

Full Paper

Dextromethorphan Inhibits the Glutamatergic Synaptic Transmission in the Nucleus Tractus Solitarius of Guinea PigsYoshiaki Ohi¹, Saori Tsunekawa¹, and Akira Haji^{1,*}¹Laboratory of Neuropharmacology, School of Pharmacy, Aichi Gakuin University, 1-100 Kusumoto, Chikusa, Nagoya 464-8650, Japan

Received January 17, 2011; Accepted March 1, 2011

Abstract. Dextromethorphan (DEX) is a widely used non-opioid antitussive. However, the precise site of action and its mechanism were not fully understood. We examined the effects of DEX on AMPA receptor-mediated glutamatergic transmission in the nucleus tractus solitarius (NTS) of guinea pigs. Excitatory postsynaptic currents (evoked EPSCs: eEPSCs) were evoked in the second-order neurons by electrical stimulation of the tractus solitarius. DEX reversibly decreased the eEPSC amplitude in a concentration-dependent manner. The DEX-induced inhibition of eEPSC was accompanied by an increased paired-pulse ratio. Miniature EPSCs (mEPSCs) were also recorded in the presence of Cd²⁺ or tetrodotoxin. DEX decreased the frequency of mEPSCs without affecting their amplitude. Topically applied AMPA provoked an inward current in the neurons, which was unchanged during the perfusion of DEX. BD1047, a σ -1-receptor antagonist, did not block the inhibitory effect of DEX on the eEPSCs, but antagonized the inhibition of eEPSCs induced by SKF-10047, a σ -1 agonist. Haloperidol, a σ -1 and -2 receptor ligand, had no influence on the inhibitory action of DEX. These results suggest that DEX inhibits glutamate release from the presynaptic terminals projecting to the second-order NTS neurons, but this effect of DEX is not mediated by the activation of σ receptors.

Keywords: dextromethorphan, nucleus tractus solitarius, excitatory postsynaptic current, patch clamp, σ receptor

Introduction

Dextromethorphan (DEX), a non-opioid dextrorotatory morphinan derivative, is one of the most widely used antitussives for suppressing cough. DEX is thought to act centrally to elevate the cough threshold (1). Besides the antitussive activity, DEX has an anticonvulsant effect by attenuating the glutamate-induced neurotoxicity (2, 3) and a neuroprotective property against the ischemia-induced brain damage (4, 5). Although DEX exhibits such useful actions in the central nervous system (CNS), the exact sites at which it acts and its mechanism are not fully understood. It has been reported that DEX inhibits ion channels including voltage-dependent Na⁺ channels (6) and Ca²⁺ channels (VDCCs) (7). Furthermore, it modifies several ligand-gated currents including glycine-

induced currents (8) and serotonin-induced inward currents (9). More intriguingly, DEX acts as an antagonist of *N*-methyl-D-aspartate (NMDA) receptors (10) or as an agonist of σ receptors (11). Drugs having the characteristics of blocking NMDA receptors (12, 13) and stimulating σ receptors (14 – 16) possess antitussive activity. Therefore, both receptors are proposed to be main targets for DEX in its antitussive action.

The caudal division of nucleus tractus solitarius (NTS) and its neighboring ventromedial area are considered as kernels constituting the cough network (17, 18). The NTS is the first synaptic site between the airway afferent fibers and second-order neurons and thought to be a first “gate” region involved in the cough reflex pathway (19). The excitatory synaptic transmission in the second-order NTS neurons is predominantly mediated by glutamate through the activation of α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA) receptors (20 – 22). In the NTS, the presynaptic modulation of synaptic transmission by activating the metabotropic

*Corresponding author. haji@dpc.agu.ac.jp

Published online in J-STAGE on April 13, 2011 (in advance)

doi: 10.1254/jphs.11008FP

glutamate receptors (23), opioid receptors (24, 25), neurokinin 1 receptors (26), or dopamine receptors (27) has been demonstrated. Furthermore, the σ -receptor (28, 29) and NMDA-receptor mechanisms (30, 31) are also suggested to modulate the release of neurotransmitter in other regions of the CNS. Recently, it has been reported that DEX dose-dependently inhibits cough without effect on the basal respiratory rate when microinjected bilaterally into the NTS (32). Since the σ -1-receptor mechanism affects the glutamatergic neurotransmission in the CNS (29) and since σ receptors are present in the NTS (33, 34), it is postulated that DEX could modulate the glutamatergic transmission in the NTS by activating the σ receptors at least partly. To address this hypothesis, we examined the effects of DEX on excitatory synaptic transmission in the second-order NTS neurons using the brainstem slice preparation of guinea pigs. Consequently, we found that DEX reduced the release of glutamate from the presynaptic terminals in the NTS, but the σ receptors were not involved in this action of DEX.

Materials and Methods

Slice preparations

This study was conducted in accordance with Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Slice preparations were made as described previously (24, 25). Briefly, male Hartley guinea pigs (200–400 g) were deeply anesthetized with inhalation of halothane and decapitated. The brainstem was excised and submerged in ice-cold low-calcium artificial cerebrospinal fluid (aCSF) containing 125 mM NaCl, 2.5 mM KCl, 0.1 mM CaCl₂, 5 mM MgCl₂, 1.25 mM NaH₂PO₄, 12.5 mM D-glucose, 0.4 mM L-ascorbic acid, 25 mM NaHCO₃. The pH was 7.4 when continuously bubbled with 95% O₂–5% CO₂. The brainstem was glued to the cutting stage of a vibrating slice cutter (DTK-1000; Dosaka, Kyoto) with the caudal side up. Two to three transverse slices of a 400- μ m thickness including the NTS region, extending from 0.5-mm caudal to 1.2-mm rostral to the obex, were made, to which the superior laryngeal nerve (SLN) afferents that transmit the laryngeal tussigenic signals project predominantly in guinea pigs (35). The slices were incubated in standard aCSF of the following composition: 125 mM NaCl, 2.5 mM KCl, 2 mM CaCl₂, 1.3 mM MgCl₂, 1.25 mM NaH₂PO₄, 12.5 mM D-glucose, 0.4 mM L-ascorbic acid, 25 mM NaHCO₃, saturated with 95% O₂–5% CO₂, for 30–40 min at 37°C, and then kept at room temperature (23 \pm 1°C) until the recording. The slice was fixed in a recording chamber (ca. 0.4 ml volume, RC-26GLP; Warner Instruments, Hamden, CT, USA) under a nylon mesh attached stainless anchor, and

then submerged in and continuously perfused with the standard aCSF at a flow rate of 1–2 ml/min. The neurons with small diameters (<15 μ m), which may receive predominantly excitatory synaptic inputs (20, 22), were visually preselected in the medial and dorsal regions of NTS with an infrared-differential interference contrast videomicroscope (BX-51WI; Olympus, Tokyo and C2741; Hamamatsu Photonics, Hamamatsu), and their images were stored on a hard disk for later analysis.

