Neuron, Vol. 20, 983–993, May, 1998, Copyright ©1998 by Cell Press

How Many Subtypes of Inhibitory Cells in the Hippocampus?

Paula Parra,*§ Attila I. Gulyás,*†§ and Richard Miles*‡
*Laboratoire de Neurobiologie Cellulaire
Institut Pasteur
25 rue de Dr Roux
75724 Paris
France
†Institute of Experimental Medicine
Academy of Sciences
POB67
H-1450 Budapest
Hungary

Summary

Hippocampal inhibitory cells are diverse. It is supposed that they fall into functionally distinct subsets defined by a similar morphology and physiology. Switching between functions could be accomplished by activating receptors for modulating transmitters expressed selectively by different subsets of interneurons. We tested this hypothesis by comparing morphology, physiology, and neurotransmitter receptor expression for CA1 hippocampal interneurons. We distinguished 16 distinct morphological phenotypes and 3 different modes of discharge. Subsets of inhibitory cells were excited or inhibited by agonists at receptors for noradrenaline, muscarine, serotonin, and mGluRs. Most cells responded to 2 or 3 agonists, and 25 different response combinations were detected. Subsets defined by morphology, physiology, and receptor expression did not coincide, suggesting that hippocampal interneurons cannot easily be segregated into a few well-defined groups.

Introduction

Single identified neurons with unique properties are known to exist in some simple nervous systems (Kupfermann and Weiss, 1978; Selverston and Moulins, 1987). In contrast, mammalian brains are thought to contain a limited number of groups of neurons within which each member expresses similar properties. For instance, each hippocampal pyramidal cell excites postsynaptic neurons by liberating an excitatory amino acid. Equally, all hippocampal inhibitory cells inhibit other cells by releasing gamma-amino-butyric acid.

In other respects, however, hippocampal inhibitory cells are rather diverse (Ramon y Cajal, 1911; Freund and Buzsaki, 1996). Axons of different groups of inhibitory cells target precise and different zones of pyramidal cell membrane (Buhl et al., 1994; Miles et al., 1996). Distinct groups of inhibitory cells contain different peptides, which may function as cotransmitters, and different calcium-binding proteins (reviewed in Freund and Buzsaki, 1996). Differences in expression of voltage-gated currents in distinct interneurons may be associated with distinct patterns of discharge that generate the temporal sequence of IPSPs impinging on postsynaptic cells (Zhang and McBain, 1995; Maccaferri and McBain, 1996).

These distinct subtypes of inhibitory cells may possess distinct computational functions within hippocampal neuronal networks (Buhl et al., 1994). Those inhibitory cells that innervate pyramidal cell somata or axon initial segments control the local generation of Na^+-dependent action potentials (Miles et al., 1996). Somatic IPSPs also tend to remove inactivation of subthreshold inward currents, and so these cells may initiate synchronous rebound firing in many pyramidal cells (Cobb et al., 1995). Dendritic IPSPs are presumed to regulate integration of afferent EPSPs and may also control the dendritic generation of Ca^+-dependent action potentials (Miles et al., 1996), deinactivate dendritic K^+ currents (Hoffman et al., 1997), as well as controlling the somatodendritic propagation of Na^+-dependent spikes (Calloway et al., 1995). The discovery that some inhibitory cells form synapses that contact only other inhibitory cells (Gulyás et al., 1996) implies that local inhibitory cell networks exist. Interactions within these networks are suggested to generate coherent inhibitory signals that, distributed to many pyramidal cells, act to pace rhythmic gamma activity (Whittington et al., 1995).

How does the brain switch between these different functions? We know that distinct groups of inhibitory cells express receptors for different modulating transmitters (Freund et al., 1990; Ropert and Guy, 1991; Behrends and ten Bruggencate, 1993; Kawa, 1994; McBain et al., 1994; Bergles et al., 1996). This suggests that subsets of interneurons with distinct roles in hippocampal computation (Buhl et al., 1994; Cobb et al., 1995; Whittington et al., 1995; Miles et al., 1996) might be differentially controlled by modulating transmitters liberated to meet specific behavioral needs (Freund et al., 1990; Nicoll, 1994; Freund and Buzsaki, 1996). To test this hypothesis, we made a horizontal study of morphological, physiological, and pharmacological properties of CA1 hippocampal interneurons. We attempted to define subsets of inhibitory cells by their axonal and dendritic distributions as well as distinct firing patterns and to confront these subsets with their responses to modulating transmitters. Responses to agonists at muscarinic, serotoninergic, and noradrenergic receptors and to metabotropic receptors for glutamate were examined. Our results reveal an unexpected diversity in interneuron properties and show that inhibitory cells are typically under the control of multiple modulating transmitter systems.

Results

Morphological Diversity of CA1 Interneurons

Three morphologically distinct types of inhibitory neuron with somata located in the stratum pyramidale have

‡To whom correspondence should be addressed.
§These authors contributed equally to this work.

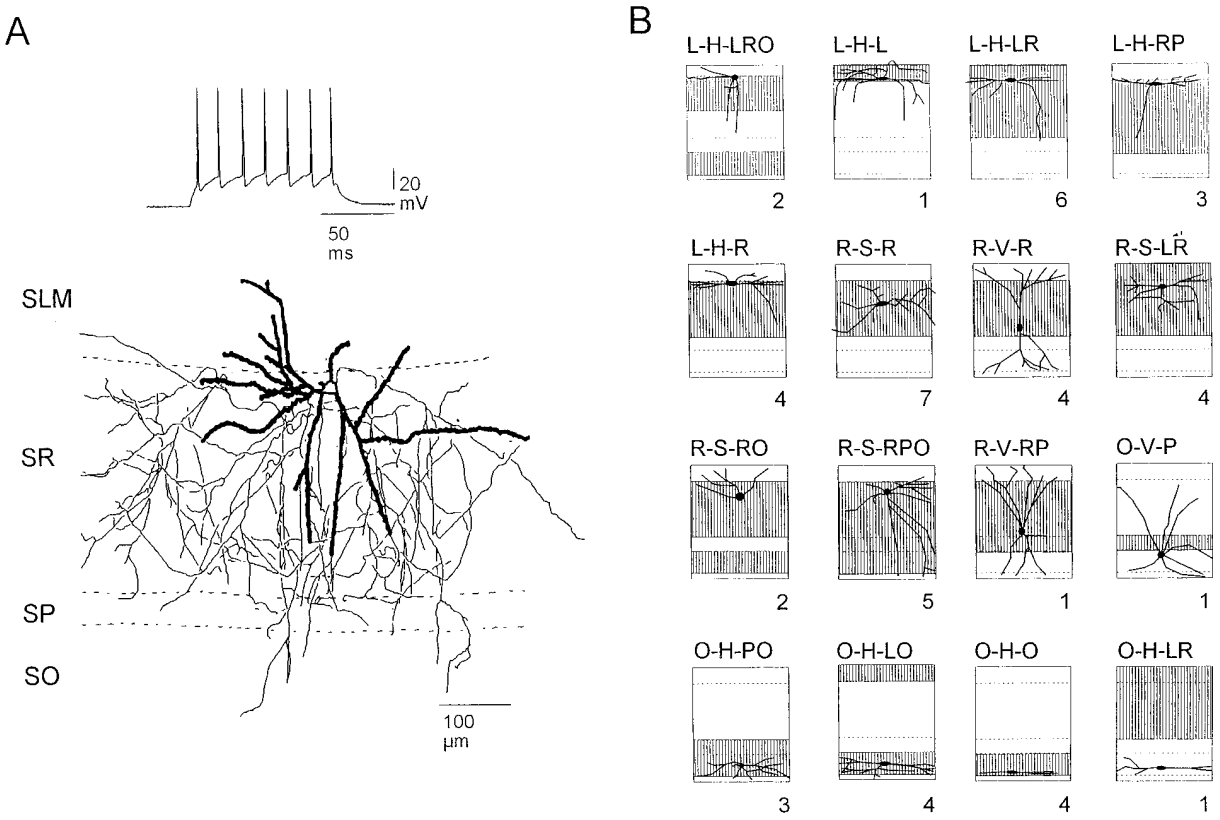

Figure 1. Diversity of CA1 Inhibitory Cell Morphologies

(A) Reconstruction of the dendritic (thick line) and axonal (fine line) arborization of a biocytin-filled CA1 cell with soma in stratum radiatum. The inset shows the response of this cell to a 500 ms depolarizing current injection. Inhibitory cells (n = 52) were classified by the location of their soma (in O, stratum oriens; P, stratum pyramidale; R, stratum radiatum; or L, stratum lacunosum-moleculare), the orientation of their dendrites (H, horizontal; V, vertical; or S, stellate) and the zones innervated by their axon (O, P, R, and L).

