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Neural Network of Structures in Which $GABA_B$ Receptors Regulate Absence Seizures in the Lethargic (*lh/lh*) Mouse Model

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In previous work we have shown that $GABA_B$ receptors are required for expression of absence seizures in the lethargic (*lh/lh*) mouse model; that *lh/lh* mice have increased numbers of $GABA_B$ binding sites compared to nonepileptic littermates (designated +/+); and that the magnitude of the increased number of $GABA_B$ receptors in *lh/lh* mice correlated positively with the frequency of absence seizures. We performed this study to delineate the neural network in which $GABA_B$ receptors regulate absence seizures in *lh/lh* mice. We designed three successive screens which had to be passed by a candidate neuronal population before it could be considered a member of the neural network in which $GABA_B$ receptors regulate absence seizures. *First*, the neuronal populations in *lh/lh* mice had to have enriched $GABA_B$ binding sites compared to homologous populations in matched nonepileptic controls; baclofen-displaceable ^{3}H-GABA binding was measured in autoradiograms for this screen. *Second*, the candidate populations had to generate spike-wave discharges (SWDs) during absence seizures in *lh/lh* mice; bipolar recording electrodes implanted into candidate neuronal structures were used in this screen. *Third*, the candidate populations had to demonstrate $GABA_B$ receptor-mediated regulation of absence seizures in *lh/lh* mice; microinjections of a $GABA_B$ agonist [(−)-baclofen] and antagonist (CGP 35348) were used for this screen. In this study we found that anterior ventral lateral thalamic nucleus (VLa), nucleus reticularis thalami (NRT), nucleus reuniens (RE) passed all three screens, and hence are members of the neural network in which $GABA_B$ receptors regulate absence seizures in *lh/lh* mice.

[Key words: absence seizure, lethargic mouse, $GABA_B$ receptor, autoradiography, microinjection, anterior ventral lateral thalamic nucleus, nucleus reticularis thalami, nucleus reuniens, neocortex]

Although the general clinical features of absence seizures have been known for decades (Jasper and Droogleever-Fortuyn, 1946; Penfield and Jasper, 1946; Williams, 1953; Pollen, 1968; Gloor, 1978), recent years have witnessed a revolution in our understanding of the underlying mechanisms at a cellular level. Absence seizures represent the synchronized burst-firing of populations of neocortical and thalamic neurons in an oscillatory fashion (Crunelli and Leresche, 1991; Steriade et al., 1993; Huguenard and Prince, 1994b). Experimental study of voltage-gated ion channels has illuminated the importance of the T-type calcium channel in synchronized thalamocortical burst-firing (Coulter et al., 1989a,b, 1990). Theoretical considerations regarding the deinactivation of T-channels led to the hypothesis that $GABA_B$ receptor activation played a role in this type of burst-firing (Crunelli and Leresche, 1991). We obtained evidence supporting this hypothesis using the lethargic (*lh/lh*) mutant mouse model of absence seizures (Hosford et al., 1992), while similar evidence was obtained in other genetic (Liu et al., 1992) and pharmacologic models (Snead, 1992). Together, these findings from diverse models suggest that $GABA_B$ receptors perform a regulatory role in absence seizures.

In a recent study we extended our earlier findings by showing that *lh/lh* mice have greater numbers of $GABA_B$ receptor binding sites in neocortical membranes, compared to matched nonepileptic controls (Lin et al., 1993). Moreover, the magnitude of the increased number of binding sites correlated with the frequency of absence seizures in *lh/lh* mice (Lin et al., 1993). We had anticipated that the number of $GABA_B$ receptors would be downregulated if they lacked a causal role in absence seizures. Instead, the finding of upregulated $GABA_B$ receptors which correlated positively with absence seizure frequency suggested that $GABA_B$ receptors were both required for and causally linked to the generation of absence seizures in *lh/lh* mice.

We performed the following study in order to delineate the neural network in which $GABA_B$ receptors regulate absence seizures in *lh/lh* mice. We surmised that the neuronal populations in which $GABA_B$ receptors regulate absence seizures would be a subset of the neuronal populations enriched in $GABA_B$ receptor binding sites. Based on this premise, we designed three successive screens which had to be passed by a candidate neuronal population before it was considered a member of the neural network in which $GABA_B$ receptors regulate absence seizures. *First*, the neuronal populations had to have enriched $GABA_B$ binding sites compared to homologous populations in matched nonepileptic controls. *Second*, the candidate populations had to generate spike-wave discharges (SWDs) synchronous with the

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absence seizure. *Third*, the candidate populations had to demonstrate $GABA_B$ receptor-mediated regulation of absence seizures. Our results suggest that anterior ventral lateral thalamic nucleus (VLa), nucleus reticularis thalami (NRT), and nucleus reuniens (RE) are members of the neural network in which $GABA_B$ receptors regulate absence seizures in *lh/lh* mice.

Materials and Methods

Mouse colonies. Colonies of *lh/lh* mice and their coisogenic, nonepileptic background strain (designated +/+: F1 progeny of C57Bl/6JEi females × C3H/HeSnJ males) were maintained in the Duke University Vivarium. Male lh/+ (heterozygote) mice were bred with female lh/+ mice to produce 25% *lh/lh* (about 40 male *lh/lh* per month) in the progeny. By 14 d of age, *lh/lh* mice were distinguished from their phenotypically normal lh/+ and +/+ littermates by the presence of an ataxic gait.

$GABA_B$ autoradiography. We modified a procedure described by Bowery and colleagues (1987). Male 8 week old *lh/lh* and +/+ mice were anesthetized with chloroform and decapitated. Brains were quickly removed and frozen in isopentane that was chilled in a dry ice/methanol bath. Sections (16 μm) were cut at −18°C and thaw-mounted onto acid-washed, gel-coated slides (Hosford et al., 1990); slides were frozen at −70°C until the day of each experiment. Fingernail polish was used to paint wells around the sections on each slide, and slides were maintained horizontally during preincubation and incubation. During preincubation, each section was covered with 200 μl of buffer (50 mM Tris-HCl with 2.5 mM $CaCl_2$, pH 7.4 at 25°C) for 45 min at 25°C. Sections were allowed to air-dry for 20 min. Sections were incubated for 45 min at 4°C by applying 200 μl of incubation buffer [buffer as above, with 20 nM ^{3}H-GABA (50 Ci/mmol) and 10 μM isoguvacine (a $GABA_A$ agonist), either in the presence (nonspecific binding) or absence (total binding) of 100 μM (−)-baclofen]. Each slide was dipped rapidly into 2.5% glutaraldehyde in acetone, and then twice into distilled water. Slides were dried with cool filtered air for 15–20 sec.

