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RAPID PUBLICATION

Clonazepam Suppresses $GABA_B$-Mediated Inhibition in Thalamic Relay Neurons Through Effects in Nucleus Reticularis

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SUMMARY AND CONCLUSIONS

1. Experiments were carried out using patch-clamp techniques in rat thalamic slices, maintained in vitro, to examine the effects of the benzodiazepine compound, clonazepam (CZP), on intrathalamic inhibition. Bath-applied CZP reduced the γ-aminobutyric acid-B ($GABA_B$) component of inhibitory postsynaptic potentials and currents (IPSPs and IPSCs, respectively) evoked in rat thalamic somatosensory relay neurons by stimulation of nucleus reticularis thalami (nRt), without consistently affecting the $GABA_A$ IPSP. Secondary IPSPs, which occur as a result of intrathalamic oscillations, were dramatically reduced.

2. Voltage-clamp experiments combined with local or bath perfusion of the $GABA_A$ antagonist bicuculline methiodide (BMI), demonstrated that nRt is a site of $GABA_A$-mediated postsynaptic inhibition that affects inhibitory output onto relay neurons. BMI enhanced both $GABA_A$ and $GABA_B$ postsynaptic inhibition in relay neurons when applied to nRt. Focal applications in the ventrobasal relay nucleus near the recording electrode blocked the $GABA_A$-mediated IPSP but had no effects on $GABA_B$ inhibitory potentials.

3. Results suggest that CZP acts to facilitate recurrent inhibition in nRt and decrease its inhibitory output onto relay neurons. Intra-nRt $GABA_A$-mediated inhibition thus has an important role in controlling thalamic excitability and in the anti-absence actions of CZP.

INTRODUCTION

Recurrent feedback within the intrathalamic circuit is thought to underlie various thalamocortical oscillations, including those involved in absence epilepsy (Avanzini et al. 1992; Buzsaki et al. 1990; Huguenard and Prince 1994; Steriade and Llinás 1988; von Krosigk et al. 1993). Thalamocortical relay cells (TCs) excite GABAergic neurons of the nucleus reticularis thalami (nRt) leading to recurrent inhibitory postsynaptic potentials (IPSPs) onto relay neurons. These IPSPs have both γ-aminobutyric acid-A ($GABA_A$)- and $GABA_B$-receptor–mediated components that hyperpolarize the postsynaptic neurons and deinactivate a transient calcium current (T current, I_T), which underlies generation of low-threshold calcium spikes (LTSs) and bursts of sodium action potentials. The bursts generated by relay neurons, in turn, feedback onto nRt neurons to re-excite them, beginning another cycle (Huguenard and Prince 1994). Because of these interactions, drugs that decrease the efficacy of T current would tend to interrupt such oscillations. This is the mode of action proposed for the succinimide anticonvulsants, which are selective drugs for treatment of petit mal epilepsy (Coulter et al. 1989a,b, 1990; Huguenard and Prince 1992). Also, according to this scheme, processes that hyperpolarize neurons in nRt and relay nuclei would tend to precipitate abnormal rhythmic discharges. Examples include *1*) light sleep, during which the 3-Hz second spike-wave discharges of petit mal are enhanced (Kellaway 1985) concurrent with decreased activity in brain stem ascending excitatory neurotransmitter systems that innervate thalamic neurons (e.g., Aston-Jones and Bloom, 1981, 1985; Lindvall et al. 1974) and *2*) administration of barbiturates that are known to exacerbate human petit mal epilepsy (Penry and So 1981), presumably by enhancing $GABA_A$-receptor–mediated inhibition (Nicoll 1972; Twyman et al. 1989) within the thalamic circuit. In this context, the beneficial actions of the benzodiazepine agonist, clonazepam (CZP), in petit mal (Dreifuss et al. 1975) are difficult to explain because benzodiazepines are known to augment $GABA_A$-mediated inhibition (Choi et al. 1981) If this drug were to enhance postsynaptic GABAergic inhibition in relay nuclei, one result would be *increased* LTSs and *more* prominent oscillations. We hypothesized that one potential site for CZP action would be within nRt. By enhancing GABAergic IPSPs that presumably result from activation of the recurrent inhibitory connections known to be present between nRt neurons, CZP might decrease inhibitory output from the nucleus onto cells in the dorsal thalamus. With this possible explanation in mind, we tested the actions of CZP on a thalamic slice preparation in which connections between nRt and the ventrobasal nuclei (VB) were intact. Results indicate that CZP can reduce mainly the $GABA_B$ component of IPSPs evoked in VB from nRt stimuli, most likely through actions on intra-nRt inhibitory circuits. Such effects in nRt appear to be far more potent that any postsynaptic actions to enhance $GABA_A$-mediated inhibition in VB.