(B) Schematic representation of 16 subclasses according to this division. The soma, axon (shading), and dendrites (solid lines) for a cell of each type were projected onto a standard plan of the CA1 region. The number of cells recovered from each class is given below each schematic diagram.

been identified in the CA1 region of rat hippocampus (Buhl et al., 1994). In this study we used infrared microscopy to identify interneurons in other strata of the CA1 region. Whole-cell recordings were made from these cells using biocytin-filled electrodes so that their axonal and dendritic arborizations could be examined. Morphological data was obtained for inhibitory cells with somata located in stratum oriens (SO; n = 13), radiatum (SR; n = 23), and lacunosum moleculare (SLM; n = 16) (Figure 1A). Figure 1A shows a complete reconstruction of the axon and dendrites of one interneuron whose somata was located in distal stratum radiatum.

We detected a large variety of interneuron morphologies. Different cells were therefore compared by constructing schematic diagrams of axonal and dendritic arborizations plotted onto a standard plan of the CA1 region (Figure 1B). Several features emerged from these diagrams. The dendrites of cells with somata located in SO and SLM were often oriented horizontally within these strata, suggesting that they receive excitatory synapses exclusively from afferents confined to these layers. In contrast, dendrites of cells from SR tended to possess a vertical or stellate distribution and to enter other layers, suggesting that they do not receive a selective excitation. The arborizations of inhibitory cell axons were often largely restricted to specific layers, suggesting that a single cell inhibits particular zones of pyramidal cell membrane (Buhl et al., 1994; Miles et al., 1996). We therefore classified inhibitory morphology according to three criteria: somatic location (L, R, or O), dendritic orientation (horizontal [H], vertical [V], or stellate [S]) and the zones innervated by their axons (L, R, P, or O). According to this classification, the 52 cells whose morphology was recovered belong to 16 distinct classes. The number of cells belonging to each group are given below the diagrams in Figure 1B.

Physiological Diversity of CA1 Interneurons

The firing pattern of an inhibitory cell generates the temporal sequence of the IPSPs, which it initiates in all of the cells it innervates. We first classified inhibitory cell physiology by spike discharges that either occured spontaneously or were induced by small, maintained current injections. At least three distinct patterns of inhibitory cell discharge were evident. Firing could be regular, irregular, or clustered into groups of action potentials (Figures 2A–2C). These distinct firing patterns resulted in characteristic interspike interval distributions (Figure 2). For the regularly firing cells (39%; n = 66), the

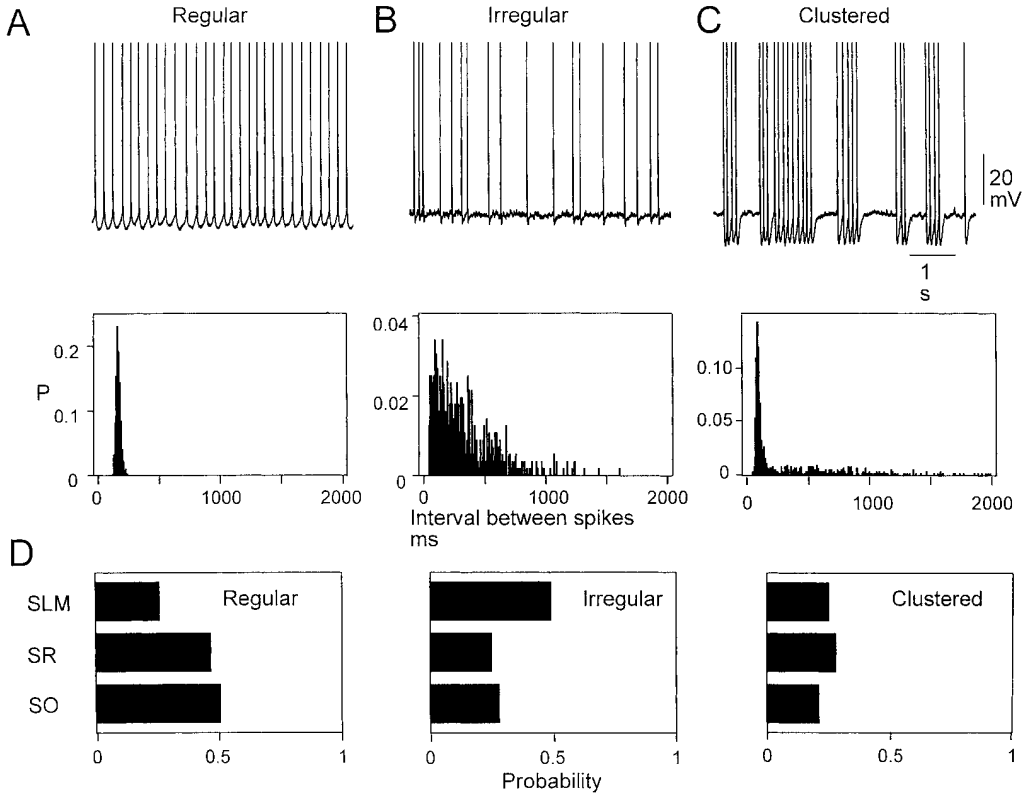

Figure 2. Diversity of Interneuron Discharge Patterns

Spontaneous activity of neurons with regular (A), irregular (B), and clustered (C) firing patterns. In each case a membrane potential trace is shown together with a histogram of interspike intervals constructed (using a program written in Labview, National Instruments) from data acquired during at least 3 min. (D) Shown is the proportion of recorded cells in stratum lacunosum-moleculare (SLM; n = 49), stratum radiatum (SR; n = 68), and stratum oriens (SO; n = 39) discharging with these patterns.

repolarization succeeding each action potential directly initiated the next spike. In contrast, for the irregular cells (36%; n = 61) the repolarization was followed by a variable delay before the next action potential occurred. In the remaining cells (25%; n = 43), clusters of three or more regularly occurring action potentials were separated by silent periods of variable duration. Each inhibitory cell firing pattern was maintained over a range of frequencies as the membrane potential of the cell was varied and did not vary during recordings of duration up to 2 hrs. Figure 2D shows the distribution of cells with these different firing patterns according to the location of their soma. Cells with somata in stratum lacunosum-moleculare tended to fire irregularly rather more often than cells in other layers, but generally firing pattern was not strongly correlated with soma location.

These differences in inhibitory cell firing pattern presumably depend on the differential expression of voltage-gated channels. We detected in different inhibitory cells distinctive potential responses associated with specific currents that shape interneuron firing patterns in the hippocampus and other structures.

Some cells (36%) exhibited voltage-dependent subthreshold oscillations (Figure 3A) in membrane potential at frequencies between 3 and 15 Hz. We included a cell in this group when the amplitude of oscillations was greater than 2–3 mV at potentials in a range of 10 mV below the threshold for action potential generation. These cells usually discharged in clusters of action potentials (n = 43), although regular (n = 9) and irregular (n = 6) discharge patterns were also observed. The oscillations were independent of synaptic events since they persisted in the presence of CNQX and APV. Furthermore, the oscillations were suppressed by tetrodotoxin (TTX; 1 mM), suggesting that they depend in part on the expression of a persistent sodium current as do similar oscillations in inhibitory cells of nucleus basalis and stellate cells of entorhinal cortex (Alonso and Llinas, 1989; Alonso et al., 1996).

