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Short communication

Tolerance to inhibition by ethanol of *N*-methyl-D-aspartate-induced depolarization in rat locus coeruleus neurons in vitro

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Abstract

Intracellullar recordings were made in a pontine slice preparation of the rat brain containing the nucleus locus coeruleus. The pressure application of *N*-methyl-D-aspartate (NMDA) produced reproducible depolarizations of stable amplitude. Superfusion with ethanol (100 mM) for 15 min inhibited the depolarizing response to NMDA; the effect of ethanol was rapidly reversed on washout. When the superfusion time of ethanol (100 mM) was increased to 60 min, its inhibitory effect disappeared after 50 to 60 min. Moreover, after the subsequent washout of ethanol a withdrawal-like increase in the sensitivity to NMDA became evident. Hence, adaptive mechanisms of locus coeruleus neurons during the long-time contact with ethanol may be modelled in an in vitro system. © 1997 Elsevier Science B.V.

Keywords: Ethanol tolerance; Ethanol withdrawal; Locus coeruleus; NMDA receptor

1. Introduction

A growing amount of experimental data indicates that the characteristic signs following ethanol consumption are due to changes of certain neurotransmitter receptors and voltage-dependent ion channels (Weight, 1992). A likely target for ethanol effects is the *N*-methyl-D-aspartate (NMDA) type of excitatory amino acid receptor (Tabakoff and Hoffman, 1996). While the acute application of ethanol inhibits NMDA-induced cationic currents in various neurons, chronic treatment with ethanol leads to an upregulation of NMDA receptor function and binding (Hoffman and Tabakoff, 1994). Such an upregulation contributes both to adaptive changes during ethanol exposure and to withdrawal reactions after its termination.

Noradrenergic neurons of the nucleus locus coeruleus are involved in a number of cognitive and emotional processes (Foote et al., 1983). Moreover, the modulation of the spontaneous discharge of locus coeruleus neurons contributes to physical aspects of opioid (Rasmussen et al., 1990) and probably also ethanol (Engberg and Hajos, 1992a,b) withdrawal. Under in vivo conditions, acutely applied ethanol inhibits the NMDA-induced increase in the firing of locus coeruleus neurons (Engberg and Hajos, 1992a), whereas following cessation of chronic ethanol treatment an enhanced sensitivity to NMDA develops (Engberg and Hajos, 1992b).

It is difficult to evaluate the interaction between ethanol and NMDA in a slice preparation, because ethanol alters the extracellularly recorded basal firing rate of locus coeruleus neurons by itself (Shefner and Tabakoff, 1985; Fröhlich et al., 1994). Hence, the conclusion that ethanol and NMDA interact in a truly antagonistic manner at the NMDA receptor-channel (Fröhlich et al., 1994) is still controversial. The aim of the present experiments was twofold. Firstly, it was investigated whether intracellular recordings from locus coeruleus neurons confirm an attenuation of the NMDA-induced depolarization by ethanol. Secondly, it was attempted to clarify whether during a longer lasting contact with ethanol the inhibition of the excitatory effect of NMDA declines culminating in a withdrawal-like increase in NMDA-sensitivity after washout.

2. Materials and methods

2.1. Brain slice preparation

Mid-pontine slices of the rat brain were prepared and maintained as previously described (Nieber et al., 1995). In

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brief, male Wistar rats (150–200 g) were anaesthetized with ether and decapitated. Coronal slices about 400 μm thick, containing the caudal part of the locus coeruleus, were prepared in oxygenated medium at 1–4°C with a Lancer vibratome. A single slice was placed in a recording chamber and superfused at a rate of 2 ml/min with medium saturated with 95% O_2–5% CO_2 and kept at 35–36°C. The medium was composed of (in mM): NaCl, 126; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.3; $CaCl_2$, 2.4; $NaHCO_3$, 25 and glucose, 11. Ascorbic acid (0.3 mM) and EDTA (0.03 mM) were present in order to prevent the oxidation of noradrenaline.

2.2. Identification of locus coeruleus neurons and intracellular recording

Locus coeruleus neurons were identified both visually and by their electrophysiological properties, including a hyperpolarizing response to noradrenaline (Nieber et al., 1995). Recording of the membrane potential was carried out with glass microelectrodes filled with KCl (2 M; tip resistance, 60–100 MΩ) using a high impedance preamplifier and a bridge circuit (Axoclamp-2A; Axon Instruments). In order to prevent spontaneous firing, the membrane potential of locus coeruleus neurons was hyperpolarized by about 20 mV from rest by injecting a constant current. In some experiments, hyperpolarizing current pulses of constant amplitude and 250 ms duration were delivered at a frequency of 0.5 Hz. The apparent input resistance was calculated from the peak potential change produced. The membrane potential was displayed on a Gould RS 3200 pen recorder and was determined on withdrawal of the microelectrode from the cell at the end of each experiment.

