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# Presynaptic Specificity of Endocannabinoid Signaling in the Hippocampus

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## Summary

**Endocannabinoids are retrograde messengers released by neurons to modulate the strength of their synaptic inputs. Endocannabinoids are thought to mediate the suppression of GABA release that follows depolarization of a hippocampal CA1 pyramidal neuron—termed "depolarization-induced suppression of inhibition" (DSI). Here, we report that DSI is absent in mice which lack cannabinoid receptor-1 (CB1). Pharmacological and kinetic evidence suggests that CB1 activation inhibits presynaptic $Ca^{2+}$ channels through direct G protein inhibition. Paired recordings show that endocannabinoids selectively inhibit a subclass of synapses distinguished by their fast kinetics and large unitary conductance. Furthermore, cannabinoid-sensitive inputs are unusual among central nervous system synapses in that they use N- but not P/Q-type $Ca^{2+}$ channels for neurotransmitter release. These results indicate that endocannabinoids are highly selective, rapid modulators of hippocampal inhibition.**

## Introduction

Several recent studies have indicated that endogenous cannabinoids function as rapid, retrograde signaling molecules in the central nervous system (Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001). This pathway is initiated by depolarizing stimuli which open voltage-dependent calcium channels (VDCCs) in the postsynaptic neuron. Cytoplasmic $Ca^{2+}$ then triggers the synthesis of endocannabinoids, possibly anandamide or 2-arachidonylglycerol (Piomelli et al., 1998; Ameri, 1999). Endocannabinoid molecules exit the postsynaptic cell by an unknown mechanism, either by diffusing directly through the plasma membrane or else via the action of carrier proteins. Endocannabinoids then diffuse backward across the synapse to activate a G protein-coupled receptor, presumed to be CB1. Cannabinoid receptor activation then leads to a decrease in the probability of release of a vesicle of neurotransmitter from the axon terminal (Hajos et al., 2000; Hoffman and Lupica, 2000; Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001).

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In the hippocampus, CB1 is only expressed by GABAergic interneurons (Katona et al., 1999, 2000; Tsou et al., 1999), suggesting that the endocannabinoid system functions specifically to depress GABA release. Accordingly, brief depolarization of CA1 pyramidal cells causes a transient decrease in GABAergic (but not glutamatergic) synaptic transmission—a process termed "depolarization-induced suppression of inhibition" (DSI) (Pitler and Alger, 1992; Wagner and Alger, 1996). The recent insight that DSI is probably mediated by cannabinoids was based on the findings that (1) DSI is blocked by CB1 antagonists and that (2) DSI is mimicked and occluded by a synthetic CB1 agonist (WIN55212-2) or by blocking uptake of endocannabinoids, which are tonically produced by the slice (Wilson and Nicoll, 2001; Ohno-Shosaku et al., 2001). DSI also has properties consistent with the anatomy and biochemistry of the endocannabinoid system, including a presynaptic locus of expression (Pitler and Alger, 1992; Alger et al., 1996; Morishita and Alger, 1997), an absolute requirement for postsynaptic $Ca^{2+}$ (Pitler and Alger, 1992), and a nonvesicular mechanism of release for the retrograde signal (Wilson and Nicoll, 2001).

Here, we focus on elucidating the presynaptic mechanisms of synaptic depression by endocannabinoids. We find that hippocampal DSI is completely absent in *CB1*$^{-/-}$ mice, strongly implying that DSI is mediated by cannabinoid release, and that its presynaptic target is indeed exclusively the brain-specific cloned cannabinoid receptor. Furthermore, we find that the downstream effector of CB1 in this context is likely to be direct inhibition of $Ca^{2+}$ channels by G proteins, rather than a diffusible second messenger in the presynaptic terminal. This direct mechanism is likely to account for the remarkably rapid induction of synaptic depression, which we find begins within about 1.2 s after postsynaptic depolarization. Finally, we find that endocannabinoids exhibit a striking specificity in targeting a distinct class of interneurons, which are distinguished by their synaptic kinetics, connection strength, excitability profile, and exclusive use of the N-type $Ca^{2+}$ channel for neurotransmitter release. These findings provide important clues as to the function of endocannabinoids in the hippocampal network and suggest possible mechanisms for the disruption of memory by cannabis derivatives.

## Results

### DSI Is Absent in CB1-Deficient Mice

Previous work (Wilson and Nicoll, 2001; Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001) demonstrated that hippocampal DSI and a related phenomenon in the cerebellum are blocked by SR141716, which is considered to be a specific CB1 antagonist (Pertwee, 1997). However, endogenous cannabinoids have SR141716-sensitive actions in the cardiovascular system that are independent of either CB1 or CB2 (Wagner et al., 1999; Járai et al., 1999), suggesting that there might be

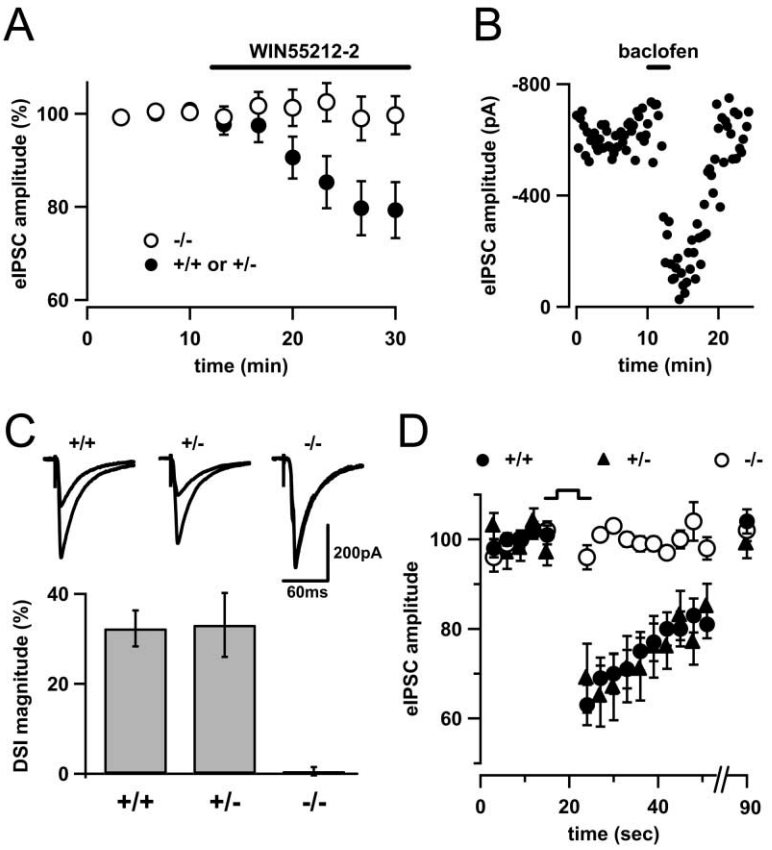


Figure 1. DSI Is Absent in CB1-Deficient Mice

(A) The synthetic CB1 agonist WIN552121-2 (800 nM) depresses eIPSCs in the CA1 region of $CB1^{+/+}$ and $CB1^{+/-}$ mice but has no effect on eIPSCs in slices from $CB1^{-/-}$ mice.

