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DOI: <https://doi.org/10.1007/s11064-014-1303-5>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-171992>

Journal Article

Published Version

Originally published at:

Möhler, Hanns (2014). Endogenous Benzodiazepine Site Peptide Ligands Operating Bidirectionally In Vivo in Neurogenesis and Thalamic Oscillations. *Neurochemical Research*, 39(6):1032-1036.

DOI: <https://doi.org/10.1007/s11064-014-1303-5>

Endogenous Benzodiazepine Site Peptide Ligands Operating Bidirectionally In Vivo in Neurogenesis and Thalamic Oscillations

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Received: 22 November 2013/Revised: 31 March 2014/Accepted: 1 April 2014/Published online: 9 April 2014
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Abstract By binding to the benzodiazepine site, diazepam binding inhibitor (DBI) is associated with negative allosteric modulation (NAM) of GABA_A receptors (Costa and Guidotti in *Life Sci* 49:325–344, 1991). However, the demonstration of a true physiological role of DBI and its fragments has only recently been reported. Based on DBI gain- and loss-of-function experiments in vivo, DBI and its fragment ODN were found to promote neurogenesis in the subventricular zone in vivo. Acting as NAM on GABA_A receptors of precursor cells, DBI counteracted the inhibitory effect of GABA and thereby enhanced the proliferation of these cells (Alfonso et al. in *Cell Stem Cell* 10:76–87, 2012). Conversely and most remarkably, in similar gain- and loss-of-function experiments in the thalamus, the DBI gene products acted as positive allosteric modulators (PAM) of GABA_A receptors in prolonging the duration of IPSCs, an effect which was specific for GABA transmission within the reticular nucleus (nRT) (Christian et al. in *Neuron* 78:1063–1074, 2013). Since intra-nRT potentiation of GABA transmission by benzodiazepine drugs exerts powerful anti-oscillatory effects, DBI might be endogenously effective by modulating seizure susceptibility. It remains to be seen by which mechanism both NAM and PAM activity can arise from the *Dbi* gene. Nevertheless, the results open new perspectives on the

regionally distinct endogenous modulation of GABA transmission via the benzodiazepine site.

Keywords GABA_A receptors · Benzodiazepine receptor · Endogenous ligand · Neurogenesis · Absence epilepsy

The Quest for Endogenous Ligands

Benzodiazepines have remained one of the most commonly prescribed medications in the field of psychiatry and neurology. Thanks to their potency, efficacy, short onset of action and low toxicity, benzodiazepines are widely used as anti-anxiety, anticonvulsant, sedative and muscle-relaxing drugs. All these effects are due to increasing the amplitude or duration of inhibitory postsynaptic currents mediated by GABA_A receptors, thereby enhancing inhibitory synaptic transmission by interacting with a specific benzodiazepine drug target site of the receptors, originally termed benzodiazepine receptor [4].

The discovery of the benzodiazepine receptor [5, 6] led to the hypothesis that the brain produces endogenous ligands that bind to this site and serve as endogenous allosteric modulators of GABA_A receptors with the potential of contributing to human disease. Endogenous ligands were perceived to potentially act as positive allosteric modulators (PAM, also termed agonists) or negative allosteric modulators (NAM, also termed inverse agonists) [1, 7]. Several naturally occurring candidate compounds were isolated from brain tissue such as diazepam binding inhibitor (DBI) [1, 7, 8], oleamides [9] and non-peptidic endozepines [10], of which DBI was most widely studied. DBI fragments were recently proposed to serve as regionally selective, physiological regulators acting at the benzodiazepine site of GABA_A receptors, as summarized below.

