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Excitatory afferents to CA3 pyramidal cells display differential sensitivity to CB1 dependent inhibition of synaptic transmission

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ABSTRACT

Recent advances in immunohistochemical techniques have, contrary to earlier reports, positively identified CB1 receptors on glutamatergic terminals in the hippocampus. Further work has implicated these receptors in modulation of susceptibility to kainic acid induced seizures. Based on these results, the current study was designed to test the hypothesis that both exogenous and endogenous cannabinoids can selectively modulate glutamatergic afferents to CA3 pyramidal cells, and that such modulation is mediated by cannabinoid type 1 (CB1) receptors. Towards that end we employed either conventional or two-photon guided minimal stimulation techniques to isolate mossy fiber and/or associational/commissural (A/C) inputs to CA3 pyramidal cells. We report that bath application of WIN55,212-2 selectively inhibits minimally evoked A/C inputs to CA3 pyramidal cells, without significantly altering simultaneously recorded mossy fiber inputs. Further, we find that WIN55,212-2 mediated inhibition of A/C inputs is completely blocked by the CB1 selective antagonist AM-251 and absent in $CB1^{-/-}$ animals, suggesting a dependence on CB1 receptors. Finally, we demonstrate that depolarization of CA3 pyramidal cells leads to calcium dependent release of endogenous cannabinoids that transiently inhibit A/C mediated responses, and that this effect is also sensitive to both AM-251 and the muscarinic acetylcholine receptor antagonist atropine. To our knowledge this represents the first demonstration of depolarization induced suppression of excitation in area CA3 of the hippocampus. Collectively, these results provide new information relevant to developing a thorough understanding of how ECs modulate excitatory transmission in an area that is both essential for the acquisition of new memories and intimately involved in epileptogenesis.

1. Introduction

In recent years it has become clear that endogenous cannabinoids (ECs) are released from many neurons in an activity dependent fashion. Subsequent to activity (or depolarization) dependent release, ECs act as retrograde messengers capable of inhibiting transmitter release through activation of presynaptic cannabinoid receptors (Kreitzer and Regehr, 2001; Ohno-Shosaku et al., 2001; Wilson and Nicoll, 2001). When this process occurs at GABAergic terminals it is known as depolarization induced suppression of inhibition (DSI), while a similar effect on excitatory (usually meaning glutamatergic) terminals is referred to as depolarization induced suppression of excitation (DSE). While actions of endogenous cannabinoids have been well documented at both glutamatergic and GABAergic synapses in many areas of the CNS, it is fair to say that, to date, the strong majority of well described EC effects in the hippocampus depend on activation of presynaptic CB1 receptors expressed on GABAergic terminals. These include not only activity dependent short term effects such as DSI (Hofmann et al., 2006; Isokawa and Alger, 2005; Wilson and Nicoll, 2001), but also longer term inhibition where postsynaptic metabotropic receptors for glutamate or acetylcholine appear to be intimately involved in EC release (Chevaleyre and Castillo, 2003, 2004; Edwards et al., 2006). Cumulatively these results have made a compelling case that ECs play an integral role in regulation of inhibition in the hippocampus.

By contrast, it has been somewhat more difficult to determine the role of ECs in modulating excitatory transmission in this area. A lack of immunohistochemical evidence for CB1 on glutamatergic terminals in the hippocampus has, until very recently, been difficult to reconcile with clear evidence of antiepileptic effects of CB agonists (Blair et al., 2006; Wallace et al., 2001), and numerous (but often conflicting) reports of cannabinoid dependent modulation (or even DSE) of the Schaffer collateral pathway (Domenici et al., 2006;

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Hajos and Freund, 2002b; Hajos et al., 2001; Hoffman et al., 2005; Kawamura et al., 2006; Németh et al., 2008; Ohno-Shosaku et al., 2002; Takahashi and Castillo, 2006). Very recently, immunohistochemical studies using novel C-terminal antibodies have positively identified CB1 on a subset of glutamatergic terminals in the hippocampus, and provided evidence implicating them in modulating the threshold for kainate induced seizures (Katona et al., 2006; Monory et al., 2006). The current study was motivated by these recent immunohistochemical findings, and designed to directly test the hypothesis that isolated glutamatergic inputs to CA3 pyramidal cells will show differential sensitivity to CB1 agonists. Towards that end, we used both conventional and two-photon guided minimal stimulation techniques to examine the effects of CB1 agonists on minimally evoked mossy fiber (MF) and associational/commissural (A/C) projections to CA3 pyramidal cells. Our results indicate that CB1 dependent inhibition of A/C mediated transmission occurs subsequent to both bath application of exogenous agonists and depolarization mediated release of endogenous agonists. By contrast, MF projections to CA3 pyramidal cells appear to lack functional CB1 receptors. Portions of this study have been previously presented in abstract form (Hofmann and Frazier, 2007).

