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Nociceptin Reduces Epileptiform Events in CA3 Hippocampus via Presynaptic and Postsynaptic Mechanisms

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The opiate-like peptide nociceptin/orphanin FQ (Noc) and its receptor [opiate receptor-like receptor (ORL-1)] are highly expressed in the hippocampus. Noc has inhibitory postsynaptic actions in CA1, CA3, and the dentate and seems to lack the disinhibitory, excitatory actions demonstrated for some opiate peptides in the hippocampus. The CA3 hippocampal region is important in the generation of hippocampal seizures. Therefore, we tested the action of Noc on spontaneous epileptiform activity recorded extracellularly or intracellularly in CA3 and generated by removal of Mg^{2+} from the bathing solution or by raising extracellular K^+ from 3.5 to 7.5 mM. Superfusion of Noc robustly depressed spontaneous bursting without desensitization. The ORL-1 antagonist [$Phe^1\Psi(CH_2\text{-}NH)Gly^2$]NC(1–13)$NH_2$ (1–2 μM) greatly attenuated the reduction of spontaneous bursting by Noc. To characterize the cellular mechanism of action of Noc, we recorded intracellularly from CA3 pyramidal neurons. Noc reduced EPSCs evoked by stimulating either mossy or associational/commissural fibers. Analysis of miniature EPSCs using whole-cell voltage-clamp recording suggests that Noc acts presynaptically to inhibit glutamate release. This is the first demonstration of a presynaptic effect for Noc in the hippocampus. Noc also increased K^+ currents in CA3 pyramidal neurons, including the voltage-sensitive M-current. Blocking the M-current with linopirdine increased the duration of individual CA3 bursts but did not attenuate Noc-mediated inhibition of bursting. Thus, Noc acts via multiple mechanisms to reduce excitation in CA3. However, Noc inhibition of epileptiform events is not dependent on augmentation of the M-current.

Key words: nociceptin; ORL-1; epilepsy; epileptiform; slice; miniature EPSC; CA3; M-current; electrophysiology

The hippocampus is an important structure in generating and transmitting temporal lobe seizures, a common type of epileptic event in humans. The modulatory actions of neuropeptides on seizures have received much attention. Neuropeptide expression is altered by seizures in humans and in animal models of epilepsy (Sperk et al., 1986; de Lanerolle et al., 1989; Mazarati et al., 1998), and neuropeptides have both proepileptic [substance P and CRF (Marrosu et al., 1987; Liu et al., 1999a)] and antiepileptic [neuropeptide Y, galanin, somatostatin, and dynorphin (Baraban et al., 1997; Bausch et al., 1998; Mazarati et al., 1998; Tallent and Siggins, 1999)] actions. Furthermore, mice with overexpression of or null mutations in peptide genes have profound alterations in sensitivity to chemoconvulsants (Baraban et al., 1997; Liu et al., 1999b; Mazarati et al., 2000). With the development of nonpeptide ligands with blood–brain barrier permeability (Schulz et al., 1996; Rohrer et al., 1998), neuropeptide receptors could become important targets for antiepileptic drugs.

The opiate peptides have wide-ranging neuromodulatory actions in the hippocampus. The μ and δ receptor ligands have excitatory actions on CA1 pyramidal neurons via inhibition of GABAergic interneurons (Zieglgänsberger et al., 1979; Madison and Nicoll, 1988). The action of dynorphin via κ receptors is more exclusively inhibitory in the dentate, CA3, and CA1 (Caudle et al., 1990; Wagner et al., 1993; Weisskopf et al., 1993; Moore et al., 1994; Madamba et al., 1999a). Accordingly, κ-selective agonists have antiseizure activity in the hippocampus in both *in vivo* and *in vitro* models (Siggins et al., 1986; Bausch and Chavkin, 1997; Bausch et al., 1998).

Nociceptin/orphanin FQ (Noc) is an opiate-like peptide originally characterized as the endogenous ligand for the opiate receptor-like receptor (ORL-1) (Meunier et al., 1995; Reinscheid et al., 1995). Among opiate peptides, Noc exhibits the highest homology to dynorphin (Meunier et al., 1995), and ORL-1 may be analogous to the κ_3 receptor identified pharmacologically (Pan et al., 1998). Noc and ORL-1 are abundantly expressed throughout the hippocampus (Florin et al., 1997; Neal et al., 1999a; Letchworth et al., 2000), where Noc has postsynaptic augmenting actions on K^+ currents of principal neurons in CA1, CA3, and the dentate (Ikeda et al., 1997; Yu and Xie, 1998; Madamba et al., 1999b; Amano et al., 2000). As with dynorphin, no disinhibitory actions of Noc have been reported in the hippocampus.

We showed recently that in CA1 pyramidal neurons Noc augmented the M-current, a voltage-sensitive, noninactivating K^+ current important in regulating neuronal excitability (Madamba et al., 1999b). The M-channel consists of KCNQ2 and KCNQ3 subunits that coassemble to form heteromers (Wang et al., 1998). These two subunits are mutated and hypofunctional in benign familial neonatal convulsions (Charlier et al., 1998; Singh et al., 1998), suggesting that the M-current is important in preventing seizures. KCNQ2 and KCNQ3 are highly expressed in CA3, where they are localized on the soma and dendrites of pyramidal neurons (Cooper et al., 2000). The CA3 is critical in generating hippocampal seizures, because of a dense network of recurrent connections that interconnect these neurons, enabling them to burst synchronously (Traub and Wong, 1982). We report here that Noc has antiepileptiform activity in CA3, via synergistic presynaptic and postsynaptic actions. However, although Noc

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augments the M-current, this mechanism does not appear to contribute significantly to antiepileptic actions of Noc in the models tested here.