Whole-cell transmembrane current recording

Recordings were made at room temperature. The composition of the intracellular solution was as follows: 120 mM potassium gluconate, 6 mM NaCl, 5 mM CaCl₂, 2 mM MgCl₂, 2 mM MgATP, 0.3 mM NaGTP, 10 mM EGTA, 10 mM HEPES, pH adjusted to 7.2 with KOH. The tip resistance of the electrodes ranged from 4 to 6 M Ω when filled with the pipette solution. After establishing the cell-attached configuration with a seal resistance of 1–10 G Ω , the whole-cell mode was established with a brief negative current and pressure pulse. The series resistance (<30 M Ω) and membrane capacitance were compensated and checked regularly during the recording. At a holding potential of –60 mV, the transmembrane current was recorded with a patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, USA) with a high-cut filter at 2 kHz. The membrane current was sampled on-line at 4 kHz (PowerLab; AD Instruments, Castle Hill, Australia) and stored on the hard disk of a computer.

A stainless concentric bipolar electrode was placed on the tractus solitarius (TS) ipsilateral to the recorded neuron (20). The distance between the two poles was 100 μ m. The intensity of stimulation was set to a minimal voltage with which every pulse of the TS stimulation constantly induced a clear monosynaptic excitatory postsynaptic current (EPSC) peak without failure. Usually, the stimulation intensity was 2.0–20 V and the pulse duration was 0.1 ms. Stimulation was given every 10 s. The evoked EPSC (eEPSC) with a latency of less than 7.5 ms with little variation was judged to be monosynaptic (20). For calculation of the paired-pulse ratio (PPR), two serial eEPSCs were induced by double stimulus pulses at an inter-pulse interval of 20–30 ms to the TS (36). The peak amplitudes of the first and second eEPSCs were determined. The PPR was defined as the peak amplitude of the second eEPSC divided by that of the first eEPSC. For recording miniature EPSCs (mEPSCs), either Cd²⁺, which blocks the VDCCs in the nerve terminal, or tetrodotoxin (TTX), which blocks action potential-dependent transmitter release from the presynaptic terminal, was added to the aCSF.

Drugs

The following drugs were dissolved in aCSF: dextromethorphan hydrobromide monohydrate (DEX, 0.1 – 1.0 mM; Wako Pure Chemical, Osaka), naloxone (10 μ M; Sankyo, Tokyo), 6-cyano-7-nitroquinoline-2,3-dione disodium (CNQX, 10 μ M; Sigma, St. Louis, MO, USA), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA, 0.1 mM; Research Biochemicals international, Natick, MA, USA), picrotoxin (0.1 mM, Sigma), CdCl₂ (0.1 mM, Wako Pure Chemical), tetrodotoxin (TTX, 1.0 μ M; Wako Pure Chemical), SKF-10047 (*N*-allyl-normetazocine; Tocris, Ballwin, MO, USA) and BD1047 [*N*-(2-(3,4-dichlorophenyl)ethyl)-*N*-methyl-2-(dimethylamino)ethylamine dihydrobromide; Tocris]. Haloperidol hydrochloride (Tocris) was dissolved in DMSO at a final concentration of 0.4%. The subtype selectivity for σ receptors and concentrations of SKF-10047 (0.3 mM), BD1047 (0.1 mM), and haloperidol (0.1 mM) were taken from previous reports (32, 37 – 39). Application of all drugs except AMPA was delivered for 5 – 6 min by gravity feed from 60-ml reservoirs bubbled with 95% O₂ – 5% CO₂. The neuronal recording during the first 60 s was not included in the data analysis to compensate for dead space of tubing between the bath and reservoirs. The aCSF containing AMPA was applied for 5 s locally to the slice with a glass pipette (0.7-mm i.d.) whose tip was placed at the most upstream of the recording chamber and never moved throughout the recording. Bath perfusion with aCSF was discontinued during application of AMPA and restarted after application.

Data analysis

The recorded membrane currents were analyzed off-line with Chart 5 and Scope 4 (AD Instruments). The amplitude of eEPSC was calculated as the difference between the post-stimulus through current and the pre-stimulus mean current over 10 ms. Averaged traces of eEPSCs were made by adding 5 to 10 sampled data using stimulus pulses as a trigger. The mEPSCs were detected by ORIGIN software (Origin Lab, Northampton, MA, USA) where the threshold for detection was set just above baseline noises of the recordings, which was 3 – 5 pA. For the group analysis, the number and amplitude of mEPSCs occurring for 1 min were compared before, during DEX (4 min after onset of DEX perfusion), and during washout (10 min after washout). Group values are expressed as the mean \pm S.E.M. Derived parameters were compared using a one-way analysis of variance (ANOVA) followed by multiple comparisons (Duncan's test), paired *t*-test, or Kolmogorov-Smirnov test with the level of significance set at $P < 0.05$.

