Endogenous Positive Allosteric Modulation of GABA<sub>A</sub> Receptors by Diazepam binding inhibitor

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SUMMARY

Benzodiazepines (BZs) allosterically modulate γ-aminobutyric acid type-A receptors (GABA<sub>A</sub>Rs) to increase inhibitory synaptic strength. Diazepam binding inhibitor (DBI) protein is a BZ site ligand expressed endogenously in the brain, but functional evidence for BZ-mimicking positive modulatory actions has been elusive. We demonstrate an endogenous potentiation of GABAergic synaptic transmission and responses to GABA uncaging in the thalamic reticular nucleus (nRT) that is absent in both nm1054 mice, in which the Dbi gene is deleted, and mice in which BZ binding to α3 subunit-containing GABA<sub>A</sub>Rs is disrupted. Viral transduction of DBI into nRT is sufficient to rescue the endogenous potentiation of GABAergic transmission in nm1054 mice. Both mutations enhance thalamocortical spike-and-wave discharges characteristic of absence epilepsy. Together, these results indicate that DBI mediates endogenous nucleus-specific BZ-mimicking (“endozepine”) roles to modulate nRT function and suppress thalamocortical oscillations. Enhanced DBI signaling might serve as a therapy for epilepsy and other neurological disorders.

INTRODUCTION

Allosteric modulation can profoundly regulate the function of ion channels and G protein-coupled receptors in either a positive or negative direction (Conigrave and Franks, 2003; Schwartz and Holst, 2007) and is of increasing interest for both physiology and pharmacology. Benzodiazepines (BZs) act as allosteric modulators on type-A receptors for the inhibitory neurotransmitter γ-aminobutyric acid (GABA). BZs act as either positive allosteric modulators (PAMs) and prolong currents through GABA<sub>A</sub>Rs to increase the duration and strength of inhibitory signals, or as negative allosteric modulators (NAMs, or inverse agonists) (Sieghart, 1995). The discovery of BZ sites on GABA<sub>A</sub>Rs (Braestrup and Squires, 1977; Möhler and Okada, 1977; Gavish and Snyder, 1980) led to the hypothesis that the brain might synthesize its own endogenous BZ site ligands (Iversen, 1977). In the intervening years, however, functional evidence for endogenous PAM effects has been quite elusive.

The family of peptides derived from the 10 kDa protein diazepam binding inhibitor (DBI) (Guidotti et al., 1983; Alho et al., 1985), also known as acyl-CoA binding protein (ACBP) (Knudsen, 1991), has been suggested to play such roles. Most evidence, however, has indicated NAM actions, such as facilitation of anxiety behaviors (Guidotti et al., 1983; De Mateos-Verchere et al., 1998), increased aggression (Kavaliers and Hirst, 1986), and decreased sleep (Dong et al., 1999). DBI and a DBI fragment peptide, octadecaneuropeptide (ODN), also promote neurogenesis in the subventricular zone (SVZ) via negative modulation of GABA signaling (Alfonso et al., 2012). DBI is synthesized by both neurons and glia (Alho et al., 1989), and its proteolytic peptide products bind to both GABA<sub>A</sub>R and mitochondrial BZ sites (Papadopoulos et al., 1991). Functional evidence for endogenous PAM actions that would suppress neural excitability, however, has not been demonstrated.

Absence seizures, which are characterized by staring spells and brief lapses of consciousness that occur hundreds of times per day, are driven by abnormal oscillatory activity in thalamocortical (TC) networks (Crunelli and Leresche, 2002; Beenakker and Huguenard, 2009). The thalamic reticular nucleus (nRT) is functionally and anatomically poised to play a critical gating role in this circuitry, which is normally involved in sleep rhythms and sensory processing (Steriade et al., 1993). nRT receives excitatory input from both corticothalamic and TC axons and provides GABAergic input onto TC relay cells in dorsal thalamus, such as the ventrobasal nucleus (VB), as well as intranuclear inhibition via recurrent collaterals (Cox et al., 1996; Pinault et al., 1997; Shu and McCormick, 2002). Reductions in intra-nRT inhibition result in hypersynchronous epileptiform oscillations between nRT and VB and promote absence seizures (von Krosigk et al., 1993; Huguenard and Prince, 1994a; Huntsman et al., 1999; Sohal and Huguenard, 2003). Conversely, a gain of intra-nRT inhibition dampens oscillatory duration and power (Schofield et al., 2009). Modulation of intra-nRT inhibition can thus shape TC circuit activity, thereby influencing seizure susceptibility and duration.
In mature nRT, the predominant GABA$_A_R$ $a$ subunit is $a_3$, whereas $a_1$ is highly expressed in dorsal thalamus (Wisden et al., 1992; Fritschy and Mohler, 1995). Experiments utilizing mice bearing point mutations in either $a_3$(H126R) or $a_1$ subunits ($a_1[H101R]$) that selectively abolish BZ binding in GABA$_A_R$s containing these subunits (Rudolph et al., 1999; Löw et al., 2000) demonstrated that BZs act via specific enhancement of intra-nRT inhibition to suppress TC oscillations (Sohal et al., 2003). A human GABA$_A_R$ $g_2$ subunit mutation that alters BZ binding is associated with absence seizures (Wallace et al., 2001), suggesting a role for a PAM in regulating seizure activity, but this mutation also affects receptor trafficking (Kang and Macdonald, 2004) and GABA deactivation and desensitization kinetics (Bowser et al., 2002).

