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Role of Ca^{2+} Ions in Nicotinic Facilitation of GABA Release in Mouse Thalamus

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Presynaptic nicotinic acetylcholine receptors (nAChRs) are present in many regions of the brain and potentially serve as targets for the pharmacological action of nicotine *in vivo*. To investigate their mechanism of action, we performed patch-clamp recordings in relay neurons from slices of thalamus sensory nuclei. In these nuclei, nAChR activation facilitated the release of the inhibitory neurotransmitter GABA. Micromolar concentrations of nicotinic agonists increased the frequency of miniature GABAergic synaptic currents and decreased the failure rate of evoked synaptic currents. These actions of nicotinic agonists were not observed in knock-out mice lacking the β2 nAChR subunit gene. Nicotinic effects were dependent on extracellular calcium ions, and they persisted when calcium was replaced by strontium or barium but not by magnesium. Furthermore, in high extracellular calcium concentrations, nicotinic agonists evoked an increase in spontaneous release lasting for minutes after removal of the agonist. This supports the view that presynaptic nAChRs facilitate the release of neurotransmitter by increasing the calcium concentrations in presynaptic nerve endings. With use of cadmium and nickel ions as selective blockers, it was found that in different sensory nuclei the presynaptic influx of calcium could result either from the activation of voltage-dependent calcium channels or from a direct influx through nAChR channels. Finally, we propose that the nicotinic facilitation of GABAergic transmission may contribute to the increase of signal-to-noise ratio observed in the thalamus *in vivo* during arousal.

Key words: nicotinic acetylcholine receptors; presynaptic; facilitation; GABA; calcium; thalamus (sensory nuclei)

Since the early proposal that nicotinic acetylcholine receptors (nAChRs) regulate neurotransmitter release from peripheral nerve terminals (Koelle, 1961), the presence of these receptors has been ascertained in many types of axon terminals in the CNS. Pathway lesions combined with receptor labeling have indicated the presence of nAChRs in retinal terminals in the tectum of various species (Henley et al., 1986; Prusky and Cynader, 1988; Sargent et al., 1989; Britto et al., 1992), in substantia nigra dopaminergic terminals in the rat striatum (de Belleroche et al., 1979; Clarke and Pert, 1985), in thalamic terminals in rat and cat cortex (Prusky et al., 1987; Sahin et al., 1992), in medial-habenula terminals in rat interpeduncular nucleus (Clarke et al., 1986), and in a general manner in catecholamine terminals in the brain (Schwartz et al., 1984). On the other hand, experiments in synaptosomes from various CNS regions have demonstrated that nicotinic agonists are able to trigger the release of dopamine, noradrenaline, GABA, and acetylcholine (see references in Wonnacott et al., 1989).

Despite the abundance of indications for the existence of presynaptic nAChRs throughout the nervous system, only a few electrophysiological studies have examined their function. The presynaptic nAChRs have been proposed in different structures to facilitate (King, 1990; Vidal and Changeux, 1993; McGehee et al., 1995) or depress (Brown et al., 1984; Mulle et al., 1991) neurotransmitter release. Recent studies dealing with the modulation of spontaneous neurotransmitter release have given insights into the mechanisms of action of presynaptic nAChRs. In a first type of synapse, sodium channels have been proposed to relay presynaptic nAChRs to elicit the release of GABA; these nAChRs have been referred to as "preterminal" (Léna et al., 1993; McMahon et al., 1994b). In other synapses, nAChRs were shown to increase the release of GABA, glutamate, or acetylcholine in the presence of the sodium channel blocker TTX, and they therefore qualified as "terminal" (McMahon et al., 1994a; McGehee et al., 1995). The purpose of this paper is to further examine the mechanisms of action of nAChRs that increase the release of neurotransmitter in the absence of sodium channel activity.

Using the patch-clamp technique, we have recorded GABAergic postsynaptic currents (IPSCs) in relay cells from the ventrobasal complex (VB; somatosensory thalamus) and from the dorsolateral geniculate (DLG; visual thalamus) of the mouse thalamus. We present evidence that presynaptic nAChRs increase the release of GABA by producing an influx of calcium in the presynaptic compartment, and that this influx occurs via different pathways in the DLG and the VB.

MATERIALS AND METHODS

Preparation of the slices and solutions. Six- to 15-d-old C57Black6 mice (CERJ) and β2-subunit knockout mice (Picciotto et al., 1995) were anesthetized with ether and then decapitated. The brain was removed rapidly and placed in ice-cold Krebs solution containing (in mM): 126 NaCl, 26 $NaHCO_3$, 25 glucose, 1.25 NaH_2PO_4, 2.5 KCl, 2 $CaCl_2$, and 1 $MgCl_2$ bubbled with 95% O_2/5% CO_2. Slices (300 μM thick) were obtained using a DSK-1000 slicer (Dosaka, Kyoto, Japan) and kept submerged on a net in 200 ml of Krebs solution. Recordings were performed under an Axioscop microscope (Zeiss). The neurons could be easily

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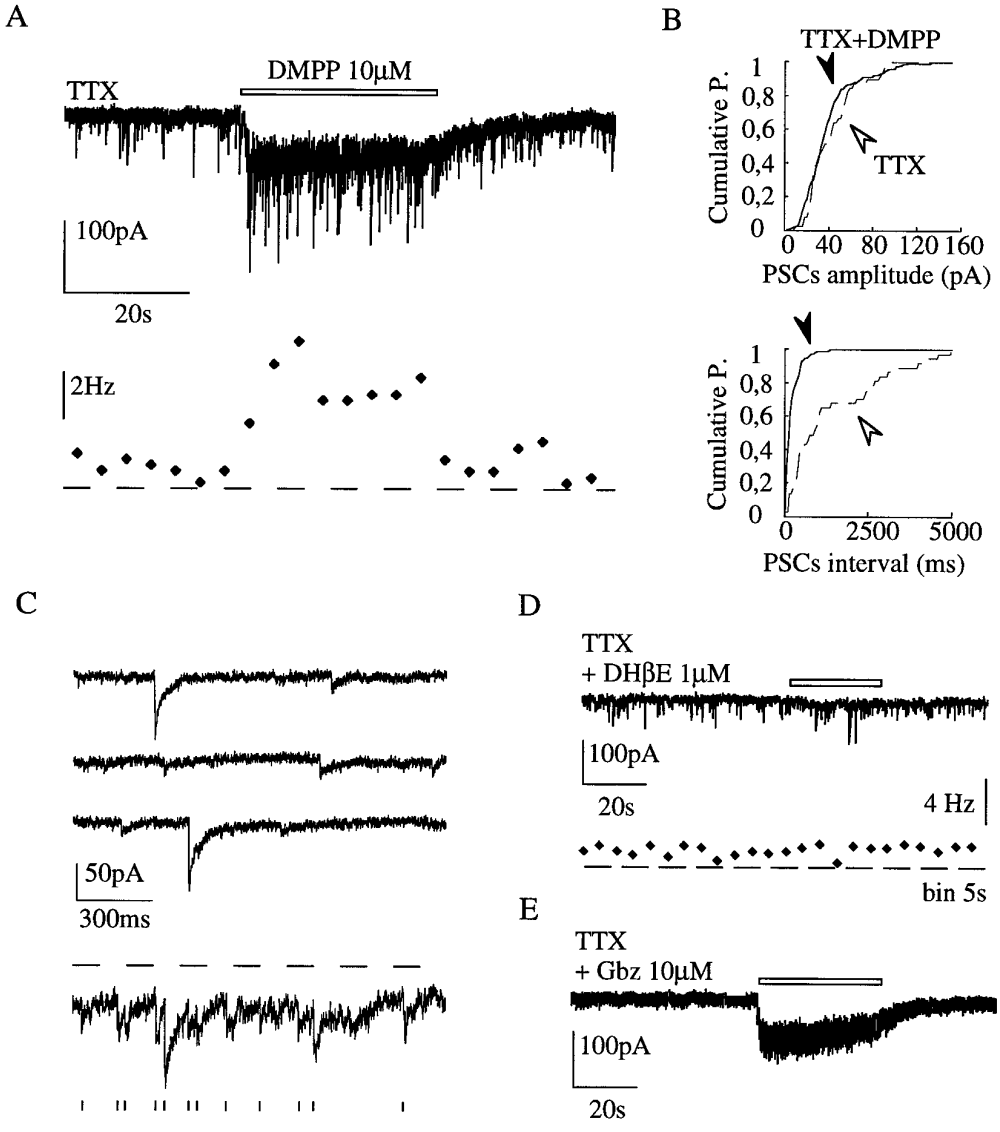