Autoradiograms were generated by apposing the slides alongside tritium standards against ^{3}H-Ultrofilm (Amersham) at 4°C for an exposure time of 3–4 weeks. The autoradiograms were digitized and analyzed using a computer-assisted image analysis system (RAS/R1000; Loats, Inc.). Optical density (OD) measurements of the tritium standards allowed conversion of OD measurements into units of fmol/mg protein.

Electrode implantation and EEG recordings. In each of six groups of mice, bipolar recording electrodes were implanted into frontal neocortex and one of six different subcortical sites. Male 8 week old *lh/lh* mice (19–22 gm) were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and placed into a stereotaxic holder fitted with a mouse incisor bar. Burr holes were drilled through the left side of the skull over the frontal neocortex (1.5 mm anterior to bregma, 1.5 mm lateral to the midsagittal suture), and through the right side of the skull over one of the following neuronal structures: VLa (1.3 mm posterior to bregma, 1.8 mm lateral); RE (0.9 mm posterior to bregma, 1.0 mm lateral); NRT (0.9 mm posterior to bregma, 2.0 mm lateral); hippocampal formation (HIP; 1.6 mm posterior to bregma, 1.8 mm lateral); caudate-putamen (CPU; AP at bregma, 2.5 mm lateral); and lateral amygdaloid nucleus (AL; 1.6 mm posterior to bregma, 3.8 mm lateral) (coordinates modified from Slotnick and Leonard, 1975). Bipolar teflon-coated microelectrodes (constructed from monopolar electrodes with a 0.011" outer diameter) were lowered into each structure per the following coordinates: frontal neocortex (0.8 mm below dura); VLa (3.2 mm); RE (3.7 mm, directed off vertical by 16° toward the midline); NRT (3.0 mm); HIP (1.8 mm); CPU (3.0 mm); and AL (4.4 mm). Four screws, placed at the periphery of the skull, served as grounds and helped to anchor a dental acrylic cap. After these surgical procedures, mice were allowed to recover for 7 d before the EEG recording session.

During 3 hr EEG recording sessions, records were examined for SWDs arising from the neocortex. Epileptiform bursts were counted as SWDs only if they met all of four criteria (modified from Hosford et al., 1992): (1) bursts had a duration >0.6 sec, (2) bursts were comprised of epileptiform spikes (i.e., <70 msec per spike), (3) bursts had a frequency typical for that animal (5–6 Hz in all cases), (4) bursts were recorded simultaneously from both poles of the electrode (bipolar recording) and from one pole of the electrode referenced to ground (monopolar recording). When bipolar recordings from the frontal neocortex demonstrated SWDs, the record was then examined for the presence of simultaneous SWDs in bipolar recordings from the candidate structure. The simultaneous bipolar recording of SWDs from the neocortex and the candidate structure was considered evidence that SWDs occurred in both structures. Conversely, the presence of SWDs in bipolar recording from the neocortex but not from the candidate structure was considered evidence that SWDs did not occur in the candidate structure (but *only* if the candidate electrode was verified to function properly, by monopolar recordings of SWDs from one pole of the candidate electrode). Electrode placements were verified histologically after the mice were sacrificed. Data were analyzed only from mice in which electrodes were properly positioned in the targeted structure.

Cannula implantation and microinjections. In each of five groups of mice, a bipolar recording electrode was implanted in left frontal neocortex, and guide cannulae were directed towards one of five subcortical targets. Male 8 week old *lh/lh* mice were anesthetized with sodium pentobarbital and placed into a stereotaxic holder as described above. Burr holes were drilled over the left frontal neocortex and over the following neuronal structures *bilaterally*, except in RE: VLa (same coordinates as above, but bilaterally), RE (per above), NRT (per above, but bilaterally), CPU (per above, but bilaterally); and cerebellum (6.2 mm posterior to bregma, 1.1 mm lateral, bilaterally) (coordinates modified from Slotnick and Leonard, 1975). A bipolar Teflon-coated microelectrode was lowered into the frontal neocortex (0.8 mm below dura). Subcortical guide cannulae (22 gauge; Plastic Products) were implanted through their burr holes to the following depths, each of which is 0.5 mm dorsal to their intended targets: VLa (2.6 mm below dura), RE (3.2 mm, directed off vertical by 16° toward midline), NRT (2.5 mm), CPU (2.5 mm), and cerebellum (1.0 mm). Guide cannulae were plugged with blunt stylets when not in use. After surgical procedures, mice were allowed to recover for 7 d before experiments.

One week after implantation, each group of mice (n = 8 male 8 week old *lh/lh* mice) underwent a series of 14 daily 3 hr EEG recording sessions. Thirty minutes after the beginning of each session mice were administered either vehicle [0.9% NaCl (days 1, 3, 5, 7, 9, 11, and 13)] or successively increasing doses of test drug [(−)-baclofen, 4.7–1400 pmol/cannula (days 2, 4, 6 and 8); or CGP 35348, 1.33–13.3 nmol/cannula (days 10, 12, and 14)] via injection cannulae. Injection cannulae were lowered to their final targets (0.5 mm beyond the end of guide cannulae) under gentle hand-held restraint. A Harvard infusion pump attached to two Hamilton syringes was used to infuse drug or vehicle through each cannula at a rate of 0.25 μl/min, to a total volume of 0.25 μl/cannula. Cannulae were left in place an additional 2 min before they were withdrawn. At the end of each series of experiments, mice were sacrificed and cannulae positions were verified histologically. The following data analyses were performed only from mice in which cannulae were properly positioned in the targeted structures.

EEGs were divided into 12 15 min epochs and the seizure frequency was determined. Epileptiform bursts were counted as seizures only if they met the four criteria listed above. Use of these criteria helped to exclude recording artifacts. To analyze effects produced by a test compound over time, the seizure frequency of an animal during each of these 12 15 min epochs was divided by the seizure frequency in the corresponding epoch after vehicle administration. This normalized the effect in each animal against its own baseline seizure frequency, reducing interanimal variability. The mean change in seizure frequency during the epochs was then calculated for all the animals in the group, and plotted as a function of time after drug administration. To statistically compare effects produced by test drug and vehicle, the sum of seizure frequency for 150 min following each dose of test drug was calculated for each mouse. The mean sum for the group was then compared to the corresponding sum following vehicle administration.

Pharmacologic compounds and reagents. ^{3}H-GABA was procured from Du Pont-NEN (Boston, MA). Isoguvacine was purchased from Tocris Cookson (Langford, Bristol, UK). (−)-Baclofen and CGP 35348 were the kind gifts of Dr. Helmut Bittiger of Ciba-Geigy AG (Basel, Switzerland). Sodium pentobarbital was obtained from Durham V.A. Hospital Pharmacy (Durham, NC). All other standard compounds and reagents were procured from Sigma (St. Louis, MO).