2. Materials and methods

Male Sprague–Dawley rats or C57BL6/J mice aged 18–25 days post-natal were given an IP injection of ketamine (80–100 mg/kg) and decapitated using a small animal guillotine. The brain was quickly removed and placed into ice cold artificial cerebral spinal fluid (ACSF) consisting of (in mM): 124 NaCl, 2.5 KCl, 1.2 NaH_2PO_4, 2.5 $MgSO_4$, 10 D-glucose, 1 $CaCl_2$, and 25.9 $NaHCO_3$. Horizontal hippocampal slices were cut at 300 μm, incubated at 30–35 °C for 30 min, and then allowed to equilibrate to room temperature. All animal procedures were approved by the IACUC at the University of Florida and conformed to animal welfare guidelines issued by the National Institutes of Health.

For whole-cell patch-clamp experiments, slices were perfused with oxygenated ACSF heated to 30 °C at a rate of 2 mL/min, and containing (in mM): 126 NaCl, 3 KCl, 1.2 NaH_2PO_4, 1.5 $MgSO_4$, 11 D-glucose, 2.4 $CaCl_2$, and 25.9 $NaHCO_3$. Carbachol (CCh, 3 μM) was added to the ACSF for all experiments that involved depolarization induced release of endogenous cannabinoids, but was absent in experiments with exogenous CB1 agonists. Recording pipettes had tip resistance of 3–5 MΩ when filled with an internal solution containing (in mM): 140 Cs-$MeSO_3$, 1 $MgCl_2$, 3 NaCl, 0.2 Cs-EGTA, 10 HEPES, 4 Na_2-ATP, 0.3 Na-GTP, 5 Qx-314-Cl, and ~0.063 sulforhodamine 101 or 0.1 Alexa 594, pH adjusted to 7.3. Voltage clamp experiments were conducted using an Axon Multiclamp 700A or 700B amplifier. Access resistance was typically between 10 and 30 MΩ and uncompensated. All data were sampled at 20 kHz, filtered at 2 kHz, and recorded using Clampex version 9/10 (Molecular Devices, Sunnyvale, CA).

$CB1^{-/-}$ and WT C57 mice were obtained by crossing homozygous littermates derived from heterozygous parents (WT and $CB1^{-/-}$ animals were therefore cousins). Our original heterozygous breeders were descendents of the line developed by A. Zimmer at NIMH, and were a generous gift from Dr. Carl Lupica (NIDA).

In our initial experiments (Fig. 1C,D), evoked EPSCs were elicited at 0.33 Hz using a bipolar stimulator placed in the dentate granule cell layer. For all other experiments involving evoked EPSCs, MF and A/C responses were isolated using minimal stimulation techniques similar to those previously described by our lab (Nahir et al., 2007). In brief, responses displaying a sharp stimulus threshold were evoked with 0.1 ms stimulation through a glass pipette (~1 μm tip diameter) containing ACSF. Typical stimulus intensity was <100 μA, and response amplitudes were stable with modest additional increases in stimulus intensity (Fig. 2D). MF mediated responses were evoked with a minimal stimulator placed in stratum lucidum, showed strong frequency facilitation, and were reduced by at least 60% following bath application of the group II mGluR agonist DCG-IV. A/C mediated responses were evoked with a minimal stimulator placed in stratum radiatum (typically between 100 and 150 μm from the cell soma), lacked similarly strong frequency facilitation, and were insensitive to DCG-IV. In some cases, stimulators were placed under visual control in close proximity to distal dendrites or proximal spines actively illuminated with 2-photon based epifluorescence microscopy (Prairie Technologies, Middletown, WI, for original description of this technique see Balu et al., 2007). Overall, 2-photon guided minimal stimulation significantly reduced the time and effort required to isolate MF mediated responses. Stimuli were delivered at 0.2 Hz except in experiments involving DSE (0.33 Hz). In those cases the effect of depolarization was calculated by comparing the response amplitude in the four sweeps immediately following depolarization (from −70 to 0 mV, 5 s) to the 8 sweeps immediately prior. For all experiments with minimally evoked EPSCs (meEPSCs) individual traces contaminated by spontaneous EPSCs were manually eliminated from analysis. For experiments focusing on spontaneous EPSCs, events were detected using MiniAnalysis v. 6.03 (Synaptosoft, Decatur, GA), and further analyzed in OriginPro 7.5 (OriginLab, Northampton, MA) using custom software written by CJF. In some experiments 20 μM DNQX and 40 μM APV were bath applied to verify the isolated responses were in fact glutamatergic in origin.

Statistical significance was generally assessed using the Student's *t*-test or (in Fig. 1) the Kolmogorov–Smirnov (K-S) test. All drugs used in this study were purchased from either Sigma (St. Louis, MO) or Tocris (Ellisville, MO). For all figures the error bars represent the SEM. No attempt was made to wash out WIN55,212-2 in experiments that involved bath application of this lipophilic CB1 agonist.

