Noradrenaline Increases High-Frequency Firing at the Calyx of Held Synapse During Development by Inhibiting Glutamate Release

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Leão, Ricardo M. and Henrique von Gersdorff. Noradrenaline increases high-frequency firing at the calyx of Held synapse during development by inhibiting glutamate release. J Neurophysiol 87: 2297–2306, 2002; 10.1152/jn.00761.2001. The mammalian auditory brain stem receives profuse adrenergic innervation, whose function is poorly understood. Here we investigate, during postnatal development, the effect of noradrenaline (NA) at the calyx of Held synapse in the rat medial nucleus of the trapezoid body (MNTB). We observed that NA inhibits the large glutamatergic EPSC, evoked by afferent fiber stimulation, in a dose-dependent manner. The inhibition was maximal (approximately 48%) at the concentration of 2 μM. It was antagonized by yohimbine and mimicked by the α2-adrenergic specific agonist UK14304. Both AMPA and NMDA receptor-mediated EPSCs were inhibited in parallel by NA, suggesting a presynaptic effect. Presynaptic recordings showed that NA inhibits the action potential (AP) generated Ca current by about 20%; however, NA did not significantly affect the presynaptic AP waveform. We thus conclude that the calyx of Held presynaptic terminal expresses α2-adrenergic receptors that inhibit its Ca current and thus glutamate release. Noradrenaline was effective in all cells tested from postnatal days 6 to 7 (P6–P7), and thereafter the number of responsive cells diminished, although half of the P14 cells tested still had EPSCs that were inhibited by NA. By contrast, activation by 1,2-amino-5-phosphonovaleric acid-sensitive metabotropic glutamate receptors strongly inhibited the EPSCs of all cells tested from P6 to P14. The effect of NA on postsynaptic action potential firing was dependent on the stimulus frequency. At 10 Hz, NA had no effect on firing probability; however, NA helped MNTB cells fire more action potentials during a 100-Hz train of stimuli, even though it did not increase the steady-state depressed EPSC, because it produced a smaller N-methyl-D-aspartate (NMDA) receptor-activated depolarizing plateau. We therefore suggest that the reduction by NA of the first few EPSCs in a train leads to a smaller NMDA depolarizing plateau and thus to increased firing probability at 100 Hz in young synapses. Surprisingly, the inhibition of glutamate release by NA can thus actually increase the excitability of MNTB neurons during early postnatal development.

INTRODUCTION

Many neurotransmitters and modulators in the central and peripheral nervous system act on presynaptic terminals inhibiting the release of neurotransmitters via activation of metabotropic receptors (Wu and Saggau 1997). Three mechanisms proposed to account for the presynaptic inhibition of transmitter release are: inhibition of presynaptic Ca channels, activation of presynaptic ion channels (e.g., K channels), and regulation of the synaptic vesicle fusion complex by modulation of the proteins involved in exocytosis (Jones and Elmслиe 1997; Miller 1998). One main problem in addressing the question of which mechanism a given transmitter uses to inhibit transmitter release is the inaccessibility of most presynaptic bouton-type terminals to electrophysiological approaches.

Few preparations allow direct recordings of the presynaptic terminal. The calyx of Held, a giant synaptic terminal in the mammalian auditory brain stem (Rowland et al. 2000), is involved in binaural sound localization (Guinan and Li 1990; Oertel 1999; Spirou et al. 1990), and its large size allows direct electrophysiological recordings (Borst et al. 1995; Forsythe 1994; Sakaba and Neher 2001). Glutamate release from the rat calyx of Held is mediated mainly by P/Q-type calcium channels after postnatal day 10 (Forsythe et al. 1998; Iwasaki and Takahashi 1998; Wu et al. 1999). Direct recordings of the calyx demonstrated that activation of presynaptic metabotropic GABAB (Isaacson 1998; Takahashi 1998; Wu et al. 1999) and glutamate (Takahashi et al. 1996) receptors inhibits glutamate release via inhibition of presynaptic calcium channels.

Noradrenaline alters neuronal excitability and transmitter release by G-protein-coupled receptors in central and peripheral synapses (Boehm 1999; Dunlap and Fischbach 1981; Kamisaki et al. 1992; Kondo and Marty 1998; Lipscombe et al. 1989; Madison and Nicoll 1986; Scanziani et al. 1993). The mammalian auditory brain stem, and in particular the medial nucleus of the trapezoid body (MNTB) (Wynne and Robertson 1996), receives extensive adrenergic input (Jones and Friedman 1983; Klepper and Herbert 1991; Kössl et al. 1988; Vincent 1988). However, the physiological function of this adrenergic innervation is not well understood. Noradrenaline (NA) can profoundly affect the response to auditory stimuli of neurons in the adult bat cochlear nucleus because it reduced spontaneous neuronal activity and it caused a twofold decrease in the latency jitter of the first tone-evoked spikes (Kössl and Vater 1989). In rats, NA applied iontophoretically increases the discharge of the neurons in the cochlear nucleus (Ebert 1996). In addition, NA also alters the excitability of neurons in the ventral nucleus of the trapezoid body by decreasing K+ conductances (Wang and Robertson 1997) and in the MNTB by modulating the size of a postsynaptic hyperpolarization-activated current Ih (Banks et al. 1993).