Results

$GABA_B$ autoradiography

Specific (−)-baclofen-displaceable ^{3}H-GABA binding (referred to as $GABA_B$ binding throughout the remainder of this article) was used as our first method to screen candidate neuronal structures. The anatomic distribution of specific $GABA_B$ binding in

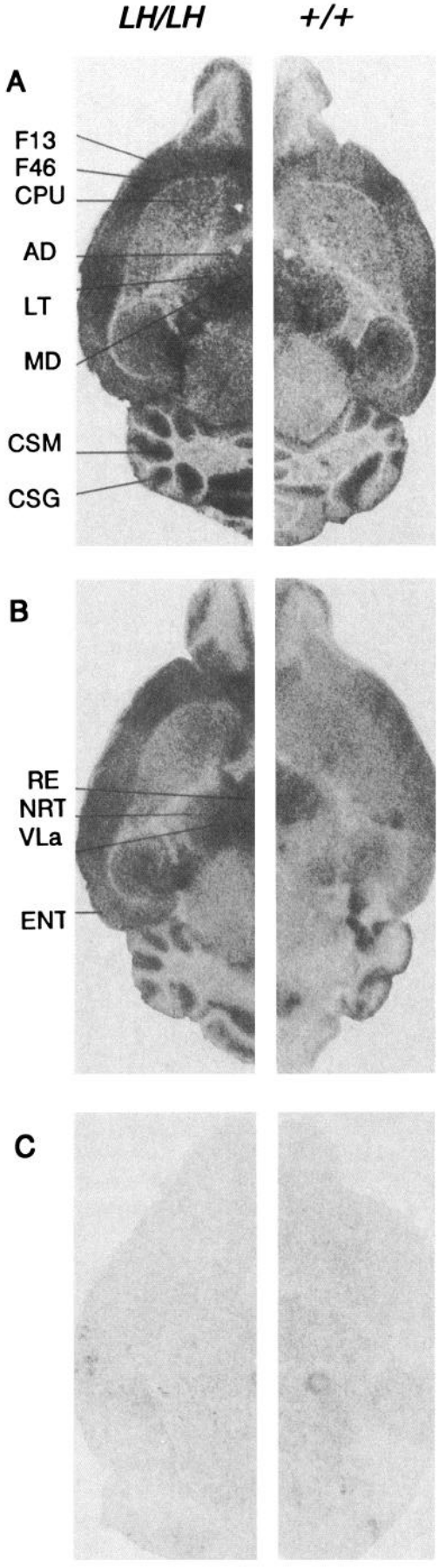

Table 1. Baclofen-displaceable ^{3}H-GABA binding: *lh/lh* versus +/+ mice

Neuronal structure	Specific binding[a] lh/lh	+/+
Frontal neocortex (n = 12)[b]		
Layers 1–3 (F13)	345 ± 30***	214 ± 31
Layers 4–6 (F46)	308 ± 26*	205 ± 32
Entorhinal cortex (ENT) (n = 12)	244 ± 20	185 ± 26
Caudate-putamen (CPU) (n = 7)	162 ± 14*	102 ± 19
Thalamic nuclei (n = 7)		
Anterodorsal (AD)	249 ± 12*	189 ± 23
Lateral (LT)	336 ± 16*	243 ± 28
Mediodorsal (MD)	306 ± 9***	195 ± 26
Nuc. reticularis (NRT)	314 ± 9**	183 ± 44
Reuniens (RE)	536 ± 37***	293 ± 61
Anterior vent. lat. (VLa)	605 ± 23**	370 ± 70
Cerebellum (n = 7)		
Stratum granulosum (CSG)	97 ± 19	48 ± 26
Stratum moleculare (CSM)	249 ± 25	187 ± 36

[a] All values are mean ± SEM; units are fmol/mg protein.

[b] Number of paired sections for each structure given in parentheses (see Fig. 1 for representative original data).

*, $p < 0.05$; ** $p < 0.02$; *** $p < 0.01$; (*lh/lh* vs +/+; two-tailed independent t test).

lh/lh and +/+ brains was qualitatively similar, and was highest in diverse thalamic nuclei, moderate in neocortex and stratum moleculare of cerebellum, and lowest in stratum granulosum of cerebellum (Fig. 1, Table 1). This pattern is similar to that of specific $GABA_B$ binding in rat brain (Bowery et al., 1984, 1987).

Specific $GABA_B$ binding in discrete neuronal populations of *lh/lh* brain was 32–104% greater than binding in corresponding populations of +/+ brain (Table 1). Specific $GABA_B$ binding was significantly greater in *lh/lh* than +/+ in the following neuronal populations (Fig. 1, Table 1): frontal neocortex ($p < 0.05$); multiple thalamic nuclei [anterodorsal (AD), $p < 0.05$; lateral (LT), $p < 0.05$; mediodorsal (MD), $p < 0.01$; NRT, $p < 0.02$; RE, $p < 0.01$; and VLa, $p < 0.02$], and CPU ($p < 0.05$). Although specific $GABA_B$ binding was also greater in other neuronal populations (e.g., entorhinal cortex and cerebellar populations) in *lh/lh* compared to +/+, these increases were not significant at the 0.05 level (Table 1). The results of this first screen thus implicated frontal neocortex, multiple thalamic nuclei, and

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Figure 1. Baclofen-displaceable ^{3}H-GABA autoradiography in *lh/lh* and +/+ mice. Shown are autoradiograms of total (*A, B*) and nonspecific (*C*) baclofen-displaceable ^{3}H-GABA binding in horizontal sections of brain at level of dorsal (*A*) and ventral thalamus (*B*). To facilitate visual comparison, corresponding hemisections of 8 week old male *lh/lh* (*left*) and +/+ (*right*) brain are shown in *A–C*. Structures analyzed at level of dorsal thalamus are labeled as follows in *A*: *F13* (laminae 1–3 of frontal neocortex); *F46* (laminae 4-6 of frontal neocortex); *CPU* (caudate-putamen); *AD* (anterodorsal thalamic nuc.); *LT* (lateral thalamic nuc.); *MD* (mediodorsal thalamic nuc.); *CSM* (stratum moleculare of cerebellum); and *CSG* (stratum granulosum of cerebellum). Structures analyzed at level of ventral thalamus are labeled as follows in *B: RE* (nucleus reuniens); *NRT* (nucleus reticularis thalami); *VLa* (anterior ventral lateral thalamic nucleus); and *ENT* (entorhinal cortex). Nonspecific binding was homogeneously low in *lh/lh* and +/+ brain at both levels; *C* shows representative *lh/lh* and +/+ autoradiograms at level of dorsal thalamus. See Table 1 for analyses of these data.

