuron. (Bar = 10 μm.) (A_2) Schematic drawing of its terminal ramifications. Some of them (at least five) were in direct contact with the M-cell soma; in the axon cap (AC, outlined by a circle) their only possible targets are M-cell cap dendrites (13). Synaptic knobs are represented by dark circles, and blind end terminals, as T shaped. Since the latter could issue several boutons, histological n is at least 15. Corresponding processes are indicated in A_1 and A_2 by arrowheads. LD, lateral dendrite; S, soma; VD, ventral dendrite. (B_1 and B_2) Same presentation as above, for a collateral interneuron; synaptic boutons shown in B_1 were tightly clustered and their exact number could be determined only by focusing at different planes; this cluster is labeled b in B_2. The total synaptic complement of this cell was at least 37, but only endings impinging on the soma (i.e., 27 of them) are illustrated here; the remaining ones synapsed on the ventral dendrite.

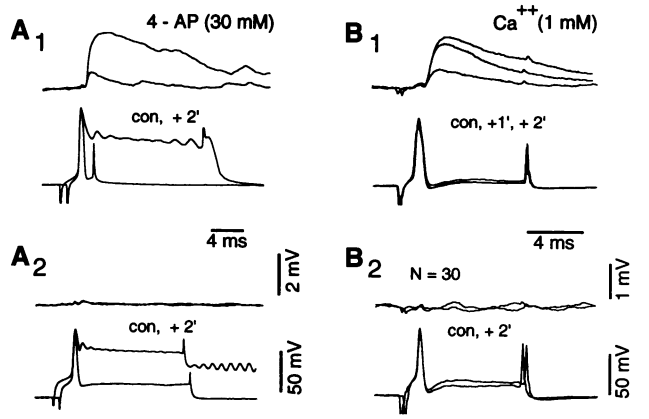

FIG. 3. Weak connections are not unblocked by presynaptic injections of either 4-AP or Ca^{2+}. (*A*) Comparison of the effect of 4-AP on a potent cell (A_1) and a weak cell (A_2). Superimposed averaged recordings of IPSPs (n = 30) evoked in the M-cell (upper sweeps) by direct stimulation of two different presynaptic interneurons (lower sweeps) immediately after their impalement with a 4-AP-containing microelectrode (con) and at the indicated time (e.g., 2 min) thereafter. The experiment shows that the diffusion of 4-AP in the potent cell (A_1) was followed by a marked increase of the postsynaptic response, whereas there was no effect in the case of the weak contacts (A_2). (*B*) Same representation as in *A*, but for Ca^{2+} injections. Here Ca^{2+} potentiates the postsynaptic effect of the potent cell (B_1) but not of the weak cell (B_2).

synaptic transmission was not facilitated (Fig. $3A_2$), except once where the response increased from 0.3% to as little as 0.79% of V_{coll}.

Second, another set of 31 axons was impaled with Ca^{2+}-containing microelectrodes, the rationale being that diffusion to the terminals might lead to a larger peak Ca^{2+} concentration following an impulse. Indeed (Fig. $3B_1$) in 14 of 17 cases

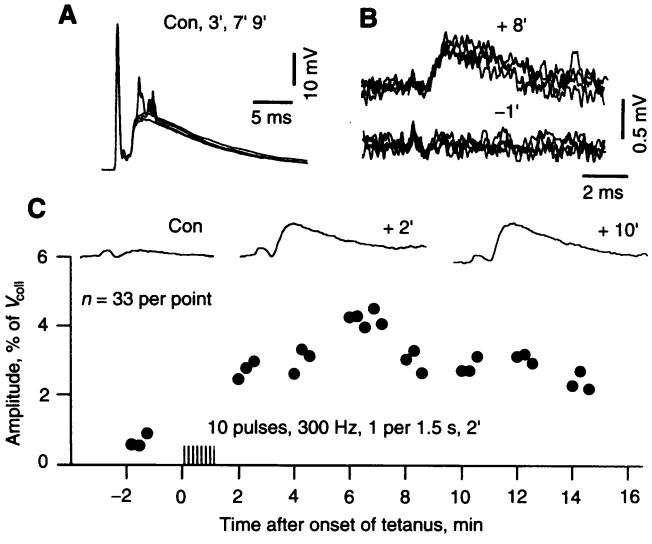

FIG. 4. Unmasking of synaptic transmission after tetanic stimulation. (*A*) Superimposed averages (n = 3) of antidromic spikes and subsequent collateral IPSPs, with a slight decrease of the latter throughout the recording period. Con, control before tetanus. (*B*) Superimposed unitary IPSPs recorded at high gain 1 min before (lower) and 8 min after (upper) tetanization of the contralateral eighth nerve (stimulus parameters are indicated in *C*). (*C*) Time course of the synaptic potentiation obtained during the same experiment; amplitudes are normalized as a percentage of V_{coll}. Averaged responses (n = 99) evoked at the rate of 2 per s are shown above the graph. Note that averaging all control sweeps revealed a small IPSP that was indistinguishable from the background instrumental noise in single traces (same experiment as in Fig. 2*B*).

where the electrode contained 1 mM $CaCl_2$, unitary IPSPs evoked from functional neurons began to increase within 10–60 s after penetration, finally doubling in amplitude (m = 190% ± 87%). With 0.5 mM (n = 6) the success rate and mean potentiation decreased to 66% and 133% ± 16%, respectively. Again, results were different when the mean control response was less than 1% of V_{coll}: concentrations of 0.5 mM were ineffective in two axons, as was 1 mM (Fig. $3B_2$) in five of the six latent interneurons.