Results

Effects of DEX on eEPSCs in NTS neurons

The second-order NTS neurons showed monosynaptic eEPSCs in response to electrical stimulation of the TS. The mean amplitude of eEPSCs was 158 ± 14 pA ($n = 43$) at a holding potential of -60 mV. The eEPSCs were completely abolished by CNQX (10 μ M) application in 12 neurons tested (data not shown), indicating that the neurons analyzed here receive predominantly glutamatergic synaptic inputs through the activation of non-NMDA or AMPA receptors. Polysynaptic inhibitory currents were not observed with or without picrotoxin (0.1 mM). The mean resting membrane potential was -63.1 ± 5.3 mV ($n = 43$). Bath application of DEX (0.3 mM) decreased the peak amplitude of eEPSCs in second-order neurons (Fig. 1A). This effect of DEX gradually waned after reperfusion of normal aCSF and the eEPSC amplitude recovered to the control level about 10 min after washout of DEX (Fig. 1B). The inhibitory action of DEX on eEPSCs is statistically significant and concentration-dependent (Fig. 1C). The PPR (the peak amplitude of second eEPSC divided by that of first eEPSC) was calculated when double stimulus pulses were applied to the TS. DEX decreased the first eEPSC more strongly than the second one (Fig. 1: A, D). Consequently, the PPR significantly increased from 0.62 ± 0.06 to 0.87 ± 0.08 ($P < 0.01$, $n = 14$) during the perfusion of DEX.

The site of action of DEX

From the above-described results, it is postulated that DEX acts at the presynaptic terminal synapsing to the NTS neuron rather than at the postsynaptic membrane. To address this issue, two experiments were performed: 1) determination of the effects of DEX on mEPSCs and 2) determination of the effects of DEX on AMPA-induced currents in the NTS neurons. The effects of DEX on mEPSCs were investigated in 12 neurons in the presence of Cd²⁺ (0.1 mM, $n = 7$) or TTX (1.0 μ M, $n = 5$). The mean frequency and amplitude of mEPSCs were 14.6 ± 2.5 events/s and 10.0 ± 0.9 pA in the control condition, respectively. The mEPSCs were completely abolished by perfusion of CNQX (10 μ M, data not shown). Figure 2 illustrates a typical example of DEX's action under perfusion of Cd²⁺. Application of DEX (0.3 mM) decreased the frequency of mEPSCs but had no effect on their amplitude (Fig. 2A). Cumulative distribution plots of the amplitude and inter-event interval (IEI) of mEPSCs also show that the former curve remained unchanged ($P > 0.05$) and the latter curve significantly shifted rightward ($P < 0.01$) during the perfusion of DEX (Fig. 2B). Percent changes in the mean frequency and amplitude of mEPSCs in the presence of Cd²⁺ are shown

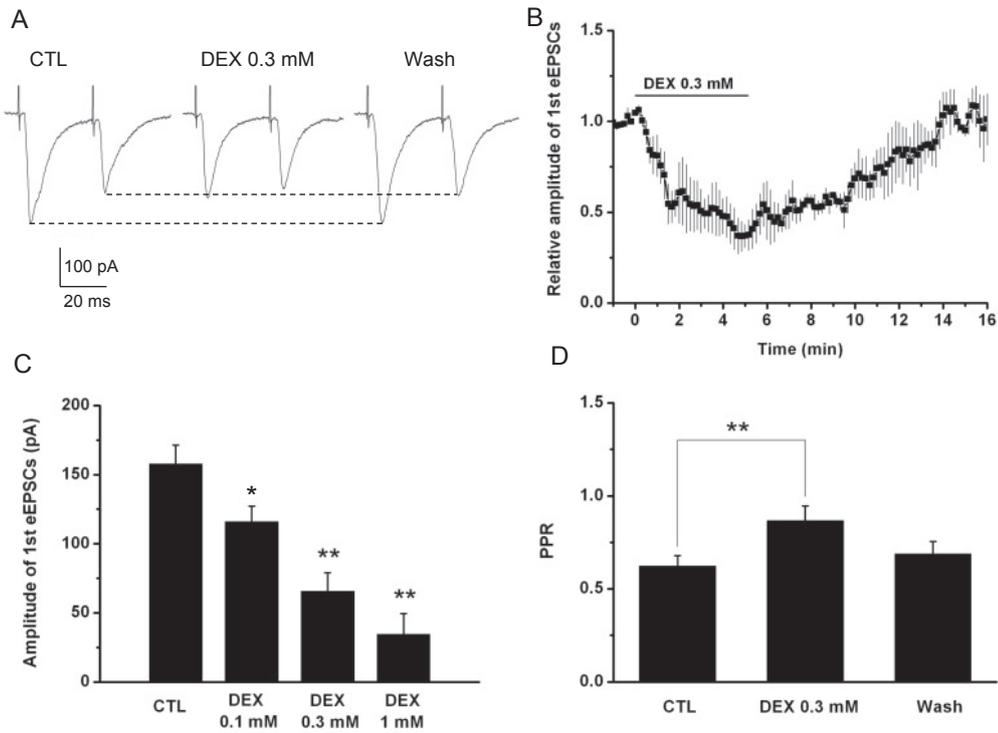


Fig. 1. Effects of DEX on the eEPSCs in second-order NTS neurons. A) Traces of eEPSCs during control (CTL), 4 min after the onset of DEX (0.3 mM), and 10 min after washout (Wash). Dashed lines indicate the control level of the first and second eEPSCs. B) Time course of the change in eEPSC amplitude. DEX (0.3 mM) was applied during a horizontal bar. Data are reported as the mean \pm S.E.M. ($n = 7$). C) Changes in the amplitude of first eEPSCs. Data are reported as the mean \pm S.E.M. ($n = 14$, * $P < 0.05$, ** $P < 0.01$ vs. control, ANOVA followed by Duncan's test). D) The paired-pulse ratio (PPR) during the control period (CTL), 4 min after the onset of DEX (DEX 0.3 mM), and 10 min after washout (Wash). Data are reported as the mean \pm S.E.M. ($n = 14$, ** $P < 0.01$ vs. control, paired t -test).