Here we investigated the effects of NA on the calyx of Held fibre stimulation, in a dose-dependent manner. The inhibition was maximal (approximately 48%) at the concentration of 2 μM. It was antagonized by yohimbine and mimicked by the α2-adrenergic specific agonist UK14304. Both AMPA and NMDA receptor-mediated EPSCs were inhibited in parallel by NA, suggesting a presynaptic effect. Presynaptic recordings showed that NA inhibits the action potential (AP) generated Ca current by about 20%; however, NA did not significantly affect the presynaptic AP waveform. We thus conclude that the calyx of Held presynaptic terminal expresses α2-adrenergic receptors that inhibit its Ca current and thus glutamate release. Noradrenaline was effective in all cells tested from postnatal days 6 to 7 (P6–P7), and thereafter the number of responsive cells diminished, although half of the P14 cells tested still had EPSCs that were inhibited by NA. By contrast, activation by 1,2-amino-5-phosphonovaleric acid-sensitive metabotropic glutamate receptors strongly inhibited the EPSCs of all cells tested from P6 to P14. The effect of NA on postsynaptic action potential firing was dependent on the stimulus frequency. At 10 Hz, NA had no effect on firing probability; however, NA helped MNTB cells fire more action potentials during a 100-Hz train of stimuli, even though it did not increase the steady-state depressed EPSC, because it produced a smaller N-methyl-D-aspartate (NMDA) receptor-activated depolarizing plateau. We therefore suggest that the reduction by NA of the first few EPSCs in a train leads to a smaller NMDA depolarizing plateau and thus to increased firing probability at 100 Hz in young synapses. Surprisingly, the inhibition of glutamate release by NA can thus actually increase the excitability of MNTB neurons during early postnatal development.

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synapse. We found that NA inhibits glutamate release by inhibiting presynaptic Ca channels via activation of α2-adrenoceptors and that this effect is developmentally regulated, being strongly present in immature synapses. Surprisingly, presynaptic inhibition of glutamate release by NA allows the postsynaptic MNTB neuron to fire more action potentials (APs) during a 100-Hz stimulus train due to a reduction in the NMDA receptor-activated depolarizing plateau.

METHODS

Slice preparation

Brain stem slices were obtained from postnatal day 6 (P6) to P15 Sprague-Dawley rats. After rapid decapitation, the brain stem was immersed in ice-cold low-calcium artificial cerebral spinal fluid (ACSF) containing (in mM) 125 NaCl, 2.5 KCl, 3.0 MgCl₂, 0.1 CaCl₂, 25 glucose, 25 NaHCO₃, 1.25 Na₂HPO₄, 0.4 ascorbic acid, 3 myo-inositol, and 2 Na-pyruvate. pH = 7.3 when bubbled with carbogen (95% O₂–5% CO₂). Transverse slices (170- to 200-μm thick) were cut from the caudal to rostral direction. Slices were rapidly transferred to an incubation chamber containing normal ACSF bubbled with carbogen and maintained at 37°C for 30–50 min and thereafter at room temperature. The normal ACSF was the same as the low-calcium ACSF except that 1.0 mM MgCl₂ and 2.0 mM CaCl₂ were used.

Electrophysiology

Whole cell patch-clamp recordings were performed in normal ACSF at room temperature (21–23°C). The standard patch pipette solution consisted of (mM) 130 K-gluconate, 20 KCl, 5 Na₃-phosphocreatine, 10 HEPES, 5 EGTA, 4ATP-Mg, and 0.5 GTP, pH = 7.3 with KOH. To record N-methyl-d-aspartate receptor (NMDAR)-mediated currents at depolarized resting membrane potentials, CsCl was substituted for K-gluconate and KCl and 10 mM TEA-Cl was added. During experiments, the slices were continuously perfused with normal ACSF solution via a gravity-fed system. Neurons were visualized by infrared-differential interference contrast (IR-DIC) microscopy (Leica LDMLFS, Leica, Weltzar, Germany) through a ×40 water-immersion objective (Leica APO LU-V,1, Leica) and a CCD camera (C79, Hamamatsu, Japan). A bipolar stimulation electrode was placed on the brain stem midline. Connected cells were preselected by the presence of evoked extracellular APs with a patch pipette containing normal ACSF solution via a gravity-fed system. Neurons were visualized by infrared-differential interference contrast (IR-DIC) microscopy (Leica LDMLFS, Leica, Weltzar, Germany) through a ×40 water-immersion objective (Leica APO LU-V,1, Leica) and a CCD camera (C79, Hamamatsu, Japan). A bipolar stimulation electrode was placed on the brain stem midline. Connected cells were preselected by the presence of evoked extracellular APs with a patch pipette containing normal ACSF (Borst et al. 1995; Guinan and Li 1990).