DBI mRNA is widely expressed throughout the brain, including the thalamus (Lein et al., 2007). Previous immunohistochemical studies have observed varying profiles of protein expression for DBI and fragment peptides in the CNS of various species (Tonon et al., 1990; Slobodyansky et al., 1992; Lihrmann et al., 1994), likely due to use of different antisera and other methodological differences, but in some cases higher expression was observed in nRT (Alho et al., 1985, 1989). We therefore hypothesized that DBI may exert endogenous effects within the thalamus, thereby modulating seizure susceptibility. Here, we investigate this by comparing inhibitory transmission, effects of BZ site blockade, and seizure profiles in wild-type (WT), $a_3$(H126R), and $nm1054$ (new mutation 1054) mice, which harbor a 400 kb deletion on chromosome 1 that includes the $Dbi$ gene (Ohgami et al., 2005). Furthermore, we tested the ability of viral transduction of DBI into the thalamus to rescue the effect of the $nm1054$ mutation. We also examine allosteric modulation of responses to focal GABA uncaging in “sniffer” patches pulled from VB neurons and placed back in the slice in either VB or nRT.

**RESULTS**

**IPSC Duration in nRT Is Reduced in $a_3$(H126R) Mutant Mice**

The primary PAM effect of BZs on GABA$_A_R$s is to increase the duration of inhibitory postsynaptic currents (IPSCs) (Mody et al., 1994). Therefore, we focused on this parameter in assessing whether endogenous BZ site PAMs alter intrthalamic inhibition. We recorded spontaneous IPSCs (sIPSCs) and evoked monosynaptic intra-nRT IPSCs (eIPSCs) in nRT cells from...
C57BL/6 WT and α3(H126R) mice. As in previous reports (Huntsman and Huguenard, 2000), sIPSC duration progressively decreased through early development, reaching maturity by P20; therefore, all experiments used age-matched comparisons. α3(H126R) cells in both young and adult mice showed briefer sIPSCs (p < 0.001) and eIPSCs (p < 0.01) compared to WT (Figures 1A–1D and S1; Table S1). The decay phase of sIPSCs could be best described by a double exponential function, and both fast and slow decay time constants were shortened by the α3(H126R) mutation, while the relative contribution of fast and slow decay was unaffected (Table S1). This suggests that both components of IPSC decay are dependent on BZ-sensitive GABAARs. There was no difference in unitary conductance or numbers of channels mediating events as revealed by non-stationary variance analysis (Sigworth, 1980; De Koninck and Mody, 1994; Schofield and Huguenard, 2007; Figures 1E–1G). Outside-out membrane patches pulled from WT and α3(H126R) cells showed similar responses to laser photolysis of caged GABA (1 mM) (Figures 1H–1J), indicating that differences in sIPSC duration are not due to altered GABA affinity or chloride conductance, but rather are due to the modulatory actions of a constitutively released BZ site PAM.

Flumazenil Reduces IPSC Duration in nRT, but Not VB

To test the hypothesis that in WT mice an endogenous BZ site PAM constitutively potentiates IPSC duration, we examined the effect of 10 min bath application of flumazenil (FLZ, 1 μM), a BZ site antagonist (Hunkeler et al., 1981). FLZ reduced sIPSC (p < 0.001) and eIPSC (p < 0.05) duration, along with decay rates, in WT nRT cells (Figures 2A–2E; Table S1) but had no effect on sIPSC duration in α3(H126R) cells (Figures 2F and 2G), confirming that these effects depend on the BZ site on the α3 subunit.

BZs and DBI-derived peptides can increase the production of neurosteroids via upregulation of the mitochondrial BZ receptor, also known as peripheral BZ receptor (PBR) or 18 kDa translocator protein (TSPO) (Papadopoulos et al., 1991; Tokuda et al., 2010). To examine if the FLZ-sensitive potentiation of sIPSCs reflect actions of neurosteroids, we used the 5α-reductase inhibitor finasteride (1 μM) to block neurosteroidogenesis. Finasteride alone reduced nRT sIPSC duration, indicating a level of constitutive neurosteroid expression (Figures 3A and 3B), but did not affect the response to FLZ, indicating that FLZ-induced reductions in sIPSC duration do not reflect blockade of neurosteroid actions (Figures 3C and 3D).

