Adrenergic Facilitation of GABAergic Transmission in Rat Entorhinal Cortex

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Abstract

Adrenergic facilitation of GABAergic transmission in the superficial layers of the entorhinal cortex (EC) was investigated. Application of noradrenaline (NE) dose-dependently increased the frequency and amplitude of spontaneous inhibitory postsynaptic currents (IPSCs) recorded from the principal neurons in layer II/III. NE-mediated facilitation of GABAergic function was independent of PLC, protein kinase C, and tyrosine kinase activities. Our results suggest that NE-mediated facilitation of GABAergic function contributes to its antiepileptic effects in the EC.

INTRODUCTION

The entorhinal cortex (EC) mediates the majority of connections between the hippocampus and other cortical areas (Witter et al. 1989, 2000). Sensory inputs converge onto the superficial layers (layers I–III) of the EC (Burwell 2000), which give rise to dense projections to the hippocampus; the axons of the stellate neurons in layer II of the EC form the perforant path that innervates the dentate gyrus and CA3 (Steward and Scoville 1976), whereas pyramidal neurons in layer II/III provide the primary input to CA1 regions (Steward and Scoville 1976; Witter et al. 2000). Moreover, neurons in the deep layers of the EC (layers IV–VI) relay a large portion of hippocampal output projections back to the superficial layers of the EC (Dolfo and Amaral 1998a, b; Kohler 1986; van Haeften et al. 2003) and to other cortical areas (Witter et al. 1989). The EC is part of a network that is closely related to the consolidation and recall of memories (for reviews, see Dolcos et al. 2005; Haist et al. 2001; Squire et al. 2004; Steffenach et al. 2005), Alzheimer’s disease (Hyman et al. 1984; Kotzbauer et al. 2001), schizophrenia (Arnold et al. 1991; Falkai et al. 1988; Joyal et al. 2002; Prasad et al. 2004), and temporal lobe epilepsy (Spencer and Spencer 1994).

The EC receives innervations from the cortical mantle and from the brain stem. The locus coeruleus sends strong noradrenergic projections to the EC (Fallon et al. 1978; Palkovits et al. 1979; Wilcox and Unnerstall 1990). The EC also expresses α1 (Stanton et al. 1987), α2 (Boyajian et al. 1987; Unnerstall et al. 1984, 1985), and β (Booze et al. 1993) adrenergic receptors. In accordance with the structural innervations of noradrenergic fibers and the expression of adrenergic receptors in the EC, application of norepinephrine (NE) inhibits excitatory synaptic transmission in the EC (Pralong and Magistretti 1994, 1995) and reduces epileptiform discharges induced by bicuculline (Stoop et al. 2000) through α2 receptors. Furthermore, NE has been reported to block low Mg2+–induced epileptiform activity through α1 receptors in the EC (Stanton et al. 1987). Because bicuculline-induced epileptic model is produced by inhibition of GABAergic transmission, whereas low Mg2+–induced epileptic model is caused by an overactivation of N-methyl-d-aspartate (NMDA) type of glutamate receptors, these results suggest that NE modulates inhibitory and excitatory synaptic transmission through distinct adrenergic receptors. However, the effects of NE on inhibitory synaptic transmission have never been determined. In this study, we examined the effects of NE on GABAergic transmission in the EC. Our results indicate that NE increases GABAergic transmission in the superficial layers of the EC through activation of α1 receptors. NE-mediated increase in GABA release is independent of Ca2+, phospholipase C (PLC), protein kinase C (PKC), and tyrosine kinase activities. NE-mediated facilitation of GABAergic function likely contributes to its antiepileptic effects in the EC.

METHODS

Slice preparation

Horizontal brain slices (400 μm) including the EC, subiculum, and hippocampus were cut using a vibrating blade microtome (VT1000S, Leica, Wetzlar, Germany), usually from 13- to 20-day-old Sprague-Dawley rats as described previously (Deng and Lei 2006, 2007; Deng et al. 2006, 2007). After being deeply anesthetized with isoflurane, rats were decapitated, and their brains were dissected out in ice-cold saline solution that contained (in mM) 130 NaCl, 24 NaHCO3, 3.5 KCl, 1.25 NaH2PO4, 0.5 CaCl2, 5.0 MgCl2, and 10 glucose, saturated with 95% O2-5% CO2, pH 7.4. Slices were initially incubated in the preceding solution at 35°C for 40 min for recovery and kept at room temperature for 1 hr before use.
temperature (~24°C) until use. All animal procedures conformed to the guidelines approved by the University of North Dakota Animal Care and Use Committee.