Presynaptic calcium currents were recorded in calyces identified visually by fluorescence labeling with Lucifer yellow (0.25 mg/ml) included in the internal solution. The extracellular solution was ACSF with 20 mM TEA-Cl, substituted equimolarly for NaCl, plus TTX (1 μM). The internal solution for calcium currents recordings consisted of (mM) 90 Cs-methanesulphonate, 20 CsCl, 1 MgCl₂, 5 Na₃-phosphocreatine, 40 HEPES, 10 TEA-Cl, 0.5 EGTA, 4 ATP-Mg, and 0.2 GTP, pH = 7.3 with CsOH. The osmolarity of the internal solution was adjusted to approximately 310 mOsm with CsCl. The calyx terminal was held at −70 mV. The Ca²⁺ currents displayed in Fig. 5A were evoked by a pair of 15-ms square pulses to −10 mV, delivered at 0.1 Hz, with a 100-ms depolarizing prepulse of 100 mV in between to induce relief of G-protein inhibition of the calcium current (Elmsie et al. 1990). The relationship between first (I1) and second Ca current (I2; elicited after the depolarizing prepulse to 100 mV) was used to confirm the voltage-dependent inhibition and to discard effects due to the slow and spontaneous run-down of the current that occurred in some terminals. Leak subtraction was done using a Pn protocol. In some cells, we recorded calcium currents evoked by a presynaptic AP waveform, which was recorded previously from a calyx terminal of the same age in fast current-clamp mode (EPC-9 amplifier).

Patch pipettes were pulled from soft thin-walled glass (WPI, Sarasota, FL) using a Narishige puller (PP-830, Japan). Patch pipettes had an open tip resistance of 1.5–3.0 MΩ for postsynaptic recordings and 2.5–7.0 MΩ for presynaptic recordings. Postsynaptic access resistance Rₑ was around 2–5 MΩ and Rₑ compensation was set to 75–90% (10 μs lag). Presynaptic terminals had a Rₑ around 10–15 MΩ and were also electronically compensated (about 60–70%). Principal cells were voltage-clamped at a holding potential of −70 mV if not stated otherwise. No corrections were made for liquid junction potentials.

Pre- and postsynaptic APs were recorded in the fast current-clamp mode of the EPC-9 after adjusting for the fast-capacitance cancellation while in cell-attached mode. After break-in, the Rₑ value was determined in the voltage-clamped cell at −70 or −80 mV. Current-clamp recordings were continued only if the initial uncompensated Rₑ was <10 MΩ. For current-clamp AP recordings, presynaptic terminals were identified by choosing connected cells that, after whole cell, presented “action currents” instead of excitatory postsynaptic currents (EPSCs) when electrically stimulated (Forsythe 1994; Taschenberger and von Gersdorff 2000). Presynaptic recordings were unequivocally confirmed afterwards via Lucifer yellow fluorescence.

Afferent fiber stimulation was applied through a Master-8 stimulator (AMPI, Jerusalem, Israel) and had a duration of 100 μs and amplitudes of 2 to 25 V. Stimulation pulses were controlled using Pulse software (HEKA, Germany), and signals were recorded via an EPC-9 (HEKA) patch-clamp amplifier. Sampling intervals were 20 or 50 μs for AMPA or NMDA-EPSC recordings, and 10 μs for calcium current recordings. Data were low-pass filtered at 2.9 kHz (Bessel).

Drugs and off-line analysis

Yohimbine hydrochloride, UK 14304, t-2-amino-5-phosphonovaleric acid (t-AP4), and (RS)-α-cyclopentyl-4-phosphonophenylglycine (CPPG) were obtained from Tocris-Cookson (Bristol, UK). TTX was from Alomone Labs (Jerusalem, Israel). All other salts and chemicals were from Sigma (St. Louis, MO). All drugs were kept as 1,000-fold concentrated stock solutions and added to the barrels with oxygenated ACSF during the experiment. A fresh stock solution of NA was prepared every 10 days and kept protected from light. NA was added to the ACSF immediately prior to its perfusion in the bath to avoid oxidation and light degradation.

Off-line analysis was done with PulseFit (HEKA, Germany) or IgorPro software (Wavematicks, Lake Oswego, OR). Statistical analysis and curve fitting were also performed with Microsoft Excel and Prism 3.0a (GraphPad, San Diego, CA). Paired and unpaired t-tests were performed to access statistical significance of the data, and means with two-tail P values less than 0.05 were considered significantly different. Data are reported as mean ± SE values.

For quantifying the mean postsynaptic NMDA receptor plateau depolarization in Fig. 10, C and D, during a 100-Hz train, we choose to take the midpoint between the initial depolarization caused by the first EPSP and the maximum value attained by the plateau depolarization during the train. This was important because in many occasions the initial depolarization was substantially more affected by NA than application of the maximum plateau depolarization.

RESULTS

NA inhibits the glutamatergic EPSC

Afferent fiber stimulation at 0.1 Hz elicited AMPA receptor-mediated EPSCs in the principal cells of the MNTB with a mean peak amplitude of 4.8 ± 0.6 nA (n = 64). The perfusion of noradrenaline (NA) in the recording chamber decreased the EPSC amplitude in 77.2% of the 101 cells tested (Figs. 1A and 2A). The effect was reversible, and re-application of the same concentration of NA produced the same magnitude of effect (Fig. 1A). The effect was dose dependent, and was observed in

J Neurophysiol • VOL 87 • MAY 2002 • www.jn.org
Effect of NA is dependent on development

During the range of ages used in this work, the rat calyx of Held synapse undergoes a series of morphological (Kandler and Friauf 1993) and physiological (Chuhma and Omori 1998; Iwasaki and Takahashi 1998, 2001; Taschenberger and von Gersdorff 2000) changes. The expression of mGluR subtypes is also dependent on age (Elezgarai et al. 1999). Because not all cells were affected by NA, we asked whether this is due to a developmental process.