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DBI and Its Multiple Pharmacological Actions

DBI, also known as acyl-CoA binding protein [11], is a small cytosolic protein (10 kDa) expressed predominantly in glia and ependymal cells lining the ventricles [2, 12] that is secreted into the extracellular space via a non-conventional secretory pathway [13]. DBI and its proteolytic peptide products octadecaneuropeptide (ODN) and triakontatetrapeptide (TTN) bind to the central benzodiazepine site of GABA_A receptors and/or the so-called peripheral benzodiazepine receptor site [1] a 18 kDa mitochondrial translocator protein (TSPO) involved in the regulation of steroid synthesis [14]. DBI and ODN interacted with high affinity with the central benzodiazepine receptor site of GABA_A receptors ($K_i = 4$ micromolar in 3H-diazepam binding), while the TTN fragment of DBI had a preferential affinity for the peripheral benzodiazepine site ($K_i = 5$ micromolar in 3H Ro5-4864 binding) [1, 7, 8, 15]. When injected icv into rats, DBI, ODN and TTN all showed anxiogenic activity [16]. However, in keeping with the target distinction, the effect of DBI and ODN was antagonized by flumazenil while that of TTN was antagonized by PK11195, the peripheral site antagonist, but not by flumazenil [16]. Correspondingly, TTN and similarly DBI, but not ODN, enhanced steroidogenesis [17]. Thus, DBI gives rise to functionally distinct biologically active fragments. However, their functional role in vivo was only recently discovered.

Negative Allosteric Modulation of GABA_A Receptors by ODN in Neurogenesis In Vivo

The subventricular zone (SVZ) of the lateral ventricles is the largest neurogenic niche of the postnatal brain. New SVZ-generated neurons migrate via the rostral lateral stream to the olfactory bulb where they integrate in pre-existing circuits. Non-synaptic GABA signaling is known to inhibit SVZ-derived neurogenesis [18, 19] with GABA being released from neuroblasts [2].

The GABA_A receptor modulator DBI is highly expressed in the SVZ. To test its potential regulatory role in neurogenesis, DBI loss- and gain-of-function experiments were performed in vivo at the peak of postnatal neuron generation in mice (P4–P8) [2]. DBI knockdown in vivo by retroviral injection of shRNA impaired proliferation in the SVZ niche as shown by a decrease of cells stained positive for BrdU, a marker of dividing cells, as determined 12 days post injection. The specificity of the DBI knockdown effect was verified by a rescue of the impaired proliferation through retroviral expression of DBI in shRNA-DBI-infected cells. A gain-of-function experiment in vivo further substantiated the role of DBI in neuronal proliferation. Injection of a DBI

overexpressing virus into the SVZ of mice (P4) increased proliferation of neuronal SVZ progenitor cells. Thus, DBI loss- and gain-of-function experiments had opposite effects, in line with DBI being a positive regulator of subventricular neurogenesis.

The enhanced postnatal neuronal proliferation induced by DBI was mediated via the central benzodiazepine site of GABA_A receptors, not the peripheral/mitochondrial benzodiazepine site [2]. In primary cultures containing neurospheres of SVZ progenitor cells, DBI expression induced proliferation, in agreement with the in vivo results. The DBI effect was blocked by flumazenil but not PK11195, suggesting that DBI acted via GABA_A receptors.

Remarkably, ODN replicated the DBI effects in vivo and similarly induced neuronal proliferation following retroviral overexpression of ODN in the SVZ [2]. This effect was based on ODN acting as NAM of GABA_A receptors as shown in patch clamp experiments in fast proliferating progenitor cells. Thus, in the regulation of neurogenesis, DBI is expressed and released from neural stem cells and fast proliferating progenitor cells and processed to ODN which attenuates GABA_A receptor mediated inward currents on neural progenitors. ODN thereby counteracts the GABAergic brake on subventricular neuronal proliferation [2]. Thus, for the first time evidence for a physiological function of DBI and ODN as NAM of GABA_A receptors has been provided in a defined cellular context in vivo.