(B) A representative experiment showing that baclofen (3 μM) elicits a robust depression of eIPSCs in $CB1^{-/-}$ mice, demonstrating that presynaptic inhibition by GABA-B receptors is intact in the mutant animals.

(C) DSI is normal in $CB1^{+/+}$ and $CB1^{+/-}$ mice but completely absent in $CB1^{-/-}$ mice. DSI is expressed as a percentage depression in eIPSC amplitude during the first 10 s after depolarization, averaged across four to five trials per sampled cell. DSI for each genotype is averaged across all cells sampled. Insets show eIPSCs from representative experiments for each genotype, with averaged traces (12 to 15 sweeps) obtained just before and just after depolarization overlaid.

(D) Average time course of eIPSC amplitudes after depolarization in $CB1^{+/+}$, $CB1^{+/-}$, and $CB1^{-/-}$ mice. Square step indicates 5 s depolarization from −60mV to 0mV.

an uncloned cannabinoid receptor which is sensitive to SR141716. Consistent with this possibility, both anandamide and the cannabinoid receptor agonist WIN55212-2 still stimulate [$^3$H]GTPγS binding in the brains of $CB1^{-/-}$ mice, and this binding is blocked by SR141716 (Breivogel et al., 2001). Therefore, we used $CB1^{-/-}$ mice (Zimmer et al., 1999) to determine whether hippocampal DSI is indeed mediated by CB1 activation.

We first investigated the effects of WIN55212-2, a relatively nonselective CB1/CB2 agonist, in $CB1^{-/-}$ mice (Figure 1A). We monitored evoked inhibitory postsynaptic currents (eIPSCs) in whole-cell voltage-clamp recordings from hippocampal CA1 pyramidal cells. WIN55212-2 (800 nM) depressed eIPSCs by 21% ± 6% in slices from $CB1^{+/+}$ and $CB1^{+/-}$ mice (n = 10 +/+ and 2 +/−) compared to 0% ± 4% in $CB1^{-/-}$ slices (n = 7, $p < 0.05$, t test). This agrees with a previous study which showed that WIN55212-2 has no effect on eIPSCs recorded in dentate granule cells of $CB1^{-/-}$ mice (Hajos et al., 2000). Baclofen (3 μM) could still elicit a robust depression of eIPSCs in $CB1^{-/-}$ slices (average depression 69% ± 9%; n = 3; Figure 1B), showing that other components of presynaptic inhibition via G proteins are still intact in these animals.

We then assessed DSI in these animals by recording from one CA1 pyramidal neuron per slice and averaging over four to five trials per cell during the first 10 min of recording. DSI is expressed as a percentage depression in eIPSC amplitude. Using this method, average DSI magnitude in wild-type mice (32% ± 4%, n = 33) and heterozygotes (33% ± 7%, n = 9) is comparable to DSI measured in hippocampal slices from juvenile rats (Figures 1C and 1D; compare with controls in Figure 2A). In $CB1^{-/-}$ mice, however, DSI is entirely absent (0.6% ± 0.9%, n = 25; $p < 10^{-9}$, t test; Figures 1C and 1D). These data demonstrate that CB1 is indeed an absolute requirement for hippocampal DSI and is likely to be the direct presynaptic target of the retrograde signal.

### Presynaptic Inhibition by Endocannabinoids Is Likely to Involve Direct Interaction of G Proteins with Calcium Channels

Next, we investigated the signaling pathway downstream from the CB1 receptor. Synaptic depression by endocannabinoids in the hippocampus and cerebellum is caused by inhibition of presynaptic voltage-dependent $Ca^{2+}$ channels (VDCCs) (Hoffman and Lupica, 2000; Kreitzer and Regehr, 2001). CB1 is likely to be acting via G proteins in this case, as DSI is pertussis-toxin sensitive (Pitler and Alger, 1994), but inhibition of VDCCs may be caused either by a direct action of Gβγ on VDCCs (Mackie and Hille, 1992; Herlitze et al., 1996; Ikeda, 1996) or else by a second messenger cascade involving inhibition of adenylate cyclase (Howlett and Fleming, 1984). We found that DSI is resistant to staurosporine (5 μM; n = 5 treated, 5 control), a broad-spectrum inhibitor of serine/threonine kinases, as well as the phosphatase inhibitors FK506 (10 μM; n = 6 treated, 5 control) and calyculin A (100 nM; n = 6 treated, 5 control) (Figure 2A). In agreement with other investigators (Morishita et al., 1998), we also found that DSI is unaffected by for-

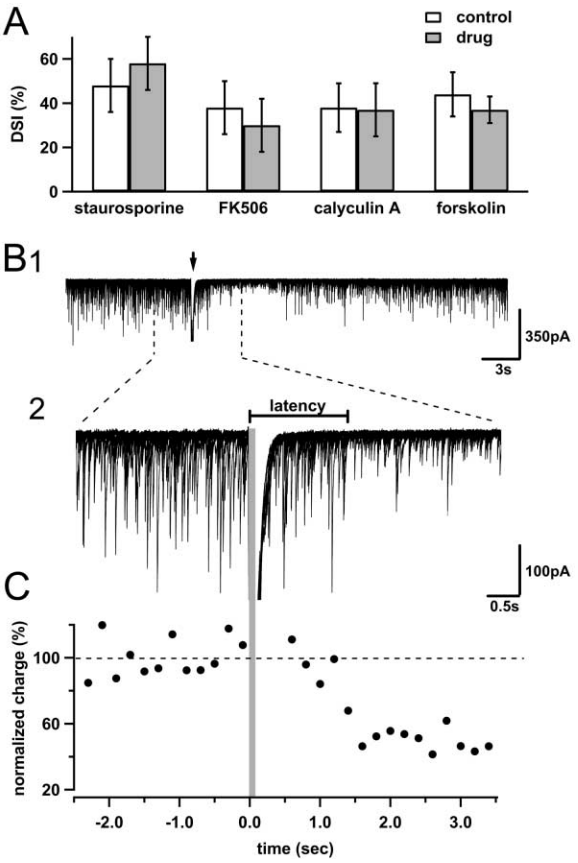


Figure 2. Presynaptic Inhibition by Endocannabinoids Is Likely to Involve Direct Interaction of G Proteins with Calcium Channels

(A) DSI is not affected by staurosporine (5 μM), a broad spectrum kinase inhibitor, or the phosphatase inhibitors FK-506 (10 μM ) or calyculin A (100 nM). DSI is also not affected by forskolin (10 μM), which activates adenylate cyclase. Slices were preincubated and recorded in either the drug or a solvent control, and DSI was sampled in one cell per slice.