MATERIALS AND METHODS

Slice preparation. We prepared hippocampal slices as described previously (Pittman and Siggins, 1981; Schweitzer et al., 1993). Briefly, male Sprague Dawley rats (100–200 gm) were anesthetized with halothane (4%) and decapitated, and the brain was rapidly removed. Transverse hippocampal slices (350–400 μM) were cut on a McIlwian brain slicer or a Campden vibraslicer and placed in artificial CSF (ACSF), which was gassed with 95% O_2/5% CO_2 (carbogen), of the following composition (in mM): 130 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 1.5 $MgSO_4 \cdot 7H_2O$, 2 $CaCl_2 \cdot 2H_2O$, 24 $NaHCO_3$, and 10 glucose. After ~30 min of incubation with their upper surfaces exposed to warmed, humidified carbogen, the slices were completely submerged and continuously superfused with ACSF (31°C) at a constant rate (2–3 ml/min) for the remainder of the experiment. The inner chamber had a total volume of 1 ml; at the superfusion rates used, 90% replacement of the chamber solution could be obtained within 1–1.5 min. Drugs and peptides were added to the bath from stock solutions at known concentrations. We obtained Noc, Noc (1–13) amide, and the ORL-1 antagonist [Phe$^1\Psi$(CH_2-NH)Gly^2]NC(1–13)NH_2 (hereafter called ORLAn) from Tocris Cookson (St. Louis, MO) and/or Bachem (Torrance, CA); 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) from Tocris Cookson; and D,L-2-amino-5-phosphonovaleric acid (APV) and norbinaltorphimine (nBNI) from Research Biochemicals (Natick, MA). All other chemicals were from Sigma (St. Louis, MO).

Extracellular recording. We recorded extracellular epileptiform bursts by conventional means in the CA3 pyramidal layer using glass extracellular pipettes (1–3 MΩ tip resistance when filled with 3 M NaCl) and an Axon Instruments Axoclamp 2B amplifier. Recordings were filtered at 3–10 kHz and digitized using pClamp software (Axon Instruments). Two models were used to elicit spontaneous epileptiform bursting extracellularly: superfusion of Mg^{2+}-free ACSF or increasing extracellular K^+ (from 3.5 to 7.5 mM). Bursts were recorded over 1 min trials acquired via computer and continuously monitored on a chart recorder. Burst frequency was analyzed using Mini 4.3 and 5.02 software (Synaptosoft, Leona, NJ); bursts were detected using both amplitude and area as detection parameters.

Intracellular recording. We used discontinuous single-electrode voltage-clamp (switching frequency, 3–4 kHz) or current-clamp techniques with sharp intracellular micropipettes (3 M KCl; 50–80 MΩ) as described previously (Tallent and Siggins, 1997; Madamba et al., 1999b). To block $GABA_A$-mediated IPSCs, 10–15 μM bicuculline or 50 μM picrotoxin was included in the bath, and when a $GABA_B$ receptor component was apparent, 1 μM CGP 55845A was added. We evoked associational/commissural (A/C) EPSCs in CA3 hippocampal pyramidal neurons (HPNs) by stimulating in the stratum radiatum toward the CA1. Mossy fiber (MF) EPSCs were generated by stimulating in the stratum lucidum proximal to the recording electrode. In some experiments, two stimulating electrodes were placed in the slice, and in others only a single pathway was stimulated. Because MF EPSCs can be difficult to isolate, we took precautions to avoid polysynaptic A/C contamination (Williams and Johnston, 1991; Claiborne et al., 1993; Tallent and Siggins, 1999); (1) recordings were done in ACSF containing 7 mM Mg^{2+}, 4 mM Ca^{2+}, and 30 μM APV to block polysynaptic events (Williams and Johnston, 1991), and (2) we discarded recordings of EPSCs with a variable latency, a slow rising phase, or a complex falling phase (Williams and Johnston, 1991; Claiborne et al., 1993). Two traces were averaged for each stimulus intensity.

To record spontaneous epileptiform bursting intracellularly, we recorded in current-clamp mode and superfused picrotoxin with 0.75 mM external Mg^{2+} and linopirdine (10 μM), a selective M-current blocker. In some experiments, the membrane potential was manually adjusted with positive or negative current injection. As with extracellular bursting, trials were recorded on a computer and also continuously monitored with a chart recorder.

Voltage-sensitive currents were recorded in voltage clamp in 1 μM tetrodotoxin (TTX); we assessed current–voltage relationships by stepping to hyperpolarized and depolarized potentials for 1.5 sec from a holding potential of −60 mV. For M-current analyses, the neuron was depolarized to −45 to −50 mV in the presence of nifedipine (10 μM) to block L-type Ca^{2+} currents that interfere with analysis of M-currents in CA3 (Moore et al., 1994). A series of hyperpolarizing steps (5–25 mV; 1 sec duration) was given, and the M-current was observed as the slow inward current relaxation after the ohmic current drop (Moore et al., 1988, 1994; Madamba et al., 1999a). To measure the current relaxation, we fitted the peak of the initial current after the capacitive transient (5–20 msec after the onset of the voltage step) to the peak steady-state current just before the offset of the command step, using Clampfit software (Madamba et al., 1999a).

We performed statistical analysis using two-factor ANOVA with or without replication or Student's t test, depending on appropriateness, using Microsoft Excel or Crunch (Crunch Software Corporation, Oakland, CA). Data are reported as the mean ± SEM and considered statistically significant at $p < 0.05$.

Whole-cell patch-clamp recordings of miniature EPSCs. To obtain the necessary resolution for recording miniature EPSCs (mEPSCs), we recorded in CA3 pyramidal neurons using the "blind" method of whole-cell patch clamp (Blanton et al., 1989) in the presence of 15 μM bicuculline and 1 μM TTX. Data were acquired using continuous voltage clamp at a sampling frequency of 20 kHz with an Axopatch 200B amplifier. The patch solution contained (in mM): 130 K-gluconate, 7 KCl, 10 HEPES, 2 $MgCl_2$, 0.5 EGTA, 5 ATP, and 1 GTP (the latter two added fresh on the day of the recording). We pulled patch electrodes on a Flaming/Brown puller from borosilicate glass (input resistance of 2–3 MΩ when filled). Access resistance was 15–30 MΩ immediately after breaking into the cell, and we rejected neurons in which this value increased by >15% during the course of an experiment. The junction potential was nulled with amplifier circuitry. We analyzed miniature events using the Mini 4.3 software (Synaptosoft, Leona, NJ). The threshold for detection of mEPSPs was 5–7 pA and was maintained constant for an individual neuron, and automatic detection was verified by visual analysis. Drug effects on frequency and amplitude within individual neurons were evaluated using cumulative probability analysis, with statistical significance determined using the Kolmogorov–Smirnov nonparametric two-sample test (Van der Kloot, 1991) ($p < 0.05$ is considered significant).