In 52% of cells tested, a voltage-dependent sag was apparent in response to hyperpolarizing current injections (Figure 3B). Cells were classified as possessing a sag when the membrane potential at the end of a 0.2 nA, 300–500 ms hyperpolarizing pulse from rest was reduced by more than 15% of its maximal value during the step. This voltage-dependent sag was suppressed by 2 mM Cs^+ (Figure 3B), indicating that it resulted from the activation of an I_H current (Maccaferri and McBain, 1996). The presence of an I_H current was not associated with a specific discharge pattern. From 75 cells in which a voltage-dependent sag was evident, 33 discharged regularly, 22 fired irregularly, and 20 discharged in clusters of action potentials.

The presence of a low-threshold calcium current, I_T,

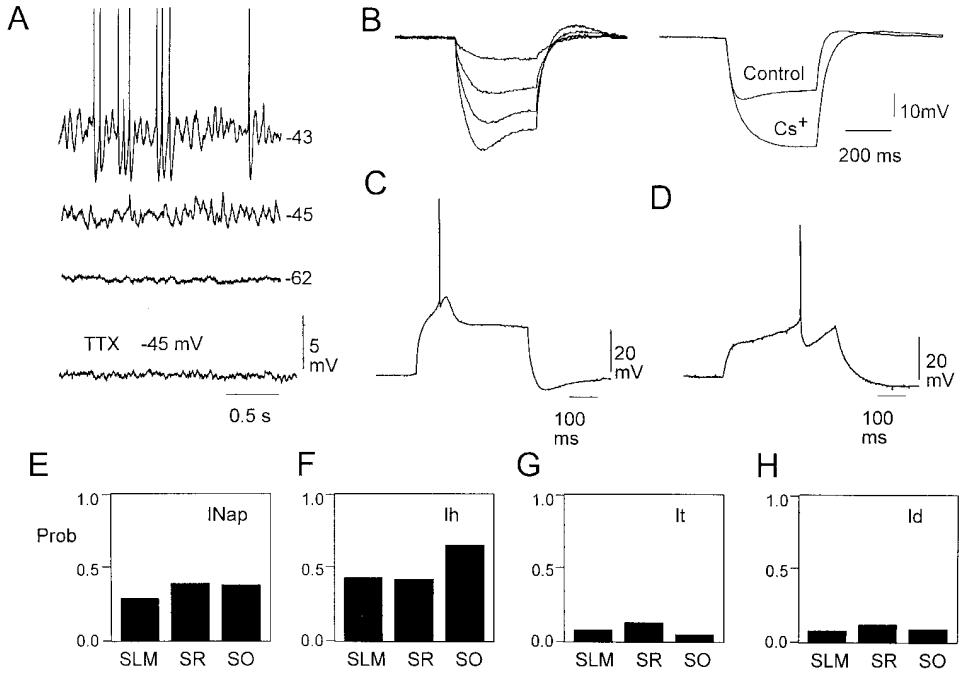

Figure 3. Evidence that Interneurons Express Diverse Intrinsic Currents

(A) Subthreshold voltage-dependent oscillations in membrane potential recorded in the presence of CNQX (10 μM) and APV (100 μM) were abolished by 1 μM tetrodotoxin (TTX), suggesting that they were dependent in part on a Na^+ current.

(B) Presence of I_H inferred from a sag in responses to hyperpolarizing current injection that was suppressed by 2 mM Cs^{2+}.

(C) Slow potential wave induced by depolarizing currents applied from hyperpolarized holding potentials consistent with the presence of a low-threshold Ca^{2+} current, I_T.

(D) Delay in spike generation in response to depolarizing current steps suggestive of the presence of a slowly inactivating K^+ current, I_D.

(E and F) Proportions of cells sampled from stratum lacunosum-moleculare (SLM), stratum radiatum (SR), and stratum oriens (SO) presenting evidence (as in A–D) for the presence of a persistent sodium current (E), an I_H (F), an I_T (G), and an I_D (H).

(Fraser and MacVicar, 1991; Kawaguchi, 1993) was inferred in a minority of cells tested (8%) when depolarizing steps applied from hyperpolarized holding potentials initiated a slow transient wave (Figure 3C). In these cells step somatic depolarizations from hyperpolarized potentials could trigger bursts of 2–4 action potentials. Bursts of action potentials were not evident, however, in the spontaneous firing of these cells, which could be either regular (n = 3), irregular (n = 2), or clustered (n = 1).

Finally, I_D, a slowly inactivating potassium current (Storm, 1988; Kawaguchi, 1995), was evident in a few cells (8%) as a ramp-like subthreshold depolarization that delayed spike generation in response to depolarizing current steps (Figure 3D). 4-aminopyridine (4-AP) is a specific antagonist of the I_D current at low concentrations. We found that in the presence of 50 μM 4-AP, depolarizing steps induced in these cells a continuous discharge rather than a delayed firing (n = 2; data not shown). Cells that expressed I_D tended to discharge in groups of spikes (4 out of 6 cells).

This selective expression of voltage-gated channels, sometimes in small minorities of cells, increases the variability in inhibitory cell physiology. Figures 3E–3H show the somatic locations of cells that expressed subthreshold oscillations, voltage-dependent sag, I_T, and I_D. Cells with somata located in stratum oriens were more likely to show a sag than other interneurons, but otherwise there was little tendency for these currents to be selectively expressed.

Diverse Responses to Neurotransmitter Agonists

Diverse axonal and dendritic arborizations and distinct modes of spontaneous discharge suggest different inhibitory cells might control pyramidal cell activity in different ways (Buhl et al., 1994; Miles et al., 1996). A selective expression of neurotransmitter receptors that excite or inhibit different interneurons could be a way to switch between different modes of inhibitory control (Freund et al., 1990; Nicoll, 1994; Freund and Buzsaki, 1996). In its most simple form, this hypothesis implies that each inhibitory cell should express receptors for just one modulating transmitter (Nicoll, 1994).

We examined the effects of four neurotransmitter agonists: (1S, 3R)-aminocyclopentane-1,3-dicarboxylic acid (1S, 3R-ACPD), noradrenaline (NA), muscarine (M), and serotonin (5-HT). They were applied via the bath perfusion with an exchange time of 40–65 s. Applications lasted for 2–3 min and were separated by 3–6 min depending on the time taken to return to control conditions. These experiments were made in the presence of CNQX (10 μM) and APV (100 μM) to eliminate changes in interneuron membrane potential due to alterations in synaptic input from pyramidal cells (Figures 4A–4D).

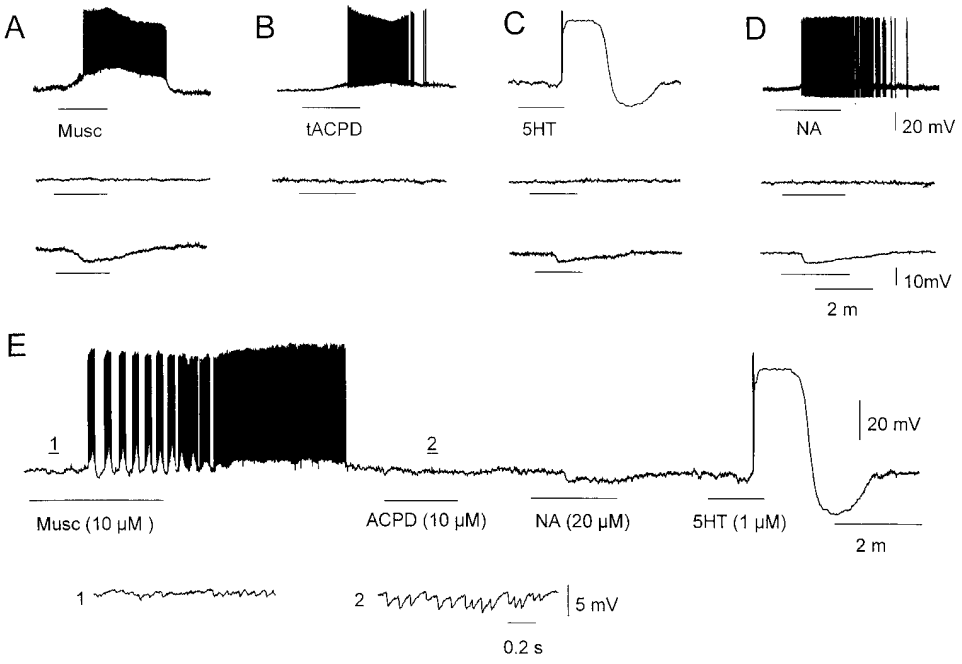

Figure 4. Diversity of Expression of Neurotransmitter Receptors by CA1 Inhibitory Cells

(A–D) Examples of different responses of inhibitory cells to bath application of each of the transmitter agonists tested. Depolarizing, null, and hyperpolarizing responses are shown for M (A), 5-HT (C), and NA (D). 1S, 3R-ACPD elicited only depolarizing or null responses. In each case horizontal lines mark the duration of the application.