Figure 1. DMPP (10 μM) increases the frequency of miniature GABAergic IPSCs without altering their amplitude distribution in the thalamic relay neurons. *A* (*top*), Current trace from a neuron in the VB. The nicotinic agonist DMPP elicits an inward current, an increase in baseline noise, and an acceleration of the frequency of miniature IPSCs; (*bottom*) frequency plot of miniature IPSCs corresponding to the *current trace above*; bin is 4 sec. *B*, Cumulative probability plot corresponding to *trace A* (2 min of control condition and 30 sec in the presence of DMPP). The amplitude distribution was unchanged ($p > 0.1$) from control conditions in the presence of DMPP, whereas the frequency was increased significantly ($p < 0.001$). *C*, Part of *trace A* at an expanded time-scale in control condition (*three top traces*) and in DMPP condition (*bottom trace*). The *short bars* indicate the detection of an IPSC by the analysis program. *D*, Same as *A* in the presence of the nicotinic antagonist DHβE: all of the effects of DMPP are abolished. *E*, Same as *trace A* in the presence of the $GABA_A$ antagonist Gabazine (*Gbz*) all of the IPSCs are blocked, but not the postsynaptic response to DMPP.

visualized without the help of phase-contrast optics. Drugs were applied either in the bath or using a broken patch pipette (tip ~50 μM diameter) placed at the surface of the slice; this pipette allowed for either an outward flow of drug or an inward flow of extracellular medium. This system exchanged solution close to the cell in the second range. As a result, fast desensitizing nAChR currents may have been missed. When applied through the pipette, the drugs were dissolved in solution containing (in mM): 150 NaCl, 10 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, and 2.5 KCl, pH 7.3 with NaOH. Applications of nicotinic agonists desensitized the nicotinic responses over tens of minutes (unpublished observations). Most of the experiments were thus conducted with applications of 10 μM dimethylphenylpiperazinium (DMPP) interspaced by 20–30 min.

Changes in the external KCl concentration were obtained by isosmotic replacement of sodium with potassium and changes in the external calcium concentration were obtained by isosmotic replacement of calcium with the ions, as indicated in text. A minimum delay of 5 min was necessary to reach steady state when application was by bath perfusion.

Electrophysiological recordings. The patch pipettes were pulled from thin, hard glass tubes (Masfeld, Hilgenberg, Germany) with a P-87 Sutter Instruments puller, wax-coated, and filled with (in mM): 135 CsCl, 10 BAPTA, 10 HEPES, 5 MgCl2, 4 NaATP, pH 7.3, yielding a 2–3 MΩ resistance. Voltage-clamp experiments were performed with an Axopatch1D amplifier (Axon Instruments, Foster City, CA). Gigaseals were obtained without cleaning the cells. All neurons exhibited a multiphasic capacitance transient indicating the presence of multiple electric compartments (Llano et al., 1991). During whole-cell recordings, series resistance and capacitance were compensated to 80%. Just after the patch was broken, input resistance ranged from 250 to 800 MΩ, and capacitance ranged from 20 to 50 pF. Some neurons in the DLG (<10%) had a smaller capacitance (5–15 pF) and a very small response (<20 pA) to 10 μM DMPP. These neurons were discarded from the analysis. The holding

Table 1. Pre- and postsynaptic nicotinic responses are absent in β2 knock-out mice

	Wild type		β2 knock-out	
	Postsynaptic	Presynaptic	Postsynaptic	Presynaptic
VB	100% (45/45)	62% (28/45)	0% (0/12)	0% (0/12)
DLG	96% (218/226)	48% (106/226)	0% (0/16)	0% (0/16)
Reticularis thalamus	100% (15/15)	0% (0/15)	0% (0/4)	0% (0/4)

Percentage of cells having a pre- or postsynaptic response in the VB, DLG, and *reticularis* thalamus. Numbers in parentheses correspond to (number of cells with a pre- or postsynaptic response)/(number of cells tested). The occurrence of pre- and postsynaptic response to 10 μM DMPP was tested in four knock-out mice (7, 8, 9, and 11 d old). Nicotinic responses in the medial habenula persisted in knock-out animals (Picciotto et al., 1995) and therefore were used as a control for the correct functioning of the perfusion system.

membrane potential was usually set to −70mV; current traces were saved on a DAT recorder and simultaneously acquired at 3.33 kHz on a computer with the PClamp-6 program (Axon Instruments). Synaptic currents were evoked by stimulating presynaptic elements in the vicinity of the recorded cell either with a patch pipette filled with the buffer used for drug application (monopolar stimulation) or with two tungsten electrodes separated by 30–50 μm (bipolar stimulation).

Data analysis. Data analysis was performed with the help of the DetectIvent program (Ankri et al., 1994) in the Labview (Scientific Instruments, Hawthorne, NY) environment. This program detects the events having a rising slope above a manually set threshold (10–20 pA/msec). The threshold was determined with traces devoid of synaptic currents (for example, after blockade of the GABAergic IPSCs) to avoid detecting events that were postsynaptic noise. We probably missed some small events, and the frequency during the application of agonist might have been underevaluated. The decay rate of the IPSCs was determined by a single exponential fit performed with the PClamp-6 program. Changes in the frequency and amplitude of the miniature IPSCs were evidenced with the help of cumulative diagrams of the miniature IPSC interval and amplitude, and ascertained with the Kolmogorov–Smirnov test. For this test, the level of significance was set to values of $p < 0.001$. Values in text are mean ± SD.

Drugs and chemicals. All drugs were from Sigma (St. Louis, MO) except CNQX, SR-95531 (Gabazine; RBI, Natick, MA), and methyllycaconitine (MLA; Latoxan, Rosans, France).

RESULTS

Whole-cell recordings were performed in >200 relay neurons from VB and DLG slices. Unless specified otherwise, all experiments were conducted in the presence of 200–300 nM TTX. Spontaneous synaptic activity varied from cell to cell, but the frequency of miniature IPSCs tended to increase with the age of the animals. In most cells, the miniature IPSCs had a fast time to peak (1.5 ± 0.4 msec; $n = 20$ cells), a slow decay rate (29 ± 4 msec; 200 events from four different cells), and a mean amplitude of 30 ± 5 pA ($n = 20$ cells), and they were blocked by 10 μM $GABA_A$ antagonist Gabazine ($n = 8$). The occurrence of fast-inactivating synaptic currents (decay rate of <10 msec) was less frequent and was prevented by 10 μM CNQX, a non-NMDA glutamatergic antagonist. No spontaneous synaptic currents were observed in the presence of CNQX and Gabazine in any of the cells recorded.