RESULTS

Noc reduces spontaneous epileptiform bursting

After superfusion of Mg^{2+}-free ACSF, spontaneous epileptiform "interictal" bursting that represents the synchronous, recurrent firing of CA3 neurons can be recorded extracellularly in CA3 (Traub et al., 1994). Superfusion of 500 nM Noc reversibly blocked bursting in six of seven slices within 2–3 min of application. Mean burst frequency was decreased 98% by Noc, from 0.52 ± 0.03 to 0.01 ± 0.01 Hz, with recovery to 0.46 ± 0.05 Hz after washout (20–30 min). When 2 μM ORLAn was coapplied, 500 nM Noc reduced bursting by only 46 ± 7% ($n = 4$); thus at these concentrations ORLAn can partially block the actions of Noc. In the presence of ORLAn alone (2 μM; $n = 7$), burst rate was inhibited by only 4.4 ± 8% ($p > 0.05$); thus little partial agonist activity was detected. In contrast, 500 nM Noc completely blocked CA3 bursting when coapplied with the κ antagonist nBNI (500 nM; $n = 3$). Thus Noc does not interact with a κ-like receptor to reduce bursting in CA3.

A lower concentration of Noc (100 nM) reduced bursting frequency by 54 ± 1% ($n = 6$) (Fig. 1). When 1 μM ORLAn was coapplied with 100 nM Noc, bursting was not significantly depressed (7.3 ± 8%; $p > 0.05$). No desensitization was observed when a second application of 500 nM Noc was superfused within 30 min of the first (96 ± 5% inhibition with the first application and 99 ± 1% inhibition with the second application; $n = 4$). A truncated Noc analog, Noc (1–13) amide [an endogenous cleavage product of pronociceptin (Sandin et al., 1999)], also very potently inhibited bursting (Fig. 1).

Noc depresses CA3 EPSCs generated at both mossy fiber and A/C synapses

To determine more precisely the cellular mechanisms by which Noc inhibited epileptiform bursting, we performed intracellular

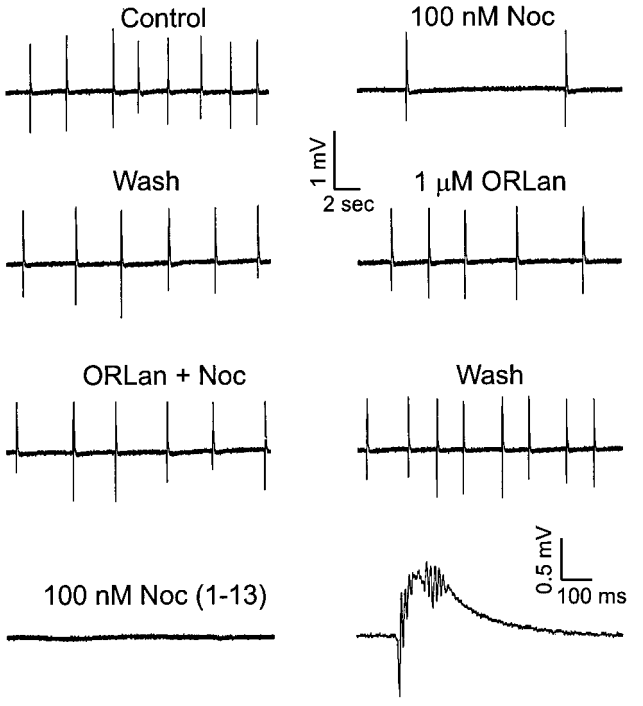

Figure 1. Noc reduced spontaneous bursting recorded in CA3. Extracellular recordings in Mg^{2+}-free ACSF. In this slice, Noc reduced the burst rate from 0.3 to 0.07 Hz, with recovery after washout (*Wash*). This effect was blocked by coapplication of ORLAn, the ORL-1 antagonist. Inhibition of bursting during a subsequent application of Noc (1–13) amide showed that no desensitization occurred. *Bottom right*, An individual burst with an expanded time base. Noc did not consistently affect the shape of individual bursts (data not shown).

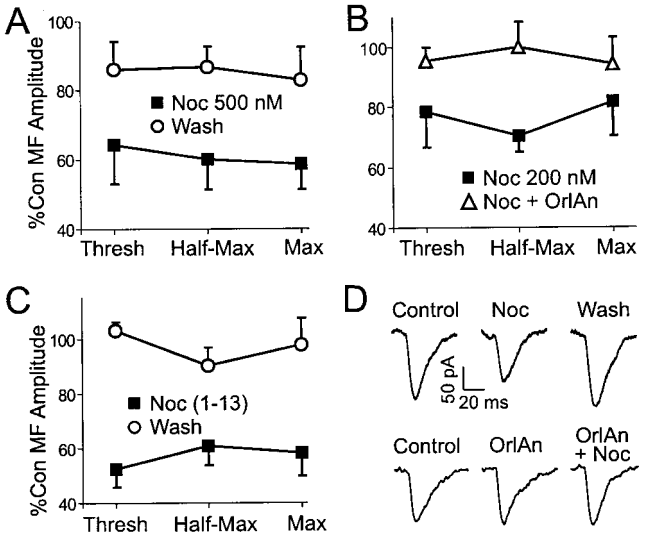

Figure 2. Noc reduced MF-generated EPSCs. *A–C*, Plots of mean EPSC amplitudes versus stimulation strength are shown. *A*, Noc (500 nM; *closed squares*) reduced the mean amplitude of EPSCs generated by stimulating MFs in the stratum radiatum. This effect reversed after washout (*open circles*; $n = 6–9$ cells). *B*, A lower concentration of Noc (200 nM) also reduced the mean amplitude of MF EPSCs (*closed squares*; $n = 5$). This effect was blocked when Noc was coapplied with 1 μM ORLAn (*open triangles*; $n = 5$). *C*, The truncated analog Noc (1–13) amide (500 nM; *closed squares*) also effectively reduced mean MF EPSC amplitude ($n = 5$), with complete recovery after washout (*open circles*). *D*, Representative current traces recorded from two CA3 neurons are shown. *Top row*, MF EPSCs generated at half-maximal stimulus intensity were attenuated by 200 nM Noc, with recovery after washout. *Bottom row*, In a different neuron, ORLAn (1 μM) alone did not alter the evoked EPSC but blocked the effect of 200 nM Noc when coapplied. Noc and Noc with ORLAn were tested in different neurons to avoid desensitization issues. *Con*, Control; *Max*, maximal; *OrlAn*, ORLAn; *Thresh*, threshold.