Positive Allosteric Modulation of GABA_A Receptors by DBI in Suppressing Thalamic Activity In Vivo

In contrast to DBI acting as NAM as described above, a physiological role of DBI acting as endogenous PAM of GABA_A receptors was recently identified [3]. The thalamocortical (TC) circuit is normally involved in sleep rhythms and sensory processing [20] and abnormal oscillatory activity in this circuit can promote absence seizures [21]. The thalamic reticular nucleus (nRT) is posed to play a critical role in gating this circuit. The nRT receives excitatory input from both cortico-thalamic and thalamocortical axons (Fig. 1). The nRT provides GABAergic inhibitory input onto TC relay cells in dorsal thalamus such as the ventrobasal nucleus (VB) and enforces intranuclear inhibition via recurrent collaterals [21] (Fig. 1).

A reduction in intra-nRT inhibition results in hypersynchronous epileptiform oscillations between nRT and VB [22]. Conversely, a gain of intra-nRT inhibition dampens oscillatory duration. Modulation of intra-nRT inhibition can thus shape TC circuit activity and thereby influences seizure susceptibility and duration. Benzodiazepines can suppress these thalamocortical oscillations by enhancing inhibition

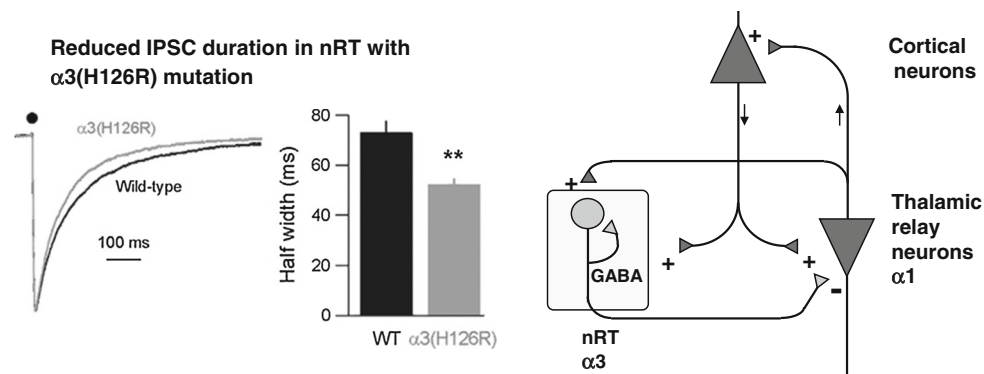


Fig. 1 Duration of GABA-induced IPSC shortened in the thalamic reticular nucleus (nRT) of α_3 (H126R) GABA_A receptor mutant mice. *Left:* Averaged eIPSC traces across thalamic reticular nucleus cells recorded from WT mice (lower trace) and α_3 (H126R) mutant mice (upper trace) and the corresponding bar graph. *Black dot* indicates

time of electrical stimulation applied to nRT [3]. *Right:* Scheme of the TC circuit. Excitatory thalamic relay neurons largely express α_1 GABA_A receptors. GABA neurons (*light gray*) within the reticular nucleus express α_3 GABA_A receptors [21] respectively

within nRT via α_3 GABA_A receptors [22]. Individuals with a mutation of the γ_2 subunit of the GABA_A receptor, which disrupts the benzodiazepine receptor site commonly develop absence seizures [23]. It was therefore hypothesized that an endogenous PAM resides within the nRT to enhance synaptic inhibition and thereby limiting TC oscillations.

Evidence of a PAM residing in the nRT, which potentiates GABAergic transmission was based on loss-and gain-of function experiments [3]. In slices from mice with a point mutated α_3 subunit [α_3 (H126R)] which renders the benzodiazepine site diazepam-insensitive, whole cell recordings in nRT revealed a reduced duration of GABA-induced and spontaneous IPSCs compared to wild type (Fig. 1). The response to GABA itself was unaltered by the point-mutation as shown by the responses of outside-out patches to laser-evoked GABA uncaging, suggesting that the GABA-dependent receptor operation was comparable in wt and mutant animals and did not account for the differences observed in IPSCs. In addition, flumazenil reduced the IPSC duration in slices of wt but not mutant mice. These findings were in line with the presence of an endogenous PAM in the nRT [3]. Remarkably, when examining the adjacent VB thalamic nucleus, flumazenil failed to affect the duration of the IPSCs. To exclude the possibility that differences in the composition of GABA_A receptors in nRT and VB would account for the different responses, sniffer patch experiments were performed in thalamic slices combined with GABA uncaging. A patch pulled from the VB neurons exposed to the nRT, displayed an increased duration of the GABA response compared to VB suggesting that differences in receptor composition did not account for the distinctive response to flumazenil between nRT and VB [3].