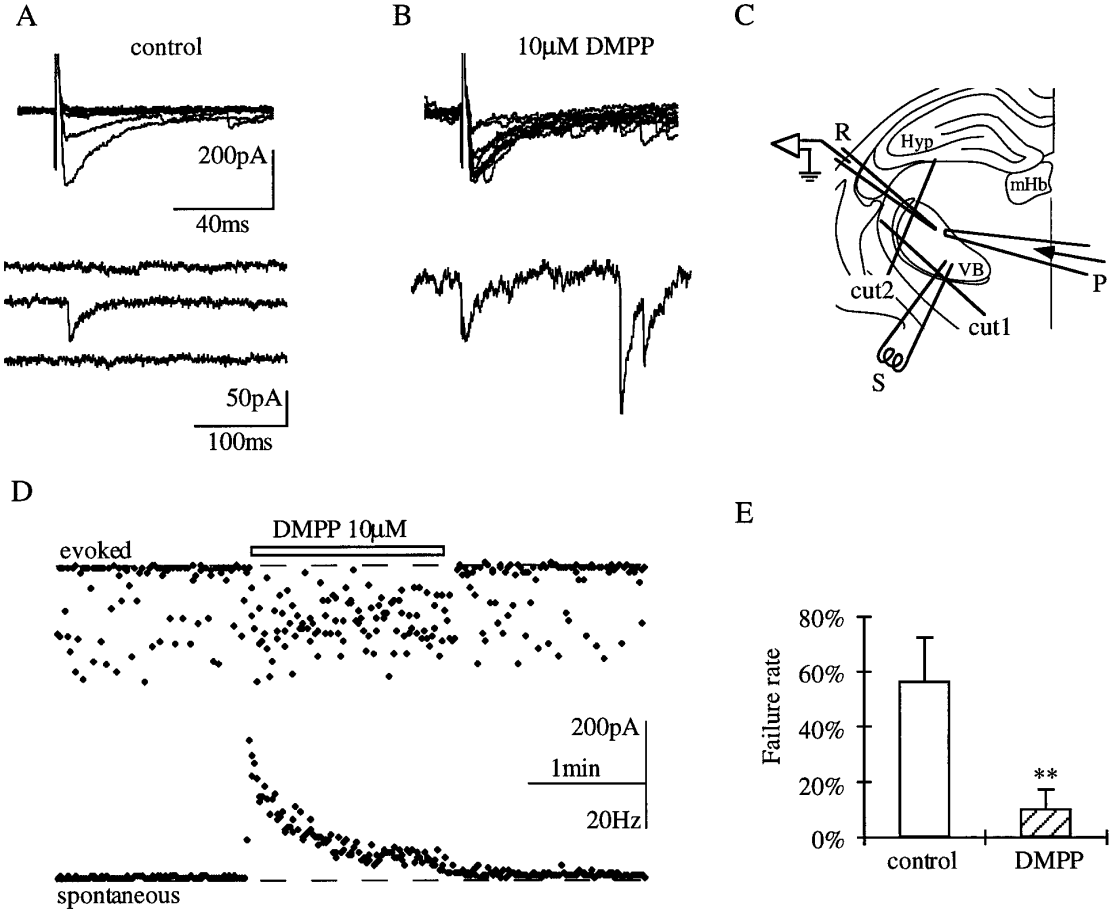

Figure 2. DMPP reduces the number of failures in the GABAergic transmission in the VB. *A*, Current traces of 10 successive evoked GABAergic synaptic currents (*top*) and spontaneous activity recorded between the electrical stimulations (*bottom*) in control condition. The failure rate has been increased by raising the extracellular magnesium concentration to 4 mM. *B*, Same as in *A* in the presence of 10 μM DMPP. Note the dramatic reduction in the number of failures in evoked synaptic currents. Calibration is the same as in *A*. *C*, Scheme of the preparation. The VB is isolated from the *reticularis* thalamus GABAergic neurons with two cuts (*cut1*, *cut2*) at its borders with a razor blade. The stimulation with a bipolar tungsten electrode (*S*) is performed at random positions in the nucleus. The perfusion pipette (*P*) is placed in front of the recording electrode (*R*). *D*, Amplitude of evoked synaptic currents (*top*: one point is one event; the amplitude is plotted downward) and frequency of spontaneous IPSCs (*bottom*: bin = 1 sec) during the same experiment. Failures are rare in the presence of DMPP. There is no dramatic change in the amplitude distribution of the successfully evoked IPSCs. (*A*, *B*, and *D* are from the same cell); *E*, decrease of the failure rate of evoked IPSCs before and during the application of DMPP ($n = 6$). Matched *t* test indicated a significance probability of $p = 0.0004$. The contribution of spontaneous IPSCs that would occur together or in place of evoked IPSCs was evaluated by using the protocol used for detecting evoked IPSCs between the stimulations instead of during the stimulation episodes. In 2/6 cells we found that we may have overestimated by 10–15% the number of successfully evoked IPSCs because of the high frequency of spontaneous IPSCs, whereas in 4/6 cells we found a possible 2–5% overestimation. When corrected for these biases, however, the decrease in failure rate was still statistically significant ($p = 0.0008$).

