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# Serotonergic control of the hippocampus via local inhibitory interneurons

(feedforward inhibition/disinhibition/γ-aminobutyrate/calbindin/parvalbumin)

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ABSTRACT **Information flow and processing in hippocampal neuronal networks is determined by a wide range of inhibitory mechanisms [e.g., feedforward or feedback, γ-aminobutyrate (GABA) A or B receptor-mediated, perisomatic shunting, or distal dendritic inhibition], each subserving specialized functions. These forms of local inhibition are mediated by morphologically and neurochemically well-defined, mostly GABA-containing, interneurons, which control large populations of principal cells through their extensive axonal arborizations. These neurons can serve as ideal targets for subcortical pathways, such as those originating in the septum or raphe, which exercise a global control over hippocampal activity. This intriguing possibility prompted us to study whether the profound effect of the serotonergic raphe–hippocampal pathway is mediated by inhibitory interneurons or whether a direct diffuse action on the principal cells is dominant. We demonstrate that axons of this pathway form multiple synaptic contacts with hippocampal GABAergic interneurons. Interestingly, the serotonergic afferents selectively innervate the somata and dendritic trees of GABAergic neurons that contain the 28-kDa calcium-binding protein calbindin $D_{28K}$, but never those that contain another calcium-binding protein, parvalbumin. These results show that the mechanism by which the serotonergic pathway may exert a powerful influence on hippocampal function involves the modulation of local inhibitory circuits. Furthermore, the selectivity in the choice of target GABAergic interneurons suggests a strong functional specialization among inhibitory circuits, as well as among the subcortical input pathways originating in the septum and raphe.**

A wide variety of inhibitory mechanisms regulate information flow and processing in hippocampal neuronal networks (1–5). Inhibition is mediated by extensively arborized, mostly γ-aminobutyrate (GABA)-containing, interneurons that control large numbers of principal cells. These neurons can serve as targets for subcortical pathways (6), such as those originating in the septum (7, 9) or raphe. The serotonergic raphe–hippocampal pathway has a powerful effect on hippocampal electrical activity (8, 10), synaptic plasticity (11), and cognitive behavior (12, 13). Its function was suggested to include a behavioral state-dependent control of hippocampal activity (10, 14). Electrophysiological and pharmacological studies have shown that the effects of serotonin are mediated by at least six different types of receptors (15)—of which the 5-$HT_{1A}$ receptor has the highest concentration in the hippocampus—and that a typical effect of serotonin on hippocampal neurons is a hyperpolarization evoked by an increase in $K^+$ conductance, although a depolarization and reduction of afterhyperpolarization were also reported (16–19). However, no consensus has been reached as to which, if any, of these postsynaptic mechanisms was responsible for the observed functional effects. One reason for this uncertainty in interpretation is the sparse knowledge of the termination pattern and synaptic organization of the serotonergic afferents in the different layers and subfields of the hippocampus.

Another major subcortical pathway that has a profound effect on hippocampal electrical activity in spite of arising from a small number of neurons is the septohippocampal pathway (9). Buzsáki *et al.* (6) proposed that this pathway may be able to influence the firing pattern of large populations of principal cells via the feedforward activation of hippocampal inhibitory interneurons. Freund and Antal (7) showed that the GABAergic component of the septohippocampal pathway selectively innervates GABAergic interneurons in the hippocampal formation, forming multiple contacts with the majority of them. This synaptic organization is consistent with a powerful disinhibitory function and may explain the great efficacy of the septal input in driving hippocampal electrical activity.

In this study we aimed to establish whether the mechanism by which the serotonergic pathway controls hippocampal function also involves the modulation of local inhibitory circuits and, if so, whether the types of inhibitory interneurons mediating the functional effects of the raphe–hippocampal projection are the same as those innervated by the septohippocampal pathway.