The effect of NA is clearly dependent on the developmental stage of the synapse as can be seen in Fig. 2A. We observed that the number of NA-responsive cells decreased with increasing age. All cells from P6 and P7 rats responded to NA (n = 26), but the percentage of responsive cells started to decrease progressively from P8 (93.3% of responsive cells, n = 15) to P15 (25% of responsive cells; n = 4).

The average effectiveness of NA did not seem to change considerably with development as can be seen in Fig. 2B. NA was still very effective in inhibiting the EPSC of responsive cells from P14 rats (61 ± 3% inhibition; n = 4). However, the mean inhibition at P7 was significantly higher than at other ages that had a similar number of tested cells (61.7 ± 4.4% at P7, n = 16; 46.4 ± 5% at P8, n = 13; 39.0 ± 3.5% at P9, n = 17; Fig. 2B). The amount of NA inhibition was not correlated with the EPSC peak amplitude (data not shown), and the potency (IC50) of NA did not seem to change significantly during development.

### FIG. 1. Effect of noradrenaline (NA) on the AMPA receptor mediated excitatory postsynaptic current (EPSC).

**A**: time course of a typical experiment where each point represents the peak amplitude of the EPSC evoked by afferent fiber stimulation at the frequency of 0.1 Hz. ●, the bath perfusion of artificial cerebrospinal fluid (ACSF) containing NA (2 μM). Experiment done in a slice from a P7 rat. **B**: comparison of the EPSC waveforms of the experiment shown in A. **Left**: an average of 4–5 EPSCs immediately before (●) and after (○) the perfusion of NA. **Right**: both EPSCs normalized to the peak. **C**: dose-response curve for the inhibition of the EPSC peak amplitude by NA. The number of cells used for each point is shown in parentheses. The points were fitted with a logistic equation, which gave an IC50 value of 0.19 μM, a Hill coefficient of 1.2, and a maximum inhibition value of 56.4%. Data collected from slices of P6 (2), P7 (6), P8 (4), P9 (3), P10 (5), and P14 (1) rats.

Concentrations as low as 50 nM, and reached a plateau around 2 μM (Fig. 1C). The mean amount of inhibition produced by 2 μM of NA (46.7 ± 4%; n = 33) or 20 μM of NA (48.8 ± 2%; n = 37) was not significantly different (P = 0.56). These saturating concentrations inhibited the AMPA EPSCs by 47.8 ± 2% (n = 70). The amount of the inhibition ranged from 7.0 to 90.4%. The half-effective concentration (IC50), estimated by fitting a logistic function to the dose-response data, was 0.19 ± 0.05 μM.

The NA inhibited EPSC had the same average kinetics as the control EPSC (rise time control: 0.27 ± 0.02 ms; NA: 0.29 ± 0.04 ms; half-width control 1.6 ± 0.3 ms, NA 1.5 ± 0.2 ms; P > 0.05; n = 5 cells from slices of rats from P7 to P14). This lack of effect is clearly demonstrated when the EPSCs were normalized (Fig. 1B).

### FIG. 2. Developmental decrease of NA effect.

**A**: percentage of NA-responsive cells with age. At the top of each bar is the number of responsive cells over the number of cells tested. We have taken as positive a cell in which the AMPA or NMDA EPSC was clearly inhibited to any extent by any dose of NA. **B**: magnitude of NA effect with age. Each point represents the individual percentage of inhibition of the AMPA EPSC peak amplitude by saturating concentrations of NA (2–20 μM). The lines between the points mean the average of all points in each age. The dashed line represents the mean of AMPA EPSC peak inhibition (47.8%) of 72 cells.
Effect of NA is mediated by α2-adrenergoreceptors

The type of receptor involved in the effect of NA was investigated using the antagonists of β, α1, and α2 receptors, propranolol, prazosin, and yohimbine, respectively (Fig. 3A). After application of 2 μM NA, subsequent addition of propranolol (1 μM) or prazosin (1 μM) did not antagonize the effect of NA (Fig. 3B), ruling out the participation of adrenoceptors of β and α1 types in this effect. In contrast, after the addition of yohimbine (20 μM), the EPSC amplitude returned to control (Fig. 3A and B). Application of yohimbine without previous application of other antagonists completely antagonized the NA inhibition of the EPSC (n = 6; not shown), confirming that the antagonism was not due to a delayed effect of propranolol or prazosin. Further evidence for a role of α2 receptors in this effect was obtained with the specific α2 agonist UK 14304 (1 μM), which could mimic the effect of NA, albeit less efficiently (Fig. 3, C and D). Subsequent application of 2, 5, or 20 μM UK 14304 did not increase the degree of inhibition in two cells tested. The effect of UK 14304 was fully antagonized after application of 10 μM yohimbine (Fig. 3, C and D). In cells that did not respond to NA, subsequent application of UK 14304 also had no effect (data not shown), showing that the lack of effect of NA in slices from older rats is not due to an increase in the uptake or oxidation of NA. In this regard, note that the recording solution routinely contained 0.4 mM ascorbic acid, which should prevent NA oxidation. These results thus indicate that α2 adrenergic receptors mediate the effect of NA on the EPSC.