(E) Responses of an SR inhibitory cell from stratum radiatum to applications of all agonists. This cell was excited by M and 5-HT, inhibited by NA and was not sensitive to 1S, 3R-ACPD. Expanded membrane potential traces from the control period (1) and during application of 1S, 3R-ACPD (2) reveal that IPSP frequency increased, suggesting that this agonist excited other inhibitory cells in the slice.

In these experiments we classed a cell as being excited when a transmitter agonist induced a depolarization of more than 3–5 mV from a holding potential of −55 to −65 mV. Conversely, a hyperpolarization of more than 3–5 mV from rest corresponded to an inhibition. We found that 1S, 3R-ACPD (1–10 μM), which activates metabotropic glutamate receptors, excited 39% ($n = 32$) of inhibitory cells tested. NA (1–20 μM) excited 52% of cells tested ($n = 41$) and inhibited 6% ($n = 5$). 5-HT (1–10 μM) excited 52% of inhibitory cells ($n = 43$) and inhibited 12% ($n = 10$). Finally, M (1–10 μM) excited 61% ($n = 52$) and inhibited 5% ($n = 4$) of cells tested.

Indirect inhibition of a recorded cell was sometimes evident (Figure 4E) as an increase in frequency of spontaneous IPSPs, presumably due to excitation of other inhibitory cells in the slice. However, such indirect inhibition was readily distinguished from direct hyperpolarizing effects. Other indirect actions might involve liberation of neuroactive substances other than GABA. To control against such actions, cellular responses to 1S, 3R-ACPD and M were examined in the absence and then in the presence of TTX (0.5 μM). TTX, which should block Na^+ action potential–dependent release, did not suppress responses ($n = 4$ cells; data not shown). However, since TTX would not suppress calcium-dependent release (Manzoni et. al., 1994), we also made experiments using a low Ca^{2+}/high Mg^{2+} solution (0.2 and 8 mM, respectively). Also under these conditions, responses to the agonist persisted ($n = 2$; data not shown), suggesting that indirect effects can probably be excluded.

The effects of all four transmitter agonists were examined in 64 cells (Figures 4E and 5A–5C). Most inhibitory cells were excited by two (37.5%) or three (21.9%) agonists. In contrast, a minority of cells responded to none (12.5%), one (12.5%), or to all four agonists (15.6%). These results show that a simple model in which each inhibitory cell is excited by a single modulating transmitter (Nicoll, 1994) does not hold true. Furthermore, they reveal another degree of diversity in that different inhibitory cells express different combinations of receptors for modulating transmitters. If 5-HT, M, and NA can excite, inhibit, or have no influence on inhibitory cells while 1S, 3R-ACPD is excitatory or has no effect, then 54 ($= 3 \times 3 \times 3 \times 2$) different combinations of receptor expression are possible. In 64 cells tested, we found 25 of these 54 possible combinations. The two nonspecific patterns where either all or none of the agonists excited a single cell occurred in seven and eight cells, respectively. Two specific combinations were observed in five cells each, one pattern in four cells, six combinations in three cells, four combinations with two cells, and nine response patterns were seen in just one cell (Figures 5A–5C). In contrast, pyramidal cell ($n = 8$) responses to the same four transmitter agonists were less variable (Figure 5D), suggesting that the properties of this cell type may be less diverse than those of inhibitory cells.

A cross desensitization occurs between responses

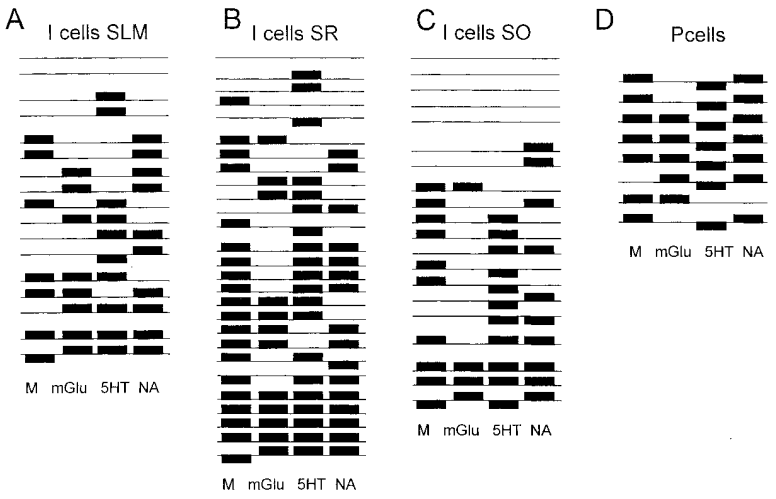

Figure 5. Diversity of Interneuron Expression of Receptors for Four Modulating Transmitters

(A–D) Inhibitory and pyramidal cell responses to neurotransmitter agonists are represented as a bar code with each horizontal line corresponding to a different cell. A bar above the line represents an excitation, a bar below the line an inhibition, and no bar indicates no effect. Tests were made at holding potentials between −60 and −55 mV, and a change in membrane potential of more than 5 mV was counted as an effect. Interneurons are separated according to location in stratum lacunosum-moleculare (SLM; n = 17), stratum radiatum (SR; n = 27), and stratum oriens (SO; n = 20). Responses of eight CA1 pyramidal cells to the same neurotransmitters are also shown under P cells. While pyramidal cell responses to these transmitter agonists were rather constant, 25 distinct combinations of responses were detected from the 63 interneurons tested.

mediated by mGluRs and muscarinic receptors (Guerineau et al., 1997). We examined whether this phenomenon may have generated false null responses in experiments in which 1S, 3R-ACPD and M were applied at differing intervals. In 15 cells that were excited by both transmitters, a cross-desensitization generated a false null response in only one case at an interval (1.5 min) that was shorter than the normal interval between tests (3–6 min). Comparison of responses measured at short (1–3 min) and long (6–7 min) intervals sometimes showed reduced responses at the shorter interval but would always have satisfied our detection criteria (n = 7 cells; data not shown). These results show that cross-desensitization did not mask the true responses at the agonist concentrations we used.

Correlations between Subsets of Inhibitory Cells?

Our data suggest that CA1 inhibitory cells possess at least 16 distinct morphologies, discharge in 3 distinct modes, and can express 25 or more combinations of receptors for the neurotransmitters we have examined. Comparisons across these classifications increase further the number of distinct cell types that must be envisaged. Thus, cells possessing the same combination of transmitter responses could be located in different layers and have different axonal arborizations. Equally, cells with similar morphologies and firing patterns usually expressed different combinations of transmitter receptors. Table 1 summarizes the properties of 26 cells for which a complete data set was collected. Each cell was different. From additional cells where our data was not complete, we calculate that at least 52 distinct cell types exist.

An order might emerge from this complexity if a given transmitter excited or inhibited cells whose axon targeted specific zones of pyramidal cell membrane regardless of the location of their soma. We examined this hypothesis by mapping transmitter actions—excitation, inhibition, or no effect—onto a plot of interneuron axonal arborizations. Figure 6 shows this mapping for 35 cells for which both morphology and responses to all four agonists were obtained. The results did not support the hypothesis that inhibitory actions onto particular zones of pyramidal cell membrane were selectively enhanced or suppressed by the actions of the agonists we examined. For instance, of 26 cells with axons ramifying in stratum radiatum, 14 were excited by M, 7 were excited by 1S, 3R-ACPD, 14 were excited and 2 inhibited by 5-HT, while 10 were excited and 1 inhibited by NA. Similar nonspecific distributions were evident for axons arborizing in other strata.

