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Long-term transformation of an inhibitory into an excitatory GABAergic synaptic response

(*Hermissenda*/associative learning/Cl^- channel/K^+ channel/Ca^{2+} imaging)

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ABSTRACT **For a constant membrane potential, a predominantly inhibitory GABAergic synaptic response is shown to undergo long-term transformation into an excitatory response after pairing of exogenous γ-aminobutyric acid (GABA) with postsynaptic depolarization or pairing of pre- and postsynaptic stimulation. Current- and voltage-clamp experiments suggest that this synaptic transformation is due to a shift from a net increase of conductance to a net decrease of conductance in response to GABA. GABA-induced elevation of intracellular calcium is prolonged after the same stimulus pairing and may, therefore, contribute to this synaptic transformation via Ca^{2+}-activated phosphorylation pathways. This synaptic transformation, which does not follow unpaired stimulus presentations, occurs in a neuronal compartment spatially separated from the soma, which also changes during stimulus pairing.**

Pavlovian conditioning exhibits a number of distinguishing features for a wide range of species including humans, other mammals, arthropods, and even the snail *Hermissenda crassicornis* (1). Because the visual–vestibular pathways of *Hermissenda* mediate Pavlovian conditioning and are now known in great detail, we decided to further characterize the synaptic compartment of the type B cell, whose soma compartment (60–80 μm away from the synaptic region) has been extensively analyzed.

Hair cells (HCs) in the caudal portion of the *Hermissenda* statocyst produce synaptic inhibition of photoreceptors within the ipsilateral eye by a presumed monosynaptic effect (1). More recently (2, 3), a number of experiments suggested the monosynaptic release of γ-aminobutyric acid (GABA) (4–6) from presynaptic HC terminals onto postsynaptic type B photoreceptor branches. GABA was localized in the presynaptic cells by HPLC, immunocytochemical, and mass spectrophotometric criteria (3). Because the type B cell and the HC synaptic input to the type B cell are activated during Pavlovian conditioning (1), we examined the long-term effects of pairing GABA (or presynaptic activation) with light or postsynaptic depolarization. Additional experiments were designed to suggest possible ionic and second messenger mechanisms for future research.

MATERIALS AND METHODS

Electrophysiology. Two-microelectrode recording techniques for *Hermissenda* neurons have been described (7–9). The type B photoreceptor was impaled with two intracellular somatic microelectrodes and connected to a bridge amplifier for conductance measurements or to a two-electrode voltage-clamp amplifier (Pelagic Electronics, Falmouth, MA) to measure ionic currents. To measure the decay of passively spreading electrical potentials, one microelectrode was inserted into the type B cell soma, a second was inserted in its axon (simultaneously), and a third was inserted in a neighboring type B cell (as described in ref. 7). This configuration confirmed a voltage decay for nonactive potentials of 20–25% from soma to axon or axon to soma.

For measurements of pre- and postsynaptic electrical signals, a microelectrode was inserted into the type B cell soma and, simultaneously, a second microelectrode was inserted into an ipsilateral caudal HC.