(B) Kinetics of DSI onset are rapid. Spontaneous IPSCs elicited by carbachol (5 μM) are transiently suppressed following a 100 ms depolarization from −60mV to 0mV (arrow). Eight sweeps representing individual DSI trials from a single cell are overlaid to show the average time course of DSI (B1). The region surrounding the depolarizing step is displayed below on an expanded time scale (B2) to illustrate the short latency between the step and suppression of synaptic activity.

(C) Group data showing average kinetics of DSI onset. Spontaneous IPSCs from 37 DSI trials (four cells) were detected and measured, and the total charge transfer associated with every IPSC was summed within 200 ms bins and then normalized to average charge transfer during the baseline period. Overall, charge transfer began to drop about 1.2 s after the start of the depolarizing step. Gray bar (B and C) indicates 100 ms depolarization.

skolin (50 μM), which activates adenylate cyclase and should interact with any process mediated by a change in cAMP levels (Figure 2A). These results raise the possibility that DSI involves a direct action of Gβγ on VDCCs by a fast, membrane-delimited pathway. These data do not rule out a more subtle, modulatory effect of kinases or phosphatases on DSI.

A previous study has qualitatively described a latency period before DSI onset (Pitler and Alger, 1994). We decided to examine the kinetics of DSI onset more precisely, in order to determine if they were consistent with direct inhibition of presynaptic VDCCs. Carbachol (5 μM) was added to the bath to increase the frequency of spontaneous IPSCs (sIPSCs) to 10–15 Hz (Pitler and Alger, 1992) to allow visualization of DSI onset at higher temporal resolution, and very short depolarizing steps (100 ms) were used to elicit DSI. We observed that the depolarizing step was followed by a short delay before sIPSCs were depressed. On average, this delay lasted 1.2 s (Figure 2B). Whereas phosphorylation-induced changes in channel activity typically require many seconds, the membrane-delimited pathway of presynaptic inhibition is much faster (Hille, 1992; Hescheler and Schultz, 1993), consistent with the brief latency observed here. Together, these pharmacological and kinetic results are evidence that DSI inhibits VDCCs by the direct, membrane-delimited action of Gβγ.

### Differential Involvement of Presynaptic VDCC Subtypes in Endocannabinoid Signaling

Next, we asked whether endocannabinoid-mediated inhibition showed any specificity for a particular presynaptic $Ca^{2+}$ channel subtype. GABA release from hippocampal interneurons is mediated by both N and P/Q subtypes of VDCCs in the presynaptic terminal (Doze et al., 1995). According to a previous report (Lenz et al., 1998), DSI is blocked by a specific antagonist of N-type channels, ω-conotoxin GVIA (ω-CTx-GVIA). This was interpreted as a postsynaptic requirement for N-type channels as the mediators of the depolarization-evoked, postsynaptic calcium spike which triggers DSI. We wondered whether DSI might require N-type VDCCs presynaptically.

We monitored eIPSCs and DSI while washing ω-CTx-GVIA (250 nM) into the bath. ω-CTx-GVIA irreversibly depressed eIPSCs by 74% ± 9% (Figure 3A; $n = 4$) and abolished DSI (Figures 3A and 3C; 3% ± 4% DSI in ω-CTx-GVIA compared to 50% ± 7% during baseline period; $p < 0.005$, paired t test). Surprisingly, ω-CTx-GVIA also blocked the depressant effects of WIN55212-2 (Figures 3A and 3D; eIPSC amplitude depressed by 6% ± 5% compared to 28% ± 5% in control slices, $n = 17$, control data not shown; $p < 0.05$, t test). This result suggests that there may be a presynaptic requirement for N-type calcium channels in the regulation of GABAergic transmission by cannabinoids.

We then repeated these experiments using ω-agatoxin TK (ω-Aga-TK), a selective antagonist of P/Q-type calcium channels. ω-Aga-TK (250 nM) irreversibly depressed eIPSCs by 70% ± 6% (Figure 3B; $n = 5$). In agreement with a previous report (Lenz et al., 1998), ω-Aga-TK actually increased the magnitude of DSI, from 50% ± 6% to 68% ± 4% (Figures 3B and 3C; $p < 0.005$, paired t test). Based on our results with ω-CTx-GVIA, we suspected that ω-Aga-TK might be acting presynaptically to remove a component of GABA release that is insensitive to DSI. Consistent with this hypothesis, eIPSCs in the presence of ω-Aga-TK were extremely sensitive to WIN55212-2 (Figures 3B and 3D; eIPSC amplitude depressed by 83% ± 9%; $p < 0.05$ compared to controls, t test).

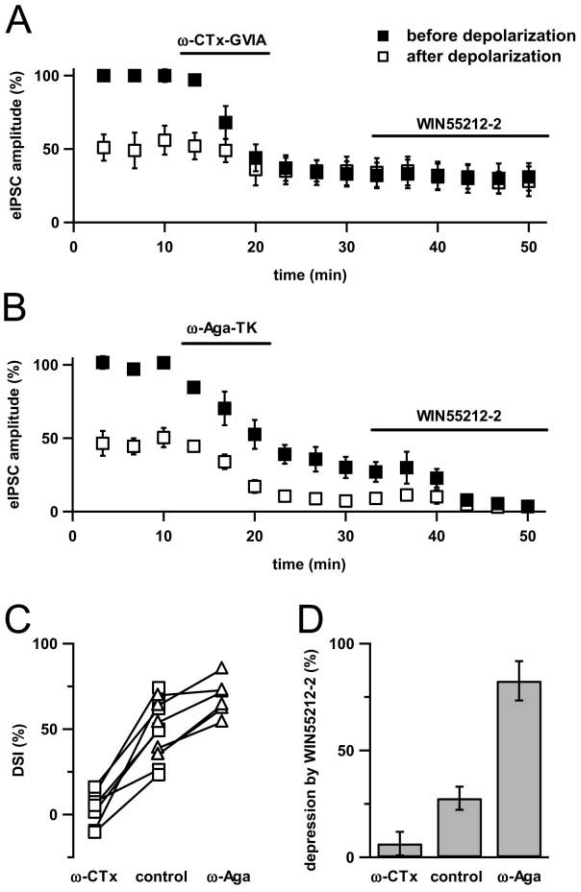


Figure 3. Presynaptic N-Type $Ca^{2+}$ Channels Are Required for Endocannabinoid Signaling

(A) DSI was monitored by comparing eIPSC amplitudes just before (filled symbols) and just after (open symbols) depolarizing steps. After a stable baseline period, the N-type VDCC antagonist ω-conotoxin GVIA (ω-CTx-GVIA) was washed onto the slice, causing a depression of basal IPSC amplitude and a complete block of DSI. Subsequent wash-in of WIN55212-2 had no effect, indicating that N-type VDCCs are required for presynaptic inhibition by cannabinoids.