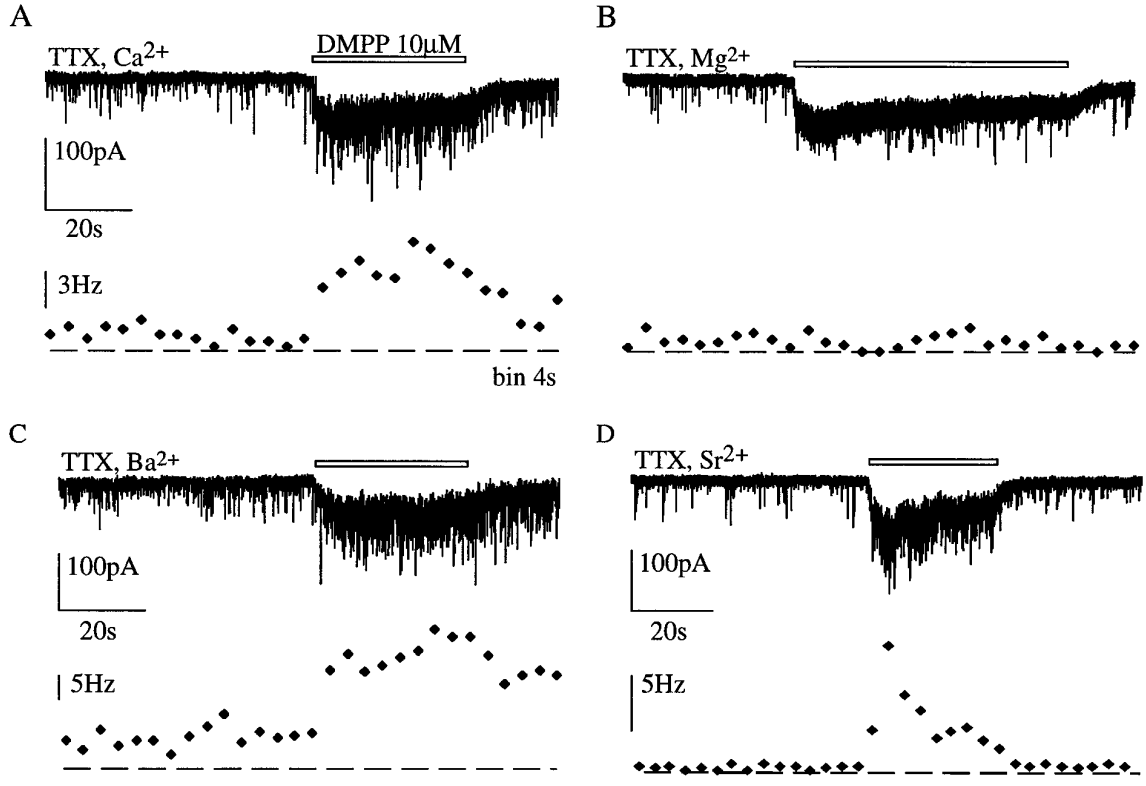

Figure 3. The presynaptic effect is dependent on extracellular calcium. *A*, Control application of DMPP and frequency plot. *B*, Application after replacement of 90% of extracellular calcium by magnesium. DMPP does not elicit any increase in frequency of miniature IPSCs in 200 μM Ca^{2+} and 2.8 mM Mg^{2+}. The amplitude of the postsynaptic response is unchanged from *A* to *B* (*A* and *B* are from the same cell). *C, D*, Same as in *A* but 90% of calcium is replaced by barium or strontium; the control applications are not shown. DMPP is still able to elicit a presynaptic effect in 200 μM Ca^{2+}, 1 mM Mg^{2+}, and 1.8 mM Ba^{2+} or Sr^{2+}. *C* and *D* are from different cells.

Nicotinic agonists increase the spontaneous release of GABA

Micromolar concentrations of the nicotinic agonist DMPP elicited a steady inward current (postsynaptic response) and an acceleration of the frequency of the miniature IPSCs (presynaptic response) in a large fraction of the DLG and VB neurons (Fig. 1, Table 1); 10 μM DMPP increased the frequency of the miniature IPSCs 4.3 ± 3.1 times (ranging from 1.5 to 20; $n = 50$), without noticeably modifying their amplitude distribution (Fig. 1*A–C*). The postsynaptic response (ranging from 20 to 500 pA) was accompanied by an increase in the baseline noise that blurred the miniature IPSCs, even if only cells with a small postsynaptic response (<100 pA) were analyzed. In cells with a large presynaptic response, the frequency of miniature IPSCs remained elevated after the end of the postsynaptic response (see below). These IPSCs could be accurately compared to IPSCs before the application of DMPP. They exhibited the same mean amplitude, amplitude distribution, time to peak, and decay time as the IPSCs in control condition. Gabazine (10 μM) completely blocked the IPSCs elicited by DMPP (Fig. 1*E*). The presynaptic effect was still present after 4 hr of dialysis of the postsynaptic neuron with 10 mM BAPTA in the intracellular medium. It is thus unlikely that the presynaptic effect requires a postsynaptic G-protein-dependent or any Ca^{2+}-dependent production or release of a retrograde messenger.

No drug was found that distinguished the presynaptic from the postsynaptic responses. Various nicotinic agonists (1–20 μM nicotine, carbachol + 10 μM atropine, anatoxin-a, 30 nM epibatidine) had similar potency on the pre- and postsynaptic responses. The nicotinic agonists cytisine and lobeline were poor agonists of the postsynaptic response ($n = 5$) and failed to produce a presynaptic response ($n = 3$). All of the responses to DMPP were blocked by the nicotinic antagonist 1 μM DHβE (Fig. 1*D*) (5/5 neurons) but not by 20 nM MLA (0/5 neurons), an antagonist known to block α-Bgt-sensitive nAChRs.

There is no remaining presynaptic response in β2 knock-out mutant mice

The effect of nicotinic agonists was investigated in slices from homozygous mutants lacking the gene coding for the β2 nAChR subunit (Picciotto et al., 1995). In these slices, the GABAergic activity in relay neurons was similar to that of the wild-type preparation; however, no pre- or postsynaptic nicotinic responses could be recorded in any thalamic neuron tested (Table 1).

DMPP increases the evoked release of GABA

Presynaptic nAChRs have been shown to either facilitate or impair electrically evoked synaptic transmission (see reference citations in introductory remarks). The effect of presynaptic nAChRs on evoked GABAergic IPSCs could be studied easily in the VB. In this structure, the GABAergic innervation is extrinsic and originates from the lateral neighboring nucleus, the *reticularis* thalamus. The VB was isolated from the presynaptic GABAergic neurons when its borders were cut with a razor blade (Fig. 2*C*). After this treatment, GABAergic miniature IPSCs were still recorded in the VB, and local bipolar electrical stimulation evoked (in the absence of TTX) GABAergic IPSCs (Fig. 2*A*, *top*).

The application of 10 μM DMPP increased the frequency of spontaneous IPSCs as in experiments carried out in the presence of TTX (Fig. 2*A,B,D bottom*). The action of DMPP on the evoked

IPSCs depended strongly on the occurrence of failures in control conditions. When the stimulation robustly activated IPSCs, no changes in their amplitude distribution were noticed, as if the probability of release was already maximal. On the contrary, in cases in which failures occurred, DMPP reduced their number. This was analyzed by increasing the concentration of extracellular magnesium up to 4 mM to reduce the probability of release at GABAergic synapses. In six cells, 10 μM DMPP reduced the failure rate from 56% ± 15% to 10% ± 7%, whereas the amplitude distribution of the successfully evoked IPSCs did not change significantly (Fig. 2*A,B,D top*, and *E*). Presynaptic nAChRs thus increased simultaneously the spontaneous and evoked release of GABA in the VB.

The increase in miniature IPSCs frequency is attributable to a sustained increase in presynaptic calcium concentration

The mechanism of action of presynaptic nAChRs in the VB and the DLG was investigated further by analyzing the changes in frequency in miniature IPSCs. Neuronal nAChRs possess channels that display high Ca^{2+}/Na^{2+} permeability ratios (see references in Rathouz and Berg, 1994); furthermore, increases in presynaptic calcium concentration enhance neurotransmitter release. The role of extracellular calcium ions was thus examined by partial exchange with other divalent cations. In 9/9 cells from the VB and the DLG, replacement of 90% of extracellular calcium ions with magnesium ions resulted in a complete extinction of the presynaptic effect of DMPP (Figs. 3*B*, 4). Replacement experiments were also attempted with the divalent cations barium and strontium. Their influx in nerve terminals was known to increase the spontaneous release of neurotransmitter (Zengel and Magleby, 1981). Indeed, these ions consistently supported the presynaptic action of nAChRs ($n = 4$) (Fig. 3*C,D*).

The extracellular calcium concentration was then set at values in the 1–4 mM range (while keeping the extracellular magnesium concentration at 1 mM). In the presence of 1–4 mM extracellular calcium, 10 μM DMPP increased the frequency of miniature IPSCs (Fig. 5*A*). The most striking effect of a change in extracellular calcium concentration was observed after the end of the application of DMPP (Fig. 5). In 1 mM calcium, the frequency of miniature IPSCs returned rapidly to baseline together with the postsynaptic response. On the other hand, a sustained high frequency of miniature IPSCs was observed for a few minutes after the end of the postsynaptic response in 2–4 mM calcium.

These data are consistent with the notion that nAChR activation causes a sustained influx of extracellular calcium in the presynaptic compartment. The possible contribution of a calcium-induced calcium release in the presynaptic compartment was not investigated extensively; yet, 4 μM thapsigargin failed to block the presynaptic effect of nAChRs ($n = 3$; data not shown).

