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Roles of K⁺ and Cation Channels in ORL-1 Receptor-mediated Depression of Neuronal Excitability and Epileptic Activities in the Medial Entorhinal Cortex

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Abstract

Nociceptin (NOP) is an endogenous opioid-like peptide that selectively activates the opioid receptor-like (ORL-1) receptors. The entorhinal cortex (EC) is closely related to temporal lobe epilepsy and expresses high densities of ORL-1 receptors. However, the functions of NOP in the EC, especially in modulating the epileptiform activity in the EC, have not been determined. We demonstrated that activation of ORL-1 receptors remarkably inhibited the epileptiform activity in entorhinal slices induced by application of picrotoxin or by deprivation of extracellular Mg²⁺. NOP-mediated depression of epileptiform activity was independent of synaptic transmission in the EC, but mediated by inhibition of neuronal excitability in the EC. NOP hyperpolarized entorhinal neurons via activation of K⁺ channels and inhibition of cation channels. Whereas application of Ba²⁺ at 300 μ M which is effective for the inward rectifier K⁺ (Kir) channels slightly inhibited NOP-induced hyperpolarization, the current-voltage (I-V) curve of the net currents induced by NOP was linear without showing inward rectification. However, a role of NOP-induced inhibition of cation channels was revealed after inhibition of Kir channels by Ba²⁺. Furthermore, NOP-mediated augmentation of membrane currents was differently affected by application of the blockers selective for distinct subfamilies of Kir channels. Whereas SCH23390 or ML133 blocked NOP-induced augmentation of membrane currents at negative potentials, application of tertiapin-Q exerted no actions on NOP-induced alteration of membrane currents. Our results demonstrated a novel cellular and molecular mechanism whereby activation of ORL-1 receptors depresses epilepsy.

Keywords

hyperpolarization; hippocampus; synaptic transmission; glutamate; K⁺ channels; epilepsy; action potential; cortex; neuropeptide

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1. Introduction

Recurrent uncontrolled seizures or epilepsy is a common neurological disorder that is characterized by excessive excitation of many brain regions including the entorhinal cortex (EC) and hippocampus. The conventional antiepileptic drugs, while somewhat effective, have side effects and target a limited number of mechanisms, necessitating the identification of novel therapeutic strategies. Nociceptin (NOP) or orphanin FQ is an endogenous opioid-like peptide that selectively activates the opioid receptor-like (ORL-1) receptors without effects on the traditional mu, delta and kappa opioid receptors (Bunzow et al., 1994; Fukuda et al., 1994; Lachowicz et al., 1995; Mollereau et al., 1994; Wick et al., 1994). Recent evidence suggests that NOP modulates epilepsy. For example, NOP inhibits kindling epileptogenesis (Bregola et al., 2002a; Gutierrez et al., 2001) and epileptic activity in hippocampal slices (Tallent et al., 2001); NOP depresses epileptic activities in a variety of epilepsy models (Carmona-Aparicio et al., 2007; Feng et al., 2004; Rubaj et al., 2002); the expressions of NOP and NOP receptors are altered in epilepsy (Aparicio et al., 2004; Bregola et al., 2002b). Whereas all the above lines of evidence suggest that NOP exerts an inhibitory role in epilepsy, NOP-deficient mice exhibit delayed epileptogenesis (Binaschi et al., 2003). Therefore, the exact roles and underlying mechanisms of NOP in epilepsy are still elusive.

The entorhinal cortex (EC) serves as the interface to control the flow of information into and out of the hippocampus. Afferents from the olfactory structures, parasubiculum, perirhinal cortex, claustrum, amygdala and neurons in the deep layers of the EC (layers V–VI) converge onto the superficial layers (layer II/III) of the EC (Burwell, 2000; Witter et al., 1989). The axons of principal neurons in layer II form the major component of perforant path that innervates the dentate gyrus and CA3 (Steward and Scoville, 1976), whereas those of the pyramidal neurons in layer III form the temporoammonic pathway that synapses onto the distal dendrites of the pyramidal neurons in the CA1 and subiculum (Steward and Scoville, 1976; Witter et al., 2000). The output from the hippocampus is then projected to the deep layers of the EC that relay information back to the van Haeften et al., 2003) and to other cortical areas (Witter et al., 1989). The EC is an essential structure in the limbic system that is closely related to emotional control (Majak and Pitkanen, 2003), consolidation and recall of memories (Dolcos et al., 2005; Steffenach et al., 2005), Alzheimer's disease (Hyman et al., 1984; Kotzbauer et al., 2001), schizophrenia (Joyal et al., 2002; Prasad et al., 2004) and especially temporal lobe epilepsy (Avoli et al., 2002; Spencer and Spencer, 1994).

Whereas both the EC and NOP are related to epilepsy and the EC expresses high densities of NOP receptors (Carmona-Aparicio et al., 2007; Shimohira et al., 1997; Sim-Selley et al., 2003), the functions of NOP in the EC, especially in modulating the epileptiform activity in the EC, have not been determined. Here, we studied the roles of ORL-1 receptor activation in modulating the neuronal excitability and the epileptiform activity induced by bath application of the GABA_A receptor channel blocker, picrotoxin (PTX) or deprivation of extracellular Mg²⁺ in entorhinal slices. Our results indicate that activation of ORL-1 receptors by NOP depresses epileptiform activity by inhibiting neuronal excitability in the EC. We further demonstrate that NOP-elicited depression of neuronal excitability in the EC is mediated by activation of K⁺ channels and reduction of cation channels. Our results

provide a novel cellular and molecular mechanism whereby NOP dampens epileptiform activity in the EC.

2. Materials and Methods

2.1. Slice preparation

Horizontal brain slices (400 μm) were prepared from Sprague-Dawley rats (15–22 days old) as described previously (Cilz et al., 2014; Ramanathan et al., 2012; Wang et al., 2013; Xiao et al., 2009) with slight modification (Xiao et al., 2014). After being deeply anesthetized with isoflurane, animals were decapitated and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 N-methyl-D-glucamine (NMDG)-Cl, 24 NaHCO_3 , 3.5 KCl, 1.25 NaH_2PO_4 , 0.5 CaCl_2 , 5.0 MgCl_2 , and 10 glucose, saturated with 95% O_2 and 5% CO_2 (pH 7.4, adjusted with HCl). Slices were then incubated in the above solution except NMDG-Cl was replaced with NaCl at 35°C for 1 h for recovery and then kept at room temperature (~24°C) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

2.2. Immunocytochemistry

Detailed experimental procedures for immunocytochemistry were described previously (Deng et al., 2009; Ramanathan et al., 2012; Xiao et al., 2014; Xiao et al., 2009). Briefly, rats (18-day-old) were anaesthetized with pentobarbital sodium (50 mg/kg) and then perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were rapidly removed and postfixed in the same fixative for additional 2 h. After postfixation, brains were cryoprotected with 30% sucrose in PBS for 12 h and then cut into 20 μm slices in thickness horizontally in a Leica cryostat (CM 3050 S) at -21°C. Slices were washed in 0.1 M PBS and then treated with 0.3% H_2O_2 to quench endogenous peroxidase activity. After being rinsed in 0.1 M PBS containing 1% Triton X-100 and 1.5% normal donkey serum for 30 min, slices were incubated with the primary antibodies (rabbit anti-ORL-1, sc-15309, Santa Cruz Biotechnology) at a dilution of 1:200 at 4°C for 12 h. Slices were incubated at room temperature initially with biotinylated donkey anti-rabbit IgG (ABC Staining System, Santa Cruz Biotechnology Inc.) for 1 h, and then with avidin-biotin complex (ABC Staining System) for 30 min. After each incubation, slices were washed three times for a total of 30 min. Diaminobenzidine (ABC Staining System) was used for a color reaction to detect the positive signals. Finally, slices were mounted on slides, dehydrated through an alcohol range, cleared in xylene and covered with cover-slips. Slides were visualized and photographed with a Leica microscope (DM 4000B). We stained 5–6 nonadjacent sections and each staining was repeated by using 3 rats.

2.3. Recordings of the spontaneous epileptiform activity

Spontaneous epileptiform activity was induced by including PTX (100 μM) (Deng et al., 2006; Kurada et al., 2014; Wang et al., 2013) or no Mg^{2+} (Deng and Lei, 2008; Zhang et al., 2014) in the extracellular solution as described previously. An electrode containing the extracellular solution was placed in layer III of the medial EC to record epileptiform activity. After stable spontaneous epileptiform activity occurred, NOP (0.3 μM) was applied in the bath. The epileptiform events were initially recorded by Clampex 10.04 and subsequently

analyzed by Mini Analysis 6.0.1. The data recorded by Clampex 10.04 were re-saved as type “ABF1.8(integer)(*.abf)” in Clampfit 10.04 so that Mini Analysis 6.0.1. could read them. The parameters required by Mini Analysis 6.0.1 to identify epileptiform events were measured by Clampfit 10.04.