**Recordings of spontaneous, miniature, and evoked GABA<sub>A</sub> receptor-mediated IPSCs**

Whole cell patch-clamp recordings using two Multiclamp 700B amplifiers (Molecular Devices, Sunnyvale, CA) in voltage-clamp mode were made from the principal neurons in layer II/III of the EC visually identified with infrared video microscopy (BX51WI, Olympus, Tokyo, Japan) and differential interference contrast optics (Deng and Lei 2007; Deng et al. 2007). The recording electrodes were filled with the following solution (in mM): 100 cesium gluconate, 0.6 EGTA, 5 MgCl<sub>2</sub>, 8 NaCl, 2 ATP<sub>2</sub>Na, 0.3 GTP<sub>Na</sub> and 1 QX-314; pH 7.3. The extracellular solution contained (in mM) 130 NaCl, 24 NaHCO<sub>3</sub>, 3.5 KCl, 1.25 Na<sub>2</sub>HPO<sub>4</sub>, 1.5 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, and 10 glucose, saturated with 95% O<sub>2</sub>-5% CO<sub>2</sub>; pH 7.4. To record GABA<sub>A</sub> receptor–mediated spontaneous inhibitory postsynaptic currents (sIPSCs), the external solution was supplemented with l-2-amino-5-phosphonovaleric acid (l-APV; 100 μM) and 6,7-dinitroquinoxaline-2,3-(1H, 4H)-dione (DNQX; 10 μM) to block NMDA and AMPA receptor–mediated responses, respectively. Under these conditions, the recording inhibited currents had a reversal potential of approximately −30 mV and were completely blocked by bicuculline. To adjust pH to 7.4, HCl was used to replace the same concentration of NMDG, and HCl was used to acidify the holding solution for the recordings of action potentials from interneurons in layer III. The pipettes were filled with the above K<sup>+</sup>-gluconate solution. NE was applied after the action potentials or the firing activity had been stable for 5~10 min. The frequency of the action potentials or firing activity was calculated by Mini Analysis 6.0.1.

**Recordings of holding current**

Holding current at −55 mV was recorded from interneurons in layer III in the extracellular solution containing TTX (1 μM) to block action potential firing. The intracellular solution was the above K<sup>+</sup>-gluconate solution containing 0.2% biocytin. Because gradual dialysis of K<sup>+</sup> into cells changed the holding current, we began our recordings after waiting for ~15 min from the formation of whole cell configuration. Holding currents at −55 mV were recorded every 3 s and averaged per minute. We subtracted the average of the holding current recorded for the last 5 min before the application of NE from those recorded at different time-points to zero the basal level of the holding current for better comparison.

**Histological staining of interneurons**

After recordings, slices were fixed in 0.1 M PBS containing 4% paraformaldehyde and 0.2% picric acid for 24 h at 4°C. After an extensive wash in 0.1 M PBS, slices were incubated with Texas red–conjugated streptavidin (1:200) for 2 h at room temperature. After wash, slices were mounted on slides and coverslipped. Slides were visualized with an Olympus Fluoview 300 confocal microscope and photographed.

**Breeding and genotyping of mutant mice**

Heterozygous mating pairs (F1 hybrid crosses from 129 PLC<sup>β1+/−</sup> X C57BL/6 J PLC<sup>β1−/−</sup>) were obtained from the Korea Institute of Science and Technology. The breeders were used to derive wild-type, heterozygous, and homozygous pups for experimental analysis. PCR genotyping from purified genomic DNA was performed as described previously (Deng et al. 2006; Kim et al. 1997).

**Data analysis**

Data are presented as the means ± SE. Concentration–response curve of NE was fit by Hill equation: \( I = I_{\text{max}} \times (1/(1 + [\text{EC}_{50}/(\text{lignand})^n])) \), where \( I_{\text{max}} \) is the maximum response, \( \text{EC}_{50} \) is the concentration of ligand producing a half-maximal response, and \( n \) is the Hill coefficient. Student’s paired or unpaired t-test or ANOVA was used for statistical analysis as appropriate; \( P \) values are reported throughout the text, and significance was set as \( P < 0.05 \). For sIPSC or mIPSC cumulative probability plots, events recorded 5 min before and 5 min after reaching the maximal effect of NE were selected. Same bin size (25 ms for frequency and 2 pA for amplitude) was used to analyze data from control and NE treatment. Kolmogorov-Smirnoff test was used to assess the significance of the cumulative probability plots. \( N \) in the text represents the cells examined.

**Chemicals**

Corynathine, yohimbine, propranolol, genistene, and U73122 were purchased from TOCRIS (Ellisville, MO). Calphostin C and Ro318220 were from BIOMOL (Plymouth Meeting, PA). 1-O-Octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine (edelfosine) was purchased from Avanti Polar Lipids (Alabaster, AL). 