voltage- and current-clamp studies in CA3 HPNs. Neurons were held at −75 mV, and we evoked synaptic responses by stimulating either the MF or A/C pathways (see Materials and Methods). These neurons had a mean resting membrane potential (RMP) of −73 ± 1 mV, input resistance of 112 ± 2 MΩ, and spike amplitude of 101 ± 1 mV. EPSCs were generated in the presence of APV to block NMDA receptors, so that MF responses could be better isolated (Claiborne et al., 1993). MF-generated EPSCs were sensitive to Noc (Fig. 2). Noc (500 nM; 3–8 min superfusion) significantly reduced ($F_{(1,5)} = 5.5$; $p < 0.05$) the peak EPSC amplitude at all three stimulus intensities (threshold, half-maximal, and maximal) (Fig. 2*A*), an effect reversible after washout of the peptide. A lower concentration of Noc (200 nM; 4–8 min) also significantly reduced the mean EPSC amplitudes ($F_{(1,4)} = 11.0$; $p < 0.005$) (Fig. 2*B*). Inhibition by 200 nM Noc was significantly blocked when 1 μM ORLAn was coapplied ($F_{(1,4)} = 9.6$; $p < 0.01$) (Fig. 2*B,D*). This concentration of ORLAn had no effect on mean EPSC amplitudes when applied alone (103 ± 5% of mean control EPSC amplitude measured at half-maximal stimulation intensity). Noc (1–13) amide (500 nM) also inhibited MF EPSCs (500 nM; $F_{(1,5)} = 47.5$; $p < 0.001$) (Fig. 2*C*) to a slightly but not significantly greater degree than did the same concentration of Noc.

Noc also inhibited A/C-generated EPSCs. The mean peak amplitudes of A/C-generated EPSCs were significantly reduced by both 500 nM Noc ($F_{(1,6)} = 8.28$; $p < 0.01$) (Fig. 3*A*) and 200 nM Noc ($F_{(1,3)} = 8.60$; $p < 0.01$) (Fig. 3*B*). In the presence of 1 μM ORLAn, the reduction of the peak amplitude by 200 nM Noc was completely blocked ($F_{(1,6)} = 29.3$; $p < 0.001$) (Fig. 3*B,D*). As with MF EPSCs, 500 nM Noc (1–13) amide also attenuated A/C EPSCs to a significant degree ($F_{(1,4)} = 23.9$; $p < 0.005$) (Fig. 3*C*).

Noc decreases the frequency but not the amplitude of miniature EPSCs

To determine the site of action of Noc on EPSCs, we recorded mEPSCs in five CA3 HPNs using whole-cell patch clamp in the presence of TTX and bicuculline. These five neurons had a mean RMP of −72 ± 3 mV immediately after breaking into the cell and were subsequently held at −70 mV. The majority of mEPSCs were mediated by AMPA receptors, because they were blocked by 30 μM CNQX (data not shown). Superfusion of 500 nM Noc reduced the frequency of mEPSCs to 65 ± 3% of control (Fig. 4*D*) and significantly shifted the cumulative frequency distribution to longer interevent intervals in all five cells (Fig. 4*B*). In contrast, we detected no significant shift in the distribution of mEPSC amplitude in four of the five neurons ($p > 0.05$) (Fig. 4*C*). In the other neuron Noc decreased the mean amplitude of the mEPSCs by 18% and shifted the cumulative amplitude distribution to lower amplitudes. The mean amplitude of the mEPSCs after superfusion of 500 nM Noc for all five neurons was 93 ± 5% of control ($p > 0.05$) (Fig. 4*E*).

Noc hyperpolarizes CA3 neurons by activating an outward current

As with our observations in CA1 (Madamba et al., 1999b) and those reported by others for CA3 (Ikeda et al., 1997; Amano et al., 2000), superfusion of 500 nM Noc elicited a robust steady-state

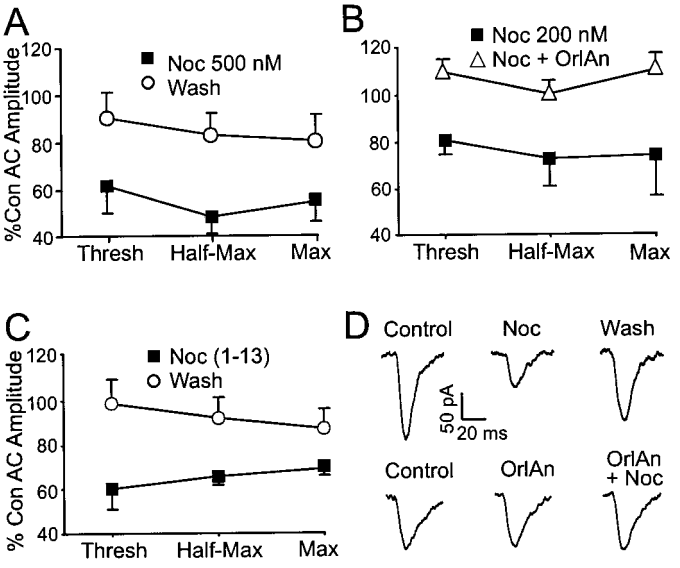

Figure 3. Noc reduced A/C (*AC*)-generated EPSCs. *A–C,* Mean EPSC amplitudes versus stimulation strength are shown. *A*, Noc (500 nM; *closed squares*) reversibly reduced the mean amplitude of EPSCs generated by stimulating A/C fibers in the stratum radiatum. This effect was reversible after washout (*open circles*; $n = 7$–10). *B*, A lower concentration of Noc (200 nM) also reduced the amplitude of A/C EPSCs (*closed squares*; $n = 5$). This effect was completely blocked when Noc was coapplied with 1 μM ORLAn (*open triangles*; $n = 5$). *C*, Noc (1–13) amide (500 nM) also effectively reduced mean EPSC amplitude (*closed squares*; $n = 5$), with recovery after washout (*open circles*). *D*, Representative current traces from CA3 neurons show A/C EPSCs. *Top row*, Noc (200 nM) reduced the EPSC generated at half-maximal stimulus intensity, an effect that washed out. *Bottom row*, In contrast, in a different neuron, 1 μM ORLAn alone or coapplied with 200 nM Noc did not alter the evoked A/C EPSCs.