(B) The same experiment as in (A) was performed using the P/Q-type VDCC antagonist ω-agatoxin TK (ω-Aga-TK). ω-Aga-TK also depressed basal IPSC amplitude but increased DSI magnitude. Subsequent wash-in of WIN55212-2 blocked most of the remaining IPSC, indicating that the component of release mediated by N-type VDCCs is highly sensitive to cannabinoids.

(C) Summary data showing that, in experiments where ω-CTx-GVIA was added (squares), DSI was blocked, whereas, in experiments where ω-Aga-TK was added (triangles), DSI was increased.

(D) Summary data showing that after ω-CTx-GVIA addition, eIPSCs are not depressed by WIN55212-2. In contrast, after ω-Aga-TK addition, eIPSCs are depressed more strongly than in control conditions (control WIN55212-2 time course not shown).

### Interneuron Subtypes Selectively Targeted by Endocannabinoids

The differential involvement in DSI of two types of presynaptic VDCCs suggests two possible scenarios. One possibility is that CB1 is expressed on GABAergic terminals that use both N- and P/Q-type calcium channels for transmitter release but that CB1 activation affects only the N-type channels in those terminals. It seems likely that there are at least some GABAergic terminals having both types of channels, since the algebraic sum of the effects of ω-CTx-GVIA and ω-Aga-TK (144%) exceeds 100%, attributable to the supralinear relationship between calcium influx and neurotransmitter release (Dodge and Rahamimoff, 1967). Selective presynaptic inhibition of N-type versus P/Q-type channels by G protein-coupled receptors has been reported at excitatory synapses in the hippocampus, where these channel subtypes are colocalized in the same presynaptic boutons (Wu and Saggau, 1994). An alternative possibility is that CB1 is expressed on a subpopulation of terminals that use only N-type channels for transmitter release. Indeed, two studies of GABAergic connections among cultured hippocampal neurons (Ohno-Shosaku et al., 1994; Poncer et al., 1997) found that some interneurons use only N-type channels for transmitter release, whereas others use only P/Q-type channels.

In order to distinguish between these two hypotheses, we recorded from connected interneuron-pyramidal cell pairs in the CA1 region. Interneurons were recorded in whole-cell current-clamp mode, and presynaptic action potentials were elicited by brief (5 ms) current injections. We recorded from 343 interneuron-pyramidal cell pairs; we detected an interneuron-to-pyramidal cell synaptic connection in 30 of these. We found that unitary IPSCs (uIPSCs) recorded in these connections varied considerably in their kinetics, generating a bimodal histogram of uIPSC rise times (Figures 4A and 4D). We termed these two groups "GABA-$A_{fast}$" and "GABA-$A_{slow}$," following the convention established by a previous study (Pearce, 1993). GABA-$A_{fast}$ inputs also had faster time constants of decay (19 $\pm$ 1 ms) than GABA-$A_{slow}$ inputs (40 $\pm$ 4 ms). These kinetic differences cannot be explained by higher access resistance in GABA-$A_{slow}$ recordings ($R_{access}$ = 13 $\pm$ 1 MΩ for GABA-$A_{slow}$ compared to 16 $\pm$ 2 MΩ for GABA-$A_{fast}$). Also, cable filtering, which should produce a smooth distribution, seems unlikely to account for all of the kinetic differences we observed. In this respect, our data agrees with evidence that these two groups of synapses use different GABA-A receptors, which could account for the bimodal distribution, with few synapses showing intermediate kinetics (Pearce, 1993; Banks et al., 1998).

We also observed a bimodal distribution of connections in terms of their DSI susceptibility (Figures 4B and 4D). Synapses in one group were strongly depressed by postsynaptic depolarization (average DSI = 88% $\pm$ 5%, n = 7 connections). All of these connections had fast rise times (1.6 $\pm$ 0.2 ms) and are termed here "GABA-$A_{fast\ I}$." The other population with fast kinetics, which we termed "GABA-$A_{fast\ II}$" (average rise time 1.5 $\pm$ 0.2 ms) was completely insensitive to DSI (average DSI = $-$6% $\pm$ 5%, n = 8 connections). All GABA-$A_{slow}$ connections were resistant to DSI (average DSI = 1% $\pm$ 2%, n = 14 connections). GABA-$A_{fast\ I}$ inputs were also distinguished by their large uIPSC amplitudes compared to GABA-$A_{fast\ II}$ ($p < 0.001$) and GABA-$A_{slow}$ ($p < 0.001$, Bonferroni t tests) (Figures 4C and 4D).

We also examined the intrinsic excitability profile of these interneurons. Consistent with previous work (Parra et al., 1998), we found a large diversity in excitability profiles which does not seem to map perfectly onto other physiological properties. Each of the three groups included several types of action potential waveforms

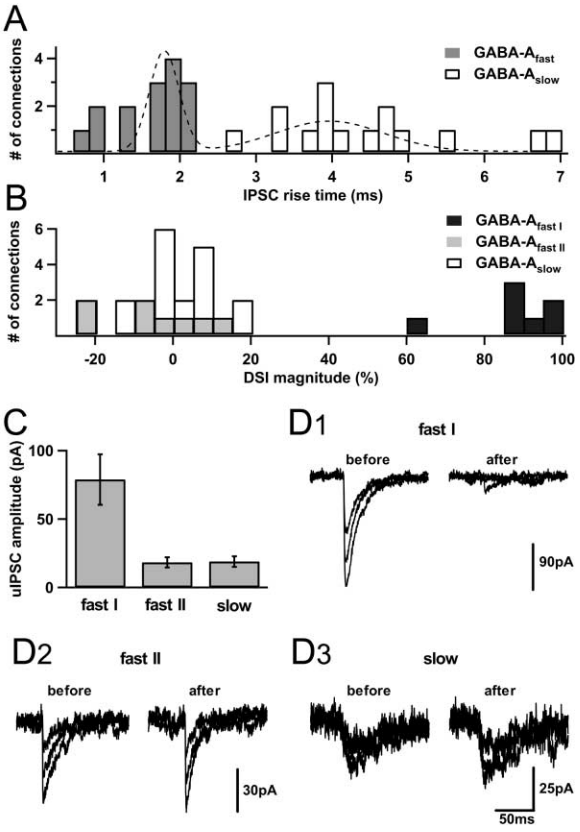


Figure 4. Endocannabinoids Selectively Affect an Interneuron Subtype with Distinctive Synaptic Properties