## METHODS

Eleven adults rats (Wistar, male and female) were used for the *Phaseolus vulgaris* leukoagglutinin (PHAL)-tracing experiment, and four for the serotonin/calbindin $D_{28K}$ and serotonin/parvalbumin double-immunostaining experiment. Raphe–hippocampal axons were visualized by anterograde transport of PHAL (2.5%; Vector Laboratories) iontophoresed into the median raphe nucleus. Six to eight days later the animals were perfused first with saline (0.9% NaCl) for 1–2 min and then with a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 0.2% picric acid in 0.1 M phosphate buffer (pH 7.4) for 30 min. Animals for serotonin immunocytochemistry were perfused first with saline (1 min), then with 4% paraformaldehyde and 0.05% glutaraldehyde (5 min), and then with 4% paraformaldehyde and 1% glutaraldehyde (25 min) in 0.1 M phosphate buffer (pH 7.4). Blocks of the hippocampi and also the injection sites from the PHAL-injected animals were immersed in 10% and 20% sucrose, freeze–thawed in liquid nitrogen, sectioned on a vibrating microtome at 60–80 μm, and then extensively washed and processed for double immunocytochemistry (20).

**PHAL-Tracing Experiment.** Sections were incubated with primary antisera for 2–3 days: biotinylated goat anti-PHAL

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Abbreviations: GABA, γ-aminobutyrate; PHAL, *Phaseolus vulgaris* leukoagglutinin; DAB, 3,3'-diaminobenzidine.

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(1:200; Vector Laboratories) plus rabbit anti-calbindin $D_{28K}$ (R 202, dilution 1:1000; ref. 21), or biotinylated anti-PHAL (1:200; Vector Laboratories) plus rabbit anti-parvalbumin (R 301, 1:500; ref. 21). The second layer (4–6 hr) was avidin–biotinylated horseradish peroxidase complex (1:100; Vector Laboratories) plus goat anti-rabbit IgG (1:50; ICN). The first immunoperoxidase reaction was developed using 3,3′-diaminobenzidine (DAB) as a substrate and was intensified with ammonium nickel sulfate (black reaction product). The third layer (≈12 hr) was rabbit peroxidase-antiperoxidase complex (1:100; Dakopatts). The second immunoreaction was developed with DAB alone (brown reaction product). Tris (0.05 M)-buffered saline (pH 7.4) containing 1% normal goat serum and 0.5% Triton X-100 was used for all the washes and antibody dilutions. The sections were dehydrated on slides and embedded for light microscopy.

**Serotonin Immunocytochemical Experiment.** The immunostaining procedure was preceded by a 30-min treatment with 1% sodium borohydride. The first primary antiserum (for 2 days) was rabbit anti-serotonin (1:10000; ref. 22), followed by goat anti-rabbit IgG (6 hr, 1:50; ICN) and then by rabbit peroxidase-antiperoxidase complex (≈12 hr, 1:100; Dakopatts). The first immunoperoxidase reaction was developed using nickel-intensified DAB, as above. Staining for the second antigen was preceded in some experiments by an elution of the antibodies of the first sequence (23), but the results were identical to those seen without elution—i.e., no color mixing or crossreactivity was seen. The second primary antiserum was either rabbit anti-calbindin $D_{28K}$ (as above) or rabbit anti-parvalbumin (as above), followed by the same sequence of antisera as for serotonin. The second immunoreaction was developed with DAB alone (brown reaction product), and the sections were dehydrated and embedded for light microscopy. The incubation buffer was the same as in the tracing experiment. Some sections were incubated for serotonin alone without Triton, then treated with osmium tetroxide, dehydrated, and embedded in Durcupan (ACM; Fluka). Selected areas containing serotonin-immunoreactive axons were sectioned for electron microscopy, and alternate ultrathin sections were immunostained for GABA by the antiserum/immunogold procedure of Somogyi and Hodgson (24, 25).