3. Results

3.1. DSE is present in area CA3

In an early set of experiments, we observed that both spontaneous and stimulus evoked EPSCs recorded from CA3 pyramidal cells are subject to DSE. Specifically, in the presence of 3 μM carbachol (CCh), a muscarinic acetylcholine receptor agonist (mAChR), depolarization of CA3 pyramidal cells from −70 mV to 0 mV reduced both frequency and amplitude of spontaneous EPSCs by 14.5 ± 4.39% and 10.4 ± 1.47% ($n = 7$, $p < 0.01$, Fig. 1B). Similarly, identical depolarization depressed EPSCs generated with a bipolar stimulator placed in the granule cell layer (0.1 ms, 200–900 μA) by 23.1 ± 3.88% of baseline ($n = 15$, $p < 0.01$, Fig. 1C). DSE of stimulus evoked EPSCs was eliminated by bath application of the CB1 antagonist AM-251 (5 μM) and the mAChR (5 μM) antagonist atropine, but was insensitive to simultaneous bath application of the $GABA_A$ antagonist PTX (50 μM) and the $GABA_B$ antagonist CGP (10 μM) (% change was -0.80 ± 6.19%, $n = 3$, $p = 0.9$, Fig. 1D; 1.93 ± 3.81%, $n = 5$, $p = 0.6$, Fig. 1E; and 16.3 ± 1.62%, $n = 4$, $p < 0.01$, Fig. 1E; respectively). These results strongly suggest that at least a subset of excitatory afferents to CA3 pyramidal cells are modulated by endocannabinoid dependent retrograde signaling. However, they are insufficient to distinguish reliably between specific types of excitatory inputs, as evidenced by clear activation of recurrent connections via granule cell stimulation in prior studies (Nicoll and Schmitz, 2005; Xiang and Brown, 1998).

3.2. WIN55,212-2 selectively inhibits glutamate release from A/C but not MF inputs to CA3 pyramidal cells via activation of CB1 receptors

In order to more precisely identify which glutamatergic inputs to CA3 pyramidal cells were susceptible to DSE, we used both conventional and 2-photon guided minimal stimulation techniques to isolate either MF or A/C afferents to CA3 pyramidal cells (see Section 2). We found that bath application of the CB1 agonist WIN55,212-2 (5 μM) reduced the amplitude of A/C mediated EPSCs (evoked with a minimal stimulator placed in stratum radiatum) in 18 of 22 cases (by 34.42 ± 4.67%, $p < 0.001$, Fig. 2C). This effect was associated with a clear increase in the coefficient of variation (from 0.31 ± 0.02 to 0.43 ± 0.05, $n = 18$, $p = 0.01$, data not shown) suggesting that the site of action is likely presynaptic. By sharp contrast, we found that identical application of WIN55,212-2 had no effect on MF mediated EPSCs that were evoked with a minimal stimulator placed in stratum lucidum (% reduction: 8.43 ± 7.57%, $n = 8$, $p = 0.30$, Fig. 2C). This differential sensitivity to CB1 agonists was also strikingly apparent in a series of experiments in which two separate minimal stimulators were used to simultaneously isolate both an A/C and a MF input to a single CA3 pyramidal cell (Fig. 2A). Under those conditions, bath application of WIN55,212,2 again clearly inhibited the A/C (stratum radiatum evoked and DCG-IV insensitive) but not the MF (stratum lucidum evoked and DCG-IV sensitive) meEPSCs (% reduction: 50.2 ± 6.09% vs. 18.1 ± 8.29% respectively, $n = 6$, $p = 0.02$, Fig. 2B). We next determined that WIN55,212-2 mediated inhibition of isolated A/C inputs to CA3 pyramidal cells was blocked by pre-incubation with AM-251 (%