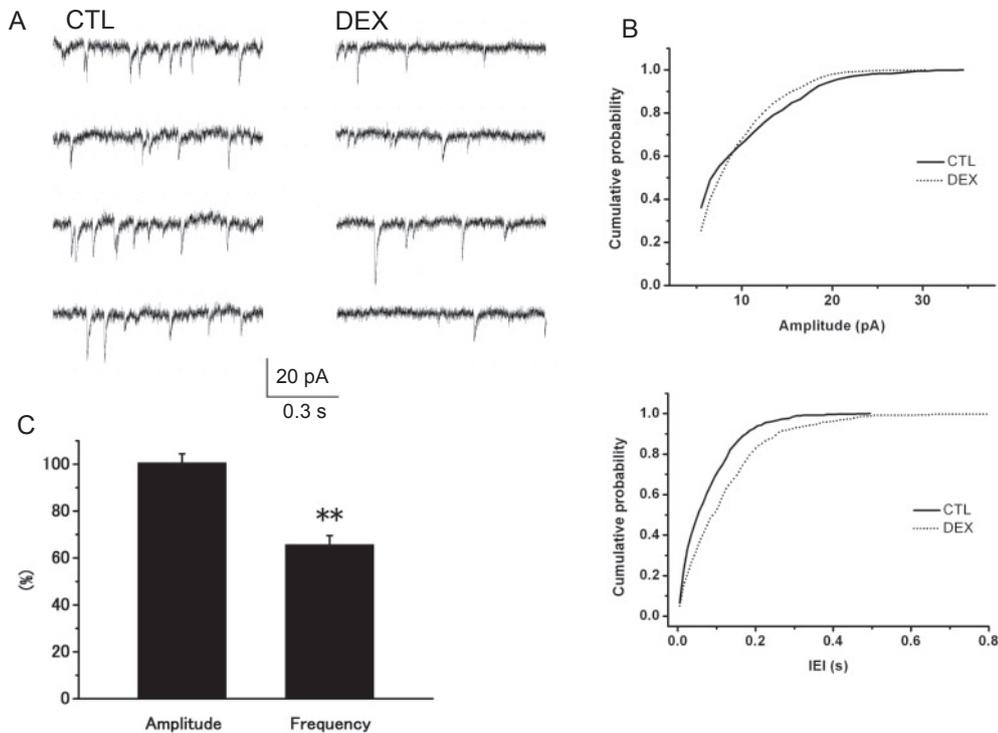


Fig. 2. Effects of DEX on the mEPSCs in second-order NTS neurons. A) Traces of mEPSCs during the control period (CTL) and 4 min after the onset of DEX (0.3 mM) in the presence of Cd^{2+} (0.1 mM). B) Cumulative probabilities of the amplitude ($P > 0.05$, Kolmogorov-Smirnov test) and inter-event intervals (IEI) ($P < 0.01$, Kolmogorov-Smirnov test) of mEPSCs in the neuron in A. C) Relative changes in the amplitude and frequency of mEPSCs by DEX. Data are the mean \pm S.E.M. ($n = 7$, ** $P < 0.01$ vs. control, paired t -test).

in Fig. 2C. Furthermore, DEX had a similar inhibitory effect on mEPSCs in the presence of TTX. The mean frequency and amplitude of mEPSCs during perfusion of DEX under TTX were, respectively, 9.5 ± 1.8 events/s

($P < 0.05$) and 9.5 ± 1.0 pA ($P > 0.05$). The effects of DEX on the membrane current induced by topically applied AMPA (0.1 mM) were examined (Fig. 3). Exogenous AMPA provoked a large inward current, of which

the amplitude was 246.9 ± 34.8 pA ($n = 7$). DEX even at the highest concentration (1.0 mM) used in this experiment had no detectable effect on the AMPA-induced current ($P > 0.05$).

Effects of σ -receptor ligands on the action of DEX

σ Receptors are divided into at least two subtypes, σ -1 and -2 receptors (40). We firstly examined the effects of

a σ -1-receptor agonist, SKF-10047 (0.3 mM), on the eEPSCs in four neurons (Fig. 4A). SKF-10047 significantly decreased the amplitude of eEPSCs. This inhibitory effect of SKF-10047 was blocked by BD1047 (0.1 mM), a σ -1-receptor antagonist, suggesting that the σ -1-receptor mechanism modulates the glutamatergic transmission in the NTS. As shown in Fig. 4B, however, DEX had an inhibitory effect on the eEPSCs during the

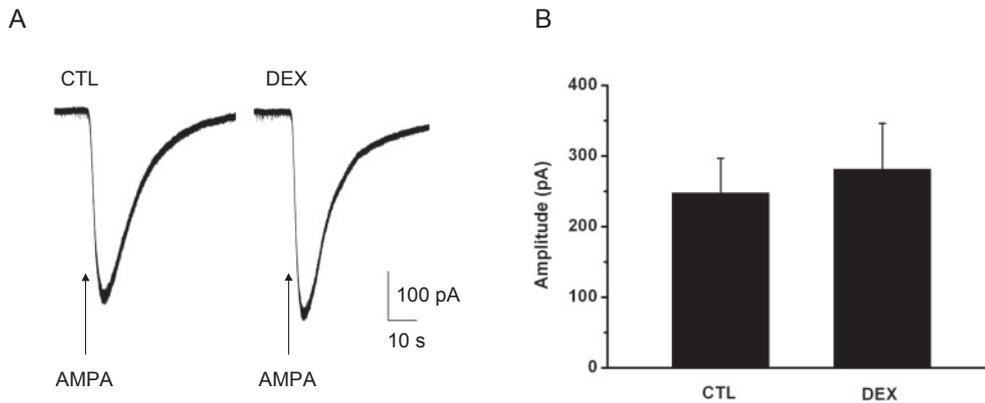


Fig. 3. Effects of DEX on the AMPA-induced currents in second-order neurons. A) Traces of the inward currents during the control period (CTL) and 4 min after the onset of DEX (1.0 mM). AMPA (0.1 mM) was topically applied (indicated by arrows). B) The mean amplitude of AMPA-induced currents. Data are the mean \pm S.E.M. ($n = 7$ vs. control, $P > 0.05$, paired t -test).