METHODS

Methods have been previously described in detail (Huguenard and Prince 1994). Briefly, rat pups, 10–19 days old, were anesthetized (50 mg/kg ip pentobarbital sodium), decapitated, and the brains were removed rapidly and placed in chilled (4°C) slice solution. Horizontal 400 μm-thick slices were cut on a vibratome, incubated at 30°C in gassed slice solution (below) and transferred as needed to an in vitro recording chamber and maintained at 34 ± 1°C (mean ± SD). Slices containing adjacent portions of nRt and VB were selected for study.

The standard extracellular superfusion solution consisted of (in mM) 126 NaCl, 26 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $MgCl_2$, 2 $CaCl_2$, and 10 glucose. Intracellular recordings were made with patch-clamp electrodes filled with the following solution (in

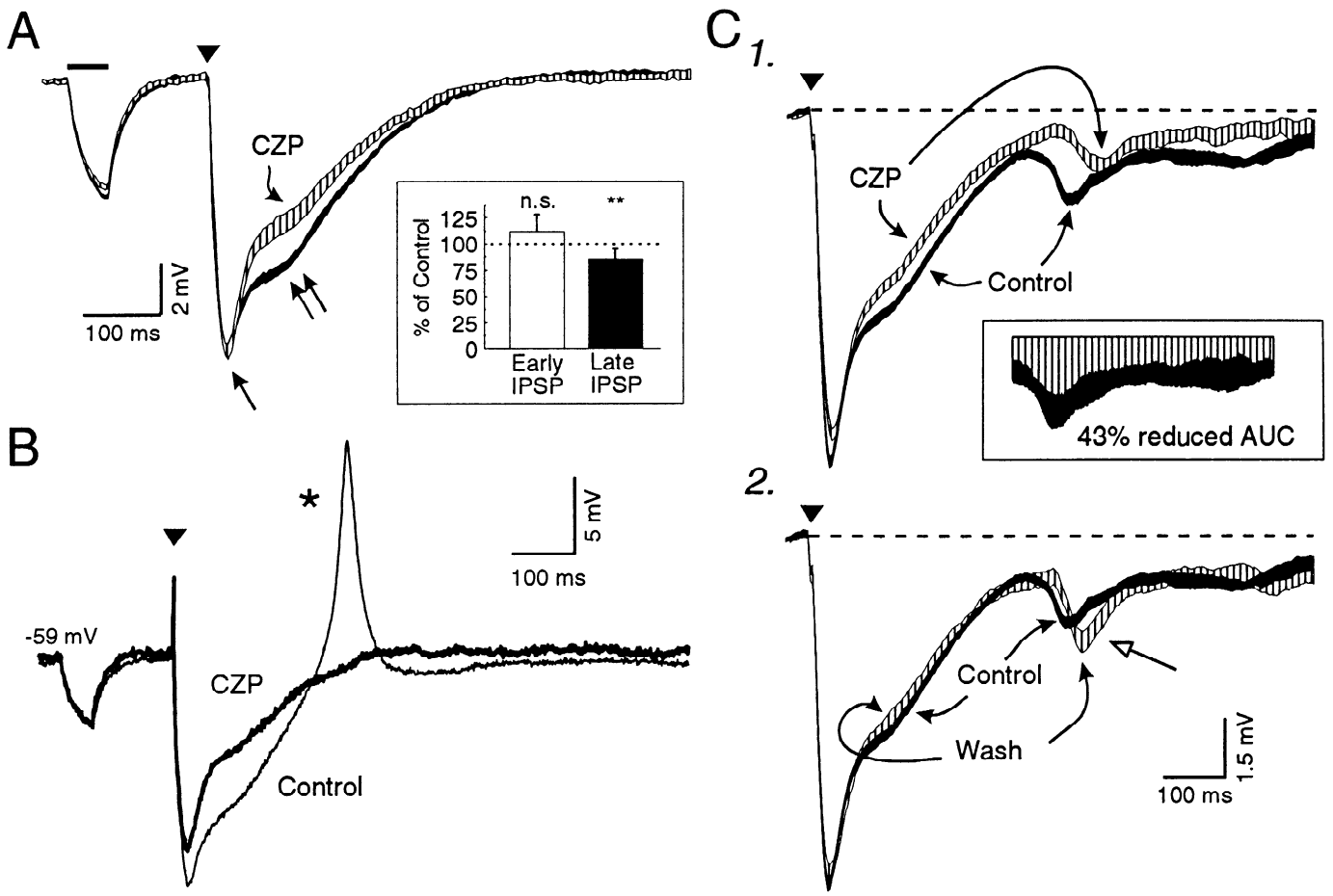

FIG. 1. Bath application of clonazepam (CZP) reduces inhibitory poatsynaptic potentials (IPSPs) in ventrobasal nuclei (VB) neurons. Traces in *A* and *C* are each the average of 6 trials. The thickness of the line indicate ±1 SE. *A*: the late γ-aminobutyric acid-B (GABA$_B$) IPSP (↖↖) evoked by nucleus reticularis thalami (nRt) stimulation (▼) was reduced to 24% of control by 300 nM CZP (striped trace), whereas the early GABA$_A$ IPSP (↖) and the input resistance were not similarly affected. Initial deflection in *A* and *B*: responses to −50 pA intracellular current injections. *Inset*: only the late IPSP was reduced by CZP. $^{**}P < 0.01$; error bars, SD. *B*: nRt-evoked IPSP generates a rebound low-threshold calcium spike (LTS; *) in another neuron. Addition of 300 nM CZP (thick line) results in depression of both GABA$_A$ and GABA$_B$ IPSPs and blocks the rebound LTS. *C*: secondary IPSPs are depressed to a greater extent than primary IPSPs by CZP. *C1*: nRt stimulation (▼) evokes a biphasic primary IPSP and a secondary IPSP at a latency of 350 ms (open arrow in *C2*). CZP (striped trace) reduces the peak GABA$_A$ IPSP by 7%, the GABA$_B$ IPSP by 15%, and delays and diminishes the secondary IPSP. *Inset*: secondary inhibition was measured by integrating the IPSC from the onset of the secondary event. Peak latencies in control and CZP were aligned. The integrated area under the curve (AUC) in CZP (striped area) was 43% reduced compared with control (solid area). *C2*: the reduction in all 3 components is reversible.