## RESULTS AND DISCUSSION

Axons originating from the median raphe nucleus that contain mostly serotonergic projection neurons were visualized throughout the hippocampal formation by an anterograde tracer, PHAL. The labeled fibers were most numerous in stratum lacunosum moleculare of the CA1 to CA3 regions, and in the hilus of the dentate gyrus in a 50-μm-thick band below the granule cell layer. The majority of the fibers had small *en passant* varicosities, but a separate population was studded with relatively large boutons (26). The latter type of fiber occurred more frequently in stratum radiatum and oriens of CA1 and in the dentate gyrus, whereas the former was concentrated at the border region of stratum lacunosum moleculare and radiatum. Both fiber types had a rather straight passage through the neuropil; close contacts with cell bodies were rarely observed, and thus the possible targets could not be predicted at the light microscopic level. However, in sections double-stained for PHAL and calbindin $D_{28K}$ a striking pattern of innervation became apparent. The PHAL-labeled fibers of both types outlined the cell bodies and followed the dendrites—often for more than 150 μm—of calbindin-positive neurons in all strata, most frequently in stratum radiatum and oriens (Fig. 1 *a–d*). Up to 40 PHAL-labeled boutons could be counted on a single calbindin-positive neuron. In the CA1 subfield of the dorsal hippocampus 71% (127 out of 179), and in the CA3 subfield 63% (55 out of 88), of the calbindin-positive nonpyramidal cells received multiple innervation from PHAL-labeled afferents in the animal where the labeling of raphe-hippocampal axons was the most abundant. In sections double-stained for PHAL and parvalbumin, the PHAL-labeled axons were never seen to establish multiple contacts with the parvalbumin-positive neurons; the axons usually crossed the dendrites of these cells at right angles. It should be noted that parvalbumin and calbindin $D_{28K}$ are present in distinct populations of GABAergic interneurons in the hippocampal formation and neocortex (21, 27, 28).

To confirm that the axons anterogradely labeled by PHAL from the median raphe nucleus were largely serotonergic, we repeated the double-staining experiment in another group of animals, using an antiserum against serotonin instead of the PHAL tracing. The results were essentially similar in that the varicose serotonergic axons often formed pericellular arrays around the somata and dendrites of calbindin-positive neurons (Fig. 1 *e* and *f*), but never around parvalbumin-immunoreactive neurons. The general distribution of serotonin-immunoreactive fibers in the hippocampal formation was similar to that described earlier (29). It was also similar to the pattern of PHAL-positive axons observed in the present tracing experiment, although the stained axons were far more numerous in the serotonin-immunostained material. The proportion of calbindin-positive cells receiving multiple contacts from serotonin-immunoreactive fibers varied from animal to animal, and also among the different subfields. The former variation was clearly due to technical reasons, e.g., slight differences in the perfusion or staining intensity of the fibers. The two animals showing the strongest serotonin immunoreactivity were used to calculate the proportion of calbindin-immunoreactive neurons receiving a climbing-type contact from serotonin-immunoreactive axons: 69% (188 out of 273) in the CA1 region and 49% (50 out of 102) in the CA3 region of the dorsal hippocampus. The highest proportion (up to 95%) of contacted calbindin-positive cells was found at middle temporal levels, toward the subicular end of the CA1 subfield. In the CA3 region the proportion of contacted nonpyramidal cells was always lower than in the CA1 subfield. The large calbindin-positive neurons in stratum oriens of CA3 were rarely seen to receive multiple contacts from the serotonergic axons.

In spite of the larger number (although fainter immunoreactivity) of stained afferents in the serotonin-immunostained material, the multiple contacts with calbindin-positive neurons were slightly less frequent than in the PHAL-immunostained material (69% vs. 71% in the CA1 region, and 49% vs. 63% in the CA3 region). This may be due to the reduced visibility of serotonin-immunoreactive axons in the double-stained sections, especially in association with other immunostained elements, or may indicate that mesencephalic raphe neurons containing transmitters other than serotonin (30) also contribute to the innervation of hippocampal interneurons. Further studies are required to identify the neurotransmitter(s) and termination pattern of the non-serotonergic raphe–hippocampal pathway. Furthermore, this comparison of serotonin immunostaining (revealing both median and dorsal raphe projections) and PHAL tracing (revealing mostly the median raphe projection) suggests that the termination pattern of dorsal raphe afferents is rather diffuse compared to the median raphe, which is largely responsible for the innervation of the calbindin-containing nonpyramidal cells.

In sections stained for serotonin alone, basket-like arrays, such as those seen in the cat neocortex (31), were rarely observed. The association of serotonergic axons with interneuronal processes became apparent only when the target neurons were also visualized by immunostaining for calbindin $D_{28K}$.