(A) Recordings from connected interneuron-pyramidal cell pairs yielded 30 GABAergic connections. A histogram of uIPSC rise times produced a bimodal distribution, which we termed GABA-$A_{fast}$ and GABA-$A_{slow}$. Dotted line is a fit to the sum of two gaussian functions, and events are classified (open and gray bars) according to which gaussian better describes them.

(B) A histogram of DSI magnitude shows that all connections were either very sensitive to DSI or else completely insensitive. All the DSI-sensitive connections belonged to the fast kinetic group and are referred to here as GABA-$A_{fast\ I}$, whereas the DSI-insensitive group included both fast synapses (termed here GABA-$A_{fast\ II}$) and all slow synapses (GABA-$A_{slow}$).

(C) Average uIPSC amplitude is significantly larger for GABA-$A_{fast\ I}$ connections compared to either of the other two groups.

(D1–D3) Raw traces from representative connections. Three overlaid raw sweeps acquired just before depolarization are displayed next to three overlaid raw sweeps acquired just after depolarization. Note the different vertical scaling for (D1)–(D3); horizontal scaling is the same for all three groups. GABA-$A_{fast\ I}$ connections (D1) show both failures and small-amplitude successes after depolarization, whereas connnections from the other two groups (GABA-$A_{fast\ II}$ [D2] and GABA-$A_{slow}$ [D3]) are not affected by depolarization.

(Figure 5A) and discharge patterns (Figure 5B). On average, however, interneurons forming GABA-$A_{fast\ II}$ connections were able to fire at a higher maximum initial rate than the other types of connections and showed less accommodation. We also noted that GABA-$A_{fast\ I}$ interneurons had significantly smaller after-hyperpolarizations than the other two interneuron types. GABA-$A_{slow}$ interneurons were distinguished by their broad action potentials and high input resistances (Table 1).

In a subset of these connections where uIPSC amplitude was at least 10 pA and relatively stable, we were able to examine the involvement of different VDCC subtypes in GABA release (Figure 6). All GABA-$A_{fast\ I}$ synapses that we tested were completely blocked by ω-CTx-GVIA (99% ± 0.4%; n = 4). When ω-Aga-TK was washed in prior to ω-CTx-GVIA, it had no effect on the uIPSC (0% ± 9%; n = 2). Conversely, all GABA-$A_{fast\ II}$ synapses were completely blocked by ω-Aga-TK (99% ± 0.3%; n = 5) but insensitive to ω-CTx-GVIA (0% ± 8%; n = 3). Finally, all GABA-$A_{slow}$ connections relied on both N- and P/Q-type channels in roughly equal proportions. When ω-CTx-GVIA was washed on first, it blocked these synapses by 71% ± 3%, and subsequent addition of ω-Aga-TK blocked 96% ± 4% of the remaining response (n = 3). When ω-Aga-TK was washed on first, it blocked GABA-$A_{slow}$ synapses by 68% ± 7%, and subsequent addition of ω-CTx-GVIA blocked 97% ± 1% of the remaining response (n = 3).

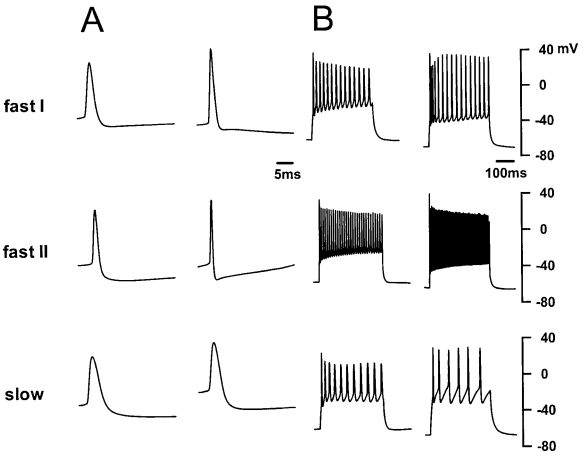


Figure 5. Intrinsic Excitability Differs for the Three Types of Interneurons

The left side of (A) shows single action potentials from two representative cells for each synapse type. These sweeps correspond to a 350 ms current injection producing the lowest (nonzero) spiking rate for that cell. The right side of (B) shows trains of action potentials for the same six cells. These sweeps correspond to a 350 ms current injection producing the maximal spiking rate for that cell. Note the different horizontal scaling for single action potentials and trains; vertical scaling and alignment is the same for all traces (see guide, in mV, on the right).

## Discussion

In the present study, we have shown that DSI is completely absent in *CB1*$^{-/-}$ mice, providing strong evidence that DSI represents the release of endocannabinoids from the postsynaptic neuron. The molecular identification of the cloned receptor CB1 with the presynaptic target of endocannabinoids should now permit a unified discussion of DSI's electrophysiological properties in light of the large literature on CB1 anatomy and signal transduction. Second, we present evidence that CB1 activation is unlikely to inhibit $Ca^{2+}$ channels via inhibition of adenylate cyclase, since DSI is not affected by kinase or phosphatase inhibitors or by forskolin. Rather, CB1 activation is more likely to decrease $Ca^{2+}$ influx

Table 1. Intrinsic Excitability Profiles for Three Interneuron Subtypes

| | Fast I (4) | Fast II (8) | Slow (11) |
|---|---|---|---|
| Interspike interval (ms) | | | |
| Initial | 15 ± 4 | 12 ± 1 | 17 ± 1 |
| After 175 ms | 30 ± 3 | 18 ± 3[a] | 34 ± 3[a] |
| After 350 ms | 34 ± 5 | 19 ± 3[b] | 41 ± 5[b] |
| Fast AHP (mV) | −9 ± 1[c,d] | −17 ± 2[c] | −17 ± 1[d] |
| Spike width (ms) | 1.9 ± 0.2 | 1.6 ± 0.2[e] | 2.6 ± 0.2[e] |
| Input resistance (MΩ) | 252 ± 26[f] | 251 ± 36[g] | 358 ± 24[f,g] |