mM): 120 K-gluconate, 1 $MgCl_2$, 1 $CaCl_2$, 11 KCl, 10 *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid (HEPES), and 11 ethylene glycol-bis(β-aminoethyl ether)-*N, N, N', N'*-tetra-acetic acid (EGTA). The pH was adjusted to 7.3 with KOH and osmolality to 290 mosM with distilled H_2O. Voltages were corrected for liquid junction potentials.

Blind whole-cell patch-clamp recordings were obtained (Blanton et al. 1989), and an Axoclamp 2A (Axon Instruments, Foster City, CA) was used for bridge mode voltage recordings as well as switched single-electrode voltage-clamp recordings. The headstage was continually monitored to ensure complete decay of the current transient before voltage measurement. Constant current extracellular stimuli (250 μA, 40–100 μs) were delivered through a pair of 1–10Ω sharpened tungsten electrodes (FHC, Maine) that had a tip separation of ~100 μm. All chemicals were obtained from Sigma (St. Louis, MO) except 6-cyano-7-nitroquinoxaline-2,3-dione and D-2-amino-5-phosphonopentanoic acid (D-AP5) (Research Biochemicals International, Natick, MA). Local perfusion was by pressure application. Trains of 1–10, 50- to 100-kPa pulses were delivered through a patch pipette positioned within the top 100 μm of the slice. Drugs were dissolved in normal saline.

Using these techniques, we obtained stable voltage- or current-clamp recordings in 34 relay neurons. To quantify IPSPs and inhibitory postsynaptic currents (IPSCs), GABA$_A$ and GABA$_B$ components were measured at separate latencies from the onset of extracellular stimulus. Because the GABA$_B$ component had a delayed onset (Huguenard and Prince 1994; Otis et al. 1993), there was essentially no contamination of the GABA$_A$ component, when measured at its peak (20 ms; Huguenard and Prince 1994). On the other hand, at the peak of the GABA$_B$ response (100–150 ms) the decay of the picrotoxin-sensitive GABA$_A$ component was ~95% complete (Huguenard and Prince 1994), so responses measured at 150 ms largely reflect the GABA$_B$-mediated component. Population data are given as means ± SD.