Calcium channel blockers do not block the nAChRs in presynaptic neurons and act differentially in the VB and the DLG

We then investigated whether the presynaptic nAChRs depolarize the nerve terminals and activate voltage-dependent calcium channels to produce the facilitation of neurotransmitter release. This was tested by blocking calcium entry through calcium channels. For this purpose, the preparation was bathed with 50 μM cadmium (Cd^{2+}) or nickel (Ni^{2+}), which preferentially block high-threshold and low-threshold voltage-dependent calcium channels, respectively (see references in Hille, 1992; Randall and Tsien, 1995). The high-threshold but not the low-threshold calcium channels

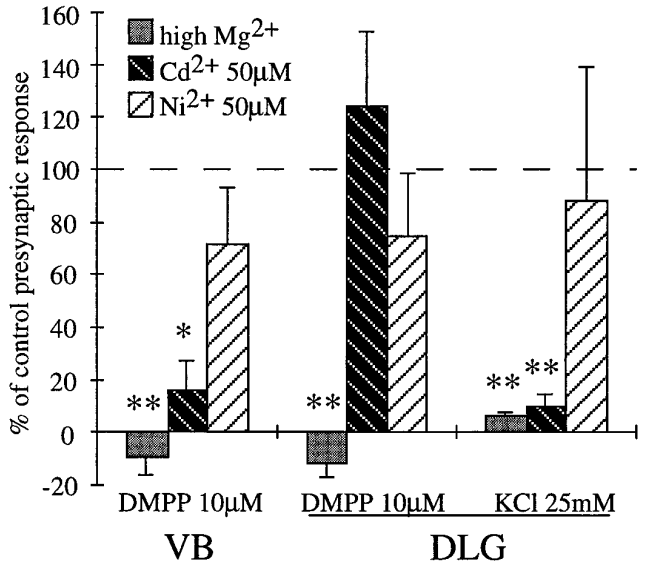

Figure 4. Quantification of the remaining presynaptic effect of DMPP and KCl after treatment with low $[Ca^{2+}]$, Cd^{2+}, and Ni^{2+} in the VB and DLG. Low $[Ca^{2+}]$ solutions block the increase of frequency of IPSCs produced by DMPP in the VB and the DLG, or by KCl in the DLG; 50 μM Cd^{2+} blocks the effect of potassium in the DLG and of DMPP in the VB but not in the DLG; 50 μM Ni^{2+} has no significant effect on any of the conditions tested. The remaining presynaptic effect after the treatments was evaluated with the formula $(X2 - 1)/(X1 - 1)$ where $X1$ and $X2$ are the frequency of IPSCs on DMPP application normalized to the frequency before the application, in control conditions and in treatment conditions. In most cases, $X1$ was the average of the effect in the control applications performed before and after the treatment application. A value of 100% means that the treatment did not affect the increase in frequency, whereas a value of 0% corresponds to its complete blockage. The large deviation in the results corresponds to the variability in the responses of the cells rather than a variable effect of the treatments. Similar values of the deviation were obtained by comparing successive control applications in the cells. There is a continuous distribution of the values around the mean value and no evidence for the existence of subsets. Statistical differences were tested by a nonparametric paired comparison test (Wilcoxon) between the pairs ($X1$, $X2$). The differences in remaining presynaptic effect of DMPP in the VB and DLG neurons in the presence of Cd^{2+} was tested with a nonparametric test (Mann–Whitney) and yielded a value of $p = 0.01$. The plot represents mean ± SEM ($^*p = 0.03$, $^{**}p < 0.01$). The number of points represented in each *bar* of the histogram is (*left* to *right*) 4,6,5, 5,11,10, 4,7,5.

are generally assumed to be responsible for the presynaptic calcium influx causing neurotransmitter release (Dunlap et al., 1995). At this point, the presynaptic effect of DMPP exhibited similar properties in the VB and the DLG, but the experiments with Cd^{2+} and Ni^{2+} revealed differences between these two structures.

Before the action of Cd^{2+} and Ni^{2+} was tested on nicotinic presynaptic effects, we tested their action on the nAChRs in presynaptic neuronal cell bodies in the *reticularis* thalamus, following the hypothesis that the same nAChRs are present in presynaptic cell soma and terminals. This structure provides the GABAergic input to the VB and in part to the DLG. In these neurons, nicotinic agonists (nicotine, cytisine, and DMPP) elicited large postsynaptic responses. In some cells, DMPP increased the frequency of glutamatergic (CNQX sensitive) miniature IPSCs but not GABAergic miniature IPSCs. The replacement of calcium by magnesium ($n = 4$) or the application of Cd^{2+} ($n = 6$) or Ni^{2+} ($n = 3$) poorly affected the amplitude of the response to 10 μM DMPP (Fig. 6). Thus, the action of such treatments on the presynaptic effect of DMPP could not be attributed to a direct action on nAChRs.

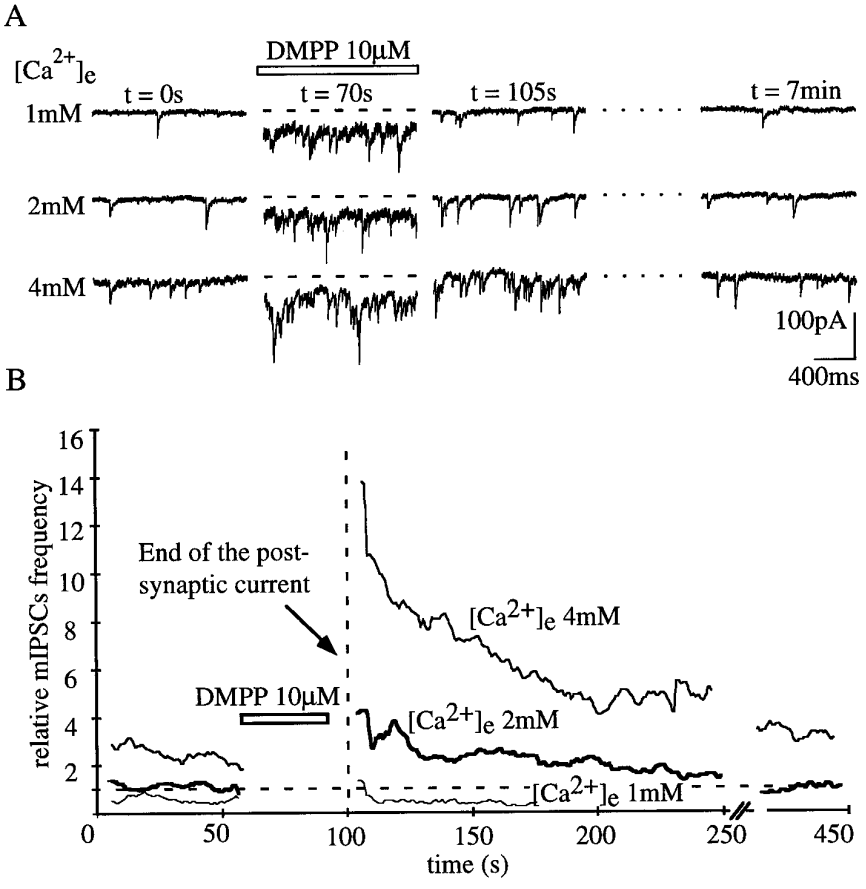

Figure 5. DMPP causes an increase in frequency of miniature IPSCs lasting longer than the postsynaptic current in the DLG. *A*, Samples of current trace from a single cell. Note the presynaptic effect of DMPP in 1 mM, 2 mM, and 4 mM extracellular calcium. *B*, Running average of the frequency in 1 mM ($n = 5$), 2 mM ($n = 7$), and 4 mM ($n = 5$) calcium. The traces were normalized to the basal frequency in 2 mM extracellular calcium before averaging. Most cells were recorded from 2-week-old animals that had a larger presynaptic effect but also a large postsynaptic current that hindered an accurate measure of the miniature IPSCs frequency during the application. Therefore, the frequency during the application is not plotted in this figure.