**ADRENERGIC INHIBITION IN THE ENTORHINAL CORTEX**

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RESULTS

NE increases the frequency and amplitude of sIPSCs

Stellate and pyramidal neurons are the two major types of neurons in the superficial layers of the EC. In this study, we identified these two types of neurons by their morphology and location because the characteristic electrophysiological property of stellate neurons (depolarizing voltage sag in response to hyperpolarizing current pulses; Deng and Lei 2007; Deng et al. 2007) could not be observed when Cs⁺ and QX-314 were included in the intracellular solution to record GABA_A receptor–mediated synaptic currents. Stellate neurons are usually located in layer II or the border of layer II and III, and they have larger and polygonal soma with variable number of main dendrites radiating out from the cell body, but are devoid of a clearly dominant dendrite. Pyramidal neurons have a pyramidal or elongated soma with dendrites orientated in a bidirectional way; one (sometimes 2) thick apical dendrite that runs to the surface of the cortex and several (3–5) basal dendrites extending toward the deeper layers. We recorded sIPSCs from both stellate and pyramidal neurons in layer II/III of the EC. Application of NE (100 μM) significantly increased the frequency (157 ± 11% of control, P < 0.001; Fig. 1, A–C) and amplitude (152 ± 12% of control, P = 0.011; Fig. 1D) of sIPSCs in five of five stellate neurons examined. Similarly, application of NE (100 μM) significantly increased the frequency (163 ± 12% of control, P < 0.001; Fig. 1, E–G) and amplitude (162 ± 19% of control, P = 0.03; Fig. 1H) of sIPSCs in five of five pyramidal neurons examined. Because there were indistinguishable differences for NE-induced increases in sIPSC frequency (P = 0.69, Student’s unpaired t-test) and amplitude (P = 0.66, Student’s unpaired t-test) recorded from stellate neurons and pyramidal neurons, we performed the rest of the experiments on both stellate and pyramidal neurons. The frequency of sIPSCs after application of NE became so high (Fig. 1, A and E) that it prevented reliable comparison of the decay kinetics of sIPSCs. The EC₅₀ value was measured to be 4.4 and 5.0 μM when the percentage of increase in frequency (Fig. 2A) or amplitude (Fig. 2B) was plotted versus the concentrations of NE, respectively.

Involvement of α₁ receptors

NE possesses high potency for α₁ and α₂ but has weak activity on β₁ adrenergic receptors. We next examined the roles of these receptors in the effects of NE on sIPSCs. Application of a specific α₁ receptor blocker, corynathine (100 μM), completely blocked the NE-induced increase in sIPSC frequency (101 ± 7% of control, n = 5, P = 0.91; Fig. 2C) and amplitude (97 ± 9% of control, n = 5, P = 0.64), whereas application of yohimbine (100 μM), a α₂ receptor antagonist, or propranolol (100 μM), a β receptor antagonist, had no effects on NE-induced increases in sIPSC frequency (yohimbine: 168 ± 11% of control, n = 5, P = 0.003; Fig. 2D; propranolol: 158 ± 18% of control, n = 5, P = 0.018; Fig. 2E) and amplitude (yohimbine: 152 ± 13% of control, n = 5, P = 0.02; propranolol: 164 ± 23% of control, n = 5, P = 0.04). Furthermore, application of phenylephrine (100 μM), a α₁ adrenergic receptor agonist, increased the frequency (177 ± 21% of control, n = 5, P = 0.02; Fig. 2F) and amplitude (163 ± 22% of control, n = 5, P = 0.04) of sIPSCs. Together, these results indicate that the effects of NE on sIPSCs are mediated through activation of α₁ adrenergic receptors in the EC.
NE increases the frequency with no effects on the amplitude of mIPSCs

sIPSCs recorded in the absence of TTX are believed to be action potential- and Ca\(^{2+}\)-dependent. We next examined the effects of NE on mIPSCs recorded in the presence of TTX (1 \(\mu\)M). Application of NE (100 \(\mu\)M) significantly increased the frequency of mIPSCs (155 \(+\) 8\% of control, \(n = 5\), \(P = 0.002\); Fig. 3, A–C), while the amplitude remained unchanged (107 \(+\) 5\% of control, \(n = 5\), \(P = 0.22\); Fig. 3D). These results suggest that NE increases presynaptic GABA release with no effects on postsynaptic GABA\(_A\) receptors. We compared the kinetics of the averaged mIPSCs before and after the effect of NE reached maximal. NE significantly slowed the decay of mIPSCs (control: 23.3 \(\pm\) 1.6 ms, NE: 31.8 \(\pm\) 4.4 ms, \(n = 5\), \(P = 0.04\); Fig. 3E).

Heterogeneous effects of NE on evoked IPSCs

We examined the effects of NE on IPSCs evoked by placing a stimulation electrode locally in the EC to stimulate GABAergic inputs. Because the normal variation of evoked IPSC amplitude was \(~10\%\) in our recording condition, we defined that synapses showing changes in evoked IPSC amplitude by \(>15\%\) in response to the application of NE (100 \(\mu\)M) as responsive synapses. Of the 18 synapses examined, 5 synapses exhibited an increase (148 \(\pm\) 13\% of control, \(n = 5\), \(P = 0.023\); Fig. 4, A and D), 6 synapses showed no change (96 \(\pm\) 3\% of control, \(n = 6\), \(P = 0.21\); Fig. 4, B and D), and 7 synapses displayed a decrease (66 \(\pm\) 4\% of control, \(n = 7\), \(P < 0.001\); Fig. 4, C and D) in evoked IPSC amplitude in response to the application of NE (100 \(\mu\)M). The biophysical mechanisms underlying NE-induced heterogeneity of evoked IPSCs...
may include the selective expression of α1 adrenergic receptors or other relevant release machineries at the stimulated presynaptic terminals and changes in presynaptic release pool at the active zone (see Discussion). Consistent with our results, heterogeneity of NE-induced changes in evoked IPSCs has been observed in different neurons (Bennett et al. 1998; Braga et al. 2004; Hirono and Obata 2006; Madison and Nicoll 1988).