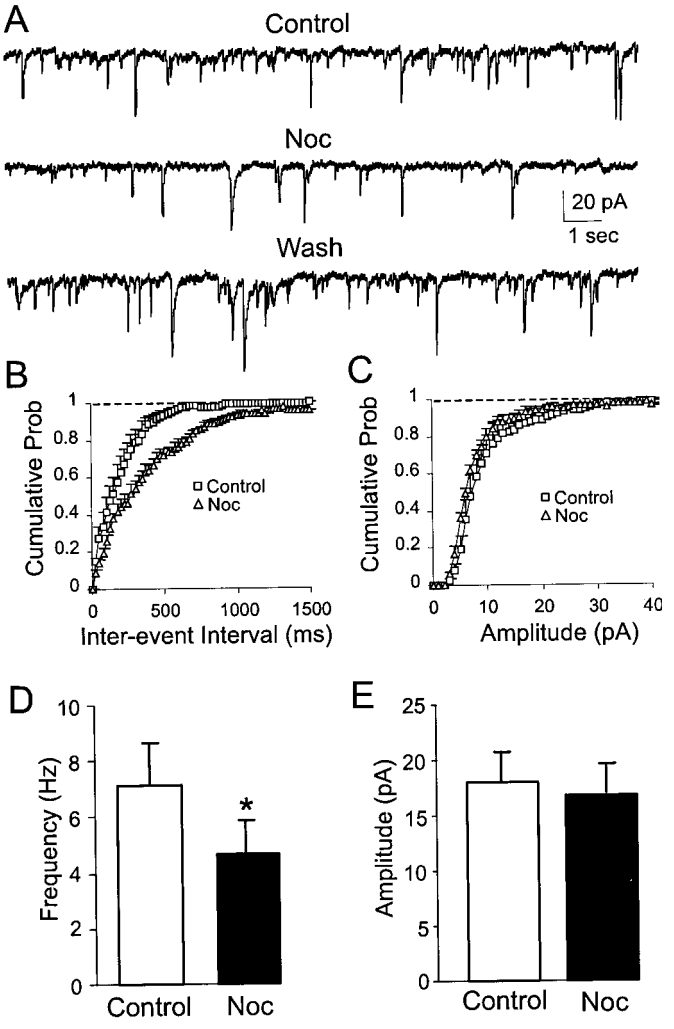

Figure 4. Noc reduced the frequency of mEPSCs. *A*, Whole-cell voltage-clamp recordings from a representative CA3 neuron in TTX and bicuculline. Note the Noc-induced decrease in the frequency of the mEPSCs. *B*, Cumulative frequency histogram for a representative neuron showing a shift to longer interevent intervals (lower frequencies) after application of Noc (500 nM). Data were plotted in 25 msec bins and averaged from three different 20 sec recording intervals each for control and Noc. *C*, Cumulative amplitude graph from the same neuron showing no change in the distribution of mEPSC amplitudes. Data shown are means from three 20 sec recordings plotted in 1 pA bins. *D*, Pooled data showing mean inhibition of mEPSC frequency by 500 nM Noc ($n = 5$). The *asterisk* denotes statistical significance ($p < 0.05$). *E*, Mean amplitudes of mEPSC from the same five neurons. Noc (500 nM) did not significantly affect mean mEPSC amplitude. *Prob*, Probability.

current throughout the *I–V* curve and increased input conductance (Fig. 5). Analysis of the control-subtracted current obtained from *I–V* plots revealed that the Noc-sensitive current reversed at −97 mV ($n = 5$) (Fig. 5*B*, *left*), near the reversal potential for K^+. Unlike our observations in CA1 (Madamba et al., 1999b), superfusion of 1–2 μM ORLAn alone did not evoke currents in CA3 HPNs and had very little effect across the range of voltages tested (Fig. 5*B*, *right*). When coapplied with Noc, ORLAn blocked most of the action of 500 nM Noc, especially in the range from −60 to −100 mV.

Noc augments the M-current

To characterize the postsynaptic actions of Noc further, we recorded M-currents in CA3 neurons. In the presence of nifedipine to block L-type Ca^{2+} currents (Moore et al., 1994) and 1 μM TTX to block Na^+ channels, Noc (0.5 μM) increased M-current amplitudes (Fig. 6*A*). Mean data from five cells showed that Noc significantly ($F_{(2,48)} = 64.96$; $p < 0.0001$) increased M-current amplitudes with recovery after washout (Fig. 6*B*). In a different set of five neurons, we tested the action of linopirdine, an M-current blocker (Schnee and Brown, 1998; Schweitzer, 2000). At a concentration that is selective for the M-current [10 μM (Schnee and Brown, 1998; Schweitzer, 2000)], linopirdine prevented the Noc-induced increase of M-current (Fig. 6*C*). Despite the M-current blockade by linopirdine, superfusion of 0.5 μM Noc still significantly ($F_{(1,44)} = 7.279$; $p < 0.01$) induced steady-state currents (Fig. 6*D*), although to a much lesser extent than with Noc alone. The residual Noc-induced current in linopirdine also reversed near the K^+ equilibrium potential and showed some inward rectification, suggesting that Noc may activate the inward rectifier K^+ current in rat CA3 neurons, as has been reported for mouse (Ikeda et al., 1997).

Effect of M-current blocker on inhibition of epileptiform bursting by Noc

We next examined the contribution of the M-current to the antiepileptic actions of Noc by superfusing 0 Mg^{2+} ACSF and recording spontaneous bursting extracellularly. After the burst rate stabilized, we superfused on linopirdine (10 μM) for at least 30 min before examining Noc effects. Linopirdine alone did not significantly affect the burst rate but increased the duration of individual bursts (Fig. 7*A*, *inset*). In the continued presence of linopirdine, 500 nM Noc reduced the rate of bursting to 5.5 ± 6%