The degree of constitutive BZ site activation in nRT was estimated at ~60%, based on the maximal modulation produced by a saturating concentration (100 nM) of clonazepam (CZP).
FLZ had no effect on VB cell sIPSCs (p > 0.2) (Figures 2 A–2C and S2) but reversed the effects of CZP (Figures 4 E and 4F), indicating that VB neurons do respond to FLZ, but only in the presence of exogenous BZs; i.e., endogenous BZ site PAMs are not functionally active in VB.

Dbi Gene Products Mediate Endogenous BZ-Mimicking Actions in nRT

One molecular candidate that may mediate the endogenous PAM effects in nRT is DBI. To test the role of DBI in mediating these effects, we compared nm1054 mice to WT littermates. Immunocytochemical staining confirmed that DBI protein expression in the thalamus is essentially abolished in nm1054 mice (Figures 5 A and 5B). As with the α3(H126R) mutation, the duration, charge transfer, and fast and slow decay time constants of sIPSCs in nRT cells from nm1054 mice was reduced compared to WT (p < 0.001) (Figures 5C and 5D; Table S2). These results suggest that loss of the Dbi gene reduces endogenous allosteric potentiation of GABAAergic currents in nRT.

Figure 3. Endozepine Effects in nRT Are Distinct from Neurosteroid Actions
(A) Averaged sIPSC traces from nRT cells in control conditions (n = 14 cells) and cells preincubated and recorded in finasteride (n = 13 cells) recorded at ages P17–25, normalized to peak amplitude. (B) Probability distributions for >1,300 events per group comparing sIPSC half width in control (black line) versus finasteride conditions (purple line). (C) sIPSC half width in individual nRT cells in control (n = 10) or finasteride conditions (n = 9) before (Con) and during (FLZ) in vitro FLZ treatment. Open boxes indicate mean ± SEM. (D) Mean ± SEM for percentage change in sIPSC half width in response to FLZ in control and finasteride conditions. **p < 0.01, ***p < 0.001 versus control (B and C); ns, not significant.

Figure 4. Constitutive Activation of BZ Sites in nRT Is Approximately 60% of Maximal, and VB Neurons Are Sensitive to BZs
(A) Representative averaged traces of sIPSCs recorded in an individual WT nRT cell under control conditions (black trace), after 10 min bath application of 100 nM clonazepam (CZP, gray trace), and in CZP with subsequent 10 min bath application of FLZ (CZP+FLZ, red trace), normalized to peak amplitude. (B) sIPSC half width in individual WT nRT cells (n = 8) at P20–29 in control conditions (Con), during CZP treatment (CZP), and during subsequent FLZ treatment (CZP+FLZ). Open boxes indicate mean ± SEM during control (black), CZP (gray), and CZP+FLZ conditions (red). (C) Mean ± SEM for percentage change in sIPSC half width from control to CZP conditions (gray bar), CZP to CZP+FLZ conditions (red bar), and from control to CZP+FLZ conditions (black bar). (D) Mean ± SEM for the within-cell ratio of the percentage change from control to CZP+FLZ conditions compared to the percentage change from CZP alone to CZP+FLZ. (E) Averaged sIPSC traces from control VB cells (black trace) and cells in slices either preincubated in 100 nM clonazepam (CZP, gray trace), or in CZP with subsequent 10 min bath application of FLZ (CZP+FLZ, red trace). Note that FLZ treatment does not reduce sIPSC duration past control values, as compared to nRT cells. **p < 0.01, ***p < 0.001 versus control (B and F); ns, not significant.
To determine whether this deficit in the nm1054 mutant was due to loss of Dbi gene products, we tested the effect of infecting nRT cells with an AAV vector expressing DBI and green fluorescent protein (GFP) (Figure S3). Injection of AAV-DBI-GFP into nRT of nm1054 mice both increased sIPSC duration and conferred responsiveness to FLZ treatment that was not observed in nm1054 mice injected with control AAV-GFP (Figures 5E–5K). Injection of AAV-GFP into WT nRT was confirmed to not affect responsiveness to FLZ (Figures 5I–5K). Thus the endogenous PAM actions in nRT are mediated by products of the Dbi gene.