Evidence on the molecular nature of the PAM in nRT came from mice lacking a 400 kb region of chromosome 1

(mm1054) which comprises the *Dbi* gene. Like the α_3 (H126R) mice, the mm1054 mice showed a reduced duration of the IPSC in the nRT [3]. Importantly, this IPSC deficit was rescued by viral expression of DBI, demonstrating that the loss of this gene was sufficient to account for the reduced IPSC duration [3]. These findings provide strong evidence that the *Dbi* gene encodes the endogenous PAM which acts within the nRT as physiological modulator of GABA_A receptors. In addition, this is first evidence of a physiologically relevant PAM to arise from the *Dbi* gene. Furthermore, the results led to the suggestion that PAM release within the TC circuit may reduce seizure susceptibility and severity through a slowing of the seizure oscillations, which may destabilize them.

An Endogenous PAM Regulating Vigilance in Hypersomnia?

In a recent study [24], in which flumazenil normalized vigilance in patients with non-hypocretin-deficient primary hypersomnias, the presence of a peptidergic PAM was found in CSF. A CSF fraction of 500–3,000 Da enhanced, in a trypsin-sensitive manner, GABA-induced chloride currents, acting preferentially at α_2 subunit- compared to α_1 subunit-containing recombinant GABA_A receptors (176.4 ± 41.3 vs. 71.4 ± 36.4 %). However, this potentiation did not affect a potentiation by midazolam and partly persisted in α_1 (H101R) GABA_A receptors, indicating that it may not be a classical benzodiazepine-mimicking agent [24]. The chemical nature of this CSF constituent and its potential role in the pathophysiology of hypersomnia remains to be resolved.

An improvement in vigilance by administration of flumazenil had been reported earlier in some patients with hepatic encephalopathy [25, 26], sleep deprivation [27, 28]

and idiopathic recurrent stupor [29]. These findings remained inconclusive with regard to an endogenous PAM since flumazenil itself, depending on the paradigm in question, can display partial inverse agonistic activity or partial agonistic activity [31]. Nevertheless, in the case of recurrent stupor, a small molecular weight, non-peptidergic bioactivity, termed endozepline-4, was increased in CSF during ictal periods up to 300-fold [30]. Although endozepline-4, like other endozeplines of this type, were purified from rat and human brain and potentiated GABA evoked chloride currents [10], their chemical nature was not resolved and was partly attributed to environmental/nutritional sources [32].

Future Directions

The search for the elusive endogenous ligand for the benzodiazepine site of GABA_A receptors has closed in on a longstanding suspect, DBI and its proteolytic fragments [2, 3]. Most remarkably, the endogenous ligands exploit the ability of GABA_A receptors to undergo positive (in the case of nRT) as well as negative (in the case of neurogenesis) allosteric modulation, which up to now seemed to be a prerogative for pharmacological ligands. Several questions remain [33]. In thalamus, PAM effects were only detectable within the nRT although the *Dbi* gene is likewise expressed in the VB. How are regionally and functionally distinct peptides derived from the *Dbi* gene? Are there cell-specific peptide products being generated? Could post-translational modifications result in opposing effects? Is DBI or a DBI fragment operative as endogenous ligand in other parts of the CNS and PNS? Answers to these questions would substantiate the claims for DBI and may open a new chapter in the regional regulation of GABA transmission. The findings may also shed new light on a potential regional efficacy modulation by drugs acting at the benzodiazepine site.

Conflict of interest The author declares that he has no conflict of interest.

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