**NE-induced increase in GABA release is Ca\(^{2+}\) independent**

mIPSCs recorded in the presence of TTX are independent of action potential, and they are generated by spontaneous vesicle fusion. Whereas the result that NE still increased the frequency of mIPSCs in the presence of TTX suggests that voltage-gated Ca\(^{2+}\) channels are unlikely to be responsible for the effects of NE, we still tested the possibility that NE might inhibit resting K\(^+\) channels to generate membrane depolarization resulting in opening of low-threshold Ca\(^{2+}\) channels to increase GABA release. We initially recorded sIPSCs in the absence of TTX and switched to the extracellular solution containing two non-specific Ca\(^{2+}\) channel blockers, Cd\(^{2+}\) (100 μM) and Ni\(^{2+}\) (100 μM), to block Ca\(^{2+}\) influx by voltage-gated Ca\(^{2+}\) channels. Application of the Ca\(^{2+}\) channel blockers significantly reduced sIPSC frequency (59 ± 10% of control, n = 5, P = 0.016; Fig. 5A) and amplitude (76 ± 7% of control, n = 5, P = 0.02). Subsequent application of TTX (1 μM) after Ca\(^{2+}\) channels were blocked did not further significantly reduce the frequency (94 ± 3% of control, n = 5, P = 0.11; Fig. 5A) and amplitude (96 ± 3% of control, n = 5, P = 0.2) of IPSCs, suggesting that voltage-gated Ca\(^{2+}\) channels contribute significantly to the generation of sIPSCs. In the presence of Cd\(^{2+}\), Ni\(^{2+}\), and TTX, application of NE (100 μM) still significantly increased the frequency (163 ± 10% of control, n = 5, P = 0.003; Fig. 5A) without changing the amplitude (102 ± 4% of control, n = 5, P = 0.5) of IPSCs, suggesting that voltage-gated Ca\(^{2+}\) channels are unlikely to be involved in NE-induced increases in GABA release. We performed a similar kind of experiment by omitting Ca\(^{2+}\) in the extracellular solution. Exclusion of Ca\(^{2+}\) in the extracellular solution remarkably reduced IPSC frequency (52 ± 2% of control, n = 6, P < 0.001; Fig. 5B) and amplitude (61 ± 7% of control, n = 6, P = 0.002). Subsequent inclusion of TTX (1 μM) in the extracellular solution failed to change significantly IPSC frequency (100 ± 5% of control, n = 6, P = 0.95; Fig. 5B) and amplitude (98 ± 3% of control, n = 6, P = 0.5) of IPSCs, suggesting that voltage-gated Ca\(^{2+}\) channels are unlikely to be involved in NE-induced increases in GABA release. We performed a similar kind of experiment by omitting Ca\(^{2+}\) in the extracellular solution. Exclusion of Ca\(^{2+}\) in the extracellular solution remarkably reduced IPSC frequency (52 ± 2% of control, n = 6, P < 0.001; Fig. 5B) and amplitude (61 ± 7% of control, n = 6, P = 0.002). Subsequent inclusion of TTX (1 μM) in the extracellular solution failed to change significantly IPSC frequency (100 ± 5% of control, n = 6, P = 0.95; Fig. 5B) and amplitude (98 ± 3% of control, n = 6, P = 0.5).

**FIG. 4.** Heterogeneity of NE on evoked IPSCs. A: bath application of NE (100 μM) increased amplitude of evoked IPSCs at an inhibitory synapse in EC. Top: traces averaged from 10 IPSCs taken at time-points indicated in bottom panel. Stimulation artifact was blanked for each trace for clarity. Bottom: time-course of NE-mediated increase in IPSCs. Holding potential was +30 mV. B: application of NE (100 μM) did not obviously change amplitude of evoked IPSCs at an inhibitory synapse in EC. Figure was arranged in the same way as in A. C: application of NE (100 μM) depressed amplitude of evoked IPSCs at an inhibitory synapse in EC. Figure was arranged in the same way as in A and B. D: scatter plot of amplitude of evoked IPSCs from 18 synapses.

**FIG. 5.** NE-mediated facilitation of GABA release is Ca\(^{2+}\) independent. A: application of Ca\(^{2+}\) channel blockers, Cd\(^{2+}\) (100 μM) and Ni\(^{2+}\) (100 μM), failed to block NE-induced increase in mIPSC frequency (n = 7). B: omission of extracellular Ca\(^{2+}\) did not change NE-induced increase in mIPSC frequency (n = 5). C: replacement of extracellular Na\(^+\) with the same concentration of N-methyl-D-glucamine (NMDG) failed to alter NE-mediated increase in mIPSC frequency (n = 5). D: application of BAPTA-AM (100 μM) did not block NE-induced increase in mIPSC frequency (n = 5). E: application of thapsigargin (10 μM) failed to block NE-induced increase in mIPSC frequency (n = 5). F: application of ryanodine (100 μM) failed to block NE-induced increase in mIPSC frequency (n = 5).
more, bath application of thapsigargin (10 μM) still increased the frequency (157 ± 13% of control, n = 6, P = 0.007; Fig. 5B) with no effects on the amplitude (104 ± 2% of control, n = 6, P = 0.14) of IPSCs, further excluding the involvement of voltage-gated Ca2⁺ channels.