2.3. Application of drugs

NMDA (10 mM; Sigma, Deisenhofen, Germany) was dissolved in medium and was applied by pressure pulses (60 kPa) from a micropipette (tip diameter, 10–20 μm). The duration of the pulses (10–580 ms) was adjusted so that the amplitude of depolarization was about 20 mV. NMDA was applied every 5 min throughout, except when superfusion with an ethanol-containing (or in control experiments an ethanol-free) medium was started or ceased. Ethanol (100 mM; Riedel de Haen; Seelze, Germany) was applied by changing the superfusion medium by means of three-way taps. At the constant flow rate of 2 ml/min about 30 s were required to reach the bath.

At the beginning of each experiment NMDA was applied three times. Then, ethanol was added to the superfusion medium 5 min before the next agonist application for 15 or 60 min in total and was washed out subsequently for 20 and 55 min, respectively. The amplitudes of the NMDA-induced depolarizations were normalized with respect to depolarizations recorded immediately before su-

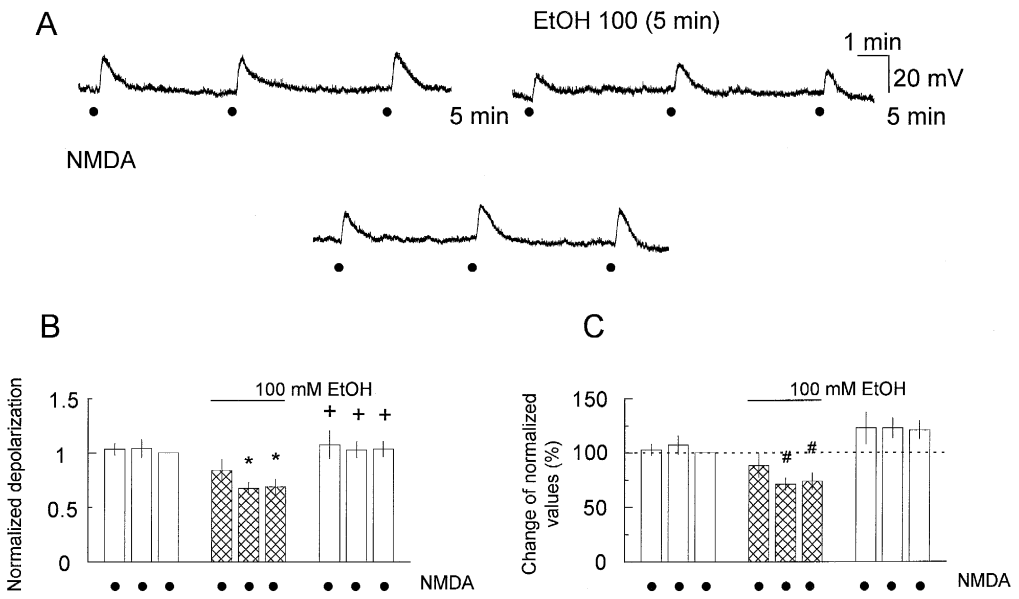

Fig. 1. Depolarization of rat locus coeruleus neurons by pressure application of NMDA and interaction with ethanol superfused for 15 min. (A) Representative tracings. NMDA (10 mM) was applied every 5 min from a micropipette (●). The duration of the pulses was adjusted so that the amplitude of depolarization was about 20 mV. The concentration of ethanol (EtOH) is expressed in mM; the superfusion time is bracketed. The periods between tracings are indicated. (B) Means ± S.E.M. of 7 experiments similar to those shown in (A). The amplitudes of the NMDA-induced depolarizations were normalized with respect to the depolarization recorded immediately before superfusion with ethanol (100 mM) (18.2 ± 0.9 mV; $n = 7$). (C) The normalized responses to NMDA in B were compared with the time-matched controls in Fig. 2A and expressed as percentage changes; the dotted line indicates 100%. Ethanol (100 mM) was present in the superfusion medium over the period marked by the horizontal bars. * $P < 0.05$; significant differences from the effect of NMDA before the application of ethanol. $^{+}$ $P < 0.05$; significant differences from the effect of NMDA immediately before the washout of ethanol. $^{\#}$ $P < 0.05$; significant differences from time-matched controls in Fig. 2A.

perfusion with ethanol. Then, the normalized responses to NMDA measured in the presence of ethanol (Fig. 1B and Fig. 2B) were compared with the time-matched controls obtained in its absence (Fig. 2A) and expressed as percentage changes (e.g., Fig. 2C).