2.4. Recordings of action potentials, resting membrane potentials and holding currents from entorhinal neurons

Whole-cell patch-clamp recordings using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA) in current- or voltage-clamp mode were made usually from the pyramidal neurons in layer III of the medial EC visually identified with infrared video microscopy (Olympus BX51WI) and differential interference contrast optics unless stated otherwise. The recording electrodes were filled with (in mM) 100 K⁺-gluconate, 0.6 EGTA, 2 MgCl₂, 8 NaCl, 33 HEPES, 2 ATPNa₂, 0.4 GTPNa and 7 phosphocreatine (pH 7.4). The extracellular solution comprised (in mM) 130 NaCl, 24 NaHCO₃, 3.5 KCl, 1.25 NaH₂PO₄, 2.5 CaCl₂, 1.5 MgCl₂ and 10 glucose, saturated with 95% O₂ and 5% CO₂ (pH 7.4). Data were filtered at 2 kHz, digitized at 10 kHz, acquired on-line and analyzed after-line using pCLAMP 10.04 software (Molecular Devices, Sunnyvale, CA). The whole-cell recording configuration was used to record action potential (AP) firing from layer II stellate neurons and layer III pyramidal neurons. The stellate neurons in layer II usually do not exhibit spontaneous AP firing. A small portion of layer III pyramidal neurons displayed sparse spontaneous AP firing, whereas most of them were silent at their RMPs. We therefore injected a constant positive current to bring the membrane potential to around -40 mV to elicit stable and persistent firing. The preceding extracellular solution was supplemented with bicuculline (10 μM) and CGP55845 (1 μM) to block GABA_A and GABA_B responses, respectively, and DNQX (10 μM) and *d,l*-APV (50 μM) to block glutamatergic transmission. Under these circumstances, the observed effects in response to the activation of ORL-1 receptors should be a direct action on the recorded neurons. NOP (0.3 μM) was applied after the AP firing had been stable for 5~10 min. To avoid potential desensitization induced by repeated applications of the agonist, one slice was limited to only one application of NOP. Frequency of APs was calculated by Mini Analysis 6.0.1 (Synaptosoft Inc., Decatur, GA). The data recorded by Clampex 10.04 were re-saved as type “ABF1.8(integer)(*.abf)” in Clampfit 10.04 so that Mini Analysis 6.0.1. could read them. The parameters required by Mini Analysis 6.0.1 to count APs were measured by Clampfit 10.04. Resting membrane potentials (RMPs) and holding currents (HCs) at -60 mV were recorded from layer III pyramidal neurons in the extracellular solution supplemented with tetrodotoxin (TTX, 0.5 μM) to block synaptic transmission. I-V curves were obtained by using a ramp protocol from -100 mV to -40 mV at a speed of 0.002 mV/ms. We compared the I-V curves recorded before and during the application of NOP for 5–7 min when its effect was maximal.

2.5. Recordings of AP firing by perforated patches from layer V pyramidal neurons

Because we observed significant rundown of the AP firing frequency in many layer V pyramidal neurons using whole-cell recording configuration, we utilized perforated patch-clamp recording configuration to record APs from layer V pyramidal neurons as described previously (Deng and Lei, 2007; Deng et al., 2010). Recording pipettes were tip-filled with the above- mentioned K⁺-gluconate-containing intracellular solution and then back-filled

with the intracellular solution containing freshly prepared amphotericin B (200 µg/ml, Calbiochem, San Diego, CA). Patch pipettes had resistance of 6–8 MΩ when filled with the preceding solution. A 5-mV hyperpolarizing test pulse was applied every 3 s to monitor the changes of the series resistance and the process of perforation. For those cells showing abrupt reduction in series resistance during membrane perforation suggesting the simultaneous formation of whole-cell configuration, experiments were terminated immediately. Perforated-patch configuration was verified by examining the series resistance again at the end of the experiments. Data were included for analysis only from those cells showing <15% alteration of series resistance. Because layer V pyramidal neurons did not display spontaneous AP firing, we injected a constant positive current to bring the membrane potential to around –40 mV to induce stable and persistent AP firing.

2.6. Recordings of synaptic currents from layer III pyramidal neurons

Synaptic currents were recorded from layer III pyramidal neurons. The recording electrodes were filled with the following solution (in mM): 100 Cs⁺-gluconate, 0.6 EGTA, 2 MgCl₂, 8 NaCl, 33 HEPES, 2 ATPNa₂, 0.4 GTPNa, 7 phosphocreatine and 1 QX-314 (pH 7.4). The above-mentioned extracellular solution was supplemented with DNQX (10 µM) and *d,l*-APV (50 µM) to record IPSCs (holding potential: +30 mV) or bicuculline (10 µM) and CGP55845 (1 µM) to record EPSCs (holding potential: –60 mV). For the recordings of evoked IPSCs (eIPSCs) or evoked EPSCs (eEPSCs), a stimulation electrode was placed in layer III (~200 µm from the recorded cell). Spontaneous IPSCs (sIPSCs) or spontaneous EPSCs (sEPSCs) were recorded in the extracellular solution described above, respectively. Miniature IPSCs (mIPSCs) or miniature EPSCs (mEPSCs) were recorded in the above-mentioned extracellular solution containing TTX (0.5 µM). The amplitudes of eIPSCs or eEPSCs were measured by Clampfit 10.04, whereas the spontaneous and miniature synaptic currents were quantified by Mini Analysis 6.0.1.

2.7. Data analysis

Data are presented as the means ± S.E.M. NOP concentration-response curve was fit by using the Hill equation: $I = I_{\max} \times \{1/[1 + (EC_{50}/[\text{ligand}])^n]\}$, where $I_{\max}$ is the maximum response, EC_{50} is the concentration of ligand producing a half-maximal response, and n is the Hill coefficient. Student's paired or unpaired *t* test or analysis of variance (ANOVA) was used for statistical analysis as appropriate; *P* values are reported throughout the text and significance was set as *P* < 0.05. *N* number in the text represents the cells or slices examined.

3. Results

3.1. Expression of ORL-1 receptors in the EC

The EC can be divided into 6 layers (layer I–VI) (Mulders et al., 1997). Whereas high density of ORL-1 receptors has been detected in rat EC (Sim-Selley et al., 2003), the distribution of ORL-1 receptors in each layer of the EC has not been examined. We therefore examined the distribution of the immunoreactivity of ORL-1 receptors in the EC. As shown in Fig. 1, high density of immunoreactivity of ORL-1 receptors was detected in the soma of layer II to layer VI of the EC.

3.2. Activation of ORL-1 receptors depresses epileptic activity in the EC

Because the EC is an important structure involved in epilepsy, we studied the roles of NOP in epilepsy by bath perfusion of PTX or Mg^{2+} -free extracellular solution in entorhinal slices. As demonstrated previously (Kurada et al., 2014; Wang et al., 2013), stable epileptiform activity could be recorded from layer III of the EC in the horizontal slices after perfusion of PTX (100 μ M) for ~20 min. After recordings of stable basal epileptiform activity for 10 min, NOP (0.3 μ M) dissolved in the PTX-containing extracellular solution was applied to the slices for ~7 min. Application of NOP completely inhibited the epileptiform activity ($n=7$ slices, $P<0.001$, Fig. 2A and 2B). NOP-induced inhibitory effect on epileptiform activity was only partially reversible after wash in NOP-free external solution for >20 min (Fig. 2B). Because the above experiments were performed on horizontal slices containing the EC, subiculum and hippocampus, one would argue that the inhibitory effect of NOP on the epileptiform activities recorded from layer III of the EC could be an indirect effect of NOP on the hippocampus or other structures. Whereas this is unlikely the case because the seizure activity in the horizontal slices containing the above structures originates from the EC (Nagao et al., 1996), we cut the whole EC out from the horizontal slices (denoted as EC mini slices in Fig. 2C) under a microscope and recorded the epileptiform activity induced by PTX from layer III of the EC in the mini slices. Application of NOP (0.3 μ M) also completely inhibited the epileptiform activities in the EC mini slices ($n=10$ slices, $P<0.001$, Fig. 2C) indicating that the inhibitory effect of NOP is in the EC. Because there was no appreciable difference for the effect of NOP between the horizontal and the EC mini slices, we used the horizontal slices for the rest of experiments simply for the convenience of experiments. The EC_{50} value of NOP was measured to be 35 nM (Fig. 2D). We used 0.3 μ M NOP for the rest of experiments because this is a near saturating concentration.

We then tested the role of ORL-1 receptors in NOP-induced depression of epileptiform activity. We selected two ORL-1 receptor blockers, J113397 and JTC 801. Because pre-incubation for ~40 min is required for these inhibitors to achieve their maximal inhibition (Marti et al., 2003), we incubated the slices in the extracellular solution without PTX for 20 min and then placed the slices into the recording chamber and perfused with the extracellular solution containing PTX (100 μ M) and the ORL-1 blockers for additional 20 min. In this way, slices were bathed with the ORL-1 receptor blockers for 40 min and stable epileptiform activities were induced as well. Pretreatment of slices with and continuous bath application of J113397 (1 μ M) completely blocked NOP-induced depression of epileptiform activity ($125\pm16\%$ of control, $n=7$ slices, $P=0.18$, Fig. 2E). Similarly, pretreatment of slices with and continuous bath application of JTC 801 (10 μ M) completely blocked NOP-induced depression of seizure activity ($95\pm14\%$ of control, $n=12$ slices, $P=0.71$, Fig. 2F). These results demonstrate that NOP inhibits the epileptiform activity via activation of ORL-1 receptors. We further probed the roles of ORL-1 receptors by using two ORL-1 receptor agonists: NNC 63–0532 and $[Arg^{14},Lys^{15}]Nociceptin$ ($[Arg^{14},Lys^{15}]NOP$). Application of NNC 63–0532 (5 μ M) significantly inhibited the epileptiform activity ($64\pm6\%$ of control, $n=7$ slices, $P=0.001$, Fig. 2G) and so did $[Arg^{14},Lys^{15}]NOP$ (0.3 μ M) ($40\pm11\%$ of control, $n=10$, $P<0.001$, Fig. 2G), further confirming the involvement of ORL-1 receptors. We also tested whether the anti-seizure activity of NOP is relevant to other opioid receptors. Slices were pretreated with naloxone (10 μ M), a broad-spectrum opioid receptor antagonist, and

the same concentration of naloxone was continuously bath-applied. Under this condition, application of NOP (0.3 μ M) still completely inhibited the epileptiform activity ($n=5$ slices, $P<0.001$, Fig. 2H) suggesting that other opioid receptors are not involved in NOP-induced depression of epileptiform activity. We also tested the antiepileptic effects of NOP by using the Mg^{2+} -free seizure model (Deng and Lei, 2008; Zhang et al., 2014). Application of NOP significantly depressed the frequency of the epileptiform activity induced by deprivation of extracellular Mg^{2+} ($35\pm7\%$ of control, $n=11$ slices, $P<0.001$, Fig. 2I–J), further supporting that activation of ORL-1 receptors exerts antiepileptic activity in the EC.