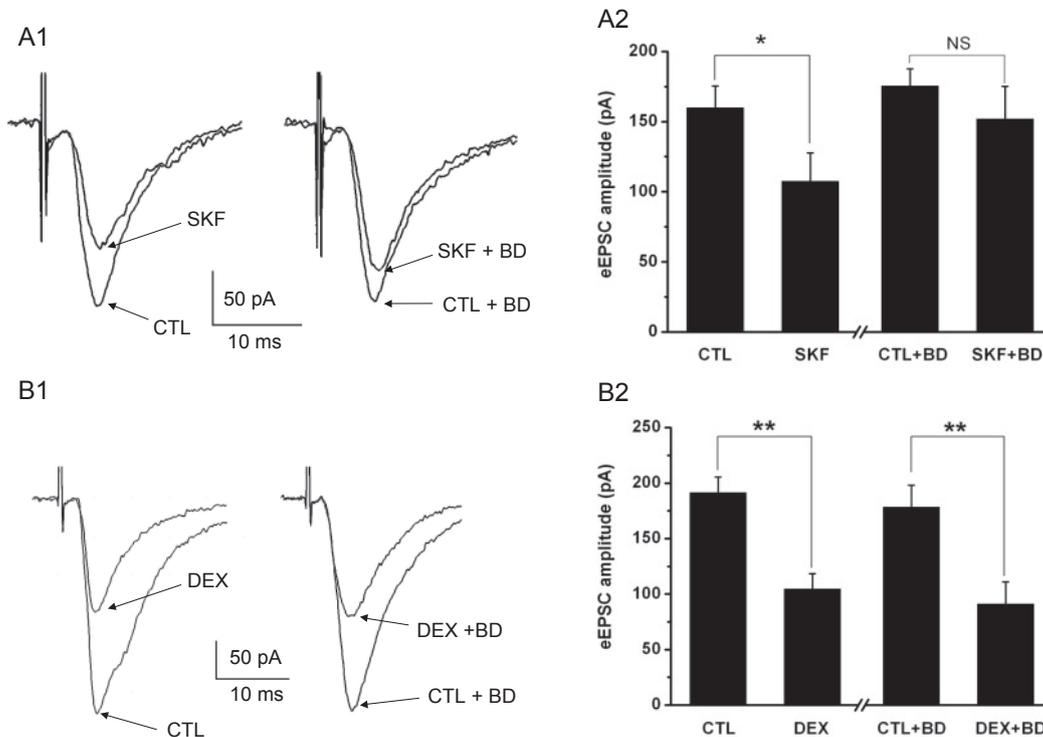


Fig. 4. Effects of BD1047 (0.1 mM) on the inhibitory actions of SKF-10047 (0.3 mM) and DEX (0.3 mM). A1) Left panel: Traces of eEPSCs during the control period (CTL) and 4 min after the onset of SKF-10047 (SKF). Right panel: Traces of eEPSCs during the control period (CTL + BD) and 4 min after the onset of SKF-10047 (SKF + BD) in the presence of BD1047. A2) The mean amplitude of eEPSCs. Data are the mean \pm S.E.M. ($n = 4$, $*P < 0.05$ vs. control, paired t -test, NS; not significant). B1) Left panel: Traces of eEPSCs during the control period (CTL) and 4 min after the onset of DEX. Right panel: Traces of eEPSCs during the control period (CTL + BD) and 4 min after the onset of DEX (DEX + BD) in the presence of BD1047. (B2) The mean amplitude of eEPSCs. Data are the mean \pm S.E.M. ($n = 11$, $**P < 0.01$ vs. control, paired t -test).

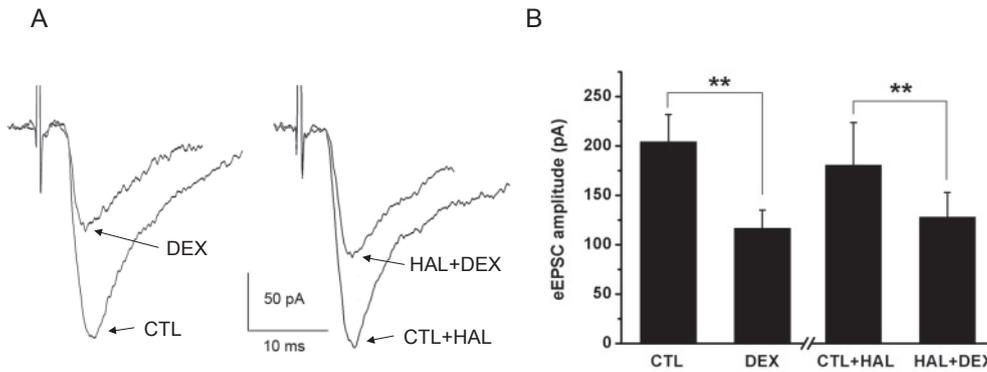


Fig. 5. Effects of haloperidol (0.1 mM) on the inhibitory action of DEX (0.3 mM). A) Left panel: Traces of eEPSCs during the control period (CTL) and 4 min after the onset of DEX (DEX). Right panel: Traces of eEPSCs during control (CTL + HAL) and 4 min after the onset of DEX (HAL + DEX) in the presence of haloperidol. B) The mean amplitude of eEPSCs. Data are reported as the mean \pm S.E.M. ($n = 7$, $**P < 0.01$ vs. control, paired t -test).

perfusion of BD1047 in a similar manner to the condition without this antagonist ($n = 11$). To assess involvement of the σ -2-receptor mechanism in the inhibition of eEPSCs induced by DEX, the influence of haloperidol, which binds both σ -1 and -2 receptors (41), on the DEX's action was examined (Fig. 5). The inhibitory effect of DEX on the eEPSC amplitude was not blocked by haloperidol ($n = 7$). Neither BD1047 nor haloperidol alone had any significant effect on the eEPSCs. Additionally, DEX decreased the amplitude of eEPSCs from 161 ± 16 pA to 67 ± 9 pA ($P < 0.01$, $n = 5$) in the presence of naloxone ($10 \mu\text{M}$). The magnitude of inhibition was similar to that without naloxone (see Fig. 1), suggesting that opioid receptors are not involved in the inhibitory effects of DEX on the eEPSCs.

Discussion

The present study demonstrated that DEX reversibly and concentration-dependently decreased the amplitude of eEPSCs in the second-order NTS neurons. It increased the PPR resulting from a stronger inhibition of the first eEPSC amplitude than the second one. Furthermore, DEX decreased the frequency of action potential- and VDCC-independent mEPSCs without any change in their amplitude. These results suggest that DEX acts at the presynaptic terminals and inhibits the release of glutamate in the NTS. It is supported by the evidence that the inward current induced by exogenously applied AMPA was unaltered during the perfusion of DEX. This central action of DEX in the NTS neurons could lead, at least partly, to suppression of the cough production. The presynaptic action of DEX has been also demonstrated in the hippocampal neurons (42) and in the cerebrocortical synaptosomes (43).