**GABA Uncaging Responses in Sniffer Patches Pulled from VB Neurons Demonstrate nRT-Dependent Potentiation**

The nucleus-specificity of FLZ effects may result from differential localization of PAMs and/or different GABAAR subunit composition in nRT and VB. To test these possibilities directly, we pulled outside-out membrane patches containing GABAARs from VB cells, which were then placed back into the slice to function as “sniffer patches” (Isaacson et al., 1993; Allen, 1997; Banks and Pearce, 2000). We then tested the response

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**Figure 5. AAV-Mediated Expression of DBI Rescues Effects of nm1054 Mutation on nRT sIPSC Duration**

(A and B) Fluorescence images illustrating immunocytochemical staining for DBI and the nucleic acid marker DAPI in striatum and thalamus in WT (A) and nm1054 (B) mice. Dotted and dashed lines indicate approximate borders of striatum (Str), internal capsule (IC), and thalamic nuclei.

(C) Averaged sIPSCs from nRT cells recorded from WT (n = 14 cells) and nm1054 mice (n = 13 cells) at P22–29, normalized to peak amplitude.

(D) Probability distributions (>1,300 events/group) comparing sIPSC half width.

(E) GFP fluorescence in mouse thalamus injected with AAV-DBI-GFP vector.

(F–H) DBI and GFP staining in nRT of nm1054 mice injected with control AAV-GFP (F) or AAV-DBI-GFP vector (G and H).

(I) Probability distributions (>800 events/group) comparing sIPSC half width.

(J) sIPSC half width in nRT cells from WT mice injected with control AAV-GFP (left, n = 7 cells), and nm1054 mice injected with AAV-GFP (middle, n = 7) or AAV-DBI-GFP vector (right, n = 11) before (Con) and during FLZ treatment. Open boxes indicate mean ± SEM.

(K) Mean + SEM for percentage change in sIPSC half width in response to FLZ.

Calibration: 100 μm (A and B); 200 μm (E); 25 μm (F–H). **p < 0.01, ***p < 0.001 versus WT (D, I, and K) or Con (J); ##p < 0.01, ###p < 0.001 versus nm1054 AAV-DBI-GFP (I and K). See also Figure S3 and Table S2.
In WT slices, sniffer patches moved to nRT exhibited an increased uncaged IPSC duration compared to patches placed in VB (p < 0.00001) (Figure 6A). Both FLZ treatment and the nm1054 mutation largely blocked the nRT-dependent potentiation (~25% enhancement remaining in FLZ or nm1054 versus 72% in control, p < 0.01), and FLZ had no effect on responses in nm1054 slices (p > 0.9), suggesting that the nm1054 mutation removes a source of potentiating actions at BZ sites. This was confirmed further by occlusion of the nRT-dependent potentiation to a similar degree (~13% potentiation remaining) by the presence of CZP (Figures S4A and S4B). Combined application of GABA transporter (GAT) antagonists and FLZ in WT slices blocked all nRT-dependent potentiation (p > 0.9), which was preserved in the presence of GAT antagonists alone (p < 0.001) (Figures S4C–S4E), demonstrating that the residual potentiation in the presence of FLZ/CZP and in nm1054 mutants results from tissue-dependent differences in GABA uptake. Dbi gene products that are endogenous PAMs are thus constitutively released and bind to the extracellular BZ binding domain on GABA<sub>A</sub> receptors in nRT, but not VB. Furthermore, z1-containing GABA<sub>A</sub>Rs in VB are sensitive to endogenous PAMs (i.e., these actions do not depend on z3 subunits per se) but do not normally respond to FLZ treatment due to the absence of endogenous ligand in this nucleus.

**Altered Spike-and-Wave Discharge Activity in z3(H126R) and nm1054 Mice**

Intra-nRT inhibition plays a critical role in regulating thalamic oscillations and absence seizure activity in the thalamocortical circuit. To examine whether the endogenous PAM actions observed in nRT modulate seizure susceptibility, we performed electroencephalogram (EEG) recordings in adult mice and assessed both spontaneous and pharmacologically induced spike-and-wave discharges (SWDs, a characteristic of absence epilepsy). SWDs in human absence epilepsy patients typically display ~3 Hz internal frequency (Steriade et al., 1993; Crunelli and Leresche, 2002), but in many rodent models the internal frequency is in the range of 4–6 Hz (Noebels and Sidman, 1979; Ryan and Sharpless, 1979; Hosford et al., 1992). Both z3(H126R) and nm1054 mice showed a higher incidence of spontaneous 4–6 Hz SWDs compared to the very rare events in WT counterparts (p < 0.01; Figures 7A–7D), suggesting a level of constitutive BZ site activation that is antiepileptic.