2.4. Statistics

Means ± S.E.M. are given throughout. Multiple comparisons with a control value were performed by one-way analysis of variance followed by Bonferroni's *t*-test. All other comparisons with a control value were made by the unpaired Student's *t*-test. A probability level of 0.05 or less was considered to be statistically significant.

3. Results

A total of thirty-one locus coeruleus neurons included in this study had a resting membrane potential of 53.8 ± 1.9 mV. The generation of spontaneous action potentials was prevented by passing continuous hyperpolarizing current via the recording electrode.

When NMDA was applied every 5 min by pressure from a micropipette onto locus coeruleus neurons, depolarizations of a rather slow time-course were evoked (Fig. 1A). These responses had a tendency to slightly decrease over a period of 130 min, although statistical significance was reached only after 120 min (Fig. 2A). Although ethanol (100 mM) appeared to transiently hyperpolarize the locus coeruleus neuron shown in Fig. 1A, it did not alter the mean membrane potential of 18 neurons; measurements were made both before and 5 min after the application of ethanol (0.3 ± 0.6 mV; $P > 0.05$). After 10 min of exposure, ethanol (100 mM) inhibited the depolarizing effect of NMDA by 29.0 ± 5.7% ($n = 7$; $P < 0.05$; Fig. 1C) of the time-matched depolarization obtained in drug-free medium ($n = 13$; Fig. 2A). The inhibitory effect did not increase further after 15 min of exposure to ethanol (100 mM; Fig. 1C). When ethanol (100 mM) was washed out, the NMDA-induced depolarizations had a tendency to increase with respect to the time-matched control values (Fig. 1C).

In the following experiments the superfusion time of ethanol (100 mM) was increased from 15 to 60 min (Fig. 2B). Although the NMDA-induced depolarization was inhibited by 34.6 ± 5.4% ($P < 0.05$) after 10 min, this inhibition disappeared after 50 to 60 min (2.7 ± 19.8% at 60 min; $P > 0.05$; $n = 11$ each) (Fig. 2C). Furthermore, after the washout of ethanol most responses to NMDA tended to be larger than under control conditions, although a statistically significant difference was reached only in the case of