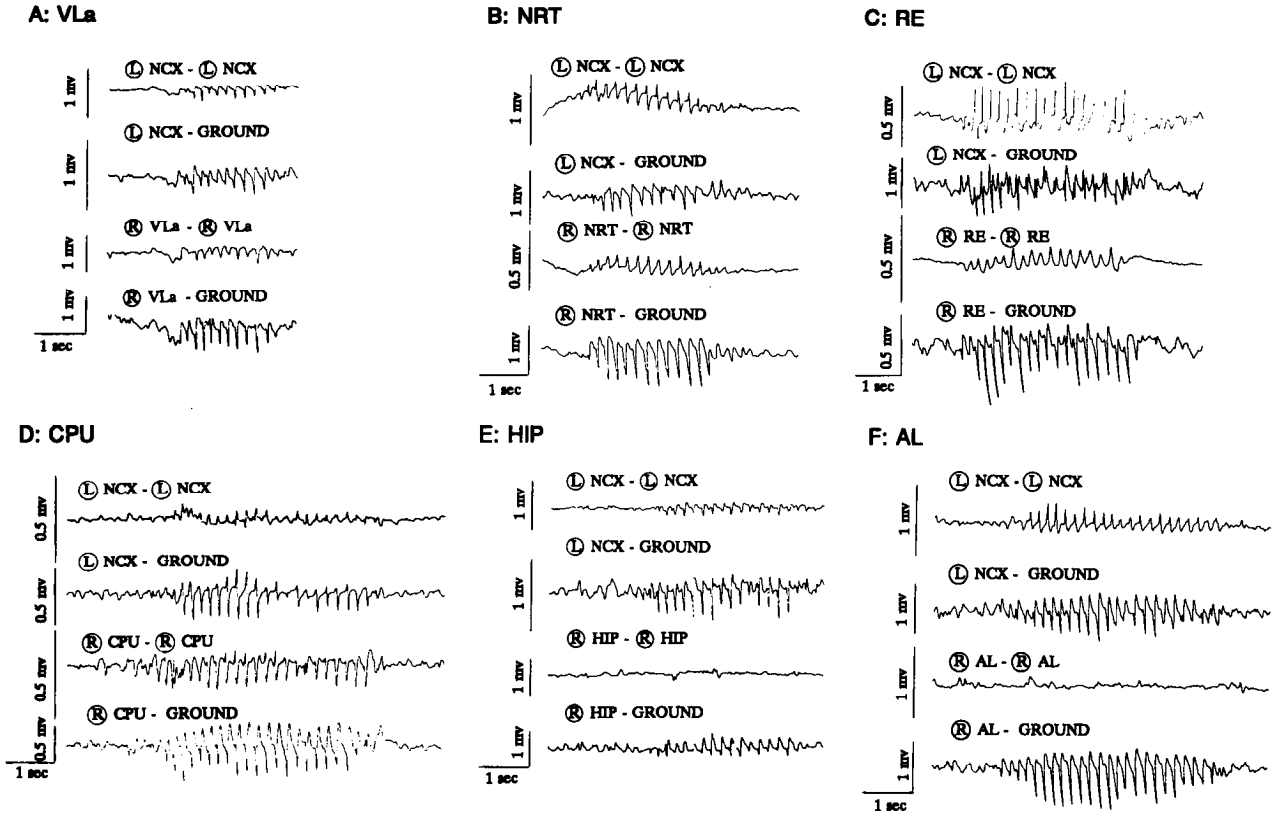

Figure 2. Electroencephalographic recordings of seizures in neocortex and six candidate neuronal populations. In each panel are four simultaneously recorded traces during a seizure; traces are from (top to bottom): left neocortical bipolar electrode; one pole of neocortical electrode referenced to ground; bipolar electrode in subcortical structure of interest in right hemisphere; one pole of subcortical electrode referenced to ground. Durations and amplitudes are marked by bars in each panel. Panels depict recordings in: VLa thalamic nuc. (*A*), NRT (*B*), RE (*C*), CPU (*D*), hippocampal formation (*HIP*) (*E*), and lateral amygdaloid nuc. (*AL*, *F*). Low- and high-frequency filters were set at 0.3 and 35 Hz, respectively. See Table 2 for summary of these data.

CPU as candidate structures to submit to the second screen of subcortical EEG recordings.

Subcortical EEG recordings

For the second level of screening we determined if candidate neuronal structures with significantly greater $GABA_B$ binding could generate SWDs, by recording from bipolar EEG electrodes implanted within each structure (Fig. 2, Table 2). EEG recordings using bipolar electrodes revealed synchronous SWDs in frontal neocortex and VLa (n = 6 of 6; Fig. 2*A*), NRT (n = 7 of 7; Fig. 2*B*), RE (n = 6 of 6; Fig. 2*C*), and CPU (n = 5 of 5; Fig. 2*D*).

As a control we also implanted electrodes into HIP and AL, structures which have moderate $GABA_B$ receptor binding but no currently known role in the generation of absence seizures. EEG recordings revealed no synchronous SWDs in bipolar derivations from HIP (n = 0 of 7; Fig. 2*E*) or from AL (n = 0 of 6; Fig. 2*F*). Importantly, the subcortical electrodes used to obtain these negative results were demonstrated to be functional, by recording SWDs from one pole of the subcortical electrode and a reference electrode (monopolar recordings).

Thus, this screening method revealed that frontal neocortex generated SWDs that were synchronous with SWDs in VLa, NRT, RE, and CPU (Table 2). These structures were candidates for our third screen, involving microinjections of (−)-baclofen and CGP 35348.

Table 2. Ability of structures to generate seizures in synchrony with neocortex

Neuronal structure	Number[a]	% with seizures[b]
Neocortex	>100	95%
Thalamic nuclei		
Anterior vent. lat. (VLa)	6	100%
Nuc. reticularis (NRT)	6	100%
Reuniens (RE)	6	100%
Caudate-putamen	5	100%
Hippocampal formation	7	0%
Lateral amygdaloid nuc.	6	0%

[a] Number of *lh/lh* mice with bipolar recording electrodes implanted in left neocortex and designated subcortical structure in right hemisphere.

[b] Percentage of mice in which seizures were recorded synchronously in designated subcortical structure and neocortex (see Fig. 2 for representative original data).

Microinjections of baclofen and CGP 35348

For the third level of screening we began to identify if these candidate structures had $GABA_B$ receptors that regulated absence seizures, by microinjecting baclofen or CGP 35348 into the structures. To permit meaningful interpretation of these results, it was first necessary for us to measure the volume of diffusion of (−)-baclofen from its microinjection site during the recording period. Accordingly we bilaterally microinjected into

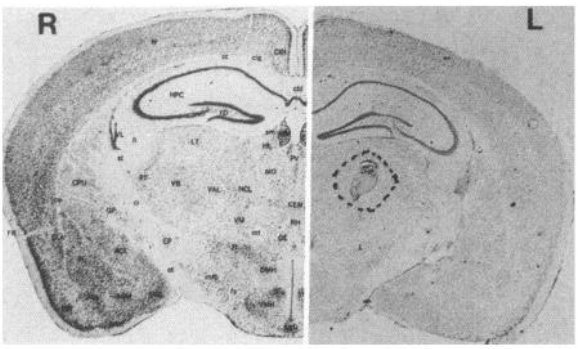

Figure 3. Extent of diffusion of ^{3}H-baclofen after microinjection into VLa thalamic nucleus. A cresyl violet–stained coronal section of *lh/lh* brain is shown (anatomic left hemisection) adjacent to the corresponding atlas level (anatomic right hemisection; Slotnick and Leonard, 1975). The *lh/lh* section shows the microinjection site in the left VLa thalamic nucleus. Outlined around the injection site is the extent of measurable diffusion of ^{3}H-baclofen (0.25 μl cocktail containing 200 ng (−)-baclofen "spiked" with 0.2 μCi of ^{3}H-baclofen), occurring during a 120 min period after injection into VLa. This was assessed by apposing to x-ray film the section adjacent to the one shown here.