Presynaptic effect of NA

To know if NA is acting postsynaptically in the AMPA receptors or presynaptically, we studied the effect of NA on the AMPA EPSC and NMDA EPSC simultaneously (von Gersdorff et al. 1997). The cells were kept at a depolarized holding potential (+30 mV) to relieve the block of NMDA receptors by magnesium. Application of NA produced a simultaneous and similar decrease in the amplitudes of both the AMPA and the NMDA-EPSCs (30 ± 6% in the AMPA EPSC and 38 ± 6% in the NMDA EPSC; n = 8; Fig. 4, A and B), and the effect on both EPSCs was fully antagonized by application of yohimbine. Although NA was slightly (8%) more efficient in blocking the NMDA-EPSC, this decrease correlated very well with the decrease in the AMPA-EPSC. This suggests that NA is acting presynaptically by inhibiting glutamate release.

NA inhibits presynaptic Ca channels

Because inhibition of Ca channels on neuronal somas is a common mechanism of action of the adrenoceptors (Boehm and Huck 1996; Dunlap and Fischbach 1981; Lipscombe et al. 1989), we asked whether NA is also acting on presynaptic Ca channels. To directly investigate the action of NA on presynaptic Ca channels, we recorded presynaptic Ca currents in calyces morphologically identified with Lucifer yellow fluorescence (P6-P8 rats; Fig. 5).

We used a double pulse protocol to study the voltage-dependent inhibition of Ca channels by NA (Bean 1989; Dolphin 1998; Elmslie 1990; see METHODS and Fig. 5A). When we

![Figure 3](http://jn.physiology.org/)

**FIG. 3.** Pharmacological characterization of the NA effect. A: effect of the antagonists of the different adrenergic receptors. ■, the bath perfusion of ACSF with NA (2 μM) and the different antagonists, propranolol (prop, 1 μM), prazosin (praz, 1 μM), and yohimbine (yohimb, 20 μM). Each point represents the peak amplitude of the EPSC evoked by afferent fiber stimulation at the frequency of 0.1 Hz. Experiment performed in a slice from a P8 rat. B: summary of the effects of NA, and sequential application of propranolol, prazosin, and yohimbine on the EPSC amplitude. Each bar represents the mean ± SE of the normalized peak amplitudes in relation to the control amplitude (−−−−): NA, 61 ± 7%; + prop, 60 ± 7%; + praz, 57 ± 6%; + yohimb, 104 ± 5%; n = 6; * significantly different from NA; P < 0.05. C: effect of the specific α2-adrenergic agonist UK 14304 on the EPSC amplitude. Each point represents the peak amplitude of the EPSC evoked by afferent fiber stimulation at the frequency of 0.1 Hz. ■, the bath perfusion of ACSF containing NA (20 μM) UK 14304 (UK, 1 and 5 μM), and yohimbine (yohimb, 20 μM). Experiment done in a slice from a P14 rat. D: summary of the effects of NA, UK, and sequential application of yohimbine on the EPSC amplitude. Each bar represents the mean ± SE of the normalized peak amplitudes in relation to the control amplitude (−−−−): NA, 52 ± 12%; UK, 62 ± 7%; UK + yohimb, 100 ± 12%; n = 4. * significantly different from NA and UK; P < 0.05.
Perfused NA (20 μM), we observed a depression of the peak calcium current (I1) of 10.9 ± 1.5% (Fig. 5; from 0.94 ± 0.06 to 0.84 ± 0.06 nA; n = 11; P < 0.01). Characteristic of a G protein inhibition, NA inhibition of the Ca current was almost completely relieved by the 100-mV depolarizing prepulse (Fig. 5; current at I2 from 0.88 ± 0.07 nA to 0.86 ± 0.07 nA; n = 11; P > 0.05). Accordingly, when the ratio I2/I1 was compared, we observed that it significantly increased from 0.93 ± 0.04 to 1.02 ± 0.03 (P < 0.01).

In cells from older animals (P9–P12), NA had a similar effect inhibiting Ca channels by 10.2% (P < 0.01; n = 5). In three of eight P9–P12 terminals tested, the Ca current was insensitive to NA application (I2/I1 control = 1.0 ± 0.05; I2/I1 NA = 1.0 ± 0.06; n = 3) as expected by the developmental decrease of responsive cells observed in Fig. 2A. Terminals from animals older than P12 were not tried due to the heavy myelination of fibers present in the brain stem slices after P12 that severely impairs visualization of the terminals.

Because the effect of NA and other G-protein-linked transmitter receptors affects mainly the activation phase of the current, we supposed that in a calcium current evoked with a AP waveform NA should have a more pronounced effect (Brody et al. 1997; Park and Dunlap 1998). In fact, when we evoked the calcium current with a presynaptic AP waveform recorded from a P7 calyx (Fig. 6A), NA was almost twice as potent in inhibiting the peak of the AP-evoked Ca current than the peak of the square-pulse-evoked Ca current in the same P7/8 terminals (8.6 ± 1.9%, square pulse vs. 16.6 ± 1.7%, AP evoked; P < 0.01; n = 3; Fig. 6B).

In the rat calyx of Held synapse, neurotransmitter release is triggered mainly by activation of P/Q-type voltage-dependent Ca channels with a significant contribution of N- and R-type voltage-dependent Ca channels in young animals (Iwasaki and Takahashi 1998; Wu et al. 1998, 1999). Because we observed a substantial effect of NA in animals older than P10 when the calyx Ca-channel is exclusively of the P/Q type (Iwasaki and Takahashi 1998), this suggests that the decrease of NA responsive cells with age is not due to the observed decline of N- or R-type Ca channel with increasing age.