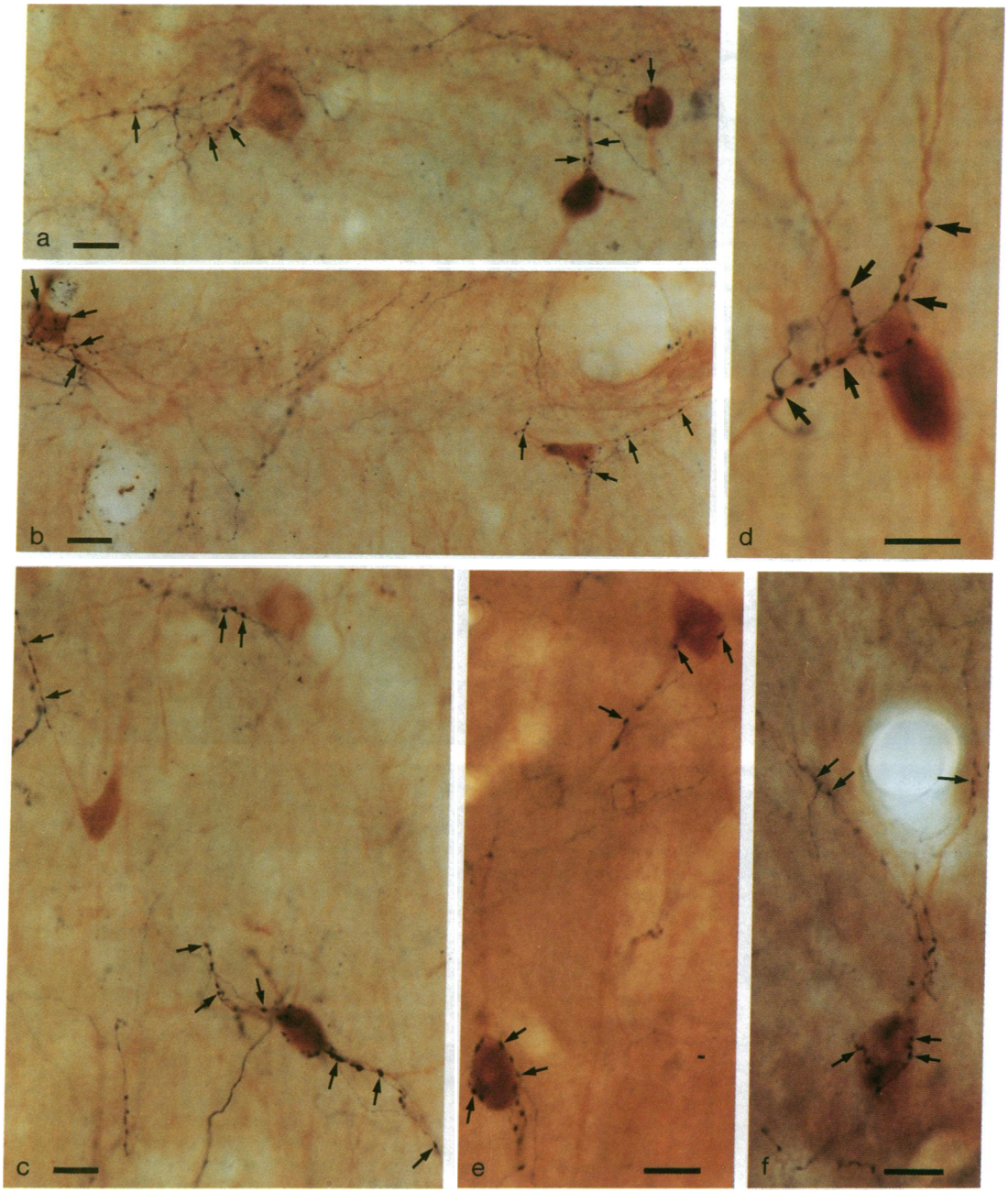


FIG. 1. (*a–d*) Afferent axons originating in the median raphe nucleus were labeled by PHAL transport (black, nickel–DAB reaction) and found to form multiple contacts with interneurons immunoreactive for calbindin $D_{28K}$ (brown, DAB reaction) in the hippocampus. The varicose (arrows) raphe axons climb along proximal as well as distal dendrites and cell bodies of calbindin-immunoreactive interneurons in stratum radiatum of the CA1 region (*b–d*) and the CA3 region (*a*) and in stratum lacunosum moleculare of the CA1 region (*b*). (*e* and *f*) Sections of the hippocampal CA1 region double-stained for serotonin (black fibers, nickel–DAB) and calbindin $D_{28K}$ (brown, DAB). The serotonergic axons form multiple contacts (arrows) with dendrites and somata of calbindin-immunoreactive interneurons, confirming the results obtained with PHAL tracing (Bars = 10 $\mu$m.)