VLa a 0.25 μl solution of (−)-baclofen that was "spiked" with ^{3}H-baclofen. At the end of a typical recording period we sacrificed these mice, removed their brains, and prepared adjacent sections for either autoradiographic or histologic analyses. When autoradiograms were juxtaposed with adjacent stained sections, it was evident that baclofen diffused up to 500 μm from the center of its injection site during the 3 hr EEG recording period (Fig. 3). Knowledge of this volume of diffusion helps to ascertain the extent to which the following microinjection results are derived from drug effects that are confined to their targeted injection sites. All of these injection sites were histologically verified before they were included in these results.

Microinjection of (−)-baclofen (4.7–1400 pmol/cannula in 0.25 μl) into VLa (Fig. 4) produced a dose-dependent (Fig. 4*A*) and significant increase ($p < 0.001$) in SWDs to the point of status epilepticus (Fig. 4*B*). The onset of this effect was rapid (<15 min) and persisted for the duration of the 3 hr recording period (Fig. 4*B*). Conversely, microinjection of CGP 35348 (1.33–13.3 nmol/cannula) into VLa significantly ($p < 0.05$) suppressed SWDs in a dose-dependent (Fig. 4*A*) and prolonged manner (Fig. 4*B*).

In a qualitatively similar fashion, microinjection of (−)-baclofen into NRT (Fig. 5) or RE (Fig. 6) produced rapid, dose-dependent, significant ($p < 0.005$) and prolonged (>150 min) increases in SWDs to the point of status epilepticus. Conversely, CGP 35348 produced dose-dependent significant ($p < 0.05$) suppression of SWDs after microinjection into NRT or RE (Figs. 5, 6).

Although microinjection of the highest dose of (−)-baclofen (1400 pmol/ cannula) into CPU produced an increase in SWDs (Fig. 7*A*), this increase required 75 min to fully develop (Fig. 7*B*) and did *not* reach the 0.05 level of significance. *More importantly*, microinjection of the highest dose of CGP 35348 (13.3 nmoles/cannula) did not suppress SWDs (Fig. 7), in contrast to its significant effects after microinjection into VLa (Fig. 4), NRT (Fig. 5), and RE (Fig. 6).

As a control, we also studied the effect of microinjections into cerebellum. This structure has no currently known role in generating SWDs, but does exhibit a moderate level of $GABA_B$ binding that is mildly enriched in *lh/lh* mice (Fig. 1, Table 1). Microinjection of the highest doses of (−)-baclofen or CGP 35348 into cerebellum had no effects on SWDs (Fig. 8).

Thus, neuronal structures in which $GABA_B$ receptors appear to regulate SWDs include VLa, NRT and RE. The rapid onset of significant effects after (−)-baclofen or CGP 35348 was microinjected into each of these neuronal structures supports the contention that a major portion of these effects were produced within the microinjected structures; however, we cannot exclude a contribution by adjacent structures into which drugs may have diffused during the recording period. In contrast, microinjection of (−)-baclofen into CPU produced late-developing, nonsignificant effects and microinjections of a $GABA_B$ antagonist into CPU did not suppress seizures. It is probable that the late-developing effect of (−)-baclofen was the result of diffusion into

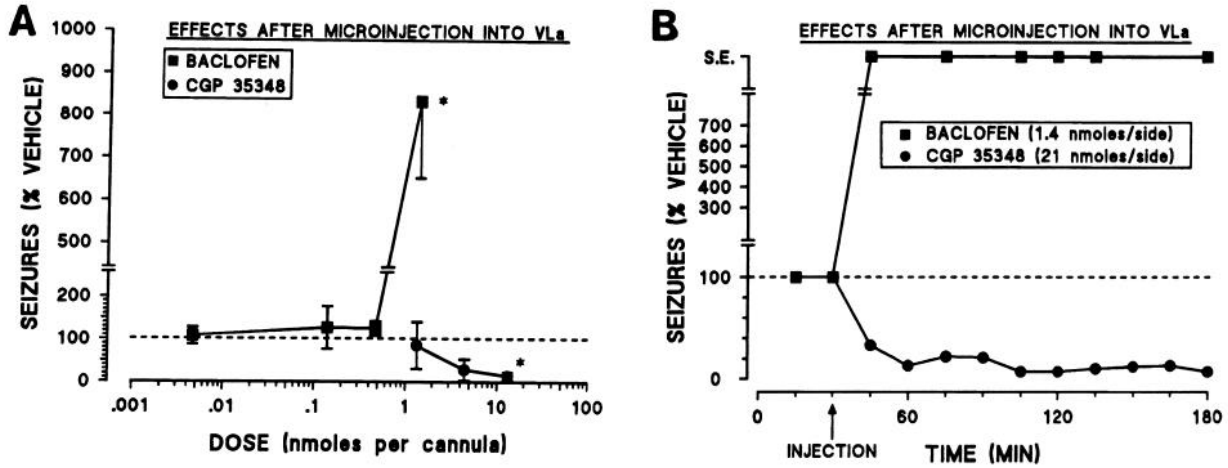

Figure 4. Effects of (−)-baclofen and CGP 35348 on seizure frequency after microinjection into VLa thalamic nucleus. The graph in *A* depicts the dose-dependent and significant (denoted by "*") enhancement of seizures by baclofen ($p < 0.001$; Student's independent two-tailed *t* test) and suppression of seizures by CGP 35348 ($p < 0.05$) after microinjection into VLa. The y-axis shows seizure frequency as a percent of the frequency following vehicle administration. The *dotted line* represents 100% of seizure frequency following vehicle. The graph in *B* depicts the time course of these effects after administration (denoted by *arrow*) of the highest doses of baclofen (1.4 nmol/side) and CGP 35348 (21 nmol/side). Note the full development of baclofen's effect by 15 min after injection. SE denotes the development of status epilepticus.