Low dose pentylenetetrazol (PTZ) is a common means of chemically inducing absence seizures and SWD in rodents (Snead, 1998). In WT and z3(H126R) mice on the C57BL/6 background, however, PTZ induced generalized tonic-clonic seizures rather than absence seizures, precluding examination of strain-dependent differences in SWDs (see Supplemental Information). Therefore, we also examined z3(H126R) mutants on the 129X1/SvJ background, which we found to be less susceptible to tonic-clonic seizures. In this strain, experimental absence seizures initiated rapidly and were characterized by prominent ~4–5 Hz SWDs that peaked approximately 5 min after injection, reaching a similar peak incidence in both genotypes, but persisted at a much higher rate over time in the z3(H126R) mutants (Figure S5). SWD internal frequency showed a progressive slowing from ~4 to 3 Hz during the course of repeated
seizures in WT, but remained constant at /C24
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a
3(H126R)
mutants (p > 0.8) (Figures 7 E and 7G). Similarly, nm1054
mice
failed to display the slowing of SWD internal frequency following
PTZ injection (p > 0.6) that was observed in WT (Figures 7 F and
7H). These results suggest that PAM release within the TC circuit
reduces seizure susceptibility and severity through a slowing of
the seizure oscillations, which may destabilize them.

DISCUSSION

Following the discovery of BZ binding sites on GABA ARs 35
years ago (Braestrup and Squires, 1977; Möhler and Okada,
1977; Gavish and Snyder, 1980), it was thought that the brain
likely produces cognate endogenous ligands for these sites. In
the intervening years, however, the search for an endogenous
PAM proved elusive. The studies presented here provide func-
tional evidence for endogenous BZ-mimicking ("endozepine")
PAM actions and indicate that these effects are mediated by
Dbi gene products. This was demonstrated by the deficits in
IPSC potentiation exhibited in nm1054 mice, which was rescued
by local viral transduction of DBI into nRT. Although the PAM
effects in nRT depend on the a3 subunit BZ binding site, as
demonstrated by the lack of modulation in a3(H126R) mice,
GABA ARs in VB neurons respond to endozepines when placed
in nRT. This indicates that the nucleus specificity of the observed
effects results from nRT-specific localization of PAMs within the
thalamus. Finally, we present in vivo evidence that SWD activity
is altered in both a3(H126R) and nm1054 mice, suggesting that
local PAM actions in nRT can exert seizure-suppressive effects
on TC circuitry as a whole. Although it remains to be determined
whether DBI itself or a peptide fragment act as the BZ site
agonist, our data provide functional evidence that indeed
peptides in the DBI family can act as PAMs.

Constitutive Endogenous Positive Allosteric Modulation
of GABAergic Transmission by Endozepines in nRT

The principal findings of this study are the existence and
functional consequences of endozepines in nRT, which act to
potentiate GABAergic postsynaptic currents in this nucleus.
a3(H126R) mutant mice, in which classical BZ binding to the
GABAAR a3 subunit is effectively abolished (Löw et al., 2000),
exhibited shorter durations of both sIPSCs and eIPSCs, indicat-
ing that in WT mice, constitutive modulation via this binding
site acts to prolong inhibitory signals. Another GABA AR
mutation associated with BZ binding and absence seizures (Wallace
et al., 2001) has been reported to affect basic receptor

Figure 7. Endozepines Modulate Spike-and-Wave Discharge (SWD) Activity Characteristic of Absence Seizures In Vivo

(A and B) Example traces of spontaneous SWDs recorded from a3(H126R) mice and respective WT (A) and nm1054 mice and respective WT (B). Note that SWD amplitude (power) was not significantly different between groups (p > 0.2). Therefore, examples were chosen to illustrate typical SWD waveforms in each condition, which were not affected by SWD power.

(C and D) Mean ± SEM for spontaneous 4-6 Hz SWD rate and percentage of time spent in spontaneous SWD activity in WT (n = 17) and a3(H126R) mice (n = 9) (C) and WT (n = 7) and nm1054 mice (n = 4) (D).

(E and F) Mean ± SEM for SWD period over time following PTZ injection (Time 0, 40 mg/kg) in WT (n = 7) and a3(H126R) mice (n = 4) (E) and WT (n = 7) and nm1054 mice (n = 4) (F).

(G and H) Linear regression of lines of best fit for points plotted in (E-F) shows positive correlations (i.e., SWD slowing) with time after PTZ injection in WT, but not in a3(H126R) nor nm1054 mice.

*p < 0.01, **p < 0.001 versus WT; ##p < 0.01, ###p < 0.001, repeated-measures ANOVA (E and F) or Pearson correlation (G and H) across 1 min bins after PTZ injection. Gray bars in (E) and (F) indicate times at which values differed between mutants and controls (p < 0.05). See also Figure S5.
properties such as receptor trafficking and expression and response to GABA (Bowser et al., 2002; Kang and Macdonald, 2004). Here, the effects of the z3(H126R) mutation on sIPSCs were confined to the duration of events, with no difference in either amplitude or frequency; thus it does not appear that this mutation leads to large differences in receptor expression, localization, or function besides the loss of BZ or endozepine binding. Both fast and slow decay time constants were reduced, suggesting that both fast and slow decay time constants were localized, or function besides the loss of BZ or endozepine either amplitude or frequency; thus it does not appear that receptors in nRT are responsive to endozepines (Huntsman and Huguenard, 2006; Hentschke et al., 2009). Furthermore, when nRT GABAA Rs in excised patches were removed from the slice and thus no longer exposed to putative endogenous modulators within the slice, the response to GABA uncaging was identical between WT and z3(H126R) mutant mice. Together, these results support the interpretation that the H126R mutation does not lead to differences in fundamental receptor properties apart from the effects on BZ/endozepine binding in z3(H126R) mutant receptors.