A blunt micropipette filled with 30 mM GABA was placed near the synaptic endings (60–80 μm away from the B cell body) of both the type B cells of the eye and the caudal HC of the statocyst. GABA puffs along the proximal axon or cell body or on axotomized B cells failed to elicit responses. Three-second pressure pulses of 10 psi (1 psi = 6.9 kPa) ejected GABA solution droplets of 600 pl. The preparation was kept at 22°C in a chamber of about 1 ml and continuously perfused with a pump (Micron Systems, Bethesda, MD) at about 5 ml/min. It was estimated from the rate of bath volume replacement due to continuous external perfusion and ionic mobility that GABA pressure pulses or "puffs" with a pressure injector (General Valve, Fairfield, NJ) caused a final concentration of ≈10 μM in the vicinity of the branches. The extracellular medium (and the GABA solutions) was artificial seawater (ASW) containing 10 mM KCl, 50 mM $MgCl_2$, 10 mM $CaCl_2$, 430 mM NaCl, and 10 mM Hepes (pH 7.4). Osmolarity was 990 milliosmolar. Zero Na^+ solutions were made by replacing NaCl with equimolar concentrations of tetramethylammonium chloride, and 0 Ca^{2+} solutions were made by replacing Ca^{2+} with Ba^{2+}. For current-clamp experiments, pulse generation and data acquisition and analysis were performed with PCLAMP and AXOTAPE (Axon Instruments, Burlingame, CA). Only pulse generation and timing were controlled by PCLAMP in voltage-clamp experiments. None of the voltage-clamp records were digitized. The current records in the figures are the undigitized signals recorded on tape and then fed into a Brush pen recorder. Immediately after impalement, a resting membrane potential of −45 mV was measured, and after 10 min of dark adaptation, it reached −60 mV. Voltage and current GABA responses were elicited at different membrane potentials from their resting level or holding potential of −60 mV.

Calcium Imaging. Fura-2 was iontophoretically injected into the B cells with a black-coated microelectrode, the tip of which contained 6 mM fura-2 pentapotassium salt (Molecular Probes) in 200 mM KOAc and whose barrel was filled with 3 M KOAc (10). A constant hyperpolarizing current of −2.0 nA for 10–15 min produced adequate staining of the axonal terminal branches, located 80 μm away inside the neuropile.

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Abbreviations: GABA, γ-aminobutyric acid; HC, hair cell; ASW, artifical seawater.

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The experiments were performed in 0 Na^+ ASW to minimize the voltage-dependent calcium influx induced by the spread of action potentials.

Cell fluorescence was measured by using a Ca^{2+}-imaging system (ARGUS 50, Hamamatsu Photonics, Hamamatsu, Japan), which consists of an inverted microscope with a 100-W xenon lamp, a rotating filter changer, a SIT (silicon-intensified target) camera, and an imaging processor under the control of an IBM compatible computer. For excitation, 340-nm and 380-nm bandpath filters and a neutral-density filter were used. Images of fluorescence were obtained by using a 400-nm dichroic mirror and a 510-nm long-pass barrier