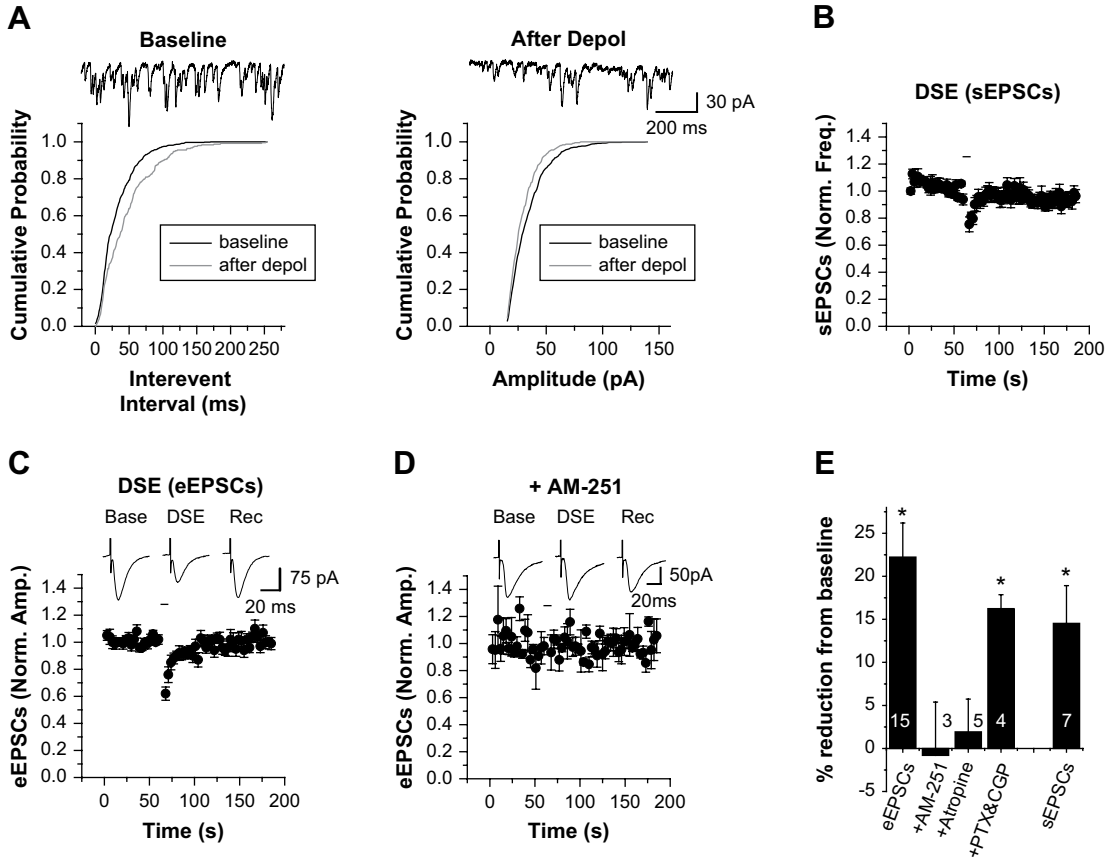

Fig. 1. DSE is observed in CA3 pyramidal cells and requires activation of the CB1 receptor. (A) Cumulative probability histograms of the interevent interval (left) and amplitude (right) in a representative cell. Depolarization induced a statistically significant decrease in both frequency and amplitude of spontaneous EPSCs ($p \leq 0.001$ in both cases, K-S test). Insets: Raw traces from the representative cell before (Baseline) and after (After Depol) depolarization. (B) DSE of spontaneous EPSCs (sEPSCs) recorded in the presence of 3 μM CCh. (C) DSE of evoked EPSCs (eEPSCs) stimulated at 0.33 Hz with a bipolar stimulator placed in the granule cell layer. (D) AM-251 sensitivity of stimulus evoked DSE. Bars in (B)–(D) indicate a 5-s depolarization from −70 to 0 mV. Insets in (C) and (D) are averages of 4–8 consecutive sweeps for each time period taken from a representative cell. (E) Summary plot indicating magnitude of DSE in various experimental conditions. Numbers on/by each bar are *n* values. $^{*}p < 0.01$.