We next tested the possibility that NE opens a cationic conductance to increase GABA release. If so, the extracellular Na⁺ should be the major cations to facilitate GABA release. Replacing the extracellular Na⁺ with the same concentration of NMDG reduced remarkably IPSC frequency (47 ± 4% of control, n = 5, P < 0.001; Fig. 5C) and amplitude (69 ± 2% of control, n = 5, P < 0.001). Subsequent application of TTX failed to further change IPSC frequency (105 ± 4% of control, n = 5, P = 0.31; Fig. 5C) and amplitude (111 ± 10% of control, n = 5, P = 0.39). In this recording condition, application of NE (100 μM) still increased the frequency (152 ± 10% of control, n = 5, P < 0.001; Fig. 5C) without significantly altering the amplitude (103 ± 4% of control, n = 5, P = 0.49) of IPSCs, suggesting that NE-induced increases in GABA release are unlikely to be mediated through cationic channels.

We finally tested whether an increase in intracellular Ca2⁺ concentration was required for NE-induced increase in GABA release. Inclusion of the membrane-permeable Ca2⁺ chelator, BAPTA-AM (100 μM), in the extracellular solution reduced sIPSC frequency to 54 ± 4% of control (n = 7, P < 0.001; Fig. 5D) and amplitude to 72 ± 4% of control (n = 7, P < 0.001). Subsequent application of TTX (1 μM) did not significantly reduce sIPSC frequency (94 ± 4% of control, n = 7, P = 0.2; Fig. 5D) and amplitude (96 ± 2% of control, n = 7, P = 0.12). In this recording condition, application of NE (100 μM) still significantly increased the frequency (150 ± 8% of control, n = 7, P < 0.001; Fig. 5D), with no effects on the amplitude (101 ± 3% of control, n = 7, P = 0.65) of IPSCs. Furthermore, bath application of thapsigargin (10 μM), a potent inhibitor of sarco-endoplasmic reticulum Ca2⁺-ATPases, significantly reduced sIPSC frequency (84 ± 5% of control, n = 5, P = 0.045; Fig. 5E) and amplitude (73 ± 4% of control, n = 5, P = 0.002). In the presence of thapsigargin, application of TTX (1 μM) further reduced sIPSC frequency to 65 ± 5% of control (n = 5, P = 0.002; Fig. 5E) and amplitude to 52 ± 5% of control (n = 5, P = 0.005). After application of NE (100 μM) still significantly increased the frequency (166 ± 8%, n = 5, P = 0.001; Fig. 5E) without altering the amplitude (104 ± 7% of control, n = 5, P = 0.61) of IPSCs. Similarly, bath application of ryanodine (100 μM) reduced sIPSC frequency to 87 ± 3% of control (n = 5, P = 0.02; Fig. 5F) and amplitude to 81 ± 3% of control (n = 5, P = 0.002). In the presence of ryanodine, application of TTX (1 μM) further reduced the frequency (63 ± 11% of control, n = 5, P = 0.03; Fig. 5F) and amplitude (66 ± 8% of control, n = 5, P = 0.013). After application of NE (100 μM) still significantly increased IPSC frequency (157 ± 10% of control, n = 5, P = 0.005; Fig. 5F) with no effects on IPSC amplitude (106 ± 4% of control, n = 5, P = 0.19). Together, these results indicate that the NE-induced increase in GABA release is Ca2⁺-independent.

NE does not modulate the excitability of the interneurons in layer III of the EC

Principal neurons in layer II/III receive GABAergic innervations from local interneurons. We next tested whether NE changes the excitability of the interneurons by recording action potential firing and holding current (at −55 mV) from GABAergic interneurons in layer III of the EC. We identified interneurons by referring to the criteria set by Kumar and Buckmaster (2006). Interneurons in layer III had smaller capacitance (24.6 ± 1.6 pF, n = 10), higher membrane resistance (349 ± 28 MΩ, n = 10), and apparent spike afterhyperpolarization amplitude (−14.7 ± 1.2 mV, n = 10). The identities of the interneurons were further confirmed by ad hoc biocytin staining of the recorded interneurons (Fig. 6D). Application of NE (100 μM) changed neither the frequency of action potentials (96 ± 7% of control, n = 5, P = 0.57; Fig. 6, A and B) nor the holding current recorded at −55 mV in the presence of 1 μM TTX (0.19 ± 0.95 pA, n = 5, P = 0.85; Fig. 6C), suggesting that NE does not influence the excitability of the interneurons.

Because the above experiments were performed with whole cell recordings, one could argue that the intracellular molecules required for the effects of NE might have been dialyzed out in whole cell configuration. We therefore used cell-attached patches and recorded action potential firing. The pipettes con-

FIG. 6. NE does not change excitability of interneurons in layer III of EC. A: action potentials recorded from an interneuron in layer III of EC in whole cell configuration before (top) and during (bottom) application of NE (100 μM). Note that NE had no effects on action potential firing. B: summarized time-course of action potential firing frequency from 5 cells. C: NE did not change holding current recorded from layer III interneurons (n = 5). D: biocytin-labeled interneuron. E: firing activity recorded from an interneuron in layer III of EC in cell-attached patch before (top) and during (bottom) application of NE (100 μM). Event indicated by arrow was an event plotted in enlarged scale. F: summarized time-course of firing frequency recorded from 7 cells by cell-attached patches.
tained the K\(^{+}\)-gluconate internal solution and cell-attached patches were formed on interneurons in layer III. In this recording condition, bath application of NE (100 \(\mu\)M) still did not change the frequency of action potential firing (101 \(\pm\) 5\%) of control, \(n = 7\), \(P = 0.85\); Fig. 6, E and F). Together, these results suggest that NE does not influence the excitability of the interneurons in the EC.