The ability of FLZ, a BZ binding site antagonist (Hunkeler et al., 1981), to reduce sIPSC duration also suggested an endogenous augmentation of IPSC duration in nRT. Similarly, FLZ has been observed to reduce evoked inhibitory postsynaptic potential (IPSP) amplitude in hippocampal CA1 pyramidal neurons (King et al., 1985) and eIPSC duration in dissociated cortical neurons (Vicini et al., 1986). Some reports have indicated agonist effects of FLZ (Skerritt and Macdonald, 1983; De Deyn and Macdonald, 1987; Weiss et al., 2002), including at receptors carrying z3 subunits (Ramerstorfer et al., 2010). This would not explain the reductions in duration and decay time observed here, although FLZ increased sIPSC amplitude and slightly increased charge transfer in both WT and z3(H126R) nRT cells, potentially indicating a nonspecific effect representing actions on presynaptic terminals (Table S1). Partial agonist effects of FLZ have also been described at receptors containing z4 or z6 subunits (i.e., receptors that do not respond to classical BZs) (Hadingham et al., 1996; Knoflach et al., 1996; Whitemore et al., 1996). Here, however, z3(H126R) GABAA Rs did not respond to FLZ, indicating that disruption of BZ binding to these receptors does not change the direction of response to BZ binding site activation.

A recent report indicates that the CSF of hypomnemotonic patients can potentiate currents evoked by GABA application in a FLZ-sensitive manner (Rye et al., 2012). This potentiation, however, is not occluded by the BZ midazolam and persists in z1(H101R) GABAA Rs, indicating that it does not represent a true BZ-mimicking endozepine effect.

**Dbi Gene Products Mediate Endozepine Effects in nRT**

The experiments in z3(H126R) mice examined the potential effects of endozepines at the level of postsynaptic GABAA Rs. To investigate this question at the level of the ligand, we tested the role of *Dbi* gene products in mediating endozepine actions by exploring intra-nRT GABAergic transmission in *nm1054* mice, which lack the *Dbi* gene (Ohgami et al., 2005). The other known genes deleted by the mutation are: primary ciliary dyskinesia protein 1 (*Pcdp1*); secretin receptor (*Sctr*); neuronal voltage-gated calcium channel γ-like subunit (*Pr1*); and six-transmembrane epithelial antigen of the prostate 3 (*Steap3*). *Steap3* has been described at receptors containing z3 subunits (Ferrero et al., 1986). It is also possible that nRT-specific receptor-associated proteins are required to obtain a PAM effect, though this is unlikely to be solely responsible as VB receptors tor-associated proteins are required to obtain a PAM effect, though this is unlikely to be solely responsible as VB receptors placed in nRT also exhibit a PAM response, as demonstrated in the sniffer patch studies (Figure 6). In addition, the dose response curve for stimulation of intracellular calcium levels in cultured astrocytes by ODN and octapeptide (OP), a smaller fragment, is bell-shaped with peak stimulation at 10 nM (Leprince et al., 1998). Therefore, it is possible that extracellular concentrations of endogenous DBI peptides in discrete areas such as nRT are at a lower level that is more likely to exert PAM rather than NAM effects.

In contrast to the stark nRT versus VB differences in physiological PAM effects of DBI, immunoreactivity to DBI was observed in both nRT and VB (Figure 5). In this regard, it is important to note that DBI is also known as ACBP and the *Dbi/Acbp* gene is thought to ubiquitously serve housekeeping functions (Knudsen et al., 1993), generating an intracellular protein critically involved in facilitating intracellular transport of Acyl-CoA. We hypothesize that the nRT versus VB differences in the electrophysiological findings likely represent nucleus-specific differences in the extracellular release and processing of DBI.