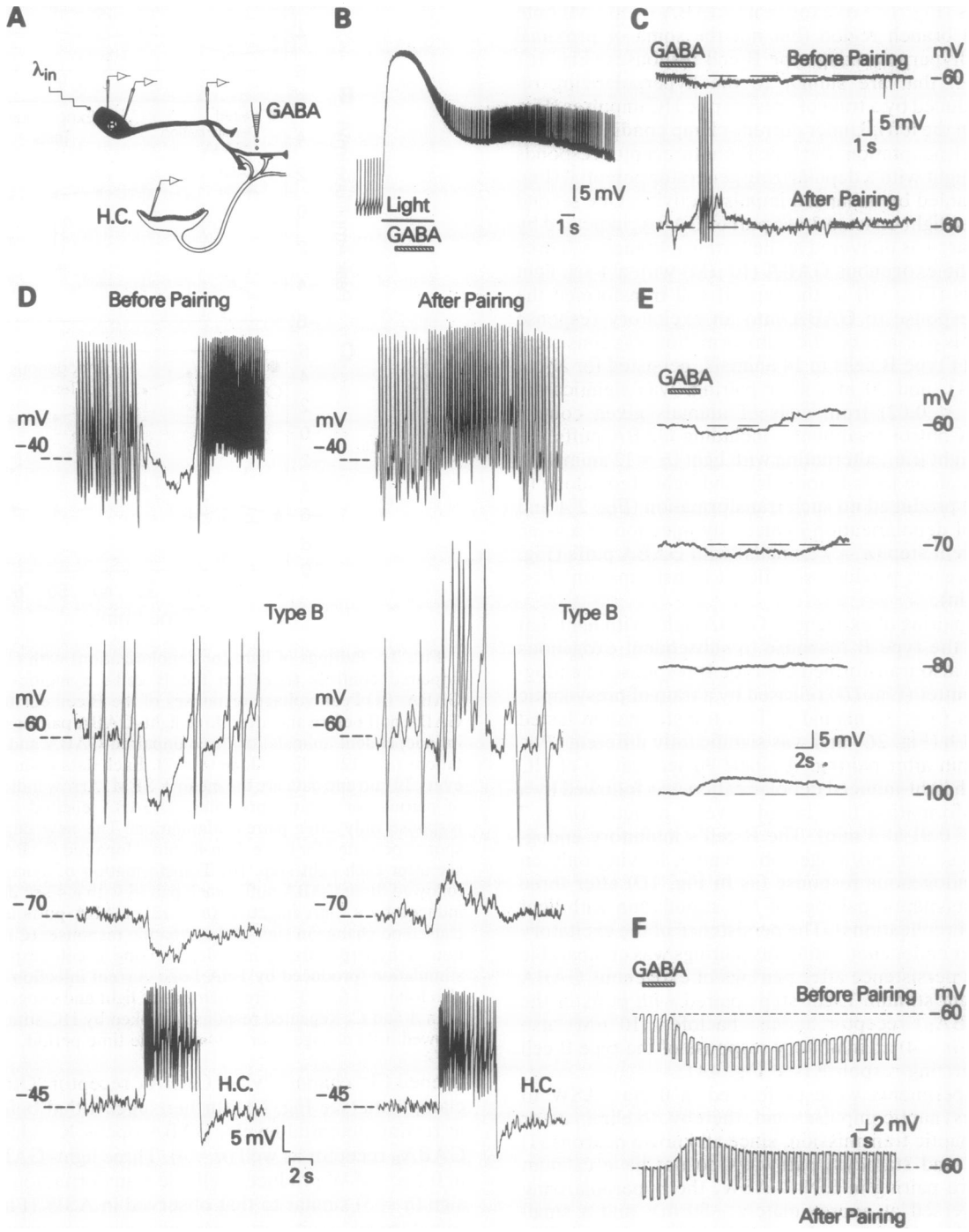

FIG. 1. Type B responses to GABA and HC impulses are transformed from inhibitory to excitatory after light–GABA pairings. (*A*) Diagram of the experimental preparation (7). (*B*) Current-clamp recording of type B response to light and GABA. (*C*) After three light–GABA applications at 90-sec intervals, the initial hyperpolarizing response to GABA (upper trace) was transformed into an excitatory response (lower trace). (*D*) After three light–GABA pairs, the B cell's endogenous hyperpolarizing response (left traces) to HC stimulation with 1-sec 1-nA current pulses (bottom traces) was transformed into depolarization (right traces). Both hyperpolarizing and depolarizing responses became negligible at about −80 mV. The traces here are typical examples of 12 replications in 12 animals. Some impulse peaks were filtered from the records. (*E*) Current-clamp recordings of the postsynaptic response to GABA in 0 Na^+ ASW. The early hyperpolarization had a reversal potential of about −80 mV, and the later depolarizing phase was minimal at potentials more negative than −60 mV. (*F*) Hyperpolarizing responses (before pairing) evoked by GABA puffs are accompanied by a decrease of input resistance of the B cell, whereas depolarizing responses (after pairing) are accompanied by an increase of input resistance measured by 200-msec hyperpolarizing (−1 nA) pulses. Dashed lines indicate level (−60 mV) of resting membrane potential.

filter. The objective lens was a 10× Nikon UV fluor, and images were enlarged by 8 times on the monitor to focus images of the B cell's axonal branches.