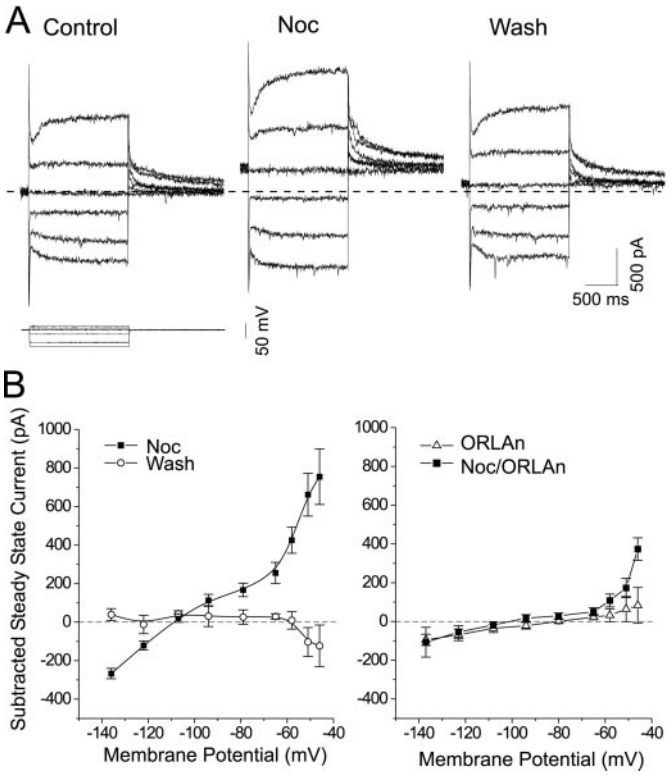

Figure 5. Intracellular voltage-clamp recordings from CA3 pyramidal neurons showing current–voltage relationships of Noc effects. *A*, Current traces from a representative neuron. Superfusion of 0.5 μM Noc (5 min) increased steady-state currents across the range of voltages tested, with recovery after washout (21 min). RMP was −70 mV, and V_H was −62 mV. Voltage commands are shown at *lower left*. *B, left*, Current–voltage (*I–V*) plot for mean net (control-subtracted) current elicited by 0.5 μM Noc ($n = 5$). The nociceptin-induced current reversed at −97 mV, suggesting that the Noc current is carried by K^+ ions. *Right*, Mean control-subtracted currents for ORLAn alone (1 μM) and for 0.5 μM Noc plus 1 μM ORLAn ($n = 6$–7). Note that at this concentration, ORLAn has very little partial agonist activity but blocks almost all of the action of Noc.

of control (Fig. 7*A*) ($n = 4$), with a complete block of bursting in three of four slices. This is not significantly different from the Noc inhibition of bursting in the same epileptiform model without linopirdine ($p > 0.05$). Thus, Noc actions on the M-current do not appear to contribute significantly to its ability to reduce the burst rate in this epileptiform model. We also examined Noc actions on spontaneous bursting elicited by superfusing high K^+. In this model, all neurons in the network are depolarized by ~10 mV (Jensen et al., 1994; Jensen and Yaari, 1997); thus, it is possible that the M-current might play a larger role at this membrane potential. However, in the presence of linopirdine, Noc still completely blocked bursting under these conditions (Fig. 7*B*) ($n = 4$).

To determine whether membrane potential in an individual neuron could affect the actions of Noc in linopirdine, we recorded intracellularly in current-clamp mode and elicited spontaneous bursting by superfusing 0.75 mM Mg^{2+}, 50 μM picrotoxin, and 10 μM linopirdine. We compared the effects of Noc at the resting membrane potential (−69 to −74 mV) and at more depolarized potentials (−58 to −62 mV) in the active range of the M-current. As with extracellular recordings, linopirdine alone increased burst duration (Fig. 7*C*, *inset*). Noc plus linopirdine still completely blocked bursting in all four cells; furthermore, there was no effect of membrane potential on the actions of Noc (Fig. 7*C*). Thus, using these specific models, with M-current blockade, Noc inhibition of bursting is not compromised.

DISCUSSION

Noc has been reported to have presynaptic and/or postsynaptic actions in several different brain regions (Schlicker et al., 1998; Wagner et al., 1998; Connor et al., 1999). For example, a recent study in hypothalamic slices showed that Noc activated an inwardly rectifying K^+ conductance and presynaptically inhibited EPSCs in arcuate neurons (Emmerson and Miller, 1999). These dual actions of Noc should synergize to reduce excitability in these neurons. In the dentate gyrus, Noc hyperpolarizes granule cells and reduces NMDA EPSCs via an apparent postsynaptic mechanism, and no presynaptic actions were detected (Yu and Xie, 1998). Therefore, whereas the reported actions of Noc are primarily inhibitory, it appears to have discrete mechanisms of action in different brain regions. We chose to examine the effects of Noc in the CA3 hippocampus because this region is critical in the generation of seizure events. Bursting in CA3 is initiated at positive feedback synapses that interconnect the principal pyramidal neurons (Wong and Traub, 1983). When spontaneous glutamate release at these synapses reaches a critical level, bursting is initiated. Therefore, presynaptic inhibition of glutamate release would decrease the propensity of the network to burst. Likewise, a postsynaptic action such as hyperpolarization of the pyramidal neurons would decrease the likelihood of firing and would also reduce the probability of the synchronization required for bursting.

Our results show that Noc acting on ORL-1 has inhibitory actions on epileptiform activity in CA3 and has both presynaptic and postsynaptic sites of action. At the cellular level, Noc hyperpolarizes CA3 pyramidal neurons via augmentation of K^+ currents [see also Ikeda et al. (1997) and Amano et al. (2000)], moving these neurons away from their threshold for firing. Furthermore, Noc reduces EPSCs generated by stimulating either mossy or A/C fibers. Inhibition of EPSCs appears to be via presynaptic inhibition of glutamate release and to be independent of postsynaptic actions on glutamate receptors, because Noc reduces the frequency of mEPSCs without altering their amplitude distribution. This is the first demonstration of a presynaptic action for Noc in the hippocampus. Thus, presynaptic and postsynaptic actions of Noc on CA3 pyramidal neurons would act in concert to reduce excitability and the spread of seizure events through the hippocampus.

That Noc reduces mEPSC frequency in TTX suggests that its presynaptic actions are "downstream" of Ca^{2+} entry into the cell, because spontaneous release of glutamate in TTX is Ca^{2+} independent. This might also be reflected in the relative insensitivity of the Noc inhibition of evoked EPSCs to stimulus intensity (Figs. 2, 3), because this also suggests that the actions of Noc are independent of the amount of Ca^{2+} in the presynaptic terminal. Similar actions have been reported for μ opioid ligands, which also decrease mEPSC frequency in the CA3 of culture hippocampal slices (Capogna et al., 1993). The mechanism via which these peptides inhibit Ca^{2+}-independent glutamate release is unknown, although it could involve inhibition of adenylyl cyclase (Tzounopoulos et al., 1998) or direct interaction with synaptic machinery, as has been reported for muscarinic receptors (Linial et al., 1997).