Pairs of superscript letters indicate pairwise comparisons that are significantly different at $p < 0.05$ (Bonferroni t tests). Number of observations are in parentheses. Interspike intervals were measured using 350 ms current injections where the interneuron is spiking at its maximal rate (Figure 5B). The amplitude of the fast AHP was measured following single spikes, using the 350 ms current injection that produced the minimal spiking rate (Figure 5A). Spike width was measured at the half-maximum amplitude.

via the fast, membrane-delimited pathway whereby Gβγ interacts with VDCCs directly. Consistent with this hypothesis, the kinetics of DSI onset are relatively rapid, with a delay of only ~1.2 s after postsynaptic depolarization. This is considerably faster than phosphorylation-induced changes in channel activity, which typically require many seconds (Hille, 1992; Hescheler and Schultz, 1993). Measurement of this latency period is also valuable becauses it places an upper limit on the amount of time required for the synthesis of endocannabinoids in relevant concentrations and their diffusion to presynaptic target receptors. Prior to the present study, the upper limit on the kinetics of this process had been approximately 30 s (Evans et al., 1994), since previous investigators had been limited by the poor temporal resolution of biochemical methods. Thus, our findings

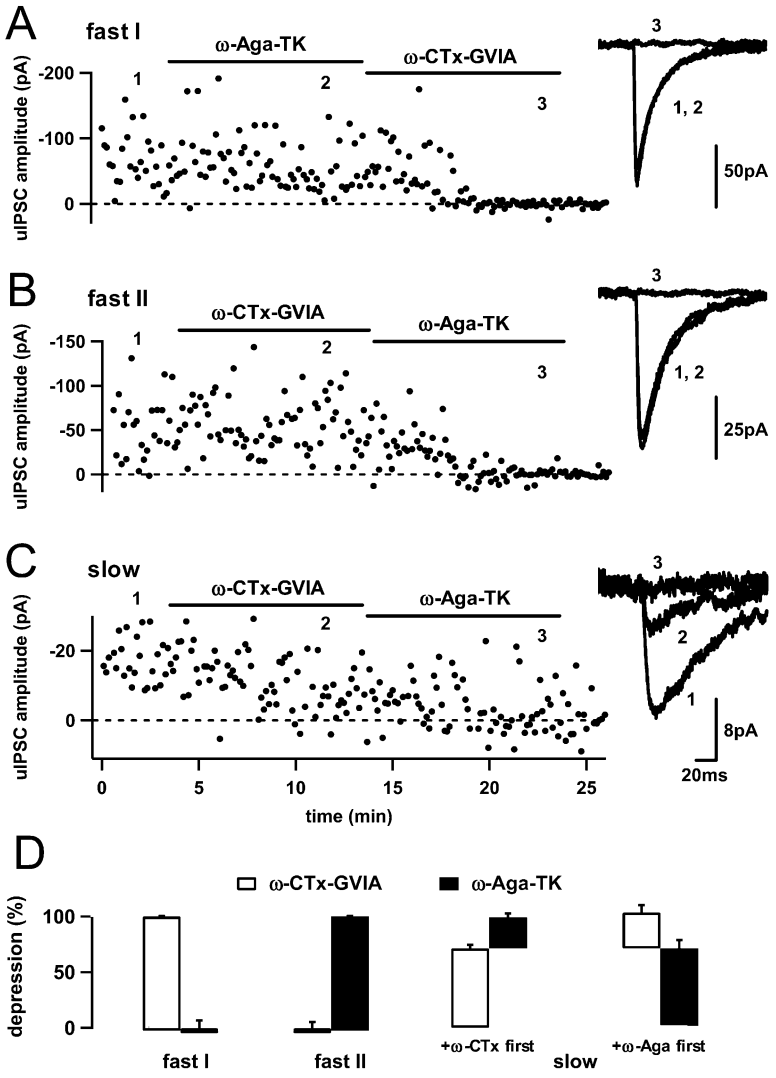


Figure 6. Cannabinoid-Sensitive and -Insensitive Interneurons Use Different $Ca^{2+}$ Channel Subtypes at the Presynaptic Terminal

(A–C) Representative experiments for each connection type showing the effects of specific VDCC antagonists on synaptic transmission. Insets show averaged traces (30–60 sweeps) for each experiment corresponding to baseline (1), effect of the first toxin (2), and effect of the second toxin (3). Note different vertical scaling in (A)–(C) insets; horizontal scaling is the same.

(D) Group data showing that GABA-$A_{fast\ I}$ connections are completely blocked by ω-CTx-GVIA but resistant to ω-Aga-TK, whereas GABA-$A_{fast\ II}$ connections are completely blocked by ω-Aga-TK but resistant to ω-CTx-GVIA. GABA-$A_{slow}$ connections are blocked partially by both toxins, with the first toxin application eliminating about 70% of the IPSC.

suggest that endocannabinoids may represent a novel class of neural signaling molecules, from a kinetic standpoint—slower than classical neurotransmitters, like glutamate and GABA, but faster than the actions of some classical neuromodulators, such as the monoamines. Endocannabinoids are also intermediate between these classical extremes in terms of their spatial range of action (Wilson and Nicoll, 2001), which is more diffuse than the typical actions of neurotransmitters and more local than many neuromodulators.

Anatomical studies have demonstrated that CB1 receptors are expressed by a subclass of hippocampal interneurons that arborize mainly in stratum pyramidale and to a lesser extent in radiatum close to stratum pyramidale (Tsou et al., 1998; Katona et al., 1999, 2000; Hajos et al., 2000). This is also the class of interneurons which release the neuromodulatory peptide cholecystokinin (CCK) (Tsou et al., 1999; Marsicano and Lutz, 1999). These interneurons have been classified as basket cells by morphological criteria; another class of basket cells (expressing the immunocytochemical marker parvalbumin [PV]) is CB1 negative. Consistent with the anatomical restriction of CB1 expression, prior investigators have also shown that individual GABAergic connections show marked differences in their sensitivity to DSI (Alger et al., 1996) and to WIN55212-2 (Hajos et al., 2000; Ohno-Shosaku et al., 2001).

Using paired recordings in acute slices, we now have extended these investigations to demonstrate that the hippocampal endocannabinoid system targets a subclass of interneurons with distinctive physiological properties. First, these interneurons form powerful connections with their postsynaptic targets, generating uIPSCs that are on average 4-fold greater than the cannabinoid-insensitive connections. Thus, cannabinoid signaling is likely to play a more important role in hippocampal function than the relatively small number of CB1-immunopositive interneurons would suggest.