RESULTS AND DISCUSSION

Transformation of Synaptic Voltage Responses. Pressure-ejected puffs (Fig. 1*A*) of exogenous GABA (≈10 μM) onto the terminal branch region (but not the soma or proximal axon) elicit hyperpolarizing type B cell responses (Fig. 1*C*, upper traces) that are similar to the hyperpolarizing responses produced by a train of presynaptic HC impulses (Fig. 1*D*, traces on the left). Under current-clamp conditions, after 10 min of dark adaptation, the type B photoreceptor responds to a flash of light with a depolarizing generator potential (Fig. 1*B*) accompanied by enhanced impulse activity. One second after the 4-sec light onset, a 3-sec puff of GABA, indicated by horizontal bar, was delivered at the terminal branches. Three pairings of the exogenous GABA (10 μM) with a 4-sec light step stimulus (Fig. 1*B*) to the type B cell transformed the inhibitory response to GABA into an excitatory response (Fig. 1*C*). This pairing-specific transformation was consistent for each of 14 type B cells in 14 animals, persisted for about 1 h (Fig. 2*A*), and 30 min after pairing, was significantly different ($P < 0.02$) from cells of animals given control treatments. Control treatments, including GABA puffs unpaired with light [i.e., alternating with light ($n = 12$ animals)], GABA puffs alone ($n = 8$ animals), and light steps alone ($n = 7$ animals) produced no such transformation (Fig. 2 *A* and *B*). Pairing of depolarization induced by injection of a 4-sec positive current step ($n = 7$ animals) with GABA puffs (Fig. 2*B*) did, however, produce significant transformation ($P < 0.05$ at 30 min).

The same pairing of exogenous GABA puffs with light that transformed the type B response to subsequent exogenous GABA puffs also transformed the type B response to endogenous transmitter (Fig. 1*D*) released by a train of presynaptic HC impulses ($n = 12$ animals). This transformation lasted longer than 1 h (Fig. 2*C*) and was significantly different ($P < 0.01$) at 30 min after pairing. A single 30-sec pairing of HC impulses with light-induced depolarization was followed by a similar transformation that lasted several minutes ($n = 7$ animals; $P < 0.01$ at 3 min). The B cell's inhibitory endogenous response was no longer observable, leaving only an excitatory endogenous response (as in Fig. 1*D*) after three pre- and postsynaptic pairings of HC stimulation with light stimuli ($n = 4$ replications). The persistence of the excitatory response after endogenous stimulus pairings was comparable ($n = 4$) to its persistence after pairings of exogenous GABA puffs with light stimuli. Light steps paired with puffs of the type B ($GABA_B$) receptor agonist baclofen (10 μM) also transformed ($n = 4$) an inhibitory response of the type B cell into a depolarizing response (see Fig. 4*A*).

Similar experiments were performed in 0 Na^+ ASW to eliminate presynaptic impulses and, thereby, to eliminate or minimize synaptic transmission, since all known neurons (1) in the visual and statocyst pathways do not have calcium spikes. Before pairing in 0 Na^+ ASW, the hyperpolarizing response reversed at approximately −80 mV and a small delayed depolarization was absent (Fig. 1*F*) or minimal (Fig. 1*E*) at potentials less than or equal to −60 mV. These potentials were confirmed ($n = 4$) by using two microelectrodes within the soma of same type B cell, one for recording membrane potential and the other for injecting current through a balanced bridge circuit and for recording potential. Consistent with previous observations (2), the type A ($GABA_A$) receptor blocker bicuculline (100 μM) markedly reduces ($n = 5$) the early hyperpolarizing response to GABA, as did the Cl^- channel blocker picrotoxin (10 μM). These current-clamp data suggest that before pairing GABA first