RESULTS

In thalamic slices that retained connectivity between nRt and VB, extracellular stimulation of nRt evoked IPSPs with

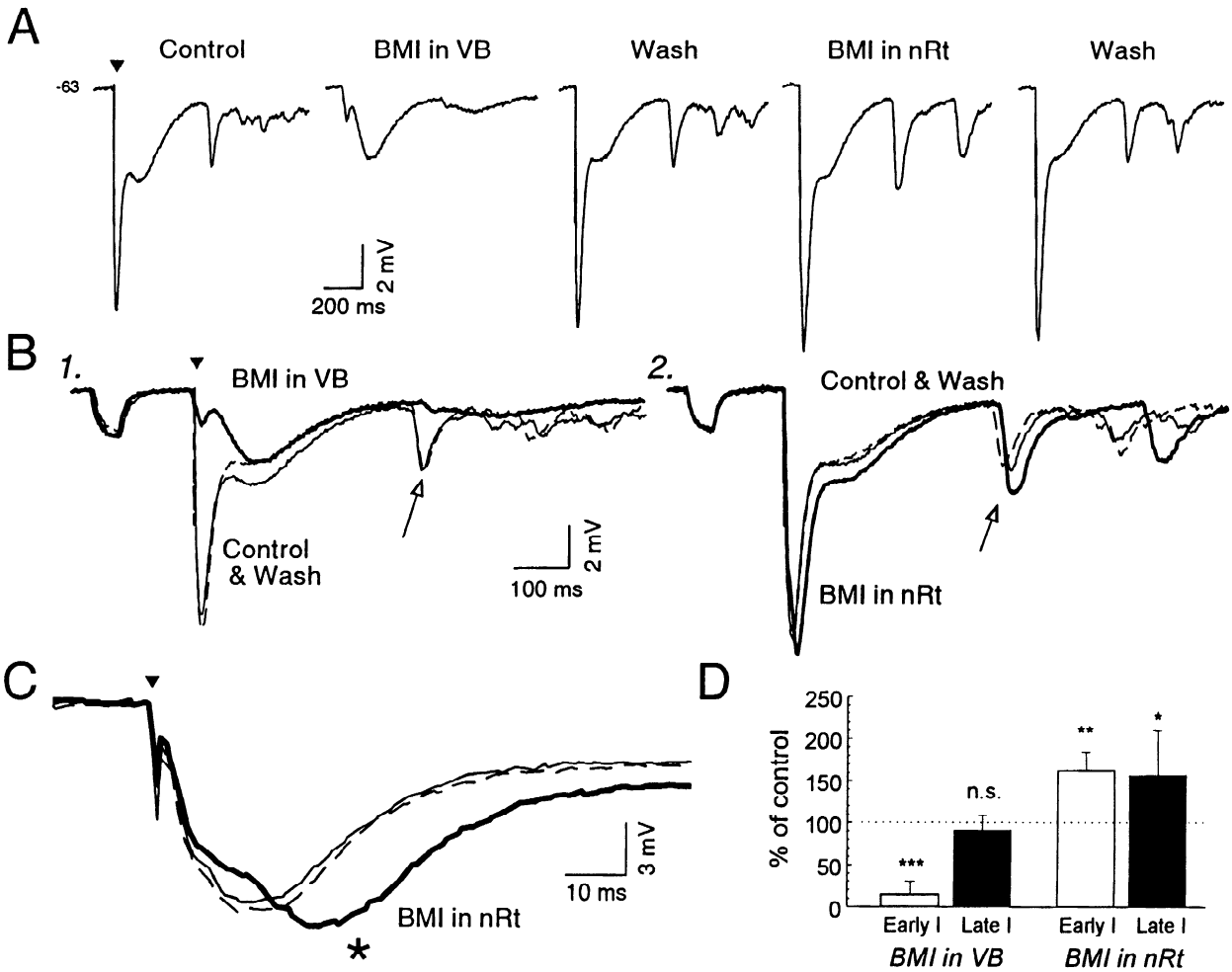

FIG. 2. Effects of local perfusion of bicuculline methiodide in VB and nRt [bicuculline methiodide (BMI), puffer concentration 50 μM] on IPSPs recorded in a VB neuron. *A*: multiple secondary IPSPs occur after a primary IPSP with a dominant $GABA_A$ component (control). BMI applied to VB (2nd segment) reversibly reduces $GABA_A$ IPSPs while leaving $GABA_B$ components intact. After washout of VB application (3rd segment), application of BMI in nRt (4th segment) increases both $GABA_A$ and $GABA_B$ components, and this effect is reversible (5th segment). Each trace is the average of 5 trials. *B*: overlaid sweeps from *A*. In this figure and in Fig. 3, traces obtained during BMI application are indicated with thick lines and wash by dashed lines. *B1*: both primary and secondary $GABA_A$ IPSPs were decreased by BMI in VB. The slight reduction in the $GABA_B$ IPSP after BMI application appeared to be due to wash out of the response during perfusion with pipette solution, because it never returned to control levels (note that the $GABA_B$ IPSP obtained during BMI application to VB was indistinguisible from either wash condition). *B2*: BMI application to nRT increased the size of primary and secondary (open arrow) components. BMI application in either location had no effect on input resistance. *C*: the early $GABA_A$ IPSP is prolonged by BMI application to nRt and a second peak is evident (*). *D*: population data for local perfusion experiments. n = 6–14, error bars, SD. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s., not significant.