**NE-induced increase in GABA release is independent of PLC, PKC, and tyrosine kinase activities**

\(\alpha_1\)-Adrenergic receptors are G protein–coupled receptors that are coupled to \(G_{\text{q/11}}\) (Hein 2006). Activation of \(G_{\text{q/11}}\) increases the activity of PLC, which hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP\(_2\)) to produce inositol triphosphate (IP\(_3\)) to facilitate intracellular Ca\(^{2+}\) release and diacylglycerol to activate PKC. We next tested the roles of this pathway in NE-induced increases in GABA release. Slices were pretreated with U73122 (20 \(\mu\)M), a PLC inhibitor, and the same concentration of U73122 was bath applied. In the presence of U73122, application of NE (100 \(\mu\)M) significantly increased the frequency (162 \(\pm\) 14\%) of control, \(n = 7\), \(P = 0.005\); Fig. 7A) and amplitude (153 \(\pm\) 15\%) of control, \(n = 7\), \(P = 0.014\) of sIPSCs. We also used another PLC inhibitor, edelfosine (Horowitz et al. 2005; Powis et al. 1992). Slices were pretreated with edelfosine (20 \(\mu\)M), and the same concentration of edelfosine was bath applied. In the presence of edelfosine, application of NE (100 \(\mu\)M) still significantly increased the frequency (181 \(\pm\) 10\%) of control, \(n = 5\), \(P = 0.001\); Fig. 7B) and amplitude (169 \(\pm\) 6\%) of control, \(n = 5\), \(P < 0.001\) of sIPSCs. To test whether U73122 and edelfosine were effective at inhibiting PLC activity, we performed a positive control experiment. Because brain-derived neurotrophic factor (BDNF) has been reported to inhibit GABAergic transmission in CA1 region of the hippocampus through activation of Trk-B and PLC-\(\gamma\) (Tanaka et al. 1997), we recorded the evoked IPSCs from CA1 pyramidal neurons by placing a stimulation electrode in the stratum pyramidale. Bath application of BDNF (100 ng/ml) significantly inhibited the amplitude of evoked IPSCs (56 \(\pm\) 9\%) of control, \(n = 6\), \(P = 0.005\). However, application of the same concentration of BDNF failed to significantly inhibit the amplitude of evoked IPSCs in slices pretreated with U73122 (20 \(\mu\)M, 93 \(\pm\) 5\%) of control, \(n = 6\), \(P = 0.21\) or edelfosine (20 \(\mu\)M, 90 \(\pm\) 5\%) of control, \(n = 6\), \(P = 0.12\). These results suggest that the activity of PLC is not required for NE-induced increase in GABA release in the EC. Because G protein–coupled receptors are coupled to PLC\(\beta\) and among the four isoforms of PLC\(\beta\) (PLC\(\beta\)1–4), only PLC\(\beta\)1 is expressed in the hippocampal formation (Watanabe et al. 1998), we tested the role of PLC in the effects of NE by using PLC\(\beta\)1 knockout mice (Deng et al. 2006). Application of NE (100 \(\mu\)M) increased the frequency and amplitude of sIPSC in both wild-type and PLC\(\beta\), knockout mice (Fig. 7C). Together, these results indicate that PLC is unlikely to be involved in NE-mediated increase in GABA release. We also tested whether the activity of PKC was necessary for NE-induced increase in GABA release. Application of calphostin C (1 \(\mu\)M), a specific PKC inhibitor, significantly inhibited the basal sIPSC frequency (83 \(\pm\) 4\%) of control, \(n = 5\), \(P = 0.008\); Fig. 7D) and amplitude (79 \(\pm\) 3\%) of control, \(n = 5\), \(P = 0.002\). In the presence of calphostin C, application of NE (100 

\(\mu\)M) still significantly increased sIPSC frequency (170 \(\pm\) 14\%) of control, \(n = 5\), \(P = 0.009\); Fig. 7D) and amplitude (151 \(\pm\) 6\%) of control, \(n = 5\), \(P < 0.001\). We also used another specific PKC inhibitor, Ro318220. Application of Ro318220 (1 \(\mu\)M) significantly depressed sIPSC frequency (77 \(\pm\) 6\%) of control, \(n = 7\), \(P = 0.007\); Fig. 7E) and amplitude (71 \(\pm\) 3\%) of control, \(n = 7\), \(P < 0.001\). However, after application of NE (100 \(\mu\)M) still significantly enhanced sIPSC frequency (155 \(\pm\) 11\%) of control, \(n = 7\), \(P = 0.002\); Fig. 7E) and amplitude (145 \(\pm\) 12\%) of control, \(n = 7\), \(P = 0.008\). Together, these data suggest that the activity of PKC is unnecessary for NE-induced increase in GABA release.