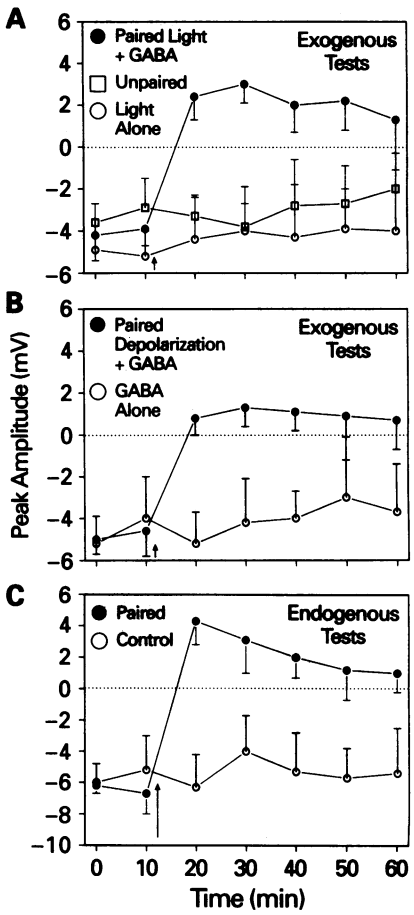

FIG. 2. Pairings of light (or depolarization) with GABA but not unpaired controls transform the B cell's synaptic responses to GABA. (*A*) Peak voltage responses of the B cell elicited by a 3-sec GABA puff before and after three light–GABA pairings ($n = 14$ cells in independent animals) or three unpaired GABA and light presentations ($n = 12$) or light alone ($n = 7$). Each data point was obtained every 10 min and data are the mean ± SEM. Arrow indicates the time of pairings or control procedures. GABA elicited a depolarizing response only after paired stimulation. The new depolarizing response persisted for more than 40 min. Unpaired GABA occurred 40 sec after each light step. (*B*) Transformation of synaptic sign also followed three GABA puffs, each paired with a 4-sec depolarization induced by a 2-nA injection ($n = 12$). GABA puffs alone ($n = 8$) caused no change in sign of the synaptic response. (*C*) Transformation of hyperpolarizing into depolarizing B-cell responses to HC stimulation (produced by 1-nA 1-sec current injection into the HC) also followed ($n = 12$) three pairings of light and exogenous GABA, as in *A* and *C*. Repeated responses evoked by HC stimulation alone showed little change over a comparable time period.

opened Cl^- channels via a $GABA_A$ receptor. Other current-clamp data (see Fig. 4*A*) further suggest that before pairing, at resting potential levels, GABA opens K^+ channels via a $GABA_B$ receptor as well ($n = 4$). Three light–GABA pairings in 0 Na^+ ASW produced reliable transformation of synaptic sign ($n = 5$) similar to that observed in ASW (Fig. 1*F*). The transformed excitatory response was accompanied by a prolonged decrease in conductance (Fig. 1*F*) that is apparent immediately after the peak depolarizing response (which itself may contribute to some voltage-dependent rectification of the membrane). In contrast, the untransformed inhibitory response was accompanied by an increased conductance (Fig. 1*F*).

Ionic Currents During Synaptic Responses. For measurement of ionic currents (Fig. 3), all GABA responses were recorded after the voltage-clamp system had brought the type B cell membrane potential to a steady-state level. Because of