Discussion

It seems unlikely that any two mammalian neurons have exactly the same pattern of afferent and efferent innervation. Even so, although each spinal motorneuron may innervate a different muscle, it is useful to consider motorneurons as a group of cells with similar properties. This report suggests that hippocampal inhibitory cells cannot be considered like this. We have detailed a wide variability in their morphology as well as in the expression of voltage-dependent channels and neurotransmitter receptors. Furthermore, groups of cells defined according to these different criteria did not coincide. Thus, these results suggest that either there is a very large but finite number of subtypes of hippocampal inhibitory cells or that each hippocampal interneuron is different.

Technical Problems

Before accepting this conclusion, we should consider whether aspects of our technique may have introduced a spurious variability into our results.

First, developmental changes may have contributed to the variability. GABAergic inhibition in the hippocampus approaches its adult electrophysiology at about 10 days after birth (Leinekugel et al., 1997). In the cortex, changes in inhibitory cell phenotype occur but are complete toward the end of the third postnatal week (Alcantara et al., 1996). Since our experimental animals were aged between 18 and 35 days, it seems probable that inhibitory cell properties were close to their adult state.

Table 1. Inhibitory Cell Diversity

	Morphology						Discharge			Transmitter Responses			
Cell Number	Soma	Dendrite	Axon					Vm (mV)	Rin (MΩ)	Musc	mGlu	5-HT	NA
82	L	H	L				Clustered	−69	250	+	+	+	0
49	L	H	L	R			Irregular	−60	260	+	0	+	0
40	L	H		R	P		Regular	−70	400	0	0	+	0
21	L	H		R			Regular	−58	250	0	+	+	0
76	R	S		R			Regular	−58	400	+	0	+	−
201	R	S		R			Regular	−62	170	+	+	+	0
62	R	S		R			Irregular	−53	150	+	0	+	+
58	R	S		R			Clustered	−63	200	0	0	−	0
188	R	S		R			Clustered	−66	350	+	0	+	+
190	R	V		R			Regular	−72	190	+	0	−	0
38	R	V		R			Irregular	−58	250	+	0	0	0
187	R	V		R			Clustered	−62	300	+	0	+	+
145	R	S	L	R			Irregular	−52	200	0	+	+	0
148	R	S	L	R			Irregular	−43	160	0	0	0	0
52	R	S		R		O	Irregular	−56	150	0	0	+	0
37	R	S		R	P	O	Irregular	−69	250	+	+	0	0
134	R	S		R	P	O	Clustered	−72	350	0	0	0	0
43	R	V		R	P		Clustered	−65	160	+	0	0	+
42	O	H			P	O	Regular	−53	150	0	0	−	−
147	O	H			P	O	Irregular	−60	300	+	0	−	0
128	O	H	L			O	Regular	−52	220	−	+	−	+
22	O	H	L			O	Irregular	−43	150	+	0	+	0
204	O	H				O	Regular	−72	350	0	0	−	−
203	O	H				O	Regular	−61	220	−	+	+	+
146	O	H	L	R			Irregular	−54	180	0	0	0	+

Morphology: interneuron somata were located in stratum oriens (O), stratum radiatum (R), or in stratum lacunosum-moleculare (L). Their dendrites were oriented horizontally (H), vertically (V), or were stellate (S). Their axon ramified located in stratum oriens (O), stratum radiatum (R), stratum pyramidale (P), or in stratum lacunosum-moleculare (L). Firing patterns were classified as regular, irregular, or clustered as in Figure 2. Resting potential (Vm) and input resistance (Run) are given for each cell. Neurotransmitter responses were classified as depolarization (+), no effect (0), or hyperpolarization (−) as in Figure 5.

Second, inhibitory cell axonal arbors were not completely contained within the 200–300 μm thick slices used in this study. We assume that the arborization present in a slice was representative of the entire axonal spread. By following each axon to its cut ends, we attempted to ensure this assumption was reasonable. We also had some doubts on the limits between morphological classifications. Some dendritic arbors were difficult to classify as either stellate or vertical, while axons could arborize very strongly in some strata and weakly in others. For example, the cell shown in Figure 1 was classified as having an axonal arbor limited to stratum radiatum even though it made small projections into strata pyramidale and lacunosum-moleculare. Taking these differences into account would have increased still further the number of morphological cell types.

Third, whole-cell recording, where there is exchange of the electrode solution and cytoplasmic contents, may have affected our physiological results. However, evidence for these same voltage-dependent currents and discharge patterns has been obtained in records made with sharp electrodes from inhibitory cells in slices from adult animals (Miles et al., 1996; unpublished data), suggesting that the diversity in firing patterns does not arise from the whole-cell recording configuration.

Fourth, washout of cytoplasmic contents modifies G protein–mediated signaling. We did in some records lasting tens of minutes see a reduction in amplitude of responses to repeated stimuli with the same transmitter (data not shown). However, the responses did not disappear completely even in records lasting more than 1 hr, and the data presented in Figures 4 and 5 were all obtained in the first 30 min after break-in. We note also from other work that inhibitory cell responses to agonists of mGluRs (McBain et al., 1994), of noradrenergic receptors (Bergles et al., 1996), and of muscarinic (Behrends and ten Bruggencate, 1993) receptors persist in the whole-cell recording configuration.

We attempted to exclude another potential uncertainty in experiments to detect whether cross-desensitization between M and mGluRs (Guerineau et al., 1997) could have introduced false-negative responses to agonists for either of these receptors. Our results suggest that at the agonist concentrations we used, cross-desensitization was not sufficiently marked to obscure true responses.

Finally, bath agonist application might depolarize cells other than the recorded neuron, initiating release of other neuroactive substances with indirect actions on the recorded cell. Such an effect was responsible for increases in the frequency of fast IPSPs seen occasionally (Figure 4). The kinetics of other indirect actions should have been rather slower than the responses we normally observed. Furthermore, experiments showing that responses recorded in control conditions persisted in the presence of TTX or of high Mg/low Ca^{+} suggest that other such indirect actions may be excluded.

Interneuronal Morphologies

In a previous study, we distinguished six different arborization patterns for axons of inhibitory cells whose somata were located close to stratum pyramidale of the

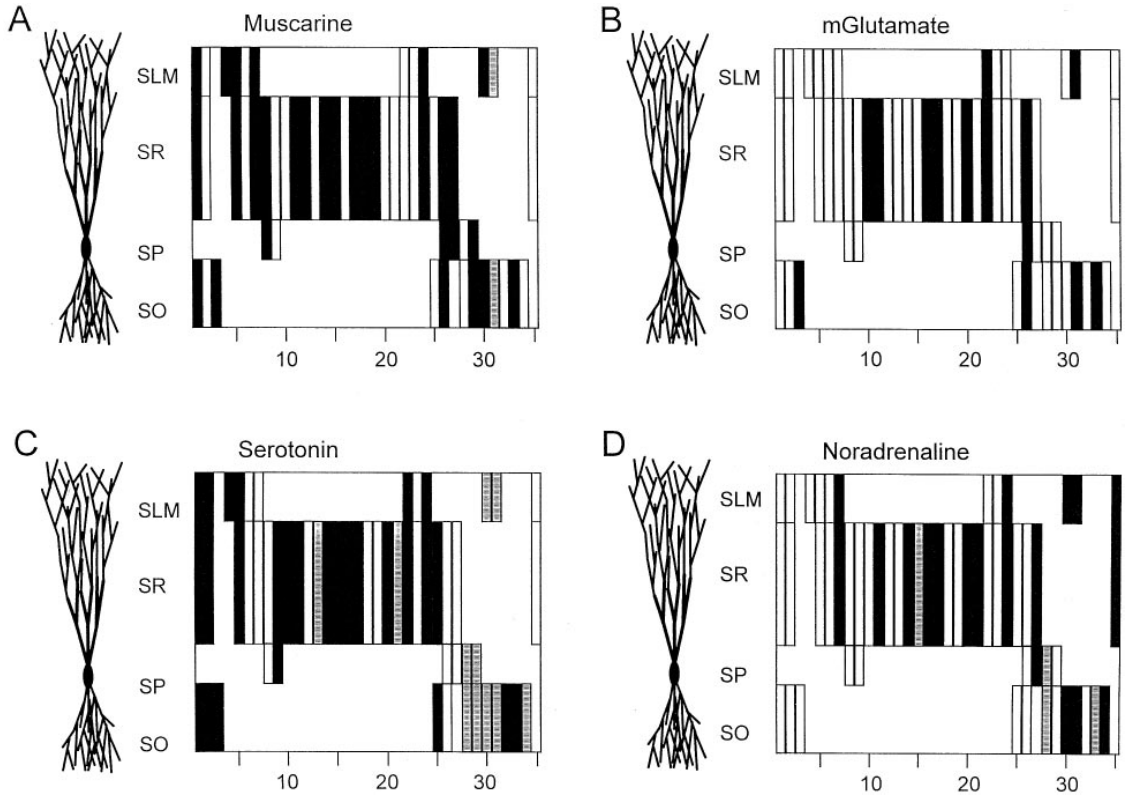

Figure 6. Mapping Modulating Transmitter Actions onto Inhibitory Axon Arborizations