3.3. Activation of ORL-1 receptors does not modulate synaptic transmission in the EC

The above results demonstrate that NOP exerts powerful inhibitory effects on epileptiform activity. We then studied the cellular and molecular mechanisms whereby NOP inhibits seizure activity. One possible mechanism whereby NOP depresses epileptiform activity is modulating glutamatergic and/or GABAergic transmission. Whereas the results that NOP inhibited the epileptiform activity induced by PTX, which has already inhibited GABA_A receptors, do not favor a role of GABA in NOP-mediated inhibition of seizure, we still examined the potential effects of NOP on GABAergic transmission onto layer III pyramidal neurons. We chose layer III pyramidal neurons because neurons in layer III of the EC are selectively lost in epileptic animals and patients (Du and Schwarcz, 1992; Du et al., 1993), indicating that they are especially important for epilepsy. Bath application of NOP failed to change significantly the amplitudes of eIPSCs ($94\pm4\%$ of control, $n=7$, $P=0.18$, Fig. 3A₁–A₂). Because neuromodulators modulate GABAergic transmission in distinct modes (Deng and Lei, 2008), we also examined the effects of NOP on sIPSCs and mIPSCs recorded from layer III pyramidal neurons. Similarly, bath application of NOP did not alter significantly the frequency ($103\pm7\%$ of control, $n=5$, $P=0.72$, Fig. 3B₁–B₂) and amplitude ($101\pm3\%$ of control, $n=5$, $P=0.73$, data not shown) of sIPSCs and those of mIPSCs (frequency: $100\pm3\%$ of control, $n=5$, $P=0.99$, amplitude: $100\pm5\%$ of control, $n=5$, $P=0.98$, Fig. 3C₁–C₂). These data together indicate that NOP does not modulate GABAergic transmission in the EC.

We then probed the effects of NOP on glutamatergic transmission onto layer III pyramidal neurons. Application of NOP did not alter significantly the amplitude of eEPSCs ($99\pm5\%$ of control, $n=6$, $P=0.88$, Fig. 3D₁–D₂), nor did it change the frequency ($101\pm5\%$ of control, $n=6$, $P=0.87$, Fig. 3E₁–E₂) and amplitude ($95\pm8\%$ of control, $n=6$, $P=0.49$, data not shown) of sEPSCs and those of mEPSCs (Frequency: $104\pm4\%$ of control, $n=5$, $P=0.33$, Amplitude: $94\pm10\%$ of control, $n=5$, $P=0.36$, Fig. 3F₁–F₂). These data together demonstrate that NOP-mediated inhibitory effects on the epileptiform activity are not mediated by modulating synaptic transmission in the EC.

3.4. Application of NOP inhibits neuronal excitability in the EC

Because a high density of ORL-1 receptors has been detected in the entorhinal neurons (Fig. 1), we then tested the hypothesis that NOP depresses epileptiform activity by inhibiting neuronal excitability in the EC. We examined the effects of NOP on AP firing frequency in the principal neurons of each layer. The extracellular solution contained DNQX (10 μ M) and *d,l*-APV (50 μ M) to block glutamatergic, and bicuculline (10 μ M) and CGP55845 (1 μ M) to block GABAergic transmission. Because the stellate neurons are the principal cell type in

layer II (Alonso and Klink, 1993), we studied the actions of ORL-1 receptor activation on the excitability of the stellate neurons. Stellate neurons were identified by their morphology, significant sag response and the persistent rhythmic subthreshold membrane potential oscillations (Alonso and Klink, 1993; Deng and Lei, 2007). Bath application of NOP completely inhibited the AP firing frequency of the stellate neurons (Control: 1.88 ± 0.19 Hz, NOP: 0 ± 0 Hz, $n=10$, $P<0.001$, Fig. 4A₁–A₃). The inhibitory effect of NOP was partially reversed after wash for ~28 min (0.71 ± 0.32 Hz, $34 \pm 16\%$ of control, $n=10$, $P=0.003$ vs. control baseline, Fig. 4A₂–A₃).

Because pyramidal neurons are the principal cell type in layer III of the EC (Dickson et al., 1997), we further examined the effects of NOP on the excitability of layer III pyramidal neurons. Bath application of NOP completely depressed the AP firing frequency of layer III pyramidal neurons (Control: 2.06 ± 0.14 Hz, NOP: 0 ± 0 Hz, $n=8$, $P<0.001$, Fig. 4B₁–B₃). The depressant effect of NOP was partially reversible after wash for 28 min (0.67 ± 0.18 Hz, $30 \pm 7\%$ of control, $n=8$, $P<0.001$ vs. control baseline, Fig. 4B₂–B₃). We also tried to use whole-cell recordings to examine the effects of NOP on the excitability of layer V pyramidal neurons. However, many layer V pyramidal neurons displayed significant rundown of the basal AP firing frequency in whole-cell recording configuration. We therefore used perforated patch recordings to record AP firing from layer V pyramidal neurons. In this recording configuration, application of NOP significantly inhibited the AP firing frequency of layer V pyramidal neurons (Control: 1.82 ± 0.11 Hz, NOP: 0 ± 0 Hz, $n=10$, $P<0.0001$, Fig. 4C₁–C₃). The NOP-induced depression of AP firing frequency was partially reversible after wash for 28 min (0.50 ± 0.13 Hz, $28 \pm 7\%$ of control, $n=10$, $P<0.001$ vs. control baseline, Fig. 4C₂–C₃). These results together demonstrate that activation of ORL-1 receptors inhibits the excitability of entorhinal neurons in each layer of the EC. For the remaining experiments, we focused on layer III pyramidal neurons to determine the underlying mechanisms because layer III pyramidal neurons are selectively lost in epileptic animals and patients (Du and Schwarcz, 1992; Du et al., 1993), highlighting their importance in epilepsy. We further assessed the inhibitory effects of NOP on neuronal excitability by constructing the current–AP firing frequency curve in layer III pyramidal neurons. A series of positive currents ranging from 30 to 210 pA at an interval of 30 pA with a duration of 600 ms were injected to layer III pyramidal neurons before (Fig. 4D₁) and during (Fig. 4D₂) the application of NOP. Application of NOP significantly reduced the number of APs ($n=12$, $P<0.001$, two-way ANOVA, Fig. 4D₃), further supporting the notion that NOP depresses neuronal excitability.

3.5. NOP generates membrane hyperpolarization, reduces neuronal input resistance and membrane constants but increases rheobase of layer III pyramidal neurons in the EC

We further probed the mechanisms underlying NOP-induced inhibition of neuronal excitability by recording from layer III pyramidal neurons. Depression of AP firing could be attributed to NOP-induced membrane hyperpolarization. We therefore recorded RMPs in current-clamp mode in the presence of TTX ($0.5 \mu\text{M}$). Application of NOP hyperpolarized layer III pyramidal neurons by 5.0 ± 0.7 mV (Control: -60.3 ± 0.8 mV, NOP: -65.4 ± 1.2 mV, $n=24$, $P<0.001$, Fig. 5A₁–A₂). Similarly, application of NOP induced a significant outward HC recorded at -60 mV (55.9 ± 6.4 pA, $n=10$, $P<0.001$, Fig. 5B₁–B₂). We further recorded in current clamp and injected a negative current (-50 pA for 500 ms) every 5 s to assess the

changes of input resistance. Under these conditions, application of NOP significantly reduced the input resistance (Control: $370 \pm 28 \text{ M}\Omega$, NOP: $211 \pm 22 \text{ M}\Omega$, $n=5$, $P=0.002$, Fig. 5C₁–C₂). The membrane time constant obtained by fitting a single exponential function to the voltage transient (130 ms from the baseline) induced by -100 pA current step (Fig. 5D₁–D₃) was significantly reduced (Control: $44.4 \pm 4.4 \text{ ms}$, NOP: $33.9 \pm 3.5 \text{ ms}$, $n=7$, $P<0.001$, Fig. 5D₃). We also measured the rheobase (defined as the minimum current inducing at least one spike during the step application) before and during the application of NOP by injecting a series of positive currents at an interval of 10 pA (Fig. 5E₁–E₃). NOP significantly increased the rheobase (Control: $41.7 \pm 9.8 \text{ pA}$, NOP: $91.7 \pm 10.1 \text{ pA}$, $n=6$, $P=0.004$, Fig. 5E₃).