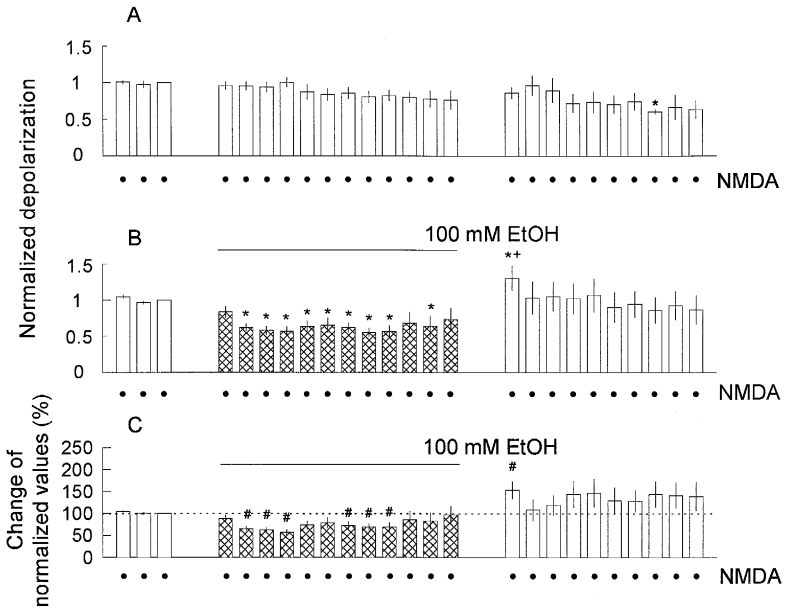

Fig. 2. Depolarization of rat locus coeruleus neurons by pressure application of NMDA and interaction with ethanol superfused for 60 min. NMDA (10 mM) was applied every 5 min from a micropipette (●). The duration of the pulses was adjusted so that the amplitude of depolarization was about 20 mV. (A) Responses to NMDA recorded in the absence of ethanol throughout. The amplitudes of the NMDA-induced depolarizations were normalized with respect to the 3rd depolarization (19.0 ± 0.7 mV; $n = 13$). (B) Responses to NMDA recorded before, during and after superfusion with ethanol (100 mM). The amplitudes of the NMDA-induced depolarizations were normalized with respect to the 3rd depolarization recorded immediately before superfusion with ethanol (19.7 ± 0.7 mV; $n = 11$). (C) The normalized responses to NMDA in B were compared with the time-matched controls in A and expressed as percentage changes; the dotted line indicates 100%. Ethanol (100 mM) was present in the superfusion medium over the period marked by the horizontal bars. * $P < 0.05$; significant differences from the effect of the 3rd application of NMDA immediately before the application of ethanol. $^{+}$ $P < 0.05$; significant differences from the effect of NMDA immediately before the washout of ethanol. $^{\#}$ $P < 0.05$; significant differences from time-matched controls in (A).

the first response which was facilitated by 53.0 ± 19.1% ($P < 0.05$; $n = 11$) (Fig. 2C). Possible non-selective effects of ethanol (100 mM) on the membrane properties of locus coeruleus neurons were excluded by the measurement of the input resistance both before (119.1 ± 13.2 MΩ) and 10 min (128.5 ± 14.0 MΩ; $P > 0.05$) or 60 min (128.5 ± 12.1 MΩ; $P > 0.05$; $n = 4$ each) after superfusion with ethanol (100 mM). Apparently the input resistance did not change either during the long-lasting presence of ethanol (100 mM) or after its washout (120.0 ± 16.2 MΩ at 5 min; 112.0 ± 9.1 MΩ at 50 min; $P > 0.05$ and $n = 4$ each).

4. Discussion

The present results confirm the observation that ethanol inhibits NMDA receptor-channels of locus coeruleus neurons (Engberg and Hajos, 1992a; Fröhlich et al., 1994). However, a somewhat higher concentration of ethanol was needed to interact with the depolarizing responses to NMDA than in most other neuronal systems (Weight, 1992). In agreement with this, our previous extracellular study showed an attenuation of the NMDA-evoked increase in the firing rate of locus coeruleus neurons at 10 to 100 mM ethanol only (Fröhlich et al., 1994). The ability of ethanol to inhibit NMDA-evoked activities varies regionally in the brain (Simson et al., 1993), probably according to the different subunit composition of NMDA receptors (Kuner et al., 1993). Hence, ethanol had a relatively low potency in the locus coeruleus, but still within the pharmacologically relevant concentration range; ethanol at 40 to 100 mM has behavioural effects in the rat that range from sedation through ataxia, with loss of the righting reflex at the higher end of this range (Majchrowicz and Hunt, 1976).

Locus coeruleus neurons are known to provide extensive projections to both cortical and subcortical structures (Foote et al., 1983). NMDA receptors may be situated both on the cell bodies of these noradrenergic neurons and at their nerve terminals in various areas of the brain. The activation of presynaptic receptors by NMDA increases the release of [^{3}H]noradrenaline and is concentration-dependently inhibited by ethanol (Fink et al., 1992). In the presence of Mg^{2+} in the superfusion medium and at a relatively hyperpolarized membrane potential, the major fraction of the excitatory postsynaptic potential (EPSP) evoked by focal electrical stimulation is due to the release of glutamate onto somatic non-NMDA receptors of locus coeruleus neurons (Cherubini et al., 1988). Although under these conditions the contribution of NMDA receptors is rather small, it may increase in the absence of Mg^{2+} or after repetitive nerve stimulation. In other areas of the brain, such as the hippocampus, EPSPs mediated by NMDA receptors are thought to be involved in neuronal plasticity, cognitive functions and certain forms of behaviour; their blockade by ethanol may interfere with these functions (Lovinger et al., 1989).

In our present study, locus coeruleus neurons developed, within a period of 50 to 60 min, tolerance to the inhibitory effect of ethanol on NMDA-induced depolarizations. When ethanol was subsequently washed out, a withdrawal-like increase in the sensitivity to NMDA became evident. Although the firing rate of locus coeruleus neurons is regulated both by intrinsic membrane properties and extrinsic synaptic mechanisms (Cherubini et al., 1988), ethanol may affect the discharge of action potentials mostly via modulation of the external excitatory input (Aston-Jones et al., 1982). Hence, alterations by ethanol of NMDA receptor function in the locus coeruleus is probably important for ethanol tolerance and dependence; the cellular mechanisms of adaptation may be acutely modelled in an in vitro test system.

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