(A–D) Data from 36 cells for which responses to all four transmitters and a complete axonal arborization were determined. The axonal arborization of each cell is represented by vertical bars corresponding to SLM, SR, SP, and SO. For each transmitter, bars filled in black correspond to an excitation of that cell, empty bars to no effect, and bars shaded in gray correspond to an inhibition. The figure suggests that none of the agonists excited (or inhibited) subsets of inhibitory cells that innervated spatially well-defined zones on pyramidal cell membrane.

CA3 region (Gulyás et al., 1993). At least three different morphological cell types were evident in a similar study made in the CA1 region (Buhl et al., 1994). The present data suggests that CA1 interneurons with somata located in other layers possess an even greater morphological diversity. Some of the cell types we detected have been reported previously (McBain et al., 1994; Bergles et al., 1996; Freund and Buzsaki, 1996). Among the novel cell types are neurons of stratum lacunosum-moleculare with horizontally oriented dendrites and with axons terminating in lacunosum-moleculare, in radiatum and oriens, in radiatum and pyramidale, or in stratum radiatum alone (Figure 1; L-H-LRO, L-H-RP, and L-H-R). This subdivision may be justified, since the differing innervation pattern presumably corresponds to diverse physiological actions on pyramidal cells. Another previously unrecognized cell group are interneurons of stratum oriens with horizontal dendrites and axons restricted either to stratum oriens or to both oriens and pyramidale (Figure 1; O-H-PO and O-H-O). These cells might correspond to stratum oriens neurons with axons projecting to stratum lacunosum-moleculare (McBain et al., 1994; Figure 1; O-H-LO) in which these major myelinated axons were either cut in slice preparation or were incompletely filled. Close inspection of these specimens, however, convinces us that this is not the case and that they should be considered as a distinct group of interneurons.

Interneuronal Discharge Patterns

Eccles was the first to suggest that hippocampal interneurons fire differently than pyramidal cells, typically at very high frequency (Eccles, 1969). More recent work, especially since the advent of techniques for recording from visualized inhibitory cells in slices (Kawaguchi, 1993, 1995; Macaferri and McBain, 1996; Cauli et al., 1997), suggests that firing patterns differ. We chose to classify patterns of spontaneous interneuron discharges. This revealed a group of cells possessing subthreshold Na^+-dependent oscillations in membrane potential, which could result in a characteristic clustered firing pattern (Figures 2C and 3A). The discharge of this cell group is similar to that of stellate neurons of entorhinal cortex (Alonso and Llinas, 1989) and GABAergic cells of nucleus basalis (Alonso et al., 1996) but has not previously been reported from hippocampus. We also provide evidence that specific currents including I_H, I_D, and I_T are expressed in subsets of inhibitory cells (Figure 3). Previous reports suggest that specific groups of hippocampal or cortical inhibitory neurons express these currents (Fraser and MacVicar, 1991; Kawaguchi, 1995; Maccaferri and McBain, 1996), whereas our data point to a more

widespread but patchy distribution. The origin of these differences is not clear, although it is notable that the hippocampal studies were made on cells from a single layer. As in the cortex (Kawaguchi, 1993, 1995), more detailed studies of different neuronal subtypes may refine our knowledge on interneuron physiology.

Selective Expression of Receptors of Modulating Transmitters

Although the identity of the receptors involved in exciting and inhibiting inhibitory cells was not explored systematically, our results tend to agree with previous reports. Thus, 1S, 3R-ACPD excites inhibitory cells by an action at either mGluR1 or mGluR5 receptors (McBain et al., 1994; Poncer et al., 1995). Immunocytochemical studies show mGluR1α is expressed selectively by cells with somata located in stratum oriens (Baude et al., 1993), while mGluR5 is expressed by interneurons with somata in other layers of the CA1 region (Lujan et al., 1996). Serotonin excites a subpopulation of inhibitory cells by an action at 5-HT3 receptors (Ropert and Guy, 1991; Kawa, 1994) and inhibits other inhibitory cells via 5-HT1 receptors (Schmitz et al., 1995). NA acts at α receptors to excite interneurons located in all layers of the CA1 region but inhibits others with somata located in stratum radiatum and lacunosum-moleculare acting via β adrenergic receptors (Bergles et al., 1996). Finally, M has been shown to excite CA1 interneurons (Behrends and ten Bruggencate, 1993), but the receptor subtype that mediates this response is not certain, and a muscarinic inhibition of hippocampal interneurons has not yet been described.

Absence of Correlations

The most interesting aspect of this work is that subsets of inhibitory cells defined by morphology, electrophysiology, and expression of neurotransmitter receptors do not coincide. In this study, these three parameters have been correlated for a large sample of interneurons from all layers of the hippocampal CA1 region. Previous work has either examined responses to one transmitter (e.g., McBain et al., 1994; Bergles et al., 1996) or the electrophysiology of morphologically defined interneurons with somata located in a single layer of this region (e.g., Buhl et al., 1994; Macaferri and McBain, 1996).

Two studies have compared physiological characteristics of morphologically defined hippocampal inhibitory cells. Action potential durations and membrane time constants were statistically different for CA1 basket cells and bistratified cells (Buhl et al., 1996). In contrast, classifications of dentate interneurons (Mott et al., 1997) based on axonal arborization and on adaptation of spikes initiated by step depolarizations did not coincide. Another study on cortical interneurons has correlated electrophysiology with multiplexed RT–PCR to detect whether mRNAs for three calcium-binding proteins and four peptides were present in the recorded cell (Cauli et al., 1997). While some tendencies were apparent (parvalbumin mRNA was often found in fast-spiking interneurons, for instance), overall there was a wide variability in patterns of calcium-binding protein and peptide expression such that 92 cells examined fell into 34 distinct groups.

Such a division of neurons into classes is difficult since in some respects each cell may be different. It seems probable that for each cell exact axonal or dendritic arborizations are different. Equally, the detailed location, expression, and functional state of membrane ion channels and receptor-operated channels seem likely to vary dynamically even in the same cell. However, it is conceptually useful to form classes of cells that behave similarly in many respects. Our data suggest that inhibitory cells of the CA1 region of the hippocampus cannot easily be grouped into a small number of cell classes. Instead three possibilities exist. First, there might exist a large number of inhibitory cell groups, perhaps more than 20–30, within each of which cellular properties are identical. Second, each interneuron may be different, and further horizontal studies examining other cellular properties may reveal a larger diversity than that we have described. Third, a small number of groups of hippocampal inhibitory cells might exist with most, but not all, cellular properties identical within each group. This final possibility raises the question of whether two neurons belong to different classes when they differ in expression of a single molecule such as a calcium-binding protein or a neurotransmitter receptor.