Second, cannabinoid-sensitive interneurons generated uIPSCs with fast kinetics. These connections, together with a group of cannabinoid-insensitive fast synapses, probably represent the group of furosemide-sensitive synapses termed GABA-$A_{fast}$ (Pearce, 1993). What might be the functional significance of the observation that DSI disproportionately affects fast synapses? It has been proposed that GABA-$A_{fast}$ interneurons are responsible for controlling oscillations in the $\gamma$ band (40 Hz) (Banks et al., 2000). $\gamma$ oscillations occur synchronously in multiple brain regions and have been proposed to be involved in binding simultaneous perceptions (Gray, 1994). Consistent with the idea that endocannabinoids regulate the $\gamma$ rhythm, WIN55212-2 decreases the power of $\gamma$-range oscillations induced in hippocampal slices by kainate (Hajos et al., 2000).

Furthermore, GABA-$A_{fast}$ synapses are known to be segregated onto the somata and proximal dendrites of pyramidal cells, whereas the population termed GABA-$A_{slow}$ is located on distal dendrites (Pearce, 1993; Banks et al., 1998; Maccaferri et al., 2000). This is consistent with the morphological identification of CB1-immunopositive cells as basket cells, which arborize selectively in stratum pyramidale and proximal radiatum (Freund and Buzsáki, 1996). Basket cells also exhibit small afterhyperpolarizations, narrow spikes, and low input resistances (Buhl et al., 1995; Sik et al., 1995; Vida et al., 1998), consistent with our data on the excitability profile of GABA-$A_{fast\ I}$ cells. It appears, therefore, that endocannabinoids regulate primarily somatic inhibition. This is likely to have important functional consequences, as somatic synapses account for most tonic, action potential-independent inhibition (Soltesz et al., 1995).

We have found that a third distinguishing mark of cannabinoid-sensitive connections is that they use exclusively N-type $Ca^{2+}$ channels for vesicular release. This was never observed at cannabinoid-resistant synapses, and so it is tempting to speculate that these two unusual properties are somehow related. Both N- and P/Q-type channels are inhibited by G$\beta\gamma$; however, N-type channels are twice as sensitive to inhibition by this mechanism (Zhang et al., 1996; Currie and Fox, 1997). Selective targeting of N-type VDCCs to these terminals might therefore explain why endocannabinoids are able to cause such a profound inhibition of GABA release at these synapses.

We found that cannabinoid-insensitive synapses fell into two broad categories. One category, GABA-$A_{fast\ II}$ synapses, have fast kinetics indistinguishable from GABA-$A_{fast\ I}$ synapses. Like the cannabinoid-sensitive interneurons, these interneurons have relatively narrow action potentials and low input resistance. However, their excitability profile is distinguished by higher firing rates and less spike accommodation than the other interneuron types. GABA-$A_{fast\ II}$ interneurons are therefore likely to include CB1-negative (PV-positive/CCK-negative) basket cells and possibly bistratified/trilaminar cells as well (Freund and Buzsáki, 1996). PV-positive hippocampal interneurons tend to have unusually high firing rates and narrow action potentials (Kawaguchi et al., 1987; Du et al., 1996), similar to the properties we have observed in interneurons forming GABA-$A_{fast\ II}$ synapses. Similarly, bistratified/trilaminar cells, which may be included in the GABA-$A_{fast\ II}$ group, exhibit high in vivo firing rates, sometimes $>$300 Hz (Sik et al., 1995). Strikingly, all GABA-$A_{fast\ II}$ synapses we recorded used exclusively P/Q-type $Ca^{2+}$ channels for neurotransmitter release. The reasons for this selectivity are unclear but might relate to the high, sustained firing rates that these neurons are capable of. It might be advantageous for these cells to use only P/Q-type VDCCs at the presynaptic terminal because this VDCC subtype shows much less inactivation than N-type channels (Usowicz et al., 1992) and thus may permit sustained neurotransmitter release under depolarizing conditions of high firing rates. Consistent with this idea, P/Q-type channels support most of the miniature IPSCs elicited by strong depolarization (high external potassium) in the CA1 region (Doze et al., 1995); this might account for why miniature IPSCs recorded under these conditions are only modestly depressed by endocannabinoids (Wilson and Nicoll, 2001). Also, reconstruction of interneurons that use only P/Q-type channels for neurotransmitter release has shown that the axonal varicosities of these neurons are consistently larger in size than those of other filled interneurons (Poncer et al., 1997), suggestive of boutons with large reserve pools of vesicles well-suited to repetitive release.

Finally, a second type of cannabinoid-insensitive synapse (GABA-$A_{slow}$) showed quite different properties,

namely, slow uIPSC rise and decay kinetics. Based on these kinetics, interneurons responsible for this type of synapse may include LM cells displaced into stratum radiatum and also O-LM cells (Maccaferri et al., 2000; Freund and Buzsáki, 1996). These interneurons also show broad action potentials and high input resistance, consistent with their identification as LM cells forming predominantly small, slow synapses (Williams et al., 1994; Vida et al., 1998). We have shown that these interneurons use both N- and P/Q-type channels for neurotransmitter release, which is typical of CNS neurons in general (Takahashi and Momiyama, 1993). GABA-$A_{slow}$ synapses are likely to originate in the distal regions of the dendritic tree (Pearce, 1993; Banks et al., 1998), and it has been suggested that interneurons responsible for GABA-$A_{slow}$ synapses are responsible for oscillations in the $\theta$ band (5–10 Hz) (Banks et al., 2000), which are prominent during exploratory behaviors (Gray, 1994). Endocannabinoids might therefore be predicted to suppress $\gamma$ oscillations selectively while preserving $\theta$ oscillations, thus altering the temporal output of the hippocampus. Whereas endocannabinoid signaling is not only interneuron specific but also local (Wilson and Nicoll, 2001) and transient (Pitler and Alger, 1992), marijuana and hashish flood the brain with CB1 agonists and may cause a global and sustained suppression of $\gamma$ oscillations.

A tacit assumption of this study has been that the synapses of a single interneuron onto different pyramidal cells will have similar properties. Our results are consistent with this assumption, as the three interneuron classes we have defined by synaptic criteria also turn out to exhibit distinctive intrinsic properties (Table 1). However, it may be the case that our synaptic description of these interneurons does not extend to other (nonpyramidal) postsynaptic cell types. Target-specific expression of presynaptic phenotypes has been well-documented in the hippocampus and cortex (Tóth and McBain, 2000). It will be interesting to see, for example, whether synapses formed by GABA-$A_{fast\ I}$ interneurons onto other interneurons are endocannabinoid sensitive.