3.6. K⁺ channels are involved in NOP-mediated hyperpolarization

We next determined the ionic mechanisms whereby NOP hyperpolarizes layer III pyramidal neurons. Because Ca^{2+} is involved in modulating numerous ion channels and NOP has been shown to inhibit Ca^{2+} channels (Borgland et al., 2001), especially the low-threshold T-type Ca^{2+} channels (Abdulla and Smith, 1997; Moran et al., 2000), we then tested whether extracellular Ca^{2+} is required for NOP-induced hyperpolarization by substituting extracellular Ca^{2+} with the same concentration of Mg^{2+} and adding EGTA (1 mM) to chelate potential tracing Ca^{2+} . In this situation, bath application of NOP still induced a comparable hyperpolarization ($-8.1 \pm 0.8 \text{ mV}$, $n=5$, $P<0.001$ vs. baseline, $P=0.07$ vs. control, Fig. 6A, 6F), suggesting that extracellular Ca^{2+} is not required for NOP-induced hyperpolarization. We further determined whether intracellular Ca^{2+} is required for NOP-induced hyperpolarization by including BAPTA (10 mM) in the recording pipettes. After the formation of whole-cell recording configuration, we waited for $\sim 20 \text{ min}$ to allow the perfusion of BAPTA into the cells. Under these circumstances, application of NOP induced a statistically indistinguishable hyperpolarization ($-6.2 \pm 0.4 \text{ mV}$, $n=5$, $P<0.001$ vs. baseline, $P=0.47$ vs. control, Fig. 6B and 6F). These results demonstrate that NOP-induced hyperpolarization is Ca^{2+} -independent.

We then measured the reversal potential of the current generated by NOP to further determine the involved ionic mechanisms. The extracellular solution contained TTX ($1 \mu\text{M}$) and 0 Ca^{2+} to exclude the contaminations of voltage-gated Na^+ and Ca^{2+} channels and the above K^+ -gluconate-containing intracellular solution was used. We used a ramp protocol (from -100 mV to $\sim -40 \text{ mV}$) to measure the I-V curve before and during the application of NOP. Under these circumstances, the I-V curve was almost linear with a reversal potential of $-87.4 \pm 3.4 \text{ mV}$ ($n=5$, Fig. 6C–D), close to the calculated K^+ reversal potential (-85.5 mV) when the extracellular K^+ concentration was 3.5 mM . When the extracellular K^+ concentration was raised to 10 mM , the NOP-induced current showed a reversal potential of $-58.9 \pm 3.1 \text{ mV}$ ($n=7$), which is close to the calculated K^+ reversal potential (-58.7 mV) at this K^+ concentration. Moreover, replacement of extracellular K^+ with the same concentration of Na^+ to deplete extracellular K^+ to increase the driving force for K^+ significantly increased NOP-mediated hyperpolarization ($-16.9 \pm 1.9 \text{ mV}$, $n=6$, $P<0.001$ vs. control, Fig. 6E–F). These data together demonstrate that activation of ORL-1 receptors opens a K^+ conductance to generate hyperpolarization.

We further determined the properties of the K^+ channels involved in NOP-mediated hyperpolarization. NOP has been reported to activate the inward rectifier K^+ (Kir) channels in a variety of neurons (Allen et al., 1999; Chee et al., 2011; Connor et al., 1996; Emmerson and Miller, 1999; Luo et al., 2001; Meis and Pape, 1998; Pan et al., 2000; Parsons and Hirasawa, 2011; Vaughan et al., 1997). In CA1 (Madamba et al., 1999) and CA3 (Tallent et al., 2001) pyramidal neurons of the hippocampus, NOP increases the voltage-dependent M-currents. Because the activation threshold of M-channels is close to or positive to RMPs and M-channels are outwardly rectified, one possibility is that NOP activated both Kir and M-channels and the neutralization of these two channels resulted in the almost linear I-V curve of the net currents. We therefore included the selective M-channel blocker, linopirdine (30 μ M), in the above-mentioned extracellular solution and constructed the I-V curve. If NOP activates both Kir and M-channels, blockade of M-channels would unveil the inward rectification property of Kir channels. However, the I-V curve was still linear in the presence of linopirdine (Fig. 6G–H), suggesting that M-channels contribute little to NOP-induced hyperpolarization in the EC.

We then tested the roles of Kir channels in NOP-mediated hyperpolarization in layer III pyramidal neurons. Because micromolar concentration of Ba^{2+} (100–300 μ M) has been shown to block Kir channels by at least 80% (Carr and Surmeier, 2007; Lacey et al., 1988; Slugg et al., 1999), we included 300 μ M Ba^{2+} in the extracellular solution to inhibit Kir channels. Bath application of 300 μ M Ba^{2+} induced a significant depolarization of layer III pyramidal neurons (7.9 ± 1.2 mV, $n=17$, $P<0.001$, Fig. 7A). To exclude the influence of the depolarization evoked by Ba^{2+} , we inject a negative current to bring the membrane potential back to the initial level. In the continuous presence of Ba^{2+} , application of NOP still induced a significant hyperpolarization (-2.9 ± 0.8 mV, $n=17$, $P=0.001$ vs. baseline, Fig. 7A, 7G). The hyperpolarization induced by NOP in the absence and presence of Ba^{2+} was slightly but significantly different ($P=0.049$, Fig. 7G). Because Ba^{2+} at this concentration used is very specific for Kir channels, this result suggests that Kir channels are related to NOP-mediated hyperpolarization of layer III pyramidal neurons.

We further characterized the isoforms of the Kir channels involved in NOP-induced hyperpolarization. There are seven Kir channel subfamilies that can be classified into four functional groups: i) Kir2 subfamily including Kir2.1, Kir2.2, Kir2.3 and Kir2.4 form the classical Kir channels and are constitutively active; ii) Kir3 subfamily comprising Kir3.1, Kir3.2, Kir3.3 and Kir3.4 constitute the G protein-gated GIRK channels; iii) Kir6 subfamily encompassing Kir6.1 and Kir6.2 form the ATP-sensitive K^+ (K_{ATP}) channels; iv) K^+ transport channels include Kir1.1, Kir4.1, Kir4.2 and Kir7.1 (Hibino et al., 2010). Tertiapin-Q is an antagonist of GIRK ($K_i=13.3$ nM) and Kir1.1 ($K_i=1.3$ nM) (Jin et al., 1999; Jin and Lu, 1999). We therefore used tertiapin-Q and tested the roles of GIRK and Kir1.1 channels in NOP-induced hyperpolarization. Application of tertiapin-Q (250 nM) did not significantly affect NOP-induced hyperpolarization (-4.3 ± 0.9 mV, $n=12$, $P=0.51$ vs. control, Fig. 7B, 7G). Similarly, NOP-mediated hyperpolarization was not significantly altered by bath application of SCH23390 (20 μ M, -4.6 ± 0.8 mV, $n=14$, $P=0.7$ vs. control, Fig. 7C, 7G), another GIRK channel blocker (Chee et al., 2011; Kuzhikandathil and Oxford, 2002), and ML133 (30 μ M, -4.2 ± 0.5 mV, $n=6$, $P=0.57$ vs. control, Fig. 7D, 7G), a specific antagonist for Kir2 subfamily (Ford and Baccei, 2016; Huang et al., 2018; Kim et al., 2015; Sonkusare

et al., 2016; Wang et al., 2011). Because our intracellular solution contained 2 mM ATP which completely blocked K_{ATP} channels, it is unlikely that NOP hyperpolarizes entorhinal neurons by activating K_{ATP} channels. Consistent with our speculation, application of the selective K_{ATP} channel blocker, glibenclamide (10 μ M) failed to alter significantly NOP-mediated hyperpolarization (-4.3 ± 1.1 mV, $n=10$, $P=0.54$ vs. control, Fig. 7E, 7G). Because NOP has been reported to activate M-channels in CA1 (Madamba et al., 1999) and CA3 (Tallent et al., 2001) pyramidal neurons, we also tested the roles of M-channels. Application of the selective M-channel blocker, linopirdine (30 μ M) failed to change significantly NOP-mediated hyperpolarization (-5.0 ± 0.4 mV, $n=8$, $P=0.98$ vs. control, Fig. 7F–G), suggesting that M-channels are not involved in NOP-mediated hyperpolarization in the EC.

3.7. Roles of K^+ and cation channels revealed by recordings of membrane currents from layer III pyramidal neurons

The above-mentioned experiments were performed by measuring the RMPs of layer III pyramidal neurons to assess the effects of NOP on membrane potentials. The RMPs of the layer III pyramidal neurons are usually between -60 to -70 mV. If Kir channels are involved, the effects of NOP on Kir channels may have not been observed at the RMPs because of the voltage dependence of the Kir channels, i.e., more extent of opening at negative potentials than at positive potentials. We therefore characterized the effects of NOP on the K^+ channel currents recorded from layer III pyramidal neurons at different voltages. The extracellular solution contained TTX (0.5 μ M) to block voltage-gated Na^+ currents and extracellular Ca^{2+} was replaced with the same concentration of Mg^{2+} to exclude the contamination of voltage-gated Ca^{2+} channels because the effect of NOP was Ca^{2+} -independent (Fig. 6). Cells were held at -60 mV and stepped from -140 mV to -40 mV for 400 ms at a voltage interval of 10 mV in every 10 s. Steady-state currents were measured within 5 ms before the end of the step voltage protocols. Under these circumstances, the currents recorded before and during the application of NOP showed inward rectification (Fig. 8A₁–A₂, $n=6$), suggesting that Kir channels are expressed in layer III pyramidal neurons. Subtraction of the I–V curve in control condition from that during the application of NOP demonstrated a slightly outward I–V curve induced by NOP (Fig. 8A₃, $n=6$).