prominent $GABA_A$ and $GABA_B$ components in VB neurons (Huguenard and Prince 1994). An example of such an IPSP is shown in Fig. 1*A*, where the nRt stimulus evokes a short-latency $GABA_A$ (↖) and a later slow $GABA_B$ component (↖↖). Bath application of CZP (300 nM) reduced the size of $GABA_B$ IPSP, but did not alter the $GABA_A$ component. On average, CZP did not affect input resistance (in CZP R_n was 97.6 ± 7.6% of control, n = 9) and thus the reduced IPSP was probably the result of activation of fewer $GABA_B$ receptors, and not due to shunting of the synaptic current. The effects of CZP were subtle and variable. In six of eight cells that responded the reduction in $GABA_B$, IPSP was 20 ± 6%. In the other two cells there was no effect of CZP on the $GABA_B$ response, but even including these cells, the overall depression (15 ± 10%) was significant ($P <$ 0.01, Fig. 1*A, inset*). $GABA_A$ IPSPs were slightly enhanced, but not significantly (12 ± 16% increase, $P > 0.05$). We recently described a network oscillation that occurs in horizontal rat thalamic slices (Huguenard and Prince 1994) because nRt-evoked IPSPs can trigger rebound low-threshold spikes (LTSs) in relay neurons (Fig. 1*B*), and the output from relay neurons can re-excite nRt neurons to generate another IPSP, and thus restart the cycle. An example of the early portion of this oscillation is seen in a VB neuron in the control trace of Fig. 1*C1*, where a secondary IPSP is evoked by the nRt stimulus with a latency of ~375 ms. By reducing the size of the primary IPSP, CZP can block rebound LTS generation (Fig. 1*B*) and therefore exert an anti-oscillatory action. This is clearly seen in the CZP reduction of the secondary IPSP in Fig. 1*C1*. In this neuron, CZP slightly reduced the $GABA_A$ and $GABA_B$ components (7–15%), but dramatically reduced the strength of the sec-