It is important to note we did not consider variations in the extent to which a cell showed a particular property. Thus, we did not distinguish between cells that showed a strong and a weak response to a particular transmitter agonist. Rather, we attempted to set criteria that could reasonably separate cells that, for instance, did or did not express mGluRs or whose axons did or did not innervate stratum oriens. Following this approach, it is of course possible that we may have misclassified cells that expressed a particular property to a small extent, but we are confident that this did not increase the number of cell groups. Then, it is possible that as more cellular properties are examined, the fragmentation of classes of hippocampal and cortical interneurons will continue. This balkanization raises two further questions. How is the diversity generated and what does it mean for the function of inhibitory cells in hippocampal networks?

How Is Inhibitory Cell Diversity Generated?

Both cell lineage (Chess et al., 1994) and responses to environmental cues (Turner et al., 1990) might contribute to this inhibitory cell diversity. In rat cortex, cell fate becomes progressively more and more restricted during successive divisions of initially multipotent cells (McConnell, 1995; Reid et al., 1995). Clonal analysis suggests that by E16 or E17, developing cells are committed to release either GABA or glutamate in their adult state (Mione et al., 1994; Reid et al., 1995). However, the expression of calcium-binding proteins, which discriminates between subsets of inhibitory cells (Freund and Buzsaki, 1996), is heterogeneous in clonally related inhibitory cells, suggesting lineage does not completely determine interneuron phenotype (Mione et al., 1994). Indeed, parvalbumin and calbindin may be colocalized in cortical and hippocampal interneurons until P14 (Alcantara et al., 1996), suggesting that inhibitory cell phenotype does not stabilize until quite late in development.

This stabilization may depend on environmental cues such as innervation by specific afferent fibers (Rocamora et al., 1996), activation of receptors for nerve growth factors (Marty et al., 1996), and the level of activity in hippocampal networks (Marty and Ontoniente, 1997). It remains to be determined how environmental cues influence the properties examined in this report.

What Are the Functional Consequences of Inhibitory Cell Diversity?

What does this degree of diversity imply for the role of inhibitory cells in hippocampal computation? It remains attractive to suppose that different inhibitory cell types have different functions (Buhl et al., 1994; Cobb et al., 1995; Whittington et al., 1995; Miles et al., 1996), but if so our data suggests that the number of inhibitory cell functions may be larger than previously supposed. Furthermore, the expression of different combinations of transmitter receptors by different groups of hippocampal interneurons may permit a very subtle matching of inhibitory cell activity to the computational needs of different behavioral states, resulting in a high flexibility of response.

Experimental Procedures

Slice Preparation

Rats aged between 18 and 28 days were anesthetized with a 1:1 mixture of 10% ketamine and 35% chloralose (1 ml/200 g). Under anesthesia they were perfused through the heart with a solution containing (in mM) 130 NaCl, 2.7 KCl, 20 $NaHCO_3$, 1 $CaCl_2$, 10 $MgCl_2$, 1.3 NaH_2PO_4, 27.7 D-glucose, 0.4 ascorbic acid, and 0.05 ketamine at 4°C. After perfusion, the brain was removed and transverse hippocampal slices 200–350 μm thick were prepared. Only slices from the middle third of the hippocampus were taken. Slices were submerged in an incubation chamber in a solution containing (in mM) 130 NaCl, 2.7 KCl, 20 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, 1.3 NaH_2PO_4, and 27.7 D-glucose maintained at 30°C and bubbled with 5% CO_2 in O_2. After at least 1 hr, single slices were transferred to a recording chamber mounted on the stage of a microscope (Zeiss Axioskop FS with a 40×, 0.75 NA objective). This recording chamber was perfused with the same solution as that used in the incubation chamber at a temperature of 32°–35°C.

Whole-Cell Recordings

Hippocampal interneurons in strata oriens, radiatum, and lacunosum moleculare were visualized using a CCD camera and controller (Hamamatsu 2400–07) with incident light filtered to pass visible and infrared. Pipettes for whole-cell recording contained (in mM) 120 K-gluconate, 10 HEPES, 10 EGTA, 3 $CaCl_2$, 2 $MgCl_2$, 4 ATP-$(Na)_2$, 0.4 GTP-Na, 10 KCl, and 0.5 biocytin and had resistances of 3–6 MΩ. The pH of the pipette solution was adjusted to 7.4 and its osmolarity to 290 mOsm. Somatic whole-cell recordings were made in current clamp mode using an Axoclamp-2A amplifier that was zeroed before seal formation. Recordings were accepted when access resistance was less than 20 MΩ, action potentials overshooting 0 mV were generated, and resting potentials were more negative than −40 mV. The mean resting membrane was 61 ± 2.7 mV (mean ± SEM; n = 144) and the mean input resistance was 230 ± 17 MΩ (mean ± SEM; n = 144) Neither of these parameters varied systematically with the other properties that we examined.

Drug Application

1S, 3R-ACPD, M, NA, and 5-HT were made up as concentrated stocks and diluted to their final concentrations in the external solution before use. These agonists were applied via bath perfusion. The order of their application to each cell was varied but not systematically randomized.

Morphology

Recordings with biocytin-containing electrodes were normally made from a single cell in each slice. After recording, slices were fixed in a solution containing 4% paraformaldehyde, 0.05% glutaraldehyde, and 15% picric acid in 0.1 M phosphate buffer for 18 hr at 4°C. Slices were resectioned at 80 μm using a vibratome, and these sections were freeze-thawed above liquid N_2 in a 0.1 M phosphate buffer containing 10% glycerol and 15% sucrose. Neurons were visualized using the avidin–biotinylated horseradish peroxidase complex reaction (Vector Labs, Elite ABC kit) with 3–3′-diaminobenzidine (Sigma) as chromogen and intensified with ammonium nickel sulphate. Sections were treated with 1% OsO_4, dehydrated, and embedded in resin (Durcupan ACM, Fluka). Axonal and dendritic arborizations of filled cells were reconstructed from multiple sections with a camera lucida using a 50× oil immersion objective. Axonal and dendritic processes were followed either to their termination or to the point where they contacted the cut surface of the slice.

Acknowledgments

We thank T. F. Freund, C. Sotelo, D. S. Faber, B. Barbour, and K. Toth for helpful comments. This work was supported by grants from the HFSP, ESF, NIH (MH54671), INSERM and MRT (France), HSF and OTKA (T16942 and T23261, Hungary), and Colciencias (Colombia).

Received October 2, 1997; March 18, 1998.

References

Alcantara, S., de Lecea, L., del Rio, J.A., Ferrer, I., and Soriano, E. (1996). Transient colocalization of parvalbumin and calbindin D28K in the postnatal cerebral cortex: evidence for a phenotypic shift in developing nonpyramidal neurons. Eur. J. Neurosci. *8*, 1329–1339.

Alonso, A., and Llinas, R. (1989). Subthreshold Na^+-dependent theta-like rhythmicity in stellate cells of entorhinal cortex layer II. Nature *342*, 175–177.

Alonso, A., Khateb, A., Fort, P., Jones, B.E., and Muhlethaler, M. (1996). Differential oscillatory properties of cholinergic nucleus basalis neurons in guinea pig brain slice. Eur. J. Neurosci. *8*, 169–182.

Baude, A., Nusser, Z., Roberts, J.D.B., Mulvihill, E., McIlhinney, R.A.J., and Somogyi, P. (1993). The metabotropic glutamate receptor (mGluR1α) is concentrated at perisynaptic membrane of neuronal subpopulations, as detected by the immunogold reaction. Neuron *11*, 771–787.

Behrends, J.C., and ten Bruggencate, G. (1993). Cholinergic modulation of synaptic inhibition in the guinea pig hippocampus in vitro: excitation of GABAergic interneurons and inhibition of GABA-release. J. Neurophysiol. *69*, 626–629.

Bergles, D.E., Doze, V.A., Madison, D.V., and Smith S.J. (1996). Excitatory actions of norepinephrine on multiple classes of hippocampal CA1 interneurons. J. Neurosci. *16*, 572–585.

Buhl, E.H., Halasy, K., and Somogyi, P. (1994). Diverse sources of hippocampal unitary inhibitory postsynaptic potentials and the number of synaptic release sites. Nature *368*, 823–828.

Buhl, E.H., Szilagyi, T., Halasy, K., and Somogyi, P. (1996). Physiological properties of anatomically identified basket and bistratified cells in the CA1 area of the rat hippocampus in vitro. Hippocampus *6*, 294–305.