Activation of Weak Connections. We looked for a possible enhancement of efficacy at these synapses in 13 experiments during which V_{coll} remained stable or was slightly smaller after tetanization of the contralateral eighth nerve (see *Material and Methods*). For 9 of them (6 commissural and 3 collateral), synaptic transmission was already potentiated by the end of the tetanus, and this enhancement persisted as long as the penetrations could be maintained (m = 7.7 ± 3.5 min after tetanus onset). In 6 cases, a small control postsynaptic depolarization (m = 0.73% ± 0.25% of V_{coll}) was present, and no response was detected in the 3 other connections. The maximal potentiated amplitudes of the IPSPs ranged from 1.50% to 6.7% of the collateral inhibition, with a mean of 4.14% ± 1.9% (n = 9). It should be noted that these values approached those obtained at nonconditioned functional connections (2). Results from the longest of these experiments are shown in Fig. 4. Two additional cells, which were first shown to be unaffected by 1 mM intraaxonal Ca^{2+}, became active after a tetanus, demonstrating the functional identity of the inhibitory neurons studied here.

DISCUSSION

Latent neurons may be common in the central nervous system, since in the absence of electrophysiological criteria which allowed them to be identified unambiguously as presynaptic, they would have been considered as not connected, and discarded. Here they form a subset of inhibitory interneurons which are functionally disabled, a conclusion derived from the discrepancy between their morphological connectivity with the postsynaptic cell and the absence or extremely small size of evoked responses. In an attempt to determine if these neurons were truly silenced, we reexamined successive sweeps by eye in five experiments where averaging had revealed small putative responses, and we constructed separate averages of those that contained a waveform resembling a unitary IPSP and those that did not. In four of them the first group yielded a small mean response with appropriate kinetics which could not be explained by the random occurrence of a few large isolated spontaneous events. Averages of the apparent failures did not have sufficiently stable baselines to allow a definitive conclusion as to the absence or presence of a hidden response. Thus, although some of these connections may not be totally ineffective, they are still distinct from those studied previously (12). Indeed, given the range of the number of contacts (7 to 75) found for the weak interneurons, one would, from previous work (2), expect the normalized IPSP to be distributed between 2% and 37% of the collateral response. Furthermore, it is important to note that an extensive ultrastructural study in the peripheral part of the axon cap (1) did not reveal any heterogeneity (e.g., with respect to the number of presynaptic specializations, or glycine receptors facing release sites) that could point to a population of defective junctions, and all the glycinergic boutons contained at least one active zone with a unimodal distribution of areas (15).

The most likely origin of the weak transmission at these connections is presynaptic, since their terminals are intermingled with those of potent ones, and it could be due to a low or zero probability of release in most synaptic endings. Ineffective synapses have been described, such as the M-cell mixed excitatory connections (16), and some Ia-to-motoneuron syn-

apses (17, 18), although in both of these cases transmission is restored by 4-AP (16, 19). The inability of 4-AP and Ca^{2+} to potentiate IPSPs at weak connections leads us to suggest that a number of release sites are not functional and that the deficit most likely involves the biochemical cascades implicated in triggered release (20) or vesicle docking. Alternatively, a possible postsynaptic mechanism includes an unresponsiveness of glycine receptors which was revealed by a synergism between functional and "silent" synapses (6).

Inhibitory transmission in the system studied here can be enhanced for prolonged periods after tetanic stimulation of vestibular afferents (21). This phenomenon, which has been recently found in the developing visual cortex (22), was also called inhibitory LTP, because although it is not clear whether it is in all respects equivalent to classical LTP of excitatory junctions as in the hippocampus (23, 24), it exhibits the same temporal characteristics and synapse specificity. While the *in vivo* paired recordings thus far did not last sufficiently long to establish the reality of long-term changes of unitary responses, it is interesting to speculate that potentiation of weak connections could play a important role in this form of network plasticity. Furthermore, it cannot be excluded that activation of parallel pathways (e.g., serotoninergic fibers, see ref. 25) contributes to this phenomenon.

At excitatory synapses it has been shown that connections having a low probability of release, P, are more likely to be facilitated than those with a high one (26) and that the number of failures of transmission decreases during LTP (27). This notion is consistent with observations suggesting that P values can be heterogeneous (28, 29) and that connections with lower ones are more sensitive to drugs that enhance transmitter release. Hence, it was postulated that they provide the basis for activity-dependent changes in synaptic efficacy (ref. 29, but see ref. 28). Indeed, we found that facilitation was more frequent and more effective at weak than at "normal" connections; in the latter, potentiation occurred in 7 of 18 pairs, with a mean increase of 64% ± 32% relative to the control (8), versus a mean enhancement of 375% ± 182% (n = 6) in 9 of 12 weak neurons, when the presence of a small initial IPSP made comparisons possible.

Finally, it may be suggested that central connections enter a latent state, for instance when levels of activity are low or due to active processes supporting depression (30). The distribution of synaptic strengths in a population of neurons would thus constantly be subject to dynamic shifts between two distinct states. In the case of the M-cell, this process would in turn result in continuous adjustments in the magnitude of inhibition capable of opposing the excitatory sensory input that triggers the escape response.

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