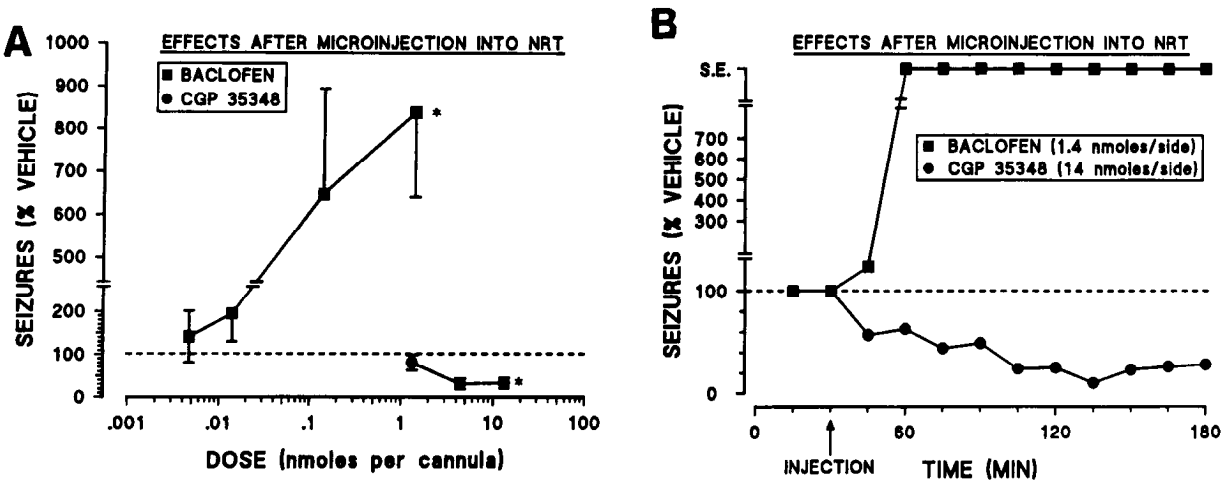

Figure 5. Effects of (−)-baclofen and CGP 35348 on seizure frequency after microinjection into NRT. *A* depicts the dose-dependent and significant (denoted "*") enhancement of seizures by baclofen ($p < 0.005$) and suppression of seizures by CGP 35348 ($p < 0.005$) after microinjection into NRT. *B* depicts the time course of these events. Axes and markings in each graph are similar to those described in Figure 4.

adjacent structures such as neocortex or lateral thalamic nuclei. Thus, it is likely that $GABA_B$ receptors in CPU do not regulate SWDs.

Discussion

Principal findings

Three findings emerged from this study. First, autoradiographic measurements revealed significantly greater binding to $GABA_B$ sites in frontal neocortex, diverse thalamic nuclei, and CPU of *lh/lh* mice compared to matched +/+ mice. Second, subcortical EEG recordings in these $GABA_B$-enriched structures revealed SWDs that were generated synchronously in frontal neocortex and VLa, NRT, RE, and CPU. Third, the rapid onset of significant blockade of SWDs after microinjection of CGP 35348 into VLa, NRT, and RE demonstrated that $GABA_B$ receptors in these neuronal populations regulated absence seizures in *lh/lh* mice. Together, these three findings reveal that VLa, NRT, and RE are part of the neural network in which $GABA_B$ receptors regulate absence seizures in the lethargic (*lh/lh*) mouse model.

In the following discussion it is important to remember that VLa, NRT, and RE are not the only neuronal structures in this neural network. The resolution of our autoradiographic and EEG recording techniques may prevent detection of subpopulations of neurons which harbor $GABA_B$ receptors that regulate absence seizures. Furthermore, the diffusion of compounds beyond their sites of microinjection (see Fig. 3) may have allowed neuronal populations in neighboring neuronal structures to participate in the observed effects. Finally, it is certain that neocortical pyramidal neurons also participate in this network because of their role in thalamocortical oscillatory burst-firing (Crunelli and Leresche, 1991; Steriade et al., 1993), significantly increased numbers of $GABA_B$ binding sites, and generation of SWDs (Hosford et al., 1992; screens 1 and 2 of this study). However, the large volume of neocortex precluded the microinjection studies of screen 3. Nevertheless, the identification of VLa, NRT, and RE as part of this neural network in *lh/lh* mice provides a first step in studies exploring the molecular basis for regulation of absence seizures by $GABA_B$ receptors.

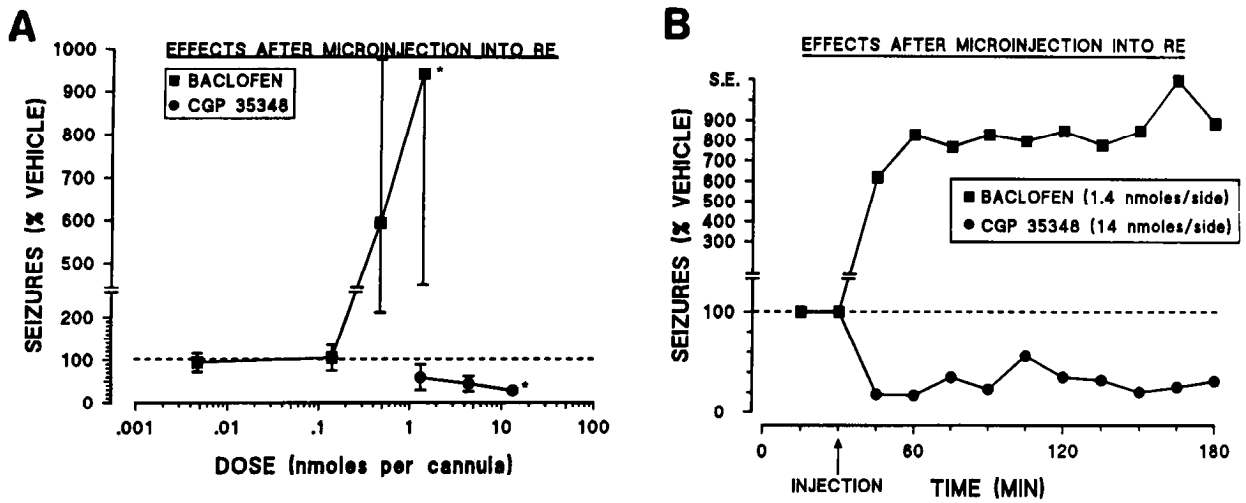

Figure 6. Effects of (−)-baclofen and CGP 35348 on seizure frequency after microinjection into RE. *A* depicts the dose-dependent and significant (denoted "*") enhancement of seizures by baclofen ($p < 0.01$) and suppression of seizures by CGP 35348 ($p < 0.05$) after microinjection into RE. *B* depicts the time course of these events. Axes and markings in each graph are similar to those described in Figure 4.