We previously demonstrated that a Na^+ -permeant cation channel contributes to the maintenance of RMPs in subicular neurons (Hu et al., 2017). Here, we also tested whether Na^+ -permeant cation channels contribute to the maintenance of RMPs in layer III pyramidal neurons of the EC. In the continuous presence of TTX to block voltage-gated Na^+ channels, replacing extracellular NaCl with the same concentration of NMDG-Cl to minimize Na^+ influx induced an outward current at -60 mV (37.0 ± 10.4 pA, $n=5$, $P=0.024$, data not shown), suggesting that Na^+ influx via cation channels contributes to the maintenance of RMPs in entorhinal layer III pyramidal neurons. If so, inhibition of cation channels could be a potential mechanism underlying NOP-induced hyperpolarization. We thus replaced extracellular NaCl with the same concentration of NMDG-Cl to test the roles of Na^+ influx in NOP-mediated augmentation of membrane currents (Fig. 8B₁–B₃). Substitution of extracellular NaCl with NMDG-Cl did not significantly alter NOP-induced facilitation of membrane currents ($n=7$, $F_{(1,11)}=1.99$, $P=0.19$, Fig. 8B₃), suggesting that activation of K^+ channels is a predominant mechanism responsible for NOP-induced hyperpolarization.

We then tested the effects of Ba^{2+} on NOP-mediated alteration of membrane currents. Application of Ba^{2+} (300 μ M) indeed inhibited a current showing inward rectification (Fig. 9A₁–A₃, n=9), suggesting that Ba^{2+} at this concentration inhibited Kir channels. In the presence of Ba^{2+} , NOP-elicited currents were persistently outward (Fig. 9B₁–B₃, n=9), suggesting that the contribution of cation channels in NOP-mediated hyperpolarization was revealed after Ba^{2+} -mediated inhibition of Kir channels. However, the cation channels inhibited by NOP were unlikely to be the hyperpolarization-activated cyclic nucleotide-gated (Ih) channels because inclusion of the selective Ih channel blocker, ZD7288 (50 μ M, n=9), in the recording pipettes to inhibit Ih channels did not significantly alter NOP-mediated currents ($F_{(1,16)}=0.08$, $P=0.78$, Fig. 9B₃).

We further examined whether the persistent outward currents induced by NOP in the presence of Ba^{2+} was due to NOP-mediated inhibition of cation channels by replacing extracellular NaCl with NMDG-Cl (Fig. 9C₁–C₃). In the continuous presence of Ba^{2+} , substitution of extracellular NaCl with NMDG-Cl significantly reduced NOP-mediated facilitation of membrane currents (n=6, $F_{(1,13)}=6.87$, $P=0.02$, Fig. 9C₃), revealing the contribution of cation channel inhibition in NOP-induced hyperpolarization.

We further identified the roles of Kir subfamilies in NOP-mediated hyperpolarization by using their selective blockers. Application of tertiapin-Q (250 nM) significantly inhibited Kir channel currents only at potentials negative to –120 mV (n=7, Fig. 10A₁–A₂) and the net currents sensitive to tertiapin-Q displayed an inward rectification (Fig. 10A₂). However, application of tertiapin-Q did not significantly alter NOP-induced currents ($F_{(1,11)}=0.003$, $P=0.96$, Fig. 10A₃–A₄). Furthermore, application of SCH23390 (20 μ M) did not significantly alter Kir currents ($F_{(1,20)}=0.25$, n=11, $P=0.62$, Fig. 10B₁–B₂). One plausible explanation for this result is that the SCH23390-sensitive Kir channels are not constitutively active. However, application of NOP in the presence of SCH23390 significantly increased the membrane currents only at potentials positive to –80 mV and no significant currents were elicited by NOP at potentials negative to –80 mV (Fig. 10B₃–B₄). Moreover, application of ML133 (30 μ M) significantly inhibited Kir channel currents at potentials negative to –90 mV (n=6, Fig. 10C₁–C₂) and application of NOP in the presence of ML133 increased membrane currents only at potentials positive to –70 mV (Fig. 10C₃–C₄).

4. Discussion

Our results demonstrate that a high density of ORL-1 receptors is expressed in the soma of the neurons in layer II to layer VI of the medial EC of rats. Activation of the ORL-1 receptors by NOP concentration-dependently induces robust depression of the epileptiform activity induced by bath application of PTX or by deprivation of extracellular Mg^{2+} . NOP exerts no effects on either glutamatergic or GABAergic transmission in the EC, whereas it remarkably inhibits the excitability of entorhinal neurons by hyperpolarizing these neurons. NOP-mediated hyperpolarization is Ca^{2+} -independent but related to the activation of a K^{+} conductance and the inhibition of a cation channel. Our results provide a novel cellular and molecular mechanism whereby activation of ORL-1 receptors inhibits epilepsy in the EC.

NOP decreases glutamatergic transmission in the hippocampus (Tallent et al., 2001; Yu and Xie, 1998), the amygdala (Kallupi et al., 2014; Meis and Pape, 2001), the spinal cord (Ahmadi et al., 2001a; Ahmadi et al., 2001b; Faber et al., 1996; Liebel et al., 1997; Luo et al., 2002; Zeilhofer et al., 2000), the hypothalamus (Gompf et al., 2005) and the thalamus (Meis et al., 2002). NOP also inhibits GABAergic transmission in the nucleus ambiguus (Venkatesan et al., 2002), the ventral tegmental area (Zheng et al., 2002), the suprachiasmatic nucleus (Gompf et al., 2005) and the amygdala (Meis and Pape, 2001; Roberto and Siggins, 2006). Furthermore, NOP inhibits the long-term potentiation (LTP) (Bongsebandhu-phubhakdi and Manabe, 2007; Manabe et al., 1998; Yu and Xie, 1998). However, we failed to observe any effects of NOP on either glutamatergic or GABAergic transmission in the EC. There are several explanations for the discrepancy of our results. First, we only examined the effects of NOP on synaptic transmission onto layer III pyramidal neurons which receive inputs outside the EC (Canto et al., 2012) and from the neurons in the deep layers (layer V/VI) (Canto and Witter, 2012). It is possible that ORL-1 receptors are not expressed at the terminals innervating the layer III pyramidal neurons. This possibility is supported by our immunostaining showing that ORL-1 receptors are densely expressed in the soma with less expression in the neurites because layer I of the EC where outside axonal inputs converge into the EC displayed less immunostaining intensity (Fig. 1). If NOP decreases synaptic transmission by inhibiting Ca^{2+} channels at the axonal terminals, deficiency of ORL-1 receptors at the axonal terminals could explain the incompetence of NOP-mediated modulation of synaptic transmission. Second, our results demonstrate that the major effect of NOP is to hyperpolarize neurons and inhibit AP firing. However, if the neurons do not show spontaneous AP firing or are silent in physiological condition, the contribution of NOP to synaptic transmission is likely trivial. Layer III pyramidal neurons are innervated by the neurons in the deep layers (layer V/VI) and these neurons are silent in physiological condition (Canto and Witter, 2012). However, the inability of NOP to modulate synaptic transmission in physiological condition does not preclude its roles in pathological conditions such as epilepsy. Epilepsy is an increased excitation of neuronal network activity in which AP firing likely plays an important role. NOP-mediated inhibition of AP firing could thus exert inhibition on neuronal network activities. Third, we used Cs^{+} -containing intracellular solution to probe the potential roles of NOP in synaptic transmission. Intracellular Cs^{+} could block the postsynaptic K^{+} channels and NOP-mediated activation of K^{+} channels was not exhibited in this situation. As demonstrated previously, many effects of NOP on synaptic transmission (Farhang et al., 2010; Meis et al., 2002; Yu and Xie, 1998) and LTP (Bongsebandhu-phubhakdi and Manabe, 2007; Yu and Xie, 1998) are mediated postsynaptically. Thus, more thorough studies are required to determine the effects of NOP on synaptic transmission in the EC.

Our results indicate that facilitation of a K^{+} conductance is involved in the hyperpolarization of entorhinal neurons in response to ORL-1 receptor activation based on the following lines of evidence. First, bath application of NOP decreases the input resistance and the membrane time constant of layer III entorhinal neurons suggesting that activation of ORL-1 receptors increases a membrane conductance. Second, the reversal potential of the I-V curve is close to the K^{+} reversal potential and elevation of extracellular K^{+} concentration shifts the reversal potential to the right. Third, depletion of extracellular K^{+} concentration increases the extent

of NOP-induced hyperpolarization, suggesting that increasing the driving force of K^+ facilitates NOP-mediated hyperpolarization. NOP has been shown to activate Kir channels in neurons from a variety of structures including the suprachiasmatic nucleus (Allen et al., 1999), arcuate nucleus (Emmerson and Miller, 1999; Farhang et al., 2010; Wagner et al., 1998), ventromedial nucleus of the hypothalamus (Chee et al., 2011; Emmerson and Miller, 1999), locus coeruleus (Connor et al., 1996), substantia gelatinosa (Luo et al., 2001), amygdala (Meis and Pape, 1998), brainstem (Pan et al., 2000), melanin-concentrating hormone neurons (Parsons and Hirasawa, 2011), and periaqueductal gray neurons (Liao et al., 2011; Vaughan et al., 1997). However, the roles of Kir channels in NOP-induced hyperpolarization in the EC are subtle. The I-V curve of the net currents induced by NOP was linear without showing inward rectification in normal condition (Fig. 6D and Fig. 8A₃) or when the extracellular NaCl was replaced by NMDG-Cl to eliminate the contamination of cation channels (Fig. 8B₃). Therefore, the I-V curve of NOP-induced net currents is inconsistent with Kir channels. Furthermore, bath application of Ba^{2+} at a concentration (300 μ M) which is effective for Kir channels, only slightly reduced NOP-mediated hyperpolarization assessed by recording RMPs (Fig. 7G). However, application of Ba^{2+} at 300 μ M indeed inhibited Kir channel currents (Fig. 9A₃) and NOP-mediated inhibition of cation channels was revealed after inhibition of Kir channels by application of Ba^{2+} (Fig. 9B₁-B₃, Fig. 9C₁-C₃). These results together suggest that Kir channels play a permissive role in NOP-mediated hyperpolarization.