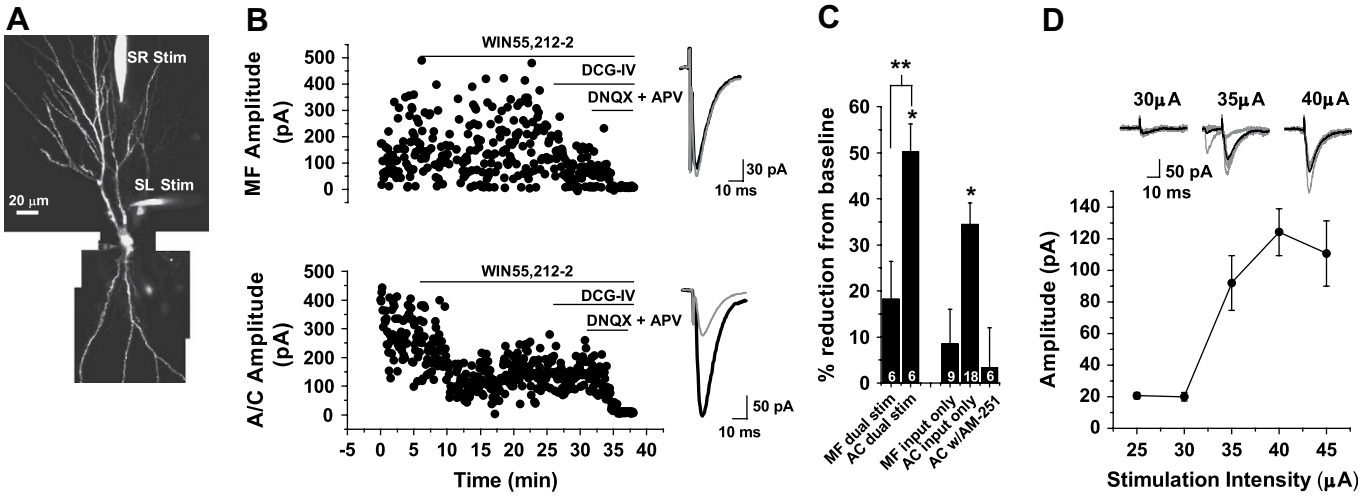

Fig. 2. Functional expression of the CB1 receptor on A/C but not MF afferents to CA3 pyramidal cells. (A) 2-photon image of a CA3 pyramidal cell indicating typical stimulator placement for dual stimulation experiments. SR, stratum radiatum. SL, stratum lucidum. (B) Simultaneous recording of minimally evoked MF (top) and A/C (bottom) afferents from another CA3 pyramidal cell clearly demonstrate differential sensitivity to both 5 μM WIN55,212-2 and 1 μM DCG-IV. Insets: averages of 6 consecutively accepted sweeps during baseline (black) and following application of WIN55,212-2 (gray). (C) Summary plot. 'Dual stim' refers to experiments as in (B) where an A/C and MF input were recorded simultaneously. In other experiments either a MF or an A/C input (but not both) were recorded. (D) Representative plot depicting the sharp threshold found in a minimally evoked isolated A/C afferent. Insets: overlay of raw data (gray) and average (black) for depicted stimulation intensity. All experiments contained 50 μM picrotoxin in the external solution. $^{*}p < 0.001$. $^{**}p = 0.02$.

reduction: 3.37 ± 8.65%, $n = 6$, $p = 0.71$, Fig. 2C), suggesting that the effect is dependent on activation of CB1 receptors. Consistent with that interpretation we noted that WIN55,212-2 had no effect on isolated A/C afferents to CA3 pyramidal cells in CB1$^{-/-}$ mice, and yet was still effective on wild type controls (% reduction: 11.93 ± 10.63%, $n = 7$ vs. 41.96 ± 6.33%, $n = 4$, respectively, $p < 0.01$, Fig. 3A). Finally, because a previous study reported an effect of persistently activated cannabinoid receptors on a subpopulation of hippocampal interneurons (Losonczy et al., 2004), we decided to test for an endocannabinoid mediated inhibitory tone at A/C inputs to CA3 pyramidal cells. Our results indicated that minimally evoked A/C mediated EPSCs were insensitive to bath application of 5 μM AM-251 (% reduction: 1.10 ± 14.23%, $n = 9$, $p = 0.99$, data not shown), indicating there is no apparent tonic CB1 receptor activation at these synapses in vitro.

3.3. Endocannabinoid mediated retrograde signaling at isolated A/C inputs to CA3 pyramidal cells depends on the CB1 receptor, postsynaptic calcium influx, and activation of mAChRs

Next we demonstrated that depolarization of CA3 pyramidal cells in the presence of 3 μM CCh reduces A/C mediated meEPSC amplitude by 30.1 ± 2.89% ($n = 9$, $p < 0.01$, Fig. 4A). This effect is significantly more robust than DSE of spontaneous events ($p < 0.01$) and larger than DSE of EPSCs generated with a bipolar stimulator in the granule cell layer ($p = 0.11$). Within 90 s of depolarization, meEPSC amplitude had recovered to 94.4 ± 3.38%, $p = 0.12$, suggesting there is not a long lasting component to this plasticity.

Further characterization of minimally evoked A/C-CA3 DSE revealed that it is largely blocked by preincubation with AM-251 (% reduction: 8.12 ± 6.37%, $n = 6$, $p = 0.23$, Fig. 4B), indicating a dependence on CB1 receptors, and that it is eliminated by application of 10 mM BAPTA via the recording pipette (% reduction: 1.64 ± 5.76%, $n = 7$, $p = 0.78$, Fig. 4C), indicating a dependence on postsynaptic calcium influx. We further report that DSE of minimally evoked A/C afferents to CA3 pyramidal cells (again in the presence of 3 μM CCh) was blocked by bath application of 5 μM atropine (control: 31.3 ± 4.60%, atropine: 1.33 ± 6.35%, $n = 4$, $p < 0.05$, Fig. 5A). This effect was not due to rundown of the EC system as DSE could be maintained over an identical time period in the absence of atropine (first 4–5 sets: 27.8 ± 2.25%, last 4–5 sets: 26.6 ± 8.08%, $n = 2$, $p = 0.88$, data not shown). Finally, we note that CCh dependent DSE of A/C inputs to CA3 pyramidal cells is not blocked by subsequent bath application of 100 μM L-NAME, a nitric oxide (NO) synthase inhibitor (control: 30.6 ± 4.44%, L-NAME: 27.8 ± 3.16%, $n = 4$, $p = 0.49$, Fig. 5B). This potentially further distinguishes CCh dependent DSE of A/C inputs to CA3 pyramidal cells from DSI of evoked IPSCs recorded from CA1 pyramidal cells in the presence of CCh, the latter of which has recently been demonstrated to be sensitive to disruption of NO synthesis (Makara et al., 2007).