**NA does not affect the presynaptic AP waveform**

Manipulations of the presynaptic K currents can alter the shape of the presynaptic AP (Ishikawa and Takahashi 2000; Wang and Kaczmareck 1998), while the presynaptic Ca current...
does not take part in the shaping of the presynaptic AP waveform (Borst et al. 1995). So it is possible that the NA effect could also reduce release by shortening the duration of the presynaptic AP by activation of presynaptic K⁺ channels. We therefore recorded presynaptic APs evoked by afferent fiber stimulation. Figure 5B shows an example of two presynaptic APs before and after the application of NA. As can be seen, NA did not alter the presynaptic AP waveform. In four calyces tested from P6 to P9 rats, NA application did not change the amplitude (control, 97 ± 9 mV; NA, 93 ± 12 mV; P > 0.05), the 20–80% rise time (control, 0.21 ± 0.03 ms; NA, 0.21 ± 0.03 ms; P > 0.05), or the half-width (control, 0.8 ± 0.14 ms; NA, 0.9 ± 0.15 ms; P > 0.05) of the AP. In addition, the presynaptic resting potential was also not changed by NA (control −67 ± 3 mV; NA, −66 ± 3 mV; P > 0.05, n = 4). We conclude that NA has no significantly effect on presynaptic APs or in the presynaptic resting membrane potential.

**NA does not inhibit all G-protein-sensitive Ca channels in the calyx**

Activation of metabotropic glutamate receptors by the agonist L-AP4 also leads to inhibition of glutamate release (Barnes-Davies and Forsythe 1995) by inhibition of presynaptic calcium channels (Takahashi et al. 1996). We observed that application of L-AP4 (50 μM) inhibited the EPSC amplitude more potently (70.2 ± 3.5%; n = 16) than NA. In accordance with this more-potent inhibitory effect, the sequential application of L-AP4 after NA application produced a further inhibition in the EPSC amplitude (NA 46.1, ± 6.9%; NA + L-AP4, 76.4 ± 3.2, P < 0.01; n = 5; Fig. 7A). Application of yohimbine (20 μM) did not relieve the effect (NA + L-AP4 + yohimbine, 73.5 ± 6.8%; n = 4; P > 0.05 when compared with NA and L-AP4 or L-AP4 alone) showing that L-AP4 and NA have nonadditive effects. Inversely, application of the mGluR group II/III antagonist CPPG, reverts the effect of L-AP4 on the EPSC after co-application with NA (Fig. 7B), and the remaining inhibition is reverted completely by yohimbine (Fig. 7B). In fact after inhibition of presynaptic Ca currents by NA, L-AP4 is able to further inhibit the Ca current (Fig. 7C), and L-AP4 occludes the NA effect in inhibiting the EPSC when applied first (Fig. 7B). We conclude that L-AP4 acts in the same pool of Ca channels accessible to NA but can inhibit another additional pool of Ca channels that are not affected by NA in the calyx of Held.

We also observed that the effect of L-AP4 is not dependent on development. Cells where the EPSC or the presynaptic Ca current did not respond to NA still invariably responded to L-AP4 (P6-P14 cells; data not shown). This demonstrates that the developmental decline of the effect of NA is a phenomenon specific to its receptor and not due to some general change in the G-protein machinery.

**Effects of NA during 10- and 100-Hz trains of stimuli**

So far we have studied the effect of NA in EPSCs evoked at the frequency of 0.1 Hz, but the physiological frequencies of discharge of this synapse may be much higher even in immature animals (Spirou et al. 1990; Wu and Kelly 1993). For example, in adult cats the spontaneous rate of firing of the calyciferous axon varied from 10 to 110 Hz (Spirou et al. 1990). However, at frequencies as low as 10 Hz, the EPSCs may already present some depression that is especially severe (more than 90%) in young rat pups (Borst et al. 1995; Taschenberger and von Gersdorff 2000; von Gersdorff et al. 1997). We tested the effect of NA on the EPSCs elicited during 10- and 100-Hz frequencies trains of stimuli. We observed that NA is less effective in inhibiting the amplitude of the depressed steady-state EPSCs (ssEPSCs) at 10 Hz (16.3 ± 4.7% inhibition; n = 15; P < 0.05; Fig. 8A) and ineffective at depressed ssEPSCs generated by a 100-Hz train (4.9 ± 6.4% inhibition; n = 13; P > 0.05; Fig. 9). A similar result was also observed when L-AP4 (50 μM) was applied to the slice (data not shown).

Thus in contrast to the avian nucleus magnocellularis, an auditory region that also receives a calycal input, we did not observe a general enhancement of the depressed ssEPSCs after presynaptic inhibition (Brenowitz et al. 1998).