Whereas there are many subfamilies of Kir channels, few blockers selective for these channels are available. Tertiapin (Jin et al., 1999; Jin and Lu, 1999) and SCH23390 (Kuzhikandathil and Oxford, 2002) are selective blockers for GIRK channels, and ML133 is a specific antagonist for Kir2 subfamily (Ford and Baccei, 2016; Huang et al., 2018; Kim et al., 2015; Sonkusare et al., 2016; Wang et al., 2011). Whereas application of 300 μ M Ba^{2+} slightly and significantly attenuated NOP-mediated hyperpolarization, application of tertiapin-Q, SCH23390 and ML133 had no significant effects on NOP-induced hyperpolarization recorded at the RMPs (Fig. 7G). Because Kir channels are voltage-dependent with more opening at negative potentials and less opening at the potentials close to or positive than the RMPs, one possibility for the inability of these Kir channel blockers is that they exert no actions at the voltage range of RMPs. We therefore also examined the effects of these blockers on NOP-mediated facilitation of membrane currents recorded at different voltages. Our results demonstrate that tertiapin-Q-mediated depression of Kir channels is voltage-dependent, i.e., only significantly at voltages negative to -120 mV (Fig. 10A₁). One possible explanation for this result is that the isoforms inhibited by tertiapin-Q have little expression in the EC and thus contribute trivially to the Kir currents recorded from the layer III pyramidal neurons. Consistent with this conjecture, NOP-mediated facilitation of membrane currents was not significantly altered in the presence of tertiapin-Q (Fig. 10A₄). Furthermore, application of SCH23390 did not significantly alter Kir channel currents (Fig. 10B₁-B₂). One possibility for the ineffectiveness of SCH23390 is that GIRK channels may not be constitutively active, leaving no currents to be inhibited by SCH23390. Application of NOP significantly increased the membrane currents at potentials positive to -80 mV, but had no significant actions on the membrane currents at potentials negative to -80 mV (Fig. 10B₃-B₄). These results suggest that the SCH23390-sensitive Kir channels are

involved in NOP-induced facilitation of Kir channels. Because Kir channel currents are larger at negative potentials than at positive potentials, the ineffectiveness of SCH23390 on NOP-induced facilitation of membrane currents at positive potentials may be due to the effect of NOP on cation channels (see below). ML133 is a blocker selective for Kir2 subfamily which is constitutively active (Hibino et al., 2010). Consistent with this scenery, application of ML133 significantly inhibited the currents at potentials negative to -90 mV (Fig. 10C₁), suggesting that the ML133-sensitive channels are constitutively active. Similarly, application of NOP in the presence of ML133 facilitated the membrane currents only at potentials positive to -70 mV (Fig. 10C₃-C₄). One explanation for these results is that ML133 inhibited Kir channels at negative potentials, revealing the NOP-mediated action on cation channels at positive potentials.

In addition to the Kir channels, activation of ORL-1 receptors has also been reported to activate Ca^{2+} -dependent K^+ channels (Chin et al., 2002; Deak et al., 2013; Klukovits et al., 2010; Shirasaki et al., 2001). However, our results demonstrate that it is unlikely that activation of ORL-1 receptors hyperpolarizes entorhinal neurons by activating the Ca^{2+} -dependent K^+ channels because depletion of extracellular Ca^{2+} or intracellular application of BAPTA failed to alter significantly NOP-mediated hyperpolarization. Furthermore, activation of ORL-1 receptors also increases the voltage-sensitive M-channels in CA1 (Madamba et al., 1999) and CA3 (Tallent et al., 2001) pyramidal neurons of the hippocampus. However, the K^+ channels activate by NOP appear not to be M-channels based on the following lines of evidence. First, application of the selective M-channel blocker, linopirdine, has no effects on NOP-mediated hyperpolarization (Fig. 7G). Second, M-channels are voltage-dependent and the activation threshold of M-channels is close to or positive to RMPs, whereas NOP-facilitated K^+ currents showed an almost linear I-V curve (Fig. 8A₃), i.e., opening at membrane potential negative to the RMPs. Third, because M-channels are outwardly rectified and if NOP activates M-channels, blocking the outwardly rectified component in the presence of linopirdine should reveal an inward I-V curve. However, the I-V curve of NOP-induced net currents obtained in the presence of linopirdine was still linear. We therefore conclude that the effects of NOP on M-channels are trivial in layer III pyramidal neurons of the EC.

Our results also indicate a contribution of cation channel inhibition to NOP-mediated hyperpolarization in layer III pyramidal neurons. When Kir channels were inhibited by Ba^{2+} , NOP-mediated inhibition of cation channels was revealed (Fig. 9B₃) and replacement of extracellular NaCl with NMDG-Cl in the continuous presence of Ba^{2+} remarkably reduced NOP-induced persistent outward currents (Fig. 9C₃). Consistent with the effects of Ba^{2+} , NOP-induced inhibition of cation channels was uncovered when Kir channels were inhibited by application of SCH23390 (Fig. 10B₄) and ML133 (Fig. 10C₄). While the properties of cation channels inhibited by the $\text{G}_{i/o}$ protein-coupled ORL-1 receptors in the EC are still awaiting for identification, $\text{G}_{i/o}$ protein-induced inhibition of cation channels including TRPM1 (Shen et al., 2012) and TRPM3 (Quallo et al., 2017) has been reported.

NOP and ORL-1 receptors are involved in epilepsy although there are conflict results as to whether they are antiepileptic or pro-epileptic. The following lines of evidence support that NOP depresses epilepsy; application of NOP to hippocampal slices inhibits spontaneous

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epileptiform activity recorded in CA3 region induced by removal of extracellular Mg^{2+} or by raising extracellular K^+ concentration (Tallent et al., 2001); intracerebroventricular injection of NOP inhibits kindling- induced epileptogenesis (Gutierrez et al., 2001) and epilepsy in a variety of animal models of experimental epilepsy (Rubaj et al., 2002) including the penicillin-induced seizures (Feng et al., 2004). However, NOP may also play a pro-epileptic role. For example, NOP facilitates ictal activity in rats with partial seizures, whereas in animals with generalized seizures, it induces inhibitory effects on afterdischarge and enhances the postictal period (Carmona-Aparicio et al., 2007); NOP knock-out mice are less susceptible to kainate seizures (Binaschi et al., 2003; Bregola et al., 2002b); Moreover, epilepsy is associated with increased NOP release (Aparicio et al., 2004) but decreased expression of ORL-1 receptors (Bregola et al., 2002a; Rocha et al., 2009). Whereas NOP has been demonstrated unequivocally to be involved in epilepsy, the underlying cellular and molecular mechanisms are elusive. While NOP increases M-channel currents in CA3 pyramidal neurons of the hippocampus, application of M-channel blockers has no effects on NOP-induced depression of epileptiform activity in this region (Tallent et al., 2001), suggesting that mechanisms other than inhibition of M-channels are involved. Because the EC is an indispensable brain region involved in the generation and propagation of epileptic activity (Avoli et al., 2002; Spencer and Spencer, 1994), studies of the effects of NOP on the EC are likely to be important as to determine the mechanisms of NOP in epilepsy. One potential mechanism whereby NOP depresses the epileptiform activity is to modulate synaptic transmission in the EC. In the present work, we initially examined the actions of NOP on both glutamatergic and GABAergic transmission and our results demonstrate that NOP has no effects on either glutamatergic or GABAergic transmission. Because we have also detected a high density of ORL-1 receptors in the soma of neurons in layer II to layer VI of the EC, we examined the effects of NOP on neuronal excitability in the EC. Our results demonstrate that NOP robustly inhibits neuronal excitability in the EC. We therefore conclude that NOP depresses epileptiform activity majorly by inhibiting neuronal excitability in the EC. Because we have further demonstrated that NOP decreases neuronal excitability by activating a K^+ conductance possibly belonging to the Kir family and inhibiting a cationic conductance, it is reasonable to speculate that NOP depresses epileptiform activity likely by interacting with these channels. Consistent with our results, epilepsy is associated with Kir2.1, Kir4.1 and Kir6.2 (Villa and Combi, 2016) and GIRK2 (Signorini et al., 1997) channels. The polymorphism and dysfunction of KCNJ10 which encodes Kir4.1 affect seizure susceptibility (Buono et al., 2004; Dai and Wasay, 2007; Ferraro et al., 2004; Sicca et al., 2016); A novel mutation in the KCNJ2 which encodes Kir2.1 in monozygotic twins displaying autism-epilepsy phenotype suggests a role of Kir2.1 in epilepsy (Ambrosini et al., 2014); GIRK2 knockout mice showed spontaneous seizures (Signorini et al., 1997), whereas GIRK activators depresses epilepsy (Kaufmann et al., 2013; Mazarati et al., 2006). Because our results showed that the contribution of cation channels in NOP-mediated depression of neuronal excitability in the EC could only be revealed after inhibition of Kir channels, it is difficult to assess the roles of cation channels in NOP-induced depression of epileptiform activity. Whereas the identities of the cation channels inhibited by ORL-1 receptors in the EC have not been characterized, TRPM1 (Shen et al., 2012) and TRPM3 (Quallo et al., 2017) are inhibited by $G_{i/o}$ coupled-receptors. However,

the roles of cation channels in NOP-mediated depression of neuronal excitability and epilepsy still need further investigation.