In the VB, 50 μM Cd^{2+} significantly reduced (and in three cells, totally blocked) the presynaptic action of nAChRs ($n = 6$), whereas 50 μM Ni^{2+} ($n = 5$) had no effect (Figs. 4, 7). Thus the nAChR-mediated calcium influx into the GABAergic terminals was produced by a depolarization followed by the activation of high-threshold voltage-dependent calcium channels in the VB.

In contrast, neither 50 μM Cd^{2+} ($n = 11$) nor 50 μM Ni^{2+} ($n = 10$) significantly reduced the presynaptic response in the DLG (Figs. 4, 8). The lack of effect of Cd^{2+} suggested either that a different type of calcium channel (Cd^{2+} insensitive) was responsible for neurotransmitter release in the DLG or that nAChR-stimulated calcium influx in the nerve terminals occurred even in the absence of calcium channels.

The involvement of Cd^{2+}- or Ni^{2+}-insensitive calcium channels in synaptic neurotransmission in the DLG was examined by local monopolar stimulation of GABAergic IPSCs in the absence of TTX. Cd^{2+} readily blocked the evoked IPSCs, whereas Ni^{2+} had no effect ($n = 3$; not shown). It could be argued, however, that a sustained depolarization induced by the activation of nAChRs during several seconds activates a set of calcium channels different from the one activated during the fast transient depolarization caused by the action potential. The action of Cd^{2+} and Ni^{2+} thus was tested in slices depolarized by a high extracellular concentration of potassium. In the presence of TTX, bath application of 25 mM potassium caused a large, calcium-dependent ($n = 4$) increase in frequency of miniature IPSCs (Figs. 4, 9*A*). This increase was reversibly blocked by Cd^{2+} ($n = 7$), whereas Ni^{2+} was ineffective ($n = 5$) (Figs. 4, 9*B*). Thus, a sustained depolarization increased the frequency of miniature IPSCs by activating Cd^{2+}-sensitive calcium channels in the DLG, and the increase in frequency of IPSCs resulting from activation of nAChRs and from depolarization exhibited a different sensitivity to Cd^{2+} in the DLG.

DISCUSSION

We have shown in mouse sensory thalamic neurons from VB and DLG that presynaptic nAChRs potentiate the spontaneous and electrically evoked synaptic release of GABA. In agreement with previous studies in cultures of chick neurons (McGehee et al., 1995), this effect is likely to be attributable to an increase in presynaptic calcium concentration. Our results suggest that this increase is produced either by the activation of voltage-dependent calcium channels in VB or by the influx of calcium through the channel of the nAChRs in DLG, and it might last several minutes after the removal of the agonist.

Molecular type of nAChRs in the thalamus

nAChRs are present in the somatodendritic compartment of VB and DLG relay neurons, in the GABAergic terminals contacting the relay neurons, and in the *reticularis* thalamus neurons. The pharmacological characteristics of these nAChRs are similar: 10 μM nicotine evokes a steady current, and nicotine is as potent as DMPP and more potent than cytisine; the currents of nAChRs are blocked by 1 μM DHβE but not by 20 nM MLA. This resembles the characteristics of nAChRs containing the β2 subunit (Luetje and Patrick, 1991) but not of nAChRs containing the α7 subunit (Alkondon and Albuquerque, 1993; Seguela et al., 1993). Consistently, all of the nicotinic responses observed in this study were absent in mice lacking the gene coding for the β2 nAChR subunit

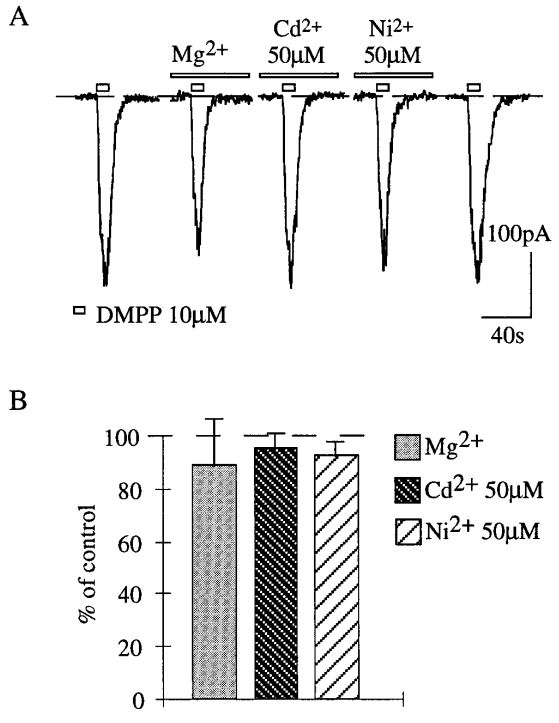

Figure 6. The nicotinic postsynaptic currents in the *reticularis* thalamus are insensitive to the replacement of calcium by magnesium or by Cd^{2+} or Ni^{2+} treatment. *A*, Examples of current traces from *reticularis* thalamus neurons; 10 μM DMPP is applied in control condition, after the replacement of calcium by magnesium, or after the addition of Cd^{2+} or Ni^{2+}. *B*, Average of the responses to DMPP after the different treatments and normalization to the control responses. The number of points averaged is (from the *left* to the *right bar*) 4, 6, 3.

(Picciotto et al., 1995). This is the first strong evidence that $\beta 2$ is involved in a functional presynaptic receptor.

The $\beta 2$ subunit may be associated with the $\alpha 4$ subunit, which is expressed at very high levels in the sensory and *reticularis* thalamus (Wada et al., 1989), or with the $\alpha 3$ subunit, whose expression in the thalamus has been proposed by some authors (Wada et al., 1989) but not confirmed by later studies (Zoli et al., 1995). Recent *in situ* hybridization studies have shown that subunit $\alpha 6$ and $\beta 3$ of the nAChRs are also expressed in the *reticularis* thalamus (LeNovère et al., 1997); however, these subunits have not been proven to form functional nAChRs oligomers, and the pharmacology of such nAChRs is not known. In regard to these results and the present data, most of the nicotinic currents recorded in our study are likely to correspond to a single class of oligomers containing subunits $\alpha 4$ and $\beta 2$ of the nAChRs.

Where do nicotinic agonists act to produce their presynaptic effect?

The nicotinic agonists likely increase the frequency of miniature GABA IPSCs by activating nAChRs at the presynaptic level. Cutting the GABAergic afferents from the *reticularis* thalamus did not alter the presynaptic effect in the VB. Because GABAergic interneurons are absent in the VB (Ohara and Lieberman, 1993), presynaptic nAChRs in this structure are probably localized in GABAergic axon terminals rather than in presynaptic neuronal soma.

