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 Mg2+ 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 [Phe1-Gly10]-Noc (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 δ and μ receptor ligands have excitatory actions on CA1 pyramidal neurons via inhibition of GABAergic interneurons (Zieglgansberger 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 κ1 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 (Marrosu 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...
augments the M-current, this mechanism does not appear to contribute significantly to antiepileptic actions of Noc in the models tested here.

METHODS AND MATERIALS

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 McIlwain brain slicer or a Catslab slicer (Clifton, NJ) and placed in artificial CSF (2 mM MgCl2, 2 mM CaCl2, 115 mM NaCl, 30 mM KCl, 25 mM HEPES, pH 7.4). The slices were superfused at 0.75 ml/min with artificial CSF (2 mM MgCl2, 2 mM CaCl2, 115 mM NaCl, 30 mM KCl, 25 mM HEPES, pH 7.4) as described previously (Tallent et al., 1994). 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 twosample test (Van der Kloo, 1991) (p < 0.05 is considered significant).

RESULTS

Noc reduces spontaneous epileptiform bursting

After superfusion of Mg2+-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 nBN1 (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).

These neurons had a mean resting membrane potential (RMP) of 72 ± 3 mV. The majority of mEPSCs were recorded in different neurons to avoid desensitization issues. Con: Control; Max: maximal; ORLAn: ORLAn; Threshold: threshold.

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. 4D) and significantly shifted the cumulative frequency distribution to longer interevent intervals in all five cells (Fig. 4B). In contrast, we detected no significant shift in the distribution of mEPSC amplitude in four of the five neurons (p > 0.05) (Fig. 4C). 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. 4E).

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
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 $-97 \text{ mV}$ ($n = 5$) (Fig. 5B, left), near the reversal potential for $K^+$. Unlike our observations in CA1 (Madamba et al., 1999b), superfusion of 1–2 $\mu M$ ORLAn alone did not evoke currents in CA3 HPNs and had very little effect across the range of voltages tested (Fig. 5B, right). When coapplied with Noc, ORLAn blocked most of the action of 500 nM Noc, especially in the range from $-60$ to $-100 \text{ mV}$.

**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 $\mu M$ TTX to block Na$^+$ channels, Noc (0.5 $\mu M$) increased M-current amplitudes (Fig. 6A). 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. 6B). 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 $\mu M$ (Schnee and Brown, 1998; Schweitzer, 2000)], linopirdine prevented the Noc-induced increase of M-current (Fig. 6C). Despite the M-current blockade by linopirdine, superfusion of 0.5 $\mu M$ Noc still significantly ($F_{(1,14)} = 7.279; p < 0.01$) induced steady-state currents (Fig. 6D), 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 $\mu M$) 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. 7A, inset). In the continued presence of linopirdine, 500 nM Noc reduced the rate of bursting to $5.5 \pm 6\%$.
of control (Fig. 7A) (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. 7B) (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 Mg²⁺, 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. 7C, 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. 7C). 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 inward-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 Ca²⁺ entry into the cell, because spontaneous release of glutamate in TTX is Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-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
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). Postsynthetically, dynorphin acts only on the M-current and does not activate a K⁺ current near the RMP (Moore et al., 1994), whereas presynthetically 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 dentritic 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.
After Noc superfusion, membrane potential was manually adjusted to the RMP (74 mV; 30 min), Noc (0.5 μM) still had a full inhibitory effect in linopirdine when high extracellular K⁺ was still able to block spontaneous bursting completely, with recovery after washout.


REFERENCES


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).

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 Mg²⁺-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 Mg²⁺-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 Mg²⁺ (75 mM Mg²⁺), 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). 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.


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