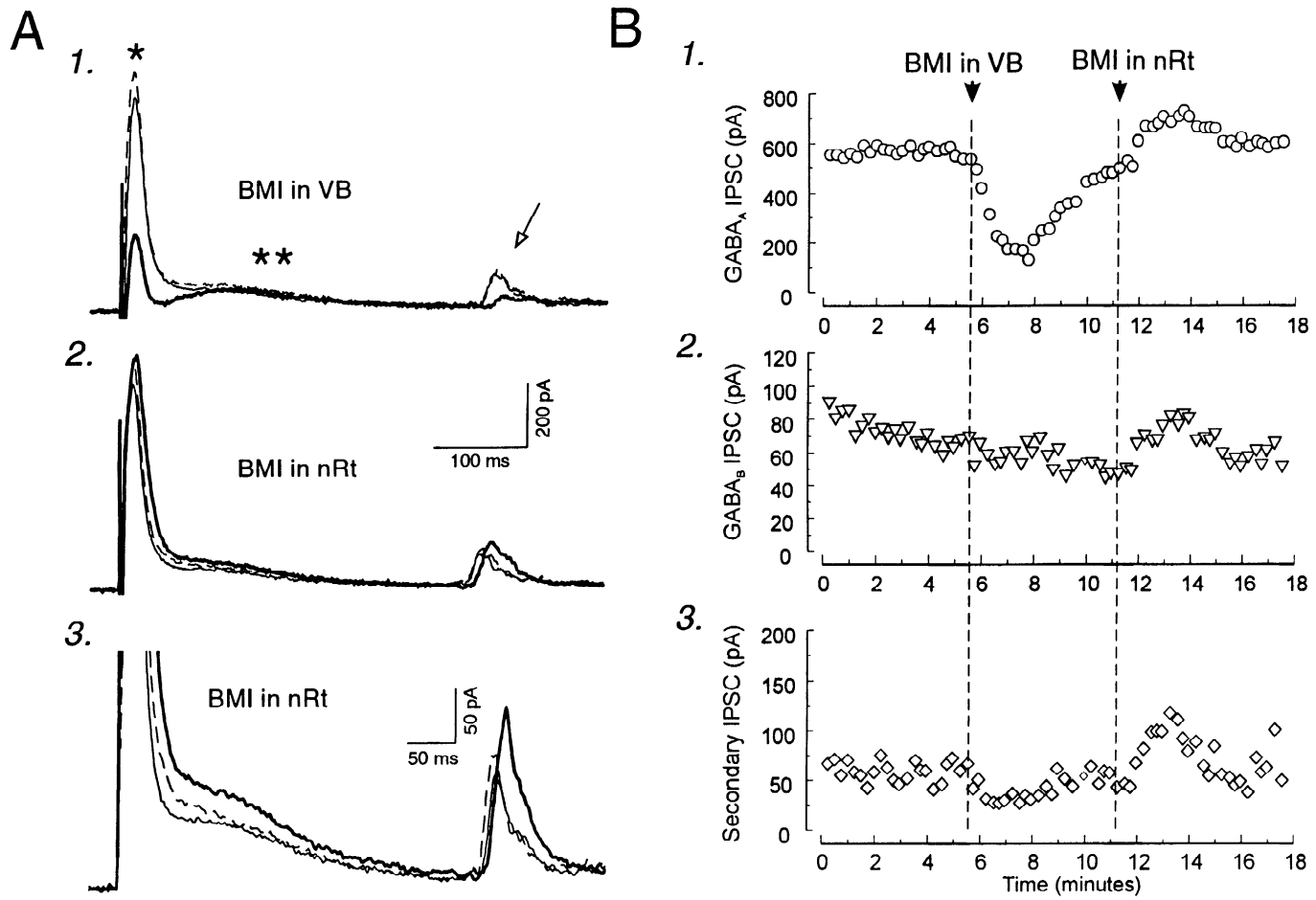

FIG. 3. Local BMI application in nRt increases IPSCs in VB. *A*: VB neuron was voltage clamped to a holding potential of −50 mV and IPSCs with prominent early GABA$_A$ (*) and late GABA$_B$ (**) components evoked every 15 s with nRt stimulation. Secondary IPSCs (open arrow) occurred 380 ms after the stimulus. Each trace is the average of 4 trials. *A1*: BMI applied to VB reduced the GABA$_A$ IPSC and secondary event. *A2*: GABA$_A$ and GABA$_B$ IPSCs (measured at 20 and 150 ms) were increased (7 and 28%, respectively) by BMI application to nRt. *A3*: the traces from *A2* are displayed at higher gain to show the enhancement of the secondary IPSC and the primary GABA$_B$ IPSC. *B*: time course of the experiment from *A*. *B1*: GABA$_A$ IPSCs were stable in control, reversibly reduced by a bolus BMI application to VB, and enhanced by BMI application to nRt. *B2*: theGABA$_B$ IPSC was less stable; it gradually ran down throughout the course of the experiment. BMI had no effect on the amplitude of the GABA$_B$ IPSC when applied to VB, but increased it when applied to nRt. *B3*: amplitude of the secondary IPSP was decreased by local BMI perfusion in VB and enhanced by nRt perfusion.

ondary IPSP (43%), and this effect was reversible (Fig. 1*C2*). In this cell the total duration of the circuit oscillation was reversibly reduced by 41% from 2.2 to 1.3 s (not shown).