In the DLG, presynaptic nAChRs control the release of GABA either from *reticularis* thalamus terminals or from GABAergic interneurons. The different sensitivity to Cd^{2+} of the presynaptic effect of DMPP in the VB and the DLG is an indication of a difference between the presynaptic structures. For example, presynaptic nAChRs in the DLG might increase GABA release from interneurons rather than from *reticularis* thalamus afferents. A more accurate subcellular localization of the presynaptic nAChRs probably could be achieved by electron microscopic (EM) studies with specific antibodies (Hill et al., 1993).

The mechanism of action of presynaptic nAChRs

Changes in external concentrations of divalent cations support the view that the nAChR-evoked facilitation is mediated by an influx of calcium in the presynaptic terminal. Replacing Ca^{2+} with Mg^{2+} did not significantly change the function of the nAChRs (Fig. 6; however, see Mulle et al., 1992b; Vernino et al., 1992) but did suppress the nicotinic presynaptic response. On the other hand, Ba^{2+} and Sr^{2+} could be substituted for Ca^{2+} without blocking the nicotinic presynaptic effect. Finally, an increase in the extracellular concentration of Ca^{2+} caused a sustained increase in frequency of IPSCs on application of nicotinic agonist that lasted minutes after the removal of the agonist. In this respect, the nAChR-evoked facilitation described in our paper shares a number of characteristics with the stimulation-evoked facilitation studied extensively at the neuromuscular junction (Miledi and Thies, 1971; Magleby and Zengel, 1976; Zengel and Magleby, 1981). A large body of work (for review, see Zucker, 1989) supports the hypothesis (called "the residual-calcium hypothesis") that the electrically evoked increase in spontaneous and evoked release of neurotransmitter is attributable to a long-lasting increase in presynaptic calcium concentration. That presynaptic nAChRs exert their facilitatory effect by increasing the presynaptic calcium concentration is consistent with these views.

The activation of nAChRs has been demonstrated in various preparations to cause an elevation in intracellular calcium concentration either by depolarizing the cell membrane to potentials where voltage-dependent calcium channels open (Noronha-Blob et al., 1989; Vijayaraghavan et al., 1992; Rathouz and Berg, 1994; Zhang and Melvin, 1994; Sorimachi, 1995) or by producing a direct influx of calcium through nAChRs (Mulle et al., 1992a; Trouslard et al., 1993; Zhou and Neher, 1993; Rathouz and Berg, 1994; Vernino et al., 1994; Rogers and Dani, 1995). The presynaptic action of nAChRs is thus expected to be attributable to a balance between theses two effects, depending on whether the nAChR-elicited depolarization reaches the threshold of the voltage-dependent calcium channels in the nerve terminal.

In this paper, we have used Cd^{2+} as a potent blocker of the high-threshold calcium channels (see references in Hille, 1992; Randall and Tsien, 1995) to evaluate their contribution to the presynaptic effect of nAChRs. Fifty micromolar Cd^{2+} blocked the release of GABA caused by electrical stimulation or by potassium depolarizations in the thalamus. On the other hand, Cd^{2+} blocked the nAChR-induced presynaptic facilitation in the VB but not in the DLG. Cd^{2+} might block the presynaptic nAChRs in the VB; however, our experiments with nAChRs from presynaptic neurons of the *reticularis* thalamus failed to demonstrate such an action (although nAChRs on the soma and on the terminals may differ). This suggests that nAChRs depolarized the GABAergic terminals (and activated voltage-dependent calcium channels) in the VB but not the DLG. We have also used Ni^{2+}, a blocker of the low-threshold voltage-gated calcium channels. These channels do not seem to be involved in neurotransmission (for review, see Dunlap et al., 1995). In agreement with this, Ni^{2+} failed to block neurotransmission and the nicotinic presynaptic effect studied in this paper.

Different mechanisms can account for the fact that in contrast

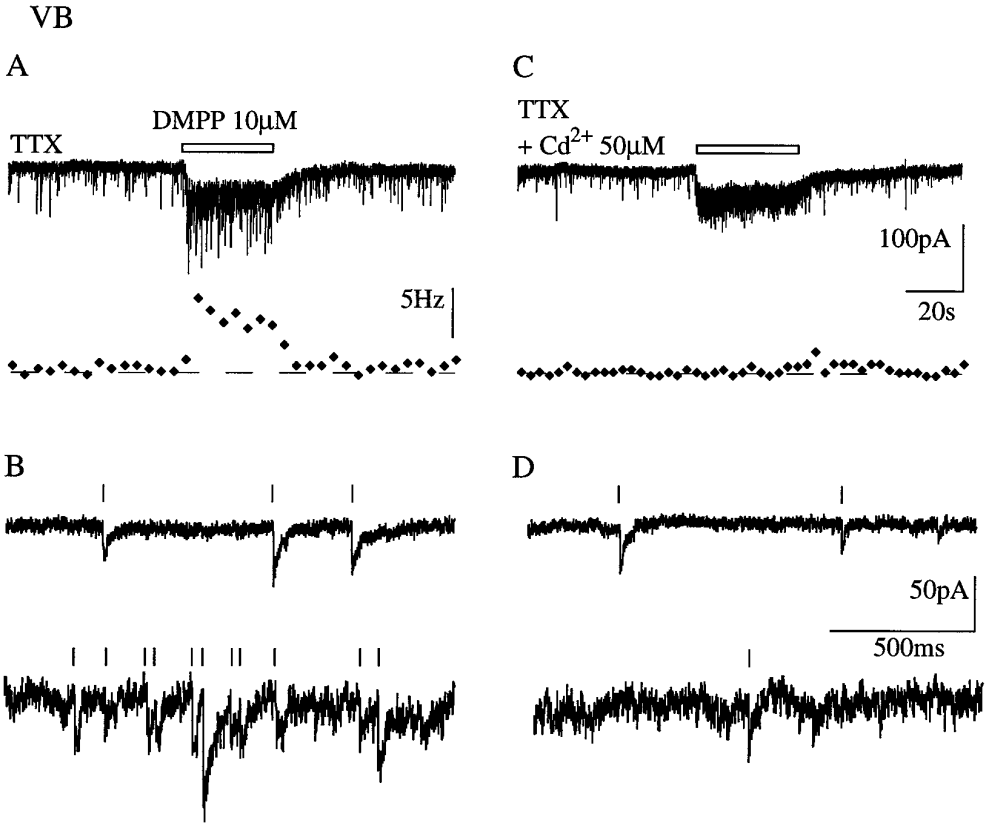

Figure 7. The presynaptic effect of DMPP is blocked by Cd^{2+} in the VB. *A, B*, Control application of DMPP; *C, D*, application in the presence of Cd^{2+} in the same cell. *A*, Current traces and frequency plot (bin = 4 sec). *B*, Traces from *A* at expanded time scale in control condition (*top*) and during application (*bottom*). The occurrence of the synaptic currents is indicated by a *bar*. *C*, Same as in *A* in the presence of Cd^{2+}. *D*, Traces from *C* at an expanded time scale in the presence of Cd^{2+} during control (*top*) and application (*bottom*).

to the experiments in the VB, nAChRs in the DLG increase the presynaptic calcium concentration without depolarizing the nerve terminals. First, there may be nAChRs that are more permeable to calcium, or there may be a different density of nAChRs in the GABAergic terminals in the DLG than the VB. Second, the terminals may have a different control of membrane potential in the DLG. They may be less prone to depolarization (having a lower membrane resistance than in the VB), or they may contain calcium-activated potassium channels that can keep the membrane potential at hyperpolarized values (Wong and Gallagher, 1991; Fuchs and Murrow, 1992), thereby increasing the influx of calcium through the nAChRs (Mulle et al., 1992a). Finally, the influx of calcium through nAChRs can be relayed by the release of calcium from intracellular pools (Sasakawa et al., 1986; Zhang