It has been reported that compounds including dizocilpine and DEX, which are functional antagonists against the NMDA receptors (10, 44), exhibit antitussive activity (12). Recently, we have demonstrated using the *in vivo* cat model that blockade of NMDA receptors by dizocilpine suppresses the fictive cough by elevating the cough threshold and suggested that NMDA-receptor mechanisms are involved in depolarization of augmenting expiratory neurons during the expulsive phase of fictive cough (13). Furthermore, Woodhall and coworkers (30) revealed that activation of the presynaptic NMDA receptors facilitated glutamatergic synaptic transmission in the entorhinal cortical neurons. However, the functional significance of presynaptic NMDA receptors has not been clarified in the NTS, although immunohistochemical evidence for distribution of NMDA receptors on the vagal afferent terminals was reported (45). Since both eEPSCs and mEPSCs recorded in the NTS neurons are mediated by CNQX-sensitive non-NMDA receptors and unchanged in the presence of dizocilpine (24, 25), the blocking action of DEX against the NMDA receptors is excluded in the present case.

DEX is 11–40 times more effective when given directly into the left vertebral artery than intravenously in the cat cough model (46, 47). Moreover, it has been reported that DEX acts on the σ receptors as an agonist (1, 11) and that agonists of σ receptors show an antitussive activity (15, 16). These previous results suggest that DEX preferentially acts within the brainstem cough network rather than at the peripheral sites by activating the σ receptors. In the present study, however, the specific σ -1-receptor antagonist BD1047 did not block the inhibitory action of DEX on the eEPSCs in the second-order neurons. Therefore, it seems unlikely that DEX acts as a σ -1-receptor agonist in the NTS. Nevertheless, the σ -1-

receptor mechanism may play a role in modulation of the glutamate release in the NTS, because the σ -1 agonist SKF-10047 decreased the eEPSC amplitude and BD1047 antagonized its inhibitory action. Recently, Brown et al. (14) demonstrated the peripheral component of antitussive action of DEX, where inhalation of aerosolized BD1047 prevented the antitussive action of DEX given intraperitoneally. This indicates that the peripheral action of DEX is mediated by the σ -1-receptor mechanism. It is conceivable that a systemically administered drug may have both the peripheral and central sites of action and that the mode of action with which DEX interacts is different between the two types of sites. It is shown that DEX binds with relatively high affinity to σ -1 receptors and with very low affinity to σ -2 receptors in mouse cells (44). Indeed, haloperidol neither affected the eEPSCs nor blocked the DEX-induced inhibition of eEPSCs in the present study. There is yet no positive evidence available to indicate that the σ -2 receptors contribute to the inhibitory effects of DEX on the excitatory transmission in the NTS. Over all, both σ -1 and -2 receptors are not involved in the DEX-induced inhibition of glutamatergic transmission in the NTS. Furthermore, naloxone, an antagonist of opioid receptors, could not block the DEX action, suggestive of no contribution of opioid receptors as well.

This is the first report that DEX presynaptically inhibited the glutamatergic synaptic transmission in the NTS. Several lines of evidence suggest that DEX inhibits the VDCCs (7, 48). Lin and coworkers (43) have shown an interesting result using the synaptosomal model that DEX inhibits the release of glutamate from presynaptic terminals by directly inhibiting N- and P/Q-type Ca^{2+} channels. In the NTS, the spontaneously occurring EPSCs are characteristic in the second-order neurons, which are generated by excitatory connections between intrinsic neurons (20–22). Most of the spontaneous EPSCs are action potential-dependent because they are TTX-sensitive, and the remaining EPSCs, that is, mEPSCs, are VDCC-independent because they are Cd^{2+} -insensitive. From the present result that DEX reduced only the frequency of mEPSCs with no change in their amplitude in the presence of TTX or Cd^{2+} , we conclude that DEX does not modify the Ca^{2+} channels, or Ca^{2+} entry, at the presynaptic terminal. Unfortunately, the mechanisms by which DEX inhibited eEPSCs and mEPSCs in the NTS neurons could not be determined in this study and more research would be needed to identify the precise mechanism of DEX's action.

Two limitations exist in the present study. Firstly, since many visceral afferent inputs including cardiovascular, respiratory, or gastrointestinal processing-related information are known to terminate in the NTS at several subnuclei (49), it is difficult to discriminate accurately

the projection sites of cough-related afferents and the subdivision of cough-related NTS from others. Previously we have investigated the projection sites of the SLN, to which electrical stimulation can induce the cough reflex *in vivo*, in the NTS by using a fluorescent dye in the guinea pig (35). The SLN afferents terminated preferentially in the ipsilateral region, extending from 0.5-mm caudal to 1.2-mm rostral to the obex. The medial and dorsal regions of NTS including the interstitial nucleus were densely stained. Based on that histochemical result, we selected second-order neurons located at the medial and dorsal part of the NTS. However, we could not rule out the possibility that DEX inhibits excitatory transmission in the NTS neurons receiving other visceral afferents. On the other hand, it is suggested that the second-order NTS neurons receive inputs from a number of sensory afferent fibers with varying indirect effects on cough threshold, frequency, and intensity (50, 51). For example, acute stimulation of peripheral chemoreceptors and nasal sensory afferents up-regulates the cough reflex, while stimulation of bronchopulmonary C-fiber, cardiac, and abdominal afferents may down-regulate it. If some of the second-order neurons recorded in this study receive information other than tussigenic information, they might modulate the cough production indirectly. Secondly, the concentration of DEX used in this report (0.1–1.0 mM) is considerably higher than the IC_{50} (10–100 μM) for the NMDA-induced current and the voltage-gated Na^+ and Ca^{2+} currents in cultured neurons (4). DEX is shown to be highly lipophilic and the octanol/water distribution coefficient is 40 (52), suggestive of a good brain penetration of DEX (53). More directly, the concentrations of DEX in the blood and brain were measured after DEX (30 mg/kg) was administered intraperitoneally in the rat (54). In that report, the brain/plasma ratio of DEX was 8 and the concentration of DEX in the brain was around 86 μM . Since DEX exhibits antitussive activity when a dose of 30–60 mg/kg is administered intraperitoneally in the guinea pig *in vivo* (14), the concentration of DEX in the brain is estimated to be 0.09–0.18 mM. This is comparable with the concentrations used in our study. However, the relevant free brain concentration of DEX is unknown and likely to be much lower. Therefore, there remains a suspicion that the action of DEX observed here may not be involved directly in cough suppression.