In the current studies, we used a viral strategy to examine whether *Dbi* gene products are necessary and sufficient to produce endozepine actions in nRT. Although it is possible that viral introduction of DBI into a system in this way induces changes in its modulatory effects, it is unlikely that this explains the PAM actions observed here, as a recent report using similar viral vectors to express DBI and ODN in the SVZ exclusively observed
NAM effects (Alfonso et al., 2012). The mechanism(s) underlying these differences remain to be determined, though one possible explanation is differential intra- or extracellular processing of DBI, the nature of which is specific to certain areas or cell types. It is certainly conceivable that some DBI fragments exert PAM effects, whereas others are NAMs. In addition, preliminary evidence suggests that exogenous application of DBI does not alter sIPSCs in VB (C.A.C. and J.R.H., unpublished data), in contrast to the robust potentiation of uncaging responses when VB sniffer patches are placed in nRT. This further supports a working model in which nRT-specific processing of Dbi gene products underlies the PAM actions.

Functional Endozepines Are Absent in VB

Strikingly, endogenous BZ-mimicking potentiation was observed in nRT, but not in adjacent VB thalamus. This raises the question of why such effects would be specifically localized to nRT, and not VB. Perhaps selection pressure led to evolution of an adaptive specific subcircuit modulation in nRT that reduces the possibility of seizure occurrence. Synaptic inhibition in nRT exerts a prominent desynchronizing effect to reduce the propensity for oscillatory activity (von Krosigk et al., 1993; Huntsman et al., 1999; Schofield et al., 2009), and potentiation of synaptic inhibition by BZs further suppresses oscillations (Huguenard and Prince, 1994a; Sohal and Huguenard, 2003; Sohal et al., 2003). In VB, by contrast, postsynaptic inhibition via GABA A exerts a prominent desynchronizing effect to reduce the propensity for oscillatory activity (von Krosigk et al., 1993; Huntsman et al., 1999; Schofield et al., 2009), and potentiation of synaptic inhibition by BZs further suppresses oscillations (Huguenard and Prince, 1994a; Sohal and Huguenard, 2003; Sohal et al., 2003). In VB, by contrast, postsynaptic inhibition via GABA A and GABA B receptors interacts with prominent T-type calcium conductances in VB relay neurons to drive robust postinhibitory rebound burst firing (Llinás and Jahans, 1982; Huguenard and Prince, 1994b; Sohal and Huguenard, 2003) and paradoxically promote oscillatory activity. Therefore, endogenous potentiation of intra-nRT inhibition is poised to exert an endogenous antioscillatory seizure-suppressing effect, whereas such potentiation in VB could be disadvantageous. It should be noted, however, that VB neurons are BZ-sensitive, as demonstrated here and in previous studies (Oh et al., 1995; Peden et al., 2008), although systemic treatment with BZs would influence both nRT and VB inhibition such that activity throughout the circuit would be globally suppressed. Indeed, constitutive activation in nRT was ~60% of maximal, indicating that although there is a substantial degree of endogenous modulation, there is still an extent of enhancement that can be exploited by exogenous BZs, likely explaining the therapeutic efficacy of these drugs.

Focal GABA Uncaging on Sniffer Patches Reveals Nucleus-Specific Differences in Endozepine Potentiation and GABA Uptake

Here, we introduce a methodology combining the “sniffer patch” recording configuration (Isaacson et al., 1993; Allen, 1997; Banks and Pearce, 2000) with laser GABA uncaging, which is used to examine nucleus-specific differences in endogenous BZ site modulation. The main advantage of this method is that GABA exposure to the patches can be normalized independent of patch placement, so that region-specific differences in modulation of GABAergic signaling can be assessed. Combined application of GAT antagonists and FLZ was sufficient to completely block the nRT-dependent potentiation. This potentiation is thus mediated in large part by endozepines, and to a lesser extent by nucleus-specific differences in rates of GABA uptake, with more robust uptake in VB than in nRT. This is consistent with the postulated role for GATs in removing GABA from VB extracellular space in order to prevent excessive GABA 3R activation and oscillatory seizure activity (Beenakker and Huguenard, 2010).

The durations of uncaging responses for both nRT and VB patches are longer than those for spontaneous or evoked IPSCs, in accordance with previous studies on patches from thalamic, hippocampal, and cortical neurons (Galarreta and Hestrin, 1997; Jones and Westbrook, 1997; Banks and Pearce, 2000; Schofield and Huguenard, 2007). Therefore, this appears to be an effect of the pulled patch configuration rather than the use of uncaging to apply GABA. Nevertheless, the application of GABA by laser photolysis replicates the nRT versus VB differences in receptor affinity for GABA and kinetics of IPSC decay (Zhang et al., 1997; Mozryzmas et al., 2007; Schofield and Huguenard, 2007).  }