We found that much of the postsynaptic action of Noc in voltage ranges depolarized from rest is via activation of the

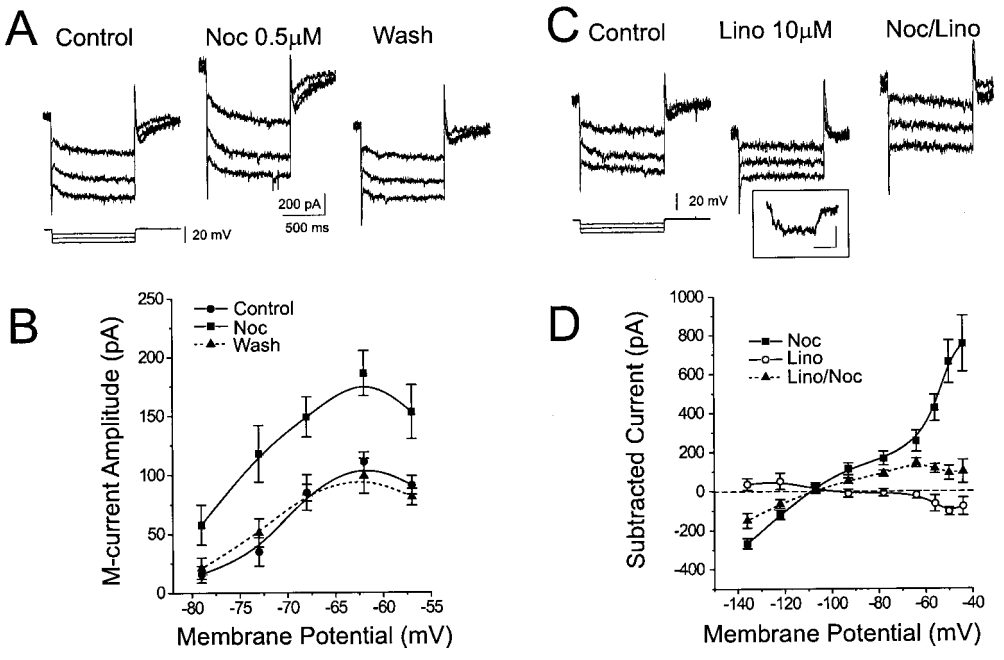

Figure 6. Noc increased the M-current in CA3 neurons; this effect was blocked by linopirdine. *A*, M-current current record of a CA3 neuron in the presence of 10 μM nifedipine and 1 μM TTX is shown. Superfusion of 0.5 μM Noc for 6 min induced an outward current and increased the M-current with recovery after washout (34 min). Voltage command steps are shown at *lower left*; $V_H = -49$ mV; RMP = -71 mV. *B*, *I–V* analysis of pooled data from five cells shows a significant Noc enhancement of M-current amplitudes with complete recovery after washout. *C*, In another cell 10 μM linopirdine (*Lino*), an M-current blocker, induced a small inward baseline current consistent with the observed blockade of the M-current (note flat current traces). When superfused with linopirdine, 0.5 μM Noc had no effect on the M-current but still induced a small steady-state outward current. *Inset*, The middle current for linopirdine subtracted from the control current isolates the linopirdine-sensitive component (M-current). Scale units are the same as for *A*. Voltage command steps are shown at *lower left*; $V_H = -40$ mV; RMP = -67 mV. *D*, Analysis of pooled, control-subtracted steady-state values from five cells indicates that Noc still induced a significant, but greatly reduced, outward current in linopirdine. The Noc-induced steady-state current (without linopirdine) is from the same five cells shown in *B*. The residual Noc-induced current in linopirdine reversed near the K^+ equilibrium potential. These findings suggest that Noc activates two different K^+ currents.

M-current. Linopirdine, a drug with previously demonstrated selectivity in CA1 (Aiken et al., 1995; Schnee and Brown, 1998; Schweitzer, 2000), also appears to block selectively the M-current in CA3 at the concentration tested (10 μM). The M-current has been implicated recently in an inheritable form of epilepsy, benign familial neonatal convulsions. Mutations in two genes for subunits of M-type K^+ channels (KCNQ2 and KCNQ3) that lead to hypofunctional channels (Schroeder et al., 1998) have been found in families with this disease (Charlier et al., 1998; Singh et al., 1998). Interestingly, in the epilepsy models used in our study, blocking M-currents with linopirdine did not significantly affect the ability of Noc to reduce epileptiform activity. Even when neurons were depolarized to voltages in which the M-current would normally contribute significantly to the postsynaptic action of Noc, the peptide still completely inhibited bursting with the M-current blocked. These results suggest that the ability of Noc to augment the M-current does not contribute significantly to its antiepileptic actions in the models used in this study. We do show, however, that blocking the M-current leads to an increase in the duration of individual bursts without consistently altering burst rate. Thus, the M-current does appear to be involved in the regulation of burst duration. It is possible that in other epilepsy models, such as those with prolonged depolarizing ictal events, M-current enhancement could play a larger role in mediating antiepileptic actions of Noc. Presynaptic inhibition of glutamate release and enhancement of linopirdine-insensitive K^+ currents [i.e., inward rectifier (Ikeda et al., 1997)] by Noc are most likely to account for antiepileptic actions of Noc in the tested models.

Peptidergic modulation of limbic seizures may be an important compensatory mechanism in the hippocampus. Noc acts via more diverse mechanisms to reduce CA3 excitability than have been reported for other neuropeptides. For example, neuropeptide Y does not appear to have a postsynaptic action on CA3 pyramidal neurons (Colmers et al., 1988) and does not inhibit mEPSCs recorded in TTX but instead inhibits activity-dependent spontaneous EPSCs (McQuiston and Colmers, 1996). Postsynaptically, dynorphin acts only on the M-current and does not activate a K^+ current near the RMP (Moore et al., 1994), whereas presynaptically dynorphin inhibits MF EPSCs but not A/C EPSCs (Weisskopf et al., 1993). The postsynaptic actions of somatostatin in CA3 have not been characterized in detail, although it hyperpolarized neurons near the RMP (Tallent and Siggins, 1999). Presynaptically, somatostatin acts at A/C synapses to inhibit EPSCs, whereas MF EPSCs are insensitive to somatostatin (Tallent and Siggins, 1999). The μ opioid agonists inhibit mEPSCs in cultured hippocampal slices (Capogna et al., 1993) but have no postsynaptic actions (Moore et al., 1994). Thus Noc, by depressing EPSCs at both MF and A/C synapses and by activating K^+ currents across a wide range of voltages, is an especially robust inhibitor of CA3 excitability.