4. Discussion

There are two central conclusions of this study. The first is that recurrent connections between CA3 pyramidal cells express functional CB1 receptors while mossy fiber inputs do not. This conclusion is based in large part on experiments in which meEPSCs were recorded from individual CA3 pyramidal cells. We found, in brief, that mossy fiber mediated meEPSCs were not significantly altered by bath application of a CB receptor agonist, while A/C inputs were strongly inhibited in an AM-251 sensitive manner. Further, the effect of CB1 activation on A/C mediated synaptic transmission was absent in CB1$^{-/-}$ animals and yet still present in wild type controls. The second central conclusion of this study is that CB1 positive A/C inputs to CA3 pyramidal cells are subject to DSE that depends on presynaptic CB1 receptors, postsynaptic calcium influx, and interestingly, activation of mAChRs. Cumulatively, these results provide clear physiological evidence indicating that glutamatergic inputs to CA3 pyramidal cells have differential sensitivity to EC mediated and CB1 dependent retrograde inhibition.

While this is the first study to use physiological techniques to directly examine the EC sensitivity of either A/C or MF inputs to CA3 pyramidal cells, significant effort has been made to characterize EC sensitivity of A/C inputs to CA1 pyramidal cells, often with mixed results. For example, several studies have reported not only that A/C inputs to CA1 pyramidal cells are inhibited by bath application of WIN55,212-2, but also that this inhibition is retained in CB1$^{-/-}$ animals suggesting the presence of a novel CB receptor. Further, this effect of WIN55,212-2 in CB1$^{-/-}$ animals was noted to be insensitive to AM-251 but antagonized by SR141716A (Hajos and Freund, 2002a,b; Hajos et al., 2001). By contrast, other studies have reported clear sensitivity of A/C inputs to CA1 pyramidal cells that are both sensitive to AM-251 and absent in CB1$^{-/-}$ animals (Kawamura et al., 2006; Takahashi and Castillo, 2006), while others yet have reported species and strain specific differences in EC dependent modulation of Schaffer collaterals (Haller et al., 2007; Hoffman et al., 2005). Although the reasons for these discrepancies remain largely unclear, one recent report has provided evidence suggesting that WIN55,212-2 can directly inhibit N-type voltage gated calcium channels in a concentration range often used in EC studies

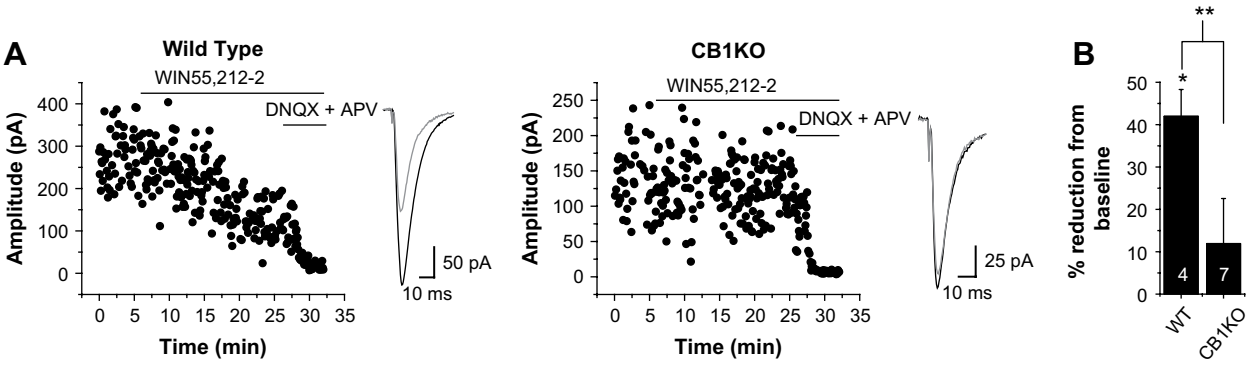

Fig. 3. WIN55,212-2 decreases the amplitude of minimally evoked A/C afferents in wild type but not CB1$^{-/-}$ mice. (A) Representative experiments indicating that 5 μM WIN55,212-2 reduced meEPSCs of an isolated A/C afferent in a WT mouse (left), and yet failed to reduce A/C mediated meEPSCs in a CB1$^{-/-}$ mouse (right). These responses were isolated in the continual presence of 1 μM DCG-IV and 50 μM picrotoxin. (B) Summary plot from experiments with wild type and CB1$^{-/-}$ mice. Insets: averages of 6 consecutively accepted sweeps during baseline (black) and following application of WIN55,212-2 (gray). $^{*}p < 0.01$. $^{**}p < 0.01$.