Because G protein–coupled receptors are also coupled to tyrosine kinase such as src in some neurons (Marinissen and Gutkind 2001), we examined the roles of tyrosine kinase activity in NE-induced increases in GABA release. Application of genistein (50 \(\mu\)M), a tyrosine kinase inhibitor, did not significantly change sIPSC frequency (88 \(\pm\) 5\%) of control, \(n = 6\), \(P = 0.09\); Fig. 7F) and amplitude (94 \(\pm\) 7\%) of control, \(n = 6\), \(P = 0.43\). After application of NE (100 \(\mu\)M) still
significantly increased sIPSC frequency (158 ± 11% of control, n = 6, P = 0.003; Fig. 7F) and amplitude (152 ± 10% of control, n = 6, P = 0.002). The inability of genistein to block the effects of NE was not caused by the ineffectiveness of tyrosine kinase inhibition, because application of genistein (50 μM) blocked BDNF (100 ng/ml)-induced inhibition of evoked IPSCs (94 ± 4% of control, n = 5, P = 0.19) recorded from CA1 pyramidal neurons of the hippocampus.

**NE inhibits the excitability of principal neurons in the EC**

If NE increases GABAergic transmission onto the principal neurons, it should reduce the excitability of the principal neurons in the EC. We next recorded from the principal neurons in the EC and tested the effects of NE on action potential firing. Application of NE (100 μM) significantly reduced the frequency of action potentials to 56 ± 5% of control (n = 6, P < 0.001; Fig. 8), showing that NE inhibits neuronal excitability in the EC.

**DISCUSSION**

The EC receives noradrenergic projections from the locus coeruleus of the pons (Fallon et al. 1978; Palkovits et al. 1979; Wilcox and Unnerstall 1990), although the function of NE in the EC is still elusive. In this study, we examined the effects of NE on GABAergic transmission, and our results showed that NE facilitates GABA release through activation of α1 receptors. We also showed that NE-induced increase in GABA release is independent of Ca2+ influx, PLC, PKC, and tyrosine kinase activities. NE-induced GABA release inhibits the neuronal excitability of principal neurons.

Our results showed that NE enhances GABAergic transmission in the EC by increasing presynaptic GABA release without altering postsynaptic GABA receptors because NE only increased the frequency without altering the amplitude of mIPSCs, although it increased both the frequency and amplitude of sIPSCs. Our results exclude a role of Ca2+ influx through voltage-gated Ca2+ channels because application of the Ca2+ channel blockers, Cd2+ and Ni2+, and deprivation of extracellular Ca2+ failed to change NE-induced increase in mIPSC frequency. It is also unlikely that NE opens a cationic conductance on the presynaptic membrane to increase GABA release because replacing the extracellular Na+ with NMDG did not prevent NE-induced increase in mIPSC frequency. The results that application of BAPTA-AM, thapsigargin, and ryanodine failed to change NE-induced increase in mIPSC frequency suggest that Ca2+ released from intracellular stores is not required for NE-induced increase in GABA release. Together, these results indicate that NE-mediated facilitation of GABA release is Ca2+-independent and mediated by an interaction with GABA release machinery. Consistent with our results, a variety of G protein–coupled receptors including adenosine A1 (Capogna et al. 1996; Scanziani et al. 1992), GABA_B (Capogna et al. 1996; Scanziani et al. 1992), somatostatin (Boehm and Betz 1997), muscarinic (Scanziani et al. 1995), and metabotropic glutamate (Scanziani et al. 1995; Tyler and Lovinger 1995) receptors modulate transmitter release through a direct interaction with the secretory apparatus on the presynaptic terminals.

Dependent on the brain regions, activation of adrenergic receptors modulates GABAergic transmission through at least three distinct ionic mechanisms. First, NE increases the frequency of sIPSCs with no effects on mIPSCs in CA1 pyramidal neurons of the hippocampus (Bergles et al. 1996), the frontal cortex (Kawaguchi and Shindou 1998), and the hypothalamic paraventricular nucleus (Han et al. 2002). The effects of NE in these brain regions are likely caused by NE-induced depression of K+ channels in presynaptic GABAergic interneurons (Bergles et al. 1996). This mechanism, however, is not applicable for NE-induced facilitation of GABAergic transmission in the EC because depression of resting membrane K+ channels increases action potential firing and Ca2+ influx through voltage-gated Ca2+ channels, whereas in the EC, NE does not modulate the excitability of interneurons. Second, NE increases the frequency and amplitude of sIPSCs, but only increases the frequency of mIPSCs in sensory motor cortex (Bennett et al. 1998) and in Purkinje cells of the mouse cerebellum (Hirono and Obata 2006). The effects of NE in these brain regions resemble our results, suggesting that they may share the similar ionic mechanism, although the underlying ionic mechanisms of NE in those brain regions have not been determined yet. Based on our results, it is reasonable to speculate that NE may also facilitate GABA release by interacting with the release machinery in those areas. Third, NE increases mIPSC frequency in the accessory olfactory bulb through Ca2+ influx mediated by Ca2+ channels because NE-induced increase in mIPSC frequency is sensitive to Ca2+ channel blockers, Cd2+ and Ni2+ (Araneda and Firestein 2006). Our results suggest that this is not likely the mechanism for NE-mediated increase in GABA release in the EC because application of these Ca2+ channel blockers had no effects on NE-induced increase in mIPSC frequency.