5. Conclusion

Our results demonstrate that high densities of ORL-1 receptors are expressed in layer II to layer VI of the EC and activation of ORL-1 receptors robustly inhibited the epileptiform activity in entorhinal slices induced by PTX or deprivation of Mg^{2+} from the extracellular solution. NOP-mediated depression of epileptiform activity was not mediated by alteration of synaptic transmission, but due to NOP-induced depression of neuronal excitability in the EC. NOP hyperpolarized entorhinal neurons via activation of K^+ channels and inhibition of cation channels. Our results provide a novel cellular and molecular mechanism by which activation of ORL-1 receptors depresses epilepsy.

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Highlights

- Activation of ORL-1 receptors by nociceptin inhibits epileptiform activity in the entorhinal cortex
- Nociceptin-mediated depression of epileptiform activity is not mediated by modulation of synaptic transmission
- Activation of ORL-1 receptors inhibits epileptiform activity by decreasing neuronal excitability
- Nociceptin decreases neuronal excitability in the entorhinal cortex by activation of K⁺ channels and inhibition of cation channels

Fig. 1. High immunoreactive densities of ORL-1 receptors are expressed in the soma of layer II to layer VI of the medial EC. The right panel is the enlargement of the selected region in the left panel.

Fig. 2.

Activation of ORL-1 receptors inhibits epileptiform activity induced by PTX or deprivation of extracellular Mg^{2+} . **A**, Epileptiform activity induced by PTX before, during and after the application of NOP. **B**, Pooled time course of the frequency of PTX-induced epileptiform activity in response to NOP recorded from layer III in whole horizontal slices. **C**, Bath application of NOP inhibited the frequency of epileptiform activity recorded from layer III of the EC in “mini slices” for which the hippocampus and other cortices were cut away. **D**, Concentration-response curve of NOP. Numbers in the parentheses were the numbers of slices recorded. **E**, Pretreatment of slices with and continuous bath application of the selective ORL-1 receptor antagonist, J113397 (1 μM), blocked the depression of epileptiform activity induced by NOP. **F**, Pretreatment of slices with and continuous bath application of another selective ORL-1 receptor antagonist, JTC 801 (10 μM), blocked the depression of epileptiform activity induced by NOP. **G**, Application of two ORL-1 receptor agonists, NNC 63-0532 (5 μM) or $[Arg^{14},Lys^{15}]NOP$ (0.3 μM), inhibited PTX-induced epileptiform activity. **H**, Pretreatment of slices with and continuous bath application of naloxone (10 μM), a broad-spectrum opioid receptor antagonist, did not significantly alter NOP-induced depression of epileptiform activity. **I**, Epileptiform activity induced by deprivation of extracellular Mg^{2+} before (*left*), during (*middle*) and after (*right*) the

application of NOP. **J**, Summary data showing NOP-induced depression of epileptiform activity induced by deprivation of Mg^{2+} .

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Fig. 3.

Activation of ORL-1 receptors does not modulate GABAergic or glutamatergic transmission. **A₁-A₂**, Bath application of NOP failed to alter GABA_A receptor-mediated evoked IPSCs (eIPSCs) recorded from layer III pyramidal neurons. **A₁**, Averaged trace of eIPSCs recorded before and during the application of NOP. **A₂**, Pooled time course of the amplitude of eIPSCs in response to bath application of NOP. **B₁-B₂**, Bath application of NOP did not alter GABA_A receptor-mediated spontaneous IPSCs (sIPSCs) recorded from layer III pyramidal neurons. **B₁**, Sample traces of sIPSCs recorded before and during the application of NOP from a layer III pyramidal neuron. **B₂**, Pooled time course of the frequency of sIPSCs in response to bath application of NOP. **C₁-C₂**, Bath application of NOP did not alter GABA_A receptor-mediated miniature IPSCs (mIPSCs) recorded in the presence of TTX from layer III pyramidal neurons. **C₁**, Sample traces of mIPSCs recorded before and during the application of NOP from a layer III pyramidal neuron. **C₂**, Pooled time course of the frequency of mIPSCs in response to bath application of NOP. **D₁-D₂**, Bath application of NOP failed to alter AMPA receptor-mediated evoked EPSCs (eEPSCs) recorded from layer III pyramidal neurons. **D₁**, Averaged eEPSCs recorded from a layer III pyramidal neuron before and during the application of NOP. Note that application of NBQX

(10 μ M) at the end of the experiments blocked EPSCs confirming they were mediated by AMPA receptors. **D₂**, Pooled time course of the amplitude of eEPSCs in response to bath application of NOP. **E₁-E₂**, Bath application of NOP did not alter AMPA receptor-mediated spontaneous EPSCs (sEPSCs) recorded from layer III pyramidal neurons. **E₁**, Sample traces of sEPSCs recorded before and during the application of NOP from a layer III pyramidal neuron. Application of NBQX (10 μ M) at the end of experiments completely blocked the recorded events. **E₂**, Pooled time course of the frequency of sEPSCs in response to bath application of NOP. **F₁-F₂**, Bath application of NOP did not alter AMPA receptor-mediated miniature EPSCs (mEPSCs) recorded in the presence of TTX from layer III pyramidal neurons. **F₁**, Sample traces of mEPSCs recorded before and during the application of NOP from a layer III pyramidal neuron. **F₂**, Pooled time course of the frequency of mEPSCs in response to bath application of NOP.

Fig. 4.

Activation of ORL-1 receptors depresses the AP firing frequency in entorhinal neurons. **A₁-A₃**, Application of NOP inhibited the AP firing frequency of layer II stellate neurons. **A₁**, Voltage responses (*upper panel*) generated by current injection at an interval of 0.1 nA (*lower panel*) recorded from a stellate neuron in layer II. The RMP of this neuron was shown on the left. **A₂**, AP firing recorded before, during and after application of NOP from the stellate neuron. The membrane potential elevated by continuous injection of a positive current to induce stable firing was shown on the left. **A₃**, Pooled time course of AP firing frequency from 10 stellate neurons in response to NOP. **B₁-B₃**, Application of NOP inhibited the AP firing frequency of layer III pyramidal neurons. **B₁**, Voltage responses (*upper panel*) generated by current injection at an interval of 0.1 nA (*lower panel*) recorded from a pyramidal neuron in layer III. The RMP of this neuron was shown on the left. **B₂**, APs

recorded for the layer III pyramidal neuron before, during and after the application of NOP. The membrane potential elevated by continuous injection of a positive current to induce stable firing was shown on the left. **B₃**, Pooled time course of AP firing frequency from 8 layer III pyramidal neurons before, during and after the application of NOP. **C₁-C₃**, Application of NOP significantly inhibited the AP firing frequency of layer V pyramidal neurons recorded by perforated patches. **C₁**, Voltage responses (*upper panel*) generated by current injection at an interval of 0.1 nA (*lower panel*) recorded from a pyramidal neuron in layer V. The RMP of this neuron was shown on the left. **C₂**, APs recorded for the layer V pyramidal neuron before, during and after the application of NOP. The membrane potential elevated by continuous injection of a positive current to induce stable firing was shown on the left. **C₃**, Pooled time course of AP firing frequency from 10 layer V pyramidal neurons in response to NOP. **D₁-D₃**, NOP depressed the excitability of layer III pyramidal neurons assessed by injecting a series of positive currents. **D₁**, Overlays of voltage responses (*upper*) evoked by injection of currents from 0 to 210 pA at an interval of 30 pA with a duration of 600 ms (*lower*) in a layer III pyramidal neuron in control condition. The RMP of this neuron was -60 mV. Note that injection of current at +30 pA began to evoke AP firing in this condition. **D₂**, Overlays of voltage responses (*upper*) evoked by injection of currents from 0 to 210 pA at an interval of 30 pA with a duration of 600 ms (*lower*) in the same layer III pyramidal neuron in the presence of NOP. Note that NOP hyperpolarized the RMP of the neuron and injection of positive currents >+90 pA began to evoke AP firing. **D₃**, Relationship of the numbers of APs and the corresponding currents injected from the same population of layer III pyramidal neurons (n=12) before and during the application of NOP. Note that the current-AP curve shifted to the right in the presence of NOP suggesting that NOP decreases neuronal excitability.

Fig. 5.