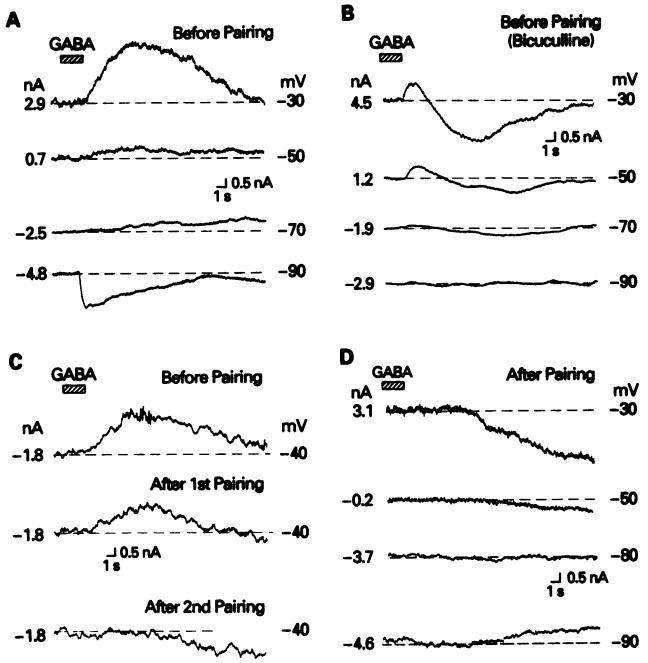

FIG. 3. GABA-induced currents are modified by pairing. (*A*) Early outward currents recorded in 0 Na^+ ASW. Cells were held in steady state at the membrane potentials indicated on right with the holding currents indicated on left of each record. GABA puffs elicited outward currents that reversed at −80 mV and were greatly reduced by 10 μM picrotoxin or 100 μM bicuculline. Dashed line indicates level of steady-state current. (*B*) In bicuculline, a small early outward transient current remained followed by a later inward current. Both currents were eliminated by replacing Ca^{2+} with Ba^{2+} and were negligible at potentials less than or equal to −80 mV. (*C*) Pairing of GABA with light caused the outward current to progressively decrease and become inward. Note the long latency of the inward current. (*D*) After pairing, GABA elicited an inward current that reversed at −80 mV. All records are typical of at least four replications.

the slow kinetics and moderate amplitudes of the responses recorded at steady-state membrane potentials, the dynamics of the voltage-clamp system were more than adequate to maintain isopotentiality and to follow the currents induced with the necessary temporal resolution.

In 0 Na^+ ASW, GABA induced an outward current that reversed at approximately −80 mV (Fig. 3*A*). Application of bicuculline or picrotoxin left a small early outward current that became negligible at approximately −80 mV and a sustained delayed inward current that became negligible at potentials less than or equal to −70 mV (Fig. 3*B*). Replacement of Ca^{2+} with Ba^{2+} in 0 Na^+ ASW containing bicuculline eliminated the GABA-induced early outward current and the later inward current. In the absence of blockers, the GABA-induced outward current progressively decreased and became inward with light–GABA pairings (Fig. 3*C*). This post-pairing inward current reversed at approximately −80 mV (Fig. 3*D*). In bicuculline, GABA produced an isolated post-pairing inward current that also reversed at approximately −80 mV.

Puffs of baclofen elicited an outward current followed by a delayed inward current that was particularly apparent at holding potentials more positive than −60 mV. In 0 Na^+ ASW, three light–baclofen pairings eliminated any early outward current, leaving the baclofen-induced inward current, which became negligible at approximately −80 mV (Fig. 4*B*). These currents elicited by baclofen were eliminated when Ba^{2+} (to which the type B cell's Ca^{2+} channels have been shown permeant; see ref. 9) was used instead of Ca^{2+} in the external medium (Fig. 4*C*).