We observed that NE slowed the decay of the averaged mIPSCs in the EC. There are two plausible explanations for NE-induced change in mIPSC kinetics. First, our results suggest that the effects of NE are mediated through a direct interaction with the release machinery. If the action site of NE is on the fusion pore, the kinetics of mIPSCs could possibly be altered. Second, NE-induced slowing of mIPSC decay kinetics might be caused by NE-mediated increases in the number of mIPSCs that overlay on the decay phase of previous mIPSCs, resulting in a slowdown of the decay kinetics of the averaged mIPSCs.

We also observed that NE generates heterogeneous responses on evoked IPSCs. Application of NE increased, did not change, or decreased the amplitude of evoked IPSCs at individual synapses in the EC. Consistent with our results, NE has been shown to depress evoked IPSCs in the hippocampus.

**FIG. 8.** NE reduces excitability of principal neurons in EC. A: action potentials recorded from a principal neuron in EC before and during application of NE (100 μM); B: summarized time-course of action potential firing frequency from 6 cells.
(Madison and Nicoll 1988), but increase evoked IPSCs in Purkinje cells of mouse cerebellum (Hirono and Obata 2006) and in basolateral amygdala neurons (Braga et al. 2004). NE-mediated increase, decrease, and no change in evoked IPSCs have been observed in rat sensorimotor cortex (Bennett et al. 1998). Whereas the exact mechanisms underlying the heterogeneous effects of NE on the evoked IPSCs remain to be determined, we propose three possible mechanisms to explain our results. First, NE-mediated increase in evoked IPSC amplitude at some synapses may reflect the true effects of NE, i.e., enhancement of GABA release. At these synapses, the presynaptic terminals of the stimulated fibers are likely to express α1 adrenergic receptors and other required machineries. Second, different from sIPSCs and mIPSCs, which are likely from many different synapses onto the recorded neurons, evoked IPSCs are generated by a few fibers that are stimulated exogenously. If the stimulated fibers do not express α1 adrenergic receptors or other release machineries required by NE, application of NE would generate no responses. This may explain the results that NE had no effects on the amplitude of evoked IPSCs at some synapses. Third, NE-induced increase in spontaneous GABA release at many release sites resembles a condition named asynchronous release. NE-induced increases in asynchronous release may have reduced the size of the release pool at the active zone resulting in a reduction in evoked IPSCs (synchronous release). Whereas the heterogeneous effects of NE on evoked IPSCs may be generated by distinct biophysical mechanisms, our result that NE increased sIPSC frequency and amplitude in every cell examined (Fig. 1) suggest that NE makes considerable contribution to the inhibition in the EC because sIPSCs represent a more natural transmission in vivo.

Our results show that NE increases GABA release through activation of α1 adrenergic receptors without the requirement of α2 or β receptors, consistent with the results from other synapses (Araneda and Firestein 2006; Bennett et al. 1998; Bergles et al. 1996; Braga et al. 2004; Han et al. 2002; Hirono and Obata 2006). α1 receptors are coupled to Gs11, resulting in activation of the PLC pathway. We showed that the function of PLC is unnecessary for NE-mediated facilitation of GABA release because application of NE still increased GABA release in the presence of two PLC inhibitors (U73122 and edelfosine) and in PLCβ1 knockout mice. Because a general caveat for the experiments of knockout animals is that the knockout animals can potentially produce compensatory signals, we cannot rule out this possibility for the experiments involving PLCβ1 knockout mice, although PLCβ1 is the type of PLCβ expressed in the hippocampal formation (Watanabe et al. 1998) and there has been no report in the literature suggesting that the PLCβ1 knockout mice generate other PLCβ isoforms to compensate the deleted PLCβ1. Our results do not support any roles of the two downstream targets of PLC (IP3-mediated intracellular Ca2+ release and PKC) in NE-mediated increase in GABA release because application of BAPTA-AM to chelate intracellular Ca2+ and two PKC inhibitors (calphostin C and Ro318220) failed to change NE-mediated GABA release. Together, these results suggest a mode in which G proteins activated by α1 receptors directly interact with the release machinery to facilitate GABA release. Consistent with this notion, Gβγ released by activation of G protein-coupled serotonin receptors modulates transmitter release through direct interaction with exocytotic fusion machinery (Blackmer et al. 2001, 2005; Gerachshenko et al. 2005; Photowala et al. 2006).

In addition to these findings that NE facilitates GABAergic transmission through α1 receptors, NE also inhibits glutamatergic transmission through α2 receptors in the EC (Pralong and Magistretti 1994, 1995). The inhibitory effects of α2 receptors on excitatory synaptic transmission in the EC may explain NE-mediated antiepileptic actions in a bicuculline-induced seizure model (Stoop et al. 2000). Nonetheless, NE has been reported to block low Mg2+-induced epileptiform activity through α1 receptors in the EC (Stanton et al. 1987). Our results can explain the discrepancy of these results, because NE-induced facilitation of GABAergic transmission through α1 receptors was overwhelmed in a bicuculline-induced seizure model, whereas it was functional in a low Mg2+-induced epileptic model. Therefore NE-mediated facilitation of GABAergic transmission is likely to be an important player in NE-induced inhibition of epilepsy in the EC.

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REFERENCES


ADRENERGIC INHIBITION IN THE ENTORHINAL CORTEX