To establish whether the multiple contacts between serotonergic axons and GABAergic interneurons were conventional synaptic contacts, we combined pre-embedding serotonin and post-embedding GABA immunocytochemistry at the electron microscopic level. The staining in this material was substantially reduced for both antigens, due to the necessary compromise made with the fixation (see *Methods*). Nevertheless, a sample of 16 serotonin-immunoreactive boutons was reconstructed from serial ultrathin sections, 5 of which were found to establish conventional symmetrical synaptic contacts with dendritic shafts (Fig. 2) and a cell body. Three of the postsynaptic dendrites and the cell body in stratum radiatum were shown to contain immunoreactive GABA (Fig. 2). Synaptic contacts on dendrites showing the characteristics of pyramidal cells were not found, but a detailed study of the serotonergic innervation of identified pyramidal cells has still to be done. Nevertheless, considering the data available today, the possibility that pyramidal cells are effected by serotonin released from the large number of apparently nonsynaptic varicosities (26) cannot be excluded.

The significance of the present findings for hippocampal function is twofold: (*i*) the serotonergic, brainstem control of hippocampal electrical activity is mediated, to a large extent,

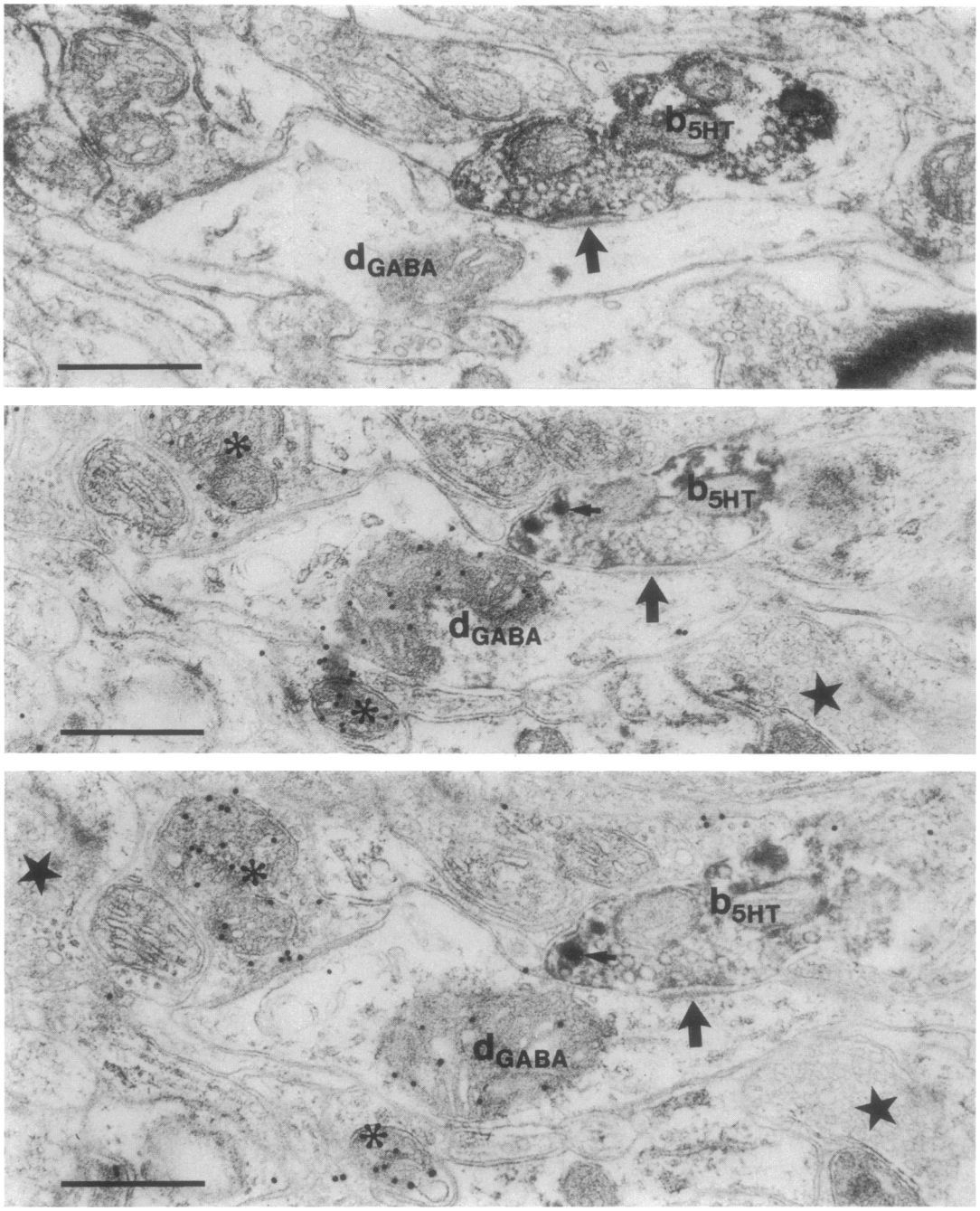


FIG. 2. A serotonin-immunoreactive bouton ($b_{5HT}$) is in symmetrical synaptic contact (large arrows) with a varicose dendrite ($d_{GABA}$) of a GABAergic neuron, as shown in serial ultrathin sections. The lower two sections were immunostained for GABA by a post-embedding immunogold procedure. GABA immunoreactivity is seen as an accumulation of colloidal gold particles over the positive profiles. The dendritic shaft ($d_{GABA}$) postsynaptic to the serotonin-immunoreactive bouton ($b_{5HT}$) is positive for GABA as well as two axon terminals (asterisks) establishing symmetrical synapses. Other boutons (stars), making asymmetrical synapses, are negative for GABA. Small arrows in the serotonin-positive terminal indicate large dense-core vesicles. (Bars = 0.5 $\mu$m.)

by GABAergic inhibitory interneurons; (*ii*) the target inhibitory interneurons form a chemically distinct subpopulation of GABAergic cells.