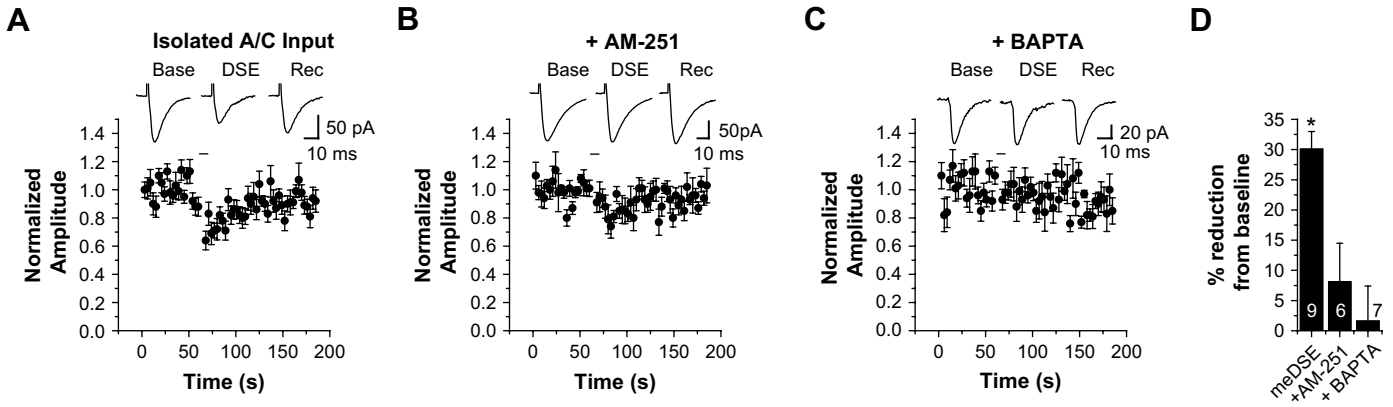

Fig. 4. DSE is observed in minimally evoked A/C afferents and requires both the CB1 receptor and post synaptic calcium influx. All experiments contained 3 μM CCh, 50 μM picrotoxin, and 1 μM DCG-IV in the external solution. (A) Normalized average of meEPSCs elicited at 0.33 Hz. Cells were depolarized from −70 to 0 mV for 5 s (bar). DSE was blocked by 5 μM AM-251 (B) and by 10 mM BAPTA, a calcium chelator, in the internal solution (C). (D) Summary plot depicting the percent reduction in all experimental conditions. Insets: average trace for 4–8 consecutive sweeps for each time period from a representative cell. $^{*}p < 0.01$.

(including ours), and that this CB receptor independent effect of WIN55,212-2 may explain some previous reports that were interpreted to indicate the presence of a novel CB receptor (Németh et al., 2008). In the present study, we noted no consistent evidence of WIN55,212-2 mediated inhibition of MFs, of A/Cs in CB1$^{-/-}$ animals, or of A/Cs in slices pre-treated with AM-251. Further, it remains unclear whether a direct action of WIN55,212-2 on voltage gated calcium channels could account for the SR141716A sensitive effect of WIN55,212-2 in CB1 knockout animals previously described. On the other hand, in experiments where WIN55,212-2 was effective, we did note some variability in the magnitude of the effect. For example, the average effect of WIN55,212-2 on A/C inputs in the absence of AM-251 varied between 34 and 50% inhibition in our dual stimulation, single stimulation, and wild type mouse experiments; however there was no significant difference between these groups when compared on a one-way ANOVA. Similarly, the effect of WIN55,212-2 on isolated MF mediated EPSCs in our single stimulation experiments ranged from 39% potentiation to 27% inhibition, but were not significantly different than the null hypothesis on average ($8.43 \pm 7.57\%$, $n = 78$, $p = 0.30$, see Section 3). While such variability is not unexpected in experiments that rely on meEPSCs, we cannot definitively rule out the hypothesis that CB1 receptor independent sites of action for WIN55,212-2 exist in area CA3. It is possible that changes in the cutting solution, incubation procedure, and flow rate such as described by Németh et al., 2008 would help to reveal such additional mechanisms of action. However at present our results in CA3 most closely parallel the subset of studies in CA1 that conclude that the CB1 receptor in particular is both necessary and sufficient to mediate the effects of WIN55,212-2 on action potential dependent glutamate release from A/C fibers (Kawamura et al., 2006; Takahashi and Castillo, 2006).

Perhaps one of the most unusual features of the A/C-CA3 DSE reported here is the apparent high level of dependence on activation of mAChRs. In many original studies of EC mediated retrograde signaling, low concentrations of bath applied CCh were used primarily with the intention of facilitating EC sensitive spontaneous IPSCs. However, later work in area CA1 of the hippocampus has