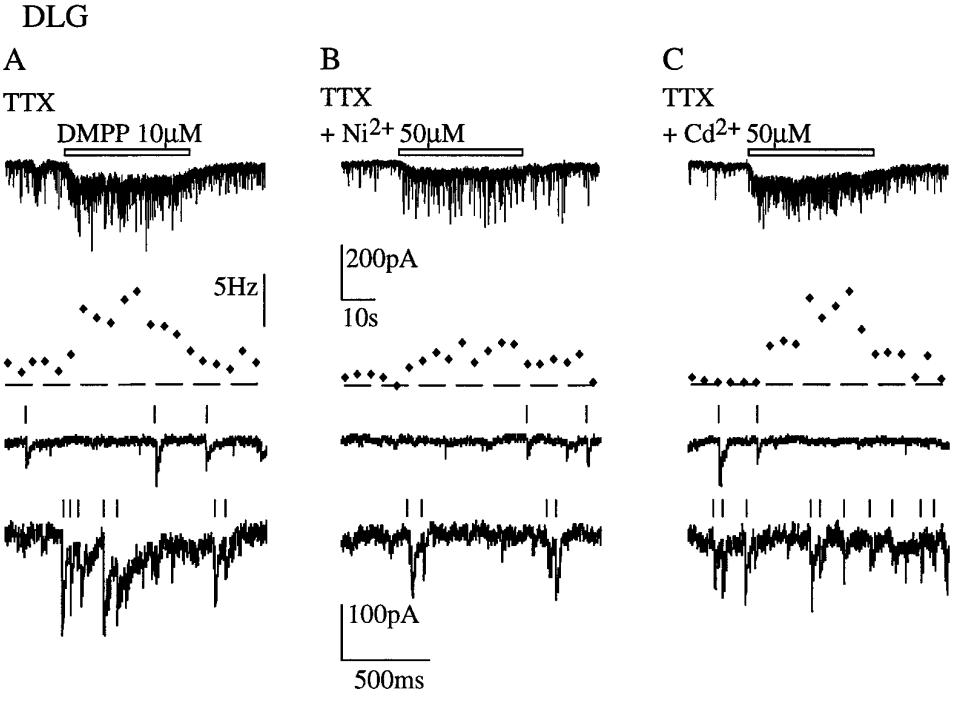

Figure 8. The presynaptic effect of DMPP is unchanged by Cd^{2+} in the DLG. *A*, Control application of DMPP; *B*, application in the presence of Ni^{2+}; *C*, application in the presence of Cd^{2+}. Same vertical organization as in Figure 7.

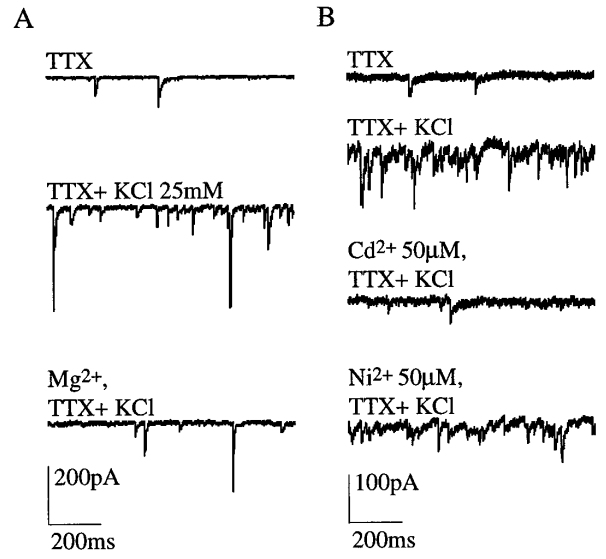

Figure 9. The increase in frequency of IPSCs induced by 25 mM potassium is calcium-dependent and blocked by Cd^{2+}. *A*, Samples of current traces in control conditions (*top*), in 25 mM potassium (*middle*), and in 200 μM Ca^{2+}, 2.8 mM Mg^{2+}, and 25 mM potassium (*bottom*). *B*, Current traces in (from *top to bottom*) control condition, 25 mM potassium, 25 mM potassium + 50 μM Cd^{2+} or Ni^{2+}. *A* and *B* are from different cells.

and Melvin, 1994; Mollard et al., 1995); preliminary experiments, however, showed that thapsigargin did not block the presynaptic effect of nAChRs in the DLG, but additional experiments are needed to rule out any contribution of Ca^{2+} intracellular pools.

One of the striking results of our study is that in the VB, where the presynaptic nAChRs depolarize the nerve terminal up to the threshold of activation of calcium channels, the electrically evoked IPSCs are facilitated by presynaptic nAChRs. As a matter of fact, activation of presynaptic nAChRs should shunt the presynaptic action potential, and the depolarization of the terminal should (at least partially) inactivate sodium and calcium channels, resulting in a reduction of the presynaptic action potential and of the subsequent calcium influx (Graham and Redman, 1994). Our results show that the increase in presynaptic calcium concentrations overcomes these depressing effects. An alternative explanation of the facilitatory effect of the presynaptic depolarization would be that it relieves the blockade of action potential propagation attributable to hyperpolarization of the nerve terminals; however, this fails to explain why the facilitatory effect of presynaptic nAChRs is more contrasted (or only present) when the failure rate is augmented by an increase of the extracellular magnesium concentration.

Physiological role of the nAChRs in thalamus

The rodent thalamic nuclei receive most of their cholinergic input from the midbrain pedoculopontine and laterodorsal tegmental fields (Hallanger et al., 1987). EM studies in the rat failed to demonstrate cholinergic axo–axonic synapses in the DLG and the VB (Hallanger et al., 1990). The apparent lack of cholinergic synapses onto GABAergic terminals suggests that acetylcholine reaches the presynaptic nAChRs by long-range diffusion. Extrasynaptic release of acetylcholine has been proposed to occur in the rat cortex (Umbriaco et al., 1994), but the possible occurrence of this kind of release was not considered in EM studies in the rat thalamus.

Acetylcholine in the thalamus plays a critical role in the sleep/wake transitions and in attentional processes (Steriade and Llinas, 1988; Williams et al., 1994). *In vivo* and *in vitro* experiments have shown that acetylcholine and brainstem stimulations both disinhibit and excite the thalamic relay cells through the activation of muscarinic receptors (for review, see McCormick, 1993). Functional studies in the cat visual thalamus, however, have shown that the application of acetylcholine increased the signal-to-noise ratio observed during arousal (Livingstone and Hubel, 1981; Sillito et al., 1983; Eysel et al., 1986). This increase is attributable to an augmentation of the action of local interneurons. Because the muscarinic agonists inhibit the interneurons (McCormick and Pape, 1988), presynaptic nAChRs in DLG interneurons thus could be responsible for the observed acetylcholine-dependent increase in local inhibition.

Finally, in a more general point of view, our experiments demonstrate that presynaptic nAChRs increase the probability of spontaneous and evoked neurotransmitter release by increasing the presynaptic calcium concentration. This is in contrast to the effect of most G-protein-linked presynaptic receptors that reduce the probability of release (see Vidal and Changeux, 1993). The presynaptic nAChRs thus could relieve such presynaptic inhibition, thereby setting the nerve terminals as an integrative unit in the nervous system. Because presynaptic nAChRs can cause a sustained facilitation of neurotransmitter release, they also may play a role in synaptic plasticity.

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