Despite the depression of the EPSC, the postsynaptic cell
can reliably fire APs when subjected to a 10-Hz train of stimuli. But we observed that EPSC inhibition by NA has no effect on the firing of APs elicited by a 10-Hz train of stimuli (Fig. 8B). Because the noradrenergic AMPA-EPSC amplitude inhibition per se is not sufficient to prevent the postsynaptic cell from firing, could NA affect the firing of the postsynaptic cell by other means? Although the postsynaptic cell is able to reliably fire APs during a high-frequency train (100 Hz or more) at synapses older than P14, at immature synapses (P5–P8), high-frequency trains of stimuli produce a plateau depolarization due to the activation of NMDA receptors that impairs the firing of APs (Futai et al. 2001; Taschenberger and von Gersdorff 2000). When postsynaptic cells from P6 to P9 rats in current-clamp were subjected to a 100-Hz train of 20 stimuli, we observed that the number of APs fired in the train was inversely correlated to the mean amplitude of the NMDA-plateau depolarization as can be seen in Fig. 10C. We thus

FIG. 7. Partially overlapping effects of NA and l-2-amino-5-phosphonovaleric acid (l-AP4). A: l-AP4 effect is not occluded by NA preapplication. ■, the sequential bath perfusion of NA (20 μM), l-AP4 (50 μM), and yohimbine (yohimb, 20 μM). Each point represents the peak amplitude of the EPSC evoked by afferent fiber stimulation at the frequency of 0.1 Hz. Experiment done in a slice from a P7 rat. B: l-AP4 occludes the effect of NA. ■, the sequential bath perfusion of l-AP4 (50 μM), NA (20 μM), CPPG (group III mGluR antagonist; 300 μM), and yohimbine (yohimb, 20 μM). Each point represents the peak amplitude of the EPSC evoked by afferent fiber stimulation at the frequency of 0.1 Hz. Experiment performed on a slice from a P8 rat. C: example of a presynaptic calcium current elicited by a 15-ms step to −10 mV and the effect of NA (20 μM) and l-AP4 (50 μM). Slice from a P11 rat.

FIG. 8. Effect of NA on the firing of the postsynaptic cell during a 10-Hz train of stimuli. A: EPSCs elicited by the 10-Hz train before and after application of NA (20 μM). B: postsynaptic APs elicited by a 10-Hz stimuli train, in the same cell as in A, before and after application of NA. Slice from a P7 rat.
suggest that the size of the NMDA-plateau depolarization regulates the number of APs fired in a 100-Hz train.

Because the amplitude of the NMDA-EPSC decays in a developmental time frame similar to that observed for the NA effect, we asked whether the presynaptic inhibition of glutamate release by NA can increase the firing of P6–P8 postsynaptic cells by diminishing the amplitude of the plateau depolarization. From 11 cells tested, 8 fired less than the maximum response (Fig. 10A) and 3 fired maximally (20 APs or slightly more due to some aberrant firing; Fig. 10B) (see Futai et al. 2001). In the cells that fired less than the maximum rate, application of NA reduced significantly the size of the mean plateau depolarization (from $-39 \pm 4$ to $-45 \pm 3$ mV; $P < 0.01$; Fig. 10, A and D), and the number of APs fired was concomitantly increased (ratio APs/EPSPs from 0.38 ± 0.1 to 0.5 ± 0.1; $P < 0.01$; Fig. 10, A and D). In cells that already fired at the maximum rate (Fig. 10B), NA had no effect in increasing the firing (ratio APs/EPSPs from 1 ± 0.02 to 1 ± 0.04), although it decreased the NMDA-plateau depolarization (from $-54 \pm 0.1$ to $-58 \pm 2$ mV; $P > 0.05$; Fig. 9B), and it significantly increased the AP amplitude of these cells. These 100-Hz effects and the lack of effect at 10 Hz (Fig. 8), a frequency that does not produce a large NMDA plateau depolarization, suggest that the increase in firing observed at 100 Hz was not due to an increase of the general excitability of the postsynaptic cell. Also corroborating this, we observed that the relationship between the number of APs fired and the size of the plateau depolarization was the same either in the absence or in the presence of NA (Fig. 10C). Because we did not observe a potentiation of the depressed EPSCs after presynaptic inhibition of glutamate release, as observed by Brenowitz et al. (1998), it is not likely that an increase in the depressed EPSCs amplitude is responsible for the increased firing. We suggest instead that the large depolarizing plateau produced in the absence of NA probably prevents the recovery from inactivation of the postsynaptic sodium channels (as suggested also by the decreased AP amplitude, Fig. 10B), and this then prevents the firing of APs during rapid stimulation.

**DISCUSSION**

We have characterized the effect of NA in the calyx of Held synapse in the auditory brain stem during early development. NA inhibits glutamate release at the calyx by activation of $\alpha_2$-adrenoreceptors that inhibit presynaptic Ca channels. This effect is clearly associated with immature synapses because the number of responsive cells decreases from 100% in immature P6–P7 calyces to 25% in the more mature P15 calyx. NA was relatively weak at inhibiting presynaptic Ca channels because the activation of mGluRs by the agonist L-AP4 is able to inhibit a larger fraction of Ca channels, including those inhibited by NA. Finally, we observed that presynaptic inhibition by NA in immature calyces makes some postsynaptic neurons fire significantly more APs during a 100-Hz train of stimuli. Immature calyces have large NMDA receptor EPSCs that produce a plateau depolarization that can inactivate postsynaptic Na channels when the synapse is firing at a high-frequency. So, presynaptic inhibition of glutamate release in immature calyces would lead to a smaller NMDA receptor plateau depolarization and thus to less Na channel inactivation and thereby to more postsynaptic firing.