Because benzodiazepines specifically interact with GABA$_A$ receptors, the most parsimonious mechanism for the effects on IPSPs in VB would be enhancement of GABA$_A$-mediated responses. The next set of experiments was designed to determine the site of action of the CZP effect. Local drug perfusion in nRt or VB was used to test whether this was a pre- or postsynaptic action. Preliminary experiments with CZP indicated that local perfusion (1 μM) in nRt reduced the size of the IPSP in VB neurons, whereas perfusion in VB had little effect (not shown, $n = 3$), implicating a presynaptic site of CZP action in nRt. To demonstrate a robust GABA$_A$-mediated presynaptic action in nRt, we used bicuculline methiodide (BMI) rather than CZP because it is a complete antagonist of GABA$_A$ responses rather than a modulator. We found that the effects of BMI (10 μM in the bath, $n = 4$; or locally applied in nRt, $n = 6$) were opposite to those of bath-applied or locally applied CZP. An example of a local perfusion current-clamp experiment during recording from a VB neuron is shown in Fig. 2. In control, nRt stimulation evoked a biphasic IPSP, which in this case had a relatively small GABA$_B$ component and several secondary IPSPs. As expected, local perfusion of BMI in VB (Fig. 2*A*, 2nd sweep; Fig. 2*B1*, thick trace; and Fig. 2*D*) nearly completely blocked the GABA$_A$ components of both the primary and secondary IPSPs, without affecting the GABA$_B$ components. On the other hand, when BMI was applied to nRt, all IPSP components were enhanced (Fig. 2, *B2* and *D*), but the effect on the secondary IPSP was generally greater than that on the primary IPSP (Fig. 2*A*, 4th sweep, and Fig. 2*B2*, open arrow). The shape of the early IPSP was sometimes altered by BMI applied to nRT (e.g., Fig. 2*C*); it was no longer monophasic, but exhibited early and late (*) peaks, indicating that excitability within nRt had been altered.

Voltage-clamp experiments confirmed that the effects of BMI and CZP were the result of altered postsynaptic GABA

conductances ($n = 5$). An example is shown in Fig. 3. BMI application to VB reduced the $GABA_A$ current (Fig. 3, *A1*, *, and 3*B1*), and the secondary IPSC (Fig. 3*A1*, open arrow, and 3*B3*), but did not affect the $GABA_B$ current (Fig. 3, *A1*, **, and 3*B2*). When BMI was applied to nRt, there was a slight enhancement of $GABA_A$ response (Fig. 3, *A2* and *B1*), but a relatively larger increase in $GABA_B$ and secondary IPSCs (Fig. 3, *A3*, *B2*, and *B3*).

DISCUSSION

These results indicate that $GABA_A$-mediated mechanisms exist within the thalamus that can up- or down-regulate the inhibitory output from nRt to dorsal thalamus. Several sites of action of CZP or BMI could account for our observations. The effects of bath applications of CZP to decrease IPSPs and IPSCs in VB neurons could conceivably be due to augmentation of $GABA_A$-mediated presynaptic inhibition of nRt terminals in VB. Suchreceptors are present in spinal cord (Rudomin 1990) and a recent report demonstrates presynaptic $GABA_A$ inhibition of nerve terminals in the pituitary (Zhang and Jackson 1993). The observation that BMI applied locally in VB did not significantly augment the $GABA_B$ component of the IPSP or IPSC argues against such an effect and also shows that the drug perfusion was localized only to the site of application. Our data cannot exclude existence of $GABA_A$ presynaptic receptors in nRt, because we did not test the effects of BMI on $GABA_B$-mediated responses in nRt neurons.

The most likely explanation for the data is a predominant effect of CZP on recurrent inhibitory synapses within nRt. Enhanced $GABA_A$-mediated inhibition at this site could decrease the output of nRt neurons in several ways, depending on the relationship of GABA equilibrium potential (E_{GABA}) to membrane potential (V_m) and to the steady-state inactivation curve for I_T (Huguenard and Prince 1992). If E_{GABA} is significantly depolarized in nRt neurons, as has been reported in rat (Spreafico et al. 1988) and if there is significant background GABA release (Otis et al. 1991), CZP might augment $GABA_A$-mediated depolarization of nRt neurons, increase the steady-state inactivation of T current, and decrease inhibitory burst output onto VB neurons. Alternatively, if E_{GABA} were significantly hyperpolarized to V_m, as is the case in VB neurons (Huguenard and Prince 1994), CZP could increase V_m into a range where more intense excitation would be required to evoke an nRt output. In either case, increased GABA-activated conductance would shunt excitatory postsynaptic potentials and currents.