Cauli, B., Audinat, E., Lambolez, B., Angulo, M.C., Ropert, N., Tsuzuki, K., Hestrin, S., and Rossier, J. (1997). Molecular and physiological diversity of cortical nonpyramidal cells. J. Neurosci. *17*, 3894–3906.

Calloway, J.C., Lasser-Ross, N., and Ross, W.N. (1995). IPSPs strongly inhibit climbing fiber activated [Ca2+]i increases in the dendrites of cerebellar Purkinje neurons. J. Neurosci. *15*, 2777–2778.

Chess, A., Simon, I., Cedar, H., and Axel, R. (1994). Allelic inactivation regulates olfactory receptor gene expression. Cell *78*, 823–834.

Cobb, S.R., Buhl, E.H., Halasy, K., Paulsen, O., and Somogyi, P.

(1995). Synchronization of neuronal activity in hippocampus by individual GABAergic interneurons. Nature *378*, 75–78.

Eccles, J.C. (1969). The Inhibitory Pathways of the Central Nervous System (Liverpool, UK: Liverpool University Press).

Fraser, D.D., and MacVicar, B.A. (1991). Low-threshold calcium current in rat hippocampal lacunosum-moleculare interneurons: kinetics and modulation by neurotransmitters. J. Neurosci. *11*, 2812–2820.

Freund, T.F., and Buzsaki, G. (1996). Interneurons of the hippocampus. Hippocampus *6*, 345–470.

Freund, T.F., Gulyás, A.I., Acsady, L., Gorcs, T., and Toth, K. (1990). Serotonergic control of the hippocampus via local inhibitory interneurons. Proc. Natl. Acad. Sci. USA *87*, 8501–8505.

Guerineau, N.C., Bossu, J.-L., Gahwiler, B.H., and Gerber, U. (1997) G-protein mediated desensitization of metabotropic glutamatergic and muscarinic responses in CA3 cells in rat hippocampus. J. Physiol. *500*, 487–496.

Gulyás, A.I., Miles, R., Hájos, N., and Freund, T.F. (1993). Precision and variability in postsynaptic target selection of inhibitory cells in the hippocampal CA3 region. Eur. J. Neurosci. *5*, 1729–1751.

Gulyás, A.I., Hájos, N., and Freund, T.F. (1996) Interneurons containing calretinin are specialized to control other interneurons in the rat hippocampus. J. Neurosci. *16*, 3397–3411.

Hoffman, D.A, Magee, J.C., Colbert, C.M., and Johnston, D. (1997). K^+ channel regulation of signal propagation in dendrites of hippocampal pyramidal neurons. Nature, *368*, 869–875.

Kawa, K. (1994). Distribution and functional properties of 5-HT3 receptors in the rat hippocampal dentate gyrus: a patch-clamp study. J. Neurophysiol. *71*, 1935–1947.

Kawaguchi, Y. (1993). Groupings of nonpyramidal and pyramidal cells with specific physiological and morphological characteristics in rat frontal cortex. J. Neurophysiol. *69*, 416–431.

Kawaguchi, Y. (1995). Physiological subgroups of nonpyramidal cells with specific morphological characteristics in layer II/III of rat frontal cortex. J. Neurosci. *15*, 2638–2655.

Kupfermann, I., and Weiss, K.R. (1978). The command neuron concept. Behav. Brain Sci. *1*, 3–39.

Leinekugel, X., Medina, I., Khalilov, I., Ben-Ari, Y., and Khazipov, R. (1997). Ca^{2+} oscillations mediated by the synergistic excitatory actions of GABAA and NMDA receptors in the neonatal hippocampus. Neuron *18*, 243–255.

Lujan, R., Nusser, Z., Roberts, J.D.B., Shigemoto, R., and Somogyi, P. (1996). Perisynaptic location of metabotropic glutamate receptors mGluR1 and mGluR5 on dendrites and dendritic spines in the hippocampus. Eur. J. Neurosci. *8*, 1488–1500.

Maccaferri, G., and McBain, C.J. (1996). The hyperpolarization-activated current (I_h) and its contribution to pacemaker activity in rat CA1 hippocampal stratum oriens-alveus interneurones. J. Physiol. *497*, 119–130.

Manzoni, O.J., Manabe, T., and Nicoll, R.A. (1994). Release of adenosin by activation of NMDA receptors in the hippocampus. Science *265*, 2096–2101.

Marty, S., and Ontoniente, B. (1997). The expression pattern of somatostatin and calretinin by postnatal hippocampal interneurons is regulated by activity-dependent and independent determinants. Neuroscience *80*, 79–88.

Marty, S., Berninger, B., Carroll, P., and Thoenen, H. (1996). GABAergic stimulation regulates the phenotype of hippocampal interneurons through the regulation of brain-derived neurotrophic factor. Neuron *16*, 565–570.

McBain, C.J., DiChiara, T.J., and Kauer, J.A. (1994). Activation of metabotropic glutamate receptors differentially affects two classes of hippocampal interneurons and potentiates excitatory synaptic transmission. J. Neurosci. *14*, 4433–4445.

McConnell, S.K. (1995). Strategies for the generation of neuronal diversity in the developing central nervous system. J. Neurosci. *15*, 6987–6998.

Miles, R., Toth, K., Gulyás, A.I., Hájos, N., and Freund, T.F. (1996). Differences between somatic and dendritic inhibition in the hippocampus. Neuron *16*, 815–823.

Mione, M.C., Danevic, C., Boardman, P., Harris, B., and Parnevalas, J.G. (1994). Lineage analysis reveals neurotransmitter (GABA and Glutamate) but not calcium-binding protein homogeneity in clonally related cortical neurons. J. Neurosci. *14*, 107–123.

Mott, D.D., Turner, D.A., Okazaki, M.M., and Lewis, D.V. (1997). Interneurons of the dentate-hilus border of the rat dentate gyrus: morphological and electrophysiological heterogeneity. J. Neurosci. *17*, 3390–4005.

Nicoll, R.A. (1994). Cajal's rational psychology. Nature *368*, 808–809.

Poncer, J.-C., Shinozaki, H., and Miles, R. (1995). Dual modulation of synaptic inhibition by distinct metabotropic glutamate receptors in the rat hippocampus. J. Physiol. *485*, 121–134.

Ramon y Cajal, S. (1911). Histologie du Systeme Nerveux de L'Homme et des Vertebres, Vol. 2 (Paris: Maloine).

Reid, C.B., Liang, I., and Walsh, C. (1995). Systematic widespread clonal organization in cerebral cortex. Neuron *15*, 299–310.

Rocamora, N., Pascual, M., Acsady, L., De Lecea, L., Freund, T.F., and Soriano, E. (1996). Expression of NGF and NT3 mRNAs in hippocampal interneurons innervated by the GABAergic septohippocampal pathway. J. Neurosci. *16*, 3991–4004.

Ropert, N., and Guy, N. (1991). Serotonin facilitates GABAergic transmission in the CA1 region of rat hippocampus in vitro. J. Physiol. *441*, 121–136.

Schmitz, D., Empson, R.M., and Heinemann, U. (1995). Serotonin reduces inhibition via 5-HT_{1A} receptors in area CA1 of rat hippocampal slices in vitro. J. Neurosci. *15*, 7217–7225.

Selverston, A.I., and Moulins, M. (1987). The Crustacean Stomatogastric System (Berlin: Springer).

Storm, J.F. (1988). Temporal integration by a slowly inactivating K^+ current in hippocampal neurons. Nature *336*, 379–381.

Turner, D.L., Snyder, E.Y., and Cepko, C.L. (1990). Lineage-independent determination of cell type in the embryonic mouse retina. Neuron *4*, 833–845.

Whittington M. A., Traub, R.D., and Jefferys, J.G.R. (1995). Synchronized oscillations in interneuron networks driven by metabotropic glutamate receptor activation. Nature *373*, 612–615.

Zhang, L., and McBain, C.J. (1995). Potassium conductances underlying repolarization and after-hyperpolarization in rat CA1 hippocampal interneurones. J. Physiol. *488*, 661–672.