References

- 1 Tortella FC, Pellicano M, Bowery NG. Dextromethorphan and neuromodulation: old drug coughs up new activities. *Trends Pharmacol Sci.* 1989;10:501–507.
- 2 Choi DW, Peters S, Viseskul V. Dextrorphan and levorphanol selectively block N-methyl-D-aspartate receptor-mediated neurotoxicity on cortical neurons. *J Pharmacol Exp Ther.* 1987;242:

- 713–720.
- 3 Ferkany JW, Borosky SA, Clissold DB, Pontecorvo MJ. Dextromethorphan inhibits NMDA-induced convulsions. *Eur J Pharmacol.* 1988;151:151–154.
 - 4 Netzer R, Pflimlin P, Trube G. Dextromethorphan blocks N-methyl-D-aspartate-induced currents and voltage-operated inward currents in cultured cortical neurons. *Eur J Pharmacol.* 1993;238:209–216.
 - 5 Prince DA, Feeser HR. Dextromethorphan protects against cerebral infarction in a rat model of hypoxia-ischemia. *Neurosci Lett.* 1988;85:291–296.
 - 6 Trube G, Netzer R. Dextromethorphan: cellular effects reducing neuronal hyperactivity. *Epilepsia.* 1994;35 Suppl 5:S62–S67.
 - 7 Carpenter CL, Marks SS, Watson DL, Greenberg DA. Dextromethorphan and dextrorphan as calcium channel antagonists. *Brain Res.* 1988;439:372–375.
 - 8 Takahama K, Fukushima H, Isohama Y, Kai H, Miyata T. Inhibition of glycine currents by dextromethorphan in neurones dissociated from the guinea-pig nucleus tractus solitarii. *Br J Pharmacol.* 1997;120:690–694.
 - 9 Ishibashi H, Kuwano K, Takahama K. Inhibition of the 5-HT(1A) receptor-mediated inwardly rectifying K⁺ current by dextromethorphan in rat dorsal raphe neurones. *Neuropharmacology.* 2000;39:2302–2308.
 - 10 Church J, Lodge D, Berry SC. Differential effects of dextrorphan and levorphanol on the excitation of rat spinal neurons by amino acids. *Eur J Pharmacol.* 1985;111:185–190.
 - 11 Chau TT, Carter FE, Harris LS. Antitussive effect of the optical isomers of mu, kappa and sigma opiate agonists/antagonists in the cat. *J Pharmacol Exp Ther.* 1983;226:108–113.
 - 12 Kamei J, Tanihara H, Igarashi H, Kasuya Y. Effects of N-methyl-D-aspartate antagonists on the cough reflex. *Eur J Pharmacol.* 1989;168:153–158.
 - 13 Haji A, Ohi Y, Tsunekawa S. N-methyl-D-aspartate mechanisms in depolarization of augmenting expiratory neurons during the expulsive phase of fictive cough in decerebrate cats. *Neuropharmacology.* 2008;54:1120–1127.
 - 14 Brown C, Fezoui M, Selig WM, Schwartz CE, Ellis JL. Antitussive activity of sigma-1 receptor agonists in the guinea-pig. *Br J Pharmacol.* 2004;141:233–240.
 - 15 Kamei J, Iwamoto Y, Kawashima N, Hitosugi H, Misawa M, Kasuya Y. Involvement of haloperidol-sensitive sigma-sites in antitussive effects. *Eur J Pharmacol.* 1992;224:39–43.
 - 16 Kamei J, Iwamoto Y, Misawa M, Kasuya Y. Effects of rimcazole, a specific antagonist of sigma sites, on the antitussive effects of non-narcotic antitussive drugs. *Eur J Pharmacol.* 1993;242:209–211.
 - 17 Bolser DC, Davenport PW. Functional organization of the central cough generation mechanism. *Pulm Pharmacol Ther.* 2002;15:221–225.
 - 18 Shannon R, Baekey DM, Morris KF, Lindsey BG. Ventrolateral medullary respiratory network and a model of cough motor pattern generation. *J Appl Physiol.* 1998;84:2020–2035.
 - 19 Widdicombe JG. Afferent receptors in the airways and cough. *Respir Physiol.* 1998;114:5–15.
 - 20 Champagnat J, Denavit-Saubié M, Grant K, Shen KF. Organization of synaptic transmission in the mammalian solitary complex, studied in vitro. *J Physiol.* 1986;381:551–573.
 - 21 Fortin G, Champagnat J. Spontaneous synaptic activities in rat nucleus tractus solitarius neurons in vitro: evidence for re-excitatory processing. *Brain Res.* 1993;630:125–135.
 - 22 Kawai Y, Senba E. Organization of excitatory and inhibitory local networks in the caudal nucleus of tractus solitarius of rats revealed in in vitro slice preparation. *J Comp Neurol.* 1996;373:309–321.
 - 23 Chen CY, Ling EH, Horowitz JM, Bonham AC. Synaptic transmission in nucleus tractus solitarius is depressed by group II and III but not group I presynaptic metabotropic glutamate receptors in rats. *J Physiol.* 2002;538:773–786.
 - 24 Haji A, Ohi Y. Inhibition of spontaneous excitatory transmission induced by codeine is independent on presynaptic K⁺ channels and novel voltage-dependent Ca²⁺ channels in the guinea pig nucleus tractus solitarius. *Neuroscience.* 2010;169:1168–1177.
 - 25 Ohi Y, Kato F, Haji A. Codeine presynaptically inhibits the glutamatergic synaptic transmission in the nucleus tractus solitarius of the guinea pig. *Neuroscience.* 2007;146:1425–1433.
 - 26 Sekizawa S, Joad JP, Bonham AC. Substance P presynaptically depresses the transmission of sensory input to bronchopulmonary neurons in the guinea pig nucleus tractus solitarii. *J Physiol.* 2003;552:547–559.
 - 27 Kline DD, Takacs KN, Ficker E, Kunze DL. Dopamine modulates synaptic transmission in the nucleus of the solitary tract. *J Neurophysiol.* 2002;88:2736–2744.
 - 28 Mtchedlishvili Z, Kapur J. A presynaptic action of the neurosteroid pregnenolone sulfate on GABAergic synaptic transmission. *Mol Pharmacol.* 2003;64:857–864.
 - 29 Cobos EJ, Entrena JM, Nieto FR, Cendan CM, Cel Pozo E. Pharmacology and therapeutic potential of sigma₁ receptor ligands. *Cur Neuropharmacol.* 2008;6:344–366.
 - 30 Woodhall G, Evans I, Cunningham MO, Jones RSG. NR2B-containing NMDA autoreceptors at synapses on enterohinal cortical neurons. *J Neurophysiol.* 2001;86:1644–1651.
 - 31 Corlew R, Brasier DJ, Feldman DE, Philpot BD. Presynaptic NMDA receptors: newly appreciated roles in cortical synaptic function and plasticity. *Neuroscientist.* 2008;14:609–625.
 - 32 Canning BJ. Central regulation of the cough reflex: therapeutic implications. *Pulm Pharmacol Ther.* 2009;22:75–81.
 - 33 Alonso G, Phan V, Guillemain I, Saunier M, Legrand A, Anoaï M, et al. Immunocytochemical localization of the sigma-1 receptor in the adult rat central nervous system. *Neuroscience.* 2000;97:155–170.
 - 34 Walker JM, Bowen WD, Walker FO, Matsumoto RR, De Costa B, Rice KC. Sigma receptors: biology and function. *Pharmacol Rev.* 1990;42:355–402.
 - 35 Ohi Y, Yamazaki H, Takeda R, Haji A. Functional and morphological organization of the nucleus tractus solitarius in the fictive cough reflex of the guinea pigs. *Neurosci Res.* 2005;53:201–209.
 - 36 Debanne D, Guérineau NC, Gähwiler BH, Thompson SM. Paired-pulse facilitation and depression at unitary synapses in rat hippocampus: quantal fluctuation affects subsequent release. *J Physiol.* 1996;491:163–176.
 - 37 Johannessen M, Ramachandran S, Riemer L, Ramos-Serrano A, Ruoho AE, Jackson MB. Voltage-gated sodium channel modulation by sigma-receptors in cardiac myocytes and heterologous systems. *Am J Physiol Cell Physiol.* 2009;296:C1049–C1057.
 - 38 Massamiri T, Duckles SP. Interactions of sigma and phencyclidine receptor ligands with the norepinephrine uptake carrier in both rat brain and rat tail artery. *J Pharmacol Exp Ther.* 1991;256:519–524.