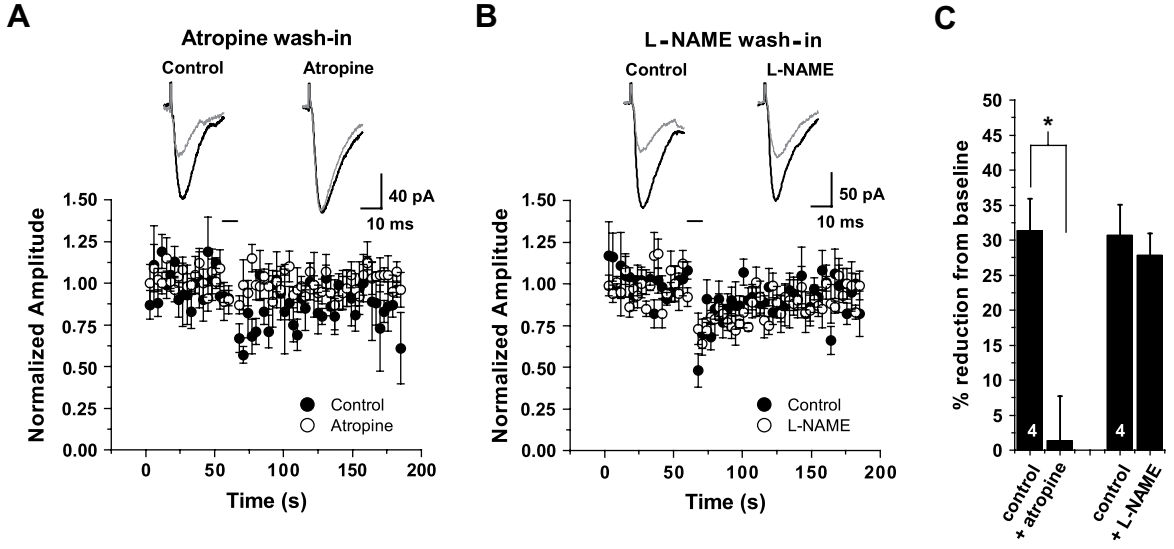

Fig. 5. DSE of minimally evoked A/C afferents requires mAChR activation and is unaffected by inhibition of nitric oxide synthase. All experiments contained 3 μM CCh, 50 μM picrotoxin, and 1 μM DCG-IV in the external solution. (A) DSE is present in control conditions (closed circles) but is blocked after application of 5 μM atropine (open circles). (B) DSE is present in control conditions (closed circles) and is unaltered after application of 100 μM L-NAME, an NO-synthase inhibitor (open circles). Bars in (A) and (B) represent cell depolarization from −70 to 0 mV for 5 s. (C) Summary plot depicting the effects of atropine and L-NAME on DSE. Insets: average of 4–8 sweeps before (black line) and after (gray line) depolarization from representative cells. $^{*}p < 0.05$.

revealed two specific mechanisms through which postsynaptic mAChRs may facilitate EC signaling. In one mechanism, described by Kano and colleagues, mAChR dependent activation of a calcium sensitive PLC subunit results in greater calcium dependent EC synthesis and release upon robust depolarization (Hashimotodani et al., 2005). However, another mechanism has been suggested by a recent study which intriguingly noted that DSI evoked in the presence of CCh has a high dependence on NO mediated signaling, while DSI evoked in the absence of CCh does not (Makara et al., 2007). In the present study we have tested the hypothesis that mAChR dependent DSE might depend on NO by establishing DSE and then bath applying an NO-synthase inhibitor. Our results indicated that A/C-CA3 DSE, although atropine sensitive, is apparently not NO dependent. Thus further work will be necessary to fully characterize the mechanism of mAChR action in this system.

In closing, it is worth noting that much of the work described above was done during a time when CB1 receptor expression on glutamatergic terminals in the hippocampus was not expected. In fact, until recently, detailed immunohistochemical studies had clearly concluded that CB1 expression in the hippocampus was largely if not exclusively confined to the axon terminals of CCK positive GABAergic neurons (for review see Frazier, 2007). This common viewpoint was dramatically altered very recently when new immunohistochemical studies using novel C-terminal antibodies unambiguously revealed CB1 receptor expression on glutamatergic terminals (Katona et al., 2006; Monory et al., 2006). This expression was indeed lower than on GABAergic terminals, but was eliminated by elegant technology that allowed for creation of CB1 knockouts targeted specifically at glutamatergic neurons (see also Marsicano et al., 2003). Importantly, the CB1 expression on glutamatergic terminals was found to be high in extrapyramidal and extragranular cell layers but was virtually absent in stratum lucidum of area CA3, implying that mossy fiber transmission might be insensitive to CB1 dependent modulation. The potential significance of CB1 on glutamatergic terminals was indicated by increased susceptibility of glutamate specific CB1 knockouts to kainic acid induced seizures (Marsicano et al., 2003; Monory et al., 2006). Although the results of these studies were quite striking, they still largely lacked direct functional tests of their conclusions at a cellular level, particularly in area CA3. Thus, the significance of the current study is also derived, in part, from its ability to use physiological approaches at a cellular level to both confirm and extend conclusions based on recent immunohistochemical advances. We believe that these types of results are rapidly bringing the GABAergic centric view of EC signaling in the hippocampus to an end. In fact, in addition to the results presented here and discussed above there is also new evidence indicating that glutamatergic axon terminals in the inner molecular layer of the dentate gyrus are sensitive to CB agonists, and that hilar mossy cells express CB1 receptors (Chiu and Castillo, 2008; Monory et al., 2006). Further, a synthetic enzyme for anandamide has now been identified in apparently CB1 negative mossy fiber terminals (Nyilas et al., 2008). This finding reinforces the important point that the lack of CB1 mediated effects on MF inputs apparent in our work does not necessarily rule out other potential roles for MFs in EC dependent signaling. Collectively, these results suggest a strong role for ECs in modulating recurrent excitatory circuits in the hippocampus, a rich future for research on EC dependent modulation of excitability, and real potential to make significant strides towards a better understanding of neurological disorders of both memory and excitability.

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