In sum, endocannabinoid signaling appears to be a specific mechanism for rapidly turning off a distinct class of hippocampal interneurons. In vivo physiological and behavioral experiments, as well as a better understanding of interneuron classification and function, should help unravel the role of this system in the cognitive functions of the hippocampus.

## Experimental Procedures

### Slice Preparation and Electrophysiology

Transverse hippocampal slices (300 microns) were obtained from Sprague-Dawley rats (P16-30) or C57BL6J mice (see below) and maintained in artificial cerebrospinal fluid (ACSF) for at least 1 hr prior to recording. ACSF contained (in mM) NaCl, 119; $NaHCO_3$, 26; glucose, 10; KCl, 3; $CaCl_2$, 2.5; $MgSO_4$, 2; $NaH_2PO_4$, 1; NBQX, 0.005; and CPP, 0.002; and was equilibrated with 95% $O_2$ and 5% $CO_2$ at 20°C–22°C. CA1 pyramidal cells were recorded in whole-cell voltage-clamp mode, using electrodes (2–3.5 MΩ) filled with a solution of (in mM) $CsCH_3SO_3$, 100; CsCl, 60; QX-314 chloride, 5; HEPES, 10; EGTA, 0.2; $MgCl_2$, 1; MgATP, 1; and $Na_3GTP$, 0.3 (pH 7.3, 275 mOsm). Synaptic currents were filtered at 2 kHz and collected at 5 kHz. When series resistance exceeded 35 MΩ or input resistance fell below 100 MΩ, experiments were terminated. IPSCs were elicited using bipolar tungsten electrodes in or near CA1 stratum pyramidale. DSI tests, performed every 120 s, consisted of 30 stimuli at 0.33 Hz, with depolarization from −60mV to 0mV for 5 s after the 13th stimulus. DSI was calculated using the mean of the five eIPSCs just before the depolarization ($amp_{baseline}$) and the three eIPSCs just after the depolarization ($amp_{test}$): DSI (%) = $100(1 - (amp_{test}/amp_{baseline}))$.

It is thus possible to obtain small negative values for DSI (see for example Figure 4B) as a result of statistical noise, especially when the measurement is based on a small number of trials in unitary connections which have a high variability in IPSC amplitude.

### CB1-Deficient Mice

The generation of CB1-deficient mice is described elsewhere (Zimmer et al., 1999). *CB1*$^{+/+}$ (n = 6), *CB1*$^{+/-}$ (n = 2), and *CB1*$^{-/-}$ (n = 5) mice were of both genders and varied in age from 6 weeks to 8 months. Most of these animals had siblings in one of the other genetic groups of experimental animals. In all cases, the experimenter was blind to genotype. The mouse was selected (according to a coin toss) and decapitated by a person other than the experimenter, in order to avoid possible behavioral indications of genotype. Inquiries regarding CB1-deficient mice should be directed to a.zimmer@uni-bonn.de.

### Pharmacology

For experiments in Figure 2A, slices were preincubated with the kinase/phosphatase inhibitors for 45–120 min or forskolin for 20–60 min, and recordings were performed in the presence of the same concentration of the drug. Drug-treated slices were interleaved with control slices from the same animal incubated for an equivalent period of time in the same concentration of the solvent (DMSO or water) used to make the drug stock. Overall, about 60% of cells have significant DSI, with DSI in these cells averaging about 50% in magnitude; thus, DSI averaged about 30% in control experiments. For the acute applications of $Ca^{2+}$ channel antagonists (Figure 3), cells were discarded immediately if they did not show DSI of at least 30% during the 10 min baseline period. Drugs were from Calbiochem (staurosporine, FK-506, calyculin A, ω-conotoxin GVIA), RBI (WIN55212-2, carbachol), Sigma (CPP, ω-agatoxin TK), and Tocris (NBQX disodium, forskolin).

### Paired Recordings

Interneurons were recorded in whole-cell current-clamp mode, using electrodes filled with a solution of (in mM) K-gluconate, 140; HEPES, 10; EGTA, 1; MgATP, 4; and $Na_3GTP$, 0.3 (pH 7.3; 275 mOsm). A constant hyperpolarizing current was used to maintain a resting potential of −60mV to −70mV. Single action potentials were elicited with 5 ms current injections sufficient to bring the cell just above threshold. Interneurons were selected for recording if their somata were <100 μm from stratum pyramidale and do not represent a random sample of all CA1 interneurons, as we generally avoided somata in stratum lacunosum-moleculare, as well as very small or pyramidal-shaped somata. Most (77%) of the interneurons we recorded which were participating in synaptic connections had their somata in stratum radiatum. Three (two GABA-$A_{fast\ I}$ and one GABA-$A_{slow}$) had somata in stratum oriens, and three (two GABA-$A_{fast\ II}$ and one GABA-$A_{slow}$) had somata in stratum pyramidale. NBQX and CPP were added to the ACSF for all experiments, so, none of the connections we detected can be glutamatergic. All unitary PSCs were also observed to reverse near $E_{Cl}$ (−18mV), consistent with a GABA-A receptor-mediated response. Postsynaptic GABA-B receptor-mediated currents were blocked by QX-314 and cesium in the electrode-filling solution used in pyramidal cell recordings.

Unitary IPSC rise time was measured from 20% to 80% of the peak IPSC amplitude. The falling phase of the IPSC was in all cases well-fit with a single exponential, and the decay time was expressed as $\tau$, where current = (peak current)$e^{-t/\tau}$. The three types of connections did not differ in their average paired-pulse ratios (GABA-$A_{fast\ I}$, 0.82 ± 0.6; GABA-$A_{fast\ II}$, 0.81 ± 0.6; GABA-$A_{slow}$, 0.82 ± 0.6). Paired-pulse ratios ($amp_{IPSC\ 2}/amp_{IPSC\ 1}$) were measured using an interpulse interval of 55 ms. For experiments testing the effects of ω-Aga-TK and ω-CTx-GVIA on unitary connections, uIPSCs were elicited continuously at 0.15–0.2 Hz, and the effects of each toxin were measured beginning 6 min after the initial application, when wash-in

was complete. If application of the first antagonist (ω-CTx-GVIA or ω-Aga-TK) did not block the uIPSC completely, the other antagonist was then applied.

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**Note Added in Proof**

After submission of our manuscript, a paper appeared that draws similar conclusions regarding the heterogenous sensitivity of interneurons to DSI: Martin, L.A., Wei., D.-S., and Alger, B.E. (2001). Heterogeneous susceptibility of $GABA_A$ receptor-mediated IPSCs to depolarization-induced suppression of inhibition in rat hippocampus. J. Physiol. *532*, 685–700.