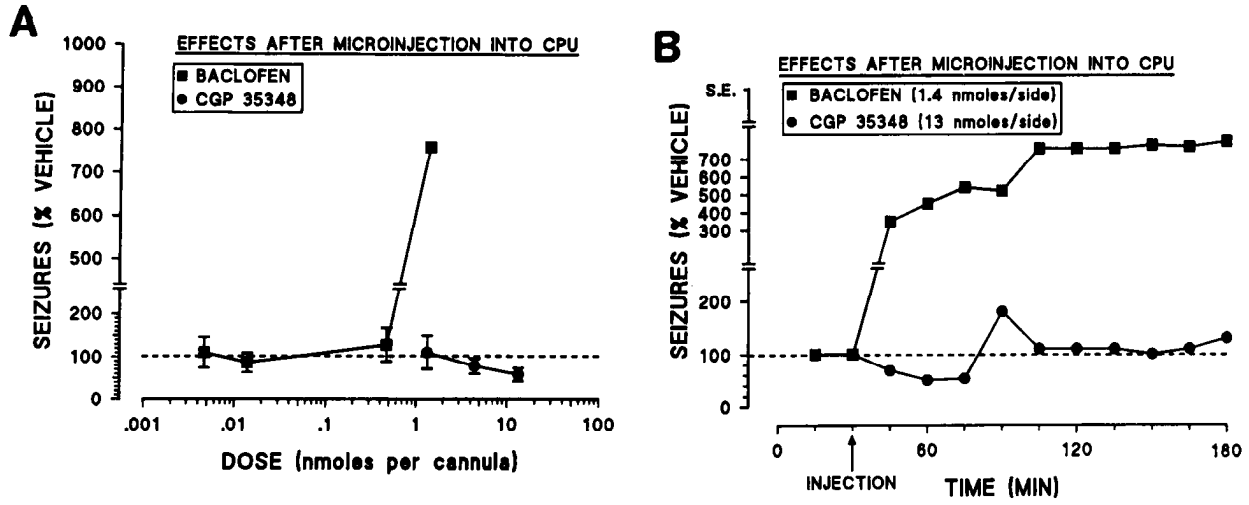

Figure 7. Effects of (−)-baclofen and CGP 35348 on seizure frequency after microinjection into CPU. *A* depicts the late-developing enhancement of seizures by baclofen and the *lack* of suppression of seizures by CGP 35348 after microinjection into CPU. *B* depicts the time-course of these events; note the 75 min required before the effect of baclofen was fully developed. Axes and markings in each graph are similar to those described in Figure 4.

Functional consequences of the neural network in which $GABA_B$ receptors regulate absence seizures

It is not surprising that neuronal populations within the thalamus *generate* absence seizures. Extensive data from humans (Williams, 1953) and from animal models have demonstrated the contribution of both cortical and thalamic neurons to these seizures (Jasper and Droogleever-Fortuyn, 1946; Quesney et al., 1977; Avoli et al., 1983; Vergnes et al., 1987; Gloor and Fariello, 1988; Vergnes et al., 1990; Vergnes and Marescaux, 1992). Likewise, the role of thalamic relay neurons in the thalamocortical circuit of synchronized burst-firing has been clearly documented (Steriade and Llinś, 1988; Crunelli and Leresche, 1991; Steriade et al., 1993; von Krosigk et al., 1993; Huguenard and Prince, 1994a,b). However, only one other study has attempted to identify discrete neuronal populations in which $GABA_B$ receptors *regulate* absence seizures (Liu, 1992), and our study is the first to demonstrate the importance of NRT and RE in this regard.

What is the functional consequence of VLa in absence seizures? Before addressing this question we must first consider the anatomic projections from neurons in VLa. There is a surprising lack of information regarding projections from VLa in mouse (Jones, 1985). In monkey, neurons in VLa project to supplementary motor cortex (Asanuma et al., 1983a,b). In cat, these neurons project to both supplementary motor and motor cortex (Strick, 1970, 1973), and in hedgehog, nucleus ventralis thalami projects to widespread areas of neocortex including somatosensory cortex (Valverde et al., 1986). These findings demonstrate a widespread projection from VLa to motor and perhaps somatosensory neocortex in diverse mammalian species. This widespread projection may underlie observations that electrical stimulation of nucleus ventralis thalami of rat (Kohler and

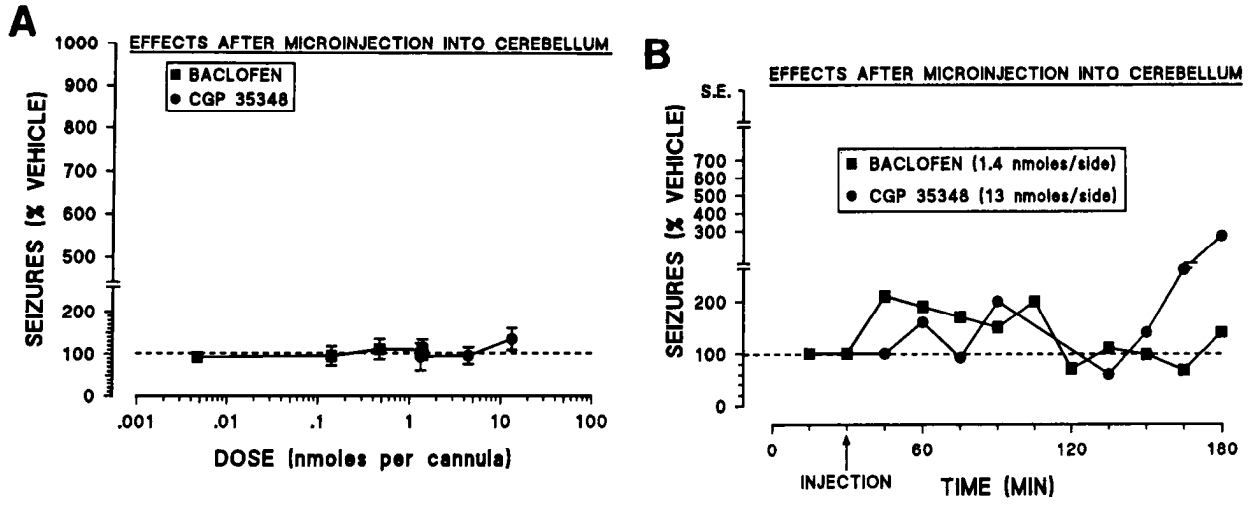

Figure 8. Effects of (−)-baclofen and CGP 35348 on seizure frequency after microinjection into cerebellum. *A* depicts the lack of effect of baclofen and CGP 35348 on seizure frequency after microinjection into cerebellum. *B* depicts the time course of these experiments. Axes and markings in each graph are similar to those described in Figure 4.