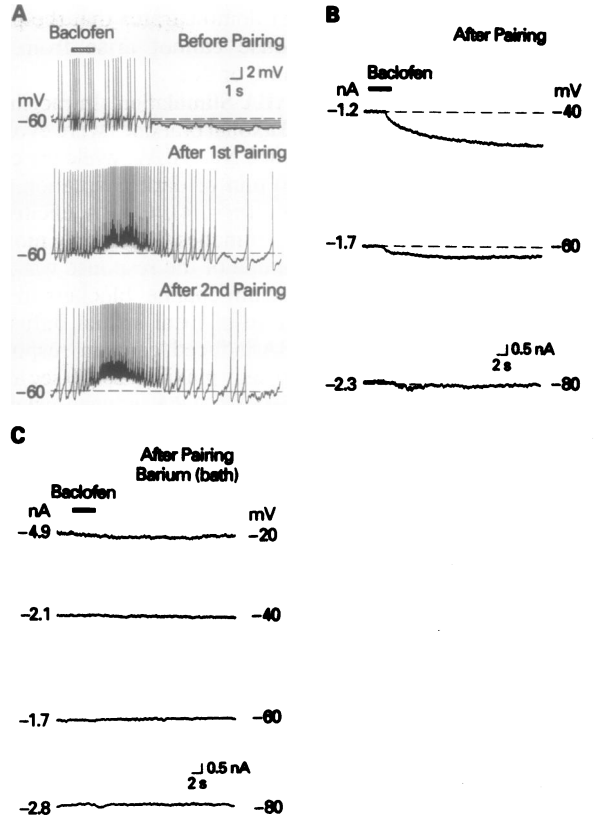

FIG. 4. Change in the synaptic sign was also observed when baclofen (10 μM) instead of GABA was paired with light. (*A*) Under current-clamp conditions, an initial hyperpolarizing response was transformed into a depolarization after one and then two light–baclofen pairings. (*B*) $GABA_B$-induced currents by baclofen are inward after pairing and are Ca^{2+}-dependent. Post-pairing inward currents elicited by baclofen become negligible at −80 mV. (*C*) Replacement of external Ca^{2+} with 10 mM Ba^{2+} causes the inward current to be reversibly abolished, suggesting the absence of a GABA-induced inward Ca^{2+} current.

These voltage-clamp data suggest that before pairing, GABA acts via $GABA_A$ receptors to increase Cl^- conductance and via $GABA_B$ receptors to increase K^+ conductance. The data further suggest that, before pairing, GABA decreases K^+ conductance via a $GABA_B$ receptor at holding potentials more positive than the resting level. This K^+ conductance decrease is suggested because (*i*) before pairing, GABA elicits a delayed depolarization at holding potentials greater than or equal to −60 mV, (*ii*) in bicuculline, GABA elicits an inward current with a reversal at −80 mV, and (*iii*) baclofen elicits a delayed inward current particularly apparent at holding potentials greater than or equal to −60 mV. Stimulus pairing appears to eliminate the $GABA_A$-mediated increase of Cl^- conductance and the GABA-mediated increase of the K^+ conductance but apparently leaves a $GABA_B$-mediated K^+ conductance decrease. This shift from a net increase of conductance to a net decrease of conductance in response to GABA appears to be responsible for transforming the sign of the synapse from inhibition to excitation. All of the data in current-clamp and voltage-clamp recordings indicate underlying conductances with a reversal potential more negative than the resting level (−60 mV). It is not possible, therefore, for the post-pairing depolarizing response associated with a conductance decrease to arise from increased conductance changes to an ion (e.g., Ca^{2+} or Na^+) with an equilibrium potential more positive than the resting level. The finding that no GABA response occurs in 0 Na^+ ASW containing Ba^{2+} instead of Ca^{2+} (through Ba^{2+}-

permeant Ca^{2+} channels) further demonstrates that the pairing-specific depolarizing response cannot arise from increased conductance to Ca^{2+} and/or Na^+.

Calcium Responses During GABA Stimulation. Intracellular calcium responses at the terminal axonal branches were evoked by 3-sec pulses of 10 μM GABA in 0 Na^+ ASW, while the cells were held at −60 mV during 10 min of dark adaptation. As shown in Fig. 5*A*, before pairing (n = 4), GABA elicited a localized calcium elevation in the branches that rapidly moved toward the cell body. The magnitude of the response was not changed with low calcium or calcium channel blockers in the external solution (n = 5). Because of local spatial diffusion barriers, the onset of the GABA-induced calcium response showed some delay, but it was typically maximal 20–30 sec after GABA stimulus offset. The onset of GABA-induced calcium elevation closely followed the onset of the hyperpolarizing voltage responses recorded with the intracellular microelectrode. Calcium responses were monophasic and lasted for periods no longer than 1 min. This kind of GABA-induced calcium response has been previously reported in rat cerebellar granule cells (11) and in newborn rat visual cortex slices (12). After pairing, GABA induced significantly longer calcium responses (70–80 sec) but did not change their magnitude or localization (n = 4). Unpaired controls (n = 4) did not induce changes of the calcium responses.