Endozepines Modulate TC Circuit Activity In Vivo

Potentiation of intra-nRT GABAergic transmission by BZs is capable of exerting powerful antoscillatory effects by reducing synchronization in intra-thalamic networks (Huguenard and Prince, 1994a; Huguenard, 1999; Sohal et al., 2003). Similarly, the EEG recordings here indicate that PAM potentiation of GABAergic transmission, presumably within nRT as observed in vitro, can modulate in vivo rhythmic activity in TC circuitry in a manner consistent with suppression of seizure susceptibility and severity. In particular, both α3(H126R) and nm1054 mice exhibit similar phenotypes with respect to an increased incidence of spontaneous SWDs and a lack of slowing of SWD period following PTZ treatment that is observed in WT mice. This suggests that endogenous activation of BZ binding sites on GABA ARs containing the α3 subunit by DBI ligands in nRT exerts two primary effects: (1) a reduction in the propensity for the circuit to generate SWDs, and (2) a reduction in the intensity and severity of absence seizures once initiated. Although the α3 subunit is expressed in other brain regions, including cortical layers V and VI (Pirker et al., 2000), the complementary in vitro experiments implicate actions that originate in thalamus, and specifically in nRT, in underlying these differences.

Summary

Our results describe the molecular identification of an endogenous DBI-related PAM acting via GABA AR BZ binding sites, which may represent a novel endogenous anti-seizure mechanism. The highly specific release and processing within nRT (and presumably other restricted brain regions) may explain why previous studies using α3(H126R) and similar knockin mice have not observed overt phenotypes beyond those of subtype-specific BZ actions (Rudolph and Möhler, 2004). Given the important role of nRT as a locus of control in TC circuitry, targeted release of Dbi-derived endozepine peptides in nRT may be fruitful in the development of therapies against absence seizures. Furthermore, regulation of DBI release or function may provide a safer alternative to long-term BZ administration in the treatment of epilepsy and other neurological disorders.
EXPERIMENTAL PROCEDURES

Additional information on these methods can be found in Supplemental Information. All procedures were approved by the Administrative Panel on Laboratory Animal Care at Stanford University.

Brain Slice Preparation
Mice were anesthetized with pentobarbital sodium and killed via decapitation and the brain was quickly removed and placed in ice-cold oxygenated sucrose slicing solution. Horizontal thalamic slices (250 μm thickness) were prepared as previously described (Huguenard and Prince, 1994a). Slices were incubated and continuously oxygenated in warm (32°C) artificial cerebrospinal fluid for 1 hr and then transferred to room temperature (21°C–23°C) for at least 15 min prior to recording.

Patch-Clamp Electrophysiology
Patch-clamp recordings were made using a MultiClamp 700A amplifier with Clampex 9.2 software. Patch pipettes were filled with a 135 mM CsCl-based solution. To isolate GABAergic IPSCs, ionotropic glutamate receptors were blocked with either kynurenic acid (1 mM) or a combination of D-(-)-2-amino-5-phosphonovaleric acid (APV, 100 μM) plus 6,7-dinitroquininalene-2,3-dione (DNQX, 20 μM).

IPSCs
In whole-cell recordings, membrane potential was clamped at −60 mV. Evoked IPSCs were elicited by extracellular tungsten electrode stimulation in nRT.

GABA Uncaging
CNB-caged GABA (Invitrogen) was added to a recirculating 10–20 ml bath solution containing APV and DNQX. Outside-out patches of membrane from nRT or VB cells were maintained in voltage-clamp mode at a membrane holding potential of −30 mV. Laser photolysis (DPSS Lasers) was achieved via 1 ms ultraviolet laser exposure.

EEG Recordings
EEG recordings were obtained from either metal skull screws or silver wires implanted above the left and right frontol and parietal cerebral cortices. Experimental absence seizures were induced via s.c. injection of PTZ. EEG activity and simultaneous video were recorded for up to 90 min post-PTZ injection.

Virus Injections
Bilateral stereotaxic injections of either control or DBI-expressing AAVs were performed under isoflurane anesthesia between P48-50. Brain slices were prepared for electrophysiology at 2–3 weeks postinjection. Infected cells expressing GFP were visualized using epifluorescence microscopy.

Data Analysis
sIPSCs were analyzed using the custom software programs wDetecta and WinScanSelect (J.R.H.). eIPSC and uncaging recordings were analyzed using Clampfit. EEG recordings were analyzed using a continuous wavelet transform method in MATLAB to isolate SWD events (Schofield et al., 2009). Comparisons between groups were made using two-tailed independent or paired t tests, nonparametric Mann-Whitney rank-sum tests, or one-way ANOVA with Tukey’s post hoc means comparison tests. Cumulative probability distributions were constructed using up to 100 randomly selected sIPSCs (events) per cell and compared using two-sample Kolmogorov-Smirnov (KS) goodness of fit tests. Differences within each genotype for EEG parameters across different time points after PTZ injection were assessed using one-way repeated-measures ANOVA. Statistical significance was set at p < 0.05 for means comparisons, and p < 0.001 for KS tests.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures, two tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.neuron.2013.04.026.

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REFERENCES

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