Activation of ORL-1 receptors induces hyperpolarization, reduces the input resistance and membrane time constant but increases the rheobase in layer III pyramidal neurons. **A₁-A₂**, Application of NOP hyperpolarized layer III pyramidal neurons. **A₁**, RMP recorded from a layer III pyramidal neuron in response to bath application of NOP. **A₂**, Summary data showing that NOP induced hyperpolarization of layer III pyramidal neurons. Empty circles represent the values from individual cells and the solid circles are their averages. **B₁-B₂**, Bath application of NOP induced outward HCs. **B₁**, HC recorded at -60 mV from a layer III pyramidal neuron. **B₂**, Time course of the HCs in response to NOP averaged from 10 cells. **C₁-C₂**, Activation of ORL-1 receptors reduced input resistance. **C₁**, A negative current (-50 pA for 500 ms) was injected every 5 s to assess the changes of input resistance. Insets are the voltage traces taken before (a) and during (b) the application of NOP. Note that NOP

induced membrane hyperpolarization and reduced the voltage responses induced by the negative current injections suggesting a reduction in input resistance. To exclude the influence of NOP-induced membrane hyperpolarization on input resistance, a constant positive current (+30 pA indicated by the horizontal bar) was injected briefly to elevate the membrane potential to the initial level. Under these circumstances, the voltage responses induced by the negative current injections (−50 pA) were still smaller compared with control suggesting that NOP-induced reduction in input resistance is not secondary to its effect on membrane hyperpolarization. **C₂**, Summary data for NOP-induced reduction of input resistance. **D₁-D₃**, Activation of ORL-1 receptors decreased membrane time constant. **D₁**, Overlays of the voltage responses elicited by injection of −100 pA before and during the application of NOP. The traces within the box was fit by one exponential function. **D₂**, Voltage response induced by −100 pA during the application of NOP as shown in the box in **D₁** was scaled to the voltage response before NOP application to demonstrate NOP-induced decreases of membrane time constant. **D₃**, Summary data for NOP-induced reduction of membrane time constant. **E₁-E₃**, NOP increased rheobase. **E₁**, Voltage responses evoked by positive current injections (from 0 to +30 pA at an interval of 10 pA) in control condition. Note that the cell began to fire APs at +30 pA in control condition. **E₂**, Voltage responses evoked by positive current injections (from 0 to +110 pA at an interval of 10 pA) from the same cell as shown in **E₁** during the application of NOP. Note that the cell began to fire APs at +110 pA. **E₃**, Summary data for NOP-induced increases in rheobase.

Fig. 6. Hyperpolarization induced by NOP is related to the opening of a K^+ conductance. **A**, Depletion of extracellular Ca^{2+} by replacing extracellular Ca^{2+} with Mg^{2+} and including EGTA (1 mM) in the extracellular solution failed to alter NOP-induced hyperpolarization. **B**, Inclusion of BAPTA (10 mM) in the recording pipettes did not change NOP-induced hyperpolarization. **C**, I-V curves recorded by a ramp protocol in the extracellular solution containing 3.5 mM K^+ in control condition and during the application of NOP. **D**, I-V curves of the net currents isolated by subtraction of the I-V curves recorded in control condition from those recorded during the application of NOP in the extracellular solution containing 3.5 mM or 10 mM K^+ . Note that NOP-elicited I-V curve was shifted to the right in 10 mM extracellular K^+ . **E**, Depletion of extracellular K^+ by substitution of extracellular K^+ with the same concentration of Na^+ enlarged NOP-induced hyperpolarization. **F**, Summary data.

G, I-V curves recorded by the same ramp protocol in the extracellular solution containing the M-channel blocker, linopirdine (30 μ M), before and during the application of NOP (n=6). **H**, I-V curve of the net current isolated by subtraction of the I-V curves recorded in control condition from those recorded during the application of NOP in the extracellular solution containing linopirdine (n=6).

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Fig. 7.

Effects of K^+ channel blockers on NOP-induced hyperpolarization. **A**, Bath application of Ba^{2+} (300 μM) induced depolarization and following application of NOP still hyperpolarized the layer III pyramidal neuron. **B**, Bath application of tertiapin-Q (250 nM) had no apparent effect on RMP and application of NOP in the continuous presence of tertiapin-Q still hyperpolarized the recorded layer III pyramidal neuron. **C**, Bath application of SCH23390 (20 μM) exerted little effect on RMP and subsequent application of NOP in the presence of SCH23390 still induced hyperpolarization in the recorded neuron. **D**, Application of ML133 (30 μM) did not block NOP-mediated hyperpolarization. **E**, Application of glibenclamide (10 μM) failed to affect NOP-elicited hyperpolarization. **F**, Application of linopirdine (30 μM) did not block NOP-induced hyperpolarization. **G**, Summary data. Note that among all these K^+ channel blockers, only Ba^{2+} slightly, but significantly reduced NOP-induced hyperpolarization.

Fig. 8.

Voltage-current relationship of NOP-induced currents assessed by measuring membrane currents elicited by voltage steps. **A₁-A₃**, NOP-elicited net currents did not display inward rectification ($n=6$). **A₁**, Membrane currents (*Upper*) elicited by application of a voltage protocol (*Lower*) before and during the application of NOP. **A₂**, I-V curves before and during the application of NOP. **A₃**, NOP-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP. **B₁-B₃**, Substitution of extracellular NaCl with the same concentration of NMDG-Cl failed to alter significantly NOP-induced facilitation of membrane currents. **B₁**, Membrane currents (*Upper*) elicited by application of a voltage protocol (*Lower*) before and during the application of NOP when extracellular NaCl was replaced by NMDG-Cl. **B₂**, I-V curves before and during the application of NOP. **B₃**, Plot of the NOP-elicited I-V curves of the net currents isolated by subtraction of the I-V curve before from that during the application of NOP in two different extracellular solutions, normal extracellular solution ($I_{NOP} - I_{Control}$) and extracellular solution in which NaCl was replaced with NMDG-Cl ($I_{NMDG+NOP} - I_{NMDG}$).

Fig. 9.

NOP-mediated inhibition of cation channel currents is revealed after inhibition of Kir channels by Ba²⁺. **A₁-A₃**, Application of Ba²⁺ (300 μM) inhibited Kir channels. **A₁**, Membrane currents (*Upper*) elicited by application of a voltage protocol (*Lower*) before and during the application of Ba²⁺. **A₂**, I-V curves before and during the application of Ba²⁺. **A₃**, Ba²⁺-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of Ba²⁺. Note that Ba²⁺-sensitive currents showed inward rectification. **B₁-B₃**, Blocking Kir channels by Ba²⁺ (300 μM) revealed the contribution of cation channel inhibition in NOP-mediated hyperpolarization. **B₁**, Membrane currents (*Upper*) elicited by application of a voltage protocol (*Lower*) before and during the application of NOP in the continuous presence of Ba²⁺. **B₂**, I-V curves before and during the application of NOP in the continuous presence of Ba²⁺. Note that there was no intersection

of the two I-V curves. **B₃**, NOP-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP in the continuous presence of Ba²⁺ was persistently outward, demonstrating the participation of cation channels. Inclusion of ZD7288 (50 μM, denoted as ZD in the figure) in the recording pipettes failed to significantly alter NOP-induced inhibition of cation currents. **C₁-C₃**, Blocking Kir channels by Ba²⁺ and replacing extracellular NaCl with NMDG-Cl blocked NOP-mediated augmentation of membrane currents. **C₁**, Membrane currents (*Upper*) elicited by application of a voltage protocol (*Lower*) before and during the application of NOP in the continuous presence of NMDG and Ba²⁺. **C₂**, I-V curves before and during the application of NOP in the continuous presence of NMDG and Ba²⁺. **C₃**, Plot of NOP-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP in two different extracellular solutions, normal extracellular solution containing Ba²⁺ and extracellular solution containing Ba²⁺ and NMDG.

Fig. 10.

Effects of blockers for subfamilies of Kir channels on NOP-mediated facilitation of membrane currents. **A₁-A₄**, Application of tertiapin-Q (250 nM) significantly reduced Kir channel currents at potentials negative to -120 mV, but had no significant effects on NOP-induced currents. **A₁**, I-V curves before and during the application of tertiapin-Q. **A₂**, Tertiapin-Q-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of tertiapin-Q. **A₃**, I-V curves constructed during the application of tertiapin-Q alone and during the application of both tertiapin-Q and NOP. **A₄**, Plot of NOP-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP in two different extracellular solutions, normal extracellular solution and extracellular solution containing tertiapin-Q (TQ). **B₁-B₄**, Application of SCH23390 (20 μM) by itself failed to significantly alter Kir channel currents, but blocked NOP-induced increases in membrane currents at potentials negative to -80 mV. **B₁**, I-V curves before and during the application of SCH23390. **B₂**, SCH23390-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of SCH23390. **B₃**, I-V curves constructed during the application of SCH23390 alone and during the application of both SCH23390 and NOP. **B₄**, Plot of NOP-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP in

two different extracellular solutions, normal extracellular solution and extracellular solution containing SCH23390 (SCH). **C₁-C₄**, Application of ML133 (30 μ M) alone significantly inhibited Kir channel currents at potentials negative to -90 mV and blocked NOP-induced increases in membrane currents at potentials negative to -70 mV. **C₁**, I-V curves before and during the application of ML133. **C₂**, ML133-elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of ML133. **C₃**, I-V curves constructed during the application of ML133 alone and during the application of both ML133 and NOP. **C₄**, Plot of NOP- elicited I-V curve of the net currents isolated by subtraction of the I-V curve in control condition from that during the application of NOP in two different extracellular solutions, normal extracellular solution and extracellular solution containing ML133.