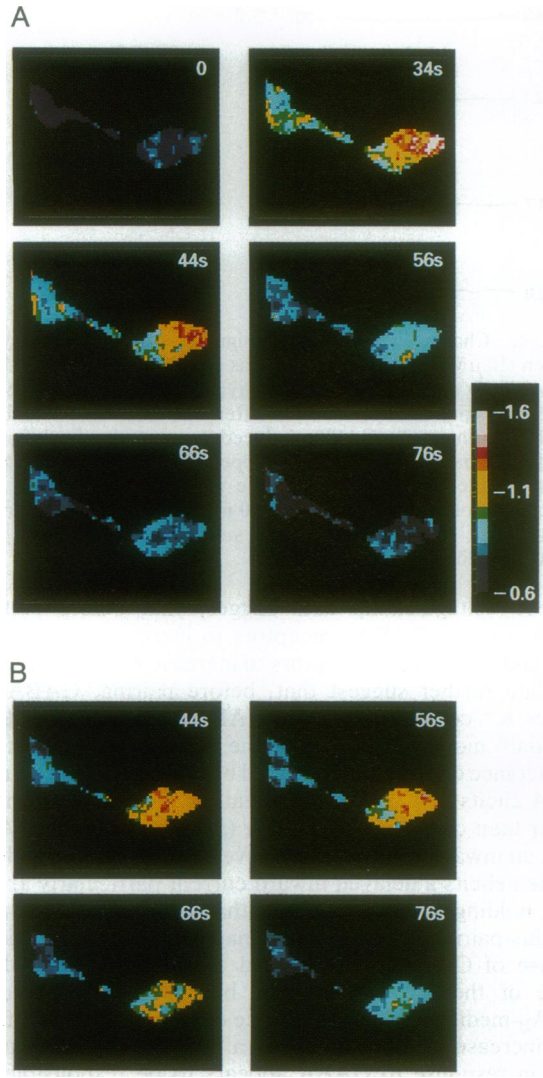

FIG. 5. GABA-induced calcium responses in terminal branches of a type B cell are prolonged after pairings. Fluorescence radio images (340 nm/380 nm) were obtained every 2 sec for this single fura-2-injected type B cell bathed in 0 Na^+ ASW. Individual B-cell terminal branches are not resolved but appear instead as a solid oval. A 3-sec 10 μM GABA pulse was delivered in the vicinity of the axonal branches at 10 sec, and responses were maximal in the terminal branches at 34 sec. (*A*) Before pairing, the calcium signal moved toward the cell body and ended at about 60 sec. (*B*) The signal in the terminal branches persists about 70 to 80 sec after pairings of GABA with light steps.

The prolonged rise of intracellular calcium after GABA paired with depolarization could contribute to the observed shifts of synaptic sign. Furthermore, recent experiments (D.L.A., C.C., and E.I., unpublished observations) showed that pharmacologic blockade of Ca^{2+}-activated kinases with staurosporine prevents the GABA-induced Ca^{2+} signals. We speculate that GABA-elicited opening of Cl^- and K^+ channels is reduced or eliminated by prolonged activation of Ca^{2+}-activated phosphorylating pathways (cf. refs. 13–15). Such calcium-mediated reduction of ionic currents has been implicated in long-term associative memory of molluscan (16) and mammalian (17) preparations.

Previously observed synaptic sign changes were not pairing-specific and lasted only 10–20 sec (18) or could not be localized to a single synapse (19). In our study, three pairings, chosen to approximate stimulus pairings presented during training of the living animals (20, 21), cause transformation lasting more than 1 h. Considering that animals are trained with 90 such pairings on each of three successive days, this GABA synapse transformation is clearly implicated in associative memory formation. Furthermore, changes at this GABA synapse occur in addition to previously observed changes in the soma compartment of the same type B neuron. These two compartments, separated by 60–80 μm, could interact during acquisition and storage of an associative memory. Given the vast number of GABAergic synapses in the mammalian cerebral cortex (22), the pairing-specific GABA-mediated transformation may have significance for mechanisms of mammalian associative memory as well.

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