The effects of BMI perfusion or local application into nRt are compatible with action on the recurrent inhibitory circuit in nRt. Enhancement of both IPSP components would be expected if nRt neurons became disinhibited, especially if there were a tonic activation of GABA receptors in nRt at rest (Spreafico et al. 1988). The discrepancy between CZP effects on the $GABA_A$ versus $GABA_B$ IPSP components in VB could be due to concurrent effects in VB to enhance GABA action and in nRt to decrease GABA release, so that the resultant $GABA_A$ IPSP would be little changed in amplitude (e.g., Fig. 1*A*). Because GABA receptors are thought to be maximally activated during evoked IPSPs (Edwards et al. 1990; Otis and Mody 1992), effects of CZP on IPSP amplitude would be less likely than an increase in the time constant of decay of the GABA response (Choi et al. 1981; Otis and Mody 1992).

Because the conductances of the $GABA_A$- and $GABA_B$-mediated events overlap, further experiments on isolated $GABA_A$ components will be required to determine whether there would be a prolongation of the $GABA_A$ IPSP in VB by benzodiazepines. In light of the significantly different $GABA_A$ receptor subunits in nRt and relay nuclei (Wisden et al. 1992), and the occurrence of a δ subunit in the ventral posterior nuclei but not in nRt, it may be that the failure of CZP to significantly augment $GABA_A$ IPSP components in VB reflects relative insensitivity of VB $GABA_A$ receptors to benzodiazepines (Shivers et al. 1989). In any case, it is unlikely that prolonging the $GABA_A$ IPSP could account for the augmentation or depression of the $GABA_B$-mediated hyperpolarization seen with BMI in nRt or CZP bath applications, respectively. We performed current-clamp simulations, based on a published model of relay neurons (McCormick and Huguenard 1992), to test whether increased conductance during a prolonged $GABA_A$ IPSP could shunt a $GABA_B$ response. By increasing the time constant of IPSP decay fourfold from 7 to 28 ms (a conservative test because benzodiazepines are associated with only a twofold change in kinetics (Choi et al. 1981), we were able to prolong the $GABA_A$ component, but this rapidly decayed so that control and "benzodiazepine" curves were identical after ~150 ms from onset of the IPSP. This is clearly different from the experimental results where the clonazepam depressed $GABA_B$-mediated events over a period of several hundred milliseconds (Figs. 1, *A* and *C*, 2*B2*, and 3*A3*).

We conclude that mechanisms exist within nRt that normally produce recurrent inhibition of nRt neurons by nRt neurons. There is strong anatomic basis for such circuitry in rat nRt (Scheibel and Scheibel 1966; Spreafico et al. 1988), where it may be important in synchronizing nuclear output (Wang and Rinzel 1993). Normally such inhibition would prevent some nRt cells from firing in response to the extracellular stimulation. Blocking the inhibition with BMI would enhance responsiveness of nRt neurons to excitatory drives and secondarily increase their inhibitory output onto VB (Fig. 2*C*, *), whereas by enhancing recurrent inhibition among nRt neurons, CZP would tend to reduce GABAergic nRt output to dorsal thalamus. Furthermore, secondary IPSPs tended to be more strongly affected than the primary response (Figs. 1*C1*, 2*B2*, and 3, *A3* and *B3*), so that a modest decrease in primary response (Fig. 1*A*) led to a strong circuit desynchronization (cf. Huguenard and Prince 1994). Because powerful GABAergic responses in the thalamus (especially those mediated through $GABA_B$ receptors) may be an important factor in generalized absence epilepsy (Hosford et al. 1992; Liu et al. 1992; Snead 1992), the enhancement of intra-nRt inhibition by CNZ may explain its clinical effectiveness (Dreifuss et al. 1975). The recurrent GABAergic circuitry within nRt is a potential target for drugs that would promote or block intrathalamic oscillations underlying normal rhythms or petit mal discharges.

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