Klingberg, 1969) or cat (Quesney et al., 1977) elicited robust spindle activity in neocortex. If similar projections are found in mouse, then the involvement of relay neurons in VLa may account for the propagation of absence seizures to central neocortex, with progressively decreased representation in posterior regions of neocortex (Morison and Dempsey, 1942; Williams, 1953). Our demonstration that $GABA_B$ receptors in VLa regulate absence seizures in *lh/lh* mice underscores the importance of this neuronal population in the propagation of absence seizures.

What are the functional consequences of our finding that $GABA_B$ receptors in NRT regulate absence seizures? The important role of NRT in synchronized burst-firing was recognized after lesion studies in cat (Steriade et al., 1987). NRT neurons, which are exclusively GABAergic (Houser et al., 1980), project to diverse relay nuclei (Steriade et al., 1990; Shibata, 1992) and serve as the primary means of synchronizing thalamocortical burst-firing (von Krosigk et al., 1993; Huguenard and Prince, 1994a,b). The ability of NRT neurons to exhibit spontaneous oscillatory burst-firing is dependent on membrane potential (Avanzini et al., 1989; Bal and McCormick, 1993), and may be most effective at relatively hyperpolarized potentials (Wallenstein, 1994). Consequently, our observation that activation of $GABA_B$ receptors in NRT enhances the frequency of absence seizures in *lh/lh* mice may be due in part to $GABA_B$ receptor-mediated hyperpolarization of NRT neurons. Alternative mechanisms through which $GABA_B$ receptors may regulate absence seizures are addressed in the following section.

What are the functional consequences of RE in absence seizures? The ability of $GABA_B$ receptors in RE to regulate absence seizures is a surprising result that is not adequately explained by the known neuroanatomic connections between RE and other neuronal populations. RE projects primarily to entorhinal cortex and hippocampal area CA1 (Wouterlood et al., 1990), and to subiculum and parasubiculum (van Groen and Wyss, 1990). Sparse projections to nucleus accumbens and olfactory tubercle have also been described (Berendse and Groenewegen, 1990). Because hippocampal neuronal populations are not thought to contribute to the propagation of absence seizures, the physiologic relevance of the connections between RE and hippocampal areas is uncertain. Our finding that seizures recorded in neocortex of *lh/lh* mice did not correlate with any epileptiform discharge recorded from bipolar electrodes in the hippocampal formation is consistent with this idea. Nevertheless, the presence of mossy fiber sprouting in the hippocampal formation of stargazer (*sg/sg*), a mutant mouse model of absence seizures, suggests that hippocampal neurons may suffer pathologic consequences of absence seizures (Qiao and Nocbels, 1993). Projections from RE to area CA1 may provide the pathophysiologic basis for this finding.

Clearly not all neuronal populations enriched in $GABA_B$ receptors play a role in the generation or regulation of absence seizures. For example, hippocampal formation, AL and cerebellum contain neuronal populations in which $GABA_B$ receptor binding sites were enriched. However, subcortical recordings showed that none of these structures generated absence seizures that were synchronous with those generated by neocortex. Thus, these neuronal structures are not part of the network through which absence seizures are generated or propagated.

Likewise, CPU is an example of a neuronal population which has enriched $GABA_B$ receptor binding sites and which undergoes synchronized burst-firing during an absence seizure, yet lacks regulation by $GABA_B$ receptors of these seizures. There are two possible explanations for this finding. First, if the expression of absence seizures is dependent solely on the presence of thalamocortical SWDs, then the propagation of SWDs beyond this critical circuitry may no longer affect the seizure itself. Thus, although the rich excitatory afferents to CPU from neocortex would favor the propagation of thalamocortical SWDs into CPU, $GABA_B$ receptors in CPU neurons may have no effect on the expression of absence seizures. Second, $GABA_B$ receptors in CPU neurons may not be coupled to the intracellular mechanisms (e.g., T-channels) which link receptor activation to burst-firing. The mechanisms coupling $GABA_B$ receptor activation to thalamocortical burst-firing will be considered in the next section.

Possible role of $GABA_B$ receptors in absence seizures

By identifying that VLa, RE, and NRT are among the neuronal structures in which $GABA_B$ receptors regulate absence seizures in *lh/lh* mice, it should be feasible to explore the mechanism(s) by which $GABA_B$ receptors regulate the synchronized burst-firing of absence seizures at a cellular level. A diversity of postsynaptic or presynaptic mechanisms could underlie this role of $GABA_B$ receptors. One possible role of postsynaptic $GABA_B$ receptors relates to the biophysical properties of T-type calcium channels. This type of voltage-dependent calcium channel endows thalamic relay neurons with the capacity to undergo burst-firing. Supporting the critical role of T-channels in absence seizures, ethosuximide appears to produce its antiabsence effects by suppressing the currents (T-currents) which are elicited by activation of T-channels (Coulter et al., 1989a,b; Huguenard and Bercutt, 1994; Huguenard and Prince, 1994b). Once activated, T-channels are quickly inactivated and they require a lengthy, intense hyperpolarization to remove their inactivation (a process termed *deinactivation*). The requisite hyperpolarization can be provided by $GABA_B$ receptors that are present on thalamic relay neurons (Crunelli and Leresche, 1991). Hence, this type of role could be played by $GABA_B$ receptors on cells in VLa.

In a similar fashion, postsynaptic $GABA_B$ receptors in NRT cells could help to deinactivate T-channels. This process could help synchronize the output from NRT cells to thalamocortical cells, and thereby could regulate the oscillatory tendencies of the thalamocortical circuit.

Presynaptic $GABA_B$ receptors may also participate in the pathogenesis of absence seizures. For example, strategic localization of $GABA_B$ receptors on recurrent collaterals of NRT cells, which are exclusively GABAergic (Houser et al., 1980), could inhibit neurotransmission between adjacent NRT cells, and thereby enhance the output of tonically active NRT cells to thalamocortical cells. The net effect would be an increased likelihood of oscillatory firing within the network. Several studies have provided evidence favoring enhanced presynaptic $GABA_B$ receptor-mediated function in pharmacologic (Banerjee et al., 1993; Banerjee and Snead, 1994; Liu et al., 1994) and *lh/lh* models (Lin and Hosford, 1994; Lin et al., 1995) of absence seizures.

Possible mechanisms underlying absence seizures in other models

It is now clear that numerous molecular targets besides $GABA_B$ receptors can be altered to generate or suppress absence seizures in different models. In the GAERS model absence seizures do not appear to be linked to changes in $GABA_B$ receptors (Knight and Bowery, 1992), but instead these seizures may be linked to

altered expression of β2 and β3 subunits of $GABA_A$ receptors (Spreafico et al., 1993) or to altered biophysical properties of T-channels in NRT neurons (Tsakiridou et al., 1995). In the tottering (*tg/tg*) mouse model there may be a regulatory role for noradrenergic mechanisms (Levitt and Noebels, 1981; Noebels, 1984). We hope that information forthcoming from future studies in *lh/lh* and other models will facilitate the development of new therapies for humans with absence seizures.

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