Earlier electrophysiological and pharmacological studies reported both hyperpolarizing and depolarizing effects of serotonin on the principal cells (16–19). Interneurons were considered as possible targets in a study by Segal (18), who reported that sufficiently low concentrations of serotonin selectively reduced the slow inhibitory postsynaptic potential, which is likely to be mediated by $GABA_B$ receptors (32, 33). Following serotonin application, the postsynaptic sensitivity of the recorded cell to GABA or baclofen did not change; thus serotonin was likely to inhibit the GABAergic interneuron directly. A similar conclusion was drawn from results achieved by using serotonin releasers (34).

The selective serotonergic modulation of one but not the other type of GABAergic neurons strongly suggests a functional difference between inhibition mediated by parvalbumin-containing interneurons and that mediated by calbindin-containing interneurons. The parvalbumin-containing interneurons, which are mainly basket and axo-axonic cells, terminate in the perisomatic region of the principal cells (35). They leave most of the dendritic tree free to be innervated by other types of GABAergic interneurons—i.e., by those lo-

cated in stratum radiatum, oriens, and lacunosum moleculare. In these strata, nonpyramidal cells, most of which contain calbindin $D_{28K}$, have been shown to arborize extensively in the apical dendritic region of pyramidal neurons (5, 36, 37). Besides their location, an additional difference between perisomatic and distal dendritic inhibition is that $GABA_B$ receptor-mediated feedforward inhibition occurs preferentially in the dendritic region, whereas feedback inhibition is $GABA_A$ receptor-mediated and is likely to occur predominantly in the perisomatic region (1–5). The present findings, taken together with these earlier results, lead to the conclusion that serotonin can influence—via the calbindin-containing cells terminating largely in the dendritic fields of pyramidal cells—the feedforward, $GABA_B$ receptor-mediated inhibition. This type of inhibition may determine the degree of long-term potentiation by decreasing the probability of *N*-methyl-D-aspartate receptor activation or, as shown in the CA3 region of slice cultures (38), may regulate the excitability of the hippocampus by limiting recurrent synaptic excitation. The degree of population synchrony and output controlled most effectively by perisomatic feedback inhibition should be largely unaffected by serotonin. However, the parvalbumin-containing neurons mediating this latter form of inhibition are strongly innervated by the GABAergic septohippocampal pathway (7). Thus, it appears that septal and median raphe afferents can differentially regulate hippocampal information processing by innervating different types of specialized inhibitory interneurons.

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