A series of evidences was presented that suggest this is the mechanism involved in the increase of firing of the postsynaptic neuron and not some other change in the postsynaptic membrane excitability that may produce. First, NA did not change the general relationship between the plateau depolarization amplitude and the number of APs fired. Second, at the frequency of 10 Hz, which does not produce a large sustained plateau depolarization, NA has no effect on the number of APs fired. Third, NA has no effect in the amplitude of the depressed ssEPSCs during the 100-Hz train, showing that the potentiation observed by Brenowitz et al. (1998) cannot contribute to the increased firing.

We have also demonstrated pharmacologically that NA acts via $\alpha_2$-adrenoreceptors. Calcium channel inhibition by these receptors and others is due to the direct interaction of the $\beta Y$ subunits of the G protein with the presynaptic Ca channels (Dolphin 1998). Subtypes of the $\alpha_2$-adrenoreceptors ($\alpha_{2A/D}$, $\alpha_{2B}$, and $\alpha_{2C}$) are known from biochemical and genetic studies (MacKinnon et al. 1994), but a pharmacological distinction is difficult, due to a lack of highly selective agonists and antagonists suitable for functional studies. Genetically engineered...
Noradrenergic inhibition of glutamate release

In the calyx of Held, the presynaptic inhibition of glutamate release by mGluR and GABA_{\beta} receptors has been attributed to the inhibition of presynaptic Ca channels, without any participation of potassium channels (Isaacson 1998; Takahashi et al. 1996, 1998), although one cannot exclude completely a direct action on the release machinery, like that recently demonstrated by Blackmer et al. (2001). We observed an approximately 10% inhibition of the peak of depolarizing-step evoked presynaptic Ca current by NA. In Ca currents evoked by an AP waveform, the peak inhibition was almost twice as potent (1.9 times). If we suppose a power-relation between intracellular Ca and transmitter release of around 3–4, as has been demonstrated for the calyx of Held (Bollmann et al. 2000; Borst and Sakmann, 1999; Schneckgenburger and Neher 2000; Wu et al. 1999), a 17–19% inhibition of the peak AP-evoked calcium current will produce a 43–53% inhibition of the transmitter release in the range of what we observed (average of 48%).

We observed that the agonist for group III mGluR receptors, L-AP4 is more effective than NA in blocking Ca current and glutamate release. Their effects were not additive showing that they are sharing the same mechanism. We suggest that NA acts on a smaller pool of Ca channels than L-AP4 and that L-AP4 acts in a pool of channels that engulf the NA-sensitive pool of Ca channels. Possibly the number of mGlu receptors is bigger than the number of \alpha_2-adrenoreceptors, producing more free \beta_{y} subunits that can inhibit more Ca channels, or they are specifically targeted to more Ca channels than the adrenergic receptors. In agreement with this last hypothesis, NA did not inhibit the R-type current, on which L-AP4 is effective (not shown) (Wu et al. 1998). Interestingly, at another calyx-type synapse in the chick ciliary ganglion, NA has a very different effect on synaptic transmission. In contrast to our present results in the calyx of Held, NA potentiated the size of the EPSC and this was due to a GMP-dependent mechanism that increased the Ca^{2+} sensitivity of the exocytotic process (Yawo 1999).

In contrast to L-AP4, the effect of NA decays with development. This suggests that the EPSP inhibition by NA may have some role in the development of this auditory synapse. At present, we do not know if this effect disappears completely with age or if it becomes restricted to some subset of cells. However, we emphasize that in some P14 calyces (an age when calyces are morphologically mature) (Kandler and Friauf 1999) NA was still able to produce a strong inhibition of release. Interestingly, using transgenic animals it has been shown that NA has a role in mouse brain development (Thomas et al. 1995) and that the \alpha_{2D} adrenoreceptor subtype is particularly important (Kable et al. 2000).

What may be the role of NA during development? The onset of hearing in rats occurs at P12 (Blatchey et al. 1987), and an important developmental change in this synapse is the marked reduction of the size of the NMDA-EPSC with increasing age (Futai et al. 2001; Taschenberger and von Gersdorff 2000). Here we report that there also occurs an almost parallel reduction of the NA effect. In addition, we demonstrated that during high-frequency stimulation the presynaptic inhibition of glutamate release makes the postsynaptic cell fire more impulses. Presynaptic inhibition might thus be of physiological relevance in the MNTB pathway during this critical period when the auditory brain stem adapts itself to high-frequency transmission. In addition, the presynaptic inhibition could decrease the amount of Ca entering the postsynaptic cell via NMDA receptors; this accounts for approximately 30% of the total Ca that enters the principal cell during an EPSP in P8–P10 rats (Bollmann et al. 1998). Interestingly, we also observed that NA inhibits the somatic Ca currents of the MNTB principal cell (data not shown); this accounts for approximately 70% of the total Ca that enters the cell during an EPSP (Bollmann et al. 1998). Because calcium influx can affect gene expression in neurons (Gallin and Greenberg 1995), the conjunction of these effects could strongly regulate developmental changes.

Noradrenaline may thus be playing an important developmental role in the maturation of this synapse. These results are somewhat surprising given the canonical view of this synapse as operating solely as a fail-safe relay that simply follows its massive calyceal input. Contrary to this view, our results suggest that the output of this synapse can be modified by hormones and neuromodulators during development.

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