- 39 Smith KJ, Butler TR, Prendergast MA. Inhibition of sigma-1 receptor reduces N-methyl-D-aspartate induced neuronal injury in methamphetamine-exposed and -naive hippocampi. *Neurosci Lett*. 2010;481:144–148.
- 40 Quirion R, Bowen WD, Itzhak Y, Junien JL, Musacchio JM, Rothman RB, et al. A proposal for the classification of sigma binding sites. *Trends Pharmacol Sci*. 1992;13:85–86.
- 41 Lever JR, Gustafson JL, Xu R, Allmon RL, Lever SZ. Sigma 1 and sigma 2 receptor binding affinity and selectivity of SA4503 and fluoroethyl SA4503. *Synapse*. 2006;59:350–358.
- 42 Annels SJ, Ellis Y, Davies JA. Non-opioid antitussives inhibit endogenous glutamate release from rabbit hippocampal slices. *Brain Res*. 1991;564:341–343.
- 43 Lin TY, Lu CW, Wang SJ. Inhibitory effect of glutamate release from rat cerebrocortical synaptosomes by dextromethorphan and its metabolite 3-hydroxymorphinan. *Neurochem Int*. 2009;54:526–534.
- 44 Wong BY, Coulter DA, Choi DW, Prince DA. Dextrorphan and dextromethorphan, common antitussives, are antiepileptic and antagonize N-methyl-D-aspartate in brain slices. *Neurosci Lett*. 1988;85:261–266.
- 45 Aicher SA, Sharma S, Pickel VM. N-methyl-D-aspartate receptors are present in vagal afferents and their dendritic targets in the nucleus tractus solitarius. *Neuroscience*. 1999;91:119–132.
- 46 Chou DT, Wang SC. Studies on the localization of central cough mechanism; site of action of antitussive drugs. *J Pharmacol Exp Ther*. 1975;194:499–505.
- 47 Domino EF, Krutak-Krol H, Lal J. Evidence for a central site of action for the antitussive effects of caramiphen. *J Pharmacol Exp Ther*. 1985;233:249–253.
- 48 Church J, Fletcher EJ. Blockade by sigma site ligands of high voltage-activated Ca²⁺ channels in rat and mouse cultured hippocampal pyramidal neurones. *Br J Pharmacol*. 1995;116:2801–2810.
- 49 Andresen MC, Kunze DL. Nucleus tractus solitarius-gateway to neural circulatory control. *Annu Rev Physiol*. 1994;56:93–116.
- 50 Pratter MR. Overview of common causes of chronic cough: ACCP evidence-based clinical practice guidelines. *Chest*. 2006;129:59S–62S.
- 51 Widdicombe JG, Singh V. Physiological and pathophysiological down-regulation of cough. *Respir Physiol Neurobiol*. 2006;150:105–117.
- 52 Roos FJ, Zysset T, Reichen J. Differential effect of biliary and micronodular cirrhosis on oxidative drug metabolism. In vivo-in vitro correlations of dextromethorphan metabolism in rat models. *Biochem Pharmacol*. 1991;41:1513–1519.
- 53 Wills RJ, Martin KS. Dextromethorphan/dextrorphan disposition in rat plasma and brain. *Pharm Res*. 1988;5 Suppl:S-193.
- 54 Wu D, Otton SV, Kalow W, Sellers EM. Effects of route of administration on dextromethorphan pharmacokinetics and behavioral response in the rat. *J Pharmacol Exp Ther*. 1995;274:1431–1437.