Noc-containing interneurons are found throughout the hippocampus, in stratum radiatum and stratum lucidum interneurons of CA1 and CA3 and in the polymorphic and molecular layers of the dentate gyrus (Neal et al., 1999a). Unlike many other neuropeptides, no Noc-containing hilar interneurons have been identified (Neal et al., 1999a), although one group found Noc mRNA in the hilar region (Ikeda et al., 1998). Furthermore, parahippocampal regions such as the subiculum and entorhinal cortex express high levels of Noc. ORL-1 binding and mRNA expression are also distributed throughout the hippocampus. Expression of ORL-1 mRNA appears primarily limited to principal neurons in CA1, CA3, and the dentate (Ikeda et al., 1998; Neal et al., 1999b). In contrast, autoradiography shows that binding is highest in dendritic layers (Neal et al., 1999b; Letchworth et al., 2000), suggesting that the receptor protein is transported to dendrites and/or terminals. Thus Noc and its receptors are critically localized to regulate excitatory activity in hippocampal efferents and afferents.

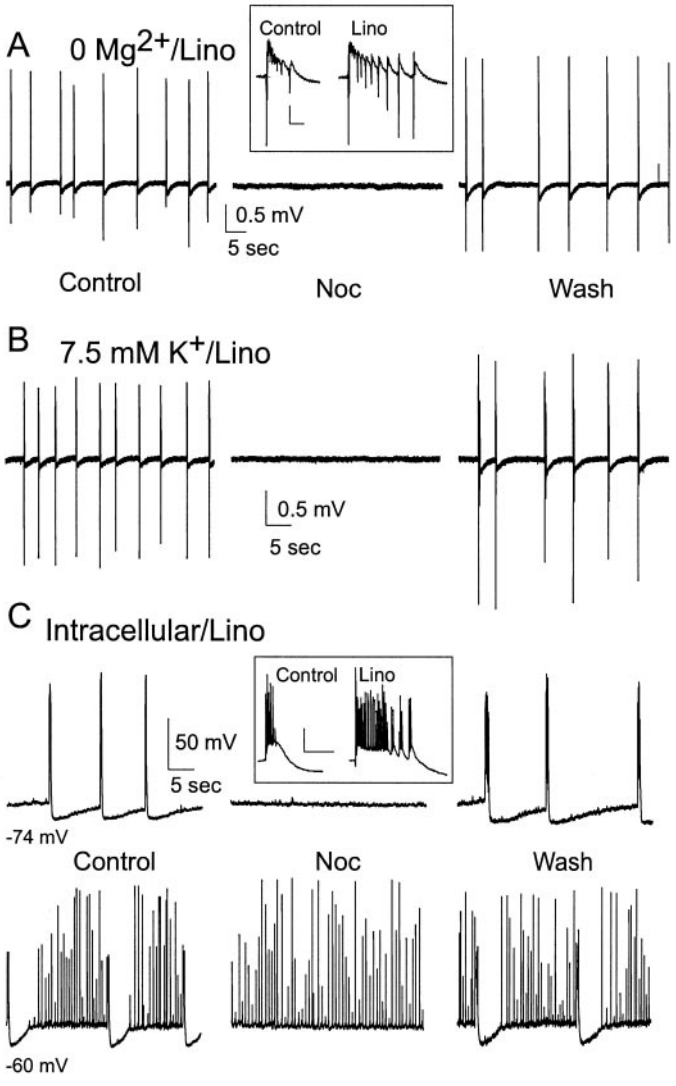

Figure 7. Linopirdine did not attenuate Noc inhibition of burst frequency in epileptiform models. *A*, Representative voltage traces recorded extracellularly in the CA3 cell layer are shown. In Mg^{2+}-free ACSF containing linopirdine (10 μM; 30 min), Noc (0.5 μM) was still able to block spontaneous bursting completely, with recovery after washout. After Noc superfusion, membrane potential was manually adjusted to the control level with current injection. *Inset*, Spontaneous extracellular bursts in Mg^{2+}-free ACSF are shown with an expanded time scale. Calibration: 100 msec, 0.5 mV. Application of linopirdine (30 min) resulted in an increase in the duration of the burst, with the appearance of multiple secondary afterdischarges. *B*, Extracellular recordings show that Noc (0.5 μM) still had a full inhibitory effect in linopirdine when high extracellular K^+ (7.5 mM) was used to induce spontaneous bursting. *C*, Intracellular current-clamp recordings of spontaneous bursting in 0.75 mM Mg^{2+} and picrotoxin (50 μM) are shown. *Top*, In the presence of linopirdine, Noc completely blocked bursting at the RMP (−74 mV; current injection was used to keep the membrane potential constant after Noc application). *Bottom*, Even when the neuron was depolarized by 14 mV with current injection, blocking M-currents with linopirdine did not interfere with the ability of Noc to suppress bursting. Note that bursts can be identified by large afterhyperpolarizations. When the neuron was depolarized beyond the threshold for action potentials, single spikes are observed that Noc did not affect (membrane potential was held constant after Noc application by current injection). *Inset*, Single spontaneous burst recorded intracellularly is shown with an expanded time scale. Calibration: 250 msec, 25 mV. As with extracellular recordings, linopirdine (35 min) increased the duration of individual bursts and increased the number of afterdischarges.

Peptide release is thought to require high-frequency activation of the peptidergic neuron, as would occur during a seizure event (Vezzani et al., 1992). Although there is currently no direct evidence that hippocampal seizures result in Noc release or regulation (Bregola et al., 1999), activation of peptidergic interneurons by seizure events and enhanced release of peptides after seizures have been frequently demonstrated. For example, augmentation of somatostatin release and expression after seizures have been shown in several different animal models (Vezzani et al., 1992; Schwarzer et al., 1996), and seizures activate somatostatin and enkephalin-containing interneurons (Pretel et al., 1995, 1996). Similar results have been found for dynorphin, neuropeptide Y, and CRF (Sperk et al., 1992; Smith et al., 1997). Although little is known about the possible colocalization of Noc with any of these other peptides, because of the high expression of Noc in hippocampal interneurons, it seems likely that some Noc-containing neurons would be activated during seizure events. Our results suggest that such activation leading to Noc release would act both presynaptically and postsynaptically to reduce the spread of seizures. An unexplored question is whether there are synergistic interactions among the numerous inhibitory hippocampal neuropeptides that may be released during seizures, since many peptides appear to have distinct cellular actions (Zieglgänsberger et al., 1979; Colmers et al., 1993; Moore et al., 1994; Tallent and Siggins, 1999).

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